Biochimica et Biophysica Acta, 509 (1978) 90—99

BBA 77996

MAGNETIC RESONANCE STUDIES OF EUKARYOTIC CELLS

III. SPIN LABELED FATTY ACIDS IN THE PLASMA MEMBRANE

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(Received August 16th, 1977)

Summary

XC Sarcoma, Vero and *Aedes aegypti* plasma membranes have been studied in viable cells and in purified membrane of XC Sarcoma cells by the spin label method. The temperature dependence of the order parameter of fatty acid spin labels is found to be linear in all three cells and membrane and shows no evidence of a lipid phase transition. The order parameter of the fatty acid labels substituted at the 5-position is shown to increase as a function of the cholesterol : phospholipid molar ratio in cells that have been studied to date. Cells attached to their growing surface are studied for the first time by electron paramagnetic resonance spectroscopy (EPR). The resulting spectra are anisotropic due to the non-spherical shape of the cells and show that these labels orient preferentially perpendicular to the cell surface. The viscosity of the extracted XC cell membrane is estimated to be 2.5 P from rotational correlation time measurements of the spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO).

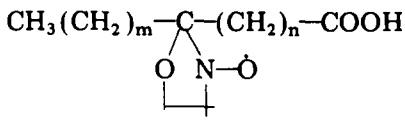
Introduction

Of the many physical techniques that are used to study model membrane systems and intact membranes, electron paramagnetic resonance spectroscopy (EPR), especially of nitroxide spin labels, has been one of the more widely applied and important ones [1—4]. Recently, this technique has become utilized in the important, but intrinsically more complicated problem of the membrane(s) in viable eukaryotic cells [5—10]. In this paper we report the third in a series of spin label investigations in cells. Previously, lipid chain

Abbreviations used: EPR, Electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl. TEMPONE, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl.

flexibility versus cell morphology was studied in Sarcoma 180 cells [7] and in XC Sarcoma cells [8].

The fatty acid spin labels, I(m, n),



have been used in all eukaryotic cell studies [5—10] reported to date and in numerous model membrane studies [4]. These labels reorient in an anisotropic manner which has been interpreted to be due to the ordering of the lipids that make up the lipid bilayer portion of the membrane. In this way, these label report, indirectly, the order of lipids themselves. A wide variety of useful information has thus been derived in model systems and purified membranes which poses the question: to what extent is this information applicable to the structure of the plasma membrane of an intact eukaryotic cell? With this in mind, we have studied the plasma membrane of intact XC Sarcoma cells and the purified plasma membrane of these cells. We have also investigated XC Sarcoma cells still attached to their growing surface to pursue a similar question: what effect does the common practice of removing cells, grown in vitro, from their growing surface have on the structure of the lipids? In addition, it was possible in this latter study to show that the orientation of the labels is perpendicular to the membrane surface in intact cells as is known to be true in model studies.

We have also studied the plasma membrane of intact Vero and *Aedes aegypti* cells.

The viscosity of the hydrophobic portion of the lipid bilayer in purified plasma membrane of XC Sarcoma cells was measured using the spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO).

Finally, we show that the order induced in the lipids of intact eukaryotic cells near the polar head groups seems to be influenced by cholesterol, as has been shown to be the case in model systems.

Materials and Methods

Materials

The fatty acid spin labels I(1, 14), I(5, 10) and I(12, 3) were purchased from Syva Assoc., Palo Alto, Calif., and were used without further purification. A 0.1 M stock solution in 100% ethanol was prepared of each label and was stored in a refrigerator. TEMPO was prepared by Dr. J.D. Medina according to published procedures [11] and 5 mM solutions in 100% ethanol or in water were prepared and stored in refrigerator. Reagent grade $K_3Fe(CN)_6$ (Carlo Erba), dibutyryl cyclic AMP (Sigma), testosterone (Schwartzmann, Orangeburg, N.J.), were used as received.

Cell cultures and membrane preparation

Serially propagated *Aedes aegypti* [12—13] cells were maintained at 28°C in disposable tissue culture flasks (Falcon Plastics, Oxnard, Calif.), used in

passages 100—200, and grown in the medium described by Kitamura [14]. The Vero cell line (green monkey kidney) was grown and maintained at 37°C in the medium of Macpherson and Stoker [15]. XC Sarcoma cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's medium supplemented with 10% serum with and without the presence of 1 mM dibutyryl cyclic AMP and 15 μM testosterone. The purpose of this latter agent is to increase the effect of the dibutyryl cyclic AMP [16].

Plasma membranes were prepared from XC cells approx. two-thirds confluent. The cells were detached by brief exposure to 0.5 mM EDTA in phosphate-buffered saline (pH 7.0, 37°C). After low-speed centrifugation, the cells were resuspended to 15-times their packed volume in 1 mM $ZnCl_2$ and homogenized in a tight-fitting Kontes homogenizer with approx. 30 strokes [17]. Cell rupture and preservation of nuclei was monitored by phase contrast microscopy and subsequent isolation of the plasma membrane fraction was carried out by the 2-phase procedure [18].

Spin labeling

Vero and *Aedes aegypti* cells were washed once with phosphate-buffered saline and XC cells were washed with serum-free Dulbecco's medium. The desired amount, of the order of 1 μl, of the stock solution of I(m, n) was added to the buffer covering the cells (5—10 ml) during slight agitation of the solution by hand. In routine experiments, the cells were incubated at 37°C for 15 min with the label. The cells were then washed with their respective label-free solutions, detached with a rubber policeman, centrifuged at low speed and sealed into 50 μl pipettes which served as EPR sample tubes. Attached XC cells were prepared for EPR measurements by growing the cells on thin glass plates placed in the bottom of the petri dishes, following the labeling procedure above, forming a loose sandwich of 3—7 plates and enclosing the sandwich in parafilm. The sandwich was held in the EPR sample cavity by a specially constructed holder.

Extracted plasma membranes from XC cells were labeled with the labels I(m, n) by placing the desired amount of a 10-fold dilution of the stock solution into a tube, evaporating the ethanol under vacuum, adding the membrane suspension and swirling for a few min. The same procedure was followed with TEMPO in ethanol. Alternatively, TEMPO in water was added directly to the membrane suspension. The labeled membrane suspension was then sealed into 50 μl capillary tubes for the EPR measurements.

EPR measurements were carried out as before [7].

Results

Spin label incorporation

The labels I(m, n) were readily incorporated into all three cell types studied as evidenced by their rapid uptake and by the almost total absence of the sharp three-line spectrum often observed [9] that is due to label in the aqueous fraction superimposed on the broader anisotropic spectra due to the label in the membrane (see Fig. 1).

The EPR signal intensity due to all three labels I(m, n) in all the cells reported

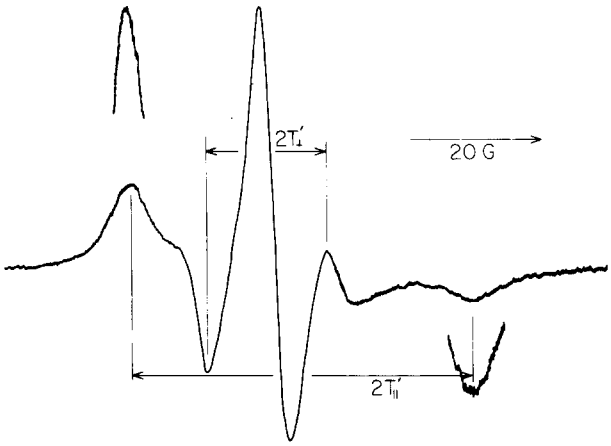


Fig. 1. EPR spectrum of I(12, 3) in cells and dibutyryl cyclic AMP at 37°C taken 2.5 h after 15 min exposure to spin label. The method of measuring $T'_{\parallel}$ and $T'_{\perp}$ used in the calculation of the order parameter is indicated. In many runs, the important high and low field features were recorded at higher gain as shown.

here and in plasma membrane prepared from XC Sarcoma cells, declined as a function of time. The signal falls to one-half its original strength in approx. 0.5 h in cells and in approx. 1 day in extracted membrane.

After the EPR signal had disappeared, we applied the technique of Kaplan et al. [5] to preferentially observe the EPR signal due to labels in the surface membrane by adding $K_3Fe(CN)_6$ to the cell sample. This salt revives the signal. It is important to note that the surface labels produce the same order parameter (next section) as the order parameter observed throughout the experiment.

Order parameters

In Fig. 1, a spectrum of I(12, 3) in XC cells is shown. The spectra due to these labels in Vero and *Aedes aegypti* were very similar in all respects. All spectra were analized from the experimental line separations $T'_{\parallel}$ and $T'_{\perp}$ defined in Fig. 1, using the order parameter formalism as before [7], that is

$$S = \frac{T'_{\parallel} - T'_{\perp} - C}{T'_{\parallel} + 2\,T'_{\perp} + 2\,C} \cdot 1.723 \tag{1}$$

where the correction factor $C = 1.4\ \text{G} - 0.053(T'_{\parallel} - T'_{\perp})$, where $T'_{\parallel}$ and $T'_{\perp}$ are in gauss. In the case of labels I(5, 10) and I(1, 14), the spacing $T'_{\parallel}$ is not resolved, nevertheless, one may estimate an order parameter by measuring $T'_{\perp}$. Following Gaffney [9] we write

$$S = \frac{43.7\ \text{G} - 3T'_{\perp}}{46.1\ \text{G}} \cdot 1.723 \tag{2}$$

The order parameter has been discussed extensively in the literature [4] but we wish to emphasize that the numerical value of S depends on the choice of nitroxide molecular fixed principal values of the magnetic coupling tensors

TABLE I

HYPERFINE CONSTANTS AND ORDER PARAMETERS OF I (12, 3) IN EUKARYOTIC CELLS AND PLASMA MEMBRANE. T = 22°C *

Sample	$T'_{/\!/}$	$T'_{\perp}$	Order parameter	Ref.
XC cells				
attached ⊥ **				
with dibutyryl cyclic AMP	28.9 ± 0.30	9.0 ± 0.20	0.71 ± 0.02 (3)	This work
without dibutyryl cyclic AMP	28.6 ± 0.30	9.1 ± 0.20	0.69 ± 0.02 (5)	This work
attached // **				
with dibutyryl cyclic AMP	28.1 ± 0.30	8.9 ± 0.20	0.69 ± 0.02 (4)	This work
without dibutyryl cyclic AMP	27.8 ± 0.40	8.8 ± 0.20	0.69 ± 0.02 (4)	This work
detached				
with dibutyryl cyclic AMP	28.1 ± 0.29	8.8 ± 0.16	0.70 ± 0.013 (6)	8
without dibutyryl cyclic AMP	27.7 ± 0.35	8.8 ± 0.12	0.69 ± 0.013 (20)	8
Vero cells	27.7 ± 0.30	8.9 ± 0.15	0.68 ± 0.016 (12)	This work
Aedes aegypti cells	27.7 ± 0.36	8.9 ± 0.20	0.68 ± 0.027 (5)	This work
XC plasma membrane	27.9 ± 0.38	9.0 ± 0.10	0.68 ± 0.01 (3)	8

* Hyperfine coupling constants given in gauss. Order parameters calculated from Eqn. 1. The errors are observed standard deviations in the number measurements given in the parentheses.

** The symbols // and ⊥ mean that the plane of the growing surface was parallel with or perpendicular to the external magnetic field, respectively. Note that the order parameters calculated in the case of attached cells are only approximate (see text).

and on the method used to calculate C [9]; thus, if the order parameters measured in various cell membranes are to be compared, care must be taken that the order parameters have been calculated under the same assumptions.

The results are given in Tables I and II. These results together with those of Tables I and II of ref. [7] are all calculated under the same assumptions using the original data and comprise all the data in viable eukaryotic cells of which we are aware.

Temperature dependence of the spectra

No evidence of a lipid phase transition was found in XC cells (10—43°C),

TABLE II

ORDER PARAMETER OF I (5, 10) AND I (1, 14) IN EUKARYOTIC CELLS AND PLASMA MEMBRANE. T = 22°C *

Sample	Order parameter I (5, 10)	Order parameter I (1, 14)	Ref.
XC cells			
attached (// or ⊥)			
with dibutyryl cyclic AMP	0.51	0.29	8
without dibutyryl cyclic AMP	0.51	0.36	8
detached			
with dibutyryl cyclic AMP	0.51	0.33	8
without dibutyryl cyclic AMP	0.51	0.32	8
Vero cells		0.29	This work
Aedes aegypti cells		0.29	This work
XC plasma membrane		0.33	This work

* Calculations made using Eqn. 2. Estimated errors are ±0.01 except for the attached cells in which case the errors are estimated to be ±0.02.

** Note that the order parameter in the case of attached cells is only approximate (see text).

TABLE III

PARAMETERS DESCRIBING THE LINEAR LEAST SQUARES FIT OF EQN. 3 TO DATA FOR FATTY ACID SPIN LABELS IN CELLS

Cell	Label	S (37°)	α (°C^{-1})	Correlation coefficient
XC *	I (12, 3)	0.62	—0.0062	0.985
XC **	I (1, 14)	0.25	—0.0049	0.994
Vero *	I (12, 3)	0.59	—0.0059	0.935
Vero **	I (1, 14)	0.19	—0.0039	0.948
Aedes aegypti **	I (1, 14)	0.21 (5)	—0.0048	0.967
XC membrane *	I (12, 3)	0.57	—0.0074	0.987

* Eqn. 1 used.
** Eqn. 2 used.

Vero (0—25°C), *Aedes aegypti* (0—25°C) or in XC plasma membrane (0—45°C). In fact, in each case studied in detail the order parameter was found to vary linearly with temperature over the above-mentioned temperature ranges. Thus, the order parameter as a function of temperature may be written

$$S(T) = S(37°) + \alpha(T - 37) \tag{3}$$

where $S(37°)$ is the order parameter at 37° C and α is a constant. The linear least squares fit of the data yielded the values of $S(37°)$ and α given in Table III. The final column gives the correlation coefficient of the linear least squares fit.

EPR spectra of cells attached to their growing surfaces

EPR spectra of I(12, 3) in XC cells attached to their growing surfaces are shown in Fig. 2. These spectra are similar to spectra of I(12, 3) in detached XC cells, but careful inspection shows that there are slight differences. The two

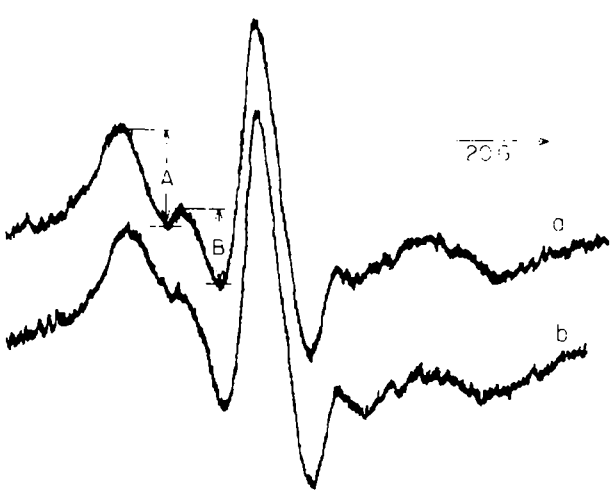


Fig. 2. EPR spectra of I(12, 3) in XC cells attached to their growing surface at 22°C. The upper (lower) trace corresponds to the magnetic field being perpendicular to (parallel with) the plane of the growing surface. The intensities A and B are defined as indicated (see text).

traces in Fig. 2 were taken with the plane of the growing surface oriented perpendicular to (upper trace) and parallel with (lower trace) the external magnetic field. The symbols '*A*' and '*B*' in Fig. 2 define line intensities that are qualitative measures of the populations of spin labels whose time-averaged cylindrically symmetric hyperfine axis is parallel with and perpendicular to the magnetic field axis, respectively. The change in the ratio A : B as a function of the change in the orientation of the sample with respect to the magnetic field then can be taken as a qualitative measure of the departure from an isotropic distribution of orientations. From the measured ratios for each orientation one can calculate a parameter: $F = (A/B)_\perp/(A/B)_\parallel$. Labels oriented, in general, perpendicular to the cell surface of a flattened cell would produce $F > 1$ and labels parallel with the surface, $F < 1$. Labels oriented in membranes which had no particular orientational relationship with the growing surface, would give $F = 1$. For XC cells attached to their growing surface, $F = 2 \pm 0.5$. The error is the standard deviation observed in ten samples. For one sample $F = 2 \pm 0.2$ where the error is the standard deviation in five measurements of the same sample and $F = 3 \pm 0.2$ after physically squeezing the sample. For XC membranes squeezed between microscope slides, $F = 4.1$.

We assume that the membrane fragments are preferentially oriented with their surfaces parallel with the glass plates. Thus, the fact that F is greater than 1 both in cells and in the partially-oriented membrane fragments demonstrates that these labels orient with their chain perpendicular to the membrane surface in cells as well as in membrane fragments. There are also small differences in the values of $T'_\parallel$ and $T'_\perp$ in the two orientations of the growing surface, which one would measure according to Fig. 1. Clearly, Eqn. 1 would not yield accurate values of the order parameter using these values, but we tabulate in Tables I and II the values of S given by Eqn. 1 to indicate that they are remarkably close to those found in detached cells.

TEMPO in XC plasma membrane

When the spin label TEMPO is introduced into a suspension of XC cell plasma membrane in buffer, the EPR spectrum depends on the relative amounts of buffer and membrane present. At high water concentrations a sharp three-line nitroxide spectrum is observed with $a = 17.1 \pm 0.1$ G, $g = 2.0057 \pm 0.0001$, and rotational correlation, τ_c less than $1 \cdot 10^{-10}$ s due to TEMPO in the aqueous fraction. At low water concentrations, the corresponding parameters observed are $a = 15.7$ G, $g = 2.0060$ and $\tau_c = 3.8 \cdot 10^{-10}$ s due to the label in the membrane. To estimate the rotational correlation times we have used the method of Morse et al. [19].

The rotational correlation time of a similar molecule, TEMPONE in mixtures of water and glycerol was measured as a function of viscosity by Morse et al. [19]. Using their results, assuming that the two labels reorient similarly, we estimate the viscosity to be 2.5 P. This value is rather typical for membranes as measured by various methods [20].

Discussion

Previously [7], we summarized the arguments that suggest that the observed order parameter of the fatty acid spin labels is that due to labels in the plasma

membrane of Sarcoma 180 cells, and we have applied those arguments to the cells studied here including the method of Kaplan et al. [5]. We have further studied this question in XC cells by studying high spin label concentrations. The details of the method will be given elsewhere but the basic idea is to introduce an amount of spin label that would lead to high label : lipid concentration based on the assumption that all of the label resides in the plasma membrane, and to a rather low concentration based on the assumption that the label is equally distributed among all cellular membrane. The results show that the label is concentrated in approx. 10% of the total membrane (Bales, B.L. and León, V., unpublished studies). Thus, the facts are:

(1) the label, in the first stages of the experiment, resides in a small fraction of the total cellular membrane;

(2) after the signal disappears, a substantial amount of reduced label still resides in the surface membrane; and

(3) the order parameter of the 'revived signal' due to the labels in the surface membrane is the same as the order parameter observed in the early stages, indeed in all stages, of the experiment.

Thus, these experiments suggest that we are indeed studying the plasma membrane. Also, the fact that the spectrum of Fig. 1 is quite well resolved supports this idea. One would expect that a spectrum made up of a superposition of spectra due to the label in various membranes each with a slightly different order parameter would lead to a broadened spectrum.

Table III shows that there are indeed small differences in the order parameter and its temperature dependence of I(12, 3) in the plasma membrane of XC cell and in the purified plasma membrane. The temperature dependence is linear in both cases and shows no evidence of a phase transition in either case. In the case of I(1, 14), which probes the region farther away from the head group, the results were the same in cells and purified membrane at $T = 22°C$. Thus, we conclude that the order in the lipids is similar but not identical in the cell and the purified membrane, the former showing slightly more order in the region near the polar head groups. We note that this difference would be nicely explained if some cholesterol were lost in the membrane preparation process (see below).

The lipid order in the intact eukaryotic cells studied to date [5—10] is remarkably similar. Nevertheless, there are small differences. In order to attempt to interpret these differences in terms of the physical properties of the cell membranes we have compiled the molar ratio of the cholesterol to phospholipid in the plasma membrane of all the cells that we could find in the

TABLE IV

CHOLESTEROL : PHOSPHOLIPIDS MOLAR RATIOS IN EUKARYOTIC CELLS

Cell	Cholesterol : phospholipid	Reference
Human erythrocyte	0.95	22
Human lymphocyte	0.75	23
L cells	0.69	24
Chick embryo fibroblast	0.6	25
Sarcoma 180	0.42	26

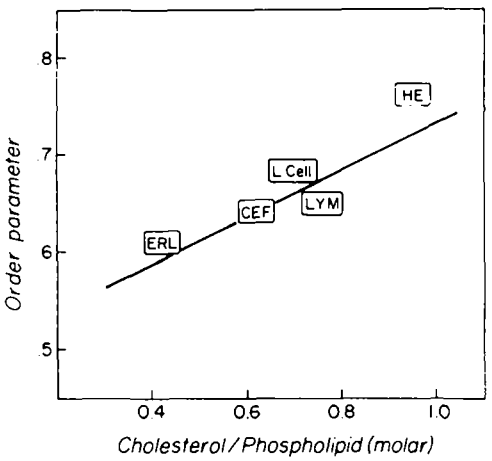


Fig. 3. The order parameter vs. the molar cholesterol : phospholipid ratio from Table IV. The symbols are: ERL, Sarcoma 180 ascites; CEF, chick embryo fibroblasts; L cell, mouse L cells; LYM, human lymphocytes; and HE, human erythrocytes. Spin label I(12,3) at 22°C.

literature that have been studied with the labels I(m, n). These are tabulated in Table IV and the order parameter of I(12, 3) at 22°C measured in the five cells is plotted vs. this ratio in Fig. 3. The error bars are still rather large on both axes but the data nevertheless are encouraging in that they show that the lipid order increases as the cholesterol content increases. This is similar to the results in the model systems [4].

Plasma membrane composition measurements are time-consuming; thus, if a behaviour as indicated in Fig. 3 is valid, this would provide a quick way to estimate the important parameter cholesterol-phospholipid.

Attempts to arrive at systematic descriptions of the results from I(1, 14) and I(5, 10) in terms of cholesterol content were not successful.

It is known that cholesterol tends to 'wash out' lipid phase transitions in model systems [21], thus, the lack of a phase transition in the cells studied here may be a reflection of their high cholesterol content.

Except for the effect that we have attributed to the anisotropic distribution of membrane orientation due to the flatness of the cells, we find no large difference in the order parameter in attached and detached cells because even the approximate order parameters calculated in these cells are within experimental error of those calculated in detached cells. Measurements with attached cells are difficult, so this is a result of practical importance, although one which should be viewed with some caution because the error bars are still rather large.

Acknowledgements

The authors wish to thank the following for their contribution to this work: Dr. J.D. Medina for synthesizing the TEMPO; Dr. J. Esparza for providing Vero and *Aedes aegypti* cells; Dr. M. Rieber for providing facilities to grow XC cells, and Drs. L. Mateu, J. Esparza and M. Rieber for advice, discussion and encouragement.

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