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Authors
Keith, Alec
Bulfield, Grahame
Snipes, Wallace.

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DISCLAIMER

SPIN-LABELED NEUROSPORA MITOCHONDRIA

Alec Keith and Grahame Bulfield†
Department of Genetics

Wallace Snipes*
Donner Laboratory and Lawrence Radiation Laboratory

University of California
Berkeley, California 94720

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ABSTRACT Spin-label studies were carried out on Neurospora mitochondria under in vivo and in vitro labeling conditions. A long-chained spin-labeled fatty acid was incorporated by Neurospora and was found in mitochondrial phospholipids. The molecular motion at various temperatures was different from that for the same spin label under in vitro labeling conditions. The results for spin-labeled mitochondria were compared with those from isolated lipids and with those from aggregates of spin-labeled fatty acid and isolated mitochondrial structural protein. These comparisons suggest that there is a maximum interaction between lipid elements and a minimum interaction between the lipid and protein elements in the intact mitochondrial membranes.

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†Present address: Institute of Animal Genetics, University of Edinburgh.

*Present address: Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802.

INTRODUCTION

Electron spin resonance (ESR) employing spin labels (nitroxides) is well suited to studying biological membranes. Potentially, information can be obtained about the freedom of molecular motion, local environment, anisotropic motion, and physical state of the spin-labeled molecule (see Refs. 1-3 for reviews). An appropriate spin label will relatively accurately reflect these data about the skeleton of the molecule to which the spin label is attached.

There are several reports of experiments in which a spin label was added directly to a biological sample, and much useful information has been obtained in this way. Under ideal conditions it is also possible to supply a suitable spin-labeled molecule and have it incorporated during growth by the organism. A spin-labeled fatty acid was previously added in vivo to study Neurospora mitochondria (4). This sytem is explored in greater depth in this report in an attempt to obtain useful information about membranes.

Many ideas and models concerning membrane structure and function have been expressed during recent years. The best known of the structural models is the bilayer model, which depicts a minimum association, polar in nature, between the protein and lipid elements of membranes (reviews 5-7). We have compared our results with this widely accepted model. Several other workers have expressed a variety of viewpoints about the interaction between the lipid and protein elements of membranes (reviews 8, 9). Consequently, even though many of the investigations on which these models were based were highly sophisticated, the fundamental problems remain unsolved. One of these, the nature

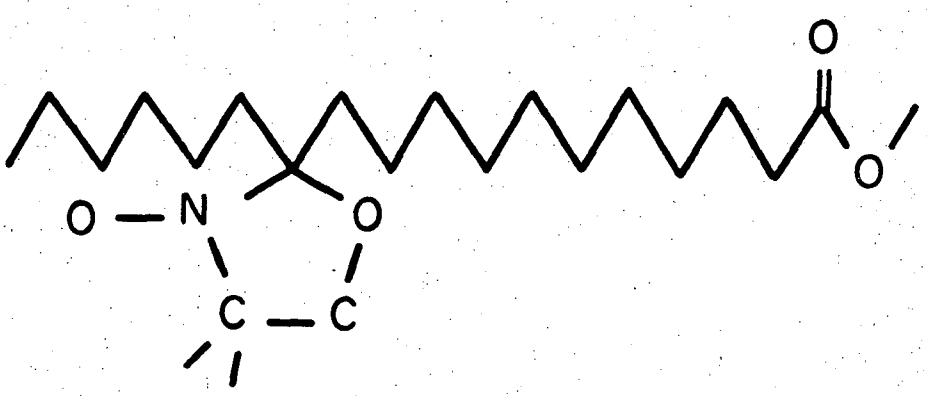
of the association of the lipid and protein elements of membranes, is investigated by the use of spin-labeled lipids, and is the subject of this report.

MATERIALS AND METHODS

Fifteen hundred ml of Neurospora minimal medium, supplemented with 0.25% yeast extract and 1% Tergitol (Union Carbide NP-40), was incubated with 7×10^5 conidia per ml. Fifty mg of 12 nitroxide stearate (12NS) in 1 ml of ethanol was added at zero time. Neurospora was grown for 16 hours in a rotary shaker at 34° C. The mycelium from the growth medium was strained through cheesecloth and squeezed dry. The average weight of mycelium under these conditions was about 20 g. The mitochondrial isolation procedure is presented in detail elsewhere (10); this procedure is briefly described below. The mycelium was then ground with 6 g of washed glass beads per g of mycelium and 200 ml of 0.25 M sucrose in an Eppenbach micromill for one minute at speed 110 and a pore setting of 64. The homogenization was carried out at 4° C and the mitochondrial extraction at as near 0° C as possible. The homogenate was vacuum-filtered through several layers of toweling to remove glass beads and mycelial fragments. The mitochondria were separated from this brei by differential centrifugation. Heavy contaminating particles were sedimented at low speed; the mitochondria were sedimented from the supernatant at high speed and were further purified on a continuous sucrose gradient followed by a final wash and centrifugation. Marker enzymes were used to show that this mitochrondrial preparation was not contaminated by other cell fractions (10).

Structural protein isolated from Neurospora mitochondria was generously supplied by Prof. Patricia St. Lawrence.

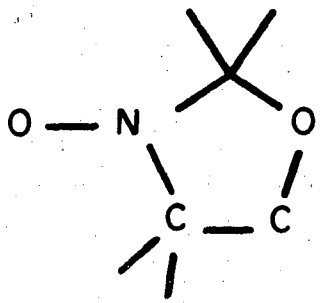
The synthesis, general chemical properties, and biological use of 12NS



(12NS)

XBL6911-6271

are described elsewhere (11). The same synthetic procedure, with acetone as the starting material, was used for 2 nitroxide propane (2NP):



(2NP)

XBL6911-6270

A Varian X-band electron paramagnetic resonance spectrometer equipped with the Varian variable-temperature accessory was used for all measurements. The temperature accessory was calibrated with an iron-constantan thermocouple to an estimated accuracy of ± 1.5° C.

RESULTS

Molecular freedom of motion is related quantitatively in ESR to rotational correlation time (τ_c) of the nitroxide spin-labeled molecule. The general theory and derivation of τ_c expressions were given by McConnell (12) and later, in more detail, by Kivelson (13). One form of these equations is

$$\tau_c = \frac{\pi\sqrt{3}\, W_0}{b}\left[\frac{4\Delta\gamma H}{15}+\frac{b}{8}\right]^{-1}\left[\frac{W_{-1}}{W_0}-1\right]. \qquad (1)$$

Here $\Delta\gamma$ is a constant that depends on the anisotropic g values, b is a constant that depends on the anisotropic nitrogen hyperfine couplings (A^N), H is the laboratory magnetic field, and W_0 and W_{-1} are the widths of the mid- and high-field lines. When the parameters of Griffith et al. (14) for b and $\Delta\gamma$ are used and H is maintained at 3400 gauss, this expression reduces to

$$\tau_c = 6.5\times10^{-10}\, W_0\left[\frac{W_{-1}}{W_0}-1\right], \qquad (2)$$

where W_0 is in gauss. For convenience of measurement the ratio W_{-1}/W_0 can be replaced by $(h_0/h_{-1})^{1/2}$, where h_0 and h_{-1} are the heights of the mid- and high-field lines on a first-derivative absorption spectrum. It is empirically estimated that the actual numerical values may be off by as much as a factor of two or three but that the relative

values for purposes of comparison are quite accurate.

Molecular Motion.

Figure 1 shows examples of ESR spectra that reflect different states of molecular motion. Figure 1a gives a typical spectrum of quite rapid motion in water and Fig. 1b gives the same general type of spectrum of the same nitroxide spin label in octadecane. The only difference between the two spectra is that the coupling constant for Fig. 1a is considerably greater than that for Fig. 1b. This reflects the polarity of the local environment of the nitroxide spin label. Figure 1c illustrates a spectrum that has considerably reduced molecular motion. The observations on line shape reported in this paper all fall between the two extremes of Fig. 1a and 1c. The spin-labeled fatty acid (12NS) was incorporated into mitochondria by growing Neurospora mycelium, and resulted in the same general ESR spectra as previously reported (4). The spin-labeled fatty acid gave an ESR signal reflecting a relatively fluid local environment of the spin label in the mitochondria (Fig. 2). As an example of what this signal means in terms of molecular motion, the ESR signal taken at 30° C from mitochondria that were spin-labeled during growth (in vivo), can be approximately duplicated by the signal from 12NS in glycerol at 60° C. This comparison might not be completely valid, since the bulk viscosity may not coincide with the local viscosity; however, it is helpful to consider that the viscosity of glycerol at 60° C approximates the viscosity of the local environment of 12NS in the mitochondria.

As temperature increased, the motional freedom of 12NS increased both in the mitochondria and in all other preparations, and decreased as

temperature decreased. No sharp discontinuities or anything that could be interpreted as phase transitions in response to temperature changes were observed in any of the samples. Differences were noted, however, between the in vivo and in vitro spin-labled mitochondria (Fig. 6). Lyophilization did not seem to alter the signal from the in vivo preparation, but considerably altered the in vitro ones (Fig. 3); in fact, τ_c values show that lyophilizing the in vitro labeled mitochondria reduced the motional freedom considerably. However, when the in vivo labeled mitochondria were air dried at room temperature the motional freedom was affected. This perhaps indicates that extensive denaturation of the mitochondria may have occurred during air drying.

The dried total lipids extracted from mitochondria that were spin-labeled in vivo resulted in ESR signals only slightly more free in molecular motion than the mitochondrial preparations. The preparation of total lipids was similar to mitochondria and responded to temperature changes in the same general way (Fig. 4). The phospholipids extracted from in vivo spin-labeled mitochondria and resuspended in water gave ESR signals almost identical to the total dried lipids, indicating that the aqueous phase had a minimal effect on the spin-labeled mitochondria (Fig. 4).

12NS bound tightly to structural protein, and yielded a signal considerably more restricted in molecular motion than the mitochondrial preparation (Fig. 5). However, it must be emphasized that this general type of signal is not unique, since spectra with very similar

states of immobilization were obtained when 12NS was gently agitated in solution with bovine serum albumin (Sigma), lysine-rich histone (Sigma), argenine-rich histone (Sigma), crude snake venom, and Carnation low-fat milk solids. Lyophilizing the structural protein-12NS preparation gave a signal even more restricted in motion (Fig. 5). No signal of this type was observed in the mitochondrial preparations (see Discussion).

Molecular Environment.

The coupling constant (A^N) is used as a measure of the polarity of the local environment of a defined nitroxide radical (Fig. 1). This value has been shown to vary with dielectric constant. For example a small, much used nitroxide--2, 2, 6, 6-tetramethyl-1-oxylpiperdinol (TEMPOL)--has A^N = 17.1 gauss in H_2O at 25° and A^N = 15.2 gauss in hexane. Since A^N values can easily be measured, it is useful in studying heterogeneous systems such as membranes to determine the polarity of the local environment of the nitroxide. 12NS has an A^N of 14.2 gauss in octadecane and 15.7 gauss in 20% ethanol; therefore, it gives the same general response to solvent polarity as other nitroxides (Table 1). A structurally closely related spin label, 2-nitroxide propane, which is soluble in either octadecane or water, gives A^N values of 14.2 gauss in octadecane and 16.1 gauss in water.

The A^N values for the mitochondrial and other preparations shown in Table 1 must be considered as approximate, since the hyperfine lines of the ESR signal were broadened to the extent that they were somewhat overlapping. The A^N values were taken at 60° C on most samples to minimize this overlap, and it was necessary to heat the dried structural protein sample to 150° C to achieve a signal in which

an A^N could be measured.

All the preparations were measured as having an A^N of 14.2 gauss (the same as in octadecane) except the extracted spin-labeled phospholipid resuspended in water, which was 14.5 gauss (Table 1). A value of 14.5 gauss indicates either an interface area or a hydrocarbon zone that does not have total water exclusion. The observation that a hydrocarbon coupling occurred even when 12NS was associated with structural protein may at first be somewhat surprising. Still, it seems plausible that a protein of 20,000–60,000 molecular weight might have at least one hydrocarbon-rich channel capable of cradling a fatty acid molecule.

Destruction of Signal.

Nitroxides have a certain heat lability that is dependent upon the solvent properties. For example, 12NS in octadecane is stable for half an hour at 90° C without detectable loss of signal, and for months in most organic solvents or in water at room temperature and neutral pH. Nitroxides demonstrate a general sensitivity to acid pH but are quite stable in basic media.

Although we have not precisely quantified the rate at which Neurospora destroys the signal from 12NS during growth, it is extensively destroyed over a period of several hours. Also, if the freshly extracted mitochondria are left standing at room temperature for several hours or stored in the refrigerator for 2 days no signal remains. When the mitochondria were advanced through a temperature range, detectable destruction occurred at 50-60° C, and at 70° C about half the signal

was destroyed in 5-10 minutes. This was most obvious in the in vivo labeled preparations, but was also true in the in vitro ones. No noticeable destruction occurred in the lyophilized or air-dried mitochondria at these temperatures. Furthermore, no loss of signal was observed in either lipid preparation or structural protein preparation. The addition of Nystatin (10^2 units/ml), antimycin a (10^{-3}M), and sodium azide (10^{-2}M) to the mitochondrial preparations reduced or prevented loss of signal in the same temperature range in which destruction was otherwise observed. These preliminary observations indicated that the signal destruction may be enzymic in nature (see Discussion).

The addition of 10 molar equivalents of reduced vitamin C caused loss of signal in all aqueous samples, except the structural protein preparation, in less than 5 min at room temperature (the concentration of 12NS in the in vivo labeled mitochondria was estimated by comparing signal intensities). Vitamin C (10 equivalents) caused loss of signal in aqueous environments or aqueous dispersions of phospholipids; however, extraction of the spin label into organic solvent restored the signal. Spin label treated in this manner could be restored to fresh aqueous medium with no loss of signal. On the other hand, similar extraction of the mitochondria after biological destruction of spin yielded no restoration of signal. Therefore, the loss of signal in mitochondrial preparations was probably by a mechanism different from that for vitamin C destruction.

DISCUSSION

Most traditional methods for studying membranes present a time-averaged or static image. Spin labels, in contrast, are ideal for obtaining quantitative information about molecular mobility. Currently, relatively accurate rotational correlation times (τ_c) can be measured between 10^{-8} and 10^{-10} sec; when adequate theory or computer-simulated spectra (or both) are developed this can be extended to 10^{-7}-10^{-10} sec. Although these and other measurements from spin labels can be quantified there remains a valid criticism in using these values in membrane studies. For, at best, the organism will accept a nitroxide-containing molecule as an intruder. 12NS is acylated by Neurospora into complex lipids (mostly phospholipids)(4) and we have made the assumption that these nitroxide-containing structures are not treated with extensive prejudice by the organism. If our assumption is true then these observations reflect the environment and behavior of a native constituent fatty acid.

Motion.

The freedom of motion in Neurospora mitochondria as viewed by spin-labeling is consistent with either a "unit membrane" or "subunit" model for membrane structure. The data show the 12NS was in a semiviscous hydrophobic environment. A closer inspection of the data reveals that even though 12NS hydrophobically associates with structural protein *in vitro*, no such binding was observed in the mitochondrial preparations (Figs. 2 and 3). Structural protein bound 12NS tighter and offered far more protection from the effects of vitamin C than the mitochondrial or

phospholipid samples. The 12NS in the structural protein–12NS sample was about 1% of the protein weight; consequently, it was not typical of the lipid: protein ratio in mitochondria. Nonetheless, if similar lipid–protein associations were present in the mitochondria then this immobolized signal would have remained after vitamin C had destroyed the nonprotein associated signal. No such signal was observed even when the spectrometer sensitivity was increased as much as 100 times following vitamin C treatment.

Fig. 6b shows that the τ_c values for the in vivo preparation were much closer to the phospholipid values but still had some "protein" influence, since the τ_c values were always between the phospholipid and structural protein–12NS preparations. It may also be noted that the in vitro incorporated 12NS resulted in spectra almost identical to that of the phospholipid preparation, indicating that under in vitro conditions 12NS is dissolved in phospholipid-rich zones. Therefore there may be some additional structural limitations on fatty acids that are in vivo incorporated. If 12NS accurately reflected the environment of the hydrocarbon portions of fatty acid–containing structures, then there probably is no (or very little) hydrophobic association between the lipid and protein elements of these membranes.

Environment.

The coupling constant (A^N) data (Table 1) suggest the 12NS was localized in regions of water exclusion in all the mitochondrial preparations, yet vitamin C at about 10 molar equivalents completely destroyed the signal. Therefore, even though 12NS was in a hydrophobic environment

it was still accessible to vitamin C. The explanation of this is not clear, but we imagine that some type of exchange event could be responsible for the ability of vitamin C, a water-soluble compound, to destroy a signal mediated from a hydrocarbon environment. Even more surprising is the observation that 10 equivalents of vitamin C resulted in no detectable destruction of the signal arising from the structural protein–12NS aqueous preparation, even in 48 hours at room temperature, indicating that this protein has at least one hydrocarbon-lined channel in which to cradle a fatty-acid molecule. This association must envelop the fatty acid with hydrocarbon-rich groups so tightly that vitamin C is excluded and the fatty acid is unable to move independently. If the assumption is made that 12NS is rigidly fixed to the protein structure then it is possible to estimate the radius (r) of the rotating particle by using Stokes' equation for viscosity,

$$\tau_c = \frac{4\pi\eta r^3}{3kT} , \qquad (3)$$

where η is the viscosity of water, k is Boltzmann's constant, and T is the temperature in °K. Using the value of τ_c for the structural protein–12NS preparation at 60° C, one can calculate the radius to be about 22.3 Å. By assuming a spherical particle with a density (ρ) of 1.2 g/cm^3 an estimate of the molecular weight of the structural protein–12NS complex can be made. This is given by

$$MW = 0.8\pi\rho r^3. \qquad (4)$$

This yields a protein with a molecular weight of about 33,400, which seems to be a reasonable value. The motion observed in the structural protein–12NS preparation can thus be accounted for by the motion of the

aggregate, and need not be accounted for by independent motion of 12NS relative to the structural protein molecule.

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TABLE 1

NITROGEN HYPERFINE COUPLINGS FOR 12NS AND 2NP IN VARIOUS ENVIRONMENTS

Spin Label	Matrix	A^N	Temp. (°C)
12NS	octadecane	14.2	25
12NS	octadecane	14.2	60
2NP	octadecane	14.2	25
2NP	water	16.1	25
12NS	20% Ethanol	15.7	25
12NS	Mitochondria in vivo (H_2O)	14.2	60
12NS	Mitochondria in vitro (H_2O)	14.2	60
12NS	Mitochondria in vivo (lyoph.)	14.2	60
12NS	Mitochondria in vitro (lyoph.)	14.2	60
12NS	Phospholipid (H_2O)	14.5	60
12NS	Structural protein (H_2O)	14.2	80
12NS	Structural protein (dry)	14.2	150

FIGURE LEGENDS

Fig. 1. First-derivative ESR absorption of 2NP in different environments, showing the effects of medium polarity and viscosity on the spectra.

Fig. 2. Effect of temperature on the ESR spectra of Neurospora mitochondria spin-labeled *in vivo*.

Fig. 3. Effect of temperature on the ESR spectra of Neurospora mitochondria spin-labeled *in vitro*.

Fig. 4. Effect of temperature on the mobility of 12NS in Neurospora total lipids (dried) and an aqueous suspension of Neurospora phospholipids.

Fig. 5. Effect of temperature on the mobility of 12NS in mitochondrial structural protein from Neurospora.

Fig. 6. Rotational correlation times as a function of temperature for various spin-labeled mitochondrial preparations and extracts.

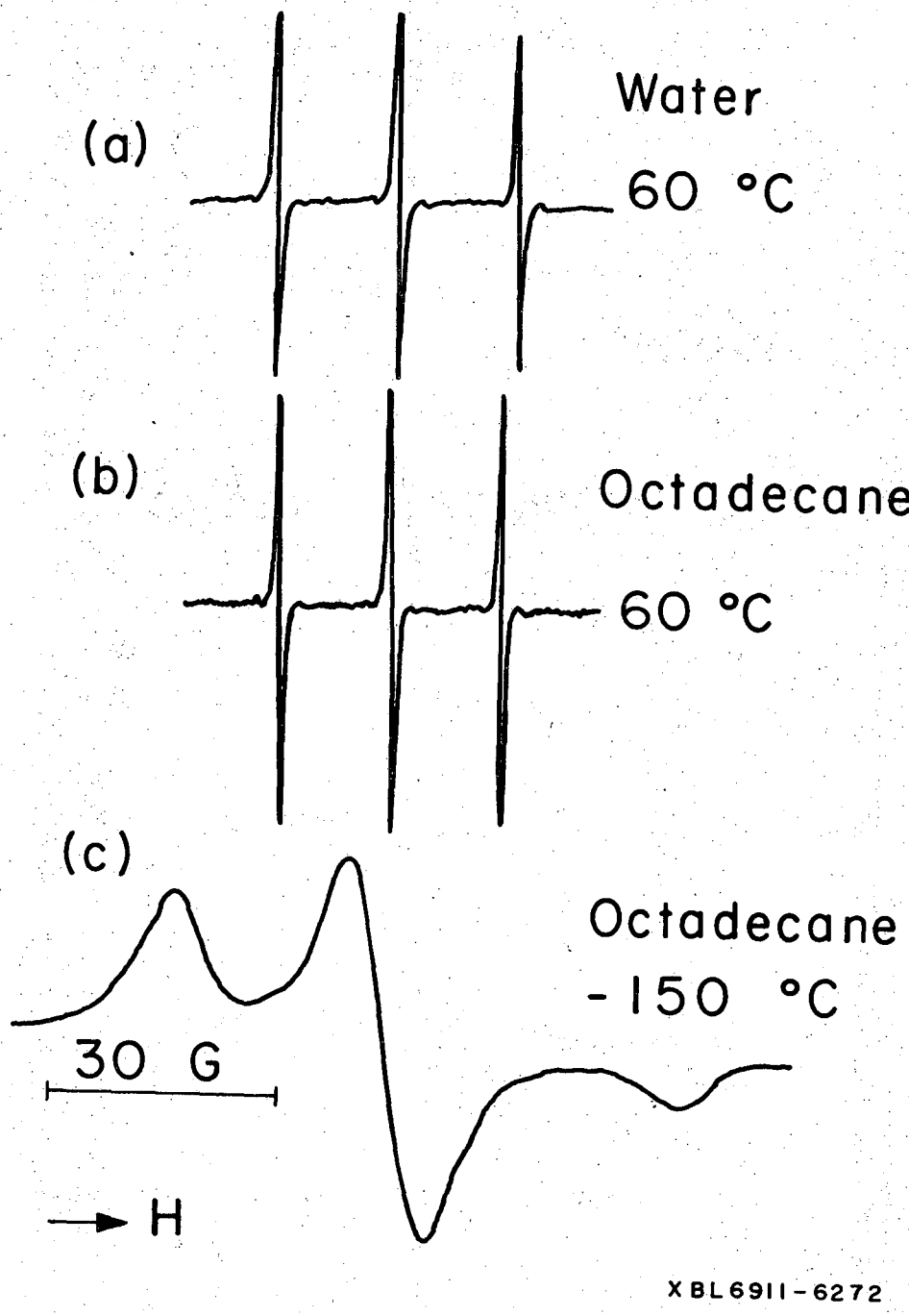
(a)
Water
60 °C
(b)
Octadecane
60 °C
(c)
Octadecane
-150 °C
30 G
H
XBL6911-6272

Fig. 1

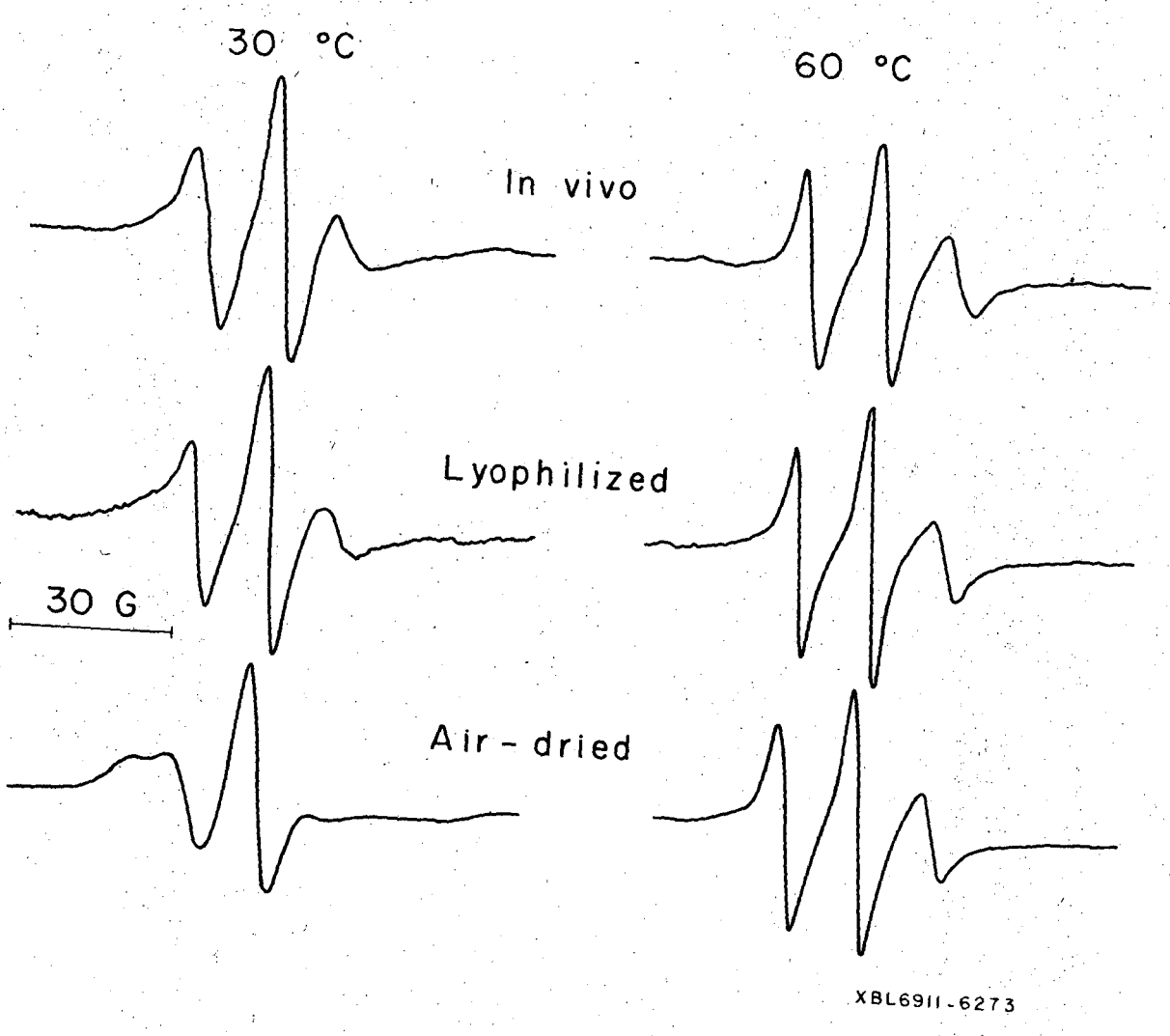

Fig. 2

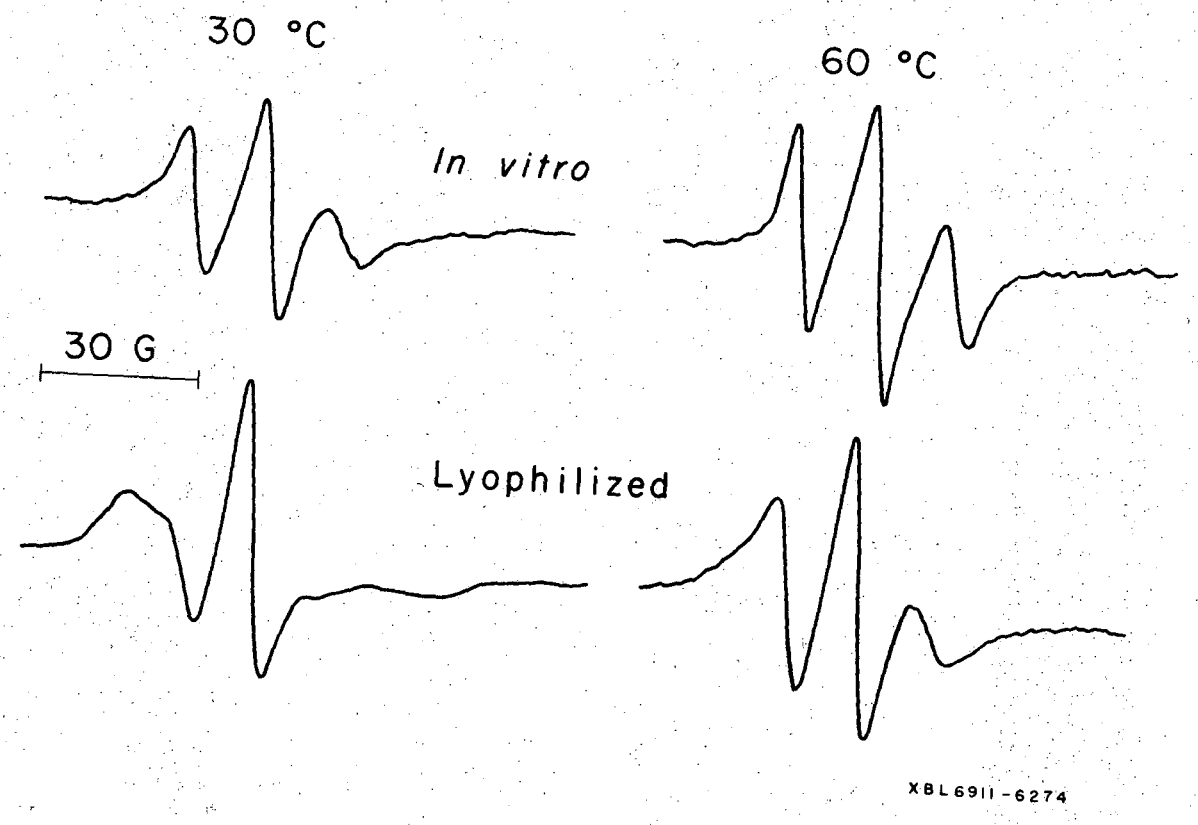

Fig. 3

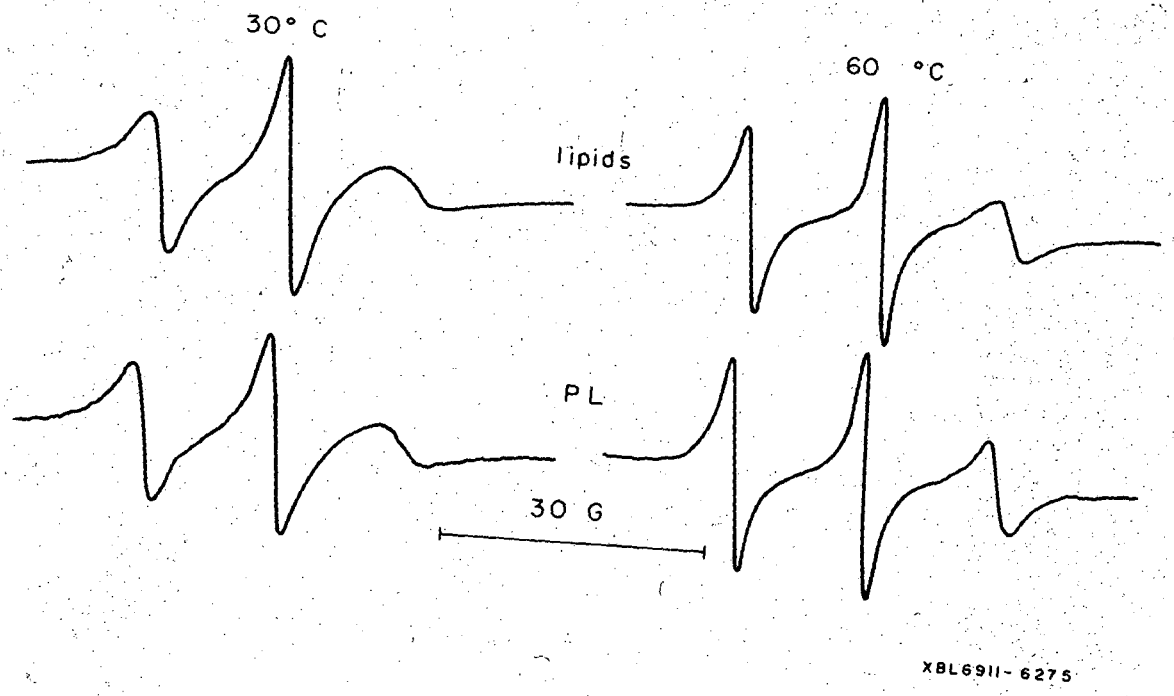

Fig. 4

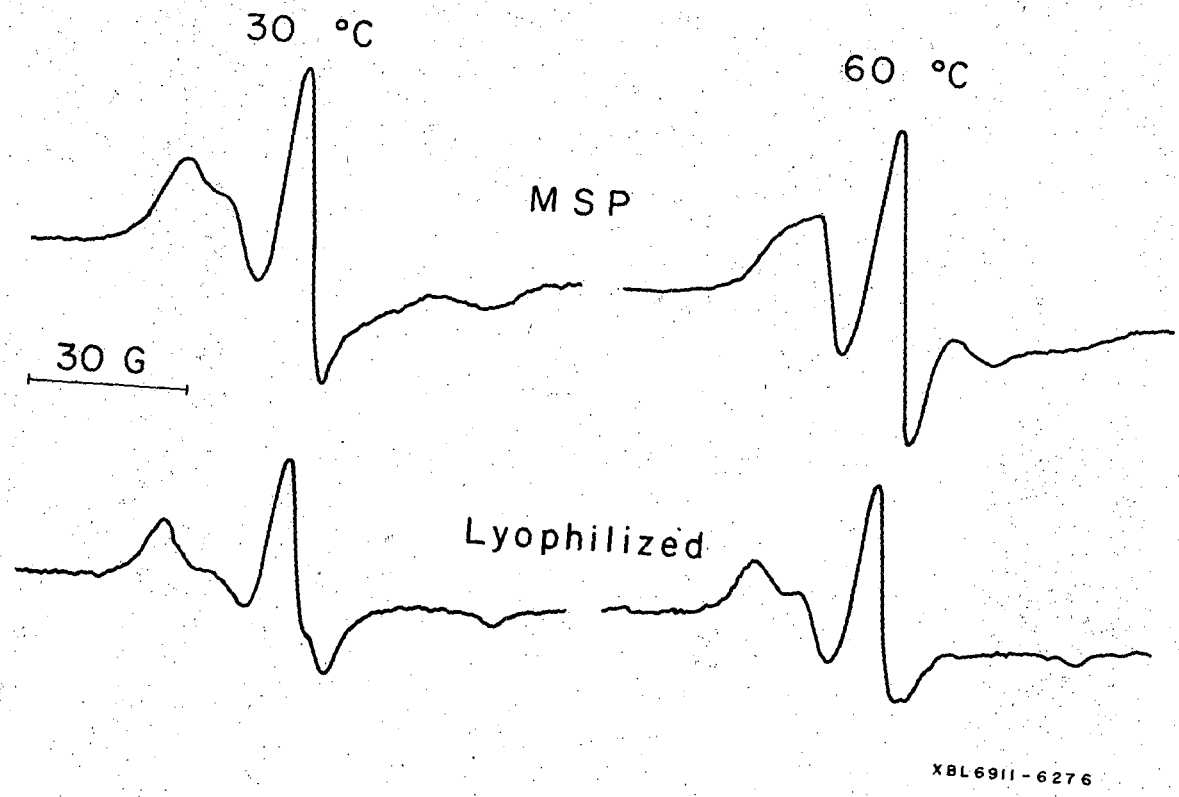

Fig. 5

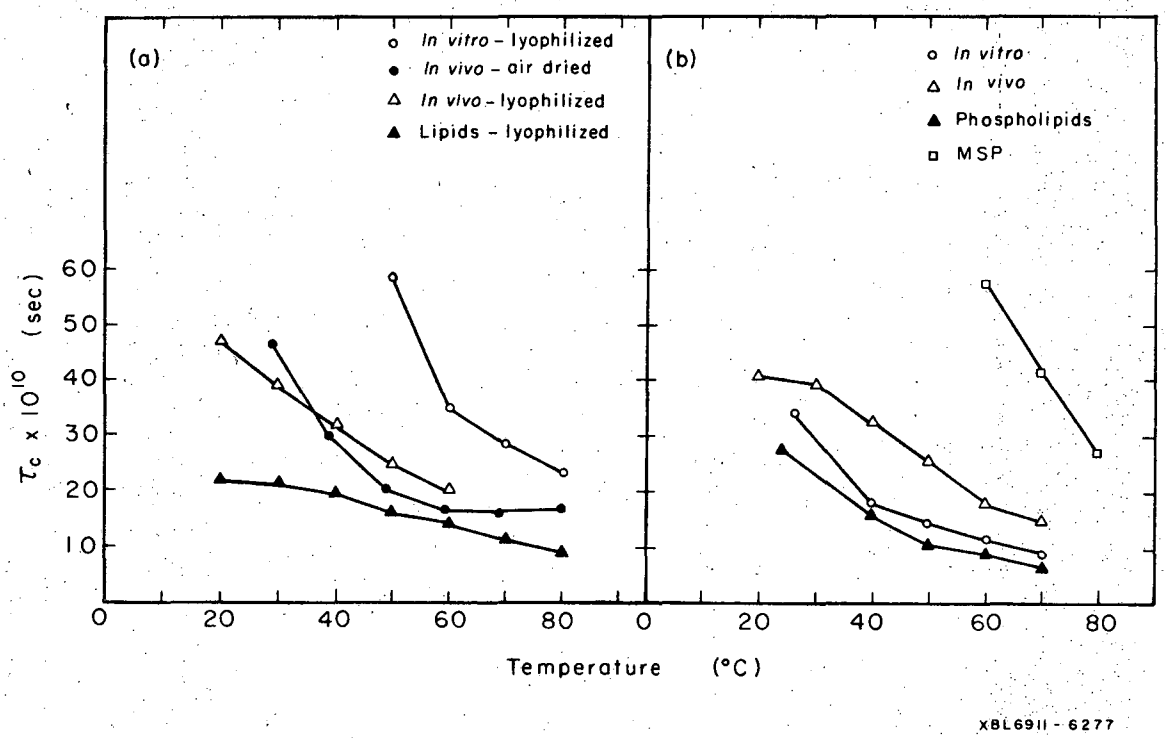
(a)
In vitro – lyophilized
In vivo – air dried
In vivo – lyophilized
Lipids – lyophilized
(b)
In vitro
In vivo
Phospholipids
MSP
$\tau_c \times 10^{10}$ (sec)
60
50
40
30
20
10
0
20
40
60
80
Temperature (°C)
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Fig. 6

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