SPIN-LABELED *NEUROSPORA* MITOCHONDRIA

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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 bovine serum albumin. These comparisons suggest that the hydrocarbon portions of membranes are relatively fluid and are not extensively restricted in motion by association with proteins.

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 references 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 system 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 (see references 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 (see references 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

1500 ml of *Neurospora* minimal medium, supplemented with 0.25% yeast extract and 1% Tergitol (Union Carbide NP-40 [Union Carbide Corp., Chemicals Div., New York]), was incubated with 7×10^5 conidia per ml. 50 mg of 12-nitroxide stearate (12NS) in 1 ml of ethanol was added at zero time. *Neurospora* was grown for 16 hr 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;[1] 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 1 min 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 mitochondrial preparation was not contaminated by other cell fractions.[1]

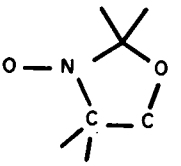
The in vitro spin labeling was carried out by adding 12NS (stock solution 10^{-1} M in ethanol) directly to mitochondrial suspensions containing 20–25 mg of mitochondrial protein as determined by the method of Lowry (10). The mitochondrial suspensions were gently agitated and were then immediately used for ESR analysis. The solubilized bovine serum albumin (BSA) preparation was spin labeled in the same way (crystallized [Sigma Chemical Co., St. Louis, Mo.]). The in vivo labeled mitochondria were stored at 0°C until analyzed. This was always as quickly as possible (within 1–4 hr) except with dehydrated preparations. All aqueous preparations were buffered with 0.01 M *N*-2-hydroxyethyl piperazine-*N'*-2-ethylsulfonic acid at pH 7.4 in 0.5 M sucrose. Air drying was carried out at room temperature for 48 hr and lyophilization was at the temperature of dry ice for 48 hr. All dried preparations were sealed under vacuum in ESR tubes for analysis. No destruction of signal was observed in any of these samples over a several week period.

The Folch (11) solvent system was used in lipid extractions. Mitochondria were placed in chloroform:methanol (2:1) and stirred over night. After going through the Folch washing procedure, the total lipid extract was dried under vacuum, redissolved in chloroform, and streaked on preparative thin-layer plates of predeveloped Silica Gel-G (Merck & Co., Rahway, N. J.) on glass. Phospholipids and nonphospholipids were separated by developing the plate in diethyl ether. Under these conditions nonphospholipids migrate with the solvent front. The location of lipid zones was detected by fluorescence and was verified by charring

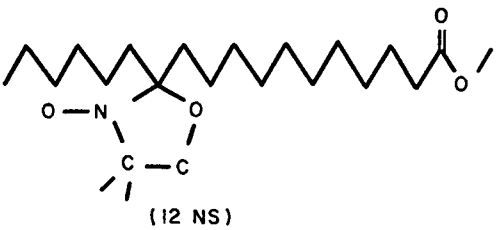
[1] Bulfield, G., and A. D. Keith. Manuscript in preparation.

a sample plate with sulfuric acid. The neutral lipids were collected by scraping off the appropriate zone followed by elution through sintered glass funnels with diethyl ether and removal of the solvent by vacuum. The origin was scraped off and exhaustively eluted with methanol through sintered glass funnels covered with one-half inch of anhydrous sodium sulfate powder to prevent Silica Gel-G leakage through the funnel. This eluate was dried under vacuum and then divided into two parts. One part was for direct ESR analysis and the other part was restreaked onto another thin layer plate. This time the activated plate was developed in chloroform:methanol:water (65:25:4). The zones corresponding to phosphatidyl choline and phosphatidyl ethanolamine were scraped off together, eluted, and dried. These three lipid fractions were dissolved in measured amounts of chloroform and were prepared for ESR in 10-λ microtubules. This general procedure has been carried out some 12 times during the past several months in our laboratory.

The synthesis, general chemical properties, and biological use of 12NS are described elsewhere (12). The same synthetic procedure, with acetone as the starting material, was used for 2-nitroxide propane (2NP).



(2NP)



(12 NS)

A Varian X-band electron paramagnetic resonance spectrometer (Varian, Palo Alto, Calif.) 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 (13) and later, in more detail,

by Kivelson (14). One form of these equations is

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

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, Cornell, and McConnell (15) for b and $\Delta\gamma$ are used and H is maintained at 3400 gauss, this expression reduces to

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

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. The expressions based on line broadening presented by Kivelson (14) assume isotropic motion. Where the ESR spectra reflect partial immobilization and the axial ratio of the spin label is not equal to one, as in most spectra presented in this report, the net motion is almost certainly somewhat anisotropic and therefore results in inaccuracies in τ_c calculations. Consequently, 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.

Neurospora Spin-Labeled Lipids.

Of the several lipid analyses (12 total) dealing with the distribution of 12NS in *Neurospora* mitochondria (including those given in reference 4), the following percentages were arrived at. Spin in phospholipids (total polar lipids) averaged 58% of the total. The remaining percentage was in neutral lipids (not analyzed further except that their thin-layer chromatographic (TLC) behavior was different from that of the methyl ester of 12NS). From 75 to 89% of the spin in the total polar lipids could be accounted for as migrating on TLC plates with the phosphatidyl choline and phosphatidyl ethanolamine fractions. Basic hydrolysis of these two phospholipid classes yielded only one spin-labeled product which gave identical TLC behavior to the acid of 12NS.

Organism Growth.

The same apparent growth rates were achieved in the presence of 12NS as in its absence; however, precise growth measurements were not carried out. When 12NS is present, within about 10% the same number of grams of hyphae result from 7×10^6 conidia per ml in 16 hr of growth in 1500 ml of medium. Since *Neurospora*

only grows aerobically and is dependent upon mitochondrial function for energy metabolism, it was reasoned that 12NS did not seriously impair mitochondrial function.

Molecular Motion.

Fig. 1 shows examples of ESR spectra that reflect different states of molecular motion. Fig. 1 *a* gives a typical spectrum of quite rapid motion in water and Fig. 1 *b* 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. 1 *a* is considerably greater than that for Fig. 1 *b*. This reflects the polarity

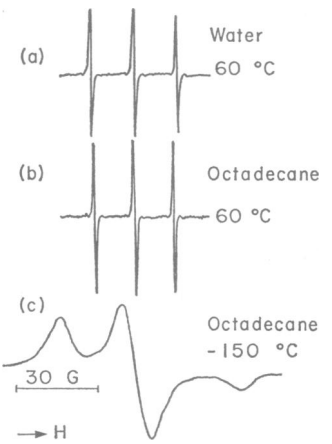


FIGURE 1 First-derivative ESR absorption of 2NP in different environments, showing the effects of medium polarity and viscosity on the spectra. G: gauss.

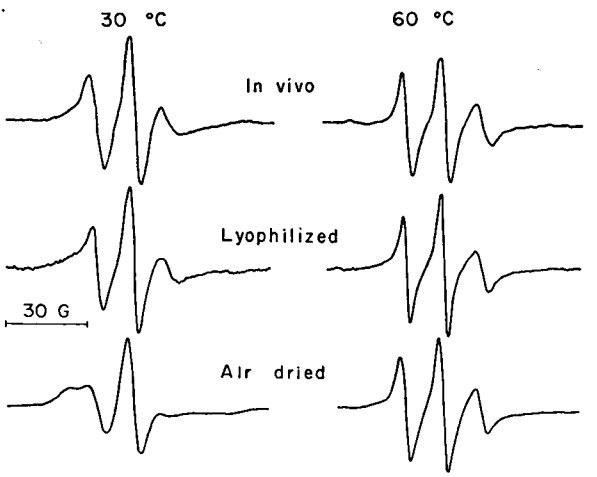


FIGURE 2 Effect of temperature on the ESR spectra of *Neurospora* mitochondria spin labeled in vivo.

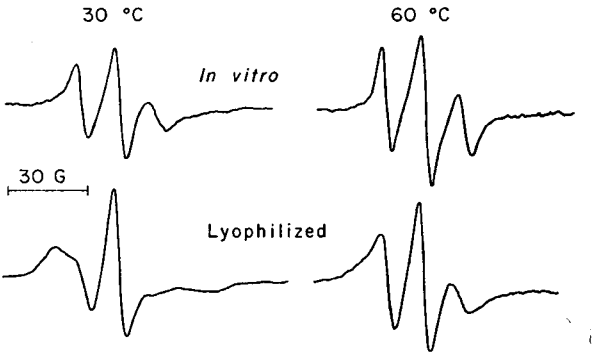


FIGURE 3 Effect of temperature on the ESR spectra of *Neurospora* mitochondria spin labeled in vitro.

of the local environment of the nitroxide spin label. Fig. 1 *c* 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 Figs. 1 *a* and *c*. 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-labeled 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

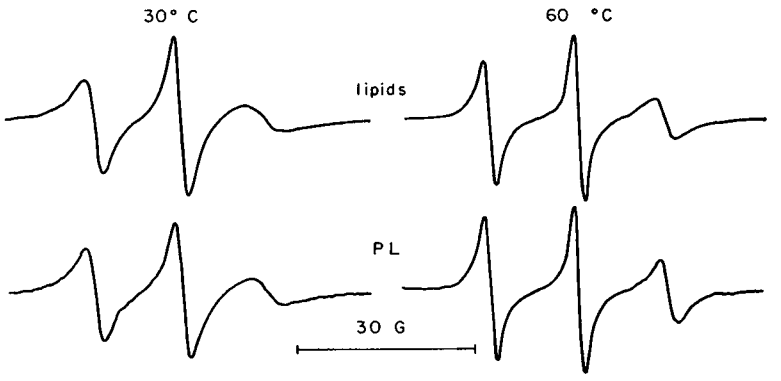


FIGURE 4 Effects of temperature on the mobility of 12NS in *Neurospora* total lipids (dried) and an aqueous suspension of *Neurospora* phospholipids (PL).

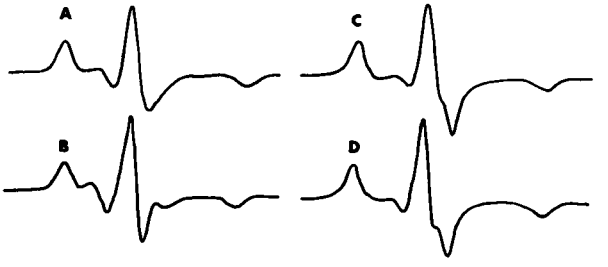


FIGURE 5 Effect of temperature on the mobility of 12NS bound to bovine serum albumin. (A) BSA at 25 mg-ml in solution at 30°C; (B) same as (A) except at 60°C; (C) BSA lyophilized at 30°; (D) the same as (C) except at 60°C.

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 motion of spin-labeled molecules in the mitochondrial lipids (Fig. 4).

12NS bound tightly to BSA, and yielded a signal considerably more restricted in molecular motion than the mitochondrial preparation (Fig. 5). It is well known that BSA binds fatty acids (16) although no data has been published indicating the tightness of binding in terms of molecular motion. However, it must be emphasized that this general type of ESR signal is not unique, since spectra with very similar states of immobilization were obtained when 12NS was gently agitated in solution with lysine-rich histone (Sigma Chemical Co.), arginine-rich histone (Sigma Chemical Co.), crude snake venom, and Carnation low-fat milk solids

(Carnation Co., Los Angeles, Calif.). Lyophilizing the BSA:12NS preparation gave a signal at least as 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-l-oxylpiperidinol (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 (having both polar and nonpolar zones) 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 I). 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 I 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.

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 I). A value of 14.5 gauss indicates either an interface area or a hydrocarbon zone that does not have total water exclusion.

TABLE I

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

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 min. 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 the BSA preparation. The addition of Nystatin (10^2 units/ml) (Grand Island Biological Co., Grand Island, N.Y.), 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 in dicated 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 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 (more than half in phospholipids)([4] and Results) 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.

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 that 12NS was in a semiviscous hydrophobic environment. A closer inspection of the data reveals that even though 12NS hydrophobically associates with BSA in vitro, no such binding was observed in the mitochondrial preparations (Figs. 2 and 3). We feel that the tight binding of 12NS to isolated BSA (and several other proteins) shows the effect on molecular motion imposed by tight protein binding to lipids. Since this type of immobilization is not observed in *Neurospora* mitochondria, this type of binding also must not occur to any marked extent in the mitochondrial membranes. Membrane proteins are components of complex aggregates and may have these "tight" hydrophobic binding sites occupied by other groups than lipids in native membranes (or 12NS may be specifically excluded); consequently, it is possible that lipids may still associate with proteins in membranes and be allowed more independent motion than we observe in the in vitro binding of 12NS to isolated proteins. The 12NS in the BSA, 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 the immobilized part of the signal would have been visible. No such signal components were observed even when the spectrometer sensitivity was increased as much as 10 times.

Fig. 6 shows the way τ_c changes with temperature in the various preparations. Probably the major point is that the mitochondrial preparations are always in between the lipid and BSA, 12NS preparations although closer to that of the lipids. Since the in vivo and in vivo lyophilized preparations were almost identical, the aqueous bath of the mitochondria must have very little influence on the motional state of the lipids. The phospholipid dispersion is also very similar in τ_c vs. temperature to that of the dried total lipids, again indicating that the aqueous phase had little influence on motion.

The coupling constant (A^N) data (Table I) suggest that 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.

If the assumption is made that 12NS is rigidly fixed to a protein structure, then

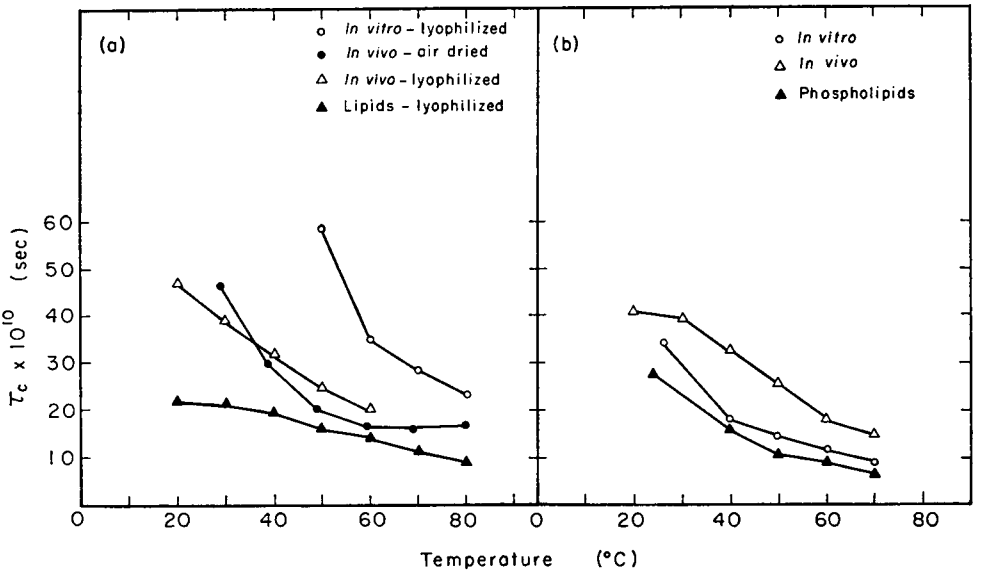


FIGURE 6 Rotational correlation times as a function of temperature for various spin-labeled mitochondrial preparations and extracts.

it is possible to estimate the radius (r) of the rotating particle by using Stokes' equation for viscosity,

$$\tau_c = \frac{4\pi\eta r^3}{3kT}, \tag{3}$$

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 BSA:12NS preparation at 60°C, one can calculate the radius to be about 20.5 A. An estimate of τ_c was arrived at by comparison with computer simulations of spectra yielding the same line shapes (17, 1). By assuming a spherical particle with a density (ρ) of 1.2 g/cm^3, an estimate of the molecular weight of the BSA:12NS complex can be made. This is given by

$$\text{mol wt} = 0.8\,\pi\rho r^3, \tag{4}$$

and yields a protein with a molecular weight of about 26,000 which is less than the reported value of 66,500. Nonetheless, this reflects the motion of a particle much larger than can be accounted for in terms of independent motion of 12NS. The motion observed in the BSA:12NS preparation can thus be accounted for by the motion of an aggregate, and need not be accounted for by independent motion of 12NS relative to the protein molecule.

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