Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3412–3415, November 1972

Existence of Phospholipid Bilayer Structure in the Inner Membrane of Mitochondria

(spin label/multibilayers/liposomes)

J. C. HSIA, W. L. CHEN, R. A. LONG, L. T. WONG, AND W. KALOW

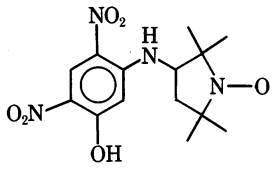
Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto 181, Ontario, Canada

Communicated by David E. Green, September 11, 1972

ABSTRACT **The presence of ordered phospholipid lamellar structure in inner membranes of mitochondria was detected with the use of a spin-labeled 2,4-dinitrophenol. A phospholipid bilayer may be an important structural and functional component of the inner mitochondrial membrane.**

Current research on the mechanism of energy conservation in mitochondria has focused on the structural relationship of the lipid and protein components within the inner mitochondrial membrane (1, 2). While a great deal of information is available concerning the structure and function of individual membrane components, a detailed knowledge of lipid–protein interactions and the structural organization of the membrane is generally lacking. In recent years, several models have been proposed for the structure of the mitochondrial membrane (3–8). The major difference among these models, and indeed an important unsolved question, is concerned with the actual location and arrangement of phospholipid within the repeating units (9).

In this communication data are presented showing that a spin-labeled 2,4-dinitrophenol (Dnp) is localized in the polar



Dnp-SL(5)

head-group regions of phospholipid bilayers and exhibits rapid anisotropic motion. Similar motion of the spin-labeled 2,4-dinitrophenol in intact mitochondria and the inner mitochondrial membrane was detected. These results suggest the existence of phospholipid bilayer structure in the inner membrane of mitochondria.

MATERIALS AND METHODS

Materials. Spin-labeled 2,4-dinitrophenol[1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-amino-pyrrolidinyl)-2,4-dinitrobenzene: Dnp-SL(5)] was prepared as described (10). The pK of Dnp-SL(5) was 4.8 (10). The lipids were obtained from the following sources: egg phosphatidylcholine (Pierce Chemical Co.); phosphatidylethanolamine, phosphatidylinositol, and cardiolipin (Serdary Research Laboratories, London, Ontario).

Methods. Rat-liver mitochondria were isolated by the method of Hagihara (11), and the inner membrane of mitochondria was prepared by the procedure of Parsons *et al.* (12). Oxygen uptake was studied to measure the uncoupling activity of Dnp-SL(5) in intact mitochondria. It was found that Dnp-SL(5) possesses about the same activity as Dnp in the uncoupling of oxidative phosphorylation in rat-liver mitochondria (10). "Lipid-depleted" mitochondria were prepared by the method of Fleischer by extraction of mitochondria with a solution of 10% water in acetone containing ammonia (0.003% w/v) (13).

Planar phospholipid multibilayers containing Dnp-SL(5) (phospholipid to spin label ratio of 150:1) were prepared on the inner surfaces of a flat quartz cell, dimensions 4 cm × 1 cm × 0.25 mm (14). The multibilayers were hydrated with isotonic phosphate buffer (0.44% NaCl in 67 mM phosphate buffer, pH 7.4). Liposomes were prepared by hand dispersion of a dry film of phospholipid and Dnp-SL(5) in isotonic phosphate buffer (pH 7.4) to final concentrations of 15 mM and 50 μM, respectively. Mitochondrial membranes were spin-labeled by the following procedure. All steps were done at 0–4° unless stated otherwise. Isolated mitochondria, inner mitochondrial membranes, or "lipid-depleted" mitochondria were suspended in an ice-cold medium containing 10 mM phosphate buffer pH 7.4, 50 mM Tris·HCl buffer pH 7.4, 50 mM KCl, 50 mM $MgSO_4$, and 0.15 M sucrose, to a concentration of 15–20 mg of protein per ml, and were mixed with a solution of 0.9% NaCl containing Dnp-SL(5) (final spin-label concentration of 50 μM). The mixture was centrifuged at 18,000 × *g* for 10 min. The mitochondrial pellet was taken up in a 50-μl disposable glass pipette and immediately used for electron spin resonance (ESR) spectral measurements at 22°. All spectra were recorded on a Varian X-band E-6 ESR spectrometer.

RESULTS AND DISCUSSION

Resonance spectra of Dnp-SL(5) in model membranes

The orientation and motional characteristics of Dnp-SL(5) in membranes can be best understood from the resonance spectra of the label in planar phospholipid multibilayers (14–17), where the plane of the bilayers can be rotated with respect to the laboratory magnetic field. Analysis of ESR spectra of spin labels in oriented multibilayers or phospholipid dispersions or both has been discussed by several authors (14–

Abbreviation: Dnp-SL(5), spin-labeled 2,4-dinitrophenol.

20). For the special case of rapid anisotropic motion of a label about some axis, the hyperfine tensor components of the nitroxide will be preferentially time averaged. The magnitude of the hyperfine splitting parallel and perpendicular to the symmetry axis is designated $T'_{||}$ and $T'_{\perp}$. These two parameters may be related to an order parameter S_3 (18, 19).

$$S_3 = (T'_{||} - T'_{\perp})/(T_{zz} - T_{xx})$$

where T_{zz} and T_{xx} are the hyperfine tensor components of the nitroxide calculated from $T'_{||}$ and $T'_{\perp}$ as described by Seelig (18). S_3 is a measure of the degree of alignment of the z axis of the nitroxide with the symmetry axis of motion.

The spectra of Dnp-SL(5) in hydrated phosphatidylcholine multibilayers (excess buffer phase removed) are shown in Fig. 1A. The maximum (minimum) hyperfine splitting $T'_{||}$ ($T'_{\perp}$) was observed when the normal of the planar bilayers was parallel (perpendicular) to the magnetic field direction. The values of $T'_{||}$ and $T'_{\perp}$ obtained from spectra of Dnp-SL(5) were 23.0 and 10.9 gauss, respectively. The derived order parameter (S_3 = 0.45) indicates a preferential alignment of the z axis of the nitroxide parallel to the normal of the bilayers. Similar resonance spectra were obtained with multibilayers of phosphatidylethanolamine, phosphatidylinositol, or cardiolipin; the data are summarized in Table 1.

The resonance spectrum of Dnp-SL(5) in egg phosphatidylcholine liposomes is shown in Fig. 1B. The separation of the outer hyperfine extrema is a good measure of $2T'_{||}$ according to Hubbell and McConnell (19), and the magnitude of $T'_{\perp}$ may be estimated by separation of the inner hyperfine extrema. The hyperfine splittings of Dnp-SL(5) in the liposomes at pH 7.4 are $T'_{||}$ = 23.0 gauss and $T'_{\perp}$ = 10.4 gauss. A correction factor of +0.5 gauss was applied to the values for $T'_{\perp}$ from dispersed vesicular membranes in order to correlate the order parameters to that obtained from the well-defined planar model membrane system (Table 1). The large order parameter ($S_3 \simeq 0.45$) of Dnp-SL(5) in phosphatidylcholine bilayers suggests that the spin label is probably located near the highly ordered polar head-group region rather than the fluid hydrocarbon interior of the bilayer (17, 19). The polar nature of the spin label's environment is substantiated by the magnitude of the isotropic splitting constants (Table 1) as calculated from the relation $a'_N = 1/3\,(T'_{||} + 2T'_{\perp})$ (18).

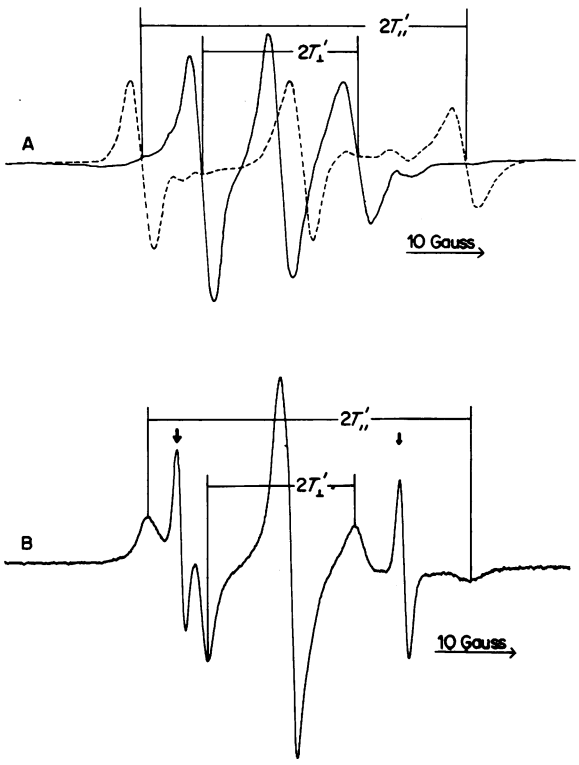


FIG. 1. (A) ESR spectra of Dnp-SL(5) in hydrated planar phosphatidylcholine multibilayers, after draining the aqueous phase that effectively removes the isotropic triplet due to the partitioning of Dnp-SL(5) into the buffer phase seen in (B). Spectra were recorded with the normal of the plane of the bilayers parallel (– – –) and perpendicular (——) to the applied magnetic field. (B) ESR spectra of Dnp-SL(5) in an aqueous dispersion of phosphatidylcholine. The two sharp lines indicated by *arrows* arise from labels present in the aqueous phase.

TABLE 1. *ESR spectral parameters of Dnp-SL(5) in model and mitochondrial membranes at pH 7.4*

Type of membrane	$T'_{||}$	$T'_{\perp}$	S_3	a_N'
Planar phosphatidylcholine bilayers	23.0	10.9	0.45	14.9
Phosphatidylcholine liposomes	23.0	10.9*	0.45	14.9
Planar phosphatidylethanolamine bilayers	22.3	12.0	0.38	15.4
Planar cardiolipin bilayers	21.8	13.1	0.31	16.0
Planar phosphatidylinositol bilayers	22.4	12.7	0.34	15.9
Intact mitochondria	23.6	10.6	0.49	14.9
Inner mitochondrial membrane	24.1	11.0	0.48	15.3

* The measured value of $T'_{\perp}$ is $\simeq$ 0.5 gauss smaller in liposomes than in the planar multibilayers (see text). Thus, values for $T'_{\perp}$ in liposomes and mitochondrial membranes have been arbitrarily increased by 0.5 gauss. Hubbell and McConnell found the correction factor to be about 0.8 gauss for spin-labeled lipids in phospholipid dispersions (19).

Lipids can exist in several different phases (21), and, therefore, it is of interest to determine if the Dnp-SL(5) is sensitive to these structural arrangements. Dnp-SL(5) in phospholipid bilayers (lamellar phase) invariably results in highly anisotropic motion of the label, S_3 ranging from 0.31 to 0.45. In contrast, the ESR spectra of Dnp-SL(5) solubilized in a cardiolipin–Ca^{++} complex (hexagonal phase) (22) or hydrated lipid films of mono- and di-glycerides (micelles) reveal no significant anisotropic motion of the label, $S_3 < 0.01$. These observations therefore imply that the Dnp-SL(5) label can be used to differentiate between various structural arrangements of lipids.

Distribution of Dnp-SL(5) in model membranes

In the previous section we have accounted for the resonance peaks in Fig. 1B that are due to solubilization of Dnp-SL(5) in the membrane phase. The rapid isotropic tumbling of the label in the bulk aqueous phase results in averaging of the electron and nuclear spin dipolar interaction so that the spectrum consists of three sharp lines of about equal intensity with a hyperfine splitting, a_N = 15.8 gauss. In the presence of membrane phase, only the high- and low-field peaks of the

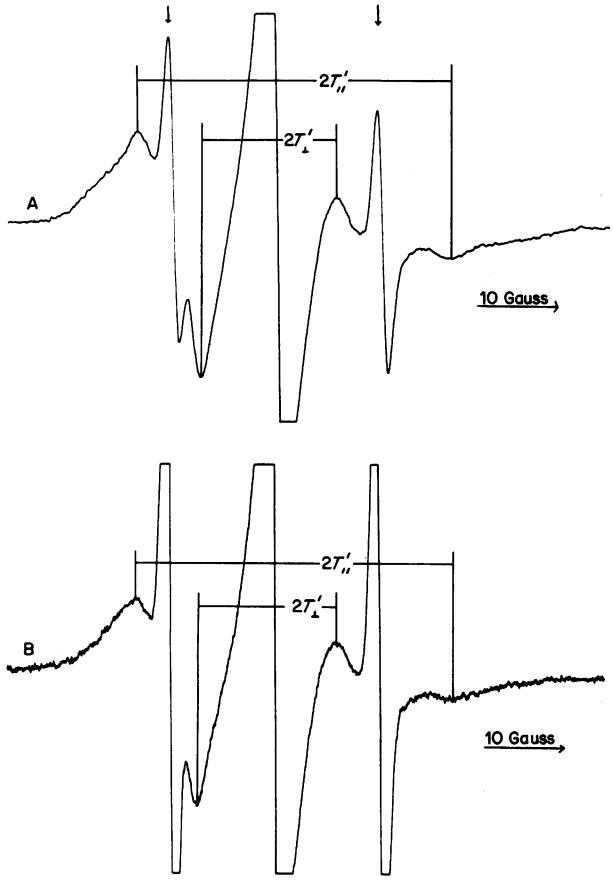


FIG. 2. ESR spectrum of Dnp-SL(5) in the presence of (*A*) intact rat-liver mitochondria and (*B*) inner mitochondrial membrane preparation. The lines indicated by *arrows* are components of the spectrum of those labels that are in the aqueous environment.

isotropic triplet are resolved due to overlap of the center peaks of the membrane-bound and "free" spectra (Fig. 1*B*). Since the high-field line of Dnp-SL(5) in the aqueous phase is distinctly separated from the membrane-bound peaks, we have used its intensity in the presence and absence of membrane phase to estimate the partition coefficient of Dnp-SL(5). The measured partition coefficients for Dnp-SL(5) between phosphatidylcholine and buffer are 500 at pH 7.4 and 2600 at pH 2.2 and 22°. Partition coefficients between other phospholipids and buffer obtained in the same manner are listed in Table 2. These partition coefficients suggest that Dnp-SL(5) is very soluble in the membrane phase. To discern the distribution of Dnp-SL(5) between the polar and apolar regions of the phospholipid bilayers, we measured the partition coefficients of Dnp-SL(5) between hexane and buffer, assuming that the apolar regions of the bilayers have the same hydrophobic characteristics as hexane. The partition coefficients of Dnp-SL(5) between buffer and *n*-hexane were determined from the decrease in ESR signal intensity in the buffer phase after equilibration with equal volume of *n*-hexane. We found partition coefficients of 0.05 at pH 7.4 and 5.6 at pH 2.2 for Dnp-SL(5) between hexane and buffer at 22°. The difference in the partition coefficients in phospholipids and *n*-hexane suggests that at any given time more than 95% of the membrane-soluble Dnp-SL(5) is located in the polar region of the phospholipid bilayers, presumably due to favorable dipole–dipole interactions between Dnp and the polar head groups of the phospholipids.

TABLE 2. *Partition coefficients of Dnp-SL(5) for phospholipid dispersions in isotonic phosphate buffer (pH 7.4) at 22°*

Phospholipid	% of Total phospholipid in inner mitochondrial membrane*	Partition coefficient† (membrane/buffer)
Phosphatidylcholine	41	500
Phosphatidylethanolamine	35	310
Phosphatidylinositol	2	135
Cardiolipin	21	130

* Data from L. Ernster & Kuylenstierna, B. (1970) in *Membranes of Mitochondria and Chloroplasts*, ed. Racker, E. (Academic Press, New York), pp. 172–212.

† The density of each of the lipids was taken as 1 g/cm³.

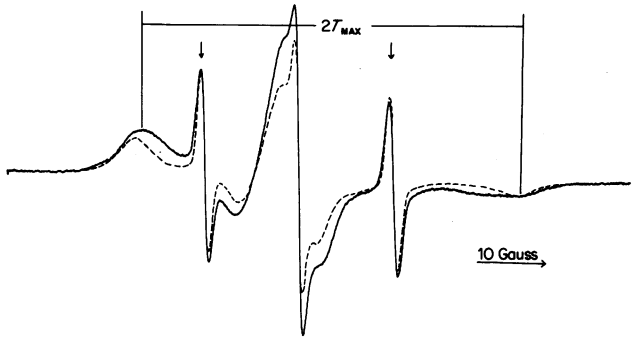


FIG. 3. ESR spectrum of Dnp-SL(5) in the presence of "lipid-depleted" mitochondria (——) and bovine-serum albumin (10 mg/ml) (– – –) in isotonic phosphate buffer pH 7.4. In both cases labels interact with protein(s) and are "strongly immobilized" ($2T_{max} \simeq 64$ gauss). The sharp signals in the spectra indicated by *arrows* are due to Dnp-SL(5) in aqueous solution in equilibrium with bound labels.

Resonance spectra of Dnp-SL(5) in mitochondrial membranes

The membrane-bound spectra of Dnp-SL(5) in intact mitochondria and the inner mitochondrial membrane are shown in Fig. 2*A* and *B*. The spectra closely resemble the spectrum observed in the liposome preparation (Fig. 1*B*), although the outer peaks are somewhat broader. The broader peaks could be a result of a slower rotational frequency of some of the labels in the mitochondrial membrane due to protein binding. The spectral data derived from Figs. 1 and 2 are summarized in Table 1, and they indicate that great similarity exists between the motional characteristics of Dnp-SL(5) in phospholipid bilayers and mitochondrial membranes. The observed similarity in the spectra of spin-labeled 2,4-dinitrophenol in highly ordered phospholipid bilayers and in the inner mitochondrial membrane strongly suggests the presence of a phospholipid bilayer structure in the inner membrane of mitochondria. Phospholipid bilayers may be an important feature of the membrane structure since 94% of all the lipid in the inner mitochondrial membrane is phospholipids (23).

As a further demonstration that the rapid anisotropic motion of Dnp-SL(5) in mitochondrial membranes (Fig. 2) is a consequence of a phospholipid bilayer in the membrane, we investigated the interaction of the label with mitochondrial membrane protein(s) using "lipid–depleted" mitochondria. The mitochondrial protein-bound spectrum (Fig. 3) is significantly different from the spectra obtained with intact mitochondria and the inner mitochondrial membrane (Fig. 2). From Fig. 3, the peak-to-peak separation, $2T_{MAX} \simeq 64$ gauss, indicates that labels interacting with protein(s) are "strongly immobilized", and thus exhibit no detectable anisotropic motion.

The present results are consistent with the view that the inner mitochondrial membrane is basically formed by a phospholipid bilayer, with various transport proteins and the respiratory chain embedded in the bilayer matrix through specific charge and hydrophobic interactions (1,2). We propose that one of the functional roles of the phospholipid bilayer structure is to serve as a diffusion barrier for maintaining the electrical and chemical gradients across the inner mitochondrial membrane.

This research was supported in part by Grant MA-4129 and MRC-DA6 from the Medical Research Council and the Department of National Health and Welfare of Canada.

1. Green, D. E. & Baum, H. (1970) *Energy and the Mitochondrion*, (Academic Press, New York), pp. 1–205.
2. Racker, E. (ed) (1970) *Membranes of Mitochondria and Chloroplasts*, (Van Nostrand Reinhold Co., New York), pp. 127–171.
3. Green, D. E. & Perdue, J. F. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 1295–1302.
4. Benson, A. A. (1966) *J. Amer. Oil Chem. Soc.* **43**, 265–270.
5. Vanderkooi, G. & Green, D. E. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 615–621.
6. Branton, D. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 1048–1056.
7. Vanderkooi, G. & Green, D. E. (1970) *BioScience* **21**, 409–415.
8. Lucy, J. A. (1964) *J. Theor. Biol.* **7**, 360–373.
9. MacLennan, D. H. (1970) in *Current Topics in Membranes and Transport*, eds. Bronner, F. & Kleinzeller, A. (Academic Press, New York), Vol. 1, pp. 177–232.
10. Hsia, J. C., Chen, W. L., Long, R. A., Wong, L. T. & Kalow, W. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1273–1279.
11. Hagihara, B. (1961) *Biochim. Biophys. Acta* **46**, 134–142.
12. Parsons, D. F., Williams, G. R. & Chance, B. (1966) *Ann. N.Y. Acad. Sci.* **137**, 643–666.
13. Fleischer, S. & Fleischer, B. (1967) *Methods Enzymol.* **10**, 406–433.
14. Hsia, J. C., Schneider, H. & Smith, I. C. P. (1970) *Biochim. Biophys. Acta* **202**, 399–402.
15. Libertini, L. J., Waggoner, A. S., Jost, P. C. & Griffith, O. H. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 13–19.
16. Hsia, J. C., Schneider, H. & Smith, I. C. P. (1971) *Can. J. Biochem.* **49**, 614–622.
17. Jost, P., Libertini, L. J., Hebert, V. C., & Griffith, O. H. (1971) *J. Mol. Biol.* **59**, 77–98.
18. Seelig, J. (1970) *J. Amer. Chem. Soc.* **92**, 3881–3887.
19. Hubbell, W. L. & McConnell, H. M. (1971) *J. Amer. Chem. Soc.* **93**, 314–326.
20. Williams, J. C., Mehlhorn, R. & Keith, A. D. (1971) *Chem. Phys. Lipids* **7**, 207–230.
21. Luzzati, V. (1968) in *Biological Membranes*, ed. Chapman, D. (Academic Press, New York), pp. 71–123.
22. Rand, R. P. & Sengupta, S. (1972) *Biochim. Biophys. Acta* **255**, 484–492.
23. Fleischer, S., Klouwen, H. & Brierley, G. P. (1961) *J. Biol. Chem.* **236**, 2936–2941.