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SPIN LABEL EVIDENCE FOR THE ROLE OF LYSOGLYCEROPHOSPHATIDES IN CELLULAR MEMBRANES OF HIBERNATING MAMMALS

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SUMMARY

The phospholipid composition of ground squirrel heart muscle changes during hibernation: more lysoglycerophosphatides are found in the hibernating state than in the active state. Phase transitions inferred from spin label motion occur in the usual manner typical of mammalian mitochondria for the mitochondria and mitochondrial lipids from active squirrels. However, a conspicuous absence of a spin label-detectable phase transition is observed in equivalent preparations from hibernating animals. The addition of lysolecithin to preparations from active squirrels removes the break and induces a straight line in the Arrhenius plot. The lack of a spin label-detectable phase transition in hibernating animals, therefore, is attributed to an increased content of lysoglycerophosphatides present in the phospholipids during hibernation.

INTRODUCTION

Previous studies have demonstrated that the temperature dependence of oxygen uptake by mitochondria from chilling-sensitive plants and homeothermic organisms results in a break on an Arrhenius plot while the plot for chilling-resistant and poikilothermic organisms is exhibited as a straight line [1, 2]. Such breaks or changes in Arrhenius activation energies have been correlated with visual melts of defined lipid systems [3], biological function [4, 5] and are inferred, but not proven, to coincide with physical lipid phase transitions in biological membranes [6]. Such physical sphase transitions, inferred from spin label motion [6, 7], enzyme activity [8] and oxygen uptake activity [2], have a strong correlation with temperature tolerance or sensitivity of bacterial, plant and animal tissues. Below the midpoint of the phase transition range, bacterial growth is retarded or inhibited [9], chill-sensitive

Abbreviations: 2N8, 2-hexyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 2N12, 2-decyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 5N10, 5-butyl-2,2-dimethyl-5-pentyloxazolidine-*N*-oxyl.

plants are damaged [10] and physiological functions, such as heart muscle action potentials, are abolished in homeothermic organisms [11].

Hibernating or heterothermic organisms normally maintain body temperatures of about 37 °C, but can temporarily abandon the homeothermic state, lower their temperature to about 1 °C and maintain all vital membrane functions [12]. The temperature dependence of oxygen uptake by squirrel mitochondria results in a break in the Arrhenius plot for squirrels in the active state and in no break for squirrels in the hibernating state [13]. The general observation that there is a loss of a phase transition in the mitochondrial membranes concomitant with hibernation indicates a role of the membrane phospholipids in temperature sensitivity and tolerance. Our previous investigation does indeed indicate significant alterations in membrane phospholipid class composition during the hibernating state in the hearts of the ground squirrel, *Citellus lateralis* [14].

This investigation relates the membrane lipid composition to spin label detection of membrane physical state in the hibernating and active ground squirrel, *Citellus lateralis*.

MATERIALS AND METHODS

Whole hearts from eight active and three hibernating ground squirrels were homogenized with chloroform/methanol, 2 : 1, containing 0.01 % butylated hydroxytoluene as antioxident, in a Beckman Polytron homogenizer, filtered and the residue reextracted with chloroform/methanol, 4 : 1, containing 5 % of 28 % ammonium hydroxide [14].

Hearts and livers were removed within 90 s of decapitation from 2 active and 2 hibernating ground squirrels and mitochondrial preparations were made. The tissues were homogenized about 45 s in a Potter Elvehjem homogenizer in 0.025 M sucrose, 0.001 % EDTA, 0.024 M Tris buffer at pH 7.6, containing 0.5 mg/ml bovine serum albumin. The homogenate was centrifuged at $600 \times g$ for 10 min: this pellet was discarded, and the supernatant was recentrifuged at $12\,000 \times g$ for 10 min. This pellet was resuspended, recentrifuged and then frozen in liquid nitrogen.

Phospholipids were extracted from the mitochondrial samples with deoxygenated chloroform/methanol, 2 : 1, and washed according to Folch et al. [30]. The sample was dried under a stream of nitrogen and passed through a small silica gel G column, eluted with degassed diethyl ether and then with degassed methanol. Nitrogen gas bubbled through the solvent for 5 min was sufficient to deoxygenate and eliminate the oxygen-dependent paramagnetic broadening of the spin label lines. Solvents were degassed to avoid oxidation problems with unsaturated fatty acids. The use of spin labels in organic solvent is a convenient method of determining if oxygen is present since molecular oxygen is paramagnetic and broadens spin label lines. The methanol eluate was then weighed and made into vesicles at 25 mg/ml in water.

Phospholipid vesicle dispersions were prepared by mild sonication for 10 min in a nitrogen atmosphere or by drying to film under nitrogen on the bottom of a screw-cap test tube, adding the appropriate amount of water and heating to 80 °C with intermittent vortexing. Both preparative procedures resulted in preparations which gave the same spin label behavior.

Spectra were taken on a Varian 4500 electron paramagnetic resonance spectrom-

eter fitted with a laboratory-constructed variable temperature device calibrated and continuously monitored to an accuracy of approx. ± 0.2 °C. Spectra were taken using a 5 gauss sweep and measuring one spectral line at a time. The general procedure consisted of measuring the mid-field line on a forward sweep and the high-field line on a reverse sweep, followed immediately on the same time sequence by first measuring the high-field line on a reverse sweep and the mid-field line on a forward sweep. When averaged together, spectral pairs of this type cancel any spin label destruction mediated by reduction processes which occur linearly in time. These spectral pairs were carried out three times and averaged to result in the points shown in Figs 1 and 2. At higher temperatures, above 35 °C, a detectable amount of spin reduction was observed, but by adherence to the procedure outlined we believe no inaccuracies in spectral measurements were incurrred.

For certain calculations, we define approximate values for the average phospho lipid molecular weight to be about 800 and for lysophospholipid to be about 500. These values may not be strictly accurate, but for present purposes they are adequate.

In the mitochondrial samples it was necessary to add potassium ferricyanide $K_3Fe(CN)_6$ as an anti-reductant to prevent reduction of the spin labels. Lysolecithin from egg yolk was obtained from Sigma Chemical Company, St. Louis.

Rotational correlation time (T_c) measurements can be carried out using a constant (K) derived from the Kivelson treatment [15] where

$$T_c = KW_0 \, (h_0/h_{-1})^{\frac{1}{2}} - 1$$

K is a constant, W_0 is the mid field first derivative line width, h_0 is the mid field first derivative line height and h_{-1} is the high field first derivative line height. This treatment assumes isotropic motion, no hyperfine or g-value mixed anisotropes caused by solvent heterogeneity, and that molecular motion is in the fast tumbling range. We set the constant K equal to one and call resulting value an empirical motion parameter, R_i. R_i values assume only that the integrated intensity under the mid and high field lines are equal. We use R_i measurements only where spectral measurements of integrated intensity are equal within limits of measurements. R_i values are valid for comparative purposes where the same spin label is used in two different systems. R_i values can be converted into T_c values although these T_c suffer the same inaccuracies as before: $T_c = K(\text{approximately } 6.5 \cdot 10^{-10} \text{ s}) \cdot R_i$. The R_i values for 5N10 (5-butyl-2,2-dimethyl-5-pentyl-oxazolidine-*N*-oxyl) in the present system start to have mid field and high field lines which are unequal at R_i values greater than three. All of our measurements are well below this limit.

RESULTS

Spin label analysis was carried out on the intact mitochondria from both hibernating and active animals. Fig. 1 shows data taken from a liver mitochondrial sample of an active animal. The rotational motion of a hydrocarbon spin label solubilized in the mitochondrial preparation shows an Arrhenius break in the neighborhood of 20 °C (○, Fig. 1). The extracted phospholipids from the same preparation show a break at 23 °C. Previous results demonstrated that the lysoglycerophosphatide content of the hearts of hibernating ground squirrels was increased to about 8 mol% [14]. To simulate the general composition of the phospholipids from the

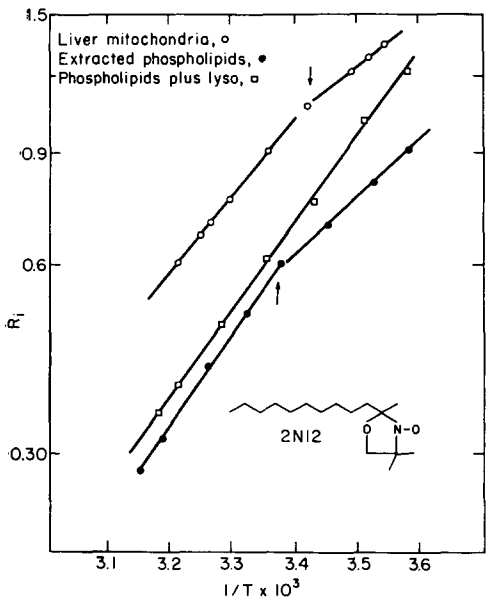

Fig. 1. Mitochondria and extracted phospholipids from active squirrel liver. All samples were spin labeled with 2N12 immediately before analysis. ○, liver mitochondria; ●, phospholipids extracted from liver mitochondria; □, same extracted liver mitochondrial phospholipids with 7 mol% of lysolecithin added.

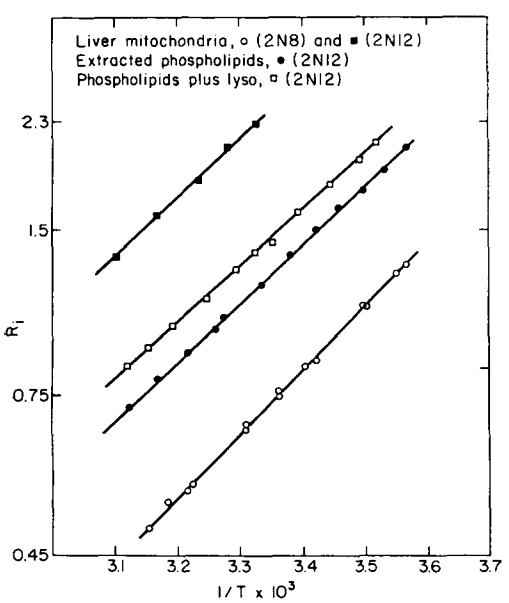

Fig. 2. Mitochondria and extracted phospholipids from hibernating squirrel liver. ○, liver mitochondria analyzed with 2N8; ■, liver mitochondria analyzed with 2N12; ●,extracted mitochondrial phospholipids; □, extracted phospholipids with an additional 7 mol% of lysolecithin added.

hibernating animals, 7 mol% of lysolecithin was added to the extracted phospholipids from active animals. The preparation treated in this manner (Fig. 1, □) clearly demonstrates that the discontinuity was eliminated and no departure from linearity was observed on the Arrhenius plot.

This entire procedure was repeated on the heart mitochondria and extracted phospholipids from the same squirrel, the heart and liver mitochondria and extracted phospholipids from an additional active squirrel, as well as the phospholipids of the whole heart from a pooled sample of eight active ground squirrels, with essentially the same results. In the various samples analyzed, the active preparations always exhibited a discontinuity similar to that shown in Fig. 1. However, the extrapolated intercept varied from 17 to 26 °C. In every case the addition of 7 mol% of lysolecithin to the phospholipids removed the discontinuity and established a straight line Arrhenius plot with a single activation energy throughout the entire temperature range. Lysolecithin was not added to isolated mitochondria.

Fig. 2 shows comparable analyses taken from a hibernating animal. Spin label motion is more restricted in the preparations from hibernating animals. The spin label, 2N12 (2-decyl-2,5,5-trimethyloxazolidine-*N*-oxyl), could not be used for a complete temperature profile of the mitochondria from hibernating animals since motion was too restricted at reduced temperatures to permit accurate determinations. Consequently, the smaller but otherwise similar spin label, 2N8 (2-hexyl-2,5,5-trimethyloxazolidine-*N*-oxyl), was used where accurate measurements in the fast tumbling range could be carried out. The mitochondria from the hibernating squirrels (○ for 2N8, ■ for 2N12) show linearity throughout the entire range of temperature. The extracted phospholipids and phospholipids with an additional 7 mol% of lysolecithin also result in linear plots, but the additional lysolecithin increases R_i at comparable temperatures. Again, similar results of a straight line Arrhenius plot were obtained from heart and liver mitochondria and extracted mitochondrial phospholipids from two hibernating ground squirrels, as well as from the heart phospholipids from a pooled sample of three hibernating ground squirrels. Without exception, everytime lysolecithin was added to an extracted phospholipid preparation the relative R_i increased.

DISCUSSION

Spin labels used to probe phospholipid or membrane systems yield information relative to the physical state of the local environment of the spin label. 2N12 does not have adequate water solubility to yield an aqueous signal. For a variety of other similar spin labels, and for 2N12, the isotropic hyperfine coupling (A_n) indicates that the polarity of the local enironment seen by the *N*-oxyl group in mitochondrial preparations is nonpolar. 2N12 has anisotropic hyperfine coupling of 14.3–14.4 G in the various preparations, which is characteristic of a nonpolar environment for this spin label.

Our results indicate that the non-polar hydrocarbon regions of the extracted phospholipids of the hearts, and heart and liver mitochondria from active ground squirrels undergo a physical state change to a more ordered state below their transition temperature. The addition of 7 mol% lysolecithin abolishes this transition. These results correlate with the greatly enhanced content of lysoglycerophosphatides

(8 mol%) found in the hearts of ground squirrels during hibernation [14] and strongly indicate that lysoglycerophosphatides play a vital role in maintenance of tissue and cellular integrity at reduced temperatures.

Phosphatidylcholine can be made into bilayer vesicles of about 300 Å in diameter containing about 7000 molecules [17]. Although the precise packing geometry is difficult to visualize, this indicates that the cross sectional area of the polar zone of a single phospholipid molecule is not greatly different from that of the hydrocarbon zone, which contains two fatty acyl residues. Lysolecithin, in contrast, forms a micelle of approximately 50 Å in diameter with about 100 or less molecules arranged with a hydrocarbon core [18] and cannot form bilayer vesicles. The radius of curvature of this micelle is great compared to that of the phosphatidylcholine bilayer visicle indicating a considerably greater cross-sectional residence of the polar zone compared to the hydrocarbon zone.

These considerations indicate that bilayers of diacyl phosphatides should have their physical properties altered in the presence of an admixture of lysoglycerophosphatides. Assuming that the polar groups are unchanged and continue to occupy as much space whether bound to one or two acyl chains, then admixtures of lysoglycerophosphatides could, in principle, create "vacancies". Due to the dynamic nature of lipid bilayers, these vacancies would average in space to result in an overall lower hydrocarbon density. For example, 8 mol% of lysoglycerophosphatide would lower the hydrocarbon density by about 4 %, or would increase the average interchain hydrocarbon close order spacing by about 0.1 Å. Such vacancies may well disorder the hydrocarbon zone sufficiently that comparatively less molecular order is sustained at reduced temperatures. The data presented here indicate that the degree of hydrocarbon disorder is increased by the action of lysolecithin, to the extent that the break in the Arrhenius plot of spin label motion observed with phospholipids from active ground squirrel tissues is abolished completely, or at least lowered to a temperature below about 5 °C. This indicates that lysoglycerophosphatides may be a natural means of preventing the highly ordered solid state of the membrane lipids which would usually ensue at low temperatures.

The precise meaning of the observation that spin labels are more immobilized by squirrel phosphatides when an admixture of lysophosphatides are also present seems complicated. The R_i exhibited by a spin label is determined by its own properties and by its interaction with the host matrix. Any structural change that causes the spin label to fit more tightly with the matrix molecules will probably cause the R_i of the spin label to increase. The expected disordering effect of lysophosphatides may well cause the phospholipid or membrane matrix to become a glass at lower temperatures, rather than paracrystalline. Spin labels trapped in a glass matrix usually exhibit highly restricted motion. The general property of glass formation may also facilitate slowing down of metabolism while the squirrels are in the hibernating state.

The lateral and perpendicular distribution of 2N12 and 2N8 are unknown. There may be unequal distribution of 2N12 with respect to outer and inner mitochondrial membranes, with respect to outer and inner halves of membrane bilayers and with respect to local zones of relatively high solubility in the lateral plane of either membrane or both membranes. Distribution of these spin labels is also unknown in the phospholipid vesicles. The distribution of spin label also may be different at different temperatures. The introduction of lysophosphatides may substantially

modify the spin label distribution. It is of interest to note that the Arrhenius breaks seen on Fig. 1 are not at the same temperatures for the mitochondria and for the extracted lipids.

Previous studies on bacteria [19, 20], plants [21–23] and cold blooded animals [24–26] adapted for growth at low temperatures have indicated a compensatory increase in membrane lipid unsaturation, which theoretically sustains membrane fluidity [4, 5, 27] and enzyme activity [28, 29] at these temperatures. We believe that lysophosphatides in such membranes as those of hibernators provide a mechanism for cold tolerance whereby the organism can interconvert between the hibernation and the active state quickly and efficiently without necessarily changing fatty acid composition.

In contrast to our study, Cannon [31] using mitochondria from brown adipose tissue from hamster and rat of cold-adapted animals found no ESR-detectable non-linearity which disappeared in the cold-adapted state. No phospholipid differences were found by Cannon either, suggesting that cold-adapted rats and hamsters may not constitute the same physiological state as hibernating squirrels. Details of the spin label analysis differed: the organisms were different and the physiological states were probably different therefore the two studies are not directly comparable.

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