Calorimetric and Spectroscopic Studies of Lipid Thermotropic Phase Behavior in Liver Inner Mitochondrial Membranes from a Mammalian Hibernator†

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ABSTRACT: Arrhenius plots of various enzyme and transport systems associated with the liver mitochondrial inner membranes of ground squirrels exhibit changes in slope at temperatures of 20–25 °C in nonhibernating but not in hibernating animals. It has been proposed that the Arrhenius breaks observed in nonhibernating animals are the result of a gel to liquid-crystalline phase transition of the mitochondrial membrane lipids, which also occurs at 20–25 °C, and that the absence of such breaks in hibernating animals is due to a major depression of this lipid phase transition to temperatures below 4 °C. In order to test this hypothesis, we have examined the thermotropic phase behavior of liver inner mitochondrial membranes from hibernating and nonhibernating Richardson's ground squirrels, *Spermophilus richardsonii*, by differential scanning calorimetry and by ^{19}F nuclear magnetic resonance and fluorescence polarization spectroscopy. Each of these techniques indicates that no lipid phase transition occurs in the membranes of either hibernating or nonhibernating ground squirrels within the physiological temperature range of this animal (4–37 °C). Moreover, differential scanning calorimetric measurements indicate that only a small depression of the lipid gel to liquid-crystalline phase transition, which is centered at about −5 °C in nonhibernating animals and at about −9 °C in hibernators, occurs. We thus conclude that the Arrhenius plot breaks observed in some membrane-associated enzymatic and transport activities of nonhibernating animals are not the result of a lipid phase transition and that a major shift in the gel to liquid-crystalline lipid phase transition temperature is not responsible for seasonal changes in the thermal behavior of these inner mitochondrial membrane proteins.

Hibernation is a seasonal adaptation by which some mammalian species survive low environmental temperatures and a scarcity of food by drastically lowering their body temperatures, thereby dramatically reducing their energy requirements. Mammalian species capable of and prepared for seasonal hibernation can tolerate body temperatures near freezing for several weeks at a time without apparent ill effects. In contrast, nonhibernating mammals cannot tolerate severe hypothermia, and if body temperatures fall below about 20 °C, death results. Interestingly, mammals capable of but not prepared for hibernation are susceptible to hypothermia, indicating that these species are not intrinsically resistant to the lethal effects of low body temperatures. Moreover, hibernating mammals retain the ability to spontaneously arouse, elevate their body temperature, and resume normal activity between bouts of deep hibernation. Thus, whatever the nature of the biochemical and physiological adaptations involved in preparing an animal for hibernation, these adaptations apparently do not result in any loss of ability to function at normal body temperatures. There is also considerable evidence that alterations in the functions of cellular membranes are important in hibernation and hypothermia (Aloia et al., 1986; Cossins et al., 1986; Rahman, 1986; Willis, 1986).

Lyons and Raison (Lyons & Raison, 1970; Raison & Lyons, 1971) have proposed that membrane lipid phase transitions play a critical role in membrane function during hypothermia and hibernation. Their hypothesis is based on both functional and structural observations. These workers report that the succinate oxidase activity in liver mitochondria from nonhibernating ground squirrels exhibits an Arrhenius plot break at about 23 °C while such a break is absent in mitochondria from hibernating ground squirrels. The conclusion that the Arrhenius break at 23 °C may be related to the capacity to hibernate is further strengthened by the finding of similar breaks in the succinate oxidase activity of liver mitochondria of homeothermic but nonhibernating species such as the rat and their absence from poikilothermic animals such as fish, which can function normally at low environmental temperatures. These workers also report that Arrhenius plots of the rotational correlation time of a fatty acid ester spin-label incorporated into these mitochondria exhibit a break at about 23 °C in nonhibernating ground squirrels but no break is seen in hibernating animals (Raison et al., 1971). The Arrhenius break at 23 °C in the electron spin resonance (ESR)[1] spectroscopic parameter observed only in the mitochondria from nonhibernating ground squirrels was intepreted as being due to a lipid phase transition. Thus, Lyons and Raison proposed that the function of the succinate oxidase enzyme complex was perturbed in nonhibernating animals by the formation of gel-state lipid in the mitochondrial membrane. They further postulated that no break in the enzyme activity occurred in hibernating animals because the gel to liquid-crystalline lipid phase transition had been reduced to temperatures below the

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[1] Abbreviations: ESR, electron spin resonance; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; SR, sarcoplasmic reticulum; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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physiological range. A corollary of this hypothesis is that mammalian hibernators are capable of a fairly efficacious "homeoviscous adaptation" (Sinensky, 1974) or "homeophasic adaptation" (Silvius et al., 1980) of their membrane lipids in preparation for hibernation. Subsequent work has supported the finding that several enzyme activities (such as succinate–cytochrome *c* reductase and succinate dehydrogenase) and transport functions (such as calcium uptake and proton efflux) also exhibit Arrhenius plot breaks near 20 °C in mitochondria from nonhibernating but not from hibernating ground squirrels (Augee et al., 1984; Pehowich & Wang, 1984). However, the intepretation of the ESR spectroscopic results as indicative of a lipid phase transition has been controversial. Two types of criticisms have been made of this interpretation, one relating to the methodology employed in analyzing the ESR spectra (Cannon et al., 1975; Bigelow et al., 1986) and the other to the perturbing nature of the extrinsic spin probe itself (Seelig & Niederberger, 1974; Seelig & Seelig, 1974; Cadenhead et al., 1975; Schrier et al., 1978; Taylor & Smith, 1980). Whatever its basis, it is clear that ESR methods of the type employed sometimes do yield results which are at variance with those of other thermodynamic, structural, and spectroscopic techniques in some other biological membrane systems (McElhaney & Souza, 1976; Raison et al., 1982; McElhaney, 1984a,b).

In order to clarify the functional importance of the membrane lipid phase state in seasonal hibernators, we have examined the thermotropic behavior of the liver inner mitochondrial membranes from hibernating and nonhibernating ground squirrels using three separate but complementary physical techniques: differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) spectroscopy, and steady-state fluorescence polarization spectroscopy. The inner membrane was chosen because it is the site of several catalytic events specific to electron transport and oxidative phosphorylation, including the activities of succinate dehydrogenase and succinate–cytochrome *c* reductase, enzymes which show seasonal variations in thermal response (Augee et al., 1984; Pehowich & Wang, 1984). Membranes were examined in preference to extracted lipids in order to preserve relevant lipid–lipid and lipid–protein interactions, such as localized concentrations of specific proteins within a lipid domain or lipid clusters within the membrane bilayer. In addition, the membranes from animals from all phases of an annual hibernation cycle were examined with DSC to determine if alterations in membrane structure are a consequence of the low body temperatures experienced during hibernation or if any such changes precede the commencement of hibernation.

Materials and Methods

Animals. Adult Richardson's ground squirrels (*Spermophilus richardsonii*) were captured near Edmonton, Alberta. Squirrels designated as spring animals were trapped in April, about 2 weeks after emergence from hibernation, and were killed and examined within 1 week. Squirrels designated as summer animals were trapped in June–July and killed within 1 week as well. Other animals trapped in July were housed in individual cages under a 12-h light–12-h dark photoperiod at 18–20 °C and provided with food and water ad libitum. A number of these animals, designated warm-acclimated, were kept under these conditions until they were killed in October–November. The remainder were placed in a 4 °C cold room the first week in September with a 2-h light–22-h dark photoperiod. Of those animals which entered hibernation, half were killed after 3 days or longer into a well-established hibernation bout (hibernating body temperature = 4 °C), and the remainder, designated as aroused, were killed and examined after spontaneous arousal and after they had been euthermic (body temperature = 37 °C) for 6–9 h. Animals that could not be induced to hibernate and that remained euthermic, designated as cold-acclimated, were killed in October–November, a minimum of 8 weeks after exposure to 4 °C and a 2-h light–22-h dark photoperiod. All animals were killed as near to the same time of day (0900) as possible.

Isolation of Inner Mitochondrial Membrane. Animals were killed by decapitation, and liver mitochondria were isolated as described previously (Pehowich & Wang, 1984). Briefly, livers were minced in ice-cold isolation medium and then homogenized with a motor-driven Teflon pestle (0.7-mm clearance) in a Potter–Elvehjem-type tissue grinder. Homogenization was accomplished with 4–5 strokes of the pestle rotating at 1500 rpm. An inner membrane matrix fraction was prepared by removal of the outer membrane with digitonin (Schnaitman & Greenawalt, 1968). The mitochondrial pellet was resuspended in 70 mM mannitol, 120 mM sucrose, 2 mM HEPES, pH 7.4, and 0.5 mg of BSA/mL to a final concentration of 100 mg of protein/mL. Digitonin (Sigma) dissolved in 1 volume of isolation medium was added to the mitochondrial stock to a final concentration of 0.15 mg/mg of mitochondrial protein. The suspension was stirred at 0 °C for 20 min and then homogenized with a Potter–Elvehjem tissue grinder with a tight-fitting glass pestle. The inner membrane fraction was sedimented on centrifugation at 12000*g* for 15 min. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, centrifuged at 20000*g* for 10 min, and used immediately for high-sensitivity DSC analysis or frozen in dry ice–acetone, lyophilized, flushed with N_2, and stored at −70 °C in Teflon-lined screw cap tubes until analysis. The purity of inner membrane preparations was assessed by measuring the activity of monoamine oxidase, an outer membrane marker (Schnaitman & Greenawalt, 1968).

The digitonin used in the isolation of ground squirrel liver inner mitochondrial membranes is supposed to interact only with the cholesterol-containing outer membrane under the experimental conditions employed. However, in order to rule out the possibility that some digitonin might associate with the inner membrane and alter the physical properties of the membrane lipids, we also prepared inner mitochondrial membranes by the freeze–thaw hypotonic lysis method of Caplan and Greenawalt (1966), a method which does not employ detergents or other lipid-perturbing agents. Mitochondrial inner membranes prepared by this alternate isolation procedure gave results identical with those obtained by the digitonin procedure of Schnaitman and Greenawalt (1968) when analyzed by DSC or by DPH fluorescence polarization spectroscopy. These results indicate either that no residual digitonin is present in our inner mitochondrial membranes or that it is present in such low amounts that the physical properties of the membrane lipids are not significantly affected.

Differential Scanning Calorimetry. Lyophilized mitochondrial inner membrane preparations were rehydrated in 100–150 μL of 50% ethylene glycol in 0.05 M Tris-HCl, pH 7.4, which had been degassed with N_2. Calorimetric analysis was then performed on a Perkin-Elmer DSC-2C low-sensitivity differential scanning calorimeter equipped with a thermal analysis data station. The calorimeter was routinely calibrated with water, indium, or dipalmitoylphosphatidylcholine. Stainless-steel sample pans containing 50–75 mg of membrane and a reference pan containing 50% ethylene glycol and buffer were run through at least two heating–cooling cycles (−40 to +40 °C) in order to fully hydrate the lipid component.

Table I: Thermal Properties of Liver Inner Mitochondrial Membrane from Richardson's Ground Squirrels in Different Phases of a Hibernation Cycle[a]

	heating scan[b]				cooling scan			
phase	onset temp (°C)	transition temp (°C)	final temp (°C)	ΔT	onset temp (°C)	transition temp (°C)	final temp (°C)	ΔT
summer (7)	−11.7 ± 0.77	−1.8 ± 0.55	+7.0 ± 1.45	18.7 ± 1.40	+1.7 ± 2.40	−6.9 ± 1.38	−15.2 ± 1.08	16.9 ± 0.93
hibernating (7)	−13.0 ± 0.96	−5.7 ± 0.41[c]	+3.3 ± 0.60	16.3 ± 0.95	−12.4 ± 0.46[c]	−17.2 ± 0.39[c]	−31.2 ± 0.86	18.8 ± 1.10
aroused (4)	−11.7 ± 1.22	−4.1 ± 0.42	+3.6 ± 0.85	15.3 ± 1.20	−9.4 ± 1.17[c]	−15.3 ± 2.24[c]	−25.6 ± 4.61	16.2 ± 1.52
cold-acclimated (4)	−12.3 ± 1.06	−5.6 ± 0.62[d]	+3.4 ± 0.73	15.7 ± 1.06	−9.3 ± 0.98[c]	−16.5 ± 1.12[c]	−31.3 ± 0.92	22.0 ± 0.89
warm-acclimated (4)	−12.7 ± 0.99	−4.6 ± 0.72[d]	+4.0 ± 0.94	16.7 ± 0.96	−10.4 ± 0.88[c]	−17.0 ± 1.11[c]	−29.9 ± 1.27	19.5 ± 1.1
spring (4)	−12.6 ± 0.63	−4.3 ± 0.16[d]	+2.4 ± 0.85	15.0 ± 1.62	−9.0 ± 0.25[c]	−16.9 ± 0.19[c]	−30.2 ± 0.79	21.2 ± 0.80

[a] Values are means ± SE. Numbers in parentheses = n. [b] Samples were heated or cooled at a scan rate of 10 °C/min. Statistically significant difference from summer group. Determined by two-tailed Student's t test. ΔT = temperature range from the onset to the completion of the lipid phase transition. [c] $P < 0.001$. [d] $P < 0.05$.

Thermal scans, either heating or cooling, were programmed at a scan rate of 10 °C/min with sensitivity set at 0.2 mcal/s full-scale. Samples were equilibrated at the starting temperature until a stable base line was recorded (5–10 min) and then scanned from −40 to 90 °C, cooled to −40 °C, and heated again to +90 °C. Thermally induced phase transitions were characterized by the point at which the recording trace deviated significantly from base line (onset temperature) and when it returned to base line (completion temperature). The phase transition temperature, T_m, was taken as the temperature of maximum excess specific heat. Scans of membranes rehydrated in 0.05 M Tris-HCl showed large water transitions near −3 °C which almost completely masked the lipid transitions. Under these conditions, no other transitions were detected above 0 °C.

Thermal analysis of freshly isolated inner mitochondrial membranes from hibernating and nonhibernating squirrels was also performed on a Microcal MC-2 high-sensitivity differential scanning calorimeter. Samples were suspended in 50 mM Tris-HCl, pH 7.4, at a final concentration of approximately 200 mg of protein/mL. Thermograms were obtained on first heating from 0 to 75 °C at a scan rate of 20 °C/h and then, after cooling, by heating again under the same conditions.

^{19}F Nuclear Magnetic Resonance Spectroscopy. 6-Fluoropalmitic acid was synthesized from its keto acid methyl ester as described by McDonough et al. (1983). Lyophilized inner mitochondrial membrane preparations were suspended in 0.154 M NaCl, 0.05 M Tris-HCl, pH 7.4, and 20 mM β-mercaptoethanol and diluted 20-fold with 95% deuterium oxide. Preparations were incubated at 37 °C for 30 min under N_2 to intercalate the monofluoropalmitate into the membrane bilayer. ^{19}F NMR spectra were obtained at −3, +7, +17, +27, and +37 C at 254.025 MHz on a Bruker HXS-270 NMR spectrometer, essentially as described by Macdonald et al. (1984). Computer-simulated best-fit lines were derived from the experimental line spectra in order to calculate the orientational order parameter, S_{mol}. However, in the present study, an experimentally determined maximum chemical shift anisotropy value of 52 ppm for the monofluoropalmitic acid was employed in calculations of S_{mol}, rather than the maximum chemical shift anisotropy value of Teflon (82.2 ppm) used previously. Use of this more appropriate value has the effect of increasing the calculated S_{mol} values in the liquid-crystalline state by about 60% relative to those reported previously, thus bringing these values much closer to those determined by ^2H NMR.

Fluorescence Polarization Spectroscopy. Inner mitochondrial membranes were isolated from summer-active and winter-hibernating ground squirrels as described above. Preparations were suspended in 0.25 M sucrose–2.0 mM HEPES and frozen on dry ice under vacumm. The frozen samples were sealed in Teflon screw-capped vials under N_2 and kept at −70 °C until analysis. Just before spectroscopy, samples were thawed under N_2, and an aliquot was suspended in 2.4 mL of 0.05 M phosphate buffer, pH 7.6, to give an $A_{500} = 0.1 \pm 0.01$. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was dissolved in tetrahydrofuran under N_2, and a 2-μL aliquot was added to the membrane suspension with vigorous mixing. The preparations were incubated at 37 °C for 15 min to maximize fluorescence and then cooled to 2 °C. Steady-state polarization of DPH fluorescence was measured on the computer-controlled T-format fluorometer as described by Cossins and Macdonald (1983). Polarization was automatically recorded as temperature was increased by 0.75 °C/min to give values at intervals of approximately 0.5 °C.

Results

Differential Scanning Calorimetry. Two distinct endothermic transitions were observed by conventional DSC in liver mitochondrial inner membranes from animals from all phases of the hibernation cycle when first scanned from −40 to +90 °C, one near 0 °C and the second centered near 65 °C. On cooling to −40 °C and reheating, only the low-temperature transition was observed, and this transition was nearly identical with that observed in thermograms of protein-free lipid extracted from the same membranes (data not shown). The lower temperature transition therefore corresponds to the reversible gel to liquid-crystalline phase transition of the membrane lipids, and the higher temperature transition is due to the irreversible denaturation of membrane protein.

Endothermic lipid phase transitions with an apparent midpoint (T_m) near −2 °C were detected in dispersions of inner mitochondrial membranes from summer ground squirrels upon heating (see Figure 1a). Similar transitions were seen in membranes from hibernating animals except that the T_m was lowered slightly to near −6 °C. Although the difference between the T_m's of hibernating and nonhibernating animals is not large, it is statistically highly significant, since standard errors in the determination of the T_m values are of the order of 0.5 °C. While the mean onset temperature of the transition was not significantly different between the summer and hibernating states, the completion temperature was also slightly but significantly lower in hibernating individuals (Table I). In membranes from both hibernating and nonhibernating animals, the cooperativities of the lipid gel to liquid-crystalline transitions, expressed as the temperature integral between the onset and completion of the transition, was similar. Phase transitions also occur in membrane dispersions from summer and hibernating animals on cooling of the samples, indicating that these lipid chain melting phase transitions are fully reversible, as expected (Figure 1b); however, the phase transition

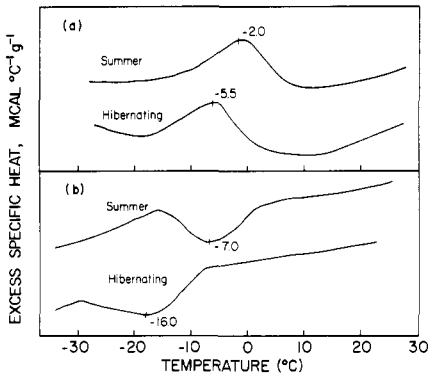

FIGURE 1: Representative conventional differential scanning calorimetry thermogram of (a) endothermic (heating) phase transition and (b) exothermic (cooling) phase transition in liver inner mitochondrial membranes from summer and from hibernating Richardson's ground squirrel. Scan rates were 10 °C/min at a sensitivity of 0.2 mcal/s full-scale.

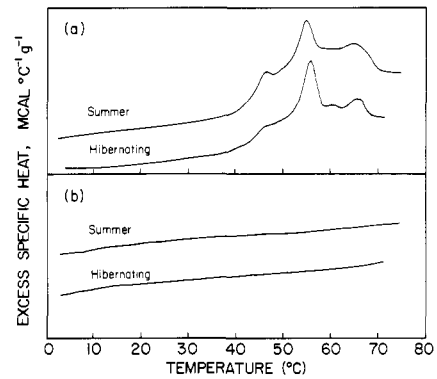

FIGURE 2: Representative high-sensitivity differential scanning calorimetry thermograms of freshly isolated liver inner mitochondrial membranes from summer and from hibernating Richardson's ground squirrels. Membranes were scanned from 0 to 70 °C at a scan rate of 20 °C/h (a), cooled, and scanned again (b).

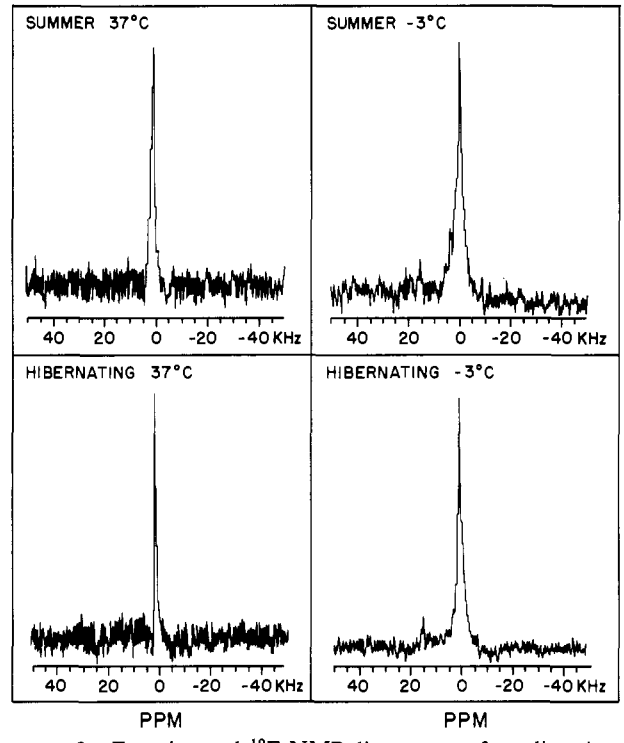

FIGURE 3: Experimental ^{19}F NMR line spectra from liver inner mitochondrial membranes from summer and from hibernating Richardson's ground squirrels. Spectra were obtained at 37 and −3 °C at 254.025 MHz using a spectral width of ±50 kHz. Samples were incubated with the monofluoropalmitate probe as described in the text.

boundaries and T_m seen on cooling are considerably lower than observed upon heating, especially in the case of the hibernating animals (Table I). At the high heating and cooling scan rates required in order to obtain sufficient instrumental sensitivity with conventional DSC instruments, we estimate that the true equilibrium (zero scan rate) T_m and onset and completion temperatures are overestimated by 3–4 °C on heating and are underestimated by at least 5 °C on cooling. Thus, the actual T_m's of the lipids of summer and hibernating membranes are probably −5 to −6 and −9 to −10 °C, respectively, with the upper boundaries being about +3 to +4 and 0 °C. Thus, in membranes from both summer and hibernating animals, the lipids are completely in the liquid-crystalline state over the physiologically relevant temperature range (4–37 °C). These results are confirmed by the absence of any low-temperature deflections from base line in the high-sensitivity DSC scans, which indicates that the lipid transitions are essentially complete by +3 to +4 °C (see below). Conventional DSC heating and cooling scans of liver inner mitochondrial membranes from spring, aroused, and warm- and cold-acclimated animals are not statistically different from those animals killed in hibernation, but all are different from summer animals (Table I). These results indicate that the slightly lower T_m of hibernating as compared to summer animals is not the result of the low body temperature experienced during bouts of hibernation, since the lipid transition temperatures of the mitochondrial inner membranes in the other animal groups which had remained euthermic were also similarly reduced. Moreover, the fact that cold-acclimated but nonhibernating ground squirrels also exhibit a slightly reduced T_m in comparison to summer animals suggests that this modest change in membrane lipid thermotropic phase behavior precedes entry into hibernation.

High-sensitivity DSC thermograms of freshly isolated inner mitochondrial membranes scanned over the temperature range 3–75 °C revealed only a series of irreversible endothermic transitions between 37 and 70 °C in both the summer and hibernating states (Figure 2). These transitions are due to the thermal denaturation of the inner mitochondrial membrane proteins. The protein denaturation profile is essentially identical in membranes from summer and hibernating animals. As mentioned above, there was no evidence of lipid phase transitions near 20 °C even at the highest possible instrumental sensitivity.

^{19}F Nuclear Magnetic Resonance Spectroscopy. The experimental ^{19}F NMR spectra increased in width as temperature was decreased for membranes from both summer and hibernating animals (see Figure 3). This is indicative of a progressive increase in acyl chain ordering with decreasing temperature. Generally, the orientational order parameter S_{mol} approaches unity as acyl chains become completely ordered (as in an oriented crystal) and decreases to near zero in a completely disordered state (such as liquid paraffin). In natural membranes, the S_{mol} value for the particular probe utilized varies from about 0.25 to 0.40 in the liquid-crystalline state to above 0.80 in the gel state, depending to some extent on the fatty acid composition of the membrane and the proximity to the lipid phase transition temperature (Macdonald et al., 1983, 1984). At temperatures from about 7 to 37 °C, the relatively low S_{mol} values observed and their weak dependence on temperature indicate that essentially all of the lipid in mitochondrial inner membranes from both summer and hibernating animals exists entirely in the fluid state (see Figure 4). However, the slightly but significantly lower order exhibited by the mitochondrial membranes from hibernating

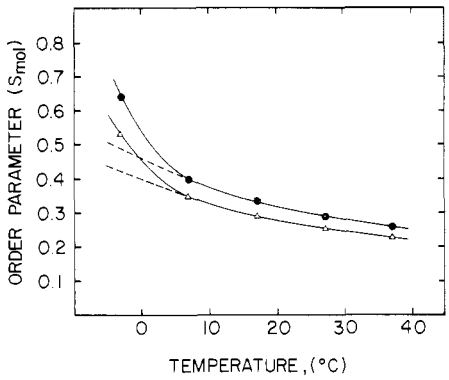

FIGURE 4: ^{19}F NMR orientational order parameter (S_{mol}) profiles of liver inner mitochondrial membranes from summer (●) and from hibernating (△) Richardson's ground squirrels. S_{mol} was derived from computer simulations of best-fit lines for experimental line spectra (n = 3); the standard error was ±3%. The dashed lines indicate the projected S_{mol} values in the absence of any conversion from the liquid-crystalline to the gel state.

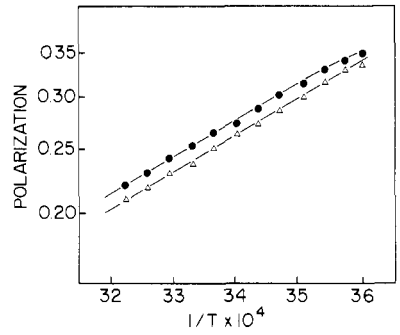

FIGURE 5: Arrhenius plot of polarization of the fluoroprobe 1,6-diphenyl-1,3,5-hexatriene incorporated into liver inner mitochondrial membranes from summer (●) and from hibernating (△) Richardson's ground squirrels. Plots represent 60 determinations between 2 and 40 °C from pooled animals in each group (n = 9). Only every fifth temperature is plotted.

animals indicates a slightly greater degree of fluidity than in summer animals throughout this temperature range. At temperatures below 7 °C, the progressively higher S_{mol} values and their greater dependency on temperature indicate the formation of increasing quantities of gel-state lipid in both membranes as temperature is lowered. Since the average S_{mol} value in the presence of both gel and liquid-crystalline lipid is simply the weighted average of the S_{mol} value for each phase (Macdonald et al., 1983, 1984), one can estimate the relative proportion of solid and fluid lipid present at any temperature from a knowledge of the S_{mol} values at temperatures above and below the phase transition boundaries. Such an analysis reveals that even at −3 °C more than half of the membrane lipids from summer animals, and a higher proportion from hibernating animals, remain in the liquid-crystalline state. The lower S_{mol} values observed below 7 °C for the hibernating animals indicate that the lipid phase transition midpoint temperature must be somewhat reduced in comparison to summer animals, thus confirming the DSC results.

DPH Fluorescence Polarization Spectroscopy. The effect of temperature on the steady-state fluorescence polarization of DPH incorporated into the inner mitochondrial membrane of summer and hibernating ground squirrels is shown in Figure 5. At 4 °C, the polarization was 0.344 ± 0.006 (mean ± SE) in summer animals and 0.337 ± 0.004 in hibernators, indicating that the structural order of the hibernating membrane bilayer was slightly lower. With increasing temperature, polarization decreased, indicating an increasing angular excursion of the DPH probe within the membrane. This is interpreted to represent a decrease in orientational order and thus an increase in average fluidity with temperature. At 37 °C, polarization was slightly but significantly higher in summer animals than in hibernators (0.221 ± 0.009 vs 0.212 ± 0.007), again indicating a less fluid lipid environment in the former. Arrhenius plots of DPH steady-state polarization values of both summer animals and hibernating animals were linear with no apparent breaks. As DPH is specifically confined to the hydrocarbon region of the lipid bilayer, the low polarization values and the absence of any marked increases in the microviscosity in the region of the probe indicates that the inner mitochondrial membrane is predominantly fluid between 4 and 37 °C and that no phase transitions occur in either hibernating or summer animals, again corroborating the DSC results.

Discussion

The major findings of this study are that the lipids from liver mitochondrial inner membranes from nonhibernating as well as from hibernating animals do not undergo a phase transition within the physiological temperature range and that the membrane lipid phase transition temperature of hibernating animals undergoes only a small decrease compared to summer animals. The appearance of the lipid gel to liquid-crystalline phase transition at low temperatures (−5 to −10 °C) is not unexpected given the relatively high content of polyunsaturated fatty acids in the phospholipids of the inner mitochondrial membrane (Platner et al., 1976; Aloia, 1980). Indeed, the calorimetrically detected lipid phase transition of liver mitochondrial inner membranes from nonhibernating mammals such as the rat or cow occurs at about the same temperature (−10° to 0 °C) as found here for the ground squirrel (Hackenbrock et al., 1976; Blazyk & Newman, 1980; Madden et al., 1980). Further, in these nonhibernating species, no lipid phase transition was found to occur near 20 °C, either in native whole mitochondria (Blazyk & Steim, 1972) or in isolated inner membranes (Hackenbrock et al., 1976; Blazyk & Newman, 1980; Madden et al., 1980). Moreover, no lipid phase transitions occurring in the temperature range 5–40 °C were detected by ESR spectroscopy in mitochondrial membranes from warm- or cold-acclimated rats or hamsters (Cannon et al., 1975). It thus seems clear that the breaks in the Arrhenius plots of enzyme or transport activity which have been reported to occur near 20 °C in intact mitochondria are not caused by a lipid phase transition.

The hypothesis of Lyons and Raison also predicts a major seasonal shift in the lipid phase transition temperature of liver inner mitochondrial membranes, from 20–25 °C in summer to less than 4 °C in hibernating animals. However, instead of a phase transition temperature shift of 20 °C or more, we found a decrease of only about 4 °C in the hibernating animals. The small decrease in the lipid phase transition temperature of the hibernators was reflected as well in only a small decrease in order (increase in fluidity) of the liquid-crystalline membrane lipids throughout the physiological temperature range, as detected by ^{19}F NMR and DPH fluorescence polarization spectroscopy. In fact, the decrease in order detected by these spectroscopic techniques would correspond to a decrease in the lipid phase transition temperature of 6–7 °C (^{19}F NMR) or 3–4 °C (DPH fluorescence polarization), in good agreement with the calorimetric results. Again, this small decrease in the phase transition temperature of the inner mitochondrial membrane lipids of hibernating animals is consistent with the small changes in phospholipid polar head group and fatty acid composition found in hibernating as compared to summer ground squirrels (Platner et al., 1976; Aloia, 1980). It is also

consistent with the recent ESR spectroscopic results of Aloia et al. (1986), who reported no significant difference in the fluidities of the mitochondrial membranes from summer and hibernating ground squirrels of another species. One should note that this small shift of about 4 °C in lipid phase transition temperature observed here is not nearly sufficient to compensate for the large decrease in body temperature during hibernation, which is of the order of 35 °C. Thus, these ground squirrels have only a very limited ability to regulate the physical properties of their liver mitochondrial inner membrane lipids in response to changes in body temperature. It remains to be determined whether or not the small decrease in the lipid phase transition temperature which probably precedes the entry of summer animals into hibernation has any physiological significance.

The molecular basis for the correlation observed between the Arrhenius plot break temperatures of some enzyme or transport activities and ESR spectroscopic empirical spectral parameters is presently unclear. However, some recent studies on the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum (SR) membrane may be relevant to the less well-studied liver mitochondrial inner membrane [for a review, see Hidalgo (1985)]. The Ca^{2+}-ATPase in native SR membranes also exhibits a break near 20 °C in its Arrhenius plot, and several early studies also indicated a break in Arrhenius plots of lipid ESR spectral parameters at this same temperature, although other studies employing ESR or DPH fluorescence polarization spectroscopy failed to detect any Arrhenius breaks near 20 °C. Moreover, calorimetric and X-ray diffraction studies of SR membranes have shown the absence of a lipid phase transition above 10 °C. In order to resolve this controversy, Bigelow and co-workers (Bigelow et al., 1986) have investigated the relationship between Ca^{2+}-ATPase function and molecular dynamics of both the lipid and the Ca^{2+}-ATPase protein in SR membranes, using conventional and saturation-transfer ESR spectroscopy to probe rotational motions of spin-labels attached either to fatty acid hydrocarbon chains or to the Ca^{2+}-ATPase sulfhydryl groups. ESR studies were also performed on aqueous dispersions of extracted SR lipids in order to study intrinsic lipid properties independent of the protein. While an Arrhenius plot of the Ca^{2+}-ATPase activity exhibits a clear change in slope at 20 °C, Arrhenius plots of lipid hydrocarbon chain mobility are linear, indicating that an abrupt thermotropic change in the lipid hydrocarbon phase is not responsible for the Arrhenius break in enzymatic activity. The presence of protein was found to decrease the average hydrocarbon chain mobility, but linear Arrhenius plots were observed both in the intact SR membranes and in extracted lipids when ESR spectra were analyzed by procedures that prevent the production of artifactual breaks in the Arrhenius plots. Although evidence for the possible importance of protein mobility in ATPase function was presented, these studies provide no evidence that the changes observed in protein mobility or conformation are caused by lipid phase transitions in the SR membrane.

One should also mention that there is abundant evidence that the Ca^{2+}-ATPase protein itself undergoes a change in conformation and mobility near 20 °C, both in native SR membranes and when reconstituted with various phospholipids or even detergents [see Hidalgo (1985)]. It is possible, therefore, that the Arrhenius break in both the rate of ATP hydrolysis and calcium transport could be due to a temperature-induced conformational change in the Ca^{2+}-ATPase which is intrinsic to the protein molecule as its exists in a fluid, hydrophobic environment. This hypothesis could explain the fact that Arrhenius plot breaks have been reported for spin-labeled lipid hydrocarbon chains covalently attached to the Ca^{2+}-ATPase molecule but not in similar probes free to diffuse in the lipid bilayer [see Hidalgo (1985)]. It is therefore possible that the ESR Arrhenius breaks detected by fatty acid spin-labels, if real, are secondary consequences of a local alteration of the organization of the phospholipid molecules induced by an intrinsic, thermally induced conformation change in the Ca^{2+}-ATPase protein rather than the other way around. If this view is correct, future studies of membrane adaptations for hibernation could very profitably be directed toward an understanding of the conformation and dynamics of membrane proteins and how their thermotropic properties appear to be altered during hibernation.

There is good evidence for the existence of a boundary lipid annulus around integral transmembrane proteins such as those present in the inner mitochondrial membranes of liver [see Bloom and Smith (1985), Marsh (1985), and McElhaney (1986)]. This boundary lipid is usually thought to be composed of a single layer of phospholipid molecules whose structure and dynamics are perturbed by the irregular surface of the hydrophobic domain of the protein with which they are in transient contact. The lipid molecules residing in this boundary layer at any given instant are considered to be motionally restricted and orientationally disordered compared to liquid-crystalline phospholipid molecules which are not in contact with the protein. It has been suggested that the Arrhenius plot breaks observed for many membrane-associated enzymes and transport systems may be triggered by a "phase transition" in the boundary lipid annulus rather than in the bulk lipid phase (Hidalgo, 1985; Charnock et al., 1982). We believe this suggestion to be extremely implausible for a number of reasons. Calorimetric studies have revealed that the boundary lipid surrounding most integral membrane proteins either does not undergo a cooperative gel to liquid-crystalline phase transition at all or undergoes a rather uncooperative, partial hydrocarbon chain-melting process at temperatures *below* the bulk lipid phase transition [see McElhaney (1986)]. This result has been confirmed by numerous spectroscopic studies, which have shown as well that the bulk and boundary lipids undergo an extremely rapid exchange of the order of 10^6–10^7/s (Bloom & Smith, 1985; Marsh, 1985). Thus, the physical behavior of the boundary lipids, although different in detail from that of the bulk lipids, reflects the overall physical properties of the bulk lipid with which they are in dynamic equilibrium. Therefore, in the case of the liver mitochondrial inner membrane of nonhibernating ground squirrels, the possibility of the existence of a discrete, highly cooperative, boundary lipid phase transition, which would occur some 25 °C *above* the phase transition temperature of the bulk lipids, would seem very remote. Moreover, even if such a phase transition did occur, it seems unlikely that it would not also have been detected by the ^{19}F NMR and DPH fluorescence polarization techniques employed here, which in other membrane systems can monitor both the bulk and boundary lipid domains. It would also be difficult to explain how a boundary lipid phase transition could be selectively and completely abolished in the membranes of hibernating animals when the composition and physical properties of the bulk (and presumably also the boundary) lipids are almost identical in both summer and hibernating animals.

In summary, we conclude that the Arrhenius plot breaks observed in enzymatic and transport activities of the liver inner mitochondrial membranes of nonhibernating squirrels are not the result of a lipid phase transition and that a major shift in

the gel to liquid-crystalline lipid phase transition temperature is not responsible for seasonal changes in the thermal behavior of these inner mitochondrial membrane proteins.

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