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FLUORESCENCE POLARIZATION STUDY OF LIPIDS AND MEMBRANES PREPARED FROM BRAIN HEMISPHERES OF A HIBERNATING MAMMAL

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SUMMARY : The physical behavior of total lipids, microsomes and microsomal lipids prepared from brain hemispheres of European Hamsters (*Cricetus cricetus*) was approached by the measure of the fluorescence polarization of the probe 1,6-diphenyl 1,3,5-hexatriene. We compare in this study the results obtained for two critical periods for a hibernator : winter (torpid state) and summer (active state). An increase in fluidity was noticed in the winter lipid and membrane preparations. The difference was however of very low magnitude, suggesting that only the microenvironment of some proteins was involved, rather than the bulk membrane fluidity.

The study of membrane fluidity, by a variety of physical techniques, has shown that it was markedly temperature-dependent : a decrease in temperature results in a decrease of membrane fluidity. When studied at the same temperature, the membranes of cold-adapted organisms are more fluid than the membranes of warm-adapted organisms. This has been shown in bacteria (1, 2), in yeasts such as *Tetrahymena* (3), in plants (4) and in poikilothermic vertebrates such as Goldfish (5). Therefore, cold-adapted organisms are able to maintain the fluid liquid-crystalline state of their membranes. This phenomenon has been called homeoviscous adaptation by Sinensky (6). This adaptation may result from several mechanisms. An increase of the unsaturation of membrane phospholipid acyl groups correlates with the lowering of the acclimation temperature in numerous species (3, 4, 7-10) and is considered to be responsible for the maintenance of the fluid liquid-crystalline state of the membranes of these organisms. Membrane fluidity may also be increased by other mechanisms such as reduction of hydrocarbon chain length (11), insertion of spacer molecules like cholesterol (12) or modification of the proportion of phospholipid polar head groups (13).

If such a membrane adaptation can exist in mammals, one can expect to find in hibernators similar results as in other organisms. Hibernating mammals are able to lower their central temperature in winter, down to 6-7°C. However, the changes in lipid composition noticed during the circannual rhythm in hiber-

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nator tissues are, when present, of very low magnitude, especially in brain (14-18) and it is doubtful if these changes are high enough to be responsible for changes in bulk membrane fluidity. The variations of membrane fluidity in hibernators has only been studied in brain of Hamsters by Goldman and Albers (19), who observed an increase in fluidity in hibernating animals ; by Raison et al. (20) in Ground Squirrels myocardial membranes, which similarly exhibit an increase of fluidity in winter ; and by Cossins and Wilkinson (21) who observed no change in membrane fluidity between active and torpid Golden Hamsters brain synaptosomes and kidney microsomes. We have already presented the lipid modifications encountered in the brains of European Hamsters during the course of the year (18) ; we present here the variations in membrane fluidity, as measured by fluorescence polarization of diphenylhexatriene, in summer (active) and winter (torpid) European Hamsters brain lipids and membranes. Fluorescence polarization is a widely used method to study the movements of a fluorescent probe embedded in a phospholipid bilayer (22).

MATERIALS AND METHODS

Animals. The wild European Hamsters (*Cricetus cricetus* L.) used in this study are those which were used in our previous study (18). One hemisphere was used for the extraction of lipids, and the other one for the subcellular fractionation. The study was limited to the animals sacrificed in summer, between July 12 and July 24 and in winter (torpid state only) between January 29 and February 12.

Lipid and membrane preparations. Lipids were extracted according to Folch et al. (23) as already described (18). Lipid phosphorus was assayed according to Macheboeuf and Delsal (24) . Brain microsomes were prepared according to De Robertis et al. (25) with the minor modifications already described.

Fluorescent labelling of lipids and membranes. Aliquots containing 5 mg of total brain lipids were taken to dryness and the residue suspended in 1 ml of 20 mM phosphate buffer, pH 7.5, containing 1 mM EDTA (buffer A) by vigorous stirring on a Cyclo-mixer. To this suspension, we added 10 µl of 6 mM 1,6-diphenyl 1,3,5-hexatriene (DPH, Ega Chemie) dissolved in tetrahydrofuran (THF, Merck). The mixture was protected from light and vigorously stirred. 50 µl of this preparation were added to 3.95 ml of buffer A and placed in a cuvette. For microsomal membranes, 10-20 µl of 0.2 mM DPH in THF were added to 0.5 ml of the microsomal preparation containing 1.5 to 3 mg proteins per ml, so that the expected molar ratio between membrane lipids and DPH was > 250, assuming a weight ratio phospholipids/proteins of 0.5. An incubation was preformed at 37°C during 20 min to ensure equilibration of DPH in membranes. 100-200 µl of this mixture were added to 3.80-3.90 ml of buffer A and placed in a cuvette. For microsomal lipids, 1-1.5 mg of lipids were taken to dryness and suspended in 1 ml of buffer A. 20-40 µl of 0.2 mM DPH in THF were added. 200-400 µl of this preparation was completed to 4 ml with buffer A and placed in a cuvette.

Fluorescence polarization spectroscopy. The measurements were performed on a Jobin-Yvon spectrofluorometer, model JY 3. Excitation was performed at 360 nm (slit 10 nm) ; emission was detected at 450 nm (slit 10 nm). The excitation light was polarized vertically and the polarized emission was observed through an analyzer oriented either in a parallel direction (I_{VV}) or perpendicularly (I_{VH}) to the direction of the excitation light. The fluorescence polarization primary value was defined by the equation : $P_M = I_{VV} - I_{VH} / I_{VV} + I_{VH}$. This value must be corrected by the value P_T obtained when the excitation light was polarized horizontally and the polarized emission was observed through an analyzer oriented either perpendicularly (I_{HV}) or in a parallel direction (I_{HH}) to the excitation light : $P_T = I_{HV} - I_{HH} / I_{HV} + I_{HH}$. The corrected

fluorescence polarization P_V was given by the equation : $P_V = P_M - P_T / 1 - P_M.P_T$. We have then calculated the fluorescence anisotropy r which was given by the formula : $r = 2 P_V / 3 - P_V$. A precise control of the temperature of the mixture was provided by a water bath circulating around the jacketted cuvette (Huber) ; a digitalic thermistance (Comark) was placed in the liquid of the cuvette just above the excitation beam. Measurements were done at 2°C intervals between 0°C and 50°C. Exposure to the excitation beam was limited to approximately 10 sec for each measurement in order to prevent photoisomerization. Light scattering was measured and evaluated to ca. 1 % of the fluorescence intensity values, and was not taken into account for the calculations. The optical density of the microsomal preparations was measured at 360 nm ; it was found between 0.2 and 0.3 for all preparations. We have verified that these optical densities did not modify significantly the P_V values by measuring this value for the same preparation at 5 different dilutions (optical densities ranging from 0.04 to 0.36) and 5 different temperatures. Identical P_V values were obtained over the whole range of dilutions for each temperature.

RESULTS

The fluorescence anisotropy values obtained for brain total lipids, microsomes and microsomal lipids from active and torpid European Hamsters are plotted on a logarithmic scale (figures 1-3) as a function of the inverse of the absolute temperature (Arrhenius plotting). Each point is the mean of the values obtained from the brain lipids of three animals or from the brain microsomes and microsomal lipids of four animals. For each preparation, determinations were performed in triplicate or in duplicate.

For each type of preparation, the Arrhenius curves obtained in both physiological states of the European Hamster are parallel. In each case, the winter

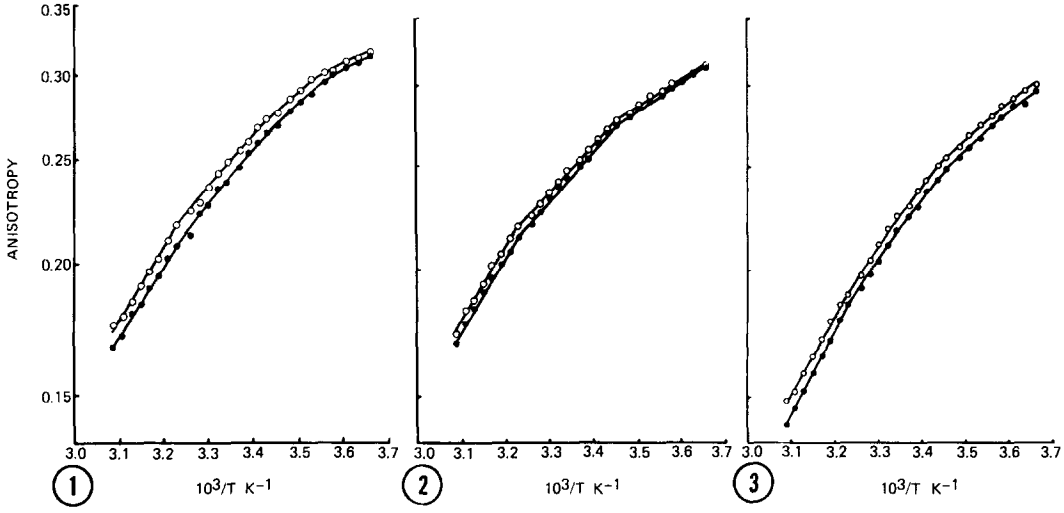

Figure 1 : Arrhenius plots of the DPH anisotropy in whole brain total lipids of hibernating torpid Hamsters (●) and active Hamsters (○).

Figure 2 : Arrhenius plots of DPH anisotropy in brain microsomal membranes of hibernating torpid Hamsters (●) and active Hamsters (○).

Figure 3 : Arrhenius plots of DPH anisotropy in brain microsomal lipids of hibernating torpid Hamsters (●) and active Hamsters (○).

curves are shifted to lower r values ; these differences between summer and winter animals are significant when the paired values are compared with the Student's t test ($P < 0.0001$). It must be underlined however that the difference is of very low magnitude and reaches only 2.5-5 % of the value of r for each type of preparation.

DISCUSSION

In the various organisms ranging from bacteria to poikilothermic vertebrates, it has been shown that the lipid membrane viscosity, as measured by physical techniques, is decreased when the organism studied is adapted to lower environmental temperatures (1-5). When using fluorescence spectroscopy, this is evident both by a shift of the curve to lower anisotropy values and by a decrease of the temperature of the break points noticed on the curve.Several interpretations have been given to the meaning of the Arrhenius breaks of such curves ; they can be considered to characterize a phase separation process occurring in membrane lipids. We noticed that a slight shift was present between the curves obtained for DPH anisotropy in lipids and membranes from torpid and active Hamsters brains. The shift of the Arrhenius plots is about 2-4°C for a difference in body temperature of 30°C and may not be interpreted as a homeoviscous adaptation. This difference between torpid and active Hamster brain lipids and membranes may be related to the differences in acyl group unsaturation or phospholipid polar head group distribution we had noticed (18). One can also hypothesize that the differences observed in cholesterol level between summer and winter brain lipids could be responsible for the differences in DPH anisotropy curves.

Goldman and Albers (19), working on DPH polarization in Golden Hamster ATPase-enriched brain membranes, observed a shift of the Arrhenius curves in hibernating animals, both for lipid or phospholipid preparations or for membranes. The difference between the winter and the summer curves is in the same range as that we observed for brain lipids and membranes of European Hamsters. These differences between active and torpid hibernators are much lower than those observed in lipids or membranes of Goldfish adapted to various environmental temperatures (5). The work of Raison et al. (20) also compared active and torpid hibernators. They observed an important increase in fluidity in membranes isolated from myocardial membranes of hibernating Ground Squirrels. Cossins and Wilkinson (21) studied DPH polarization in Golden Hamsters brain synaptosomes and kidney microsomes. They noticed no difference at all between active and torpid animals, and they concluded that hibernators did not use this process of homeoviscous adaptation of their membranes during hibernation. We think however that some tissues and some membrane fractions are subjected to low magnitude changes, which may be evidenced by chemical analysis and physical

properties. It is likely that these changes affect rather the microenvironment of some proteins than the bulk membrane fluidity, as suggested by Aloia (15).

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