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A STEADY STATE AND DIFFERENTIAL POLARISED PHASE FLUORIMETRIC STUDY OF THE LIVER MICROSOMAL AND MITOCHONDRIAL MEMBRANES OF THERMALLY ACCLIMATED GREEN SUNFISH (*LEPOMIS CYANELLUS*)

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Summary

The liver mitochondrial and microsomal membranes of green sunfish and rat were examined by steady state polarisation and differential polarised phase fluorimetry to determine the effects of seasonal adaptation of membrane dynamic structure to temperature. Steady state polarisation studies indicated that the liver mitochondria of green sunfish acclimated to different temperatures showed a greater partial compensation of membrane fluidity for the altered acclimation temperature than did liver microsomal membranes. The fatty acid composition of both membrane preparations generally became more unsaturated at lower acclimation temperatures, though the differences between 5°C and 25°C acclimated fish were more pronounced in the mitochondrial fraction than in the microsomal fraction.

Differential polarised phase fluorimetric studies indicated that the rotations of diphenylhexatriene in mitochondrial and microsomal membranes were highly hindered, though the hindrance offered by membranes of 25°C acclimated green sunfish was far greater than that offered by the membranes of 5°C acclimated fish, thus supporting the concept of homeoviscous adaptation. The absolute rotational rate was not consistently affected by acclimation treatment.

Introduction

It is generally recognised that biological membranes play an important role in cellular adaptation to a variety of environmental stimuli. Perhaps the best

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic characterised adaptive response is the alteration in the quantity and types of membrane lipids induced during the thermal acclimation of microorganisms, plants and poikilothermic animals. In general, the proportion of *cis*-unsaturated fatty acids of the major glycerophospholipid fractions increases during cold acclimation and vice versa [1]. By inference from studies with model membrane systems [2] this alteration of membrane composition has been assumed to be an adaptive response which compensates membrane structure, and hence function, for the direct effects of the chronic temperature change [1,3,4]. Indeed, the recent development of spectroscopic techniques for the detection of molecular motion within the bilayer has permitted the direct demonstration in *Escherichia coli* [5], *Bacillus stearothermophilus* [6], *Tetrahymena pyriformis* [7,8] and in goldfish brain synaptosomes [9] of such compensatory responses in membrane fluidity.

Studies of the effects of thermal acclimation on membrane dynamic structure have used both electron spin resonance [5—7] and steady state fluorescence polarisation techniques [9—13]. The interpretation of fluorescence polarisation of membrane-bound fluorescent probes depends upon the validity of a number of assumptions which are not upheld by recent time-resolved studies [8,14—19]. Thus previous studies which demonstrated differences in the fluidity of cold and warm acclimated fish by measurements of the apparent rotational diffusion coefficient ($\overline{R}$) are open to alternative interpretation. A more valid and detailed picture of the rotational motions of diphenylhexatriene, and by inference, the characteristics of its lipid environment, may be obtained by the combined use of steady state and dynamic methods of analysis [17]. Differential polarised phase fluorimetry has been used in the present study in conjunction with a new theory by Weber [20,21] for the interpretation of the results in order to describe more fully the adaptive alterations in membrane structure.

We describe here further studies on the effects of thermal acclimation upon the lipid composition and membrane fluidity of liver microsomes and mitochondria of green sunfish. These membrane fractions are thought to be predominantly derived from a single cell type, the parenchymal cell, and comparison of their respective adaptive responses may provide some information on the nature of cellular responses to altered temperature. An important aspect of compositional and structural studies such as those presented here, is the purity of the membrane fractions, a point that has often not been considered in some previous compositional studies. Considerable difficulties were encountered with green sunfish liver in preparing a microsome-free mitochondrial preparation by the usual differential centrifugation techniques and we describe a Ficoll gradient technique which largely overcomes these problems. Studies of mitochondrial function are of particular interest because of their importance to the energy metabolism of the cell and also because they show significant adaptive responses during thermal acclimation in a variety of respects [1,22,23]. Several recent studies have demonstrated changes in lipid composition of mitochondrial glycerophospholipids with thermal acclimation [4,24—26] and some workers have attempted to correlate this with adaptations of mitochondrial function [25—28]. In contrast, Van den Thillart and Modderkolk [29] maintain that the fatty acid composition of mitochondrial total lipids was unaffected by thermal acclimation.

Methods

Animals

Green sunfish (*Lepomis cyanellus*, 6—15 cm total length) were obtained from the Illinois Natural History Survey during July and August 1978. They were maintained for at least seven days at 15 ± 1°C in the laboratory. For acclimation, fish were transferred, in the 15°C aquaria, to constant temperature rooms at either 5 ± 1°C or 25 ± 1°C where they were kept for at least 21 days. The water temperature became equilibrated to their respective acclimation temperatures within 24 h. The fish were kept under a 12 h light: 12 h dark cycle with artificial dawn and dusk, and were fed daily at 15°C and 25°C and once every two days at 5°C with Purina trout chow pellets.

Preparation of purified mitochondrial fraction

Green sunfish were killed by a blow to the head followed by bisection of the spinal cord. Livers were rapidly excised (taking great care not to disturb the gall bladder), diced with a knife and then homogenised in 10 vols. (w/v) ice-cold isolation medium (220 mM sucrose, 1 mM EDTA, 2 mM Hepes, 0.5% (w/v) fat-free bovine serum albumin (fraction V, Sigma) and KOH to pH 7.2) with 10 passes of a glass-Teflon homogeniser (Thomas type C) rotating at approx. 120 rev./min. The crude homogenate was centrifuged in 15 ml polypropylene centrifuge tubes at $600 \times g$ for exactly 10 min, in a Sorvall SS-34 rotor. The supernatant was carefully removed with a Pasteur pipette and centrifuged at $6800 \times g$ for 10 min. The surface of the loose tan pellet was gently rinsed with the supernatant to resuspend the 'fluffy' layer and the supernatant was carefully removed with a Pasteur pipette. The crude mitochondrial pellet was resuspended in 3 ml isolation medium using a Pasteur pipette and layered on a five-step Ficoll gradient (2.5 ml each of 15%, 13%, 11%, 9% and 7.5% Ficoll, all diluted with distilled water from a stock solution of 25% Ficoll in 0.25 M sucrose) which had been allowed to diffuse at room temperature for 12—15 h. The gradient was centrifuged in a Beckman SW27.1 rotor at 5°C and 12 500 rev./min ($30\,000 \times g_{max}$) for 30 min. The supernatant containing a fluffy layer was aspirated and the pellet was resuspended in a small volume of isolation medium, gently hand-homogenised in an all-glass homogeniser, diluted with 25—30 ml isolation medium and finally centrifuged at $14\,600 \times g$ for 30 min. The purified mitochondrial fraction was gently resuspended in approx. 0.5 ml isolation medium using a Pasteur pipette and stored on ice until required. Rat livers were homogenised in a high-osmolarity medium (280 mM sucrose 0.2 mM EDTA, 2 mM Hepes/KOH, pH 7.2, and 0.05% bovine serum albumin) and treated as described for green sunfish liver.

Preparation of microsomal fraction

The crude mitochondrial supernatant was centrifuged at $14\,600 \times g$ for 30 min to pellet remaining mitochondria. The supernatant was centrifuged at $105\,000 \times g$ for 60 min in a Beckman Type 40 fixed angle rotor and the pink microsomal pellet was resuspended in 0.5 ml isolation medium, hand-homogenised in an all-glass homogeniser and stored on ice until required. Care was taken not to resuspend a clear glycogen pellet that lay beneath the microsomal layer since glycogen seriously interferes with the fluorescence studies by scattering incident light.

Enzyme assays

Rotenone-insensitive NADPH : cytochrome *c* oxidoreductase was assayed at 15°C in a medium which contained 1.25 ml 0.1 M phosphate buffer pH 7.0, 0.2 ml 37.5 mM KCN, 0.05 ml cytochrome *c* (14 mg/ml), 0.01 ml 1.5 mM rotenone in ethanol and 0.01 ml 20 mM NADPH. The substrate was added last and the change in absorbance at 550 nm was monitored against a blank cuvette containing the same constituents except substrate. Succinate : cytochrome *c* oxidoreductase was assayed as above except that rotenone was omitted and 0.01 ml 1 M succinate was used as substrate. Oxygen uptake by the crude and purified mitochondrial preparations was assayed at 15°C using a Yellow Springs Oxygen Monitor. The reaction medium contained 3 ml isolation medium and 0.3 ml 100 mM phosphate buffer, pH 7.0. Sodium succinate and ADP were added sequentially to a final concentration of 10 mM and 50 mM, respectively. Respiratory control and rate of oxygen consumption were calculated as described by Chance and Williams [30].

Lipid analyses

Membrane lipids were extracted, the major glycerophospholipid fractions were purified by thin-layer chromatography and their respective fatty acid compositions were analysed by gas-liquid chromatography exactly as described previously [9,31]. The fatty acid composition is reported as mol%.

Labelling of membranous fractions

The microsomal and mitochondrial fractions were diluted in a 10 mm path length quartz cuvette with 2.2 ml of a medium containing 20 mM sucrose, 1 mM KCN and 0.1 M phosphate buffer, pH 7.2 (osmolarity 247 mosmol · kg^{-1}). 2 µl of a 2 mM all-*trans*-1,6-diphenylhexa-1,3,5-triene (Aldrich 'puriss' grade) in glass distilled tetrahydrofuran was added with vigorous stirring and the cuvettes were incubated for 10 min at room temperature.

Fluorescence polarisation

Steady state fluorescence polarisation was measured as described previously [9] on the T-format, photon-counting polarisation photometer described by Jameson et al. [32] except that the excitation wavelength (358 nm) was filtered by a Corning 7-54 broad band pass filter which considerably reduced the higher order diffraction wavelengths in the incident beam. Scattered light comprised less than 0.5% of total detected light and correction of polarisation values were not necessary. The rotational diffusion coefficient ($\overline{R}$) and the microviscosity (η) were calculated as described previously [9].

Fluorescence lifetimes

Fluorescence lifetimes were measured directly in an updated version of the cross-correlation phase and modulation fluorimeter described by Spencer and Weber [33] with improved electronics from SLM Instruments, Urbana, IL. The excitation wavelength (358 nm) was selected from a 450 Watt xenon arc lamp by a monochromator (0.25 m, bandpass approx. 2 nm) and modulated at 18 MHz by a Sears-Debye ultrasonic light modulator. Emission and excitation filters were as described for steady state polarisation measurements. Lifetimes were measured both by the phase shift and by the demodulation of the fluorescence of the diphenylhexatriene-loaded membrane suspension relative to a scattering suspension of glycogen of approximately equal emission intensity [33]. Measurements were made continuously until stable values were attained.

Differential polarised phase fluorimetry

The experimental technique of differential phase fluorimetry has been fully described elsewhere [17,18,34]. Briefly, the fluorescence observed through polarisers oriented parallel and perpendicular to the plane of polarisation of the excitation light decay at different rates. This is because the parallel polariser tends to observe those fluorophores which have emitted their photon before much rotation has occurred whereas the perpendicular polariser tends to select those fluorphores which have rotated significantly during their excited lifetime. In systems where the fluorophore is excited by a modulated light source, this difference in lifetimes is observable as a differential delay of the modulated fluorescence with respect to the excitation; that is, the phase delay or phase angle of the parallel component is less than that of the perpendicular component. This difference in phase delays is referred to as the differential tangent ($\tan \Delta$) which is related to the difference in the average lifetimes of the differently polarised components ($\Delta\tau$) by

$$\tan \Delta = 2\pi f \, \Delta\tau \qquad (1)$$

where f is the modulation frequency.

The differential lifetime of the vertically and horizontally-polarised components of the modulated fluorescence were measured on the same instrument as used for the lifetime measurements, except that the excitation light was polarised with a double Glan-Foucault prism and the emission was analysed with a single Glan-Taylor prism polariser and monitored with a Phillips XP1023 photomultiplier. Emission and excitation filters were as described previously. The instrument was used in a single-channel mode where an internal frequency synthesiser provided a reference signal. The emission polariser was first orientated parallel to the vertically-oriented excitation polariser and the phase delay was determined with reference to the internal time base. (Each value represented the average of 100 separate measurements thus improving the signal/noise ratio 10-fold.) The emission polariser was then rotated by 90° and the phase delay of the horizontally-polarised fluorescence was determined, again relative to a reference time base (average of 100 measurements). The differential phase delay between the vertically and horizontally-polarised components of the emission was determined by subtraction and the result was displayed as the differential lifetime. This procedure was repeated until a satisfactory average differential lifetime with a standard deviation of less than 0.05 ns was obtained.

The maximum theoretical values of the differential lifetime ($\Delta\tau_{\max}$) was calculated according to Equation 11 of Ref. 17. The rotational rate of the probe (R) and the non-zero limiting anisotropy (r_∞) at times which are long compared with the fluorescence lifetime, were calculated by combining steady state and differential lifetime measurements as described by Lakowicz et al. (Ref. 17, Eqns. 14 and 18). The average amplitude of the angular distribution of the probe about an axis normal to the phase of the membrane at times which are long compared with fluorescence lifetime was calculated as described by Weber (Ref. 21, Eqn. 16).

Results

Isolation of membranous preparations

The fluorescence and biochemical techniques used in this study sampled all membrane-types present in each membranous preparation. Hence, purity of each membranous fraction is crucial to the sensible interpretation of the results and much attention was paid to this factor. The separation of mitochondrial and microsomal membranes was assessed by measuring the activity of the microsomal marker enzyme rotenone-insensitive NADPH : cytochrome *c* oxidoreductase and the mitochondrial marker enzyme succinate : cytochrome *c* oxidoreductase (see Table I). The isolation medium had an osmotic concentration which was identical to that of green sunfish plasma (245—250 mosmol · kg^{-1}).

The simple differential centrifugation scheme described previously, proved satisfactory for the isolation of a microsomal fraction with virtually no detectable mitochondrial marker enzyme activity. Electron microscopic examination of the microsomal pellet confirmed the total absence of mitochondrial contamination. By contrast, the classical differential centrifugation scheme produced a ‘crude’ mitochondrial preparation which was heavily contaminated with microsomes such that the respective marker enzyme activities were almost equal. This mitochondrial preparation showed vigorous respiratory activity with succinate as substrate and a respiratory control index of 4—5. Modification of centrifugation speed or time gave relatively little improvement in the

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES FOR A MICROSOMAL FRACTION AND A PURIFIED MITOCHONDRIAL FRACTION ISOLATED FROM THE LIVERS OF DIFFERENTLY ACCLIMATED GREEN SUNFISH AND FROM RAT

Mitochondria were prepared by the Ficoll gradient technique and microsomes by differential centrifugation (see Methods). Values represent mean ± S.E.M. for three preparations. Values are expressed as nmol cytochrome *c*/mg protein per min. n.d., not detectable.

Acclimation temperature	Expt. No.	Mitochondria		Microsomes	
		Succinate: cytochrome *c* reductase	NADPH: cytochrome *c* reductase	Succinate: cytochrome *c* reductase	NADPH: cytochrome *c* reductase
5°C	1	104.5 ± 11.2	5.3 ± 1.5	0.2 ± 0.04	17.0 ± 4.7
	2	38.4 ± 1.0	2.7 ± 0.5	n.d.	15.5 ± 3.2
25°C	1	39.6 ± 5.9	3.0 ± 1.0	n.d.	8.5 ± 1.2
	2	17.1 ± 4.4	1.7 ± 0.4	n.d.	11.1 ± 1.9
Rat		64.8 ± 2.4	4.5 ± 1.0	n.d.	20.6 ± 1.9

separation and it proved necessary to resort to a density gradient centrifugation scheme using a discontinuous Ficoll gradient prepared by dilution with distilled water of 24% Ficoll in 0.25 M sucrose. Presumably, a simpler Ficoll gradient would suffice to pellet the purified mitochondrial preparation although this was not attempted. Adjustment of the Ficoll osmolarity with sucrose to produce an isosmotic gradient with respect to the isolation medium resulted in poor separation of mitochondria and microsomes. Some variability in solution colour was experienced with different Ficoll lots which appeared to result in inferior separation of mitochondria and microsomes. Only Ficoll solutions which were totally colourless were used. The mitochondrial preparation isolated by the Ficoll gradient technique, possessed high succinate : cytochrome *c* oxidoreductase and considerably reduced rotenone-insensitive NADPH : cytochrome *c* oxidoreductase, which indicated at least a 10-fold improvement in mitochondrial purity compared to the crude mitochondrial fraction. The contaminating microsomes usually formed a band at approx. 7—9% Ficoll and were easily decanted leaving a tightly packed mitochondrial pellet. The purified mitochondria exhibited vigorous state IV respiration which, however, was not stimulated by addition of ADP.

Steady state polarisation studies

The steady state polarisation and fluorescence lifetimes were measured simultaneously on microsomal and mitochondrial preparations isolated from the liver of 5, 15, 25 and 34°C acclimated green sunfish and from rat. The computed average values for the rotational diffusion coefficient ($\bar{R}$), equivalent

TABLE II

AVERAGE ROTATIONAL DIFFUSION COEFFICIENT ($\bar{R}$), MICROVISCOSITY (η) AND FLUORESCENCE LIFETIMES BY PHASE (τ_{phase}) AND BY MODULATION TECHNIQUES (τ_{mod}) FOR DIPHENYLHEXATRIENE IN MITOCHONDRIAL AND MICROSOMAL PREPARATIONS FROM THE LIVER OF DIFFERENTLY ACCLIMATED GREEN SUNFISH AND RAT

All measurements were performed at 15°C. Values represent means ± S.E.M. of *n* preparations.

Acclimation temperature	$R \times 10^7$ (s^{-1})	η (poise)	τ_{phase} (ns)	τ_{mod} (ns)
Microsomes				
Green sunfish				
5°C (*n* = 6)	2.92 ± 0.14	1.42 ± 0.07	8.34 ± 0.06	8.54 ± 0.07
15°C (*n* = 3)	2.81 ± 0.04	1.47 ± 0.01	7.31 ± 0.09	8.10 ± 0.05
25°C (*n* = 6)	2.64 ± 0.11	1.58 ± 0.07	8.25 ± 0.05	8.51 ± 0.06
34°C (*n* = 3)	2.05 ± 0.01	2.01 ± 0.01	7.52 ± 0.06	8.25 ± 0.09
Rat (*n* = 3)	2.99 ± 0.11	1.39 ± 0.05	7.84 ± 0.10	7.84 ± 0.07
Mitochondria				
Green sunfish				
5°C (*n* = 6)	3.91 ± 0.12	1.06 ± 0.03	6.83 ± 0.12	7.50 ± 0.16
15°C (*n* = 3)	3.37 ± 0.29	1.24 ± 0.10	6.12 ± 0.08	7.02 ± 0.06
25°C (*n* = 6)	2.98 ± 0.13	1.40 ± 0.06	7.03 ± 0.06	7.89 ± 0.20
34°C (*n* = 3)	2.33 ± 0.15	1.79 ± 0.13	5.83 ± 0.13	7.15 ± 0.05
Rat (*n* = 3)	2.88 ± 0.05	1.43 ± 0.03	6.92 ± 0.05	7.29 ± 0.03

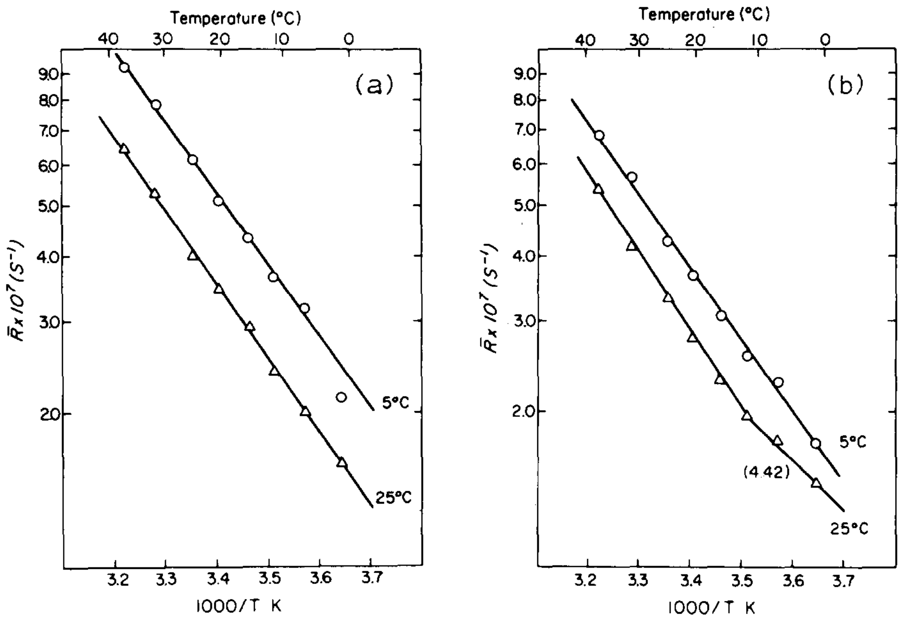

Fig. 1. Typical Arrhenius plots for the rotational diffusion coefficient ($\bar{R}$) of diphenylhexatriene in the mitochondrial (a) and microsomal fractions (b) isolated from the liver of 5° C and 25° C acclimated green sunfish. Each curve represents data obtained from a single preparation.

microviscosity (η) and fluorescence lifetimes as measured by both phase and modulation methods are all presented in Table II.

The liver mitochondria of green sunfish showed considerable differences in their average $\bar{R}$ values depending upon their previous thermal history. Thus when measured at 15°C, mitochondria from sunfish acclimated to 5°C possessed greater rotational diffusion coefficients and, by inference, a less restrictive lipid environment than mitochondria from 25°C acclimated sunfish ($t = 5.32$, $P < 0.001$). The increment in $\bar{R}$ for a given change in acclimation temperature was relatively constant over the entire 5—34°C range of acclimation temperature. The difference in lifetimes measured by the phase and modulation methods remained relatively constant at approx. 0.6—0.9 ns, which as before [9,10] is taken to indicate only a small degree of heterogeneity of diphenylhexatriene environments. Fig. 1a illustrates the dependence of $\bar{R}$ upon measurement temperature for single preparations isolated from 5°C and 25°C acclimated green sunfish. The difference in $\bar{R}$ between acclimation groups noted previously was maintained over the entire 5—38°C range of measurement temperatures. Preparations of 5°C and 25°C acclimated green sunfish exhibited linear Arrhenius plots with very similar Arrhenius activation energies of 28.7 and 27.5 kJ · mol^{-1}, respectively.

Liver microsomes showed much reduced differences in $\bar{R}$ values except at the highest acclimation temperatures (Table II). Thus, microsomes of 5°C and 25°C acclimated sunfish were not significantly different ($t = 1.6$, $P > 0.1$) but microsomes of 34°C acclimated sunfish were significantly less fluid at 15°C than those of 25°C acclimated green sunfish ($t = 3.66$, $P = 0.01—0.001$). The differences in fluorescence lifetimes determined by phase and modulation methods were very small for 5°C and 25°C acclimated fish (<0.3 ns) but were somewhat larger for the 15°C and 34°C acclimated fish (approx. 0.7 ns). Indeed, for many preparations the lifetimes determined by the two methods were identical within statistical error, suggesting that the heterogeneity of probe environments was considerably smaller or even absent in microsomes compared to mitochondria. Fig. 1b presents linear Arrhenius plots of $\overline{R}$ for microsomes of both 5°C and 25°C acclimated green sunfish, with similar activation energies of 26.7 and 27.9 kJ · mol^{-1}, respectively. Below 12°C, the slope for the 25°C acclimated preparation was somewhat reduced to 18.5 kJ · mol^{-1}.

For comparative studies, the microsomal and mitochondrial membrane fractions of rat liver were prepared by identical techniques except that an isolation medium of higher osmolarity was used. For rat liver mitochondria the average $\overline{R}$ value at 15°C fell in between those of 25°C and 34°C acclimated green sunfish, whilst for liver microsomes it was greater than for the corresponding membrane fraction of 5°C acclimated green sunfish. Notably, the lifetimes derived by phase and modulation methods were identical for rat liver microsomes and were only slightly different for mitochondria.

TABLE III

THE FATTY ACID COMPOSITION (mol%) OF THE MAJOR MEMBRANE PHOSPHOLIPID FRACTIONS ISOLATED FROM THE LIVER MICROSOMAL FRACTION OF 5°C AND 25°C ACCLIMATED GREEN SUNFISH AND RAT

Values represent the mean ± S.E.M. for *n* separate preparations.

Fatty acid	Choline phosphoglycerides			Ethanolamine phosphoglycerides		
	5°C Green sunfish (*n* = 4)	25°C Green sunfish (*n* = 4)	37°C Rat (*n* = 3)	5°C Green sunfish (*n* = 3)	25°C Green sunfish (*n* = 4)	37°C Rat (*n* = 3)
16:0	20.2 ± 1.2	21.9 ± 0.7	21.1 ± 3.4	7.5 ± 1.6	13.9 ± 0.8	16.1 ± 1.0
16:1	10.2 ± 0.8	5.1 ± 0.5	0.7 ± 0.5	3.0 ± 1.1	3.5 ± 0.6	0.7 ± 0.5
18:0	6.0 ± 0.6	6.4 ± 0.5	25.1 ± 2.8	8.9 ± 0.5	13.3 ± 1.5	28.3 ± 0.2
18:1	18.4 ± 0.5	11.5 ± 0.8	7.4 ± 1.0	22.4 ± 0.7	13.4 ± 0.7	3.9 ± 0.0
18:2ω6	7.3 ± 0.4	15.4 ± 0.8	12.5 ± 1.0	5.9 ± 0.7	10.0 ± 1.4	5.5 ± 0.2
18:3ω3	2.0 ± 0.2	2.5 ± 0.3	—	7.7 ± 0.3	4.6 ± 0.5	0.1 ± 0.0
20:4ω6	0.8 ± 0.1	2.5 ± 0.3	23.9 ± 1.8	3.0 ± 0.6	5.0 ± 0.4	23.6 ± 1.0
20:5ω3	0.7 ± 0.1	1.3 ± 0.1	0.4 ± 0.1	1.4 ± 0.4	1.3 ± 0.2	0.8 ± 0.2
22:5ω3	1.6 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 1.1	1.6 ± 0.1
22:6ω3	28.8 ± 1.9	26.5 ± 0.8	6.9 ± 0.2	32.4 ± 1.7	27.6 ± 1.6	15.8 ± 0.4
Others *	4.0	6.7	1.4	6.9	6.6	3.6
Total saturated	26.7 ± 1.8	29.5 ± 0.7	46.8 ± 0.6	16.8 ± 1.8	28.0 ± 2.3	46.2 ± 0.6
Total monounsaturated	30.0 ± 0.7	17.6 ± 0.5	8.4 ± 0.4	27.5 ± 1.8	17.6 ± 1.0	5.7 ± 1.1
Total polyunsaturated	43.2 ± 2.5	52.9 ± 0.5	44.9 ± 1.0	55.7 ± 3.6	54.3 ± 2.8	48.1 ± 0.8
Unsaturation index **	245.7 ± 12.2	248.4 ± 4.6	177.5 ± 5.8	293.9 ± 13.2	270.0 ± 10.2	221.3 ± 2.9

* Other components present in trace quantities include 17:0, 17:1ω9, 20:2ω6, 22:0, 20:4ω3, 22:3ω6, 22:4ω6 and 22:5ω6.

** Unsaturation index was calculated as the sum of the mol% multiplied by the number of olefinic bonds for each fatty acid in the mixture.

Fatty acid composition

Earlier studies [9,10,12] have indicated that changes in the bulk fluidity of biological membranes with temperature acclimation were associated with shifts in fatty acid composition of the major glycerophospholipid classes and do not correlate well with changes in the serine and inositol phosphoglyceride classes. Thus the fatty acid compositions (mol%) of the choline phosphoglyceride and ethanolamine phosphoglyceride fractions only are presented in Tables III and IV for microsomal and mitochondrial fractions, respectively. For convenience, the data have been summarised as the total saturated, monounsaturated and polyunsaturated fatty acid fractions and as an unsaturation index.

Considering first the liver microsomes (Table III), the acyl composition of choline phosphoglycerides of 5°C sunfish was different in detail from 25°C sunfish, although the grouped data show very similar proportions of saturated and unsaturated fatty acids and almost identical unsaturation indices. There were some differences in the mono and polyunsaturated fatty acid fractions. For ethanolamine phosphoglycerides, the 5°C-acclimated sunfish exhibited a

TABLE IV

THE FATTY ACID COMPOSITION (mol%) OF THE MAJOR MEMBRANE PHOSPHOLIPID FRACTTIONS ISOLATED FROM THE LIVER MITOCHONDRIAL FRACTION OF 5°C AND 25°C ACCLIMATED GREEN SUNFISH AND RAT

Values represent the mean ± S.E.M. for *n* separate preparations.

Fatty acid	Choline phosphoglycerides			Ethanolamine phosphoglycerides		
	5°C Green sunfish (*n* = 4)	25°C Green sunfish (*n* = 4)	37°C Rat (*n* = 3)	5°C Green sunfish (*n* = 3)	25°C Green sunfish (*n* = 4)	37°C Rat (*n* = 3)
16:0	15.6 ± 1.4	20.6 ± 0.6	19.2 ± 1.9	9.3 ± 0.7	16.7 ± 1.1	15.3 ± 0.6
16:1	7.0 ± 1.0	5.0 ± 0.2	1.2 ± 0.1	1.8 ± 0.2	2.7 ± 0.9	0.4 ± 0.4
18:0	4.9 ± 0.5	5.9 ± 0.2	25.0 ± 1.4	12.3 ± 0.5	14.4 ± 0.6	28.7 ± 1.2
18:1	17.7 ± 0.6	11.7 ± 0.6	6.8 ± 0.3	17.0 ± 0.4	10.5 ± 0.6	4.3 ± 0.5
18:2ω6	7.0 ± 0.2	15.0 ± 0.9	11.6 ± 1.0	4.7 ± 0.1	9.6 ± 0.6	4.9 ± 0.5
18:3ω3	2.8 ± 0.3	2.7 ± 0.2	0.1 ± 0.1	6.4 ± 0.5	3.9 ± 0.3	0.1 ± 0.1
20:4ω6	0.9 ± 0.1	2.7 ± 0.2	24.6 ± 1.3	2.6 ± 0.2	5.0 ± 0.2	24.6 ± 1.0
20:5ω3	0.7 ± 0.1	1.5 ± 0.2	—	1.3 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
22:5ω3	2.0 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.3 ± 0.1
22:6ω3	37.7 ± 1.6	27.3 ± 0.5	7.4 ± 0.2	38.7 ± 1.0	30.2 ± 2.1	16.8 ± 0.5
Others *	3.7	6.3	3.4	5.1	5.5	2.8
Total saturated	21.4 ± 1.9	28.1 ± 0.5	46.2 ± 0.7	22.6 ± 1.2	32.6 ± 1.1	45.6 ± 0.9
Total monounsaturated	25.4 ± 0.9	17.5 ± 0.7	8.5 ± 0.2	19.6 ± 0.2	14.0 ± 1.7	5.3 ± 0.9
Total polyunsaturated	53.2 ± 2.1	54.5 ± 0.3	45.4 ± 0.7	57.9 ± 1.2	53.4 ± 1.6	49.1 ± 0.2
Unsaturation index **	297.4 ± 10.8	256.9 ± 3.0	180.8 ± 4.2	311.8 ± 6.7	264.4 ± 9.7	227.7 ± 1.26

* Other components present in trace quantities include 17:1ω9, 20:0, 20:2ω6, 22:0, 22:3ω3, 22:3ω6, 22:4ω6 and 22:5ω6.

** Unsaturation index was calculated as the sum of the mol% multiplied by the number of olefinic bonds for each fatty acid in the mixture.

much reduced proportion of saturated fatty acids and increased proportions of monounsaturated fatty acids compared to 25°C-acclimated sunfish, although the total polyunsaturated fatty acids were similar in both acclimation groups. The unsaturation index was only slightly greater in the 5°C acclimated sunfish.

The differences in lipid composition of differently acclimated sunfish were considerably greater in liver mitochondria (Table IV). For the choline phosphoglycerides, the preparations of 5°C acclimated sunfish contained much smaller proportions of saturated fatty acids and larger proportions of monounsaturated fatty acids compared to the corresponding preparations of 25°C acclimated sunfish. The proportions of total polyunsaturated fatty acids were almost identical, although alterations in the proportion of 22 : 6ω3 and 18 : 2ω6 resulted in a dramatic increase in the unsaturation index of the 5°C acclimated sunfish. A similar situation exists with the ethanolamine phosphoglycerides.

The corresponding data for rat liver microsomes and mitochondria are also presented in Tables III and IV for comparative purposes. In all instances rat membranes contained considerably greater proportions of saturated fatty acids, smaller proportions of unsaturated fatty acids and much smaller unsaturation indices than the sunfish membranes. The polyunsaturated fatty acids are composed mainly of arachidonic acid (20 : 4ω6) with much smaller proportions of 22 : 6ω3 than sunfish membranes.

Differential phase fluorimetric studies

The effect of temperature upon the differential lifetime ($\Delta\tau$) for single microsomal and mitochondrial preparations isolated from the liver of 5°C and 25°C acclimated green sunfish and for rat are presented in Figs. 2 and 3. For the green sunfish preparations the curves had a symmetrical bell-shape which passed through a maximum at temperatures between 10°C and 15°C (Table V), whilst for both rat preparations the dependence of $\Delta\tau$ upon temperature was a good deal more complex, with a dramatic increase in $\Delta\tau$ between 10°C and 18°C followed by a gradual decrease above 18°C. The preparations of 5°C acclimated green sunfish showed significantly greater values of $\Delta\tau$ over almost the entire temperature range compared to the corresponding preparations of 25°C acclimated green sunfish. The maximal observed values of $\Delta\tau$ are presented in Table V together with the corresponding theoretical values that were calculated assuming isotropic, unhindered rotations (see Methods). In all preparations the observed $\Delta\tau_{\max}$ achieved only half of their theoretical value (Table V) suggesting that the assumed unhindered rotational model for diphenylhexatriene in biological membranes, as in artificial bilayers [17,18], is inappropriate. Interestingly the observed $\Delta\tau_{\max}$ values for both microsomal and mitochondrial preparations of 5°C acclimated green sunfish were very similar as were the corresponding preparations of 25°C acclimated green sunfish.

Rotational rates (R) and the limiting anisotropy (r_∞) were calculated by combining data from both steady state (r) and differential phase measurements ($\Delta\tau$ and τ) using the theory of Weber [21] as extended by Lakowicz et al. [17]. The effect of temperature upon rotational rates are presented in Fig. 4 as Arrhenius plots which for all preparations were curvilinear. For the mitochondrial preparation, (Fig. 4b) the Arrhenius plots for the 5°C and 25°C

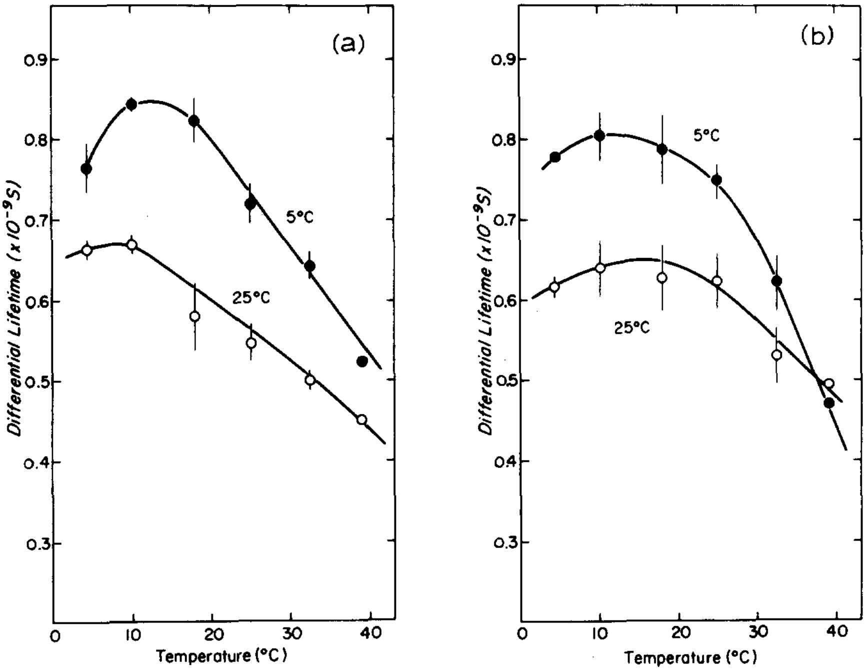

Fig. 2. Effect of temperature upon the differential lifetime ($\Delta\tau$) for diphenylhexatriene incorporated into the microsomal fraction (a) and the mitochondrial fraction (b) of the liver of 5° C and 25° C acclimated green sunfish. Values represent the mean of 3—7 determinations on a single preparation except for the value at 39° C which was measured only once. Error bars denote ± standard deviation.

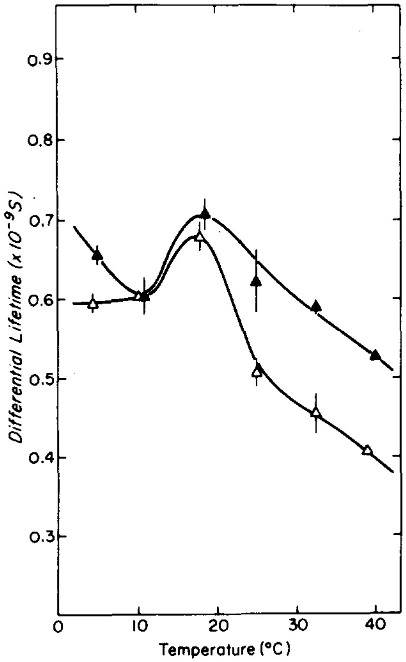

Fig. 3. Effect of temperature upon the differential lifetime ($\Delta\tau$) for diphenylhexatriene incorporated into microsomal (△) and the mitochondrial fractions (▲) of rat liver. Values represent the mean of 3—6 determinations on a single preparation except for the value at 39° C which was measured only once. Error bars denote ± standard deviation.

TABLE V

MAXIMAL DIFFERENTIAL LIFETIMES AND TANGENT DEFECT OBTAINED BY THE PHASE SHIFT TECHNIQUE FOR THE MICROSOMAL AND MITOCHONDRIAL FRACTIONS ISOLATED FROM 5°C AND 25°C ACCLIMATED GREEN SUNFISH AND RAT

Membrane fraction	Source	Temp. (°C)	$\Delta\tau_{max.}$ (ns)		Tangent defect (%)	$<\theta>_{max}$ (15°C)
			Observed	Calculated		
Microsomes	5°C Green sunfish	12 [a]	0.85 [b]	1.62 [c]	47.7 [d]	44.2 [e]
	25°C Green sunfish	10	0.67	1.32	49.2	39.4
	Rat	18	0.68	1.55	56.1	42.2
Mitochondria	5°C Green sunfish	12	0.80	1.50	46.3	44.6
	25°C Green sunfish	15	0.65	1.33	50.9	41.0
	Rat	18	0.71	1.41	50.0	42.1

[a] Temperature at maximum $\Delta\tau$ obtained from Figs. 2 and 3.

[b] Also obtained from Figs. 2 and 3.

[c] Calculated according to Eqn. 11 of Ref. 17.

[d] Calculated as (observed $\Delta\tau_{max.}$/calculated $\Delta\tau_{max.}$) × 100.

[e] Calculated as described in Eqn. 16 of Ref. 21 using r_∞ at 15°C from Fig. 4.

acclimated green sunfish and rat were quite indistinguishable over the temperature range 4.5°C to 39°C. For the microsomal preparations, however, the values of *R* obtained for 5°C acclimated green sunfish were somewhat lower than those of the 25°C acclimated green sunfish (Fig. 4a).

The calculated values of the limiting anisotropy, r_∞ are plotted against temperature in Fig. 5a and b, where it can be seen that r_∞ decreased smoothly with increased temperature in all preparations. For both microsomal and mitochondrial preparations the values of r_∞ obtained for 5°C acclimated green sunfish were much smaller than the corresponding values for the 25°C acclimated green

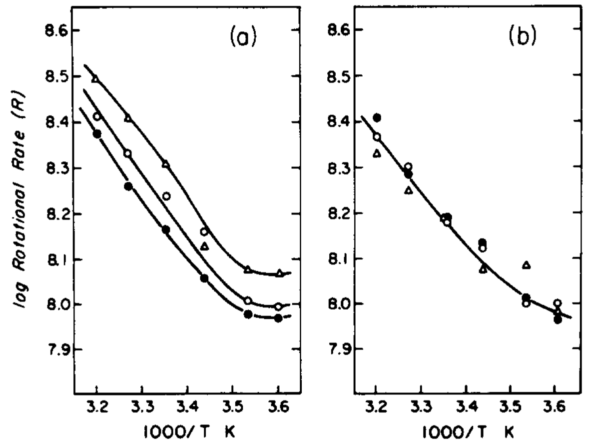

Fig. 4. The effect of temperature upon the rotational rate (*R*) for diphenylhexatriene incorporated into the liver microsomal (a) and mitochondrial (b) fraction of 5°C acclimated green sunfish (●), 25°C acclimated green sunfish (○) and rat (△).

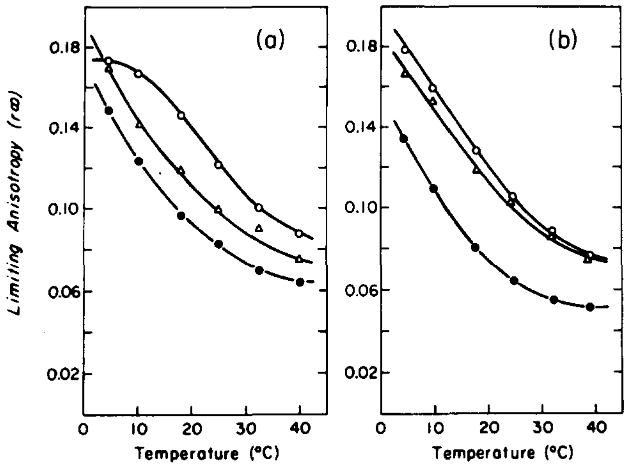

Fig. 5. The effect of temperature upon the limiting anisotropy values (r_∞) for diphenylhexatriene incorporated into the liver microsomal (a) and the mitochondrial (b) fraction of 5°C acclimated green sunfish (●), 25°C acclimated green sunfish (○) and rat (△). Values were calculated as described in Methods.

sunfish. Thus at 18°C, r_∞ for the mitochondria of 5°C acclimated green sunfish was 0.08 whilst for 25°C acclimated green sunfish it was 0.128. The curves for rat mitochondria were similar to that obtained for the mitochondria of 25°C acclimated green sunfish whilst the curve for rat microsomes lay inbetween those of the corresponding 5°C and 25°C green sunfish preparations. The differences in wobbling motion of diphenylhexatriene in the various membranous preparations can be more readily understood by examination of $\langle\theta\rangle_{\max}$, the average angular distribution of the probe about a plane normal to the membrane, at times which are long compared with the fluorescence lifetime (Table V). The membranes isolated from 5°C acclimated fish allowed greater amplitude of wobbling motion by diphenylhexatriene than the corresponding membranes of 25°C acclimated fish.

Discussion

The steady state polarisation technique has previously been useful in detecting differences in the dynamic structure of membranes isolated from organisms with different thermal histories [9,10,12,13]. In the present study, liver mitochondria of green sunfish have been shown to compensate for the effects of acclimation temperature over the range 5°C to 34°C, although, like brain synaptosomes of goldfish [9] the adaptation was only partial in that the fluidity (as measured by $\bar{R}$) was not identical at each acclimation temperature. The lipid composition of the mitochondrial fractions were also different with the usual trends of greater proportions of the saturated fatty acids, smaller proportions of the monounsaturated and some polyunsaturated fatty acids and a significantly lower unsaturation index in 25°C acclimated fish compared to 5°C acclimated fish. The lipid analysis of mitochondria agrees broadly with the results of previous workers [4,24,25]. Wodtke [25] also noted differences in the proportion of 16 : 0, 18 : 1, 20 : 4 and 22 : 6 of liver mitochondrial phosphatidylcholine of 10°C and 25°C acclimated carp. The proportion of polyunsaturated fatty acids was almost identical in the two preparations whereas the proportions of saturated and monounsaturated fatty acids varied as described here for green sunfish liver mitochondria. However, in contrast to the present results, the unsaturation index of the differently acclimated preparations were identical. Van den Thillart and Modderkolk [29] observed no consistent differences in the total mitochondrial lipids of various tissues of differently acclimated goldfish and the authors conclude that the unsaturation index is not relevant in studies of this sort. However, differences in fatty acid composition may not be evident in total lipid extracts which may contain variable amounts of neutral lipids but differences are apparent in purified phospholipid fractions [31].

Although there was a trend of decreasing membrane fluidity for microsomes measured at 15°C with increasing acclimation temperature, the differences between the preparations of 5°C, 15°C and 25°C acclimated fish were not statistically significant. However, microsomes of 34°C acclimated fish were significantly different with respect to those fish acclimated to lower temperatures. The demonstration of somewhat diminished adaptive responses of liver microsomal membranes to altered acclimation temperature lends further support to the suggestion [9,10] that the homeoviscous response is not ubiquitous even for the various membrane types within a cell. The fatty acid composition of the microsomes of 5°C and 25°C acclimated green sunfish were different in some respects, but rather similar in others. Thus, the proportion of saturated fatty acids was slightly greater and the proportion of monounsaturated and polyunsaturated fatty acids was smaller in the 25°C acclimated fish compared to the 5°C acclimated fish, whilst the unsaturation indices of the choline phosphoglyceride fraction, at least, were almost identical. Christiansen [24] has demonstrated differences in the fatty acid composition of liver microsomes of 15°C and 25°C acclimated green sunfish similar to those described here for 5°C and 25°C acclimated fish. Indeed, the unsaturation index for the total microsomal lipids of differently acclimated fish were rather similar.

In view of the lack of information on the influence of individual fatty acids or combinations of fatty acids upon the physical properties of membranes, it is difficult to evaluate the significance of the observed compositional differences. It is evident that biological membranes with distinct lipid compositions may have quite similar physical properties; for example, the membrane fractions of rat have much higher proportions of saturated fatty acids and lower unsaturation indices than the green sunfish preparations and yet their estimated fluidities, as revealed by the steady state and differential polarised phase fluorimetric techniques, are not very different. Lipid composition is known to be affected by a variety of influences including seasons [24], photoperiod [31], hormones and diet [35—37], some of which may act in concert with seasonal changes in temperature. Therefore, an altered lipid composition per se should not be considered to be a reliable indicator of altered membrane properties and separate functional and/or structural parameters must be measured.

In previous studies [9,11] it was suggested that the ratio of saturated to unsaturated fatty acids was an important determinant of membrane properties.

For choline phosphoglycerides the difference in proportion of saturated fatty acids for mitochondria of 5°C and 25°C acclimated green sunfish was over twice as great as the corresponding difference of microsomes. Another important parameter may be the accumulated number of olefinic bonds or unsaturation index for mitochondrial choline phosphoglycerides of 5°C and 25°C acclimated fish were quite different whilst the unsaturation index for the microsomal choline phosphoglycerides of the 5°C and 25°C acclimated fish were almost identical. In these two respects the fatty acid compositional data agree with the conclusion of the fluorescence measurements.

Following Weber [20] the existence of tangent defects of approx. 50% indicates that the assumptions of isotropic, unhindered rotations by the fluorophore in the liver membranes of green sunfish and rat are invalid. The observed tangent defect is thought to be the result of either the combination of separate $\Delta\tau$/temperature plots for several populations of diphenylhexatriene each with appreciably different rotational rates, or alternatively, the restrictions upon the free rotations of diphenylhexatriene imposed by its highly anisotropic lipid environment. This latter possibility has been elaborated into a theory of hindered rotations [21,38] which permits the calculation of the rotational rate (R) and the limiting anisotropy (r_∞) from the phase delay of the differently polarised components of the fluorescence. The rotational rate, R, is an aggregate term reflecting the average rate of motion (radians $\cdot$ s^{-1}) of the rod-shaped probe within a cone that is defined by the highly anisotropic hydrocarbon environment. The limiting anisotropy, r_∞, is the lowest value of anisotropy attainable at times that are long compared to the fluorescent lifetime, reflecting the degree of constraint imposed upon the wobbling motion of the fluorophore by its neighbouring molecules. The rotational diffusion coefficient, $\overline{R}$, derived by the steady state polarisation technique is an aggregate of both R and r_∞ and hence is a considerably less well defined parameter.

According to the theory of hindered rotations [38], the dramatic difference of r_∞ in cold and warm acclimated preparations indicates that the mitochondrial membranes of the cold acclimated fish permit rotation of the probe through a much greater angle (see Table V) and is, by inference, more fluid than the corresponding membranes of the warm-acclimated fish, all of which is consistent with the hypothesis of homeoviscous adaptation. The microsomal preparations of the 5°C acclimated green sunfish also exhibited lower values of r_∞ than the 25°C acclimated green sunfish and a similar conclusion concerning the adaptation of fluidity seems valid. However, it should be noted that the values of rotational rate were greater in the 25°C acclimated fish compared to the 5°C acclimated fish, which is contrary to what one might have expected, and that the steady state polarisation values for this 5°C acclimated preparation were somewhat below the range encountered in the steady state studies described earlier (see Table II) and perhaps are not a true representation of the situation in the 5°C acclimation group. Nevertheless, the data illustrates the utility of the differential phase fluorimetric technique for detecting differences in the dynamic structure of the hydrophobic core of different membrane preparations.

It is also instructive to compare the data obtained for microsomes with those obtained for mitochondria. The differences in fluorescent lifetime derived by the phase and modulation techniques give some indication of the heterogeneity of fluorophore properties since the phase technique weights those fluorophores in the population with short lifetimes more heavily whereas the opposite is true of the modulation technique [33]. For the microsomal membranes the difference in lifetimes obtained by phase and modulation techniques was usually between 0.2 and 0.4 ns. whereas the difference for mitochondria was typically 0.5—1.0 ns, suggesting that the former possess a much reduced microheterogeneity of probe environments. These findings are also supported by consideration of the temperature profile of $\Delta\tau$ (Figs. 2 and 3) since one possible cause of broadening of the symmetrical bell-shaped curve and, incidentally, of tangent defect, is the combination of the separate $\Delta\tau$/temperature plots for different populations of fluorophores. The slightly flatter-topped curve for mitochondria compared to that for microsomes may reflect the more heterogeneous state of the mitochondrial membrane. This proposed difference in degree of heterogeneity for microsomes and mitochondria is, of course, not surprising in view of the fact that the latter are composed of two distinct membranes each with a characteristic protein and lipid composition and with a different membrane fluidity [39].

The temperature profiles of $\Delta\tau$ for the rat liver preparations were somewhat more complex than those of the green sunfish and following Lakowicz et al. [17] it is tempting to speculate that the sudden increase in $\Delta\tau$ at between 12 and 18°C represents some sort of phase transition or separation. Despite this suggested complexity, the derived values of $\bar{R}$, r_∞ and R indicate that rat liver mitochondrial membranes have an average bulk fluidity that is close to that of the mitochondria of 25°C acclimated green sunfish, whereas rat liver microsomes have an average fluidity that lies between that of the microsomes of 5 and 25°C acclimated green sunfish. In both cases, the expectation of major differences in fluidity between the different species which compensate for their diverse body temperatures [9] has not been fulfilled and there are presumably other factors which confound this interspecies comparison.

To summarise, the differential lifetime studies support the theory of isotropic but restricted rotations of diphenylhexatriene in biological membranes as well as in artificial bilayers [17] and considerably extend the previous steady state polarisation studies of biological membranes isolated from thermally acclimated fish [9]. The major difference between the membranes of differently-acclimated green sunfish is quite clearly in the degree of hindrance imposed on the rotations of the fluorophore, whereas the average rotational rate R of the fluorophore was not consistently affected by acclimation treatment. Using steady state polarisation techniques, it was shown [9] that for goldfish synaptosomes an increase in acclimation temperature from 5°C to 25°C produced only a 6°C shift in the $\bar{R}$/temperature curves on the temperature axis. In contrast, in the present study using differential phase fluorimetric techniques the shift in the r_∞/temperature curves for liver membranes of green sunfish was approximately 14°C for a 20°C increase in acclimation temperature. Whether this difference is real or due to the use of different fluorescence techniques is not known.

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