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ADAPTATION OF BIOLOGICAL MEMBRANES TO TEMPERATURE

THE EFFECT OF TEMPERATURE ACCLIMATION OF GOLDFISH UPON THE VISCOSITY OF SYNAPTOSOMAL MEMBRANES

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Summary

The fluidity of synaptosomal membrane preparations isolated from goldfish acclimated to 5, 15 and 25°C and from rat has been estimated using the fluorescence polarisation technique with 1,6-diphenyl-1,3,5-hexatriene as probe. Membranes of cold-acclimated goldfish were more fluid than those of warm-acclimated goldfish when measured at an intermediate temperature, indicating a temperature-dependent regulation of this parameter. Similarly, membranes of warm-acclimated goldfish were more fluid than those prepared from rat brain. Liposomes prepared from the purified phospholipids of goldfish and rat synaptosomal preparations showed differences similar to those of the native membranes. Increased membrane fluidity of cold-acclimated goldfish was correlated with a decrease in the proportion of saturated fatty acids of the major phospholipid classes and an increased unsaturation index in choline phosphoglycerides. Rat membranes showed a substantial reduction in unsaturation index and an increase in the proportion of saturated fatty acids compared to the membranes of 25°C-acclimated goldfish. The cholesterol content of synaptosomal membranes of goldfish was unaffected by acclimation treatment.

The role of homeoviscous adaptation in the compensation of the rates of membrane processes during thermal acclimation, and upon the resistance adaptation of poikilotherms to extreme temperatures is discussed.

Introduction

Recent conceptual advances in the study of biological membranes have revealed the dynamic nature of membrane structure [1] and much evidence

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Abbreviation: BHT, 2,5-di*tert*-butyl-*p*-cresol.

indicates that the thermodynamic state of the hydrophobic portion of the membrane plays an important role in a number of physiological functions and properties associated with membranes, such as membrane-bound enzyme kinetics [2—5], transport of low molecular weight substances [6—8], protein synthesis by membrane-bound ribosomes [9] and microbial cell growth [10]. The physical state of the membrane interior varies with temperature due to the altered molecular mobility of the hydrocarbon chains of membrane phospholipids [11], and it may be expected that continued perturbation by a changed cellular temperature will lead to homeostatic responses by the organism which would preserve membrane function and properties in their "optimal" state. Indeed, such homeoviscous adaptations have been described in *Escherichia coli* [12] and in *Tetrahymena pyriformis* [13] where compensation in membrane fluidity has been correlated with marked changes in the degree of saturation of membrane phospholipids.

Poikilothermic animals also respond to decreased environmental temperature by changes in the fatty acid composition of membrane phospholipids (see ref. 14) and it has been suggested that this also represents an adaptation towards the maintenance of an optimal membrane "fluidity" [15,16]. The present communication seeks to demonstrate this compensation in physical terms by estimating the viscosity of the hydrophobic interior of membranes isolated from animals acclimated to different environmental temperatures. The results indicate that a goldfish brain synaptosomal preparation exhibits a partial compensation (Precht type 3) for changed environmental temperature and that this is related mainly to changes in the degree of saturation of membrane phospholipids.

Materials and Methods

Animals

Common goldfish (*Carassius auratus*, 5—7 inch) were obtained from a commercial source and were maintained for at least one week at 15°C in 8 gallon glass aquaria filled with unchlorinated well water. The water was constantly filtered and aerated. Fish were subsequently acclimated for at least 21 days in 5 ± 1°C or 25 ± 1°C constant temperature rooms with a 12L : 12D photoperiod and artificial dawn and dusk provided by a Conviron Sunrise-Sunset Simulator. Fish were fed twice daily at 25°C, one daily at 15°C and on alternate days at 5°C.

Isolation of brain synaptosomes

All procedures were carried out at 0—4°C. Goldfish were decapitated and their brains rapidly dissected out and freed of sub-arachnoid fatty material, major cranial nerves and spinal cord. The brain was placed in 5 ml ice-cold isolation medium (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris · HCl, pH 7.1) and homogenized by 10 passes of a Thomas type B glass-teflon homogenizer. After centrifugation at 1000 × *g* for 10 min the supernatant was centrifuged at 20 000 × *g* for 30 min. The white pellet was resuspended by homogenization in 5 ml 0.32 M sucrose (8 passes) and layered on a discontinuous sucrose gradient consisting of 6 ml 1.2 M sucrose and 6 ml 0.8 M

sucrose [17]. This was centrifuged at 25 000 rev./min for 90 min in a Beckman SW27.1 rotor. The membrane fraction occupying the interface between the 0.8 M and 1.2 M sucrose solutions was removed with a Pasteur pipette, diluted with a minimum of 10 volumes of 0.32 M sucrose and centrifuged at 105 000 $\times g$ for a further 60 min. The resulting synaptosomal pellet was resuspended in approximately 200 μl of 0.32 M sucrose and gently homogenized in an all-glass hand homogenizer to give a 1 mg protein/ml suspension. Rats were killed with a blow to the head and the brain was rapidly excised. Brain synaptosomes were isolated as above except that each brain was homogenized in 20 ml isolation medium. Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as standard.

Lipid analyses

The total lipid fraction of the synaptosomal preparation was extracted using a method modified from Folch, Lees and Sloane-Stanley [19] by addition of 20vols chloroform/methanol (2 : 1, v/v) containing 0.005% 2,5-di*tert*-butyl-*p*-cresol (BHT). Where possible, all operations were performed under an atmosphere of pure, dry nitrogen. After 30 min at room temperature, 0.2 vol of 0.9% aqueous KCl was added and the mixture was vigorously shaken. The emulsion was separated by centrifugation in glass tubes at 100 $\times g$ for 30 min. The aqueous layer was discarded and the chloroform layer was decanted, reduced in volume under nitrogen and stored at −20°C in glass ampoules which had been flushed with nitrogen and sealed.

Phospholipid classes were separated by two-dimensional thin layer chromatography as described previously [20]. The phospholipid spots were aspirated into a Pasteur pipette with a glass wool plug at one end and the phospholipids were eluted with two 1-ml portions of chloroform/methanol (2 : 1, v/v, with 0.005% BHT). Fatty acid methyl esters were prepared using the boron trifluoride/methanol technique of Morrison and Smith [21]. The composition Chromasorb W AW (Supelco, Bellefonte, Pennsylvania). Nitrogen carrier gas chromatograph with a 1500 × 2 mm glass column packed with 10% SP-2340 on Chromasorb W AW (Supelco, Bellefonte, Pennysylvania). Nitrogen carrier gas flow rate was 25 ml/min and column temperature was 180°C. Peaks were tentatively identified with graphical procedures as well as after argentation chromatography as described previously [20] and quantitated with an electronic integrator.

Phospholipids were separated from neutral lipids by one dimensional thin layer chromatography as described earlier [20]. Liposomes were prepared by drying the purified phospholipids in a 50 ml round bottom flask on a rotary evaporator at 20°C and then resuspending them in 0.4 ml 20 mM KCl, 1 mM EDTA. Glass beads (5 mm diameter) and hand warming of the flask aided liposome formation. Cholesterol was determined by the method of Zlatkis and Zak [22], and lipid phosphorus as described by Cossins [20].

Fluorescence polarisation

Fluorescence polarisation measurements were made on a T-format fluorometer (Jameson, D., Spencer, R.D., Mitchell, J. and Weber, G., unpublished) similar to that described by Weber [23] but with improved optics, double Glan-Foucault polarisers, and photon-counting electronics.

The synaptosomal preparation (25—50 μl) was added to 2.2 ml 0.1 M potassium phosphate pH 7.1 in a 10 mm path length quartz cuvette. 2 μl of a 2 mM 1,6-diphenyl-1,3,5-hexatriene (Aldrich 'puriss' grade) solution in glass-distilled tetrahydrofuran were added with vigorous stirring. The A_{450} of the final solution was approximately 0.10. The fluorescence signal reached a maximum intensity within 10 min at room temperature during which time the polarisation of fluorescence was constant. Phospholipid liposomes were suspended in 20 mM KCl, 1 mM EDTA and treated similarly. Diphenyl hexatriene was excited through a 0.5 m Bausch and Lomb monochromator (1.6—3.2 nm bandpass) at 357 nm with a 450 W xenon arc lamp and the emission was detected through a 2 mm layer of aqueous 2 N sodium nitrite followed by a Corning CS 3-73 glass filter. Steady-state fluorescence polarisation was measured, and was corrected for light-scattering artifact using an identical sample but without added probe, both as described by Shinitzky et al. [24]. Scattered light typically comprised less than 1% total detected light. Replicate polarisation measurements for each sample were usually ±0.001 of a mean value. Addition of a further 2 μl aliquot of diphenyl hexatriene did not alter the polarisation value, indicating that excitation transfer between diphenyl hexatriene molecules does not occur [25]. A Lauda Super K/2R thermostated bath was used to control the cuvette temperature to ±0.1°C at temperatures from 3 to 40°C. Measurement temperature was recorded to ±0.05°C with a precision mercury thermometer immersed in a blank cuvette.

The rotational diffusion coefficient of the probe (R) and the derived viscosity (η) of the probe's environment were calculated using the theory of Perrin [26] as described by Shinitzky et al. [24].

$$\left(\frac{1}{p}-\frac{1}{3}\right)\Big/\left(\frac{1}{p_0}-\frac{1}{3}\right) = 1 + 6\,R\tau \tag{1}$$

$$\left(\frac{1}{p}-\frac{1}{3}\right)\Big/\left(\frac{1}{p_0}-\frac{1}{3}\right) = 1 + C(r)\,\frac{T\tau}{\eta} \tag{2}$$

where p is the measured polarisation and p_0 is the limiting polarisation, which for diphenyl hexatriene in propylene glycol at −51°C was found to be 0.485. T is the absolute temperature, and τ the average lifetime of the excited state, which was measured directly with a modified version of the cross-correlation phase fluorometer described by Spencer and Weber [27]. The lifetimes derived by modulation and phase methods were averaged. $C(r)$ is a constant which depends upon the shape of the probe molecule and has a value of $8.6 \cdot 10^5$ poise $\cdot$ deg^{-1} $\cdot$ s^{-1} [25].

Results

Isolation of brain synaptosomes

The identities of the membrane fractions collected from the sucrose gradient were established by electron microscopy, use of enzyme markers and examination of lipid components. Examination of the 'synaptosomal' fraction (0.8 M and 1.2 M sucrose interface) of goldfish brain by electron microscopy revealed

a rather homogeneous collection of membranous sacs enclosing electron-dense cytoplasm and other membranous structures. Some of the membrane vesicles (less than 5%) were approximately twice the size of the nerve-ending particles and devoid of cytoplasmic contents. The 'myelin' fraction (0.32 M and 0.8 M sucrose interface) consisted of large irregular multilamellar whorls with no cytoplasmic contents.

Approximately 50—70% of the total lactate dehydrogenase and acetylcholinesterase activity recovered from the sucrose gradient was located in the synaptosomal fraction, the remainder occurred mainly in the myelin fraction. Lactate dehydrogenase is considered a general cytoplasmic marker enzyme whereas acetylcholinesterase is specific for the post-synaptic membrane [28]. Lipids isolated from the myelin and synaptosomal fractions were tentatively identified by two-dimensional thin layer chromatography. Only the lipid extract from myelin contained spots that corresponded to cerebrosides or sulphatides, confirming that myelin was essentially absent from the synaptosomal fraction.

Estimation of membrane viscosity

The steady state fluorescence polarisation for diphenyl hexatriene incorporated into synaptosomal preparations was measured at temperatures between 0 and 40°C. The results of typical experiments with preparations isolated from 5-, 15- or 25°C-acclimated goldfish and from rat (37°C, see ref. 29) are illustrated in Fig. 1. For all preparations, as the temperature increased the polarisation decreased. Arrhenius plots of polarisation of the goldfish membranes consisted of two intersecting straight lines, the temperature of the break increasing with higher acclimation temperatures. The graphs for preparations isolated from differently acclimated goldfish had similar slopes both above and below the intersection although their positions were translated to higher temperatures with an increased acclimation temperature. The occurrence of discontinuities in polarisation-Arrhenius plots was not a characteristic of all fish species since green sunfish/bluegill hybrids (*Lepomis* sp.) acclimated to 5°C and to 25°C exhibited linear Arrhenius plots over the same temperature range as used for goldfish (Cossins, A.R., unpublished observation). Apart from this difference, the polarisation values for these latter fish were similar to corresponding graphs for 5- and 25°C-acclimated goldfish preparations over the same temperature range.

The corresponding data for a rat synaptosomal preparation (Fig. 1) are likewise best described by two straight lines at the extremes of the temperature range studied, although the transition between them was smooth and occurred over a 20°C temperature range. In addition, the graph was considerably shifted towards higher temperatures compared to the corresponding plots for goldfish synaptosomes.

The lifetimes of the excited state for diphenyl hexatriene incorporated into synaptosomes isolated from 5°C- and 25°C-acclimated goldfish was measured at temperatures between 0 and 40°C by cross-correlation phase fluorometry. The lifetime as measured by the phase method was typically 0.5—0.8 ns shorter than that measured by the modulation method at any particular measurement temperature (Table I). This indicated a small heterogeneity of probe lifetime,

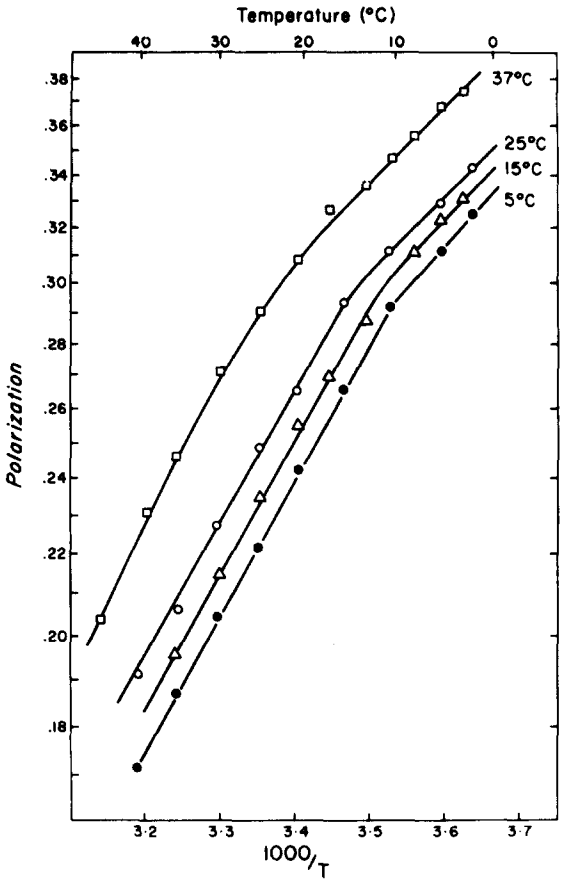

Fig. 1. Typical Arrhenius plots for polarisation of diphenyl hexatriene in synaptosomes isolated from 5-, 15- and 25° C-acclimated goldfish and from rat (37° C). T, absolute temperature, K.

presumably caused by some heterogeneity of the probe environment [30]. The small standard errors for measurements on different preparations indicated excellent reproducibility of these observations. Lifetimes measured by phase and modulation methods were averaged and the mean fluorescence lifetime for several preparations isolated from 5- and 25° C-acclimated goldfish are plotted against measurement temperature (Fig. 2). The mean fluorescence lifetimes for the former preparations were somewhat shorter than those of the 25° C-acclimated fish at all measurement temperatures, correlating with the increased polarisation values of the latter group. In addition, there was a discontinuity in the slope for both acclimation groups at 18—20° C.

The rotational diffusion coefficient (R) was calculated from the polarisation and lifetime analyses and typical Arrhenius plots for preparations isolated from 5-, 15- and 25° C-acclimated goldfish and from rat are illustrated in Fig. 3. The plots for all goldfish preparations exhibited no breaks or discontinuities (compare Fig. 1) suggesting that there was no change in the nature of the probe environment over the entire temperature range. The breaks observed in the goldfish polarisation-Arrhenius plots (Fig. 1) may be a manifestation of the

TABLE I

FLUORESCENCE LIFETIME VALUES AT 3.5, 20 AND 35°C FOR DIPHENYL HEXATRIENE IN SYNAPTOSOMAL PREPARATIONS ISOLATED FROM THE BRAINS OF GOLDFISH ACCLIMATED TO 5°C OR TO 25°C

Acclimation temperature	3.5 °C			20°C			35°C		
	τ phase	τ modulation	τ average	τ phase	τ modulation	τ average	τ phase	τ modulation	τ average
5°C (n = 3)	9.72 * ± 0.11	10.42 ± 0.05	10.07 ± 0.08	9.07 ± 0.14	9.80 ± 0.11	9.44 ± 0.13	8.15 ± 0.14	8.77 ± 0.14	8.43 ± 0.13
25°C (n = 3)	10.03 ± 0.08	10.52 ± 0.10	10.27 ± 0.09	9.38 ± 0.11	10.05 ± 0.09	9.72 ± 0.09	8.45 ± 0.15	9.10 ± 0.15	8.77 ± 0.15

* Values (ns) represent the mean ± S.E.M.

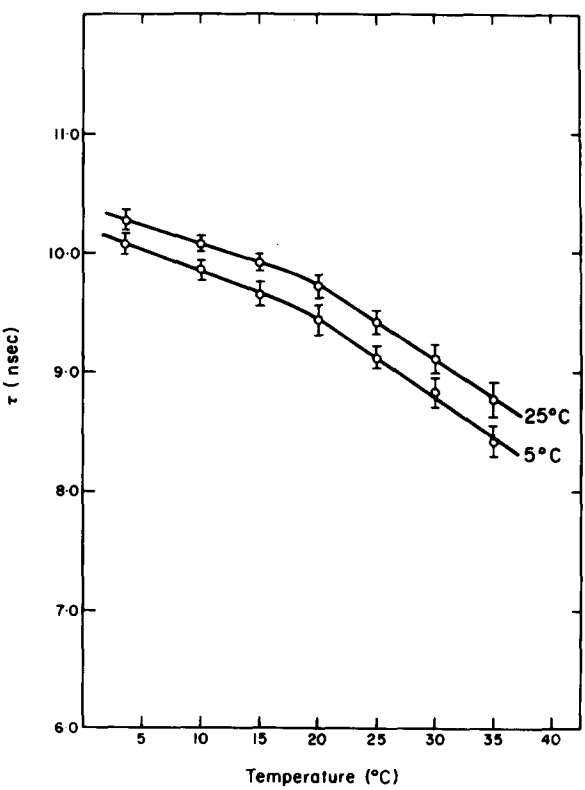

Fig. 2. Effect of temperature upon the mean fluorescence lifetime for DPH in synaptosomes isolated from goldfish acclimated to 5 and to 25°C. Lifetimes derived by the phase and modulation methods were averaged. Values represent the mean for three preparations and the error bars denote the standard error of the mean.

changed slope of the fluorescence lifetime vs. temperature relationship (Fig. 2) caused perhaps by some temperature-dependent change in the quenching or thermalization process. The position of each R-Arrhenius plot was shifted to higher temperatures with an increased acclimation or body temperature, such

that the synaptosomal membranes of 5° C-acclimated goldfish exhibited higher R values at all measurement temperatures than did the corresponding membranes of 15° C-acclimated goldfish. Similarly, 15° C-acclimated goldfish membranes showed higher R values than 25° C-acclimated goldfish membranes. This indicates that acclimation of goldfish to higher environmental temperatures resulted in a more restrictive probe environment (i.e. a more viscous membrane interior) and vice versa. The absolute polarisation, lifetime and derived R were remarkably reproducible from preparation to preparation over a period of days or weeks (Table II), although significant differences became apparent when comparing different preparations several months apart. These differences are throught to have their origin in some seasonal effects upon the animal prior to and during its laboratory experience, since the absolute performance of the polarisation spectrometer was repeatedly checked using standard fluorophores and scattering solutions. A change in the preparative or analytical solutions had no demonstrable effect upon polarisation values.

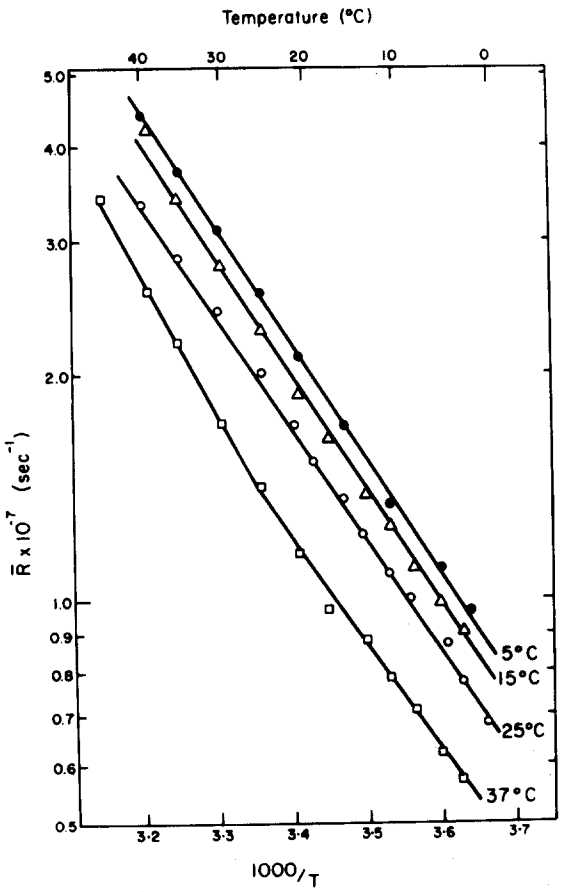

Fig. 3. Typical Arrhenius plots for the rotational diffusion coefficient (R) of diphenyl hexatriene in synaptosomes isolated from 5-, 15- and 25° C-acclimated goldfish and from rat (37° C). R was calculated from polarisation and fluorescence lifetime data as described in Methods. T, absolute temperature, K.

TABLE II

AVERAGE ROTATIONAL DIFFUSION COEFFICIENT ($\bar{R}$) AND MICROVISCOSITY AT 5, 15 AND 25°C AND ACTIVATION ENERGY (E_a) FOR DIPHENYL HEXATRIENE IN SYNAPTOSOMAL PREPARATIONS ISOLATED FROM THE BRAINS OF 5, 15 AND 25°C-ACCLIMATED GOLDFISH AND FROM RAT

	$R \times 10^{-7}$ (s^{-1})				Microviscosity (poise)			
	5°C	15°C	25°C	E_a (kcal · mol^{-1})	5°C	15°C	25°C	E_a (kcal · mol^{-1})
5°C-acclimated goldfish (n = 4)	1.077 * ± 0.017	1.672 ± 0.022	2.551 ± 0.021	6.865 ± 0.132	3.654 * ± 0.033	2.470 ± 0.030	1.714 ± 0.032	6.238 ± 0.139
15°C-acclimated goldfish (n = 2)	0.962	1.483	2.252	7.176	4.205	2.787	1.900	6.594
25°C-acclimated goldfish (n = 4)	0.859 ± 0.020	1.324 ± 0.026	1.982 ± 0.037	6.945 ± 0.107	4.624 ± 0.115	3.114 ± 0.063	2.154 ± 0.039	6.352 ± 0.114
Rat (n = 1)	0.611	0.960	1.464	7.200	6.525	4.307	2.923	6.612

* Values represent the mean ± S.E.M.

Derived microviscosity values are included in Table II for comparative purposes.

The R-Arrhenius plot for rat synaptosomes (Fig. 3) showed a gradual change in slope between 20 and 30°C, the slope below these temperatures being parallel to the goldfish preparations. Again, the position of the line showed a clear shift to higher temperatures, indicating a lower membrane fluidity at a given temperature compared to goldfish membranes.

Viscosity of phospholipid liposomes

A total lipid fraction was extracted from synaptosome preparations isolated from 5- and 25°C-acclimated goldfish and from rat. The phospholipids were separated from neutral lipids as described in Methods. Phospholipid liposomes were prepared and polarisation and lifetime measurements were made at

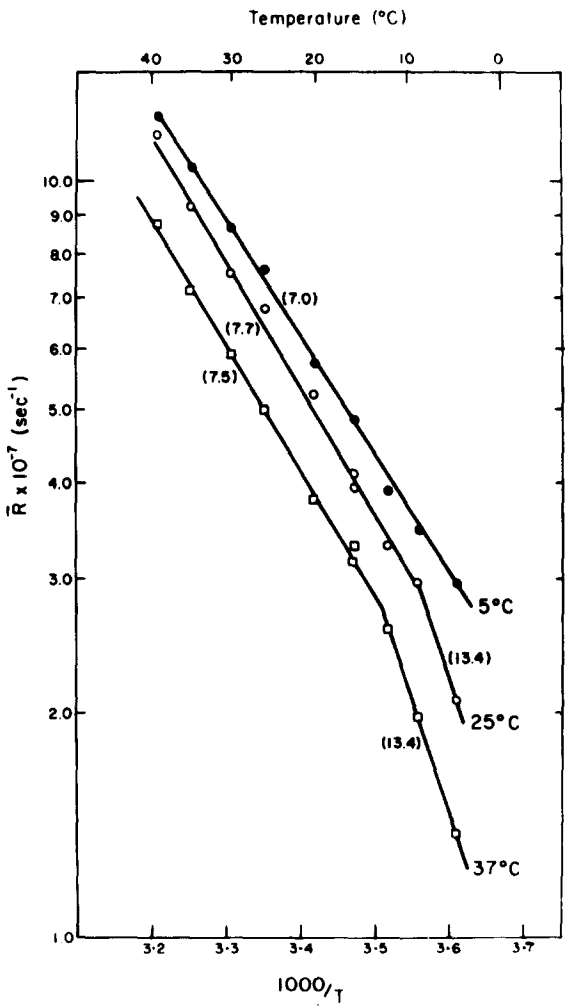

Fig. 4. Arrhenius plots for the rotational diffusion coefficient (R) of diphenyl hexatriene in pure phospholipid liposomes prepared from synaptosomes of 5- and 25°C-acclimated goldfish and rat (37°C). Values in brackets refer to the Arrhenius activation energy (kcal · mol^{-1}) for the adjacent graph.

temperatures between 4 and 40°C. The results are presented in Fig. 4 as Arrhenius plots of R. In all cases R was over two times greater in the pure phospholipid model system than in the synaptosomal membrane, indicating a more restricted probe environment in the latter. This is consistent with the well-documented effects of cholesterol and protein on membrane dynamics [31].

In addition, there was a sizeable shift of the Arrhenius plot towards higher temperatures with an increased cell temperature, as had been observed for the native synaptosomal membranes (Fig. 3). The membrane interior of liposomes prepared from rat brain synaptosomes were markedly less fluid than liposomes of 25°C-acclimated goldfish, and these were in turn less fluid than liposomes prepared from 5°C-acclimated goldfish. The Arrhenius plots for the rat preparation exhibited a marked inflexion at 13°C with activation energies of approx. 7.5 kcal · mol^{-1} and 13.4 kcal · mol^{-1} above and below the break temperature, respectively. The plot for the 25°C-acclimated goldfish liposomes was linear between 40°C and 9°C but the value at 4°C deviated considerably, suggesting the presence of a different slope below 9°C with an activation energy of 13.4 kcal · mol^{-1}. The plot for the 5°C-acclimated goldfish liposomes was linear from 4°C to 40°C. It is suggested that the phospholipid membrane systems of rat and 25°C-acclimated goldfish may have undergone a phase transition over a discrete temperature range whereas the preparation from 5°C-acclimated goldfish remained in the more fluid condition down to the lowest measurement temperature.

Fatty acid composition of membrane phospholipids

The fatty acid composition of the major phospholipid classes (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine/phosphatidylinositol) for the synaptosomal membrane preparations of 5°C and 25°C acclimated goldfish and rat are presented in Table III. In general, warm acclimation of goldfish led to increased proportions of saturated fatty acids in each phospholipid class with decreased proportions of monounsaturated (in phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol) and polyunsaturated fatty acids (phosphatidylcholine, phosphatidylethanolamine). For each phospholipid class, different fatty acids contributed to the overall changes observed. In phosphatidylserine/phosphatidylinositol and phosphatidylethanolamine, 18 : 0 and 18 : 1ω9 showed major differences whereas in phosphatidylcholine, 16 : 0 and 16 : 1ω9, showed large differences and 18 : 0, 18 : ω9 and 22 : 6ω3 smaller differences. Paradoxically, phosphatidylethanolamine of 25°C-acclimated goldfish membranes contained a larger proportion of 22 : 6ω3 than 5°C-acclimated goldfish synaptosomes, a feature that has been observed in a number of other studies (see ref. 20). Rat synaptosomal phospholipids showed profound differences in fatty acid composition compared to 25°C-acclimated goldfish synaptosomal phospholipids with a considerably smaller proportion of 22 : 6ω3 and increased proportions of arachidonate (20 : 4ω6) oleate (18 : 1ω9), palmitate and/or stearate.

The fatty acid composition of each phosphoglyceride fraction is summarised in Table III as a saturation ratio and an unsaturation index. The ratio of saturated to unsaturated fatty acids showed marked increases with higher acclimation or body temperatures particularly in phosphatidylcholine and

TABLE III

THE FATTY ACID COMPOSITION OF MEMBRANE PHOSPHOLIPIDS ISOLATED FROM THE SYNAPTOSOMAL PREPARATIONS OF 5- AND 25°C-ACCLIMATED GOLDFISH AND RAT

Fatty acid	Choline phosphoglycerides			Ethanolamine phosphoglycerides			Serine/inositol phosphoglycerides		
	5°C-Acclimated goldfish (n = 4)	25°C-Acclimated goldfish (n = 3)	Rat (n = 3)	5°C-Acclimated goldfish (n = 4)	25°C-Acclimated goldfish (n = 3)	Rat (n = 3)	5°C-Acclimated goldfish (n = 4)	25°C-Acclimated goldfish (n = 3)	Rat (n = 3)
16:0	30.37 ± 0.46 *	35.22 ± 0.94	43.06 ± 1.41	9.01 ± 0.82	9.59 ± 0.64	9.47 ± 0.21	7.02 ± 1.29	4.50 ± 0.44	5.85 ± 0.76
16:1ω9	8.98 ± 0.72	4.28 ± 0.72	1.93 ± 0.20	3.13 + 0.61	1.69 ± 0.62	1.51 ± 0.25	3.08 ± 0.82	1.32 ± 0.09	1.63 ± 0.43
18:0	6.54 ± 0.43	8.78 ± 0.35	10.93 ± 0.28	13.93 ± 0.99	22.38 ± 1.36	28.17 ± 0.57	22.39 ± 3.20	31.40 ± 2.97	32.36 ± 1.47
18:1ω9	23.58 ± 1.18	26.71 ± 0.33	26.67 ± 0.50	13.33 ± 0.90	8.99 ± 0.58	13.68 ± 0.29	11.65 ± 3.07	8.29 ± 1.98	15.71 ± 0.68
18:2ω6	1.48 ± 0.07	0.76 ± 0.20	1.05 ± 0.01	2.06 ± 0.29	0.66 ± 0.34	0.76 ± 0.12	1.23 ± 0.25	1.20 ± 0.89	0.94 ± 0.29
18:3ω3	1.01 ± 0.08	0.93 ± 0.01	1.21 ± 0.07	2.03 ± 0.16	0.92 ± 0.27	0.47 ± 0.02	1.61 ± 0.23	1.15 ± 0.48	0.47 ± 0.06
20:4ω6	3.02 ± 0.19	2.39 ± 0.07	6.04 ± 0.34	9.15 ± 0.74	6.29 ± 0.17	12.63 ± 0.50	6.27 ± 1.02	5.38 ± 2.06	9.81 ± 0.56
20:5ω3	0.85 ± 0.13	0.43 ± 0.07	—	1.52 ± 0.43	0.45 ± 0.06	0.94 ± 0.55	1.65 ± 0.70	0.27 ± 0.11	1.03 ± 0.89
22:5ω6	0.54 ± 0.19	0.62 ± 0.10	0.28 ± 0.07	1.44 ± 0.07	1.46 ± 0.03	0.75 ± 0.88	1.97 ± 0.62	2.08 ± 0.40	0.89 ± 0.08
22:5ω3	0.42 ± 0.04	0.17 ± 0.01	0.15 ± 0.06	1.22 ± 0.10	0.62 ± 0.04	0.14 ± 0.01	1.49 ± 0.26	1.05 ± 0.20	0.08 ± 0.02
22:6ω3	17.97 ± 0.76	15.06 ± 0.66	5.33 ± 0.40	37.67 ± 1.81	43.29 ± 1.75	24.63 ± 0.38	35.54 ± 3.22	37.05 ± 4.22	25.44 ± 1.30
Others **	5.28	4.81	3.38	5.45	3.67	6.83	6.07	6.37	5.77
Total saturated	39.74 ± 0.43	45.04 ± 1.25	54.73 ± 1.48	25.36 ± 1.19	33.62 ± 1.79	39.36 ± 0.41	31.44 ± 2.28	38.78 ± 2.03	39.85 ± 0.46
Total monoun-saturated	33.40 ± 0.88	31.84 ± 0.76	29.44 ± 0.76	16.98 ± 0.45	11.23 ± 1.11	15.39 ± 0.57	16.23 ± 4.03	10.40 ± 2.62	17.59 ± 0.77
Total polyun-saturated	26.90 ± 0.99	23.28 ± 1.92	15.51 ± 0.23	57.60 ± 2.07	55.16 ± 1.35	45.04 ± 0.34	52.30 ± 2.70	50.88 ± 2.81	42.41 ± 0.62
Ratio saturated : unsat-urated	0.66	0.82	1.22	0.34	0.51	0.65	0.46	0.63	0.66
Unsatu-ration index ***	172.54 ± 4.66	149.47 ± 5.86	98.53 ± 3.91	317.22 ± 9.89	316.44 ± 9.60	250.48 ± 7.34	294.34 ± 13.97	284.84 ± 18.94	233.41 ± 6.73

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

** Other components present in trace quantities include 15:0, 15:1ω9, 17:0, 17:1ω9, 20:0, 20:2ω9, 20:2ω6, 20:4ω3, 22:3ω6 and 22:4ω6.

*** Unsaturation index is computed as the sum of the % weight multiplied by the number of olefinic bonds for each fatty acid in the mixture.

phosphatidylethanolamine. The unsaturation index, which gives a relative measure of the number of olefinic bonds in each phospholipid class (see Table III) showed marked increases with decreased acclimation or body temperature in phosphatidylcholine. In phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol, however, the values for 5- and 25°C-acclimated goldfish were similar; although there were changes in the saturation ratio of phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol during thermal acclimation in the goldfish, the overall unsaturation of these phosphoglycerides did not vary greatly. By contrast, rat membranes exhibited a substantially lower unsaturation index and an increase in the ratio of saturated to unsaturated fatty acids in all phosphoglyceride fractions compared to goldfish membranes.

Assuming the synaptosomal membrane was composed of 55% phosphatidylcholine, 35% phosphatidylethanolamine and 10% phosphatidylserine/phosphatidylinositol, the overall unsaturation ratio increased from 0.528 for 5°C-acclimated goldfish to 0.693 for 25°C-acclimated goldfish, whilst the overall unsaturation indices were relatively similar at 235.36 and 221.45, respectively. The relative constancy of this indicates an increase in the average number of olefinic bonds on each unsaturated fatty acid, of 3.60 for 5°C-acclimated goldfish to 3.75 for 25°C-acclimated goldfish, caused mainly by an exchange of monounsaturated for saturated fatty acids with a greater degree of retention of polyunsaturated fatty acids (Table III). Indeed, the incorporation of a first olefinic bond into a fatty acid results in large effects upon bilayer properties of the phosphatide whilst further desaturation of an already highly polyunsaturated fatty acid has only a small effect [32]. Changes in bilayer properties can be most effectively elicited by exchange of saturated with monounsaturated fatty acids. Changes of membrane viscosity during acclimation correlated better with changes in the saturation ratio than with the unsaturation index.

Rat membranes exhibited an overall saturation ratio of 0.965 with an average number of olefinic bonds per unsaturated fatty acid of 3.245, indicating not only an increased proportion of saturated fatty acids compared

TABLE IV

LIPID PHOSPHORUS AND CHOLESTEROL CONCENTRATIONS AND PHOSPHORUS/CHOLESTEROL MOLAR RATIO FOR SYNAPTOSOMAL MEMBRANES ISOLATED FROM 5- AND 25°C-ACCLIMATED GOLDFISH

Cholesterol and phosphorus analyses were performed on lipid extracts of membrane preparations which had previously been analysed for protein content.

Acclimation group	μM Lipid phosphorus/ mg protein	μM Cholesterol/ mg protein	Lipid phosphorus/ cholesterol molar ratio
5°C (n = 9)	1.089 ± 0.064 *	0.690 ± 0.109 *	1.776 ± 0.166 *
25°C (n = 9)	1.013 ± 0.078	0.824 ± 0.142	1.408 ± 0.155
P **	> 0.1	> 0.1	> 0.1

* All values represent mean ± S.E.M.

** Probability for Student's t calculated for grouped data.

to goldfish, but also a greatly decreased membrane unsaturation due mainly to reduced proportions of polyunsaturated fatty acids. (Table III).

Cholesterol content

Table IV presents the cholesterol and lipid phosphorus concentration expressed as a function of synaptosomal protein concentration and the molar ratio of lipid phosphorus to cholesterol for synaptosomal preparations isolated from 5° C- and 25° C-acclimated goldfish. The values for the two acclimation groups were not significantly different.

Discussion

The experimental approach adopted here has several limitations. First, the position of the probe within the membrane is not known with any certainty and the precise environments being sampled are not known. Diphenyl hexatriene is certainly located within a hydrophobic environment since it does not fluoresce in aqueous media [33,34], but it may be distributed through a wide variety of positions within the phospholipid bilayer as well as a number of different microenvironments provided by phase separations or by the presence of protein. Viscosity parameters derived from the fluorescence polarisation technique relate to the weighted average for all the many different environments within the membrane that are sampled by probe molecules and must be regarded as giving information on the bulk membrane properties only. The technique gives no data on specific membrane microenvironments and for this reason precise correlations of bulk membrane viscosity with specific membrane functions will be difficult to achieve.

Second, the introduction of an exogenous hydrophobic probe molecule will result in some disturbance of membrane structure and the derived information may be biased by this perturbation effect [35]. A number of studies have established that diphenyl hexatriene in chemically defined model systems faithfully reflects the properties of its membranous environment as established by independent techniques [36,37], and that probe incorporation does not cause severe disturbance of those properties. The fluorescence polarisation technique probably has its greatest value not so much in absolute measurement as in comparative studies of similar membrane preparations where problems of unknown probe environment and membrane perturbation are of reduced importance.

Third, the results described earlier are for a synaptosomal membrane preparation which contains an unknown distribution of membrane types. Although the majority of particles were of synaptic origin they consisted of pre- and post-synaptic membranes with their associated complexes, neurilemma, inner and outer membranes of intrasynaptic mitochondria, synaptic vesicle membranes as well as other contaminating membranes.

The highly reproducible values of R obtained for synaptosomal membranes indicates that the technique is capable of great precision and, more importantly, that the viscosity of this membrane preparation is a highly regulated parameter. This latter conclusion supports the hypothesis that certain membranes possess an optimal viscosity for structural and/or functional purposes, and that the

preservation of this thermodynamic state is of physiological importance. The experiments described previously demonstrate that continued perturbation of the optimal viscosity by a changed environmental temperature leads to a homeostatic response which compensates bulk membrane viscosity or the ratio of fluidus to solidus partially for the temperature shift. This phenomenon has been termed "homeoviscous adaptation" [12], and represents a Type 3 adaptation or Type IIA translation [38]. The fact that synaptosomal membranes of rat have a bulk viscosity which is higher than that of 25° C-acclimated goldfish suggests that the viscosity adaptation may also occur over the evolutionary time scale and that the relationship between cell temperature and membrane viscosity crosses phylogenetic boundaries.

The partial nature of the compensatory process in synaptosomal membranes may be the result of the heterogeneity of membrane types. Other types of membrane are known to be non-compensating (Cossins, A.R., Christiansen, J. and Prosser, C.L., unpublished) and the viscosity values may be a weighted average for both fully compensating and non-compensating components. On the other hand, the adaptive process may be truly partial, suggesting that the 'optimal' state towards which the animal is adapting, may change with temperature. It is not possible to rule out changes in the distribution of probe molecules within the membranes of differently acclimated goldfish, although this would appear unlikely. Changes over the evolutionary time period almost certainly resulted in biochemical modifications in addition to those in the fatty acid composition of membrane phospholipids which may affect the types and distribution of microenvironments available to the fluorescent probe.

Although the bulk membrane viscosity is influenced by all membrane constituents and their interactions, it is clear that differences in the viscosity of the synaptosomal membranes isolated from variously acclimated goldfish and from rat, may be explained in large part by changes in the biochemical composition of their constituent phosphatides. Changes in membrane cholesterol levels are generally not involved in thermal compensation [20]. The incorporation of an elevated proportion of *cis*-unsaturated fatty acids during cold acclimation agrees with a number of previous studies [14,39] and is thought to result in a greater molecular mobility or flexibility of hydrocarbon chains through increased disruption of London-Van der Waals interactions between neighbouring membrane constituents [40], compensating at least in part for the increased hydrocarbon interactions at lower environmental temperatures. Changes in the reverse direction occur on acclimation to higher temperatures. The magnitude of the viscosity compensation observed in the present study was substantially smaller than that observed in *E. coli* [12], and *Bacillus stearothermophilus* [41] where membrane viscosity, appeared to be similar at each growth temperature, but was of a similar magnitude to that described for the protozoan *Tetrahymena pyriformis* [13]. This correlates with the more pronounced differences in the fatty acid composition of bacteria cultured at different growth temperatures.

The functional consequences of the homeoviscous responses relate to all processes which are dependent upon membrane viscosity. These include compensations in the passive permeability of the membrane to ions and other metabolites as well as the activity and kinetics of membrane-bound enzymes.

TABLE V

THE MICROVISCOSITY OF SYNAPTOSOMAL PREPARATIONS OF 5- AND 25°C-ACCLIMATED GOLDFISH AT TEMPERATURES WHICH CAUSE HYPEREXCITABILITY, LOSS OF EQUILIBRIUM AND COMA

	Onset of hyperexcitability		Loss of equilibrium		Heat coma	
	Temp. ** (°C)	η *	Temp. ** (°C)	η *	Temp. ** (°C)	η *
5°C-acclimated goldfish (n = 4)	29.7	1.46 ± 0.03	32	1.35 ± 0.03	33	1.30 ± 0.03
25°C-acclimated goldfish (n = 4)	34.5	1.55 ± 0.03	37.6	1.40 ± 0.03	39	1.34 ± 0.03
P ***	0.05—0.1		< 0.2		< 0.2	

* Microviscosity (poise) was calculated from regression analysis of data presented in Table I. Values represent mean ± S.E.M.

** Values from ref. 43.

Probability calculated for Student's t analysis for comparison of values for 5- and 25°C-acclimated goldfish.

In addition, there is much evidence linking cellular heat injury and heat death of poikilotherms to a breakdown in the structural integrity of specified membrane-types, presumably due to excessive molecular mobility of membrane constituents [38,41,42]. The resistance of animals to the disruptive effects of high temperature may be modified by thermal acclimation in an apparently adaptive manner. For example Cossins et al., [43] have demonstrated that 25°C-acclimated goldfish become comatose at 39°C whereas the corresponding value for 5°C-acclimated goldfish is 33°C. In this case, heat coma appears to be related to block of synaptic transmission of brain centres [44]. The compensation of membrane fluidity demonstrated here for goldfish synaptic membranes, provides a means by which organisms can modify the resistance of the membrane to the effects of lethal high temperatures, such that the membranes of warm-acclimated organisms will become disrupted at higher temperatures than those of cold-acclimated organisms.

The membrane microviscosity of the synaptosomal preparations at the temperatures which cause certain behavioural deficits (e.g. hyperexcitability, loss of righting response and coma) in both 5°C- and 25°C-acclimated goldfish are not significantly different (Table V), indicating that the magnitude of the viscosity compensation is sufficient to account for the behavioural resistance adaptation (see also ref. 43). The temperature at which the rat synaptosomal membrane preparation achieves the same microviscosity as that which is associated with coma in goldfish, is approximately 45°C, which agrees reasonably closely with the values for final tolerance temperatures of small mammals determined by Frankel (quoted in ref. 45).

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