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HOMEOVISCOUS THEORY UNDER PRESSURE

II. THE MOLECULAR ORDER OF MEMBRANES FROM DEEP-SEA FISH *

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The molecular order of brain and liver membranes isolated from deep sea and continental shelf fish species have been estimated and compared using the fluorescence polarization technique in order to determine whether life in a high pressure habitat is associated with an adjustment of membrane order. Fish were trawled at depths between 200 m and 4000 m, liver and brain membranes were fractionated, and fluorescence polarization was measured at 4°C and ambient pressure. Polarization of the brain myelin fraction provided a statistically significant regression with depth of capture ($P < 0.001$) with a slope of -0.004 km^{-1}. This change in polarization with depth was sufficient to offset approximately half of the pressure-induced increase in polarization and thus represents the first structural evidence of homeoviscous adaptation to pressure. Polarization of the brain synaptic and liver mitochondrial fraction was not significantly related to depth. This may be due, at least in part, to a high individual variability of polarization compared to laboratory-acclimated freshwater fish.

Introduction

Hydrostatic pressure has significant effects upon the molecular order of model and natural membrane bilayers. A hydrostatic pressure of 1000 atmospheres orders these membranes by an amount equivalent to a 15–25°C decrease in temperature [1–4]. Nevertheless, animals and bacteria occupy the deep sea at pressures of up to nearly 1100 atmospheres and temperatures less than 4°C [5].

A reasonable prediction concerning the physiological adaptations of abyssal organisms is that the molecular order of their membranes at depth is similar to that of the corresponding membranes of comparable shallow water fish. This hypothesis implies that the biochemical composition of the membranes of deep sea fish are modified to offset or compensate for the combined ordering effects of low temperature and high pressure. This is an extension of the concept of homeoviscous adaptation [6], which in fish has been studied by comparing the membranes of fish from different temperatures. In particular brain membranes of fish from the arctic or from hot desert springs [7] and from eurythermal fish acclimated to high or low temperatures have been compared [8,9]. In both cases, the membranes of the cold-adapted fish were somewhat more fluid than those of warm-adapted fish and this difference partially offsets the ordering effects of the cold. The difference in molecular order has been correlated with differences in membrane lipid composition [7,8].

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In postulating homeoviscous adaptation to pressure we predict that, at atmospheric pressure, the molecular order of membranes from deep sea fish should be less than that of shallow-water species. Accordingly, the molecular order of membranes isolated from species collected at different depths from 200 m to 4000 m has been estimated at 4 °C and 1 atmosphere using the steady-state polarization technique with 1,6-diphenyl-1,3,5-hexatriene as fluorescent probe [11]. A preliminary account of this work has been published [12].

Methods

Fish

Fish were collected during two cruises on R.R.S. Challenger during September 1981 and September 1982 to the Porcupine Sea Bight off southwestern Eire, centred on 50 °N, 13 °W. Fish were trawled at various depths using a semi-balloon otter trawl (1350–4000 m) and an Aggasiz trawl (200–1000 m). The depth of the trawl on the sea floor was measured acoustically. The temperature varied from 6–9 °C at 1000 m to 3–3.6 °C at 3000 m, to 2.5 °C at 4000 m [13].

Typically, trawling at 200 m depth required only 20 min hauling whilst trawling at 4000 m required up to 3 h. Fish from 200 m were alive and could be maintained in aquaria. Fish from 1000 m were frequently moribund but usually possessed spinal reflexes, a beating heart and opercular movements. Fish from 2000–4000 m were usually unresponsive to bissection of the spinal cord and lacked a heart beat. The deeper fish showed increased concentrations of Mg^{2+} and Ca^{2+} in their plasma (Shelton, C., Pequeux, A., and Macdonald, A.G., unpublished data). Following arrival of the trawl on deck, selected specimens were transferred to a 3 °C laboratory, dissected and then either preserved in buffered formalin or frozen. Subsequently the specimens were identified by Dr. N.E. Merrit, Institute of Oceanographic Sciences, Wormley or by Dr. J. Hislop, The Marine Laboratory, Aberdeen.

Isolation of membrane fractions

Brain. The brain of each fish was dissected free of cranial nerves and spinal cord, carefully washed free of sub-arachnoid fatty material and homogenised in 2 ml of ice-cold brain isolation medium (320 mM sucrose, 1 mM EDTA, 10 mM imidazole-HCl (pH 7.6 at room temperature)) using a glass-teflon homogeniser (20 passes, approx. 100 rpm). Where possible all procedures were carried out at 0–5 °C. The preparative scheme used was an abbreviated version of that used in previous studies (Cossins 1977, Cossins and Prosser 1982) to take into account the exigencies of ship-board experimentation.

The homogenate was centrifuged in the cold at $1000 \times g$ for 10 min (MSE 'Minor' Centrifuge) and the supernatant was carefully removed with a Pasteur pipette and centrifuged at 15 000 rpm for 30 min at 5 °C (Damon-IEC Centra 3RS centrifuge or a Sorvall SS-1 centrifuge). The pellet was resuspended by homogenisation with a tight-fitting glass homogeniser (Kontes, 'A' pestle) in lysing medium (10 mM imidazole-HCl (pH 7.6 at room temperature), 1 mM EDTA) and recentrifuged at 15 000 rpm for 30 min. The pellet was resuspended in 4 ml lysing medium, layered on 3 ml 0.8 M sucrose and centrifuged at 15 000 rpm for 60 min. The white material at the interface of the sucrose solution and the lysing medium (the brain myelin fraction) was carefully removed by Pasteur pipette and diluted approximately 3-fold with lysing medium. The pellet (the brain synaptic fraction) was resuspended in 5 ml lysing medium. Both membrane fractions were centrifuged at 15 000 rpm for 30 min, resuspended in 0.2–0.5 ml lysing medium and then stored at 0 °C until used. Samples of the fractions were frozen at −20 °C for subsequent electron microscope examination.

Liver. Samples of liver (1–2 g) were homogenised in 5 ml liver isolation medium (320 mM sucrose, 1 mM EDTA, 1% (w/v) bovine serum albumin (fraction V, Sigma Chemical Co.), 10 mM imidazole-HCl (pH 7.6 at room temperature)) using a glass-teflon homogeniser (15–20 passes, 100 rpm approximately). The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was removed with a Pasteur pipette and centrifuged at 10 000 rpm for 10 min. For those samples which contained large quantities (up to 50% (v/w)) of fatty material, the low speed centrifugation was repeated up to three times to ensure a complete separation of the aqueous supernatant from the overlaying fatty material. The tan pellet (the liver

mitochondrial fraction) was washed twice in liver isolation medium. The pellet was finally suspended in 0.2–0.5 ml liver isolation medium and stored at 0 °C until used. Samples were frozen for electron microscope examination.

Fluorimetry

Steady-state polarization of diphenylhexatriene fluorescence was measured with an analogue T-format fluorimeter (Applied Photophysics Ltd.), similar to that described by Jameson et al. [14] but with modified excitation optics, analogue photomultipliers and a ratiometric amplifier. A thermostatted cuvette chamber was constructed to include provision for the gassing of cuvette surfaces with dry nitrogen gas at sub-ambient temperature to prevent condensation in a humid atmosphere. The fluorimeter was mounted on a 1/4 inch aluminum optical bench and this was bolted with steel girders to a wooden laboratory bench to prevent relative movement of the optical paths with motion of the ship.

An unexpected problem of shipboard measurements of polarization was the varation of the photomultiplier voltage with ship's motion. This variation was approximately ± 1–2% of the total signal from each photomultipler, but was somewhat smaller for the ratio of the two photomultipler voltages. The variation was smoothed by continuously averaging the polarization ratio over several cycles of ship motion using a BBC microcomputer (model B, Acorn Computers Ltd., Cambridge) connected to an Acorn 12-bit analogue-to-digital converter. The computer was programmed to provide an average of 256 individual conversions which were themselves continously averaged until a stable value to three decimal places was obtained.

Membrane preparations were diluted into 0.1 M phosphate buffer (pH 7.6 at room temperature) or into 0.133 M citrate/0.266 M disodium hydrogen phosphate buffer (pH 7.5 at room temperature) to give an absorbance of 0.1 at 500 nm. The suspension was labelled with diphenylhexatriene (Aldrich 'puriss' grade) as described previously [8]. The labelled suspension was incubated at room temperature for at least 10 min before cooling to the measurement temperature (4 °C). Excitation wavelength was 360 mm. The excitation path was filtered with a Corning 7-54 broad bandpass filter and the emission path with a Corning 3-73 sharp-cut filter. Cuvette temperature was maintained ±0.1 °C with a Julabo thermostatted circulator and cuvette temperature was measured with a thermistor. Polarization was calculated as described previously [8,10]. Corrections for light-scattering artefact were not made.

The performance of the fluorimeter was checked using a suspension of glycogen in water (polarization at 500 nm = 0.97–0.98) and diethylnaphthalene sulphonate in water (polarization = 0.002). An optical misalignment on the 1981 cruise neccesitated a small correction. The correction factor was experimentally determined by measuring the polarization of fluorescein in a solution of glycogen/water (90 : 10, v/v, approximately) using the correct and the misaligned optical arrangements. This accounts for the differences in the results presented here to those presented in a preliminary report [15].

Results

The membrane fractions were examined by transmission electron microscopy. The brain myelin fraction consisted mainly of loose myelin whorls and a certain amount of large membranous vesicles. Only slight mitochondrial contamination was observed. The brain synaptic fraction consisted mainly of small and large membranous vesicles, synaptic vesicles, mitochondria and a few intact synaptosomes. There were no myelin whorls. The liver mitochondrial fraction consisted predominently of disrupted mitochondria although substantial quantities of membranous vesicles were also noted. Lipid droplets were not observed in any fractions.

A summary of the diphenylhexatriene polarization values obtained for each species is presented in Table I. In each case polarization at 4 °C decreased in the order myelin, synaptic and liver mitochondrial, much as has been observed in previous studies of goldfish [7] and green sunfish [15].

Intraspecific variation for brain myelin was noticeably smaller than observed in the other fractions, and was similar to that experienced with laboratory-conditioned goldfish [7]. By contrast, the variability for brain synaptic and, more partic-

ularly, liver mitochondrial fractions was substantially greater than observed in the studies of goldfish [7] or green sunfish [15].

In some cases sufficient individuals were caught in separate trawls to determine intertrawl and interyear variability. One such case was *Antimora rostrata* during the 1981 cruise in which virtually identical results were obtained in two separate

TABLE I

A SUMMARY OF DIPHENYLHEXATRIENE POLARIZATION VALUES MEASURED IN VARIOUS MEMBRANE FRACTIONS OF BRAIN AND LIVER OF FISH CAUGHT DURING TWO CRUISES ON R.R.S. CHALLENGER

Values represent mean ± S.E.; numbers in brackets refer to the number of individual fish measured.

Species	Depth of capture (m)	Species depth range [a] (m)	Membrane fraction		
			Brain myelin	Brain synaptic/ mitochondrial	Liver mitochondrial
1981					
Nezumia aequalis	1200	200–2320	–	–	0.279 ± 0.005 (7)
Coryphenoides rupestris	1300	400–2000	0.352 ± 0.011 (6)	0.310 ± 0.007 (6)	0.275 ± 0.013 (4)
Hoplostethus atlanticus	1300	900–1700	0.369 ± 0.007 (4)	0.324 ± 0.004 (4)	–
Antimora rostrata	2000	700–3000	0.355 ± 0.014 (4)	0.308 ± 0.007 (4)	0.274 ± 0.004 (7)
Antimora rostrata	2000	700–3000	0.355 ± 0.005 (4)	0.308 ± 0.010 (4)	0.278 ± 0.004 (4)
Conocara murrayi	2000	1900–2400	0.352 ± 0.007 (4)	0.326 ± 0.007 (4)	0.274 ± 0.008 (4)
Histiobranchus bathybius	4000	1800–4800+	0.351 (1)	0.305 (1)	0.262 (1)
Coryphenoides armatus	3800–4000	2200–4800+	0.339 ± 0.005(15)	0.320 ± 0.003(15)	0.288 ± 0.003(15)
1982					
Lepidorhombus whiffiagonis	200	200– 540	0.356 ± 0.004 (7)	0.308 ± 0.006 (7)	0.264 ± 0.008 (7)
Lophius budegassa	200		0.354 ± 0.007 (3)	0.317 ± 0.004 (3)	0.275 ± 0.013 (3)
Lophius piscatorius	200–1170	100–1170	0.359 ± 0.005 (3)	0.319 ± 0.006 (3)	0.320 ± 0.013 (3)
Helicolenus dactylopterus	200	200– 950	0.357 ± 0.003 (4)	0.309 ± 0.002 (4)	0.292 ± 0.015 (4)
Phycis blennoides	800	150–1030	0.340 ± 0.002 (4)	0.305 ± 0.003 (4)	0.234 ± 0.007 (4)
Lepidion eques	800	510–2420	0.367 ± 0.003 (3)	0.328 ± 0.004 (3)	0.299 ± 0.015 (3)
Lepidion eques	850–900	510–2420	0.346 ± 0.001 (7)	0.309 ± 0.003 (7)	0.258 ± 0.006 (7)
Mora moro	1000	500–1400	0.355 ± 0.005 (3)	0.303 ± 0.003 (3)	0.274 ± 0.014 (4)
Alephocephalus bairdii	1000	650–1900	0.351 ± 0.002 (6)	0.317 ± 0.003 (7)	0.307 ± 0.010 (7)
Nezumia aequalis	1000	200–2320	0.361 ± 0.002 (5)	0.332 ± 0.005 (5)	0.275 ± 0.010 (5)

[a] Range of depths at which each species has been collected in the North-East Atlantic (Merrit, N.M.E., personal communication)

TABLE II

THE LINEAR REGRESSION ANALYSIS OF DIPHENYLHEXATRIENE POLARIZATION WITH DEPTH OF CAPTURE FOR FISH TRAWLED DURING THE 1981 AND 1982 CRUISES

Values represent the slope ± S.E. of the slope calculated according to Ref. 16. *n* represents the number of individual observations. The probability was determined by calculating Student's '*t*' as described by Bailey [16].

Membrane fraction	*n*	Slope	*P*	*Y*-intercept
Brain myelin	82	−0.004 ± 0.001	< 0.001	0.358
Brain synaptic	83	+0.002 ± 0.001	0.05–0.1	0.312
Liver mitochondrial	88	+0.003 ± 0.002	> 0.1	0.275

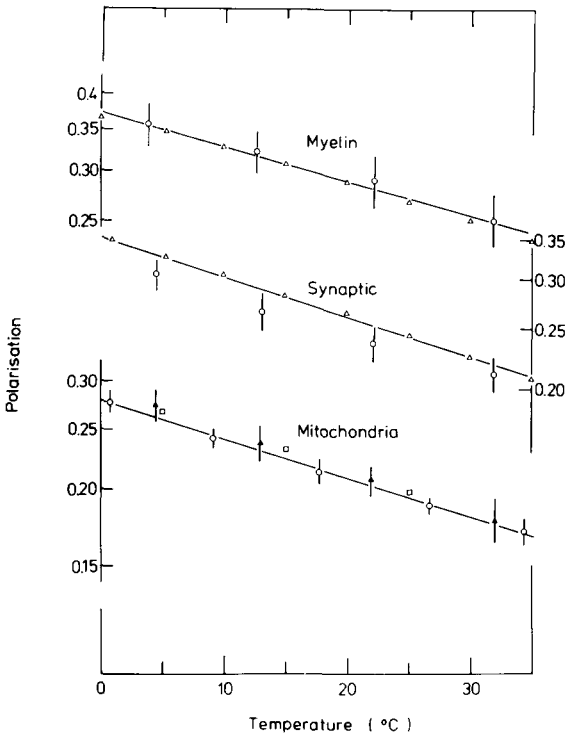

Fig. 1. A comparison of the temperature dependence of the fluorescence polarization of diphenylhexatriene in membranes from deep sea fish and eurythermal freshwater fish. Values and bars represent the mean and standard deviation of *n* individuals. Note that polarization is plotted on a log scale. ○, *Antimora rostrata* (*n* = 4) from 2000 m depth. ▲, *Conocara murrayi* (*n* = 4) from 2000 m depth. △, Goldfish (*Carrasius auratus*, *n* = 1) acclimated to 5 °C. Data from Ref. 7. □, green sunfish (*Lepomis cyanellus*, *n* = 1) acclimated to 5 °C. Data from Ref. 15.

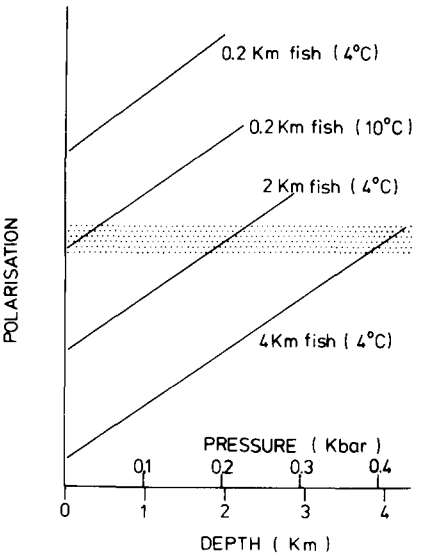

Fig. 2. A hypothetical graph showing how the predicted homeoviscous adaptation to high pressure may be detected by measurements of diphenylhexatriene polarization at 4 °C and atmospheric pressure in membranes from fish trawled at depths down to 4000 m.

groups of animals. Similarly, the liver mitochondrial fraction of *Nezumia aequalis* caught during the 1981 cruise gave almost identical values to fish caught during 1982. On the other hand, *Lepidion eques* caught during the 1981 cruise at 800 m was somewhat different to individuals caught in a subsequent trawl at 850–900 m.

Table II presents the linear regression analysis of diphenylhexatriene polarization with depth of capture using data for individual fish. The brain myelin fraction provided a regression with a statistically significant slope ($P < 0.001$). In this case the regression coefficient was negative; that is, as depth increased the membrane bilayers become less ordered when measured at 4 °C, atmospheric pressure. This relationship is well illustrated by the only intrageneric comparison in Table I, namely the lower polarization values in *Coryphenoides armatus* than in the more shallow water *Coryphenoides rupestris*. The regressions for the brain synaptic and liver mitochondrial preparations were not statistically significant. In all three fractions the extrapolated polarization at zero depth (i.e., *y*-intercept, Table II) agreed closely with the values observed in goldfish brain frac-

tions [7] and in liver mitochondrial membranes of green sunfish [15] when acclimated to 5 °C.

The temperature dependence of fluorescence polarization of membranes from some abyssal species is compared in Fig. 1 with that observed in previous studies of membranes from goldfish and green sunfish. It is clear that there are no substantial differences in the temperature-dependence of polarization between the abyssal and freshwater species.

Discussion

In principle, two extreme types of membrane adaptation to hydrostatic pressure may occur. Firstly, the membranes of abyssal fish may be relatively pressure-insensitive compared to surface-dwelling fish. Secondly, the pressure-dependence of membrane order of deep-sea and surface-dwelling species may be similar but their respective degree of ordering may be sufficiently different to provide for similar orders in their respective habitats. Preliminary studies with a pressurised cuvette chamber [17] suggest that the brain membranes of abyssal fish are not less sensitive to pressure than those of the goldfish (unpublished observations), so that the first alternative is unlikely.

Fish at 4000 m suffer a significant hydraulic decompression when brought to the surface so their polarization values at atmospheric pressure and 4 °C would be lower than those prevailing at depth. Furthermore, if their polarization values at depth are the same as those of comparable membranes in shallow water species, then the membranes of deep sea species should have lower polarization values than those of shallow water species when measured under identical conditions (atmospheric pressure and 4 °C); the difference depending upon the pressure-dependence of polarization (illustrated in Fig. 2). The effect of pressure upon polarization has been determined in goldfish synaptic membrane preparations as a decrease of 0.055–0.075 for a pressure increase of 1 Kbar [1] and a similar value has been obtained for brain myelin fraction of goldfish (Chong and Cossins, unpublished observations). Animals at a depth of 4000 m (approx. 0.4 kbar) will therefore suffer an increase in diphenylhexatriene polarization of 0.02–0.03 relative to surface-dwelling species and any depth-related variations in diphenylhexatriene polarization must be of this magnitude in order to compensate for this effect. We have found that replicate shipboard measurements of polarization were possible with a precision of ± 0.002 which is well inside this expected range of biological variation.

A significant negative regression was obtained between polarization and depth of capture for the brain myelin fraction with a slope of -0.004 km^{-1} or -0.040 $kbar^{-1}$. This is consistent with the prediction and amounts to approx. 50–80% of that required to offset the direct ordering effects of hydrostatic pressure over the 1000–4000 m pressure range as determined from goldfish synaptic membranes [1]. This partial compensation is similar in magnitude to the partial homeoviscous compensations observed during seasonal acclimation and evolutionary adaptation to temperature [7,9] and represents the first evidence of homeoviscous adaptation to pressure.

In contrast, the liver mitochondrial and brain synaptic fractions did not show significant regressions with depth of capture. This is somewhat surprising in that the presumed physiological benefits of homeoviscous adaptation seem to be more obvious and clear-cut in these fractions than for the myelin fraction. However, the intraspecific variability of polarization in these two fractions was substantially greater than similar membrane fractions isolated from laboratory-conditioned fish [7,15]. In view of the obvious intraspecific and interspecific variability in gross morphology, pigmentation and size of the liver it seems reasonable to expect that there may be some variability in the membrane-type composition of the liver membrane fraction and perhaps in the order of each membrane-type. A similar arguement may hold also for the brain synaptic fraction. On the other hand, the highly consistent values obtained for the brain myelin fraction of each species was similar to the consistency observed in laboratory conditioned goldfish [7]. This may result from the more successful separation of myelin membranes from other membrane-types with the simple sucrose-gradient employed in these studies.

There is also an adaptive arguement for the different responses observed in myelin and in liver mitochondria. Certain deep sea species have a very

low metabolic rate, which may be of selective advantage in their food-scarce environment [18]. Enzyme studies have led to the suggestion that the reduced metabolic rate generally derives from less efficient enzymes which are present in low intracellular concentrations. However, brain tissue is an exception in not possessing the depth-related, reduced enzyme activity. Thus whilst a compensatory response is not necessarily the most appropriate adaptive response for those tissues involved in energy metabolism (such as liver) it may be important in the brain.

Finally, we should not overlook the complication presented by variations of temperature within the vertical distribution of the fish. In the area where the fish were collected, the temperature below a depth of 1000 m varied little (see Methods) but nevertheless constitutes an important factor when estimating membrane order in situ. The higher temperatures in shallower waters will tend to increase the differences bectween the membranes of species from different depths, as shown in Fig. 2, but will not greatly affect the conclusions drawn from this study.

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