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FLUIDITY OF NATURAL MEMBRANES AND PHOSPHATIDYLSERINE AND GANGLIOSIDE DISPERSIONS

EFFECTS OF LOCAL ANESTHETICS, CHOLESTEROL AND PROTEIN

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SUMMARY

The microviscosity of artificial lipid membranes and natural membranes was measured by the fluorescence polarization technique employing perylene as the probe. Lipid dispersions composed of brain gangliosides exhibited greater microviscosity than phosphatidylserine (268 cP vs 173 cP, at 25 °C). Incorporation of cholesterol (30–50 %) increased the microviscosity of lipid phases by 200–500 cP. Cholesterol's effect on membrane fluidity was completely reversed by digitonin but not by amphotericin B. Incorporation of membrane proteins into lipid vesicles gave varying results. Cytochrome b_5 did not alter membrane fluidity. However, myelin proteolipid produced an apparent increase in microviscosity, but this effect might be due to partitioning of perylene between lipid and protein binding sites since the latter have a higher fluorescence anisotropy than the lipid. The local anesthetics tetracaine and butacaine increased the fluidity of lipid dispersions, natural membranes and intact ascites tumor cell membranes. The effect of the anesthetics appears to be due to an increased disordering of lipid structure. The fluidity of natural membranes at 25 °C varied as follows: polymorphonuclear leukocytes, 335 cP; bovine brain myelin, 270 cP; human erythrocyte, 180 cP; rat liver microsomes, 95 cP; rat liver mitochondria, 90 cP. In most cases the microviscosity of natural membranes reflects their cholesterol : phospholipid ratio. The natural variations in fluidity of cellular membranes probably reflect important functional requirements. Similarly, the effects of some drugs which alter membrane permeability may be the result of their effects on membrane fluidity.

INTRODUCTION

It is now widely appreciated that the dynamic properties of the fluid lipid bilayer structure may profoundly influence membrane functions related to ion and

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water permeability, and enzymatic activity [1–5]. It has therefore become of increasing interest to be able to study dynamic changes in membrane physical state and to correlate this with functional effects. Furthermore, the specialized properties of individual cell types and intracellular organelles may be determined to a degree by the functional properties of their membranes. Thus, one would expect that the differences in the chemical composition of membranes such as myelin, and from cells of such widely diverse functional properties as erythrocytes and leukocytes, would be reflected in different physical properties such as membrane fluidity. A number of recent studies have appeared which demonstrate the utility of fluorescent probe techniques for measuring membrane fluidity [6–9].

In this study we have investigated, by fluorescence polarization techniques, the microviscosities of various biological membranes and artificial lipid disperisons. The effects of incorporation of cholesterol or specific membrane proteins (e.g. cytochrome b_5 and the basic protein and proteolipid of myelin) on the fluidity of lipid dispersions were investigated, and we have also been concerned with the effects of local anesthetics on the fluidity of membranes. Electron spin resonance studies with a variety of spin probes have yielded varying results regarding the effects of anesthetics on lipid structure. Hubbell et al. [10] observed that local anesthetics increased the mobility of the lipids in red cell membranes. Butler et al. [11] also observed a disordering effect of tetracaine on brain lipid films, but under some experimental conditions they observed the reverse effect.

METHODS AND MATERIALS

Preparation of membranes

Lipid dispersions were prepared by sonication. Lipid films were deposited on the surfaces of glass beakers by evaporation of benzene or chloroform solutions under nitrogen. 0.1 M Tris · HCl, pH 7.4, was added and the suspensions, in an ice bath or maintained at room temperature, were sonicated at 70 W (Branson Sonifier) under N_2 for from 10 min to 1 h. Large particles were removed by centrifugation at 40 000 × g for 1 h. Transparent dispersions could be produced in this way at the low lipid concentrations employed ($\approx 4 \cdot 10^{-4}$ M). Erythrocyte ghosts were prepared from fresh human blood by the method of Dodge et al. [12]. Myelin was prepared by the method of Rumsby et al. [13] and its chemical composition and properties have been described by Feinstein and Felsenfeld [14, 15]. A highly homogeneous population of polymorphonuclear leukocytes (90–95 %) was collected from the peritoneal cavity of rabbits according to the method of Cohn and Hirsh [16]. The membranes were isolated on a discontinuous sucrose gradient according to the method of Oliver et al. [17]. Mouse ascites tumor cells of the Nelson [18] variety were equilibrated with perylene (10^{-6} M) for 1 h at room temperature, collected by centrifugation, washed once and resuspended in normal saline to make a concentration of 10^6 cells/ml. Rat liver mitochondria and microsomes were isolated by the method of Cinti and Schenkman [19]. Myelin basic protein was prepared by the method of Oshiro and Eylar [20] and myelin proteolipid by the method of Folch-Pi and Stoffyn [21]. Cytochrome b_5, purified from rat liver microsomes by the method of Spatz and Strittmatter [22], was donated by Dr. D. L. Cinti. Phosphatidylserine was obtained from Applied Science Laboratories and Supelco, brain gangliosides from Sigma containing principally one

N-acetylneuraminic acid group/ceramide group (Gammack [23], fraction III); Perylene (Gold Label) was obtained from Aldrich. The purity of these substances was verified by thin-layer chromatography and chemical analysis.

Fluorescence measurement

A Hitachi MPF-2A fluorescence spectrophotometer was employed to measure steady-state fluorescence intensities and anisotropies and the latter were corrected for scattering contributions (typically less than 5 %). Perylene at 10^{-6} M was used as the fluorescent probe. This dye appears to be distributed evenly in the hydrocarbon interior of lipid dispersions [6]. The dye was excited at 436 nm and emission was measured at 474 nm. Fluorescence decay curves were obtained with a calibrated Ortec 9200 ns fluorimeter and lifetimes were calculated therefrom by a method of moments [24]. The calculation of membrane microviscosity, $\bar{\eta}$, was based on the following relationship [25]

$$\frac{r_0}{r} = 1 + \frac{KT\tau}{\bar{\eta}V(\mathrm{r})}$$

where r_0 is the limiting anisotropy and r is the anisotropy measured under the conditions of interest. τ is the mean fluorescence lifetime, K is Boltzman's constant, T is the absolute temperature and $V(\mathrm{r})$ is the effective rotational molecular volume. Since this latter parameter is not known, an empirical standardization procedure with oils of known viscosity [6] is employed to calculate microviscosities. Fluorescence lifetimes were determined at 25 °C and were assumed to vary with temperature exactly as fluorescence intensity.

Decay of anisotropy

The decay of fluorescence polarized parallel ($I_{||}$) and perpendicular ($I_{\perp}$) relative to the polarization of the exciting light were recorded with the Ortec ns fluorimeter. The time dependence of the emission anisotropy is calculated from the deconvoluted $I_{||}(t)$ and $I_{\perp}(t)$ curves according to the equation

$$A(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)}$$

Whereas for a rigid spherical fluorescent complex the decay of anisotropy can be characterized by a single rotational correlation time, in the more general case of a molecule of arbitrary symmetry the decay is multi-exponential. Since in the present study we are interested in observing a change of anisotropy decay with experimental conditions, rather than to characterize the decay in terms of its multi-exponential components (which would be expected since the perylene molecule is planar rather than spherical and membranes are not isotropic), and because of the limited precision of the data, we have analyzed the $A(t)$ curves only in terms of a single exponential and thus we speak of a mean rotational correlation time (ϕ).

RESULTS

Microviscosity of artificial lipid dispersions and natural membranes

Table I summarizes the values of the microviscosities at 25 °C of various

TABLE I

VALUES OF FLUORESCENCE LIFETIMES AND MICROVISCOSITIES OF PERYLENE IN VARIOUS MEMBRANE PREPARATIONS AT 25 °C

Material	Lifetime (ns)		Microviscosity ($\bar{\eta}$, cP)	
	Control	+0.6 mM tetracaine	Control	+0.6 mM tetracaine
(a) Brain ganglioside	5.9	6.5	268	215
+cholesterol (1 : 0.5)	9.2		762	
(b) Phosphatidylserine (0.35 mg/ml)	4.9		173	
+proteolipid (0.028 mg/ml)	4.4		222	
+basic protein (0.01 mg/ml)	4.8		193	
+cholesterol (1 : 1)	6.4		406	
+cholesterol (1 : 1)+digitonin			156	
+cholesterol (1 : 1)+amphotericin B			506	
(c) Bovine brain myelin	6.4	6.5	270	207
(d) Human erythrocyte membrane	7.0	7.0	180	157
(e) Rabbit polymorphonuclear leukocyte membrane	7.4		335	
(f) Rat liver mitochondria	7.0		90	
(g) Rat liver microsomes	7.0		95	
(h) White oil	5.6		115	
(i) Quinine in 0.05 M H_2SO_4	19.3			

dispersions of lipids, mixtures of lipids and membrane proteins, red blood cell membranes, myelin and rabbit polymorphonuclear leukocyte membranes and rat liver mitochondria and microsomes. Lifetime values of perylene fluorescence in these membranes are included. The effect of the anesthetic tetracaine · HCl on both the values is also given in some cases.

The anisotropy of perylene fluorescence and microviscosity of various biological membranes and lipid dispersions were determined as a function of temperature. The results are summarized in Figs 1–3. In Fig. 1, data are presented from measure-

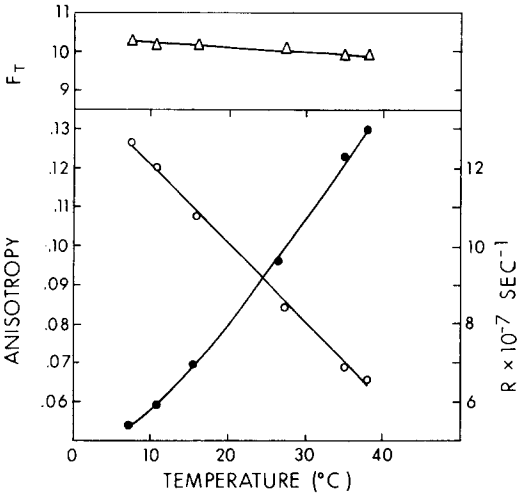

Fig. 1. Temperature dependence of perylene fluorescence anisotropy (○), total fluorescence emission (F_T) and R, the rate of rotation (●). Perylene (10^{-6} M) was incorporated into brain ganglioside (0.4 mg/ml) dispersions by sonication.

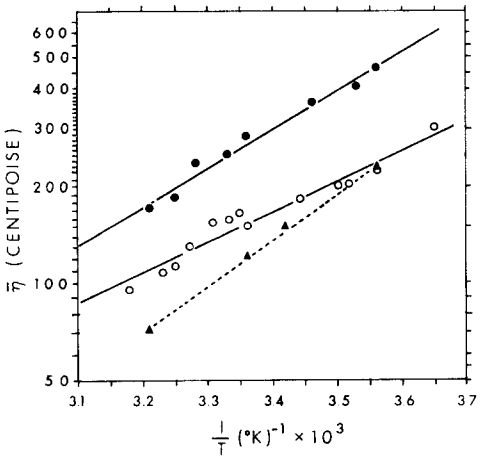

Fig. 2. Temperature dependence of the microviscosity of ganglioside (●) and phosphatidylserine (○) dispersions as determined by labelling with 10^{-6} M perylene. The data of Cogan et al. [6] for egg lecithin (▲) is included for purposes of comparison.

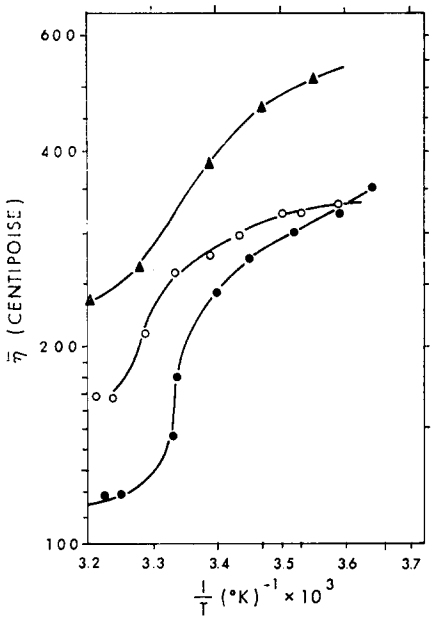

Fig. 3. Temperature dependence of the microvisocisty of polymorphonuclear leucocyte (▲), myelin (○) and erythrocyte (●) membranes as determined by labelling with 10^{-6} M perylene.

ments of perylene fluorescence in ganglioside dispersions. Over the temperature range from 8 to 38 °C the fluorescence emission intensity fell progressively to a small extent due to the increase in rate constants for various quenching processes. With increasing temperature the lipid environment of the dye became more fluid so that the anisotropy of the dye decreased, and the average rotation rate of dye molecules increased 2.4-fold. The microviscosity of phosphatidylserine was found to be lower than that of a brain ganglioside dispersion (Fig. 2). The natural logarithm of the viscosities of both lipids varied linearly with the reciprocal of the absolute temperature, and no sharp transitions were observed. Thus, throughout the temperature range studied the membranes were apparently in the liquid-crystalline state. In this respect they are similar to egg lecithin membranes, as opposed to dipalmitoyl phosphatidylcholine. The latter undergoes a very marked phase transition [6] owing to the melting of its saturated fatty acid chains in the temperature range of 25–37 °C. The lack of a gel → liquid-crystalline transition in the 0–40 °C range for phosphatidylserine is characteristic for lipids containing unsaturated fatty acids. The same result was obtained with ganglioside despite the fact that the glycolipid has a very high content of stearic acid (> 80 %) [26]. Both of these lipids form dispersions having microviscosities which are higher than that of egg lecithin (Fig. 2). In contrast to lecithin, both phosphatidylserine and the brain gangliosides bear a net negative surface charge at the pH of the experiment. The apparent enthalpy (ΔH) for perylene in ganglioside and phosphatidylserine dispersions was 5.41 and 4.69 kcal/mol, respectively (Table II). The latter is close to the value of 4.24 kcal/mol reported by Vanderkooi et al. [7] for L-α-dipalmitoyl phosphatidylcholine below 38 °C, employing 12-(9-anthroyl) stearic acid as the fluorescent probe.

TABLE II

EFFECT OF INCORPORATION OF CHOLESTEROL, MYELIN BASIC PROTEIN OR PROTEOLIPID, AND TETRACAINE ON THE ENTHALPY AND CHANGE OF ENTROPY OF THE SYSTEM

Liposome composition	Enthalpy ΔH (kcal/mol)	Entropy change (cal/degree per mol)
Phosphatidylserine	4.69	
+cholesterol	4.00	−4.2
+basic protein	4.77	−0.3
+proteolipid	3.43	−5.2
Ganglioside	5.41	
+tetracaine	6.61	+4.6

The natural membranes exhibited interesting differences in microviscosity. Unlike the lipid dispersions the relationship of ln $\bar{\eta}$ vs $1/T$ was non-linear for natural membranes. This was most evident for myelin and erythrocyte membranes, but less so for the polymorphonuclear leukocyte membrane (Fig. 3). The region of increased slope suggests an enhanced rate of melting of some of the lipid hydrocarbon chains between 25 and 37 °C. The magnitude of the viscosity change over this temperature range indicates that the degree of melting was much less than was observed for dipalmitoyl phosphatidylcholine membranes [6], suggesting that only a small fraction of the membrane lipid undergoes a phase change, and that there is some heterogeneity of lipid state in natural membranes. In the case of dipalmitoyl phosphatidylcholine, the viscosity change was about 7-fold from 27 to 45 °C.

Over almost the entire temperature range (0–37 °C) the microviscosity of the leukocyte membrane was greater than the microviscosities of the lipids or other natural membranes we studied. At 37 °C the microviscosity of the hydrocarbon interior of the erythrocyte membranes was 119 cP, (in excellent agreement with the data of Rudy and Gitler [8]), myelin microviscosity was 165 cP, and that of rabbit polymorphonuclear leukocyte membranes was 240 cP. Below 10 °C the microviscosities of myelin and the erythrocyte membranes were nearly equal, but the leukocyte membrane remained strikingly less fluid than the other membranes. At 37 °C the polymorphonuclear leukocyte membrane microviscosity was about 2-fold greater than that reported for human peripheral lymphocyte membranes by Rudy and Gitler [8]. Rat liver mitochondria and microsomes exhibited the lowest microviscosity of all membranes studied. The viscosities of all these natural membranes, except for mitochondria and microsomes, were greater than that of phosphatidylserine at all temperatures. On the other hand, at 37 °C the microviscosity of brain ganglioside was about equal to that of myelin, and at low temperatures (0-20 °C) it nearly approached the viscosity of polymorphonuclear leukocyte membranes. The phospholipid, glycolipid and natural membranes all exhibit a significantly greater microviscosity than a pure fluid hydrocarbon phase such as white oil.

Cholesterol effects

The addition of cholesterol to dispersions composed of phosphatidylserine or ganglioside resulted in an increase in microviscosity (see Table I). Subsequent addition

of digitonin reduced the microviscosity back to its precholesterol value. On the other hand, amphotericin B slightly increased the viscosity in the presence of cholesterol. Thus, qualitatively at least, the incorporation of cholesterol into these lipid dispersions brings about the same results as previously reported for lecithin [6], but the magnitude of the effect is much less than that obtained by addition of the sterol to lecithin dispersions. At a molar concentration ratio of egg lecithin/cholesterol of 1.5 to 1.0 the microviscosity increased by an order of magnitude over that of the phospholipid alone at all temperatures between 10 and 37 °C [6]. The absolute values for the increase in viscosity are striking. At 37 °C the net increase was 567 cP and at 25 °C it was 1180 cP! The effect of cholesterol on ganglioside and phosphatidylserine fluidity, at lipid/cholesterol ratios of 1 : 1 to 1.5 : 1, was significantly less, amounting to a net increase of 494 cP for ganglioside and 233 cP in the case of phosphatidylserine at 25 °C.

Effects of local anesthetics

The effect of the local anesthetics tetracaine and butacaine on the microviscosity of lipid dispersions or isolated natural membranes was measured. In these experiments, a small volume of concentrated local anesthetic solution was added to a test cuvette containing lipid or natural membrane containing perylene, to give the desired anesthetic concentration. An equal volume of 0.1 M Tris · HCl buffer solution (pH 7.4) was added to another cuvette used as a control. For light scattering measurements

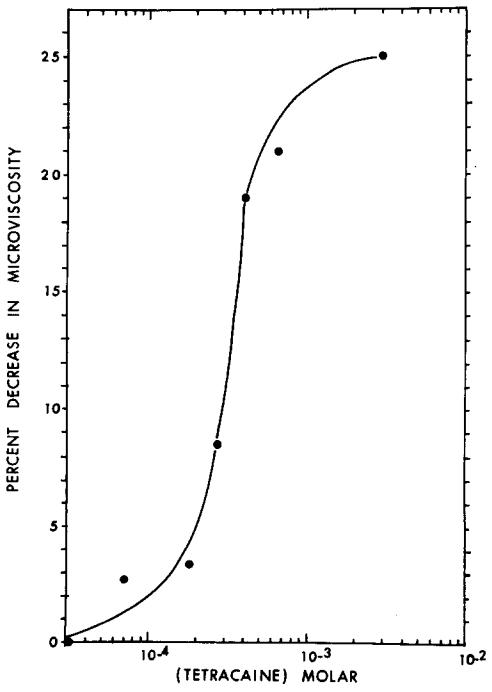

Fig. 4. Effect of tetracaine on the microviscosity of ganglioside dispersion as determined by labelling with perylene (10^{-6} M). Temperature was 25 °C.

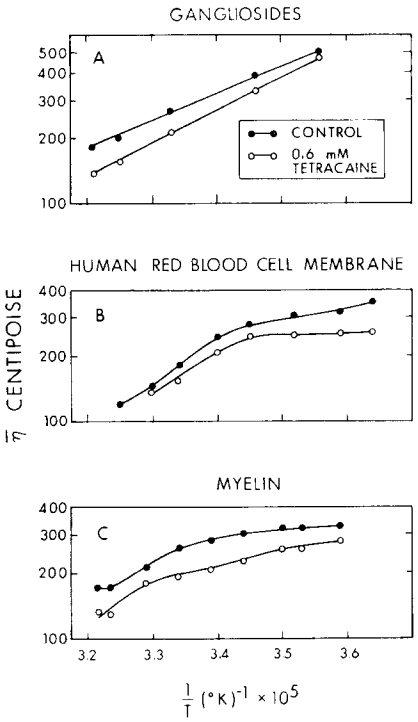

Fig. 5. Temperature dependence of microviscosity of ganglioside, red cell membranes and myelin membranes as determined by labelling with 10^{-6} M perylene. Controls (●) are compared with the same membrane preparations in the presence of $6 \cdot 10^{-4}$ M tetracaine (○).

an identical membrane dispersion without perylene was treated in exactly the same way, i.e. titrated with buffer or local anesthetic solution.

Tetracaine at concentrations between 0.06 and 3 mM produced a fall in microviscosity of ganglioside dispersions which amounted to 25 % at the higher concentration (Fig. 4). At a concentration of 0.6 mM tetracaine reduced the microviscosity of the hydrocarbon interior of myelin and erythrocyte membranes as well (Table I). The reduced viscosity was apparent throughout the temperature range studied (Fig. 5) and amounted to between 40 and 60 cP for ganglioside and myelin membranes. In erythrocyte membranes a fall of 95 cP was produced at 0 °C by 0.6 mM tetracaine, but the effect became progressively less as the temperature increased and the initial microviscosity of the membrane fell. At 37 °C the reduction in viscosity amounted to about 10–20 cP. Another local anesthestic, butacaine sulfate, over the concentration range of 0.23–1.8 mM also reduced the membrane microviscosity, but to about half the extent produced by tetracaine.

We have obtained the nuclear magnetic resonance spectra of tetracaine (10 mM) in ^2H_2O containing gangliosides (0.4 %). Marked broadening of the signals from the aromatic protons of the benzene ring (a, b), the *N*-aryl protons (h, i), and the *N*-alkyl protons (c, d, f) was evident and the proton spin-lattice relaxation times (T_1), for a and b were decreased from 1.30 and 1.15 s, respectively, to 0.30 s (Fig. 6). At lower concentrations of tetracaine (1 mM) signals a and b (aromatic protons) and f

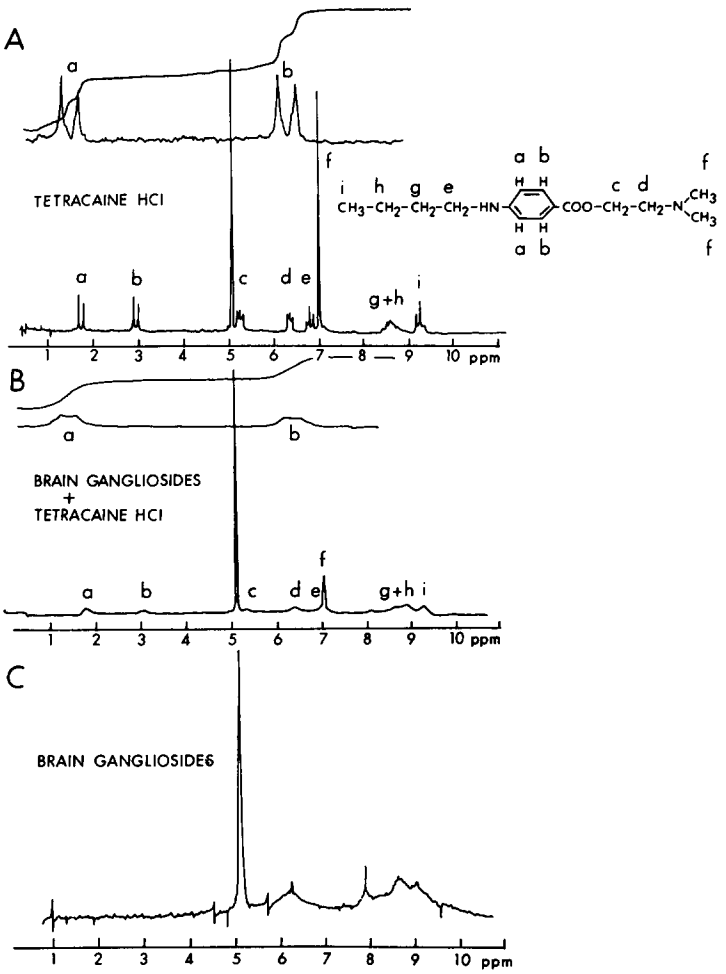

Fig. 6. The NMR spectra of (A) 10 mM tetracaine · HCl in ^2H_2O; (B) 10 mM tetracaine plus 0.4 % brain ganglioside in ^2H_2O; and (C) brain ganglioside (0.4 %) in ^2H_2O. Temperature 28 °C. The expanded records (three times vertical, four times horizontal) of proton signals a and b, as well as their integrated amplitudes are shown in (A) and (B) above the NMR spectra.

(*N*-alkyl protons) were so broadened as to be nearly lost in the baseline. Thus, the tetracaine molecules are strongly immobilized in the glycolipid membrane. The data also suggests that the *N*-alkyl (methyl) protons (f) may be involved in H-bonding at the polar surface of the membrane. Hauser et al. [27] observed line broadening, especially for the aromatic protons, when procaine and tetracaine were mixed with phosphatidylserine, but not with phosphatidylcholine, and Cerbón [28] noted the same effect employing a mixed egg phospholipid preparation.

The fluidity of the cellular membrane of intact living cells was assessed by measuring the decay of anisotropy of perylene fluorescence from which one can calculate directly the mean rotational correlation time (ϕ) and the rotational relaxation time (ρ). The decay of anisotropy curves for perylene in Ehrlich ascites cells are

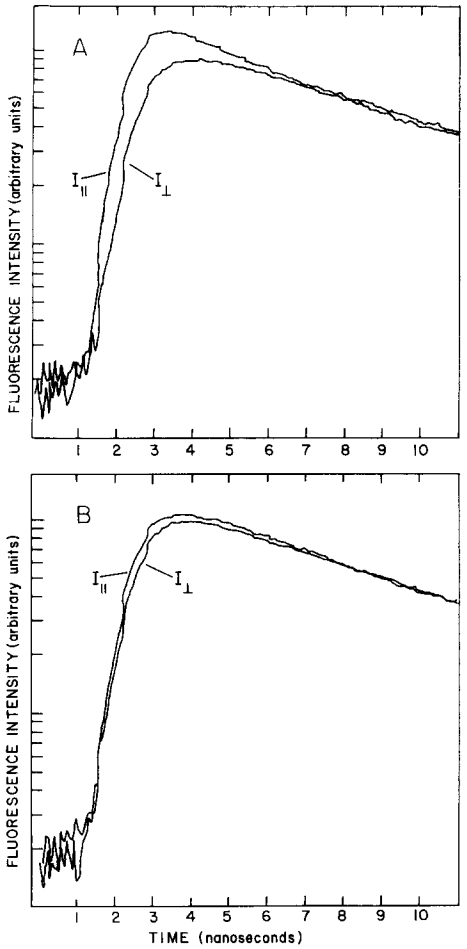

Fig. 7. Decay of anisotropy of perylene fluorescence in Ehrlich ascites tumor cells. (A) Fluorescence decay curves taken in control cells with emission polarizers in parallel ($\parallel$) and perpendicular ($\perp$) orientation to the plane of exciting light. (B) After the addition of 0.6 mM tetracaine anisotropy is decreased. The calculated rotational correlation time (ϕ) in (A) is 8.7 ns and in (B) ϕ = 6.5 ns.

shown in Fig. 7. The effect of 0.6 mM tetracaine added to the tumor cells was to decrease ϕ for perylene from 8.7 to 6.5 ns, and therefore ρ from 26.1 to 19.5 ns. Thus, the anesthetic increased the mobility of the dye in the cell membrane indicating that the viscosity of the dye environment was decreased.

Effects of proteins

The addition of the myelin proteins to phosphatidylserine dispersions resulted in an increase in the anisotropy of dye fluorescence and calculated microviscosity as seen in Fig. 8. The effect of the basic protein was significantly less than that of the proteolipid and was maximal at a concentration of 10 μg protein to 0.35 mg phosphatidylserine, a molar ratio of about 1 : 1000. The increase in anisotropy and calculated microviscosity was apparent over the entire temperature range 0–37 °C. The molar

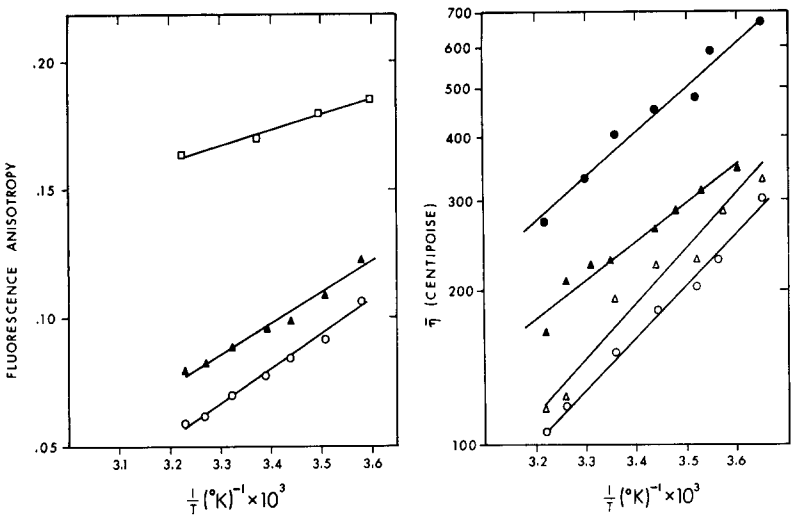

Fig. 8. Temperature dependence of (A) fluorescence anisotropy of perylene bound to proteolipid (□), phosphatidyl serine (○) and phosphatidylserine and proteolipid (▲) and (B) the calculated microviscosity of phosphatidylserine liposomes (○) sonicated in the presence of myelin basic protein (△), myelin proteolipid (▲) and cholesterol (●). The proteins are present at the concentrations listed in Table I. Cholesterol is present at a 1 : 1 molar ratio. Perylene concentration was 10^{-6} M. See text for explanation of apparent changes in microviscosity due to protein incorporation into membranes.

ratio of proteolipid to lipid was about 1 : 1000 assuming a molecular weight for the proteolipid of about 25 000–30 000 [28]. The proteolipid comprised 7 % of the total lipid plus protein weight of the membrane, whereas in myelin this protein accounts for about 15 % of the dry weight of the membrane.

Perylene binds to the proteolipid in aqueous solution with an anisotropy of dye fluorescence emission of 0.17 at 25 °C. The increase in fluorescence anisotropy which accompanied the incorporation of proteolipid into the phospholipid dispersions was 0.015 to 0.023 over the temperature range of 3–37 °C (Fig. 8). The magnitude of the increase in r was such that it could be accounted for by the binding of about 9–14 % of the dye present in the membrane to the protein. The fluorescence intensity when dye and protein were mixed in solution was only 15 % of that attained with the lipid alone. Thus, if any substantial partition of dye from lipid to protein occurred in the membrane a fall in fluorescence intensity would be expected. But no change was observed. Nevertheless, we have no certain way of determining in this case how much of the anisotropy change resulted from alterations of the lipid environment, as opposed to shifting of lipid-bound dye to protein binding sites. The slope of the fluorescence anisotropy vs temperature plots were significantly different for protein as compared to membrane binding sites. However, the expected change in slope for the lipid-protein membrane would be barely detectable if only 10 % of the membrane-bound dye were attached to protein sites.

Upon incorporation of cytochrome b_5 into phosphatidylserine dispersions (molar ratio 1 : 70) the anisotropy of perylene fluorescence was 0.105 as compared to 0.108 for purely lipid vesicles. The fluorescence anisotropy of perylene bound to the

protein was 0.090. Thus, the presence of cytochrome b_5 in the lipid membrane had a neglibible effect on calculated microviscosity (Table I).

Enthalpy and Entropy of the System

The apparent changes in enthalpy (ΔH) of the system upon the incorporation of cholesterol, basic protein, and proteolipid may be computed in a straightforward manner by using the following equation:

$$\bar{\eta} = k e^{+\Delta S^{\ddagger}/R} e^{-\Delta H/RT}$$

in which $\bar{\eta}$ is the viscosity, k is a constant and R and T have their usual meaning. ΔH can be calculated from the slope of the line ln $\bar{\eta}$ vs $1/T$. ΔS cannot be calculated directly since we do not know the value of k. However, the change in ΔS upon the incorporation of cholesterol, protein or a local anesthetic into a lipid dispersion can be calculated as follows: let the subscript 1 refer to the case in which the dispersions are made up of phosphatidylserine only; the subscript 2 refers to the cases in which cholesterol, protein or local anesthetic are incorporated in the system. From the above equation the change in entropy upon the incorporation of cholesterol for example ($\Delta S_2^{\ddagger} - \Delta S_1^{\ddagger}$) can be calculated from the intercept of the line ln $\bar{\eta}$ vs $1/T$ by the following equation:

$$\Delta S_2^{\ddagger} - \Delta S_1^{\ddagger} = R(I_1 - I_2)$$

where I refers to the intercept of the line ln $\bar{\eta}$ vs $1/T$. The results are summarized in Table II. Cholesterol and the proteolipid decreased the entropy. The basic protein had a much smaller entropy effect but it is in the same direction as that of the proteolipid. Tetracaine on the other hand increased the disorder, or randomness of the system as indicated by the increase in entropy produced.

DISCUSSION

Membrane fluidity vitally influences permeability, the transport of metabolites and ions and membrane enzymatic activity [1, 2, 29]. The fluorescence anisotropy method for measurement of membrane microviscosity is useful for revealing the differences in membrane fluidity which may be related to specific cellular properties, and for elucidating those factors which affect the overall fluidity of membrane lipids. It is important to realize, however, that, when the steady-state fluorescence method for measuring membrane microviscosity is employed, an average anisotropy of dye fluorescence is determined. Thus, regional variations in microviscosity, which may be large, will not be detected. Furthermore, our results indicate that in some cases the partitioning of a fraction of the dye molecules into protein binding sites may introduce some error into the calculation of the microviscosity of the lipid phase of the membrane.

Effects of cholesterol on membrane fluidity

As a general rule we conclude that the presence of cholesterol in membranes leads to a decrease in the fluidity of the hydrocarbon chains of lipid membranes which are in the liquid-crystalline state. Quantitatively the effects of cholesterol on membrane

structure and permeability are dramatically influenced by the fatty acid composition of the lipids, as well as the charged groups of the membrane surface [5].

Shah [30] has shown how the effect of cholesterol on surface film pressure and area varies with the initial film pressure (and thereby the interlipid distances), the degree of unsaturation of the fatty acyl chains, and the length of the fatty acids. Depending upon these factors potential cavities of varying size exist between lipid molecules which can accommodate cholesterol. If such cavities easily accommodate the sterol molecule, little change in surface area occurs and an apparent condensation of the film area occurs, as had indeed been reported [29]. However, should the cavities be too small, then the insertion of cholesterol into the lipid film will actually increase the area, or increase the film pressure if the area is limited [30]. An expanded state, due to charge repulsion at the polar surfaces of membranes composed of gangliosides or phosphatidylserine, is expected because both bear a net negative charge arising from the presence of sialic acid and serine carboxyl groups, respectively. From our experiments it is apparent that the microviscosity of lipid membranes is affected by the incorporation of cholesterol in a manner dependent upon surface charge, since the negatively charged phosphatidylserine and ganglioside membranes are not as greatly altered as neutral lecithin membranes. Consistent with this result is the observation that the ability of cholesterol to decrease glucose permeability of liposomes was reduced by the presence of small accounts of charged lipids such as dicetyl phosphate or phosphatidic acid [5].

Digitonin decreases the resistance of membranes [31], an effect which may result from a change in membrane fluidity. We observed that addition of digitonin to lipid dispersions containing cholesterol caused the microviscosity to fall to the value obtained in the absence of cholesterol. The unsaturated lactone ring of digitonin interacts with the -OH group of cholesterol and 1 : 1 stoichiometric associations occur [32]. We propose that these digitonin-cholesterol complexes cluster in the membrane so that phospholipid molecules have little cholesterol interspersed between them. The perylene fluorescence anisotropy reports the fluidity of this purely phospholipid environment. The calculated microviscosity is therefore characteristic of a cholesterol-free phospholipid membrane.

Amphotericin B also increases membrane permeability and modifies membrane structure by interaction with cholesterol. Although the formation of clusters of cholesterol and filipin (a polyene related to amphotericin B) has been reported to occur in membranes [33] neither we nor Puchwein et al. [34] have observed any fall in membrane microviscosity with either filipin or amphotericin B. It is probable that the polyene-cholesterol complexes remain scattered throughout the lipid bilayer.

Anesthetic effects on membrane fluidity

The interactions of local anesthetics with phosphatidylserine and ganglioside have been found to be particularly noteworthy [35–39]. Both lipids are furthermore thought to be important in nerve function, and could represent important binding sites for local anesthetics in axons or synaptic regions. No previous work on local anesthetic reactions with glycolipids has been reported except for their effect on anilinonaphthalenesulphonate binding to the glycolipid dispersions [38].

Tetracaine and butacaine produced a fall in the microviscosity of lipid dispersions and natural membranes based upon measurements of steady-state anisotropy

of perylene fluorescence. This finding is further confirmation of conclusions drawn about the membrane disordering effects of anesthetics through the use of NMR spectroscopy [40, 41]. Hubbell et al. [10] and Butler et al. [11] measured the ESR spectra of spin labels in membranes and also concluded that anesthetics could produce a disordering effect. An advantage of the fluorescence method with respect to previous NMR and ESR studies is that a more quantitative estimate of membrane fluidity can be obtained. In addition, the effects of anesthetics were apparent at lower concentrations than were required in the ESR and NMR studies.

The increase in rotational mobility of perylene in the membranes of intact ascites tumor cells, calculated by measuring the decay of anisotropy, showed that tetracaine decreased membrane microviscosity. This effect of an anesthetic, measured for the first time by the fluorescence method in an intact living cell, is consistent with our observations on artificial lipid dispersions and isolated cell membranes. The use of this dynamic method of measurement of membrane microviscosity may make it possible in the future to concurrently study permeability changes and membrane viscosity in the same intact cells.

Tetracaine increased the entropy of the system (Table II). Comparable entropy changes have been observed with a variety of volatile anesthetics in liposomes [42] and with chlorpromazine in erythrocyte ghosts [43]. It is likely that the membrane disordering effects of anesthetics are the basis for the marked degree of membrane expansion they induce [44]. The insertion of the shorter anesthetic molecules between the lipid chains would result in the formation of cavities between hydrocarbon chains at their extremities. This would be expected to provide greater degrees of freedom for molecular motion of the lipid chains and to lessen van der Waals-London interaction forces.

The effects of anesthetics on ionic movements in artificial membranes [39, 42] may result from the changes in microviscosity which we have reported. However, in natural membranes the effects of lipid disordering on peptide or protein ionophores, or gating mechanisms may also have to be considered. With respect to the amine local anesthetics (e.g. tetracaine) an additional mechanism is probably of great importance, namely ionic or electrostatic interactions with lipid polar or charged groups [35–37]. These interactions would affect the ζ-potential of the membrane [39] and thereby the ionic concentrations at the membrane surface. Such a mechanism has been invoked to explain the inhibitory effect of local anesthetics on ionophore-induced conductance changes in lipid bilayer membranes [45].

Effects of protein on membrane fluidity

The results obtained with the myelin proteolipid, which indicate that the protein increase membrane microviscosity, are inconclusive because of the uncertainty concerning the extent to which perylene molecules are bound to protein in the artificial membranes. However, in the case of cytochrome b_5 it has been possible to demonstrate more conclusively that insertion of a hydrophobic protein into a lipid membrane does not necessarily increase the microviscosity of the bulk lipid phase. A strong immobilization of spin-labelled lipid (doxylstearic acid and doxylphosphatidylcholine) bound to cytochrome b_5 has recently been demonstrated [46]. In these experiments the protein : lipid molar ratio was 8 : 1. When located in a liposome the presence of cytochrome b_5 resulted in the appearance of two populations of spin-labelled lipid,

one highly immobilized and presumably bound to the protein, and another with a mobility characteristic of pure lipid membranes. It was calculated [46] that each mol of cytochrome b_5 probably immobilizes 2–4 mol of lipid in a membrane. Thus, only 3–6 % of the lipid in our experiments would be expected to be affected. The negligible change in membrane viscosity we have noted upon incorporation of cytochrome b_5 is therefore consistent with the above-noted findings since the fluorescence anisotropy measurements represent the average for the entire population of perylene molecules. As the protein concentration was 6 nmol/ml compared to about 420 nmol/ml phospholipid, we can reasonably conclude that the dye was primarily associated with purely lipid regions remote from the protein molecules. Thus, in the immediate region of lipid-protein interaction the microviscosity could be much higher than the measured average value which predominantly reflects normal lipid regions.

Fluidity of natural membranes

The relative microviscosities of myelin, mitochondrial and microsomal membranes correlate well with their respective cholesterol : phospholipid ratios. The high viscosity of myelin favors its effectiveness as an insulator, and the low viscosity of microsomal and mitochondrial membranes probably facilitates mobile protein interactions concerned with electron transport. The explanation for the high viscosity of the polymorphonuclear leukocyte membrane is not apparent, since the cholesterol : phospholipid ratio in this membrane is not significantly different from that of erythrocyte membrane. However, the high viscosity of the polymorphonuclear leukocyte membrane is reflected in a very low water permeability ,0.1 that of erythrocytes [47]. It is likely that this low water permeability confers greater cellular stability in the face of potential osmotic stresses that are imposed by the digestion and incorporation of large amounts of phagocytized foreign materials [48]. It is known that extensive degradation of bacterial lipids, nucleic acids and protein occurs upon phagocytosis by polymorphonuclear leukocytes. Re-incorporation of bacterial constituents into the leukocyte occurs, accompanied by the appearance of large amounts of inorganic phosphate [48].

In conclusion, we have shown that there are significant variations in the fluidity of natural membranes, which must be of considerable functional significance. Furthermore, membrane fluidity is in some cases quite susceptible to alteration by substances which are known to affect membrane permeability. The nature of the lipid composition of membranes, with respect to charged groups, degree of unsaturation and length of fatty acid chains, and relative cholesterol content appear to be the most important parameters controlling fluidity. It is already apparent that membrane lipid abnormalities in disease states may induce changes in membrane fluidity. For example, in Duchenne type muscular dystrophy there are significant changes in membrane viscosity [49] which are associated with altered function of membrane-bound enzymes [50].

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