Microviscosity and Order in the Hydrocarbon Region of Phospholipid and Phospholipid–Cholesterol Dispersions Determined with Fluorescent Probes

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ABSTRACT: Microviscosity, existence of phase transitions, and structural organization in the hydrocarbon region of lipid dispersions of biological importance were investigated by means of fluorescence polarization techniques. Dispersions of egg lecithin, dipalmitoyllecithin, and lecithin–cholesterol mixtures as well as egg lysolecithin micelles were labeled with perylene, 2-methylanthracene, or 9-vinylanthracene and the microviscosities which represent the harmonic mean of the internal viscosities opposing the in-plane and out-of-plane rotations of the nonpolar fluorescence dyes were evaluated by measuring the degree of the fluorescence depolarization. In the phospholipid systems studied, microviscosity values obtained were lowest for egg lysolecithin, followed by egg lecithin, and were highest for dipalmitoyllecithin. Cholesterol, when incorporated into lecithin dispersions, caused a marked increase in the internal viscosity. A phase transition between liquid crystalline and gel states was detected by determining the dependence of the microviscosity (η) on the absolute temperature (T). Deviation of ln η *vs.* $1/T$ plots from linearity or sigmoidal response of the plots was taken to indicate the presence of a phase transition. Such was found to be the case for dipalmitoyllecithin and dipalmitoyllecithin–cholesterol dispersions. No deviation from linearity was observed for egg lecithin and egg lecithin–cholesterol dispersions or lysolecithin micelles. The degree of order or disorder in the interior of lipid dispersions was estimated from the relative rates of the in-plane and out-of-plane rotations. Egg lecithin and lysolecithin were found to possess a largely isotropic hydrocarbon interior. The incorporation of cholesterol into egg lecithin transformed the disordered interior into an ordered structure. The cholesterol effect appeared to be an additive phenomenon, *i.e.*, the anisotropic character of the interior increased with increase in the level of the incorporated cholesterol.

Lipids, mostly phospholipids, play an essential role in the structural organization of lipoproteins and membranes, as well as in their function and properties (Rouser *et al.*, 1968). In addition to phospholipids, cholesterol is a basic constituent of myelin, erythrocyte membranes, and various plasma membranes (Korn, 1969). Proper arrangement and orientation of protein, phospholipid, and cholesterol appears to determine the physical characteristics of the membrane such as mechanical strength, flexibility, and prevention of free diffusion (Chapman and Wallach, 1968). Because of the complexity of membrane structure, lipid monolayers, bilayers, and dispersions have been widely employed as model systems for the study of the role of phospholipid and cholesterol in membranes. The present communication provides some quantitative information on the fluidity or microviscosity of the interior of various phospholipid and phospholipid–cholesterol dispersions determined by measuring fluorescent depolarization of hydrophobic dyes (Shinitzky *et al.*, 1971) incorporated into the hydrocarbon regions. In addition, a recent theory which describes the pattern of fluorescence depolarization of asymmetric molecules due to Brownian rotations (Weber, 1971) is used to determine the anisotropic character of the rotational motion inside these lipid entities.

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Experimental Section

Materials. Perylene (Aldrich Gold label) was used without further purification. 2-Methylanthracene and 9-vinylanthracene (Aldrich) were crystallized from ethanol. White Oil USP 35 was obtained from the American Oil Co. Purity as well as spectral properties of the dyes and oil were previously established (Shinitzky *et al.*, 1971). Highly purified triolein was obtained from the Hormel Institute (Austin, Minn.) and was used without further purification. Egg lecithin was isolated according to the method of Rhodes and Lea (1957), and was purified by silicic acid column chromatography as described by Hanahan *et al.* (1957). Dipalmitoyllecithin was purchased from General Biochemicals (Chagrin Falls, Ohio) and was purified by the same chromatographic method. Egg lysolecithin was obtained from the enzymic hydrolysis of egg lecithin with *Crotalus admanteus* venom and purified by silicic acid chromatography according to the procedure of Hanahan *et al.* (1954, 1960). All phospholipid preparations exhibited well-defined single spots by thin-layer chromatography on silica gel G plates with chloroform–methanol–water (65:25:4, v/v) as the developing solvent. Cholesterol of high purity (>99%) was obtained from Sigma Chemical Co. and used without further purification. Cetylbetaine was prepared from redistilled cetyldimethylamine (K & K Laboratories Inc., Plainview, N. Y.) according to Beckett and Woodward (1963). Cetyldimethylamine oxide was also prepared from redistilled cetyldimethylamine according to Hoh *et al.* (1963) and Lake and Hoh (1963) and was kept as a 30% aqueous gel.

Buffer. Experiments were performed in phosphate buffer of ionic strength 0.1, pH 7.4, containing 0.025% EDTA unless specified otherwise in the text.

Preparation of Lipid Dispersions. Lipid dispersions of leci-

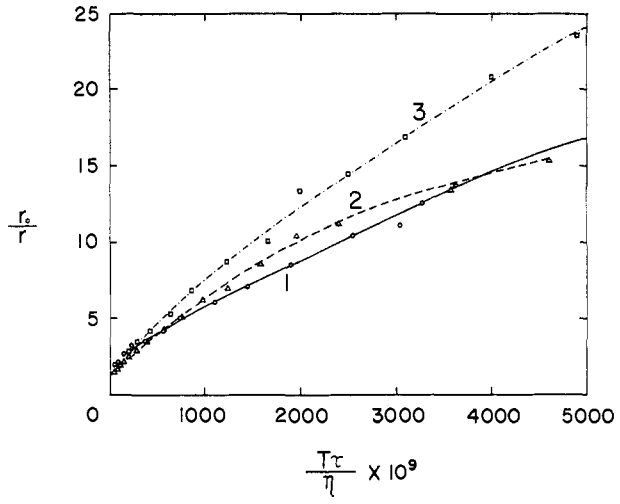

FIGURE 1: The dependence of r_0/r on $T\tau/\eta$ for perylene (curve 1), 2-methylanthracene (curve 2), and 9-vinylanthracene (curve 3). Anisotropy (r) was determined in White Oil USP 35 and limiting anisotropy (r_0) in propylene glycol at $-50°$. Perylene, 10^{-6} M, was excited at 436 nm; 2-methylanthracene, 10^{-5} M, at 380 nm; and 9-vinylanthracene, 10^{-5} M, at 392 nm.

thin or lecithin–cholesterol mixtures were prepared by sonifying desirable lipids or lipid mixtures under nitrogen in 10 or 15 ml of 0.025% EDTA (pH 7.4) using a Branson sonifier (Model S-75, 20 kcycle/sec, Branson Instruments, Inc., Danbury, Conn.) as described previously (Muesing and Nishida, 1971). With dispersions of egg lecithin, the temperature was kept between 30 and 37°, while with dispersions containing dipalmitoyllecithin the temperature during sonication and storage was maintained above 40° in order to prevent aggregation. Due to the low concentration of lipids used (0.04–0.12%), sonication of 10–20 min was found satisfactory to produce transparent dispersions. Large aggregates were removed by centrifugation for 30 min at 65,000*g*. When labeled with perylene, the final concentration of lipids was adjusted to 0.02% in the phosphate buffer. With 2-methylanthracene or 9-vinylanthracene, the final lipid concentration was 0.1%. Lipid phosphorus and cholesterol were determined as described previously (Muesing and Nishida, 1971).

Labeling Lipid Dispersions with Fluorescent Dyes. Labeling was achieved by sonicating lipids mixed with a measured amount of dye. The amounts of dyes added were equivalent to approximately 1 molecule/375,000 daltons of lipids for perylene and 1 molecule/200,000 daltons of lipids for 9-vinylanthracene and 2-methylanthracene. Under these conditions, undesirable fluorescence depolarization due to nonradiative or radiative energy transfer between probe molecules was practically eliminated. For each preparation, a control dispersion containing the same lipid components, but lacking the probe, was prepared under identical conditions to serve as a blank.

Fluorescence Polarization Measurements. The instruments and methods employed for the evaluation of fluorescence polarization of the scattering lipid dispersions were previously described (Shinitzky *et al.*, 1971). The following relationship was used to obtain the fluorescence anisotropy, r, of the probe

$$r = \frac{(I_{\parallel} - I_{\perp}) - (I_{\parallel} - I_{\perp})_s}{(I_{\parallel} + 2I_{\perp}) - (I_{\parallel} + 2I_{\perp})_s}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the exciting beam, and the subscript s stands for the scattering component. Corrections for scattering obtained with dye-free control lipid dispersions were relatively small and of minor importance with excitations in the vicinity of the last absorption maximum of the dye. However, with excitations at shorter wavelengths which were used for obtaining anisotropy spectra, corrections for scattering became very significant.

Lifetime Measurements. A cross-correlation-phase fluorometer designed by Spencer and Weber (1969) was used to evaluate fluorescence decay times of the dyes in the various environments. Lifetimes (τ) were corrected for scattering when the contribution of scattering to fluorescence intensity exceeded 1%. The corrections of τ obtained by the phase and modulation methods were made as follows, assuming a heterogeneous system of emission with $\tau = 0$ for the scattering component.

τ BY PHASE METHOD

$$\tan \phi = \frac{(\Sigma A_i \sin \phi_i \cos \phi_i)}{(\Sigma A_i \cos^2 \phi_i)}$$

$$= \frac{A \sin \phi_F \cos \phi_F}{A \cos^2 \phi_F + (1 - A)}$$

$$= \frac{(1 - \cos^2 2\phi_F)^{1/2}}{\cos 2\phi_F + \left(\frac{2}{A} - 1\right)}$$

Solving for $\cos 2\phi_F$ yields

$$\cos 2\phi_F = \frac{-\left(\frac{2}{A} - 1\right)\tan^2 \phi \pm \left[\tan^2 \phi \frac{4}{A}\left(1 - \frac{1}{A}\right) + 1\right]^{1/2}}{\tan^2 \phi + 1}$$

where A represents the fraction of total emission intensity given by the fluorescent component; ϕ, the observed phase angle; ϕ_F, the corrected phase angle; and the subscript i, the ith component.

τ BY MODULATION METHOD

$$\bar{m}^2 = (\Sigma A_i \cos^2 \phi_i)^2 + (\Sigma A_i \cos \phi_i \sin \phi_i)^2$$

$$= [A \cos^2 \phi_F + (1 - A)]^2 + A^2 \sin^2 \phi_F \cos^2 \phi_F$$

where $\bar{m}$ is the observed modulation and the rest of the notation is the same as described for the phase method. Solving for $\cos \phi_F$ yields

$$\cos \phi_F = \left[\frac{\bar{m}^2 - (1 - A)^2}{A(2 - A)}\right]^{1/2}$$

All lifetimes were measured at 25°. To estimate the lifetime of the excited state at any given temperature, it was assumed that both the lifetime and the fluorescence intensity have the same temperature dependence, *i.e.*, $\tau_T/\tau_{25} = F_T/F_{25}$, where τ_T denotes decay time of the fluorescence at temperature T, τ_{25} is the measured decay time at 25°, and F_T and F_{25} are the fluorescence intensities of the dye at temperature T and 25°, respectively. The excited states lifetime values of the three dyes used in the reference solvent and in the various lipid dispersions or micelles are given in Table I.

TABLE I: Lifetime Values of Perylene, 2-Methylanthracene, and 9-Vinylanthracene in White Oil, Triolein, and Lipid Dispersions of Micelles.[a]

Dye	Material	Lifetime (nsec) at 25°[c]		
		Phase Method	Modulation Method	Av
Perylene	White oil	4.41 ± 0.21	4.57 ± 0.10	4.5
	Triolein	4.67 ± 0.11	4.77 ± 0.12	4.7
	Egg lecithin	4.71 ± 0.14	5.97 ± 0.03	5.3
	Egg lecithin–cholesterol (1.5:1)[b]	6.38 ± 0.19	6.80 ± 0.06	6.6
	Dipalmitoyllecithin	5.37 ± 0.61	5.45 ± 0.09	5.4
	Dipalmitoyllecithin–cholesterol (3:1)[b]	5.02 ± 0.15	7.00 ± 0.21	6.3
	Dipalmitoyllecithin–cholesterol (6:1)[b]	6.68 ± 0.23	6.10 ± 0.07	6.6
	Egg lysolecithin	5.46 ± 0.44	6.29 ± 0.20	5.9
9-Vinylanthracene	White oil	7.67 ± 0.60	8.65 ± 0.22	8.2
	Triolein	7.50 ± 0.29	9.50 ± 0.07	8.5
	Egg lecithin	12.05 ± 0.39	10.18 ± 0.08	11.1
2-Methylanthracene	White oil	3.29 ± 0.14	4.36 ± 0.07	3.8
	Egg lecithin	2.96 ± 0.17	3.90 ± 0.09	3.4

[a] Excitation wavelengths for perylene, 9-vinylanthracene, and 2-methylanthracene were 413, 392, and 380 nm, respectively. Dye concentrations in white oil and triolein were 10^{-6} M with perylene and 10^{-5} M with 9-vinylanthracene and 2-methylanthracene. In dispersion or micelle preparations, the amounts of dyes incorporated were equivalent to approximately 1 molecule/375,000 daltons of lipids for perylene and approximately 1 molecule/200,000 daltons of lipids for 9-vinylanthracene and 2-methylanthracene. Lipid concentration was 0.02% when labeled with perylene and 0.1% with the other two dyes. [b] The values in parentheses represent the molar ratios of lecithin to cholesterol. [c] Only perylene in white oil and triolein showed homogeneous emission; in all other cases, the emission is from fluorophores with more than one fluorescence lifetime since the values from phase and modulation methods show differences outside the experimental errors. These differences may be due to the formation of more than one type of molecular complexes with the solvent, brought about by the presence of groups of different character in the solvent, the fluorophore, or both.

Evaluation of Microviscosities. Microviscosities were determined essentially according to the method previously described (Shinitzky *et al.*, 1971) using the relationship: $r_0/r = 1 + (kT\tau/\eta V(r))$, where r_0 is the limiting anisotropy of the dye at the wavelength employed; r, observed anisotropy; k, Boltzman constant; T, absolute temperature; η, viscosity of the environment of the dye; $V(r)$, effective rotational molecular volume; and τ, decay time of the excited state. Standard curves which relate anisotropy to microviscosity were obtained using white oil in the following manner. The anisotropy of each dye dissolved in the white oil was measured as a function of temperature. Also determined was the viscosity of the white oil at various temperatures. The ratio r_0/r for each dye was then plotted against $T\tau/\eta$ to give standard curves as shown in Figure 1. The curvature of the curves apparently reflects the nonspherical nature of the dyes. To determine microviscosities of lipid dispersions, the anisotropies of incorporated dyes were measured as a function of temperature. The value, $T\tau/\eta$, corresponding to each r_0/r value was obtained from the standard curve and upon insertion of T and τ, the viscosity, η, of the dye environment was obtained. It was assumed that when a dye molecule has the same degree of depolarization in two different environments (the reference white oil and a lipid dispersion or micelle in the present study), the effective molecular rotational volumes in the two solvents are identical. The validity of this assumption has previously been discussed (Shinitzky *et al.*, 1971).

Results

The microviscosity of egg lecithin was determined as a function of temperature with perylene, 9-vinylanthracene, and 2-methylanthracene (Figure 2). The viscosity values obtained, as well as their temperature dependence, were not appreciably different with the three dyes used. Linear response of log η *vs.* $1/T$ plots obtained with the dyes indicated that the change of η with temperature followed the exponential form: $\eta = Ae^{\Delta E/RT}$ which is typical for pure liquids. Fusion activation

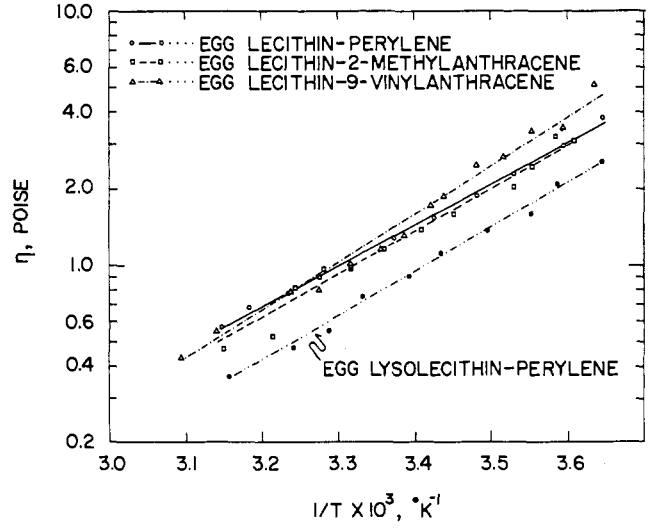

FIGURE 2: Temperature dependence of the microviscosity of egg lecithin dispersions as determined by labeling with perylene, 2-methylanthracene, and 9-vinylanthracene, and of egg lysolecithin micelles labeled with perylene. Lipids and dye concentrations were the same as those given in Table I.

TABLE II: Microviscosities and Fusion Activation Energies of Triolein and of Various Lipid Dispersions or Micelles as Determined by Labeling with Perylene.[a]

Materials	Microviscosity (P) at 10°	20°	25°	37°	45°	ΔE (kcal/mol)
Triolein	3.4	1.8	1.3	0.64	0.36	10.6
Egg lecithin	2.4	1.5	1.2	0.73	0.53	7.3
Egg lecithin–cholesterol (1.5:1)[b]	26	15	12	6.4	4.4	8.5
Dipalmitoyllecithin	12	10.5	9.4	3.9	0.94	
Dipalmitoyllecithin–cholesterol (6:1)[b]	52	44	36	11	1.8	
Dipalmitoyllecithin–cholesterol (3:1)[b]	91	69	53	19	3.2	
Egg lysolecithin	1.6	0.97	0.77	0.45	0.33	8.3
Cetylbetaine	1.12	0.69	0.55	0.33	0.24	7.7
Cetyldimethylamine oxide		0.69	0.59	0.34	0.24	7.7

[a] Perylene was excited at 413 or at 436 nm; lipid and dye concentrations were the same as those given in Table I. [b] The values in parentheses represent the molar ratios of lecithin to cholesterol.

energies, ΔE, obtained with perylene, 2-methylanthracene, and 9-vinylanthracene were similar, the values being 7.3, 8.1, and 8.8 kcal per mol, respectively. A high degree of fluidity of the interior of lecithin dispersions became evident upon comparison of the microviscosity of lecithin with the values obtained with isotropic triolein under similar conditions. Comparable values were obtained in both systems with perylene at 25–37°, although temperature response of the viscosity was somewhat greater for triolein (Table II). As was expected, egg lysolecithin micelles exhibited lower microviscosity; the values were approximately 30–40% smaller than those of egg lecithin in the temperature range studied (Figure 2 and Table II).

The temperature dependence of the microviscosity of dipalmitoyllecithin was found to be considerably different from that of egg lecithin (Figure 3). The plot of log η *vs.* $1/T$ gave a sigmoidal curve indicating a phase transition in the state of the hydrocarbon region in the temperature range of 25–50°. The hydrocarbon chains are likely to be in a liquid crystalline state above 50° and in the gel state below 20°, the midpoint of the transition being in the vicinity of 39°. In the entire temperature range studied, dipalmitoyllecithin was found to possess considerably higher microviscosity than egg lecithin, the difference being more pronounced below the temperature at which phase transition begins to occur. The slope of the log η *vs.* $1/T$ plot above 44° is essentially the same as that for egg lecithin but is considerably reduced below 25° which may be characteristic of the gel phase.

The incorporation of cholesterol into egg lecithin dispersions resulted in a marked increase in the microviscosity (Figure 4). Egg lecithin–cholesterol dispersions appeared to be in the liquid crystalline phase in the entire temperature range covered; no phase transition was observed. The microviscosity of dipalmitoyllecithin dispersion was also elevated

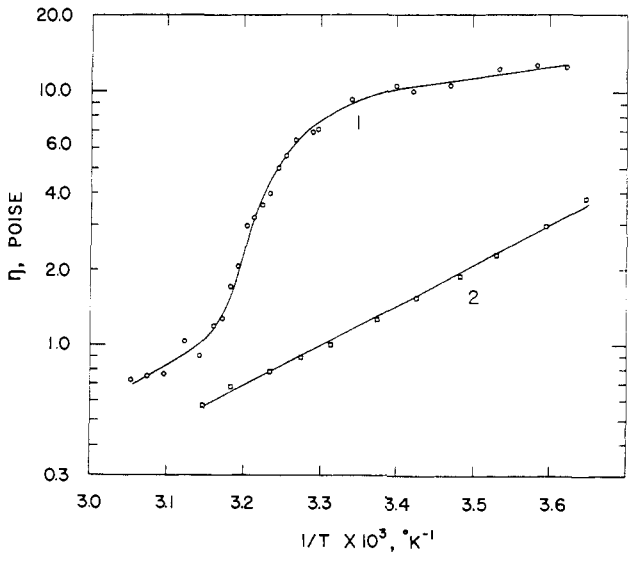

FIGURE 3: Temperature dependence of the microviscosities of dipalmitoyllecithin dispersion (curve 1) and of egg lecithin dispersion (curve 2) as determined by labeling with perylene. Lipid and dye concentrations were the same as those given in Table I.

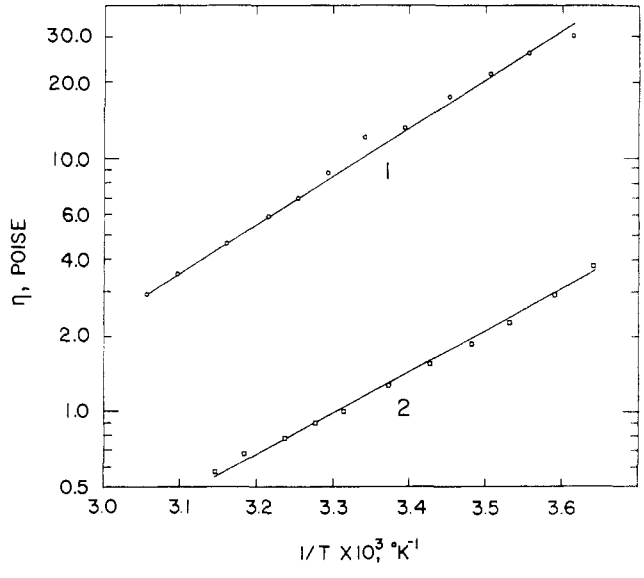

FIGURE 4: Temperature dependence of the microviscosity of egg lecithin–cholesterol dispersion (curve 1) as determined by labeling with perylene. The lecithin to cholesterol molar ratio was 1.5. The plot for egg lecithin (curve 2) is shown for comparison. The same lipid and dye concentrations as those given in Table I were used.

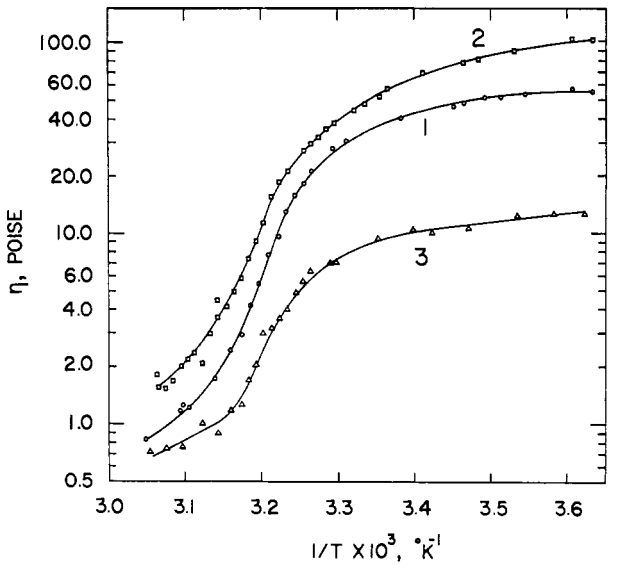

FIGURE 5: Temperature dependence of the microviscosity of dipalmitoyllecithin–cholesterol dispersions as determined by labeling with perylene. Lecithin to cholesterol molar ratios were 6 (curve 1) and 3 (curve 2). The plot for dipalmitoyllecithin (curve 3) is given for comparison. Lipid and dye concentrations were the same as those given in Table I.

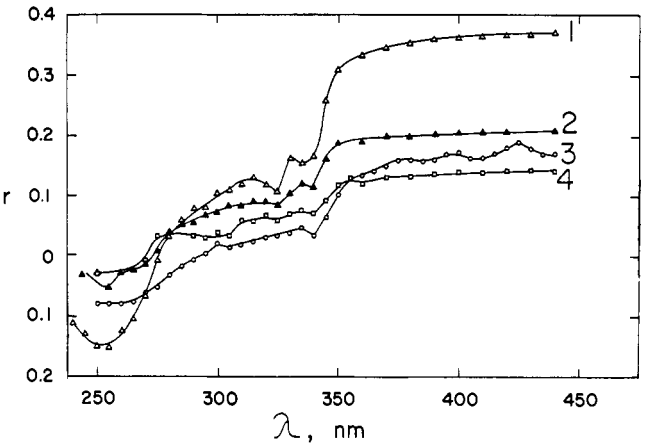

FIGURE 6: Anisotropy spectra of perylene in propylene glycol at −50° (curve 1), in propylene glycol at −14° (curve 2), in egg lecithin–cholesterol dispersion with lecithin to cholesterol molar ratio of 1 at −10° (curve 3) and in egg lysolecithin micelles at −11° (curve 4). Concentration of perylene in propylene glycol was 10^{-6} M; concentration of lipids and dye were the same as those given in Table I. Spectra of perylene in lipid dispersions were made in phosphate buffer–3 M NaCl of pH 7.4.

by the incorporation of cholesterol (Figure 5), the values being greater at a higher molar ratio of cholesterol to lecithin. The presence of the cholesterol, however, did not alter the sigmoidal shape of the log η *vs.* $1/T$ curve and the temperature range at which transition took place. We did not study dispersions having lecithin to cholesterol molar ratio below 3 since such dispersions prepared by sonication did not possess sufficient stability.

In order to obtain further information on the internal structure of phospholipid dispersions, anisotropy spectra were obtained for perylene in the following systems: propylene glycol at −50° and at −14°, egg lysolecithin micelles at −11°, egg lecithin dispersion at −10°, and egg lecithin–cholesterol dispersions having lecithin to cholesterol molar ratios of 1.0, 1.5, 3.0, and 5.0 at −10°. The anisotropy spectra of perylene in the phospholipid dispersions were monitored in 3 M NaCl at low temperatures, thus limiting the extent of depolarization. This condition was necessary for the evaluation of the rate of rotation, R, from the expression (Shinitzky *et al.*, 1971) $R = [(r_0/r) - 1]/6\tau$, and also for obtaining better resolution of anisotropy spectra. In the absence of extrinsic depolarization (propylene glycol at −50°) the anisotropy spectrum of perylene extended between r_0 values of 0.370 and −0.152 and crossed the zero point ($r_0 = 0$) at 277 nm (Figure 6). When extrinsic depolarization was allowed (propylene glycol at −14°) the zero point crossing occurred at a shorter wavelength (about 274 nm) indicating that the rate of in-plane rotations was greater than that of out-of-plane rotations (Weber, 1971). A similar observation was made for the lysolecithin micelles for which the zero point crossing occurred at about 268 nm. However, in the lecithin–cholesterol dispersion with lecithin to cholesterol molar ratio of 1.0, the spectrum crossed the zero point at a longer wavelength (about 293 nm) than that obtained with the perylene in propylene glycol at −50°, indicating that the rate of out-of-plane rotations was greater than that of in-plane rotations. It was observed that the spectra of lecithin–cholesterol dispersions with the molar ratio of 1.5, 3.0, and 5.0 and of lecithin dispersions all crossed the zero point at wavelength between 293 and 268 nm, the zero point crossing being at lower wavelength with the increase in lecithin to cholesterol molar ratio.

The magnitude of differences in the rates of the in-plane and out-of-plane rotations of perylene in the different solvent environments can best be illustrated by plotting the apparent rate of rotation, R, against the degree of limiting polarization, p_0 (Figures 7 and 8). The R values corresponding to $p_0 = -0.333$, 0.143, and 0.5 represent the rate of in-plane and out-of-plane rotations and their average, respectively (Shinitzky *et al.*, 1971). Generally the R *vs.* p_0 plots for lysolecithin micelles (Figure 7, curve 2) and lecithin dispersions (Figure 8A) resembled the plot obtained for isotropic liquid, propylene glycol (Figure 7, curve 1); the perylene in these environments

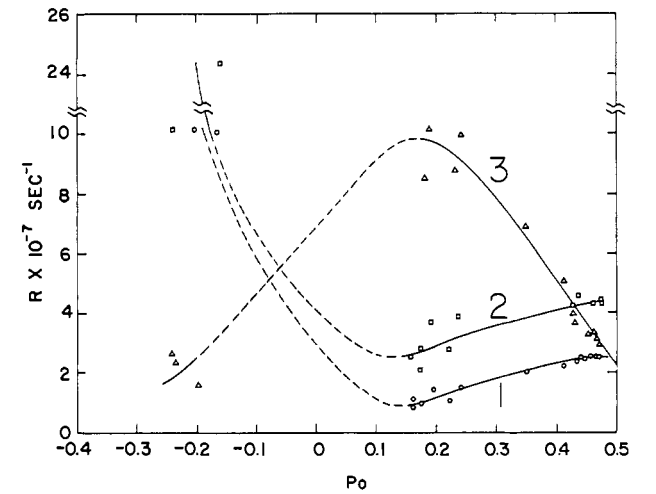

FIGURE 7: The dependence of the apparent rate of rotation, R, of perylene on the limiting polarization, p_0, in propylene glycol at −14° (curve 1), in egg lysolecithin micelles at −11° (curve 2) and in egg lecithin–cholesterol dispersion with lecithin to cholesterol molar ratio of 1 at −10° (curve 3). Concentration of perylene in propylene glycol was 10^{-6} M; concentrations of lipids and dye were the same as those given in Table I. Measurements with lipid dispersions were made in phosphate buffer–3 M NaCl of pH 7.4.

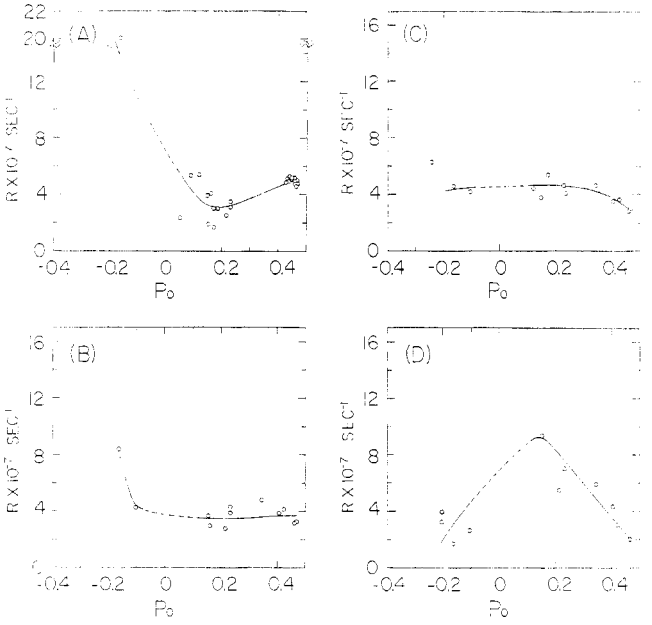

FIGURE 8: The dependence of the apparent rate of rotation, R, of perylene on the limiting polarization, p_0, in egg lecithin dispersion (A) and in egg lecithin–cholesterol dispersions with lecithin to cholesterol molar ratios of 5 (B), 3 (C), and 1.5 (D). Concentration of lipids and dye were the same as those given in Table I. Measurements were made at $-10°$ in phosphate buffer–3 M NaCl of pH 7.4.

exhibited low values of R at p_0 values near 0.143 and large R values as p_0 approached the value -0.333. It was thus apparent that the interior structure of lysolecithin micelles and lecithin dispersions is similar to that of isotropic liquid in which the rate of in-plane rotations is considerably higher than that of out-of-plane rotations.

An opposite trend was observed for the perylene incorporated into lecithin–cholesterol dispersions having lecithin to cholesterol molar ratio of 1.0 (Figure 7, curve 1) and 1.5 (Figure 8D); the R *vs.* p_0 plot showed high rates of rotation at p_0 corresponding to out-of-plane rotations. As was expected, the plots obtained for lecithin–cholesterol dispersions having the molar ratios of 5.0 and 3.0 (Figure 8B,C) indicated that the internal structures of these dispersions are intermediate between those of lecithin or lysolecithin which favor in-plane rotations and those of lecithin–cholesterol dispersions of higher cholesterol content which favor out-of-plane rotations. The apparent rates of rotation for the samples with the molar ratios of 3.0 and 5.0 were not profoundly influenced by the change of p_0 unlike those which showed preferential in-plane or out-of-plane rotations. It was thus apparent that the presence of cholesterol reduces the rate of in-plane rotations and increases the rate of out-of-plane rotations; maximum effect was observed at the lecithin to cholesterol molar ratio of 1.0.

Discussion

In the present study fluorescence polarization techniques were shown to be useful means for the evaluation of the microviscosity, existence of phase transitions, and structural organization in the hydrocarbon region of lipid dispersions of biological importance. The microviscosity values obtained from the degree of fluorescence depolarization of an incorporated dye represent the harmonic mean of the effective solvent viscosities opposing the rotation of the dye molecule in all possible directions (Shinitzky *et al.*, 1971). Since the microviscosity values obtained for egg lecithin with three different dyes, *i.e.*, perylene, 2-methylanthracene, and 9-vinylanthracene, were comparable as one might expect from the pure hydrocarbon nature of the dyes and the lack of any polar functional groups, the dyes were most likely dissolved in the hydrocarbon interior of the phospholipid dispersions in a manner devoid of preferential binding or complexing to the phospholipid hydrocarbon chains and to the phospholipid polar head groups.

A considerably lower microviscosity observed with egg lysolecithin compared to that of egg lecithin is likely to reflect the difference in the organization of these two aggregates. The lamellar organization of lecithin allows more ordered arrangement and better packing of the hydrocarbon chains than the micellar organization of lysolecithin and hence exerts a higher microviscosity. It is interesting to note that the microviscosity of lysolecithin micelles was found to be 2- to 4-fold higher than that of cationic micelles of cetyltrimethylammonium bromide (Shinitzky *et al.*, 1971) and somewhat higher than that of a zwitterionic micelle of cetylbetaine and a nonionic micelle of cetyldimethylamine oxide (Table II). This indicated as one might expect that the hydrocarbon chains in the zwitterionic or nonionic micelles are considerably more densely packed than those of the cationic and also, most likely, anionic micelles in which strong electrostatic repulsion among polar head groups results in a highly fluid internal structure. It is thus conceivable that an increase in the microviscosity of anionic or cationic micelles will be observed as the ionic strength of the medium is increased.

Although no definite information can be given concerning the exact location of the dye in the hydrocarbon interior of the phospholipid dispersions and micelles studied, the observed extremely unordered state of the interior of egg lecithin and lysolecithin excludes the possibility of the dye being located close to the polar head groups; the vicinity of the polar head groups is likely to be of substantially organized structure. The possibility of the preferential location of the dyes at the tail-end junctions of the opposing phospholipid hydrocarbon chains may also be eliminated. Had the dye been located preferentially in the deepest interior where the terminal hydrocarbon chains are in isotropic state, one would not expect as much difference in the microviscosities among lecithin, lysolecithin, and cetyltrimethylammonium bromide as was observed. Thus, it seems reasonable to assume that the dye is quite evenly distributed in the hydrocarbon interior.

The differences in the microviscosity values and in their temperature dependence between egg lecithin and dipalmitoyllecithin dispersions agree with previous contention that egg lecithin dispersions are in the liquid crystalline state in a wide range of temperatures (Small, 1967) while dipalmitoyllecithin undergoes phase transition from gel to liquid crystalline phase at approximately 38° (Ladbrooke *et al.*, 1968; Lippert and Peticolas, 1971; Hubbell and McConnell, 1971), the temperature corresponding to midpoint of the transition in our log η *vs.* $1/T$ plots. It is apparent that the presence of an unsaturated fatty acid in the β position of lecithin and other glycerophosphatides is essential to prevent abrupt change in membrane fluidity in the physiological temperature range.

A marked increase in the apparent microviscosity of egg lecithin upon addition of cholesterol appeared to be the result of the restriction of motion of the phospholipid hydrocarbon chains by the presence of cholesterol, in agreement with the previous conclusion derived from the studies with nuclear magnetic resonance and electron spin resonance (Chapman

and Penkett, 1966; Oldfield and Chapman, 1971; Hubbell and McConnell, 1971). Although the addition of cholesterol to dipalmitoyllecithin below the transition temperature was previously found to increase the fluidity of the hydrocarbon chains (Ladbrooke *et al.*, 1968; Oldfield and Chapman, 1971; Lippert and Peticolas, 1971), we observed a progressive decrease in the fluidity by increasing the amount of cholesterol added to the lecithin. This difference may be explained by a preferential interaction of perylene with cholesterol. Such interaction between perylene and the rigid and bulky nucleus of cholesterol might have restricted the rotation of the probe thus yielding abnormally high viscosity values. It is possible that the interaction may enable specifically following the apparent molecular motion of cholesterol as influenced by the organization of the phospholipid hydrocarbon chains. Thus the persistence of sigmoidal response in the log η *vs.* $1/T$ plots with dipalmitoyllecithin–cholesterol dispersions suggests that even though the presence of cholesterol is likely to increase the motion of dipalmitoyllecithin hydrocarbon chains below the transition temperature, the molecular motion of the cholesterol itself is sharply reduced.

Some indications concerning the internal organization of aqueous phospholipid systems were obtained upon evaluation of the in-plane and out-of-plane rotations of the incorporated perylene by employing the method previously described (Shinitzky *et al.*, 1971). Lysolecithin micelles were found to have an isotropic, unordered structure in spite of having severalfold higher internal viscosity as compared to cationic micelles of C_{16}–C_{18} (Shinitzky *et al.*, 1971). Moreover, the plots of the rate of rotation, R, *vs.* p_0 for egg lecithin dispersion which exhibits a lamellar liquid crystalline organization of the hydrocarbon chains also resembled the plots obtained for an isotropic liquid. It should be pointed out that the in-plane and out-of-plane rates of rotation obtained from R *vs.* p_0 plots are semiquantitative in nature. However, when compared with the corresponding rates obtained for a "standard" isotropic solution, they provide an estimation of the extent of order or disorder which is characteristic of a certain medium.

Although the interior of egg lecithin dispersions and possibly of any lamellar phospholipid dispersions which are above the gel–liquid crystalline transition temperature may have some limited anisotropic character, particularly in the vicinity of the polar head groups (Veksli *et al.*, 1969; Salsbury and Chapman, 1968; Sheard, 1969), the hydrocarbon chains in the interior may primarily be in an isotropic or greatly disordered state. Apparently, it is the cholesterol molecule whose incorporation into the lecithin lamellar phase drastically transforms the largely unordered or isotropic interior into a well-organized anisotropic structure. The progressive increase in the rate of out-of-plane rotation, accompanied by the decrease in the in-plane rotation, upon increases in the cholesterol content suggests that the effect of cholesterol is an additive phenomenon and that the distribution of cholesterol within the lipid particles is uniform. It has recently been indicated that the mixtures containing gel phase lecithins and cholesterol of the concentration less than 33 mol % produce a pure lecithin phase and a mixed phase of lecithin and cholesterol (Engelman and Rothman, 1972). No such phase separation has been observed with lecithins in the liquid crystalline state (*e.g.*, egg lecithin). Apparently, cholesterol in a hydrated multibilayer structure of egg lecithin increases the degree of order as also indicated by a number of studies (Oldfield and Chapman, 1971; Hsia *et al.*, 1971; Hubbell and McConnell, 1971). Although current information concerning the degree of orientation of phospholipid hydrocarbon chains in the presence and absence of cholesterol varies considerably depending upon the methods and the structure of probes employed (Hsia *et al.*, 1971; Badley *et al.*, 1971), it can be generalized that, upon addition of cholesterol, the angle of the long axis of phospholipid hydrocarbon chains relative to the surface approaches perpendicularity. Under such a circumstance, the least-restricted motion is that of all components rotating about their axes which are perpendicular to the interface of the bilayer, thus allowing a preferential out-of-plane rotation of the perylene associated with cholesterol.

In the study of model membranes as well as biological membranes by means of fluorescent depolarization, the choice of probes may be of utmost importance in obtaining information on the molecular motion and orientation of each component. A probe which may interact with a specific membrane component can be used to investigate its mobility characteristics. For the study of the overall fluidity of the membrane interior a probe which does not interact with any membrane component is required. It was previously suggested that 8-anilinonaphthalene-1-sulfonate, when added to lecithin dispersions, could associate with the phospholipid primarily by an electrostatic interaction between the sulfonate group and the lecithin trimethylammonium group (Muesing and Nishida, 1971), indicating that the anilinonaphthalenesulfonate or compounds of similar binding properties can be used to study the rotation of the lecithin component. It appears that the use of various probes of different characteristics may be a prerequisite for the elucidation of the dynamic movement of the constituents in model membranes and in biological membranes.

Acknowledgment

The authors thank Miss Janice Warfel for skillful technical assistance during the course of this study.

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Amino Acid Sequences at Constant and Variable Regions of Heavy Chains of Monotypic Immunoglobulins G and M of a Single Patient†

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ABSTRACT: Previous work indicated that the amino (N)-terminal 34 residues (which includes one hypervariable region) of heavy chains of monotypic immunoglobulins G2-κ and M-κ from a single patient (Til) are identical, and that these two molecules share idiotypic determinants not present in their isolated light chains or in any of a large number of other immunoglobulins tested. Our present data demonstrate that the amino acid sequences of the μ and γ2 chains of this patient are also identical from residues 83 to 108, which includes two other hypervariable regions. These data furnish strong support for the concept that the constant and the variable regions of each immunoglobulin polypeptide chain are synthesized by different structural genes. Examination of amino acid sequences reported for variable regions indicates that tyrosine occurs frequently either within or at the immediate neighborhood of hypervariable regions. Thus, amino acid sequence data of monotypic immunoglobulins support the concept proposed by Singer and his colleagues that tyrosine may play an important role in antigen combining sites. Further amino acid sequence analyses show that the N-terminal 38 residues of the Fcμ fragment of Til IgM are identical with those reported for another IgM, whereas the N-terminal 60 residues of Fcγ2 fragment of Til IgG2 showed approximately 95% amino acid sequence homology at the C_H2 domain with the other three γ-chain subclasses. This degree of homology is markedly higher than that of the hinge region, where only 60% homology is observed among the four γ-chain subclasses.

In 1969, we reported on an unusual patient (Til) whose serum had greatly elevated levels of two monotypic proteins: IgG_2-κ[1] and IgM-κ (Wang *et al.*, 1969). The light chains of these two monotypic proteins were identical by several criteria, including peptide mapping, electrophoretic mobility in starch gel containing urea at pH 3 and 8, amino acid composition, amino acid sequence of the N-terminal 38 residues, optical rotatory dispersion, and circular dichroism properties (Wang *et al.*, 1969; Pink *et al.*, 1971). Further, the variable regions of the μ and the γ2 chain were identical for their N-terminal 34 residues which include one hypervariable region, and they shared idiotypic determinants not present in their isolated light chains nor in any of a large number of other immunoglobulins tested (Wang *et al.*, 1970b). The significance of these findings upon the two genes–one polypeptide chain hypothesis as well as the switch from IgM to IgG synthesis during the course of an immune response has been discussed extensively in several symposia (Fudenberg *et al.*, 1971; Nisonoff *et al.*, 1972) and a review article (Pink *et al.*, 1971). The present paper presents additional data on the amino acid residues around two other hypervariable regions (residues 83–108) which were defined by Kehoe and Capra (1971), and at the C_H2 domain for both the μ and the γ2 chains of this patient (Til). The implications of these sequence data on the genetics and evolution of immunoglobulin molecules are discussed.

Materials and Methods

Purification of Proteins. The monotypic IgG and IgM were isolated by a procedure including sodium sulfate precipitation, ion-exchange chromatography, starch block electrophoresis, and gel filtration on Sephadex columns (Wang *et al.*, 1969).

† From the Departments of Microbiology and Medicine University of California, San Francisco, California 94122. *Received August 29, 1972.* This work was supported in part by U. S. Public Health Service Grants AI-09813 and HL-05677, National Science Foundation Research Grant BG-27666, and American Cancer Society Grant IC-76F. A. C. W. is a fellow from the National Genetics Foundation.

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[1] The terminology we have used is as far as possible that recommended by the world Health Organization (*W. H. O. Bull. 33*, 721 (1965); *35*, 953 (1966); *38*, 151 (1968); *41*, 975 (1969)).