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A METHOD FOR MEASURING MEMBRANE MICROVISCOSITY USING PYRENE EXCIMER FORMATION

APPLICATION TO HUMAN ERYTHROCYTE GHOSTS

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Summary

In order to determine the microviscosity of human erythrocyte membrane suspensions, a method has been developed which is based on pyrene excimer formation. First, measurements of partitioning of pyrene into membranes, in conjunction with known values for the volume of the lipid compartment of erythrocyte ghosts are used to determine the concentration of pyrene in the membrane lipid. Secondly, reported measurements of the diffusion constants of aromatic hydrocarbons similar in structure to pyrene, are used to derive an empirical equation relating solvent viscosity and the diffusion constant of pyrene. Then, measurements of pyrene excimer formation in a series of solvents ranging up to several poise in viscosity are used to determine that the interaction diameter of the excimer formation reaction is 3 ± 1 Å. Finally all these data are brought together in order to conclude that the viscosity of the lipid in the human erythrocyte ghost is 8.0, 4.0 and 1.6 P at 10, 25 and 40°C, respectively.

Introduction

The polyaromatic hydrocarbon, pyrene, upon elevation to an excited singlet state decays either by fluorescence or by a variety of competing pathways, such

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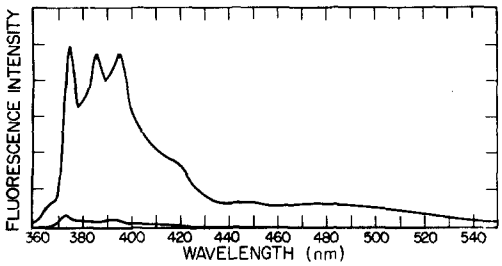

Fig. 1. Corrected spectrum of pyrene in erythrocyte ghosts at 25°C. The total concentration of pyrene was $1 \cdot 10^{-5}$ M, and the ghost concentration was 0.925 mg/ml. The small spectrum beneath the major curve shows the fluorescence remaining in the supernatant after centrifugation of the ghost suspension.

as triplet formation and internal conversion. However, if an excited pyrene monomer collides with a ground state monomer, an excited state dimer or excimer can form. Decay of pyrene excimers to the ground state by photon emission yields a broad structureless band with a maximum at 475 nm. The excimer fluorescence can be readily distinguished from the monomer emission, which occurs between 360 and 420 nm with a characteristic fine structure [1,2] as shown in Fig. 1.

The kinetics of pyrene excimer formation in organic solvents of low viscosity ($\eta \approx 0.01$ P) have been studied and an excellent review of this literature has appeared [3]. It has been shown that in these solvents the rate constant for excimer formation is inversely proportional to the solvent viscosity as expected on the basis of Von Smoluchowski's theory of diffusion-controlled reactions [4,5]. This fact has been exploited by several groups in order to measure the viscosity of the lipid phase in biological and artificial membrane systems [6—8].

The theory of Von Smoluchowski employs both the diffusion coefficient of pyrene, D, and the interaction diameter of the excimer formation reaction as explicit parameters. Consequently the use of this theoretical model to determine the viscosity requires a priori knowledge of the interaction diameter and of the relationship between D and η (e.g. a Stokes-Einstein type of relationship). Since the lipid compartment in membranes is more viscous than the model solvents that have been studied for membranes, it is desirable to validate Von Smoluchowski's theory and to determine the best value of the interaction diameter in solvents approximating the structure and viscosity of the membrane lipid compartment. In addition, an empirical relationship between D and η is needed in order to overcome the limitations of the Stokes-Einstein equation at high viscosities [9].

The present study is aimed at developing an accurate method for determining the diffusion constant of pyrene or, equivalently, the solvent viscosity, from the simplest possible series of measurements. In particular, it is desirable that fluorescence measurements on a single aliquot of pyrene-labeled membranes yield an independent determination of D. Such a scheme would allow 'before and after' comparisons to be made of the effect of various perturbants on the viscosity of the membrane lipid phase.

Materials and Methods

Chemicals. Pyrene was obtained from the Eastman Chemical Co. and recrystallized twice from ether/ethanol. Pharmaceutical grade mineral oil was obtained from the Squibb Pharmaceutical Co. All other solvents were of spectral or reagent grade. Prior to recording spectra, solutions were flushed with nitrogen for 25 min to reduce oxygen quenching to a minimum. The viscosity of the organic solvents was independently measured using a Coulter Electronics Limited Series 6063 Viscometer. The viscosity was calculated from the time required to flow a fixed horizontal distance in 0.5 mm bore tube immersed in a water bath.

Fluorimeter. Steady state spectra were obtained on a Cary spectrophotofluorimeter using front surface illumination 23° to the viewing angle. This instrument and the enhanced detection electronics have been described previously [10,11]. Fluorescence intensity is expressed in units of quanta/unit wavelength. The spectra were corrected from 350 to 600 nm against standard solutions of quinine and 2-aminopyridine according to the method of Melhuish [12]. In addition, the spectra were corrected for light-scattering background by subtracting the pyrene-free blank (solvent and erythrocyte ghosts). Excitation at 335 nm, with a bandwidth of 4.6 nm, was used for obtaining all pyrene steady state fluorescence spectra. The emission monochromator was scanned from 350 to 600 nm using a 1 mm slit, which corresponds to a 3.2 nm bandwidth at 400 nm. The excimer to monomer quantum yield ratio was obtained from the steady state spectra by taking the ratio of the integrated areas under the excimer and monomer emission, respectively, as described by Birks and Christophorou [13].

The time dependence of excimer fluorescence after delta function excitation was measured using an Ortec 9200 photon counting nanosecond fluorescence spectrometer equipped with an Ortec 6220 multichannel analyzer. A 330 nm bandpass interference filter was used to isolate the wavelength region for pyrene excitation. Excimer emission was measured using a 470 nm longpass filter. The number of ns/channel was adjusted to observe excimer fluorescence over approximately 0.5 μs. Excimer decay spectra were accumulated for 10-min periods in 512 channels of the analyzer memory. All decay spectra were corrected for background intensity by subtracting a solvent blank, or pyrene-free membrane suspension. Since the duration of the exciting pulse was approximately 2 ns, deconvolution of these spectra was unnecessary.

Preparation of erythrocyte ghosts. Erythrocyte ghosts were prepared from heparinized blood using the method of gradual hemolysis [14] with all operations conducted at 4°C. The buffers and glassware for the ghost preparation were sterilized by autoclaving. Freshly drawn human blood was diluted with 0.3 osmolar Tris-HCl, pH 7.5, and washed twice with this buffer. Subsequent washings were performed in 0.2, 0.06, and 0.03 osmolar Tris. The final erythrocyte plasma membrane pellet was stored in 0.03 osmolar Tris and all preparations were used within 48 h. The membrane content in the individual samples was determined by dry weight analysis.

Introduction of pyrene into erythrocyte ghosts. 10 mM pyrene in ethanol was diluted 1/100 in buffer. This solution was then added to suspensions of

TABLE I

PHYSICAL PARAMETERS OF ERYTHROCYTE MEMBRANE

Mass of erythrocyte ghost	$1.2 \cdot 10^{-12}$ g	Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119
Surface area of erythrocyte	$1.55 \cdot 10^{-6}$ cm^2	Westerman, M.P., Pierce, L.E. and Jensen, W.N. (1961) J. Lab. Clin. Med. 57, 819
Thickness of lipid layer in erythrocyte membrane	$40 \pm 5 \cdot 10^{-8}$ cm	Finean, J.B. (1969) Q. Rev. Biophys. 2, 1
Membrane/water partition coefficient of pyrene at 25°C	$7 \pm 2 \cdot 10^4$	This paper
Volume of pyrene compartment of erythrocyte ghost	$5.1 \pm 0.5 \cdot 10^{-4}$ ml/mg	Calculated from above parameters

erythrocyte ghosts in a manner similar to the technique of Vanderkooi et al. [7,8]. The final ethanol concentration did not exceed 0.1% of the total sample volume. Assuming there is simple partitioning of pyrene between the aqueous solution and the hydrophobic interior of the membranes, then the concentration of pyrene remaining in the supernatant, P_s, is

$$P_s = \frac{P_T}{1 + \Gamma\Omega\upsilon} \tag{1}$$

In this equation, Ω is the membrane/buffer partition coefficient of pyrene, υ is the volume of hydrophobic compartment of the membrane in ml/mg, Γ is the concentration of ghosts in mg/ml, and P_T is the total concentration of pyrene. At a P_T of 0.01 mM, measurements of P_s were obtained at various values of Γ by fluorescence assay of the supernatant following centrifugation of membrane suspensions. For $\Gamma \leqslant 0.4$ mg/ml, plots of P_T/P_s versus Γ were linear with an intercept of 1 as predicted by the above equation. The value of $\Omega\upsilon$ was obtained from the slope of these plots. From the published values for the mass, the surface area, and the thickness of the erythrocyte ghost (see Table I), the value of υ can be readily estimated as $5.1 \pm 0.5 \cdot 10^{-4}$ ml/mg. On this basis, Ω can be calculated to be $7 \pm 2 \cdot 10^4$ at 25°C.

In this study, P_T was fixed at 0.01 mM and a relatively large membrane concentration was used, $\Gamma \geqslant 1$ mg/ml, such that the internal concentration of pyrene in the membrane, P_M, can be described by:

$$P_M = \frac{P_T}{\upsilon\Gamma} \tag{2}$$

An additional consequence of the large value of Γ is that P_s was kept below 0.5 μM, which insured that excimer fluorescence from the supernatant was negligible.

Empirical relation between diffusion constant and viscosity. In order to determine the viscosity of media by this method it is necessary to relate the diffusion coefficient of pyrene and the solvent viscosity. This was done by means of the following empirical relationship:

$$D = [(1.0 + 1.07\eta)/(\eta + 0.26\eta^2)] \cdot 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \tag{3}$$

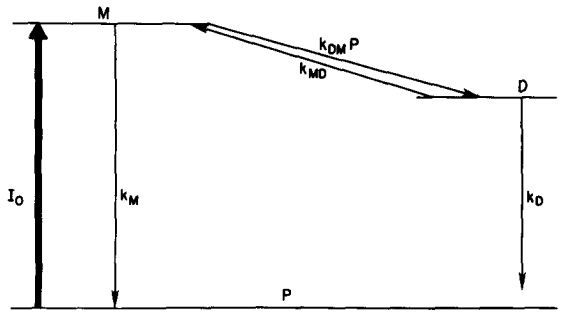

Fig. 2. A diagrammatic representation of the kinetic model of excimer formation deduced by Birks and coworkers [3,17,18]. The labels on the directed line segments of the figure indicate the notation of Birks for the rate constants of the associated transitions. Note that k_M and k_D represent overall decay rates which are the sum of the constants of fluorescence emission (k_{FM} and k_{FD}) and the rate constants for non-radiative decay.

where η is in cP. Eqn. 3 is a smoothed representation of the data of Bowen and Metcalf [15] on the diffusion constant of anthracene at 20°C in a variety of paraffin solvents ranging in viscosity from 0.5 to 200 cP. Eqn. 3 is also in good agreement with the measurements conducted by Miller et al. [16] on the diffusion constants of naphthalene, anthracene, and biphenyl in dimethyl formamide and acetonitrile. However, the diffusion constants of these substances in 75% dioxane/25% water depart significantly from Eqn. 3. These latter results indicate that Eqn. 3 breaks down in solvents in which extensive hydrogen bonding is possible.

The conversion of the diffusion constant of anthracene into the diffusion constant for pyrene was made by correcting for differences in temperature, T, and molecular weight, M, according to the common assumption.

$$D \propto TM^{-1/3}$$

This correction amounted to less than 5%.

Theory

According to the simple kinetic model of pyrene excimer formation developed by Birks and coworkers * [3,17,18], the response function of excimer fluorescence is given by the difference of two exponentials

$$i_D(t) = A[e^{-\lambda_1 t} - e^{-\lambda_2 t}] \tag{4}$$

where A is merely a constant of proportionality. The fit of Eqn. 4 to a set of typical data is illustrated in Fig. 3. λ_1 and λ_2 were determined for such data by using a non-linear least squares algorithm or by exponential peeling.

The two decay rates, λ_1 and λ_2 can be expanded asymptotically as follows:

$$\lambda_1 = \mathring{\lambda}_1 - \epsilon + 0(\epsilon^2)$$

* The notation used in this section is identical to that of Birks [3] and is illustrated in Fig. 2.

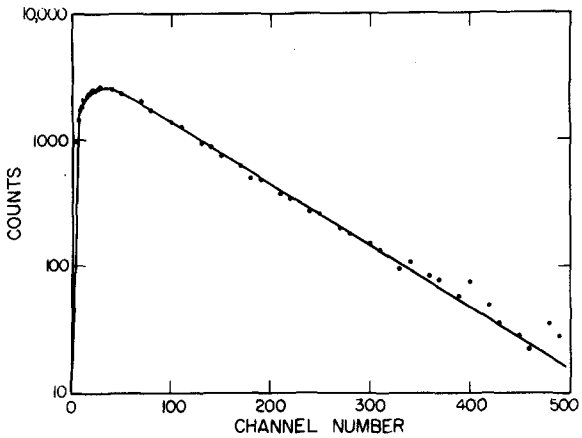

Fig. 3. Rise and decay of excimer fluorescence in erythrocyte ghosts following delta function excitation at time zero. The aliquot of ghosts was the same as was used to obtain the spectrum given in Fig. 1. The decay spectrum was corrected for light scattering and supernatant fluorescence as described in Materials and Methods. For this particular spectrum the instrument was set to record at 1.73 ns/channel. Only data for randomly selected channel numbers are shown so as not to obscure the theoretical curve showing the fit of Eqn. 4 to these data.

and

$$\lambda_2 = \mathring{\lambda}_2 + \epsilon - 0(\epsilon^2)$$

where

$$\mathring{\lambda}_1 = k_M + k_{DM}P\,, \qquad (5)$$

$$\mathring{\lambda}_2 = k_D + k_{MD}$$

and

$$\epsilon = \frac{k_{MD}k_{DM}P}{\mathring{\lambda}_2 - \mathring{\lambda}_1}$$

In Eqn. 5, k_M and k_D are the decay rates of excited pyrene monomer and excimer respectively, wheras k_{DM} and k_{MD} are the rate constants for formation (units of $M^{-1} \cdot s^{-1}$) and breakdown of excimer, respectively.

The physical significance of ignoring the terms of order ϵ and higher in Eqn. 5 is to neglect the contribution of those excited monomers which are reformed by the breakdown of excimer. Using the parameter values of pyrene fluorescence in 95% ethanol determined by Birks et al. [18] (see Table II), ϵ represents less than a 10% correction to λ_2 at a pyrene concentration of 1 mM. Since both k_{MD} and k_{DM} are inversely related to solvent viscosity, whereas k_M and k_D are independent of viscosity, ϵ will vary as $1/\eta^2$. In solvents with viscosities on the order of 10—100 times greater than the viscosity of ethanol, which are of primary interest, the higher order terms in Eqn. 5 can be safely neglected.

Under conditions of steady illumination, the ratio of excimer to monomer quantum yields is given according to the theory of Birks and coworkers

[17,18] by

$$\frac{\Phi_D}{\Phi_M} = \frac{k_{FD}}{k_{FM}} \frac{k_{DM}P}{k_{MD} + k_D} \tag{6}$$

k_{FM} and k_{FD} are the rate constants for emission of fluorescence from the excited monomer and excimer, respectively. Combining Eqns. 5 and 6 we obtain

$$k_{DM} \approx \frac{\lambda_2 \Phi_D}{P\Phi_M} \left[\frac{k_{FD}}{k_{FM}}\right]^{-1} + 0(\epsilon) \tag{7}$$

The value of k_{FD}/k_{FM}, 8.65, has been shown to be a constant independent of solvent and temperature [3,18]. Taking this number as given, Eqn. 7 was used in the present study to obtain the values of k_{DM} which are given in Tables II and III.

According to the theory of diffusion-controlled reaction rates due to Smoluchowski [3–5].

$$k_{DM} = \frac{4\pi N(2D)(pR)}{1000} [1 + pR(\pi Dt)^{-1/2}] \tag{8}$$

In Eqn. 8, N is Avogadro's number, D is the diffusion constant of pyrene, R is the interaction diameter of pyrene (i.e. the sum of the interaction radii of ground and excited state pyrene), p is a factor smaller than one reflecting the probability of reaction/encounter, and t is the time from the start of the reaction. Following the common practice of ignoring the transient term in Eqn. 8 we use this equation, in conjunction with Eqn. 3, to obtain the value of pR, the 'effective' interaction diameter for pyrene excimer formation in a series of solvents of known viscosity. With the value of pR thus fixed, Eqn. 8 can be applied in order to calculate D and consequently obtain η from measurements of k_{DM} in red cell ghosts.

Summary of the method

The overall method for determining membrane microviscosity by means of pyrene excimer formation involves the following elementary steps:

(1) Measure Φ_D/Φ_M and λ_2 by means of steady state and decay spectra of pyrene incorporated into a cell membrane suspension.

(2) Measure the concentration (mg/ml) of membrane in the suspension and estimate the volume of the pyrene compartment/mg of membrane. This information can be used to calculate the internal pyrene concentration by means of Eqn. 2.

(3) Use of the values of λ_2, Φ_D/Φ_M and the internal pyrene concentration to calculate k_{DM} by means of Eqn. 7.

(4) Use the value of k_{DM} and an independently determined value of the interaction diameter of pyrene to calculate the diffusion coefficient of pyrene by means of Eqn. 8.

(5) Use the value of the diffusion constants obtained in (4) to calculate the microviscosity by means of Eqn. 3.

TABLE II

Except for the values obtained by Birks and coworkers, the values for k_M and $k_D + k_{MD}$ include a contribution due to oxygen quenching. D was calculated using the measured value of percent by means of Eqn. 3. pR was calculated from values of D and k_{DM} by means of Eqn. 8. M.O., mineral oil.

Solvent	Temperature	k_M ($\times 10^6$ s^{-1})	$k_D + k_{MD}$ ($\times 10^6$ s^{-1})	k_{MD} ($\times 10^6$ s^{-1})	k_{MD} ** ($\times 10^6$ $M^{-1} \cdot s^{-1}$)	η (cP)	D ($\times 10^{-6}$ $cm^2 \cdot s^{-1}$)	pR ($\times 10^{-8}$ cm)
Acetone *	20	3.0	35	12	14 000	0.32	36	2.5
Cyclohexane *	20	2.3	22	6.5	6 700	0.98	16	2.6
95% Ethanol *	20	3.4	27	7.0	7 000	1.1	13	3.5
Butanol	25	3.7	37	—	3 000	2.6	8.4	2.3
Octanol	25	3.9	30	—	1 000	7.2	4.1	1.6
75% M.O. 25% Heptane	25	5.8	29	—	1 000	9.5	3.2	2.2
90% M.O. 10% Heptane	25	4.4	38	—	500	39	0.95	3.5
100% M.O.	40	4.8	38	—	450	41	0.95	3.1
100% M.O.	25	3.3	28	—	160	132	0.30	3.6
100% M.O.	10	3.1	36	—	55	396	0.10	3.6

* From Birks et al. [17,18].

** Errors in values of k_{DM} are of the order of 20%.

Results

Determination of interaction diameter of pyrene

Table II presents a summary of measurements made of pyrene fluorescence in a series of organic solvents of increasing viscosity. The parameter value in this table represent the mean of at least four measurements made at pyrene concentrations which varied by an order of magnitude or more. In order to insure that ϵ in Eqn. 5 was kept small, the maximum pyrene concentrations used were such that $\Phi_D/\Phi_M < 3$. No significant tendency of any of the parameter values to change with concentration was detected.

We point out that although the diffusion constant of pyrene varies by over two orders of magnitude in this solvent series, the effective interaction diameter of the excimer formation reaction is fairly constant (i.e. $pR = 3 \pm 1$ Å). Moreover, there does not appear to be any correlation between viscosity and interaction diameter.

Due to the highly abstract nature of Von Smoluchowski's model, which fails to take into account the chemical structure of the solvent, the agreement with the expected linear dependence of k_{DM} on D demonstrated by the data in Table II seems quite reasonable. It is interesting, however, that the value of pR deduced is fairly small compared to the larger diameter of the pyrene molecule, which is approximately 10 Å. A similar result was obtained by Osborne and Porter [9] for the case of quenching of triplet naphthalene by α-iodonaphthalene.

Determination of microviscosity of red cell ghosts

Table III presents a summary of our measurements of pyrene fluorescence in suspensions of erythrocyte ghosts. In calculating D from k_{DM}, the value of pR was taken to be 3 Å. P_M, the internal concentration of pyrene in the membrane was calculated according to Eqn. 2. The values of k_M given in Table III refer to suspensions that are equilibrated with air. Nitrogen flushing led to a considerable reduction in k_M indicating the presence of freely diffusing oxygen in the pyrene compartment of the erythrocyte ghost. However, reduction in oxygen tension did not appear to affect the value of $k_D + k_{MD}$.

According to the estimates of the volume of the pyrene compartment the concentration of pyrene was between 10 and 20 mM for the four membrane preparations. Within this limited range there was no significant concentration

TABLE III

PARAMETERS OF PYRENE FLUORESCENCE IN ERYTHROCYTE GHOSTS

Experimental errors in parameters are of the order of 20%. See Discussion for an analysis of systematic errors.

Temperature (°C)	k_M ($\times 10^6$ s^{-1})	$k_D + k_{MD}$ ($\times 10^6$ s^{-1})	k_{DM} ($\times 10^7$ $M^{-1} \cdot s^{-1}$)	D ($\times 10^{-8}$ $cm^2 \cdot s^{-1}$)	η (P)
40	7.0	48	11	25	1.6
25	5.5	34	4.4	10	4.0
10	3.6	34	2.5	5	8.0

dependence of the parameter values. Together with the evidence for simple partitioning, this indicates that at these concentrations pyrene is not a major perturbant of membrane structure.

Discussion

Vanderkooi and Callis [8] have previously presented a method for determining the microviscosity of membrane preparations using pyrene, the results of which differ by a factor of six from those given in Table III. We feel that several factors contribute to this disagreement: (1) Vanderkooi and Callis assume a value of pR = 10 Å, rather than the value of 3 Å obtained empirically in Table II; (2) these authors did not correct for the contribution that pyrene in the supernatant makes to the decay curve for pyrene monomer and to the steady state excimer/monomer ratio; (3) the method of Vanderkooi and Callis depends heavily on the validity of the time-dependent term in Eqn. 8, which has never been empirically verified; (4) even if this term were correct, the expected effects are small and easily masked by such factors as slight heterogeneities in the environment of the pyrene molecules.

In the present study we have been unsuccessful in estimating the influence of the time-dependent terms in Eqn. 8 on pyrene fluorescence in the solvents of Table II, and in erythrocyte ghosts. Consequently, we have concluded that this term is either too small to be accurately measured by our technique or it is incompletely developed.

The actual experimental variation in determining the value of k_{DM}, D and η in erythrocyte ghosts according to this method is of the order of ±20%. Far

TABLE IV

REPORTED VALUES OF THE MICROVISCOSITY OF HUMAN ERYTHROCYTE GHOSTS

Method	Temperature (°C)	Micro-viscosity (P)	Reference
Pyrene excimer formation	40	1.6	This paper
	25	4.0	
	15	8.0	
Pyrene excimer formation *	25	0.62	8
Fluorescence polarization of 1,6 diphenyl-1,3,5-hexatriene	40	2.7	19
	25	4.4	
	15	6.8	
Fluorescence polarization of 1,6 diphenyl-1,3,5-hexatriene	40	3.0	21
	25	6.0	
	15	8.0	
Calculated from permeability coefficient of methanol **	21	1.7	22
Fluorescence polarization of 12-(9-anthroyl)stearic acid	37	1.0	23
Fluorescence polarization of perylene	40	1.2	24
	15	2.3	
	25	2.6	20
		1.8	24
		1.8	25

* See Discussion for possible errors in this study.

** The Stokes-Einstein relation was used without correction for deviations at high viscosity.

more serious are the combined systematic erros introduced by uncertainty in the volume of the pyrene compartment of the erythrocyte (±30%), and in the value of the interaction diameter (another 30%). Taken together these experimental and systematic errors could change the absolute values of η by a factor of two or affect the relative changes observed by 40%. A further source of systematic error, the magnitude of which cannot be estimated, involves the implicit assumption that diffusion and excimer formation in membrane lipid are similar to the same processes in homogeneous isotropic fluids. Of course, this assumption is implicit in one form or another in all attempts to characterize membrane lipid using a concept such as 'viscosity' which is based on macroscopic phenomena.

In the final analysis, the values of the microviscosity, η, of the erythrocyte lipid phase, given in Table III, must be compared with values derived by other procedures in order to determine whether or not a characteristic property of the membrane (i.e. a property that is independent of approach) is being measured. Table IV presents a summary of recently reported measurements of erythrocyte membrane microviscosity in reverse chronological order. As can be seen from this table, measurements based on the rotational motion of fluorescent dyes or on the rate of permeation of non-electrolytes are in broad agreement with measurements based on the kinetics of excimer formation.

Acknowledgements

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