Karush, F., and Sonenberg, M. (1949), *J. Amer. Chem. Soc. 71*, 1369.
Klotz, I. M. (1953), *Proteins 1*, 727.
Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Laiken, N. (1970), Ph.D. Thesis, The Rockefeller University.
Laiken, N., and Némethy, G. (1970a), *J. Phys. Chem. 74*, 4421.
Laiken, N., and Némethy, G. (1970b), *J. Phys. Chem. 74*, 4431.
Laiken, S., and Printz, M. (1970), *Biochemistry 9*, 1547.
Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.
Ray, A., Reynolds, J. A., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2607.
Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6*, 937.
Reynolds, J. A., Herbert, S., and Steinhardt, J. (1968), *Biochemistry 7*, 1357.
Roe, R.-J. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 50.
Silberberg, A. (1962), *J. Phys. Chem. 66*, 1872, 1884.
Spector, A. A., John, K., and Fletcher, J. E. (1969), *J. Lipid Res. 10*, 56.
Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic Press.
Teresi, J. D., and Luck, J. M. (1952), *J. Biol. Chem. 194*, 823.

Microviscosity and Order in the Hydrocarbon Region of Micelles and Membranes Determined with Fluorescent Probes. I. Synthetic Micelles*

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ABSTRACT: The viscosity in micelle interiors, termed here as microviscosity, was derived from an adequate comparison of the degree of fluorescence depolarization of perylene or 2-methylanthracene when dissolved in the tested micelles and in American white oil U. S. P. 35. The latter was used as a reference system of known viscosities. In the series studied, lauryltrimethylammonium bromide, myristyltrimethylammonium bromide, cetyltrimethylammonium bromide (CTABr), and stearyldimethylbenzylammonium bromide, the determined microviscosities at 27° are all in the range of 17–50 cP. The change in microviscosity with temperature in this series was found to follow a simple exponential form with an activation energy in the range of 6.1–9.6 kcal mole^{-1}. Added salts affected only slightly the microviscosity values. Mixed micelles of perylene-labeled CTABr with cetyl alcohol or cholesterol and with sodium 1-hexadecanesulfonate, were used to test the effect of charge isolation and charge neutralization on the fluidity of the micelle interior. The microviscosity of these mixed micelles was found to increase rapidly with concentration of the admixed component, and at a molar ratio close to 1:1 microviscosities of several poises were obtained. The changes in apparent rotational diffusion with wavelength of excitation indicate that the depolarizing rotations are strongly anisotropic. In-plane rotations in perylene are ten times faster than out-of-plane rotations, independently of the medium (micelles, propylene glycol at −14°, propylene glycol–glycerol at 4°). This indicates that the resistance to the motion in the micelles must be close to isotropic. A summary of the findings presented leads to the conclusion that micelle interiors are similar in nature to aliphatic hydrocarbon solvents.

In general, the fluorescence emitted from molecules which are dispersed in a viscous medium is partially polarized. This is customarily expressed in terms of molecular anisotropy, r, or degree of polarization, p, which are measured at right angle to a polarized excitation beam and are defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

When $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the excitation beam. For a rotating fluorescent sphere the observed r or p values obey the well known Perrin (1926) equation in which r_0 and p_0 are the values

$$\frac{r_0}{r} = \frac{\frac{1}{p} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = 1 + \frac{6R_s}{\lambda} \tag{2}$$

of r and p when the emitting molecules maintain their orientation excitation and emission (*e.g.*, in a very viscous solvent), R_s is the rate of rotation of the sphere and λ is the rate of fluorescence emission. The term r_0/r is defined here as the degree of depolarization.

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Introducing the value of R, derived from hydrodynamic considerations, into eq 2 leads (Weber, 1953)

$$\frac{r_0}{r} = 1 + \frac{kT\tau}{\eta v} \tag{3}$$

where T is the absolute temperature, η is the viscosity of the medium, v is effective volume of the fluorescent sphere, τ is the average lifetime of its excited state, and k is the Boltzmann constant.

In principle, eq 3 can be used as a measure for viscosity if all other parameters are known or measurable. However, most of the fluorescence dyes, especially those employed as molecular probes, possess a planar structure for which serious deviations from eq 3 may exist.

A new derivation of the relations between fluorescence depolarization and Brownian rotations of nonspherical particles was given recently by G. Weber (to be published, 1971). This derivation was obtained by assuming three rates of rotations, R_1, R_2, and R_3, about three perpendicular axes 1, 2, and 3 fixed in the molecular backbone. R_1 is defined as the rate of rotation about the absorption oscillator and as the direction of this oscillator depends on wavelength of excitation the whole set of axes changes with wavelength of excitation accordingly. The most general expression derived is given by

$$\frac{r_0}{r} = \frac{(\lambda + 6\bar{R})^2 - 36\Delta}{\lambda[(\lambda + 6\bar{R}) + 6\langle\delta\rangle(3\cos^2\alpha - 1)^{-1}]} \tag{4}$$

where $\bar{R} = (R_1 + R_2 + R_3)/3$ = the average rate of rotation, $2\Delta = (R_1 - \bar{R})^2 + (R_2 - \bar{R})^2 + (R_3 - \bar{R})^2$ = the variance, and $\langle\delta\rangle = R_1\cos^2\alpha + R_2\cos^2\gamma + R_3\cos^2\beta - \bar{R}$. α, β, and γ are the angles between the emission oscillator and the axes 1, 2, and 3, respectively. By neglecting the terms containing Δ and $\langle\delta\rangle$ in eq 4, eq 2 is obtained.

For the most common case of rotating plates (fluorescent aromatic molecules), in which the oscillators of absorption and emission are coplanar, and if only small rotations can take place between excitation and emission ($R_i/\lambda < 1$), eq 4 assumes the following approximate form

$$\frac{r_0}{r} = 1 + \frac{6}{\lambda}\frac{R_p(2\cos^2\alpha - 1) + R_{op}\cos^2\alpha}{3\cos^2\alpha - 1} \tag{5}$$

where R_p is the rate of rotation about an axis normal to the ring plane, and is therefore an "in-plane" rate of rotation, and R_{op} is the rate of rotation about an axis contained in the ring plane at right angle to the absorption oscillator. R_{op} is therefore an "out-of-plane" rate of rotation.

Three cases of interest arise from eq 5. (1) When the oscillators of absorption and emission are parallel, as is generally the case when the dye is excited at the last absorption band, $\cos^2\alpha = 1$, $r_0 \to 0.4$ and $p_0 \to 0.5$, and the corresponding form of eq 5 is

$$\frac{r_0}{r} = 1 + \frac{6}{\lambda}\frac{R_p + R_{op}}{2} = 1 + \frac{6R_0}{\lambda} \tag{6}$$

R_0, the average rate of rotation of the plate, equals $(R_p + R_{op})/2$.

Equation 6 can be expressed in an analogous way to eq 3

$$\frac{r_0}{r} = 1 + \frac{kT\tau}{\bar{\eta}v_0}$$

The product $\bar{\eta}v_0$ in this equation is the "retarding factor" of an equivalent rotating sphere with an effective volume of v_0. $\bar{\eta}$ is termed here as the microviscosity of the system, and is the harmonic mean of the effective viscosities opposing the in- and out-of-plane rotations of the plate. (2) When $\cos^2\alpha = {}^1/_2$, $r_0 \to 0.1$ and $p_0 \to {}^1/_7$, eq 5 is then reduced to

$$\frac{r_0}{r} = 1 + \frac{6R_{op}}{\lambda} \tag{7}$$

and only the out-of-plane rotations affect the depolarization process. (3) When $\cos^2\alpha = 0$, $r_0 \to -{}^1/_4$ and $p_0 \to -{}^1/_3$, eq 5 is reduced to

$$\frac{r_0}{r} = 1 + \frac{6R_p}{\lambda} \tag{8}$$

and the observed depolarization is due only to rotation in the plane of the fluorescence molecule. Thus, from the depolarization characteristics of a fluorescent probe embedded in a medium of interest the microviscosity, expressed in macroscopic units, as well as the degree of anisotropy of the medium, can be evaluated.

In the following, the study on the hydrocarbon region in micelles with the aid of fluorescent hydrocarbon probes is presented.

Experimental Section

Materials. 2-Methylanthracene and 9-vinylanthracene (Aldrich) were crystallized from ethanol. Perylene (Aldrich Puriss grade) was used without further purification. Thin-layer chromatography showed that the above three materials were free of fluorescent impurities. White oil U. S. P. 35 was obtained from the American Oil Co. This oil does not absorb light of wavelength above 300 nm, and was found to be free of fluorescence.

Lauryltrimethylammonium bromide (LTABr),[1] and myristyltrimethylammonium bromide (MTABr) from K & K, cetyltrimethylammonium bromide (CTABr) from BDH special pure lot, and stearyldimethylbenzylammonium bromide (SDBABr) from Fine Organics, were all crystallized from methanol–ether before use.

Cetyl alcohol (CA) was purchased from Aldrich and was crystallized from methanol. Sodium 1-hexadecanesulfonate (HSNa), Aldrich Puriss grade, was used without further purification.

Optical Measurements. Absorption spectra were recorded with a Cary 15 spectrophotometer. Optical densities were measured with a Zeiss PMQ II spectrophotometer. Fluorescence spectra and intensities were determined with a spectrofluorometer described by Weber and Young (1964). Fluorescence polarizations were measured with the instrument designed by Weber and Bablouzian (1966). For systems labeled with perylene, degrees of polarization were also obtained by excitation with the isolated 436-nm line of a high-pressure mercury arc. Fluorescence decay times were measured directly with the cross-correlation-phase fluorometer designed by

[1] Abbreviations used are: LTABr, lauryltrimethylammonium bromide; MTABr, myristyltrimethylammonium bromide; CTABr, cetyltrimethylammonium bromide; SDBABr, stearyldimethylbenzylammonium bromide; CA, cetyl alcohol; HSNa, sodium 1-hexadecanesulfonate.

Spencer and Weber (1968). The change of the excited-state lifetime with temperature was assumed to follow the change in quantum yield with temperature. For a given temperature the lifetime of excited state was deduced from a calibration curve describing the change in fluorescence intensity of the labeled system with temperature.

Preparation of Labeled Micelles. Aqueous micelle solutions were prepared by dissolving the micelle forming substance in water to a concentration which is at least fivefold higher than the critical micelle concentration. Most of the subsequent work was done on micelles with hexadecyl chains among which the main system investigated was 0.01 M solution of CTABr in water. This system is very stable, transparent to ultraviolet light, and clear even in high salt concentration or at low temperatures.

The labeling of the micelles was carried out as follows. Very fine glass beads, previously washed with concentrated nitric acid, water, and acetone successively, were mixed with acetone solution of the aromatic hydrocarbon probe and dried up with continuous mixing. The weight percentage of the probe in the coated beads was 0.01–0.1%. The coated glass beads were equilibrated with the micelle solution either by stirring or by boiling the mixture, and finally were filtered off. The mixture was then diluted with the unequilibrated solution to give an optical density of 0.01–0.04 at the last absorption maximum of the dye. For such a concentration of the probe, depolarization due to trivial reabsorption of the fluorescence light, in a light path of 1 cm, is less than 1%, and the average number of probes per micelle is less than one, which eliminates fluorescence depolarization due to energy transfer between adjacent probe molecules. The solution was then boiled and cooled several times in order to assure homogeneous distribution of the probe. Because of the very large surface area of the coated beads and their simple separation, this method of labeling is very efficient and economical. When pure water was boiled up with the coated beads, not even a trace of fluorescence could be detected in it.

For preliminary observations, the labeling was carried out by dumping an acetone solution of the probe (10^{-4}–2.10^{-3} M) into the micelle solution with vigorous stirring. The final concentration of the acetone was less than 0.5%. This method is especially adequate for perylene, as it was found that when its acetone solution is dumped into pure water the solution stays clear, shows very marked hypochromism at $\lambda > 300$ nm and is practically void of fluorescence.

Mixed micelles were prepared by dissolving CA or HSNa in a 0.01 M labeled solution of CTABr. The mixtures were boiled and cooled repeatingly by which process they gradually turned clearer. To obtain different mixture ratios, the above solutions were diluted with the 0.01 M labeled solution of CTABr, and after each dilution were heated to boiling and then cooled. In the starting mixtures the maximum amount of CA or HSNa, for which the turbidity was not appreciable, was introduced.

Embedding of cholesterol into CTABr micelles was carried out by sonication under nitrogen with a Branson sonifier. Remainders of insoluble material were removed by centrifugation at 65,000*g* for 30 min.

Fluorescence Polarization Measurements in a Scattering Solution. The mixed micelle systems studied showed a slight turbidity. The main source of error is due to scattering of the polarized exciting light which results in artifactual increase of the measured polarization. Another source of error is due to the scattering of the fluorescence light which reduces the degree of polarization. The latter is much less important and was disregarded. Errors due to stray light were eliminated by interposing interference filters between the light source and the sample when excitation was carried out with a fixed wavelength. In the emission channels, cutoff filters were always used to minimize the effect of scattered light. Only in the case of the strongly scattering samples (CTABr–cholesterol micelles), a trace of scattered exciting light was detected. Corrections for the contribution of the scattered light to the polarization were then done according to the following considerations: the measured $I_{\parallel}$ and $I_{\perp}$ are composed of fluorescence and stray light and the corrected value for the anisotropy is therefore

$$r = \frac{(I_{\parallel} - I_{\parallel}^{s}) - (I_{\perp} - I_{\perp}^{s})}{(I_{\parallel} - I_{\parallel}^{s}) + 2(I_{\perp} - I_{\perp}^{s})} \tag{9}$$

where $I_{\parallel}^{s}$ and $I_{\perp}^{s}$ are the stray light contributions. Resetting eq 9 gives

$$r = \frac{(X - 1) - \frac{I_{\perp}^{s}}{I_{\perp}}(X_s - 1)}{(X + 2) - \frac{I_{\perp}^{s}}{I_{\perp}}(X_s + 2)} \tag{10}$$

where $X = I_{\parallel}/I_{\perp}$ and $X_s = I_{\parallel}^{s}/I_{\perp}^{s}$.

The values of X_s can be obtained independently with an unlabeled reference solution of the same composition, whereas the $I_{\perp}^{s}/I_{\perp}$ values are obtained by comparison between the reference and the measured solution under the same conditions in which X was measured. All solutions measured were examined and corrected for errors in polarization with the aid of an adequate reference solution. In all clear solutions the corrections applied to the measured anisotropy were less than 2%.

Evaluation of Microviscosities. American white oil U. S. P. 35, a viscous mixture of high molecular weight aliphatic hydrocarbons, were used as a reference system for the evaluation of microviscosities. The viscosities of the white oil at the temperature range of -4 to 60° were determined by flow rate measurements using Kimax 300 and 400 viscometers. Standard solutions of glycerol–water mixtures of known densities and viscosities were used for calibration of the viscometers. The viscosity of the white oil was found to be 155 cP at 25°, and a plot of ln η *vs.* 1/T was used for obtaining viscosities at different temperatures.

For the trivial case of a fluorescent sphere a comparison between the r_0/r values observed when the dye is embedded in the white oil and in a certain micelle will give the appropriate relation between the $T\tau/\eta$ products of the two systems, from which the viscosity in the micelle interior can be evaluated. In practice a planar fluorescent probe is used and the rates of rotation in- and out-of-plane are of different values, each of them depending on the product $T\tau/\eta$. However, when a planar fluorophor is excited at a wavelength for which p_0 approaches 0.5, the rates of rotation in- and out-of-plane contribute about the same to the observed depolarization (see eq 6), and the depolarization process can be ascribed to an equivalent rotating sphere with an effective volume which varies with $T\tau/\eta$. Namely, the effective volume of the equivalent sphere is a function of the observed degree of depolarization. For this case the relation given in eq 3 will become

$$\frac{r_0}{r} = 1 + \frac{kT\tau}{\eta v(r)} \tag{11}$$

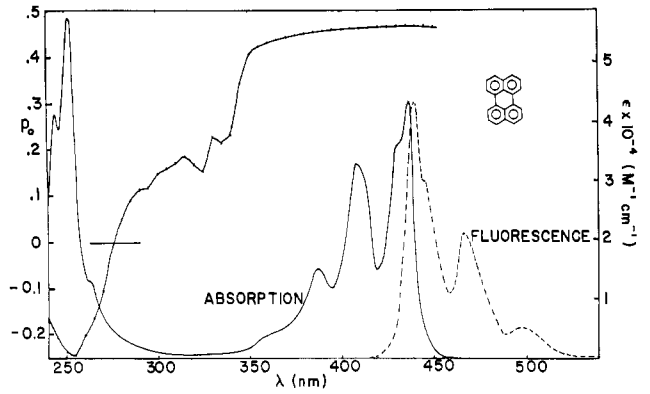

FIGURE 1: Absorption and normalized emission spectra of perylene in ethanol. The polarization spectrum of perylene (10^{-6} M) in propylene glycol at $-50°$ (·—·) is also included.

in which $v(r)$ is the variable effective volume of the equivalent sphere.

Since the interior of a micelle is of the same physical nature as the white oil, it was assumed that when a fluorescence aromatic hydrocarbon, excited at a wavelength associated with a high p_0 value, shows the same degree of depolarization in a micelle interior or in the white oil, the effective volume of the equivalent sphere, $v(r)$, in both systems is also the same. This implies that for both systems the product $T\tau/\eta$ has the same value, and thus the value of the microviscosity in the micelle interior is easily obtained by knowing all other parameters.

According to that, curves describing the change of r_0/r with $T\tau/\eta$ of the employed probes (perylene, 2-methylanthracene, and 9-vinylanthracene) in white oil, were used as comparison curves for the determination of microviscosities in micelles. These curves were obtained by measuring r, excited at a wavelength for which the limit polarization is maximal and relatively constant (see Figures 1–3), at different temperatures in the range of -10 to $+60°$. The excitation wavelengths used were 413 and 436 nm for perylene, 382 nm for 2-methylanthracene, and 392 nm for 9-vinylanthracene. The corresponding r_0 values were obtained from polarization measurements in propylene glycol at $-50°$ (see Figures 1–3). The value of $T\tau/\eta$ for the labeled micelles were obtained from the comparison curves according to the measured r_0/r. By inserting the values of T and τ of the measured system into this product, the microviscosity, $\bar{\eta}$, was derived.

The change of $\bar{\eta}$ with temperature was described in a simple exponential form

$$\bar{\eta} = Ae^{\Delta E/RT} \tag{12}$$

ΔE, termed here as the fusion activation energy, was derived either graphically from a plot ln $\bar{\eta}$ *vs.* $1/T$, or numerically by eq 13 when only two experimental points were available.

$$\Delta E = R \frac{T_1 T_2}{T_1 - T_2} \ln \frac{\bar{\eta}_1}{\bar{\eta}_2} \tag{13}$$

Results and Discussion

Fluorescence Probes for Systems with Hydrocarbon Regions Dispersed in Water. Pure aromatic hydrocarbons with three or more rings are virtually totally insoluble in water. These compounds are very soluble in hydrocarbon solvents, and when an

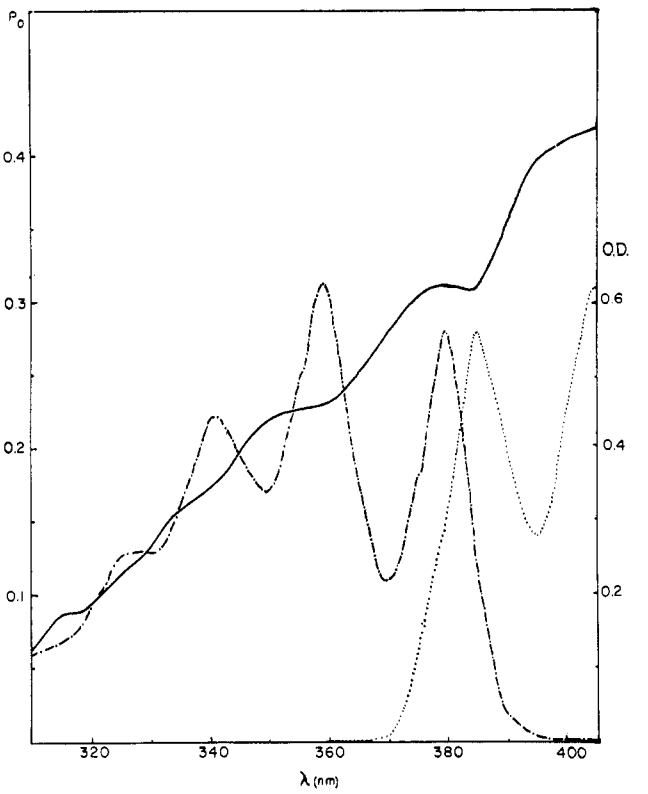

FIGURE 2: Spectra of 2-methylanthracene. (·—·) Absorption spectrum (of 10^{-4} M) in tetradecane, (·····) normalized fluorescence spectrum (of 10^{-5} M) in tetradecane, (—) polarization spectrum (of 10^{-5} M) in propylene glycol at $-50°$.

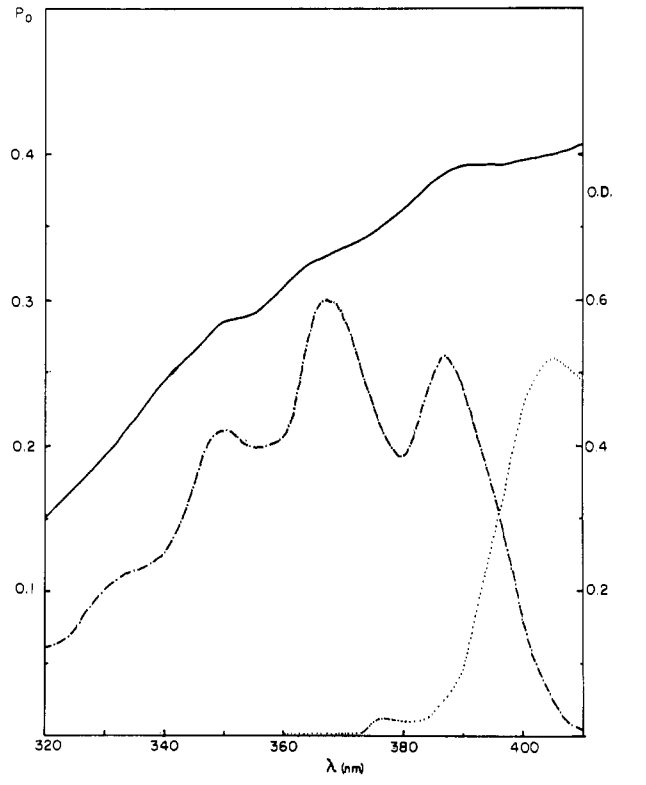

FIGURE 3: Spectra of 9-vinylanthracene. (·—·) Absorption spectrum (of 8.3×10^{-5} M) in ethanol, (·····) normalized fluorescence spectrum (of 10^{-6} M) in ethanol, (—) polarization spectrum (of 10^{-6} M) in propylene glycol at $-50°$.

TABLE I: Degree of Fluorescence Polarization, p, at 27° of 2-Methylanthracene (2MA), Excited at 382 nm, and Perylene (Per), Excited at 413 nm, Embedded in Various Micelles; and the Microviscosity, $\bar{\eta}$, Deduced from the Degree of Depolarization and the Excited-State Lifetime of the Dye.[a]

Material	Concn (mole/l.)	Probe	$p \times 100$	$\bar{\eta}$ (27°), cP	ΔE (kcal/mole)
LTABr	5×10^{-2}	2MA	5.38	26	7.2
		Per	2.08	17	9.6
MTABr	2×10^{-2}	2MA	5.10	32	7.1
		Per	2.18	21	9.5
CTABr	10^{-2}	2MA	5.73	30	6.2
		Per	2.05	19	9.6
SDBABr	10^{-2}	2MA	3.77	50	6.1
		Per	3.06	37	8.0
n-Dodecane				1.33	2.8
n-Hexadecane				2.98	3.8
White Oil U. S. P. 35				124	12.7

[a] The fusion activation energy, ΔE, was derived from the change in $\bar{\eta}$ with temperature at the range 4–27°. For comparison the table includes analogous viscosity data for *n*-dodecane, *n*-hexadecane (Rossini, 1947), and While Oil U. S. P. 35.

aqueous solution of systems containing hydrocarbonic region (*e.g.*, micelles) is equilibrated with a solid phase of aromatic hydrocarbon, the latter will penetrate and dissolve in the hydrocarbon region. This can be followed by the appearance of fluorescence in the solution.

An ideal aromatic hydrocarbon employed as a fluorescence probe for such systems should possess the following characteristics. (1) Rigid structure to avoid depolarization due to rotations of side groups. (2) High and constant p_0 values when excited in the last electronic absorption band. These properties provide a depolarization scale which is unaffected by small shifts in absorption spectrum. (3) Regions in absorption spectrum for which $p_0 = 1/7$ and $-1/3$, for the evaluations of in- and out-of-plane rates of rotation. (4) Lifetime of excited state in the range of 1–8 nsec. For such lifetimes the expected degree of depolarization r_0/r, in a system with viscosity of 1–100 cP, can be measured with good accuracy. (5) High values for the extinction coefficient and quantum yield since these will increase the fluorescence signal. (6) Minimum overlap between absorption and emission spectra, to eliminate depolarization due to energy transfer in case of high local concentration of the probe.

The above properties were examined in a large number of polynuclear aromatic hydrocarbons. Among the tested compounds perylene was found to be the most satisfactory. Except for its marked overlap between absorption and emission its other properties are almost ideal. It has a strong absorption band in the visible region and a quantum yield of 0.89 with lifetime of excited state of 4.9 nsec in dodecane at room temperature, and a polarization spectrum extended between $p_0 = -0.25$ and $p_0 = 0.47$. Some of the spectral properties of perylene are recorded in Figure 1.

Two other compounds, 9-vinylanthracene and 2-methylanthracene were found to have fairly good probe properties. Part of the absorption, emission, and the polarization spectra of these are shown in Figures 2 and 3. In hydrocarbon solvents at room temperature 9-vinylanthracene has a quantum yield of about 0.7 and excited-state lifetime of 8.2 nsec, and 2-methylanthracene has a quantum yield of about 0.3 and excited state lifetime close to 3.5 nsec. The quantum yield of both compounds is practically the same in ethanol, benzene, or hexane under the same conditions.

In the subsequent work only perylene and 2-methylanthracene were used as probes, since the relatively long lifetime of the excited state of 9-vinylanthracene leads to an undesirably large depolarization in the systems investigated.

Microviscosity in Micelle Interiors. A series of aqueous micelle solutions of quaternary ammonium salts with an aliphatic hydrocarbon chain of 12, 14, 16, and 18 carbons, which contain about 50–200 molecules/micelle (Debye, 1949), were labeled with 2-methylanthracene and perylene. The degree of polarization, the derived values for the microviscosity and its fusion activation energy are shown in Table I. The table also contains viscosity data for three relevant hydrocarbon solvents. The data given in Table I show a fairly good agreement between systems labeled with 2-methylanthracene and perylene. Part of the lower values of microviscosities obtained with perylene are probably the result of its relatively long lifetime of excited state in the micelles (5–6 nsec) which permits some contribution of micelle rotation to the observed depolarization. However, the much shorter lifetime of excited 2-methylanthracene in the micelles (1–2 nsec) probably reduces this contribution almost to zero. The small contribution of micelle rotation to the depolarization is reflected by the finding that increasing the concentration of CTABr up to 0.1 M, which gives rise to swelling of the micelles, is followed by a less than 10% increase in fluorescence polarization of embedded perylene. The data given in Table I show that the interiors of the micelles tested are of a liquid nature but less fluid than hydrocarbon solvents of similar chain length. The higher ΔE values found for the micelles, as compared to the analogous hydrocarbon solvent, are probably due to the strong interactions around the charged edges of the chains, which increase the energy required for crossing the fusion potential barrier between adjacent hydrocarbon chains. The obtained values of ΔE are all higher than 6 kcal mole^{-1}, a value predicted to be the upper limit for linear hydrocarbon liquids (Kauzman and Eyring, 1940).

The following studies were carried out with perylene-labeled CTABr micelles of reduced effective charge. These micelles certainly are of a considerably bigger size than the intact CTABr micelles and, therefore, their rotational diffusion during the lifetime of excited state of perylene was considered as negligible. The effect of salt concentration of the interior of

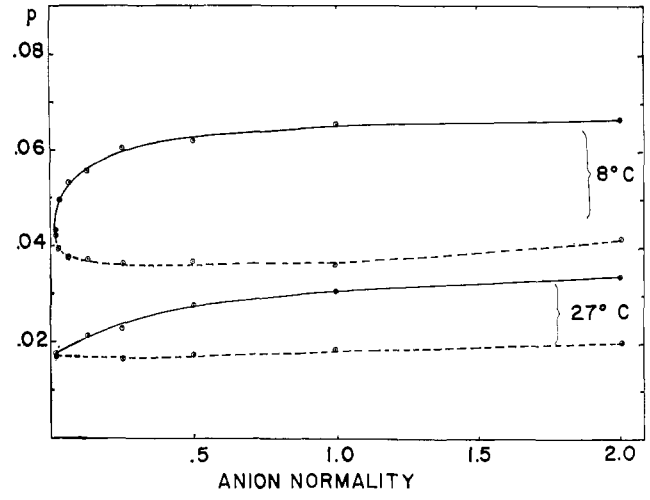

FIGURE 4: The effect of added sodium bromide (—) or sodium phosphate (– –) on the fluorescence polarization, p, of perylene-labeled CTABr micelles (10^{-2} M) at 8 and 27°.

micelles was checked with micelles of CTABr (10^{-2} M) labeled with perylene. Figure 4 shows the changes in fluorescence polarization of the CTABr–perylene system with the addition of bromide and phosphate at 8 and 27°. The increase in polarization with bromide concentration is expected, since the attraction between the bromide anions and the polycationic surface of the micelle results in partial masking of the individual positive charges which diminishes the charge repulsions between adjacent components. However, a small part of the increase in polarization may be the result of the increase in size of the CTABr micelles with salt concentration (Anacker and Ghose, 1968) which markedly reduces the contribution of micelle rotation. A reverse effect, which is emphasized at 8°, is found in the presence of phosphate at concentrations lower than 0.2 N. This can be explained by the special geometry of the interaction between the trivalent phosphate ion and three positively charged edges on the micelle surface. Such an interaction presumably causes more separation between the micelle components. At higher phosphate ion concentrations they may react as di- and monovalent ions, and the observed salt effect will approach that of the bromide. The results given in Figure 4 indeed show slight increase in fluorescence polarization at high phosphate concentrations. In addition to that, the phosphate ion unlike the bromide, probably cannot penetrate the Stern layer of the micelle and therefore the effective charge and hydration of the micelle are less affected by its presence than by the presence of bromide.

The effect of introducing uncharged hydrophilic groups into the surface of the micelle, on the fluidity of the micelle interior, was tested in CTABr micelles mixed with cetyl alcohol and cholesterol. These two alcohols are insoluble in water and can be dissolved in the presence of CTABr micelles (10^{-2} M) to form mixed micelles (see Experimental Section). In these mixed micelles the hydrocarbon moiety of the alcohol is presumably aligned with the side chains of the CTABr, whereas the hydroxyl groups are facing the water by which they isolate the charged edges. Figure 5 shows the change in fluorescence polarization at 2.5 and 27° with composition of the CTABr–cetyl alcohol mixed micelles labeled with perylene. The curves indicate that charge isolation is followed by a marked increase in microviscosity of the micelle interior. This increase is especially emphasized at small proportions of

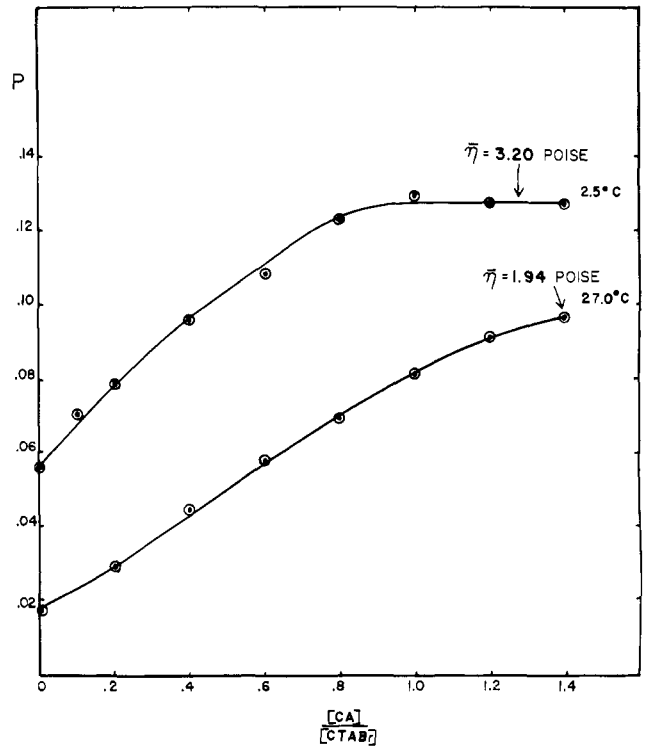

FIGURE 5: Fluorescence polarization, p, of perylene-labeled CA–CTABr mixed micelles of different proportions at 2.5 and 27.0°.

cetyl alcohol. Around a molar ratio of 1 the changes in $\bar{\eta}$ are smaller, and at 2.5° they level off at a value of 3.20 P. The change in ΔE with the micelle composition, as obtained from the data given in Figure 5 is shown in Figure 6. The given curve extrapolates to a value of about 2.5 kcal mole^{-1} for hypothetical micelles of pure cetyl alcohol, which is close to the ΔE value for *n*-hexadecane (see Table I).

Two mixed micelles of CTABr with cholesterol, at a cholesterol–CTABr molar ratio of 1:4 and 1:2, were studied. The results obtained for these systems labeled with perylene are summarized in Table II. Although qualitatively similar, the

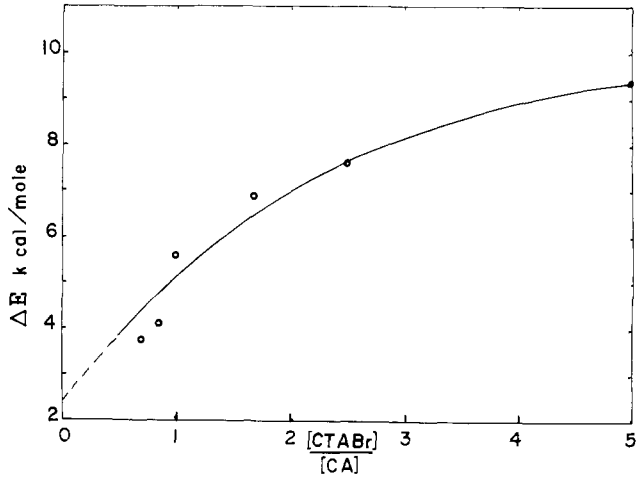

FIGURE 6: The change in fusion activation energy, ΔE, with composition of CA–CTABr mixed micelles. The ΔE values were obtained from the microviscosities at 2.5 and 27° derived from the polarization data given in Figure 5.

TABLE II: Degree of Fluorescence Polarization, p, of Perylene-Labeled CTABr Micelles (10^{-2} M) Mixed with Cholesterol; the Corresponding Microviscosity, $\bar{\eta}$, and the Fusion Activation Energy, ΔE.[a]

[Cholesterol]–[CTABr]	Temp (°C)	$p \times 100$	$\bar{\eta}$ (cP)	ΔE (kcal/mole)
1:4	27	9.6	1.61	5.2
	6	13.8	3.10	
1:2	27	14.1	3.57	5.1
	6	18.1	6.80	

[a] Excitation was performed with the 436-nm line of a mercury arc.

effect of cholesterol on the fluidity of the micelle interior is much more pronounced than that of the cetyl alcohol. On the other hand, the ΔE values for the CTABr–cholesterol micelles are somewhat smaller than those obtained for CTABr–cetyl alcohol micelles of the same proportions (see Figure 6).

Neutralization of the positive charges on the surface of CTABr micelles is accomplished in mixed micelles of CTABr (10^{-2} M) and sodium 1-hexadecanesulfonate (HSNa). Titration of perylene-labeled CTABr with HSNa is followed by a gradual increase in fluorescence polarization (see Figure 7). The titration curve shown in Figure 7 was extrapolated to complete neutralization for which the corresponding value of $\bar{\eta}$ at 27° is 4.3 P.

Estimation of Order in the Hydrocarbon Region. When a fluorescent probe in a dense medium can perform only small rotations during the lifetime of excited state, its rate of rotation, R, can be evaluated from the expression (see eq 5–8)

$$R = \frac{\frac{r_0}{r} - 1}{6\tau} \qquad (14)$$

If the rates of rotation in- and out-of-plane are the same, R

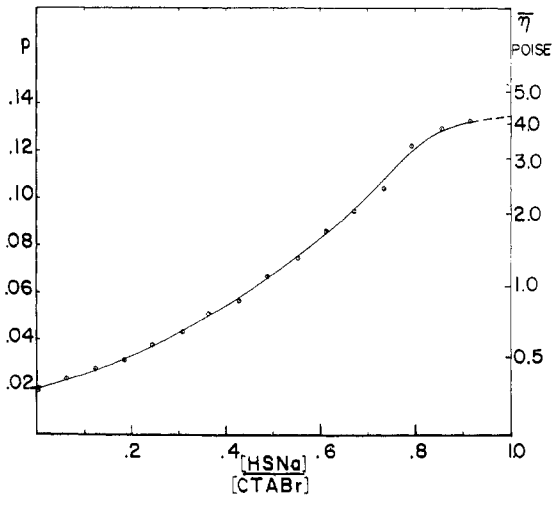

FIGURE 7: Fluorescence polarization, p, and the respective microviscosity, $\bar{\eta}$, of perylene-labeled HSNa–CTABr mixed micelles of different proportions at 27°.

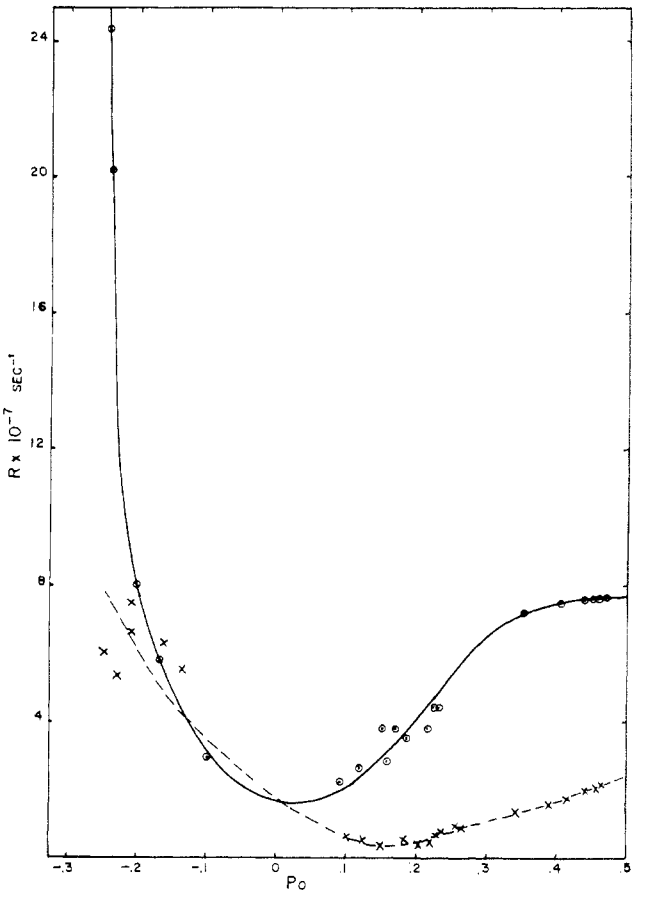

FIGURE 8: The variation of observed rate of rotation, R, of perylene with p_0. (⊙—⊙) In a mixture of 10^{-2} M CTABr and 8.5×10^{-3} M HSNa at 2°, (×— — —×) in 1:1 glycerol–propylene glycol at 4°.

should be independent of wavelength of excitation (or of p_0). However, for anisotropic rotations R will vary with p_0, and for p_0 equal to −0.333, 0.143, and 0.5 the corresponding values of R will represent the rate of rotation in-plane, out-of-plane, and their average, respectively. As a micellar system adequate for testing these predictions, we chose the perylene-labeled mixture of 10^{-2} M CTABr and 8.5×10^{-3} M HSNa at 2°. This system displays a maximum polarization value of 0.142, which is close to the highest value found for perylene-labeled micelles. Although this polarization value corresponds to a rate of rotation which might be still too large to justify the use of the relation given in eq 5, the polarization spectrum of the above system was determined, corrected for stray light, and the different values of R were derived by using eq 14. The obtained values of R are given as a function of p_0 in Figure 8. The figure shows that the rate of rotation of perylene embedded in the CTABr–HSNa micelle is highly anisotropic, R_p being at least ten times larger than R_{op}. However, this anisotropy of rotation can be the result of either anisotropic medium which surrounds the perylene molecules, or different in- and out-of-plane rates of rotation of perylene in a medium which is isotropic. To check this point the rates of rotation of 10^{-6} M perylene in glycerol–propylene glycol (1:1) at 4°, and in propylene glycol at −14°, were determined in a parallel manner. These media were chosen as they presumably are close to isotropic and on account of their high viscosity. The degree of depolarization of perylene in both systems was found always lower than 2, and the use of eq 14 for obtaining

R values for these systems was therefore more adequate. The derived values of R as a function of p_0 for perylene in glycerol–propylene glycol (1:1) at 4° are shown in Figure 8. It can be clearly seen that the changes in R with p_0 of perylene in the tested micelles and in the glycerol–propylene glycol system qualitatively follow the same pattern. For both systems the lowest values of R appear around p_0 corresponding to the out-of-plane rotation, and the highest values of R at p_0 corresponding to the in-plane rotation. R values at $p_0 = 0.5$ are somewhat lower than the respective average of the in- and out-of-plane R values. A comparison of the obtained values for R in the micelles and in the glycerol–propylene glycol system at $p_0 = 0.5$, $p_0 = -0.25$, and at the minima of the curves, shows that all three ratios of R fall in the range of 3–5. The variation of the rate of rotation of perylene in propylene glycol at −14° resembles in shape the curves given in Figure 8. An analogous comparison of the ratios of the R values of the micelles and the propylene glycol system gives values in the range of 2–3.

The above findings indicate that in the anisotropy of rotation observed for perylene molecules in CTABr–HSNa micelles, the solvent structure plays little, if any, part and therefore that the interior of these micelles is nearly isotropic and resembles in nature an aliphatic hydrocarbon solvent.

The considerable anisotropy of rotation ($R_p/R_{op} > 10$) observed in all the media must be attributed to the shape of the molecule and its relation to the nearby solvent molecules. It appears that the molecule can "slip" in its own plane much more easily than rotate out of it. Certainly the dissolved molecule cannot be conceived as anything resembling an oblate ellipsoid of revolution in a totally isotropic medium. Such a situation would demand $R_p \simeq R_{op}$ (Perrin, 1934, 1936), which is far from the present case. There is reason to believe that the assumption of a hydrodynamic ellipsoid having roughly the shape of the molecule is a valid one when the particle is large compared to the solvent molecules (10^2:1 or 10^3:1), but our present observations show that this assumption is no longer tenable when the ratio is of the order of 5:1.

References

Anacker, E. W., and Ghose, H. M. (1968), *J. Amer. Chem. Soc. 90*, 3161.
Debye, P. (1949), *Ann. N. Y. Acad. Sci. 51*, 575.
Kauzman, W., and Eyring, H. (1940), *J. Amer. Chem. Soc. 62*, 3113.
Perrin, F. (1926), *J. Phys. Radium 7*, 390.
Perrin, F. (1934), *J. Phys. Radium 5*, 497.
Perrin, F. (1936), *J. Phys. Radium 7*, 1.
Rossini, F. D. (1947), *Nat. Bur. Stand.*, 111, 112.
Spencer, R. D., and Weber, G. (1969), *Ann. N. Y. Acad. Sci. 158*, 361.
Weber, G. (1953), *Advan. Protein Chem. 8*, 415.
Weber, G., and Bablouzian, B. (1966), *J. Biol. Chem. 241*, 2558.
Weber, G., and Young, L. (1964), *J. Biol. Chem. 239*, 1424.

Comparative Structural Properties of Insect Triose Phosphate Dehydrogenases*

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ABSTRACT: Triosephosphate dehydrogenase (EC 1.2.1.12) was isolated from honey bees, four species of bumblebees (*Bombus nevadensis*, *Bombus occidentalis*, *Bombus appositus*, and *Psithyrus suckleyi*), leaf-cutting bees, fleshflies, and screwworm flies. The amino acid compositions and, except for the screwworm fly, tryptic peptide maps of these proteins were compared to one another and with the published data for lobster and pig triose phosphate dehydrogenase. The structural relationships of most of the dehydrogenases correlate with the known phylogeny of the species; however, the honeybee enzyme differs much more from the other enzymes than is expected from the phylogeny. The structure of the *Psithyrus* dehydrogenase is very similar to that of the three Bombus species; this may suggest that the genus *Psithyrus*, which comprises the bumblebees with inquiline (parasitic like) behavioral patterns, probably arose after lineages leading to at least some modern *Bombus* species had diverged from the common ancestral stock.

Although insects comprise the largest and most diverse of all taxonomic classes, there have been very few studies on the comparative structure of insect proteins. The primary sequences of only four insect proteins, all cytochromes *c*, have been published (McLaughlin, 1969). The present report describes studies on the comparative structures of triose phosphate dehydrogenases (D-glyceraldehyde 3-phosphate + phosphate + DPN^+ = 1,3-diphospho-D-glyceric acid + DPNH, EC 1.2.1.12) from six species of bees and two species of flies. The methods employed include enzyme purification, amino acid composition, and peptide mapping. This enzyme was chosen since it could be easily isolated from every insect species we tried and the primary sequences of the lobster (Davidson *et al.*, 1967) and pig (Harris and Perham, 1968) enzymes have been reported. All triose phosphate dehydrogenases yet investigated are tetramers with subunit molecular

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