Microviscosity of the Hydrocarbon Region of the Bovine Retinal Rod Outer Segment Disk Membrane Determined by Fluorescent Probe Measurements†

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ABSTRACT: The microviscosity of the hydrocarbon region of the bovine retinal rod outer segment disk membrane was determined by measuring the anisotropy of fluorescence from the probe 1,6-diphenyl-1,3,5-hexatriene. The microviscosity ranged from 1.4 P at 40 °C to 15 P at 0 °C, and no phase transition was observed in this temperature range. Bleaching of rhodopsin in the disk membrane produced no change in the microviscosity within the limits of error of our measurement (±5%). The presence of retinal in the disk membrane caused strong quenching of the probe fluorescence. Removal of retinal from bleached disks produced a threefold increase in total fluorescence intensity and only a 10% drop in fluorescence anisotropy, suggesting that large lateral gradients in microviscosity do not exist in the disk membrane. Bilayers prepared from extracted disk lipids had a microviscosity which was about one-fourth that of the intact disk membrane, demonstrating that rhodopsin hinders the mobility of the hydrocarbon chains of the disk phospholipids. In addition, vesicles formed from extracted disk phospholipids or egg phosphatidylcholine had identical microviscosities despite the much higher degree of unsaturation of the disk phospholipids.

The fluid nature of the vertebrate retinal rod outer segment disk membrane was first demonstrated by Cone (1972) by monitoring the rate of decay of induced linear dichroism of the retinal chromophore of rhodopsin. At the same time, Brown (1972) demonstrated that a permanent linear dichroism could be induced in this chromophore after fixing the membrane with glutaraldehyde. These measurements demonstrated that rhodopsin undergoes rapid rotation about an axis perpendicular to the plane of the disk membrane. Microspectrophotometric studies (Liebman and Entine, 1974; Poo and Cone, 1973, 1974) of single intact rod outer segments have shown that rhodopsin is also free to diffuse laterally in the plane of the disk membrane. The use of the rhodopsin molecule as a probe in such studies has the advantage that it is present as one of the natural components of the membrane. However, our incomplete knowledge of the shape of rhodopsin and its position in the membrane makes it difficult to obtain a quantitative estimate of the disk membrane fluidity from the calculated diffusion coefficient of rhodopsin.

In order to obtain a value for the fluidity of the disk membrane which may be quantitatively compared to other membrane systems, and also to compare the degree of fluidity encountered by an intrinsic membrane protein (rhodopsin) to that experienced by a small hydrophobic probe molecule, we have measured the microviscosity of the hydrocarbon region of the bovine retinal rod outer segment disk membrane by monitoring the anisotropy of fluorescence from the dye 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] dissolved in the membrane. The use of DPH as a probe of microviscosity in artificial and natural membranes has been previously described (Shinitzky and Barenholz, 1974; Shinitzky and Inbar, 1974). In the disk membrane we have observed a microviscosity of 3.8 ± 0.5 P at 20 °C, a simple exponential dependence of microviscosity on reciprocal temperature from 0 to 40 °C, and a fusion activation energy of 11.5 ± 0.7 kcal/mol. The microviscosity measurement is in general agreement with that estimated by Cone, while the activation energy measured with DPH is about one-half that observed in Cone's experiments. No significant difference in microviscosity between bleached and unbleached disk membranes was detected.

Methods

Disk Membrane Preparation. Retinal rod outer segment disk membranes were prepared from frozen bovine retinas (Hormel) by the method of Smith et al. (1975). The A_{280}/A_{500} ratio of the resulting material was typically 2.1 to 2.3 when solubilized in hexadecyltrimethylammonium bromide (CTAB). Spectral measurements were performed on a Cary 15 recording spectrophotometer. Protein concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard, and lipid phosphorus was assayed by the method of Bartlett (1959). When necessary, the extinction coefficient at 500 nm and the molecular weight of rhodopsin were taken to be 40 000. All procedures were performed either in darkness or under dim red light unless otherwise stated.

Disk Lipid Extraction. Disk membrane lipids were extracted by vortexing the membranes in the presence of chloroform–methanol (2:1, v/v). The lower phase was collected, dried under nitrogen, and suspended in deionized water by ultrasonic irradiation (Branson W-350, continuous mode, output setting 1) for a period of 1 min in a glass vessel immersed in stirred ice–water under a nitrogen atmosphere. An absorption spectrum of disk lipids prepared in this manner revealed the presence of retinal. In order to prepare disk lipids which did not contain retinal, the Schiff base between retinal

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[1] Abbreviations used are: DPH, 1,6-diphenyl-1,3,5-hexatriene; CTAB, hexadecyltrimethylammonium bromide; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ESR, electron spin resonance; NMR, nuclear magnetic resonance.

and opsin was reduced by a 15-min incubation of unbleached disks (4 mg of protein) in 2 ml of 0.5 M acetic acid (pH 2.6, 23 °C) containing 1% sodium cyanoborohydride (Fager et al., 1972). Following the incubation, the disk lipids were extracted and resuspended as described above. An absorption spectrum of this material contained no 365-nm band, confirming that the retinal had been removed.

Preparation of Phosphatidylcholine Vesicles. Egg yolk phosphatidylcholine was prepared according to Litman (1973) and stored in benzene at −10 °C. Tritiated rat liver phosphatidylcholine (0.1 Ci/mol) was prepared by injecting rats with [9,10-^{3}H]palmitic acid as described by Gatt (1968). Tritiated phosphatidylcholine was mixed with egg phosphatidylcholine, evaporated to dryness under vacuum, and suspended in 0.05 M KCl. The suspension was subjected to ultrasonic irradiation (Branson W-350, continuous mode, output setting 1) for 15 min in a glass vessel immersed in stirred ice-water under a nitrogen atmosphere. The sample was then centrifuged at 2 × $10^5 g$ for 2 h, and the supernatant, which consists primarily of 210 Å diameter, single-walled vesicles (unpublished results), was collected. The specific radioactivity of the final vesicle preparation was determined to be 0.008 Ci/mol by solubilizing a 0.05-ml sample of the vesicle solution in Eastman Type II scintillation fluid and counting the sample in a Beckman LS-233 scintillation counter.

Retinal Removal from Disks. A suspension of disk membranes was bleached in the presence of 0.05 M hydroxylamine in 0.1 M KH_2PO_4 buffer, pH 7, pelleted to remove excess hydroxylamine, and resuspended in phosphate buffer. At this point the 365-nm absorption band due to retinal oxime was still associated with the disks. A tenfold excess (based on phosphate analysis) of sonicated, tritium-labeled phosphatidylcholine vesicles was added to the disk suspension, and the mixture was allowed to incubate at 4 °C for 12 h. Following the incubation, the disks were pelleted (25 000g for 15 min), the supernatant containing the vesicles was removed, and the disks were resuspended and washed once in phosphate buffer. An absorption spectrum of the disks showed no 365-nm peak, while the vesicle supernatant had an absorption band at this wavelength. We estimate that 90% of the retinal was removed from the disk membranes by this p. ocedure.

To assess the degree of contamination by vesicle lipid in the retinal-free disk suspension, the sample was pelleted following the fluorescence experiment, solubilized in Eastman type II scintillation fluid, and counted in a Beckman LS-233 instrument. According to the specific radioactivity, 9% of the disk phospholipid appeared to be of vesicle origin. However, another disk sample that was subjected to incubation with vesicles experienced negligible alterations in the phosphorus/protein and lipid amino group/protein ratios (assayed by reaction of extracted disk lipids with trinitrobenzenesulfonic acid according to Litman, 1973).

Fluorescent Labeling of Membranes. Unless otherwise stated, all fluorescence measurements were performed in 0.1 M KH_2PO_4 buffer which was brought to pH 7 by addition of NaOH. Membrane concentrations of 0.05–0.1 mM lipid phosphorus were typically used. A stock solution of DPH dissolved in tetrahydrofuran was prepared, and its concentration was determined to be 1.4 mM by recording the absorption spectrum of an aliquot of the stock solution diluted into hexane (ϵ_{350} = 80 000; Shinitzky and Barenholz, 1974). Membrane suspensions were labeled with DPH by rapidly injecting small aliquots of the stock solution into the suspensions with a microliter syringe. The suspensions were mixed and incubated at 40 °C for 15–60 min to allow complete DPH incorporation. In some experiments where accurate control of DPH concentration among several samples was important, an aliquot of the DPH stock solution was first injected into phosphate buffer, and aliquots of this DPH suspension were added to the membrane samples.

Fluorescence Measurements. Fluorescence measurements were performed with a Perkin-Elmer MPF-3 instrument that was modified and adjusted for polarization measurements. A multi-wave plate wedge depolarizer (Electrooptics Industry, Rehovot, Israel) was installed between the analyzer and the emission monochrometer, and was adjusted such that no polarization was apparent in the fluorescence of *N*-methylacridinium perchlorate in methanol. For DPH fluorescence experiments the exciting and emission wavelengths were 360 and 430 nm, respectively, and the instrument's 390-nm filter was present in the emission beam to further reduce the contribution from scattered exciting light. Fluorescence intensities were recorded with the axis of the emission analyzer parallel ($I_\parallel$) and perpendicular ($I_\perp$) to that of the polarizer in the exciting beam. Scattering blanks were prepared for all membrane samples which were identical with the labeled samples except for the addition of DPH. $I_\parallel$ and $I_\perp$ of the scattering blanks were subtracted from the values obtained with the labeled samples. Scattering in the parallel mode was typically less than 20% of the signal from the labeled sample. Dilution of the samples showed that the fluorescence anisotropy was unaffected by scattering. Fluorescence anisotropy (r) and total fluorescent intensity (F) are defined as follows:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (1)$$

$$F = I_\parallel + 2I_\perp \quad (2)$$

Sample temperature was monitored by a thermistor immersed in the sample cuvette.

Calculation of Microviscosity. Microviscosity (η) was determined as previously described (Shinitzky and Barenholz, 1974) from the Perrin equation:

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\eta} \quad (3)$$

where r is the observed fluorescence anisotropy, r_0 is the limiting value of r for DPH in frozen solutions (0.362), T is the absolute temperature, and τ is the excited state lifetime. $C(r)$, a function which depends on the shape of the probe molecule, was empirically determined for DPH by calibration in White American Oil 35. The excited state lifetime of DPH was assumed to be 11.4 ns at 0 °C, and was estimated at other temperatures by assuming a direct proportionality between lifetime and total fluorescence intensity. This method was found to be valid for DPH in phosphatidylcholine or sphingomyelin liposomes, and in heavy liquid paraffin (Shinitzky and Barenholz, 1974). The lifetime for DPH in disk membranes was directly measured at 20 °C by single photon counting; this determination was in good agreement with the lifetime value obtained by the indirect method.

Quenching by Potassium Iodide. A 2-ml aliquot of a disk membrane suspension labeled with DPH in 0.1 M KH_2PO_4, pH 7, was placed in the fluorimeter, and the fluorescence intensity was recorded at 30 °C. One-half milliliter of 2.5 M potassium iodide in 0.1 M KH_2PO_4 was added to the disk suspension, and the intensity was again recorded. For comparison, equal amounts of DPH were added to two methanol samples, one of which contained 0.5 M potassium iodide, and the fluorescence intensities of the two solutions were recorded at 30 °C.

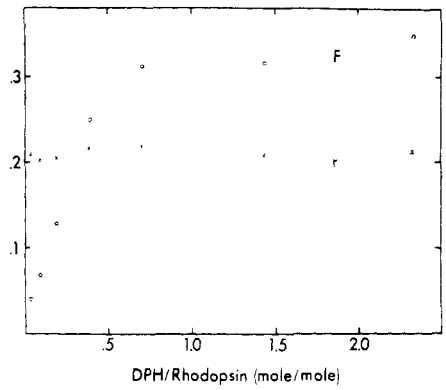

FIGURE 1: Dependence of fluorescence intensity (F) and anisotropy (r) on the DPH concentration in disk suspensions at 23 °C. Fluorescence intensity is plotted in arbitrary units.

Partitioning of DPH between Disk Membranes and Lipid Vesicles. A 4-ml sample of disk membranes (0.05 mg of rhodopsin/ml in 0.1 M KH_2PO_4, pH 7) labeled with DPH was thermostated in the fluorimeter. $I_\parallel$ and $I_\perp$ were recorded, and a small aliquot (0.005–0.1 ml) of phospholipid vesicles, prepared as described above, was added to the disk suspension. After approximately 5 min $I_\parallel$ and $I_\perp$ were again measured, and another aliquot of the vesicle solution was added. This procedure was repeated until a fourfold excess of vesicle lipid over disk lipid had been added to the disk sample. The same quantity of DPH that was used to initially label the disk membranes was also added to a separate phospholipid vesicle sample in phosphate buffer, and $I_\parallel$ and $I_\perp$ were recorded. The phospholipid concentration of the stock disk solution was determined as the amount of phosphorus obtained from the chloroform layers in two extractions of the disk membranes with 2:1:1 chloroform–methanol–water.

The partition coefficient, K, for DPH between disk membranes and vesicles was calculated by the following procedure. The total fluorescence intensities (F_{disk} and $F_{vesicle}$) and anisotropies (r_{disk} and $r_{vesicle}$) for the pure components were calculated to give the best least-squares linearization of the functions:

$$\frac{F - F_{disk}}{F - F_{vesicle}} = -K \frac{[\text{phospholipid}]_{vesicle}}{[\text{phospholipid}]_{disk}} \quad (4)$$

and

$$\left(\frac{F_{disk}}{F_{vesicle}}\right)\left(\frac{r - r_{disk}}{r - r_{vesicle}}\right) = -K \frac{[\text{phospholipid}]_{vesicle}}{[\text{phospholipid}]_{disk}} \quad (5)$$

where F is the intensity (corrected for dilution) and r is the anisotropy observed at each vesicle phospholipid to disk phospholipid ratio in the mixed sample. The values of intensity and anisotropy for the pure components calculated by the least-squares fitting procedure differed less than 10% from the measured values. The slope, K, of the above functions is the calculated partition coefficient, which is defined by

$$K = \left(\frac{[\text{DPH}]_{vesicle}}{[\text{phospholipid}]_{vesicle}}\right) \Big/ \left(\frac{[\text{DPH}]_{disk}}{[\text{phospholipid}]_{disk}}\right) \quad (6)$$

Results

DPH fluorescence may be easily observed in most lipid suspensions or natural membranes at a concentration of 1 mol/1000 mol of phospholipid. However, strong quenching of the DPH fluorescence was encountered in the disk membrane, necessitating the use of three to four times this level of DPH to obtain useful intensities. The DPH concentration dependence of the total fluorescence intensity for the disk membrane is shown in Figure 1. The intensity is linear with DPH concentration up to about 0.5 mol of DPH/mol of rhodopsin. Above this dye concentration the intensity plateaus, while the anisotropy of the fluorescence remains constant into the plateau region. Our subsequent fluorescence measurements were made in the linear portion of the DPH loading curve with label concentrations of 0.2–0.3 mol of DPH/mol of rhodopsin.

To determine the distribution of DPH within the disk membrane, the fluorescence quenching produced by potassium iodide was measured, as described under Methods. If fluorescent DPH molecules were situated in the disk membrane such that they were accessible to collision with iodide ion, then a quenching effect should be observed upon introducing KI into a DPH-labeled disk suspension. Exposure of a DPH-labeled disk suspension to 0.5 M KI produced only a 4% reduction of fluorescence intensity. By contrast, this concentration of KI produced a 32% reduction in fluorescence intensity of DPH in methanol, where all the DPH would be expected to be available to undergo collision with the iodide ion. These results suggest that very little fluorescent DPH is situated near the surface of the disk membrane, and, as is expected from its hydrophobicity, DPH seems to be localized in the hydrocarbon region of the bilayer.

The ability of DPH to partition into all the phospholipid of the disk membrane was measured by determining a partition coefficient for the distribution of DPH between phospholipid vesicles and disk membranes, as described under Methods. Four experiments at temperatures between 27 and 39 °C utilizing vesicles of phosphatidylcholine or phosphatidylcholine plus 0.2 mol fraction of egg yolk phosphatidylethanolamine yielded a partition coefficient of 0.99 ± 0.04. The same result was obtained from either the fluorescence intensity or anisotropy. Thus, all the phospholipid of the disk membrane appears to be penetrable by DPH.

Figure 2 displays the temperature dependence of the total fluorescence intensity for the following four preparations: egg phosphatidylcholine vesicles; disks; and disk lipids with and without retinal. All four curves are normalized to the same DPH concentration, and the DPH levels employed were within the linear region of the intensity vs. DPH concentration curve. Curve 1 in Figure 2, which was obtained with DPH-labeled egg phosphatidylcholine vesicles, displays roughly the same fluorescence intensity as the equivalent DPH concentration in paraffin oil, indicating that negligible amounts of the DPH remain in the aqueous phase. Extracted disk lipids that did not contain retinal exhibited comparably high fluorescence intensity (curve 2, Figure 2). By contrast, the other curves in Figure 2 for total disk lipids, which contain retinal, and for native disks have only about one-third the total fluorescent intensity of the retinal-free systems. In separate experiments, adding small aliquots of 11-*cis*-retinal dissolved in ethanol to DPH-labeled phospholipid vesicle suspensions produced a similar reduction of the fluorescent intensity at about 1 mol of retinal/100 mol of phospholipid. These results confirm that the poor quantum yield we observe for DPH in disks is due to the presence of the retinal chomophore of rhodopsin, whose absorption spectrum overlaps the DPH emission spectrum in both the bleached and unbleached states, Figure 3. Addition of hydroxylamine to bleached disks reduces the spectral overlap and increases the fluorescent intensity as expected for quenching by energy transfer. The fluorescent intensity was further increased by bleaching disks in the presence of sodium borohydride, which forms the reduced Schiff base of retinal

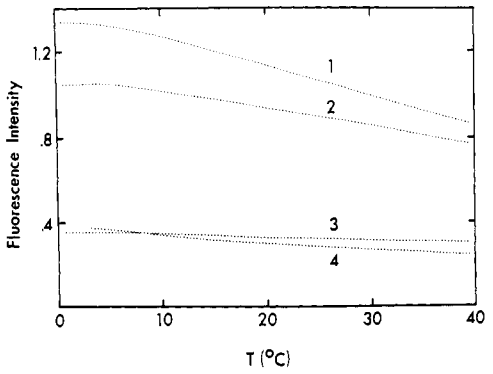

FIGURE 2: Fluorescence intensity of DPH-labeled membrane systems normalized to the intensity of an equal DPH concentration in paraffin oil at 40 °C. Curve 1, egg phosphatidylcholine; curve 2, extracted disk lipids without retinal; curve 3, total extracted disk lipids; curve 4, disk membranes.

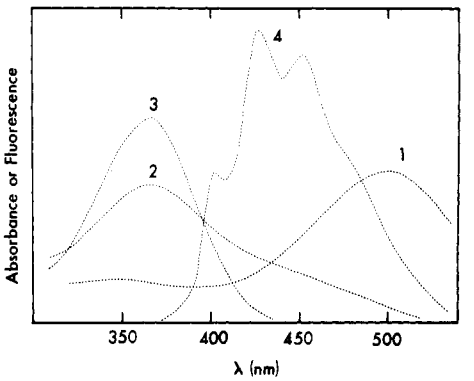

FIGURE 3: Absorption spectra of disk membranes solubilized in 1.5% CTAB, 0.1 M KH_2PO_4, pH 7, and the fluorescence emission spectrum of DPH in hexane. Curve 1, unbleached disks; curve 2, bleached disks, no hydroxylamine; curve 3, bleached disks, bleached in 50 mM hydroxylamine; curve 4, DPH fluorescence emission spectrum (Shinitzky and Barenholz, 1974).

with an absorbance maximum at 330 nm.

The anisotropy of fluorescence from the four systems described above is shown in Figure 4 as a function of temperature. The extracted disk lipid sample that did not contain retinal displayed essentially the same anisotropy as did egg phosphatidylcholine throughout the temperature range of 0–40 °C, while the sample of total extracted disk lipids showed a slightly higher anisotropy below 20 °C. In contrast to these three lipid systems, the anisotropy of fluorescence observed in the disk membrane was much greater throughout the temperature range.

As an additional test of the effect of quenching upon the anisotropy of fluorescence from disk membranes, we measured the fluorescence of DPH in bleached disks from which the retinal had been removed by incubation with phosphatidylcholine vesicles, as described under Methods. As a control, a sample of unbleached disk membranes was also subjected to incubation with phosphatidylcholine vesicles. The 500-nm absorption band of the unbleached control sample was unaffected by the incubation as expected, and quenching typical of disks was encountered when this sample was labeled with DPH and observed in the fluorimeter. However, as shown in Figure 4, the anisotropy of fluorescence from the control sample was reduced relative to untreated disk membranes. This apparent increase in fluidity may result from some unobserved alteration in the lipid composition of the disk membrane. The anisotropy of the retinal-free disk sample was only 5–10% lower than that of the control sample, but its fluorescent intensity was higher by about a factor of three. Therefore, the fluorescence quenching encountered in the disk membrane does not appear to have a large effect on the observed anisotropy.

The microviscosities calculated for the four systems previously described are displayed logarithmically vs. the reciprocal of the absolute temperature in Figure 5. The natural lipid systems studied here show no evidence of phase transitions in the 0–40 °C range. The microviscosities of the extracted disk lipid systems appear to be quite similar to that of egg phosphatidylcholine, while the disk membrane is much less fluid. Evidently, the presence of rhodopsin in the disk membrane greatly hinders the mobility of the hydrocarbon chains of the phospholipids resulting in a three- to fourfold increase in microviscosity.

The data presented here for disk membranes were obtained from preparations that had not been bleached. Absorption spectra taken following the fluorescence experiments showed that the samples had lost less than 10% of their original A_{500}.

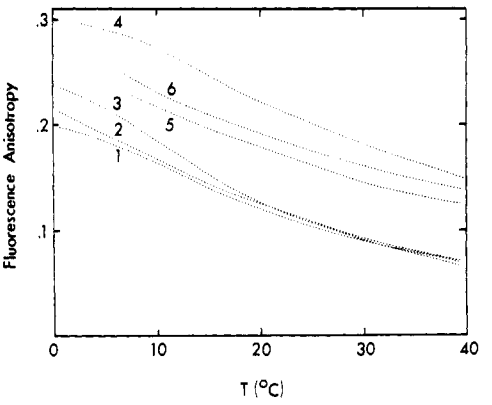

FIGURE 4: Fluorescence anisotropy of DPH-labeled membrane systems. Curve 1, egg phosphatidylcholine; curve 2, extracted disk lipids without retinal; curve 3, total extracted disk lipids; curve 4, disk membranes; curve 5, disk membranes that were bleached and incubated with phosphatidylcholine vesicles to remove retinal; curve 6, same as 5 except the sample was not bleached.

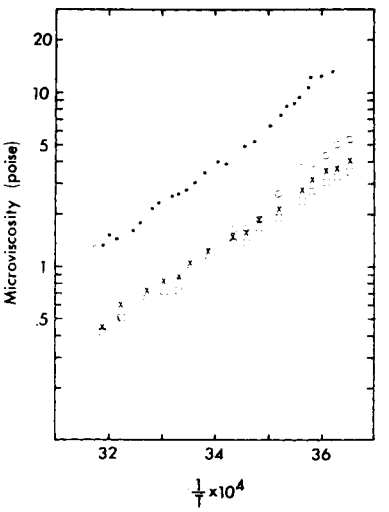

FIGURE 5: Microviscosity plotted logarithmically against the reciprocal of the absolute temperature. The excited-state lifetime was taken to be 11.4 ns at 0 °C for all samples and estimated for other temperatures in direct proportion to the total fluorescent intensity. Egg phosphatidylcholine (□); extracted disk lipids without retinal (X); total extracted disk lipids (○); disk membranes (●).

Disk samples that had been completely bleached prior to the fluorescence measurements yielded the same anisotropy values as the unbleached samples within the limits of the error of our measurements (±5%). The presence of either 1 mM EGTA, 1 mM $CaCl_2$, or 100 mM NaCl (in 50 mM Tris buffer, pH 7, 37 °C) did not yield a significantly different result from that obtained in potassium phosphate buffer.

Discussion

Fluorescence polarization has been shown to be a convenient and quantitative method of assessing the fluidity of biological membranes, when it is expressed as a microviscosity calculated from the Perrin equation (Cogan et al., 1973; Shinitzky and Barenholz, 1974; Shinitzky and Inbar, 1974; Inbar et al., 1973; Aloni et al., 1974). This equation was developed to describe rotational diffusion in an isotropic medium (Weber, 1953). Since biological membranes cannot be expected to present an isotropic environment to a probe molecule, the microviscosity determined by the fluorescence polarization technique cannot be viewed as an absolute analogue of the bulk viscosity. The microviscosities of natural and model membranes calculated from the fluorescence anisotropy of DPH do, however, correlate qualitatively with acyl chain mobility determined by other methods (Suurkuusk, et al., 1976). Therefore, we consider the microviscosity measured by DPH fluorescence to be of use as a semiempirical index for comparing the fluidity of the hydrocarbon regions of different membrane systems and for studying changes in fluidity occurring within the same system. Several questions must be addressed in order to arrive at a reliable interpretation of the data, particularly in a system such as the disk membrane where a high degree of quenching occurs.

A basic assumption of the fluorescence polarization technique is that the probe molecules are distributed uniformly throughout the lipid regions of the sample membrane, regardless of the local microviscosity. This point may not be taken for granted, since certain molecules, such as the spin probe 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo), appear to partition specifically into the more fluid regions of membranes (McConnell et al., 1972). The results of our experiments on the partitioning of DPH between disk membranes and phospholipid vesicles show that DPH distributes between the disks and vesicles strictly on the basis of their relative phospholipid concentrations. Similar partitioning experiments have been conducted in mixed populations of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine vesicles at 28 °C, where the acyl chains of the dipalmitoylphosphatidylcholine vesicles exist in the gel state and those of the dimyristoylphosphatidylcholine vesicles are in the fluid state (Lentz, Barenholz and Thompson, in preparation). In these experiments, DPH distributed uniformly over all of the phospholipid in the system, regardless of the physical state of the acyl chains. Therefore, we conclude that all of the phospholipid hydrocarbon regions of our samples are equally accessible to DPH.

Due to the overlap of the retinal absorption and DPH fluorescence emission spectra (Figure 3), the strong quenching of DPH fluorescence in the disk membrane is most likely due to dipole–dipole resonance energy transfer to retinal. According to Förster's theory for this type of quenching (Förster, 1966), the quantum yield and excited-state lifetime of DPH in the disk membrane should depend strongly on the distance from the probe molecule to the surrounding quenching centers (rhodopsin molecules in the case of unbleached disk membranes). From the spectra shown in Figure 3, we calculate a value of 48 Å for the distance of 50% quenching of the DPH fluorescence in unbleached disk membranes. Since this distance is about one rhodopsin diameter (Blasie et al., 1969), the DPH in a significant fraction of the surrounding bilayer will be subject to quenching. If a large degree of heterogeneity were introduced in the DPH excited state lifetime by the quenching, it would complicate our calculation of microviscosities from the observed fluorescence anisotropies. A direct measurement of the lifetime of DPH in the disk membrane at 20 °C was obtained by the single photon counting technique. The measured decay curve could best be fit by a major component (79%) of 9.5 ns and a minor component (21%) of 1.06 ns. The major component is in good agreement with the lifetime estimated from the temperature dependence of the fluorescence intensity. We estimate that the contribution from the minor component would produce a 10% increase in the observed anisotropy relative to that which would be observed in the absence of quenching. Therefore, the assumption of a single lifetime based on the temperature dependence on the fluorescence intensity, which was used in our microviscosity calculation, is not expected to introduce large errors. Additional support for this conclusion is found in the experiments presented here involving removal of retinal either from disk membranes or from extracted disk lipids. Removal of retinal produced a large increase in fluorescence intensity, but only a small change in anisotropy over the entire temperature range. Thus, the degree of lifetime heterogeneity does not appear to depend strongly upon temperature, and any artifacts in anisotropy caused by quenching in the disk membrane must be small compared to the difference in anisotropy between the disk membrane and the extracted lipid systems studied here.

In addition to introducing heterogeneity into the DPH excited-state lifetime, the quenching might also be expected to obscure areas of the bilayer near the quenching centers, with the result that the observed fluorescence would preferentially represent areas farther from rhodopsin in unbleached disks. If rhodopsin perturbs the motion of the acyl chains of the disk phospholipid molecules, those nearest to rhodopsin would be expected to be more strongly hindered by the presence of the protein than would those farther away. In that case, removal of retinal from the disk membrane would produce an increase in the observed anisotropy of fluorescence, as previously quenched areas of bilayer near rhodopsin become observable. Instead, this experiment produced a slight decrease in anisotropy relative to an unbleached control sample, which may be attributed to the loss of the short lifetime component. Assuming rhodopsin to be surrounded by a single boundary layer of strongly immobilized phospholipid, as was suggested for cytochrome oxidase by the ESR probe study of Jost et al. (1973), we can estimate the magnitude of the increase in fluorescence anisotropy we would expect to see upon removal of retinal from the disk membrane. Assuming rhodopsin to be a cylinder of radius 22 Å (Blasie et al., 1969), which spans the disk phospholipid bilayer, approximately 29 phospholipid molecules would be expected to contact the protein, calculated by the method of Jost et al. (1973). Since our disk membrane preparations typically contain about 75 mol of phospholipid/mol of rhodopsin, 39% of the disk phospholipid would be expected to be boundary lipid. If this boundary lipid had a microviscosity similar to that of dipalmitoylphosphatidylcholine in the gel state, we would expect DPH in this region to report an anisotropy of 0.33 at 20 °C (Lentz, Barenholz and Thompson, in preparation). Taking the weighted average of this anisotropy and that which we observe in the unbleached disk membrane at 20 °C (0.22), we arrive at an anisotropy value of 0.26 which we would expect to observe in retinal-free

disk membranes on the basis of the above model. This represents a clearly observable 18% increase in anisotropy. Since no increase in anisotropy was detected in the retinal removal experiments, we conclude that such large lateral gradients in microviscosity do not exist in the disk membrane. Hence, the perturbation of phospholipid acyl chain mobility caused by the presence of rhodopsin in the disk membrane appears to extend 2–3 phospholipid molecules from the protein, encompassing essentially all of the disk lipid.

The value of 3.8 P that we obtained for the microviscosity of the hydrocarbon region of the bovine disk membrane at 20 °C by the fluorescence polarization technique lies within the range calculated for the frog disk membrane from the rotational and lateral diffusion coefficients of rhodopsin by Cone (Cone, 1972; Poo and Cone, 1973, 1974). On the other hand, the activation energy of 11 kcal/mol for the rotation of DPH, calculated from the slope of Figure 5, is about one-half that observed by Cone for the rotation and translation of rhodopsin. The explanation for this difference may be that the motion of DPH, a small hydrophobic molecule, depends predominantly on its interactions with the phospholipid acyl chains in the disk membrane, whereas the motion of rhodopsin may be dependent on its interactions with both the phospholipid acyl chains and head groups.

The microviscosity of vesicles prepared from extracted disk lipids was one-third to one-fourth that of the disk membrane. Thus, rhodopsin appears to substantially perturb the motion of the acyl chains of the disk phospholipids. This effect was first observed by Hong and Hubbell (1972) in an ESR study of reconstituted membranes containing rhodopsin, where they observed an increase in the order parameter as the protein to phospholipid ratio was increased.

The disk membrane is notable for the high degree of unsaturation of its phospholipid acyl chains (5.2 double bonds/phospholipid molecule), which is primarily due to an unusually large percentage of 22:6 fatty acid (Borggreven et al., 1970; Nielsen et al., 1970). Despite this relatively high unsaturation, the microviscosity of vesicles prepared from extracted disk lipids was essentially the same as that of egg phosphatidylcholine vesicles which have 1.8 double bonds/phospholipid molecule (Litman, unpublished results), suggesting that the 22:6 fatty acid side chains do not impart an abnormally high degree of fluidity to the disk membrane.

Three reports have appeared in which ESR spin probes indicated a change in fluidity of the disk membrane accompanying bleaching. Verma et al. (1973), using a stearamide spin probe incorporated into disk membranes, reported that bleaching produced a 20% change in the correlation time of the probe. Delmelle and Pontus (1975) measured the partitioning of the spin probe, Tempo, between the aqueous medium and the disk membrane to evaluate membrane fluidity. They found that bleaching decreased the ratio of the aqueous probe to membrane probe ESR signal amplitudes and interpreted this result as an indication of increased membrane fluidity (McConnell et al., 1972). In addition, Pontus and Delmelle (1975) suggested that 30% of the phospholipid in the disk membrane exists as a solid phase, and that the changes observed in the Tempo signal ratio on bleaching may be caused by changes in this fraction. If DPH were similarly excluded from solid regions, this would preclude observation of such changes by the fluorescence polarization method. However, the DPH partitioning experiments discussed above demonstrate that DPH is not specifically excluded from phospholipid bilayers in the gel state or from a significant fraction of the disk membrane phospholipid.

As noted in the Results section, we found no significant changes in the microviscosity of the disk membrane upon bleaching. This result is in agreement with the ^{13}C NMR study of bovine rod outer segment membranes by Millett et al. (1973) who reported that no changes in the T_1 values of the lipid acyl chain resonances could be observed upon bleaching the sample. In addition, Poo and Cone (1974) found that the lateral diffusion coefficient of rhodopsin was not affected by bleaching, suggesting that the microviscosity experienced by rhodopsin in the disk membrane does not change on bleaching. The fact that we see no significant change in microviscosity upon bleaching, in contrast to the ESR spin probe studies cited above, may be related to the positions of the probe molecules in the membrane. The nitroxide groups of both the stearamide spin label and of Tempo are probably located near the interfacial region of the bilayer due to their dipole character. In contrast, the hydrophobicity of DPH, the dependence of its fluorescence quantum yield on local dielectric constant, and the fact that the fluorescence of DPH in the disk membrane is not quenched by addition of potassium iodide to the aqueous medium argue that the dye is located within the hydrocarbon region of the membrane. Thus, it is possible that bleaching of rhodopsin perturbs the head group region of the surrounding bilayer without influencing the mobility of the hydrocarbon chains.

Conclusions

Our fluorescence polarization measurements indicate that the presence of rhodopsin in the disk membrane hinders the mobility of the disk phospholipid acyl chains, resulting in a three- to fourfold increase in microviscosity over that observed with vesicles prepared from extracted disk lipids. The microviscosities of extracted disk lipid vesicles and egg phosphatidylcholine vesicles are the same, indicating that the high degree of unsaturation of the disk phospholipids does not impart an unusually high degree of fluidity to bilayers formed from these lipids. In our experiments no significant change in the microviscosity of the disk membrane occurred upon bleaching the sample. Since neither bleaching nor removal of retinal from bleached disks produced an increase in fluorescence anisotropy, we conclude that large lateral gradients in microviscosity do not exist in the disk membrane, and that the perturbing influence of rhodopsin extends to all of the disk phospholipid.

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Adduct of Tyrosine and the Oncogen 3-Acetoxyxanthine†

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ABSTRACT: A major product of the reaction of L-tyrosine (I) with the activated oncogen 3-acetoxyxanthine (II) at neutral pH has been characterized as 8-xanthinyl-4′-*O*-tyrosine (III). A similar product is formed with tyrosylglycine. Another major product was the hydantoin of tyrosine. 8-Xanthinyl-4′-*O*-tyrosine is hydrolyzed in acid to yield tyrosine and uric acid but is stable in base. The synthesis of 8-phenoxyxanthine is also described. A metabolite identical with III in several chromatographic systems was found in the urine of two rats after administration of radioactive 3-hydroxyxanthine. This represented about 0.05% of the urinary radioactivity.

The potent oncogen 3-hydroxyxanthine (Brown et al., 1973) is activated in vivo to a sulfate ester (Stöhrer et al., 1972) which reacts with cellular nucleophiles and is believed to be the proximate oncogen. This active ester reacts with methionine (Stöhrer and Brown, 1970) and tryptophan (Stöhrer et al., 1973) forming xanthine-substituted diastereoisomers with the latter. In this communication we describe two products from the reaction of 3-acetoxyxanthine with tyrosine. The reaction of the oncogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with aromatic amino acids has been studied by Poirier et al. (1967). Those authors isolated and characterized two adducts of tyrosine and the carcinogen. Both structures, based on color tests, contain linkages to the 3-carbon on tyrosine and are in that respect different from the tyrosine–xanthine adduct described here.

Experimental Section

8-Xanthinyl-4′-O-tyrosine (III). L-Tyrosine (500 mg, 2.75 mmol) was dissolved in 10 ml of H_2O (80 °C) with the addition of 10 N NaOH to bring the pH to 8.0. Acetoxyxanthine (1.0 g, 3.7 mmol) (Birdsall et al., 1971) was then added in portions over a period of 10 min. The solution was stirred and the pH was kept at 8.0 by the continued addition of 10 N NaOH. After completion of the reaction, 10 ml of a slurry of Sulfopropyl-Sephadex C-25 (Pharmacia Fine Chemicals, Piscataway, New Jersey), equilibrated with 0.1 N HCl, was added and stirred for another 10 min, after which the entire slurry was placed on top of a column containing 200 ml of Sulfopropyl-Sephadex. Elution with 0.1 N HCl yielded 0.72 mmol of uric acid in the eluate fraction 175–350 ml, 0.93 mmol of 3-hydroxyxanthine in the fraction 350–600 ml, an undetermined amount of xanthine together with about 1.6 mmol of unreacted tyrosine in the fraction 690–1070 ml, and 0.8 mmol of III in the fraction 1070–1700 ml. This fraction was concentrated in vacuo and re-chromatographed as above. It was then concentrated again and chromatographed over 100 ml of Dowex 1 formate. Elution with 0.1 N formic acid yielded an unknown in the fraction 91–125 ml and pure III in the fraction 185–600 ml. This fraction after evaporation and recrystallization from 0.1 N formic acid yielded 67 mg of needles of III.

Anal. Calcd for $C_{14}H_{13}N_5O_5 \cdot H_2O$: C, 48.14; H, 4.33; N, 20.05. Found: C, 48.04; H, 4.19; N, 19.91.

8-Xanthinyl-4′-O-tyrosylglycine (V). Tyrosylglycine (500 mg, 1.95 mmol) was dissolved in 12 ml of H_2O and reacted with 2 g of acetoxyxanthine as above, but at 37 °C. Workup was as above but the product (0.25 mmol) was eluted in the fraction 1170–1800 ml on the C-25 column. Re-chromatography on C-25 and finally Dowex 1 formate gave 65 mg of a yellow powder.

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