LIGHT-SCATTERING EFFECTS IN THE MEASUREMENT OF MEMBRANE MICROVISCOSITY WITH DIPHENYLHEXATRIENE

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ABSTRACT Data from several membrane systems are presented to confirm an empirical means of correcting diphenylhexatriene fluorescence for depolarization caused by sample turbidity. The depolarization proportionality constants obtained are not equal, but are shown to vary with (*a*) the physical state of the membrane, (*b*) the cholesterol content of the membrane, (*c*) the protein content of the membrane, and (*d*) the method of membrane preparation or isolation. It is concluded that depolarization corrections must always be considered when diphenylhexatriene fluorescence anisotropy is used to compare the fluidities within different membrane bilayers.

INTRODUCTION

The depolarization of fluorescence from hydrophobic fluorophores has been used to probe the fluidity or "microviscosity" within the hydrophobic region of membrane bilayers (1, 2). A very popular probe for such measurements has been 1,6-diphenyl-1,3,5-hexatriene (DPH) (3). The anisotropy of DPH fluorescence, or the derived microviscosity, has been used extensively to compare the fluidity of membranes isolated from different physiological or pathological states (4–9).

The artificial decrease in fluorescence anisotropy caused by light scattering has been noted by Teale (10), who developed approximate equations describing this effect. Although Teale tested these expressions for solutions containing glycogen and conjugates of bovine plasma albumin with either 1-dimethylaminonapthalene 5-sulfonyl chloride or fluorescein isothiocyanate, his methods have not been evaluated for turbid membrane suspensions. We report here results obtained with a variety of membrane systems which confirm the general applicability of Teale's approach to depolarization by membrane suspensions and also demonstrate the necessity of making individual and unique corrections for each specific membrane studied.

METHODS

Dipalmitoyl phosphatidylcholine, egg phosphatidylcholine, and cholesterol were obtained as previously described (11). Sarcoplasmic reticulum membrane vesicles and protein-free sarcoplasmic reticulum lipid vesicles were prepared as described elsewhere (12). Two preparations of chick embryo retinal membranes were a generous gift from D. McClay of Duke University. Zone-purified DPH was a gift from Y. Barenholz and M. Shinitzky (Weizmann Institute, Israel). DPH was introduced into membrane suspensions by injecting a small amount of a tetrahydrofuran solution of DPH into the aqueous membrane suspension to achieve a final dye to lipid ratio of 1/500 (11, 12). Fluorescence

instrumentation and methods are described elsewhere (13). The anisotropy of DPH fluorescence is taken as a measure of membrane fluidity, increased anisotropy indicating a decreased extent of phospholipid acyl chain motion within the membrane (14). Turbidities of samples containing no DPH were estimated with a GCA/McPherson 700 spectrophotometer (GCA McPherson Instrument, Acton, Mass.) at a slit of 1 mm using a temperature-controlled cell attachment. Corroborative measurements obtained with a Hitachi 100-20 spectrophotometer (Hitachi Ltd., Tokyo, Japan) gave similar anisotropy correction plots.

RESULTS

In Fig. 1, the observed anisotropies of DPH fluorescence are plotted against the optical densities of model membrane suspensions containing dipalmitoyl phosphatidylcholine and cholesterol in various molar ratios. Curves are presented for pure dipalmitoyl phosphatidylcholine both above and below its gel-to-liquid-crystalline phase transition (41.1°C [11]). These data are plotted in a form consistent with Teale's basic finding that the fractional error introduced in the anisotropy by light scattering should be proportional to the optical density of the turbid solution (10). The proportionality constant was given by Teale as 2.3. Written otherwise, this becomes:

$$r_{obs} = -r' \cdot K \cdot OD + r' \tag{1}$$

where r_{obs} is the observed anisotropy, r' is the extrapolated anisotropy at zero optical density (OD), and K is the observed proportionality constant. When plotted in this way, the data for all the model membrane suspensions fit reasonably to straight lines, thus confirming at least the qualitative ability of Teale's equations to treat scattering depolarization by membranes. From the slopes and intercepts of these lines, the proportionality constants, K, have been calculated and summarized in Table I.

In Fig. 2 the observed anisotropies for DPH fluorescence at three temperatures are plotted

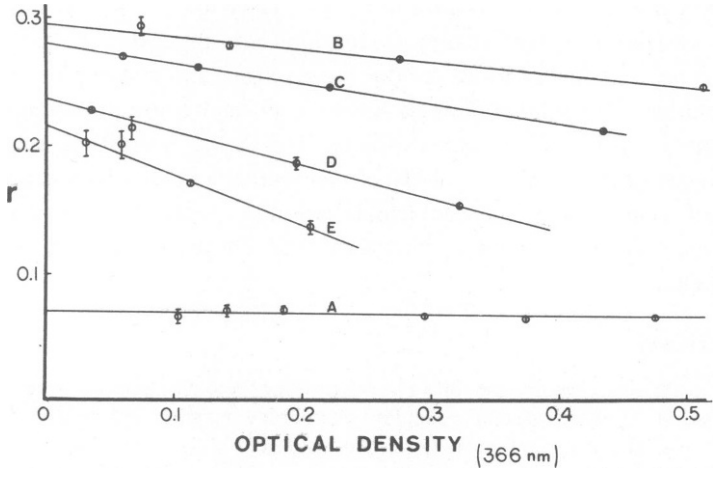

FIGURE 1 Anisotropy (r) of DPH fluorescence vs. optical density for several dilutions of dipalmitoyl phosphatidylcholine-cholesterol multilamellar vesicles. (A) 0.0 mol % cholesterol, 47°C; (B) 0.0 mol % cholesterol, 20°C; (C) 2.5 mol % cholesterol, 20°C; (D) 15 mol % cholesterol, 20°C; (E) 41 mol % cholesterol, 20°C.

TABLE I

DEPOLARIZATION OF DPH FLUORESCENCE INDUCED BY MEMBRANE PREPARATIONS

Sample	T	K	r'	Turbidity*	Δr(0.5 mM)‡
Synthetic bilayers	°C				
Multilamellar vesicles					
DPPC§	47	0.15 ± 0.12	0.070	0.476	0.005
DPPC	20	0.30 ± 0.01	0.292	0.514	0.045
DPPC	8	0.25 ± 0.04	0.310	0.751	0.058
DPPC + 2.5 mol% choles‖	20	0.551 ± 0.004	0.280	0.434	0.067
DPPC + 15 mol% choles	20	1.10 ± 0.01	0.237	0.242	0.063
DPPC + 41 mol % choles	20	1.77 ± 0.03	0.215	0.204	0.078
Single-lamellar vesicles					
DPPC	47	−1.7 ± 1.8	0.071	0.019	−0.002
DPPC	8	1.07 ± 0.12	0.317	0.286	0.097
Natural Membranes					
Sarcoplasmic reticulum vesicles with Ca-ATPase¶					
0.0 mol % Ca-ATPase	25	0.06 ± 0.2	0.101	0.330	0.002
1.2 mol % Ca-ATPase**	25	0.54 ± 0.02	0.176	0.725	0.069
1.3 mol % Ca-ATPase	25	0.47 ± 0.006	0.170	0.451	0.036
2.0 mol % Ca-ATPase	25	0.35 ± 0.05	0.212	1.05	0.078
2.9 mol % Ca-ATPase	25	0.32 ± 0.04	0.231	1.31	0.097
Chick embryonic retinal membranes					
Preparation #1	25	0.57	0.20		
Preparation #2	25	0.82	0.25		

*Observed turbidity of a 0.5-mM lipid membrane sample at 366 nm (optical density units). Note that turbidity measurements are not exactly reproducible on different spectrophotometers. Thus, K values should be determined independently in individual laboratories.

‡$\Delta r(0.5\text{ mM}) = r' - r'_{obs}$; calculated from Eq. 1, k, r', and the observed turbidity. Δr values corresponding to large turbidities (>0.6) are hypothetical (see text).

§DPPC, dipalmitoyl phosphatidylcholine.

‖ Choles, cholesterol.

¶ Ca-ATPase, Ca^{2+}-activated ATPase.

**Undelipidated sample containing proteins other than the Ca-ATPase.

against optical densities of delipidated sarcoplasmic reticulum membranes containing Ca^{2+}-activated ATPase from sarcoplasmic reticulum. For these membranes, the depolarization proportionality constants were observed to be independent of temperature, at least in a temperature range in which the membrane does not undergo a phase transition. However, the

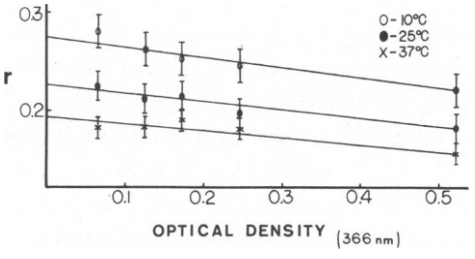

FIGURE 2 Anisotropy (r) of DPH fluorescence vs. optical density for several dilutions of delipidated (2.0 mol % Ca^{2+}-activated ATPase) sarcoplasmic reticulum vesicles, at three temperatures.

variation of the proportionality constant with protein content (see Table I) indicates that DPH anisotropies obtained from membranes of different protein content cannot be compared without proper correction for scattering depolarization.

Table I also contains depolarization constants derived from small, unilamellar vesicles and from two different preparations of chick embryonic retinal membranes. The difference in the two procedures used to isolate the chick retinal membranes is that care was taken to remove contaminating mitochondria in procedure 2. The results for the chick retinal membrane preparations clearly illustrate the necessity of characterizing individual membrane preparations for their abilities to depolarize light. In addition, comparison of the small, single-lamellar vesicle results with those for large, multilamellar vesicles demonstrates that model membranes prepared by different procedures (in this case, resulting in different size) may depolarize light quite differently.

DISCUSSION

The approximate equations given by Teale (10) adequately describe the fluorescence depolarization caused by all turbid membrane suspensions studied, at least up to turbidities of 0.5–0.6 OD. However, none of the proportionality constants derived from the data in Table I agrees with Teale's predicted value. There are three possible explanations for this. First, our measurements using standard laboratory absorption spectrophotometers produce apparent turbidities lower than the true turbidity that would be determined with a proper light-scattering instrument. Thus, even Teale's measured K value for depolarization in solutions of glycogen is smaller than predicted by his theoretical treatment. The extent to which a turbidity measurement on a standard absorption spectrophotometer will be less than the true value depends on the particular angular dependence of scattering from any particular sample. Second, it must be remembered that Teale's treatment is approximate and would not be expected to predict the depolarization constant exactly. Third, deviations of experimental depolarization constants from the predicted values may reflect scattering birefringence not taken into account by Teale's treatment of simple, isotropic scattering.

In addition to the difference between the measured and the predicted scattering depolarization constants, the constants in Table I vary considerably with the composition, structure, and preparation of the membrane samples. The reason for this variability is not clear, although all of the factors outlined above could contribute. Our findings indicate that scattering depolarization effects within different membrane preparations must be individually determined when comparing the fluidities of these membranes by DPH fluorescence depolarization. This is illustrated by considering the effect of the Ca-ATPase on the observed microviscosity within sarcoplasmic reticulum vesicles (Table I). Protein-free lipid vesicles have essentially no depolarizing effect, and the measured DPH fluorescence anisotropy yields a microviscosity of roughly 0.9 P. The uncorrected DPH fluorescence anisotropy from a membrane containing 1.3 mol % Ca-ATPase corresponds to a microviscosity of roughly 1.6 P, indicating that the inclusion of protein in the membrane made the membrane bilayer slightly more rigid. However, when the DPH fluorescence anisotropy reported from the protein-containing vesicles is corrected for depolarization, a microviscosity value is obtained (roughly 3.5 P) which shows the effect of protein to be much greater. The error in microviscosity introduced by scattering depolarization in this case is better than 50%.

It should be noted that the value of the depolarization proportionality constant, K, is not in itself a proper measure of the extent of correction necessary. For example, the K value for dipalmitoyl phosphatidylcholine small vesicles at 8°C is four times larger than the K value for multilamellar vesicles, but the actual correction required for a 0.5-mM small vesicle sample is only twice that required for a similar multilamellar sample (see Δr of Table I). This occurs because the turbidities of small, single-lamellar vesicles are considerably smaller, per mole of lipid, than the turbidities of large, multilamellar vesicles. Similar explanations may be given for the variation of K with cholesterol content in dipalmitoyl phosphatidylcholine vesicles and with protein content in delipidated sarcoplasmic reticulum vesicles. The actual anisotropy corrections required for a 0.5-mM sample (Δr in Table I) are seen to vary only slightly with cholesterol content in dipalmitoyl phosphatidylcholine multilamellar vesicles at 20°C. However, the turbidities of such vesicles decrease significantly with increasing cholesterol, thus explaining the corresponding dramatic increase in K values. Similarly, the turbidities of delipidated sarcoplasmic reticulum vesicles increase significantly with protein content, explaining why K values decrease while Δr corrections increase.

Finally, we comment on conditions under which the depolarization correction might be minimized. Because the magnitude of the correction is directly proportional to turbidity, it may be assumed that the correction can be minimized by using very dilute membrane suspensions. However, without actually measuring the dependence of anisotropy on turbidity, it is impossible to determine the concentration at which scattering effects no longer interfere with comparisons between two membranes. Thus, it would be dangerous to assume the correction to be insignificant at low concentration without demonstrating this in the particular system being studied.

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