Biochemistry

 Volume 20, Number 26 December 22, 1981

Quenching of Enzyme-Generated Acetone Phosphorescence by Indole Compounds: Stereospecific Effects of D- and L-Tryptophan. Photochemical-like Effects†

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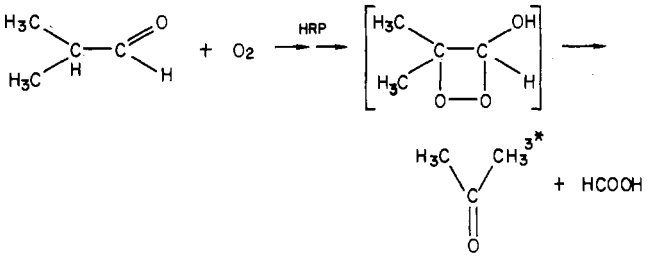
ABSTRACT: Indole compounds efficiently quench the acetone phosphorescence observed during the horseradish peroxidase catalyzed aerobic oxidation of isobutyraldehyde. Different types of Stern–Volmer plots are observed: linear, linear with two slopes, and downward- and upward-curved plots. The complexity probably stems from the operation of both dynamic and static quenching, coupled with different efficiencies of quenching of various acetone triplet populations. Binding to the enzyme may also occur, especially in the case of L-tryptophan. The different Stern–Volmer behavior of D- and L-tryptophan fully confirms that the acetone triplet is generated within the enzyme and not free in solution. This chiral discrimination toward an enzymically generated electronically excited species is novel. It is tentatively postulated that a long-range triplet–triplet exciton transfer occurs; the excited triplet indole then undergoes photochemical-like alterations.

The peroxidase-catalyzed oxidation of IBAL[1] produces



formic acid and acetone, the latter in the electronically excited triplet state (Durán et al., 1977; Faria Oliveira et al., 1978; Cilento et al., 1978; Bechara et al., 1979; Cilento, 1980a,b).

The very fact that this acetone phosphoresces indicates that triplet acetone is generated within the enzyme and considerably shielded from deactivating collisions. A study of the quenching effect of oxygen and of the sorbate ion supports this interpretation (Bechara et al., 1979). Despite protection, however, this triplet acetone can transfer its energy by a long-range mechanism, not yet fully understood (Haun et al., 1978; Augusto & Cilento, 1979; Cilento, 1980a,b; Durán & Cilento, 1980), and promote photochemical processes ("photobiochemistry in the dark") (Faljoni et al., 1978; Meneghini et al., 1978; Durán et al., 1978; Augusto et al., 1978; Rivas-Suárez et al., 1981).

Indole photochemistry is important for several reasons (Lumry & Hershberger, 1978): among these, enzyme activation (Brady & Feigelson, 1973) and splitting of pyrimidine dimers (Hélène & Charlier, 1971; Chen et al., 1976). It was thus of interest to investigate the effect of tryptophan and other indole derivatives upon enzyme-generated triplet acetone.

Experimental Procedures

Materials. HRP (Type VI) was obtained from Sigma Chemical Co.; IBAL from Carlo Erba was purified by distillation under nitrogen. The indole compounds were commercially available. D-Tryptophan and L-tryptophan were carefully purified by recrystallization from aqueous ethanol.

Methods. The standard reaction mixture was prepared as follows: 10 μL of an aqueous solution of 0.5 mM HRP was added to a mixture of 1 mL of 0.1 M pyrophosphate buffer, pH 7.4, and 1.5 mL of 1.0 M phosphate buffer, pH 7.4. This solution was thermostated at 33 °C, and the reaction was initiated by addition of 0.1 mL of a stock solution of 2.2 M IBAL in ethanol. The final reaction mixture was thus 2.0 μM in HRP, 84.6 mM in substrate, and 0.5 M in ethanol and had a total volume of 2.6 mL. The indole derivatives were studied at concentrations at which they did not interfere with the rate of reaction (O_2 uptake). The temporal behavior of the emission intensity at 435 nm was followed by using a Perkin-Elmer MPF-4 fluorescence spectrophotometer. Oxygen consumption

† From the Department of Biochemistry, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil. *Received April 27, 1981.* This work was supported by the Financiadora de Estudos e Projetos (FINEP), by the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), and by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). G.C. received a fellowship from the Guggenheim Foundation. E.R.-S. is a CNPq predoctoral fellow.

[1] Abbreviations used: IBAL, isobutyraldehyde; HRP, horseradish peroxidase; HRP-I, horseradish peroxidase compound I; IAA, indole-3-acetic acid.

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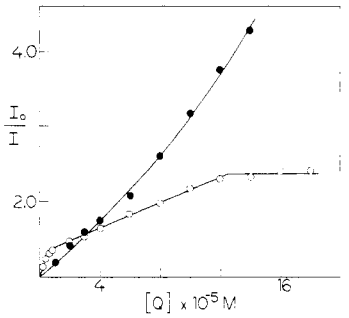

FIGURE 1: Stern–Volmer plot for the quenching of the enzymically generated acetone phosphorescence by L-tryptophan (○) and by D-tryptophan (●). Standard conditions.

was measured with a Yellow Springs Model 53 oxygen monitor. Absorption spectra were recorded on Zeiss-DMR-21 and DMR-10 recording spectrophotometers.

All Stern–Volmer plots refer to data obtained at 33 °C, which were checked for reproducibility in duplicate runs, using freshly prepared solutions.

Some comments regarding measurement of the extent of quenching under our experimental conditions are necessary. In the absence of quencher, the temporal behavior of the acetone emission intensity at 435 nm consists of an almost instantaneous rise to a plateau value that is maintained until O_2 depletion is complete, at which point the intensity drops rapidly. In the presence of most of the quenchers, the same temporal behavior was observed and the extent of quenching calculated from the decrease in the plateau value. With some of the quenchers, however, a plateau was not observed; i.e., following the initial rise, the emission decreased during the course of the reaction. This is presumably due to alteration of the quencher, resulting in the formation of a more efficient quencher species. In these cases, the extent of quenching was calculated by extrapolating the intensity data to zero time.

In a few experiments, tryptophan was exposed to the propanal–HRP–O_2 system, which generates triplet acetaldehyde (Haun et al., 1980).

Results and Discussion

Indole and substituted indoles quench the acetone phosphorescence from the IBAL–HRP–O_2 system. Indole (K_{sv} = 2.6 × 10^4 M^{-1}), *N*-methylindole (K_{sv} = 2.6 × 10^4 M^{-1}), 6-methoxyindole (K_{sv} = 4.8 × 10^4 M^{-1}), serotonine (K_{sv} = 4.3 × 10^5 M^{-1}), 5-nitroindole (K_{sv} = 9.6 × 10^3 M^{-1}), and indole-3-aldehyde (K_{sv} = 3.7 × 10^3 M^{-1}) gave linear Stern–Volmer plots. The plot for D-tryptophan (Figure 1) exhibits a slight upward curvature (initial K_{sv} is 2.2 × 10^4 M^{-1}); that for L-tryptophan (Figure 1) curves downward (initial K_{sv} is 4 × 10^4 M^{-1}) and then levels off at higher concentrations.

The Stern–Volmer plots for IAA, for 5-bromoindole, and for the tripeptide L-lysyl-L-tryptophanyl-L-lysine (Figure 2) exhibit two slopes (2.3 × 10^4 M^{-1} and 1.0 × 10^4 M^{-1}, 2.3 × 10^4 M^{-1} and 7.0 × 10^3 M^{-1}, and 2.3 × 10^4 M^{-1} and 1.0 × 10^4 M^{-1}, respectively).

In analyzing the Stern–Volmer plots, we should consider the possibility of both association and dynamic quenching. It is conceivable that if the quencher becomes bound to the acting enzyme, the quantum yield of acetone phosphorescence may be decreased. The plot will then curve downward and becomes horizontal when saturation is reached; however, if the emission is totally suppressed in the complex, the plot will be linear.

If the acetone triplet population is homogeneous, a dynamic quenching will lead to a linear Stern–Volmer plot. During the lifetime of triplet acetone within the enzyme (ca. 1 × 10^{-5} s), the latter has the opportunity to fluctuate many times among several conformations, and it is conceivable that a quencher may visit the acetone triplets in these conformations with different efficiencies. However, these differences would tend to be averaged out by the long acetone triplet lifetime. Therefore, if the quenching is of dynamic origin, the heterogeneity observed with some quenchers would imply the existence of different populations of acetone triplets, which in turn would have to be ascribed to microenvironmental heterogeneity within the enzyme. In earlier work it had already been pointed out that there must exist different populations of triplet acetone (Bechara et al., 1979).

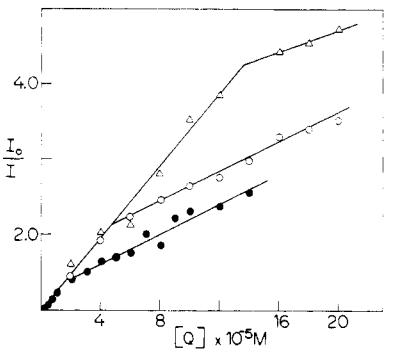

FIGURE 2: Stern–Volmer plot for the quenching of the enzymically generated acetone phosphorescence by IAA (○), 5-bromoindole (△), and the L-lysyl-L-tryptophanyl-L-lysine tripeptide (●). Standard conditions.

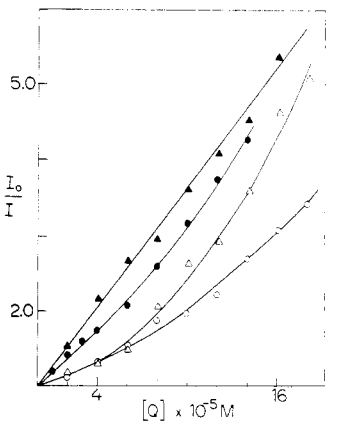

FIGURE 3: Stern–Volmer plot for the quenching of enzymically generated acetone phosphorescence by D-tryptophan in prescence (○) and absence (●) of 4 × 10^{-5} M indole. Also shown is the quenching by indole in the presence (△) and absence (▲) of 4 × 10^{-5} M D-tryptophan.

Concomitant with dynamic quenching, additional quenching may occur due to the fact that when an acetone triplet is generated it may have nearby a quencher molecule bound to or associated with the enzyme. This static quenching contribution will tend to introduce an upward curvature (Wagner, 1967).

Let us now analyze our Stern–Volmer plots in the framework of the above discussion. Let us start with plots which are linear or almost so and then proceed to more complex plots.

Some information can be obtained by the simultaneous use of two quenchers. Thus, if we add indole and run a Stern–Volmer plot for quenching by D-tryptophan, we obtain the result presented in Figure 3, where for comparative purposes the plot for quenching by D-tryptophan in the absence of indole is also presented. The smaller K_{sv} value (initial slope) observed for quenching by D-tryptophan in the presence of indole clearly indicates a shortening of τ^0 by indole and therefore dynamic quenching. The same reasoning may be applied to the reversed

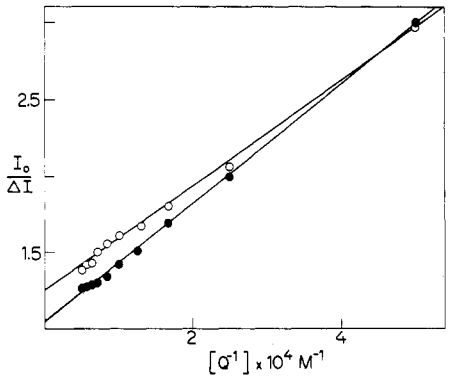

FIGURE 4: Modified Stern–Volmer plot of the quenching of enzymically generated acetone phosphorescence by IAA (○) and 5-bromoindole (●). Standard conditions.

situation, in which D-tryptophan is added and a Stern–Volmer plot is run for quenching by indole (Figure 3).

In Figure 4, data for IAA and 5-bromoindole (Figure 2) are replotted according to the modified Stern–Volmer equation (Lehrer, 1971):

$$\frac{I_0}{\Delta I} = \frac{1}{f_a k_q \tau^0 [Q]} + \frac{1}{f_a}$$

In this equation $\Delta I = I_0 - I$ is the change in acetone phosphorescence intensity corresponding to a quencher concentration, [Q], and f_a is the fraction of triplet acetone which is being quenched with quenching constant $k_q\tau^0$. According to such an analysis, IAA quenches 79% of a quenchable "population" of acetone triplets with a $k_q\tau^0$ of $2.1 \times 10^4\ M^{-1}$; 5-bromoindole quenches 95% of the quenchable acetone triplets with a $k_q\tau^0$ of $1.7 \times 10^4\ M^{-1}$.

Let us now examine quenching by L-tryptophan (Figure 1). If the plot has two slopes and then becomes horizontal, the first, tentative explanation is that L-tryptophan can readily quench a certain population of acetone triplets, can visit less easily another population, but is unable to visit any other population(s).

A second interpretation is that there is saturation of the enzyme. Apparently there would be two binding sites for L-tryptophan, one strong and the other weak. That aromatic donors including indole compounds add to native HRP is well substantiated (Morishima & Ogawa, 1979). Let us assume that they also add to HRP-I which should be the form of the acting enzyme when triplet acetone is generated (Bechara et al., 1979). L-Tryptophan itself is known to add to some heme proteins (Sono et al., 1980). In the L-tryptophan·HRP-I·^{3}*Me_2CO complex, the quantum yield of phosphorescent emission would be smaller.

When either D-tryptophan or indole is present at the 4×10^{-5} M level, quenching by L-tryptophan is very limited (Figure 5); the initial slopes (ca. $4 \times 10^3\ M^{-1}$) are much smaller than expected from a shortening of the lifetime of triplet acetone. According to the first interpretation, we must assume that D-tryptophan and indole quench preferentially that population of triplet acetone which is easily quenchable by L-tryptophan. When the situation is reversed, if L-tryptophan is present in the system at saturating concentrations (1.4×10^{-4} M), quenching by D-tryptophan or indole can still be observed (Figure 5). We must infer that D-tryptophan and indole are able to visit populations which L-tryptophan cannot.

In the framework of the second interpretation, we must infer that D-tryptophan and indole are able to penetrate the L-tryptophan·HRP-I·^{3}*Me_2CO complex. The smaller initial slope ($5 \times 10^3\ M^{-1}$ in the case of D-tryptophan) would then suggest a reduction in k_q; that is, although D-tryptophan would be able to penetrate the complex, it would do so with some difficulty.

When L-tryptophan (2×10^{-5} M) is present, practically no quenching is observed with the L-lysyl-L-tryptophanyl-L-lysine tripeptide up to a 8×10^{-5} M concentration; apparently the tripeptide visits preferentially those acetone triplets which are also more easily visited by L-tryptophan, or, alternatively, the tripeptide is unable to penetrate the L-tryptophan complex.

As expected, D-tryptophan and L-tryptophan quench triplet acetone of nonenzymic origin with the same efficiency; $k_q\tau = 2.5 \times 10^3\ M^{-1}$. In these experiments triplet acetone was generated by cleavage of tetramethyldioxetane in normally aerated water, and the acetone triplets were monitored by energy transfer to the fluorescent state of the 9,10-dibromoanthracene-2-sulfonate ion (E. J. H. Bechara, personal communication). The smaller value of $k_q\tau$ in the nonenzymic system is to be ascribed at least in part to a shorter lifetime of triplet acetone in view of the quenching effect of oxygen (Wilson, 1976) and the presence of 9,10-dibromoanthracene-2-sulfonate.

Regardless of the detailed mechanism, the different quenching behavior of D- and L-tryptophan toward enzymically generated triplet acetone is a truly remarkable finding which fully confirms that triplet acetone is not generated free in solution. This stereospecificity toward an enzyme-generated excited species is novel and potentially important. Of some interest in this regard is the recently described chiral discrimination in the interaction between horse liver alcohol dehydrogenase and the two enantiomers of methionine (Gafni, 1980). Each of these two antipodes quenches (dynamically) the fluorescence of etheno-NAD bound to the enzyme, but the rate constants for quenching by D- and L-methionine differ by a factor of nearly 3.

In the quenching of aromatic ketones by indoles, both energy transfer and electron transfer may operate (Wilkinson & Garner, 1977, 1978). In the present investigation, the effect of substituents as a whole is not very informative. Electron transfer should be considerably hampered because triplet acetone is shielded by the enzyme from deactivating collisions (Bechara et al., 1979). Electron transfer mediated by the heme group is not likely because there is no interaction between the emissive triplet acetone and the heme group, as inferred from the existence of acetone phosphorescence. At any rate, tryptophan is not oxidized by HRP·H_2O_2, implying that HRP-I, the form in which the enzyme occurs when triplet acetone is generated (Bechara et al., 1979), does not oxidize indole compounds in the absence of substrate (IBAL). Consider also that we have quenching processes with K_{sv} values above $10^4\ M^{-1}$; hence, if dynamic quenching is occurring, then since τ^0 is about 10^{-5} s, the rate constant for some processes will be higher than $10^9\ M^{-1}\ s^{-1}$ and therefore faster than diffusion controlled.

We may tentatively infer that in the enzymic system quenching occurs by a long-range energy transfer process. Radiationless transfer from triplet acetone to the first excited singlet state of the indole moiety does not meet Förster overlap criteria. On the other hand, the classical collisional triplet–triplet energy transfer is not feasible because triplet acetone is considerably shielded by the enzyme. Here, as in other systems which we have investigated, the operation of a new mechanism, presumably a long-range triplet–triplet exciton transfer (Kasha, 1979), is tentatively postulated. For such a transfer to occur, the acceptor must have heavy atoms or n,π^* character in the energetically properly located triplet level to

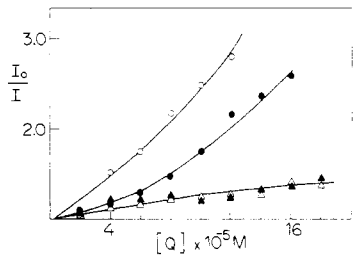

FIGURE 5: Stern–Volmer plot for the quenching of enzymically generated acetone phosphorescence by L-tryptophan in the presence of 4×10^{-5} M D-tryptophan (△) or 4×10^{-5} M indole (▲). Also shown is the quenching by D-tryptophan (●) or by indole (○) in the presence of 1.4×10^{-4} M L-tryptophan. Standard conditions.

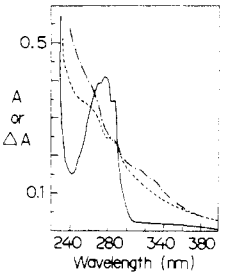

FIGURE 6: Effect of exposure to enzyme-generated triplet acetone on the absorption spectrum of 0.10 mM L-tryptophan. The solid curve represents the absorption spectrum of L-tryptophan in aqueous solution (pH 7.0); the dashed curve (- - -) is the absorption spectrum of the IBAL–HRP–O_2–L-tryptophan system taken against the IBAL–HRP–O_2 system at 40 °C after 1 min of continuous aeration in the dark. Also shown for comparative purposes is the spectrum of the L-tryptophan solution after 19-h irradiation at 290 nm, at 25 °C (- · -).

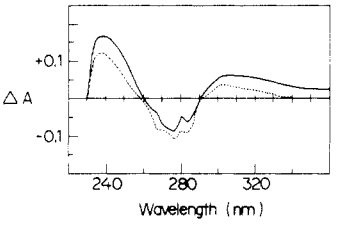

FIGURE 7: Differential absorption spectrum (—) of a 0.10 mM aqueous solution of L-tryptophan before and after 47-h irradiation at 280 nm. The dashed curve represents the differential absorption spectrum before and after exposure to the propanal–HRP–O_2 system for 10 min at 40 °C, with continuous aeration in the dark.

which it is excited; then internal conversion to the lowest triplet would ensue.

The excited indole group is not expected to phosphoresce; however, its "dark" photochemical excitation is suggested by the extensive degradation (Figure 6). The possibility of a "dark" photochemical excitation is better appreciated from Figure 7. This figure depicts the formation of products of the formylkynurenine type by irradiation of tryptophan at 280 nm for 47 h (Borkman, 1977) or by 10-min exposure to the propanal–HRP–O_2 system, which appears to generate triplet acetaldehyde (Haun et al., 1980). Here, as in other systems studied in these laboratories (Rivas-Suárez et al., 1981; Faljoni et al., 1978), a brief exposition to our enzymic reactions which generate triplet species produces photochemical-like effects which normally require prolonged irradiation.

The reaction of indole derivatives in the triplet state with oxygen, leading to formylkynurenine and related compounds, has been postulated (Amouyal et al., 1979; Pailthorpe et al., 1973). A nonphotochemical route is unlikely because, as observed with tryptophan, the indole compounds do not interfere with the enzymic reaction; indeed, there is no effect whatsoever upon either the rate of O_2 consumption (no aeration!) or the temporal behavior of the absorbance at 400, 411, and 420 nm (Bechara et al., 1979). We may further add that the alteration of the indole compound cannot be due to the superoxide ion (no protection by superoxide dismutase), to hydrogen peroxide (no protection by catalase), or to the hydroxyl and ketyl radicals (presence of 0.5 M ethanol in the reaction mixture).

Acknowledgments

We express our deep gratitude to Dr. Frank Quina for a critical reading of the manuscript and to Drs. Adelaide Faljoni-Alário and E. J. H. Bechara for their interest in this work.

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1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene: Synthesis, Fluorescence Properties, and Use as a Fluorescence Probe of Lipid Bilayers†

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ABSTRACT: 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), a cationic analogue of diphenylhexatriene (DPH), has photophysical properties that are generally similar to those of DPH. In solution the fluorescence lifetime (τ) of TMA-DPH is short (<1.5 ns), but τ increases to ~7 ns when the probe is embedded in lipid bilayers at temperatures less than the thermal transition temperature (T_c) of the lipid. The cationic charge ensures that the probe is anchored at the lipid–water interface, most likely with the DPH moiety intercalated between the upper portions of the fatty acyl chains. The profiles of changes in steady-state anisotropies (r_{ss}) and limiting hindered anisotropies (r_∞) are similar for both TMA-DPH and DPH embedded in lipid bilayers, but r_∞ values for TMA-DPH even at $T \gg T_c$ are generally >0.14, e.g., at 35 °C in 1,2-dimyristoylglycero-3-phosphocholine (DMPC) (cf. 0.03 for DPH in DMPC at 35 °C). Electrostatic interactions of the cationic probe with head groups of phospholipids do not appear to significantly influence the apparent dynamics of the probe. TMA-DPH should prove useful in the study of the dynamics of phospholipid monolayers, e.g., in native or reconsituted lipoproteins.

The popularity of diphenylhexatriene (DPH)[1] as a probe for studies of the structure and dynamics of lipid bilayers derives largely from its favorable photophysical properties; these have been reviewed in detail elsewhere (Dale et al., 1977; Lakowicz et al., 1979). Available data (Andrich & Vanderkooi, 1976) show that the DPH molecule is oriented with its symmetry axis normal to the plane of the membrane, at least in the gel phase of lipid bilayers. The absorption and emission transition moments are essentially (but not completely) collinear, one with the other and with the symmetry axis. This makes DPH an excellent probe for studies of order in the lipid bilayers since even small displacements of the symmetry axis result in depolarization of fluorescence emission which is easily detected and measured.

Diphenylhexatriene is, however, sufficiently soluble in the hydrocarbon domain of the bilayer that we cannot be sure that the location of the molecule may not change substantially when the probe is free to extensively reorientate during its fluorescence lifetime or may even undergo translational motion that could affect significantly the measured anisotropy (Engel & Prendergast, 1981). Such possibilities make it more difficult, for example, to propose models of DPH motion in bilayers. Because r_∞ values for DPH embedded in lipid bilayers at temperatures greater than the thermal transition temperature are often ≪0.1, calculations of axial depolarization values ($\langle d^x \rangle$; Eisinger et al., 1981) for DPH, so essential for energy-transfer studies in bilayers, are problematic. As Heyn (1979) has pointed out, the data from a probe anchored at the lipid interface are inherently more easily interpreted; but even the probes suggested by Heyn, namely, the parinaric acids, are still somewhat able to penetrate the membrane as the protonated (carboxyl) form.

We have sought a fluorescent probe that would at once be tethered to the lipid–water interface and yet intercalated into the lipid milieu. We have synthesized such a molecule—1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), a derivative of DPH with a cationic moiety affixed to the para position of one of the phenyl rings. Nikitina et al. (1963) and Cundall et al. (1979) have also reported on the synthesis and fluorescence properties of derivatives of DPH, but the information provided is sparse, especially with regard to the properties of TMA-DPH (Cundall et al., 1979). We have used this derivative to examine the dynamics of lipid bilayers and show that while its photophysical properties are fundamentally similar to DPH, the patterns of motion and, by inference, the region of the bilayer reported on by TMA-DPH are quite different from that of the parent molecule, diphenylhexatriene.

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[1] Abbreviations used: DPH, 1,6-diphenylhexa-1,3,5-triene; DMA-DPH, 1-[4-(dimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; DPPC, 1,2-dipalmitoylglycero-3-phosphocholine; DOPC, 1,2-dioleoylglycero-3-phosphocholine; DMPC, 1,2-dimyristoylglycero-3-phosphocholine; LOPC, lysooleoylglycero-3-phosphocholine; ^{1}H NMR, proton nuclear magnetic resonance; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Mops, 4-morpholinepropanesulfonic acid.

0006-2960/81/0420-7333$01.25/0