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# 3-[*p*-(6-Phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (PA-DPH): characterization as a fluorescent membrane probe and binding to fatty acid binding proteins

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**The negatively charged fluorophore 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (PA-DPH) was characterized by comparison with its parent compound DPH, and with cationic trimethylammonium-DPH (TMA-DPH). The molar absorption coefficient of PA-DPH (60 000 $cm^{-1} \cdot mol^{-1}$) as well as its quantum yield (0.7) and fluorescence lifetime (5 ns) in fluid phase membranes are intermediate between DPH and TMA-DPH. Steady-state fluorescence polarization studies show that PA-DPH detects the phase transition of both neutral and anionic bilayers. In fluid phase membranes the absolute values of PA-DPH polarization are considerably higher than DPH and somewhat lower than TMA-DPH. The results suggest that like TMA-DPH, PA-DPH is anchored to the surface of the membrane by its charge, but that it is probing a region somewhat deeper along the bilayer normal. PA-DPH binds to rat hepatic fatty acid binding protein (hFABP) and bovine serum albumin at PA-DPH/protein molar ratios of 1.5:1 and at least 6:1, respectively. Native oleic acid competes with PA-DPH for binding to both proteins, suggesting that the two ligands compete for similar binding sites. The affinity of PA-DPH for hFABP is similar to that of oleic acid. Thus, PA-DPH should be useful both as an anionic fluorescent membrane probe and a long-chain free fatty acid analogue.**

## Introduction

Fluorescent analogues of free fatty acids, such as the *cis*- and *trans*-parinaric acids and the anthroyloxy and pyrene derivatives of natural free fatty acids, have been used to study both membrane lipid structure and the kinetics of free fatty acid transport. For example, the series of *n*-(9-anthroyloxy) free fatty acid derivatives have been used to study membrane polarity and lipid order as a function of depth along the bilayer normal [1,2], and to monitor the intermembrane transfer kinetics of free fatty acids [3]. The excimer forming pyrenyl free fatty acid derivatives have been used to study intermembrane free fatty acid transfer [4,5], membrane fluidity [6], and lipid–protein interactions [6]. The parinaric acids have been used to monitor lipid phase transitions and the fluidity of model membranes [7,8], and to characterize the free fatty acid binding sites of serum albumin [9]. Each of these probes has certain disadvantages, however. The parinaric acids require special handling, have a relatively low quantum yield in membranes and have a polyenoic chain which is not of the unconjugated type usually found in animal membranes [7,8]. On the other hand, while the fatty acyl moieties in anthroyloxy and pyrene derivatives are naturally-occurring, the fluorescent groups are relatively large and quite hydrophobic, and may impart properties which differ from those of native free fatty acids.

The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been widely used to study the lipid order of synthetic and biological membranes [10–16], and many derivatives of DPH have been described [17,18]. A novel derivative is propionic acid DPH (PA-DPH). PA-DPH

Abbreviations: 2- or 12-AS, 2- or 12-(9-anthroyloxy)stearate; BSA, bovine serum albumin; DMF, dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMSO, dimethylsulfoxide; DPH, 1,6-diphenyl-1,3,5-hexatriene; egg PC, egg phosphatidylcholine; hFABP, hepatic fatty acid binding protein; MLV, multilamellar vesicle; PA-DPH, 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid; PnA, parinaric acid; *Q*, quantum yield; SUV, small unilamellar vesicle; $\tau$, fluorescence lifetime; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

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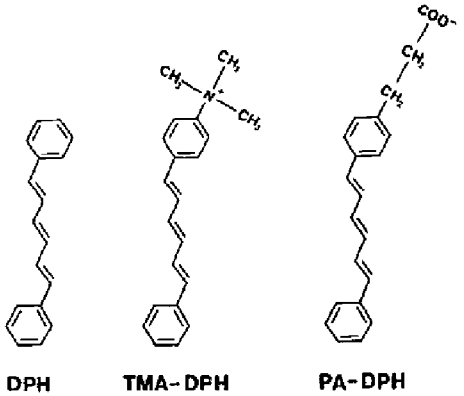


Fig 1 Structures of the neutral fluorophore DPH, cationic TMA-DPH, and anionic PA-DPH

is similar to a long-chain free fatty acid both in its anionic carboxylic acid group and in its approximate hydrocarbon chain length Moreover, PA-DPH lacks the extrinsic bulk of the large anthroyloxy or pyrene prosthetic group, and the DPH fluorophore requires little special handling PA-DPH was used to synthesize 1-palmitoyl,2-PA-DPH phosphatidylcholine [40], which has been used as a probe of membrane structure by Parente and Lentz [41] The present study was undertaken to characterize the anionic PA-DPH as a membrane probe by comparing it with DPH and a cationic derivative of DPH, trimethylammonium-DPH (TMA-DPH) (Fig 1) Further experiments were conducted to determine the utility of PA-DPH as a fluorescent analogue of long-chain free fatty acid by studying its binding to two free fatty acid binding proteins, bovine serum albumin (BSA) and rat hepatic fatty acid binding protein (hFABP).

## Materials and Methods

*Materials* 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from the Eastman Kodak Co (Rochester, NY) 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (PA-DPH) were purchased from Molecular Probes, Inc (Eugene, OR) Egg phosphatidylcholine (egg PC), L-α-dimyristoylphosphatidylcholine (DMPC), and L-α-dimyristoylphosphatidylglycerol (DMPG) were from Avanti Polar Lipids, Inc (Birmingham, AL) Bovine serum albumin (BSA), essentially fatty acid free (< 0 005%), was obtained from Sigma Chemical Co (St Louis, MO) Delipidated rat hepatic fatty acid binding protein (hFABP) was the generous gift of Dr Nathan Bass Vesicle experiments were performed in Tris-buffered saline (40 mM Tris-HCl, 100 mM NaCl) (pH 7 4), and protein binding experiments in phosphate-buffered saline (137 mM NaCl, 2 7 mM KCl, 1 5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$) (pH 7 4) For experiments in which the pH was varied Tris-buffered saline was used for pH range 7–10, and acetate-buffered saline, 40 mM sodium acetate and 100 mM NaCl, was used for pH range 3–6 All solvents were spectrophotometric grade

*Vesicle preparation* Egg PC, DMPC and DMPG small unilamellar vesicles (SUV) were prepared according to the method of Huang and Thompson [19] as described previously [3] Egg PC SUV were prepared at 4°C and DMPC and DMPG SUV were prepared at 37°C Multilamellar vesicles (MLV) were prepared by adding Tris-buffered saline buffer to the evacuated lipid film, and hand swirling. Probes were incorporated into SUV by addition of a small volume of concentrated stock solution of PA-DPH in dimethylsulfoxide (DMSO) or dimethylformamide (DMF) or DPH and TMA-DPH in ethanol, to the preformed vesicles For MLV, probes were added to the phospholipids prior to evacuation Final solvent concentration was always < 1% (v/v)

*Absorbance spectroscopy* Absorption spectra were obtained using a Beckman DU-40 or Hitachi U-2000 double beam spectrophotometer, and background corrections were made automatically The absorption coefficient of PA-DPH in DMF was estimated from the absorbance of triplicate samples for three different probe concentrations

*Fluorescence spectroscopy* Fluorescence measurements were made with an SLM 8000 fluorescence spectrophotometer. Excitation spectra for the DPH probes were obtained using an emission wavelength of 430 nm, and the emission spectra were obtained with an excitation wavelength of 360 nm Unless stated otherwise, all measurements were made at ambient temperature (22 ± 2°C)

The quantum yield, $Q$, of the DPH probes was determined relative to quinine sulfate in 0 1 N sulfuric acid Excitation of the quinine sulfate was at 352 nm Spectra were corrected for lamp and photomultiplier variation with wavelength [20], and the quantum yield for quinine sulfate was taken as 0.7 [21].

Uptake of probe into vesicles was monitored by the increase in fluorescence intensity Steady-state fluorescence polarization was determined with the SLM 8000 Ten measurements were taken at each temperature (±0 1 C°) or pH (±0 1) and averaged for each sample, and deviations were less than 0 005 polarization units Excited state lifetimes ($\tau$) were determined by the phase modulation technique [22] using an SLM 4800 fluorescence spectrophotometer Measurements were done with an excitation wavelength of 360 nm and a 390 nm long pass emission filter Lifetimes in membranes were measured at 18 mHz, and in solvents at 30 mHZ

*Protein binding* Increasing concentrations of PA-DPH were added to BSA or hFABP by the addition of aliquots from a stock solution Solvent concentration was always < 1% (v/v) PA-DPH fluorescence was measured after a 15 min incubation The inner filter effects of BSA and hFABP were negligible The dissociation constant for PA-DPH binding to hFABP was estimated using the fluorometric titration method described by Cogan et al [23]

Tryptophan emission of BSA and tyrosine emission of hFABP were monitored in the same experiment for each concentration of PA-DPH Fluorescence excitation was 280 nm, tryptophan emission was measured at 340 nm and tyrosine emission was measured at 310 nm

Competition between PA-DPH and oleic acid for binding to BSA and hFABP was studied by adding increasing concentrations of oleic acid to a solution of PA-DPH bound to protein, and measuring PA-DPH fluorescence before and at 15 min following oleate addition

## Results

### *Spectral properties of PA-DPH*

The excitation and emission spectra of PA-DPH in DMF are shown in Fig 2 The excitation maximum at 356 nm and the emission maximum at 430 nm are similar to those of DPH and TMA-DPH (Table I) Like DPH and TMA-DPH, PA-DPH shows minimal spectral shift with varying solvent polarity ([24] and data not shown) A comparison of the spectral characteristics of the three DPH probes as well as those of two fluorescent fatty acid analogues, *trans*-parinaric acid (PnA) and 12-(9-anthroyloxy)stearate (12-AS), is shown in Table I The maximum absorption coefficient of PA-DPH, 60000, is 26% lower than that of DPH, but 50% higher than that of TMA-DPH It is similar to PnA and an order of magnitude greater than 12-AS In addition, the quantum yield of PA-DPH in egg phosphatidylcholine small unilamellar vesicles (egg PC-SUV) is 0 73, intermediate between DPH and TMA-DPH, and more than 2-fold greater than that of PnA [7] Thus in comparison with PnA and 12-AS, it is noteworthy that PA-DPH possesses both a high absorption coefficient and high quantum yield, making it a strong fluorophore

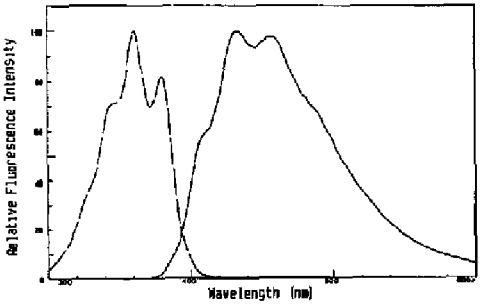


Fig. 2 Excitation (– – –) and emission (———) spectra of 2 μM PA-DPH in DMF Excitation was at 360 nm and emission at 430 nm

TABLE I

*Spectral characteristics of fluorescent membrane probes*

Absorbance and absorption coefficients were determined for DPH probes in DMF Emission and quantum yields were determined for probe in egg PC vesicles

| Probe | Absorption $\lambda_{max}$ (nm) | Absorption coefficient ($mol^{-1}$ $cm^{-1}$) | Emission $\lambda_{max}$ (nm) | Quantum yield |
|---|---|---|---|---|
| DPH | 353 | 81000 [a] | 430 | 0 80 |
| TMA-DPH | 359 | 30200 [a] | 430 | 0 65 |
| PA-DPH | 356 | 60000 | 430 | 0 73 |
| trans-PnA [b] | 314 | 65000 | 422 | 0 30 |
| 12-AS [c] | 365 | 7770 | 446 | 0 74 |

[a] Prendergast et al [24]

[b] Values in dipalmitylphosphatidylcholine (DPPC) vesicles from Sklar et al [7 8]

[c] Values in egg PC vesicles from Thulborn et al [1]

### *Membrane studies*

PA-DPH binds rapidly to egg PC-SUV The binding of both PA-DPH and TMA-DPH to egg PC-SUV results in a constant fluorescence intensity within 30 seconds (the first time point measured) Maximal binding of DPH, on the other hand, takes approx 30 min Previous studies by Shimitzky and Barenholz [25] and by Lentz et al [12] showed a similar time course for DPH binding to egg PC-SUV, and dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine SUV, respectively The following membrane studies therefore allowed maximum fluorescence to be reached before measurements were made

Steady-state polarization of the DPH probes in dimyristoylphosphatidylcholine SUV (DMPC-SUV) shows that PA-DPH, like DPH and TMA-DPH, detects the decrease in membrane lipid order which accompanies the transition from a gel to a liquid-crystalline phase (Fig 3A) All three probes show a decrease in polarization near the transition temperature ($T_c$) Above the $T_c$, the polarization of PA-DPH was consistently lower than that of TMA-DPH and considerably higher than that of DPH

The effect of membrane lipid phase on polarization of the three probes was also investigated in anionic dimyristoylphosphatidylglycerol SUV (DMPG-SUV) at pH 7 4 The transition temperature was indicated by a change in polarization of DPH, TMA-DPH and PA-DPH at 23°C (Fig 3B) As in DMPC-SUV, the absolute

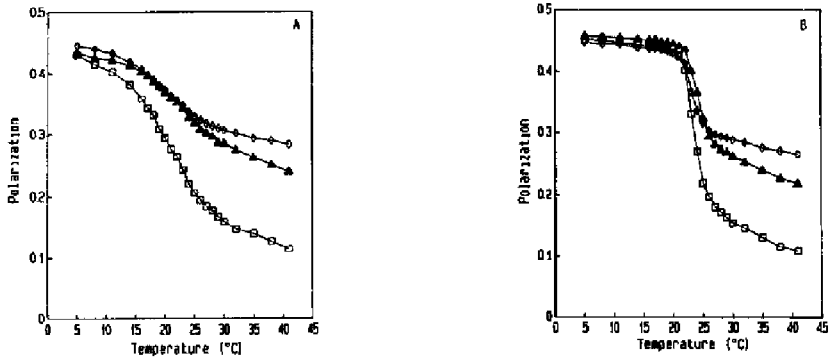


Fig 3 Variation in steady-state polarization with temperature for DPH (□), TMA-DPH (◇) and PA-DPH (▲) in (A) DMPC SUV and (B) DMPG SUV Probe concentrations were 1 μM and SUV were 0 2 mM in Tris-buffered saline (pH 7 4)

polarization of PA-DPH is intermediate between DPH and TMA-DPH in the liquid-crystalline phase The negative charge on PA-DPH did not alter the ability of the probe to detect the phase transition in these anionic liposomes Similarly, the positive charge on TMA-DPH had no effect, in agreement with the results of Prendergast et al [24]

In order to determine whether ionization of the carboxyl group would alter the spectral properties of PA-DPH in membranes, steady-state fluorescence polarization was determined in egg PC-MLV as a function of pH Fig 4 shows that pH has very little effect on polarization of DPH and TMA-DPH in these zwitterionic vesicles, while a small increase in PA-DPH polarization is observed above pH 7 In addition, while the fluorescence intensity of DPH and TMA-DPH were minimally affected by pH, the intensity of PA-DPH decreased by approx 40% when the pH was increased

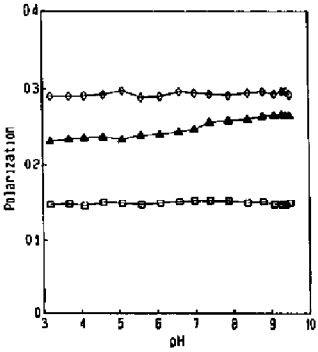


Fig 4 Effect of pH on steady-state polarization of DPH (□), TMA-DPH (◇) and PA-DPH (▲) in EPC-MLV Probe concentrations were 1 μM and EPC-MLV were 0 2 mM in acetate-buffered saline (pH 3–6 5) or Tris-buffered saline (pH 7–11)

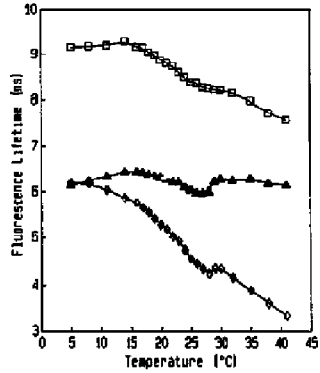


Fig 5 Variation in fluorescence lifetime with temperature for DPH (□), TMA-DPH (◇) and PA-DPH (▲) in DMPC-SUV Probes were less than 1 5% of total lipid in 0 5 mM SUV in Tris-buffered saline at pH 7 4 Only modulation lifetimes are shown, and measurements were made at 18 mHz.

from 6 to 9 (data not shown) Similar effects of pH on polarization and intensity of 2-(9-anthroyl)oxy)stearate (2-AS) and 12-AS in EPC-SUV were also observed, and

TABLE II

*Lifetimes of DPH derivatives*

| Probe | Fluorescence lifetimes [a] (ns) in | | |
|---|---|---|---|
| | egg PC [b] | DMF | $CHCl_3$/MeOH (95 5, v/v) |
| DPH | 7 65±0 07 | 3 18±0 16 | 4 87±0 15 |
| TMA-DPH | 3 16±0 09 | 0 37±0 19 | n d [c] |
| PA-DPH | 5 04±0 05 | 2 72±0 19 | 4 17±0 15 |

[a] Modulation lifetimes are shown, and measurements were made at 18 mHz in membranes and at 30 mHz in solvents

[b] Egg phosphatidylcholine small unilamellar vesicles at 23°C

[c] n d , not determined

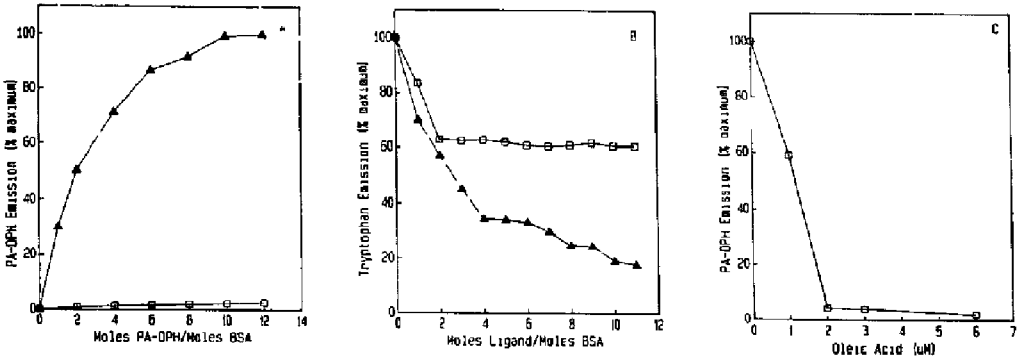


Fig. 6. Binding of PA-DPH to bovine serum albumin (BSA). (A) Titration of BSA with PA-DPH. Increasing concentrations of PA-DPH (0 to 6 μM) were added to 0.5 μM BSA in phosphate-buffered saline. ▲, PA-DPH bound to BSA; and □, PA-DPH in buffer. (B) BSA tryptophan fluorescence upon binding of PA-DPH (▲) and oleic acid (□). Increasing concentrations of PA-DPH or oleic acid were added to 0.75 μM BSA in phosphate-buffered saline. (C) Competition of oleic acid and PA-DPH for BSA binding. Increasing concentrations of oleic acid were added to 1 μM BSA which had been incubated with 6 μM PA-DPH prior to oleate addition.

the point of change was also at approximately pH 7 (data not shown).

The temperature dependence of the fluorescence lifetimes ($\tau$) of PA-DPH, DPH and TMA-DPH in DMPC-SUV is shown in Fig. 5. All three probes show a change in $\tau$ at the transition temperature of the bilayer. The $\tau$ of PA-DPH is, once again, intermediate between that of DPH and TMA-DPH (Fig. 5 and Table II). While the lifetime of TMA-DPH is markedly dependent on membrane lipid phase, DPH is somewhat less temperature sensitive, and PA-DPH in particular shows a much smaller change in $\tau$. Lifetimes of the probes in DMF and chloroform/methanol ($CHCl_3$/MeOH, 95:5 v/v) are shown in Table II. All three probes have a lower $\tau$ in these isotropic solvents than in membranes, for both DPH and PA-DPH in DMF $\tau$ is 2-fold lower, while for TMA-DPH it is 10-fold lower.

*Protein binding studies*

Natural long-chain free fatty acids have been shown to bind to bovine serum albumin (BSA) and hepatic fatty acid binding protein (hFABP) [26,27], and binding studies were done to determine whether PA-DPH interacts with these proteins. As increasing concentrations of PA-DPH are added to BSA, the PA-DPH fluorescence increases sharply, reaching approx. 90% of maximum at a ratio of 6 mol PA-DPH per mol BSA. As more probe is added, the intensity rises more gradually, leveling off at approx. 10 mol PA-DPH per mol BSA (Fig. 6A). When either natural oleic acid or PA-DPH bind to

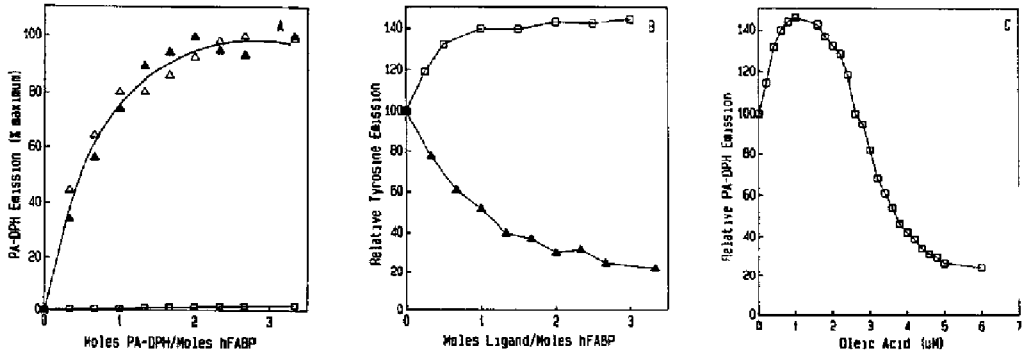


Fig. 7. Binding of PA-DPH to hepatic fatty acid binding protein (hFABP). (A) Titration of hFABP with PA-DPH. Increasing concentrations of PA-DPH were added to 3 μM hFABP in phosphate-buffered saline. ▲, △ represent data from two experiments; □, PA-DPH in phosphate-buffered saline. (B) hFABP tyrosine fluorescence upon binding of PA-DPH (▲) and oleic acid (□). Increasing concentrations of PA-DPH or oleic acid were added to 4 μM hFABP in phosphate-buffered saline. (C) Competition of oleic acid and PA-DPH for hFABP binding. Increasing concentrations of oleic acid were added to 3 μM hFABP which had been incubated with 2 μM PA-DPH prior to oleate addition.

BSA, quenching of the protein tryptophan fluorescence is observed (Fig 6B) Addition of oleate results in approx 40% quenching of tryptophan intensity at a 2 1 ligand/protein ratio Further quenching is not observed upon increasing oleate addition PA-DPH binding also causes a decrease in tryptophan fluorescence Quenching of up to 70% is seen at a 4 1 PA-DPH/BSA ratio, and further PA-DPH addition results in a more gradual decrease in intensity Competition between oleic acid and PA-DPH for BSA binding was studied by adding increasing amounts of oleic acid to BSA which had bound PA-DPH A decrease in PA-DPH fluorescence was observed upon oleate addition (Fig 6C), suggesting that PA-DPH and oleate may compete for similar binding sites on BSA A 50% decrease in maximal PA-DPH fluorescence occurs at a PA-DPH/oleic acid ratio of approximately 4 (Fig 6C)

PA-DPH also binds to hFABP, and saturable binding of PA-DPH to hFABP occurs between a 1 1 and 2 1 ratio of probe/protein (Fig 7A) Using the fluorescence titration method of Cogan et al [23] a $K_d$ of 0 3 $\mu$M for PA-DPH binding to hFABP was estimated These results are in good agreement with values reported for natural free fatty acid binding to hFABP [27] Protein tyrosine fluorescence was monitored upon ligand binding to hFABP, and Fig 7B shows that PA-DPH causes marked quenching of tyrosine fluorescence upon binding to hFABP Once again the decrease levels off at a probe/protein ratio between 1 : 1 and 2 1 Conversely, oleic acid binding (1 1 oleate/hFABP) results in a 40% enhancement of tyrosine fluorescence, suggesting a change in protein conformation [28] Competition studies show that the addition of oleic acid to 3 $\mu$M hFABP to which 2 $\mu$M PA-DPH is bound causes an initial enhancement of PA-DPH fluorescence, followed by a decrease in emission upon further oleic acid addition (Fig 7C) A 50% decrease of the maximal PA-DPH fluorescence occurs at an oleic acid/PA-DPH ratio of approximately 1 5 1, further suggesting that the affinity of PA-DPH for hFABP is similar to that of oleic acid

## Discussion

These studies were conducted in order to characterize PA-DPH both as a fluorescent membrane probe and a fatty acid analogue Cundall et al [17] have reported that the covalent attachment of various substituents to the phenyl ring of DPH generally causes only small changes in the absorbance and emission spectra of the fluorophore Similarly, Prendergast et al [24] and Cranny et al [18] have shown that substitution of DPH with the trimethylammonium group (TMA-DPH) causes only a slight red shift in the absorbance spectrum and has little effect on emission We find that modification of DPH with the propionic acid moiety also causes a slight red shift in absorbance without any effect on the emission maximum (Table I, Fig 2) As previously reported, the absorption coefficient for TMA-DPH is decreased when compared to DPH [24], and a similar but substantially smaller decrease for PA-DPH (23% decrease for PA-DPH as compared to 63% for TMA-DPH) was observed in these studies (Table I)

The quantum yield ($Q$) of both PA-DPH and TMA-DPH is lower than that of DPH (Table I), as was expected for these phenyl-substituted compounds [17] However, since we measured $Q$ in egg PC-SUV, the differences could also reflect differential quenching by the aqueous medium as a result of relative depth in the bilayer. Such an association between quantum yield and position in the bilayer has been observed for the *n*-(9-anthroyloxy) free fatty acid probes [1] Thus, while Cundall et al [17] reported that substitution of DPH on the phenyl ring causes a decrease in quantum yield in isotropic solvents, the measured quantum yield of PA-DPH in DMF is 0 37, virtually identical to the value of 0 35 measured for DPH in DMF Thus indicates that the propionic acid substituent is less perturbing to the fluorescence properties of the DPH fluorophore than are other substituents Further, this suggests that the observed difference in $Q$ for DPH and PA-DPH in bilayers (Table I) is more likely due to their relative depth in the membrane bilayer

The $\tau$ of PA-DPH in egg PC is intermediate between those of DPH and TMA-DPH (Table II) As with $Q$, this variance may reflect the relative degree of quenching by the aqueous medium The dependence of $\tau$ on lipid phase (Fig 5) might be explained similarly As was observed by Straume and Litman [10] the $\tau$ of TMA-DPH is more sensitive to temperature and hence lipid phase than is that of DPH The $\tau$ of PA-DPH is relatively unaffected by the lipid phase (Fig 5). In the gel phase the membrane is more tightly packed, and would conceivably allow less penetration of the aqueous quencher, thereby accounting for the higher $\tau$ of TMA-DPH [10,24] The data support this change for TMA-DPH, where the fluorophore is anchored in close proximity to the bilayer surface [24] The smaller effects of lipid phase on PA-DPH and DPH may be due to the greater depth of these probes along the bilayer normal and hence a greater distance from the aqueous interface The greater depth of the DPH fluorophore in PA-DPH as compared to TMA-DPH is presumably due to the propionic acid side chain (Fig 1) While DPH is known to be localized within the hydrophobic core of the membrane, it does not appear to have a unique orientation [10,12], and also seems to distribute in a wide range along the bilayer normal [29,30] It is possible that as temperature is increased, an increasing proportion of the probe may experience quenching due to probe–water interactions PA-DPH, on the other hand, is likely to be anchored in a single orientation by its

charged group as is the case with TMA-DPH [10,24] The fluorophore is removed from the surface, however, by the propionic acid chain and may therefore not experience significant aqueous quenching In addition, the variation in lipid packing density caused by increased temperature is greater in the hydrocarbon core of the membrane than in the region closer to the aqueous interface [10] PA-DPH may thus be experiencing less of an increase in molecular disorder than is DPH as temperature is raised

Several derivatives of DPH, including TMA-DPH, that have a substituent attached to the phenyl ring have a low $\tau$ in isotropic solvents [17,24] The present results show that the addition of the propionic acid moiety to DPH does not result in such a decrease in $\tau$ (Table II) Further, the $\tau$ for PA-DPH and DPH in DMF and $CHCl_3$/MeOH (95 5, v/v) are quite similar It has been suggested that the low $\tau$ of TMA-DPH in isotropic solvents relative to DPH may be due to the intrinsic properties imparted to the molecule by the addition of the trimethylammmonium substituent, or that the TMA-DPH molecules are interacting in some way resulting in a short $\tau$ [24]. Whatever the explanation, the propionic acid group on DPH does not alter the properties of the fluorophore as do other substituents [17,18,24]. Further, and as is the case with $Q$, since the $\tau$ of DPH and PA-DPH are similar in isotropic solvents, the differences seen in membranes can most likely be attributed to differential probe localization and the anisotropic nature of the bilayer rather than the fluorescence properties of the molecules per se

PA-DPH and TMA-DPH bind rapidly ( < 1 min) to phospholipid membranes Kuhry et al [31] have also shown rapid binding of TMA-DPH to L929 mouse fibroblast membranes Rapid binding of PnA [8] and the *n*-(9-anthroyloxy) free fatty acid derivatives (unpublished data) occurs as well In contrast, and as described by others [12,25], binding of DPH is slow It is possible that the uncharged DPH forms aggregates in an aqueous environment even at micromolar concentrations, and that the slow binding thus reflects the dissociation of DPH monomers from this aggregate

PA-DPH, like TMA-DPH and DPH, detects the phase transition of both zwitterionic DMPC (Fig. 3A) and anionic DMPG vesicles (Fig. 3B) In contrast, the fluorescence polarization of DPH-PC, in which PA-DPH is incorporated at the *sn*-2 position, reported an abnormally low phase transition temperature for dipalmitoylphosphatidylcholine vesicles [41] This was thought to be due to its preferential partitioning into fluid phase lipids [41], and therefore implies that PA-DPH, like DPH [13], partitions equally into gel and fluid phase lipids The higher polarization of TMA-DPH compared to DPH (Figs 3A and 3B) is thought to reflect its localization near the surface of the membrane [24] The present results show that in the liquid-crystalline phase the absolute values of steady-state polarization for PA-DPH are intermediate between DPH and TMA-DPH, but nearer to TMA-DPH (Figs 3 and 4) This again suggests that like TMA-DPH, PA-DPH may be anchored at the aqueous interface of the membrane by its charged group, but the fluorescent moiety is presumably intercalated between the phospholipid acyl chains more deeply than in the case of TMA-DPH, owing to the propionic acid chain (Fig 1) Thus, DPH, PA-DPH and TMA-DPH may prove useful for examining the gradient of lipid order in the membrane bilayer in a manner analogous to detection by the series of *n*-(9-anthroyloxy) free fatty acid probes [1,2]

pH has little effect on the polarization or fluorescence intensity of DPH and TMA-DPH in egg PC-MLV, while PA-DPH shows a small increase (approx 15%) in polarization (Fig 4) and decrease in intensity above pH 7 Two *n*-(9-anthroyloxy) free fatty acid probes examined, 2-AS and 12-AS show a similar small effect of pH on polarization and intensity above pH 7 It is likely that the observed spectral changes reflect the ionization of these probes, and that the $pK_a$ of PA-DPH is about 7 This is similar to the $pK_a$ values between 7 2 and 7 6 reported for membrane-bound natural free fatty acids [32,33] It is possible that ionization causes the PA-DPH (and *n*-(9-anthroyloxy) free fatty acids) to be shifted toward the bilayer surface and hence a more constrained environment as seen by the increase in polarization In addition the ionized probes may become more susceptible to quenching by the aqueous medium, as evidenced by the decrease in fluorescence intensity

PA-DPH binds to both BSA and hFABP in a manner similar to natural free fatty acids Spector [26] has reported that BSA has three classes of free fatty acid binding sites The primary and secondary classes, containing six free fatty acid binding sites, are high-affinity sites, and the tertiary class, estimated to contain as many as 20 to 63 sites, are more weakly binding. The present results demonstrate that binding of the first 6 mol of PA-DPH per mol of BSA accounts for 90% of the maximal increase in fluorescence, while further addition of PA-DPH (up to 12 moles) results in only small increases (Fig 7A) The first six moles of PA-DPH bound may represent PA-DPH binding to the primary and secondary sites, while the additional binding may represent binding to the tertiary sites hFABP reportedly binds between 0 6 and 2 0 mol long-chain free fatty acid per mol [27,34,35], and our results show that PA-DPH binds hFABP at probe/protein ratio of approximately 1 5 1 (Fig 7A) Thus, the data suggest that PA-DPH binding to these free fatty acid binding proteins is stoichiometrically similar to the binding of natural long-chain free fatty acids

Binding of oleic acid to BSA causes up to a 38% decrease in average tryptophan quantum yield (Fig 6B), similar to the 25% and 45% decreases reported previ-

ously [9,36] This decrease is not due to Forster energy transfer [37], but is believed to be associated with a conformational change in BSA upon free fatty acid binding [36] When PA-DPH binds to BSA, a decrease in tryptophan fluorescence is also observed (Fig 6B), and it is likely that the conformational effect of ligand binding is occurring here as well At ratios of ligand/BSA up to 2 1, the magnitude of the PA-DPH-induced decrease is somewhat greater than that caused by oleate binding In addition, while further oleate binding causes no further quenching of tryptophan, additional PA-DPH binding results in a further decrease in protein fluorescence This is likely due to energy transfer between the tryptophan(donor)-PA-DPH(acceptor) pair [37], as there is considerable spectral overlap between the absorption of PA-DPH and tryptophan emission of BSA A similar pattern of tryptophan quenching was reported by Sklar et al for PnA binding to BSA [9]

PA-DPH binding to hFABP results in a decrease in tyrosine fluorescence (Fig. 7B), suggesting energy transfer in this case as well The decrease levels off at an approximately 2 1 ratio of PA-DPH/hFABP, in agreement with the stoichiometry indicated by PA-DPH binding When native oleate binds to hFABP, however, a 40% increase in protein fluorescence is seen, suggesting that as with BSA, free fatty acid binding may result in a protein conformational change An increase in tyrosine quantum yield due to protein conformational change has been reported for several other Class A proteins, those which contain no tryptophan residues and have fluorescence properties due to tyrosine [28] Thus it is likely that the hFABP tyrosine quenching, due to energy transfer to PA-DPH, masks the conformation-induced increase in tyrosine emission The recent study of Schulenberg-Schell et al [38] shows that the $^1$H-NMR spectrum of bovine liver FABP is altered upon free fatty acid binding, also suggesting conformational changes within the protein

When PA-DPH is prebound to hFABP and increasing amounts of oleic acid are then added, a biphasic effect on PA-DPH fluorescence is seen, namely an initial increase followed by a decrease in intensity with additional oleate (Fig 7C) The initial increase levels off at approximately equal concentrations of PA-DPH and oleate A 50% decrease in PA-DPH fluorescence then occurs at an oleate/PA-DPH ratio of about 1 5 1, suggesting that PA-DPH has a similar affinity for hFABP as compared to oleate The initial increase in PA-DPH quantum yield may be due to binding of oleate to a second hFABP binding site As mentioned earlier, the present results as well as those of several other groups indicate that hFABP can bind 2 mol of free fatty acid per mol protein [27,34,35], and Keuper et al [39] have proposed that liver FABP contains a single binding site for the two fatty acids It is conceivable that when oleate and PA-DPH are simultaneously bound to hFABP, the quantum yield of the fluorophore increases, perhaps due to increased hydrophobic interactions with the oleate acyl chain Further addition of oleate then displaces the fluorophore from the binding site, resulting in a decrease in PA-DPH fluorescence Using the fluorescence titration method described by Cogan et al [23] we estimate a $K_d$ of 0 3 $\mu$M for PA-DPH binding to hFABP While such analysis assumes an equivalent intensity for PA-DPH bound to one or both sites, the $K_d$ obtained is similar to the values reported for native free fatty acid binding to hFABP [27,34,35]

Competition studies suggest that oleate also displaces PA-DPH from BSA, and further indicate that the native free fatty acid may have an average 4-fold greater affinity for BSA than does PA-DPH since PA-DPH intensity is decreased by half at a PA-DPH/oleate ratio of approximately 4 It is known that free fatty acid binding affinities differ at each of the binding sites on BSA [26] It is likely that the quantum yield of PA-DPH also varies within the different sites, as suggested by the non-linear increase in intensity when BSA is titrated with PA-DPH (Fig 6A) Thus the suggested relative affinity of oleate compared to PA-DPH is only a rough estimate for an 'average' site, and the true relative affinities undoubtedly vary at the different sites Nevertheless, the binding properties of PA-DPH and the competition with oleate suggest that PA-DPH most likely binds to the same sites as native free fatty acids on both hFABP and BSA The data also indicate that PA-DPH has a similar affinity for hFABP as do natural long-chain free fatty acids, while the average affinity for BSA may be lower than that of oleic acid

These studies were conducted to assess the utility of PA-DPH as a fluorescent free fatty acid analogue and membrane probe The results indicate that propionic acid substitution of the phenyl group of DPH results in minimal perturbation of the fluorescence properties in isotropic solvents PA-DPH binds to phospholipid membranes, detects the lipid phase transition and seems to probe a different region of the bilayer than other DPH probes Further, PA-DPH binds two physiological free fatty acid binding proteins, BSA and hFABP, in a manner similar to natural long-chain free fatty acids Since PA-DPH combines both a high absorption coefficient and quantum yield relative to other commonly used fluorescent free fatty acids, it should prove to be a useful addition to the family of fluorescent long-chain free fatty acid analogues

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## References


1 Thulborn, K R and Sawyer, W H (1978) Biochim Biophys Acta 511, 125–140
2 Thulborn, K.R., Tilley, L M , Sawyer W H and Treloar E (1979) Biochim. Biophys Acta 558, 166–178
3 Storch J and Kleinfeld, A M (1986) Biochemistry 25, 1717–1726
4 Doody, M C., Pownall, H J Kao, Y.J and Smith L C (1980) Biochemistry 19, 108–116
5 Pownall, H J , Hickson, D L and Smith, L C (1983) J Am Chem Soc 105, 2440–2445
6 Galla, H-J and Hartmann, W (1980) Chem Phys Lipids 27, 199–219
7 Sklar, L A , Hudson, B S Petersen, M and Diamond J (1977) Biochemistry 16, 813–819
8 Sklar, L A , Hudson, B S and Simoni, R D (1977) Biochemistry 16, 819–828
9 Sklar, L A , Hudson, B S and Simoni R.D (1977) Biochemistry 16, 5100–5100
10 Straume, M and Litman B J (1987) Biochemistry 26, 5113–5120
11 Kutchai, H , Chandler, L H and Zavoico G B (1983) Biochim Biophys Acta 736, 137–149
12 Lentz, B R , Barenholz, Y and Thompson T E. (1976) Biochemistry 15, 4521–4528
13 Lentz, B R , Barenholz, Y and Thompson, T E (1976) Biochemistry 15, 4529–4537
14 Jähnig, F (1979) Proc. Natl Acad Sci USA 76 6361–6365
15 Heyn, M P (1979) FEBS Lett 108, 359–364
16 Duportail, G and Weinrab, A (1983) Biochim Biophys Acta 736 171–177
17 Cundall, R.B , Johnson, I , Thomas E.W and Munro, I H (1979) Chem Phys Lett 64, 39–42
18 Cranney M., Cundall, R.B , Jones, G R., Richards, J T and Thomas, E.W (1983) Biochim Biophys Acta 735, 418–425
19 Huang, C and Thompson, T E. (1974) Methods Enzymol 32, 485–489
20 Parker C A and Rees W T (1960) Analyst 85 587–600
21 Scott T G , Spencer R D , Leonard, N.J and Weber, G (1970) J Am. Chem Soc 92 687–695
22 Spencer R D and Weber, G (1969) Ann N Y Acad Sci 158 361–376
23 Cogan, U , Kopelman M Mokady, S and Schnitzky M (1976) Eur J Biochem 65, 71–78
24 Prendergast F G Haugland R P and Callahan P J (1981) Biochemistry 20 7333 7338
25 Shinitzky, M and Barenholz, Y (1974) J Biol Chem 249 2652–2657
26 Spector, A A (1975) J Lipid Res 16, 165–179
27 Bass, N M (1985) Chem Phys Lipids 38, 95–114
28 Konev S V (1967) Fluorescence and Phosphorescence of Proteins and Nucleic Acids, pp 61–69 Plenum Press, New York
29 Fiorini, R., Valentino M Wang, S Glaser, M and Gratton E (1987) Biochemistry 26 3864–3870
30 Davenport, L , Dale, R E. Bisby R.H and Cundall, R.B (1985) Biochemistry 24, 4097–4108
31 Kuhry J G Fonteneau, P Duportail G Maechling, C and Laustriat, G (1983) Cell Biophys. 5, 129–140
32 Ptak, M Egret-Charlier M , Sanson, A and Bouloussa O (1980) Biochim Biophys Acta 600 387–397
33 Cistola, D P , Hamilton, J A Jackson, D and Small D M (1988) Biochemistry 27, 1881–1888
34 Cistola, D P Walsh M T Corey, R P Hamilton, J A and Brecher P (1988) Biochemistry 27, 711–717
35 Offner, G D , Troxler R F and Brecher P (1986) J Biol Chem 261 5584–5589
36 Spector A A and John K M. (1968) Arch Biochem Biophys 127 65–71
37 Förster, Th (1948) Ann Phys (Leipzig) 2, 55–75
38 Schulenberg-Schell, H , Schäfer, P , Keuper, H J K Stalnowski, B Hoffman, E., Ruterjans, H and Spener F (1988) Eur J Biochem 170 565–574
39 Keuper, H J K , Klein R A and Spener F (1985) Chem Phys Lipids 38, 159–173
40 Morgan, C G Thomas E.S and Yianna, Y P (1983) Biochim Biophys. Acta 728, 356–362
41 Parente, R A and Lentz. B R (1985) Biochemistry 24, 6178–6185