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THE QUENCHING OF AN INTRAMEMBRANE FLUORESCENT PROBE

A METHOD TO STUDY THE BINDING AND PERMEATION OF PHLORETIN THROUGH BILAYERS

A.S. VERKMAN

Biophysical Laboratory, Harvard Medical School, Boston, MA 02115 (U.S.A.)

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Summary

Phloretin and phloretin-like dipolar non-electrolytes strongly quench the fluorescence of several membrane-bound probes, including 1,6-diphenylhexa-1,3,5-triene and anthroyl derivatives of long-chain fatty acids. Fluorescence intensity measurements therefore provide a simple and sensitive method to study the equilibrium binding properties and permeability of phloretin-like molecules in biological and artificial membrane systems. The dissociation constants for the binding of phloretin and naringenin to phosphatidylcholine vesicle membranes are determined, assuming the Stern-Volmer relation, from the fluorescence intensity of intramembrane probes as a function of phloretin and naringenin concentrations. Results (phloretin, 9 ± 1 μM; naringenin, 21 ± 4 μM) agree with the dissociation constants obtained using absorption titration performed in the absence of fluorescent probes. Fluorescence nanosecond lifetime measurements show that the mechanism of quenching of diphenylhexatriene and 16-anthroylpalmitic acid by phloretin and naringenin is largely diffusional in nature. The transmembrane movement of phloretin through phosphatidylcholine vesicles was observed by the stopped-flow technique, in which phloretin is mixed rapidly with a vesicle solution containing a membrane-bound fluorescent probe. The time course obtained by fluorescence measurements was identical to that obtained in a parallel measurement of the time course of optical absorption of phloretin. Stopped-flow data for the permeability of phosphatidylcholine liposomes and red blood cell membranes are also presented. The use of a membrane-bound indicator greatly extends the range of concentrations and pH values as well as the types of systems which can be characterized by optical means.

Introduction

Phloretin strongly inhibits glucose transport [1], anion exchange [2] and hydrophilic non-electrolyte transport in biological membrane systems [3] and markedly alters ion conductivity in artificial systems [4]. In well-defined lipid bilayer membranes of uniform composition, phloretin binds to a single set of non-interacting sites [5,6] which are probably located in a region near each membrane/solution interface [7]. The rate of binding of phloretin to these interfacial sites is diffusion limited, while the rate of movement of phloretin across the membrane is slow (approx. 1 s^{-1}) and is the rate-determining step of phloretin permeability [8].

Previous studies of the interactions between phloretin and a bilayer membrane have been based on the observation that phloretin is a weak acid (pK_a = 7.3) in which the ionized and non-ionized species have very different optical absorption spectra [6]. Since only the non-ionized form of phloretin binds to real and artificial membranes [9,10], the amount of bound phloretin is calculated from the spectral shift that occurs as membranes are aded to a phloretin solution. Absorption measurements are relatively insensitive; they restrict the concentrations, pH values and types of compound which may be studied by optical means. The high membrane concentrations necessary for significant absorption changes limit the absorption technique to the investigation of relatively non-turbid preparations.

In this paper, a fluorescence-quenching technique is presented to study the interactions of phloretin and its analogues with membrane systems. Phloretin strongly quenches the fluorescence of several membrane-bound probes; the degree of quenching is a sensitive indicator of the amount of phloretin bound to a membrane. Several kinetic and equilibrium examples are presented to demonstrate the applications of this intramembrane quenching technique.

Materials and Methods

1,6-Diphenylhexa-1,3,5-triene was obtained from Aldrich (Milwaukee, WI) and 16-anthroylpalmitic acid was obtained from Molecular Probes (Plano, TX). The purity of 16-anthroylpalmitic acid was checked by thin-layer chromatography using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4). Phloretin was purchased from K and K Laboratories (Plainview, NY) and its purity was checked by thin-layer chromatography in $CHCl_3$/propanol (3 : 1). Naringenin was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. No significant fluorescent impurity was found in phloretin, naringenin or phosphatidylcholine vesicles.

Unilamellar vesicles were prepared by sonication using the method of Huang and Thompson [11]. Lipid stored at −20°C in ethanol was dried in a rotary evaporator to remove ethanol, lyophilized, and suspended in buffer at a lipid concentration of approx. 25 mM. The suspension was sonicated at 4°C under N_2 for 1 h using a Branson model W 185 sonicator. The vesicles were then centrifuged at 40 000 × g for 1 h to remove titanium particles and lipid debris. Vesicles were stored under N_2 at 5°C and used within 1 week of preparation.

Liposomes were prepared according to the technique of Bangham et al. [12]

by vigorously hand-shaking a dispersion of phosphatidylcholine in buffer for 1 min to arrive at a final phosphatidylcholine concentration of 40 μM. Liposomes were used within a few hours of preparation. Red blood cells were drawn freshly by venipuncture, washed five times in buffer and resuspended to a final hematocrit of 0.1%. Lipid concentration was assayed for by the method of Gomori [13].

The equilibrium binding of phloretin and naringenin to phosphatidylcholine vesicles was studied by an optical absorption titration technique which has been described previously [6]. Equilibrium fluorescence titrations were performed on a Perkin Elmer MFP-2A fluorescence spectrophotometer. Microliter aliquots of phloretin or naringenin were added to 2.0 ml of a 20 μM stirred solution of phosphatidylcholine vesicles containing 100 nM diphenylhexatriene or other fluorescent probe. The fluorescence intensity was measured after the addition of each aliquot of substrate and the concentration of bound substrate was calculated from the quenching of the intramembrane fluorescent probe. Fluorescence intensities measured at equal phloretin concentrations in duplicate titrations with this technique differed by less than 2%.

The fluorescence intensities were corrected empirically for dilution and inner filter effects by performing duplicate titrations using a fluorescent probe which did not interact with phosphatidylcholine vesicles (fluorescein) in place of the intramembrane fluorescent probe. Any change in fluorescence of a solution containing phosphatidylcholine vesicles, phloretin and a non-interacting fluorescent compound is due entirely to a dilution of the probe or to absorption of excitation or emission light by phloretin or vesicles. It turns out that there is little absorption of emission or excitation light by phloretin (less than 1% at 200 μM phloretin) for $\lambda > 350$ nm. Therefore, a calculated correction factor, which is based on dilution effects exclusively, agreed closely with the correction factor determined empirically.

Stopped-flow studies were performed on an apparatus described elsewhere [8]. Phloretin was mixed with phosphatidylcholine vesicles containing a fluorescent probe within 10 ms, and the amount of bound phloretin was determined by measuring either optical transmittance at 325 nm or the fluorescence of the intramembrane fluorescent probe. Monochromatic excitation light was passed through a 7-51 filter (Corning Glass Works, Corning, NY) to eliminate second-order reflections and the emission was filtered by two 3-144 filters (Corning) which pass light above 410 nm. Data were recorded on a waveform recorder (Biomation model 805) and transferred to a PDP 11/34 computer for analysis.

Fluorescence lifetime measurements were performed by phase-modulation fluorimetry using an SLM model 480 subnanosecond spectrofluorometer. Monochromatic excitation light was used (365 nm for diphenylhexatriene, 380 nm for 16-anthroylpalmitic acid; $\Delta\lambda = 1$ nm) and the emission light was filtered through two 3-144 filters. Each sample was referenced ten or more times to a scattering solution (glycogen) and lifetimes were corrected for scattering due to vesicles. All experiments were performed with a 30 mHz modulation frequency and no lifetime heterogeneity was detected for samples containing diphenylhexatriene or 16-anthroylpalmitic acid and phloretin or naringenin.

Results and Discussion

Diphenylhexatriene partitions strongly into a phospholipid bilayer [14,15] and more than 99.9% of the observed fluorescence intensity comes from membrane-bound diphenylhexatriene at the 200 : 1 lipid to probe ratio used in these experiments. Fig. 1 (top) shows the decrease in fluorescence intensity as phloretin and naringenin are added to a solution consisting of 100 nm diphenylhexatriene in 20 μM phosphatidylcholine vesicles in one experiment typical of three. Increasing concentrations of phloretin and naringenin quench the fluorescence of membrane-bound diphenylhexatriene in a dose-related fashion. Experiments performed using 100 nM diphenylhexatriene in 50 μM phosphatidylcholine vesicles and 10 nM diphenylhexatriene in 2 μM phosphatidylcholine vesicles gave results which were very similar to the curve shown in Fig. 1. This observation indicates that diphenylhexatriene is sensitive to the phloretin concentration and not to the lipid or diphenylhexatriene concentrations in the range studied.

It is known that phloretin binds to a single class of site on a phosphatidylcholine vesicle membrane which is described by a dissociation constant, K_D, equal to 8 μM, and a lipid per site ratio, L/S, equal to 4 [6]. It is therefore possible to test whether the observed quenching is directly related to the amount of membrane-bound phloretin. The binding of non-ionized phloretin or naringenin, [H-Sub], to sites, [S], on a phosphatidylcholine vesicle membrane is described by the bimolecular association,

$$\text{H-Sub} + \text{S} \leftrightarrow \text{bound} \qquad K_D = \frac{[\text{H-Sub}][\text{S}]}{[\text{bound}]} \tag{1}$$

For collisional quenching, the reciprocal fractional quenching, F_0/F, is related to the concentration of membrane-bound quencher, [bound], by the Stern-Volmer relation [16],

$$F_0/F = 1 + M\,[\text{bound}] \tag{2}$$

where F and F_0 are the fluorescence intensities observed in the presence and absence of quencher, respectively. M is a constant which depends upon the quenching efficiency, the rate of bimolecular collisions and the detailed spatial distributions of fluorescent probe and quencher in the membrane. Since the factors which comprise M are poorly known, M will be taken as an empirical constant.

Eqns. 1 and 2, and the conservation conditions, $[\text{S}] + [\text{bound}] = [\text{S}]_{tot}$, may be combined to give a double-reciprocal relation.

$$\frac{1}{F_0/F - 1} = \frac{K_D}{M[\text{S}]_{tot}} \frac{1}{[\text{H-Sub}]} + \frac{1}{M[\text{S}]_{tot}} \tag{3}$$

A plot of $1/(F_0/F - 1)$ vs. $1/[\text{H-Sub}]$ gives a straight line with K_D equal to the slope divided by the intercept. Double-reciprocal plots for the binding of phloretin and naringenin to phosphatidylcholine vesicles are given in the bottom section of Fig. 1. The approximation, $[\text{H-Sub}] = [\text{H-Sub}]_{tot}$, has been made since $[\text{S}]_{tot}$ is only 5 μM and K_D is 8 μM or more, implying that phloretin binding by vesicles will reduce the free phloretin concentration by less than 2%

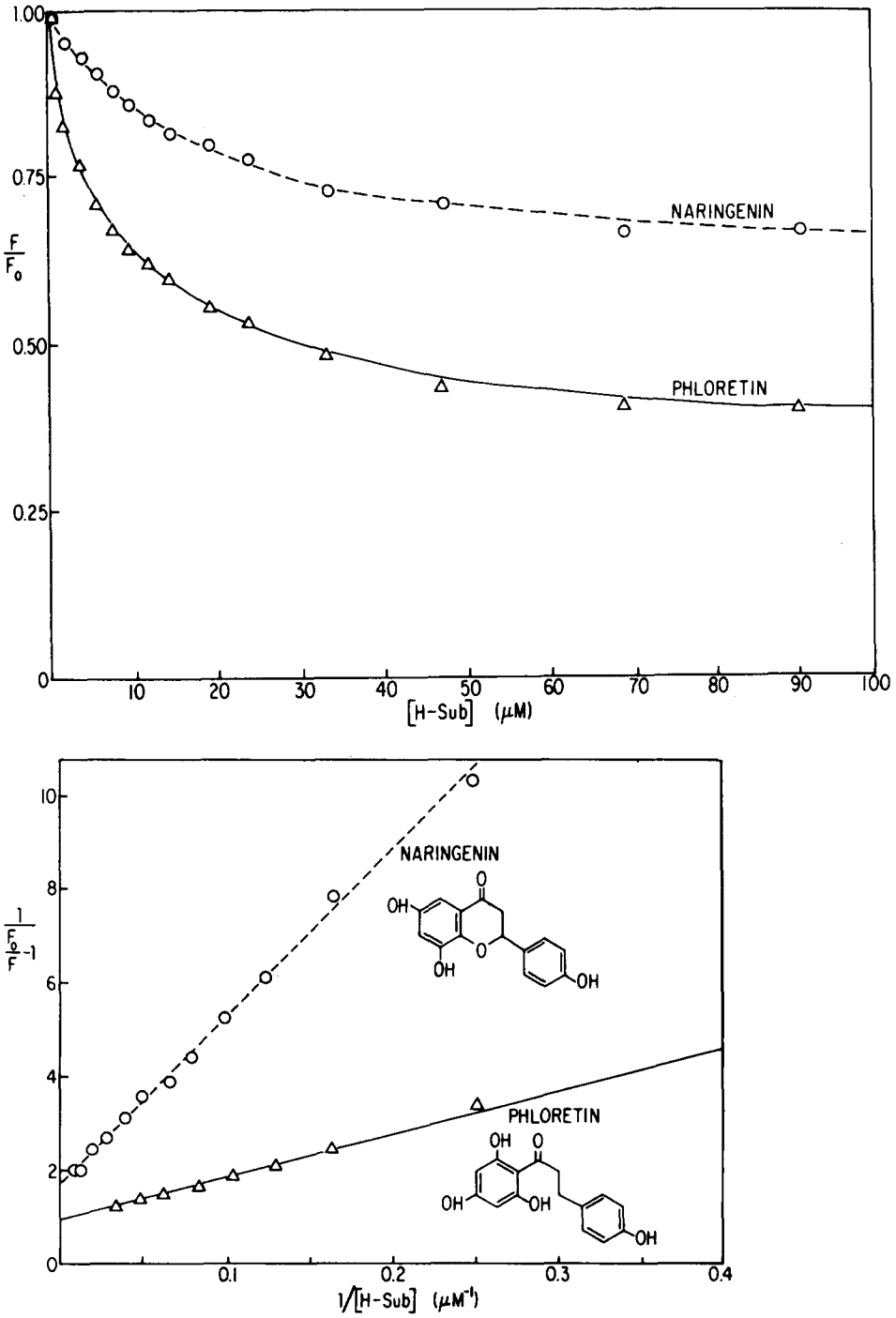

Fig. 1. Fluorescence binding titrations. Top: increasing concentrations of phloretin or naringenin, [H-Sub], are added to a solution containing 20 μM phosphatidylcholine vesicles + 100 nM diphenylhexatriene in 0.1 M Tris + 0.1 M KCl at pH 7.3, 24°C. The fractional decrease in fluorescence intensity of diphenylhexatriene, F/F_0, is due to intramembrane quenching of diphenylhexatriene by bound [H-Sub]. Bottom: the quenching data are replotted in the form of a double-reciprocal plot as described by Eqn. 3 of the text. The lines drawn through the data are least-squares fits ($r > 0.98$).

over the concentration range studied. The data fall along straight lines and Table I shows that the K_D values do not differ significantly from K_D values obtained by absorption titrations performed in the absence of fluorescent probes.

Similar titrations performed using 16-anthroylpalmitic acid are also given in Table I; results are similar to those for diphenylhexatriene. Anthroyl compounds are useful membrane probes; they are highly lipophilic, well localized in the membrane and exhibit relatively uncomplicated fluorescence properties [17—19]. The results in Table I therefore indicate that diphenylhexatriene and 16-anthroylpalmitic acid do not alter the binding affinity of phloretin to a phosphatidylcholine vesicle membrane and that intramembrane probe quenching is a well-defined indicator of phloretin binding.

Fluorescence lifetime measurements were made to distinguish between collisional quenching, as described by the Stern-Volmer relation, and static complex formation [20,21]. Fig. 2 shows the effects of phloretin and naringenin on the nanosecond lifetimes of 16-anthroylpalmitic acid and diphenylhexatriene in phosphatidylcholine vesicle membranes at a 200 : 1 lipid to probe ratio. No lifetime heterogeneity was detected for any sample as judged by the agreement of lifetimes calculated independently by modulation and phase measurements.

The lifetime of 16-anthroylpalmitic acid reaches one-half of its final value at approx. 7 μM phloretin and levels off at high phloretin concentration to a value, τ/τ_0, of 0.56. The fluorescence intensity (not shown) has the same saturation value ($F/F_0 = 0.58$) with $K_D = 8$ μM. The addition of 20 μM phloretin has no effect on the normalized excitation and emission spectra of 16-anthroylpalmitic acid in vesicles. Therefore the equality between F/F_0 and τ/τ_0, and the lack of effect of phloretin on the normalized spectra of 16-anthroylpalmitic acid in vesicles shows that the quenching of 16-anthroylpalmitic acid by phloretin occurs by a classical, collisional mechanism.

The addition of phloretin or naringenin to phosphatidylcholine vesicles containing diphenylhexatriene does not alter the normalized excitation spectrum

TABLE I

FLUORESCENCE BINDING TITRATIONS

A, absorption; F, fluorescence. All titrations were performed with 20 μM phosphatidylcholine vesicles at pH 7.3, 23°C with a 100 nM fluorescent probe concentration. Errors are 1 S.E. L/S, the number of lipid molecules per binding site, is not determined from a fluorescence titration. DPH, diphenylhexatriene; 16-AP, 16-anthroylpamitic acid.

	Probe	Technique	K_D (μM)	L/S
Phloretin	none	A	8.0 ± 0.4	4.0 ± 0.3
	DPH	A	8.3 ± 0.5	4.0 ± 0.4
	DPH	F	9 ± 1	
	16-AP	A	8.1 ± 0.5	3.8 ± 0.4
	16-AP	F	8 ± 1	
Naringenin	none	A	24 ± 1	4.0 ± 0.5
	DPH	A	22 ± 2	4.2 ± 0.5
	DPH	F	21 ± 4	

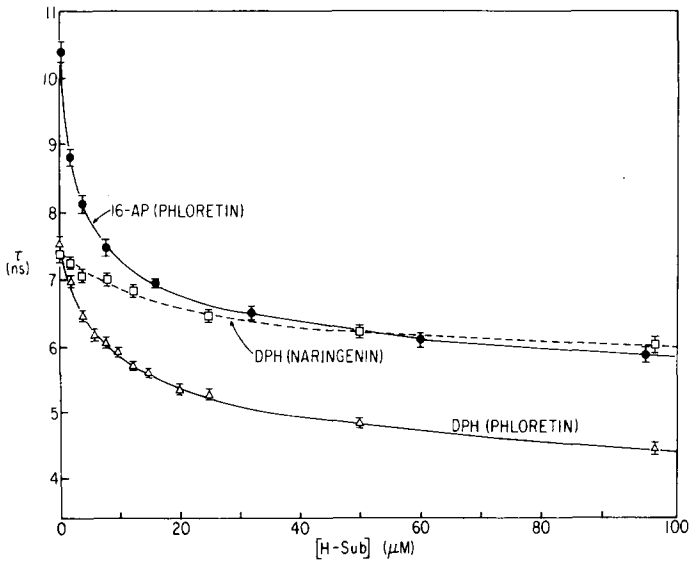

Fig. 2. Effects of phloretin and naringenin on the fluorescence lifetimes of diphenylhexatriene (DPH) and 16-anthroylpalmitic acid (16-AP). A solution containing 20 μM phosphatidylcholine vesicles + 100 nM diphenylhexatriene or 16-anthroylpalmitic acid was titrated with phloretin or naringenin as in Fig. 1. The fluorescence lifetimes represent the average of ten measurements; error bars are 1 S.E. from the mean.

of diphenylhexatriene, yet slightly flattens the red end of the emission peak at 420 nm. The K_D values predicted from the lifetime titration in Fig. 2 for phloretin (9 ± 1 μM) and naringenin (18 ± 6 μM) agree well with the intensity titration from Table I, while at high quencher concentration, the limiting values of F/F_0 (phloretin, 0.40; naringenin, 0.56) are smaller, by about a factor of 1.4, than lifetime values, τ/τ_0 (phloretin, 0.56; naringenin, 0.80). The lack of equality between F/F_0 and τ/τ_0 may be due to changes in the complex, anisotropic motions of membrane-bound diphenylhexatriene [22,23] caused by fluidity or electrical perturbations induced by phloretin.

The results of binding experiments shown in Table I and kinetic experiments to be described, show empirically that diphenylhexatriene and 16-anthroylpalmitic acid are valid indicators of the presence of phloretin in a membrane. Spectral and lifetime studies show that phloretin quenches the fluorescence of 16-anthroylpalmitic acid by a collisional mechanism. Therefore, a firm theoretical basis exists for the use of 16-anthroylpalmitic acid as a probe for phloretin binding to membranes. Although diphenylhexatriene appears to be an equally good empirical indicator of phloretin binding, the spectral and lifetime studies suggest that the mechanism of quenching may be complex. It is therefore important to demonstrate that a fluorescent molecule, which is used as an intramembrane indicator, does not interact with the chemical system and gives results which are identical to those obtained using independent methods.

Fig. 3 shows the time course of permeation of phloretin through phosphatidylcholine vesicles as observed in a stopped-flow experiment. The process is measured both by recording the depletion of phloretin from the buffer (transmittance, 325 nm) and the accumulation of phloretin in the membrane (diphenylhexatriene fluorescence). The fitted exponential time constants are

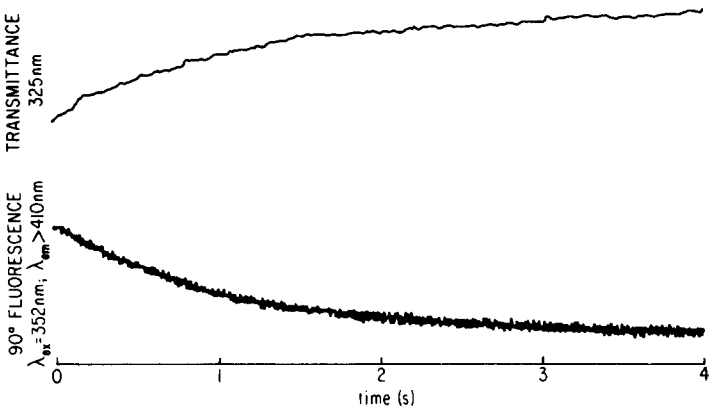

Fig. 3. Stopped-flow study of the permeation of phloretin through phosphatidylcholine vesicle membranes. In the upper trace, a solution containing 100 μM phloretin was mixed with an equal volume of solution containing 250 μM vesicles and the equilibration time course was followed at 325 nm. Increasing transmittance at 325 nm indicates increased phloretin binding to vesicles. In the lower trace, 500 nM diphenylhexatriene was equilibrated with the vesicle solution before the stopped-flow experiment. The time course of equilibration was followed by measuring the quenching of diphenylhexatriene as the phloretin concentration in the membrane rises.

999 ± 13 ms and 982 ± 8 ms (difference not significant) for the transmittance and fluorescence measurements, respectively. Thus, diphenylhexatriene acts as an indicator of the amount of phloretin bound to the membrane instantaneously and does not alter the observed permeation kinetics. Similar experiments performed with 500 nM 16-anthroylpalmitic acid in place of diphenylhexatriene and at three other phosphatidylcholine and phloretin concentrations confirm that diphenylhexatriene and 16-anthroylpalmitic acid are valid indicators of phloretin permeation kinetics.

Stopped-flow experiments have also been performed under conditions when the time course is measurable only by the fluorescence-quenching technique. Experiments performed at very low phosphatidylcholine concentrations (less than 5 μM) and low pH (4.0) have yielded data with signal-to-noise ratios exceeding 200 : 1, compared with a calculated signal-to-noise ratio of 1 : 100 for an identical experiment performed with the absorption technique. Several other phloretin analogues, including 2,4-dihydroxy and 2,4,6-trihydroxy derivatives of acetophenone, propriophenone and benzophenone also quench intramembrane diphenylhexatriene fluorescence. The quenching technique is applicable to kinetic methods other than the stopped-flow technique; it has been used successfully to follow the binding and unbinding of phloretin to phosphatidylcholine vesicles in temperature-jump and stopped-flow temperature-jump experiments.

The upper trace in Fig. 4 shows the permeation of phloretin through intact red blood cell membranes as observed by intramembrane diphenylhexatriene quenching. The concentration of phloretin which is bound to a red cell membrane equilibrates as the intracellular and extracellular solution compartments equilibrate. The measured time course ($\tau \approx 18$ s) is in agreement with the time course (τ = 10—20 s) obtained by Jennings and Solomon [10] using a pH equilibration technique. The lower trace in Fig. 4 shows the permeation of

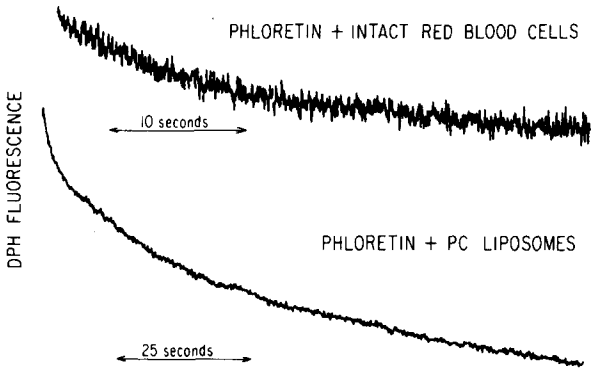

Fig. 4. The use of diphenylhexatriene (DPH) quenching to study the permeation of phloretin through red cell and phosphatidylcholine (PC) liposome membranes. In the upper trace, 500 nM diphenylhexatriene was equilibrated with a solution of human red blood cells at a 0.1% final hematocrit. The blood was mixed with a 200 μM solution of phloretin at pH 7.3 in the stopped-flow apparatus and the time course of phloretin equilibration, as shown by diphenylhexatriene quenching, is indicated. In the lower trace, a solution of 40 μM hand-shaken liposomes is pre-equilibrated with 200 nM diphenylhexatriene and then mixed rapidly with 100 μM phloretin.

phloretin through uncharacterized, hand-shaken liposomes. The time course is complex due to the presence of multiple liposome sizes. The permeation of phloretin through red blood cell and liposome membranes cannot be measured by other optical techniques.

Diphenylhexatriene and the anthroyl fatty acids are good intramembrane indicators for the binding of phloretin and phloretin-like molecules to a membrane. They are useful in equilibrium and rapid kinetic measurements. Fluorescence kinetic measurements have the advantage of excellent sensitivity and increased signal-to-noise capabilities compared with absorption measurements [24]. The use of intramembrane fluorescence quenching should prove useful in studying the interactions between substrates and real biological membranes.

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