# Use of the Fluorescent Weak Acid Dansylglycine To Measure Transmembrane Proton Concentration Gradients†

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ABSTRACT: The amphiphilic fluorescent dye *N*-[(5-dimethylamino)naphth-1-ylsulfonyl]glycine (dansylglycine) can be used to monitor the magnitude and stability of transmembrane proton gradients. Although freely soluble in aqueous media, the dye readily adsorbs to the surfaces of lipid vesicles. Because membrane-bound dye fluoresces at a higher frequency, and with greater efficiency, than dye in aqueous solution, it is easy to isolate the fluorescence emission from those dye molecules adsorbed to the lipid surface. When dansylglycine is mixed with phospholipid vesicles, the dye molecules attain a partition equilibrium between buffer and the outer, proximal surface of the vesicles. This is a rapid, diffusion-limited process that is indicated by a fast phase of fluorescence intensity increase monitored at 510 nm. In a second step, the inner, distal surface of each vesicle becomes populated with dye, a process that involves permeation through the lipid bilayer and that is generally much slower than the original adsorption step. Dansylglycine is a weak acid that permeates as an electrically neutral species; the flux of dye across the bilayer is thus strongly dependent on the degree of protonation of the dye's carboxylate moiety. When the external pH is lower than that of the vesicle lumen, the inward flux of dye is greater than that in the opposite direction, and dye accumulates in the lumen. This leads to a local elevation of dansylglycine concentration in the inner membrane monolayer, which in turn results in an elevated fluorescence intensity proportional to the membrane pH gradient.

A wide variety of experimental approaches have been applied to the measurement of proton concentration gradients ($\Delta$pH) existing across model membrane barriers [for a review, see Rottenberg (1975)], but none of these existing methods precisely matched our requirements. We wanted to study the behavior of small lipid vesicles, but the very large surface area:trapped volume ratio for these structures precludes the use of direct pH electrodes (Nozaki & Tanford, 1981) because the uptake of protons into the extremely small trapped volume of such systems generates minimal changes in external pH. The same limitation ruled out the use of optical pH indicators such as pyranine (Rossignol et al., 1982) and 5(6)-carboxyfluorescein (Thomas et al., 1979) resident (in millimolar concentrations) in the trapped space, not only because of the extremely low overall concentration of dye in suspensions of very small vesicles but also because of the possibility of complicating factors such as the generation of dye diffusion potentials and leakage into the external environment (Bramhall, 1984). The major alternative to these approaches is to monitor the redistribution of weak acids or bases in response to changes in $\Delta$pH which would be caused by net $H^+/OH^-$ movements across vesicle boundary bilayers. Although the equilibration of radioactively labeled acids and bases (Waddell & Butler, 1959) cannot easily give kinetic information about rapid changes in $\Delta$pH, the use of their optically active or paramagnetic analogues certainly can (Deamer et al., 1972; Cafiso & Hubbell, 1983). 9-Aminoacridine, a highly fluorescent weak base which freely permeates lipid bilayers, continues to be one of the most useful probes of $\Delta$pH for use with lipid vesicle systems (Deamer et al., 1972; Nichols et al., 1980; Garcia et al., 1984); however, the dye is best suited for use in systems in which the vesicle lumen is acidic with respect to the external environment (it concentrates in such vesicles, leading to quenching of fluorescence and a consequent decrease in overall sample fluorescence emission intensity). For analysis of neutral acid permeation it is experimentally convenient to monitor the diffusion of acids into vesicles rather than leakage of acids out of them (a variety of acids can be added to the external environment of a vesicle suspension rather than using several vesicle preparations each with a different trapped acid); 9-aminoacridine is not particularly well suited to such measurements. The report describes the use of the weakly acidic naphthalene derivative dansylglycine[1] in the measurement, by fluorescence spectroscopy, of the relative size and stability of proton concentration gradients existing across the boundary bilayer of small phospholipid vesicles.

## Materials and Methods

Dansylglycine was obtained from Sigma Chemical Co. (St. Louis, MO). It generally contained traces of the dansyl-free acid and was purified before use by thin-layer chromatography (TLC) on silica gel using a developing solvent of chloroform/methanol (2:1 v/v). Purified material ($R_f$ 0.24) was eluted from the adsorbent with methanol and stored as a $10^{-2}$ M solution in absolute ethanol at −20 °C.

Small unilamellar lipid vesicles were prepared by sonication of lipid suspensions in aqueous buffer solution under an atmosphere of nitrogen. Typically, 50 mg of DPPC (Sigma Chemical Co., St. Louis, MO) was sonicated in 1 mL of buffer (containing materials to be trapped when necessary). Lipid aggregates and titanium debris were removed by centrifugation at 100000*g* for 30 min. During sonication, the temperature of the lipid suspension was maintained at least 5 °C above the lipid's melting point (e.g., at 46 °C in the case of DPPC), and vesicles were stored at this temperature ($T_c$ + 5 °C) to restrict intervesicle fusion (Schullery et al., 1980). Buffer osmolarity

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[1] Abbreviations: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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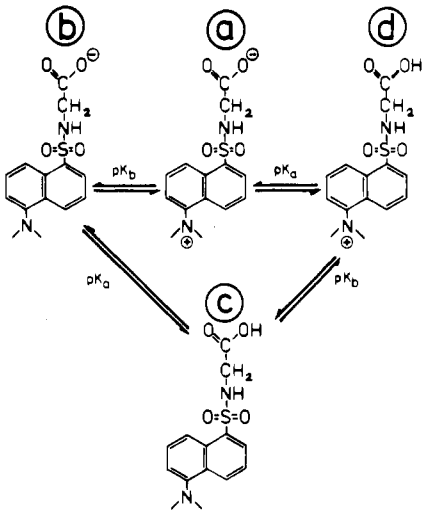


FIGURE 1: Dansylglycine: protonation states.

was determined with a vapor pressure osmometer (Wescor, Model 5100C).

Equilibrium binding experiments were performed by using plastic microdialysis chambers with a 200-$\mu$L cavity on each side of a semipermeable membrane ($M_r$ 12 000 cutoff), as previously described (Bramhall, 1984). Phospholipid vesicles prepared in 65 mM NaCl were placed in one chamber; 50 mM sodium phosphate buffer, containing $10^{-5}$ M dansylglycine, was placed in the opposite chamber. The cells were rotated as described, and percentage binding of dye was determined from the ratio of the fluorescence intensities of equal volume samples taken from the vesicle-free ($F_a$) and vesicle-containing ($F_v$) compartments (samples diluted in *n*-dioxane). The quantity of membrane-bound dye ($F_m$) was calculated from eq 1.

$$F_m = F_v - F_a \quad (1)$$

The membrane–buffer partition coefficient, $k_b$ (binding coefficient), was calculated from eq 2 as the slope of the line obtained by plotting $F_m/F_a$ vs. $V_m/V_a$, where $V_m$ and $V_a$ are the volumes of the membrane and aqueous phases, respectively. $V_m$ was calculated by assuming a partial specific volume for DMPC of 1 mL/g.

$$k_b = (F_m/F_a)(V_a/V_m) \quad (2)$$

Fluorescence and light-scattering measurements were performed on a Fluorolog II spectrometer (Spex Industries, Metuchen, NJ) equipped with temperature control accessories and magnetic stirrer; rapid sample mixing was effected with a conventional stopped-flow attachment. Sample temperature was regulated with a circulating water bath (EX-200, Neslab Inc., Portsmouth, NH) and monitored with a platinum resistance thermometer housed in the optical cuvette.

## Results and Discussion

The fluorescent dye dansylglycine appears to be ideal for use in monitoring the magnitude and stability of transmembrane proton gradients. The dye is a zwitterionic amphiphile that can exist in both charged and neutral forms. The *N*,*N*-dimethylamino function of the chromophore can become protonated (p$K \sim 3.9$) to yield charged species (a and d in Figure 1). Figure 2 shows that this protonation is accompanied by a dramatic decrease in fluorescence intensity. The carboxylate function of the glycyl moiety is also dissociable (p$K \sim 2.5$), but dissociation of this group exerts no practical influence on the fluorescence properties of the dye. The

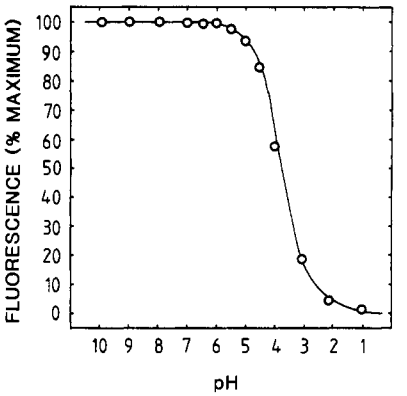


FIGURE 2: Dependence of dansylglycine fluorescence on pH. Dansylglycine was dissolved in a series of 50 mM sodium phosphate buffers with different pH values; the final dye concentration was 1 $\mu$M in each case. The fluorescence emission intensity of each solution was measured at 580 nm; excitation was at 350 nm. Measured emission intensities were expressed as percentages of the maximum emission intensity observed in the range of samples and are shown above plotted vs. experimental pH. All measurements were made at 25 °C.

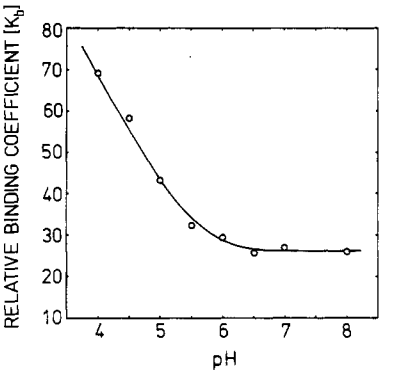


FIGURE 3: Binding of dansylglycine to membrane bilayers. DMPC vesicles, prepared in 62 mM sodium chloride and diluted in the same medium to final lipid concentrations in the range 5–100 mg/mL, were dialyzed against dansylglycine ($10^{-5}$ M) in isotonic sodium phosphate buffers of defined pH. Dialysis was carried out for 12 h at 23 °C with constant gentle agitation provided by slow rotation of the microdialysis cells used for the experiment. Following dialysis, duplicate 50-$\mu$L aliquots were taken from both the vesicle suspension and the dialysate. The samples were diluted in *n*-dioxane, and the fluorescence emission intensity (500 nm) of each sample was determined and used to calculate relative binding coefficients as described under Materials and Methods.

protonation state of both dissociable groups regulates the relative concentration of the neutral species (c), which thus varies with external pH. At pH 6.0 the relative abundance of this uncharged species is approximately 1:3000; at pH 8.0 this ratio becomes 1:300 000. Figure 3 shows that dansylglycine binds more readily to lecithin bilayers at pH 4 than at pH 8. It is not practical to measure binding below pH 4 because of the complications induced by protonation of the zwitterionic phospholipid molecules comprising the bilayer itself; as a consequence it becomes difficult to determine which protonation site of the dye is primarily responsible for the observed binding profile. It seems reasonable to speculate that neutralization of the carboxylate moiety increases the affinity of the dye for membranes and that the effects of the positive charge introduced by protonation of the dimethylamino group are minimized by extensive charge delocalization in the naphthalene ring system. It is significant to the present study that dansylglycine fluorescence and membrane binding properties are independent of pH above pH 6.0.

*Influence of pH on Passive Diffusion of Dansylglycine across Membranes.* Not only will dansylglycine bind to

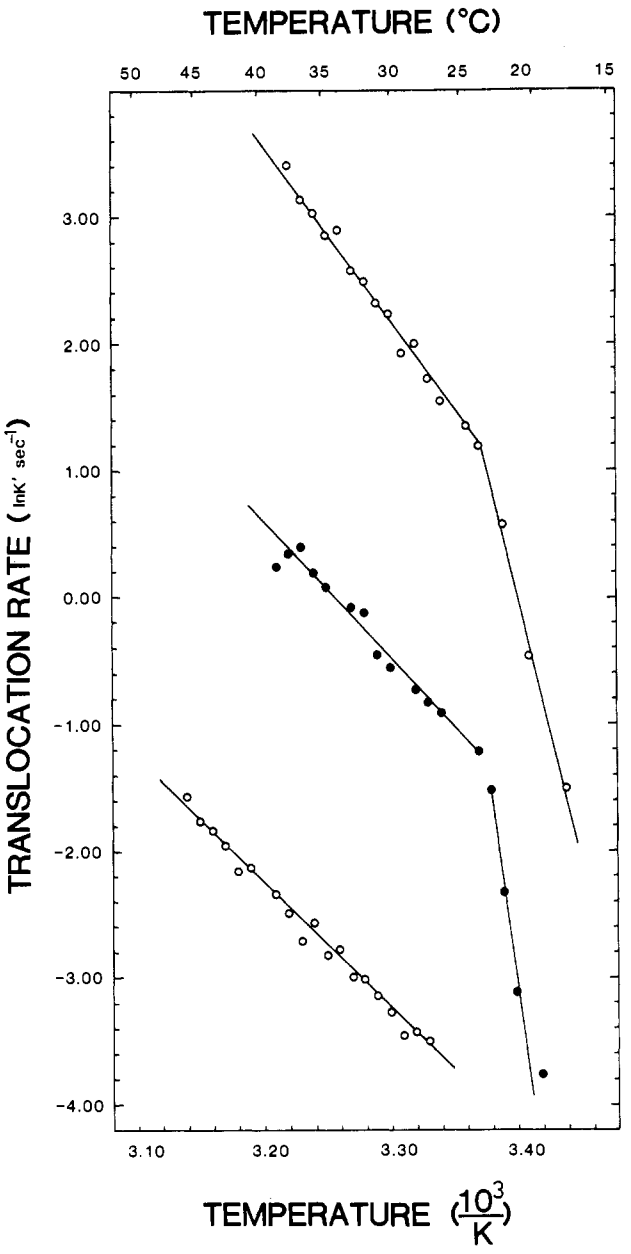


FIGURE 4: Dependence of translocation rate on temperature. Sample fluorescence intensity was monitored continuously as small DMPC vesicles were diluted into an aqueous solution of dansylglycine ($5 \times 10^{-7}$ M) to give a final lipid concentration of 1 mM with a probe:lipid ratio of 1:2000. After the initial, rapid increase in fluorescence intensity, attributable to dye adsorption to the external surfaces of the lipid vesicles, there was a subsequent, slower phase of fluorescence increase. The kinetics of this second phase were measured over a range of experimental temperatures. These data were used to calculate a rate constant for dansylglycine translocation across the vesicle bilayer at each temperature. The data are presented plotted in the form of an Arrhenius function. Measurements were made at three different pH values (defined by 100 mM sodium citrate/pyrophosphate buffer both inside and outside the vesicles): upper curve, pH 4.0; middle curve, pH 6.6; lower curve, pH 8.0. Fluorescence emission was monitored at 510 nm (excitation at 350 nM).

phospholipid bilayers, but the molecule will also permeate readily across them. I have previously demonstrated how fluorescence measurements can be used to monitor the kinetics of this translocation phase for dansylglycine, its weak base counterpart dansylethylenediamine, and a variety of related dyes (Bramhall, 1984, 1986). Figure 4 shows such bilayer translocation kinetics for dansylglycine diffusing across boundary bilayers of small unilamellar DMPC vesicles; dye translocation kinetics are markedly influenced by pH, rates

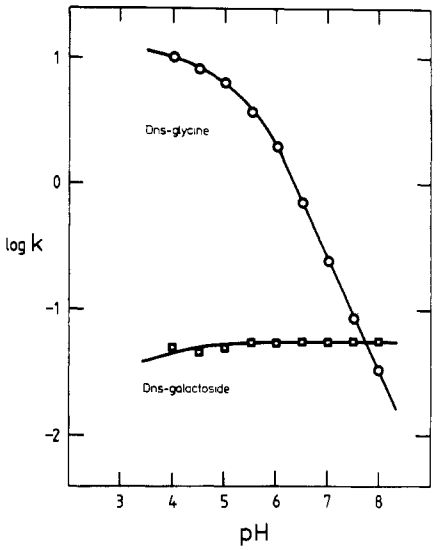


FIGURE 5: Dependence of translocation rate on pH. The rate constant for translocation of dansylglycine (○) and dansyl galactoside (□) across the boundary bilayers of small, unilamellar DMPC vesicles was measured at several pH values (defined by the same 100 mM sodium citrate/pyrophosphate buffer inside and outside the vesicles). All measurements were made at 30 °C. In the figure, the logarithm (base 10) of the translocation rate is plotted vs. experimental pH.

being directly proportional to proton concentration. Even though the dye translocation kinetics measured at pH 4 are orders of magnitude faster than those measured at pH 8, the overall similarity of the temperature dependence profiles under these different experimental conditions suggests that the mechanism of dye permeation is the same at pH 4 as at pH 8; the effect of pH is quantitative not qualitative. Furthermore, the temperature dependence profile indicates that dansylglycine permeates as an electrically neutral species (Bramhall, 1984, 1985).

Figure 5 indicates the relationship between pH and translocation rate for dansylglycine diffusing across DMPC bilayers. Rates show a linear dependence on proton concentration at pH >6.0, below this pH the protonation state of the dimethylamino function becomes a significant factor in determining rate. The data for dansylglycine contrasts with that for dansyl galactoside, which has no dissociable acid function and which shows little dependence on free proton concentration above pH 6.

Because the neutral species of dansylglycine permeates across the bilayer so much more rapidly than any dissociated species, it is to be expected that when a pH gradient exists across a bilayer, the permeation rates for dansylglycine will be different in the two directions across the membrane. In sealed vesicle system in which the trapped pH is higher than the external pH, dye will permeate into the vesicles faster than it permeates out, leading to an equilibrium accumulation of dye, which is reflected in an increase in sample fluorescence intensity. At equilibrium

$$\mathrm{pH} = \log \frac{[D^-]_i}{[D^-]_o} \qquad (3)$$

where $[D^-]_i$ and $[D^-]_o$ are the relative concentrations of the dansylglycine anion inside and outside the vesicle, respectively. Since $[D^-]_o$ is constant in any given experiment and the quantity of membrane-bound dye is directly proportional to the dye concentration of the aqueous phase, any increase in fluorescence intensity from the sample must result primarily from the increased amount of dye partitioning into the inner

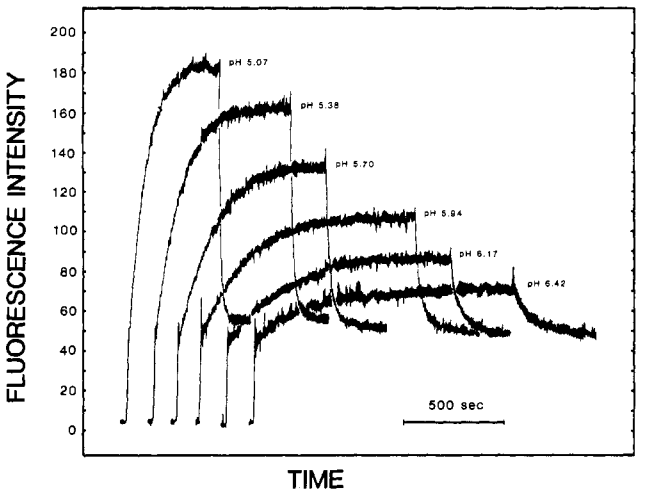


FIGURE 6: Response of dansylglycine to transmembrane pH gradients. Small, unilamellar DMPC vesicles containing 100 mM sodium pyrophosphate, pH 8.0, were diluted into 100 mM sodium pyrophosphate buffers, of varying pH, containing dansylglycine ($5 \times 10^{-7}$ M). The experimental temperature was 19 °C, and the final concentration of lipid was 1 mM. Fluorescence emission intensity (510 nm) was recorded continuously during the vesicle dilution and during a subsequent addition of ammonium acetate (final concentration 50 mM).

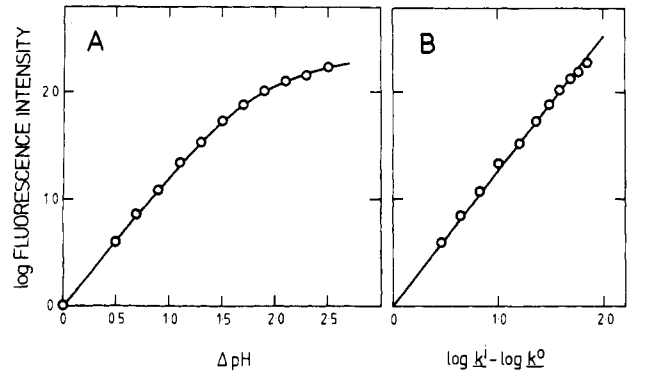


FIGURE 7: Dansylglycine fluorescence is directly proportional to the magnitude of transmembrane proton concentration gradients. Small, unilamellar DPPC vesicles containing 100 mM sodium phosphate (pH 8.0) were diluted into 100 mM sodium phosphate buffers, of varying pH, each containing $5 \times 10^{-7}$ M dansylglycine. The final lipid concentration was 1 mM, and the experimental temperature was 45 °C. In panel A the difference between the initial maximum fluorescence intensity (measured 5 s after mixing of vesicles with dye) and the final fluorescence intensity (measured after the addition of sodium acetate to a final concentration of 50 mM) is plotted vs. experimental pH. In panel B the same fluorescence values are plotted vs. inward ($k^i$) and outward ($k^o$) dye flux ratios at the various internal and external pH values used. Dye translocation rates for each pH were derived from the data given in Figure 5.

monolayer of the vesicle bilayer as $[D^-]_i$ increases; as a consequence

$$\Delta pH = k \log \Delta F_m \quad (4)$$

where $\Delta F_m$ is the intensity difference in fluorescence signal intensity from the dye resident in the vesicle boundary bilayer measured in the absence and presence of a pH gradient, and $k$ is an empirical constant of proportionality which will vary according to the response characteristics of the instrument used to detect fluorescence intensity. Figures 6 and 7 show that the relationship described by eq 4 is observed and that sample fluorescence intensity, which is dominated by emission from membrane-bound dye, is indeed directly proportional to the magnitude of the existing transmembrane proton concentration gradient. In Figure 6 each trace shows two phases of fluorescence increase initiated by the addition of lipid vesicles,

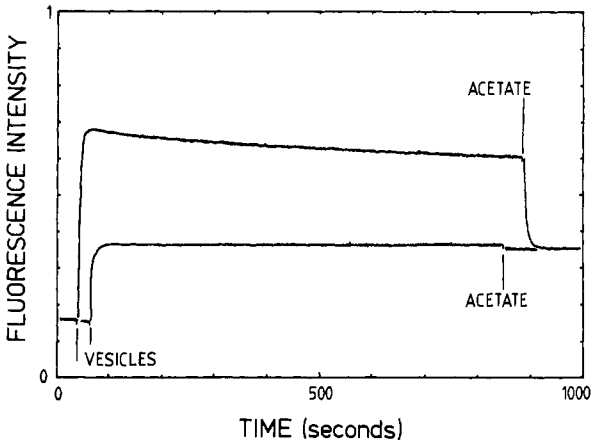


FIGURE 8: Dansylglycine reports the rate of collapse of proton gradients. Small, unilamellar DPPC vesicles suspended in 100 mM sodium phosphate buffer, pH 8.0, were diluted (1:100) into 137 mM sodium phosphate, pH 6.0 (upper trace), or into 100 mM sodium phosphate, pH 8.0 (lower trace). In each case the diluting buffer contained dansylglycine ($5 \times 10^{-7}$ M) and was maintained at a temperature of 42 °C. The fluorescence intensity of each sample was monitored continuously during and after addition of the vesicles and the subsequent addition of sodium acetate, to a final concentration of 25 mM.

the first being an instantaneous increase as dye binds (rapidly) to the external monolayer. The fluorescence intensity at the end of this first phase is only slightly influenced by the pH of the medium, which is to be expected from the data presented in Figure 3. The second phase of fluorescence intensity increase is much slower. Both the final equilibrium fluorescence intensity and the equilibration rate are markedly influenced by the external pH and are reversed by the addition of ammonium acetate, which is known to collapse transmembrane pH gradients in these vesicles (Bangham et al., 1967) without changing the external pH. Figure 7 illustrates the relationship between the decrease in fluorescence intensity triggered by the addition of ammonium acetate (collapse of pH gradient) and the magnitude of the pH gradient in existence immediately prior to that addition; over the pH range 6–8 the relationship is linear (data not shown), and over the range 4.5–7 (shown in Figure 7A) the relationship is nonlinear when $\Delta pH > 1.5$. However, Figure 7B shows that, even over these extremes, the $\Delta F_m$ associated with any given $\Delta pH$ correlates well with the value which would be predicted by the ratio of dye translocation kinetics at the two extremes of pH.

## Conclusions

For work with model membrane systems composed of small lipid vesicles, dansylglycine and its weak base counterpart dansylethylenediamine offer several distinct advantages over existing $\Delta pH$ probes, particularly when the internal trapped aqueous volume of a vesicle suspension is exceptionally small. The dye is freely soluble in water but only weakly fluorescent until it encounters the low polarity environment of the bilayer; this means that background fluorescence from the aqueous buffer suspending vesicles is minimized. Both dansylglycine and dansylethylenediamine permeate freely and rapidly across most lipid bilayers (the danylglycine equilibration half-time is <1 s with DMPC bilayers at 24 °C and pH 6) which makes them useful for monitoring relatively rapid changes in $\Delta pH$ (Figure 8). The response of dansylglycine to $\Delta pH$ shows little dependence on vesicle diameter because the feature being measured is the increase in dye concentration in the membrane phase rather than accumulation, and concomitant quenching, of dye in the vesicle lumen.

Registry No. DPPC, 2644-64-6; proton, 12586-59-3; dansylglycine, 1091-85-6.

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# Structure, Evolution, and Tissue-Specific Synthesis of Human Apolipoprotein AIV†

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ABSTRACT: Apolipoprotein AIV (apoAIV) is a protein of the lipid transport system found associated with chylomicrons, high-density lipoprotein (HDL), and the lipoprotein-free fraction of the plasma. The gene coding for the human apoAIV is closely linked with the genes coding for apolipoproteins AI (apoAI) and CIII (apoCIII). In this paper a nearly full-length apoAIV cDNA clone has been isolated by screening an adult human liver DNA library using a human apoAIV gene probe. In-frame translation of the cDNA sequence in this clone indicated that the human apoAIV consists of 396 amino acid residues including a 20 residue long signal peptide. The coding region of this cDNA sequence contains 15 nucleotide repeats, 11 of which code for amino acid repeats with potentials of forming amphipathic helices. Alignment and comparison of the human and rat apoAIV amino acid sequences indicated a five-residue deletion near the carboxy terminus of the rat protein. This comparison also indicated that these proteins are 61.8% homologous, suggesting that the rate of evolution of apoAIV is 65 accepted point mutations (PAMs) per 100 residues per 100 million years. The rates of evolution of certain amino acid repeats in apoAIV are higher than the rate of evolution of the entire protein. However, the corresponding, computer-generated, secondary structures and hydropathy profiles of these repeats are very similar between the human and rat apoAIV. The relative steady-state levels of apoAIV mRNA in various human and monkey tissues were determined by hybridization blotting analysis of total RNA from these tissues using a human apoAIV cDNA probe. This analysis showed that only fetal and adult intestine and adult but not fetal liver contain detectable amounts of apoAIV mRNA. These results indicate that the apoAIV gene evolved by amplification of an ancestral 66-bp sequence coding for a peptide with amphipathic properties and that conservation of the secondary structure and hydropathic properties of certain domains in apoAIV may be significant for the function(s) of this protein. Furthermore, these results indicate that in humans and nonhuman primates apoAIV mRNA synthesis occurs primarily in intestine while in liver apoAIV mRNA synthesis may be regulated by developmental and/or nutritional factors.

Apolipoprotein AIV (apoAIV) is a 46 000-dalton protein found initially in rat high-density lipoprotein (HDL) and chylomicrons (Swaney et al., 1974; Roheim et al., 1976) and later in human chylomicrons, very low density lipoproteins (VLDL), and in the $d > 1.21$ g/mL plasma fraction (Weisgraber et al., 1978; Beisiegel & Uterman, 1979; Green et al., 1979). Following ultracentrifugation, the majority of human apoAIV dissociates from lipoproteins and is found in the lipoprotein-free fraction of the plasma. In contrast, approximately 50% of rat and dog apoAIV remains associated with HDL under similar ultracentrifugation conditions (Roheim et al., 1976; Weisgraber et al., 1978). It has been reported that in rats the liver and intestine contribute 59% and 41%, respectively, in the plasma apoAIV pool (Wu & Windmueller, 1979). Intestinal synthesis of apoAIV has also been demonstrated in humans (Green et al., 1980). Newly synthesized intestinal apoAIV associates with chylomicrons and in humans

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