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Fluorescent Probes for Asymmetric Lipid Bilayers: Synthesis and Properties in Phosphatidyl Choline Liposomes and Erythrocyte Membranes

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Summary. We have synthesized three sets of fluorescent probes which we believe will be useful in studies of asymmetric membranes and have studied their interactions with model lipid bilayers and erythrocyte membranes. The probes were designed to partition preferentially into one face of a lipid bilayer with asymmetrically disposed phospholipids and to report lipid transitions in that monolayer.

We synthesized more than twenty probes containing anthroyl-, dansyl-, or pyrene rings with acidic, basic, and neutral functional groups and alkyl "spacers" of various lengths. The interactions of these probes with liposomes of phosphatidyl choline and with erythrocyte membranes were characterized to determine whether probe insertion was asymmetric, how deeply the probe penetrated the bilayer, and whether the probe reflected thermotropic phase transitions in model membranes. The set of variously charged anthroyl esters, analogs of local anaesthetics, appears to be promising for studies of asymmetric membranes.

Fluorescent probes have been used extensively to provide information on the lipid regions of biological membranes. Membrane fluidity, a composite of molecular packing and motion of acyl chains in lipid bilayers, has been assessed with a variety of fluorescent probes, the fluorescence of which undergoes some measurable change at the temperature of the membrane's thermotropic phase transition. A large number of fluorescent probes have been used for this purpose. Bashford, Morgan and Radda (Bashford, C.L., Morgan, C.G., Radda, G.K. 1976; *Biochim. Biophys. Acta* 426:157) and Thulborn and Sawyer (Thulborn, K.R., Sawyer, W.H. 1978; *Biochim. Biophys. Acta* **511**:125) synthesized several fatty acid derivatives in which an anthracene group is attached (in ester linkage) along the acyl chain at various positions, and have shown that this set of probes may be useful in probing membrane fluidity at different *depths* within the bilayer.

This report describes the synthesis and properties of several sets of amphipathic fluorescent probes, which may partition unequally into the two faces of an asymmetric lipid bilayer, and may therefore provide information about membranes complementary to that obtainable with existing probes.

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Fluorescent probes have been used extensively to provide information on the lipid regions of biological membranes (Azzi, 1975; Radda & Vanderkooi, 1972; Radda, 1975). This report describes the synthesis and properties of several sets of amphipathic, asymmetrically-directed fluorescent probes.

The phospholipids of several biological membranes are known to be asymmetrically disposed in the two faces of the bilayer (Rothman & Lenard, 1977). In erythrocytes, for example, the inner face of the bilayer is relatively rich in phosphatidyl ethanolamine and phosphatidyl serine and the outer face is composed mainly of phosphatidyl choline and sphingomyelin (Bretscher, 1972; Verkleij *et al.*, 1973; Bloj & Zilversmit, 1976). One consequence of this asymmetry is that the phospholipids of the inner face bear a net negative charge. A wide variety of membrane-active, hydrophobic ions interact with the erythrocyte membrane, causing characteristic shape changes of two distinctive types (Deuticke, 1968). Sheetz and Singer (Sheetz & Singer, 1974; Sheetz & Singer, 1976; Sheetz, Painter & Singer, 1976) have interpreted these effects in terms of a bilayer couple hypothesis: hydrophobic ions partition preferentially into that face of the erythrocyte bilayer in which their charged groups have the most favorable electrostatic interactions with the phospholipid headgroups. Lipophilic cations (such as tetracaine and other local anesthetics) enter the inner (negatively charged) face of the bilayer, expanding it relative to the outer face and forcing a change in cell shape ("cup formation"), while lipophilic anions stay in the outer monolayer, producing a crenated cell. We designed sets of probes, the members of which have similar fluorophores, but which bear different substituents intended to direct their preferential insertion into one face of an asymmetric lipid bilayer and report asymmetrically upon membrane properties. Specifically, we sought to determine whether the probes in a given set probed to the same depth of the bilayer, whether members of each set were equally sensitive to perturbations of lipid state such as phase transitions, and whether their distribution in the asymmetric lipid bilayer of a biological membrane was that predicted by the bilayer couple hypothesis. Since local anesthetics played a prominent role in the development of this hypothesis, a set of variously charged local anesthetic derivatives was also synthesized. We compared the effects of these compounds on erythrocyte shape with the effect predicted by the bilayer couple hypothesis to test the model.

A similar approach has been taken using spin-labeled probes; Wisnieski and Iwata (1977) compared a ubiquitously-distributing lipid probe

(a nitroxide derivative of decane) with a probe with a large polar head group (glucosamine derivative of 12-nitroxide stearic acid) which was postulated to prevent probe flip-flop. This approach indicated the existence of different physical states of lipid in the inner and outer monolayers of some animal cell surface membranes. In another study using yeast, Lepoch *et al.* (1975) showed that a sulfonic acid and a quaternary amine derivative of spin-labeled hexadecane stayed in the outer monolayer, while a similar but uncharged derivative apparently was not asymmetrically distributed.

Sheetz and Singer (1974) suggested that some membrane functions might be sensitive to changes in one side of the lipid bilayer, and our own experiments (Browning & Nelson, 1976) have indicated that excitability may be one such function. Lee (1976) recently described a model according to which local anesthetics block electrical excitability in neurons by perturbing lipids in the boundary layer surrounding ion-gating proteins. We hope to use these probes in exploring the role of lipids in membrane excitability and the effects of local anesthetics on that process.

Materials and Methods

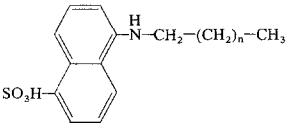
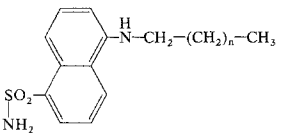
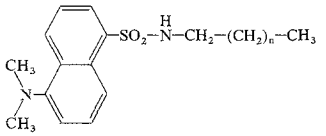
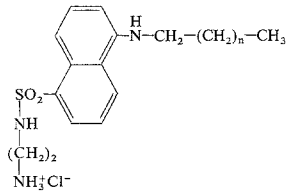
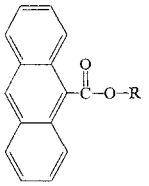
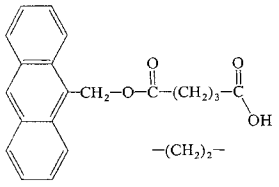
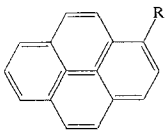
Thin layer chromatography of the various derivatives was performed on silica plates using the following solvent systems: (A) chloroform:propanol (1:4 vol/vol), (B) chloroform:methanol (4:1 vol/vol), (C) Skellysolve B (petroleum ether bp 60 to 68 °C):diethyl ether:glacial acetic acid (80:30:1 vol/vol), (D) methanol:acetone:triethylamine (100:100:3 vol/vol), (E) ethanol:glacial acetic acid:water (60:30:10 vol/vol), (F) ethanol:dioxane:benzene:NH_4OH (5:40:50:5 vol/vol), (G) chloroform:acetone (4:1 vol/vol). Visualization of spots was accomplished by fluorescence, I_2 vapor, H_2SO_4-charring, and ninhydrin spray. Ultraviolet, infrared, and NMR spectra were obtained with a Beckman Model 25 spectrophotometer, Perkin-Elmer Model 567 spectrophotometer, and a Varian T-60 spectrometer, respectively. Melting points are uncorrected. Elemental analyses (single determinations) were done by Microtech Labs (Skokie, Ill). All compounds were dried extensively *in vacuo* before analysis.

Table 1 gives the structures and abbreviations of the synthesized compounds.

N,N-Dimethyl 6-aminohexanol was obtained from K & K Fine Chemicals and 6-chloro-1-hexanol was purchased from Aldrich. N-alkyl acohols and amines were distilled when necessary before use. 4-(1-Pyrenyl) butyric acid (Eastman) was purified by chromatography over silica gel as described for 4-(1-pyrenyl) butyramide (XXV), final mp 188 to 188.5 °C. β-γ-Dimyristoyl-L-α-phosphatidyl choline and β-γ-dipalmitoyl -L-α-phosphatidyl choline were obtained from Sigma. β-γ-Distearoyl -L-α-phosphatidyl choline was purchased from Calbiochem. All lipids were used without further purification.

Fluorescence spectra were recorded with an Aminco Bowman corrected spectrophotometer in the relative energy mode. Samples were purged with nitrogen for 10 min before spectra were taken. Fluorescence measurements were made on either an Aminco Bowman SPF spectrofluorimeter or an Aminco-Keirs spectrophosphorimeter and no spectral correc-

Table 1

Structure		Abbrev.	Number
	$n=4$	6-ANSA	I
	8	10-ANSA	II
	14	16-ANSA	III
	$n=4$	6-ANSAM	IV
	8	10-ANSAM	V
	14	16-ANSAM	VI
	$n=4$	6-DANSA	VII
	8	10-DANSA	VIII
	14	16-DANSA	IX
	$n=4$	6-ANASAM	X
	8	10-ANASAM	XI
	14	16-ANASAM	XII
		PA	XIII
		SA	XIV
		DMAEA	XV
	$R=-(CH_2)_2-CH_3$	TEAEA	XVI
	$-(CH_2)_2-SO_3H$	HA	XVII
	$-(CH_2)_2-\overset{+}{N}(CH_3)_2HCl$	DMAHA	XVIII
	$-(CH_2)_2-\overset{+}{N}(CH_3CH_2)_3I^-$	TEAHA	XIX
	$-(CH_2)_5-CH_3$		
	$-(CH_2)_6-\overset{+}{N}(CH_3)_2HCl$		
	$-(CH_2)_6-\overset{+}{N}(CH_3CH_2)_3I^-$		
		AMHA	XX
		AMHS	XXI
	$R'=-(CH_2)_3-COOH$	PBA	XXII
	$-(CH_2)_3-CONH_2$	PBAD	XXIII
	$-(CH_2)_3-CH_2-NH_2$	PBAM	XXIV
	$-SO_3H$	PS	XXV
	$-NH_2$	AP	XXVI

tions were made. Plots of fluorescence *vs.* temperature were obtained directly on a *x-y* recorder. Sample temperature was controlled by a circulating water bath connected to a jacketed cuvette and monitored (±0.5 °C) inside the cuvette with a linear thermistor (Yellow Springs Instruments). Liposomes were prepared by drying a chloroform/methanol solution containing 2 mg of phospholipid and 20 µg of the desired probe (dissolved in methanol) with a stream of nitrogen. This results in a phospholipid/probe ratio of 100/1 (wt/wt). Tris-Cl buffer (0.01 M, pH 7.5 containing 0.1 M KCl) was added (2 ml) and the

solution sonicated for 12–15 min. The suspension was maintained above the lipid transition temperature during sonication by circulating water at 55 °C through a jacketed chamber.

Fresh rabbit venous blood was collected in the presence of 0.1 M Na_3citrate. The cells were gently washed three times in 0.05 M Na_3citrate, 0.01 M Tris-Cl buffer, pH 7.5, and the buffy coat was removed. Protection from osmotic lysis by various probes was tested by adjusting a solution of the wash buffer with 0.01 M Tris-Cl buffer such that 40–60% of the erythrocytes were lysed in the absence of any probe (generally the final dilution was to 0.025 M Na_3citrate, Tris-Cl buffer). An aliquot (0.2 ml) of the stock cell solution (10^9 cells/ml) was added to 2.5 ml of the hypotonic solution containing the diluted probe. The solution was immediately vortexed and, after 5 min, centrifuged for 2 min at $1500 \times g$. The absorbance of the supernatent fluid was measured at 543 nm. Control samples gave an absorbance of 0.3–0.5 OD. Probes were dissolved in either methanol or methanol/DMSO (the NASA derivatives required titration into methanol with pyridine) and diluted into the lysis buffer. Controls were checked for any effect of the solvent on erythrocyte lysis.

For the cell shape studies, fresh human venous blood was prepared as described for rabbit erythrocytes. Cells were exposed to the probe in 0.05 M Na_3citrate for 90–120 sec, placed on siliconized glass slides and cover slips, and examined by phase contrast microscopy. DMSO is effective in eliciting a cell shape change and was not used as a solvent in these studies. The levels of methanol and pyridine used in these experiments did not affect cell shape.

Synthesis

Since the local anesthetic analogs (2-carbon series of anthracene derivatives) appeared to be most useful in our studies, we include synthetic details here; the synthesis of the other compounds is described in similar detail in the accompanying supplementary material.

9-Anthracene carboxylic Acid Chloride [A-ACl]

9-Anthracene carboxylic acid (5 g, 0.022 M), chloroform (20 ml, distilled from P_2O_5) dimethylformamide (0.25 ml) and thionyl chloride (5 ml, 0.067 M) were refluxed for 1 hr. The solvent was evaporated under vacuum. This preparation was used immediately for the synthesis of esters and amides.

Propyl 9-Anthroate (XIII)

A-ACl (1 g, 4.1 mM) was refluxed with *n*-propanol (5 ml, 0.066 M) for 20 min, then concentrated. The product was crystallized twice from acetone/water. Yield was 0.85 g of yellow crystals (72%) mp 68–70 °C. The NMR spectrum ($CDCl_3$) showed the following signals: δ 1.0 (*t*, $J=7$ Hz, 3H, $-CH_3$), 1.9 (sextet $J=7$H, 2H, $-CH_2-C-$), 4.6 (*t*, $J=7$Hz, 2H, $-O-CH_2-$) and 7.3–8.4 (*m*, 9H, ring). All 9 anthroyl esters were characterized by an infrared absorption at 1710 cm^{-1} (carbonyl). *Anal.* calcd for $C_{18}H_{16}O_2$: C, 81.70; H, 6.11; O, 12.10. Found: C, 81.98; H, 6.21; O, 12.05.

Sodium 2-Sulfoethyl 9-Anthroate (XIV)

A-ACl (1 g) was added to sodium isethionate (1 g, 6.7 mM) in dry pyridine (5 ml) and refluxed with stirring for 1 hr. The solution was diluted with 20 ml of water, acidified

to pH 4.0 with concd HCl and extracted with chloroform. The chloroform solution was concentrated and extracted twice with 0.5 volumes of aqueous $NaHCO_3$ (5%). The aqueous extracts were allowed to stand at 4 °C for 1 day. Yellow crystals (0.85 g, 74%) were collected. The product was recrystallized from methanol/water. The mp was 281–284 °C. The NMR spectrum (D_2O) showed the following signals: δ 3.9 (*t*, $J=7$ Hz, $-CH_2-SO_3Na$), 5.4 (*t*, $J=7$ Hz, $-CH_2-O-$), and 7.5–8.5 (*m*, 9H, ring). The δ 5.4 signal represents a 22-Hz shift downfield following formation of the ester. *Anal.* calcd for $C_{17}H_{13}O_5S$ Na: C, 57.94; H, 3.72; O, 22.90; S, 9.10. Found: C, 56.60; H, 3.59; O, 22.78; S, 8.73.

2-Dimethylaminoethyl 9-Anthroate Hydrochloride (XV)

A-ACl (16 g, 0.065 M), dry benzene (100 ml) and N,N dimethyl amino ethanol (7.2 ml, 0.071 M) were refluxed for 90 min. The benzene was removed by evaporation and the residue was extracted twice with 100 ml of acetone. The acetone was evaporated to dryness; the resulting residue was taken up into 0.1 M HCl and lyophilyzed. The dried HCL salt was recrystallized twice from methanol. Yield 2.5 g (11%) of yellow crystals, mp 202–203 °C. The NMR spectrum (d_6-DMSO) showed the following signals: δ 2.7 (*s*, 6 Hz, $-N(CH_3)_2$) 3.6 (*t*, $J=6$ Hz, 2H, $-CH_2-N$), 4.8 (*t*, $J=6$ Hz, 2H, $-CH_2-O-$) and 7.3–8.6 (*m*, 9H, ring). *Anal.* calcd for $C_{19}H_{20}NO_2Cl$: C, 69.18; H, 6.12; N, 4.25; O, 9.70. Found C, 68.92; H, 6.19; N, 4.33; O, 9.96.

2-Triethylaminoethyl 9-Anthroate Iodide (XVI)

This compound was synthesized as previously described by Cohen, *et al.* (1974). A-ACl (5 g, 0.021 M), 2-chloroethanol (10 ml, 0.16 M), and chloroform (10 ml, distilled from P_2O_5) were refluxed for 1 hr, extracted with aqueous sodium bicarbonate, dried over sodium sulfate, and evaporated to dryness. The 2-chloroethyl 9-anthroate was taken up into acetone (50 ml), sodium iodide (6 g, 0.067 M) added, and the solution refluxed 20 hr. The NaCl was filtered off and the solution concentrated by evaporation. The residue was extracted with chloroform washed with aqueous $NaHSO_3$, dried over Na_2SO_4, and concentrated by evaporation. The product, 2-iodoethyl 9-anthroate was crystallized from chloroform/ethanol. 2-iodoethyl 9-anthroate (2 g, 5.3 mM), triethylamine (5 ml), and ethanol (10 ml) were refluxed 12 hr. The precipitate which formed on cooling was collected and recrystallized from ethanol. Yield 1.2 g (47%) of pale yellow needles, mp 228–9°. The NMR spectrum (d_6-DMSO) showed the following signals: δ 1.2 (*t*, $J=9$ Hz, 9H, $-CH_3$), 3.5 (*q*, $J=8$ Hz, 6H, $N(CH_2-CH_3)_3$, 4.0 (*t*, $J=5$ Hz, 2H, $-CH_2-N$), 5.2 (*t*, $J=5$ Hz, 2H, $-CH_2-O$) and 7.5–8.7 (*m*, 9H, ring). *Anal.* calcd for $C_{23}H_{28}NO_2I$: C, 57.85; H, 5.92; N, 2.93; O, 6.70. Found: C, 57.69; H, 5.93; N, 2.92; O, 7.55.

Results and Discussion

The R_f values from TLC of all compounds are shown in Table 5 (*Appendix*). These compounds are intended for use in applications where small signals will be detected through signal averaging. To be certain that small fluorescence changes (less than 1%) are not due to impurities, we purified these probes extensively with respect to other fluorescent impurities. Generally, no fluorescent spots other than the desired product

Table 2. Spectral properties of fluorescent probes

Compound	Absorbance[a]		Fluorescence[b] emission maximum in liposomes (nm)	Fluorescence emission maximum in methanol (nm)	Equivalent[c] dielectric constant
	λ_{max}	$\varepsilon \times 10^{-3}$			
6-ANSA	279	6.4	487	485	37
	334	1.9			
6-DANSA	247	14.1	529	535	36
	330	4.2			
6-ANSAM	256	16.5	519	538	15
	370	4.8			
6-ANASAM	256	15.4	526	550	9
	370	4.1			
PA	357	7.3	475	488	16
SA	357	7.1	470	488	9
DMAEA	357	7.1	470	488	9
TEAEA	357	7.4	478	488	20
HA	357	7.3	468	488	8
DMAHA	357	7.3	465	488	5
TEAHA	357	7.6	465	488	5
AMHA	360	7.5	395–461[d]	417	–
PBA	273	32.6	380–402–484	385–400	–
	339	35.6			
PBAD	273	33.0	380–402–484	385–400	–
	339	37.9			
PBAM	273	31.2	380–402–484	385–400	–
	339	32.8			

[a] All spectra are in methanol.

[b] Probe/phospholipid ratio was 1:100 at a final lipid concentration of 1 mg/ml.

[c] From Fig. 1, the dielectric constant correlating with the fluorescence emission maximum in dipalmitoyl phosphatidyl choline liposomes.

[d] Multiple emission peaks for AMHG, PBA, PBAD, and PBAM; the same compounds are relatively insensitive to the solvent polarity.

could be found in any of the TLC systems. Table 2 presents some ultraviolet absorption and fluorescence properties of these compounds. All the 9-anthroyl esters as well as the hexyl, decyl, and hexadecyl derivatives of the ANSA compounds had identical spectra and similar extinction coefficients.

The naphthalene derivatives were prepared from the 1,5 isomer of amino naphthalene sulfonic acid rather than the 2,6 isomer for which the Bucherer reaction proceeds more smoothly, because of the more desirable fluorescence properties of this isomer and the commercial avail-

ability of 1,5 dansyl chloride. We desired a molecule more like a fatty acid than a fluorescent dye (such as ANS or NPN), yet sufficiently mobile in the bilayer that it could orient preferentially in one face of the bilayer on the basis of charge interactions. The properties of derivatives containing a long hydrocarbon chain (i.e., 16 carbons) in a lipid bilayer would be dominated by the largely hydrophobic character of the molecule. We have synthesized probes with varying chain lengths, so that we could explore the effects of hydrophobicity upon probe distribution.

The pK_a of an acid or base can differ substantially depending upon the nature of its environment. While pK_a values were not determined for these compounds in either aqueous or nonpolar solvents, the expected values in an aqueous environment for the naphthalene derivatives are: ANSA, 0.5–0.6 (pK_a of naphthalene sulfonic acid), DANSA 4.0–4.5 (aromatic amine, pK_a of dimethylamino-naphthalene), ANSAM approx. 10 (sulfonamide) and ANASAM approx. 10 (both sulfonamide and alkyl amine). We do not expect the pK_a's of the groups (in water) on the other probes to deviate from the normal pK_a values for alkyl amines, carboxylic acids, and sulfonic acids.

How are the Probes Situated in the Membrane?

The wavelength of maximum fluorescence emission for each of the anthracene and naphthalene derivatives increasing with increased solvent dielectric constant (Fig. 1) as expected (Radda, 1975). When these same probes were cosonicated with phosphatidyl choline to form liposomes, their emission spectra indicated an environment less polar than water, and from the data in Fig. 1, we could assign an "equivalent dielectric constant" (Table 2). Seven anthracene derivatives (all except AMHA) showed relatively low equivalent dielectric constants, suggesting that the fluorophore of these probes is deeply buried in the bilayer. These data are similar to the results of Waggoner and Stryer (1970), who studied 9-anthroyl isopropane. Our 9-anthroyl derivatives with a six-carbon "spacer" appeared to have their anthroyl group in a more hydrophobic environment than the analogous compounds with two-carbon "spacers." This may mean that these probes (except HA and PA, which lack a polar group) orient with their charged groups at the bilayer surface in the region of phospholipid head group, with the anthroyl moiety extending into the hydrocarbon region. On this basis, we suppose that the anthroyl esters report from the same region of the bilayer.

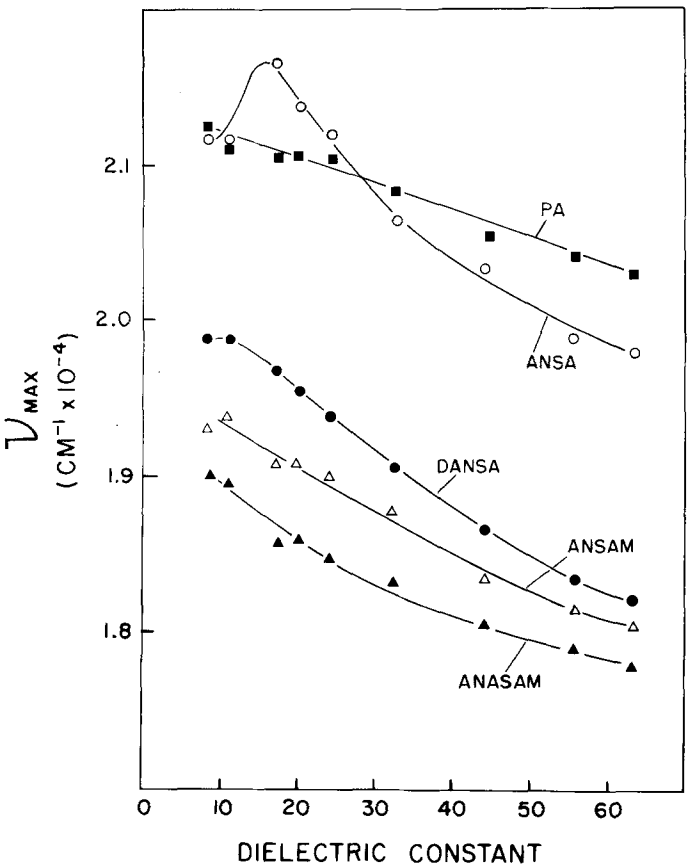


Fig. 1. Wavelength of maximal fluorescence emission (ν_{max}) as a function of the dielectric constant of the solvent. Emission spectra were recorded from each probe dissolved in *n*-decanol, *n*-octanol, *n*-butanol, *n*-propanol, ethanol, methanol and methanol/water mixtures 3:1, 1:1 and 1:2 (vol/vol). Probe concentrations were: AP (-■-) 4.7×10^{-5} M; 6-DANSA (-●-) 3.6×10^{-5} M; 6-ANSAM (-△-) 4.1×10^{-5} M; 6-ANASAM (-▲-) 3.2×10^{-5} M; and 6-ANSA (-○-) 4.0×10^{-5} M

Among the amino naphthalene sulfonic acid derivatives (ANSA series), the ANSA and DANSA fluorophores appear to be located in a more hydrophobic environment in the bilayer than either ANSAM or ANASAM. These differences are much larger than the error in separate experimental determinations, which is less than 10%.

When liposomes were prepared in D_2O, the fluorescence of several probes was enhanced over that in H_2O (Table 3), but the degree of enhancement was much less than expected if the fluorophores were directly accessible to the aqueous phase. The fluorescence of the 9-anthroyl, 9-methylene anthracene and 1-alkyl pyrene fluorophores was not en-

Table 3. Effect of D_2O on probe fluorescence

Compound	Relative fluorescence ratio D_2O/H_2O[a]		
	In solution	In liposomes	% "exposed"[b]
6-ANSA	2.1	1.1	9
6-ANSAM	4.1	1.4	13
6-ANASAM	4.1	1.5	16
6-DANSA	2.0	1.1	10

[a] Excitation and emission wavelengths were the same for measurements in water and liposomes. Wavelengths were as given in Fig. 2.

[b] $\%\ \text{exposed} = \left[\frac{\text{relative fluorescence}\,(D_2Q)-1}{\text{relative fluorescence}\,(H_2O)-1}\right] \times 100.$

hanced by D_2O. By this same criterion, all four of the ANSA probes tested were less than 20% exposed to H_2O. For comparison, the widely-used probes ANS (8-anilino naphthalene-1-sulfonic acid) and MNS (N-methyl 6-anilino naphthalene-2-sulfonic acid) showed D_2O enhancements indicating 60 and 30% exposure, respectively (Radda, 1975). These results suggest that the naphthalene derivatives are situated in the lipid bilayer with the fluorophore in the lipid bilayer but close to the polar head groups at the surface. X-ray diffraction studies on ONS (N-octadecyl 2-amino naphthalene 6-sulfonic acid) showed the sulfonic acid moiety located by the phosphate of the phospholipid head group and the chain fully extended (Lesslauer *et al.*, 1972). The spectral shift data obtained for ANSA agree well with the results obtained by Waggoner and Stryer (1970) for the related compound, ONS. Our spectral shift data for the ANSA series of fluorophores were identical with those presented by Greene (1975) for a similar set of amino naphthalene sulfonic acids. The wave-length of maximal fluorescence emission was insensitive to solvent polarity for the pyrene derivatives and AMHA.

Do the Probes Report Lipid Transitions?

Overath and Träuble (1973) and recently others have used fluorescent probes extensively in monitoring phase transitions in bilayers, and we have used their approach to characterize the sensitivity of each probe to changes in lipid structure during thermotropic phase transitions. When the anthracene or naphthalene derivatives were incorporated into phosphatidyl choline liposomes, there was a gradual decrease in fluorescence intensity with increasing temperature (Figs. 2–4) relatively independent

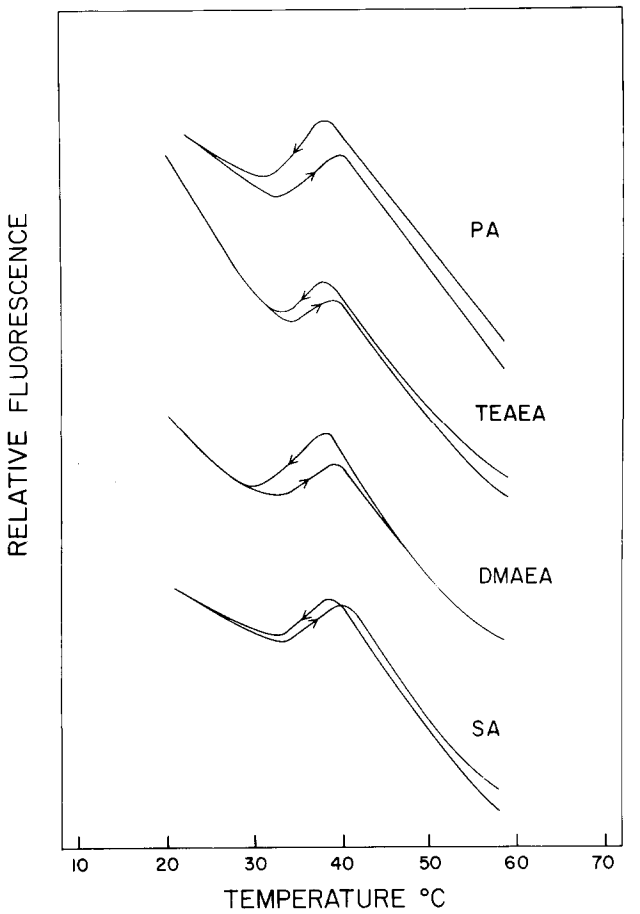


Fig. 2. Effect of charge on fluorescence of 9-anthroyl esters during the phase transition of PC vesicles. Dipalmitoyl-PC vesicles were prepared as described in *Methods*, the excitation/emission wavelengths were 370:450 nm

of the direction of the temperature scan. With most of the probes there was a marked discontinuity in the plot of fluorescence intensity *vs.* temperature at the temperature of the phospholipid phase transition. We studied these effects in liposomes composed of dimyristoyl, dipalmitoyl, and distearoyl phosphatidyl choline, which have been previously shown by spin-labeling techniques to undergo phase transitions at 23, 41, and 54 °C, respectively (Tadbrook & Chapman, 1969).

A. *9-Anthroyl Esters*. All members of the 9-anthroyl series were similar in their ability to monitor the phase transition (Fig. 2). Results very similar to those shown in Fig. 2 were obtained with HA, DMAHA and TEAHA (not shown), but AMHA, which lacks a polar substituent

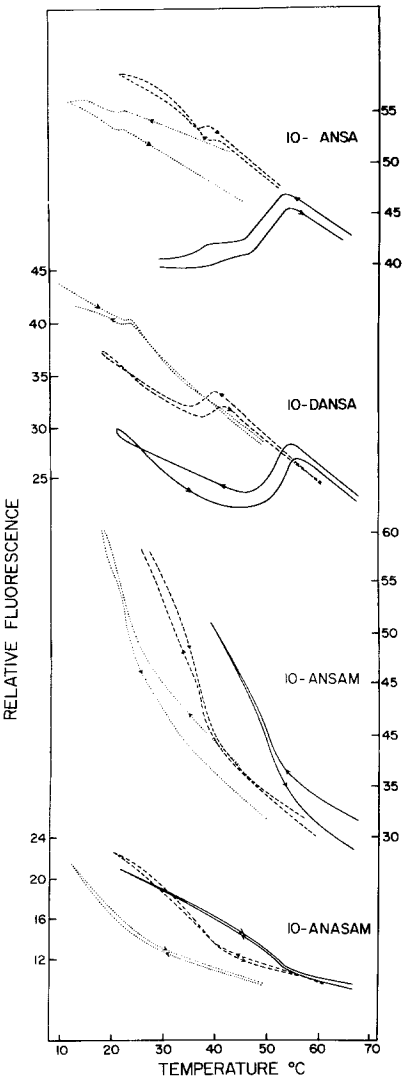


Fig. 3. Fluorescence of naphthalene derivatives in liposomes during thermal phase transitions. Liposomes containing each probe were prepared as described in *Methods*, using liposomes composed of distearoyl-PC (—), dipalmitoyl-PC (– – –), or dimyristoyl-PC (...). The excitation/emission wavelengths were: ANSA, 348:490 nm; DANSA, 345:530 nm; ANSAM, 368:522 nm; and ANASAM, 368:525 nm

on the fluorophore (the fluorescence emission maximum is insensitive to solvent polarity) showed no discontinuity in the region of the phase transition (not shown). Because they all give essentially similar reports, we believe these probes would be suitable for studies of bilayer asymmetry.

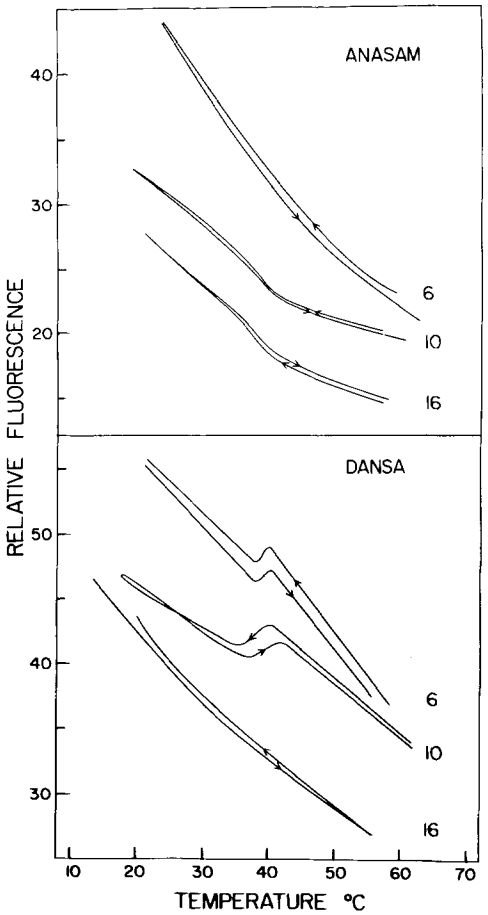


Fig. 4. Effect of alkyl chain length upon fluorescence of ANASAM and DANSA during the thermal phase transition. Dipalmitoyl-PC liposomes containing 6-, 10-, or 16-ANASAM or 6-, 10-, or 16-DANSA were prepared as described in *Methods*, and relative fluorescence was measured at the emission maximum for each probe. Numbers refer to the chain length

B. *Amino Naphthalene Sulfonic Acid Derivatives.* Each of the naphthalene derivatives (ANSA, DANSA, ANSAM and ANASAM) with 10-carbon hydrocarbon chains showed discontinuities in the fluorescence-temperature plot. The exact shapes of the curves depended upon the nature of the substituents (Fig. 3). The shape of this plot was similar for ANSA and DANSA; the two sulfonamide probes, ANSAM and ANASAM, also had similar plots, yet differed from those observed for NANSA and DANSA. The effects of varying the chain length of the hydrocarbon portion of ANASAM and DANSA are shown in Fig. 4. ANASAM (and ANSAM, not shown) displayed larger changes at the

phase transition the longer the alkyl chain relative to the thickness of the bilayer; the largest changes were seen with 16-ANASAM and 16-ANSAM in dimyristoyl-PC liposomes. In contrast, DANSA (and also ANSA, not shown) reported the phase transitions more effectively the shorter the alkyl chains, 6-ANSA or 6-DANSA in distearoyl-PC liposomes giving the largest changes at the phase transition.

Since little is known concerning which parameter(s) (quantum yield, fluorescence spectral shift, etc.) of the probe fluorescence changes during the phase transition, it is difficult to discuss the differences between the various naphthalene derivatives (especially since these probes have different fluorophores). The differing effects of increasing the alkyl chain length on the ability of ANSAM and ANASAM *vs.* ANSA and DANSA probes to report on the phase transition, suggests that these fluorophores are sensing different regions of the bilayer. ANSA and DANSA show temperature-fluorescence profiles similar to those reported for ANS and NPN (N-phenyl naphthalene) (Overath & Träuble, 1973). The ANSAM and ANASAM profiles are similar to that reported for 1-palmitoyl-2-α-parinaroyl lecithin (Sklar *et al.*, 1977). Membrane-bound ANS is believed to be located in the head-group region (Radda, 1975), while the α-parinaroyl derivative is undoubtedly sensing a phase transition in the hydrocarbon chain region. By comparison with these probes and on the basis of the spectral shift data for ANSAM and ANASAM, these two probes appear to sense a more hydrophobic region of the bilayer. Likewise, ANSA and DANSA must be reporting on a phase transition in a region closer to the phospholipid head groups.

When the alkyl chain of ANASAM or ANSAM could extend into the opposite face of the bilayer (i.e., 16-ANSAM in dimyristoyl-PC membranes), the probe was most sensitive to the phase transition. These results can be rationalized if these probes report primarily from the hydrophobic region, the long alkyl chain tending to provide a better anchor into this region. When the alkyl chain of ANSA or DANSA could extend into the opposite face (i.e., 16-ANSA in dimyristoyl -PC membranes), the fluorophore did not sense the phase change. Possibly, changes in the hydrocarbon region offset fluorescence changes in the head-group region.

C. *Pyrene Series: Excimer Formation.* Pyrene excimer formation has been used as a probe of membrane fluidity in several types of membranes (Galla & Sackmann, 1974, 1975; Vanderkooi & Callis, 1974). Pyrene excimer ("excited dimer") formation in PC liposomes was monitored by measurement of the ratio of fluorescence intensities at the emission

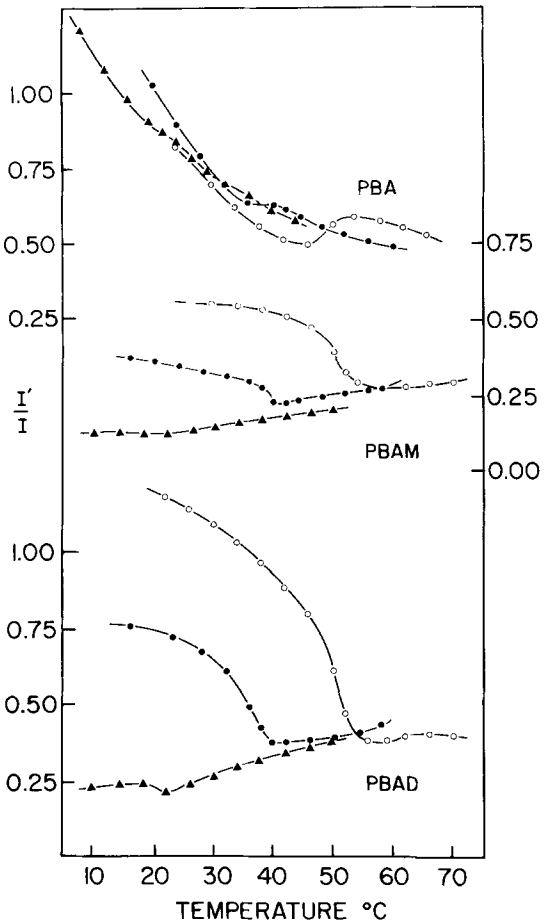


Fig. 5. Excimer formation in PC liposomes by various pyrene derivatives as a function of temperature. Liposomes containing each probe were prepared as described in *Methods*. Excimer formation was quantitated by measuring the ratio of fluorescence intensity at the dimer wavelength (480 nm), I', and the monomeric wavelength (380 nm), I. Excitation wavelength was 330 nm. Liposomes were composed of distearoyl-PC (–▲–), dipalmitoyl-PC (–●–), and dimyristoyl-PC (–○–)

wavelength of the dimer (480 nm) and monomer (380 nm). A marked discontinuity occurred in the excimer *vs.* temperature plots for all three compounds (PBA, PBAD, and PBAM), at the temperature of the phase transition (Fig. 5). Excimer formation as a function of temperature differs extensively among these three pyrene derivatives. The basic temperature dependence of excimer levels in the fluid phase (above the transition temperature) of PBAM and PBAD differed from PBA. Excimer concentration increased with increasing temperature of PBAM and PBAD, while excimer levels decreased with increasing temperature for PBA.

PBA has an excimer-temperature profile similar to that previously described for pyrene and 10-(1-pyrenyl) decanoic acid (Galla & Sackmann, 1974, 1975).

If the excimer ration, I'/I, is plotted as a function of probe concentration in dipalmitoyl PC liposomes at 55 °C, one obtains a linear plot (at low pyrene concentration), the slope of which is an index of the tendency of the probe to dimerize. We did such analyses with our probes (J. Browning, *unpublished results*) and found that pyrene formed excimers 10 times and PBAD 4 times more readily than PBA or PBAM. These results indicate that the charge of the pyrene substituent may be important in dimerization. If charge were the principle determinant, one might expect that the excimer-temperature plot for pyrene and PBAD (uncharged molecules) should be similar to each other and different from the profiles of PBA and PBAM (cases where charge-charge interactions may influence dimerization). The excimer-temperature profiles we obtained did not fit this simple pattern; possibly the charge of the pyrene substituent influences dimerization by some other mechanism than charge repulsion. Excimer formation was not observed in either AP or PS.

Interaction of Probes with Erythrocytes: Asymmetric Insertion of Probes? The bilayer couple hypothesis predicts that amphipathic cations will cause *cup-formation* in erythrocytes and that amphipathic anions will produce *crenation*. The hypothesis also predicts that quaternary amines with their fixed positive charge will be crenators, whereas tertiary amines, which can diffuse to the inner face of the bilayer as the uncharged species, will be cup-formers. Previous studies (Deuticke, 1968; Bieri, Wallach & Lin, 1974; Yoshida & Ikegami, 1974; Fortes & Ellory, 1975) showed that many hydrophobic anions induce erythrocytes to assume a crenated ("bumpy", echinocyte) shape, and that many hydrophobic cations cause erythrocytes to assume a deeply invaginated "cup" shape. The effects of two membrane probes, the spin-labeled fatty acid 5-nitroxide stearic acid and the fluorescence probe ANS on erythrocyte shape and hypotonic lysis have been described (Bieri *et al.*, 1974; Fortes & Ellory, 1975). Both the anionic ANS and 5-nitroxide stearic acid were crenators. Fortes and Ellory interpreted the action of ANS on erythrocytes as due to asymmetric insertion of the probe into the outer face of the bilayer. We tested each of our fluorescent probes for its ability to induce such shape changes by adding it to suspensions of human erythrocytes and observing the treated cells in the light microscope. Some, but not all, of our results fit these predictions (Table 4 and Fig. 6).

Many membrane-active amphipathic compounds protect erythrocytes

Table 4. Summary of the effects of variously charged fluorescent probes on erythrocytes: Protection from hypotonic lysis and cell shape

Compound	Lysis protection		Effect on cell shape	Expected ionic form
	Maximal (%)[a]	Optimal (μM)		
6-ANSA	0	–	crenator	anionic
10-ANSA	40	1–3	crenator	anionic
16-ANSA	0	–	crenator	anionic
6-DANSA	30	8–10	cup-former	neutral
10-DANSA[b]	–	–	–	neutral
16-DANSA	–	–	–	neutral
6-ANSAM	90	80–100	no effect	neutral
10-ANSAM	5	1–2	no effect	neutral
16-ANSAM	0	–	no effect	neutral
6-ANASAM	90	80–100	crenator	cationic
10-ANSAM	5	15–25	crenator	cationic
16-ANASAM	10	2–4	no effect	cationic
PA	40	>10	no effect	neutral
SA	10	10	crenator	anionic
DMAEA	100	150	cup-former	cationic
TEAEA	>80	>3000	crenator	cationic
HA	0	–	no effect	neutral
DMAHA	88	60–80	crenator	cationic
TEAHA	92	150	crenator	cationic
AMHG	>70	>1000	cup-former	anionic
	>40	>1000	cup-former	anionic
PBA	>70	>200	no effect	anionic
PBAM[b]	94	30–40	crenator	cationic
AP	–	–	cup-former	cationic
PS	–	–	crenator	anionic

[a] % = (1 − maximal rel. hemolysis)/0.01.
[b] PBAD, 10-DANSA and 16-DANSA were not tested due to poor solubility.

from hypotonic lysis, apparently by intercalating into the lipid bilayer and expanding its area (Seeman & Weinstein, 1966; Seeman, 1966, 1972; Bieri *et al.*, 1974; Fortes & Ellory, 1975). Drug-induced shape changes and protection from hypotonic lysis appear to have similar concentration dependencies (Bieri *et al.*, 1974; Sheetz & Singer, 1974). We have used the effects of the probes on hypotonic lysis as an indication of the concentration ranges in which the probes bind the erythrocyte membrane (Fig. 7).

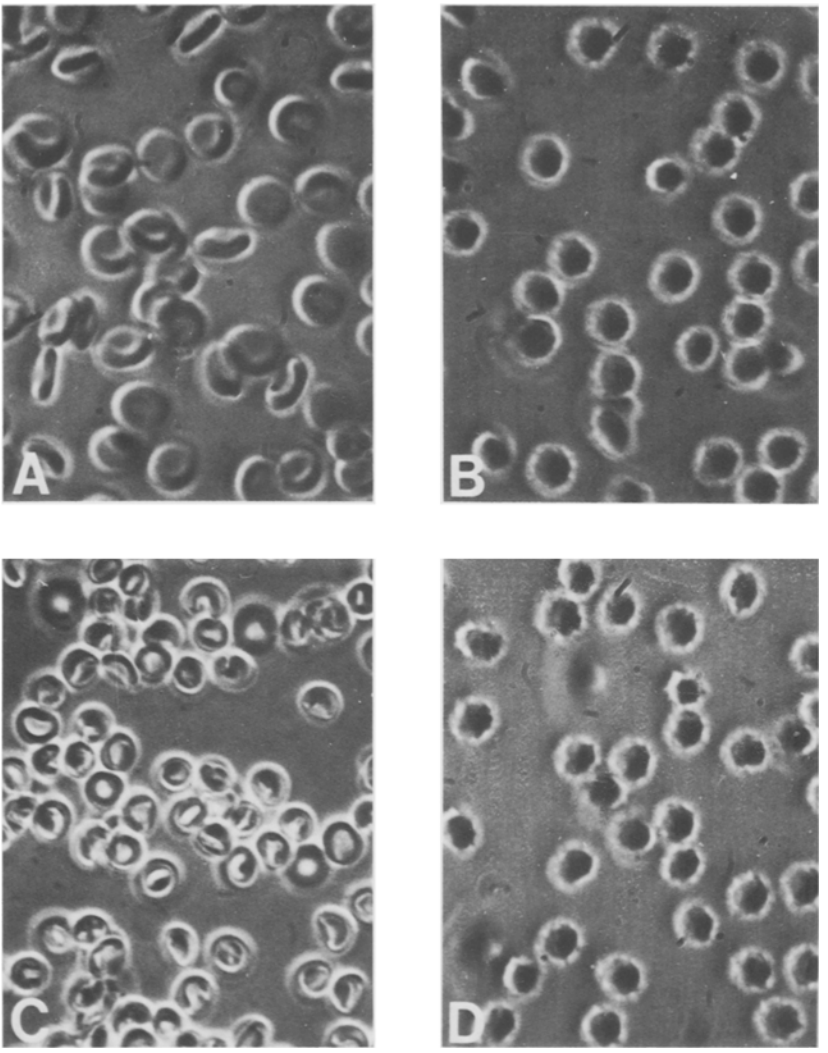


Fig. 6. Shape changes in erythrocytes induced by 9-anthroyl esters. Cells were examined by phase contrast microscopy at a magnification of 1000×, 1.5 to 2.0 min after addition of probe. (*A*): Untreated control. (*B*): SA, 3×10^{-4} M. (*C*): DMAEA, 6×10^{-4} M. (*D*): TEAEA, 5×10^{-4} M. *B* and *D* show typical crenated shape, and *C* shows cup formation

A. *9-Anthroyl esters*. In the 9-anthroyl ester set of probes, compounds with a 2-carbon separation between the charged group and the 9-anthroyl moiety (SA, DMAEA, and TEAEA) behaved *exactly* as predicted. The tertiary amine (DMAEA) was an efficient cup-former, and the quaternary amine (TEAEA) and the sulfonic acid (SA) were crenators (Table 4 and Fig. 7). However, compounds in the same set which have a 6-carbon

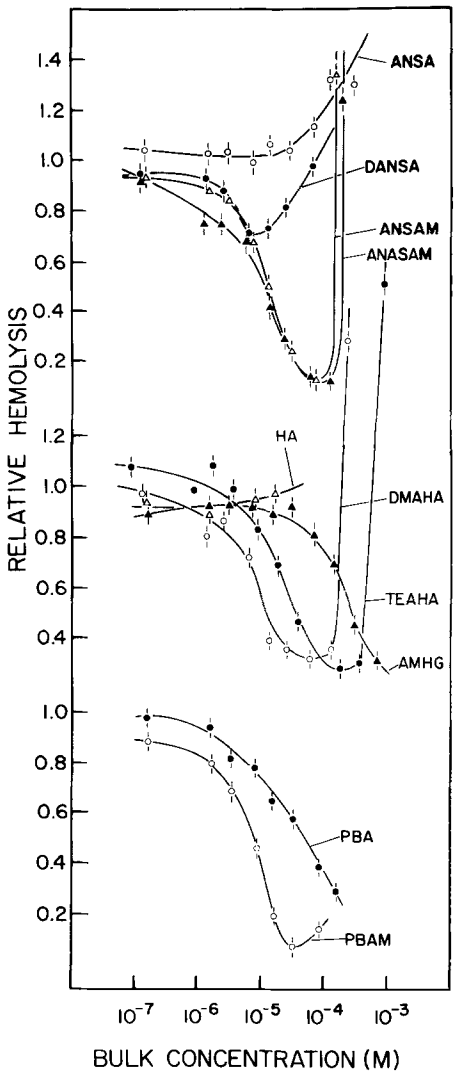


Fig. 7. Protection against hypotonic lysis of erythrocytes by fluorescent probes. Erythrocytes were diluted into a hypotonic solution containing the probe and the extent of hemolysis, measured as described in *Methods*, was compared with that of cells diluted into the hypotonic solution without probe. Relative hemolysis is the ratio of hemolysis in the presence of probe to the amount of hemolysis of the control sample. Bars represent the range of duplicate determinations. The naphthalene derivatives used were 6-ANSA, 6-DANSA, 6-ANSAM and 6-ANASAM

spacer between the charged group and the 9-anthroyl moiety (AMHA and DMAHA) did not fit the predictions: AMHG (anionic) was a cup-former and DMAHA (tertiary amine), a crenator. Likewise, a one-carbon-shorter analog of AMHA (AMHS) was also a cup-former.

Table 5. Thin-layer chromatography of fluorescent probes

Compound		R_f values solvent system						
		A	B	C	D	E	F	G
6-ANSA	I	0.59	0.34	0.00		–	–	0.00
6-ANSAM	IV	0.76	0.87	0.02	–	–	–	0.60
6-DANSA	VII	0.72	0.88	0.20	–	–	–	0.92
6-ANASAM	X	0.41	0.23	0.00	–	–	–	0.00
Anthracene-9-carboxylic acid	–	–	–	0.18	0.77	0.90	0.12	0.44
PA	XIII	–	–	0.69	0.89	0.94	0.92	0.74
SA	XIV	–	–	0.00	0.86	0.88	0.00	0.00
DMAEA	XV	–	–	0.00	0.74	0.53	0.84	0.00
TEAEA	XVI	–	–	0.00	0.07	0.47	0.00	0.00
HA	XVII	–	–	0.91	0.82	0.82	0.97	0.89
DMAHA	XVIII	–	–	0.00	0.47	0.45	0.87	0.00
TEAHA	XIX	–	–	0.00	0.17	0.39	0.09	0.04
AMHA	XX	–	–	0.10	0.51	0.77	0.06	0.38
PBA	XXI	–	0.80	0.25	0.45	0.83	–	–
PBAD	XXII	–	0.74	0.05	0.81	0.81	–	–
PBAM	XXIII	–	0.37	0.00	0.59	0.71	–	–

Compounds with tertiary amine substituents (DMAEA and DMAHA) protected best against hypotonic lysis (Fig. 7); these are the probes that most closely resemble local anesthetics in structure. Quaternary amines (TEAHA and TEAEA) were much less effective than their tertiary amine analogs [as previously shown by Sheetz and Singer (1974) for similar compounds]. Increasing the chain length separating the 9-anthroyl moiety from the charged portion of the molecule from two (DMAEA) to six carbons (DMAHA) lowered the concentration for maximal protection by an order of magnitude, indicating much better binding to the membrane. The anionic derivatives, AMHG and SA, were the least effective in protecting erythrocytes from hypotonic lysis.

These studies of cell-shape changes suggest strongly that the anthroyl esters with 2-carbon spacers do in fact partition asymmetrically into erythrocytes, as we had hoped they would.

B. *Amino naphthalene sulfonic acid derivatives.* The naphthalene series of compounds did not affect erythrocyte shape in the expected manner. The sulfonic acid derivative ANSA was a potent crenator as expected; however, the cup-forming property of DANSA was unexpected. Much of the data in this paper concerning DANSA could be explained by assuming that DANSA is in a protonated form in the bilayer. As the

aqueous pK_a for the dimethyl amino naphthalene group is 4.0–4.5, it is conceivable that this group could be protonated in a lipid environment. However, all the fluorescence data must be attributed to the free amine because the protonated form of this derivative is nonfluorescent (Pesce, Rosen & Pasby, 1971). The crenating properties of ANASAM were not expected, but the structure of this compound (several polar groups: an aryl amine, sulfonamide, and a free amine) complicates any prediction of the behavior of this compound. In the naphthalene series, 6-ANASAM and 6-ANSAM were most effective in protecting against hypotonic lysis (Fig. 7), acting optimally at 80–100 μM concentrations. The 10- and 16-carbon analogs of these two compounds were less and much less effective, respectively (Table 4).

C. *Pyrene series.* Among the pyrene derivatives, those compounds without a hydrocarbon spacer (AP and SA) behaved as predicted, but PBAM (with the spacer) was a crenator although expected to induce cup formation, and PBA, expected to be a crenator, had no effect.

Conclusions

The set of probes composed of 9-anthroyl esters with 2-carbon spacers (SA, PA, DMAEA, and TEAEA) are suitable for studies of asymmetric bilayers. They differ in charge at their "head groups," but apparently probe the bilayer to the same depth, and they give the same signal as membranes undergo a phase transition. Their effects upon erythrocyte shape strongly suggest asymmetric partitioning into the two faces of the cell membrane. Furthermore, these probes are analogs of local anesthetics, and may be useful in studies of local anesthetic action. The several naphthalene derivatives apparently do not all probe the same region of the membrane; the members of this set differ from one another in the shifts they induce in fluorescence emission maxima, they respond differently to phase changes in liposomes, and have different effects upon hypotonic lysis of erythrocytes. The two sulfonamides in this set (ANSAM and ANASAM) probably are situated in a more hydrophobic region of the bilayer than the other naphthalene derivatives. The naphthalene derivatives are therefore not well suited for studies of asymmetry.

Pyrene excimer formation has been used in studies of membrane fluidity, and we hoped our several pyrene derivatives would show preferential partitioning into one face of the bilayer. However, the anomalous

effects of charge on excimer formation in liposomes, and our evidence that asymmetric partitioning does not occur, suggest that this set of probes will also not be useful for our intended purposes.

The bilayer couple hypothesis allows predictions of the effects of amphipathic compounds with differently-charged "head groups" upon erythrocyte shape, and it was of some interest to determine whether our probes gave the predicted shape changes. Those probes with bulky rings (anthracene, pyrene, and naphthalene) linked through a *long* alkyl chain to a charged group induced shape changes not predicted by the hypothesis. It may be that the important determinant of shape changes is perturbation of the bilayer near the surface, in the region of the lipid head groups. Those probes that perturb in a more hydrophobic environment (deeper in the bilayer), such as the local anesthetic analogs with 6-carbon spacers, pyrene butyric acid derivatives, and possibly ANA-SAM) would not then be expected to fit the predictions of the bilayer couple hypothesis. Our results with the pyrene derivatives agree with this interpretation. PBAM, the compound with a spacer between ring system and amino "head group," was a crenator, but 1-amino pyrene (AP) caused cup formation as predicted. Similarly, PBA, the anion with a spacer, had little effect upon cell shape, but 1-pyrene sulfonic acid was a potent crenator. Thus when the perturbing group was close to the surface (again assuming the charged moiety orients the molecule in the bilayer) the probe's behavior was as predicted by the bilayer couple model.

Jain *et al.* (1976) reached a similar conclusion from theoretical considerations and a study of the effects of various adamantane derivatives on the phase transition in dipalmitoyl phosphatidyl choline bilayers. They postulated that a solute-induced perturbation in the transition would be maximal when the solute molecule is located nearest the phospholipid head group. Cadenhead *et al.* (1977) studied the effects of several anthroyl derivatives of fatty acids on monolayers and liposomes, and also concluded that a bulky group in the center of the bilayer perturbed bilayer structure less than the same group near the surface.

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Appendix

5-Hexylamino-1-Naphthalenesulfonic Acid (I)

The Bucherer reaction was used for coupling alkyl amines to naphthalene sulfonic acids as described by Waggoner and Stryer (1970). 5-Amino naphthalene-1-sulfonic acid (40 g, 0.16 M), sodium bisulfite (120 g, 1.17 M), N-hexylamine (120 ml, 0.91 M) and 300 ml of water were heated (100 °C) for 24 hr. The reaction mix was cooled and upon addition of 300 ml of 10 M NaOH a black oil separated. The oil was added to 1.5 liter of heptane/triethylamine (10:1), boiled, and the resultant oil was collected. Chloroform (1 liter) was added and the solution was extracted with 1.5 liter of 3 M HCl. The organic phase (including interfacial material) was concentrated and the product was precipitated from propanol/HCl/H_2O (approx. 2:1:2) after standing at −20 °C. The product was dissolved into chloroform/methanol/triethylamine (1:2:0.01, 200 ml), neutralized with HCl and concentrated to 50 ml. Upon standing at −20 °C, 3.5 g (7.5%) of purple crystals formed (mp > 290 °C).

The NMR spectrum (d_6-DMSO) showed the following signals: δ 0.9 (terminal methyl), 1.2–1.5 (methylene groups of alkyl chain), 3.4 (*t*, $N-CH_2$) and 6.5–9.0 (*m*, ring). The $\delta = 3.4$ signal represents a shift in the N-α-methylene protons from 2.7 upon N-alkylation. *Anal.* calcd for $C_{16}H_{21}NO_3S$: C, 62.50; H, 6.89, N, 4.55; O, 15.61; S, 10.43. Found: C, 62.74; H, 6.82; N, 4.51; O, 15.98; S, 10.24.

5-Decylamino-1-Naphthalenesulfonic Acid (II)

5-Amino naphthalene-1-sulfonic acid (32 g, 0.13 M), sodium bisulfite (112 g, 1.1 M) *n*-decylamine (56 ml, 0.28 M), and 300 ml of water were heated at 100 °C for 24 hr. This mixture was not stirred, since stirring led to the formation of an emulsion which seriously hampered the work-up. The reaction mix was chilled, and a dark semisolid mass was collected by filtration, dissolved into 600 ml of (boiling) methanol/conc. HCl (10:1). After cooling to room temperature, the precipitate was suspended in boiling heptane/triethylamine (10:1) and stirred until all of the solid was converted to a dark oil. The oil was collected and dissolved in 200 ml chloroform and extracted with 400 ml of 3 N HCl. The suspension was chilled overnight and the precipitated free acid was collected. The product was recrystallized twice from chloroform/methanol/water (2:1:trace). Yield 6.4 g (14%) mp 288 °C. *Anal.* calcd for $C_{20}H_{29}NO_3S$: C, 66.07; H, 7.97; N, 3.85; O, 13.20; S, 8.52. Found: C, 66.11; H, 8.15; N, 3.89; O, 12.99; S, 9.02.

5-Hexadecylamino-1-Naphthalenesulfonic Acid (III)

The reaction was performed as for (*II*), with *n*-hexadecylamine (60 g 0.25 M) substituted for *n*-decylamine. Yield 2 g (5%). mp > 290. *Anal.* calcd for $C_{26}H_{41}NO_3S$; C, 69.74; H, 9.24; N, 3.12; O, 10.72; S, 7.16. Found: C, 70.13; H, 9.39; N, 3.05; O, 10.45; S, 6.76.

5-Hexylamino-1-Naphthalenesulfonamide (IV)

6-ANSA (1.5 g, 5.3 mM) was heated to 60 °C in pyridine (45 ml, dried over CaH_2) and phosphorus oxychloride (4.5 ml, 0.028 M) was added. After 20 sec, the sulfonyl chloride was quickly added dropwise with vigorous stirring to 300 ml of NH_4OH and 100 g of

ice. The suspension was stirred for 30 min, then left at 4 °C for 8 hr. The precipitated sulfonamide was collected by centrifugation. The pellet was taken up into acetone, filtered, and crystallized twice from acetone/water (−20 °C). If the sulfonic acid was exposed to phosphorus oxychloride for longer times, polymerization was found. Yield 1.2 g (75%), mp 124 °C. The NMR spectrum ($CDCl_3/d_6$-acetone) showed the following signals: δ 0.9 (terminal methyl), 1.2–1.5 (methylenes of fatty alkyl chain), 3.4 (*t*, $J=8$ Hz, 2H, $-CH_2-N$), 4.8 (broad 1H, $-NH-$), 5.9 (*s*, 2H, $-NH_2$) and 7.5–9.0 (*m*, 6H, ring). The infrared spectrum (KBr) showed loss of the sulfonic acid bands at 1030 and 1260 cm^{-1} and the appearance of a strong absorption at 1330 cm^{-1} (amide). *Anal.* calcd for $C_{16}H_{22}N_2O_2S$: C, 62.70; H, 7.25; N, 9.14; O, 10.44; S, 10.46. Found: C, 62.74; H, 7.30; N, 9.11; O, 10.41; S, 10.46.

5-Decylamino-1-Naphthalenesulfonamide (V)

10-ANSA was reacted as for *IV*. Yield 0.65 g (63%), mp 128–129 °C. *Anal.* calcd for $C_{20}H_{30}N_2O_2S$: C, 66.24; H, 8.35; N, 7.73; O, 8.82; S, 8.84. Found: C, 66.56; H, 8.43; N, 7.82; O, 9.45; S, 7.88.

5-Hexadecylamino-1-Naphthalenesulfonamide (VI)

16-ANSA was reacted as for *IV*. Yield 0.62 g (62%), mp 126 °C. *Anal.* calcd for $C_{26}H_{42}N_2O_2S$: C, 69.89; H, 9.49; N, 6.27; O, 7.16; S, 7.18. Found: C, 70.31; H, 9.60; N, 6.17; O, 7.30; S, 7.24.

5-Dimethylamino-Naphthalene-N-Hexylsulfonamide (VII)

n-Hexylamine (2.6 ml, 0.02 M) was added to dansyl-Cl (N,N dimethyl 5-amino naphthalene-1-sulfonyl chloride) (3.54 g, 0.013 M) in 30 ml of chloroform (distilled from P_2O_5) and 1.5 ml of triethylamine. The solution was allowed to stand for 2 hr, washed with 2 volumes of saturated aqueous $NaHCO_3$, dried over Na_2SO_4 and concentrated. The oil was taken up in methanol and upon addition of H_2O the product precipitated (−20 °C). Recrystallization from methanol/water yielded 3.6 g (78%), mp 48–49 °C.

The NMR spectrum ($CDCl_3$) showed the following signals: δ 0.9 (terminal methyl), 1.2–1.5 (methylenes of fatty chain), 2.8 (*s*, 6H, $-N-(CH_3)_2$), 4.9 (*t*, $J=6$ Hz, 2H, $-N-CH_2-$) and 7.0–9.0 (*m*, ring). The 4.9 signal was shifted from 2.7 (H_2N-CH_2, of *n*-hexylamine) upon dansylation. The dansyl derivatives appeared to decompose slowly (over weeks) on exposure to light. *Anal.* calcd for $C_{18}H_{26}N_2O_2S$: C, 64.67; H, 7.78; N, 8.38; O, 9.57. Found: C, 64.68; H, 7.79; N, 8.51; O, 9.80.

5-Dimethylamino-Naphthalene-N-Decylsulfonamide (VIII)

Synthesis was as for *VII* with *n*-decylamine substituted for *n*-hexylamine. Yield 3.5 g (77%), mp 61–62 °C. *Anal.* calcd for $C_{22}H_{34}N_2O_2S$: C, 67.70; H, 8.71; N, 7.17; O, 8.20. Found: C, 67.80; H, 8.88; N, 7.36; O, 8.30.

5-Dimethylamino-Naphthalene-N-Hexadecylsulfonamide (IX)

Synthesis was as for *VII* with *n*-hexadecylamine substituted for hexylamine. Yield 3.4 g (70%) mp 65–66 °C. *Anal.* calcd for $C_{28}H_{46}N_2O_2S$: C, 70.89; H, 9.70; N, 5.90; O, 6.74. Found: C, 71.31; H, 9.96; N, 5.96; O, 6.93.

5-Hexylamino-1-Naphthalene-N-(2-Aminoethyl)Sulfonamide (X)

6-ANSA (0.5 g, 1.6 mM) in dry pyridine (20 ml) was heated to 60 °C. Phosphorus oxychloride (1.5 ml, 6.2 mM) was added and after 20 sec the sulfonyl chloride was quickly added dropwise with vigorous stirring to 100 ml of ice cold ethylenediamine. The solution was allowed to warm to room temperature, then diluted with 700 ml of cold water. After standing overnight at 4 °C, the precipitate was collected by centrifugation. The pellet was dissolved in chloroform/methanol (4:1) and extracted once with 0.05 M NaOH, then with H_2O. The organic phase was dried over Na_2SO_4, concentrated, and the concentrate was put onto a silica gel column (Biosil A, 2.5×60 cm in chloroform/methanol, 4:1). The product was eluted with chloroform/methanol (2:1), and the fractions containing product were concentrated. Excess HCl was added (as a solution of acetyl chloride in methanol) and the entire solution concentrated and dried *in vacuo*. These ethylenediamine derivatives were found to be light-sensitive and rather unstable. Yield: 0.2 g (32%) of a dark gum. NMR (d_6-DMSO/$CDCl_3$) showed the following signals: δ, 0.9 (terminal methyl), 1.2–1.4 (methylenes of the fatty chains), 1.7 ($NH-CH_2-CH_2-C$), 2.7 ($-CH_2-NH_3Cl$), 2.9 (CH_2-NH-S), 5.9 (broad, $-NH$) and 7.0–8.5 (*m*, ring). Infrared spectrum (KBr) showed loss of the sulfonic acid absorptions at 1030 and 1260 cm^{-1} and appearance of a signal at 1330 cm^{-1} (sulfonamide). TLC showed no formation of NANSA polymer or the dimeric NANSA-ethylenediamine derivative. *Anal.* calcd for $C_{18}H_{29}N_3O_2SCl_2$: C, 51.21; H, 6.87; N, 9.95; O, 7.58; S, 7.59. Found: C, 51.91; H, 7.12; N, 9.65; O, 7.32; S, 7.82.

5-Decylamino-1-Naphthalene-N-(2-Aminoethyl)sulfonamide (XI)

Synthesis was as for *X* starting with 0.5 g of 10 ANSA. Yield 0.15 g (30%) of a deep red powder. *Anal.* calcd for $C_{22}H_{36}N_3O_2SCl_2$: C, 55.37; H, 7.54; N, 8.80; O, 6.70; S, 6.76. Found: C, 55.59; H, 7.95; N, 8.79; O, 6.99; S, 5.35.

5-Hexadecylamino-1-Naphthalene-N-(2-Aminoethyl)Sulfonamide (XII)

Synthesis was as for *XI* starting with 0.5 g 16-ANSA. Yield 0.15 g (30%) of a red-brown powder. *Anal.* calcd for $C_{28}H_{49}N_2O_2SCl_2$: C, 59.92; H, 8.55; N, 7.48; O, 5.70; S, 5.71. Found: C, 61.20; H, 9.09; N, 7.64; O, 7.01; S, 5.72.

Hexyl 9-Anthroate (XVII)

A-ACl (2.16 g, 9 mM), *n*-hexanol (5 ml, 0.039 M) and chloroform (5 ml, distilled from P_2O_5) were refluxed for 2 hr. Chloroform (100 ml) was added, the solution was extracted from $NaHCO_3$, dried over Na_2SO_4, and concentrated. The product was crystallized twice from acetone/water. Yield 1.0 g (37%) of yellow crystals, mp 36–37 °C. The NMR spectrum ($CDCl_3$) showed the following signals: δ 0.9 (*t*, 3H, $-CH_3$), 1.2–2.0 (methylenes), 4.6 (*t*, $J=7$ Hz, 2H, $-O-CH_2$) and 7.4–8.5 (M, 9H, ring). *Anal.* calcd for $C_{21}H_{22}O_2$; C, 82.30; H, 7.25; O, 10.44. Found: C, 81.62; H, 7.28; O, 10.69.

6-Dimethylaminohexyl 9-Anthroate Hydrochloride (XVIII)

A-ACl (4.3 g, 0.18 M), 6-dimethylamino hexanol (3.0 g, 0.027 M), chloroform (10 ml, distilled from P_2O_5), and triethylamine (2.0 ml) were refluxed for 2 hr. The reaction mix

was concentrated by evaporation and methanol (15 ml), water (5 ml) and concd HCl (2 ml) were added. This mixture was held at −20°, and a black oil was collected. The oil was taken up into chloroform and put onto a silica-gel column (2 × 30 cm). The column was washed with chloroform, and the product was eluted with chloroform/methanol (4:1). The fractions containing product were evaporated to approx. 3 ml, 1 ml of concd HCl was added, and the solution dried with an air stream to a yellow powder. Yield 2.5 g (38%), mp 183–4°. The NMR spectrum ($CDCl_3$) showed the following signals: δ 1.2–2.0 (methylenes), 2.9 (*s*, $N-(CH_3)_3$), 3.1 (CH_2-N), 4.8 (*t*, 2H, $-CH_2-O$), and 7.5–8.4 (*m*, 9H, ring). *Anal.* calcd for $C_{22}H_{28}NO_2Cl$: C, 70.65; H, 7.56; N, 3.74; O, 8.55. Found: C, 70.47; H, 7.44; N, 3.41; O, 11.08.

6-Triethylaminohexyl 9-Anthroate Iodide (XIX)

A-ACl (6 g, 0.025 M), 6-chloro-1-hexanol (2.5 ml, 0.027 M), and chloroform (50 ml, distilled from P_2O_5) were refluxed for 1 hr and concentrated by evaporation. Acetone (50 ml) and sodium iodide (10 g, 0.067 M) were added to the above oil and refluxed for 20 hr. NaCl was filtered off and the mixture was concentrated by evaporation. The residue was taken up into chloroform, extracted with aqueous $NaHSO_3$, and dried over Na_2SO_4. The chloroform was removed by evaporation, and the oily product, 6-iodo-1-hexyl 9-anthroate was taken up into 20 ml of ethanol. Triethylamine (7 ml) was added and the mixture refluxed for 2 hr. The solution was concentrated by evaporation, and the product was taken up into chloroform. The product was put onto a silica-gel column (2 × 45 cm) which was then washed extensively with chloroform/heptane (1:1) followed by chloroform/heptane (4:1). The product was eluted with chloroform/methanol (4:1). The fractions containing product were concentrated and dried *in vacuo*. Yield 2 g (16%) of a tan powder, mp 117–119 °C. The NMR spectrum ($CHCl_3$) showed the following signals: δ 1.1 (*t*, $J=8$ Hz, $-CH_2-CH_3$), 3.1 (*q*, $J=8$ Hz, 8H, $-N-(CH_2-CH_3)_4$), 4.6 (*t*, $J=6$ Hz, 2H, $-CH_2-O$) and 7.8–8.5 (*m*, 9H ring). Anal. calcd for $C_{27}H_{36}NO_2I$: C, 60.81; H, 6.75; N, 2.62; O, 6.00. Found: C, 60.14; H, 6.64; N, 2.44; O, 6.36.

9-Anthrylmethyl Hydrogen Glutarate (XX)

9-Anthraldehyde was synthesized from anthracene using the Vilsmeier reaction as previously described (Campaigne & Archer, 1953). The aldehyde was reduced in methanol with sodium borohydride to 9-hydroxymethyl anthracene (Stewart, 1960). 9-Hydroxymethyl anthracene (5 g, 0.024 M), glutaric anhydride (4 g, 0.035 M) and pyridine (30 ml) were refluxed for 1 hr. H_2O (20 ml) and concd HCl (15 ml) were added and the solution allowed to cool. The resultant oil was collected and upon addition of ethanol and a small amount of H_2O the product crystallized out (−20 °C). Recrystallization from chloroform/Skellysolve C and again from ethanol/H_2O yielded 3.7 g of yellow crystals (39%), mp 98–99°. The NMR spectrum showed the following signals: δ 1.9 (*q*, $J=7$ Hz, $-CH_2-CH_2-CH_2-$), 2.3 (*t*, $J=7$ Hz, 4H, $-CO-CH_2-$), 6.0 (*s*, 2H, $-CH_2-O$) and 7.2–8.4 (*m*, 9H, ring). Formation of the ester shifted the NMR signal of the 9-methylene function from $\delta=5.5$ to $\delta=6.1$. *Anal.* calcd for $C_{20}H_{18}O_4$; C, 74.50; H, 5.64; O, 19.85. Found: C, 74.73; H, 5.70; O, 20.03.

4-(1-Pyrenyl)Butyramide (XXII)

4-(1-Pyrenyl)-butyric acid (XXI, 2.5 g 8.6 mM), was reacted with thionyl chloride dimethylformamide and chloroform as described for A-ACl. After removal of the solvent

and excess thionyl chloride by evaporation, the acid chloride was taken up into 20 ml of ethanol free chloroform and added dropwise to a stirred solution of dry benzene (200 ml) at 0 °C, through which (dry) ammonia gas had been bubbled for 2 hr. The solution was brought to room temperature, stirred for 10 hr, and filtered. The precipitate was extracted with boiling methanol and the extracts were added to the benzene. The solution was evaporated and the residue taken up into chloroform/methanol (4:1). The amide was placed on a silica-gel column (2 × 30 cm) in heptane/acetone (1:1) and washed extensively with heptane/acetone (1:1). The product was eluted with chloroform. Yield 1.5 g of a pale yellow powder (60%) mp 180.5–1 °C. Infrared spectrum (KBr) showed loss of the ($-COOH$) absorption at 1690 cm^{-1} and appearance of the ($-CONH_2$) absorption at 1650 and 3400 cm^{-1}. The NMR spectrum (d_6-DMSO) showed the following signals: δ 2.0–2.6 (*m*, 4H, $-CH_2-CH_2-CO$), 3.4 (*t*, 2H, ring$-CH_2-$) and 7.8–8.4 (9H, ring). Anal calcd for $C_{20}H_{17}NO$: C, 83.58; H, 5.97; N, 4.87; O, 5.57. Found: C, 83.31; H, 5.99; N, 4.79; O, 5.94.

4-(1-Pyrenyl)Butylamine (XXIII)

XXII was reduced to the amine by dropwise addition of the amide (2.0 g, 0.006 M) in 40 ml of dry tetrahydrofuran to a stirred solution of lithium aluminium hydride (1 g, 0.02 M) in 50 ml of dry tetrahydrofuran under N_2. Stirring was continued for 2 hr, then 20 ml of ethyl acetate was added. Water and sodium hydroxide were added as described (Mićovii & Mikoilović, 1953) to form a precipitate. The inorganic salts were filtered off, and the solvent was evaporated. The free amine was taken up into methanol/chloroform (1:2) and put onto a silica-gel column (2.5 × 60 cm) in chloroform. The column was washed successively with chloroform, chloroform/methanol (4:1), and chloroform/methanol (2:1), and the product was then eluted with methanol. The fractions containing product were concentrated. The product was taken up in chloroform/trace of glacial acetic acid and extracted 3× with 0.5 M NaOH and 1× with water. The organic phase was dried over Na_2SO_4, and dried to a dark oil. The oil was taken up into 3 ml of methanol and 0.5 ml concd. HCl was added. After partial evaporation of the methanol, the HCl salt precipitated out. Yield 1.2 g (73%) of a yellow powder, mp 252–256 °C. Infrared spectrum (KBr) showed disappearance of the amide absorption at 1650 cm^{-1} and a shift in the amide signal at 3400 cm^{-1} to the broader amine signal at 3450 cm^{-1}. The NMR spectrum (d_6-DMSO) showed the following signals: δ 1.8 (*m*, 4H, $-CH_2-CH_2-$), 2.9 (2H, $-CH_2-N$), 3.35 (2H, ring$-CH_2-$), and 7.8–8.4 (*m*, 9H, ring). TLC showed the appearance of a ninhydrin positive spot. Anal. calcd for $C_{20}H_{20}NCl$: C, 77.50; H, 6.52; N, 4.52. Found: C, 75.62; H, 6.58; N, 4.12.

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