Determination of the Sidedness of Carbocyanine Dye Labeling of Membranes†

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ABSTRACT: We have found that 2,4,6-trinitrobenzenesulfonate (TNBS) is an effective quencher of the fluorescence of the 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanines ($C_N$diI's). This quenching appears to occur by complex formation rather than a collisional mechanism. By use of this quenching, we have studied the transbilayer asymmetry of $C_N$diI labeling for large unilamellar membranes of egg phosphatidylcholine and the plasma membranes of human red blood cells and of ram epididymal spermatozoa. When $C_N$diI is added to membranes by ethanolic injection, only the outer leaflet labels. We have further shown that in large unilamellar vesicles of egg phosphatidylcholine, the $C_N$diI's do not appreciably "flip-flop" to the inner leaflet for at least 4 h at temperatures between 4 and 37 °C.

Carbocyanine and related dyes have been used extensively as fluorescent lipid analogues in biophysical studies of model membranes (Fahey et al., 1977; Fahey & Webb, 1978; Wu et al., 1977; Derzko & Jacobson, 1980; Klausner & Wolf, 1980; Ethier et al., 1983; Dragsten & Webb, 1978), cell membranes (Sims et al., 1974; Waggoner et al., 1977; Wolf et al., 1981a,b, 1982; Wolf & Voglmayr, 1984; Schlegel et al., 1980; Schlessinger, 1977; Peters, 1981; Peters & Richter, 1981), and lipoproteins (Barak & Webb, 1981). They have proven to be useful as probes of membrane potential (Sims et al., 1974; Waggoner et al., 1977; Dragsten & Webb, 1978) and of lipid lateral diffusion (Fahey et al., 1977; Fahey & Webb, 1978; Wu et al., 1977; Derzko & Jacobson, 1980; Wolf et al., 1981a,b, 1982; Wolf & Voglmayr, 1984; Schlessinger, 1977; Peters, 1981). Of particular usefulness have been the 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanines ($C_N$diI's)[1] (see Figure 1 for structure). These probes, particularly C_{18}diI and C_{16}diI have been widely used in fluorescence recovery after photobleaching studies [for a review, see Peters (1981)]. Comparing the ability of these and other lipid analogues to cross-intracellular tight junction, Dragsten et al. (1981) have demonstrated lipid connectivity between the apical and basal faces in the inner but not the outer leaflet of toad kidney epithelial plasma membranes. The effect of fertilization in both sea urchin (Wolf et al., 1981a) and mouse ova (Wolf et al., 1981b) on $C_N$diI diffusion depends upon the probe's alkyl chain length, N. These observations reflect the different and in both cases nonmonotonic dependence of the $C_N$diI diffusion coefficient upon chain length. Such behavior cannot be modeled by single-phase fluid or single-phase gel-state liposomes but can be modeled by liposomes that contain coexistent gel and fluid phases (Klausner & Wolf, 1981). Thus, the $C_N$diI's do not appear to distribute homogeneously in the plane of the membrane but rather as a function of their alkyl chain length to selectively partition into specific lipid domains (Klausner & Wolf, 1981; Ethier et al., 1983).

The $C_N$diI's thus have considerable potential as probes of both the lateral and transverse organization of biological membranes. Before they can be exploited further, it becomes essential to develop a method of determining the distribution of these probes between the inner and outer leaflets of the bilayer.

An obvious approach to this problem is to measure the ability of nonpermeating fluorescence quenching agents to quench the fluorescence of membrane-bound $C_N$diI. Standard quenching agents such as I^- and acrylamide are unsatisfactory for these purposes for two reasons: first because concentrations as high as 400 mM are necessary to obtain reasonable quenching levels (Eftink & Ghiron, 1976; Chaplin & Kleinfeld, 1983) and second because their membrane impermeability is questionable (Chaplin & Kleinfeld, 1983). We have found that cationic quenchers such as Co^{2+} and Cu^{2+} do not produce sufficient quenching for these fluorophores (unpublished results).

In this paper we show that 2,4,6-trinitrobenzenesulfonate (TNBS) is an effective quencher of $C_N$diI fluorescence under conditions in which it does not permeate membranes. This quenching appears to occur during complexing of TNBS with $C_N$diI. By using this agent, we show that when $C_N$diI is added by the standard method of ethanolic injection to membranes [large unilammelar vesicles (LUV) of egg phosphatidylcholine (EPC), human red blood cell (RBC) plasma membranes, and ram epididymal sperm plasma membranes], it labels on the outer leaflet only. We further show that for EPC LUV's, the $C_N$diI's do not appreciably "flip-flop" to the inner leaflet for at least 4 h at temperatures between 4 and 37 °C.

Materials and Methods

Carbocyanine Dyes. C_{10}diI and C_{16}diI were obtained as perchlorates from Molecular Probes (Eugene, OR). The purity was verified by thin-layer chromatography.

Egg Phosphatidylcholine. Egg phosphatidylcholine (EPC) was obtained from Sigma Biochemicals (St. Louis, MO), type IIIE in hexane was 99% pure, and was used without further purification.

Trinitrobenzenesulfonate. Trinitrobenzenesulfonate (TNBS) was obtained from Eastman (Rochester, NY).

Preparation of Ether Reverse Phase Vesicles. Ether reverse phase unilamellar vesicles were prepared by the method of Szoka & Papahadjopoulos (1978). A total of 50 mg of EPC (0.5 mL) was added to the flask to solubilize the lipid, fol-

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[1] Abbreviations: $C_N$diI, 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, where N is the alkyl chain length; ei, ethanol injected; EPC, egg phosphatidylcholine; F_0/F, ratio of fluorescence intensity before addition of quencher over fluorescence intensity after addition of quencher; LUV, large unilamellar vesicle; rp, ether reverse phase; TNBS, 2,4,6-trinitrobenzenesulfonate.

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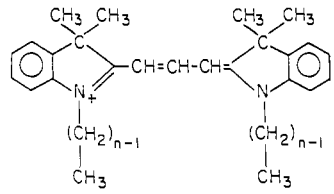

FIGURE 1: Structure of $C_N$diI's.

lowing by 1 mL of phosphate-balanced salt solution, pH 7.4. This mixture was then sonicated in a cooled bath sonicator to opalescence (~10 min). The sample was then placed in a rotoevaporator and drawn under a vacuum until all of the ether was removed. In some experiments, vesicles were prepared in buffer with 100 mM TNBS, pH 7.4.

Preparation of Ethanol-Injected Vesicles. Ethanol-injected unilamellar vesicles were prepared by the method of Kremler et al. (1977). EPC was evaporated to dryness under N_2 and then dissolved in absolute ethanol to 30 mM. A total of 75 μL of this solution was injected per mL of phosphate-buffered saline at a rate of ~50 μL/min with a Hamilton syringe while the buffer was rapidly stirred. This solution was dialyzed overnight against several changes of buffer to remove excess ethanol.

Pre- vs. Postlabeled Membranes. The two types of EPC membranes were labeled with C_{10}diI or C_{16}diI in two ways. *Prelabeled* membranes were prepared by adding 1:10^3 $C_N$diI:EPC to the membrane-forming solution. It was our a priori assumption, which was confirmed experimentally, that this labeling procedure results in an equal distribution of the $C_N$diI between the two bilayer leaflets. *Postlabeled* membranes were prepared by adding 5 μL of 1 mg/mL $C_N$diI in ethanol stock to a 1 mg/mL suspension of vesicles in buffer. This procedure gave approximately equivalent fluorescence intensity to that observed with prelabeled membranes. The $C_N$diI's do not appreciably fluoresce in aqueous phase (Sims et al., 1974). The quenching results described below were unaltered by removing any excess probe present in the aqueous phase either by multiple washes or by centrifuging excess multilamellar EPC vesicles through the suspension. Quenching results on prelabeled membranes were unaltered by the addition of 0.5% ethanol.

Quenching Measurements. Spectrofluorometric measurements were made on a Perkin-Elmer MPF 3 spectrofluorometer equipped with a water-jacketed temperature-controlled four-position sample chamber. $C_N$diI fluorescence was excited at 550 nm and monitored at 600 nm with 10-nm slit widths. A total of 100 μL of sample was first measured in micro quartz fluorescence cuvettes, and then appropriate amounts of 1, 10, or 100 mM TNBS were added. In the case of prelabeled vesicles prepared in the presence of TNBS, fluorescence was normalized to $C_N$diI concentration by solubilizing the vesicles in 3% sodium dodecyl sulfate and reading the optical density at 546 nm in a Perkin-Elmer Lambda 5 spectrophotometer.

Measurements of Liposome Permeability to TNBS. To measure the ability of TNBS to permeate liposomes, 10 mg of ether reverse phase EPC vesicles pre- or postlabeled with C_{16}diI was prepared as described above in the presence of 2 mL of 100 mM TNBS. These vesicles were run down a ~25-cm Sepharose CL-4B (200 mesh) column at 4 °C to separate them from free TNBS. The vesicles were immediately duluted to 5 mL with phosphate-buffered saline at 4 °C. A 1-mL aliquot was retained for measurement, and at ~20-min intervals, 1-mL aliquots were run a second time down the column to remove TNBS, which had permeated through the membrane during the interim. Vesicle samples were diluted

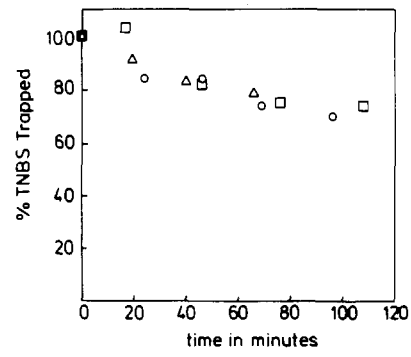

FIGURE 2: Leakage of ether reverse phase EPC vesicles prelabeled with C_{16}diI to TNBS at 4 °C. See text for experimental details. The different symbols represent separate experiments.

to approximately the same concentration on the basis of the turbidity measured at 400 nm. Vesicles were then solubilized by the addition of 25 μL of 10% sodium dodecyl sulfate/mL of sample, and the optical density was measured at 260 nm. The ratio of optical density at 260 nm to that at 400 nm was taken as a measure of entrapped TNBS.

Labeling of Cells with C_{16}diI. Cells were labeled with C_{16}diI by the standard method of ethanolic injection (synonymous with our method of postlabeling) as follows: For human red blood cells, 20 μL of a 1 mg/mL solution of C_{16}diI in absolute ethanol was added to 2.0 mL of Hank's balanced salt solution and dispersed. A 200-μL aliquot of packed washed human red blood cells was added to this solution, incubated for 8 min at room temperature, spun down at 1000*g*, and washed 2 times with Hank's balanced salt solution. Cells were then resuspended to 2.0 mL in Hank's balanced salt solution. Quenching measurements were made in a 400× dilution of this stock. For ram epididymal sperm, 50 μL of a 1 mg/mL solution of C_{16}diI in absolute ethanol was added to 5.0 mL of Krebs Ringer phosphate containing 0.2% NaN_3, and dispersed. Approximately 3.0×10^7 washed sperm cells (~10 μL) were added to this solution and incubated for 8 min at room temperature. Sperm were spun down at 500*g* and washed twice with Krebs Ringer phosphate and resuspended to 5 mL. Quenching measurements were made directly on this stock solution.

RESULTS

Permeability of EPC LUV's to TNBS. A wide variety of membranes have been shown to be essentially impermeable to TNBS at 4 °C and pH 7.4 for at least 10–20 min (Rothman & Kennedy, 1977a,b; Sleight & Pagano, 1983). To verify this for the membranes used in these studies we prepared EPC LUV's containing 0.1 mol % C_{16}diI and measured efflux of TNBS at approximately 20-min intervals with the column technique described above. The results of these experiments (Figure 2) show that negligible (<15%) TNBS permeates these membranes during time periods of 10–20 min. Similar results (data not shown) were obtained with "post"-labeled vesicles.

Determination of Distribution of $C_N$diI between Bilayer Leaflets. As shown in Figure 3, when 4.8 mM TNBS is added to EPC LUV's labeled with C_{16}diI, it rapidly quenches the probe fluorescence. This quenching plateaus at ~40% for prelabeled membranes (where $C_N$diI is present during formation of membranes) and at ~80% for postlabeled membranes (where C_{16}diI is added by ethanolic injection after membranes are formed). Similar results were obtained for EPC LUV's prepared by the method of ethanol injection (see Table I). Addition of excess TNBS (33 mM) results in ~50% quenching for prelabeled membranes and ~100% quenching for postlabeled membranes. In Table I, we summarize

Table I: TNBS Quenching of C_{10}dil and C_{16}dil Fluorescence in Membranes[a]

probe	type of membrane	method of labeling	location of TNBS inside	location of TNBS outside	% Q at [TNBS] = 4.8 mM[d]	N[e]	% Q at [TNBS] = 33 mM	N
C_{16}dil	rp EPC LUV[b]	pre	−	+	43 ± 4	3	51 ± 3	2
C_{16}dil	rp EPC LUV	pre	+	+	84 ± 8	3		
C_{16}dil	rp EPC LUV	post	−	+	84 ± 4	8	87 ± 4	1
C_{16}dil	rp EPC LUV	post	+	+	86 ± 7	3		
C_{16}dil	ei EPC LUV[c]	pre	−	+	42 ± 5	3		
C_{16}dil	ei EPC LUV	post	−	+	86 ± 3	3		
C_{10}dil	rp EPC LUV	pre	−	+	42 ± 8	5		
C_{10}dil	rp EPC LUV	pre	+	+	81 ± 7	3		
C_{10}dil	rp EPC LUV	post	−	+	85 ± 2	8		
C_{10}dil	rp EPC LUV	post	+	+	98 ± 1	3		
C_{16}dil	human red blood cells	post	−	+	82 ± 5	2	92 ± 4	2
C_{16}dil	ram epididymal sperm	post	−	+	81 ± 4	1	95 ± 4	1

[a] Data given as mean ± standard deviation except where only one measurement was made in which case uncertainty was calculated from spread in data. [b] rp = ether reverse phase. [c] ei = ethanol. [d] % Q = percent quenching. [e] N = number of measurements.

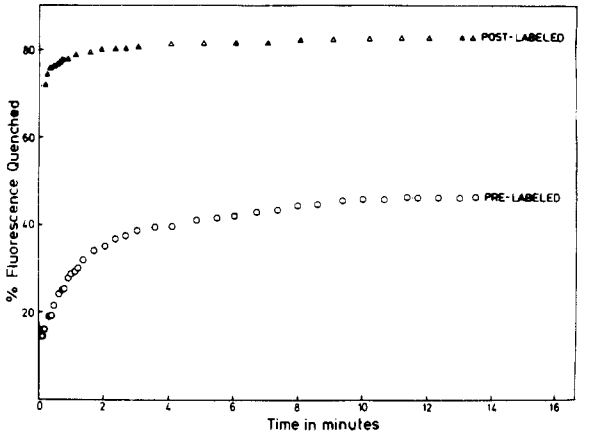

FIGURE 3: Percent quenching of C_{16}dil fluorescence as a function of time for pre- and postlabeled ether reverse phase vesicles after the addition of 4.76 mM TNBS at 4 °C.

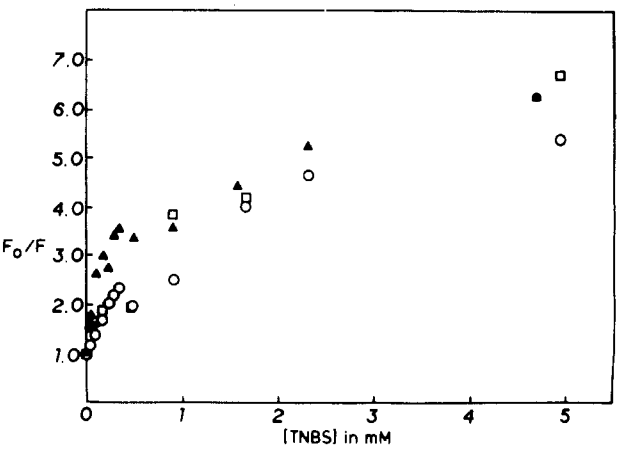

FIGURE 4: Stern–Volmer plots of TNBS quenching of C_{16}dil fluorescence in postlabeled membranes: (▲) ether reverse phase LUV's; (□) human red blood cells; (○) ram epididymal sperm. See text for details of labeling. Temperature = 4 °C.

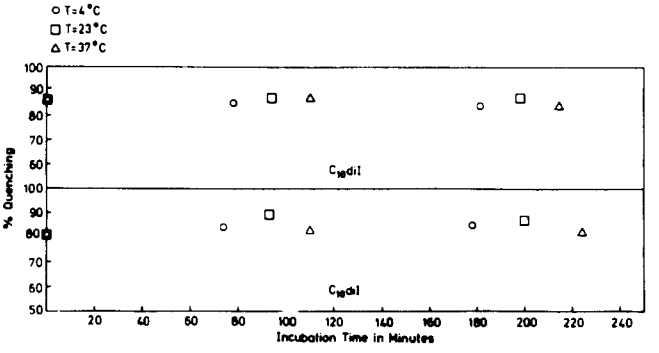

FIGURE 5: Access of C_{10}dil and C_{16}dil to 4.8 mM external TNBS in postlabeled ether reverse phase EPC LUV's. See text for experimental details. Incubation temperatures are (○) 4, (□) 23, and (△) 37 °C.

quenching results for EPC LUV's labeled by the two methods with either C_{16}dil or C_{10}dil where TNBS is either confined to the outside of vesicles (TNBS added after vesicles formed as above) or present both inside and outside (vesicles formed in the presence of TNBS). We see that for postlabeled vesicles all of the $C_N$dil is accessible to TNBS when the quencher is presented externally, while for prelabeled vesicles only half ot it is accessible. Thus, postlabeling EPC LUV's only labels the outer leaflet of the bilayer.

As summarized in Table I, we have also examined two biological plasma membranes labeled with C_{16}dil: those of human red blood cells and those of ram epididymal spermatozoa. Quenching results for these membranes are similar to those of postlabeled EPC LUV's, indicating that the probe is again confined to the outer leaflet.

The similar dependences of TNBS quenching of C_{16}dil fluorescence upon TNBS concentration are shown as Stern–Volmer plots (Stern & Volmer, 1919) in Figure 4 for postlabeled reverse-phase EPC LUV's, human red blood cells, and ram epididymal spermatozoa. As discussed below, it is important to note that in all cases a negative deviation from linearity is observed.

$C_N$dil's Do Not Flip-Flop across EPC LUV Bilayers. To determine if the $C_N$dil's flip-flop across EPC LUV bilayers, we prepared vesicles postlabeled with either C_{10}dil or C_{16}dil. An aliquot of these vesicles was quenched at 4 °C with 4.8 mM TNBS as described above. The remainder was divided into three parts and held at either 4, 23, or 37 °C, respectively. At ~1.5-h intervals, the quenching of an aliquot from each part by TNBS was measured at 4 °C. The results of these experiments are shown in Figure 5. If significant flip-flop to the inner leaflet occured, percent quenching should decrease with time. However, no significant decreases in percent quenching were observed over ~4 h at any of the three incubation temperatures. Thus, no flip-flop occurs on this time scale.

Mechanism of TNBS Quenching of $C_N$dil Fluorescence. While determination of the mechanism of TNBS quenching of $C_N$dil fluorescence was not our primary goal, several experiments were performed to begin clarifying this mechanism. In Figure 6 we compare the ability of TNBS to quench C_{16}dil fluorescence in 3% Triton, 3% sodium dodecyl sulfate, and methanol. In neutral detergent, Triton, we observe quenching similar (in both the shape of Stern–Volmer plots and the concentration of TNBS at which quenching is observed) to

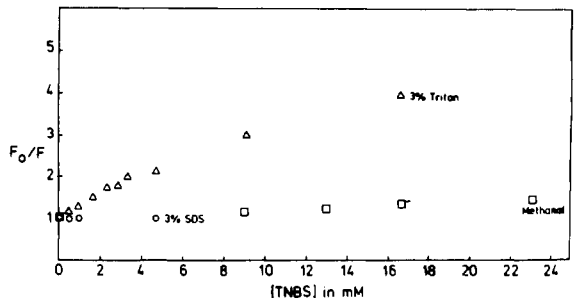

FIGURE 6: Stern–Volmer plots of TNBS quenching of C_{16}diI fluorescence in different media: (△) 3% Triton, (□) 100% methanol, and (○) 3% sodium dodecyl sulfate at 23 °C.

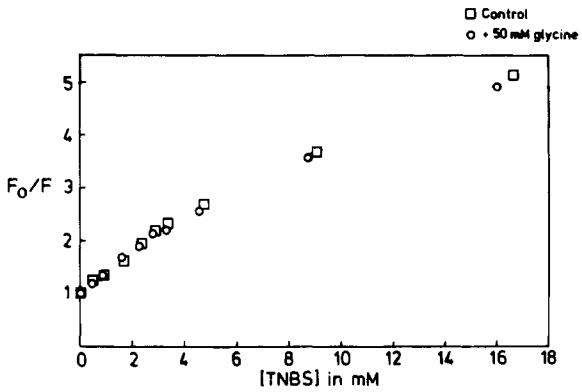

FIGURE 7: Stern–Volmer plots of TNBS quenching of C_{16}diI fluorescence at 4 °C in (□) 3% Triton and (○) 3% Triton + 50 mM glycine.

that observed on vesicles. No quenching occurs in ionic detergent, sodium dodecyl sulfate, and only weak quenching is observed in methanol. To consider whether the sulfonate of TNBS is required for quenching, we compared quenching of C_{16}diI in 3% Triton to quenching in 3% Triton + 50 mM glycine with 4.8 mM TNBS. Reaction of glycine and TNBS was confirmed from its color reaction. As shown in Figure 7, glycine does not inhibit TNBS quenching of C_{16}diI fluorescence. Similar results were obtained for C_{16}diI-labeled EPC LUV's. Furthermore, addition of 50 mM glycine following 4.8 mM TNBS does not reverse quenching of C_{16}diI fluorescence in either 3% Triton or EPC LUV's.

As will be discussed below, both the observation of deviations from linearity in Stern–Volmer plots of TNBS quenching of $C_N$diI fluorescence and the low concentrations of TNBS needed to obtain significant quenching relative to those necessary for collisional quenchers suggest a binding rather than collisional mechanism for this quenching. This is further supported by the temperature dependence of TNBS quenching. As shown in Figure 8, TNBS quenching of C_{16}diI fluorescence decreased with increasing temperature.

Discussion

We have shown that, under conditions in which it does not permeate membranes, TNBS is an effective quencher of $C_N$diI fluorescence. Using this quenching agent, we have shown that when reverse-phase or ethanol-injected EPC LUV's, human red blood cells, or ram epididymal sperm are labeled with C_{10}diI or C_{16}diI by the method of ethanol injection, only the outer leaflet of the plasma membrane is labeled.[2] For the reverse-phase LUV's no significant flip-flop to the inner leaflet

[2] Since this conclusion is based on the ratio of Q post and Q pre, the uncertainty must be obtained by error propagation for a ratio and for the data in Table I is typically 10–20%.

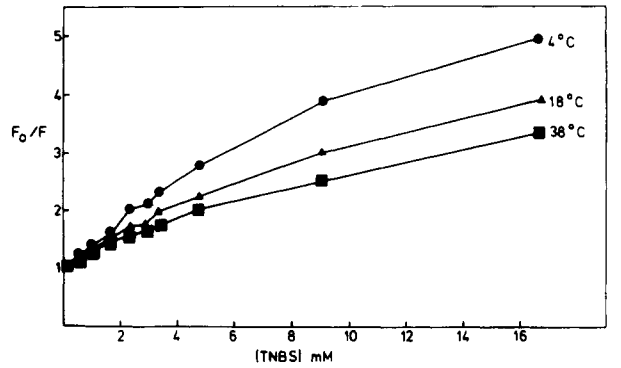

FIGURE 8: Stern–Volmer plots of TNBS quenching of C_{16}diI fluorescence in 3% Triton at (●) 4, (▲) 18, and (■) 38 °C.

occurs for either C_{10}diI or C_{16}diI for at least 4 h at 4, 23, or 37 °C.

One must be careful about generalizing this observation to all membranes. Conditions of labeling with the $C_N$diI's by ethanolic injection are variable (Wolf et al., 1981a,b). In some biological membranes, conditions exist where a given $C_N$diI will also label endomembranes (unpublished results). Since this implies a permeability of such membranes to $C_N$diI, the inner leaflet may also label under such conditions. The methods developed in this paper should be of general use in determining labeling asymmetry. For instance, TNBS should be a superior quenching agent for studying epithelial polarity than agents used previously (Dragsten et al., 1981). They may also prove useful in studying probe internalization and isolation of cytoplasmic fluorescence.

Several of our observations suggest that TNBS quenches $C_N$diI fluorescence by complex formation rather than by a simple collisional mechanism. First, the Stern–Volmer plots are nonlinear. Second, with most collisional quenchers concentrations as high as 400 mM are often required (Eftink & Ghiron, 1976; Chaplin & Kleinfeld, 1983). Third, in general, collisional quenching increases with increasing temperature, while quenching by complex formation decreases (Lakowicz, 1983). TNBS quenching of $C_N$diI fluorescence decreases with increasing temperature.

Without measurements of the effects of TNBS upon $C_N$diI fluorescence lifetime, however, a collisional mechanism cannot be ruled out. $C_N$diI fluorescence does not decay as a single exponential (Packard & Wolf, 1984). Such heterogeneity could result in nonlinearities in Stern–Volmer plots. The partition of TNBS between the bilayer and aqueous phase could enhance the local concentration of quencher at the membrane surface, resulting in more quenching at lower overall TNBS concentration. If $C_N$diI lifetime decreases rapidly with increasing temperature, then TNBS quenching could also decrease with increasing temperature. Most of these issues could be cleared up by measuring the effect of TNBS upon the lifetime of $C_N$diI fluorescence.

The precise nature of the interaction of $C_N$diI and TNBS remains uncertain. There does not appear to be a requirement for the sulfonate group at the 1-position of TNBS since quenching occurs in the presence of excess glycine and is not reversed by the subsequent addition of excess glycine. However, further testing with TNBS analogues would be required before a more definitive statement of the chemical structure requirements for quenching can be made. It should be borne in mind that the reaction of TNBS and glycine substitutes one acidic group for another at the 1-position. The fact that quenching occurs in neutral but not ionic detergent suggests an ionic aspect to the interaction of $C_N$diI and TNBS. Steiner & Kirby (1969) showed a correlation between the ability of

compounds to scavenge electrons and their ability to quench the fluorescence of indoles, indicated that the quenching occurred by electron transfer (Anbar, 196). Our results would be consistent with such a mechanism.

As noted above, the issue of quenching mechanism is secondary to the issue of fluorophore localization and flip-flop in the membrane. However, the question of mechanism is not purely abstract. The physiological significance of quenching experiments on cells in the presence of nearly molar concentrations of collisional quenchers such as Co^{2+}, Cu^{2+}, I^-, or acrylamide is suspect. Use of agents that quench at millimolar concentrations possibly by complex formation is clearly preferable. Even so, oxidizing agents such as TNBS interact with membrane lipids and proteins and can potentially alter cell structure and function. Our experiments into the mechanism of TNBS quenching of $C_N$diI fluorescence suggest that one might be able to find either a TNBS analogue or another electron scavenger that would be less reactive with native membrane components but as effective a quencher of $C_N$diI fluorescence.

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Registry No. TNBS, 2508-19-2; C_{10}diI, 84109-11-5; C_{16}diI, 84109-06-8.

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