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Reactions of Fluorescent Probes with Normal and Chemically Modified Myelin†

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ABSTRACT: The fluorescent probes 8-anilino-1-naphthalenesulfonate (ANS) and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) bind to highly purified myelin membranes obtained from bovine brain white matter. Binding of the dyes was markedly increased by environmental conditions which reduce the negative surface potential of the membrane, i.e., cations ($La^{3+} > Ca^{2+} > Na^+$, K^+), H^+, local anesthetics, and the antibiotic polymyxin B. Chemical alteration of accessible membrane charged groups affected dye binding in a manner consistent with the hypothesis that such binding is primarily dependent upon the membrane surface potential. Thus, binding was increased by blocking of carboxyl groups via carbodiimide activation and subsequent coupling with neutral amino acid esters, and even more so with a basic amino acid ester (e.g., arginine methyl ester). Dye binding was reduced by succinylation of amino groups, and by hydrolysis of choline and ethanolamine head groups of phospho- and sphingolipids by phospholipase C. Phospholipase C treatment of myelin, or sphingomyelin vesicles, reduced or abolished the augmentation of ANS and TNS binding due to cations, local anesthetics, or polymyxin B. Energy transfer from myelin tryptophan residues to bound ANS occurs, but with low efficiency. Oxidation of membrane tryptophan residues with *N*-bromosuccinimide, or alkylation with 2-hydroxy (or methoxy)-5-nitrobenzyl bromide, markedly reduced intrinsic membrane fluorescence and energy transfer to bound ANS, but did not significantly affect dye binding or the quantum yield of ANS fluorescence when excitation was at 380 nm. Proteolytic digestion removed 6–30% of myelin protein, depending upon the enzyme used, but had no effect on fluorescent dye binding. It is concluded that the binding of the anionic fluorescent probes ANS and TNS to myelin is primarily a function of the membrane surface charge density and net surface potential, as is the case with other biological membranes. Conclusions about the degree of dye binding to membrane lipids or membrane proteins cannot be drawn unless additional studies are carried out on isolated water soluble membrane proteins.

Evidence derived from polarized light, X-ray diffraction, and electron microscopy studies of myelin has been interpreted as being in accord with the Davson–Danielli–Robertson model (Robertson, 1960) for membrane structure, i.e., a laminar arrangement consisting of a bilayer lipid core with monolayers of protein at each of the surfaces of the lipid bilayer (Finean, 1953, 1965; Finean and Burge, 1962; Worthington and Blaurock, 1969; Worthington, 1970; Kirschner and Caspar, 1971). Critics (Singer, 1971, 1972) of the Davson–Danielli–Robertson model have proposed alternative models for membrane structure. They consider myelin to be chemically and functionally an atypical membrane whose structure should not be considered as typical of other cellular membranes. On the other hand, the newly discovered physical properties of the myelin proteolipid (Folchi-Pi and Stoffyn, 1972) suggest that this protein may fit the criteria proposed for integral membrane proteins. Thus, myelin may in fact be organized in the manner envisaged by the fluid lipid–globular protein mosaic model (Singer, 1971; Wallach and Zahler, 1966; Vanderkooi and Green, 1970).

In this, and the accompanying paper (Feinstein and Felsenfeld, 1975), we report on our studies of the properties and the organizational features of myelin and some of its isolated protein constituents, as revealed by the use of the fluorescent probes 8-anilino-1-naphthalenesulfonate (ANS)[1] and 2-*p*-toluidinylnaphthalene-6-sulfonate. These probes exhibit large increases in fluorescent intensity and spectral shifts when bound to certain proteins (Daniel and Weber, 1966; McClure and Edelman, 1966; Stryer, 1965), lipids (Vanderkooi and Martonosi, 1971; Feinstein et al., 1970), or cellular membranes (Wallach et al., 1970; Van-

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

derkooi and Martonosi, 1971; Rubalcava, et al., 1969; Feinstein et al., 1970).

The effects of various environmental factors on probe binding and fluorescence were studied: i.e., cation concentration, pH, and addition of drugs which are known to affect membrane function (e.g., anesthetics, polymyxin B). In addition modification of various protein and lipid functional groups has been carried out by chemical or enzymatic means, and the resulting effects on probe binding were studied. The properties of myelin revealed in these studies were much like those of other cellular membranes. A major factor affecting ANS and TNS binding to the intact membrane was found to be the nature of the fixed charges of protein and lipid at the membrane surface. However, significant differences were demonstrated between the reactions of ANS and TNS with intact myelin as compared to its isolated major protein constituents (Feinstein and Felsenfeld, 1975), particularly the proteolipid. These experiments strongly suggest that the latter may be deeply embedded in the membrane lipid bilayer, in the manner of an integral membrane protein.

Materials and Methods

Materials. Magnesium 8-anilino-1-naphthalenesulfonate (ANS), obtained from Eastman Kodak Co. or Polysciences Inc., was recrystallized as described by Weber and Young (1964). Sodium 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), obtained from Sigma Chemical Co., was used without further purification. Neither dye showed any fluorescent impurities when chromatographed on silica gel (Eastman Kodak, 6061 chromatogram sheet), in several solvent systems, and their absorption spectra corresponded to those described by Stryer (1965) and McClure and Edelman (1966). Furthermore, the fluorescence emission spectra of both dyes in absolute ethanol did not vary in form or emission maximum when excited at wavelengths from 280 to 380 nm which is confirmatory evidence for the presence of only one fluorescent species in each case (Weber, 1961).

Chemicals were obtained from the following listed commercial sources and used without further purification unless specified: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Ott Chemical Co.), glycine methyl ester and glycine ethyl ester, galactose, *N*-acetylneuraminic acid, polymyxin B, arginine, succinic anhydride, tryptophan (Sigma Chemical Co.), arginine methyl ester, Tris base, and butacaine sulfate (Schwarz/Mann), 2-methoxy-5-nitrobenzyl bromide and 2-hydroxy-5-nitrobenzyl bromide (Pierce Chemical Co.), egg lecithin, phosphatidylserine, brain cerebrosides, sphingomyelin (Supelco or Applied Sciences Lab.), sucrose (Mann, ultra pure density gradient grade), *N*-bromosuccinimide (Eastman Org. Chem.), fluorescamine (Roche Diagnostics), and procaine hydrochloride (Matheson Coleman and Bell). Cholesterol (Sigma) was crystallized three times from ethanol.

The proteins used were: bovine serum albumin, trypsin 2× crystallized (Sigma Chem. Co.), Pronase (Calbiochem), α-chymotrypsin (General Biochemicals), and pepsin (Worthington).

All salts were reagent grade chemicals and the organic solvents were obtained commercially as Spectroquality or Fluorescence grade reagents, or they were redistilled before use. The water was treated with activated charcoal and deionized.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Sodium deoxycholate (0.1%) was added to solubilize the myelin. Phosphorus was determined by the method of Taussky and Schorr (1953) after wet ashing with concentrated sulfuric acid. Cholesterol was determined by the method of Searcy and Berquist (1960), sialic acid by the method of Warren (1959), and cerebroside by the method of Svennerholm (1956). Arginine was determined by a modification of the method of Sakaguchi (1925), after solubilization of myelin in a mixture of 1-propanol–water (1:1) containing 0.5% NaOH.

Preparation of Myelin. Myelin was prepared from bovine brain white matter by a combination of the sucrose density gradient procedures A and B of Rumsby et al. (1970) modified for large batch (~300 g of white matter) preparations. The highly purified myelin was washed extensively with water and the pellets were stored in a deep freeze (−15°) or lyophilized. Samples were prepared for electron microscopy by fixation with glutaraldehyde and osmium tetraoxide.

Modification of Membrane Carboxyl Groups. The method of Hoare and Koshland (1967) for the blocking of carboxyl groups in purified proteins was applied to accessible groups of myelin. Myelin was suspended (7–9 mg of protein/ml) in 0.01 *M* sodium acetate (pH 4.75) at 0°, and sonicated for 1 min at 60 W (Heat Systems-Ultrasonics, Inc. Sonifier Cell Disruptor). The carbodiimide (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) was added (0.4 *M*) to the myelin in the presence of an amino acid ester (1.33 *M* glycine methyl ester, glycine ethyl ester, or arginine methyl ester). pH was maintained by addition of dilute HCl as necessary. After incubation for 3 hr at 22°, the pH was reduced to 2.0 for 20 min to hydrolyze phosphoamide bonds that might have formed between myelin phospholipids and the amino acid esters (Forstner and Mannery, 1971). Myelin was recovered by centrifugation at 48,000*g*, for 25 min, and was washed five times with 0.01 *M* Tris-HCl (pH 7.4).

Modification of Tryptophan Residues by Koshland Reagents I and II. Two-milliliter aliquots of the myelin suspension (9.0 mg of protein/ml) in phosphate buffer (0.01 *M*, pH 5.37) were added to 0.15 ml of 0.2 *M* 2-hydroxy-5-nitrobenzyl bromide (Koshland reagent I; Horton and Koshland, 1965) in dry acetone. After 30 min in the dark at room temperature the myelin was recovered by centrifugation at 48,000*g*, and washed five times with 0.01 *M* Tris-HCl buffer (pH 7.4).

Reaction with 2-methoxy-5-nitrobenzyl bromide (Koshland's reagent II; Horton et al., 1965) was carried out by adding 2.0 ml of a myelin suspension containing 9.0 mg of protein/ml in 0.01 *M* sodium acetate buffer (pH 4.6) to 0.1 ml of a 0.2 *M* solution of the reagent in dry acetone. After mixing for 3 hr in the dark, at room temperature, the myelin was recovered by centrifugation and washed five times with Tris-HCl buffer.

Modification of Membrane Amino Groups. Amino groups were succinylated as described by Habeeb et al. (1958). Myelin was treated with succinic anhydride in either 0.1 *M* Tris-HCl buffer at pH 8.0, or in 0.1 *M* sodium borate buffer at pH 9.2. The reaction mixture contained 40 mg of succinic anhydride per mg of myelin protein which was added in aliquots of ¼ to ½ the total amount at 10-min intervals.

Fluorescence Measurements. Fluorescence intensity and spectra were measured with the Hitachi-Perkin-Elmer MPF-2A ratio-recording spectrofluorometer. In most cases, the absorbance of the solutions in the fluorescence titration

experiments was not greater than 0.1 using 1.0-cm or 0.475-cm path-length cuvets. When the absorbance exceeded this value microcells were employed or corrections for the effect on fluorescence emission which never amounted to more than 15% were made by the method of Beyer et al. (1972).

Light scattering was minimized by a quartz polarizing filter placed horizontally in the excitation beam path or by appropriate filters inserted before the photomultiplier tube. Fluorescence intensity values were read from a Keithley digital voltmeter connected to the fluorometer output. Fluorescence lifetime measurements were made with an Ortec nanosecond fluorometer. The number of decay constants which contributed to the single decay curves actually measured was determined by the method of moments developed by Dyson and Isenberg (1969, 1971), and modified by Dr. S. Fernandez (personal communication) to correct for light scattering.

Binding parameters were determined by the methods applied to small molecule binding to proteins, described by the equation

$$\nu = nKc/(1 + Kc) \quad (1)$$

where ν = average mole ratio of bound ligand to protein, c = equilibrium free-ligand concentration, n = number of binding sites on a protein molecule, and K = intrinsic association constant of the binding sites, assuming the intrinsic association constants of all n sites are equal, and when binding to one site does not influence binding to any other site. K and n can be determined by a plot of $1/\nu$ vs. $1/c$ (Klotz, 1946), or ν/c vs. ν (Scatchard, 1949). The fraction of dye bound was determined from the ratio F/F_0 where F_0 is the actual observed fluorescence efficiency when all the dye in solution is bound. F_0 was determined by adding increasing concentrations of myelin or protein and plotting $1/F$ against 1/myelin or protein concentration (Weber and Young, 1964). Absorption spectra were obtained with a dual-beam Perkin-Elmer 356 spectrophotometer.

Results

Chemical Analysis of Myelin. The chemical composition of the myelin preparations used in these studies was comparable to that determined in several other laboratories (Autilio et al., 1964; Gonzalez-Sastre, 1970; McIlwain and Rapport, 1971): e.g., % by dry weight–cholesterol 19%, protein 25%, total cerebroside 24%, total phosphorus 1.4%, and sialic acid 0.012%. The high purity of the myelin was also evident from its nearly complete solubility in chloroform–methanol, 2:1. High power electron microscopy showed typical myelin figures with the alternating pattern of major dense lines and intraperiod lines.

Effects of Cations, pH, and Cationic Drugs on Binding of Fluorescent Probes to Myelin. Upon binding of ANS or TNS molecules to myelin the wavelength for maximum intensity of fluorescence emission shifted from 520 nm (in water, uncorrected values) to 490 nm for ANS, and from 500 to 425 nm for TNS. The addition of cations, certain local anesthetics, or the basic polypeptide antibiotic polymyxin B induced a large increase in fluorescence intensity (Figure 1). In some cases a further shift of the ANS emission peak to 470 nm occurred. The effectiveness of the cations was a function of their valency, so that $La^{3+} > Ca^{2+}$, Mg^{2+}, $Sr^{2+} > Rb^+$, Cs^+, Li^+, Na^+, K^+. The local anesthetics procaine and butacaine were comparable in effect to divalent cations, but polymyxin B increased fluorescence to a greater degree comparable to La^{3+}. The apparent cation binding constants can be derived from appropriate plots of the fluorescence titration data (Figure 1A). However, the actual concentration of cations at the membrane surface can markedly differ from that in the bulk liquid phase because of the presence of fixed negative charges at the membrane surface. This gives rise to a double-layer potential at the membrane surface. The concentration of cations at the membrane surface is a function of this potential (ψ), i.e.

$$C^* = C_i e^{-Z_i \psi F/R} \quad (2)$$

where C* is the cation concentration at the membrane surface, C_i is the cation concentration in the external solution, Z_i is the valency of the cation, and ψ is the potential difference across the double-layer (Gilbert and Ehrenstein, 1969). The fluorescence of ANS or TNS plus myelin also increased markedly as the pH was lowered (Figure 2). The effects of H^+, cations, local anesthetics, and polymyxin B would appear to be due to their ability to reduce the negative membrane double-layer potential by binding to, or shielding, membrane fixed negative charges, or by decreasing their ionization, in the case of H^+. In this way the binding of the anionic dyes (ANS and TNS) would be expected to increase as ψ, the negative double-layer potential, was reduced. The increased fluorescence enhancement by factors which reduce this potential is indeed due to increased dye binding, rather than an increase in fluorescence quantum yield (q). The calculations of q for *bound* dye (Chen, 1967) were found not to change under the various experimental conditions which enhance dye fluorescence (Figure 1B and Table I).

The affinities of the fluorescent probes for myelin and the number of binding sites available were determined from the fluorescence titration data (Figure 3). TNS binding increased from 16.3 nmol/mg of myelin dry weight (65.2 nmol/mg of myelin protein) in the absence of cations to a maximum of 62.8 nmol/mg of myelin (251 nmol/mg of myelin protein) in the presence of 10^{-3} M $LaCl_3$. The statistical average apparent dissociation constant decreased concurrently from a range of $5.2–8.4 \times 10^{-5}$ to $1.6–2.0 \times 10^{-5}$ M. Myelin bound less ANS than TNS under all conditions. The addition of 10^{-3} M $LaCl_3$ increased ANS binding from 8.5 to 18.1 nmol/mg of myelin (from 34 to 72.4 nmol/mg of myelin protein) with an apparent dissociation constant of 1.1×10^{-5} M.

Effect of Chemical Modification of Membrane Carboxyl Groups and Amino Groups. The extent to which membrane carboxyl groups contributed to the negative surface charge density of the membrane and thereby influenced the repulsion of anionic dye molecules was assessed by chemically blocking the accessible carboxyl groups in myelin by the method of Hoare and Koshland (1967). Carboxyl groups were activated by a water-soluble carbodiimide and were then subjected to nucleophilic attack by amino acid esters. In this way the free membrane carboxyl groups could be substituted by glycine ethyl or methyl esters, or arginine methyl ester. This would result in the net loss of negative charge, or the conversion of negatively charged membrane sites to positively charged guanidino groups. This method of blocking membrane COO^- groups increased the binding of ANS and TNS without significant increase in fluorescence quantum yield (Table I). The H^+-fluorescence titration curves of carboxyl-blocked myelin were shifted to the right (Figure 2). However, the basic effect of H^+ was essentially unaltered by carboxyl group blockade so that a substantial

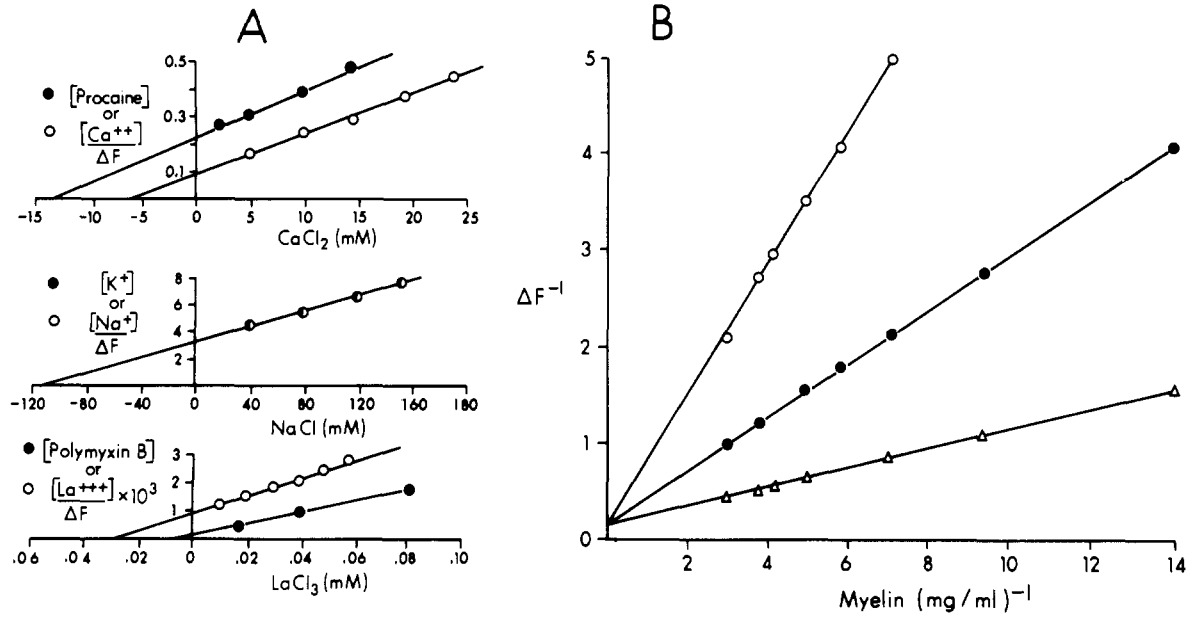

FIGURE 1: (A) The effect of cations, polymyxin B and procaine, on ANS binding to myelin. Plots of the *apparent* affinity for the membrane as measured by enhancement of ANS fluorescence. Data are plotted according to the equation: $[Me^+]/\Delta F = ([Me^+]/\Delta F_{max}) + (K_{Me^+}/\Delta F_{max})$, where $[Me^+]$ is the metal cation or cationic drug concentration, ΔF the observed fluorescence enhancement, and ΔF_{max} the fluorescence enhancement when dye binding is maximal. A plot of $[Me^+]/\Delta F$ vs. $[Me^+]$ yields a straight line with a slope of $1/\Delta F_{max}$ and an intercept on the abscissa equal to K_{Me^+}, the *apparent* average statistical dissociation constant for the Me^+-membrane complex. Myelin (30 μg of protein) in 2.0 ml of 0.01 *M* Tris-HCl (pH 7.4) containing 80 μM ANS as the magnesium salt was titrated with concentrated stock solutions of cation salts from a microburet. Fluorescence was excited at 380 *nm* and the emission intensity determined from the recorded spectra. The slits were set at 8 nm. A Polacoat uv polarizing filter arranged horizontally in the excitation path was employed to reduce Rayleigh light scatter. (B) Titrations of ANS solutions (80 μM) in Tris-HCl buffer (pH 7.4) with varying concentrations of myelin in the presence of La^{3+} (1 m*M*), Ca^{2+} (10 m*M*), or Na^+ (0.1 *M*). The intercept on the ordinate represents the extrapolated maximum fluorescence emission when all dye molecules are bound to myelin. The common intercepts indicate that the fluorescence quantum yield of bound dye is approximately the same in the presence of each of the cations.

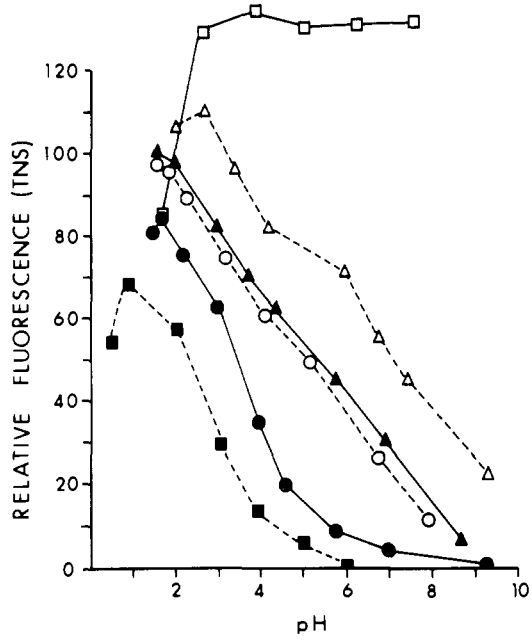

FIGURE 2: The binding of TNS to normal and chemically modified myelin as a function of pH. Quantum yield of fluorescence was not significantly altered by the various treatments nor by pH. Therefore, the fluorescence is a measure of the degree of dye binding to myelin. The responses of normal myelin alone (●) and in the presence of 10^{-3} *M* $LaCl_3$ (□) are compared with myelin treated with glycine methyl ester (○), glycine ethyl ester (▲), or arginine methyl ester (△) after carbodiimide activation to block accessible carboxyl groups. The response of succinylated myelin (■) is also shown. Membrane suspensions contained 80 μg of protein/ml and 100 μM TNS. pH was adjusted by additions of NaOH or HCl with a microburet. Fluorescence was excited at 366 nm and emission intensity measured at 430 nm. Slits were set at 8 nm.

increase in dye binding still occurred over the pH range 9–3. Incorporation of guanidino groups (from arginine methyl ester) in place of COO^- increased dye binding to a greater extent than carboxyl blockade with neutral amino acid esters. The effect of incorporated arginine methyl ester was reversed by subsequent treatment with trypsin indicating a superficial location of the blocked carboxyl groups. The number of reactive carboxyl groups was determined to be 340 nmol/mg of protein by chemical measurement of the incorporation of extra arginine residues into myelin. Dye binding to native myelin in the presence of 10^{-3} *M* $LaCl_3$ was significantly greater than the binding to carboxyl-blocked myelin preparations in the absence of La^{3+}, and remained unchanged throughout the pH range 3–8. The fall in fluorescence below pH 3 probably reflects a competition between La^{3+} and H^+ at membrane anionic sites, a conclusion supported by the measurement of a La^{3+}-induced release of H^+ (measured with a glass electrode) from myelin.

Conversion of accessible myelin amino groups to carboxyl groups, resulting in a loss of membrane (+) charged sites and an increase in (−) charged sites, was studied by treating myelin with succinic anhydride. About 70% of the membrane NH_2 groups reacting with the amino group reagent fluorescamine were blocked. Succinylated myelin exhibited reduced affinity for TNS and ANS, and a shift in the pH titration curves by nearly 1 pH unit (Figure 2).

Effect of Phospholipase C Digestion of Myelin on Fluorescent Probe Binding. Myelin suspended in Tris-HCl (0.01 *M*, pH 7.4) containing 10^{-3} *M* $CaCl_2$ was treated with phospholipase C preparations from *Clostridium perfringens* or *Clostridium welchii* at 37°, for 1–6 hr. The supernatants, obtained by centrifugation at 40,000*g*, were analyzed for water-soluble phosphorus after wet ashing. Interactions

Table I: 8-Anilino-1-naphthalenesulfonate Fluorescence Quantum Yield.[a]

	ϕ_F
Myelin (pH 7.4)	0.42
Myelin (pH 2.0)	0.44
Myelin + 0.1 *M* NaCl	0.42
Myelin + 0.01 *M* $CaCl_2$	0.44
Myelin + 0.001 *M* $LaCl_3$	0.49
Myelin–arginine methyl ester	0.42
Myelin + Polymyxin B	0.43

[a] Quantum yields were determined by comparison of the total area of the ANS fluorescence emission spectrum for bound ANS to that for the same amount of ANS in absolute ethanol gassed with nitrogen. For ANS in ethanol $\phi_F = 0.40$

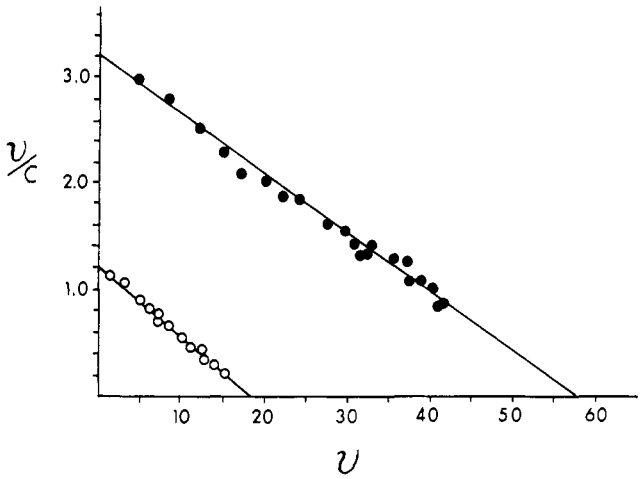

FIGURE 3: Scatchard plots of the binding of ANS (○) and TNS (●) to myelin. *V* is the average ratio nanomoles of bound ligand to mg of myelin dry weight and c is the equilibrium free-ligand concentration. The titrations were carried out in the presence of 10^{-3} *M* $LaCl_3$. In this experiment the maximum dye binding capacity was 18 nmol of ANS and 58 nmol of TNS/mg of myelin.

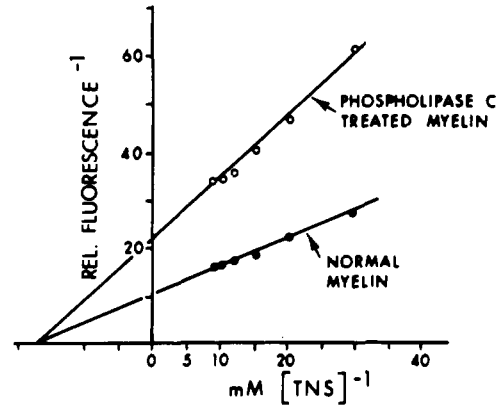

FIGURE 4: The effect of phospholipase C (*Cl. welchii*) treatment of myelin upon the binding of TNS. Myelin dispersions in 0.01 *M* Tris-HCl buffer (pH 7.4) at a concentration of 0.88 mg/ml were incubated with 1–2 units of phospholipase C and 0.001 *M* $CaCl_2$ at 37° for 2 hr. The myelin resuspended in buffer after washing three times was analyzed for protein content and then 70-μg aliquots were titrated with TNS. Myelin dispersions similarly incubated without enzyme and subsequently washed were also titrated with TNS. Fluorescence was excited at 366 nm and recorded at 420 nm, with slits set at 8 nm.

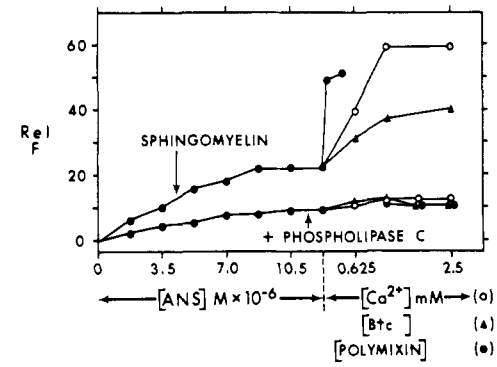

FIGURE 5: The effect of phospholipase C (*Cl. welchii*) treatment on the binding of ANS to sphingomyelin and the subsequent enhancement of dye binding by calcium, butacaine, and polymyxin B. Phospholipase C treated sphingomyelin (0.5 mg/ml) was incubated with 10 μg/ml of enzyme and 5 m*M* $CaCl_2$ at 25° for 24 hr. The solution was diluted 20-fold with buffer ($CaCl_2$ = 0.25 m*M*) prior to titration with ANS. Sonicated aqueous dispersions of sphingomyelin (25 μg/ml) in 0.01 *M* Tris-HCl (pH 7.4) were titrated with ANS to a maximum fluorescence intensity after which Ca^{2+}, butacaine, or polymyxin B were added from a microburet. Fluorescence was excited at 380 nm and the emission recorded at 480 nm with slits at 8 nm.

with fluorescent probes were studied on the three times washed, resuspended myelin. Phospholipase C produced a 63% loss of myelin phosphorus without change in myelin protein content, a similar result to that observed by McIlwain and Rapport (1971). Thin-layer chromatography of lipid extracts of myelin, after phospholipase C treatment, showed a nearly complete loss of ethanolamine and choline phosphatides, and sphingomyelin. Diglycerides in lipid extracts of myelin, which formed as a result of the enzyme treatment, were demonstrated by thin-layer chromatography on silica gel with a solvent of heptane–butanol, 70:30. Fluorescent dye binding in these membranes decreased by 35–50%, without any change in the apparent dye–myelin association constant (Figure 4). Decreased binding was also evident in the presence of calcium, local anesthetics, and polymyxin B. Hydrolysis of sonicated sphingomyelin dispersions by phospholipase C decreased the fluorescence attained upon titration with ANS by 50%, and virtually abolished the enhanced binding of the dye induced by calcium, butacaine, or polymyxin B (Figure 5). The ability of these same agents to enhance dye binding to acidic phospholipids, cerebroside, and gangliosides was not affected by phospholipase C.

Effects of Digestion of Myelin by Proteolytic Enzymes on Fluorescent Dye Binding. Previous studies have shown that ANS binding to rabbit (Rubalcava et al., 1969) or bovine (M. B. Feinstein and H. Felsenfeld, unpublished observation) erythrocyte membranes was unaffected by trypsin digestion which resulted in a loss of 50% of the membrane protein. The loss of protein from myelin under comparable conditions of proteolytic digestion was considerably less than that observed for the red cell membrane. Pronase digestion caused the loss of 30 ± 2.8% of myelin protein, and trypsin and pepsin removed even less protein, 6.0 ± 2.9 and 12.0 ± 0.62%, respectively. The proteolytic digestion had little or no effect on the subsequent binding of fluorescent probes to myelin in marked contrast to the effect of such enzyme treatment on ANS and TNS binding to isolated myelin basic protein and proteolipid (Feinstein and Felsenfeld, 1975).

Energy Transfer to Bound Fluorescent Probes. Some quenching of intrinsic protein fluorescence occurred when myelin was titrated with ANS. This could result from di-

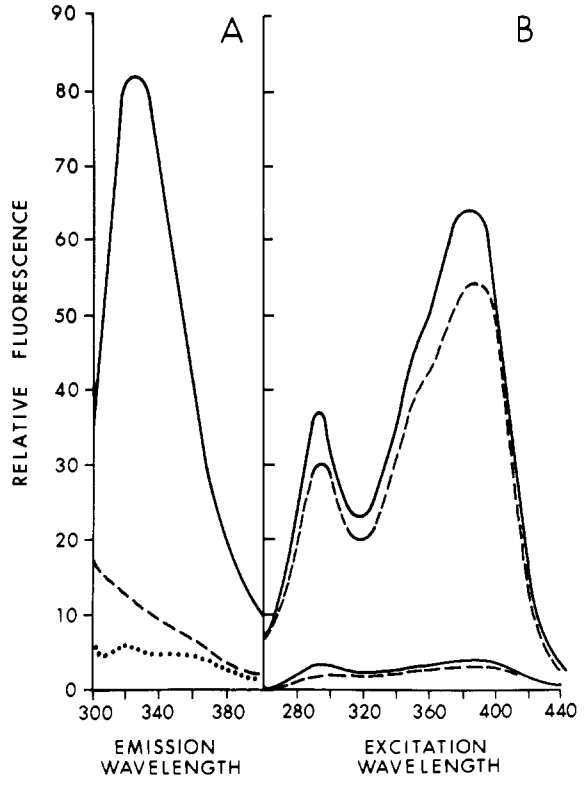

FIGURE 6: (A) Intrinsic fluorescence of native (—), 2-hydroxy-5-nitrobenzyl bromide (- - -), and 2-methoxy-5-nitrobenzyl bromide (. . . .) treated myelin. The myelin concentration was 33 μg of protein/ml in Tris buffer (pH 7.4) in each case. (B) Excitation spectra of ANS bound to native (—) and 2-methoxy-5-nitrobenzyl bromide (- - -) treated myelin. Emission was recorded at 470 nm with slits set at 8-nm bandwidth. ANS concentration was 80 μM. The lower records are in the absence of La^{3+} and the upper recordings were made after the addition of 1 mM $LaCl_3$.

pole–dipole radiationless energy transfer (Förster, 1959) from excited tryptophans to nearby bound ANS molecules. However, ANS absorbs light in the spectral regions utilized for excitation of tryptophan fluorescence, as well as in the region of the tryptophan fluorescence emission. Therefore, quenching of tryptophan fluorescence upon titration of myelin suspensions, or protein solutions with ANS, could result from so-called "inner filter" effects unrelated to radiationless energy transfer, i.e., (a) decreasing the quanta of exciting light reaching the protein chromophore, and (b) absorbing light emitted by excited tryptophan molecules. The extent of the "inner filter" effect was determined from titrations of tryptophan solutions of appropriate concentrations in the same buffers used for myelin and protein titrations. Nonradiative energy transfer efficiency, T (the ratio of the quanta emitted by myelin tryptophan in the presence and absence of bound ANS), was determined from the maximum calculated quenching of protein fluorescence after correction for the inner-filter effect from the relationship

$$T/(1 - T) = (R_0/R)^6 \quad (3)$$

(Radda and Vanderkooi, 1972). The rate of energy transfer depends upon $1/R^6$ (Förster, 1959), where R is the distance between the two species and R_0 is the distance between the donor–acceptor pair at which the rate of energy transfer is equal to all other deactivating processes for the donor. For the tryptophan–ANS pair R_0 has been calculated to be 23.3 Å (Freedman et al., 1970). The efficiency of ANS–tryptophan energy transfer was 0.16 from which an average intramolecular distance was calculated to be 31.8 Å. Because of uncertainty about the mutual orientation of donor and acceptor dipoles, and the possibility of energy transfer between multiple donor (or acceptor) molecules the calculated apparent intramolecular distances are probably only rough approximations of the true average distances. However, the low rate of energy transfer is distinctly less than that observed in erythrocyte membranes (Wallach et al., 1970) and solubilized myelin proteins (Feinstein and Felsenfeld, 1975). Alkylation of myelin tryptophan groups with 2-hydroxymethyl- or 2-methoxymethylbenzyl bromide abolished measurable energy transfer to bound ANS, and greatly reduced the intrinsic fluorescence of myelin proteins. Despite these effects, the binding of ANS or TNS, as measured by dye fluorescence excited at 380 nm, was essentially unaffected (Figure 6).

Discussion

A striking enhancement of fluorescence resulted from the binding of ANS or TNS to myelin. The quantum yield of ANS fluorescence increased more than 100-fold as compared with its value in water of 0.004. Emission maxima for both dyes also shifted markedly to the blue. These effects are characteristic for the behavior of the dyes when present in solvents of low polarity, as well as upon binding to certain proteins, lipids, or cellular membranes. It has been established by X-ray diffraction studies that ANS binds at the polar head group–hydrocarbon interface region of phospholipid micelles (Lesslauer et al., 1972). Lipid enhancement of the fluorescence of ANS and TNS appears to be related to the net surface potential of the lipid vesicles or bilayer membranes. Thus, acidic phospholipids (e.g., phosphatidylserine) bind little if any dye, unless their net surface negativity is countered by cations, H^+, or cationic drugs which are bound to the anionic sites, or which screen the negative charges by their presence in the Stern layer of the micelle surface. Lecithin and sphingomyelin which possess the quaternary nitrogen group of choline bind the anionic dyes to a much greater extent (than do acidic phospholipids) presumably because of internal electrostatic neutralization of the negative phosphoryl groups by the quaternary N groups. In a mixed phospholipid micellar dispersion containing equal amounts of phosphatidylserine and lecithin, we observed that interactions with ANS or TNS were determined by the former predominantly (Feinstein and Felsenfeld, 1975) because of the net negative surface charge imparted by the carboxyl groups of phosphatidylserine. Little or none of the anionic dyes were bound to such mixed lipid vesicles, unless cations were added to reduce the surface negativity.

The amount of fluorescent probe bound per unit weight of myelin was also greatly influenced by the ionic environment and the pH. Cations and H^+ promoted probe binding to myelin in much the same manner, quantitatively, as was observed for lipids, erythrocyte membranes (Rubalcava et al., 1969; Freedman and Radda, 1969; Feinstein et al., 1970), and membranes of the sarcoplasmic reticulum (Vanderkooi and Martonosi, 1969). The cation effect on dye binding was strongly influenced by ion valency, as predicted by eq 2 which shows the concentration of cation at the membrane surface to be a function of both cation valence and membrane surface potential. Probe binding to myelin was also increased by the addition of certain cationic molecules (over the pH range employed), such as local anesthetics (procaine and butacaine) and the antibiotic polymyxin B. Similar effects of local anesthetics and polymyxin B have

been noted previously in experiments with red cell, microsomal, and mitochondrial membranes (Vankerkooi and Martonosi, 1969; Feinstein et al., 1970; Chance and Mela, 1971). They appear to result from interactions with fixed negative charges of the membranes. The fatty acid portion of the polymyxin B side chain can penetrate into the hydrophobic portion of a liposome bilayer, and an amino group of the antibiotic (Pache et al., 1972) interacts with the phosphate group of lecithin. Local anesthetics have also been shown to bind to phospholipids, especially those with a net negative charge, thereby releasing H^+ from the lipid micelles, interfering with lipid binding of cations (Feinstein, 1964; Feinstein and Paimre, 1966) and reducing the membrane surface potential (Bangham et al., 1965). Electrostatic and H-bonding interactions of local anesthetics with phosphodiester groups are well documented (Feinstein and Paimre, 1966; Sax and Pletcher, 1969). The binding of ANS and TNS anions to a membrane is expected to be inversely proportional to the degree of negativity of the membrane surface potential. Therefore, we are led to conclude that the enhancement of fluorescent probe binding of myelin produced by polymyxin B and local anesthetics is due to interactions of the latter with membrane lipids thereby resulting in a reduction of the negative surface potential. In the accompanying paper (Feinstein and Felsenfeld, 1975) we have shown that neither polymyxin B nor local anesthetics directly affect fluorescent probe binding to extracted myelin proteins.

The decrease in fluorescence probe binding induced by phospholipase C treatment undoubtedly also results from alterations of membrane surface charges. The enzyme removed primarily the ethanolamine phosphate and choline phosphate moieties of the zwitterionic myelin lipids, with little or no effect on the acidic phospholipids or cerebrosides. This resulted not only in reduced fluorescent probe binding but also decreased the ability of certain cations, local anesthetics, and polymyxin B to enhance such binding. The effect of phospholipase C on myelin is presumably to affect binding of probe molecules to membrane lipids since these effects of the enzyme were duplicated on purely lipid membranes.

The surface potential (ψ) of a membrane is related to its fixed charge density (σ) and the ionic concentrations of the solution by the expression (Gilbert and Ehrenstein, 1969):

$$\sigma = \frac{1}{G}\left[\sum_{i=1}^{n} C_i\left(\exp-\frac{Z_i F\psi}{RT} - 1\right)\right]^{1/2}$$

where G is a parameter which is a function of the dielectric constant and temperature, and the other parameters have been previously defined (eq 2). The surface potential will in turn also affect the concentration of the anionic probes at the membrane surface. It is to be expected that chemical alteration of the membrane fixed charge density (σ) should therefore affect the surface potential in such a way as to have a predictable effect on the binding of ANS and TNS anions. These expectations were borne out in experiments designed to chemically modify the surface fixed charges of myelin. Blocking membrane carboxyl groups via carbodiimide activation and subsequent reaction with amino acid esters strikingly increased dye binding, demonstrating that the carboxyl groups had contributed to a surface negativity that repelled anionic probe molecules. This conclusion was further substantiated by the observation that substitution of positive charge, in the form of the guanidino groups of arginine methyl ester, in place of the carboxyl groups, produced an even greater enhancement of dye binding than introduction of a noncharged amino acid ester. Furthermore, the introduction of negative charge into the membrane by reaction of amino groups with succinic anhydride led, as expected, to a reduction in dye binding. It is also apparent that some inaccessible protein charged groups, but more especially lipid phosphate and sulfate groups, are also of great importance in their influence on membrane surface potential and dye binding. This conclusion is based on the marked further enhancement of dye binding to carboxyl-blocked myelin by H^+ and La^{3+}. The degree of enhancement of ANS and TNS binding to myelin by H^+ and La^{3+} is comparable to that observed with lipids. By comparison the effects of H^+ and La^{3+} on dye binding to extracted myelin proteins were weak or nonexistent (Feinstein and Felsenfeld, 1975).

The previous discussion has emphasized fluorescent probe interactions with membrane lipids, which is a well-demonstrated phenomenon. Binding of ANS and TNS to membrane proteins in situ, on the other hand, has not been demonstrated unequivocally. Although the probes have been shown to bind to soluble proteins of certain membranes (Freedman and Radda, 1969; Feinstein and Felsenfeld, 1975) some experimental observations indicate that proteins in *intact* natural membranes are not important binding sites for ANS or TNS. Binding of fluorescent probes to extracted myelin membrane proteins is abolished by proteolytic digestion, but such binding is unaffected by proteolysis in intact myelin or erythrocyte membranes. If proteins were arranged in membranes solely in the manner envisaged by the Davson–Danielli–Robertson model their potential dye binding sites would be expected to be largely accessible to the dyes, as well as to proteolytic enzymes. One possible explanation is that a large fraction of the membrane protein is situated deeply within the hydrocarbon interior of the membrane bilayer inaccessible to enzymes, and possibly to the probes as well. The small fraction of myelin protein that can be removed by proteolytic digestion (10–30%) favors an interior location for the bulk of this membrane's protein content. The dye binding sites of the membrane proteins in situ may also be inaccessible to the dyes since quantum yield, fluorescence lifetime, and efficiency of energy transfer are all significantly lower for intact myelin as compared to the solubilized myelin proteins. Furthermore, alkylation of tryptophan residues in isolated myelin proteins reduced the quantum yield of bound-ANS fluorescence to 0.15 of that in normal protein (Feinstein and Felsenfeld, 1975). Similar treatment of intact myelin drastically reduced intrinsic membrane fluorescence, but had no effect on the fluorescence yield of membrane-bound ANS. The impression gained from these experiments with fluorescent probes is that myelin has a very substantial content of *integral* proteins—as defined in the globular protein–lipid mosaic model of membrane structure (Singer, 1971).

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