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Effects of Cations and Propranolol on a Fluorescent Phospholipid Incorporated into Brain Synaptosome Membranes

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SUMMARY

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Dimethylaminonaphthalenesulfonyl-phosphatidylserine (DNS-PS), a fluorescent phospholipid, was incorporated into rat brain synaptosomes by a procedure utilizing the chaotropic agent sodium perchlorate. This procedure caused only minor changes in the electron microscopic appearance and protein composition of the membranes. Perchlorate treatment alone altered the activity of selected enzymes such as acetylcholinesterase and monoamine oxidase, but the insertion of DNS-PS within the membrane had only minor effects on the specific activities of synaptosomal enzymes. The fluorescence emission maximum of the membrane-bound DNS-PS was shifted to a slightly longer wavelength by addition of 100 mM NaCl or KCl, and the polarization of fluorescence decreased. The addition of 10 mM $CaCl_2$ shifted the emission maximum to a shorter wavelength, increased relative fluorescence 10–20%, and increased the polarization of fluorescence. Energy transfer from aromatic residues of membrane proteins to the DNS moiety was increased 11% by $CaCl_2$, while $MgCl_2$ had little effect, suggesting that Ca^{2+} may decrease the intermolecular distance between aromatic amino acids of membrane proteins and the fluorescent phospholipid. The *beta* adrenergic blocking agent propranolol reversed the changes caused by calcium and displaced at least a portion of the bound cation from the membrane.

INTRODUCTION

Phospholipids are major constituents of biological membranes, and their functional significance within the membrane has been suggested by many investigators (1–7). It has been difficult to demonstrate directly changes in the characteristics of native membrane lipids *in situ* which might be related to their function. One approach, using spin-labeled lipids (8), has strengthened the case for involvement of lipid-phase transitions in the activity of the membrane-bound $(Na^+ + K^+)$-ATPase system. Another approach has utilized fluorescent probes in the investigation of membrane architecture and function (9, 10), with changes induced in membranes being correlated with changes in the fluorescent parameters of the probe. Most fluorescent probes, e.g., 1-anilino-8-naphthalenesulfonate and 2-*p*-toluidinyl-6-naphthalenesulfonate, that have been used are noncovalently bound to the membrane, and there is considerble uncertainty as to the nature of the membrane binding site. These sites may be at a protein-lipid interface or may lie in either the lipid or protein regions of the membrane.

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In previous studies the acidic phopholipids phosphatidylserine and phosphatidylethanolamine were derivatized by reaction of their amine groups with DNS-chloride[2] to produce the fluorescent phospholipids DNS-phosphatidylserine (11) and DNS-phosphatidylethanolamine (12, 13). Also, we demonstrated that DNS-PS in micellar suspension can be substituted for micellar PS in the reactivation of the lipid-depleted rat brain ($Na^+ + K^+$)-ATPase enzyme system (11). It has been assumed that the physical characteristics of the DNS-PS as well as the PS micelles were similar to those described previously (14, 15). It was concluded that the simple mixing of micelles of DNS-PS with microsomal membranes did not lead to incorporation of the DNS-PS into the membrane matrix (11) and that activation of the ($Na^+ + K^+$)-ATPase probably occurred by interactions at or near the surface of the membrane.

The purpose of the present study was to evaluate the feasibility of using DNS-PS as a fluorescent probe to study changes within the lipid domain of the membrane. In order to do this a procedure has been developed for incorporation of the fluorescent phospholipid into the membrane matrix. Rat brain synaptosomes were chosen as the source of membranes, since they are relatively well characterized and are useful for a variety of neurochemical studies, including neurotransmission and ion transport. High concentrations of the chaotropic agent $NaClO_4$ have been shown to disrupt membranes (16, 17) as well as phospholipid dispersions (18). It was hoped that, by disrupting both the membranes and phospholipid micelles together and then gradually removing the $NaClO_4$ by dialysis, incorporation of the phospholipid into the membrane could be accomplished.

MATERIALS AND METHODS

Synthesis of DNS-PS. DNS-PS was synthesized by a slight modification of the procedure described by Waggoner and Stryer (12) and by Shechter *et al.* (13), which involved the reaction of DNS-Cl or [^{3}H]DNS-Cl with the amine group of phosphatidylserine in an anhydrous organic solvent at a basic pH. Commercially available PS (Sigma) was purified by preparative thin-layer chromatography using Camag D-O silica gel (19). Recrystallized [^{3}H]DNS-Cl (45 μmoles) was allowed to react with purified PS (30 μmoles) in 3 ml of anhydrous benzene plus 30 μl of triethylamine at 37° for approximately 8 hr. The progress of the reaction was monitored by the disappearance of the ninhydrin-positive spot on a silica gel thin-layer plate developed with chloroform–methanol–water–glacial acetic acid (65:25:4:8, by volume). After completion of the reaction, the mixture was applied to a silica gel AR cc-7 column (1.5 × 20 cm; Mallinckrodt, 200–325 mesh). The column was eluted with benzene–ether (60:30, v/v) to remove unreacted DNS-Cl, and then with chloroform–methanol (75:20, v/v), which removed most of the DNS-PS. The yield was usually greater than 80%. In some preparations contaminants were still present after the column chromatography and were removed by preparative thin-layer chromatography (see ref. 14). The specific activity of [^{3}H]DNS-Cl was determined by converting an aliquot to [^{3}H]DNS-OH with $NaHCO_3$ and measuring the optical density at 315 nm. The concentration was calculated using a molar extinction coefficient of 4.34×10^3 (20). Radioactivity was measured in a scintillation counter in Bray's solution (21). The ratio of DNS to phospholipid phosphorus of the final product was 1.04:1.

Fatty acid analysis. Fatty acid methyl esters of PS and DNS-PS were obtained using BF_3–methanol (22). Gas chromatographic analyses were done with a Hewlett-Packard 5700 system using 15% Dexsil 300 cc on Chromosorb W, 80/100 (Applied Science), and 15% EGSS-X on Gas-Chrom P, 100–120 (Applied Science). Methyl ester standards were obtained from Supelco. The PS and DNS-PS preparations used in this study had total fatty acid to phospholipid phosphorus molar rations of 2.13 (PS) and 1.98 (DNS-PS). The fatty acid composition of PS was: 16=0, 1.75%; 17=0, 0.63%;

[2] The abbreviations used are: DNS, dimethylaminonaphthaline-sulfonyl; PS, phosphatidylserine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; CTC, chlorotetracycline.

18=0, 53.32%; 18=1, 38.29%; 18=2 or 20=0, 1.39%; and 20=1, 4.63%. The composition of DNS-PS was: 16=0, 1.70%; 17=0, 0.66%; 18=0, 54.9%; 18=1, 36.30%; 18=2 or 20=0, 1.06%; and 20=1, 5.36%. This distribution of fatty acids is very similar to that previously described for bovine brain PS (23). The largest difference between the present work and the former work is in the 18=1 component. The present value was 38.3%, while the former was 30.3%. The remaining values were within ±1% of each other.

Phospholipids were dispersed in 0.01 M EGTA-Tris, pH 7.4, by ultrasonication with a Branson Sonifier. Sonication at power setting 5 was run for 30-sec periods with intervals for cooling to maintain a 4° temperature. Total sonication was 30 min at a phospholipid concentration between 15 and 20 mM. The phospholipids were then dialyzed for approximately 16 hr against 0.03 M Tris-HCl, pH 7.4. The suspensions were stored under nitrogen at 5°.

Incorporation of DNS-PS into synaptosomes. Rat brain synaptosomes (24) were used fresh or after storage for up to 2 weeks at −80°. A preparation containing 5–7 mg of protein per milliliter in 25 mM disodium EDTA, pH 7.4, was combined with a sonicated aqueous suspension of [^{3}H]DNS-PS so that the ratio of [^{3}H]DNS-PS to protein was approximately 0.25 μmole of [^{3}H]DNS-PS per milligram of protein, usually in a final volume of 5 ml. An equal volume of 4 M $NaClO_4$ (pH 7.4) was added, and the suspension was maintained at 25° for 1 hr. The solution was then dialyzed against a 1000-fold excess (changed twice) of 5 mM Tris-HCl, pH 7.4, at 4° for 16 hr. In order to separate the free [^{3}H]DNS-PS, the dialyzed sample (5 ml) was applied to the top of a discontinuous sucrose gradient consisting of 4.5–5 ml each of 1.2, 1.1, 1.0, 0.8, 0.6, and 0.4 M sucrose, and was centrifuged in an SW 25.2 rotor in a Spinco ultracentrifuge at 25,000 rpm for 2 hr at 4°. After centrifugation, opalescent bands were visible at the sucrose interfaces; when viewed under ultraviolet light, each band was fluorescent. The free DNS-PB was located at the H_2O−0.4 M surose interface, with most of the protein appearing at the 1.0 and 1.2 M sucrose interfaces (fraction 5). Control preparations without DNS-PS had a similar distribution of bands.

The various interface bands, numbered from 1 to 5 (Fig. 1), and the pellet (fraction 6) were collected using a glass capillary tube connected to a peristaltic pump. Fractions from control synaptosomes (no DNS present) were designated with the prefix C, while those with DNS-PS incorporated have the prefix D. These fractions were then diluted with water to 0.20 M sucrose and were collected by centrifugation at 20,000 rpm in a refrigerated Sorvall centrifuge for 1 hr. The pellets obtained were resuspended in 0.2 M sucrose–5 mM Tris-HCl, pH 7.4, and aliquots were frozen and stored at −80°.

Electron microscopy. Samples for electron microscopy were fixed in 2% paraformaldehyde–4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and then postfixed in 1.33% OsO_4–0.1 M *s*-collidine. Staining was done with a saturated uranyl

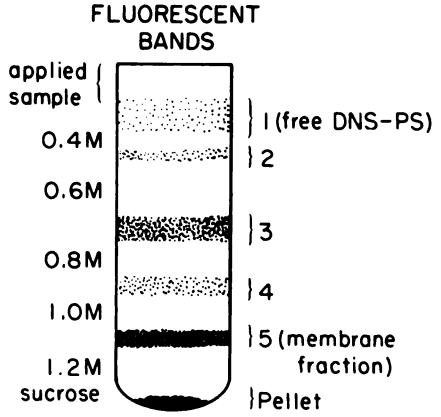

FIG. 1. *Sketch of discontinuous sucrose gradient containing synaptosomal fractions with DNS-PS incorporated*

Synaptosomes were treated with perchlorate and DNS-PS (see MATERIALS AND METHODS) and were dialyzed against 5 mM Tris, pH 7.4; they were then applied to the gradient and centrifuged for 2 hr at 25,000 rpm in a Spinco SW 25.2 rotor. A control preparation (treated with perchlorate but without DNS-PS) showed a similar distribution of bands within the gradient. Fractions were designated C-1 to C-pellet for the control preparation, and D-1 to D-pellet for the DNS-PS-treated preparation. The position of free DNS-PS was ascertained in a gradient without synaptosomes. Concentrations of sucrose within the tube are shown to the left. Bands were visualized under long ultraviolet light.

acetate–lead citrate solution (25), and sections were viewed with an AE1 801 electron microscope.

SDS–polyacrylamide gel electrophoresis. This was carried out essentially as described by Fairbanks *et al.* (26). An aliquot of each fraction was dialyzed against a solution containing 1% SDS, 10 mM Tris, and 1 mM EDTA, pH 8, overnight at room temperature, and was then incubated for 30 min at 40° in the same buffer containing 40 mM dithiothreitol. Electrophoresis was carried out in a Hoefer system (Bio-Rad model 300) at room temperature (20°), using 5 × 90 mm gels with Pyronine Y as tracking dye. The gels were run at constant current for 1 hr at 1 mamp/gel, and then for an additional 2–3 hr at 4 mamp/gel. After completion of a run, the lengths of the gels were measured and dye position was marked by insertion of a wire. The gels were stained with Coomassie blue and destained as described by Fairbanks *et al.* (26). Relative mobilities for molecular weight determinations were established according to Weber and Osborn (27) as used by Stahl (19). Densitometric scanning was done at 550 nm with a Gilford linear transport scanner at 0.5 cm/min with the 0.05 × 2.36 slit plate.

Assay and analyses. Protein (28) and phospholipid phosphorus analyses (29) were carried out by standard procedures. Acetylcholinesterase was assayed by the method of McCaman *et al.* (30), and monoamine oxidase, by the procedure of McCaman *et al.* (31). The $(Na^+ + K^+)$-ATPase assay procedure is described in detail elsewhere (32). Arylsulfatase was measured with *p*-nitrocatechol sulfate as substrate. The incubation medium contained 10 mM *p*-nitrocatechol sulfate, 0.25% Triton X-100, 0.2 mM sodium pyrophosphate, 0.6 M NaCl, 0.1 M sodium acetate (pH 4.9), and approximately 40–100 μg of synaptosomal protein in a final volume of 60 μl. The incubation was conducted for 30 min at 37°. The tubes were chilled, and 100 μl of 1 N NaOH were added. After centrifugation at 600–1000 × *g* for 15 min, the optical density of the supernatant at 515 nm was measured in a Zeiss PMQ II spectrophotometer fitted with a microcell attachment. *p*-Nitrocatechol was used to construct a standard curve, and the enzymatic reaction was linear for at least 30 min with up to 240 μg of synaptosomal protein.

The filtration procedure for measuring ^{45}Ca uptake by synaptosomes was described previously (24).

Propranolol was kindly supplied by Ayerst Laboratories. Chlorotetracycline HCl was obtained from NBC Research Biochemicals, Cleveland, and a solution was prepared fresh daily.

Fluorescence measurements. Fluorescence measurements were made with a Perkin-Elmer fluorescence spectrophotometer, model MPF-2A, using thermostated cell holders. Polarization was measured with the standard accessory. The output of the fluorometer was connected to a Houston Instruments Omnigraphic 3000 *xy* recorder through an interface that maintained the ratio capacity of the recorder normally supplied with the instrument. The *x* axis could be used either in a time base mode or as a direct display of the wavelength by means of potentiometers connected to the monochrometer drive shafts.

The typical protocol for fluorescence measurements involved preparation of a stock solution of the appropriate buffer (3 or 10 mM Tris-HCl, pH 7.4, containing 0.2 M sucrose), with synaptosomal fraction 5 containing DNS-PS (Fig. 1) so that 400 μl in each cuvette would contain between 10 and 25 μg of protein. One cuvette contained the buffer (blank), and the remaining three cuvettes, the buffer plus fraction 5 material. An initial reading of each cuvette was made and used as a correction factor if any variation was found. Then 1–4 μl of a concentrated solution of the ion or compound under investigation were added to one cuvette, with an equal volume of water added to the control cuvette. Because there was a tendency of the particulate synaptosomal membranes to settle out with time, care was taken to ensure that all samples were adequately and equally mixed.

At the completion of the experiment, mathematical corrections were made for any initial variation between samples and

for dilution (less than 5% volume increase) of the samples as a result of the addition of titrant.

There were no discernible protein light scattering artifacts on the polarization of fluorescence at the wavelength measured and protein concentration employed.

The quantum yield of the DNS-PS micelles or DNS-PS incorporated into synaptosomal membranes was not established. In the study comparing the effects of propranolol on the fluorescence of DNS-H, DNS-PS micelles, and DNS-PS incorporated into synaptosomes, samples were adjusted to have the same concentration of DNS based upon [^{3}H]DNS specific activity.

RESULTS

Characteristics of the preparation. The procedure developed for the incorporation of DNS-PS into synaptosomal membranes consists of (a) simultaneous perturbation of the membranes and phospholipid micelles with the chaotropic agent 2 M $NaClO_4$ (16), (b) removal of the perchlorate by dialysis, which presumably permits reformation of the membranes, and (c) final removal of the unbound DNS-PS by sucrose density gradient centrifugation. After the final centrifugation, the distribution of synaptosomal material was similar to that described by Whittaker *et al.* (33) for osmotically shocked synaptosomes (Fig. 1). The subcellular composition of the fractions was assumed to be similar to that previously described, and a thorough electron microscopic study was performed only on fraction 5. The general appearance of the density gradient fractions without added DNS-PS was similar to those with DNS-PS. All the recovered fractions contained approximately equal amounts of DNS-PS per milligram of protein. However, the amount of DNS-PS bound by the lightest fraction was somewhat uncertain, since this band overlapped the free DNS-PS band. The [^{3}H]DNS-PS incorporated represented between 10% and 16% of the total phospholipid in any fraction.

The enzymes listed in Table 1 were assayed to establish the degree of damage caused by the $NaClO_4$ treatment and dialysis alone and with DNS-PS added, and to see whether the presence of DNS-PS affected the distribution of enzyme markers in the gradient fraction. ($Na^+ + K^+$)-ATPase, an accepted synaptosomal plasma membrane marker, showed an increase in specific activity after treatment with 2 M $NaClO_4$, as previously reported (16), and was present in all fractions after density gradient centrifugation. A slight increase in specific activity was seen in fractions C-3, D-4, and D-5, and a slight decrease was seen in the pellets. On the other hand, treatment with 2 M $NaClO_4$ and dialysis caused a loss of more than 50% of the specific activity of acetylcholinesterase, which is also a marker for synaptosomal plasma membranes. However, acetylcholinesterase which is also a marker for synaptosomal plasma membranes. However, acetylcholinesterase was found in all fractions, and the lighter fractions had higher specific activities.

Monomamine oxidase, an outer mitochondrial membrane marker, was also very sensitive to the perchlorate treatment, with a recovery of less than 5% of the total or specific enzyme activity present in the initial synaptosomes; the pellet had no activity. The lability of this enzymatic activity made an evaluation of contamination by mitochondrial fragments inconclusive.

Arylsulfatase, used as a lysosomal marker, was only slightly affected by treatment with $NaClO_4$, but a major portion of the total activity was lost upon subsequent fractionation and storage of the individual fractions. The arylsulfatase specific activity was highest in the lighter fractions, with a uniform decrease in the heavier fractions.

We conclude that the perchlorate treatment significantly altered the activity of selected enzymes such as acetylcholinesterase and monoamine oxidase. The introduction of DNS-PS itself into the perchlorate-treated membranes appeared to have only minor additional effects on the specific activities of the enzymes studied. Introduction of DNS-PS led to 2–3-fold higher recoveries of ($Na^+ + K^+$)-ATPase, acetylcholinesterase, and arylsulfatase in fraction D-5, although the specific activities of the en-

TABLE 1

Distribution of enzyme activities in synaptosomes

The distribution of enzymatic activity within the fractions in four preparations was within ±7% of each indicated value, although the specific enzymatic activity of the material after treatment with $NaClO_4$ and dialysis had a greater variation.

Fraction	$(Na^+ + K^+)$-ATPase		Acetylcholinesterase		Monoamine oxidase		Arylsulfatase	
	μmoles P_i formed/mg protein/hr		*μmoles acetylcholine hydrolyzed/mg protein/hr*		*nmoles substrate utilized/mg protein/hr*		*nmoles p-nitrocatechol sulfate hydrolyzed/mg protein/hr*	
Original synaptosomes	4.91		5.20		189.2		365	
Perchlorate-treated synaptosomes	17.3	(100)[a]	1.20	(100)	7.55	(100)	274	(100)
C-2	12.4	(1.5)	3.25	(2.2)	8.63	(3.6)	110.4	(3.6)
C-3	19.2	(6.9)	3.98	(7.9)	0		88.1	(8.7)
C-4	17.0	(31.3)	3.63	(37.2)	5.73	(36.6)	55.9	(28.1)
C-5	13.3	(30.8)	1.45	(18.6)	7.47	(60.0)	41.7	(26.3)
C-pellet	9.4	(30.2)	1.90	(37.0)	0		37.9	(33.3)
Perchlorate-DNS-treated synaptosomes	16.6	(100)	2.39	(100)	4.11	(100)	388.9	(100)
D-2	14.4	(0.3)	0.74	(0.1)	95.9	(5.2)	26.0	(0.1)
D-3	14.4	(3.1)	2.05	(5.3)	13.6	(6.9)	143.2	(8.6)
D-4	21.7	(17.5)	2.01	(19.8)	5.2	(9.7)	88.6	(20.2)
D-5	17.5	(68.0)	1.30	(61.3)	8.7	(78.1)	53.9	(59.1)
D-pellet	12.3	(11)	1.25	(13.5)	0		47.3	(11.9)

[a] Percentage of activity recovered, based on perchlorate-treated or perchlorate- and DNS-treated synaptosomes after dialysis (see MATERIALS AND METHODS). These data were derived from a single representative experiment.

zymes were not increased proportionately.

Fractions C-5 and D-5 (1.0–1.2 M sucrose interface), which were used in the subsequent fluorescence studies, contained between 25 and 30% of the total protein, had a higher $(Na^+ + K^+)$-ATPase specific enzyme activity, lower acetylcholinesterase and arylsulfatase, and much lower monoamine oxidase activity than the intial synaptosomes.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was run on initial synaptosomes before treatment and on all the density gradient fractions. The gel scans, three of which are shown in Fig. 2, were similar for all fractions. A major band of approximately 35,000 mol wt was used as an internal marker to align all the scans. Between 19 and 21 major bands were seen in each gel. The initial synaptosomal preparation had a larger proportion of low molecular weight components than did fraction 5. The proportion of higher molecular weight components was greater in the denser gradient fractions.

Electron microscopy. Electron micrographs of 10,000- and 25,000-fold magnification (not shown) were made of the initial synaptosomes and fractions C-5 and D-5. The fractions contained empty, closed vesicles. There was no evidence of intact mitochondria or synaptic vesicles, and both C-5 and D-5 were very similar in appearance.

Removal of DNS-PS from synaptosomes. In order to establish how tightly the added DNS-PS was associated with the membrane, the procedures outlined in Table 2 were performed. An aliquot of fraction D-5 was collected by centrifugation in 0.6 M sucrose so that any free or lightly bound phospholipid would be found in the supernatant. As indicated, more than 90% of the [^{3}H]DNS-PS remained with the

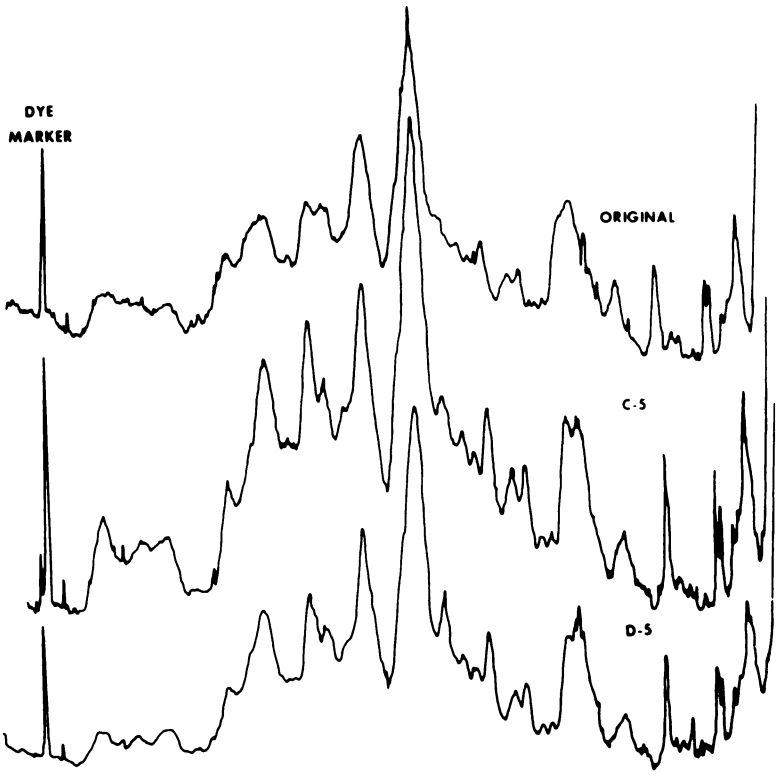

FIG. 2. *SDS-polyacrylamide gel electrophoresis of synaptosome membranes*
Gels were scanned at 550 nm, and the dye marker was Pyronine Y. The top scan was of the initial synaptosomes, the middle scan was of fraction C-5, and the bottom scan was of fraction D-5.

membranes. In a solution of high ionic strength, such as 1 M NaCl, which minimized electrostatic interactions within the membrane, essentially 100% of the protein and phospholipid was removed in the pellets. Even treatment with 2 M $NaClO_4$ did not cause appreciable dissociation of the [^{3}H]DNS-PS from the membrane. Approximately 14% of the protein and 25–30% of the incorporated phospholipid was solubilized by this second treatment with $NaClO_4$. This caused the DNS to protein ratio in the pellet to decrease by 20%. The effects of treatment with 2 M urea were similar to those with $NaClO_4$. Urea caused a larger proportion of protein to be solubilized, but the DNS to protein ratio was similar. The anionic detergent SDS caused the greatest solubilization of both protein and DNS-PS, and slightly more phospholipid than protein was lost from the pellet. This appears to show that the [^{3}H]DNS-PS is incorporated within the membrane matrix and that denaturing conditions are necessary for its removal. Such conditions are similar to those needed to separate the

TABLE 2

Retention of DNS-PS by synaptosomes

Aliquots of fraction D-5 with [^{3}H]DNS-PS incorporated (0.105 μmole of [^{3}H]DNS per milligram of protein) were dispersed in the indicated solutions, incubated at 25° for 1 hr, and then centrifuged at 25,000 rpm in a Spinco SW 39 rotor. The distribution of [^{3}H]DNS and protein in the supernatant and pellet was determined as described under MATERIALS AND METHODS.

Treatment	[^{3}H]DNS/mg of protein of pellet	Recovery of protein in pellet
	% control	*% control*
0.60 M sucrose	90–94	92
1.0 M NaCl	100	98
2.0 M $NaClO_4$	78–85	89
2.0 M urea	75–82	66
0.05% SDS	25–40	33

intrinsic membrane phospholipids from proteins (4, 34) and emphasize the importance of hydrophobic interactions in the matrix of synaptosomal membranes.

Effects of cations on fluorescence. The emission spectrum of the DNS-PS membrane fraction D-5 was studied in the presence of several cations. The emission maximum of DNS depends on the polarity of its environment, and is shifted to shorter wavelengths as the polarity of its environment is decreased (18). The broad, almost flat, emission maximum of fraction D-5 in a 3 mM Tris-HCl buffer (Fig. 3) was about 512 nm. Increasing the ionic strength to 100 mM with either KCl or NaCl changed the emission maximum to 520 nm and caused about a 5% decrease in relative fluorescence. The addition of $CaCl_2$ (10 mM, total concentration) to this solution caused a distinct shift in the emission maximum from 520 nm to 500 nm and increased the relative fluorescence about 10–20% (Fig. 3). There was no change in the excitation maximum with the above ionic variations.

The DNS-PS fluorescence of fraction D-5 in 100 mM NaCl was increased 20% by addition of 10 mM $CaCl_2$. However, additions of 10 mM $MgCl_2$ resulted in only a 4% increase at 500 nm (Fig. 4).

A molecule with electrons in the excited state may transfer a portion of this energy to a second molecule. When this energy is released by fluorescence decay, the resulting spectrum will be a composite derived from the 2 molecules, even though only the first molecule absorbed the energy. Energy transfer is a tool for determination of the distance and interactions between molecules. This phenomenon was shown to occur between the synaptosomal membrane proteins and the DNS moiety within the phospholipid. This was demonstrated

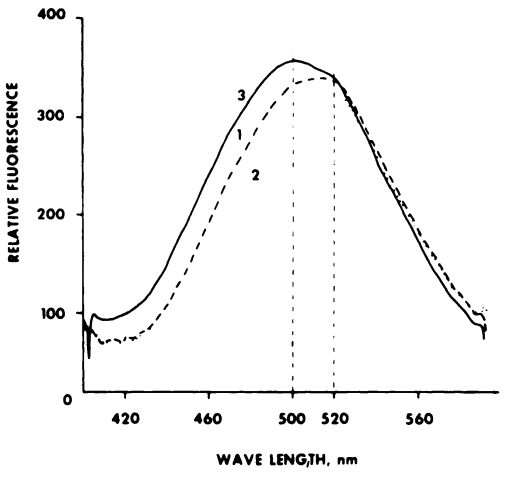

FIG. 3. *Fluorescence emission spectra of DNS-PS incorporated into synaptosomes*

Each cuvette contained 15 μg of protein (fraction D-5, 0.0016 μmol of [^{3}H]DNS-PS) in 400 μl of buffer (10 mM Tris-HCl, pH 7.4) (curve *1*), buffer plus 100 mM NaCl (curve *2*), or buffer plus 100 mM NaCl and 10 mM $CaCl_2$ (curve *3*). Samples were allowed to equilibrate at 25°. Excitation was at 350 nm, with both excitation and emission band pass at 7 nm.

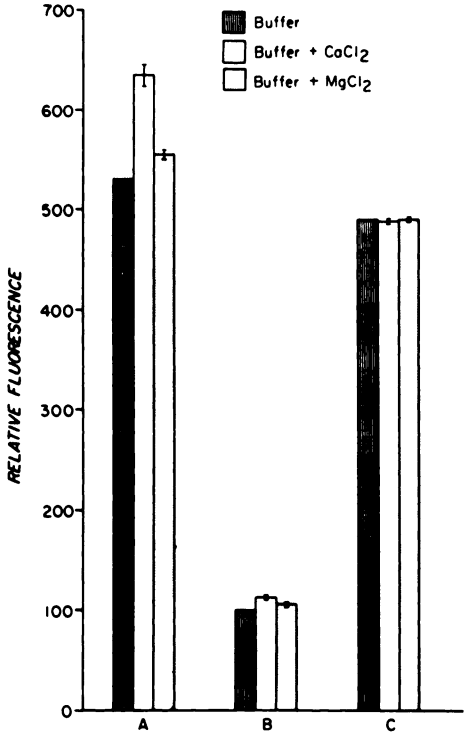

FIG. 4. *Relative increase in fluorescence in fraction D-5 in the prescence of 10 mM $CaCl_2$ and 10 mM $MgCl_2$*

Each cuvette contained 11 μg of protein (with 0.11 μmole of [^{3}H]DNS-PS per milligram of protein) in 400 μl of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.20 M sucrose. A. Major fluorescence of DNS-PS. Excitation, 350 nm; emission, 500 nm. B. Fluorescence due to energy transfer, with excitation at 285 nm and emission at 500 nm. C. Protein fluorescence, with excitation at 285 nm and emission at 340 nm. Normalized data are presented.

by the quenching of protein fluorescence at 340 nm with a parallel increase in the DNS fluorescence spectrum at 500 nm (excitation at 285 nm) (data not shown). In Fig. 4B energy transfer from protein to the DNS moiety is shown. Calcium increased this process by 11%, while magnesium had little effect. We infer that the intermolecular distance between the aromatic amino acid moiety of the membrane protein(s) and the DNS moiety was decreased by the addition of Ca^{2+}. Calcium or magnesium had little effect on the intrinsic fluorescence of membrane proteins (Fig. 4C). Calcium may therefore be important in modulating the interaction of membrane proteins and phospholipids.

Fluorescence changes caused by propranolol. There are many reports concerning the interaction of calcium with phospholipids (35–37) and the effects of local anesthetics and drugs on these interactions (38, 39). Figure 5 shows the effect of the *beta* adrenergic blocking agent propranolol on the fluorescence of DNS-PS in membrane fraction D-5.

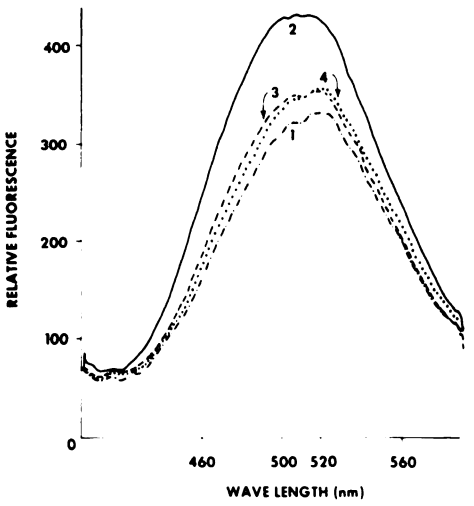

FIG. 5. *Fluorescence emission spectra of synaptosomal fraction D-5 with and without propranolol*

Each cuvette contained 24 μg of membrane protein (2.5 nmoles of [^{3}H]DNS-PS incorporated) in 400 μl of buffer (100 mM NaCl and 10 mM Tris-HCl, pH 7.4) (curve *1*), buffer plus 10 mM $CaCl_2$ (curve *2*), buffer plus 10 mM $CaCl_2$ and 1 mM propranolol (curve *3*), or buffer plus 1 mM propranolol (curve *4*). Excitation was at 350 nm, with a band pass of 7 nm for excitation and emission monochromators.

As shown in Fig. 3, fluorescence was increased by the addition of calcium, and the emission maximum of DNS-PS was shifted about 2 nm lower than a control without calcium. The addition of propranolol completely blocked the action of calcium. These effects suggest that propranolol interacts with the calcium binding site. From these spectra alone, it was difficult to determine whether the propranolol shifted the emission maximum or was affecting the entire spectrum. There was a slight overlap of the excitation spectra of DNS-PS (excitation maximum, 350 nm) and propranolol (excitation maximum, 315 nm), which may account for a small portion of the observed fluorescence changes in the membrane preparation. To resolve this point, propranolol was added to an equimolar DNS-PS micellar suspension and DNS-H to determine whether its absorption could account for the fluorescence changes observed when the DNS-PS was within the membrane. The concentration of the DNS moiety was identical in each case, as determined by [^{3}H]DNS. Although the added propranolol did cause a slight decrease in the fluorescence in the former two cases, it was not enough to account for the changes seen when DNS-PS was within the membrane.

Polarization of fluorescence. The polarization of fluorescence yields information on the freedom of rotation of the fluorochrome and is independent of the concentration of the fluorochrome. Table 3 shows the polarization of fluorescence of DNS-PS within the membranes with the ionic conditions studied in Figs. 3 and 4, and with the addition of propranolol. This preparation had a lower polarization in a medium of high ionic strength (B) than in a medium of low ionic strength (A). When calcium was added there was an apparent decrease in rotational freedom, reflected by the increase in the polarization (C). Propranolol caused a decrease in the polarization (D), indicating that the fluorescent moiety had a rotational freedom similar to that found without calcium in the medium. Although these polarization changes were small, these differences were significant ($p < 0.001$).

Fluorescence of chlorotetracycline. From

TABLE 3

Polarization of DNS-PS inserted in synaptosomal membranes

Polarization of fluorescence was measured on samples treated essentially as in Fig. 5. Excitation was at 350 nm and emission at 500 nm, with polarizing filters in the excitation and emission light paths. Measurements were made at 25°. Values are means ± standard errors for the number of determinations shown in parentheses, using three different preparations.

Preparation	Polarization of fluorescence
A. Fraction D-5 in 10 mM Tris-HCl	0.283 ± 0.0049 (23)
B. Fraction D-5 in 10 mM Tris + 100 mM NaCl	0.271 ± 0.0049 (5)
C. B + 2–10 mM $CaCl_2$	0.287 ± 0.0106 (24)
D. C + 50 μM–1 mM propranolol	0.276 ± 0.0079 (11)

the data presented it was difficult to distinguish whether propranolol was changing the calcium binding site on the membrane with the calcium still attached or was displacing the calcium from the membrane. The experiments below were performed with fraction C-5 (control membranes *without* fluorescent phospholipid) to determine whether either of the above proposals was valid. Chlorotetracycline is a probe whose fluorescence is dramatically increased when it chelates divalent cations (40, 41). Its fluorescence increases 8-fold when it binds calcium and approximately 20-fold when the calcium is also bound to a protein or membrane (40).

Figure 6 shows the increase in relative fluorescence of CTC in solution, after addition of fraction C-5, and calcium singly or together. The dashed line is the calculated sum of fluorescence of fraction C-5 and $CaCl_2$ corrected for the initial fluorescence of CTC. This combination caused a substantial increase in fluorescence over the individual components, indicating that calcium bound to the membrane. The final bar graphs indicate the fluorescence when propranolol (0.5 mM) was present. It appears that propranolol displaced at least some calcium from the membrane.

To substantiate this conclusion, experiments using ^{45}Ca were performed (Fig. 7). Synaptosomal membranes (fraction C-5) without fluorescent phospholipid but with CTC present were incubated in 0.1, 0.25, or 1 mM $^{45}CaCl_2$ with 0–1 mM propranolol. The fluorescence of CTC was measured in parallel with ^{45}Ca bound by the membranes. As in Fig. 6, the CTC-calcium-membrane complex had a high relative fluorescence which decreased upon addition of propranolol, and this was paralleled by a decrease in membrane-bound ^{45}Ca. The initial binding of ^{45}Ca to the membranes was concentration-dependent, and at 0.1, 0.25, and 1 mM the initial ^{45}Ca content was 21, 38.9, and 59.1 nmoles/mg of protein, respectively. Similar observations were also obtained when a native synaptosome preparation (no perchlorate treatment) was used (data not shown).

CONCLUSIONS

Treatment with perchlorate has differing effects on enzymes associated with synaptosomes. Certain enzymes thought to be

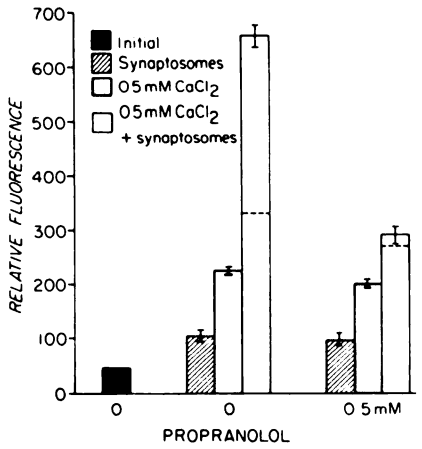

FIG. 6. *Relative fluorescence of chlorotetracycline in the presence of synaptosomes*

The effects of $CaCl_2$ and propranolol on fluorescence were monitored in an "initial" system containing of 0.2 M sucrose–10 mM Tris-HCl, pH 7.4, and 25 μM CTC, in a volume of 400 μl. When indicated, 27 μg of fraction C-5 (synaptosomes) with or without 0.5 mM $CaCl_2$ and 0.5 mM propranolol were added to the buffer-CTC solution to the same final volume. Equilibrium was attained within 2 min, and excitation was at 390 nm with emission at 530 nm. The dashed line indicates the mathematical sum of fraction C-5 plus $CaCl_2$ (see the text for details).

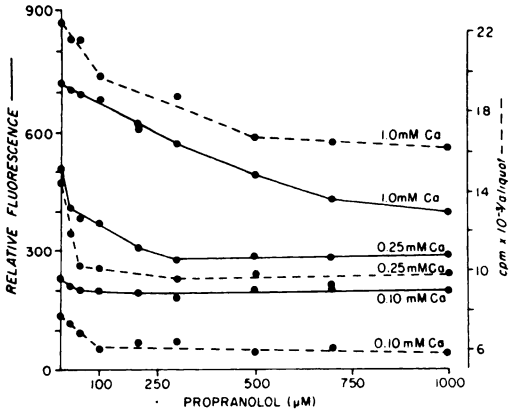

FIG. 7. *Effect of propranolol on calcium bound to rat brain synaptosomes*

Fraction C-5 (120 μg of protein) was incubated in medium containing 0.2 M sucrose–10 mM Tris-HCl (pH 7.4), 25 μM chlorotetracycline, and 0.1–1 mM ^{45}Ca (approximately 5000 cpm of ^{45}Ca per nanomole of calcium) in a volume of 500 μl. After 20 min at 25°, propranolol in H_2O (5–15 μl) was added to the cuvette and incubated for an additional 20 min. The fluorescence at 530 nm (excitation, 395 nm; bandpass, 8.8) was then measured (——), and duplicate 200-μl aliquots were withdrawn and membranes were collected on a 0.45-μm filter at 25°. Filters were then washed, dried, and counted for ^{45}Ca (24) (– – –). Fluorescence data were normalized to fit on the same scale.

extrinsic or associated with membrane surfaces (acetylcholinesterase, monoamine oxidase) were drastically inactivated by this treatment. On the other hand, $(Na^+ + K^+)$-ATPase, a core enzyme, was not inactivated by this treatment. The insertion of DNS-PS into synaptosomal membranes caused minor changes in the enzymatic character of the preparation. The procedure developed here probably incorporates the fluorescent lipid into many available regions within the membrane. The data may represent an averaging of large changes at a limited number of sites and smaller changes at the remaining sites induced by the reagents added. This would be expected to produce a high background fluorescence and may be the reason for the relatively small fluorescence changes observed. It is probable that only a few loci are required for the physiological changes necessary for membrane function, and these loci are also responsible for most of the fluorescence changes observed. It is also possible that there are numerous small effects, but the former case seems more probable.

Sodium and potassium appear to have the effect of relaxing or neutralizing the intermembrane electrostatic interactions, permitting the fluorescent probe greater rotational freedom and causing the environment to become more polar. Calcium ion showed the most dramatic effect on the fluorescence of DNS-PS. Presumably calcium changes the environment of the DNS group so that it becomes less polar and restricts the rotational freedom of the probe. These observations could be interpreted as a change in the conformation of the phospholipid. The DNS group could rotate from near the polar phosphate group at the aqueous interface to a position deeper within the glycerol–fatty acid domain of the phospholipid. An alternative explanation would be that there was a change in the microviscosity in the domain of the fluorescent probe. Either explanation would indicate that calcium has a major effect on the fluorescent phospholipid.

Propranolol interacts at or near the calcium binding site. It causes a portion of the bound calcium to be displaced from its binding site, but does not affect the fluorescent lipid in the same manner as calcium. It cannot be ascertained from this study whether this is the only action of propranol in this membrane preparation. Propranolol has a known *beta* adrenergic blocking action at the lower concentrations used in Fig. 7 (38); however, the concentration or activity of *beta* adrenergic sites in the preparation of brain synaptosome membranes used here has not been established.

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