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MECHANISM OF DRUG ACTION:
EXAMINATION OF POSSIBLE INVOLVEMENT OF LIPIDS IN THE
ACTION OF 5-HYDROXYTRYPTAMINE AND MORPHINE

by

DAVID ALLAN JOHNSON

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY

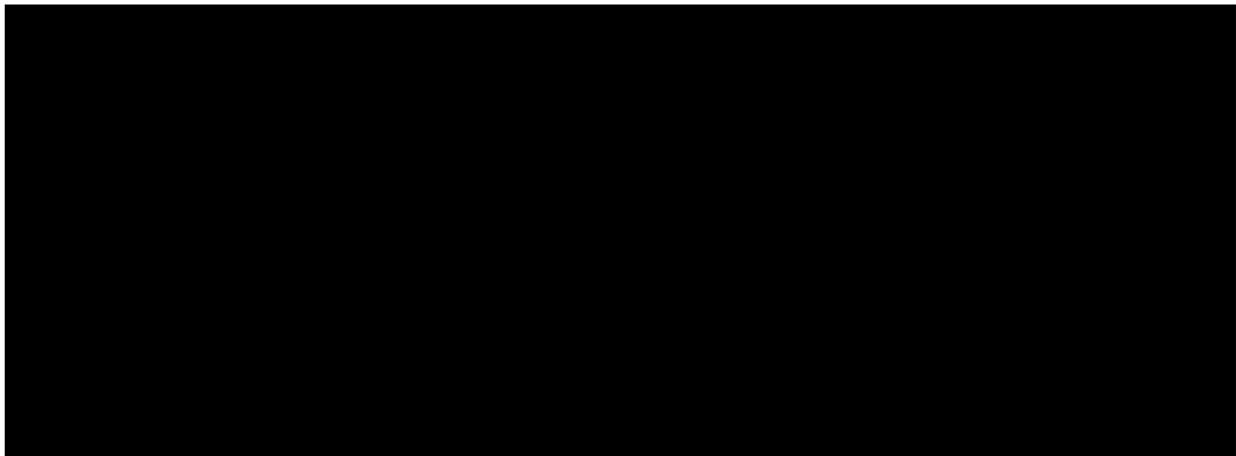
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ABSTRACT

Two general questions were examined. First, could an acidic lipid be a receptor for 5-hydroxytryptamine (5-HT)? Second, can morphine act via cerebroside sulfate to fluidize membranes? The first question was examined by demonstrating that many acidic lipids, i.e., cerebroside sulfate (CS), 1-phosphatidylinositol (PI), 1-phosphatidylinositol 4-phosphate (DPI), 1-phosphatidylinositol 4,5-bisphosphate (TPI), phosphatidic acid (PA), and phosphatidylserine (PS), can bind to tritiated 5-HT saturably and with high affinity in an organic environment, isobutanol, and that the putative 5-HT receptor isolated with Sephadex LH-20 chromatography in chloroform-methanol by Godwin and Sneddon (J. Neurochem. (1975) 25, 283-288) contains at least two acidic lipids, CS and PI, which account for over 90% of the observed binding activity in this preparation. To further investigate the possibility that an acidic lipid was a receptor for 5-HT, the affinities of 5-HT for CS, PI, DPI, TPI, PA, and PS were determined in an aqueous environment. This was done because the interaction of 5-HT with its receptor probably occurs at a water-membrane interface and not in a totally organic environment. In an aqueous environment, the affinities were observed to be in general much lower than and poorly correlated with the values measured in isobutanol. This suggested that these lipids probably are not 5-HT receptors.

The second question was examined by first determining whether opiate agonists and antagonists could alter the main phase transition temperature of lipid bilayers composed of dipalmitoylphosphatidylcholine (DPPC) and 1 mol % CS with three methods: (a) the

partitioning of the spin label TEMPO; (b) the steady state fluorescence depolarization of 1,6-diphenylhexatriene (DPH) incorporated into the bilayers, and (c) turbidic changes associated with the lipid phase transition. When it became apparent that there was no specificity in the ability of opiates to increase lipid fluidity of bilayers which contained CS, the work of Hosein *et al.* (Biochem. Biophys. Res. Comm. (1977) 78, 194-201) was repeated by monitoring fluidity with DPH. This was done because Hosein *et al.* showed, using differential scanning calorimetry (DSC), that opiates specifically increase brain lipid fluidity and that this effect required an ether precipitate rich in CS. However, even 1 mM morphine failed to alter the fluorescence polarization of DPH incorporated into bilayers composed of brain lipids, indicating that there were no alterations in bulk hydrocarbon fluidity. To show that our procedures could detect the effects of fluidizing agents, various amounts of ethanol were added. A 4-5% decrease in polarization of DPH could easily be detected with as little as 1% ethanol.

Because the measurement of the fluorescence depolarization of DPH monitors the bulk hydrocarbon region and the DSC monitors the whole system, including lipid-lipid, lipid-protein, and protein-protein interactions, our data tend to suggest that the alteration in the phase transitions observed with DSC may be due to changes in the melting of proteins or proteins interacting with lipids and not to the melting of the bulk hydrocarbon regions of the lipids.

Section 1

THE QUESTION

Even though the brain was recognized to be the center of cognitive activity and to be composed of fatty material as far back as Aristotle (Tower, 1958), and even though the basic structure of most brain lipids was worked out by the 1880's due mainly to the work of Vauquelin, Couerbe, Frémy, Gobley and, of course, J. L. W. Thudichum (Ansell, 1973), it has only been within the last decade that lipids have been recognized to play more than a static, inert structural role in brain activity. Today, few neuroscientists would argue that brain lipids are required for the functioning of membrane proteins; however, there is still some question as to what additional functions brain lipids perform besides the formation of cellular compartments or the creation of dynamic hospitable environments for membrane proteins.

Beyond their dynamic and structural roles in membranes, lipids have been implicated in many membrane functions traditionally ascribed solely to proteins. These processes include transport of ions, e.g., $\text{Na}^+\text{-K}^+$ ATPase (Karlsson, 1977), membrane fusion (Papahadjopoulos *et al.*, 1973), information transfer, e.g., neurotransmitters (Hook and Loh, 1978), and recognition of antibodies, e.g., ABO blood group antigens (Hakomori and Watanabe, 1976) and of hormones, e.g., thyrotropin (Mullin *et al.*, 1977).

Within the context of receptor pharmacology there is only one case where a reasonable amount of evidence exists which strongly links a lipid, $\text{G}_{\text{M}1}$, to a particular function; namely, a binding site

for cholera toxin (Cuatrecasas, 1973). Other lipids have been linked to other drugs, though not as clearly. Examples of some of these lipid-drug links are as follows: inositolphosphatides and acetylcholine receptor activity (Michell, 1975; Cho et al., 1978); 5-hydroxytryptamine (5-HT) and gangliosides (Woolley and Gommi, 1964); and morphine and cerebroside sulfate (Loh et al., 1978).

Because evidence supporting these other lipid-drug links is still incomplete, it was my purpose in this dissertation to further examine the relation of two drugs, 5-HT and morphine, to lipids. I had hoped in this way to contribute some answers to the general question: What direct roles do lipids play in the action of drugs?

This dissertation is basically divided into two parts. The first part deals with an examination of the possibility that an acidic lipid might be the receptor for 5-HT by determining the affinity of 5-HT for acidic lipids in an organic environment and aqueous environments and by demonstrating that certain acidic lipids can account for 90% of the receptor binding activity of one putative 5-HT receptor. The second part is an examination of the possibility that morphine can interact with cerebroside sulfate to cause an increase in lipid membrane fluidity. Each part was designed to be a separate and distinct unit.

Section 2

EXAMINATION OF THE POSSIBLE INVOLVEMENT OF ACIDIC LIPIDS

IN 5-HYDROXYTRYPTAMINE BINDING TO NERVOUS TISSUE

2.1 Binding of 5-Hydroxytryptamine to Acidic Lipids in Isobutanol

2.1.1 Introduction

Several lines of evidence have implicated some acidic lipids and proteolipids as possible 5-hydroxytryptamine (5-HT) receptors. Woolley and Gommi (1964) observed that 5-HT responsiveness of rat stomach fundus could be blocked by neuraminidase and that either a crude lipid extract or a mixture of partially purified gangliosides reversed this effect. Gangliosides, however, have been shown to have relatively low affinities for 5-HT, K_d 's from 3-5 mM (Ochoa and Bangham, 1976).

Since high affinity and saturable tritiated 5-HT binding material is extractable with butanol from rat brain (Marchbanks, 1966) and since 5-HT is a cation at physiological pH, we felt that 5-HT could bind to the anionic site on acidic lipids and could, therefore, account for at least some of the 5-HT binding material extracted with butanol. To test this possibility, we first investigated whether 5-HT would bind to purified acidic lipid in isobutanol using a two-phase partition method (Weber et al., 1971).

2.1.2 Materials and Methods

Compounds. 5-Hydroxy[side chain-1,2-³H]tryptamine with a specific activity of 5 Ci/mmol was purchased from New England Nuclear (Boston, MA). Phosphatidylserine (bovine brain) and cerebroside sulfate (bovine brain) were purchased from Analabs (North Haven, CT) and phosphatidic acid (bovine brain), monophosphoinositide (plant) and 5-HT creatinine sulfate from Sigma (St. Louis, MO). A mixture of triphosphoinositide-diphosphoinositide-monophosphoinositide (86:12:2) was extracted from fresh canine brain with chloroform-methanol-KCl (4 N) following the method of Michell et al. (1970) with one modification. Prior to the chloroform-methanol-KCl extraction, the brain was extracted four instead of three times with chloroform-methanol (2:1) (v/v). This increased the purity of the sample. [G-³H]Cerebroside sulfate with a specific activity of 9 Ci/mmol was prepared by catalytic tritium exchange by Prof. C. T. Peng (School of Pharmacy, U.C.S.F.). Analytical grade solvents were used in all procedures.

Total Phosphorus Assay. Total lipid phosphorus and Pi were determined as reported by Bartlett (1959).

Binding Procedure. Binding was determined by the method of Weber et al. (1971). Briefly, 1 ml isobutanol (saturated with water) with or without lipid (5 µg/ml) was added, in triplicate, to 1 ml [³H]5-HT (10 nM) and cold 5-HT in water (saturated with isobutanol) at pH 7.4. This was vortexed for 1 min and centrifuged at 2000 g for 10 min. A 0.5 ml aliquot was taken from the organic phase and the radioactivity was counted using liquid scintillation spectroscopy. Based on the calculated 5-HT isobutanol-water partition coefficient (P) for each

concentration of cold 5-HT added to the aqueous phase (n), the amount of 5-HT bound (B) was calculated using the following formula:

$$B = n \left[\frac{X - P(T - X)}{T} \right]$$

where X equals the observed d.p.m. in the organic phase and T equals the total d.p.m. initially added. The free concentration of 5-HT in the butanol phase (F) was calculated using the following formula:

$$F = \frac{nP(T - X)}{T}$$

A plot of B/F against B (i.e., Scatchard, 1949) was made and a least squares fit of the apparent linear portion(s) of this plot was calculated. Based on the negative reciprocal of the slope of the regression line, the apparent dissociation constant was determined. With the exception of the cerebroside sulfate, each regression line was composed of at least four 5-HT concentrations done in triplicate. The cerebroside sulfate regression line, though composed only of three 5-HT concentrations done in triplicate, was repeated in a separate experiment (data not shown) and the dissociation constant determined. The replication yielded a dissociation constant within 10% of the value shown in Table 1.

Isobutanol-Water Partition Coefficient of Lipids. The partition coefficients of the lipids were determined basically as the binding assay had been performed in the absence of 5-HT and the volume of each phase was increased to 15 ml. Aliquots of each phase were dried. The amount of lipid in each phase was determined by measuring

the total lipid phosphorus for the phospholipids and tritium for [³H]cerebroside sulfate.

TLC of Polyphosphoinositides. Multiple samples of polyphosphoinositides were spotted along with a PI standard on a silica gel H (Analabs, North Haven, CT) TLC plate which had been dipped in a 1% aqueous solution of potassium oxalate and had been heated for 1 hr at 130° C. This was developed in chloroform-methanol-ammonium hydroxide (4 N) (9:7:2) and exposed to iodine vapor. Two of the samples were sprayed with either ninhydrin or periodate-Schiff reagents. Three spots were visualized with iodine and reacted positively to the periodate-Schiff reagent but negatively to ninhydrin. One spot cochromatographed with the PI standard ($R_f = 0.73$) and the other two spots had R_f values of 0.30 and 0.19 which are in close agreement to published values of diphosphoinositide and triphosphoinositide, respectively (Gonzalez-Sastre et al., 1968). Samples which had only been exposed to iodine vapor were scraped from the plate and total lipid phosphorus determined.

2.1.3 Results

The plots of the free 5-HT vs bound to the acidic lipids in the isobutanol phase are shown in Figs. 1-5. With the exception of the polyphosphoinositides, all the lipids bind saturably and with high affinity to 5-HT. A summary of the apparent dissociation constants (K_d) is shown in Table 1. Phosphatidylserine had the highest affinity with a K_d of 0.6 μ M, followed by diphosphoinositide 0.8 μ M,

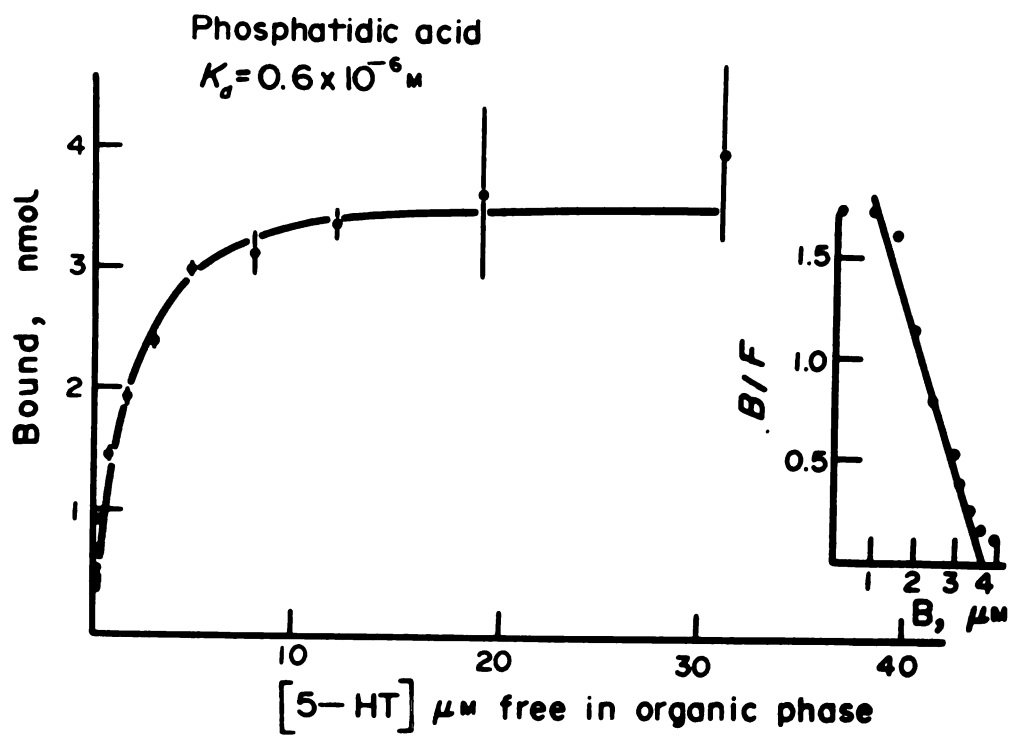


Fig. 1. The binding curve and Scatchard plot of 5-HT to phosphatidic acid in isobutanol. Bars represent \pm S.E.M.

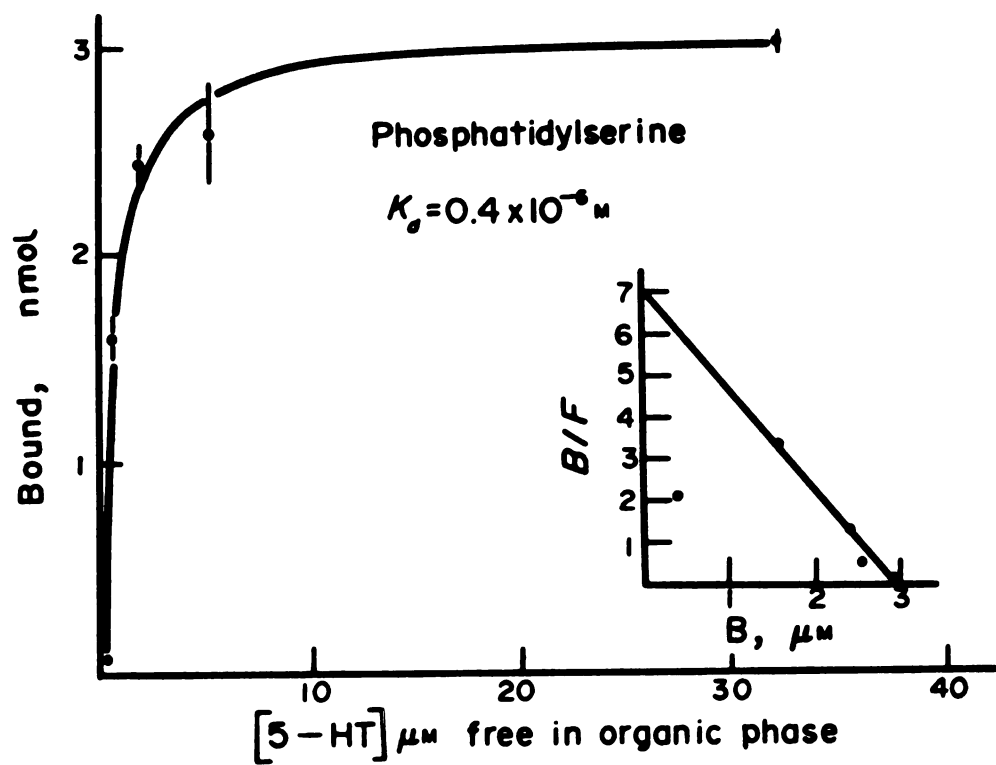


Fig. 2. The binding curve and Scatchard plot of 5-HT to phosphatidylserine in isobutanol. Bars represent \pm S.E.M.

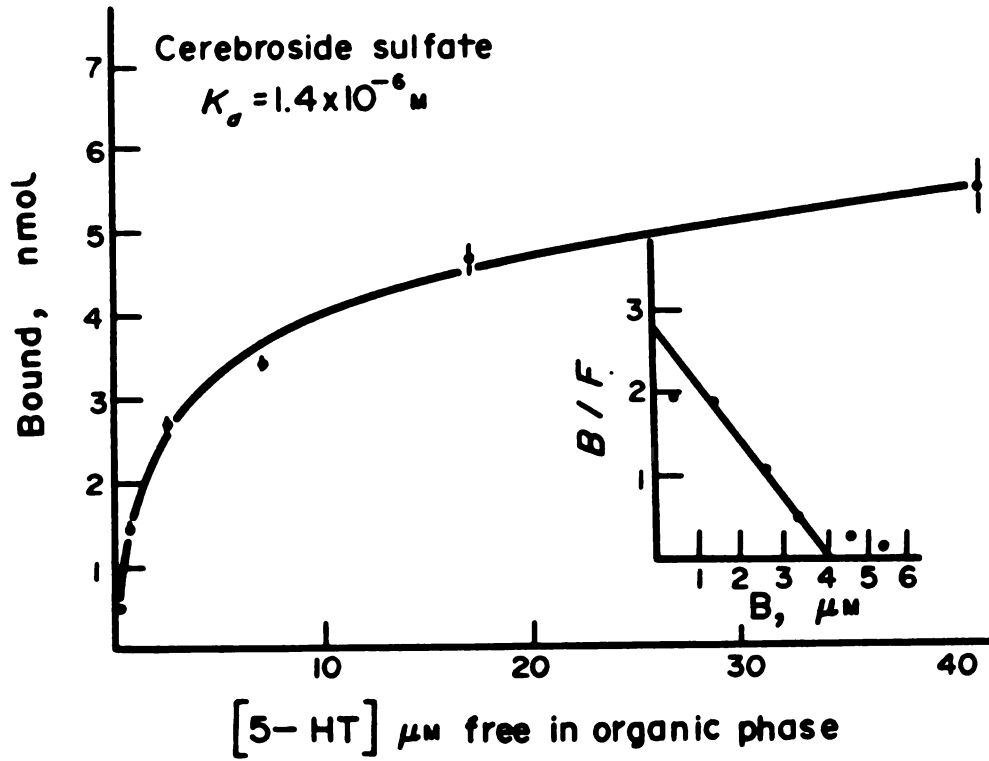


Fig. 3. The binding curve and Scatchard plot of 5-HT to cerebroside sulfate in isobutanol. Bars represent \pm S.E.M.

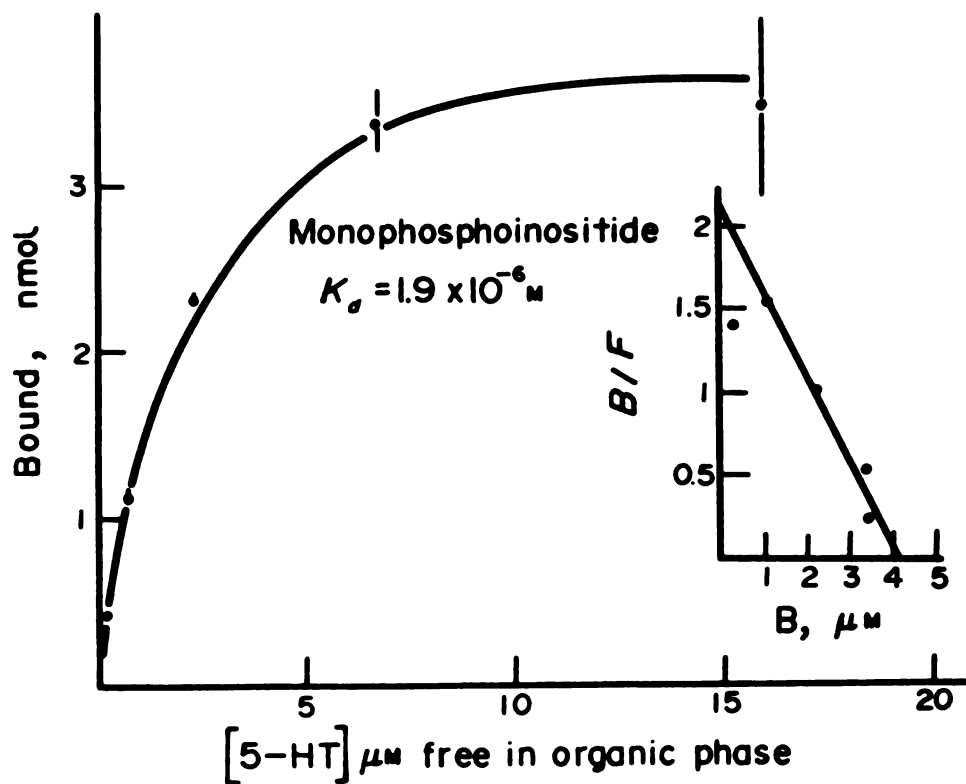


Fig. 4. The binding curve and Scatchard plot of 5-HT to monophosphoinositide in isobutanol. Bars represent \pm S.E.M.

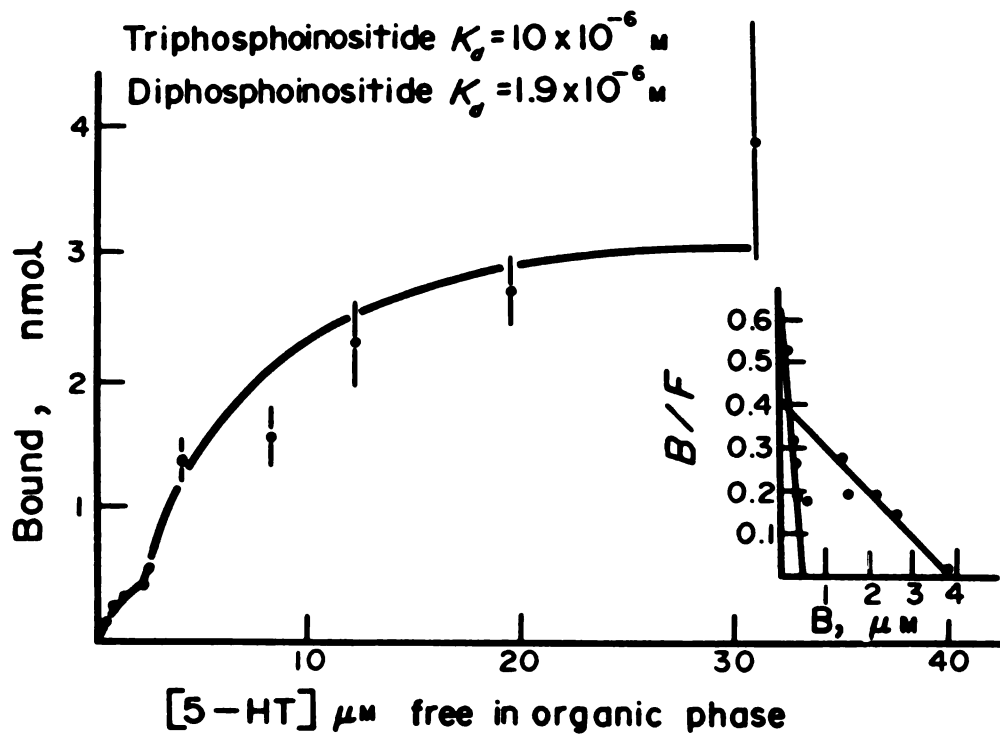


Fig. 5. The binding curve and Scatchard plot of 5-HT to a mixture of triphosphoinositide-diphosphoinositide-monophosphoinositide (86:12:2) in isobutanol. Bars represent \pm S.E.M.

Table 1. The Dissociation Constants (K_d) of 5-HT from Six Acidic Lipids in Isobutanol

Acidic lipid	K_d (μM)	$P_{B/W}^*$
Phosphatidylserine	0.4	31
Phosphatidic acid	0.6	43
Cerebroside sulfate	1.4	22
Monophosphoinositide	1.9	27
Diphosphoinositide	0.8	62†
Triphosphoinositide	10	

* The mean isobutanol/water partition coefficient ($P_{B/W}$) of the lipid was determined by measuring in triplicate the total lipid phosphorus (Bartlett, 1959) in each phase for the phospholipids and the tritium in each phase for [^3H]cerebroside sulfate.

† Because diphosphoinositide and triphosphoinositide were in a mixture, this value represents the ratio of total lipid phosphorus in each phase.

cerebroside sulfate 1.4 μM , monophosphoinositide 1.9 μM , and triphosphoinositide 10 μM .

Because diphosphoinositide and triphosphoinositide were mixed together, we distinguished between the two K_d 's on the basis of the binding capacities predicted by the x-intercepts of the two regression lines in the B/F vs B plot and of the percentage of each lipid in the mixture as measured by TLC separation and total phosphate determination of each spot. Since 86% of the phosphoinositides are triphosphoinositide and 87% of the apparent binding capacity has a K_d of 10 μM , we inferred that this was the K_d for triphosphoinositide. Likewise, 12% of the mixture of phosphoinositides is diphosphoinositide and 13% of the predicted binding capacity has a K_d of 0.8 μM , so diphosphoinositide was inferred to have a K_d of 0.8 μM .

The validity of the partition method depends on the almost exclusive distribution of the binding material into one of the phases. To check that the lipids did, indeed, distribute into the isobutanol phase, the isobutanol-water partition coefficient was determined for each lipid. The results of this experiment are shown in Table 1. All the lipids have a high partition coefficient ($> .22$). Therefore, greater than 96% of the lipid distributed into the isobutanol phase.

2.1.4 Discussion

The data demonstrate that 5-HT can bind saturably and with high affinity in an organic environment to most of the acidic lipids examined. Phosphatidylserine has the highest affinity followed by phosphatidic acid, diphosphoinositide, cerebroside sulfate, monophospho-

inositide, and triphosphoinositide. The only apparently non-saturable binding was observed with the polyphosphoinositides. This may be due to the multiple anionic sites on these lipids which could provide additional low affinity sites not saturated with the highest concentration of 5-HT used, 30 μ M.

2.2 Identification of 5-HT Binding Substances From Rat Brain Stem

2.2.1 Introduction

Since we demonstrated that 5-HT could bind acidic lipids saturably and with high affinity in an organic environment (Sec. 2.1), it seemed reasonable to examine the possibility that acidic lipids might be components of putative 5-HT receptors isolated in organic solvents.

Two laboratories (Fischer and DeRobertis, 1969; Godwin and Sneddon, 1975) using different size columns and quantities of solvent, claimed to have isolated a "special proteolipid" 5-HT receptor from the butanol extract of rat brain stem in chloroform-methanol (4:1) (v/v) using Sephadex LH-20 chromatography. Because their methods appeared to be essentially the same and because Godwin's and Sneddon's work was more recent and characterized the "receptor" binding in somewhat more detail, we followed the protocol of only Godwin and Sneddon (1975).

2.2.2 Materials and Methods

Compounds. Different batches of 5-hydroxy[side chain-1,2- 3 H] tryptamine were purchased from New England Nuclear (Boston, MA),

ranging in specific activity from 5 to 26.7 Ci/mmol. [U-¹⁴C]Mono-phosphoinositide with a specific activity of 5 mCi/mg was purchased from Applied Sciences Lab (Inglewood, CA). Cerebroside sulfate (bovine brain) was purchased from Analabs (North Haven, CT), and 5-HT creatinine sulfate and PI (plant) were obtained from Sigma (St. Louis, MO). All solvents were analytical grade and were used without further purification.

Preparation of Brain Extracts. Rat brain stems were prepared as reported by Godwin and Sneddon (1975). Briefly, male Sprague-Dawley rats (200-300 g) were decapitated, and their brains quickly removed and placed in ice-cold distilled water. The cerebral cortex and cerebellum were discarded. All procedures were carried out at 4° C unless otherwise stated. The brain stems were homogenized with a Polytron for 15 s in 20 vol of distilled water (w/v), then centrifuged at 30,000 g for 15 min. The supernatant was discarded. The pellet was rehomogenized in distilled water and centrifuged as in the previous step. The supernatant was again discarded, and the pellet was resuspended in a 50% sucrose solution (w/v) (2 ml/g wet brain stem) and extracted with 10 vol of water saturated n-butanol for 2 hr at room temperature by continuous and vigorous stirring. The mixture was centrifuged at 1000 g for 5 min and the butanol phase isolated. This was concentrated under reduced pressure at 38° C to approximately one-third of its original volume and stored at 4° C until use.

Incubation Procedures. For the discontinuous chromatographic procedures, samples of the brain extract (6.0 ml) were combined with 0.5 ml water and 3 nmol of [³H]5-HT and were incubated for 12 min

before a 6.0 ml sample was placed on the column. Purified lipids were dissolved in 6.5 ml of butanol saturated with water and incubated with 2 nmol of [³H]5-HT for 12 min. A 6.0 ml sample was then loaded on the column.

For the continuous gradient procedure, 0.5 ml of PI (2 mg/ml) and 0.1 ml [¹⁴C]PI (0.15 µCi/ml) both in chloroform-methanol (CM) 2:1 (v/v) were combined and evaporated with nitrogen in a test tube. One ml of a 2 mM solution of [³H]5-HT (65 µCi/ml) in n-butanol saturated with water was added to this test tube, and this was incubated for 12 min before being placed on the column.

Preparation of Column and Chromatography. For the discontinuous chromatographic procedure, Sephadex LH-20 (42.5 g) resin was equilibrated in chloroform and was loaded into a 2.5 cm diameter glass column. After incubation with [³H]5-HT, the sample was loaded onto the column and was eluted with the following series of solvents: 100 ml chloroform, 50 ml each of CM 15:1, 10:1, 6:1, then 300 ml CM 4:1 (v/v), and finally 200 ml methanol. Fractions of 5.0 ml were collected. A 0.2 ml aliquot was taken from each fraction to determine radioactivity by liquid scintillation spectroscopy, a 3 ml aliquot to determine lipid phosphorus by the method of Bartlett (1959) and a 1 ml aliquot to determine CS by the method of Kean (1968).

Continuous column chromatography was performed by equilibrating 20 g Sephadex LH-20 in chloroform and loading the resin into a 2.0 cm diameter column. After incubation with [³H]5-HT, a 0.8 ml sample was loaded on the column. After 20 ml of chloroform had been collected and an additional 10 ml of chloroform was placed on top of the column,

the column was connected to a closed system gradient mixer. The mixing chamber contained 100 ml chloroform into which methanol was fed. Fractions of 3.0 ml were collected directly into scintillation vials, and radioactivity was determined following evaporation of the solvent.

Analysis for Acidic Lipids. The fractions corresponding to the major peak of radioactivity following the elution of the brain extract which had been incubated with [³H]5-HT were combined and evaporated down to 2 ml. Aliquots were spotted on silica gel H (Analabs) TLC plates, which had been dipped in a 1% aqueous solution of potassium oxalate and heated for an hour at 130° C. The plates were developed in chloroform-methanol-ammonium hydroxide (4 N) (9:7:2) (Gonzales-Sastre and Folch-Pi, 1968). Acidic lipids were identified following spraying separate samples with ninhydrin or periodate-Schiff reagents and by comparison against lipid standards. Quantitative determination of the concentration of CS, PI, and diphosphoinositide in the brain extract was made by application and development of the brain extract on the above-cited TLC system, visualization of the spots with iodine, extraction of the spots corresponding to CS, PI, and diphosphoinositide standards, evaporation of the solvent, and determination of the CS concentration by the method of Kean (1968) and PI and diphosphoinositide by determination of the total lipid phosphorus as reported by Bartlett (1959).

2.2.3. Results

The elution profile of radioactivity which was obtained following incubations of [^3H]5-HT in the presence or absence of the rat brain stem extract is shown in Fig. 6. The brain extract elution pattern is essentially the same as that shown by Godwin and Sneddon (1975) with the major binding peak starting approximately at fraction number 67 and continuing over the next 12 fractions. Also, as Godwin and Sneddon (1975) had shown, the free 5-HT appeared in the methanol fractions.

An examination of the major peak of radioactivity for acidic lipids revealed the presence of CS, PI, and diphosphoinositide. The elution patterns of CS and total phosphorus from the brain extract is shown in Fig. 7. These elution patterns further confirm the presence of CS and show the probable presence of phospholipids, since at least 40 mg CS and 11 nmol phosphorus was present in each fraction composing the major peak of radioactivity. The highest levels of CS and of radioactivity, however, do not exactly correspond to each other and are separated by 4 to 7 fractions.

The total amounts of CS and PI in the brain extract loaded onto the column were found to be 4 and 2.2 mg, respectively, in two determinations. The elution pattern of radioactivity and phosphorus when this same amount of PI was incubated with [^3H]5-HT is shown in Fig. 8. This pattern of radioactivity was quite different from the brain extract pattern. All detectable phosphorus was eluted in either the chloroform or CM 4:1 fractions, while most radioactivity was eluted in the methanol near where the free [^3H]5-HT appeared. The elution

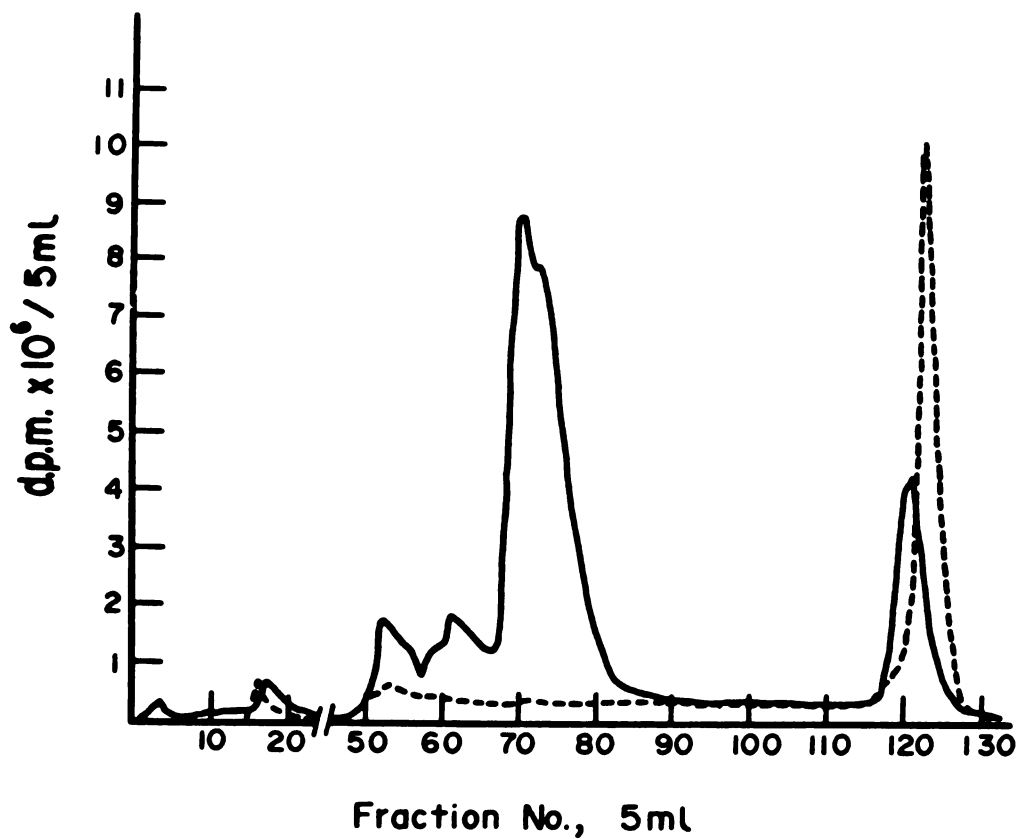


Fig. 6. Elution profile after discontinuous chromatography of radioactivity from brain extract incubated with [³H]5-HT (solid line) and elution of [³H]5-HT in butanol saturated with water (dashed line). Elution solvents: 100 ml chloroform, 50 ml each of CM 15:1, 10:1, 6:1, 300 ml CM 4:1 and 200 ml methanol. Fraction number 1 is the void volume.

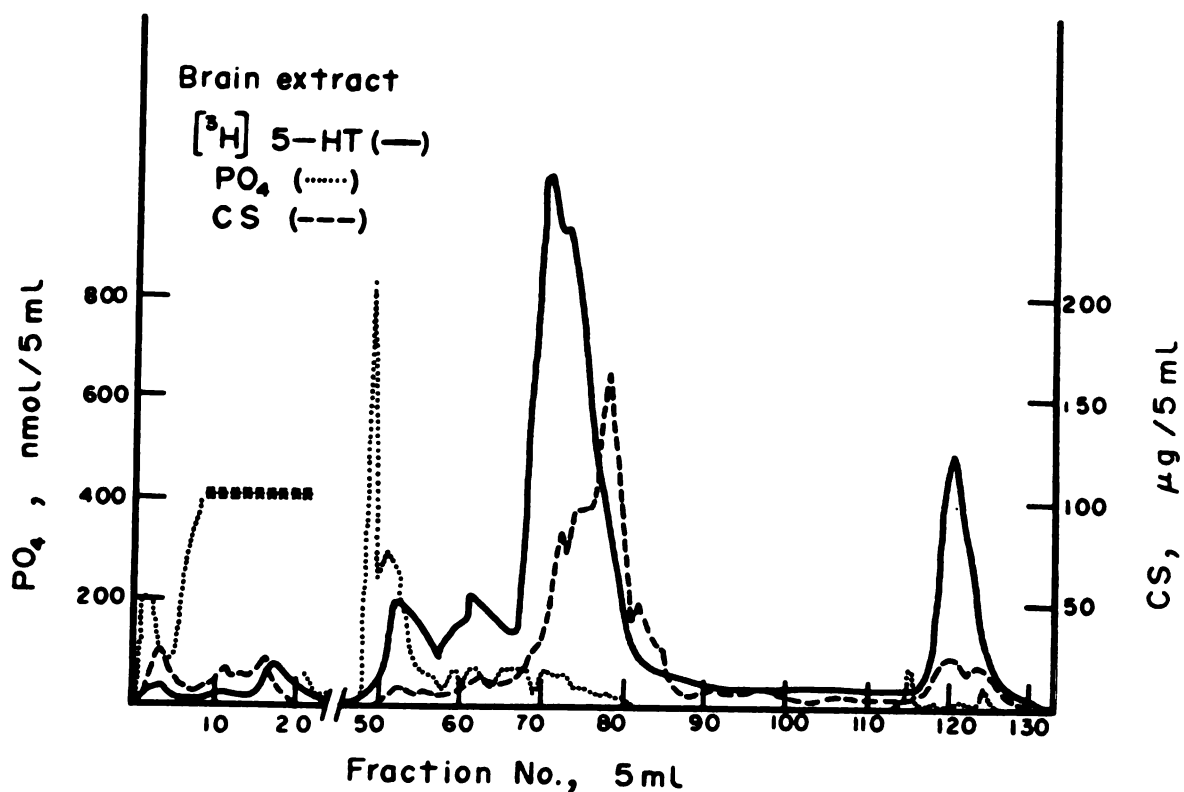


Fig. 7. Elution of brain extract showing elution profiles of lipid phosphorus (dotted line), cerebroside sulfate (dashed line) and [³H]5-HT (solid line) after discontinuous chromatography. The radioactivity corresponds to that in Fig. 6. The (*) represents those fractions in which a contaminant prevented phosphorus determination.

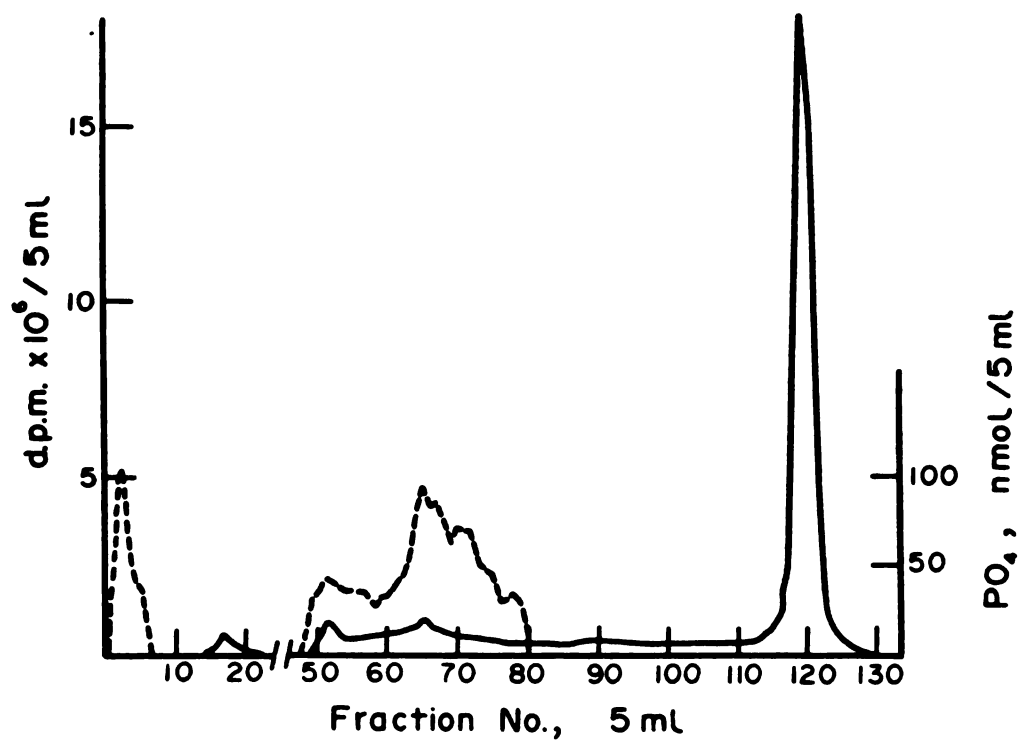


Fig. 8. Elution of purified monophosphoinositide incubated with [³H]5-HT using the discontinuous elution procedure described in Fig. 6. Radioactivity (solid line) and lipid phosphorus (dashed line) were assayed as described in methods.

pattern of the CS (4 mg) incubated with [^3H]5-HT, shown in Fig. 9, is, however, similar to that of the brain extract. The percent of radioactivity in CM 4:1 fractions, in the methanol fractions, and the total recovery (Table 2) also confirm the similarity of elution profiles. The major difference between the elution patterns of CS and of brain extract was the fraction number where the highest amount of radioactivity appeared. In three determinations the highest radioactivity appeared in fractions 71, 72 and 74 with the brain extract, while it appeared in fractions number 76 and 78 in two determinations with CS.

To determine whether PI influenced the elution pattern of CS and [^3H]5-HT, CS and PI were combined and incubated with [^3H]5-HT. This elution pattern of radioactivity (Fig. 10) is very similar to that of the brain extract. With one exception, the highest level of radioactivity appeared near the same fractions as with the brain extract in three determinations (Fractions 71, 73, and 63, compared to 71, 72, and 74). Though PI by itself did not cause any [^3H]5-HT to appear in the CM 4:1 fractions, PI appeared to interact with CS and [^3H]5-HT to cause a slightly earlier elution of [^3H]5-HT and, thereby, PI with CS produce a tritium elution pattern essentially identical to that seen with the brain extract.

Since we showed above that 5-HT binds to PI in an organic environment and that PI elutes in either the chloroform or the CM 4:1 fractions (Fig. 8), it was not anticipated that when PI and [^3H]5-HT were eluted together, the major peak of radioactivity would appear in the methanol fractions near the free 5-HT. This suggested either that PI was not binding to 5-HT or that PI was binding to 5-HT producing a

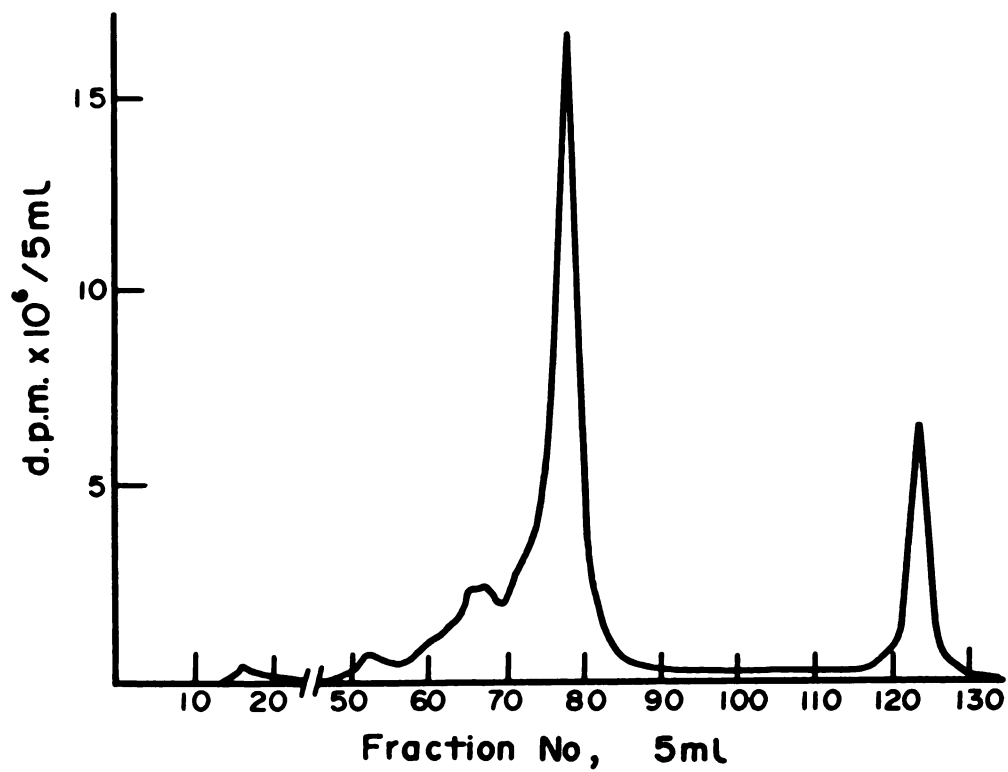


Fig. 9. Elution of purified cerebroside sulfate incubated with [³H]5-HT using the discontinuous elution procedure described in Fig. 6.

Table 2. The Mean (\pm S.E.M.) Recovery of Radioactivity Following Discontinuous Sephadex LH-20 Chromatography

	% total d.p.m. eluted	% total d.p.m. eluted in CM 4:1 (Fractions 50-110)	% total d.p.m. eluted in methanol (Fractions 111-133)	Number determinations	Fraction No. with highest radioactivity
Brain extract	96.3 \pm 1.2	81.7 \pm 2.6	17.7 \pm 2.7	3	71,72,74
CS and PI	91 \pm 2.9	76.3 \pm 3.5	6 \pm 1.5	3	62,71,73
CS	91.5 \pm 0.5	76.5 \pm 2.5	11.5 \pm 1.5	2	78,76
PI	65 \pm 3.5	28 \pm 13	37 \pm 12	2	119,119
5-HT only	82	26	53	1	123

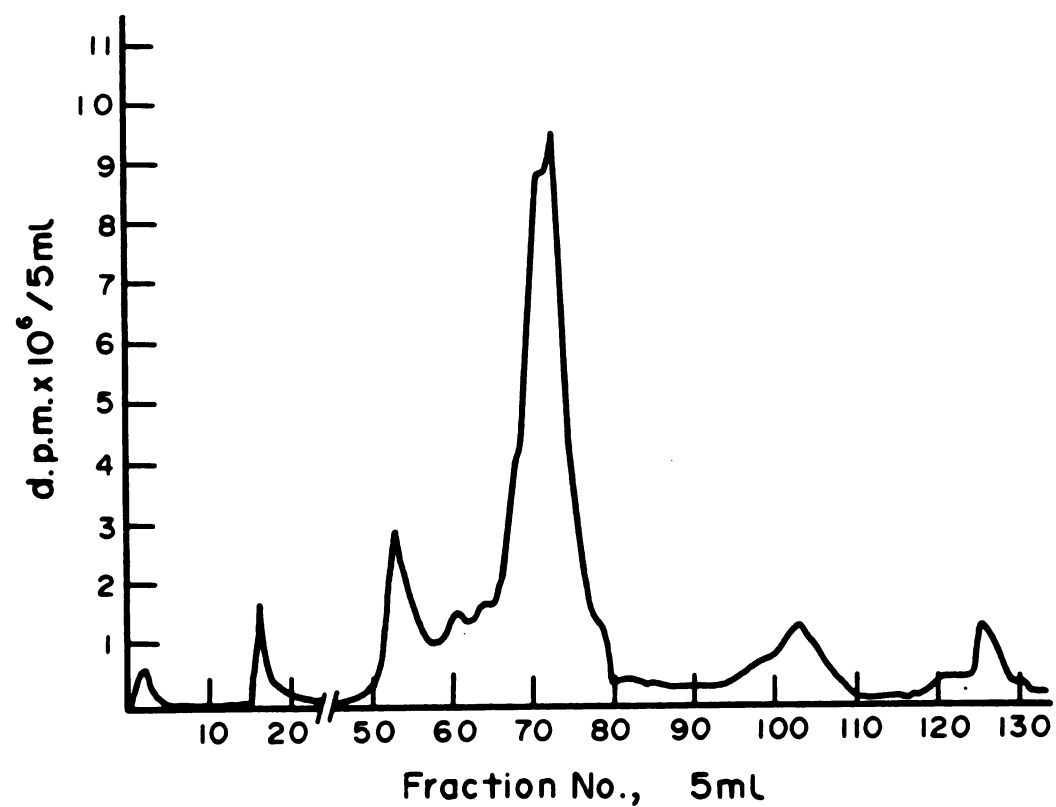


Fig. 10. Elution of purified cerebroside sulfate and monophosphoinositide incubated with [³H]5-HT using the discontinuous elution procedure described in Fig. 6.

PI-5-HT complex more polar than the unbound PI. Such a complex would thus be eluted by methanol. To differentiate between these possibilities, [^{14}C]PI and [^3H]5-HT were eluted with a continuous gradient of increasing polarity. An approximate 1:2 molar ratio of PI to 5-HT was used in order to increase the likelihood of observing free and bound peaks of 5-HT and PI. The failure to observe free and bound peaks of radioactivity in the methanol fractions when PI and [^3H]5-HT were eluted in the discontinuous chromatography could be explained if all the 5-HT was bound because of the approximate 1000-fold excess of PI compared to 5-HT.

The elution pattern from this experiment is shown in Fig. 11. There are two major peaks of [^3H]5-HT and two major peaks of [^{14}C]PI; one of the [^{14}C] peaks corresponds to the first major [^3H]5-HT peak. The molar ratio of PI to 5-HT for this later peak is 0.7 (assuming the molecular weight of PI is 860). This suggests that the first major [^{14}C] peak is free PI, the second [^{14}C] and first major [^3H] peak is a PI-5-HT complex, and the second [^3H] peak is free 5-HT. Thus, 5-HT can bind to PI on the LH-20 column and, therefore, the observed single peak of radioactivity in the methanol using the discontinuous procedure probably represents binding of PI to 5-HT.

2.2.4. Discussion

Fiszer and DeRobertis (1969) and Godwin and Sneddon (1975), using different size columns and volumes of solvent, observed that the major peak of radioactivity when [^3H]5-HT was incubated with butanol brain extracts occurred in the CM 1:4 fractions. Based on the inability of

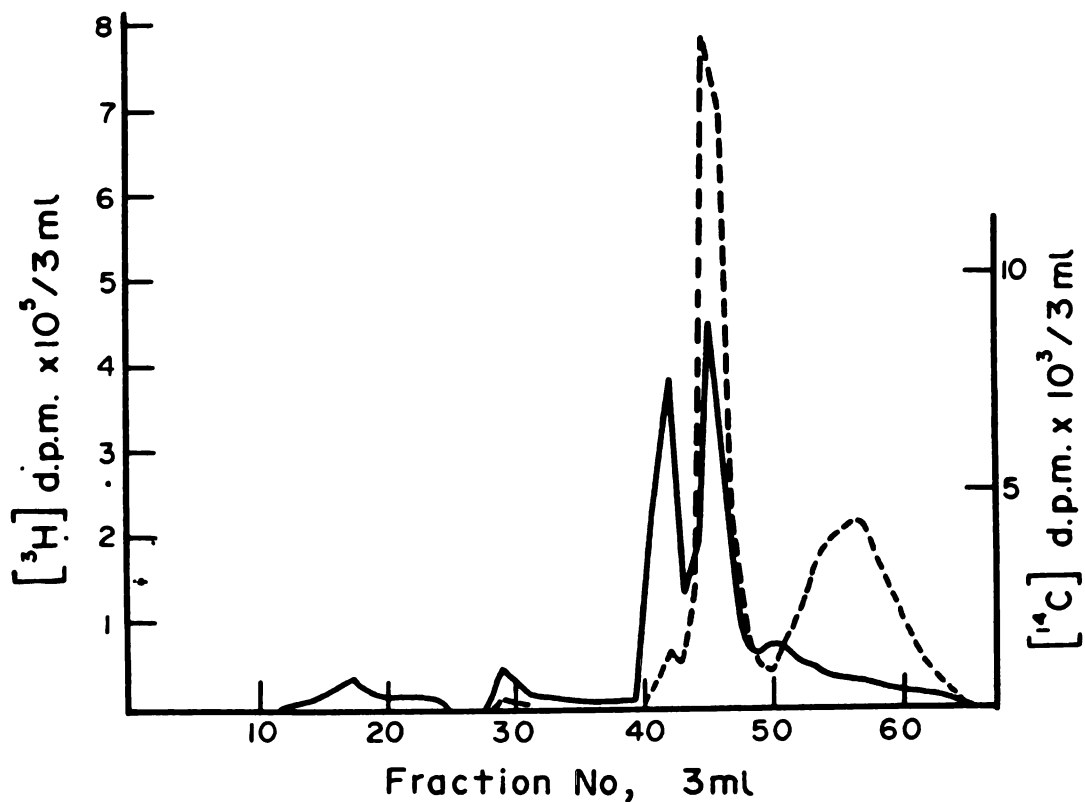


Fig. 11. Elution profile of [¹⁴C]monophosphoinositide (1 mg) (solid line) incubated with [³H]5-HT (2 μ mol) (dashed line) using a continuous gradient formed by connecting the column to a closed system gradient mixer containing 100 ml chloroform into which methanol was fed. Fraction No. 1 is the first fraction collected after sample was loaded on to column.

37% [^3H]5-HT incubated with butanol brain extract to move on silica gel G developed with chloroform:methanol:water and also based on the presence of Lowry positive material in the major radioactive peak on the LH-20 column, Fiszler and DeRobertis (1969) concluded that [^3H]5-HT was binding to a special proteolipid. Godwin and Sneddon (1975) came to the same conclusion based on the observations that 5-HT binding material was precipitated by ice-cold ether and based on the presence of Lowry positive material in the residue of the major radioactivity peak.

Our examination of this peak for acidic lipids showed the presence of CS, PI, and a little diphosphoinositide. Incubation of CS and PI with [^3H]5-HT gave essentially the same elution pattern as the brain extract. This data, coupled with our previous observation that CS and PI bind saturably and with high affinity to 5-HT, strongly implies that what has been suggested to be a proteolipid 5-HT receptor is mainly two acidic lipids, CS and PI.

This conclusion is consistent with Godwin and Sneddon's (1975) observations that the major peak of [^3H]5-HT was not saturable up to a concentration of 300 nmol 5-HT and that there was no clear relationship between the major peak of [^3H]5-HT and a discrete peak of either protein or phosphorus.

Other studies (Loh et al., 1974; Wu et al., 1977) examining what were claimed to be opioid (Lowney et al., 1974) and cholinergic (DeRobertis et al., 1969) proteolipid receptors, based on Sephadex LH-20 chromatography, have also revealed the presence of acidic lipids. Chromatography of these acidic lipids with opiates or d-

methyltubocurarine have shown that virtually all the observed binding could be accounted for by acidic lipids. This, coupled with the data presented here, casts doubt on the proteolipid nature of other putative receptors partially isolated using Sephadex LH-20 chromatography (DeRobertis, 1975).

As an aside, while we believe all future claims about the proteolipid nature of putative receptors based on Sephadex LH-20 chromatography must eliminate possible lipid binding, failure to detect lipids in the apparent binding peak is now, however, sufficient evidence to discount lipid binding. Because ligand concentrations are usually very small (a few nanomol) and because lipid-ligand complexes can exhibit chromatographic behavior quite different from unbound lipids, it would be difficult to detect 1 or 2 mol of lipid. Phosphorus determinations will not always detect phospholipid-ligand complexes due to the limited sensitivity of the procedure, as was the case with PI and [³H]5-HT (Fig. 8). Ligand concentrations in the binding peak must be high enough to detect possible lipid complexes.

2.3 Binding of 5-HT to Acidic Lipids in an Aqueous Environment

2.3.1. Introduction

So far we have shown that the putative 5-HT receptor isolated with Sephadex LH-20 chromatography in chloroform-methanol 4:1 (v/v) contains at least two acidic lipids, CS and PI, which can account for over 90% of the observed binding in this preparation. Also, 5-HT binds saturably and with high affinity to other acidic lipids, namely,

PS, PA, DPI, and TPI in an organic environment, isobutanol. The interaction of 5-HT with its receptor probably occurs at a water-membrane interface and not in a totally organic environment, so that our above observations have questionable relevance to biological systems.

Prediction of drug-receptor affinities on the basis of their affinities in organic solvents is difficult. Depending on the type of non-covalent bonding, water can either decrease or increase the apparent affinity. Ionic and dipole interactions are attenuated by water due to the reciprocal relationship between dielectric constant and ionic bonding energy, and entropic hydrophobic bonding exists only in the presence of an aqueous medium. For complex lipids, prediction of in vivo affinities for drugs on the basis of their affinities in an organic solvent is further complicated because complex lipids can exist in different physical states, i.e., monomer or one of various types of micelles, depending on the solvent, the temperature and the pressure.

The physical state of complex lipids in aqueous media has been studied for some time (Chapman, 1973). The structural characteristics of PS, PA, and PI particles swollen in aqueous salt solutions have been investigated by optical electron microscopy and X-ray diffraction methods, and it has been observed that sonicated solutions of these lipids at ambient temperatures and pressures form bilayer structures which resemble biological membranes (Papahadjopoulos and Miller, 1967). To our knowledge, DPI, CS, and 1-phosphatidylinositol 4,5-bisphosphate (TPI) have not been studied in this way, but are likely

to produce similar structures in aqueous salt solutions, since they also have a polar head group with two alkyl chains attached and in aqueous salt solutions scatter light like other phospholipids.

Since PS, PA, PI, DPI, TPI, and CS probably form bilayer structures in an aqueous salt medium similar to biological membranes, we determined whether 5-HT would bind with high affinity to these acidic lipids in an aqueous salt medium. Equilibrium binding of tritiated 5-HT to these acidic lipids in a micellar state was measured by means of the gel filtration method developed by Hummel and Dreyer (1962).

2.3.2. Materials and Methods

Compounds. Different batches of 5-hydroxy[side chain-1,2-³H] tryptamine were purchased from New England Nuclear (Boston, MA), ranging in specific activity from 21.4 to 28.4 Ci/mmol. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1-phosphatidylinositol (plant), and 5-HT creatinine sulfate were obtained from Sigma Chemical Company (St. Louis, MO), and phosphatidic acid in chloroform and phosphatidylserine were purchased from Miles Laboratories (Kankakee, IL). The phosphatidylserine was dissolved in chloroform-methanol (CM), 2:1 (v/v). N-(Trimethylsilyl)-imidazol was purchased from Aldrich (San Leandro, CA). Myo-inositol and butylated hydroxytoluene were obtained from Calbiochem (San Diego, CA). All lipids were stored at -20° C. All solvents were analytical grade and were used without further purification.

CS was prepared from CM, 2:1 (v/v), extract of canine brain as described by Fluharty et al. (1974). The purity of the CS was checked

by spotting separate samples on silica gel G TLC plates (EM laboratories, Elmsford, NY) and development in either chloroform-methanol-water (70:30:4) or in chloroform-methanol-ammonium hydroxide (4 N) (9:7:2). Only two spots were observed following visualization with iodine vapor, which cochromatographed with hydroxy and non-hydroxy forms of CS, when compared with cerebroside sulfate obtained from Supelco (Bellefonte, PA). The CS was stored in CM, 2:1 (v/v), and the concentration of this stock solution was determined by measuring total hexoses following the method of Dubois et al. (1956).

Purification of DPI and TPI. Inositolphospholipids were initially extracted from fresh canine brain with chloroform-methanol-KCl (4 N) following the method of Michell et al. (1970) with one modification. Prior to the chloroform-methanol-KCl extraction, the brain was extracted four instead of three times with chloroform-methanol, 2:1 (v/v).

DEAE cellulose chromatography of the KCl extract was based on a modification of the method of Hendrickson and Ballou (1964). One-third of the KCl extract (200 μ mol lipid phosphorus) was placed onto a 2.5 x 30 cm DEAE cellulose column (Rouser et al., 1969) equilibrated with CM, 10:1 (v/v). The column was connected to a closed system gradient mixer. The mixing chamber contained 1400 ml of chloroform-methanol-water (20:9:1) (by vol) into which chloroform-methanol-water (20:9:1) and 0.4 M-ammonium acetate was added. Twenty milliliter fractions were collected and 0.5 ml aliquots were taken for total lipid phosphorus analysis.

TLC analysis for inositolphospholipid was performed on the peaks of phosphorus. Those fractions which contained exclusively DPI or TPI were pooled. TPI was desalted by dialysis followed by lyophilization. DPI was desalted with a Sephadex G-25 column (1.0 x 10 cm) equilibrated with CM, 19:1 (v/v) (Rouser *et al.*, 1969). Both DPI and TPI were stored in CM, 19:1 (v/v), at -20° C.

The purity of DPI and TPI was examined by spotting samples (ca. 50 nmol) on silica gel H TLC plates (Analabs, North Haven, CT) which had been treated with potassium oxalate. Each plate was carefully lowered into a tray containing 1% aqueous potassium oxalate wetting all the silica gel. The plate was removed, placed upright on absorbent paper for 10 min, and then heated for 1 hr at 130° C. The plates were developed in chloroform-methanol-ammonium hydroxide (4 N) (9:7:2) (by vol) (Gonzalez-Sastre and Folch-Pi, 1968). Visualization of the spots with iodine vapor showed only single spots in the DPI and TPI samples (R_f 's = 0.30 and 0.19, respectively). Further confirmation of the purity and identity of the DPI and TPI was provided by measurement of both the total lipid phosphorus and inositol in each sample. The lipid phosphorus-inositol ratio of the DPI sample was 1.9 and 2.9 for TPI.

Measurement of Inositol. Gas chromatography was used for the inositol determinations. Samples (ca. 0.4 μ mol) were treated in sealed tubes with 1 ml of 6 N-HCl at 115° C for 44 hr. The hydrochloric acid was removed by the addition of excess silver oxide followed by centrifugation. Fatty acids were removed from the supernatant by extraction once with 2 ml heptane. The aqueous phase was

dried in a vacuum oven at 40° C. The residues were trimethylsilylated in 0.02 ml pyridine by addition of 0.2 ml N-(trimethylsilyl)imidazole for 90 min at 60° C. The products were extracted into 0.2 ml toluene after the addition of 2 ml water and 1 ml concentrated HCl. Samples (1 μ l) were analyzed on a column of 2% OV-17 on Gas Chrom Q using a model 5720A Hewlett-Packard gas chromatograph with a hydrogen flame ionization detector.

Measurement of Binding. The binding of 5-HT to the acidic lipids was measured by means of the gel filtration method of Hummel and Dreyer (1962). Ochoa and Bangham (1976) have used this method to study the binding of 5-HT to gangliosides. For all the lipids except DPI, Sephadex G-50, swollen in distilled water for 2 hr at room temperature, was placed in 0.7 cm columns to a height of 19 cm and equilibrated with 100 μ M-tritiated 5-HT and 5 mM-HEPES at pH 7.4. Because of the apparent higher affinity of 5-HT to TPI, a lower concentration of 5-HT was used so that the binding would be non-saturated, and less DPI was placed on the column so that there would be sufficient 5-HT in the column to equilibrate with the DPI. The column was equilibrated with 0.5 μ M-tritiated 5-HT and 5 mM-HEPES at pH 7.4. Also, since the 5-HT-DPI complex appeared to be unique among the lipids studied insofar as it appeared not to be totally excluded by the Sephadex G-50, Sephadex G-25 was used.

Measured amounts of the lipid stock solutions were evaporated to dryness with nitrogen and the appropriate tritiated 5-HT-HEPES buffer was added, vortexed, and sonicated with a probe type sonicator for 3 min. The final concentration of the lipids, except for DPI, was 2.2 mM; the DPI was made 0.5 mM. The lipid micelles (100 μ l) were then

applied to the top of the column. The elution rate was 0.2-0.3 ml/min with the same radioactive drug solution used to equilibrate the column. Sequential fractions (0.5 ml) were collected and aliquots removed for assay of radioactivity with lipid scintillation spectroscopy and of lipids by measurement of total lipid phosphorus (Bartlett, 1959) for the phospholipids or CS by the method of Kean (1968). Three replicate assays were performed with each of the lipids.

2.3.3 Results

The elution profiles of radioactivity and of acidic lipid, obtained when CS, PI, DPI, TPI, PA, and PS were loaded onto Sephadex columns equilibrated with tritiated 5-HT, are shown in Fig. 12. Each profile fulfills the criteria established by Hummel and Dreyer (1962) for the determination of reversible equilibrium binding; namely, the co-elution of ligand and binding substance peaks was followed by a return to baseline and then a ligand "trough" was followed by another return to baseline. The lipid recoveries are presented in Table 3.

With the assumptions that only one 5-HT molecule can bind to each lipid molecule and that the binding follows a Langmuir type of adsorption isotherm (e.g., Aveyard and Haydon, 1973, pp. 25-27), it is possible to calculate an apparent dissociation constant (K_d) of 5-HT for each of the lipids by the law of mass action for a bimolecular interaction with the following equation:

$$K_d = \frac{D_f \cdot R_F}{DR} = \frac{D_f (R_T - DR)}{DR}$$

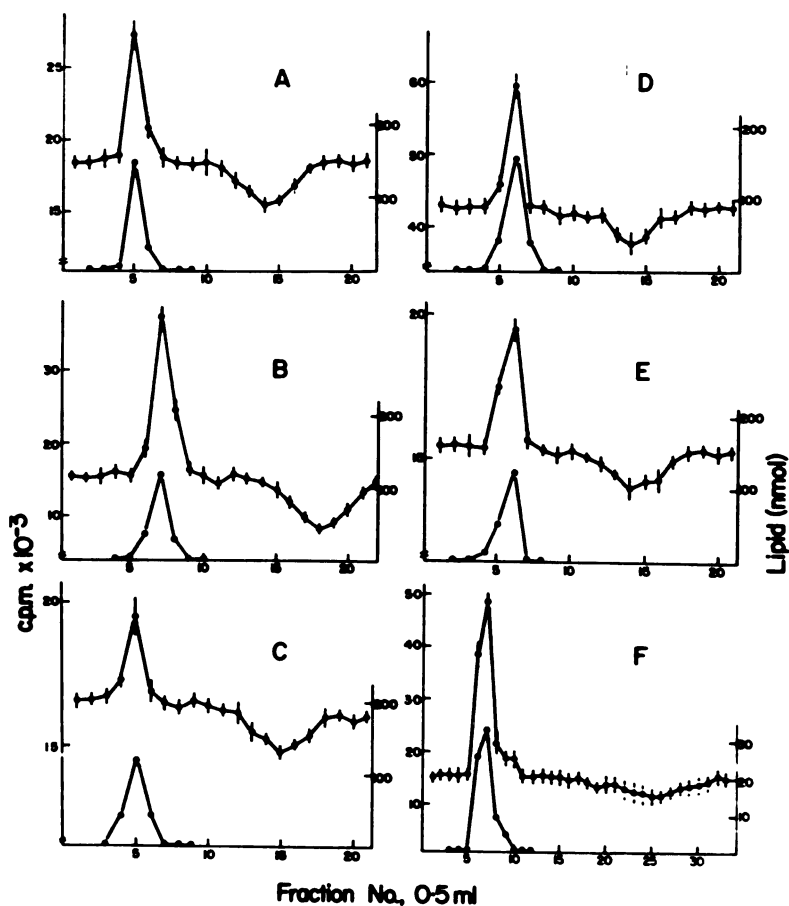


Fig. 12. Elution profiles of tritiated 5-HT (solid circles) and lipid (open circles) after 0.1 ml of tritiated 5-HT at 10^{-4} M in HEPES buffer, incubated with 216 nmol lipid (A) PI, (B) TPI, (C) CS, (D) PA, (E) PS, were loaded onto Sephadex G-50 columns, equilibrated and eluted with tritiated 5-HT at 10^{-4} M in HEPES buffer. For DPI (F), 0.1 ml of tritiated 5-HT at 5×10^{-7} M in HEPES buffer, incubated with 50 nmol of DPI, was loaded onto a Sephadex G-25 column, equilibrated and eluted with tritiated 5-HT at 5×10^{-7} M in HEPES buffer. Two 0.2 ml aliquots were taken from each 0.5 ml fraction to determine radioactivity and lipid phosphorus for PI, PA, and PS. For TPI, 0.2 ml and 0.1 ml aliquots were taken from each fraction to determine radioactivity and lipid phosphorus. For CS, 0.2 ml and 0.05 ml aliquots were taken from each fraction to determine radioactivity and CS. For DPI, 0.1 ml and 0.3 ml aliquots were taken from each fraction to determine radioactivity and lipid phosphorus. Each point represents the mean and S.E.M. of three replications.

Table 3. The Binding of Tritiated 5-HT to Acidic Lipids in Aqueous Media

Acidic lipid	[5-HT]M	nmol lipid in peak ± S.E.M.	% lipid recovery	nmol 5-HT bound ± S.E.M.	Apparent K _d (μM) in aqueous media	Apparent K _d (μM) in iso- butanol*
Cerebroside sulfate	10 ⁻⁴	205 ± 5	90	12.5 ± 1.0	1250	1.4
Phosphatidic acid	10 ⁻⁴	208 ± 2	96	25.6 ± 0.7	710	0.6
Phosphatidylserine	10 ⁻⁴	181 ± 2	84	19.3 ± 1.5	840	0.4
1-Phosphatidylinositol	10 ⁻⁴	189 ± 3	87	31.5 ± 1.8	470	1.9
1-Phosphatidylinositol 4-phosphate	5 x 10 ⁻⁷	41.4 ± 1	83	2.1 ± 0.1	9.4	0.8
1-Phosphatidylinositol 4,5-bisphosphate	10 ⁻⁴	178 ± 8	82	117.0 ± 4.0	52	10

* From Johnson *et al.* (1977b).

D_f is the concentration of free 5-HT used to equilibrate the column, R_T is the total amount of recovered lipid, R_f is amount of unbound recovered lipid, and DR is the amount of 5-HT-lipid complex (Hummel and Dreyer, 1962; Ochoa and Bangham, 1976). The calculated K_d 's of 5-HT to the acidic lipids in an aqueous medium are presented in Table 3 along with the K_d 's determined previously in isobutanol (Johnson et al., 1977b). DPI had the highest apparent affinity with a K_d of 9.4 μ M, followed by TPI, 52 μ M; PI, 470 μ M; PA, 710 μ M; PS, 840 μ M; and CS, 1250 μ M. In all cases the K_d 's determined in an aqueous medium and in isobutanol were negatively correlated with each other (Pearson product-moment correlation, $r = -0.50$), but this was not significant ($t = 1.2$, d.f. = 4).

2.3.4. Discussion

Several lines of evidence have implicated some acidic lipids as possible 5-HT receptors. Woolley and Gommi (1964) observed that 5-HT responsiveness of rat stomach fundus could be blocked by neuraminidase and that either a crude lipid extract or purified gangliosides reversed this effect. Marchbanks (1966) showed that a high affinity 5-HT binding material associated with the nerve ending particle fraction was extractable with n-butanol and that the 5-HT binding activity of this extract was inhibited by neuraminidase. Johnson et al. (1977a) found that the putative "5-HT receptor" isolated from the n-butanol extract from rat brain stem using Sephadex LH-20 chromatography (Godwin and Sneddon, 1975) contained CS, PI, and DPI. Over 90% of the observed binding of 5-HT to the butanol brain extract could be

accounted for with just purified CS and PI. Also, CS, PI, DPI, TPI, PA, and PS have been shown to bind with high affinity to 5-HT in isobutanol; K_d 's ranged from 0.4 to 10 μM (Johnson *et al.*, 1977b).

However, gangliosides have been shown to have relatively low affinities to 5-HT, K_d 's from 3 to 5 mM (Ochoa and Bangham, 1976). Moreover, in the present study we found the apparent affinities of 5-HT to other acidic lipids were in general much lower in an aqueous medium than previously reported for them in isobutanol. The K_d 's ranged from 9.4 μM for DPI to 1250 μM for CS. Though DPI had the highest apparent affinity to 5-HT of the acidic lipids tested, its affinity was approximately 10-1000 times less than what has been variously reported for the high affinity binding site of 5-HT to brain tissue (Marchbanks, 1966; McClain and Christian, 1975; Bennett and Snyder, 1976). Since the *in vivo* interaction of 5-HT with its receptor probably occurs at an aqueous-membrane interface, it is more likely that determinations of 5-HT affinities to putative receptors at aqueous-membrane interfaces provide better predictions of *in vivo* affinity than determinations in a totally organic environment. Consequently, while it may be possible that one or more of these lipids could be responsible for the high affinity binding of 5-HT to brain homogenate due to its particular location, physical state, or local chemical environment, it seems unlikely that these lipids could be 5-HT receptors based on their affinities to 5-HT in an aqueous medium.

While it is not possible to ascertain a clear understanding of the nature of the interaction energy between 5-HT and these acidic lipids from the data presented, it is reasonable to conclude that more

than a simple electrostatic interaction is involved in the binding to at least some of the lipids. If all the binding were due to electrostatic forces, then the apparent K_d 's in isobutanol and aqueous media should have been correlated, since a major difference between the two determinations of K_d 's was a change of the solvent and, therefore, a change in the dielectric constant. Moreover, if the interaction energies were primarily due to electrostatic interaction for the inositol-phospholipids in an aqueous medium, then successive addition of phosphate groups to PI should have increased the affinity of 5-HT for DPI, and still further for TPI. We observed, however, that the addition of one phosphate to PI increased the affinity approximately 50 times (DPI), but the addition of another phosphate (TPI) decreased the apparent affinity approximately 5 times compared to DPI.

Beyond the suggestion that CS, PI, DPI, TPI, PA and PS probably do not function as 5-HT receptors, the data in this study point up a pitfall in the measurement of drug affinities to biomolecules using two phase organic-water partition systems (e.g., Weber et al., 1971; Lowney et al., 1974; Cho et al., 1974). Drug affinities measured in an organic medium are not necessarily of the same order of magnitude as those measured in an aqueous medium nor are they necessarily correlated with them. In the present study, the apparent affinities of 5-HT to six acidic lipids in an aqueous medium were, in general, much lower and were poorly correlated with the affinities previously determined in isobutanol. Evaluation of drug affinities based on two phase organic-water partition systems should be done with caution.

Section 3

EXAMINATION OF POSSIBLE INVOLVEMENT OF CEREBROSIDE SULFATE IN MEMBRANE FLUIDIZING ACTION OF OPIATES

3.1 Introduction

Recently there has been renewed interest in the molecular mechanism of action of opiates in analgesia, tolerance, and physical dependence. One focus of research has been on the identification of those biomolecules directly involved in the action of opiates (Simon et al., 1978; Snyder and Simotov, 1977; Lowney et al., 1974; Terenius, 1973). A number of observations implicate an endogenous lipid, cerebroside sulfate (CS),* in opiate activity: (a) it is the major binding component in one putative receptor isolated from mouse brain (Loh et al., 1974); (b) the binding of opiates to CS is saturable, stereoselective, and the affinity of a large number of opiates for CS correlates remarkably well with their pharmacological potencies (Cho et al., 1976); (c) azure A, a dye which has high affinity for CS, competitively inhibits opiate receptor binding and increases the ED₅₀ for morphine when injected intraventricularly (Loh et al., 1978); (d) jimpy mice, who have low levels of CS, are resistant to the effects of morphine and show a decrease in the number of opiate binding sites

* The abbreviations used are: CS, cerebroside sulfate; DPH, 1,6-diphenylhexatriene; DSC, differential scanning calorimetry; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; DPPC, dipalmitoylphosphatidylcholine.

(Loh et al., 1978), and (e) cerebroside sulfatase (EC3.1.6.8) treatment of rat brain synaptic membranes abolishes about 50% of the stereospecific opiate binding (Law et al., 1978).

Because of the important role lipid head groups play in determining lipid fluidity (Chapman, 1973), and because of the suggestion that CS-agonists and CS-antagonists differ in their degree of hydration (Loh et al., 1974; Cho et al., 1976; Cho et al., 1976), an analysis of the effects of opiates on lipid fluidity of lipid bilayers which contain CS was undertaken. We thought that it might be possible to discriminate between opiate agonists and antagonists in terms of their ability to affect the interaction of polar head groups and, therefore, the fluidity of the hydrocarbon regions of lipid bilayers into which was incorporated CS. Moreover, it was recently reported using differential scanning calorimetry (DSC) that opiates specifically increase brain lipid fluidity and that this effect required an ether precipitate rich in CS (Hosein et al., 1977).

Our primary goal was to determine whether a small amount of CS incorporated into lipid bilayers (comparable to the amount in the synaptic and microsomal membranes) was sufficient to render lipid membranes sensitive to possible fluidizing action of opiates. It became apparent that some opiates affected the phase transition of lipid membranes; however, these effects occurred at 500+ times pharmacologically reasonable concentrations ($> 1 \mu\text{M}$). We next asked whether there was a qualitative difference in the nature of the interaction of opiate agonists and antagonists with CS which was reflected in their ability to fluidize lipid bilayers. We found that

the effects on the phase transition depended on the concentration of lipid and were not correlated with their analgesic activity.

Because the brain opiate receptors are located primarily in the synaptic and microsomal membranes (Pert and Snyder, 1974; Smith and Loh, 1976), and because CS composes less than 1% of the total lipid and PC is the major lipid of these membranes (Cotman et al., 1969), we studied the main phase transition temperature of lipid bilayers composed of dipalmitoylphosphatidylcholine (DPPC) and 1 mol % CS with three methods: (a) the partitioning of the spin label, TEMPO, between the aqueous and fluid hydrophobic phases (Shimschich and McConnell, 1973); (b) the steady state fluorescence depolarization of 1,6-diphenylhexatriene (DPH) incorporated into the bilayers (Shinitzky and Inbar, 1974; Amdrich and Vanderkooi, 1976; Lentz et al., 1976), and (c) turbidic changes associated with lipid phase transitions (Yi and MacDonald, 1973).

When it became apparent that we could not show any specificity in the ability of opiates to increase lipid fluidity of bilayers which contained CS, we attempted to repeat the work of Hosein et al. (1977) who showed, using DSC, that opiates specifically increase brain lipid fluidity and that this effect required a CS rich ether precipitate. We measured fluorescence depolarization of DPH incorporated in brain lipid bilayers in order to determine whether CS was the component in the ether precipitate responsible for the opiate effect. When we, however, examined the ability of as much as 1 mM morphine to alter the polarization of DPH, no changes were observed, indicating that there were no alterations in the bulk hydrocarbon fluidity. To show that

our procedures could detect the effects of fluidizing agents, various amounts of ethanol were added. A 4-5% decrease in polarization of DPH could easily be detected with as little as 1% ethanol.

Because the measurement of the fluorescence depolarization of DPH monitors the bulk hydrocarbon region and the DSC monitors the whole system, including lipid-lipid, lipid-protein, and protein-protein interactions, our data tend to suggest that the alterations in the phase transitions observed with DSC may be due to changes in the melting of proteins or proteins interacting with lipids and not to the melting of the bulk hydrocarbon regions of lipids.

3.2 Material and Methods

Materials. 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was purchased from Calbiochem; 1-phosphatidylinositol was obtained from Sigma; 3-sn-phosphatidylserine was purchased from Miles Laboratories; and 1,6-diphenyl-1,3,5-hexatriene was obtained from Aldrich. Cerebroside sulfate was purified from bovine brain following the method of Fluharty et al. (1974), 1-phosphatidylinositol 4,5-bisphosphate by the method of Johnson et al. (1978). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) was synthesized following the method of Rozantezev and Neiman (1964). Dextrophan and levorphanol tartrate were generously donated by Hoffman-LaRoche; naloxone hydrochloride by Endo Laboratories. Morphine sulfate was purchased from Mallinckrodt. All other reagents were reagent grade and were used without further purification.

Preparation of Lipid Bilayers. Lipids dissolved in an organic solvent were rotary evaporated on the walls of 25 ml pear shaped flasks, then placed under high vacuum for one hour. After removal from vacuum, the flasks were filled with nitrogen and sealed. Multilamellar bilayers were formed by adding glass beads plus buffer and by gently swirling the beads around the flask in a 50° C water bath until the lipid was dispersed.

EPR Measurements. Fifty μM TEMPO was prepared by adding 25 μL of 100 mM TEMPO in ethanol to 50 ml sodium phosphate buffer, 100 mM, pH 7.4. The lipid bilayers were prepared by mixing 14.85 μmol DPPC and 0.15 μmol of one of the acidic lipids or 15 μmol of DPPC in the absence of acidic lipids in an organic solvent prior to evaporation. Just before the EPR spectra were taken, 0.3 ml of the buffer with or without the drug was added to the flask, and the multilamellar bilayers were prepared as described above.

EPR spectra were recorded on a Varian E-3 spectrometer equipped with a variable temperature accessory. The temperature of the sample was measured by placing a thermister just above the microwave cavity inside the sample cell. The thermister was connected to a Cole-Palmer 8202-20 centigrade thermometer. The high field hyperfine line of TEMPO was recorded two minutes after the temperature in the sample cell had equilibrated to each temperature setting.

Fluorescence Depolarization Measurements. Steady state fluorescence depolarization of DPH as a function of temperature or time was performed with a fluorimeter, a schematic of which is shown on Fig. 13. Photomultiplier tubes were placed to the right and left of the

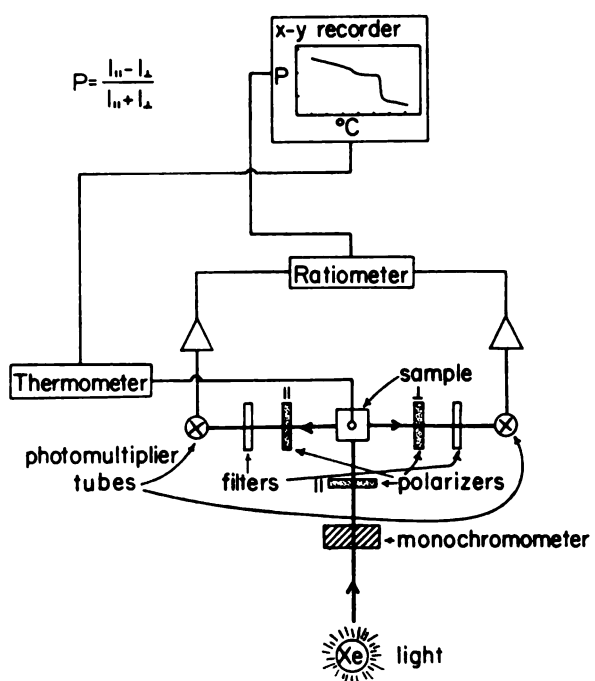


Fig. 13. Schematic diagram of fluorimeter.

sample cell: one of the emission beams was polarized parallel and one perpendicular to the plane of the 360 nm polarized excitation beam. Corning 3-74 cutoff filters were placed in the path of the emission beams. The signals from the photomultiplier tubes were fed into Pacific Instrument amplifiers and the outputs of these were fed into an Ithaco 351 Ratiometer. The ratiometer was operated in the polarization mode, $P = \frac{I_{11} - I_1}{I_{11} + I_1}$ where I_{11} is the fluorescence intensity parallel to and I_1 is the intensity perpendicular to the plane of polarization of the excitation beam. The output of this was fed into the y-axis of a Hewlett-Packard 7035B x-y recorder. Prior to running of each sample, the outputs of the photomultiplier amplifiers were balanced using a horizontally polarized excitation beam to correct for the difference in the detection efficiency of the two emission beams.

Temperature was regulated by circulating water which heated or cooled the sample cell holder. The sample was mixed with a magnetic stirrer. A thermister was placed in the sample cell just above the excitation beam, and the temperature was monitored with a Cole-Palmer 8502-20 centigrade thermometer modified to allow an analog signal proportional to the temperature to be fed into the x-axis of the recorder. When polarization was monitored as a function of time, a Hewlett-Packard 17108 AM time base unit was plugged into the x-axis of the recorder.

After the bilayers were prepared, sufficient 2 mM DPH in tetrahydrofuran was added to the buffer to make it 0.75 μ M with respect to DPH. The samples were incubated at 50° C for 90 min prior to use so that the dye would be completely absorbed into the bilayers. Drugs

were added just prior to use when temperature scans were performed, or injected directly into the sample cell while polarization was recorded and temperature held constant. Temperature scans were performed at a rate of approximately 1° C per min.

The procedure used in the fluorescence experiments is slightly different than what has previously been reported (Andrich and Vanderkooi, 1976; Lentz et al., 1976) insofar as the polarization was continuously monitored as a function of temperature or time and, consequently, the light scattering blank was not subtracted in the calculation of polarization. We judged our modification acceptable for the analysis of the effects of drugs on bilayer fluidity because of the following reasons: (1) we were only interested in changes and not the absolute values of polarization, and (2) subtraction of the apparent fluorescence intensities in the absence of dye still does not correct for depolarization of the emission beam so true polarization would not be obtainable even if the light scattering blank was subtracted.

Turbidic Measurements. A Gilford-DU model 252-1 updated spectrophotometer connected to a Gilford Model 2527 thermoprogrammer and model 6047 analog-multiplexer were used to monitor changes of optical density at 590 nm as the temperature was scanned at 1° C per min. With this system, four samples were run simultaneously so that comparisons could be readily made between the effects of drugs and/or the incorporation of CS on the main phase transition temperature of DPPC. Before the samples were placed in the sample cells, they were degassed at 50° C in order to minimize the formation of bulbs.

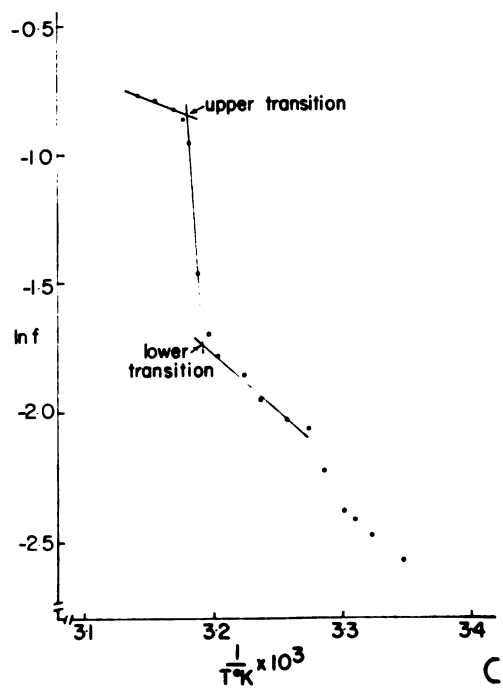
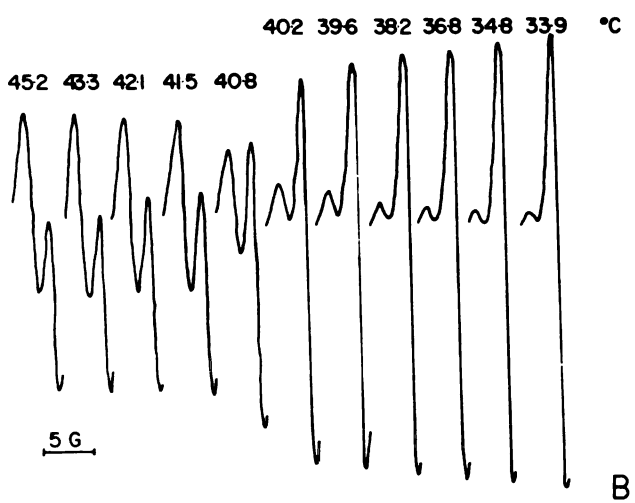
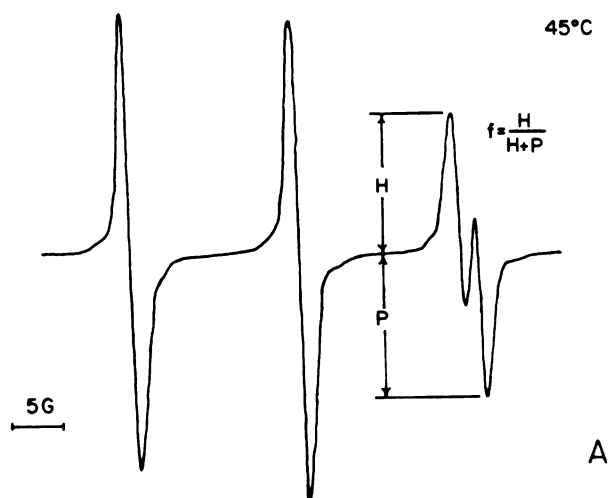
Preparation of Crude Mitochondrial Lipids. Following the method of French and Tudoroff (1971), brain mitochondria from male Sprague-Dawley rats were isolated and lipid extracted according to the method of Bligh and Dyer (24).

3.3 Results

EPR Measurements. For the EPR experiments, the phase transition temperatures were determined initially by plotting the TEMPO spectral parameter (f) as a function of temperature. This was done by measuring the amplitudes H and P of the high field nitroxide hyperfine line (Fig. 14A). To a first approximation, H is proportional to the amount of TEMPO dissolved in the lipid bilayer. As the lipid changes from the gel state to the liquid crystalline state, the solubility of TEMPO changes as does the f value. Fig. 14B shows the typical changes that occur in the high field hyperfine line of TEMPO in the presence of DPPC as the temperature is altered. The midpoint of the phase transition was determined by plotting $\ln f$ vs the reciprocal of the temperature ($^{\circ}K$) and taking the midpoint between the upper and lower transitions (Fig. 14C).

Fig. 15 shows a plot of the spectral f parameter vs temperature of DPPC plus 1 mol % CS in the presence and absence of naloxone and morphine. Even 2 mM morphine had little effect on the phase transition temperature, while 1 mM naloxone, the highest concentration we could get in our buffer, shifted the phase transition down about 1.3° , indicating an increase in hydrocarbon fluidity. Though the effect of

Fig. 14. ESR spectra of TEMPO (50 μM) in presence of DPPC (50 mM) (A) at 45° C and (B) high field hyperfine line at various temperatures. (C) Plot of the natural log of the spectral parameter (f) vs the $1/T$ ($^{\circ}\text{K}$).



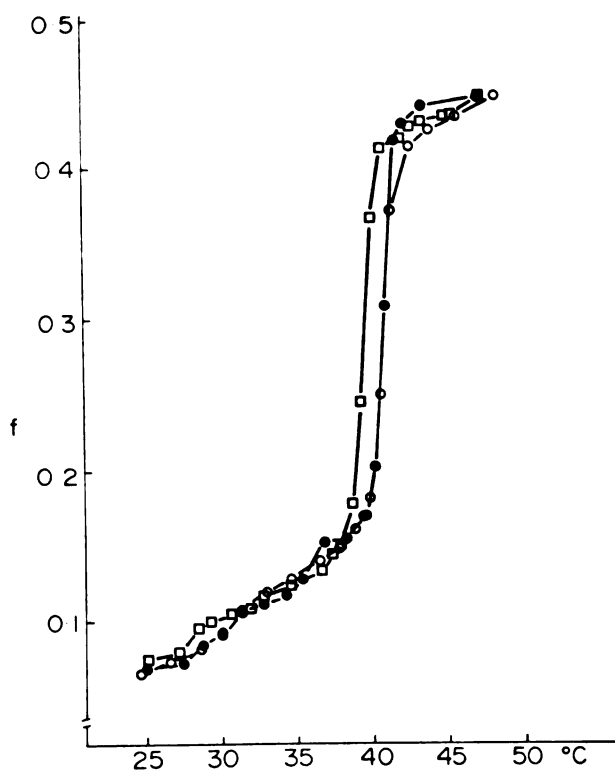


Fig. 15. Plot of the spectral parameter vs temperature of DPPC plus 1 mol % CS (50 mM) in the absence (●—●) and presence of 1 mM naloxone (□—□) and 2 mM morphine (○—○).

naloxone was only seen at pharmacologically absurd concentrations, we thought the effect may reflect a qualitative difference in the interaction of opiate agonists and antagonists with CS. To determine whether the effect was due to the presence of CS, the experiment was repeated in the absence of CS and in the presence of other acidic lipids which have been shown to bind opiates (Abood and Hoss, 1975; Hoss and Smiley, 1977; Wu et al., 1976). The results of these experiments are shown in Table 4.

In the absence of CS, neither morphine nor naloxone had a significant effect on the main phase transition temperature. This was also true when 1 mol % of 1-phosphatidylinositol was incorporated. Naloxone (1 mM) but not morphine (2 mM) had small but detectable effect (-0.3°C) when 1 mol % phosphatidylserine was incorporated. When 1-phosphatidylinositol 4,5-bisphosphate was incorporated, both morphine and naloxone lowered the main phase transition temperature, though the effects were small, 0.3°C and 0.8°C , respectively (Table 4).

Since it appeared that morphine and naloxone may interact with DPPC-CS bilayers differentially, other opiates were tested in order to determine whether the DPPC-CS bilayers could discriminate between opiate agonists and antagonists. Unfortunately, levorphanol, an agonist, and dextrophan, its inactive enantiomer, both lower the phase transition temperature about 0.7° and 0.3° , respectively. Thus, with this method it was not possible to discriminate between the interaction of opiate agonists and antagonists with CS.

Fluorescence Measurements. Because the TEMPO partitioning experiments required approximately 50 mM lipid and because drug effects

Table 4. The effect of opiate agonists and antagonists on the midpoint of the main phase transition temperature ($^{\circ}\text{C}$) of bilayers composed of DPPC and 1 mol % of various acidic lipids (~ 50 mM total lipid) by measuring the partitioning of the spin label TEMPO (average standard error on the measurements was $\pm 0.1^{\circ}\text{C}$).

Composition	No Drug	Morphine (2 mM)	Naloxone (1 mM)
DPPC only	40.8 $^{\circ}$	40.8 $^{\circ}$	40.8 $^{\circ}$
DPPC and cerebroside sulfate	40.6 $^{\circ}$	40.7 $^{\circ}$	39.3 $^{\circ}$
DPPC and phosphatidylserine	40.5 $^{\circ}$	40.4 $^{\circ}$	40.2 $^{\circ}$
DPPC and 1-phosphatidylinositol 4,5-bisphosphate	39.7 $^{\circ}$	39.4 $^{\circ}$	38.9 $^{\circ}$
DPPC and 1-phosphatidylinositol	40.5 $^{\circ}$	40.4 $^{\circ}$	40.4 $^{\circ}$

were only seen at about a one-to-one ratio of drug to CS, we thought that effects might be seen only when nearly all the CS was bound or, in other words, with at least 0.5 mM drug. At this concentration of drug, it would be difficult to pharmacologically discriminate opiate agonists from antagonists (Frank, 1976). So, the phase transition temperatures were measured using steady state fluorescence depolarization of a very hydrophobic dye, DPH. With the fluorescence depolarization, it is possible to measure transitions with 100 to 1000 times less lipid than is minimally needed in the EPR experiment.

Based on preliminary experiments, we chose to use 500 μ M lipid because this concentration appeared to provide an optimal signal to noise ratio. Fig. 16 shows the typical polarization vs temperature plot observed for DPPC plus 1 mol % CS. The main phase transition was determined by visually fitting straight lines through the linear portions of the curve above and below the main phase transition and then fitting a line through the transition. The temperature half way between the intersections of lines a, b, and c (Fig. 16) was designated T_m , the main phase transition. Repeated determinations of T_m over many days shows that the values never differed by more than 0.3° C from one another. Also, apparent in the polarization vs temperature scans was the presence of the thermal "pretransition" characteristic of multilamellar DPPC bilayers. Since the pretransition provides additional information on possible structural changes in the bilayers, the pretransition temperature, T_p , was recorded for each sample, if present, by arbitrarily taking the point where the polarization vs temperature curve departed from line a (Fig. 16). The values for T_p

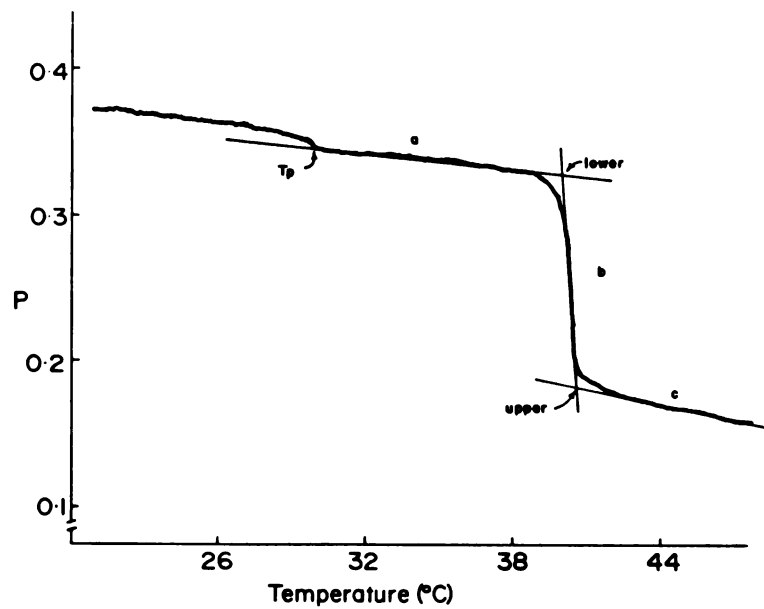


Fig. 16. Fluorescence polarization of DPH incorporated into lipid bilayers (A) composed of DPPC plus 1 mol % CS (50 μ M) as the temperature was scanned.

were not as reproducible as the T_m values, but varied not more than 1° C from one another from day to day with the same sample.

Table 5 summarizes the effects of morphine and naloxone, levorphanol, and dextrophan on DPPC-CS bilayers. As with the EPR experiments, 1 mM morphine appeared not to have a detectable effect on the main phase transition temperature, while 1 mM naloxone lowered the T_m about 1.3° C. Levorphanol (1 mM) lowered the T_m slightly as well as dextrophan (1 mM) (0.2° C). Though 1 mM morphine did not appear to affect the main phase transition, it eliminated the pretransition, above 0.5 mM. Levorphanol but not dextrophan also eliminated the pretransition.

To check that the drug effects were due to the presence of CS, the experiments were repeated in the absence of CS. Unlike the EPR experiments, 1 mM naloxone lowered the phase transition temperature of DPPC. With the exceptions that 1 mM morphine eliminated the pretransition and that the transitions occurred at slightly lower temperatures, the addition of 1 mol % CS appeared to have little specific effect on opiate induced changes in the main phase transition.

These results contradicted some of the EPR experimental results and suggested two possible explanations: First the TEMPO partitioning and the fluorescence depolarization of DPH methods are probing slightly different aspects of the behavior of the bilayers. Or, second, there was a difference in the conditions between the two experiments. Since the major difference between the EPR and fluorescence experiments was the concentration of the lipid used, 50 mM and 500 μ M, respectively, the effect of 1 mM naloxone on the main phase

Table 5. The effect of opiate agonists and antagonists on the midpoint of the main phase transition and pretransition temperatures ($^{\circ}\text{C}$) of bilayers composed of DPPC plus 1 mol % CS (500 μM total lipid) in 100 mM sodium phosphate buffer, pH 7.4, by measuring the fluorescence depolarization of incorporated DPH (average standard error of measurements for the main transition $\pm 0.1^{\circ}\text{C}$ and $\pm 0.4^{\circ}\text{C}$ for the pretransition).

DPPC and CS (1 mol %)		
Drug (mM)	Pretransition ^a ($^{\circ}\text{C}$)	Main Transition ($^{\circ}\text{C}$)
Control	30.3	40.3
Morphine		
1.0	N.D.	40.2
Naloxone		
1.0	N.D.	39.0
0.1	29.1	40.3
0.01	30.2	40.3
Levorphanol		
1.0	N.D.	40.0
Dextrorphan		
1.0	29.6	40.1

a - N.D. = transition not detectable.

transition temperature of the DPPC bilayers was determined at two concentrations of lipid and in the presence and absence of incorporated CS. Because the concentrations of lipid required for the EPR and fluorescence depolarization experiments do not overlap, we measured the turbidic changes associated with the main phase transition of the 15 mM lipid. Fifteen mM lipid and not 50 mM lipid was used because it was not possible to reliably measure the phase transition above 15 mM lipid by turbidity.

Turbidic Measurements. The midpoint in the main phase transitions of the optical density vs temperature scans were determined as were done for the fluorescence depolarization experiments. The effectiveness of naloxone to lower the main phase transition depended on the concentration of lipid used and on the presence of CS. At 500 μ M lipid, 1 mM naloxone appeared to lower the main phase transition temperature about the same amount with or without CS (1.5° C), which is consistent with the fluorescence experiments. At 30 times higher concentration of lipid (15 mM), 1 mM naloxone lowered the temperature of the main transition about 0.5° C in the absence of CS and about 1.0° C in the presence of CS. This is consistent with the EPR results which showed that with 50 mM lipid 1 mM naloxone lowered the T_m about 1.3° C in the presence of CS and had no detectable effect in the absence of CS.

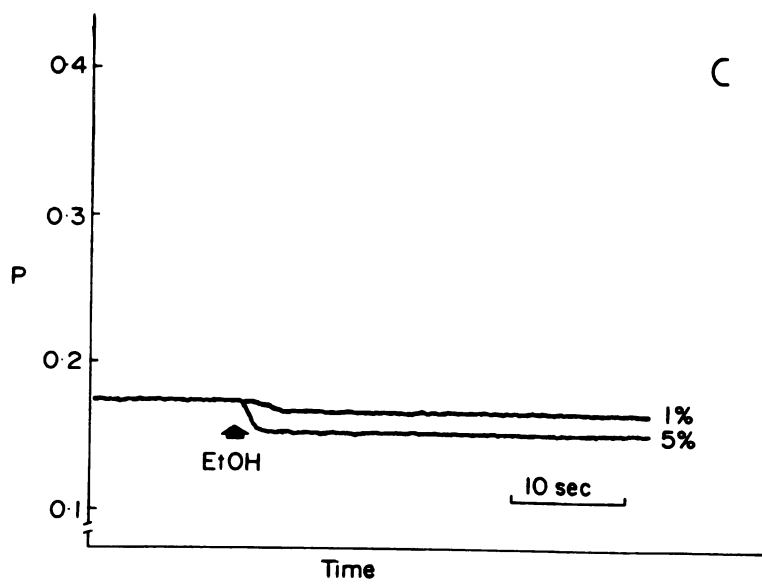
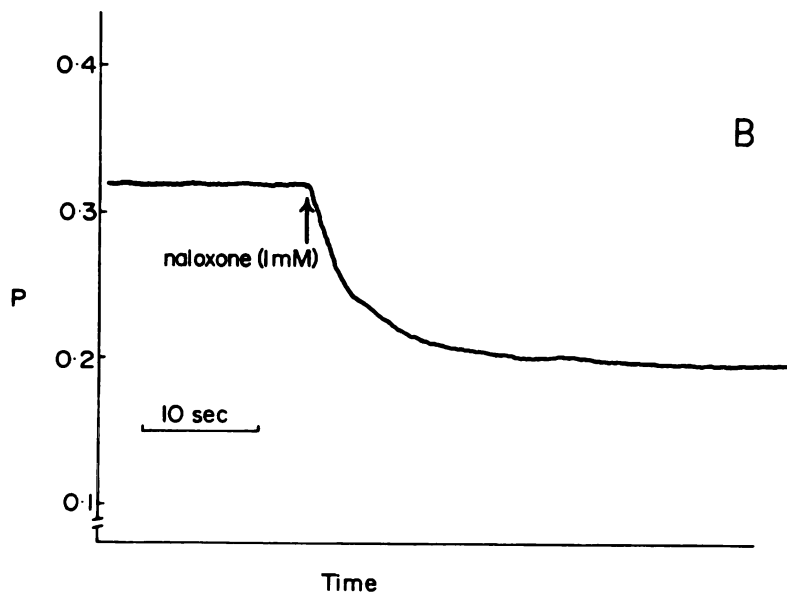
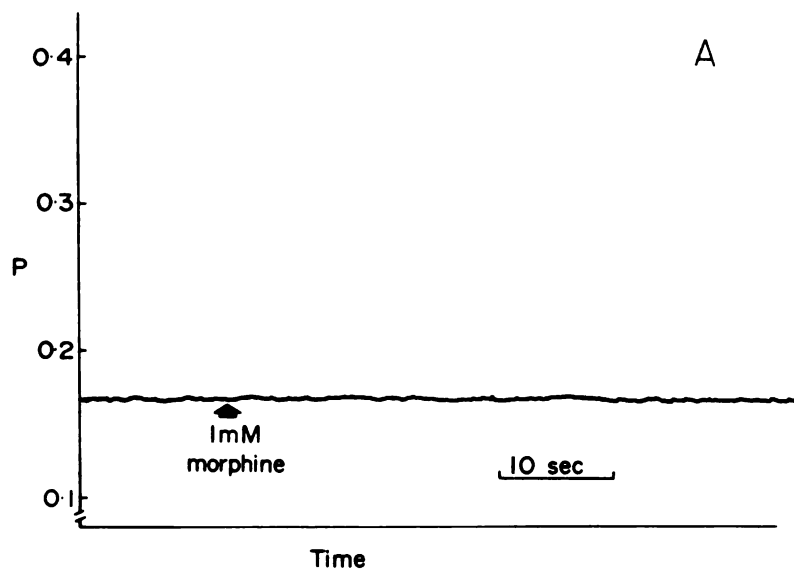
There is a slight discrepancy between the EPR and turbidity experimental results insofar as 1 mM naloxone was not observed to have any detectable effect on the T_m of DPPC in the absence of CS when measured with EPR and was observed to lower the T_m slightly (0.5° C)

when measured turbidmetrically. This discrepancy may reflect the fact that in the EPR experiments about 3 times more lipid was used.

Besides examining the effect of morphine on lipid bilayers composed of DPPC and 1 mol % CS, other bilayers which contained CS were studied. Bilayers composed of DPPC and various mol % of CS, of a mixture of neutral brain lipids and various mol % CS and of only CS were examined using fluorescence depolarization of DPH as an index of lipid fluidity with and without morphine. With our best efforts, we never observed morphine to increase bilayer fluidity even at a concentration of 1 mM (data not shown). We were, therefore, surprised at the report by Hosein et al. (1977) using DSC that morphine increased lipid fluidity and that the effect of morphine required an ether precipitable fraction previously shown to be rich in CS (Loh et al., 1974).

We attempted to repeat this work using fluorescence depolarization to determine whether CS was the component in the ether precipitate responsible for the opiate effect. Crude brain mitochondrial lipids were isolated following the procedures used by Hosein et al. (1977). The lipids were suspended in sodium phosphate buffer at a concentration of 100 μ M with respect to lipid phosphorus. Because no sharp transitions were observed when the temperature was scanned from 2° to 50° C, the temperature of the samples was held constant at 37° and various drugs were injected. When we added as much as 1 mM of morphine, we could not detect any change in polarization (Fig. 17A). To show that opiates could depolarize the DPH in bilayers when injected into the sample cuvette, naloxone was injected in the bilayers

Fig. 17. Fluorescence polarization of DPH incorporated into bilayers (A) composed of crude mitochondrial lipids (100 μ M with respect to lipid phosphorus) at 37° C while 1 mM morphine was injected into sample cuvette, (B) composed of DPPC plus 1 mol % CS (500 μ M) while 1 mM naloxone was injected, and (C) composed of crude mitochondrial lipids (100 μ M with respect to lipid phosphorus) at 37° C while 1% and 5% of ethanol were injected.



composed of DPPC and 1 mol % CS at 39.9° C. The results of this experiment are shown in Fig. 17B; naloxone depolarized the dye within 20 sec.

To show that fluidizing agents could affect bilayers composed of the crude mitochondrial lipids, various amounts of ethanol were injected. The results of this experiment are shown in Fig. 17C. As little as 1% ethanol decreased the polarization 4-5%, and 12-13% when 5% ethanol was added.

3.4 Discussion

The primary goal of this work was to determine whether an endogenous lipid, CS, which has been implicated in opiate activity, could make lipid membranes responsive to possible fluidizing effects of opiates at pharmacologically reasonable concentrations. In our various attempts at this problem, we found no evidence that opiate agonists or antagonists interacted with CS to increase lipid membrane fluidity at pharmacological concentrations. Very high concentrations of certain opiate agonists and antagonists lowered the phase transition temperatures of bilayers due to the presence of CS; however, these effects depended on the concentration of lipid and were not correlated with the analgesic activity of the drugs. Our results, therefore, suggest that opiates do not interact with CS to cause an increase in lipid membrane fluidity. This conclusion, of course, does not exclude the possibility that opiates interact with CS and, in turn, interact with protein(s) to cause an alteration in lipid membrane fluidity.

To our knowledge, there have been only two reports examining the effect of opiates on lipid membrane fluidity, one by Hosein et al. (1977) and one by Cater et al. (1974).

Hosein and his associates, using DCS of crude mitochondrial lipids from control and morphine treated rats, observed that morphine treatment caused a decrease in the temperature range and enthalpy of the phase transition. This effect was dose dependent and reversible both in vivo and in vitro by naloxone. Moreover, the ether precipitable fraction of total lipid extract was required for the opiate effect. They concluded that morphine disrupts the packing of the lipids and, thus, increases the lipid fluidity. When we attempted to repeat their in vitro experiments by monitoring the mobility of a fluorescent membrane probe, DPH, incorporated into the membranes, no changes in mobility of DPH were observed even when as much as 1 mM morphine was added, indicating no increase in lipid fluidity. One explanation for the discrepancy between our results and Hosein et al.'s (1977) is that the phase changes observed by Hosein et al. are due to changes in the melting of proteins or proteins interacting with lipids and not to the melting of the bulk hydrocarbon regions of lipid. Another explanation would be that we did not add enough morphine. The concentration of morphine in their in vitro experiments was somewhere between 25 and 50 mM (depending on the final volume); concentrations not obtainable with our experimental protocol. The fact that in their in vivo experiments morphine had apparently the same effect as the in vitro experiments may have been due to the induction of some long term change in the receptor conformation unrelated to the in vitro effects

seen only at extremely high concentrations of morphine. If Hosein et al.'s (1977) results are only obtainable at extremely high concentrations of morphine, the relevance of their work to the analgesic activity of opiates must be questioned, since the ED₅₀ concentration of morphine in the brain is about 3×10^{-7} M (Patrick et al., 1975).

Cater et al. (1974) examined the effect of opiate agonists and antagonists on phosphatidylcholine-water model membranes using DSC, NMR and ESR. Their results were consistent with ours insofar as they observed that morphine had no marked effect on the fluidity of the phosphatidylcholine bilayers and while other opiate agonists and antagonists alter the lipid fluidity, there was no relation to their analgesic activity.

THE ANSWER



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