Title
Studies on the solubilization and reconstitution of membrane opiate receptors

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Publication Date
1982

Peer reviewed|Thesis/dissertation

STUDIES ON THE SOLUBILIZATION AND RECONSTITUTION OF

MEMBRANE OPIATE RECEPTORS

by

ZOHREH TOOSSI

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: SEP 8 1982

STUDIES ON THE SOLUBILIZATION AND RECONSTITUTION OF MEMBRANE OPIATE RECEPTORS

Zohreh Toossi Farahbakhsh

September, 1982

To: My Parents,

My Husband,

My Son.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my research advisor, Dr. Horace H. Loh, without whose intellectual inspiration and guidance this dissertation could not have been completed. My deepest thanks to Dr. Nancy M. Lee for her enthusiastic help since the beginning of this research. I am sincerely grateful to all the members of Dr. Loh's research group for their thoughtful criticism and suggestions. I would like to thank Dr. David W. Deamer for his generous help in preparing the electron microscopic micrographs and valuable discussions. In addition, I wish to thank Drs. Jon R. Goerke, Ronald L. Morgan, and Andrew P. Smith for their valuable discussions and critical comments. Elizabeth Evans did an excellent job for the typing, for which I thank her.

Most importantly, I wish to express deep appreciation to my husband, Shahbaz, for his constant encouragement and determination during this long task, and my son, Pouya, for his cheerful inspiration.

ABSTRACT

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ABSTRACT

A new method utilizing lysophosphatide and acyl CoA as detergents has been used to solubilize the rat brain opiate receptor. After solubilization, lysophosphatide and acyl CoA can be completely removed by enzymatic reaction using an acyltransferase from rat liver microsome, resulting in reconstitution of the solubilized receptor. The reconstituted membrane is similar to that of the native membrane in its protein composition as judged by SDS polyacrylamide gel electrophoresis.

Morphological studies performed using negative staining and freeze fracture electron microscopy revealed that the general appearance and intramembrane particle distribution of fracture faces in the reconstituted membrane are similar to those of the native membrane; this indicates the incorporation of hydrophobic protein components of the original membrane into the reconstituted one. Reconstituted membrane, however, contained higher levels of phosphatidylcholine and lower levels of the major phospholipids and cholesterol. The activity of the membrane bound enzymes, Na^{+}-K^{+}-ATPase and Ca^{++}-Mg^{++}-ATPase in the reconstituted system, were just 24% and 3%, respectively, those of the native membrane. Although binding of opiate ligands to the reconstituted membrane is stereospecific and saturable, higher concentrations of some of the unlabeled ligands are required to inhibit binding of the radiolabeled ligands.

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Furthermore, the pH-maximum of opiate agonist binding is shifted to a lower value in reconstituted membrane.

These changes in receptor characteristics are likely due to changes in lipid composition, fluidity and/or distribution of the lipids in reconstituted membrane bilayer. This conclusion is supported by an observed increase in the affinity of opiate ligands for reconstituted membrane after adjustment of the latter's lipid composition to match more closely that of the original membrane. This was accomplished by treatment with phospholipid exchange protein to remove the phosphatidylcholine excess and by incorporation of cholesterol into the reconstituted membrane.

ABBREVIATIO

BHT	
B-ME	
C:M	
CHAPS	
CoA	
DADL	
DOC	
DTNB	
EDTA	
EGTA	
HEPES	
HPLC	
HPTLC	
leu-enkephalin	
LM	
met-enkephalin	
PC	
PE	
PI	
PS	
R	
SDS	
Sph	
SPM	

ABBREVIATIONS

BHT	butylated hydroxyltoluene
B-ME	B-mercaptoethanol
C:M	chloroform:methanol
CHAPS	3-[(3-cholamido-propyl)dimethyl-ammonio]-1-propane sulfonate
CoA	Coenzyme-A
DADL	D-ala^2-D-leu^5-enkephalin
DOC	deoxycholate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(beta-aminoethyl ether)-N-N'-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic
HPLC	High pressure liquid chromatography
HPTLC	High performance thin layer chromatography
leu-enkephalin	leucine-enkephalin
LM	liver microsomes
met-enkephalin	methionine-enkephalin
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
R	reconstituted membrane
SDS	sodium dodecyl sulfate
Sph	sphingomyelin
SPM	synaptic plasma membrane

TEMED

TLC

TEMED	N,N,N',N'-tetramethylethylendiamine
TLC	thin layer chromatography

I. INTRODUCTION

The extract of opium poppy is one of the oldest materials used in medicine with its ability to relieve pain and diarrhea known to the Ancient Greeks. The study of the major opium alkaloid, morphine, has been of great interest to biologists for decades. Despite the widespread use of opiates in medicine, the mechanism by which these drugs produce their effects, including pain relief, sedation, respiratory depression, tolerance and physical dependence is not known. However, it is believed that opiates, like other drugs, initiate their effects by association with a specific binding molecule located in responsive cells. These putative binding sites, known as receptors, have been described for dozens of other substances with physiological effects such as hormones and neurotramsmitters and somehow allow binding to be translated into the physiological response.

The opiate receptor concept evolved from extensive studies of the relationship between structure and *in vivo* pharmacologic activity in several series of opiate analgesics (Beckett *et al.*, 1965; Braenden *et al.*, 1955). More recently, the presence of a highly specific receptor is also supported by demonstration of high affinity stereo-specific binding of radioactive opiates to brain membranes. This binding has many properties similar to those of the drug pharmacological effect, including a similar correlation

of binding affinities with pharmacological potencies for many drugs (Terenius, 1973; Simon et al., 1973; Pert and Snyder, 1973).

Since this time, many attempts have been made to further characterize the chemical and physical nature of this receptor. The inhibition of stereospecific opiate binding by sulfhydryl reagents, such as N-ethylmaleimide (Terenius, 1973b) and iodoacetamide (Simon et al., 1973) indicates the importance of sulfhydryl (SH) groups to opiate binding (Ahmed and Byrne, 1980; Law et al., 1979a; Simon et al., 1980). The ability of proteolytic enzymes, such as pronase, trypsin and chymotrypsin, to inhibit binding (Pasternak and Snyder, 1974; Miller and Cuatrecasas, 1979; Law et al., 1979a) indicates the involvement of a protein component in the opiate binding site.

On the other hand, several studies have shown that lipid might also be a constituent of opiate binding sites. Phospholipase-A has been shown to decrease specific opiate binding to the brain membrane (Abood et al., 1978; Law et al., 1979a; Lin and Simon, 1978) and this decrease in binding can be restored by addition of phosphatidylserine (Abood et al., 1978). Arylsulfatase treatment, which specifically hydrolyzes cerebroside sulfate, also reduces the binding of opiates to receptor (Law et al., 1979b). The possible involvement of cerebroside sulfate in opiate bindig site was further supported by studies in which opiates

binding to cerebroside sulfate *in vitro* correlates with their pharmacological potencies (Cho *et al.*, 1976a,b).

Therefore, these indirect studies suggest that both lipids and proteins are involved in opiate binding. According to the fluid mosaic model of membrane structure (Singer and Nicolson, 1972), lipids form a fluid bilayer and proteins are embeded partially or completely into this bilayer. The lipid bilayer determines the conformation and degree of exposure of proteins, while proteins exert an ordering effect on lipids (Dehlinger *et al.*, 1974). This model thus establishes a basis for believing both components would be important to any receptor's normal functioning.

In order to fully understand opiate receptor mechanisms, however, the receptor must be purified in an active form; this enables determination of its chemical identity and characterization of the molecular nature of the reactive sites. However, the preparation of a stable active receptor after solubilization has been a major difficulty. Simon *et al.* (1975) first obtained a high molecular weight binding componenet (370,000 daltons) by detergent (Brij-36T) solubilization of brain fractions to which the radioactive opiate, ^{3}H-etorphine, had priviously been bound. Zukin and Kream (1979) modified the Simon *et al.* procedure by covalently linking ^{3}H-enkephalin to the same high molecular weight material found by Simon *et al.* They again found that the detergent-solubilized substance had an apparent gel

chromatographic molecular weight of 370,000 daltons. However, $NaDodSO_4$ gel electrophoresis, which dissociates proteins into component subunits, showed that the major radioactive peak had a molecular weight of 35,000 daltons. Neither study showed the binding component to be stereospefically active after solubilization, that is, it could not bind subsequently added opiates.

Very recently, a few reports of solubilization of an active binding component have appeared. Bidlack and Abood (1981) reported solubilization of an opiate binding component from rat brain using the non ionic detergent, Triton X-100. In this study, Triton X-100 was removed by adsorption to a special resin after solubilization, and the opiate binding characteristics then studied using equilibrium dialysis or gel filtration. It was found that the affinities of several ligands for solubilized material closely paralleled those of the native membrane, and binding was sensitive to proteolytic enzymes and phospholipase-A. In the latter case, the binding was restored by addition of phosphatidylserine.

Another detergent used for membrane solubilization was a zwitterionic detergent 3-[(3-cholamido-propyl)dimethylammonio]-1-propane sulfonate (CHAPS)(Simond et al., 1981). This detergent was able to solubilize the opiate binding material from rat brain. The solubilized component had a molecular weight similar to that reported for the material

solubilized with Brij-36T. However, only 20% of the original binding present in intact membrane was recovered after solubilization and the remaining binding was not recovered in the non-solubilized material either. It is not clear whether the low recovery of binding was due to incomplete solubilization or to changing the receptor properties due to dissociation from its specific membrane microenvironment.

Finally, solubilization of active opiate material from toad brain was reported by Ruegg et al. (1980) using digitonin. They were previously unable to solubilize the opiate binding material from rat brain membrane, using the same procedure, therefore, they concluded that important species differences exist with respect to the receptors.

A new method for solubilization of an opiate binding component from rat brain was very recently reported by Cho et al. (1981); a sonication procedure was used to disrupt the membrane and apparently release individual components. This procedure has an advantage over other solubilization methods in that no detergent is used, reducing the chance of altering receptor properties. This procedure also has relatively higher recovery than other solubilization methods and the binding properties are relatively unaltered.

Recently, partial purification of opiate receptors, solubilized by Triton X-100, was reported by Bidlack et al. (1981), by the use of affinity chromatography. An opiate ligand, 14-beta-bromoacetamidomorphine, was fixed to a

column resin, ω-aminohexyl-sepharose, and the solubilized material passed down the column and the receptors were bound. By washing the column with Tris buffer, non-specific proteins were eluted and the opiate binding materials were then eluted with 1 μM levorphanol or etorphine; three major proteins were eluted with the molecular weight of one protein being similar to that reported by Zukin and Kream (1979).

After isolation and purification of the opiate receptor the molecular structure responsible for ligand binding properties, individual sites, and also the number of components needed for activity can be determined. However, these must be correlated with opiate functions in order to show that the purified component is a true receptor. It has been suggested that cyclic nucleotides may play a role in the mode of action of opiates, for they are coupled with a mechanism similar to that of the hormone-receptor stimulation of adenylate cyclase (Blume, 1978; Law et al., (1981). Adenylate cyclase is a membrane bound enzyme and its activity is dependent on membrane lipid structure (Ross and Gilman, 1980) and it can be modulated by membrane phospholipids (Housley et al., 1976; Hanski et al., 1979). If the opiate receptor is coupled to adenylate cyclase it is thus clearly necessary to reconstitute the purified opiate receptor in a membrane environment with proper lipid composition in order to demonstrate its activity.

There are several conventional methods for reconstitution of proteins into membrane lipids which will be discussed more fully in a later section. These include sonication of lipids and proteins in an aqueous medium; solubilization of lipids and proteins in detergents and removal of detergents by dialysis against a detergent free buffer solution; and dilution of a detergent-solubilized lipid-protein mixture. All these methods, however, have disadvantages, particularly the use of non-physiological detergents, which raise the possibility of changing the receptor's native characteristics. There is also difficulty in completely removing the detergents from the medium to achieve reconstitution; this is required since the opiate receptor is thought to be very sensitive to small amounts of detergents.

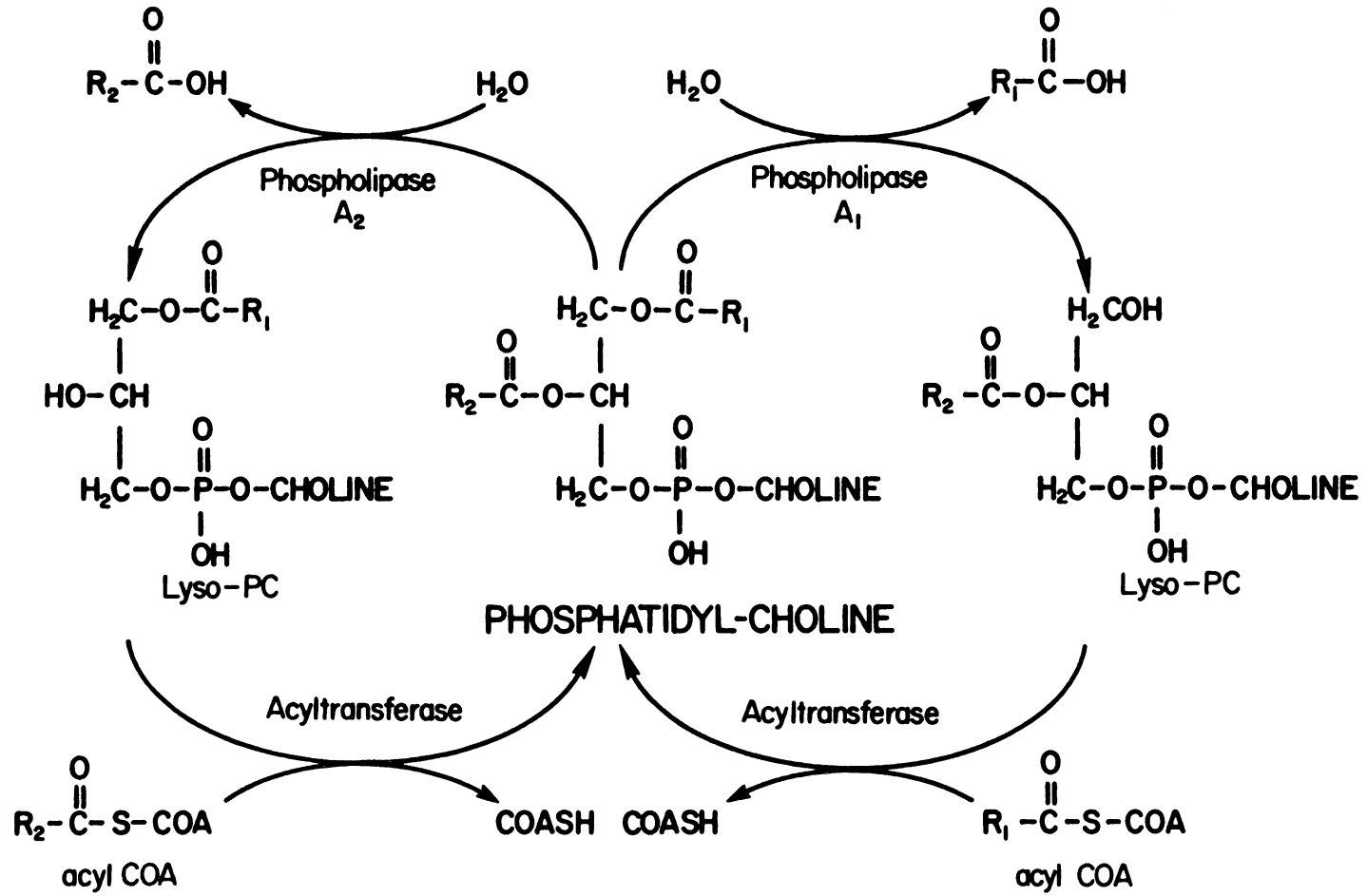
In this dissertation, a new method is described for solubilization and reconstitution of opiate receptors from rat brain membrane. This method is based on a natural process, an acylation-deacylation cycle which is a well characterized biochemical reaction. This cycle is normally involved in phospholipid metabolism and biosynthesis, as demonstrated by Lands (1960). A dynamic turnover of phosphoglycerides in biological membrane is accomplished by catabolism of existing phosphoglycerides that are constantly replaced by newly synthesized molecules. In mammalian tissues the catabolism of phosphoglycerides proceeds probably

entirely via a deacylation pathway catalyzed by phospholipase A and lipophospholipase. The resulting lysophosphatides can be reacylated by the action of selective acyltransferase. Therefore, the acylation cycle plays an important role in modulating membrane lipid composition including the biosynthesis and turnover of specific phosphoglycerides (Van Den Bosh et al., 1972) as well as net phospholipid synthesis during membrane biogenesis (Heckman et al., 1977; Higgins and Barnett, 1972) (Figure 1).

Diacylphosphoglycerides can be catabolized into their monoacylderivative by phospholipases A_1 and A_2. The resulting lysophosphoglycerides can be reacylated by specific acyltranferase in the presence of an acyl donor molecule such as acyl-CoA. Acyltransferases use lysophosphoglycerides and acyl CoA derivatives as substrate and these substrates have detergent activity and can solubilize the membrane. Thus if the solubilized fraction is incubated with acyltransferase, the detergent is completely degraded so that the reconstituted vesicular membrane can be reformed.

In studies described by Lands and Hart (1965), it was demonstrated that acylation of 1-acyl-sn-glycero-3-phosphorylcholine proceeded more rapidly with oleoyl CoA and linoleoyl CoA than with saturated acyl CoA esters, whereas 2-acyl-sn-glycero-3-phosphorylcholine was esterified preferentially with saturated fatty acids. Such specificity was

Acylation - Deacylation Cycle



in line with the asymmetric distribution of the acyl moieties in naturally occuring phospholipids. Not only 1-acyl-sn-glycero-3-phosphorylcholine, but also the 1-acyl derivative of glycero-3-phosphorylethanolamine, glycero-3-phosphorylserine, glycero-3-phosphorylglycerol and glycerol-3-phosphate can function as acceptor of a second acyl group. Acyltransferases also have different specificities toward various acyl donors and acceptors (Holub et al., 1979; Colard et al., 1980; Wise et al., 1980). Therefore, by addition of known lysophosphatide acyl CoA pairs, variation of the phospholipid content of membranes is possible and the phospholipid content of any membrane can be manipulated. Also the addition of known lipids during reconstitution can lead to formation of membrane vesicles of desirable lipid components either in position 1 or 2 of diacylphosphoglyceride and affect the asymmetric distribution of lipids. Another important advantage of this method is that exogenous proteins might also be incorporated into the membrane.

Therefore, the reconstitution of membrane based on the acylation cycle allows variation in lipid and protein composition during the reconstitution process and permits the study of phospholipid role in membrane functioning.

The successful reconstitution of liver microsomes based on the acylation cycle is described by Deamer and Boatman (1980). In their report, morphological and functional reconstitution of liver microsomes is described. Gel

electrophoresis patterns showed that the reconstituted liver microsomes contain all of the major protein components of the original microsomes. More surprisingly, a marker enzyme for liver microsomes, NADPH-cytochrome C reductase, was present in reconstituted membrane at 70% of the specific activity present in the original microsomal membrane. In freeze fracture images of reconstituted liver microsomes, intramembrane particles are prominent. This suggests that proteins are incorporated into the lipid bilayer and this procedure does not simply cause aggregation of lipids and proteins.

In this study, reconstitution of opiate receptor from rat brain membrane by using acyltransferase is described. The physical and morphological properties, protein and lipid analysis of the reconstituted membrane are evaluated. The reconstituted membrane is characterized in terms of its opiate binding characteristics.

Finally, due to the fact that the opiate receptor activity in the reconstituted membrane is quite different from that of the membrane bound receptor, attempts have been made to perform some modifications in the lipid content of the reconstituted membrane. The effects of these modifications were evaluated on membrane receptor binding and activity of the enzymes present in rat brain membranes.

II. METHODS AND MATERIALS

II-1 Methods

A) Isolation of Synaptosomal Membrane

Crude synaptosomal membranes were isolated from male Sprague-Dawley rats (180-220 g) according to the method of Law et al. (1981). Brains without cerebellum were removed and washed several times with ice-cold 0.32 M sucrose in 20 mM HEPES pH 7.7. A 10% homogenate was prepared with the above buffer using a teflon-glass tissue grinder, then pelleted at 1000 x g for 10 min in a Sorvall superspeed centrifuge. The resulting pellet was washed once with half the volume of the original homogenate. The two supernatants were pooled and centrifuged at 20,000 x g for 20 min. to produce a "crude P_2" pellet. The P_2 pellet was washed once with half the volume of the supernatant. The pellet of every brain was resuspended in 8 ml of 25 mM HEPES pH 7.7 and centrifuged at 20,000 x g for 30 minutes. Each pellet was resuspended in 20 ml of 25 mM HEPES/1mM EGTA pH 7.7 and following incubation at 37° C for 15 min, pelleted at 20,000 x g for 20 min. Each brain pellet was finally resuspended in 18 ml of 25 mM HEPES pH 7.7. All procedures were carried out at 4° C.

Protein content of this membrane homogenate was determined by the method of Lowry et al. (1951), using Bovine Serum Albumin as standard. Protein content was about 3-4 mg

per ml P_2 membrane homogenate. This membrane was divided into convenient aliquots and stored at -70° C until used.

B) Isolation of Rat Liver Microsomes

Male Sprague-Dawley rats (180-200 g) were fasted for 18-24 hours, then sacrificed by decapitation. Their livers were removed, washed with several changes of 0.9% NaCl with 1 mM EDTA at 4° C. The livers were weighed, minced and homogenized in 0.25 M sucrose, 10 mM Tris, 1 mM EDTA pH 7.3 using a Teflon-glass homogenizer (De Pierre et al., 1976). The homogenate was centrifuged twice at 10,000 x g for 20 minutes, and the pellets were discarded. The supernatant was centrifuged at 105,000 x g for 60 minutes. The pellet was resuspended in 0.15 M Tris, pH 8.0 and centrifuged again for 60 minutes. The resulting pellet was resuspended to ~25 mg protein per ml in 0.25 M sucrose, 10 mM Tris pH 7.3, divided into convenient aliquots, frozen and stored at -70° C until used. Acyltransferase activity was unchanged up to at least 2 months.

C) Acyltransferase Activity Measurements

Acyltransferase activity of isolated microsomes was measured by continuous recording of the coenzyme-A reaction with DTNB at 412 nm (Eible et al., 1969) using a Zeiss PM2D spectrophotometer (Carl Zeiss 7082, Oberkochen, West Germany). The reaction mixture contained 0.25-0.5 mg of

microsomal protein, 100 nmol of lysophosphatidylcholine (LPC), 100 nmol of oleoyl coenzyme-A and 1 nmol of DTNB, in a total volume of 2 ml of 0.1 M Tris pH 7.5. The recording started upon addition of the enzyme and continued for 35 minutes.

D) Membrane Solubilization and Reconstitution

Rat brain P_2 membrane (1 mg per ml) was solubilized by addition of 1 μmol LPC per mg protein, 0.1 mM coenzyme-A (Co-A), and 5 mM Mg^{++}-ATP, in 0.1 M Tris-HCl buffer, pH 7.5. After addition of LPC, the suspension was sonicated for 10 seconds, on output 3, model W-220F (Heat system, Ultrasonics, Inc., Plainview, New York), with a standard tapered microtip to break up aggregate. An amount of oleoyl CoA equimolar to the LPC was added in a final volume of 5 ml (Deamer and Boatman, 1980). The suspension was centrifuged for 60 minutes at 100,00 x g, or 20 minutes at 20,000 x g to remove the unsolubilized membrane fragments. A supernatant containing the solubilized membrane was incubated in the presence of rat liver microsomes as the source of the acyltransferase, 0.5-1 mg liver microsomes protein per mg of brain protein, at 37^{o} C for 45 minutes. Reformation of membrane fragments was determined by measuring the increase in optical density of the suspension before and after the incubation and electron microscopy. Reconstituted membrane was centrifuged for 20 minutes at 20,000 x g and washed

Figure 2.
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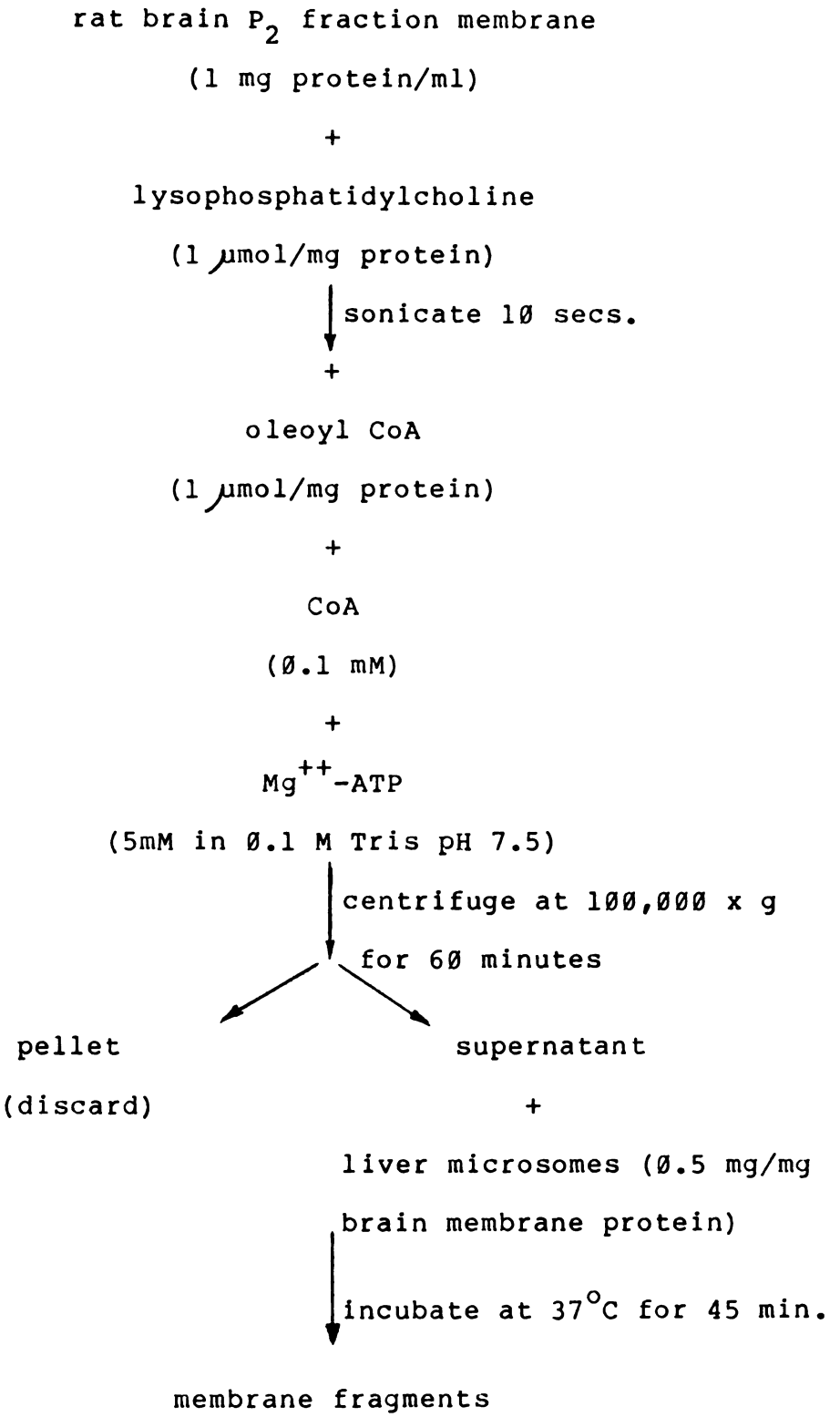

Figure 2. Solubilization and reconstitution profile of rat brain membrane

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twice in 25 mM HEPES pH 7.7 and resuspended in the same buffer to 2-3 mg protein/ml. The flow chart diagram (Figure 2) illustrates this procedure in detail.

E) Negative Staining and Freeze-fracture Electron Microscopy

Ten µl aliquots were taken from the original solubilized P_2 membrane, and from reconstituted membrane, and negatively stained by addition of 10 µl of 3% ammonium molybdate, pH 7.4, followed by draining and drying on formvar-coated grids. Stained samples were examined at 39K magnification in a Hitachi HK 11 E electron microscope.

Freeze fracture electron microscopy was also performed. Centrifuged pellets of the P_2 membrane and reconstituted membrane were infiltrated with 30% glycerol and aliquots were transferred to specimen planchettes and frozen in liquid nitrogen-cooled Freon. Freeze fracture was carried out in a Blazers apparatus (Balzers Corp., Nashua, N.H.) by standard methods (Fisher et al., 1974).

F) Slab Gel Electrophoresis of Rat Brain Membrane and Reconstituted Membrane

Slab gel electrophoresis of P_2 fractions and reconstituted brain membrane was performed under denaturing conditions essentially according to Maizel (1971). The electrophoresis was performed on a 5% to 15% polyacrylamide gradient in 0.1% SDS using the Hoffer slab gel apparatus,

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First a 5% to 15% polyacrylamide slab gel gradient was prepared by mixing 6 ml each of 5% and 15% polyacrylamide gel solutions in a gradient maker. The 5% acrylamide solution contained (final concentration): 1% glycerol v/v, 0.375 M Tris-HCl pH 8.8 at room temperature, 5% polyacrylamide, 0.13% bis-acrylamide, 0.027% ammonium persulfate, 0.025% TEMED,0.1% SDS, 4.67% glycerol v/v and 0.375 M Tris HCl, pH 8.8 (at room temperature). The 15% acrylamide solution contained 0.4% bisacrylamide, 0.03% ammonium persulfate, 0.025% TEMED and 0.1% SDS in final concentrations.

After the mixing and pouring gel solutions between slab gel plates was completed, 0.1% SDS solution was layered over the separating gel. After approximately one hour, the polymerization was completed and the SDS solution was poured off; stacking gel solution was then layered on top of the separating gel.

The stacking, upper gel, contained in final concentrations, 0.125 M Tris-HCl, pH 6.8 at room temperature, 3% polyacrylamide, 0.08% bis-acrylamide, 0.03% ammonium persulfate, 0.025% TEMED and 0.1% SDS. Immediately after layering the stacking gel, the slot former was placed in its proper position. Polymerization of the stacking gel was completed in about one hour at room temperature, after which the slot former could be removed.

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To place the samples on the stacking gel, samples were mixed with sample buffer in 1:1 ratio (v/v). The sample buffer contained in final concentrations, glycerol 5% v/v, 30 mM Tris-HCl pH 6.8, 25% B-mercaptoethanol, 1.5% SDS and 50 μg/ml Bromophenol-Blue. For each sample, 20-80 μg protein in 20-80 μl volumes were applied to each slot of the stacking gel.

Electrophoresis was run at 200 V at 4° C for about 4 hours in a chamber containing electrode buffer. The electrode buffer contained, in final concentration, 0.25 M Tris-glycine pH 8.9 and 0.1% SDS.

After completion of the electrophoresis the gels were removed and stained for 48 hours in 0.125% Coomassie Blue R-250, 50% methanol, 10% acetic acid. The gels were then destained in destaining solution I containing 50% methanol, 10% acetic acid and shaken for one hour. Then the gels were transferred to destaining solution II, containing 7% acetic acid and 5% methanol, overnight. A Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a chart recorder, was used to scan the stained gels.

G) Inhibition of Metabolic Degradation of Leu- and Met-Enkephalin

a. Tissue preparation for the in vitro studies.

Rat brain crude P_2 fraction was prepared as before.

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Synaptosomes were prepared by the method of Whittaker et al.(1964) using discontinuous sucrose density gradients consisting of two layers, 10 ml each, with 0.6 M sucrose in 20 mM HEPES pH 7.7 at the top and 1.0 M sucrose at the bottom of the gradient. The crude P_2 membrane was layered on top, and the tube centrifuged for 80 minutes at 60,000 x g. The fraction found at the interface was collected and centrifuged in a rotor 40 for 30 minutes at 100,000 x g. The pellet containing synaptic plasma membrane(SPM) was resuspended in 0.32 M sucrose buffered with 20 mM HEPES to a final concentration of 1 mg protein/ml, and this membrane fraction was used for in vitro studies.

b. In vitro incubation medium

All incubations were carried out in 1.4 ml Eppendorf polypropylene centrifuge tubes with snap-on caps. The incubation mixture consisted of 3 nM ^{3}H-leu-enkephalin or ^{3}H-met-enkephalin, 200 ul of SPM (1 mg/ ml), and different concentrations of EDTA and/or bacitracin in a final volume of one ml. Incubation was carried out for 3 hours at 4° C, beginning with addition of the peptides and terminating with immersing of tubes in a boiling water bath for 15 minutes. Tubes were then centrifuged at 8000 x g for 15 minutes. The supernatant was chilled and used for measurement of enkephalin and its degraded metabolites by high pressure liquid chromatography (HPLC) (Craves et al., 1978).

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c. Separation of enkephalin metabolites by HPLC

This consisted of a Water Associates (Milford, MA.) liquid chromatography apparatus, equipped with two model 6000 A pumps, a UK6 injector, and a model 660 solvent programmer. The column was 4 mm X 30 cm Bondapak C18, which consists of octadecyltrichlorosilane chemically bonded to 10 μ porous silica particles. A UV detector model 440 was used to monitor the absorption at 280 nm. Methanol was used as a solvent in a 0% to 100% gradient.

H) Total Lipid Extraction from Brain Membrane

Membrane lipids were extracted according to the method of Folch et al. (1957). In brief, this procedure involves extraction of membrane lipid in chloroform: Methanol (C:M, 2:1, v/v). One ml of membrane suspension was extracted with 19 ml C:M (2:1, v/v) and vortexed for 2 minutes. Then the extract was filtered and 4.0 ml of 1 M KCl was added and mixed thoroughly, and the phases allowed to separate. The upper phase containing all the non-lipid substances was discarded. The lower phase containing all the lipid components was transferred to an evaporating flask and rotor evaporated until the sample was nearly dry. The sample was resuspended in 2.0 ml C:M (2:1, v/v) and stored under nitrogen at -20° C. This lipid extract was used for phosphate determination and other phospholipid analyses.

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I) Phosphate Determination Assay

Inorganic phosphate in the lipid extract was measured according to the procedure of Bartlett (1959), using potassium phosphate as standard. The dried sample was hydrolyzed in Ø.2 ml conc. Perchloric acid (72%) for 2 hours at 18Ø° C; then it was cooled to room temperature, and 1.25 ml of reducing reagent added, mixed and 1.25 ml of molybdate reagent was added. Reducing reagent contained 1Ø g sodium bisulfite, 2 g sodium sulfite and 168 mg 1-amino-2-naphthal-4-sulfonic acid per liter, molybdate reagent was containing 4.4 g ammonium molybdate and 14 ml conc. sulfuric acid per one liter of water. After addition of these reagents, the sample was boiled for 1Ø min., cooled and read at 7ØØ nm in a Zeiss spectrophotometer. The phosphate content in the sample was determined from the standard curve by the linear regression method.

J) Sulfatide Determination

Sulfatide content of the lipid extract was measured by the method of Kean (1968). Briefly, samples were dried under nitrogen and 2.5 ml C:M (2:1, v/v), 2.5 ml of Ø.Ø5 N H_2SO_4 and Ø.5 ml Azure-A reagent solution were added. The Azure-A reagent solution contained 8Ø mg Azure-A, 1Ø mg Ø.Ø5 N H_2SO_4 in a final volume of 2ØØ ml of water, filtered before use. The mixture was vortexed and centrifuged in a bench type centrifuge. The lower phase was removed and its absorbance

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read at 645 nm. The sulfatide concentration was measured from a standard curve constructed using different concentrations of a sulfatide standard (Supelco, Inc., Bellefonte, PA.) treated the same way.

K) Phospholipid Separation by Thin Layer Chromatography (TLC)

Separation of the more common acidic and neutral phospholipids was carried out on glass plates coated with thin layers of silica gel-H, (without calcium sulfate as binder), according to the method of Skipski et al. (1964). Silica gel-H plates (0.25x20x20 cm) were activated at 150° C for one hour and cooled in a cabinet containing calcium sulfate as dessicant.

After the plates were cooled down to room temperature, the lipid solution was concentrated and spotted on the plates as compactly as possible. This was done by means of very thin capillary pipettes. the lipids were dissolved in a small volume of volatile solvent C:M (2:1, v/v) in order to allow rapid evaporation of the solvent. After the application of the samples, the plates were immediately chromatographed in glass tanks with ground glass covers, and lined with filter paper to aid in saturating the tank with solvent vapour. The tanks contained 60-80 ml of solvent which had been allowed to equilibrate with its vapour for 1-2 hours.

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acetic acid: H_2O (25:15:4:2) as a solvent system, PC, PE, sphingomyelin, and PS + PI as one spot were separated. Separation of PS from PI was accomplished by using high performance TLC plates and C:M:acetic acid:H_2O (65:43:1:3) as solvent. In all of these experiments high purity phospholipids (Supelco, Inc.) were used as standards.

Lipids were stained with iodine, circled and numbered. Spots containing the lipid and blanks of corresponding dimensions were scraped from the plates and transferred to test tubes. To each tube Ø.6 ml conc. perchloric acid was added. Tubes were covered with glass beads and digestion was performed at 18Ø° C for 2 hours. After cooling, 3.3 ml molybdate reagent and Ø.2 ml of reducing reagent was added. After rapid mixing tubes were heated 1Ø minutes in boiling water and afterward cooled to room temperature. Silica gel was removed by centrifugation and the absorbance was measured at 7ØØ nm. Reagent blanks and standard inorganic phosphate were treated similarly (Broekhayse, 1968).

L) Purification of Phospholipid Exchange Protein

Phospholipid exchange protein was purified from beef liver according to Crain et al. (198Ø) as follows:

a. pH 5.1 supernatant.

One beef liver trimmed of fat and cartilages was cut into one inch cubes and rinsed with Ø.25 M sucrose at 4° C.

A 35% homogenate was prepared in 250 mM sucrose, 1 mM EDTA 50 mM Tris, 0.02% NaN_3, pH 7.4 (SET buffer), and centrifuged for 20 minutes at 13,000 x g. The clear supernatant was adjusted to pH 7.4 with 3N NaOH and stored at -20° C. The exchange activity was stable for at least 6 months.

b. Ammonium sulfate precipitation.

The pH 5.1 supernatant (1.8 l) was thawed. Ammonium sulfate was gradually added to 40% saturation over a period of one hour and stirred for one hour. The precipitate was removed by centrifugation at 13,000 x g for 30 minutes. Ammonium sulfate was added to the supernatant over a period of one hour to 90% saturation and stirred for one hour. The pellet was collected by centrifugation at 13,000 x g for 30 minutes and resuspended in 100 ml of 25 mM sodium phosphate, 10 mM β-mercaptoethanol (β-ME) and 0.02% NaN_3, pH 7.4. This suspension was dialyzed extensively vs. 5 mM sodium phosphate, 5 mM β-ME, and 0.02% NaN_3 pH 7.4.

c. CM-cellulose column chromatography.

The dialyzed solution was applied to a CM-cellulose column (CM-52 microgranular, Bio-Rad Laboratories, Richmond, CA.) prepared from 250 ml of preswollen packed CM-cellulose pre-equilibrated in 5 mM sodium phosphate, 5 mM β-ME and 0.02% NaN_3, pH 7.4. The column was washed once with two column volumes of this buffer and eluted with two column

volumes of 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-ME, and Ø.Ø2% NaN_3, pH 7.4.

d. Heat treatment.

CM-cellulose column eluant was adjusted to pH 6.3 with 3N HCl and heated at 9Ø° C for 5 minutes. The pH was readjusted to 7.4 with 3N NaOH. This suspension was filtered to remove the precipitate. The filtrate was concentrated to 5Ø ml by ultrafiltration through a UM-1Ø membrane (Amicon Corp., Lexington, MA.).

e. Octylagarose column chromatography.

The concentrated supernatant was applied to an octyl-agarose column (1.Ø x 3Ø cm) consisting of 2Ø ml of gel preequilibrated with 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-ME and Ø.Ø2% NaN_3, pH 8.Ø. After loading the sample, the column was washed with 5Ø ml of this buffer and 5Ø ml of 5 mM sodium phosphate, 5 mM β-ME, and Ø.Ø2% NaN_3, pH 8.Ø. The column was eluted with the same buffer at pH 3.Ø and collected in 7 ml fractions. The pH was raised by addition of 7 ml 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-ME, and Ø.Ø2% NaN_3, pH 7.4. The eluted exchange activity was stable for at least 2 months at 4° C.

M) Measurement of Phospholipid Exchange Activity

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phosphatidylcholine (PC) was determined by measuring the transfer of ^{14}C PC from donor vesicles labeled with ^{14}C PC to "acceptor" liposomes containing a trace of ^{3}H-glycerol trioleate. The ^{3}H label is a non-transferable internal standard and served to determine the recovery of liposomes at the end of the incubation (Kamp et al., 1973). Donor vesicles were prepared as sonicated small unilamellar vesicles and the acceptor vesicles were prepared as large multilamellar vesicles.

Assay mixtures contained unilamellar vesicles (50 nmol phospholipids incubated with multilamellar vesicles (2 μmol lipids), at 37° C for 30 minutes in one ml of 5 mM EDTA, 50 mM Tris, and 0.02% NaN_3, pH 7.4. Phosphatidylcholine transfer was terminated by centrifugation at 40,000 x g for 20 minutes at 4° C in a Sorvall centrifuge, which selectively removed the large acceptor vesicles. Aliquots of the supernatant were counted in Liquiscint scintillation counting solution in a Beckman LS-100C scintillation counter. The small amount of transfer in the absence of exchange protein was substracted as background (Johnson et al., 1975).

The transfer of labeled PC from unilamellar vesicle to multilamellar vesicle was measured by the decrease in the ratio of labeled PC to nonexchangeable marker lipid in the supernatant after centrifugation.

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Corleto and Zilversmit (1977). Briefly, 1.8 μmol PE and Ø.2 μmol cardiolipin (9:1 ratio) were mixed, and Ø.1 mol of the antioxidant butylated hydroxy toluene (BHT) added per 1ØØ mol of lipid. Solvent was removed by rotary evaporation in vacuo, the residue redissolved in diethyl ether and redried by rotary evaporation. Then lipids were resuspended in 5Ø mM Tris-HCl, 5 mM Na_2EDTA, and Ø.Ø2% NaN_3, pH 7.4 to give a dispersions of 1Ø mg lipids/ml of buffer and the flask swirled by hand until all lipids were freed from the sides of the flask. The milky solution was allowed to swell at room temperature for two hours. These vesicles could be stored under nitrogen for several days at 4° C with no apparent changes.

Unilamellar vesicles were prepared according to the method of Johnson et al. (1975). 5Ø nmol total PE and PC (1:1), and Ø.1 mol% BHT, and trace amount of ^3H-glycerol trioleate (less than Ø.1 mol%), were mixed. The solvent was removed at $2Ø^{\circ}$ C under a stream of nitrogen. The residue was redissolved in diethylether and redried. Lipids were resuspended in 5 mM EDTA, Ø.Ø5 M Tris-HCl, Ø.Ø2% NaN_3, pH 7.4 to a final concentration of 1.67 mg/ml. The lipid suspension was mixed vigorously for 1Ø minutes and allowed to swell for one hour under a N_2 atmosphere. The test tube containing the suspension was then immersed in a Benson HD-5Ø sonication bath and sonicated at 25° C for 3Ø minutes until the sample was clear.

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N) PC-Removal from Reconstituted Membrane

Transfer of PC from reconstituted membrane was done as described above substituting reconstituted membrane for the multilamellar vesicles, and sonicated liposomes prepared from a total lipid extract from P_2 membrane as small unilamellar vesicles. Reconstituted membrane (6.5 μmol total phospholipid containing 5.1 μmol PC), used as multilamellar vesicles. Total lipid extract from P_2 membrane, 3.2 μmol, mixed with a 0.01% BHT and 0.01 nmol ^{3}H-glycerol trioleate and dried under a stream of nitrogen and lyophylized for one hr. Dried lipids resuspended in 5 mM EDTA, 50 mM Tris, 0.02% NaN_3, pH 7.4, to a final concentration of 1 μmol/ml buffer. The suspension was sonicated in a bath sonicator for 30 minutes followed by centrifugation at 20,000 g for 20 minutes to remove large multilamellar vesicles.

The incubation medium contained 6.5 μmol total phospholipid of the reconstituted membrane, 2.6 μmol sonicated liposomes in the presence of 1 ml of purified phospholipid exchange protein. The mixture was incubated for 20 minutes at 37° C followed by centrifugation at 20,000 x g for 20 minutes. The pellet was resuspended in 25 mM HEPES buffer, pH 7.7. This reconstituted membrane with lowered PC content was used for different experiments.

O) Opiate Binding Assays

Opiate binding assays were performed as described by Pert and Snyder (1973b) and Simon et al. (1973) with slight modification as made by Cho et al. Briefly, P2 membranes (Ø.6 mg protein) in 25 mM HEPES containing triated opiate ligand in the presence or absence of the unlabled drugs were incubated at 37° C for 3Ø minutes in triplicates and then cooled in an ice-bath for one hour. The P_2 membrane was collected by filtration under reduced pressure through glass fiber (GF/B) filters and the filters washed twice with 5 ml of cold 25 mM HEPES, pH 7.7. The filters were transferred to counting vials containing 1Ø ml of Scintiverse (Fisher Products) solution and radioactivity was counted in a Beckman liquid scintillation counter.

The leu- and met-enkephalin binding assay mixture contained Ø.6 mg protein, P2 membrane, 1 mM EDTA and 1ØØ ug/ml bacitracin, ^{3}H-leu- and met-enkephalin, and different concentrations of unlabeled ligands. The incubation was carried out at 4° C for 3 hours. The specific binding was measured as the mean difference between triplicate samples in the presence and absence of 1-1Ø μM of unlabeled ligands. Affinity and capacity of the opiates were determined according to the method of Scatchard (1949).

P) (Na^+-K^+)-ATPase Activity Determination

(Na^+-K^+)-ATPase activity was measured by the method of

Post and Sen (1967). The incubation mixture, 1.0 ml, contained (in final concentration) 4.5 mM Na_2ATP, 7.5 mM $MgCl_2$, 0.75 mM H_2Na_2EDTA, 30 mM imidazole and 30 mM glycylglycine solution. Then 0.1 ml of membrane suspension was added with 0.1 ml of a solution of 1.5 M NaCl and 0.3 M KCl. A duplicate set of tubes received the same additions, plus 0.1 ml of 2.5 mM ouabain. The incubation was carried out at 37° C for 20 minutes, with shaking,then stopped by addition of 2.5 ml of 0.48 M $HClO_4$, followed by vortexing. A one ml aliquot of this mixture was assayed for inorganic phosphate released from ATP. Correction was made for non enzymatic hydrolysis of ATP by using a blank containing no ATPase enzyme.

Q) <u>(Mg^{++}-Ca^{++}) ATPase Activity Measurement</u>

Activity of this ATPase was assayed by measuring inorganic phosphate released from ATP according to Ichida <u>et al</u>. (1976). The incubation mixture contained 0.2-0.3 mg protein, 50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 9 x 10^{-5} M $CaCl_2$ and 2 mM ATP-Tris in a final volume of 2 ml. Mg^{++}-Ca^{++} ATPase activity was calculated by subtracting Mg^{++}-ATPase activity determined by assaying in a medium with all the constituents of standard medium except $CaCl_2$. The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid; precipitated protein was removed by centrifugation and an aliquot of the supernatant (1 ml) was

assayed for inorganic phosphate. A correction was made for non-enzymatic hydrolysis using appropriate blanks.

R) Cholesterol Incorporation into the Membrane

Cholesterol content of the membrane was increased by a simple method based on that of Batzri et al (1973). An ethanolic solution containing cholesterol, 25 μmol cholesterol/ml ETOH, was rapidly injected through a Hamilton syringe into a membrane suspension while stirring very rapidly under a flow of nitrogen. This membrane was centrifuged at 20,000 x g for 20 minutes, and washed once with 25 mM HEPES buffer, pH 7.7 to remove ethanol.

S) Determination of Membrane Cholesterol Content

Total and exposed cholesterol in the membrane were quantitated with cholesterol oxidase (Sigma Chemical Co., St. Louis, MO.) by the method of Moore et al. (1977). In brief, cholesterol oxidase converts cholesterol to cholest-4-en-3-one, which absorbs light at 240 nm in aqueous solutions and 235 nm in hexane. The reaction mixtures contained samples with different concentrations of cholesterol, 30 μl of 10% deoxycholate (DOC) solution in water (sodium salt, Sigma Chemical Co.), 1 ml of 20 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.5 and 10 μl of cholesterol oxidase (2.5 units/ml). The reaction mixtures were incubated at 37° C for 3 hours with shaking. Each

concentration was run in triplicate and blanks contained all the reagents except the enzyme. The standard curve was established by solubilizing various concentrations of cholesterol in 10% DOC solution (60 µl DOC/mg cholesterol).

Although the standard solution could have been read directly at 240 nm, membrane protein would contribute to the absorbance in the ultraviolet spectrum. In order to reduce the background, the modified Dole procedure (Dole, 1956) was used to terminate the enzymatic reaction and to separate the neutral lipids from nonlipid and polar lipid molecules. Two ml of Dole reagent (2-propanol, hexane, H_2O) (400, 100, 10, v/v/v), was added to the reaction mixture followed by 1 ml hexane. The mixtures were extracted by vortexing, and centrifuged at 2000 rpm for 20 minutes. The upper phase, hexane, was removed and read at 235 nm in a Gilford spectrophotometer.

Measurement for exposed membrane cholesterol was carried out essentially the same as described above without the presence of DOC in the medium.

T) Fluorescent Measurements

Fluorescent measurements were performed by incorporating a nonperturbing cholesterol-like fluorescent probe into rat brain synaptosomal membranes. Incorporation of the probe into the membrane was achieved by the injection method described above for cholesterol incorporation into the

membrane. All fluorescent measurements were carried out on an Aminco-Bowman SPF fluorometer equipped with a water-jacketed cuvette holder to maintain a constant temperature. For polarization measurements, the fluorometer was fitted with three polacoat polarization filters (polished quartz disks, formula 105, 19 mm in diameter and 1.6 mm thick from 3M Company, St. Louis, MO.). These filters were installed in a fluorometer as described by Bowman and Chin (1965). One filter was placed in a horizontal polarizing direction to correct for the light scattering and the other filter was placed in the exciting beam (Giulbault, 1967).

The cholesterol probe, 20 µl from a 2 µmol/ml ethanolic solution, was injected through a Hamilton syringe into a membrane suspension containg 2-3 mg protein in one ml 0.32 M sucrose. The suspension was incubated at room temperature for 30 minutes. The degree of fluorescent polarization was measured by diluting a 0.2 ml aliquot of P_2 membrane containing the probe to 3 ml 0.32 M sucrose. The suspension was transferred to a cuvette and read at an excitation wavelength of 295 and emission wavelength of 420 nm.

To observe the effects of temperature, the diluted suspension was incubated at different temperatures for 15 minutes. For other perturbants, ethanol or other drugs, the diluted membrane was incubated in the presence of these chemicals for 15 minutes.

Value of the polarization was evaluated by the method of Chin and Bowman (1965). This procedure involved placing a horizontally polarizing filter in the excitation beam and reading the intensity with a horizontal polarizing filter in the excitation beam ($I_{||}$), as well as with a vertical filter in the emission beam ($I_{\perp}$). Values of the polarization were calculated using the standard equation:

$$P = \frac{I_{||} - C.I_{\perp}}{I_{||} + C.I_{\perp}} \qquad \text{where: } C = \frac{I_{\perp}}{I_{||}}$$

where $I_{||}$ and $I_{\perp}$ are the fluorescent intensities observed with the analyzing polarizer, parallel and perpendicular, respectively to the polarized excitation beam. The constant C is for correction of the instrument polarization. In order to measure C, a hexane solution containing the fluorescent probe was placed in the cuvette holder and readings were taken with the horizontal polarizing filer in the excitation beam and the vertical polarizing filer in the emission beam (Shinitzky et al., 1971).

The total fluorescent intensity (F) was calculated from the equation:

$$F = I_{||} + 2.I_{\perp} = (I_{||}/I_{\perp} + 2).I_{\perp}$$

For quantitation of the fluorescent probe incorporated into the membrane, the suspension containing the probe (1 ml), was centrifuged at 17,000 x g for 55 minutes. The

supernatant containing the nonincorporated probe was discarded, the pellet resuspended in one ml of Ø.32 M sucrose. To this suspension was added 1 ml of Dole reagent (hexane: 2-propanol: water, 4ØØ: 1ØØ:1, v/v/v/) and subsequently 2 ml of hexane was added and vortexed. After phase separation the hexane phase was transferred to a quartz fluorescent cuvette (1 cm by 1 cm). The intensity was measured at an excitation wavelength of 295 nm and emission wavelength of 42Ø nm. The concentration of the probe was determined by comparing the intensity reading for the samples against the standard curve. The standard curve was obtained by addition of different concentrations of the fluorescent probe to 1 ml Ø.32 M sucrose, and extracting as described above. The total fluorescent probe added to the membrane was measured as mentioned above, except that the membrane homogenate containing the probe was not centrifuged to remove the probe which is not incorporated into the membrane.

U) Data Analysis

The experimental binding data, as amount of opiate ligand bound versus concentration of free ligand, were analyzed by a weighted non-linear least square curve fitting technique (Fletcher and Schrager, 1973). This was applied to saturation isotherms (bound ligand vs. total ligand concentration). In using (bound) vs. (total) curves for

data analysis, the total ligand concentration may be regarded as an independent variable and it only is subject to pipetting error (1%) and/or counting error (Ø.1-1%) which are small in comparison with the overall experimental errors in bound/free or in (bound). After the data are analyzed, the result can be displayed in variety of coordinate systems such as bound/free vs. (bound), (bound) vs. (total), etc.

A general non-linear curve fitting program developed by Hancock et al. (1979) was used for analysis of the binding isotherm. This program is based on a model described by Feldman (1972) in which binding of any number (n) of ligands with any number of independent classes (m) of binding sites ("n by m" model) can be analyzed. The mathematical model describes the reaction of n ligands, L_i, binding to m classes of binding sites, R_j, according to the mass action law:

$$L_i + R_j \text{ ===== } L_iR_j,\ i = 1,...,n,\ j = 1,...,m$$

each with an affinity constant:

$$K_{ij} = \frac{B_{ij}}{F_i \cdot E_j}$$

where B_{ij} is the concentration of each ligand "i" bound to site "j" and F_i and E_j are the concentrations of free ligand "i" and empty site "j" respectively.

Another parameter, N_i, is incorporated into this model for nonspecific or nonsaturable binding unrelated to its

pharmacological effect. For each ligand "i", nonspecific binding is determined by:

$$NSB_i = N_i \cdot F_i$$

where N_i is equal to ratio of bound/free ligand for nonspecific binding corresponding to the horizontal asymptote on a Scatchard plot for total binding (Feldman, 1972).

In this model several assumptions are made about the ligand-binding system as follows:

1. There are two distinct classes of reactants initially present in the medium, namely ligands and binding molecules and no species acts as both ligand and binding molecule.
2. Reactions between ligand and binding molecule are reversible; the product of the reaction does not participate in another reaction; and no allosteric binding is involved.
3. Each binding reaction proceeds independently to equilibrium and the affinity constant remains unchanged by progress of the reaction, i.e. no cooperativity is involved.
4. Bound and free ligands can be separated and quantitated without disturbing the equilibrium.

The program used for data analysis (SCTFIT, Hancock et al. 1979) has the capability of using the inverse data

variance as weight for non linear least square curve fitting. Variance at each data point is approximated by:

$$\text{Var } (y) = A_{\emptyset} + A_1 . Y^{A_2}$$

where Y is the variable, bound radioligand measured; A_1 is a proportionality constant roughly equal to the square of the relative error of Y; A_2 ranges from 1 to 2 and $A_{\emptyset}$ is a small constant which prevents the variance estimate from becoming too small when Y is close to zero.

Computer modeling of each binding isotherm provides estimates and standard errors for the affinity constants, binding sites density and the coefficient for non specific binding for each ligand. Each curve can be analyzed assuming different numbers of binding sites and the statistical significance of each model is obtained by comparing the residual variance of each fit based on the "extra sum of squares" principle (Draper and Smith, 1966), using a partial F-test:

$$F = [(SS_1 - SS_2)/(df_1 - df_2)/(SS_2/df_2)]$$

where SS_1 and SS_2 are the sum of squares of residuals with model I and II, and df_1, and df_2 are the corresponding degrees of freedom (number of data points minus number of parameters estimated).

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II-2. MATERIALS:

Imidazole, glycylglycine, octylagarose, puromycin, phenanthroline, coenzyme-A, oleoyl CoA, LPC, HEPES, EGTA, EDTA, BSA-fraction V, TEMED, beta-mercaptoethanol, Coomassie Blue, Tris, SDS, sodium sulfate, sodium bisulfate, NaN_3, DOC, cholesterol oxidase, cholesta-4-en-3-one, ouabain, ATP-Tris, Ciocalteau phenol reagent, and cholesterol were from Sigma Chemical Company, St. Louis, Missouri. Glycerol, KOH, NaOH, KCl, NaCl, $MgCl_2$, $CaCl_2$, H_2SO_4, HCl, $HClO_4$, acetic acid, chloroform, methanol, 2-propanol, hexane, sucrose, ammonium sulfate and sodium phosphate were all of analytical grade from Mallinckrodt, St. Louis, Missouri. Potassium phosphate, $CuSO_4$, $5H_2O$, Na-tartarate and iodine were from Baker Reagents, Phillipsburg, New Jersey. Acrylamide, bis-acrylamide, ammonium persulfate, glycine, Bromophenol Blue and CM-cellulose were from Biorad, Richmond, California. Ammonium molybdate, DTNB, Azure-A, butylated hydroxytoluene and bacitracin were from Aldrich Chemical Company, Milwaukee, Wisconsin. Scintiverse and 1-amino-2-naphtal-4-sulfonic acid were from Fisher Scientific Fair Lawn, New Jersey. Phospholipid standards were from Supelco, Inc., Bellefonte, Pennsylvania. Ethanol was from IMC Chemical Group, Inc. Agnew, California. H-TLC plates were from Analabs (NEN), North Haven, Connecticut. HPTLC plates were from American Scientific Products, Sunnyvale, California.

Dialysis tubing was from VWR, San Francisco, California. ^{3}H-glycerol trioleate, ^{3}H-naloxone, ^{3}H-met-enkephalin, ^{3}H-Leu-enkephalin and ^{14}C-PC were from New England Nuclear, Boston, Mass. ^{3}H-etorphine and ^{3}H-dihydromorphine were from Amersham, Arlington Heights, Illinois. Morphine sulfate was from Mallinckrodt Chemical Works, St. Louis, Missouri. D-ala^{2}-D-leu^{5}-enkephalin was gift from Burrough Wellcome Company, Research Triangle Park, North Carolina. Naloxone was from Endo Laboratories, Garden City, New York. Levorphanol and dextrophan were from Hoffmann La Roche, Nutley, New Jersey. Etorphine was from American Cyanamid, Princeton, New Jersey.

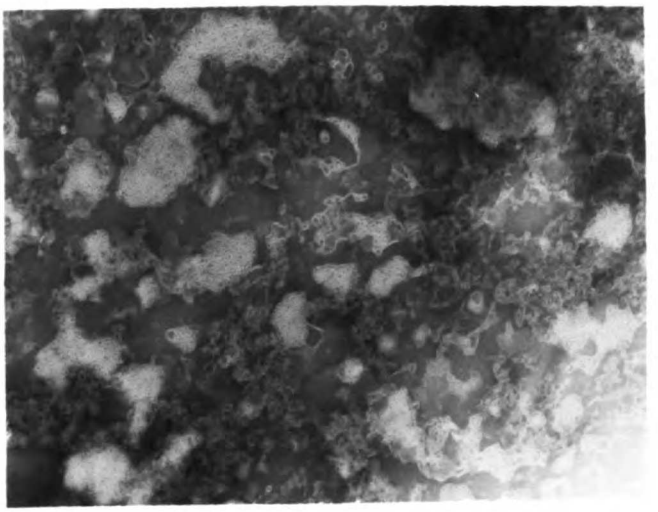
III. RESULTS

III-1. Solubilization and Reconstitution of Rat Brain Membrane

Rat brain synaptosomal membrane (P_2) was solubilized and reconstituted as described in the Methods section by the use of the acylation-deacylation cycle, using lysophosphatidylcholine and oleoyl coenzyme-A.

The solubilization of the membrane was demonstrated in two ways. 1) A change in the optical density (absorbance) of the membrane suspension at 500 nm before and after the addition of the detergents was measured. The optical density, a measure of turbidity, decreased from 1.2 in the synaptic membrane solution without detergents to 0.25 after the addition of detergents, 2) Comparison of negative stained electron microscopic picture of membrane preparations before and after solubilization. Negative staining electron microscopic picture of normal P_2 membrane showed the presence of numerous particles in the suspensions which disappeared after the detergent treatment. This observation indicates that either these membranes were solubilized, or the particle size was decreased so they could no longer be observed under the electron microscope (Figure 3).

Reconstitution of the solubilized membrane was by the



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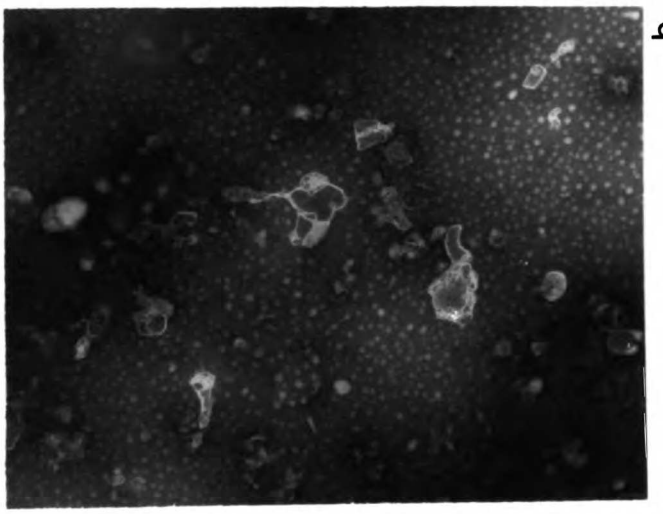
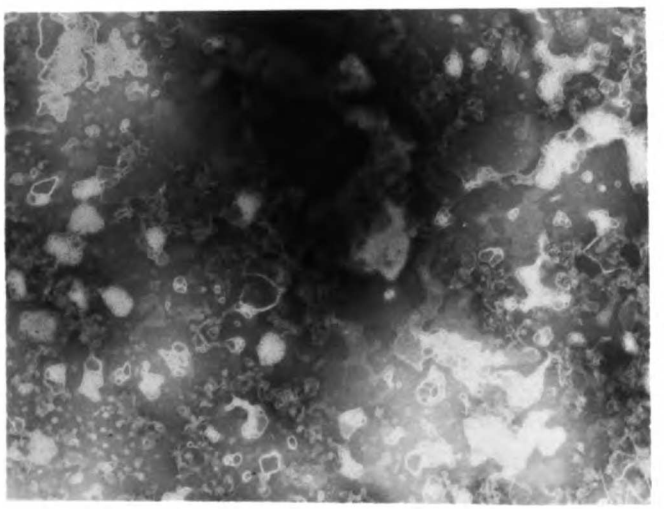


b



a

Figure 3. Negative stains of rat brain membrane synaptosomes a) before solubilization, b) after solubilization, and c) after reconstitution. Aliquots were stained with ammonium molybdate as described in method section.

reacylation of lysophosphatidylcholine and oleoyl coenzyme-A, and their conversion to phosphatidylcholine with the aid of liver microsomal acyltransferase.

In order to demonstrate that this enzyme is capable of conversion of LPC and oleoyl CoA to phosphatidylcholine and coenzyme-A, its activity was measured by the reaction product of CoA with DTNB which absorbs light at 412 nm; the increase in light absorbance was linear up to 15 minutes. This enzyme is present in liver microsomes, brain P_2 crude membrane or brain microsomes, thus the reaction can be carried out by using any of these membranes. Figures 4 and 5 show the increase in absorbance by using various amounts of brain membrane and liver microsomes as the enzyme source, respectively. A similar dose-dependent increase in light absorbance can be observed using either brain or liver microsomes. In order to demonstrate this increase in absorbance is lysophosphatidylcholine dependent, the reaction was also measured in the presence and absence of LPC. As is shown in Figure 6, using liver microsomes, there was a significant increase in light absorbance after the addition of LPC. However, there was no significant change in absorbance with or without LPC by using brain microsomes as enzyme source. This indicates that acyltransferase present in liver microsomes uses LPC specifically as substrate, the increase in absorbance by using brain microsomes as enzyme source (Figure 7) is probably due to non-LPC dependent

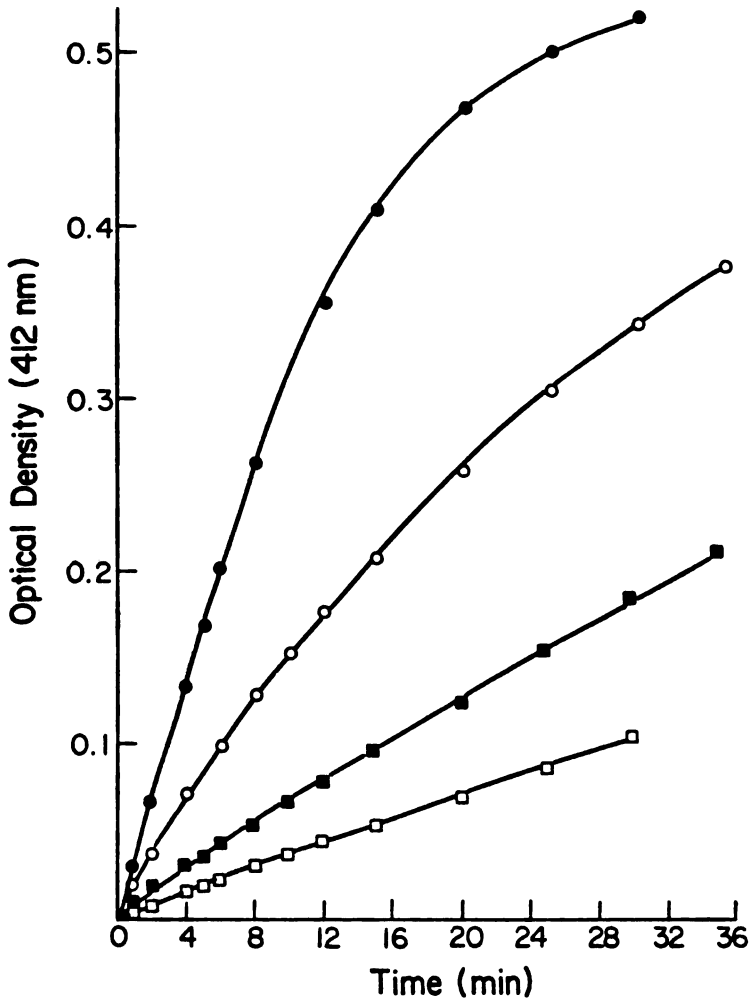


Figure 4. Acyltransferase activity of brain synaptosomes as monitored by the reaction of CoA with DTNB and which absorbs at 412 nm. Different concentrations of brain synaptosomes from Ø.Ø25 to Ø.25 mg protein were used as the source of acyltransferase enzyme. ●, Ø.25mg; o, Ø.125 mg; ■, Ø.Ø5 mg; □, Ø.Ø25 mg.

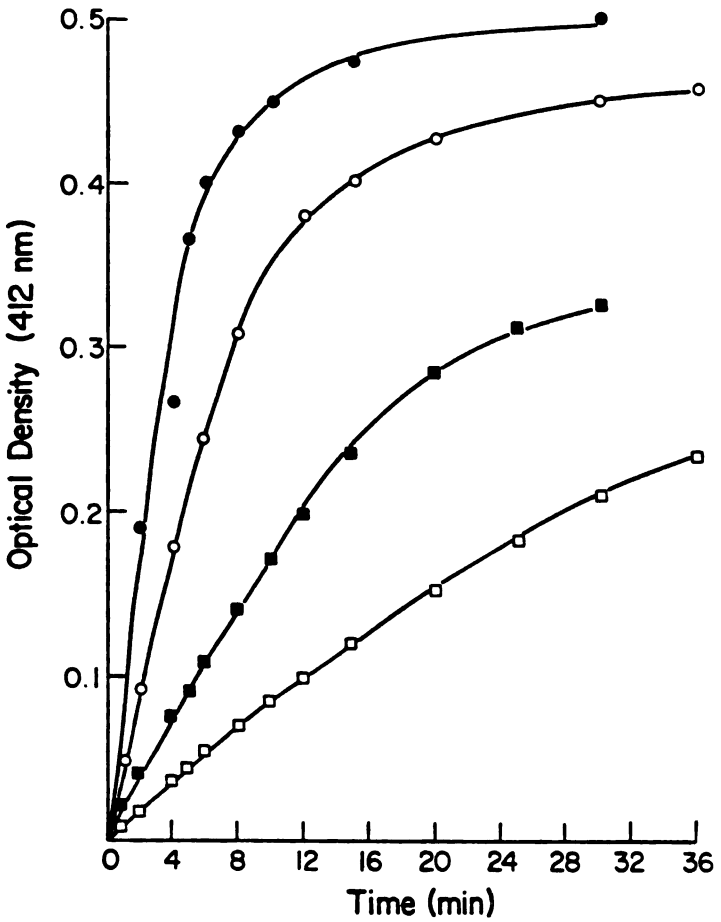


Figure 5. As in Figure 4, except liver microsomes were used as acyltransferase enzyme.

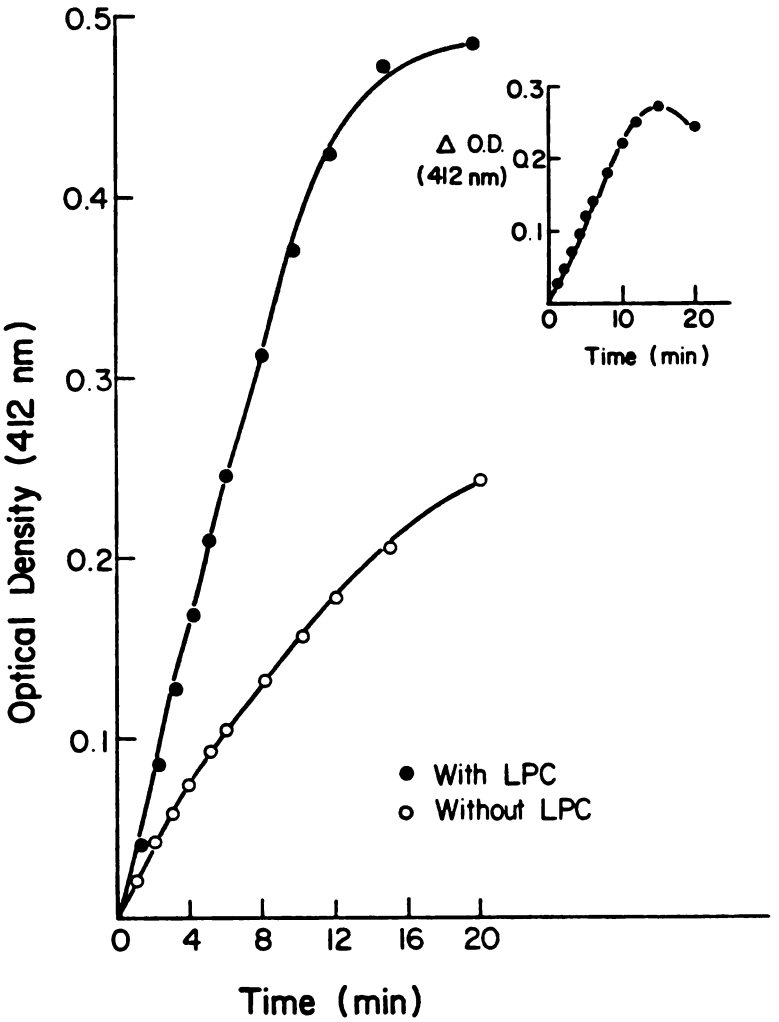


Figure 6. As in Figure 5, except activity of the acyltransferase enzyme was measured in the presence and absence of LPC. The inset shows the difference between the two values at every time point.

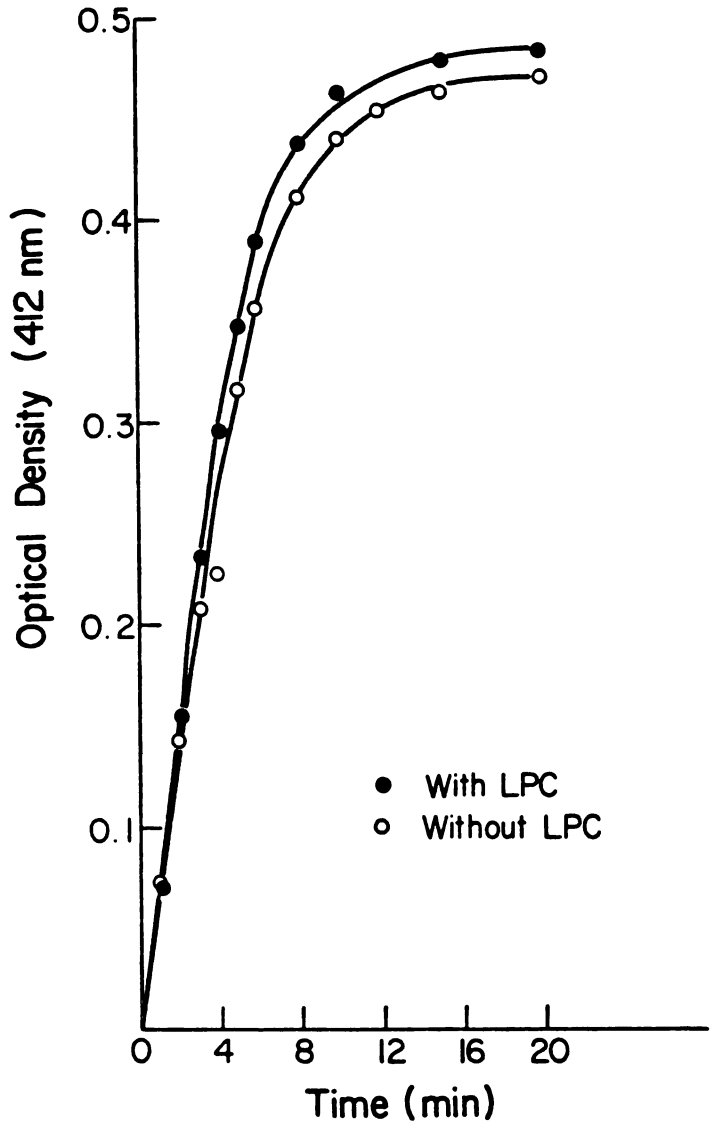


Figure 7. As in Figure 6, except brain synaptosomes used as the source of the enzyme.

production of CoA which reacts with DTNB. In the current study acyltransferase from liver microsomes was used for brain membrane reconstitution.

Incubation of solubilized membrane in the presence of liver microsomes results in reacylation of the LPC and oleoyl CoA, to form PC; the removal of these natural detergents causes reformation of membrane fragments. This process can be followed by measuring the absorbance change at 500 nm (Figure 8) at room temperature. An increase in turbidity from a nearly clear supernatant to a visibly turbid suspension can also be observed with the naked eye and the reaction is completed in one hour. On the other hand, if the complete reaction medium was stored on ice for the same length of time no increase in turbidity was observed. Using brain microsomes as the source of acyltransferase enzyme, there was either no increase or a small increase in absorbance and no membrane reformation was observed at the end of the incubation time. This lack of reconstitution by using brain microsomes correlates with the fact that acyltransferase present in brain microsomes is not specific for LPC as substrate (Figure 7). Therefore, if LPC is being used for membrane solubilization, liver microsomes should be used for reconstitution.

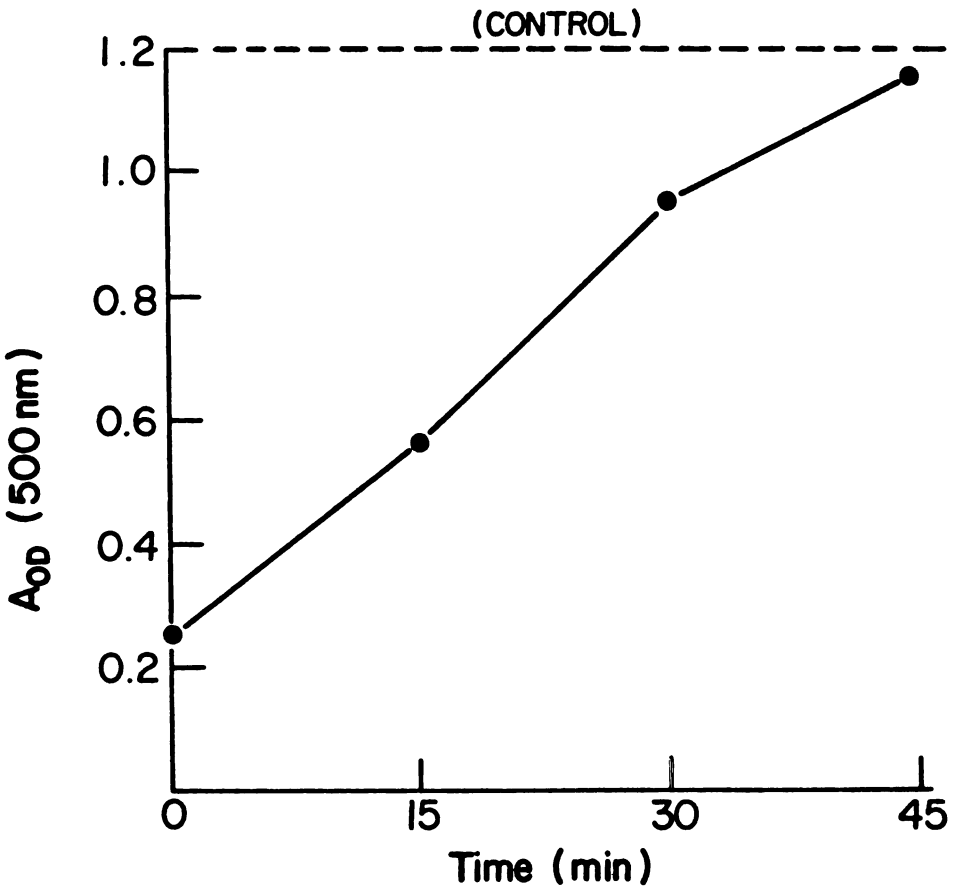


Figure 8. Turbidity changes during reacylation reaction. Turbidity was measured during a 45 min incubation period by following the absorbance change at 500 nm. Controls which were kept on ice during the incubation period had no absorbance changes.

III-2. Characterization of Native and Reconstituted Membrane

A) Morphological Studies of Native and Reconstituted Membrane by Electron Microscopy.

In order to examine the morphology of reconsituted membrane, negative staining electron microscopy was employed. As can be seen in Figure 3, solubilized samples at the beginning of the incubation were devoid of particles or membrane fragments, while numerous vesicular structures and membrane fragments resembling that of the native membrane were apparent after the enzymatic reconstruction.

In order to further characterize the reconstituted membrane, freeze fracture electron microscopy was performed, as shown in Figure 9. The fracture faces of both membranes showed prominent intramembrane particles. This suggests that the hydrophobic protein components of the original membrane are inserted into the lipid bilayer and the membranes produced by the acylation cycle can be characterized as "reconstituted" and are not a simple coagulation of lipids and proteins.

These micrographs were characterized in terms of vesicle size, intramembrane particle size and the particle distribution on both concave and convex fracture faces. The size of the vesicles measured in native and reconstituted membrane, in terms of a frequency distribution histogram, is shown in Figure 1Ø. It is observed that the larger vesicles

c

b

a

Figure 9. Freeze-fracture images of native (a), reconstituted membrane (b), and reconstituted membrane after cholesterol incorpration (c) prepared under the same conditions (see Methods). Both concave and convex fracture faces show prominent intramembrane particles.

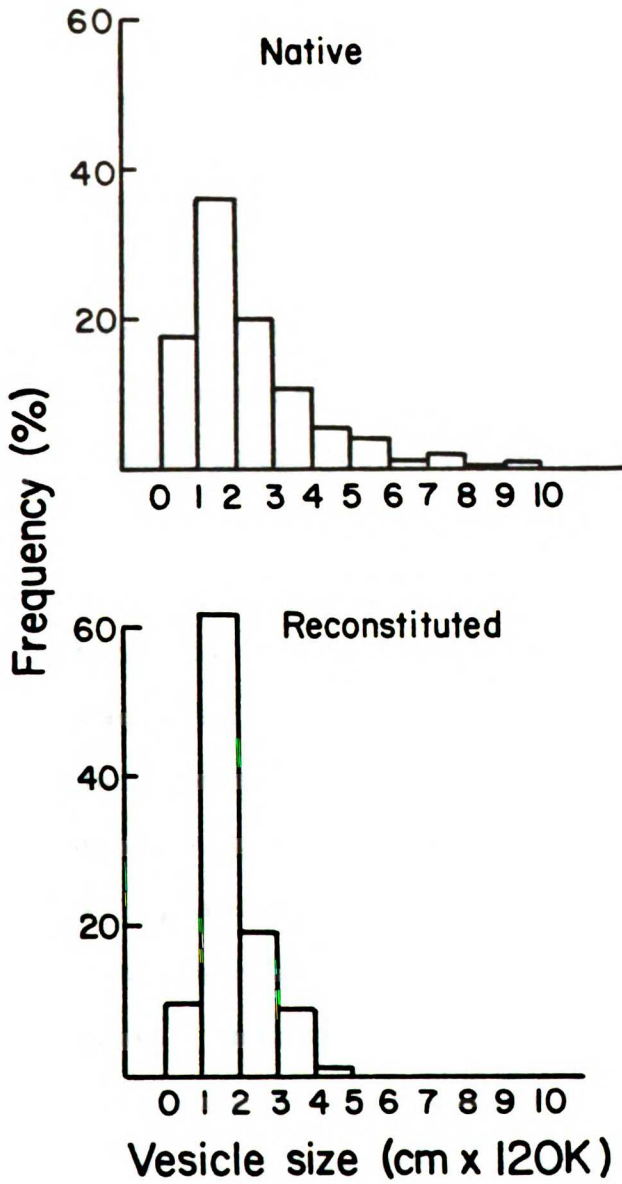


Figure 10. Frequency distribution of vesicles in native and reconstituted membrane. Data were collected from a freeze-fracture electron micrograph of 120 K magnification. The Polygon was analyzed by the chi-square method. Native membrane vesicles were larger than reconstituted membrane vesicles with a X^2 of 30.15 and df = 4 ($P < 0.001$).

are not found in the reconstituted preparation, while the number of vesicles ranging from Ø.Ø8 to Ø.16 microns (1-2 cm on 12Ø K micrograph) is greater in the latter than in controls. The difference between frequency polygons was analyzed by the chi-square method. The vesicles in the native membrane were significantly larger than that of the reconstituted membrane. This could be due to the washing procedure used after the reconstitution, which could break down the larger vesicles into smaller ones.

In order to determine if there is a non-random distribution of intramembrane particles in concave and convex fracture faces, the number of these particles were counted and compared for native and reconstituted membrane. The frequency histograms of particles distribution are shown in Figure 11, and indicate no significant differences. The same result was observed in reconstituted membrane. It can be concluded that intramembrane particles are randomly distributed between two fracture faces, and this pattern of distribution is preserved during the reconstitution. An effort was made to measure the size of these intramembrane particles, but no definitive conclusion could be drawn.

B) Separation of Proteins by Slab Gel Electrophoresis.

In order to determine whether the proteins from the solubilized membrane can be incorporated into the reformed membrane during reconstitution, slab gel-electrophoresis of

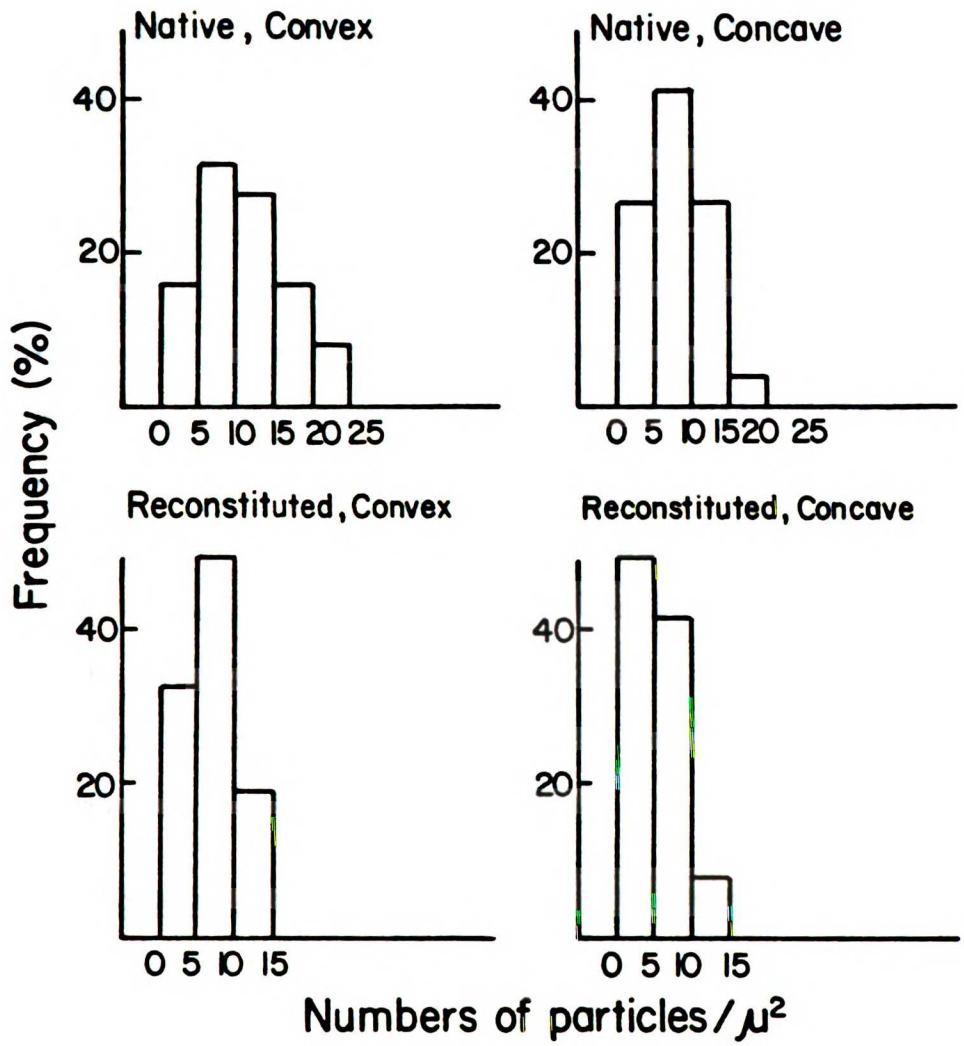


Figure 11. Frequency distribution histogram of particles in concave and convex fractured faces in native and reconstituted membrane. Number of particles were counted from 120 K magnification electron micrographs in an area of 1.2 cm^2 equivalent to 1 μ^2; their frequency distribution was analyzed by chi-squared method.

P_2 and reconstituted membranes was performed, as described in Materials and Methods. As shown in Figure 12, the protein patterns between crude P_2-membrane and reconstituted membrane were very similar, indicating that most of the major protein classes were still present in the reconstituted membrane, although some changes in quality or quantity of certain protein is possible. The densitometric scans of native and reconstituted membrane (Figure 13-A and B) reveal that there was no major difference between native and reconstituted membrane in their protein patterns, although some changes were observed in quantity. Therefore, the data suggest that most of the major protein classes present in the incubation medium can be incorporated into the membrane during reconstitution.

C) Lipid Analysis of P_2 and Reconstituted Membrane.

Membrane lipids were analyzed by using thin layer chromatography run on Silica gel-H plates in a solvent system of chloroform:methanol:acetic acid: water (25:15:4:2, v/v/v/v/). In this system, SpH, PC, and PE were separated completely from other lipids, with Rf values of Ø.172, Ø.362, and Ø.845, respectively. PI, and PS co-migrated as one spot with a Rf value of Ø.615. Cardiolipin and some other unidentified lipids migrated with a Rf value of Ø.977. A chromatogram of lipid extracts from P_2 membrane and reconstituted membrane is shown in Figure 14-A, while that

A B

Figure 12. Slab gel electrophoresis of native (A) and reconstituted (B) membrane. 5-15% polyacrylamide gradient, Ø.1% SDS.

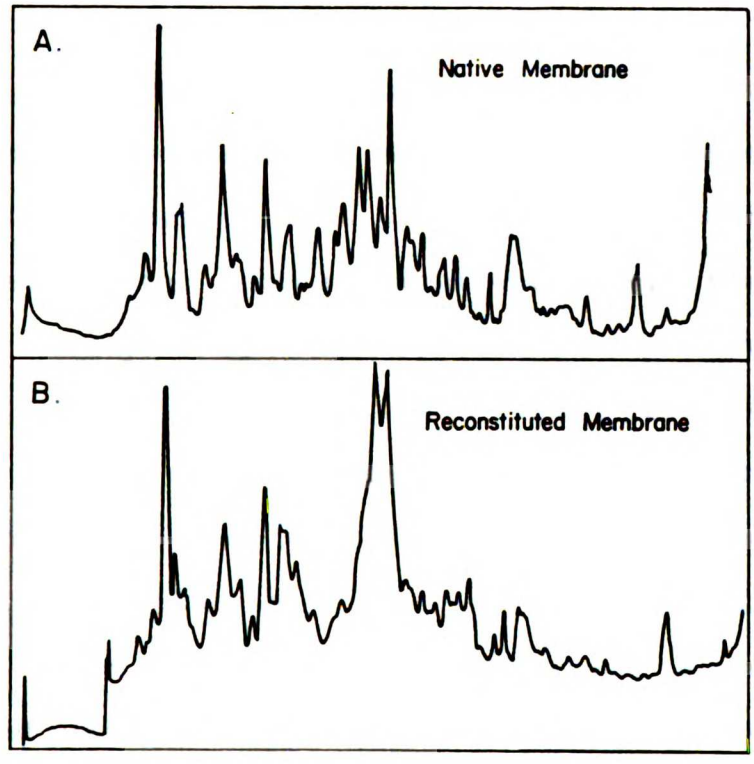


Figure 13. Densitometric tracings of gels in Figure 12 native (A) and reconstituted (B) membranes.

of standard lipids is shown in Figure 14-C. The individual spots were scraped off, extracted, and the inorganic phosphate content of each spot measured as described in Methods. As shown in Table 1, the total phospholipid content in reconstituted membrane was doubled compared to that of the native membrane; this was mainly due to an increase in PC content in the reconstituted membrane, from acylation of LPC and oleoyl CoA. In addition to the increase in PC content, about 2.8 fold, there was a 20% decrease in PE content in the reconstituted membrane. The ratio of PC/PE also increased from 0.92 in native membrane to 3.45 in reconstituted membrane.

In order to separate PS from PI, a new method was developed using high performance thin layer chromatography plates. The solvent system used was chloroform: methanol:water:acetic acid (65:43:3:1, v/v/v/v). As can be seen in Figure 14-D for standard phospholipids and Figure 14-B for P_2 and reconstituted membrane lipids, there is a clear separation of PS and PI. Phosphatidylserine and PI were separated with Rf values of 0.261 and 0.344, respectively. Sphingomyelin and PC were separated with Rf values of 0.77 and 0.178 respectively. In this system cardiolipin and PE moved together as one spot with an Rf value of 0.567. The chromatographic data for P_2 and reconstituted membrane are shown in Table 2. There is a 13% decrease in PS content of the reconstituted membrane compared to that of the native

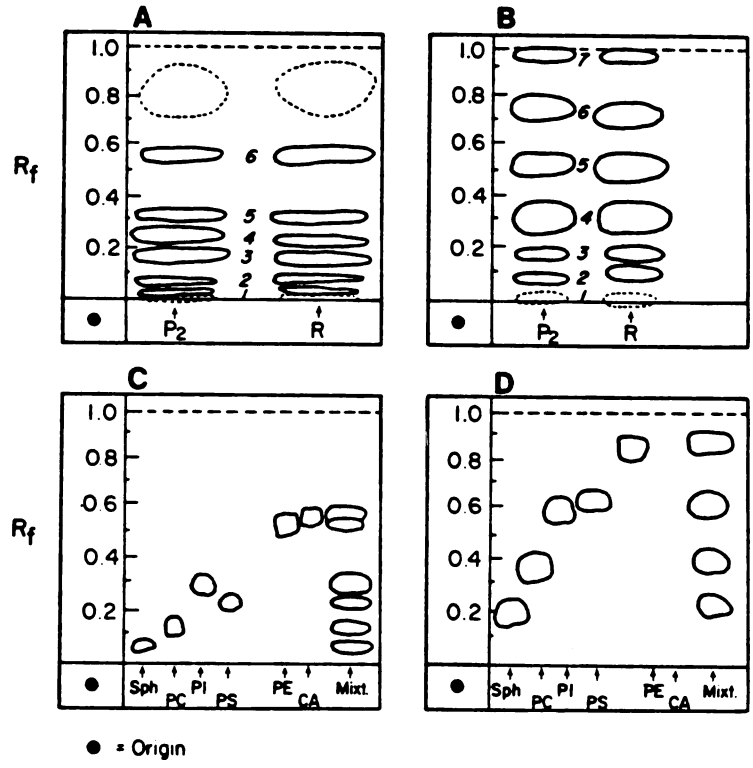


Figure 14. Thin layer chromatograms of lipids extracted from P_2 and reconstituted membrane (A) and of standard phospholipids (C). Separation on Silica gel-H plate in a solvent system of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25/15/4/2, v/v/v/v). Plate (B) is the thin layer chromatogram of lipids extracted from P_2 and reconstituted membrane on a HPTLC plate in a solvent system of $CHCl_3/ CH_3OH/CH_3COOH/H_2O$ (65/43/1/3, v/v/v/v); and plate (D) is that of standard lipids under the same condition.

Table 1

Phospholipid Content of P_2 and Reconstituted Membranes as Analyzed by Thin Layer Chromatography Using Silica-gel H Plates.

	P_2	Reconstituted
Total lipid phosphate	Ø.623 ± Ø.1Ø4 (7)	1.2Ø7 + Ø.198 (7)
Phosphatidyl-choline	Ø.162 ± Ø.Ø42 (7)	Ø.46 ± Ø.11 (7)
Phosphatidyl-ethanolamine	Ø.196 ± Ø.Ø24 (6)	Ø.154 ± Ø.Ø33 (6)
Sphingomyelin	Ø.Ø2Ø6 ± Ø.ØØ42 (4)	Ø.Ø314 ± Ø.ØØ46 (4)
Lysophospha-tidylcholine	Ø.ØØ85 ± Ø.ØØØ6 (4)	Ø.Ø646 ± Ø.Ø17 (4)
PS & PI	Ø.Ø774 ± Ø.Ø21 (3)	Ø.Ø796 ± Ø.Ø225 (3)
Cardiolipin	Ø.Ø352 ± Ø.ØØ9 (5)	Ø.Ø45 ± Ø.Ø11 (6)
Unidentified lipids	Ø.1238 ± Ø.Ø4 (6)	Ø.3Ø8 ± Ø.1335 (6)
PC/PE	Ø.92 ± Ø.165 (6)	3.448 ± Ø.85 (6)

Values are given as μmol phosphorus per mg protein with mean ± standard deviation. Numbers in parentheses are the number of experiments performed. PC/PE is the mean of 6 ratios of PC/PE in 6 different experiments.

Table 2

Phospholipid Content of P_2 and Reconstituted Membranes Analyzed by HPTLC.

	P_2	Reconstituted
Total phosphate lipid	Ø.5186	1.Ø24
Phosphatidylcholine	Ø.162	Ø.574
PE & Cardiolipin	Ø.1886	Ø.1513
Sphingomyelin	Ø.Ø19	Ø.Ø28
Lysophosphatidylcholine	Ø.Ø169	Ø.1Ø8
Phosphatidylserine	Ø.Ø62	Ø.Ø54
Phosphatidylinositol	Ø.Ø226	Ø.Ø384
Unidentified lipids	Ø.Ø475	Ø.Ø73
PC/PS	2.613	1Ø.63

Phosphorus determination of phospholipids present in P_2 and reconstituted membranes' lipid extract, stated as ^{2}umol phosphorus per mg protein, separated on HPTLC plates, under the same conditions as in Figure 14-B.

membrane. The PC/PS ratio was increased from 2.613 in native membrane to 10.63 in reconstituted membrane.

Sulfatide content of P_2 and reconstituted membrane was measured with Azure-A reagent as described previously (Keans, 1968). The concentrations found were 0.737 mg SO_4^{--}/mg protein in P_2 membrane and 0.0807 mg SO_4^{--}/mg protein in reconstituted membrane. These were not significantly different from each other.

The membrane cholesterol content was measured by using cholesterol oxidase, which converts cholesterol to cholest-4-en-3-one which absorbs light at 235 nm. The total cholesterol was measured in the presence of DOC, which solubilizes the membrane. The exposed cholesterol, on the outer surface of the membrane, was measured in the absence of DOC. The difference between the total and exposed cholesterol in membrane was taken as the amount of cholesterol which is not available for enzyme action. As can be seen in Table 3, a 30% decrease in the total cholesterol incorporation was observed during reconstitution, however, the exposed cholesterol in reconstituted membrane was 61% higher than that of the P_2 membrane. The ratio of exposed/non-exposed cholesterol is increased in reconstituted membrane from 5.5% to 13.3%.

Table 3

Cholesterol Content of P_2 and Reconstituted Membranes.

	P_2	Reconstituted
Total	Ø.533 - Ø.6Ø7	Ø.319 - Ø.496
Exposed	Ø.Ø2514 - Ø.Ø346	Ø.Ø387 - Ø.Ø56
Nonexposed	Ø.5Ø76 - Ø.5727	Ø.28 - Ø.4396
Exposed/nonexposed (%)	4.95 - 6.Ø4	12.75 - 13.8

Cholesterol content of P_2 and reconstituted membrane measured by its reaction with cholesterol oxidase and measurement of light absorbtion at 235 nm. Total cholesterol was measured by incubation in the presence of the detergent, DOC. Exposed cholesterol is the content in the absence of detergent, and the difference between the total and exposed cholesterol is considered to be nonexposed. Numbers are the values of two different experiments stated as μmol cholesterol/mg protein.

D) ATPase Activity Measurements in P_2 and Reconstituted Membranes.

It is known that there is a very specific requirement of membrane integrity for the activity of membrane-bound enzymes (Green and Tzagoloff, 1966) such as (Na^+-K^+) and (Mg^{++}-Ca^{++}) activated ATPases. The dependence of enzyme activity on membrane integrity is shown by the fact that the Na^+-K^+-ATPase activity is inhibited by treatments known to destroy or remove lipids. Solvent extraction (Jarnefelt, 1972), lipid oxidation (Tateishi et al., 1973), detergents (Grisham and Barnett, 1972, 1973; Lin, 1980) and lipolysis (Portius and Repke, 1963; Tatibana, 1963; Bigon et al., 1979); all can destroy enzyme activity. The loss of activity in delipidated ATPase can be restored by certain lipids.

Various investigators have restored the enzyme activity with PC (Tanaka and Sakamoto, 1965), PE (Taniguchi and Tonomura, 1971), PS (Wheeler and Whittam, 1970; Floreani et al., 1981; Niggli et al., 1981a), phosphatidylglycerol (Kimelberg and Papahadjopoulos, 1972; Palatini et al., 1972) and cholesterol (Jarnefelt, 1972; Levental and Rakic, 1980), indicating that a specific head group requirement for Na^+-K^+-ATPase activity. Studies by Kimelberg and Papahadjopoulos (1972) showed the requirement for unsaturated fatty acid chains, indicating that the

phospholipid fatty acid chains provide the appropriate (low dielectric) environment for the maximal activity of this enzyme. From all extensive studies, it has been concluded that a proper hydrophobic association between phospholipid and protein is required for optimal enzyme activity. It has also been shown by Levental and Rakic (1980) that PC/cholesterol ratio is important for proper enzymes functioning.

In the case of Ca^{++}-Mg^{++}-ATPase it has been shown that the purified ATPase contains approximately 600 mg lipid per mg protein with 90% phospholipid and 10% neutral lipid. Approximately 97% of the neutral lipid is cholesterol and 66% of the phospholipid is PC (MacLennan et al., 1971; Marai and Kuksis, 1973; Hoffman et al., 1980). The other phospholipids are mainly PE, PI and PS. An absolute requirement for lipid has also been shown for this ATPase activity. Phosphatidylcholine requirement was demonstrated by the fact that the ATPase activity was inhibited when treated with Phospholipase-C and restored with PC addition (Martonosi et al., 1968, 1971). Phospholipase-A treatment increased ATPase activity due to LPC liberation (Fiehn and Hasselbach, 1970), and removal of LPC with Bovine Serum Albumine inhibits this activity. There is also an increase in enzyme activity with PE (Knowles and Racker, 1975). The effect of fatty acids on enzyme activity has also been studied in which the enzyme activity is increased by

unsaturated fatty acids (Niggli et al., 1981b; Moore et al., 1981). Finally, it has been shown (Warren et al., 1974) that at least 30 lipid molecules around each molecule of the enzyme are required for activity.

So, it seems that membrane integrity, or structure, and protein/lipid ratio are important for proper functioning of the enzyme (Albert et al., 1981). Therefore, in order to examine the integrity of reconstituted membrane, Na^+-K^+-ATPase and Ca^{++}-Mg^{++}-ATPase activity were measured and compared with their values in P_2 membranes.

Table 4 shows the activity of these enzymes in P_2 and reconstituted membrane; 24% of the original Na^+-K^+-ATPase activity was recovered in reconstituted membrane, while essentially all of the Ca^{++}-ATPase activity was lost. This change in enzyme activity could be due to loss of specific phospholipids required for activity, or to a change in the protein/lipid ratio or to conformational changes of the protein. It is likely that these losses are related to the decrease in PS, cholesterol and the increase in PC incorporated into the membrane. However, the activity of Ca^{++}-Mg^{++}-ATPase is affected more than that of the Na^+-K^+-ATPase during the reconstitution procedure, suggesting it is more sensitive to lipid changes in reconstituted membrane. To support this hypothesis, the effect of adjusting cholesterol content on Ca^{++}-ATPase and Na^+-K^+-ATPase activity will be discussed later in this

Table 4

Na^+-K^+-ATPase and Ca^{++}-Mg^{++}-ATPase Activities Measurements.

	P_2	R	% Activity Recovered
Na^+-K^+-ATPase	0.398 ± 0.0213 (10)	0.097 ± 0.0049 (10)	24%
Ca^{++}-ATPase	0.048 ± 0.0052 (7)	0.0014 ± 0.0007 (7)	2.9%

Activities of Na^+-K^+-ATPase and Ca^{++}-ATPase are measured in P_2 and reconstituted membranes expressed as μmol inorganic phosphate /mg protein/min. values are mean ± standard error of means. Numbers in parentheses are the numbers of experiments performed, each assayed in triplicate. The assays were performed as described in Materials and Methods.

section.

E) Fluorescent Depolarization Measurements of P_2 and Reconstituted Membrane.

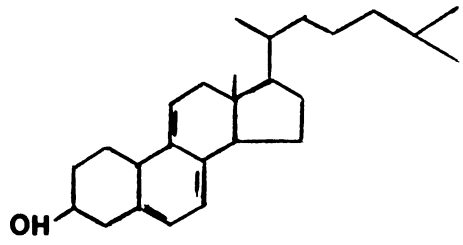
Evidence accumulated over the last several years indicates that the physiological functions of biological membranes are influenced by their lipid composition, and by their physical properties. The role of lipids is shown in many systems such as Na^+-K^+-ATPase activity, which greatly depends on specific phospholipids (Grisham and Barnett, 1973; Lin, 1980) and Mg^{++}-ATPase, which decreases as the membrane fluidity increases. It has been suggested that a high degree of order is normally required for optimal activity of this enzyme (Riordan et al., 1977). The influence of the membrane physical state on physiological function has also been shown for adenylate cyclase activity (Brivio et al., 1976). Lipid involvement is shown for many receptors, e.g., adrenergic (Fleming and Ross, 1980; De-Plaza and De-Robertis, 1972; Ochoa et al., 1972), cholinergic (Schindler and Quast, 1980), serotonin (Heron et al., 1980), opiate (Abood et al., 1979; Loh et al., 1974) and many hormone receptors (Meldolesi et al., 1977 and Pohl et al., 1971).

All these evidence suggest that a highly organized environment may be required for proper physiological function of the membrane. In this environment, lipid-lipid

and lipid-protein interactions are the main determining factors for membrane phenomena. The lipid influence on membrane function is formulated in terms of membrane fluidity or organization (Thompson, 1978). The term membrane fluidity or the ordered parameter of the membrane can usually be measured in terms of the motion of a small, hydrophobic probe that is inserted into the membrane, with its motion detected by means of nuclear magnetic resonance, electron spin resonance, or fluorescence polarization methods. The random motion of the probe within the membrane is referred to as rotational diffusion and this motion is influenced by the viscosity or the fluidity of the microenvironment in which the probe resides. Therefore, measurements of the movement of hydrophobic probe are means of assessing the fluidity or orderliness of the membrane.

The most sensitive method of measuring membrane fluidity is fluorescent depolarization, in which a fluorescent molecule is extrinsically incorporated into the membrane (Van Holde, 1971). One of the most commonly used probes for fluidity determination is diphenylhexatrien (DPH). However, this probe is non-physiological and thus may induce perturbation in its immediate environment, making the interpretation of data difficult (Birdsall et al., 1971). In order to overcome this problem a cholesterol like fluorescent probe was developed by Windaus and Linsert (1928) and has been used for fluorescent depolarization

studies in systems such as lipid bilayers (Rogers et al., 1979) and to study the interactions between sterols and polyene antibiotics in lipid vesicles (Archer, 1975) and between sterols and plasma lipoproteins (Smith and Green, 1979). This fluorescent probe has the following structure:



This probe has been prepared from 7-dehydrocholesterol by oxidation of cholesterol with mercuric acetate as described by Bergman and Stevens (1948). This probe is non-fluorescent in aqueous suspension and is only fluorescent in a hydrophobic environment (Rogers et al., 1979). Therefore, the change in polarization and increase in fluorescent intensity is an indication of probe incorporation into the membrane. The customary method used to incorporate this probe into the membrane is based on disruption of the membrane, and then reassembling in the presence of the probe. However, a new method for in vivo incorporation of this cholesterol probe into the membrane was described by Morgan et al. (1982), in which the probe was intraventricularly administered to a rat for a period of time; the brain tissue containing the probe was then isolated. However, for fluidity measurement in P_2 and

reconstituted membrane this probe must be incorporated into these membranes in vitro. Therefore, still another method was developed to incorporate the cholesterol probe into the membrane.

Incorporation of the fluorescent probe into the membrane was by injection of an ethanolic solution of the probe under a flow of nitrogen into the membrane suspension with rapid stirring. The probe was shown to be incorporated into the membrane without serious disruption of the latter. Optimum conditions for this method were determined as follows:

a. Optimum probe concentration.

Addition of successive doses of probe into one ml membrane suspension (3 mg protein per ml), beginning with 0.002 umol, results in an abrupt change in probe polarization; the intensity of polarization is also increased (Figures 15-A and B). The dose chosen for the experiments was 0.1 µmol probe/ml membrane suspension, in which there is no further change in membrane polarization and the intensity is high enough for easy measurements.

b. Time course of probe incorporation into the membrane.

The time course of probe incorporation is shown in Figure 16-A and B. Time zero is represented by the fluorescence before introducing the probe. The incubation

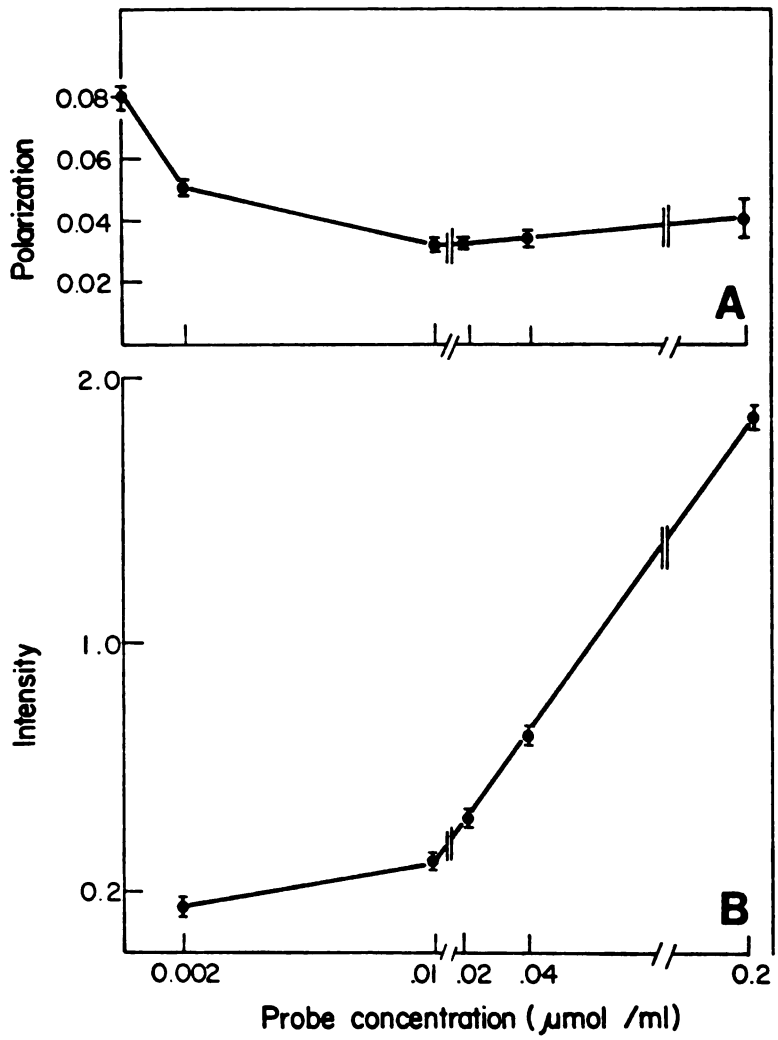


Figure 15. Change in fluorescence polarization (A) and fluorescence intensity (B) of cholesta-5,7,9,trien-3 beta-ol at different concentrations. Vertical bars indicate the standard error of the mean for 10 different experiments, each assayed in duplicate.

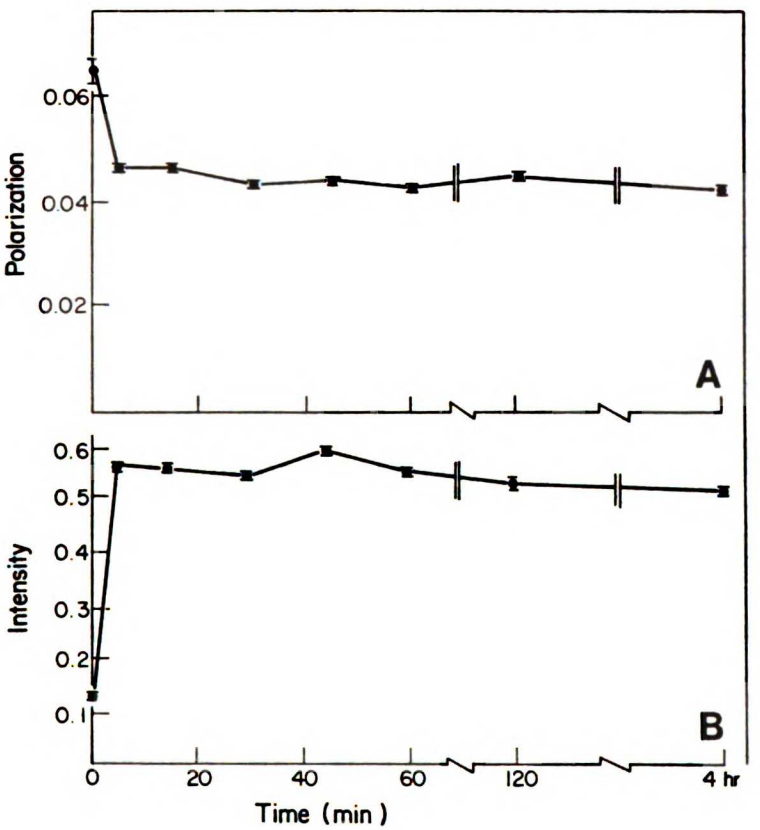


Figure 16. Time course of probe incorporation into rat brain P_2 membrane measured by changes in polarization (A) and intensity (B). Probe (Ø.1 μmol) was incorporated into Ø.25 ml of P_2 membrane (3 mg protein/ml) and incubated at room temperature for up to 4 hours. Vertical bars are the standard error of the mean for 1Ø experiments, each assayed in duplicate.

of probe with membrane suspension at room temperature causes a decrease in polarization after 5 minutes. This polarization change stays constant over 4 hours of incubation. The same time course for the intensity of fluorescence was observed. There was a sharp increase in intensity after 5 minutes, which stayed constant over a 4 hour period; therefore, an incubation time of 45 min was selected for fluorescent measurements, which allows enough time to read the samples.

c. Optimum membrane concentration.

In order to demonstrate that the probe is indeed incorporated into the membrane by this injection method, the fluorescent polarization and intensity were measured in the absence and presence of different concentrations of P_2 membrane. The probe concentration was kept constant and the mixture was incubated for 45 minutes. As shown in Figure 17-A, there is an initial drop in polarization after the incubation of the probe with less than 200 µl (0.2 mg protein) of P_2 membrane and an upturn with concentrations up to 1 mg protein. The upturn in polarization may be due to the light scattering of concentrated membrane suspensions. Figure 17-B shows the fluorescent intensity of the probe with different membrane concentrations. There is an initial sharp increase in intensity with the addition of a small amount (50 µl) of membrane correlating with polarization

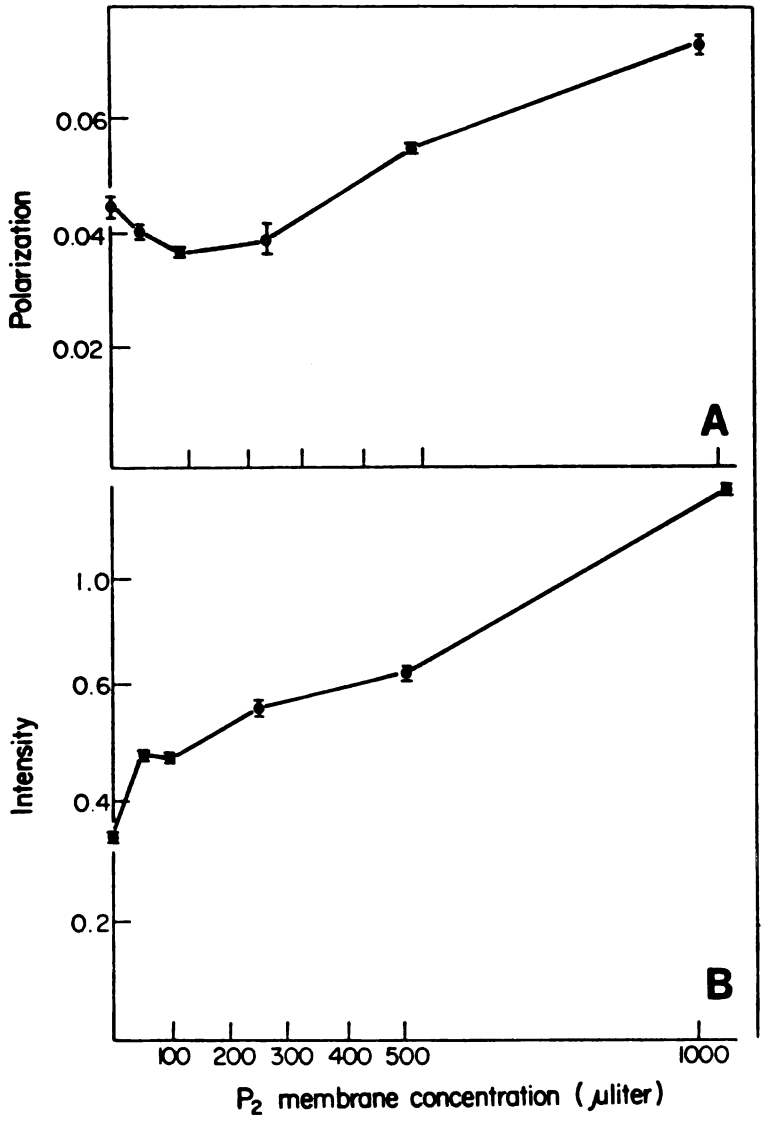


Figure 17. Changes in fluorescence polarization (A) and fluorescence intensity (B) of the cholesterol probe at different concentrations of P_2 membrane. A constant concentration of probe (0.1 μmol) was incorporated into a suspension containing different P_2 membrane concentrations, followed by incubation at room temperature for 45 minutes. Vertical bars are standard error of mean of 10 experiments, each assayed in duplicate.

changes. After this sharp increase, the intensity increase seems to correspond to the increase of protein concentration with a much slower rate of increase. Change in polarization and/or intensity of fluorescent probe by the presence of the membrane, indicate that the probe is incorporated into the membrane's hydrophobic environment. A volume of 250 µl of P_2 membrane (3 mg protein per ml) was chosen as an optimum concentration of membrane, since it has enough fluorescence for easy measurements, and minimum light scattering.

d. Optimum temperature for probe incorporation into the membrane.

The effect of temperature on probe incorporation was also investigated. A constant amount of probe (0.1 umol/ml membrane suspension) was incorporated by injection at 4°, 20°, 37° and 50° C, and incubated for 45 min at the incorporation temperature. At the end of incubation the samples were diluted in sucrose (0.2 ml of membrane into 3 ml of 0.32 M sucrose) at room temperature and fluorescent polarization and intensity was measured (Figure 18-A and B). As can be observed, there was no change in probe polarization over a wide temperature range varying from 4° C to 50° C, however, there was a steady decrease in fluorescent intensity throughout this range. Since both polarization and intensity of the fluorescent probe remained

constant over the range of 20°-37°C, all experiments carried out in this study were done at room temperature (20° C).

In summary, the optimum conditions for fluorescent measurements were obtained by incorporating the probe (0.1 μmol) into one ml of membrane suspension (containing 2-3 mg protein), and carrying out the incubation at room temperature for 45 minutes. The amount of probe incorporated into the membrane was measured as described in Methods. Approximately 20% of the probe injected into the membrane suspension was incorporated into the membrane.

Evaluation of membrane perturbation resulting from incorporation of cholesta-5,7,9-trien-3-beta-ol by the injection method *in vitro* was determined by measuring Na^+-K^+-ATPase specific activity and cholesterol organization. Since ethanol was used as solvent for probe, these measurements were performed in the membrane preincubated with ethanol or ethanol plus probe. As indicated in Table 5, there was no change in Na^+-K^+-ATPase specific activity whether the membrane was pretreated with ethanol or ethanol plus probe. Activity of Na^+-K^+-ATPase was used as a criterion because it has been demonstrated that this enzyme is sensitive to the lipid environment (Papahadjopoulos *et al.*, 1973).

When the organization of cholesterol in this membrane was examined it was found that this treatment did not significantly change the cholesterol organization with

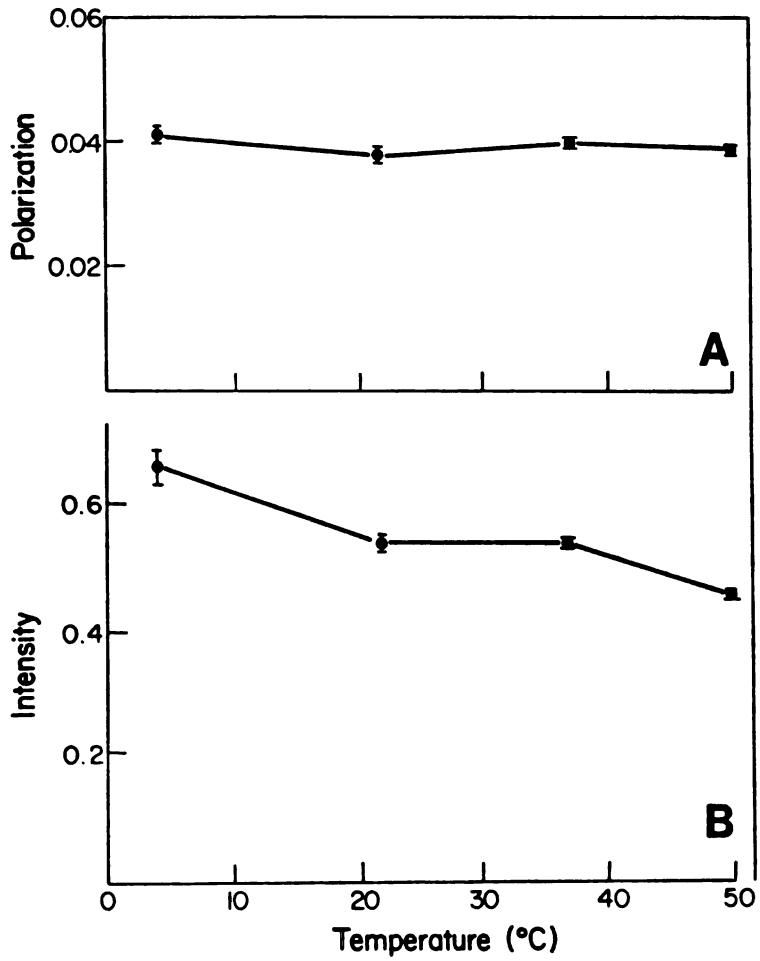


Figure 18. Effect of temperature on cholesterol probe incorporation measured by fluorescent polarization (A) and fluorescent intensity (B). A constant concentration of probe (Ø.1 μmol) was incorporated into membrane suspension at different temperatures and incubated at the same temperature for 45 minutes; samples were read at room temperature. Vertical bars are standard error of means of 1Ø experiments each assayed in duplicate.

Table 5

Effect of Cholesterol Probe Incorporation on Na^+-K^+-ATPase Activity.

Treatment	Activity (μmol p_i/min/mg protein)
Buffer (control)	0.0155 ± 0.00162 (n = 10)
Ethanol	0.0156 ± 0.00145 (n = 10)
Probe in ethanol	0.0155 ± 0.0017 (n = 10)

Values for enzyme activity are means ± standard error and the numbers in parentheses are the numbers of experiments performed, each assayed in triplicate. Enzyme activity was assayed as described in Methods.

respect to the amount of exposed and non-exposed cholesterol in the membrane; neither total nor exposed cholesterol was significantly changed in this membrane (Table 6). Based on this criteria, i.e., membrane cholesterol organization and enzymatic activity, it is obvious that the probe itself and incorporation procedure are non-perturbing to the membrane, thus this simple method has potential to be used in membrane fluidity measurement studies.

The effect of probe incorporation on membrane opiate binding was also evaluated. The binding assays were performed as described in Methods, using Ø.2 nM of ^{3}H-etorphine as a μ-agonist and 2 nM ^{3}H-D-ala^{2}-D-leu^{5}-enkephalin as an δ-agonist to label the receptor. Binding was displaced by increasing concentrations of the corresponding unlabeled agonists. The data shown in Table 7 indicate that there was no change in total bound ^{3}H-etorphine and binding affinity in the presence of ethanol alone or ethanol containing the probe. However, in the case of D-ala^{2}-D-leu^{5}-enkephalin there was a decrease of 47% in total binding in the presence of ethanol and 32% in the presence of ethanol plus probe. A two-fold decrease in affinity (K_d) for D-ala^{2}-D-leu^{5}-enkephalin binding was observed both in the presence of ethanol and ethanol plus probe (Table 7). It can be concluded that ethanol has different effects on μ and δ binding sites. This finding agreed with other data (Hiller et al., 1981) in which

Table 6

Effect of Cholesterol Probe Incorporation on Cholesterol Distribution.

Treatment	Total umol/mg protein	Exposed umol/mg protein	Percent exposed
Buffer (control)	0.5905 ± 0.0381 (n = 10)	0.0147 ± 0.0015 (n = 10)	2.49
Ethanol	0.571 ± 0.028 (n = 10)	0.0137 ± 0.002 (n = 10)	2.4
Probe in ethanol	0.549 ± 0.0286 (n = 10)	0.0159 ± 0.001 (n = 10)	2.89

Data are means ± standard error of 10 different experiments, each assayed in triplicate. Cholesterol was assayed as described in Methods.

Table 7

Binding of ^{3}H-Etorphine and ^{3}H-D-Ala^{2}-D-Leu^{5}-Enkephalin to Membrane Treated with Cholesterol Probe.

	^{3}H-Etorphine		^{3}H-D-ala^{2}-D-leu^{5}-Enkephalin	
Treatment	Total Bound (cpm)	K_d (x 10^{+9})	Total Bound (cpm)	K_d (x 10^{+9})
None (control)	2993	Ø.267	363	2.2
Ethanol	2866	Ø.346	195	4.6
Ethanol & Probe	29Ø4	Ø.275	248	4.7

Incubation medium contained 2 mg protein from membrane untreated (control) or treated either with ethanol or ethanol plus probe, Ø.2 nM labeled etorphine or 2 nM labeled D-ala^{2}-D-leu^{5}-enkephalin and 25 mM HEPES pH 7.7 in a final volume of two ml. Incubations were carried out at 37°C for 3Ø minutes followed by incubation on ice for one hour. Samples were filtered and washed twice with ice cold 25 mM HEPES and after addition of Scintiverse, the radioactivity was counted in a Beckman Scintillation Counter. Numbers are average of triplicates. K_d values were determined by displacement of bound labeled ligand with increasing amounts of the corresponding unlabeled ligand.

ethanol inhibits the binding of opiate peptides to brain membranes.

In order to further characterize the cholesterol probe, the effect of ethanol and temperature, which are known to fluidize the membrane (Chin and Goldstein, 1980), were evaluated. The effect of temperature on fluorescent polarization and intensity is shown in Figure 19-A and B. There is a decrease in fluorescent intensity over a wide range of temperature. The temperature variation shows a transition in polarization around approximately 25° C, that could be explained by the temperature-dependent transition of membrane lipids. Therefore, the fluidizing effect of temperature on membrane is evident by a decrease in probe polarization and intensity.

The effect of varying ethanol concentrations at different temperatures on fluorescent intensity is shown in Figure 20. There was no change in membrane fluorescent intensity upon ethanol treatment at 4° C, 22° C and 50° C. However, there was a concentration-dependent decrease in intensity upon ethanol treatment at 37° C. This again is in agreement with the available data concerning fluidizing effects of ethanol, and interestingly, it is observed at physiological temperature. The cholesterol probe seems to be responding to the fluidizing effect of ethanol and temperature, suggesting that this probe is suitable for use in membrane fluidity measurements. The ethanol effect on

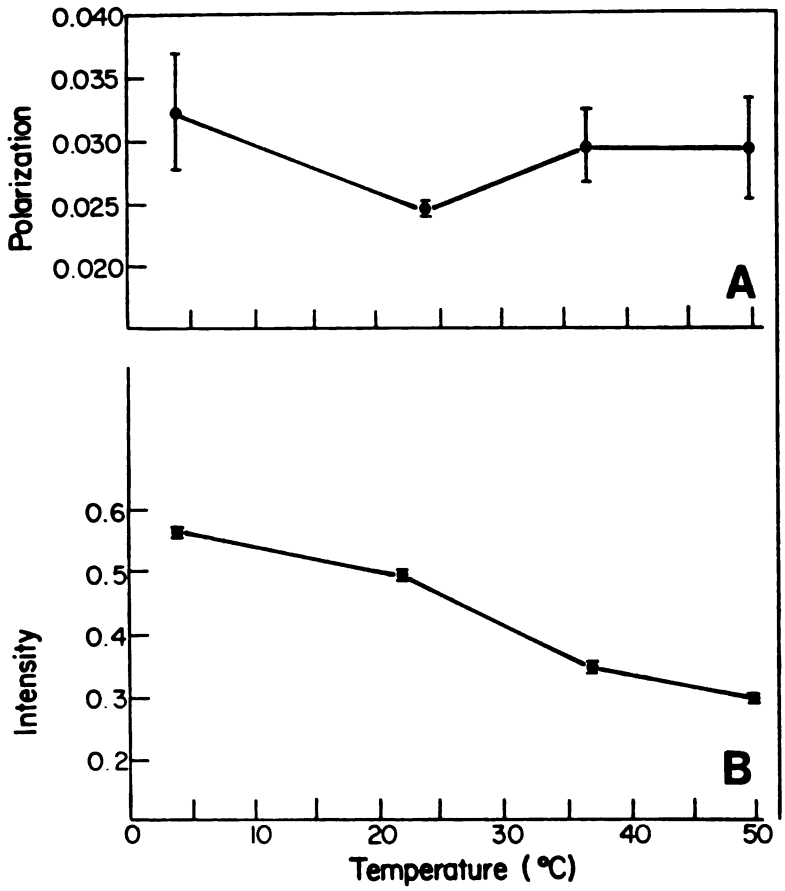


Figure 19. Influence of temperature on probe polarization (A) and fluorescence intensity (b). A constant amount of probe was incorporated into membrane at room temperature (Ø.1 μ mole/Ø.2 ml membrane suspension). Samples were incubated at different temperatures for 45 minutes and were read at the same temperature. Vertical bars are standard error of the means of 1Ø experiments each assayed in duplicate.

polarization did not give a conclusive result (data not shown).

Finally, the fluidity of P_2 membrane and reconstituted membrane were compared. Data in Table 8 show that both fluorescent intensity and polarization were lowered in reconstituted membrane compared with that of the P_2 membrane, indicating that the reconstituted membrane was more fluid. The change in fluidity in reconstituted membrane is in agreement with the lipid analysis data as explained previously. In reconstituted membrane there is an increase in oleoylphosphatidylcholine content and a decrease in cholesterol incorporated into the membrane. Membrane fluidity is strongly influenced by the composition of membrane lipids. For example, variation in acyl chain saturation is the most common determinant of membrane fluidity. Unsaturated acyl chains form a fluid disordered membrane and the reverse is true for saturated acyl chains. Membrane fluidity is also influenced by the amount of cholesterol. A decrease in cholesterol content increases the fluidity of the membrane (Cooper et al., 1978; Shinitzky and Inbar, 1976; and Vanderkooi et al., 1974). Also an increase in PC/Sph ratio increases membrane fluidity (Shinitzky and Barenholz, 1974). Since there is an increase in unsaturated acyl chains and a decrease in cholesterol content of reconstituted membrane, an increase in fluidity is expected.

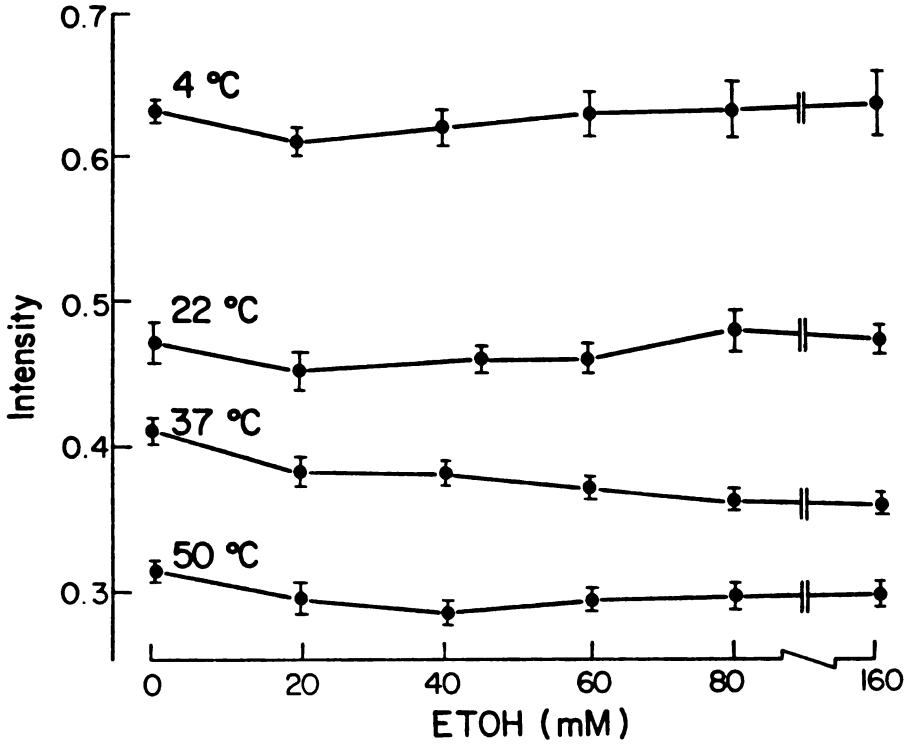


Figure 20. Effect of varying ethanol concentrations on probe fluorescence intensity in P_2 membrane under optimum conditions (0.25 ml P_2 membrane suspension, 0.1 µmol probe, incubated for 45 minutes at room temperature), followed by incubation at different ethanol concentrations at 4° C, 22° C, 37° C and 50° C. Vertical bars are standard error of means of 10 membrane preparations each assayed in duplicate.

Table 8

Fluorescent and Polarization Measurements in P_2 and Reconstituted Membranes.

	Fluorescent Intensity	Fluorescent Polarization
P_2	Ø.4377 ± Ø.Ø1Ø25 ($\bar{1}$Ø)	Ø.Ø357 ± Ø.ØØ41 ($\bar{1}$Ø)
R	Ø.3931 ± Ø.ØØ552 ($\bar{1}$Ø)	Ø.Ø2963 ± Ø.ØØ547 ($\bar{1}$Ø)
% decrease	1Ø%	17%

P_2 membrane and reconstituted membrane, Ø.25 ml, were treated with Ø.1 μmol of cholesterol probe, followed by incubation for 45 min at room temperature. Values are the means of ten membrane preparations each performed in duplicate. Values are shown with standard error of the means.

III-3. Binding Characteristic of P_2 and Reconstituted Membrane

Opiates, like other drugs, are believed to exert their pharmacological action through membrane bound receptors. In order to demonstrate that a binding site is indeed a pharmacologically relevant receptor, a number of criteria must be satisfied. The first criterion is stereoselectivity; in general, it has been found that the levorotatory opiates exhibit a thousand to ten thousand fold greater binding affinity than their corresponding dextroisomers (Pert and Snyder, 1973b; Simon et al., 1973; Simantov et al., 1978; Simon et al., 1975b; Miller et al., 1977b; Hazum et al., 1979a; Law et al., 1979a; and Terenius, 1973a). The second criterion is high-affinity binding to the receptor. Dissociation constant values of opiate binding sites are in the nanomolar ranges depending on the radioactive ligand used (Pasternak and Snyder, 1975b; Simon et al., 1973; Terenius, 1973a; Simantov et al., 1978; Law and Loh, 1978; Law et al., 1979a; Hazum et al., 1979a). Third, the drug-receptor interaction should show saturability, indicating the presence of a finite number of receptors. High-affinity, stereospecific binding usually shows this saturability, in contrast to non-specific binding which is nonsaturable, increasing linearly with increasing free ligand concentration (Pert and Snyder, 1973a; Simantov

et al.,1978; Law et al., 1979a). The fourth and the most important criterion is the ability of various unlabeled opiates to displace a labeled opiate bound to the receptor, in a rank order observed close to that of pharmacological potencies (Pert and Snyder, 1973b, 1974; Terenius, 1974; Stahl et al., 1977).

In the present studies, an effort was made to characterize binding activities in the reconstituted membrane in terms of these criteria.

A) Saturability

The saturation curve of ^{3}H-etorphine binding to P_2 and to reconstituted membrane is shown in Figure 21. Binding of ^{3}H-etorphine to P_2 membrane saturates at about 120 fmol per mg protein, which is in close agreement with 200 fmol per mg protein reported by Pert and Snyder (1973a) and Simon et al. (1973). The lower receptor content in this P_2 membrane is probably due to the use of a washed P_2 membrane. The binding of ^{3}H-etorphine to reconstituted membrane saturates at about 28 fmol per mg protein, which is 23% of that of the P_2 membrane. Binding to reconstituted membrane shows another level of saturation as well. This first part of the curve saturates at 8 fmol of drug concentration. However, this saturation occurs at a very low cpm bound to the membrane, making interpretation of the data difficult.

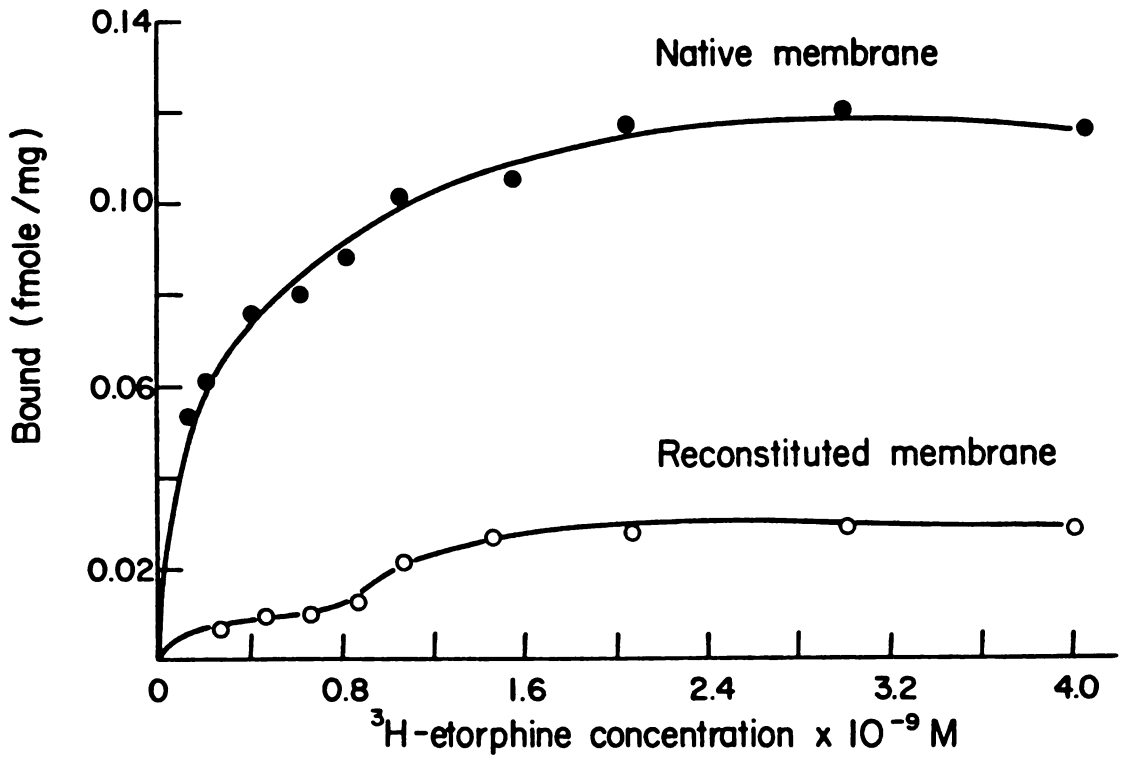


Figure 21. Saturation curve of ^{3}H-etorphine binding to native and reconstituted membrane.

B) Correlation Between the Relative Affinities of Opiates and their Pharmacological Potencies.

Saturation binding, though necessary, is not a sufficient criterion for identification of a biologically meaningful receptor. Ideally, one would like to show a correlation in the same tissue between binding and pharmacological potencies. In order to show that the reconstituted membrane still retains pharmacologically relevant opiate receptors, the relative potencies of several unlabeled drugs in reducing stereospecific ^{3}H-etorphine binding to P_2 and reconstituted membrane were evaluated. Table 9 shows the inhibition of ^{3}H-etorphine binding by several unlabeled opiate ligands. Since ^{3}H-etorphine is available at very high specific activity, about 60 ci/ mmol, and the specific to nonspecific binding ratio is relatively good, a very small concentration, 0.2 nM, can be used for binding to crude brain P_2 membrane. However, due to reduced capacity of the binding to reconstituted membrane at 0.2 nM ^{3}H-etorphine, a higher concentration of ^{3}H-etorphine, 2 nM, was required for it. As a result of this, the absolute values of all IC_{50} values were much higher in reconstituted than in original membrane; only relative potencies can be compared.

The concentration of unlabeled ligands to displace 50% of the ^{3}H-ligand bound to membrane (IC_{50} values) in P_2 and

Table 9

Inhibition of ^{3}H-Etorphine Binding by Unlabeled Ligands at pH 7.7 in Native and Reconstituted Membranes.

Drugs	IC_{50} Control	R
Etorphine	1.57 ± Ø.22 ($x10^{-8}$M) (3)	2.9 ± 1.37 ($x10^{-7}$M) (5)
Levorphanol	1.73 ± Ø.42 ($x10^{-7}$M) (3)	3.Ø5 ± Ø.42 ($x10^{-5}$M) (2)
Naloxone	3.77 ± Ø.92 ($x10^{-7}$M) (3)	3.88 ± 1.Ø ($x10^{-6}$M) (6)
Morphine	1.88 ± Ø.4 ($x10^{-6}$M) (3)	5.8 ± 1.18 ($x10^{-6}$M) (4)
DADL	1.Ø4 ± Ø.Ø57 ($x10^{-5}$M) (2)	----
Dextrophan	8.25 ± 2.29 ($x10^{-5}$M) (4)	1 x 10^{-4}M (1)

IC_{50}'s of opiate ligands to inhibit ^{3}H-etorphine binding in P_2 and reconstituted membrane. Incubation medium contained ^{3}H-etorphine (Ø.2 nM for control, 2 nM for reconstituted), membrane protein (Ø.5 mg) HEPES pH 7.7 (25 mM) and different concentrations of unlabeled drugs in a final volume of 2 ml. Incubation carried out at 37°C for 3Ø minutes followed by incubation at 4°C for 1 hour and filtration through GFB filter. Radioactivity measured in a Beckman Scintillation Counter. Number in parentheses indicates the number of experiments performed.

---- No binding detected.

reconstituted membranes were measured. The rank order of these values in P_2 membrane is in the order etorphine > levorphanol > naloxone > morphine > D-ala^2-D-leu^5-enkephalin > dextrophan (Table 9). The rank order potency of displacement of ^{3}H-etorphine binding to reconstituted membrane is in the order of etorphine > morphine > naloxone > levorphanol > dextrophan > D-ala^2-D-leu^5-enkephalin. Thus etorphine is still the most potent ligand in displacing ^{3}H-etorphine binding, and levorphanol is more potent than dextrophan, indicating stereoselectivity of binding. In the reconstituted membrane, however, levorphanol is no longer more potent than morphine as it was in P_2 membrane and the ability for D-ala^2-D-leu^5-enkephalin to displace etorphine binding was essentially lost. Loss of the peptide binding site in reconstituted membrane suggests that these sites may be dissociated from alkaloid sites during solubilization and reconstitution procedure. This finding is consistent with a model in which these sites are physically distinct (Lee and Smith, 1980).

In summary, the binding of opiates to reconstituted membranes is saturable and stereoselective and the rank order potency correlates to some degree with that of the native brain membrane, with the major exception that enkephalin binding site is not preserved.

C) pH Dependence of Opiate Binding.

Differences in the two membranes were also revealed in an analysis of the pH dependence of opiate binding. Binding to native membrane occurs maximally in a broad range of pH 6.8-8.Ø, while that to reconstituted membrane has a much sharper optimum, at about 6.Ø (Figure 22). The broad optimum in native membrane is probably the result of the combined effects of ligand protonation and binding site ionization, both of which are necessary, but which are affected oppositely by pH (Smith and Loh, 1977). The latter effect is also apparent in the secondary peak of binding in the reconstituted membrane, at higher pH.

Since the rank order studies were carried out at pH 7.7, where binding to reconstituted membrane was at a minimum, these were repeated at pH 6.Ø, which is the optimum pH for binding to the reconstituted membrane. Interestingly, a complete loss of potency correlation of ^{3}H-etorphine binding displacement by unlabeled ligands was observed at pH 6.Ø (Table 1Ø). However, under this condition D-ala^{2}-D-leu^{5}-enkephalin was able to displace ^{3}H-etorphine binding with an $IC_{5Ø}$ value of about 181.6 μM.

From these results, it is obvious that reconstituted membrane is behaving very differently from native membrane. This could very well be due to changes in lipid composition and/or distribution of the lipids and proteins in two halves

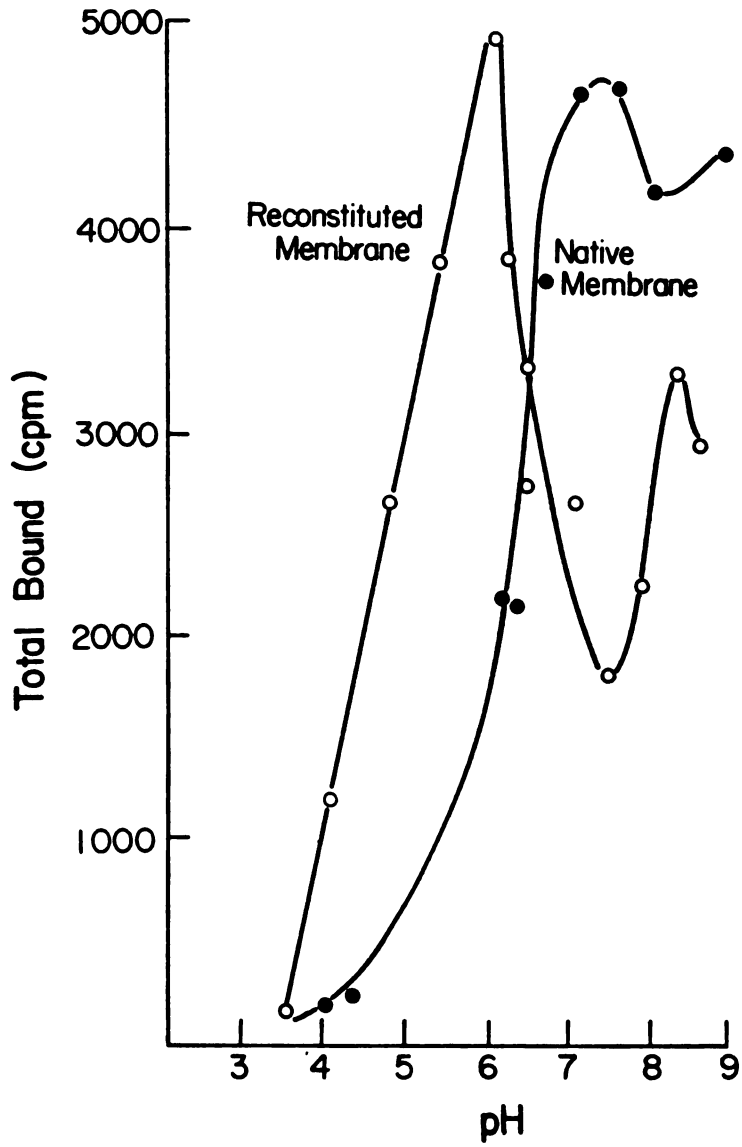


Figure 22. Effect of pH variation on ^{3}H-etorphine binding to P_2 and reconstituted membrane. The data are means of duplicate determinations.

Table 10

Inhibition of ^{3}H-Etorphine Binding (2 nM) by Unlabeled Opiates at pH 5.0 in Reconstituted Membrane.

Drugs	IC_{50} pH 7.7	pH 6.0
Etorphine	2.9×10^{-7} M	41.6×10^{-6} M
Levorphanol	3.06×10^{-5} M	80.1×10^{-4} M
Naloxone	3.88×10^{-6} M	30.2×10^{-5} M
Morphine	5.8×10^{-4} M	33.3×10^{-5} M
D-Ala^{2}-D-Leu^{5} enkephalin	1×10^{-4} M	41.6×10^{-4} M

Effect of pH variation on IC_{50} values in reconstituted membrane, experimental conditions as given in Table 9.

of membrane bilayer, such as change in PC level or the use of these lysophosphatide and oleoyl CoA as detergents which may have a primary effect on the opiate receptor. Therefore, we used a small concentration of LPC and oleoyl CoA which is not enough to solubilize the membrane to determine their effect on opiate receptor binding. In this experiment, native membrane was incubated in the presence of small concentration of LPC and oleoyl CoA, Ø.1 mM, for 45 minutes. This concentration is 1Ø fold lower than the concentration used for membrane solubilization, therefore, the membrane components are not dissociated. This is evidenced by the fact that this concentration of detergents did not cause a decrease in membrane optical density measured at 5ØØ nm, and even if there is a membrane solubilization, it is in such a small quantity that could not be detected by optical density measurements. Then the binding of ^{3}H-etorphine to detergent treated membrane was studied. It was shown that the affinity of ^{3}H-etorphine to this membrane has decreased from 41.2 nM to 28 nM and also the capacity of both high and low affinity sites has decreased 43% and 17% respectively compared to that of the native membrane (Figure 23-A and B and Table 11). This change in capacity could be due to the solubilization of receptors so they could not be trapped on the filter or the receptor environment has been changed due to solubilization of some membrane components close to the binding site,

therefore, changing the receptor binding characteristic. Another explanation is that these detergents act on receptor and directly change its binding properties.

However, in order to show that these changes in receptor binding properties are not due to receptor destruction upon detergent treatment, attempt was made to incubate the detergent-treated membrane with liver microsomes as the source of acyltransferase to reconstitute the membrane if any solubilization has been caused by the treatment of membrane with detergents. Then the membrane binding properties were studied and the data is shown in Figure 23-C and Table 11. It is shown that after reconstitution, the affinity of site 1 has increased and is close to that of the native membrane. Also, the capacity of both high and low affinity sites has increased compared to the membrane treated with detergents. This is clearly indicating that the reconstitution procedure is capable of restoring normal membrane characteristics after small concentrations of detergents and that the reconstitution procedure does not destroy opiate binding sites.

III-4. Inhibition of Metabolic Degradation of Leu- and Met-Enkephalin

Enkephalins (tyr-gly-gly-phe-met or -leu) are of great interest because of their opiate-like properties (Hughes et

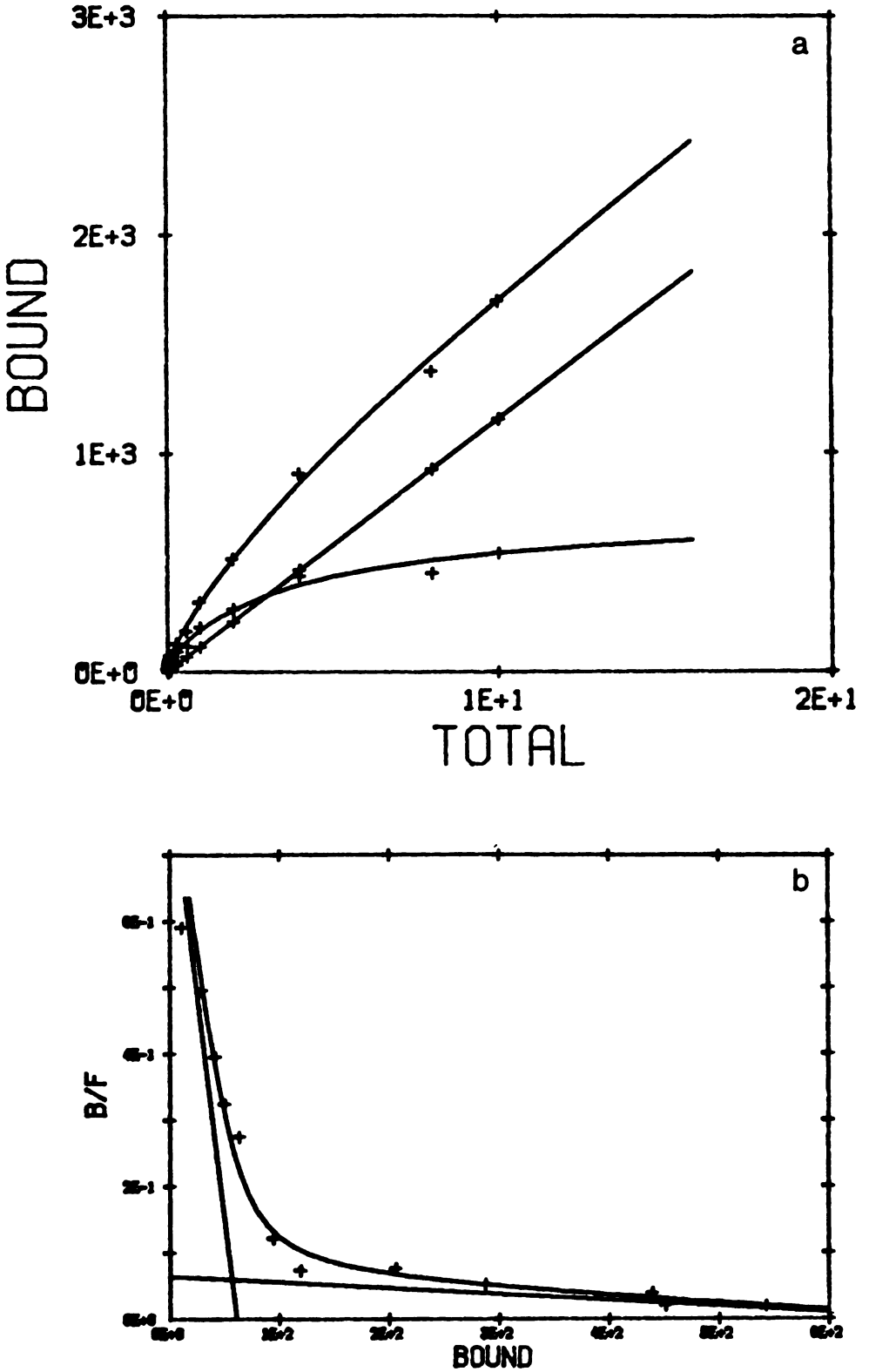


Figure 23-A. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding to native membrane.

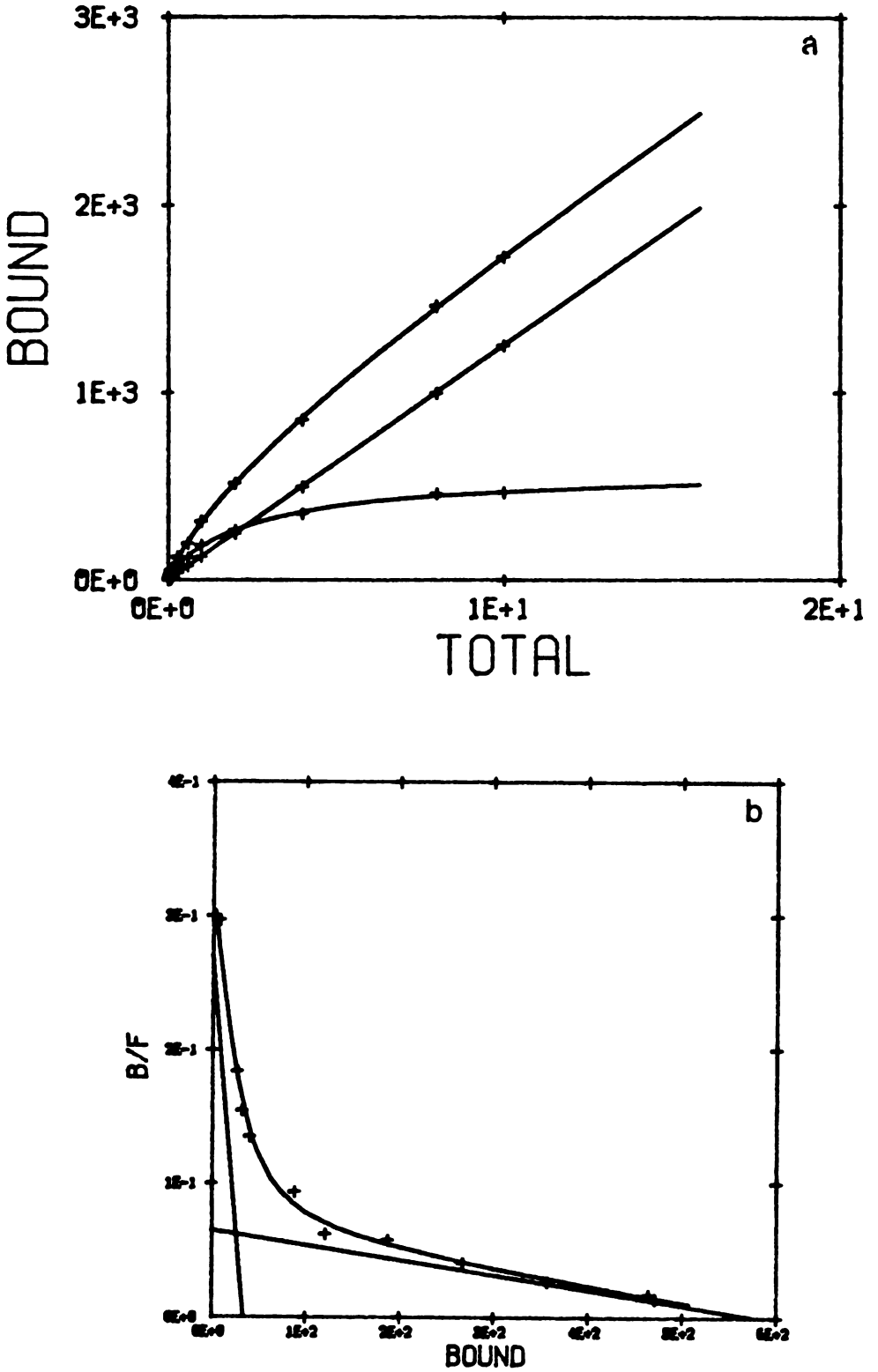


Figure 23-B. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding of membrane treated with 0.1 mM LPC and oleoyl CoA.

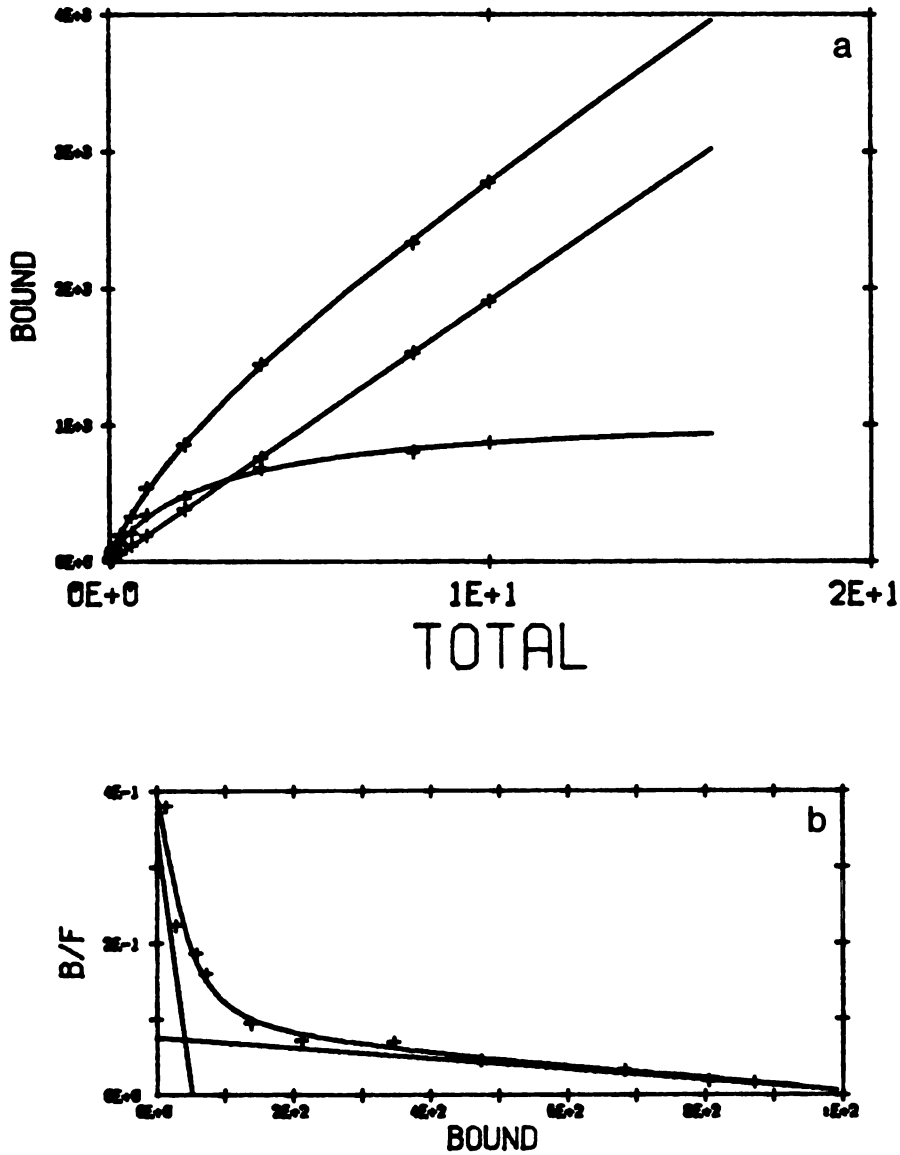


Figure 23-C. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding to the membrane treated with detergents and incubated in the presence of LM as the source of acyltransferase.

Table 11

Binding Properties of ^{3}H-Etorphine Binding to Native, Detergent Treated and Reconstituted Membranes.

Etorphine	K_1 (nM^{-1})	K_2 (nM^{-1})	R_1 (fmol/mg protein)	R_2 (fmol/mg protein)
Native	41.2 ± 13.3	0.275±0.057	61 ± 9.2	697 ± 71
Native & LPC & oleoyl CoA	28 ± 7.96	0.401±0.037	34.5± 5.3	575 ± 23
Reconstituted	52.4 ± 36.8	0.44 ± 0.11	57.8±18.8	989 ± 114

K_1 and K_2 are affinity constants of ^{3}H-etorphine binding to sites 1 and 2 respectively. R_1 and R_2 are respective capacities. Values are means ± standard errors.

al., 1975) and their possible role as endogenous neurotransmitters (Smith et al., 1976; Frederickson, 1977; Way and Glasgow, 1978). Opiate-like analgesia of these pentapeptides has not been established, however, for they produce only a weak or transient analgesia when administered intraventricularly (Belluzzi et al., 1976; Buscher et al., 1976; Chang et al., 1976). Several studies have shown that pentapeptides are subject to extremely rapid inactivation in biological tissues. Enkephalin breakdown mainly occurs through a series of amino peptidases with low specificities and affinities for enkephalins (Hambrook et al., 1976; Dupont et al., 1977; Meek et al., 1977; Marks et al., 1977; Vogel and Alstein, 1977) and a high- affinity, specific endopeptidase splitting the gly-phe bond (Malfory et al., 1978; Craves et al., 1978; Schwartz et al., 1981). Rapid metabolism of enkephalins is also shown in vitro with proteolytic enzymes (Hughes, 1975; Pasternak et al., 1975; Chang et al., 1976).

Binding studies of enkephalins in brain tissues therefore must be performed in the presence of compounds which inhibit enkephalin-degrading enzymes. Inhibitors including enkephalin fragments such as tyr-gly, tyr-gly-gly, gly-phe that are more specific for enkephalinase (Henderson et al., 1978), puromycin which is specific for aminopeptidase without any effect on enkephalinase (Vogel and Altstein, 1979) and Bacitracin, 1-10 phenanthroline, gluta-

thion (Erods, 1977) and EDTA, all have been shown to inhibit enkephalinase.

Although any of these compounds would protect enkephalin from being metabolized to a certain degree, none of them are able to protect the enkephalins 100% from degradation by amino-peptidases and/or enkephalinase. Therefore, a study was undertaken to search for compound(s) that inhibit enkephalin degradation in the presence of brain homogenates. These compounds not only have to be potent in inhibiting enkephalin metabolizing enzymes, they also must not interfere with opiate binding to membrane preparation. Enkephalin fragments with structural similarities to pentapeptides may actually compete for binding, and thus may not be good candidates for enzyme inhibition. The effects of a few compounds, which have been shown to be effective in terms of enzyme inhibition, on ^{3}H-etorphine binding are shown in Table 12. The concentrations chosen for these compounds were based on the concentrations that are effective in inhibiting enkephalin degrading enzymes. As can be seen 1-10 phenanthroline and puromycin, at the concentrations tested, inhibited ^{3}H-etorphine binding to SPM, 50% and 10% respectively. On the other hand, 1 mM EDTA and 100 µg/ml bacitracin did not interfere with opiate binding significantly, except for a slight increase. Therefore, a combination of different concentrations of EDTA and bacitracin were examined to determine the degree of

inhibition of enkephalin metabolism assayed by high performance liquid chromatography.

Briefly, synaptic plasma membrane, SPM, from rat brain was incubated with ^{3}H-leu- or ^{3}H-met-enkephalin in the presence of different concentrations of EDTA and bacitracin and 25 mM HEPES buffer pH 7.7., in a final volume of one ml, incubated at 4^{o} C for 3 hours. The reaction was terminated by immersing the mixture into boiling water for 15 minutes, followed by centrifugation at 8ØØØ x g for 15 min. The supernatants were chilled and analyzed by HPLC.

A) Inhibition of Metabolic Degradation of Leu-Enkephalin

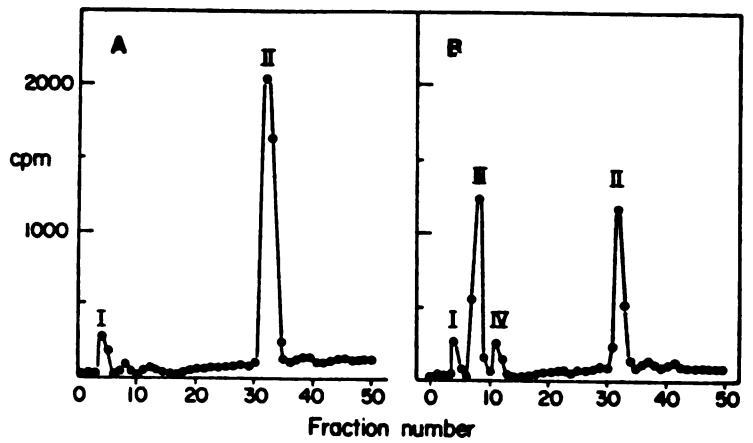
Fractions eluted from the C18 column by HPLC were collected in 1 ml volumes and the radioactivity of each fraction was measured in a Beckman scintillation counter. The elution profile of ^{3}H-leu-enkephalin incubated in the presence of brain membrane is shown in Figure 24-A; almost all the radioactivity is eluted as one major peak (II) between fractions 31-34 (3938 cpm), with a minor peak in fractions 3-5 (I). It was assumed that in this sample little or no enkephalin breakdown occurred. When the incubation is carried out in the presence of SPM, several peaks appeared, consistent with enkephalin breakdown (Figure 24-B). Figures 24-C through G show the effects of different concentrations of EDTA and bacitracin on ^{3}H-leu-enkephalin metabolism. The radioactivity present in different fractions is shown in

Table 12

Effect of Inhibitors of Enkephalin Degrading Enzymes Inhibitor on ^{3}H-Etorphine Binding.

Compound Used	CPM	% of control
None	381 ± 57	100
1-10 Phenanthroline(250 μM)	192 ± 13	51 ± 5.8
Bacitracin (100 μg/ml)	393 ± 16	103 ± 14.5
Puromycin (10 μM)	335 ± 28	90 ± 20
EDTA (1mM)	413 ± 12	110 ± 18
Bacitracin & Puromycin (100 μg/ml & 10 μM)	348 ± 79	91 ± 6.8
Bacitracin & EDTA (100 μg/ml & 1mM)	435 ± 26	115 ± 12
Puromycin & EDTA (10 μM & 1μM	383 ± 82	100 ± 29
Bacitracin & Puromycin & EDTA (100 μg/ml 10 μM & 1mM	345 ± 46	90 ± 1.66

Assay mixtures contained 0.5 nM ^{3}H-etorphine, 0.4 mg brain membrane protein and different concentrations of each compound as indicated in the table, in 25 mM HEPES, pH 7.4 in a final volume of 1 ml. Incubations were carried out at 4°C for 3 hrs, followed by filtration in vacuum through GFB filters and washing twice with 5 ml ice cold buffer. Data are means to two experiments each assayed in triplicate.


A
B
2000
cpm
1000
I
II
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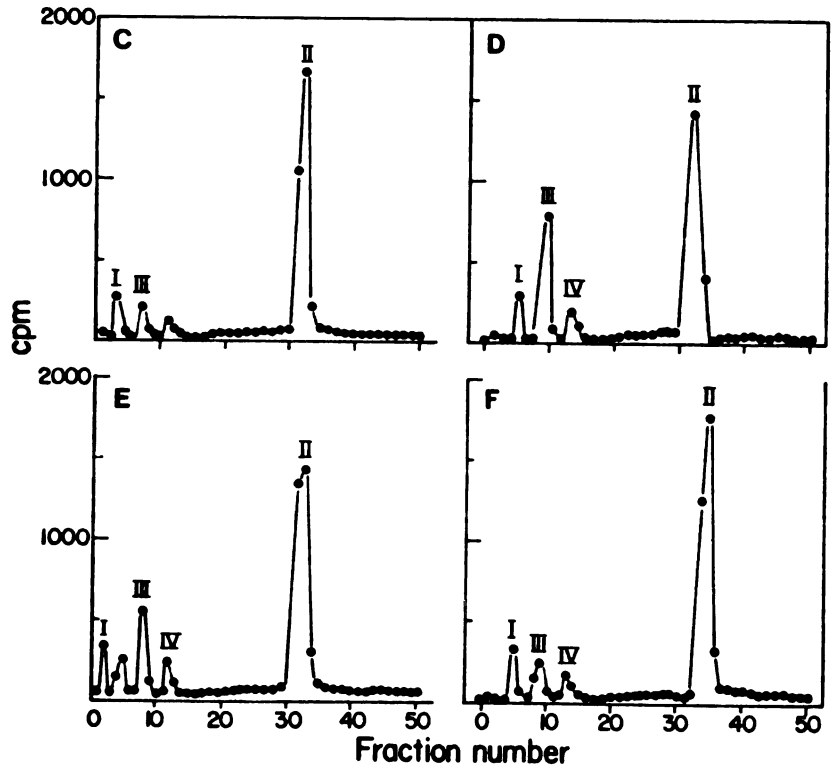
IV
0 10 20 30 40 50
Fraction number


C
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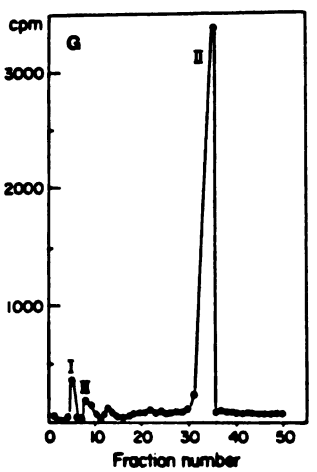
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II
0 10 20 30 40 50
Fraction number

Figure 24. Elution profile of ^{3}H-leu enkephalin from C18 HPLC column. Enkephalin incubated:

A. in the absence of brain membrane,
B. in the presence of 200 µg brain membrane protein,
C. in the presence of 200 µg membrane protein + 100 µg bacitracin,
D. in the presence of 200 µg membrane protein + 1 mM EDTA,
E. in the presence of 200 µg membrane protein + 250 µM EDTA + 25 µg/ml bacitracin,
F. in the presence of 200 ug membrane protein + 500 µM EDTA + 50 µg/ml bacitracin,
G. in the presence of 200 ug membrane protein + 1 mM EDTA + 100 µg/ml bacitracin.

Table 13

Metabolic Degradation of ^{3}H-Leu-Enkephalin Under Different Assay Conditions.

Condition	CPM I	II	III	IV
no SPM	464	3938	---	---
+ SPM	273	1886	1773	256
SPM + 100 µg bacitracin	266	2878	201	---
SPM + 1 mM EDTA	315	2082	1031	205
SPM + 250 µM EDTA + 25 µg/ml bacitracin	329	2998	525	239
SPM + 500 µM EDTA + 50 µg/ml bacitracin	326	3350	251	171
SPM + 1 mM EDTA + 100 µg/ml bacitracin	375	4066	193	---

The radioactivity eluted in fractions eluted from a C18 column from HPLC. Metabolic degradation of ^{3}H-leu-enkephalin was measured in the absence or presence of different inhibitors as described in Material and Methods. Each peak's radioactivity is the sum of the radioactivity in the fractions comprising that peak.

Table 13. It is evident that different concentrations of EDTA and bacitracin alone are not sufficient to inhibit enkephalin metabolism, even when they were used at their maximum concentrations, because peak II radioactivity was still markedly reduced. Combinations of EDTA and bacitracin, lower than 1 mM for EDTA and 100 µg/ml for bacitracin, were also unable to prevent enkephalin degradation 100%. However, 1 mM EDTA and 100 µg/ml bacitracin together were able to prevent enkephalin metabolism completely, peak II radioactivity was now equal to that observed in enkephalin not incubated with SPM.

B) Inhibition of Metabolic Degradation of Met-Enkephalin

A similar study was carried out for met-enkephalin (Figure 25). In this case, just one major peak appeared in the enkephalin not incubated with SPM, and the same degree of degradation was observed in the presence of SPM as that of the ^{3}H-leu-enkephalin (Figure 25-B). The effect of different concentrations of EDTA and bacitracin on met-enkephalin degradation is shown in Figures 25 C through 25 G, and Table 14. Again, only the combination of 1 mM EDTA and 100 ug/ml bacitracin was able to completely inhibit enkephalin degradation.

In summary, this study clearly shows that enkephalin degradation can be inhibited completely by combination of

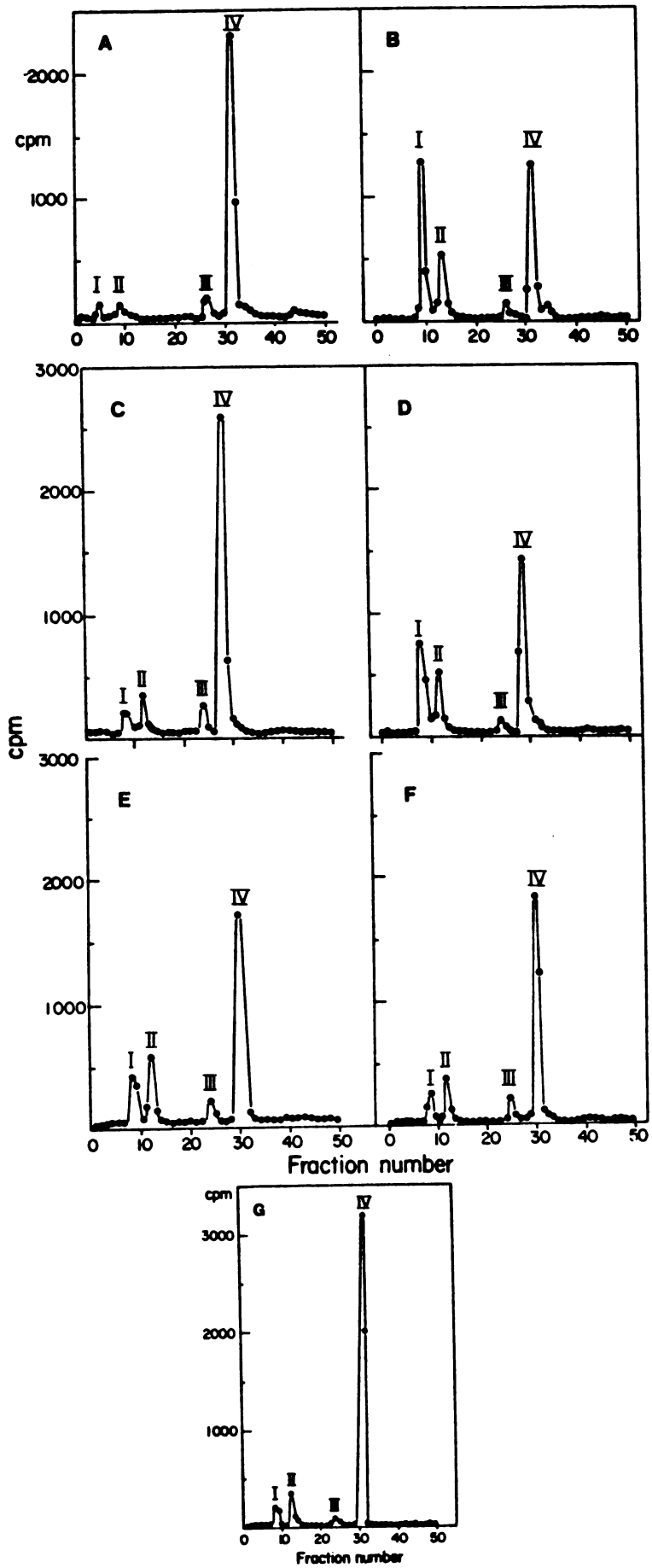
A
B
C
D
E
F
G
cpm
Fraction number
I
II
III
IV

Figure 25. Elution profile of ^{3}H-met-enkephalin from C18 HPLC column. Enkephalin incubated:

A. in the absence of SPM,
B. in the presence of 200 µg SPM protein,
C. in the presence of SPM + 200 µg membrane protein + 10 µg bacitracin,
D. in the presence of SPM + 200 µg membrane protein + 1 mM EDTA,
E. in the presence of SPM + 200 µg membrane protein + 250 µM EDTA + 25 µg/ml bacitracin,
F. in the presence of SPM + 200 ug membrane protein + 500 µM EDTA + 50 µg/ml bacitracin,
G. in the presence of SPM + 200 µg membrane protein + 1 mM EDTA + 100 µg/ml bacitracin.

SPM = Synaptic Plasma Membrane.

Table 14

Metabolic Degradation of ^{3}H Met-Enkephalin Under Different Assay Conditions.

Condition	CPM I	II	III	IV
No SPM	3554	---	---	---
+ SPM	1833	1696	855	190
SPM + 100 µg bacitracin	3190	375	340	244
SPM + 1mM EDTA	2343	1199	507	180
SPM + 250 µg EDTA + 25 µg/ml bacitracin	2840	744	555	199
SPM + 500 µM EDTA + 50 µg/ml bacitracin	3050	478	376	221
SPM + 1 mM EDTA 100 µg/ml bacitracin	3598	410	367	---

The radioactivity measurement of ^{3}H-met-enkephalin peaks, peak I to peak IV, eluted from C18 HPLC column; enkephalin incubated with synaptic plasma membrane (SPM) in the absence or presence of different inhibitors of enkephalin degrading enzymes.

appropriate concentrations of EDTA and bacitracin, without interfering with opiate binding activities *in vitro*. Therefore, in all studies that require incubation of brain membrane with pentapeptides, EDTA and bacitracin were included in the assay mixture to prevent enkephalin metabolism by enkephalinase and aminopeptidases present in brain synaptic plasma membrane.

III-5. Lipid Modification of Reconstituted Membrane.

Most biological membranes contain a phospholipid bilayer as a major structural skeleton, and this plays a static role, as a molecular "cement" for membrane construction (Day and Levy, 1969). However, a dynamic role for membrane lipids in the function of membrane-bound protein has also been suggested (Coleman, 1973).

According to the fluid mosaic model developed by Singer and Nicholson (1972), biological membranes are composed of asymmetric phospholipid bilayers. Proteins are also asymmetrically located either on the surface of the bilayer (peripheral proteins) or are partially or completely immersed in the bilayer (integral proteins). Both the membrane phospholipids and the proteins are capable of lateral mobility in the membrane plane (Edidin, 1974; Sheats and McConnell, 1978). The lipids also undergo a lateral phase separation during thermal-induced phase transition

(Smith and McConnell, 1978; Montal, 1976) which can affect the protein distribution in the membrane. Therefore, the physical state of the lipids can greatly influence membrane protein activities (Hong and Hubbell, 1973; Masotti et al., 1974).

The fact that lipid-protein interactions are essential to membrane function is apparent from studies illustrating the dependence of membrane associated enzyme activities on phospholipids. An absolute lipid requirement is shown in the case of the mitochondrial respiratory chain (Tzagoloff and MacLennan, 1965), Na^+-K^+-ATPase (Brown and Cunningham, 1982), cytochrome oxidase (Yoshida et al., 1979), glucose-6-phosphate (Duttera et al., 1968), and many other membrane enzymes. Such a dependence has been shown in intact membrane, which after delipidation exhibits a loss of enzyme activity, with the enzyme activity reactivated after certain phospholipids are added to the delipidated preparation. Lipid dependence can also be shown by enzyme sensitivity to lipid soluble agents and to phospholipases that perturb lipid-bilayers (Barrantes et al., 1975; Limbird and Lefkowitz, 1976).

The possible involvement of lipids is shown in many receptor systems in the membrane. This lipid requirement is shown for beta-adrenergic receptor (Insel et al., 1978), alpha-adrenergic receptors (Limbird and Lefkowitz, 1976), cholinergic receptor (Wu et al., 1977; Heidmann et al.,

1980), hormone receptors (Cuatrecasas, 1971; Azhar and Menon, 1976; Azhar et al., 1976) and opiate receptors (Abood and Takeda, 1976; Loh et al., 1974, 1975, 1978; Heron et al., 1980). Therefore, as was mentioned, lipids can play a very important role in membrane integrity, thus affecting physiological functions.

This lipid-protein interaction is influenced by several factors. The primary factor is the overall charge of phospholipid head groups (Weislander et al., 1980). This charge is determined mainly by the ratio of zwitterionic phospholipids (PC, PE and sphingomyelin) to anionic phospholipids (PS, PI, and phosphatidic acid). The latter account for up to 25% of the total phospholipid, and the remainder is largely PE, and ethanolamine-plasmalogen which have a small net negative charge at pH 7.0. Thus the acidic phospholipids contribute a substantial negative charge to the membrane (Nelson, 1967; Keenan and Morre, 1970). This ratio can vary widely from organism to organism (White and Hawthorne, 1970) but for a particular cell it is usually constant (Sun,1972).

The second factor influencing lipid protein interaction is the presence of a specific phospholipid ratio. The specific phospholipid ratio requirement for optimal activity has been shown for proton and electron transfer carriers with a ratio of 4PE:1PC essential for optimal activity (Kagawa and Racker, 1971); for the NADH-Co Q reductase

complex, a 4PC:1PE ratio is required for optimal activity or 1PE:1PC in the presence of low cardiolipin (Ø.Ø5%-1.5%) (Ragan and Racker, 1973).

The third factor which is important in protein lipid interaction is the state of orderliness of lipids. Many membrane protein activities are affected by the fluidity of the membrane and the immediate environment of the proteins (Heron et al., 198Ø). Membrane fluidity, in turn, is strongly influenced by the composition of membrane lipids. The presence of various phosopholipids, having different polar head groups and hydrocarbon chain length, sterol and amount of intrinsic protein influences the fluidity of biological membrane (Nicholson et al., 1977; Kimelberg, 1977). However, the main factor influencing membrane fluidity is the number of saturated double bonds within the phospholipid acyl chains. The requirement for a specific degree of saturation is shown by the affect of different phospholipases on many enzyme activities, e.g., Na^+-K^+-ATPase (Sun et al., 1971, 1975). Since the phospholipid's acyl groups are mostly unsaturated in nature (Gurr and James, 1971), their functional role could be either to provide the enzyme protein a suitable microenvironment for proper expression of enzymatic activity, or to provide the membrane a high degree of fluidity (Lenaz et al., 1975). Finally, membrane fluidity is affected by phosphatidylcholine content. Increase in PC causes an increase in the

fluidity (Nozawa et al., 1979). The increase in PC increases PC/Sph ratio and subsequent increase in the fluidity (Shinitzky and Barenholz, 1974).

Membrane fluidity is also known to be affected by cholesterol content (Nicholson et al., 1977). The interactions between sterols and phospholipids have a number of important consequences for membrane structure; for example, sterols increase the efficiency of packing of phospholipids in artificial membranes (Demel and Dekruff, 1976). The close interpositioning of sterols with phospholipids causes a degree of immobility to be imposed upon the ten acyl carbon atoms nearest to the membrane's surface, while increasing the freedom of motion deep within the hydrophobic core of the membrane (Rothman and Engelman, 1972), thus creating an intermediate fluid state in the membrane bilayers (Chapman, 1968). The physiological role of cholesterol could thus be that of a "dampening" agent or stabilizing force, needed for the overall integrity of the cell plasma membrane (Papahadjopoulos, 1973). On the other hand, partial removal of cholesterol from erythrocyte membranes results in an increase in osmotic fragility and glycerol permeability (Bruckdorfer et al., 1969).

From all of these studies it can be concluded that the mode of lipid-protein interaction in biological membranes is greatly dependent upon the charge of the membrane, on membrane phospholipid composition, specific phospholipid

ratio and the fluidity of the lipids surrounding the protein. Also it has been demonstrated that the presence of a specific phospholipid can affect the overall asymmetry of the membrane lipids.

It has been shown that the phospholipids are asymmetrically distributed in the erythrocyte membrane, with most of the PC and sphingomyelin in the outer layer and PE, PS in the inner layer (d'Hollander and Chevallier, 1972). Cholesterol preferentially associates with sphingomyelin in the mixture of this lipid with PE and/or PC, and has more affinity for PC than for PE (Demel et al., 1977; Bruckdorfer et al., 1968). On the basis of such an affinity it has been suggested that the higher levels of cholesterol and sphingomyelin in many natural membranes can be correlated with the preferential interactions of cholesterol with sphingomyelin as demonstrated by Demel et al. (1977). In the erythrocyte membrane the phospholipids with the highest cholesterol affinity, sphingomyelin and PC, are nearly exclusively located on the outer side of the membranes, whereas most of the PE and all of the PS are located on the inner side of the membrane. The different cholesterol affinities for species and classes of phospholipids might produce a non-random distribution of cholesterol in the plane of the bilayer as well as on the inner and outer side of the membrane (Demel et al.,1977). Therefore, cholesterol in the membrane might be a very important constituent in

regulating physiological function.

As mentioned above, the amount of oleoyl-PC was increased in reconstituted membrane. Since it is known that the contents and/or ratio of phospholipids are important for membrane function, an alteration of this lipid would be likely to change the lipid distribution, asymmetry and ratio of phospholipids in the membrane and thus change the membrane function. Specifically, this could be brought about in the following ways:

i) The ratio of zwitterionic to anionic phospholipids is usually constant for a specific tissue. A change in any phospholipid can cause a change in this ratio. In reconstituted membrane the PC content has increased remarkably due to acylation of lyso-PC and oleoyl CoA. Therefore, this ratio is no longer maintained constant.

ii) By using oleoyl CoA as the substrate for the acyltransferase enzyme in the acylation cycle, the oleoyl derivative of PC is generated. Increasing unsaturated oleoyl groups in the reconstituted membrane, probably causes a change in the fluidity of the reconstituted membrane, modifying lipid-protein interaction. In nerve membrane, the total concentration of saturated and unsaturated fatty acid has been measured by Tamai et al. (1971). The saturated fatty acids are 53% and unsaturated fatty acids are 45.5%. Therefore, the ratio of saturated/ unsaturated fatty acids is 1.17. This ratio for whole bovine brain is Ø.8,

indicating a higher proportion of unsaturated fatty acids in bovine brain. This ratio in reconstituted membrane has decreased due to increase in oleoyl-PC content of the membrane, therefore affecting the membrane fluidity and subsequently modify lipid-protein interaction.

iii) The phospholipid:cholesterol ratio in rat brain synaptosome membrane is about Ø.4 (White et al., 1973). This ratio has been changed in reconstituted membrane due to increase in PC content. There is evidence that partial removal of cholesterol from RBC makes the membrane fragile and leaky (Gottlieb, 1976). This may be true in reconstituted membrane and because of the lower cholesterol content, the membrane is no longer stable.

iv) Since there is a higher proportion of PC and sphingomyelin in the outer layer of the membrane (d'Hollander and Chevallier, 1972), and cholesterol has a higher affinity for sphingomyelin and PC than that of other phospholipids (Demel et al., 1977), it is possible that cholesterol is largely located on the outer layer of the membrane bilayer. This could also affect membrane properties.

In summary, since there is a considerable increase in PC content, a 3Ø% decrease in cholesterol, a 13% decrease in PS content and a 21% decrease in PE content in the reconstituted membrane compared to that of the native membrane, it is anticipated that the reconstituted membrane

would have different properties. However, due to the changes in opiate receptor characteristics in the reconstituted membrane, attempts were made to adjust the phospholipid ratio, cholesterol content, and finally to extract some of the PC from the reconstituted membrane. It was anticipated that these maneuvers would help improve opiate receptor properties in the reconstituted membrane.

A) Adjustment of Phospholipid Ratios

The ratio of different phospholipids and the cholesterol:phospholipid ratio are shown in Table 15. In order to adjust the ratio of phospholipids in the reconstructed membrane, incorporation of different phospholipids into reconstituted membrane were carried out by two different procedures. In the first, the required concentration of phospholipid was included in the incubation medium for reconstitution. In the second an ethanolic phospholipid solution was injected into reconstituted membranes as described in Methods. However, when different phospholipids, which were adjusted as to match the content in the native membrane, were incorporated into the reconstituted membrane, the opiate receptor binding characteristics were not much improved. The binding of Ø.5 nM of ^{3}H-etorphine to the reconstituted membrane was not displaced by different concentrations of unlabeled etorphine, up to 1Ø nM, indicating an absence of high-affinity

Table 15

Lipid Ratios in Native and Reconstituted Membranes.

	P_2		R	
PC:PE	Ø.92 ± Ø.16	(7)	3.18 ± Ø.53	(6)
PC:PS	2.613	(1)	1Ø.63	(1)
Cholesterol: Phospholipids	Ø.95 ± Ø.136	(3)	Ø.42 ± Ø.Ø69	(3)
Exposed: Nonexposed Cholesterol	5.5 ± Ø.77	(2)	13.28 ± Ø.74	(2)

Lipid ratios measured after quantitation of each lipid in P_2 and reconstituted membrane. Numbers are mean ± SEM. Number in parentheses indicates the number of experiments performed.

Table 16

Effect of Exogenous Cholesterol on Cholesterol Incorporation Into the Reconstituted Membrane.

Conditions	Cholesterol Content (μmol/mg protein)
P_2	Ø.415
R	Ø.15
R + 2.5 μmol cholesterol	Ø.1634
R + 5 μmol cholesterol	Ø.1595
R + 7.5 μmol cholesterol	Ø.1598
R + 12.5 μmol cholesterol	Ø.1547
R + 2Ø μmol cholesterol	Ø.133

Solubilized membrane reconstituted in the presence of 2.5 to 2Ø μmol soluble cholesterol derivative/mg protein. The total cholesterol content measured as described in Methods section.

R = reconstituted membrane.

binding characteristics.

B) Adjustment of the Cholesterol Content.

Since the cholesterol content of reconstituted membrane has also been found to be lower than that of the native P_2 membrane, different procedures were tested in an attempt to increase cholesterol content of reconstituted membrane. The first procedure tested was to add different concentrations of a soluble cholesterol derivative, polyoxyethany-cholesteryl sebacate (Sigma Chemical Company), into solubilized membrane suspensions, followed by incubation to reconstitute the membrane as described in Methods. The cholesterol content of this membrane was assayed by the use of cholesterol oxidase; unfortunately, this method did not increase the content of membrane cholesterol (Table 16). The second approach used was essentially the same as the one used to increase cholesterol in red blood cell membrane (Cooper et al., 1975) with a slight modification. Briefly, a solution of 15 µmoles PC and 30 µmoles cholesterol in 2 ml chloroform was dried by rotary evaporation. Then 2 ml of 25 mM HEPES pH 7.7 was added and the mixture was sonicated in a bath type sonicator for one hour to make single lamellar vesicles. The reconstituted membrane was incubated in the presence of these vesicles containing 2.5-12.5 µmol of cholesterol at 37° C for 4 hours, then centrifuged at 20,000 x g for 20 min; the supernatant containing single lamellar

vesicles was discarded and the pellet washed twice with 25 mM HEPES pH 7.7. This procedure has been shown to increase red blood cell membrane cholesterol content (Cooper et al., 1975). However, in this study, no significant change in cholesterol content in reconstituted membrane was observed (Table 17). This could be due to some differences in cholesterol and phospholipid distribution in red blood cell membrane and reconstituted membrane, which might affect the cholesterol absorption.

Finally, a method developed for cholesterol incorporation based on the procedure of Batzri et al. (1973) was tried. An ethanolic cholesterol solution was "injected" into membrane suspensions with a Hamilton syringe under a flow of nitrogen, while being stirred very rapidly. This simple method was found to be highly successful; 77-100% of the added cholesterol (0.5-3 μmol/mg protein) was incorporated into the membrane. Approximately 86-91% of the cholesterol incorporated was not available for cholesterol oxidase action, thus was considered non-exposed. This addition of cholesterol did not change the original ratio of the exposed/nonexposed cholesterol (Table 18), however, only total cholesterol content was altered. In view of the fact that PC levels have also been significantly increased in the reconstituted membrane, the change in cholesterol level in these membranes is desired.

Table 17

Incubation of Reconstituted Membrane with Vesicles High in Cholesterol Content.

Conditions	Cholesterol content (μmol/mg protein)
P_2	Ø.415
R	Ø.15Ø
R + 2.5 μmol cholesterol	Ø.128
R + 5 μmol cholesterol	Ø.152
R + 7.5 μmol cholesterol	Ø.15
R + 12.5 μmol cholesterol	Ø.153
R + 2Ø μmol cholesterol	Ø.17

Reconstituted membrane incubated in the presence of unilamellar vesicles containing 2.5-2Ø μmoles cholesterol at 37°C for 4 hours, followed by centrifugation at 2Ø,ØØØ x g for 2Ø min. Pellets were assayed for total cholesterol content as described in Methods section.

R = reconstituted membrane.

Table 18

Incorporation of Cholesterol Into the Reconstituted Membrane by Injection Method.

	Total Cholesterol Content	Total Cholesterol Content After Injection	Exposed Cholesterol	Nonexposed Cholesterol	Exposed/ nonexposed ratio	% of exogenous cholesterol incorporated
P_2	0.57 ± 0.052 (2)	0.030 ± 0.006 (2)	0.54 ± 0.05 (2)	5.50 ± 2.39 (2)		
R	0.32 ± 0.04 (2)	0.047 ± 0.012 (2)	0.35 ± 0.11 (2)	3.27 ± 0.74 (2)		
RI (0.3)		0.56	0.067	0.493	7.36	92
RII (1.5)		1.82	0.168	1.652	10.15	100
RIII (2.34)		2.38	0.325	2.055	15.79	88
RIV (3.37)		2.92	0.385	2.536	15.19	77

Cholesterol concentrations are μmol/mg protein. Data for P_2 and R are mean ± SEM and numbers in parentheses are the number of experiments. R = reconstituted membrane RI, RII, RIII and RIV are the reconstituted membranes in which 0.3, 1.5 2.34 and 3.37 μmol cholesterol were respectively injected.

a. Membrane enzyme activities after cholesterol incorporation.

In order to examine the characteristics of this new membrane, the activities of Na^+-K^+-ATPase and Ca^{++}-ATPase were determined. As shown in Table 19, the activity of Na^+-K^+-ATPase was not changed significantly before or after cholesterol incorporation; however, there was a remarkable increase in the Ca^{++}-ATPase activity. The Ca^{++}-ATPase activity was minimal (2.9% of native membrane) before cholesterol addition, but increased to 21.6% after. These data thus suggest that the enzyme activity is dependent very much on the cholesterol level. This is in agreement with the reported literature (Warren et al., 1974; Madden et al., 1981).

b. Membrane morphology after cholesterol incorporation

Effect of cholesterol incorporation into the reconstituted membrane was also studied in terms of vesicle size, intramembrane particle size and distribution visualized by freeze fracture electron microscopy (Figure 9C). Incorporation of cholesterol into reconstituted membrane did not significantly change any of above mentioned parameters. This indicates that cholesterol did not change morphology of the reconsituted membrane measured by electron microscopy.

Table 19

Effect of Cholesterol Incorporation on Membrane Enzyme Activities.

	P_2	R	R+Cholesterol (0.5 μmol/mg protein)	R:P_2 Activity Ratio
Na^+-K^+-ATPase	0.398 ± 0.0213 (10)	0.097 ± 0.0049 (10)	0.097 ± 0.0088 (10)	24.5%
Ca^{++}-ATPase	0.048 ± 0.0052 (7)	0.0014 ± 0.0007 (7)	0.0104 ± 0.0009 (7)	21.6%

Enzyme activities are expressed as μmol/mg protein/min. Numbers are means ± SEM and the numbers in parentheses are the number of experiments.

c. Membrane binding characteristics after cholesterol incorporation.

Binding properties of ^{3}H-etorphine to reconstituted membrane before and after cholesterol incorporation were also studied. The concentration of unlabeled ligand to displace 50% of the labeled opiate (IC_{50}) was 2.9×10^{-7} M. The IC_{50} value for cholesterol-incorporated membrane is shown in Table 20. The IC_{50} values were calculated by using the equation:

$$\% \text{ inhibition} = \frac{D \times 100}{IC_{50} + D}$$

The IC_{50} value for cholesterol-incorporated reconstituted membrane varied between 1.9-5.9 nM, compared to 1.64 nM in the case of native P_2 membrane. Thus despite the variability there was a significant improvement. This variability could be due to several steps involved in the remaking of the membrane. However, cholesterol is not known to be directly involved in opiate receptor binding, but these results clearly show it is in some way associated with the receptor.

In order to further characterize the binding properties of the native and reconstituted membrane and the effect of cholesterol incorporation, saturation binding isotherms for different opiate ligands were analyzed. A computer program

Table 2Ø

Inhibition of ^{3}H-Etorphine Binding by Unlabeled Etorphine of Reconstituted Membrane Before and After Incorporation of Cholesterol.

	$IC_{5Ø}$ (nM)	
P_2	1.64 ± Ø.373	(11)
R	29Ø ± 137	(5)
R + cholesterol (Ø.5 µmol/mg protein)	3.9 ± 2.Ø2	(8)

^{3}H-etorphine (Ø.5 nM) incubated with Ø.5 mg P_2 or reconstituted membrane in the presence of different concentrations of unlabeled etorphine in 25 mM HEPES buffer pH 7.7. The mixture was incubated at 37° C for 3Ø min., followed by one hour incubation at 4° C. Binding was then assayed by the filter method, as described above. R = reconstituted membrane

was used in this analysis which is based on a least square curve fitting technique. The saturation experiments were performed with different concentrations of the tritiated opiate ligands and non-saturable binding was determined as the binding in the presence of 1 μM of the non-labeled form of the same ligand. The difference between the total and non-saturable binding was considered to be the specific binding. The saturation curve of ^{3}H-etorphine binding and its corresponding Scatchard plot to native, reconstituted and cholesterol incorporated membrane are shown in Figures 26-A through C and their affinity constants and capacities are shown in Table 21.

The affinity of site 1, K_1, and site 2, K_2, decreased 4 and 2.5 fold, respectively, after reconstitution compared to those of the native membrane. The capacity of site 1, R_1, and site 2, R_2, was decreased 83% and 69% respectively after reconstitution. This change in receptor binding activity could be due to changes in membrane lipid composition such as a higher PC level or a lower cholesterol content. This notion was supported by the effect of cholesterol incorporation into the reconstituted membrane on receptor binding activity. It was demonstrated that cholesterol incorporation increased the affinity of site 1, K_1, and also the capacity of this site increased 83% over that of the plain reconstituted membrane. There was not a significant change in the affinity of site 2, but the capacity of this

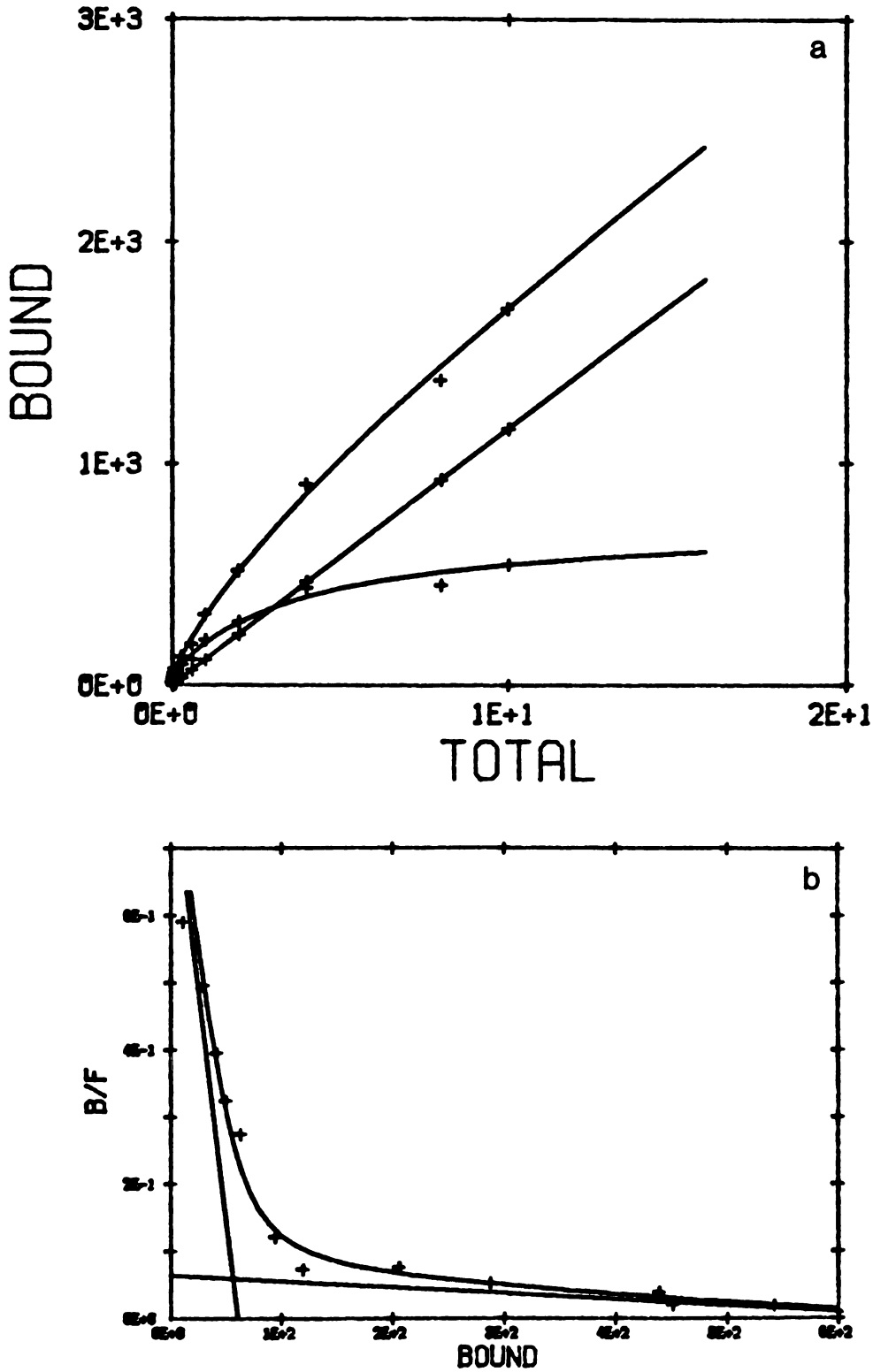

Figure 26-A. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding to native membrane.

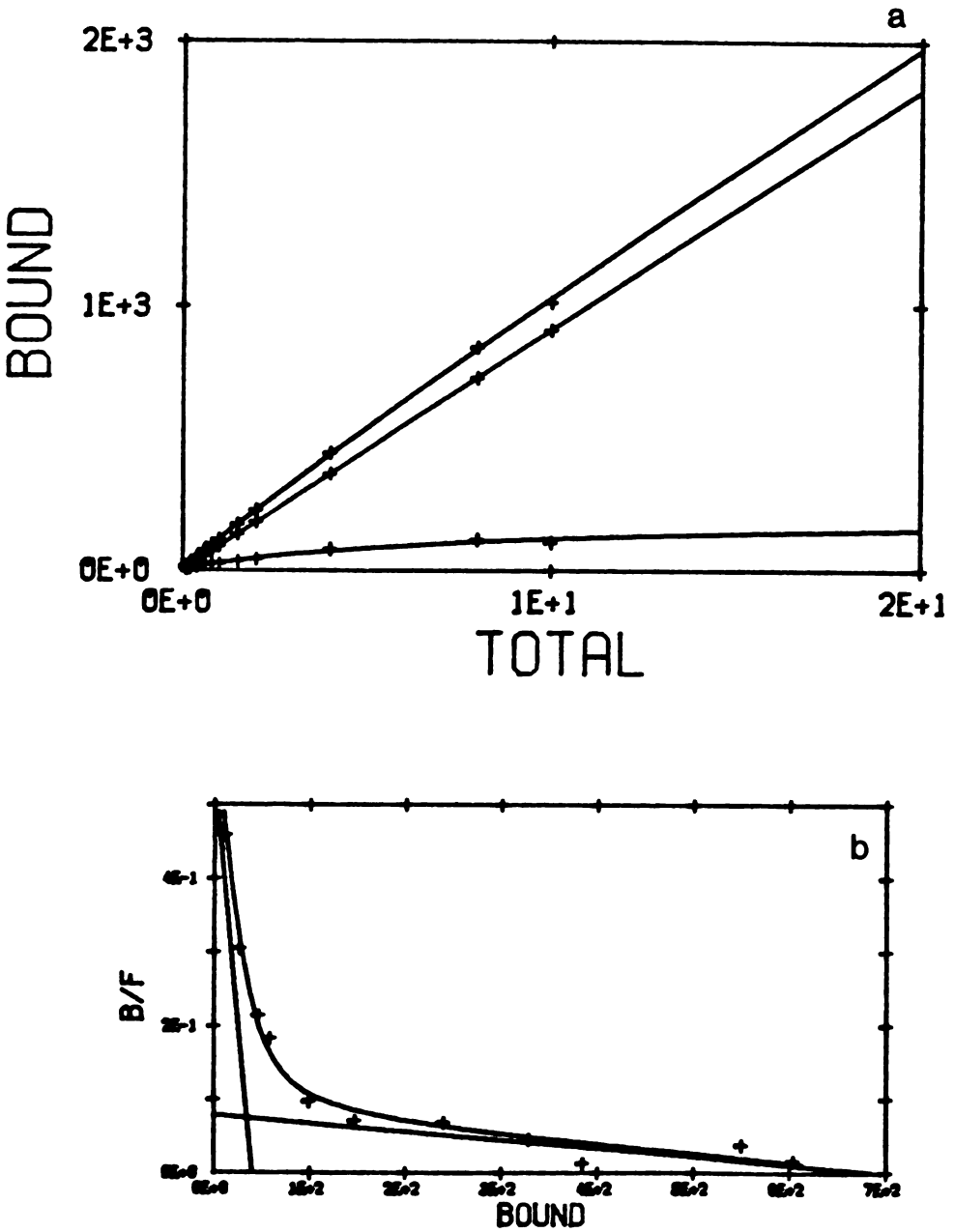

Figure 26-B. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding to reconstituted membrane.

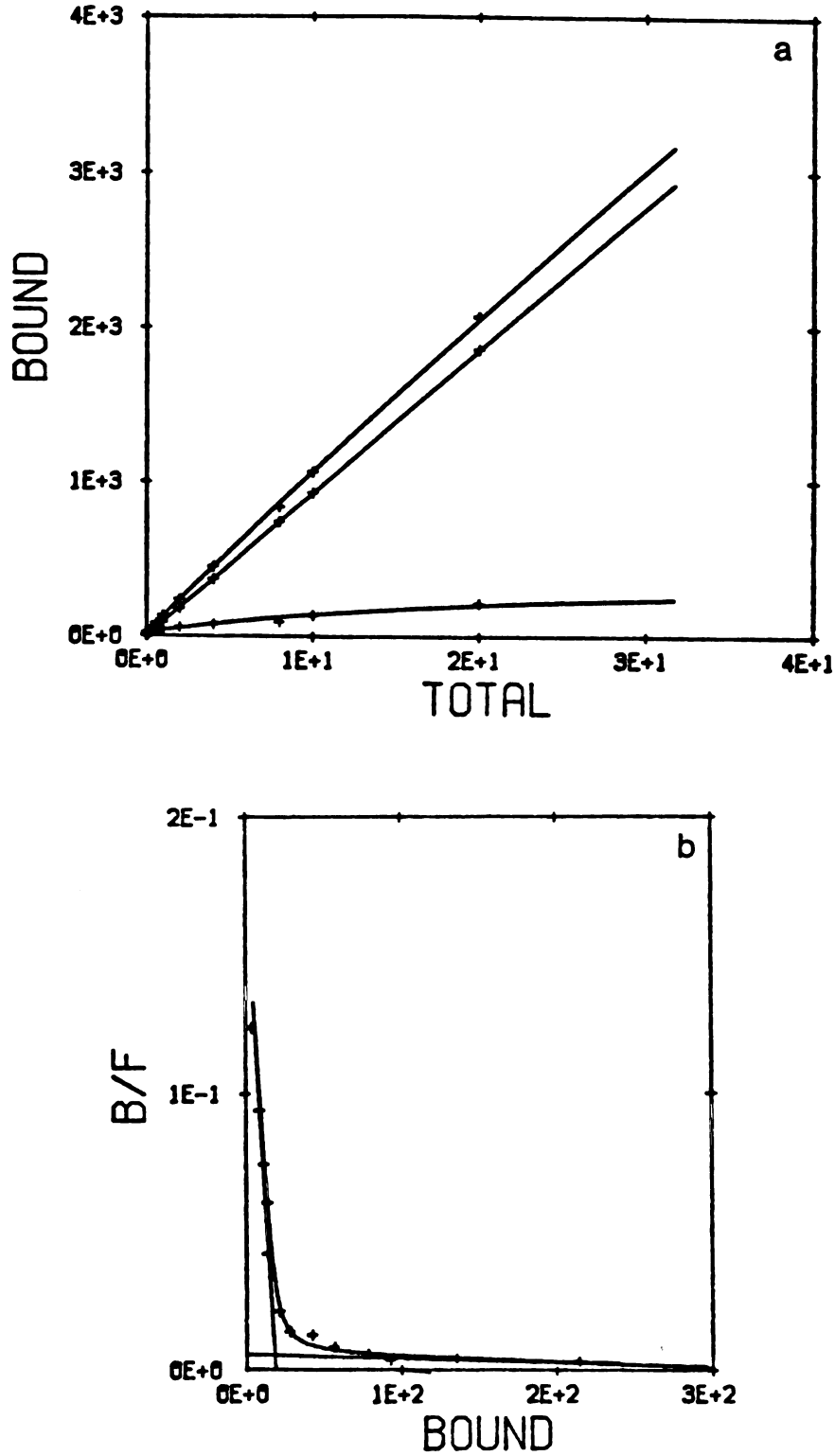

Figure 26-C. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-etorphine binding to reconstituted membrane after cholesterol incorporation.

Table 21

Properties of ^{3}H-Etorphine Binding to Native, Reconstituted and Cholesterol-Incorporated Membranes.

	K_1 (nM^{-1})	K_2 (nM^{-1})	R_1 (fmol/mg protein)	R_2 (fmol/mg protein)
Native	41.2 ± 13.3	0.275 ± 0.057	61 ± 9.2	697 ± 71
Reconstituted	10.5 ± 6.93	0.112 ± 0.037	10.2 ± 3.5	218 ± 41
Reconstituted +cholesterol	34.2 ± 8.08	0.052 ± 0.021	18.8 ± 1.8	371 ± 108

K_1 and K_2 are affinity constants of ^{3}H-etorphine binding to site 1 and 2 respectively. R_1 and R_2 are their respective capacities. Values are means ± standard errors.

site was increased 70% over that of the reconstituted membrane. These results thus support the importance of membrane composition and receptor micro-environment for proper receptor activity.

The binding of ^{3}H-dihydromorphine to native and reconstituted membrane and the effect of cholesterol incorporation on it was also studied. The data are shown in Figure 27-A through C and Table 22. It was demonstrated that after reconstitution the affinity of site 1 increased tremendously, and 98% of the capacity of this site was lost. This very high affinity binding site could not be evaluated very accurately due to very low binding at low concentrations of the radioligand and the lack of enough data points for accurate analysis. However, the data were fitted to a model consisting of two binding sites in order to fit the curve to all the data points. The affinity of site 2 was decreased 2.7 fold in the reconstituted membrane compared to that of the native membrane without a significant change in its capacity. These results again indicate the change in receptor binding properties after membrane reconstitution. The effect of cholesterol incorporation on dihydromorphine binding was evaluated and it was found that the affinity of site 2 is increased to a value similar to that of the native membrane. However, there was no change in binding activity of site 1 by cholesterol incorporation.

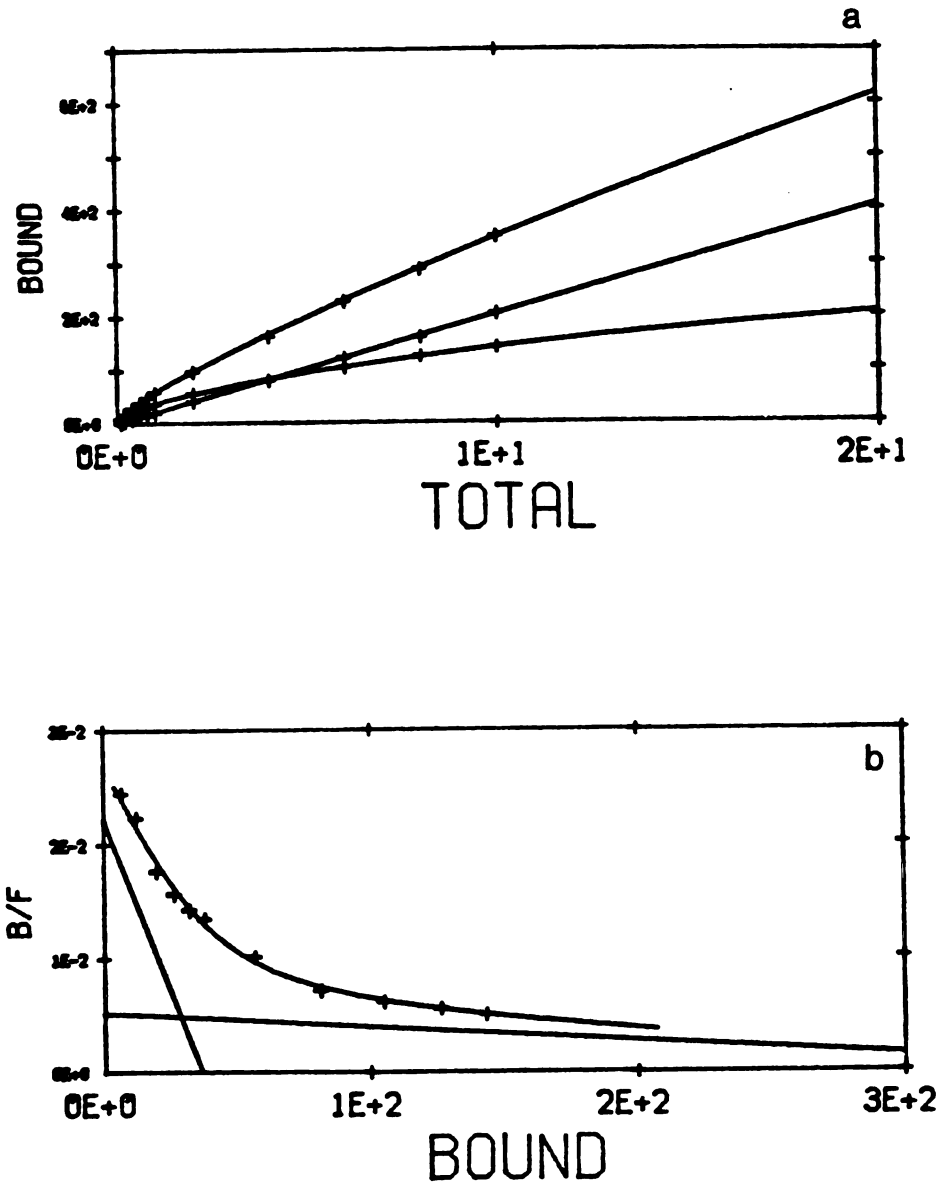

Figure 27-A. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-dihydromorphine binding to native membrane.

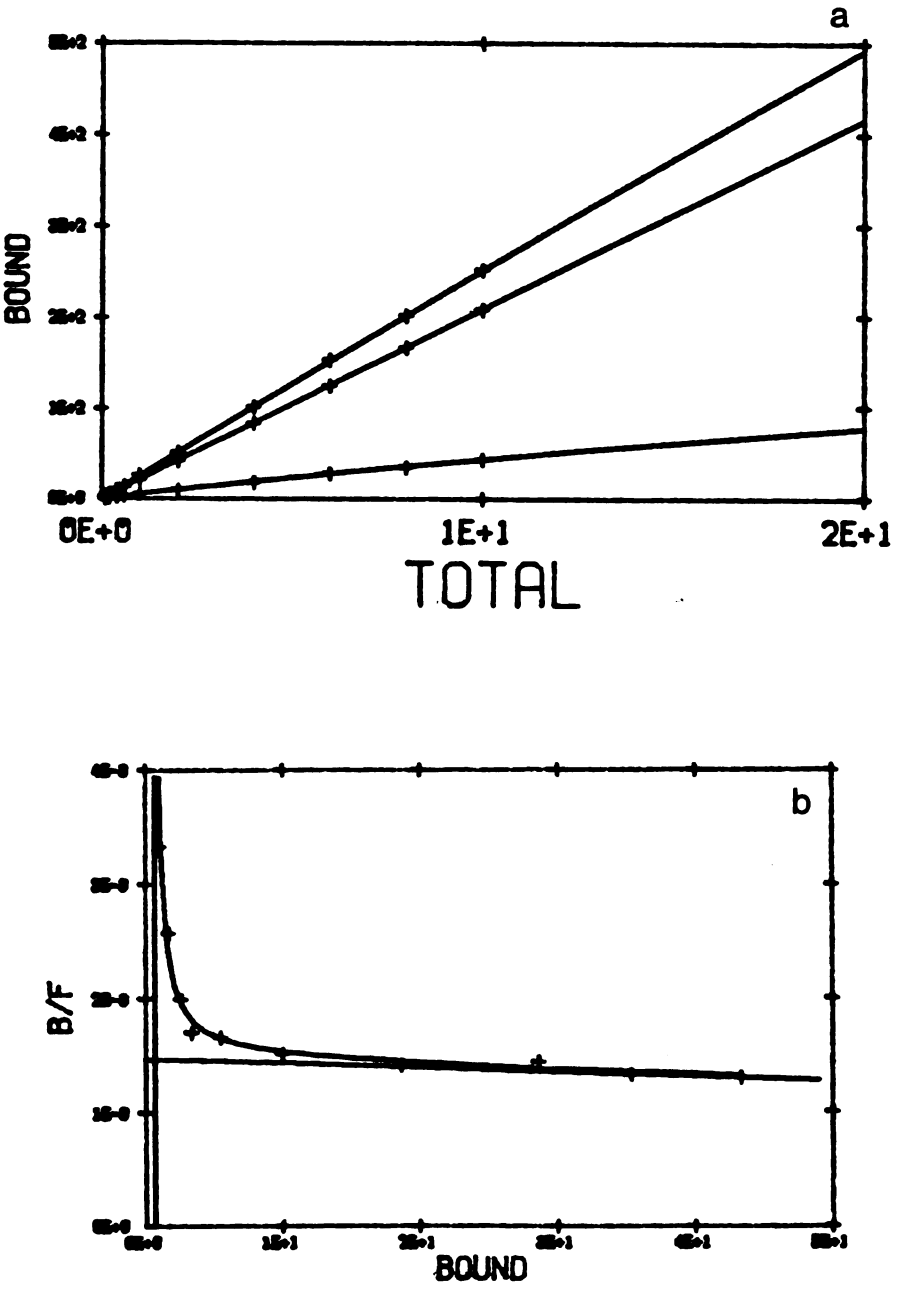

Figure 27-B. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-dihydromorphine binding to reconstituted membrane.

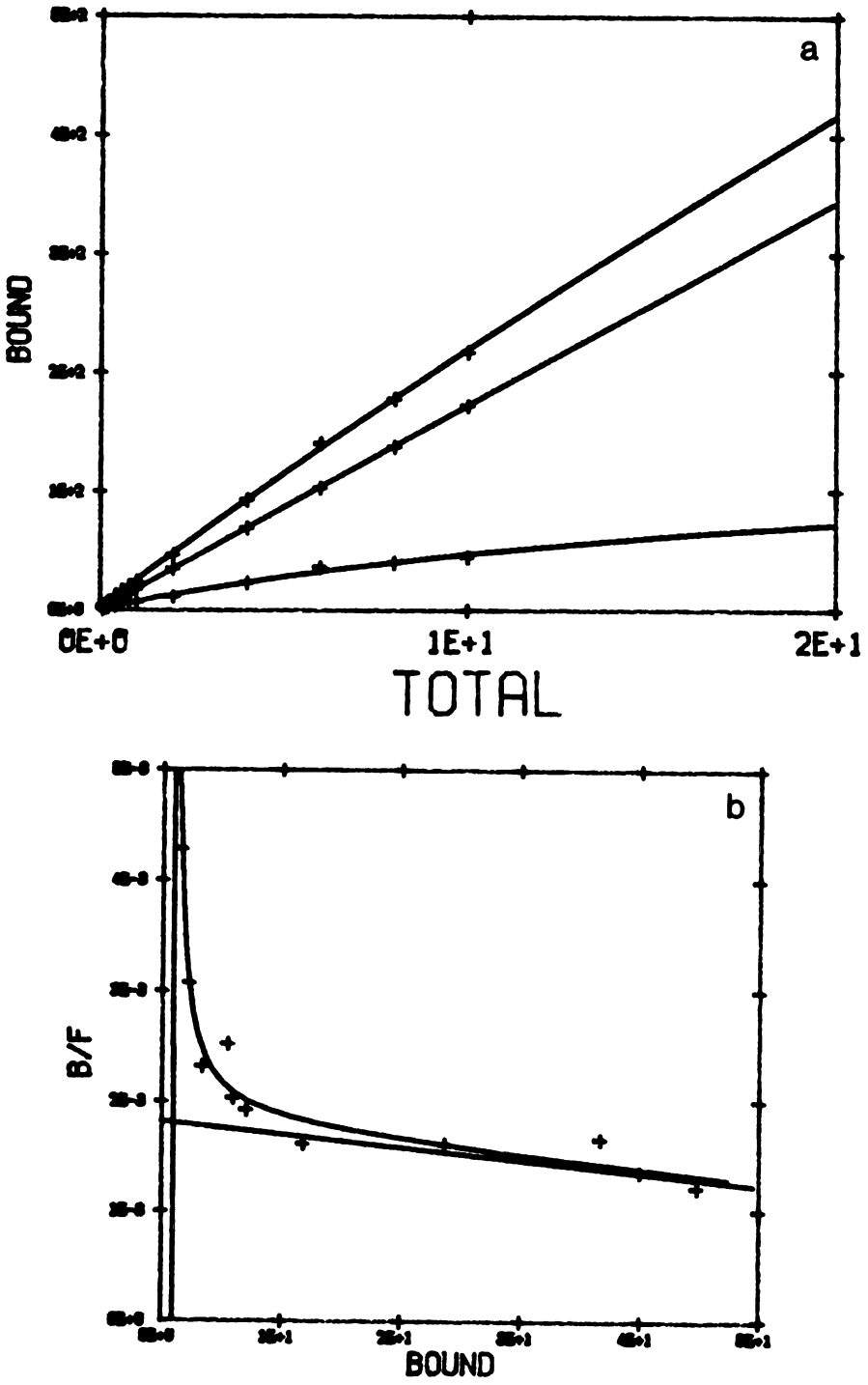

Figure 27-C. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-dihydromorphine binding to reconstituted membrane after cholesterol incorporation.

Table 22

Properties of ^{3}H-Dihydromorphine Binding to Native, Reconstituted and Cholesterol-Incorporated Membranes.

	K_1 (nM^{-1})	K_2 (nM^{-1})	R_1 (fmol/mg protein)	R_2 (fmol/mg protein)
Native	1.73 ± Ø.216	Ø.Ø356 ± Ø.Ø125	36.6 ± 4.2	42Ø ± 1Ø1
Reconstituted	448 ± 448Ø	Ø.Ø131 ± Ø.ØØ33	Ø.67 ± Ø.116	373 ± 85.7
Reconstituted +cholesterol	29Ø ± 588Ø	Ø.Ø431 ± Ø.Ø148	Ø.97 ± Ø.53	155 ± 4Ø.4

K_1 and K_2 are affinity constants of ^{3}H-dihydromorphine binding to sites 1 and 2 respectively. R_1 and R_2 are their respective capacities. Values are means + standard errors.

Furthermore the binding of an opiate antagonist, naloxone, was evaluated in native and reconstituted membrane before and after cholesterol incorporation. These data are shown in Figures 28-A through C and Table 23. There was a loss of site 1 capacity of about 96% after reconstitution and the remaining 4% had a very high affinity which is similar to the results obtained for ^{3}H-dihydromorphine binding. Again the data were fitted to two binding sites in order to include all the data points. The affinity of site 2 was decreased from Ø.175 nM to Ø.Ø12 nM, a decrease of 15 fold without a significant change in its capacity. The effect of cholesterol incorporation was also evaluated and it was demonstrated that there was no change in the capacity of site 1. However, there was an improvement in the affinity of site 2 of the reconstituted membrane after cholesterol incorporation and the affinity increased 2.3 fold.

In conclusion, it is obvious that 1) reconstituted membrane has lower affinity for binding to opiate ligands compared to native membrane, and 2) cholesterol incorporation improves the binding properties of reconstituted membrane. This is consistent with the notion of importance of receptor microenvironment for proper receptor activity.

C) Lipid Modification of Membrane by Phospholipid Exchange Protein

The PC content in the reconstituted membrane was

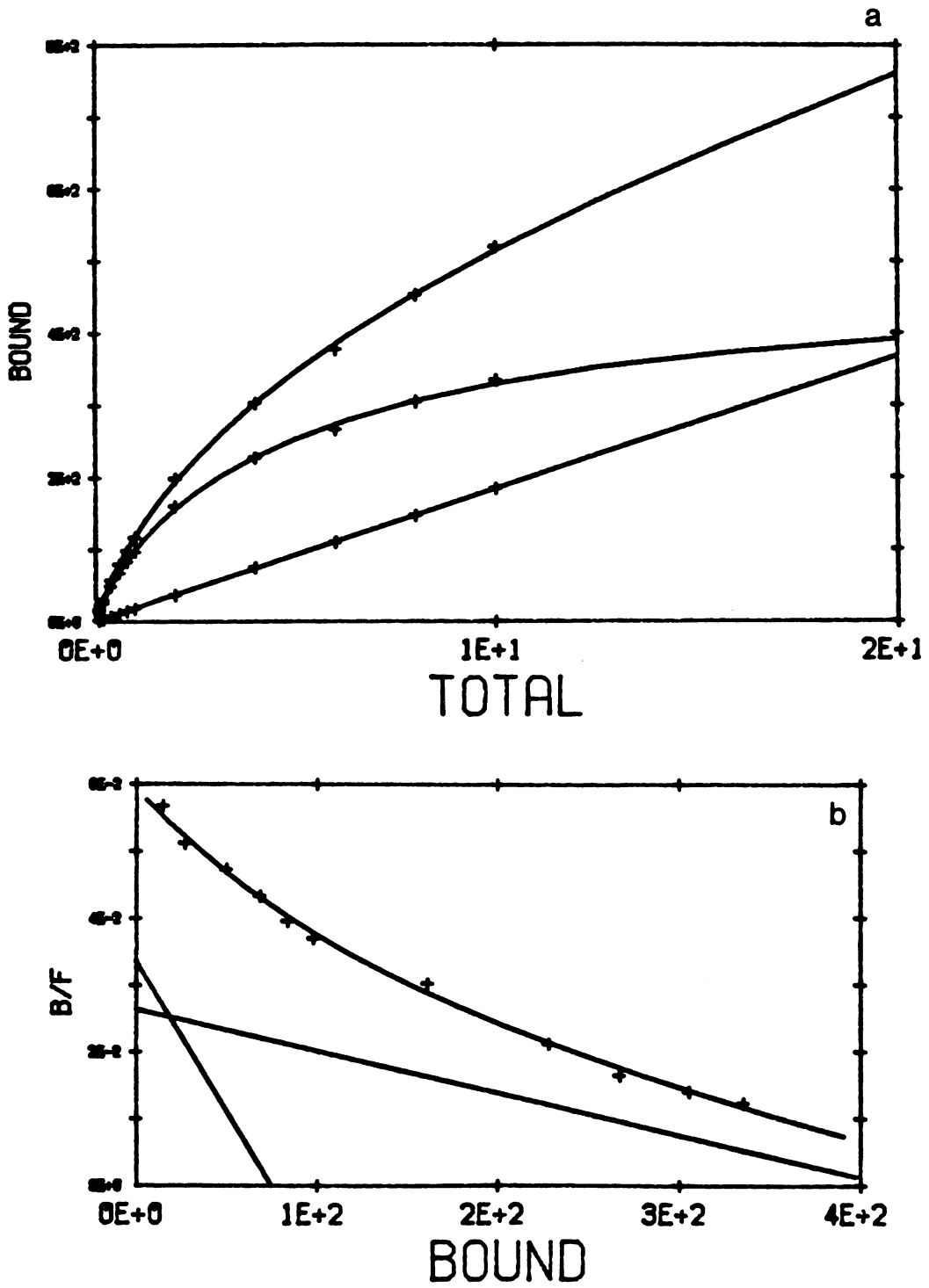

Figure 28-A. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-naloxone binding to native membrane.

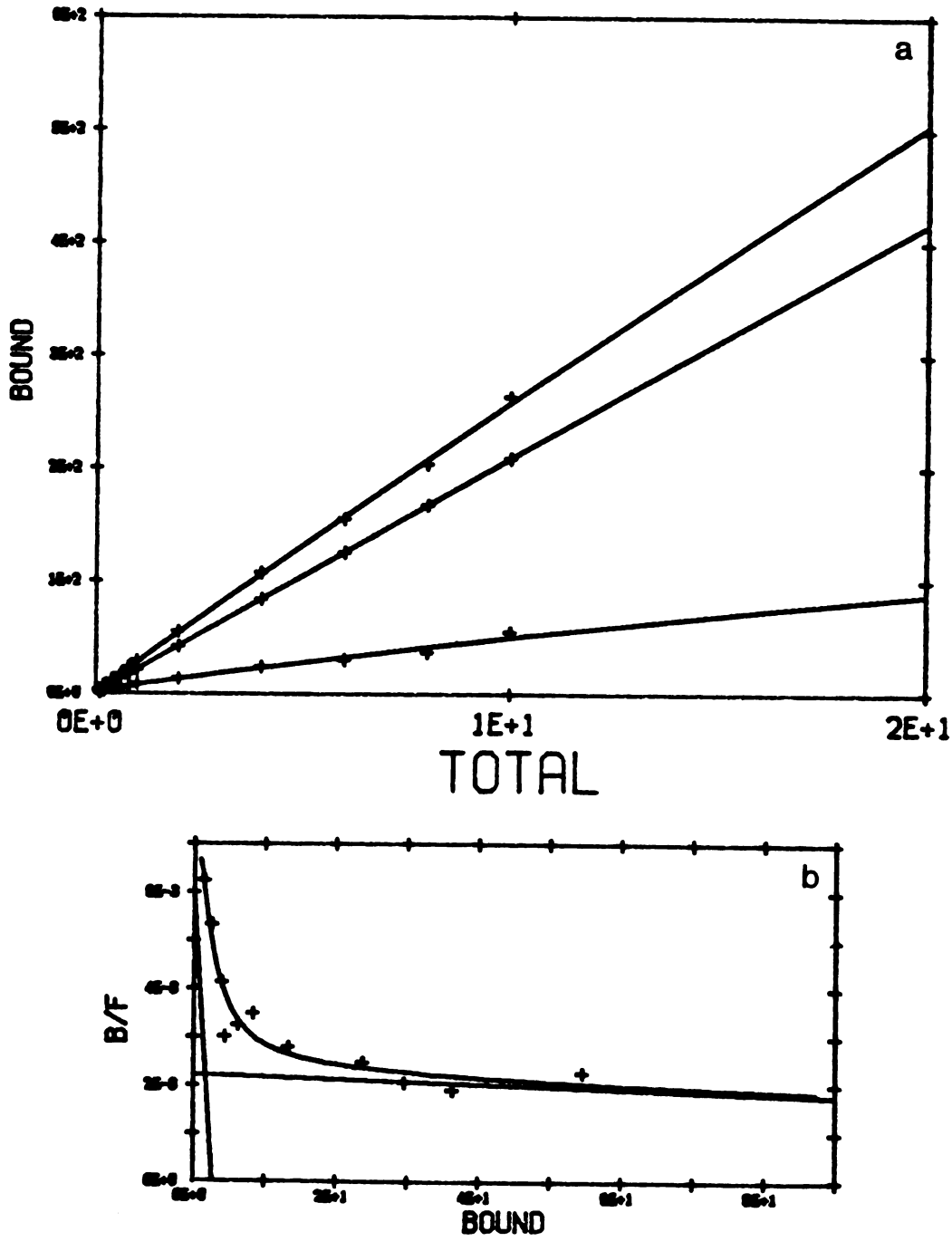

Figure 28-B. Saturation isotherm (a) and Scatchard plot (b) of ^{3}H-naloxone binding to reconstituted membrane.

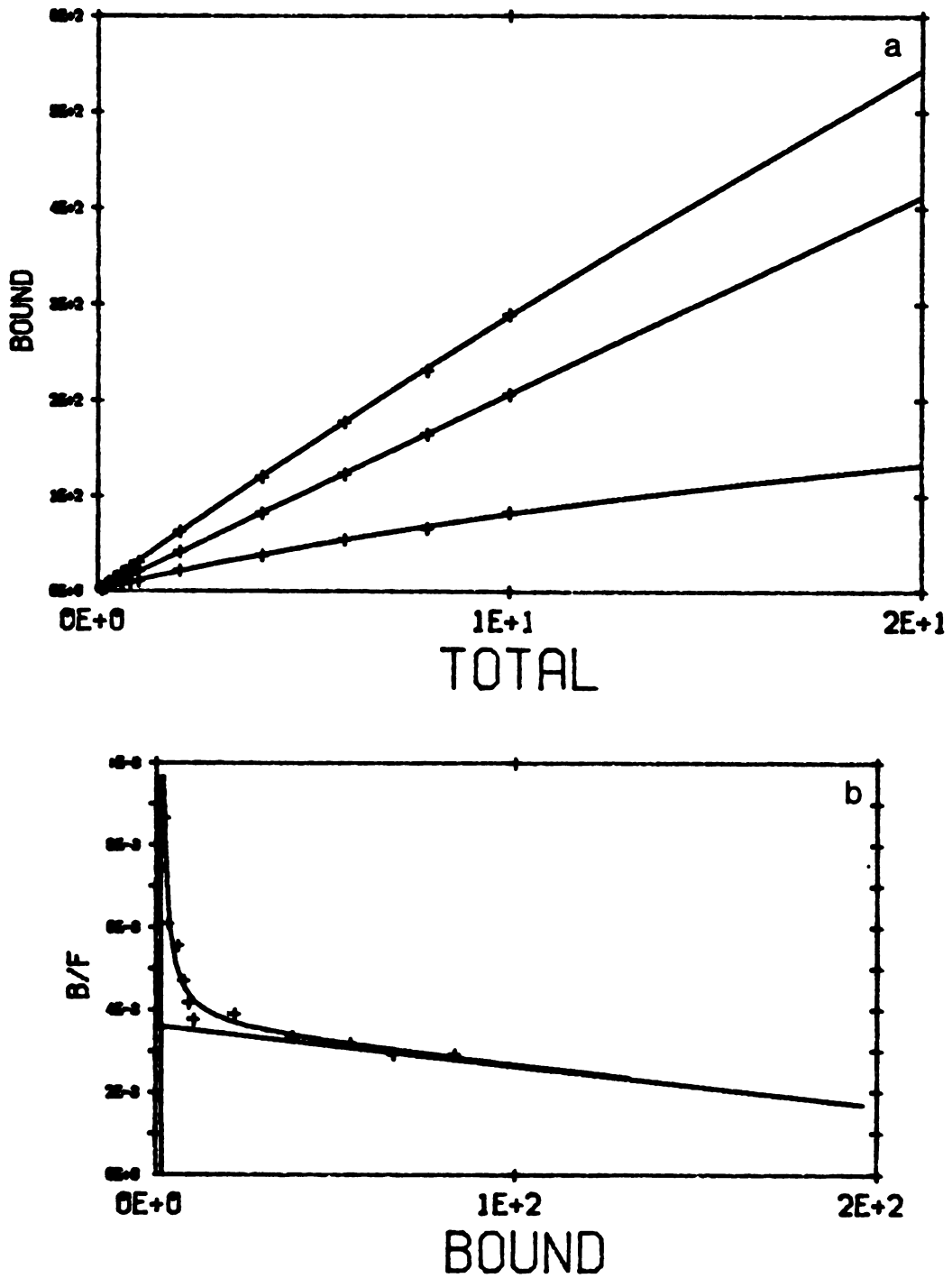

Figure 28-C. Saturated isotherm (a) and Scatchard plot (b) of ^{3}H-naloxone binding to reconstituted membrane after cholesterol incorporation.

Table 23

Properties of ^{3}H-Naloxone Binding to Native, Reconstituted and Cholesterol-Incorporated Membranes.

	K_1 (nM^{-1})	K_2 (nM^{-1})	R_1 (fmol/mg protein)	R_2 (fmol/mg protein)
Native	1.25 ± Ø.43	Ø.175 ± Ø.Ø415	74.4 ± 37.8	417 ± 19.3
Reconstituted	5.33 ± 4.75	Ø.Ø12 ± Ø.Ø189	2.74 ± 1.3	447 ± 636
Reconstituted + cholesterol	31.6 ± 34.5	Ø.Ø28 ± Ø.ØØ63	1.9 ± Ø.42	369 ± 68.9

K_1 and K_2 are affinity constants of ^{3}H-naloxone binding to sites 1 and 2 respectively. R_1 and R_2 are their respective capacities. Values are means ± standard errors.

increased remarkably due to acylation of lyso-PC and oleoyl CoA to generate PC and reconstitute the membrane. Therefore, attempts were made to remove some of the excess PC from reconstituted membrane by the use of phospholipid exchange protein.

Exchange of phospholipids between membrane fractions was first observed by Wirtz and Zilversmit (1968a,b). The low level of exchange observed when rat liver mitochondria and microsomes were incubated in the buffer, was significantly stimulated when 105,000 x g liver supernatant was added to the incubation medium. Beef liver and heart cytosol were shown to contain a heat labile, non-dialyzable factor which stimulates phospholipid exchange, referred to as phospholipid exchange protein (PLEP). This protein was first partially purified from beef heart by Wirtz and Zilversmit (1970). It has been suggested that the physiological role of these proteins is to transport phospholipids from their site of synthesis on the endoplasmic reticulum to other membranes of the cell (Dawson, 1973).

Phospholipid exchange protein is also capable of a net transfer of phospholipids between membrane fragments. Lipid exchange and lipid transfer can be considered as related events arising from the dynamic behavior of lipids in biological lipoproteins and membranes. Exchange of lipids occurs when the rate of movement of a lipid (e.g.,

cholesterol, PC) between two lipid-protein complexes is the same in both directions. Net transfer of lipid occurs when the bidirectional movement of the lipid between two lipid-protein complexes is unbalanced (Bell, 1978; Crain and Zilversmit, 1980b).

In a typical membrane system more than 60% of the available lipid can be exchanged, those available in the outer monolayer of bilayer (Johnson et al., 1975). Exchange proteins apparently do not work on the inner half of the bilayer (Johnson and Zilversmit, 1975; King and Quinn, 1980).

Exchange proteins are specific in recognizing the head group of particular phospholipids and specificities may vary with the source of the enzyme. In beef heart lipid specificity for the enzyme is in the order of PC> sphingomyelin (Enhnholm and Zilversmit, 1973); beef brain, PI>PC (Helmkamp et al., 1974); beef liver PC (Kamp et al., 1973); rat livers (M. W.> 16000) PC (Lumh et al., 1976); rat liver (M. W. > 25000) PI>>PC (Lumh et al., 1976); rat liver (M. W. 13000) PE>PC>PI>sphingomyelin (Bloj and Zilversmit, 1977) and potato tubers PI>PC>PE (Kader, 1975).

Because of their properties, the phospholipid exchange proteins have been used as membrane probes for the study of phospholipid distribution and motion in natural membranes (Bloj and Zilversmit, 1976) and in liposomes (Johnson et al., 1975; Rothman and Dawidowicz, 1975). They can also be

used to modify membrane phospholipid composition by using a specific phospholipid donor system (Barsukov et al., 1978; King and Quinn, 1980). This approach was used to reduce the PC content of reconstituted membrane, which was much higher than that of native membranes.

Phospholipid exchange protein from beef liver was purified as reported by Kamp et al. (1973), as given in the Methods section. This protein is specific for transfer of PC from a donor membrane, high in PC content, to an acceptor membrane, that has a low PC content (Wirtz et al., 1980).

In order to measure PC transfer by exchange protein, PC from "donor" (unilamellar) vesicles was labeled with ^{14}C-PC, and the "acceptor" (multilamellar) liposome contained ^{3}H-glycerol as a nontransferable marker. The transfer of labeled PC from unilamellar vesicle to multilamellar vesicles was evident from the decrease in labeled PC in the donor vesicle after incubation in the presence of exchange protein. The amount of labeled PC transferred was calculated as the ratio of $^{14}C/^{3}H$ of donor liposomes after incubation to $^{14}C/^{3}H$ of (donor liposomes + acceptor vesicles) before incubation (Johnson and Zilversmit, 1975). Concentration of exchange protein was chosen so that the transfer of ^{14}C-PC was linear for at least 30 minutes. The percentage transfer of ^{14}C-PC from unilamellar vesicles containing 25 nmol PC was 56%, a transfer of 140 nmol of PC/ml of protein solution (1.44 mg/ml)/hour.

Next, the technique was applied to the transfer of PC from reconstituted membrane. Reconstituted membrane can be spun down at 20,000 x g for 20 min., therefore, they are considered as large membrane fragments which now served as "donor." Since this exchange protein would transfer other lipids and from our reconstituted membrane we wished to transfer only PC, the concentration of all other lipids present in the acceptor membrane were maintained as closely as possible to the lipid composition of the donor vesicles. To do this, a lipid extract of P_2 membrane was used to prepare single lamellar vesicles, as described in Methods. Reconstituted membrane was incubated for 20 minutes in the presence of exchange protein, and these single lamellar vesicles. The reconstituted membrane was then centrifuged, the supernatant containing phospholipid exchange protein and unilamellar vesicles discarded, the pellet resuspended in buffer and incubated again for 20 minutes with fresh unilamellar vesicles and phospholipid exchange protein. This incubation procedure repeated up to five times, designated as RI, RII, RIII, RIV, and RV; this was necessary because the PC content in the two vesicles (donor and acceptor) reached equilibrium state in 20 minutes, at which point no further transfer could proceed.

The data are shown in Table 24. Incubation of reconstituted membrane with phospholipid exchange protein decreased the phospholipid content of the membrane with each

incubation until the fourth one, after which no further phospholipid decrease was observed. This is consistent with the report by Kornberg and McConnell (1971) and Hauser and Irons (1972), that protein mediated net transfer stopped at a depletion of 20% of PC molecule from the donor vesicles in which only 20% of the phospholipids were available for exchange.

The effect of phospholipid exchange protein on binding characteristics of reconstituted membrane is shown in Table 25. It is interesting to note that after removal of 27% of the PC from reconstituted membrane with this method, there was a change in IC_{50} values of ^{3}H-etorphine binding displacement; there was a significant improvement in IC_{50} value. This value for original reconstituted membrane was about 290 nM, but became 6.3 nM and 9.3 nM after 3 and 5 incubations, respectively, with phospholipid exchange protein.

In conclusion, it is clear that changing the membrane lipid composition can seriously interfere with the normal functioning of membrane proteins. The change in opiate receptor activity after reconstitution could be due to a change in the phosphatidylcholine and cholesterol content, and this is supported by the above data.

Table 24

Effect of Incubation of Phospholipid Exchange Protein on Phospholipid Content of Reconstituted Membrane.

	Phospholipid content (umol/mg protein)	
P_2	Ø.7458 ± Ø.Ø32	(4)
P_2 incubated 3 x 2Ø min	Ø.7458 ± Ø.324	(4)
P_2 incubated 5 x 2Ø min.	Ø.7458 ± Ø.Ø324	(4)
R	1.44 ± Ø.1Ø4	(4)
RI	1.448 ± Ø.Ø71	(3)
RII	1.387 ± Ø.Ø177	(2)
RIII	1.25 ± Ø.Ø424	(2)
RIV	1.Ø59	(1)
RV	1.Ø7	(1)

Data are means ± SEM and numbers in parentheses are the number of experiments. RI to RV are the reconstituted membrane incubated with PLEP for 1 x 2Ø minutes to 5 x 2Ø minutes.

Table 25

Effect of Incubation of Phospholipid Exchange Protein on IC_{50} Values in Reconstituted Membrane.

	IC_{50} (nM)
P_2	2.49 ± Ø.65 (4)
P_2 incubated 3 x 2Ø min	4.57 ± 1.19 (3)
P_2 incubated 5 x 2Ø min	3.68 ± Ø.11 (2)
R	2.9Ø ± 137 (5)
RI	11.3 ± 4.8 (3)
RII	ND
RIII	6.26 ± 1.76 (4)
RIV	ND
RV	9.34 ± 5.34 (2)

Data are expressed as mean ± SEM and numbers in parentheses are the number of experiments. RI to RV are the reconstituted membrane incubated with PLEP for 1 x 2Ø minutes to 5 x 2Ø minutes.

ND = not determined

IV. DISCUSSION

Opiates are thought to exert their pharmacological actions through membrane bound receptors. In order to understand opiate action it is thus essential to characterize the structure of the receptor and its mechanism of interaction with other membrane components. This work must begin with the isolation and purification of the receptor.

IV-1. Opiate Receptor Solubilization

In order to isolate the receptor, it is necessary to dissociate the receptor from other membrane components. Dissociation of proteins from lipids is to some extent possible using mild methods such as chelating agents, change in pH, ionic strength, enzymatic digestion, etc. These procedures usually lead to a partial solubilization of proteins which are loosely bound (extrinsic proteins), however, any highly bound proteins (intrinsic proteins) generally still remain with membrane matrix. Thus for a more efficient solubilization of membrane, the use of a detergent is usually required (Helenius and Simons, 1975).

Since it is important that the isolated receptor remains active, solubilization must be accomplished without denaturing the protein; this requires careful selection of the detergents. Although detergents vary considerably in

their chemical structure, they all contain certain dual features that make them both lipid- and water-soluble. Thus, theoretically, they can disperse membrane lipids and tightly bound proteins into a soluble state. Studies show that biological membranes progressively solubilize as detergent concentration increases. At low detergent concentrations (Ø.Ø1-Ø.1 mg detergent per mg membrane lipid) a small removal of lipids from the membrane is observed; at higher concentrations (Ø.1-1 mg detergent per mg membrane lipid), selective extraction of membrane protein can occur, even though the membrane bilayer remains essentially intact. If still higher detergent concentration is added (about 2 mg detergent per mg lipid), total solubilization of membrane occurs and results in formation of small detergent micelles containing lipid, protein and mixed lipid-protein complexes.

Although there are many different detergents available, they are mainly divided into two types, A and B. Type A includes the detergents which form liquid crystals at high concentration and type B, those which do not show this property. Type A surfactants are in the form of anionic, cataionic, ampholitic (zwitterionic) and non-ionic groups. The most widely used detergent of this class is sodium dodecyl sulfate, an anionic detergent, lysolecithin (ampholitic) and Brij, Lubrol and Triton (non-ionic surfactants). Surfactants of type B include bile salt

derivatives such as sodium cholate and digitonin. Ionic detergents at the concentration that have to be used for complete membrane solubilization often denature the protein; they also dissociate complex proteins into their constitutent polypeptide chains; sodium dodecyl sulfate, for example, is the standard detergent used to denature proteins. Non-ionic detergents are milder, however, and some proteins can maintain their tertiary structures in their presence.

Detergents show some selectivity in what they remove. Lysolecithin, for example, has a tendency to remove PC more efficiently than PE (Deamer, 1973). Triton-X-1ØØ has an enormous capacity to solubilize sphingomyelin and cholesterol (Yedgar et al., 1974) and it extracts choline containing phospholipids better than amino phospholipids (Simons et al., 1974). Bile salts solubilize phospholipids, but are poor solubilizers of cholesterol and other large apolar molecules (Kirkpatrick et al., 1974).

In all cases, detergent molecules replace the extracted lipids on the membrane. Since the activities of membrane proteins are mainly determined by their conformation in the native membrane (Hong and Hubbell, 1973), which in them are affected by surrounding lipids, it becomes essential to mimic this lipid with detergents as closely as possible. This goal can best be achieved by using detergents formed from endogenous phospholipids. Lysophospholipids, for

example, are formed from diacylphospholipids through the action of phospholipases, and small amounts are present in most membranes. When added in high enough concentrations to the membrane, these zwitterionic detergents solubilize the latter, and provide the next best approximation to the native membrane environment. Since the polar and apolar moieties of the phospholipids closely resemble those of the membrane diacylphospholipids, one might expect that they would be especially mild in their effects on sensitive membrane proteins.

Several attempts have been made to solubilize opiate receptors by using a variety of non-physiological detergents. One of the first was made by Simon et al. (1975) who used Brij-36-T to solubilize receptor that was pre-bound to ^{3}H-etorphine, that is, in an inactive state. The molecular weight of the inactive etorphine-macromolecule complex was reported to be 360-400 K daltons. Since this complex was associated with detergents, however, the molecular weight can not be absolutely certain. It is known that incorporation of proteins into detergent micelles causes a decrease in size of membrane fragments, and also the size of the micelles depend on the detergent/lipid ratio used for solubilization (Helenius and Simons, 1975). Therefore the correct molecular weight can not be estimated in a detergent solution.

These workers later used digitonin to solubilize active

opiate receptors from toad brain (Ruegg et al., 1980). Since they could not repeat this work with rat brain, they assumed that the opiate receptor is more stable in solubilized form in a non-mammalian system. Thus, while this detergent has an advantage over Brij 36-T, the solublized active receptor cannot be compared to the well studied membrane in mammalian brain.

Simond et al. (1981) used CHAPS, a zwitterionic derivative of cholic acid, to solubilize the opiate receptor from rat, beef brain and from membrane of neuroblastoma glioma hybrid cells (NG 108-15). The receptor solubilized by CHAPS had similar characteristic to that solubilized by digitonin. The size of the solubilized receptor was reported to be 70 A ; since this size is measured in the presence of detergent, however, it again can not be an accurate estimate. The binding affinities of opioids to the solubilized receptor was about five fold lower than those to the membrane bound receptor. Thus, there was some change in receptor properties upon solubilization. Another problem in using CHAPS to solubilize the membrane is that a proper ratio of CHAPS/protein is essential for solubilization of an active receptor, and large concentrations of CHAPS lead to receptor inactivation. Furthermore, the receptor characterization was carried out with only 300-400 cpm bound radioactivity to the receptor, making precision difficult. Finally, the recovery of the solubilized receptor was

only 10% of the receptors originally present in the membrane.

Still another detergent that has been used is Triton X-100 (Bidlack and Abood, 1980). In this procedure Triton X-100 was removed after solubilization by Bio-bead SM-2; therefore the receptor studied was probably not truly solubilized. Also the receptor properties were changed by solubilization, e.g., solubilized receptor had a greater affinity for the antagonist, naloxone, and a lower affinity for levorphanol, than that of the membrane bound receptor.

Finally, a sonication method has been used for opiate receptor solubilization (Cho et al, 1981). The major advantage of this method is the absence of detergent in the preparation, this makes changing the receptor's characteristic less likely. The receptor binding properties were in fact highly similar, in some cases, to those of the membrane bound receptor. The disadvantage of this method, however, is that the solubilized membrane was highly heterogeneous. Furthermore, certain opioid ligands, notably enkephalins, did not bind to this material at all.

In conclusion, every method used to solubilize the opiate receptor has some disadvantages: Brij isolates only inactive receptors; CHAPS yields low receptor recovery; Triton X-100 alters the receptor binding affinities for some of the opioid ligands tested. These changes in receptor characteristic could have several explanations: 1) the opiate receptor binding site is destroyed and another less

selective site is being measured, 2) the receptor undergoes conformational change during its removal from the membrane environment, 3) loss of a cofactor essential for opiate binding and 4) replacement of the natural environment surrounding the receptor present in the native membrane by the non-natural components used as detergents.

With respect to the last, the requirement for a proper lipid environment has been shown for many proteins (Kagawa and Racker, 1971; Ragan and Racker, 1973; Sun et al., 1971). The native protein conformation is greatly dependent on hydrophobic and hydrophilic forces surrounding the proteins, and these groups are also important for proper physiological functioning of the proteins. Thus, it seems preferable to use a natural detergent such as lysophosphatides, for solubilization of the opiate receptor, in this way replacing the lipids surrounding the protein by a group of lipids which more closely resemble the native membrane environment. Lysophosphatide (LPC) is one of the acylation cycle intermediates involved in phospholipid metabolism and synthesis and is usually found in very small concentration in different tissues. In high concentration LPC is toxic to cell membranes and can perturb the membrane integrity by merely solubilizing the membrane. However, since LPC is a "natural detergent" and is thought to be much milder than synthetic detergents, it has been used by several groups for solubilization of some enzyme proteins. Some membrane

enzymes are known to be destroyed by synthetic detergents, but can retain activity after LPC treatment. Rydstrom et al. (1974) reported that mitochondrial nicotinamide nucleotide transhydrogenase and succinate dehydrogenase could be preferentially solubilized with lysolecithin, thus leading to solubilized enzymes with higher specific activities compared to those removed with Triton X-100. Also, in contrast with Triton X-100, even an excess of lysolecithin does not inhibit the activities of the above mentioned enzymes. Another example of the superiority of LPC is seen in solubilization of a membrane-bound enzyme, acyl-CoA-lysolecithin acyltransferase; solubilization by Triton X-100 causes a complete loss of activity while use of lysolecithin as detergent preserves enyzmatic activity totally (Weltzien et al., 1979).

In any case, with all the effort being made to solubilize the opiate receptor in an active form, its purification seems imminent. If the receptor is purified, then the chemical and physical nature of the receptor can be better characterized. Also, the number of binding sites and eventually the number of components constituting each binding site can be determined. After the opiate receptor is isolated and purified, it must be correlated with opiate function in order to demonstrate that the purified component is a true opiate receptor. The nature of the biochemical events which are triggered by the binding of opiates to

their receptor is not well known. Evidence in the literature suggests that the cyclic nucleotides, which act as "second messengers" for many hormonally controlled phenomena, may play a role in the mode of action of opiates. It has been demonstrated that opiates inhibit adenylate cyclase activity in two model systems: the rat brain striatal membranes (Law et al., 1981) and the neuroblastoma x glioma NG 108-15 hybrid cell line (Sharma et al., 1975). In both systems the inhibition of adenylate cyclase has been shown to be dependent on Na^+ and guanine nucleotide, GTP (Law et al., 1981; Blume et al., 1979). This is similar to the hormonal regulation of adenylate cyclase which is dependent on guanine nucleotides (Childers and Snyder, 1980). Also, the opiate receptor agonist binding is regulated by guanine nucleotides (Blume, 1978). Therefore, it is very likely that the opiate receptor is coupled to adenylate cyclase by a mechanism similar to that underlying hormone-stimulated adenylate cyclase.

There is also evidence raising the possibility of Ca^{++} ion as a second messenger in the action of opiates. It has been observed by Harris et al. (1975) that the analgesic effect of subcutaneously administered morphine is antagonized by $CaCl_2$ injection into the preaqueductal gray area of rat brain. It has also been reported that Ca^{++} and cyclic nucleotide can interact in a number of ways. Calcium can regulate cAMP metabolism by affecting phosphodiestrase or

cyclase activity. The increase in intracellular Ca^{++} increases the phosphodiestrase activity through a modulator protein (Schultz, 1975). Calcium influx results in the formation of a calcium-dependent regulator complex which in turn activates adenylate cyclase and a consequent increase in cAMP occurs. Therefore, it is possible that Ca^{++} ion and/or adenylate cyclase act as second messengers in opiate receptor function.

Adenylate cyclase is a membrane- bound enzyme and its activity is dependent on membrane lipid structure (Ross and Gilman, 1980). Modulation of adenylate cyclase activity has been shown using acidic phospholipids in the glucagon receptor (Housely et al., 1976). The role of lipids has also been shown with respect to cyclase activity of the beta-adrenergic receptor (Hanski et al., 1979). This alteration of adenylate cyclase activity by alteration of lipid composition suggests the importance of the physical properties and integrity of the membrane (Ross and Gilman, 1980). Since the activity of the opiate receptor seems to be coupled to adenylate cyclase and membrane integrity is an absolute requirement for proper functioning of this enzyme, it is absolutely necessary to reconstitute the purified opiate receptor in a membrane environment with proper lipid composition in order to demonstrate functional activity of the opiate receptor. Furthermore, the direct involvement of membrane lipids on opiate receptor action has been

suggested, e.g., membrane acidic lipids can interact with opiate ligands with high affinities (Loh et al., 1974; Abood and Hoss, 1975). The hydrolysis of phospholipids with phospholipase A_2 and C decrease the opiate binding activity (Lin and Simon, 1978; Abood et al., 1978). This lipid dependence of the opiate receptor further indicates the need for incorporation of purified receptor into a membrane environment.

IV-2. Reconstitution of Purified Proteins into Membrane Lipids

A) Sonication Method.

Sonication procedure consists of exposing suspensions of phospholipids and membrane proteins in an aqueous medium to sonic oscillations. This method has been used for a variety of membrane systems (Racker, 1973) and has the advantage of rapidity and also the complete absence of detergents that may change the properties of proteins. The disadvantage of the sonication procedure is that the power output of the sonicator is difficult to reproduce and its efficiency varies considerably with the fluid volume of the sample, phospholipid composition, temperature of the bath, etc. Therefore, the material may be non- homogeneous and contain minute vesicular and linear membrane fragments (Rosenberg and McIntosh, 1968). Hence, where sonicated

material is used, there always is the possibility that true reconstitution has not been achieved, only a coalescence of membrane fragments of irregular size (Tzagoloff et al., 1968).

B) Cholate Dialysis Method.

Cholate dialysis procedure consists of solubilizing phospholipid and protein components in about 2% cholate, followed by slow removal of the detergent by dialysis against cholate-free buffer. The advantage of this method is that it is suitable for a large variety of membrane proteins (Racker et al., 1975; Racker, 1972). This method has the disadvantage of requiring dialysis from 16 hrs to several days before membrane function begins to appear and thus some protein denaturation may appear (Goldin and Tong, 1974).

C) Detergent Dilution Method.

Detergent dilution procedure consists of mixing phospholipid that had been sonicated either in the presence or absence of cholate with membrane proteins in a buffered medium. After a short incubation period, samples are diluted at least 20 fold into the assay medium. This method has been successful for reconstitution of the sarcoplasmic reticulum Ca^{++} pump (Racker, 1975). However, this method has not been successful for reconstitution of bacterio-rhodopsin

and the Na^+-K^+-ATPase of the electric eel (Racker, 1973). This method has not been thoroughly explored and it is quite possible that other detergents may yield results in systems that did not respond to cholate.

The latter two methods have a major disadvantage, in that they use a synthetic detergent to solubilize the membrane, raising the possibility of changing receptor characteristics (Simon et al., 1975; Simond et al., 1981; Bidlack and Abood, 1980). Another problem associated with detergents (in Method 2) is the difficulty in removing them from the medium to achieve reconstitution. This difficulty is due to the fact that detergents solubilize the membrane by forming a mixed detergent-protein lipid micelle, in which detergent is tightly bound to the material of interest and resistant to dialysis. Since it is almost impossible to remove all the detergents by dialysis, minute concentrations of detergent are left in the medium which generally interfere with the binding studies of opiate receptor (Pal et al., 1973). Even the presence of small concentrations of lysophospholipids seem to inhibit the opiate binding to rat brain membrane. This is suggested by the work of Lin et al. (1981), in which the treatment of brain membrane with phospholipase A, which releases lysophospholipids, inhibit opiate binding to the membrane. This inhibition can be blocked by Bovine Serum Albumin, however, which is known to remove fatty acids and lysophopholipids.

In summary, the methods available for reconstitution studies seem to be inadequate for opiate receptor reconstitution. In this dissertation a new method has been described for opiate receptor reconstitution.

IV-3. Opiate Receptor Reconstitution:

A new reconstitution method was recently developed by Deamer and Boatman (1980). A natural detergent was used to solubilize membranes from rat brain microsomes, which then were reconstituted by an enzymatic reaction based on a acylation-deacylation cycle first described by Lands et al. (1960).

A) Description of Acylation-Deacylation Cycle and Enzymatic Reconstitution:

The acylation deacylation cycle involves phosphatidylcholine (PC), phospholipases (PL) A_1, A_2, lysophosphatidylcholine (LPC) and an acyltransferase enzyme. Removal of a fatty acid molecule from a phosphoglyceride by phospholipase yields a lysophosphoglyceride (in this case LPC), which is an intermediate in phosphoglyceride metabolism and present in cells and tissues in very small amounts. It acts as a natural detergent to solubilize the membrane. The free fatty acid can later be put back on the lysoPC molecule by the action of a specific enzyme, acyltransferase. The

complete acylation cycle actually plays an important role in modulating membrane lipid composition. Thus, the detergent can be both produced and removed by natural methods, allowing the membrane to dissolve and be reformed. The advantages of this are expanded below.

B) Advantages of Enzymatic Reconstitution Method:

One of the greatest advantages of using the enzymatic reconstitution method is that there is no loss of activity of membrane bound enzymes. As mentioned before, many membrane enzymes, such as succinate dehydrogenase and nucleotide transhydrogenase (Rydstrom et al., 1974), and acyl CoA-lysolecithin acyltransferase (Weltzien et al., 1979) lose activity during solubilization with a synthetic detergent such as Triton X-100, suggesting the essential requirement of a specific lipid environment for proper protein functioning. When LPC is used to solubilize the membrane, the receptor, upon dissociation from its native membrane lipids, is still associated with a lipid, in this case LPC, which closely resembles the native membrane lipid composition. Thus the receptor is less likely to be changed in its essential native characteristics.

Another benefit is that the detergent removal by an enzymatic process is completed in a short period of time, approximately one hour. This eliminates the long dialysis

time required to remove the detergent in conventional methods of reconstitution, and the latter, complete removal of detergent can be achieved. By knowing the specific activity of acyltransferase to convert LPC and acyl CoA to PC molecule, a complete detergent removal can be achieved by addition of just enough concentration of acyltransferase.

Another important advantage of using this enzymatic method is that the lipid composition of the reconstituted membrane can be manipulated in terms of phospholipid head groups and acyl chains. This membrane lipid manipulation is possible by the use of different lyso compounds, acyl donor groups and specific acyltransferases.

Acyl-transferases are found in different tissues with different specificities. Rat liver microsomes contain 1-acyl-glycerolphosphate and 1-acyl-glycerophosphorylcholine acyltransferases (Miki et al., 1977), 2-acyl-sn-glycero-3-phosphorylinositol acyltransferase (Holub and Piekarski, 1979), diacyl- glycerol- ethanolamine- phosphotransferase (Morimoto and Kanoh, 1977) and cholesterol acyltransferase (Balasurbamaniam et al., 1978).

Rat liver mitochondria contains acyl coenzyme A-sn-glycerol-3-phosphate acyltransferase (Kelker and Pullman, 1979). The 1-acylglycerophospho-rylcholine acyltransferase has the highest specificity for 1-acyl- sn-glycero- 3-phosphorylcholine (Miki et al., 1977).

Acyltransferases are not only found in liver, but also

in other tissues such as brain microsomes containing 1-alky-sn-glycero-3-phosphate:acyl-CoA acyltransferase (Fleming and Hajra, 1977), 1-acyl-glycerophosphorylinositol (Baker and Thompson, 1973) and acyl-glycerophosphorylserine (James et al., 1979). Acylation of lysolecithin to lecithin has been reported to occur in rat brain and nervous tissue (Doherty and Rowe, 1979; Webster and Alpern, 1964) and human plasma (Subbaiah and Bagdade, 1978).

Thus, although acyltransferases are found in different organs and subcellular fractions, their specificity for acyl donor and acceptor substrates are different. By choosing a desirable acyl donor-acceptor pair and a specific acyl-transferase enzyme, the lipid content of the reconstituted membrane can be manipulated specifically. Acyltransferase activity is also dependent on the degree of saturation of the acyl donor group. The unsaturated acyl group are better donors than the saturated ones (Morimoto and Kanoh, 1978). So by using this enzymatic method, the degree of saturation of the lipid's acyl group can also be manipulated.

Finally, the evidence suggests that by using the acylation cycle a major fraction of the original membrane proteins, about 70%, are incorporated into the reconstituted membrane. Thus Deamer and Boatman (1980) found a similar SDS gel profile for original and reconstituted rat liver microsomes. This suggests that any exogenous protein can be incorporated into the membrane during the reconstitutional

process. The notion that proteins are incorporated into the lipid bilayer was further supported by freeze-fracture electron microscopy which shows that vesicles formed by reconstitution from solubilized liver microsomes contain intramembrane particles very similar to those of the native membrane. However, one possible problem must be mentioned. Reconstituted membrane is able to pump Ca^{++} ions, indicating the presence of a Ca^{++}-ATPase enzyme. However, this enzyme is also active in membrane solubilized by Triton X-100 and reconstituted membrane prepared by removal of this detergent. In order for the Ca^{++}-Mg^{++}-ATPase to be active, it must be asymmetric in the membrane. There is a controversy about the location of the asymmetric portion of the enzyme. Lin and Worthington (1974) argue that the asymmetry is explained by the presence of the ATPase protein in the inner, or cisternal, side of the vesicle membrane, while Dupont et al. (1973) consider it to be located in the outer, or cytoplasmic, portion of the membrane. The latter notion agrees with the freeze fracture studies, which indicate the ATPase location to be on the outer portion of membrane bilayer (Baskin, 1977). However, further experiments are needed to establish the random vector of the reconstituted membrane and protein asymmetry in the bilayer.

In light of all the advantages of this enzymatic reconstitution method, an attempt was made to apply it to

reconstitution of opiate receptor from rat brain membrane. Lysolecithin was used to solubilize the rat brain membrane; although other lysophosphoglycerides are also capable of solubilizing the membrane, LPC is able to solubilize the membrane by 75-80%, as shown by a decrease in optical density in the solubilized membrane. In contrast, another detergent, lysophosphatidylserine (LPS) was only able to solubilize the membrane by 50%.

After the membrane was solubilized, rat liver microsomes were used as the source of the acyltransferse enzyme needed to reconstitute the solubilized membrane. Although there are different sources of acyl transferase available in different tissues, liver microsomes use LPC as substrate for acylation most effectively. When the efficiency of other acyltransferase for using LPC as substrate was tested, e.g. rat brain microsomes, LPC was used as substrate very poorly, and reconstitution was poor.

In the acylation cycle, a donor of acyl groups is also required to reacylate the lysophosphatidylcholine used as detergent. In this study, oleoyl CoA was used as acyl group donor, because the acyltransferase present in liver microsomes has a higher specificity for using unsaturated acyl donor compared to that of the saturated acyl group (Morimoto and Kanoh, 1978).

After the membrane was solubilized by LPC and oleoyl CoA, as was both shown by a decrease in optical density and

disappearance of membrane structure visualized by negative staining electron microscopy, it was reconstituted by addition of liver microsomes as the acyltransferase enzyme source. The membrane reconstitution was confirmed by showing a time dependent increase in optical density and also by the reappearing of the membrane-like structure by negative staining electron microscopy.

The reconstituted membrane was then characterized in terms of membrane morphology, lipid-protein composition, enzyme activity, physical properties, and opiate binding activity.

IV-4. Characterization of Reconstituted Rat Brain Membrane.

A) Morphology of Reconstituted Membrane:

Native P_2 membranes and reconstituted ones were compared in terms of membrane morphology as seen by electron microscopy. Negative staining images of these membranes were very similar, indicating that this method is able to put the membrane components back together. The actual incorporation of solubilized proteins into the reconstituted membrane, moreover, was shown by freeze fracture electron microscopy, where intramembrane particles are present in both convex and concave fracture faces. There is considerable evidence indicating that these particles revealed during the splitting of the membrane, are proteins,

glycoproteins, or aggregates of these molecules (Wagner, 1980). Thus this shows that proteins are actually incorporated into membrane bilayers and the reconstituted membrane is not simply an aggregation of lipids and proteins.

Reconstituted membranes were further characterized in terms of vesicle size, intramembraneous particle size and distribution into two halves of membrane bilayers. The size of reconstituted membrane vesicles was smaller than that of the native P_2 membranes. Since reconstituted membranes went through several stages of washing, these manipulations could have had some effects on vesicle size, leading to breakdown of larger vesicles into smaller ones.

The overall distribution of particles in the two halves of native membrane and reconstituted membrane was also studied. It was observed that in native membrane there was no significant difference between the particle distribution in convex and concave halves. The same pattern was observed in reconstituted membrane. This suggests that the intramembrane particles are randomly distributed between the two fracture faces of both native and reconstituted membrane. It should be noted that this does not mean a symmetric protein distribution in native and reconstituted membrane. Since only the overall number of particles has been considered here, it is possible that one specific protein is preferentially located on one half and another

protein being located on the other half. Thus, further experiments are required to determine the distribution of a specific protein in the two halves of membrane bilayer.

B) Lipid and Protein Composition of Native and Reconstituted Membranes

As was shown in the Results section, the protein content of reconstituted membranes was very similar to that of native membranes. Further similarities were found in the gel electrophoresis patterns, which showed that the major protein components of native membranes were present in the pellet of the reconstituted membrane. The recovery of these proteins was 75-80% of the proteins which were solubilized. What proteins were lost could very well be due to the washing procedure used with reconstituted membrane.

The lipid composition of reconstituted membrane was also analyzed and compared with that of the native brain membrane. Since lysolecithin and oleoyl CoA were added exogenously to solubilize the membrane, which were reacylated to phosphatidylcholine molecule to reconstitute the membrane, it was not surprising that there was a three fold increase in phosphatidylcholine content of reconstituted membranes. The concentration of other phospholipids, such as PS, PE and PI, was 10-20% lower than that of the original membrane. The cholesterol content was 30%

lower in reconstituted membrane. The lower lipid content of reconstituted membrane could be due to the fact that lysophosphatidylcholine has a larger capacity to solubilize the PC compared to other phospholipids (Deamer, 1973). Therefore, the lipids are not solubilized from P_2 membrane to the same extent and a lower content of these lipids in reconstituted membrane is observed compared to P_2 membrane. Another possible explanation is that the size of the reconstituted vesicles is not the same in the presence of different lipids, with the vesicles formed with PC being larger than the ones made with other phospholipids. The smaller vesicles would be expected to be lost more easily during the washing procedure after membrane reconstitution.

C) Measurements of Enzyme Activities in Reconstituted Membrane

It is well known that the activity of certain membrane bound enzymes are greatly dependent upon membrane integrity, and the presence of a highly specific lipid microenvironment (Green and Tzagoloff, 1966). This dependence of enzyme activity on membrane integrity has been shown for Na^+-K^+-ATPase (Wheeler and Whittam, 1970; Jarnefelt, 1972; Grisham and Barnett, 1972, 1973) and for Mg^{++}-Ca^{++}-ATPase (Martonosi et al., 1968, 1971; Fiehn and Hasselbach, 1970). In all of these studies, the importance of membrane

integrity, structure and lipid:protein ratio for optimum activity of Na^+-K^+-ATPase and Mg^{++}-Ca^{++}-ATPase have been emphasized . Therefore, the activities of these enzymes were measured in order to achieve some idea of how well the membrane is reconstituted.

It was observed that 24% of the original Na^+-K^+-ATPase activity was recovered in reconstituted membranes, while none of the Mg^{++}-Ca^{++}-ATPase activity was recovered. This change in enzyme activity could be due to loss of specific phospholipids required for activity or to a change in a critical enzyme/lipid ratio or to conformational changes of the enzyme due to changes in enzyme micro-environment. Since there is a decrease in PS and cholesterol and an increase in PC incorporated in the membrane, these changes may very well have contributed to the changes in enzyme activity. In support of this, the activity of CA^{++}-Mg^{++}-ATPase was affected more than that of the Na^+-K^+-ATPase, and this enzyme is thought to be more sensitive to lipid changes in reconstituted membrane. To further substantiate this hypothesis, adjustments in cholesterol content and its effect on Ca^{++}-ATPase and Na^+-K^+-ATPase activity will be discussed later.

D) Fluidity of Native and Reconstituted Membranes:

There are large numbers of studies suggesting that a

high degree of membrane ordering is required for optimal activity of membrane phenomena, such as adenylate cyclase activity (Brivio et al., 1976); and Mg^{++}-ATPase (Riordan et al., 1977). Therefore, the fluidity of the native and reconstituted membrane was studied by incorporation of a non-perturbing cholesterol-like fluorescence probe into the native and reconstituted membrane by a simple injection method. By this procedure it was observed that the reconstituted membrane was about 15% more fluid than native P_2 membrane.

The change in fluidity in reconstituted membrane is in agreement with lipid analysis data presented previously. In reconstituted membrane there is an increase in oleoyl-phosphatidylcholine content and a decrease in cholesterol. Membrane fluidity is strongly influenced by the composition of the membrane lipids. Variations in acyl chain saturation are the most common determinant of membrane fluidity. Unsaturated acyl chains form a fluid disordered membrane and the reverse is true for saturated acyl chains. Membrane fluidity is also influenced by the amount of cholesterol, with a decrease in cholesterol content increasing the fluidity of the membrane (Cooper et al., 1978; Shinitzky and Inbar, 1976; Vanderkooi et al., 1974). Since there is an increase in unsaturated acyl chain and a decrease of cholesterol content of reconstituted membrane, an increase

in fluidity is expected.

E) Opiate Binding Activity of Native and Reconstituted Membranes:

The main purpose of this study was to reconstitute the opiate receptor from rat brain membrane. First of all, in order to show that the opiate receptor is not destroyed during the reconstitution process, a control experiment was performed in which the membrane was treated with very small concentration of LPC and oleoyl CoA (Ø.1 µmol/mg protein).It was observed that the membrane treated with these detergents had lower affinity and capacity for binding of ^{3}H-etorphine. The decrease in binding affinity could be due to a change in receptor microenvironment, changing the receptor binding properties. The decrease in binding capacity could be due to the same change or to solubilization of the receptor. However, after the experiment was repeated in the presence of liver microsomes, both the binding capacity and affinity approached those of the original membranes. This suggests that the opiate receptor was not destroyed by the detergent and can be recovered after reconstitution.

Subsequently, opiate binding activity of reconstituted membrane was studied. It was found that the ^{3}H-etorphine binds to reconstituted membrane in a saturable manner, thus fulfilling one important criterion of a true opiate

receptor. In addition, however, correlation between binding and pharmacological potencies must be present. In the reconstituted membrane the ability of various unlabeled drugs to displace ^{3}H-etorphine bound to the membrane is preserved, except for enkephalin analogs; the rank order of potency of different ligands to displace ^{3}H-etorphine is comparable to that of P_2 membrane except that levorphanol is less potent than morphine in the reconstituted membrane. Levorphanol is more potent than dextrophan however, indicating stereoselectivity of the binding. However, the ligand-receptor binding affinity was altered during reconstitution; higher concentrations of these ligands were required to inhibit binding of ^{3}H-etorphine to the reconstituted membrane compared with those for original P_2 membrane.

It is not known whether the inability of enkephalin to displace etorphine is due to the loss of distinct enkephalin binding sites, or whether the affinity of etorphine sites toward enkephalin was so weak that they were not detected under our experimental conditions. The loss of enkephalin binding, even while alkaloid binding is preserved, suggests that the alkaloids and enkephalins bind to two distinct binding sites. There is ample evidence in the literature supporting this opiate receptor heterogeneity. It was first demonstrated by Martin et al. (1976) that administration of three different types of opiates, morphine, ketocylcazocine

and SFK-10047, cause distinct physiological effects, suggesting the presence of three different receptors, μ, K and σ respectively. Further studies by Hutchinson et al. (1975) and Lord et al. (1976,1977) revealed that vas deferens contains another type of receptor which is different from the above mentioned three types, called the δ receptor. In these studies, the pharmacological potencies of a series of alkaloid agonists and enkephalins were compared in the vas deferens, the guinea pig ileum and in whole animal analgesia. It was demonstrated that enkephalins were more potent than alkaloids in the vas deferens. However, beta-endorphine had equal potency in all three systems. Thus, the results of these studies support the existence of separate binding sites for alkaloids and enkephalins.

Further studies are also consistent with this. Frank et al. (1978) and Urca et al. (1978) showed that enkephalins and alkaloids injected into the brain showed different effects at different doses. At low doses, enkephalins induce respiratory depression and alkaloids induce analgesia, but the reverse is true for higher doses. These differences could be due to different receptors involved or possibly to a different pharmacological action between alkaloids and enkephalins subsequent to binding. However, in studies by Lee et al. (1980), leucine and methionine enkephalin injection into the brain, while having

little or no analgesic activity by themselves, potentiated and decreased respectively morphine-induced analgesia. Since the potentiation effect is unlikely to occur due to competition at a same site, this suggests that separate binding sites exist for leucine enkephalin and morphine.

Still further support for distinct alkaloid and enkephalin binding sites was provided by Chang and Cuatrecasas (1979). This study showed that the saturating concentration of naloxone had little effect on enkephalin binding and vice versa. Furthermore, studies of Robson and Kosterlitz (1980) showed that the enkephalin or alkaloid sites can be selectively protected from an irreversible inhibitor, phenoxybenzamine, by addition of an excess concentration of corresponding unlabeled opioid.

A large number of studies have also shown that alkaloids displace labeled alkaloids better than labeled enkephalins and the reverse is true for enkephalins. Beta-endorphine displaces either labeled ligand equally well (Simantov et al., 1978; Hazum et al., 1979a; Law et al., 1979a). It was also shown that the relative potencies of enkephalins or alkaloids to displace labeled ligands are different in the vas deferens and the guinea pig ileum, while beta-endorphine is equally potent in either tissue (Lord et al., 1977). These binding studies support the notion that alkaloids and enkephalin are acting at two different binding sites.

Thus, the lack of enkephalin binding and the presence of alkaloid binding in our reconstituted membrane suggests that distinct δ and μ binding sites can be dissociated from each other during the solubilization and reconstitution process.

In any case, it is apparent that the affinity of the reconstituted membrane for all opiates is lower than that of the original membrane. Other explanations, besides the possible interaction between μ and δ binding sites, is possible for this lower receptor affinity. One is that the affinity of the site present in original membrane is presumably modulated by the specific lipid microenvironment and this could be altered independently of the receptor macromolecule itself. It is also possible that the original analgesic binding site was lost during reconstitution, and the reconstituted site is merely another binding site which happens to correlate with alkaloid function. Another possible explanation is that the receptor conformation has been changed during solubilization and reconstitution. An attempt was made to protect the original receptor conformation during the membrane solubilization by solubilizing the receptor when the latter had been pre-bound to an opiate ligand. In these studies different concentrations of ^{3}H-etorphine were pre-bound to membrane before solubilization. After the membrane was solubilized and reconstituted, it was washed several times in order to

remove the unbound ligand. It was observed that a large amount of labeled ligand remained bound to the reconstituted membrane which could not be displaced by using high concentrations of different unlabeled ligands. It is possible that the ligand receptor complex was internalized during incubation, with the receptor ligand complex inaccessible to the membrane surface.

A similar phenomenon has been observed in the case of α_2-macroglobulin and certain polypeptide hormones (Davies et al., 1980). Unoccupied receptors are diffusely distributed on the cell surface of cultured fibroblasts and they cluster and become taken up by the cell after the ligand binds. The internalization process is known to be Ca^{++}-dependent and is inhibited by primary alkylamines (Maxfield et al., 1979). A possible involvement of a transglutaminase enzyme has also been suggested; transglutaminase covalently cross-link proteins by forming ϵ-(γ-glutaminyl)-lysine cross-bridges, they require Ca^{++} for activity and can be inhibited by a variety of primary amines (Davies et al., 1980). Furthermore, studies by Davies et al. demonstrated that the receptor internalization could be inhibited by a wide variety of inhibitors of transglutaminase such as bacitracin and dansylcadaverine.

The effect of these transglutaminase inhibitors, bacitracin and dansylcadaverine, was studied on binding of an opiate ligand to native and reconstituted brain

membrane. When the membrane was incubated with opiate ligand in the presence of bacitracin and dansylcadaverine, the amount of bound radioligand was reduced compared to controls. This indicates that transglutaminase inhibitors are either decreasing the amount of ligand bound to the receptor or they are preventing receptor internalization. However, the bound radioactive ligand still could not be dissociated from bacitracin- or dansylcadaverine- treated membranes by low concentrations of unlabeled ligand, and the affinity for ligands is in the micromolar ranges, indicating that the high affinity opiate receptor can not be recovered by these treatments.

One factor probably important in the change in opiate binding characteristics as mentioned before, is a change in the receptor microenvironment. The importance of membrane microenvironment for proper functioning of different proteins or receptor has been shown with the ACh receptor, the affinity of which decreases upon solubilization and purification. However, it can be converted back to a high affinity state in the presence of different membrane lipids. In our case, the change in receptor microenvironment may be caused by an increase in the oleoyl phosphatidylcholine (PC) content of the reconstituted membrane. This increase in PC is due to conversion of lysophosphatidylcholine and oleoyl CoA, which are used to solubilize the membrane, to phosphatidylcholine by

acyltransferase.

Other data in the literature also support the importance of a proper lipid environment for normal protein functioning. This has been shown for different enzymes (Tanaka and Sakamoto, 1969; Duttera, 1968; Hong and Hubbell, 1973) and receptors (Insel, 1978; Limbird and Lefkowitz, 1976; Wu et al., 1977; Abood and Takeda, 1976; Loh et al., 1974, 1975). In many systems a requirement for a specific phospholipid ratio for optimal activity has been shown (Kagawa and Racker, 1971; Ragan and Racker, 1973). It is well known that the physical state of lipids can greatly influence membrane protein activities (Hong and Hubbell, 1973). The association of proteins and lipids is mainly determined by the overall charge of phospholipid head groups. The membrane charge is measured by the ratio of zwitterionic phospholipids (PC, PE and sphingomyelin) to anionic phospholipids (PS, PI and phosphatidic acid) (Keenan and Moore, 1970). Since there is an increase in PC content of the reconstituted membrane, the lipid ratio, charge of membrane, lipid distribution and lipid asymmetry can all be greatly influenced. By increasing the PC in the membrane the constant ratio of zwitterionic to anionic phospholipid is no longer maintained. Therefore, this can lead to a change in lipid protein association and consequently to a change in protein conformation and activity. The PC increase also, of course, has an effect on the

phospholipid/cholesterol ratio. This ratio is constant in different tissue and an increase or decrease in cholesterol content can change the membrane fluidity or stability (Nicholson et al., 1977). The change in PC content of the membrane could also have serious effects on lipid distribution and asymmetry. It has been demonstrated that the red blood cell contains PC and sphingomyelin exclusively on the outer layer of the membrane and PE and PS are mainly located on the inner side of the membrane (Demel, 1977). Cholesterol has the highest affinity for PC and sphingomyelin in comparison to the other lipids. This preferential cholesterol affinity for certain lipids might produce a non-random distribution of cholesterol in the plane of the bilayer (Demel et al., 1977). The asymmetric distribution of cholesterol might be important in regulating physiological functions of the membrane.

In fact, an effect of higher PC content on cholesterol distribution in reconstituted membrane was observed. The exposed cholesterol present in the outer layer of membrane was measured by its reaction with cholesterol oxidase and the total cholesterol was measured after complete extraction from membrane using detergent. Thus, the non-exposed cholesterol was the difference between the total and exposed cholesterol. It was found that the ratio of exposed : nonexposed cholesterol is increased 2-3 times in the reconstituted membrane compared to that of the original P_2

membrane. Since the PC molecule has a tendency to situate itself in the outer layer of the bilayer (d'Hollander and Chevallier, 1972) and cholesterol has a higher affinity for PC than for other phospholipids (Demel et al., 1977), this result is readily understandable. Thus, it is apparent that in altering a single membrane constituent, such as PC, many other changes may occur in the membrane.

The hypothesis that a different microenvironment is present in reconstituted membranes is further supported by a shift of the pH optimum for opiate binding. The shape of the peak for the pH optimum changed with a sharper peak observed in reconstituted membrane. The shift of pH optimum to a lower value close to the PK_a of phospholipids raises the possibility of phospholipid involvement or influence on the binding site. Lowering the pH may neutralize some negative charges on the membrane surface involved in the alkaloid binding site. The change in membrane charge could change the protein, receptor conformation. Therefore, a change in pH optimum is indirect support for the presence of different phospholipids, charge and microenvironments.

In reconstituted membrane the ability of enkephalins to interact with opiate binding sites is lost. However, the ability of enkephalins to displace etorphine binding is restored if the experiment is performed at the pH optimum for binding in reconstituted membrane, suggesting that the enkephalin site is present, although altered. Perhaps

changing the pH changes the conformation of the alkaloid binding site, affecting the enkephalin binding site. This indicates that the alkaloid and enkephalin binding site, while distinct from each other, can have mutual interactions.

Further evidence supporting the presence of different microenvironments in the original and reconstituted membrane is provided by fluidity measurements. During the reconstitution process, there is an increase in oleoyl PC due to acylation of oleoyl CoA and lysophosphatidylcholine and a decrease in the cholesterol incorporated into the membrane. Both of these factors are known to affect the membrane fluidity (Cooper et al., 1978). Fluidity was measured by a new method developed by incorporation of a non-perturbing cholesterol probe into the membrane. There was a 10-17% increase in fluidity of the reconstituted membrane compared to that of native P_2 membrane, as shown by a decrease in fluorescence polarization and intensity. Evidence in the literature suggests that receptor accessibility to ligands can be altered by changes in membrane fluidity and a shift in the equilibrium position of membrane proteins (Shinitzky and Souroujon, 1979; Borochov et al., 1979). Different receptors respond differently to a change in membrane fluidity, e.g., when lipid fluidity is decreased, the beta-adrenergic receptor becomes more exposed in liver cell (Bakardijieva et al., 1979), less exposed in rabbit

reticulocyte membrane (Strittmatter et al., 1979) and unaffected in turkey erythrocytes (Hanksi et al., 1979). In studies by Heron et al. (1980), a role for membrane fluidity was suggested for the serotonin and opiate receptors. An increase in cholesterol content of the membrane, which decreases membrane fluidity, caused a five fold increase in the binding of serotonin and two fold increase in the enkephalin and naloxone binding to synaptic membranes. In contrast, fluidizing the membrane with egg-lecithin causes a decrease in binding of serotonin and opiates to the membrane. In the case of opiates, increasing membrane viscosity reduces the affinity for agonist while that for antagonist increases. Thus, in the case of reconstituted membrane since there was a 10-17% increase in membrane fluidity due to an increase in oleoyl PC and a decrease in cholesterol content, a change in opiate receptor binding characteristic is expected.

All of the evidence presented so far leads to the general conclusion that the lipid microenvironment has been changed in the reconstituted membrane. Therefore, several attempts have been made to manipulate the lipids in the reconstituted membrane to mimic that of the native P_2 membrane as close as possible.

IV-5. Lipid Manipulation in the Reconstituted Membrane:

Lipid changes in reconstituted membrane include a 3-fold increase in PC content, a 10-20% decrease in the content of other phospholipids, and a 30% decrease in cholesterol content. Therefore, effects of adjustment of these lipids were studied in opiate binding characteristic.

A) Lipid Manipulation of Reconstituted Membrane by Adjustment of Phospholipid Ratios.

Since there is a change in membrane lipid composition after reconstitution the ratio of different phospholipids has been altered. When different phospholipids were incorporated into the reconstituted membrane to match their content in the original membrane, the opiate binding characteristics did not improve significantly and these attempts failed to increase the opiate receptor affinity. However, it is possible that a ratio of a specific phospholipid is required that has not been tried in this experiment, since only the major phospholipids were considered here. Also, it is likely the added phospholipids do not distribute properly into the reconstituted membrane due to the presence of high PC content. This explanation is very difficult to prove, however, due to difficulties involved in determination of phospholipids asymmetry.

B) Lipid Modification of Reconstituted Membrane by Phospholipid Exchange Protein (PLEP).

Since there is an increase in PC content of reconstituted membrane, an attempt was made to remove some of the excess. Certain proteins present in different tissues are able to transfer phospholipids from a donor to an acceptor lipid vesicle (Wirtz and Zilversmit, 1968 a, b). These proteins (PLEP) (depending on their source) have different specificities for phospholipid molecules (Enhnholm and Zilversmit, 1973, 1977; Kader, 1975; Helmkamp et al., 1974; Lumh et al., 1976; Kamp et al., 1973; Lumh et al., 1976); for example, phospholipid exchange proteins purified from beef liver are specific for transfering phosphatidylcholine (Kamp et al., 1973); these proteins are apparently capable of removing the lipids only in the outer monolayer of bilayer (Johnson et al., 1975). Since there is an increase in PC content of reconstituted membrane, this protein was used to remove the excess PC from reconstituted membrane. This resulted in an increase in opiate receptor binding affinity. In this procedure only a limited amount of PC could be removed from reconstituted membrane even after several incubations with the exchange protein, suggesting that only a fraction of PC is located in the outer layer of bilayer and is available for transfer.

The increase in opiate binding affinity by PLEP treatment was from 290 nM to 6.3 nM. This increase in affinity can be explained as due to a change in the lipid distribution throughout the membrane bilayer; a change in

the ratio of different phospholipids that might be required for a proper membrane environment for the opiate receptor, or third, the removal of oleoyl-PC, which increases the fluidity of reconstituted membrane, caused a decrease in fluidity.

C) Adjustment of Cholesterol Content of Reconstituted Membrane

Since the cholesterol content of reconstituted membrane is lower than that of the native membrane, attempts were also made to increase membrane cholesterol. Addition of different concentrations of cholesterol in the reconstitution incubation medium was expected to increase the cholesterol incorporation into the reconstituted membrane, but was not successful. This could be due to formation of small micelles or vesicles which could not be collected by centrifugation. An alternative method was to incubate the reconstituted membrane with cholesterol rich vesicles which has been successful for cholesterol enrichment of red blood cell membrane (Cooper et al., 1975); however, this procedure also did not increase cholesterol content of the reconstituted membrane. This could be due to the structural differences between red blood cell and reconstituted membrane from brain P_2 in terms of their protein and lipid composition and/or distribution. Finally, a simple and effective method was developed to incorporate exogenous

cholesterol into the membrane based on the procedure of Batzri et al. (1973). An ethanolic solution of cholesterol was injected into the membrane suspension while stirring rapidly; the membranes could effectively incorporate the cholesterol added exogenously up to 90%. Data presented showed that about 90% of the incorporated cholesterol was not available for cholesterol oxidase interaction, indicating that it was mainly incorporated into the inner layer of the membrane bilayer and was not a simple absorption of the cholesterol to the surface of the membrane. The incorporation of cholesterol into the inner layer of the membrane is similar to the pattern of cholesterol distribution in the native P_2 membrane. In native P_2 membrane only about 6-10% of the total cholesterol is exposed to interact with cholesterol oxidase and the rest of it is located in the inner layer of the membrane. Therefore, the simple method for cholesterol incorporation appears to be effective without disturbing the cholesterol distribution.

This injection method was studied in detail. Incorporation of cholesterol into the reconstituted membrane caused an increase in total cholesterol content but did not change the ratio of exposed/non-exposed cholesterol. However, when this membrane was compared with native P_2 membrane, the cholesterol level of both exposed and non-exposed compartments were increased. Since the PC level

has also been significantly increased in the reconstituted membrane, the change in cholesterol level is desirable.

Incorporation of cholesterol into the reconstituted membrane did not improve the activity of Na^+ and K^+ activated ATPase. However, there was a significant increase in the activity of Ca^{++} and Mg^{++} activated ATPase. This differential cholesterol effect on Na^+-K^+-ATPase and Ca^{++}-Mg^{++}-ATPase activity indicates the requirement for a different lipid environment for their activity. The lipid dependence of Na^+-K^+-ATPase has been shown by restoration of the activity of a delipidated ATPase by certain lipids such as PC (Tanaka and Sakamoto, 1965); PE (Taniguchi and Tonomura, 1971); PS (Wheeler and Whittam, 1970); phosphatidylglycerol (Kimelberg and Papahadjopoulos, 1972) and cholesterol (Jarnefelt, 1972). An absolute requirement for lipids has also been shown for Ca^{++}-Mg^{++}-ATPase such as PC (Martonosi et al., 1968, 1971); PE (Knowles and Racker, 1975).

The effect of cholesterol incorporation into the reconstituted membrane was further studied with respect to opiate binding characteristics. The binding properties of ^{3}H-etorphine to reconstituted membrane before and after cholesterol incorporation was studied and the concentrations of unlabeled ligands needed to displace 50% of the ^{3}H-etorphine (IC_{50}) were evaluated. The IC_{50} value of etorphine to displace ^{3}H-ligand from reconstituted membrane

was 290 nM before incorporation of cholesterol, but decreased to 1.9-5.9 nM after cholesterol incorporation which is close to the original value (1.64 nM). The importance of cholesterol has also been demonstrated for proper functioning of other receptor systems such as the acetylcholine receptor (Dalziel et al., 1980), in which ACh R is reconstituted in a defined system containing PE and PS (3:1 molar ratio). This reconstituted receptor is ordinarily not very effective in agonist induced ion flux, but becomes so when reconstituted vesicles contain 25-50 mol% cholesterol. This concentration of cholesterol is similar to that of native AChR enriched membrane, about 45 mol% cholesterol (Schiebler and Hucho, 1978). Under these conditions, the low affinity carbamylcholine binding site could be converted to a high affinity site by preincubation with carbamylcholine, and the binding properties became very similar to that of the native membrane (Dalziel et al., 1980). This finding, along with the cholesterol effect on reconstituted opiate receptor, provides evidence that both AChR ion permeability control and opiate receptor binding characteristics are very sensitive to the lipid environment.

In order to further characterize opiate receptor binding characteristics in cholesterol-incorporated-reconstituted membrane, the saturation isotherms of binding of several opiate agonists and an antagonist were analyzed

before and after incorporation of cholesterol. It was observed that in reconstituted membrane ^{3}H-etorphine binding sites are lower in affinity and capacity than those of the native membrane. However, after cholesterol incorporation there was an increase in the affinity and capacity of these sites and also an increase in the capacity of low affinity ^{3}H-etorphine binding sites. The affinity of these low affinity sites was affected by cholesterol incorporation only in the case of ^{3}H-dihydromorphine and ^{3}H-naloxone binding. Also, cholesterol incorporation was not able to recover enkephalin binding in the reconstituted membrane. Therefore, it is clear that the low affinity sites of ^{3}H-etorphine, ^{3}H-dihydromorphine and ^{3}H-naloxone binding are all affected by cholesterol incorporation; however, cholesterol has an effect on the high affinity binding sites binding to etorphine only, which may indicate that these sites are different from those binding to naloxone and dihydromorphine (μ sites). These data also suggest that the cholesterol effect is not due to non-specific changes of the membrane because it is different in the case of different ligands. It is possible that cholesterol has a specific effect on opiate receptors, exerted through their immediate lipid microenvironment.

In conclusion, adjustment of cholesterol by this procedure to the level comparable to that of the P_2 membrane significantly increased the affinity of the opiate receptor

for ^{3}H-etorphine into the order of nanomolar range. Several explanations could be suggested for this cholesterol effect on opiate binding. First, the cholesterol may decrease the membrane fluidity, which has increased in reconstituted membrane due to an increase in oleoyl acyl chain derivatives of PC. Second, the phospholipid/cholesterol ratio has been adjusted to a value closer to that of the original membrane. Third, an increase in cholesterol level affects the phospholipid distribution in a manner that may cause a change in receptor configuration and therefore receptor binding characteristics. At any rate, the cholesterol effect further supports the importance of lipids and the microenvironment for proper receptor functioning.

Finally, a possible effect of cholesterol on changing protein distribution was tested by examining intramembraneous particle distribution under freeze fracture electron microscopy. In these experiments, the distribution of intramembraneous particle was studied in convex and concave fractured faces before and after cholesterol incorporation. It was observed that cholesterol does not affect the overall protein distribution compared to that of the reconstituted membrane before cholesterol incorporation. However, it should be noted that in these studies only the overall distribution could be studied and since we are unable, at the present time, to study the opiate receptor distribution specifically, the possibility of cholesterol

affecting the opiate receptor distribution can not be ruled out.

In summary, all the data presented in this dissertation provide support for a role of lipids in membrane functions. The alkaloid binding site seems to be reincorporated into the membrane during reconstitution and is dependent on lipid microenvironment for its proper functioning. However, the enkephalin binding site is not functioning in reconstituted membrane and lipid modifications do not seem to affect its expression of activity. This suggests that the alkaloid and enkephalin binding sites are dissociated during the solubilization and reconstitution procedure. Alternatively, it is possible that we have not found the required lipid environment for the enkephalin site or that a certain cofactor is required for the activity of enkephalin binding site. Hopefully, by improving the lipid micro-analytical method a very careful analysis of membrane lipids and phospholipids will eventually become possible.

The enzymatic reconstitution used in the study offers several clear advantages over reconstitution methods available; it uses a natural, gentle detergent which can be completely removed and provides the opportunity to modulate membrane lipid composition by using a known lysophosphatide-acyl CoA pair. This allows the manipulation of phospho- lipid head groups and acyl chain simultaneously. Finally, the freeze fracture electron microscopy studies

revealed that the intramembrane particle distribution is also preserved. Therefore, this method is an attractive alternative to the reconstitution methods that are available at the present time.

V. REFERENCES

Abood, L. G. and Hoss, W. (1975). Stereospecific morphine adsorption to phosphatidylserine and other membraneous components. Eur. J. Pharmacol 32, 66-75.

Abood, L. G., Salem, N., MacNeil, M. and Butler, M. (1978). Phospholipid changes in synatpic membranes by lipolytic enzymes and subsequent restoration of opiate binding with phosphatidylserine. Biochem. Biophys. Acta. 530, 35-46.

Abood, L. G. and Takeda, F. (1976). Enhancement of stereospecific opiate binding to neural membrane by phosphatidylserine. Eur. J. Pharmacol 39, 71-77.

Ahmed, M. S. and Byrne, W. L. (1980). Opiate receptor binding studies. Influence of a reversible sulfhydryl reagent. In: Endogenous and Exogenous Opiate Agonists and Antagonists, E. L. Way (ed.) (Plenum, New York), pp. 51-54.

Albert, A. D., Lund, M. and Yeagle, P. L. (1981). Evidence for the influence of the protein phospholipid interface on sarcoplasmic reticulum Ca^{++} Mg^{++} ATPase activity. Biophys. J. 36, 393-407.

Archer, D. B. (1975). The use of a fluorescent sterol to investigate the mode of action of amphotericin methyl ester, a polyene antibiotic. Biochem. Biophys. Res. Commun. 66, 195-201.

Azhar, S., Hajra, A. K., and Menon, K. M. J. (1976). Gonadotropin receptors in plasma membranes of bovine corpus luteum. II. Role of membrane phospholipids. J. Biol. Chem. 251, 7405-7412.

Azhar, S. and Menon, K. M. J. (1976). Gonadtropin receptors in plasma membrane of bovine corpus luteum. I. Effect of phospholipases on the binding of ^{125}I-Choriogonadotrophin by membrane-associated and solubilized receptors. J. Biol. Chem. 251, 7398-7404.

Bakardijieva, A., Galb, H. J. and Helmreich, E. J. M. (1979). Modulation of the beta-receptor adenylate cyclase interaction in cultured Chang liver cells by phospholipid enrichment. Biochemistry, 8, 3016-3023.

Baker, R. R. and Chang, H. Y. (1981). A comparison of lysophosphatidyl choline acyltransferase activities in neuronal nuclei and microsomes isolated from immature rabbit cerebral cortex. Biochem. Biophys. Acta. 666, 223-229.

Baker, R. R. and Thompson, W. (1973). Selective acylation of 1-acylglycero- phosphorylinositol by rat brain microsomes.

J. Biol. Chem. 218, 7060-7065.

Balasurbamaniam, S., Mitropoulos, K. A. and Venkaiesan, S. (1978). Rat brain acyl-CoA: cholesterol acyltransferase. Eur. J. Biochem. 90, 377- 383.

Barranetes, F. J., Changeux, J. P., Lunt, G. G. and Sobel, A. (1975). Differences between detergent-extracted acetylcholine receptor and "cholinergic proteolipid". Nature, 256, 325-327.

Barsukov, L. I., Kulikov, V. I., Simakova, I. M., Tikhonova, G. V., Ostrovskii, D. N. and Bergelson, L. D. (1978). Manipulation of phospholipid composition of membranes with the aid of lipid exchange proteins. Eur. J. Biochem. 90, 331-336.

Bartlett, G. R. (1959). Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466-468.

Baskin, R. J. (1977). The Ca^{++}-Mg^{++} ATPase protein of sarcoplasmic reticulum. In: Membrane Proteins and their Interactions with Lipids, R. A. Capaldi (ed.) (Marcel Dekker, Inc., New York and Basel), pp. 151-188.

Batzri, S. and Karn, E. D. (1973). Single bilayer liposomes prepared without sonication. Biochem. Biophys. Acta. 298, 1015-1019.

Beckett, A. H. and Casy, A. F. (1954). Synthetic analgesics: stereo chemical considerations. J. Pharm. Pharmacol. 6, 986-999.

Bell, F. P. (1978). Lipid exchange and transfer between biological lipid-protein structure. Prog. Lipid Res. 77, 207-243.

Belluzzi, J., Grant, N., Grasky, V., Sarantakis, D., Wise, C. and Stein, L. (1976). Analgesia induced in vivo by central administration of enkephalin in rat. Nature (Lond.) 260, 626-626.

Bergman, W. and Stevens, P. G. (1948). Conversion of ergosterol to adrenal cortical hormones. J. Org. Chem 13, 10-20.

Bidlack, J. M. and Abood, L. G. (1980). Solubilization of the opiate receptor. Life Sci. 27, 331-340.

Bidlack, J. M., Abood, L. G., Osei-Gyimah, P. and Archer, S. (1981). Purification of the opiate receptor from rat brain. Proc. Natl. Acad. Sci. U.S.A. 78, 636-639.

Bigon, E., Boarato, E., Bruni, A., Leon, A. and Toffano, G. (1979). Pharmacological effects of phosphatidylserine liposome: The role of lysophosphatidylserine. Br. J. Pharmac. 67, 611-616.

Birdsall, N. J. M., Hulme, E. C., Bradbury, A. F., Smyth, D. G. and Snell, C. R. (1976). The binding of the C-fragment of lipoprotein and methionine-enkephalin to brain opiate receptors. In: Opiates and Endogenous Opioid Peptides, H. W. Kosterlitz (ed.) (Elsevier/North Holland Biomedical Press, Amsterdam), pp. 19-26.

Birdsall, N. J. M., Lee, A. G., Levine, Y. K. and Metcalf, J. C. (1971). F19-NMR of monofluorostearic acids in lecithin vesicles. Biochem. Biophys. Acta 241, 693-696.

Bloj, B. and Zilversmit, D. B. (1976). Asymmetry and transposition rates of phosphatidylcholine in rat erythrocyte ghosts. Biochemistry 15, 1277-1283.

Bloj, B. and Zilversmit, D. B. (1977). Rat liver proteins capable of transferring phosphatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. J. Biol. Chem. 252, 1613-1619.

Blume, A. J., Boone, G., and Lichtshtein, D. (1979). Regulation of the neuroblastoma X glioma hybrid opiate receptors by Na^+ and guanine nucleotides. Adv. Exp. Med. Biol. 116, 163-174.

Blume, A. J., Shorr, J., Finberg, J. P. and Spector, S. (1977). Binding of the endogenous nonpeptide morphine-like compound to opiate receptors. Proc. Natl. Acad. Sci. U.S.A. 74, 4927-4931.

Borochov, H., Abbott, R. E., Schachter, D. and Shinitzky, M. (1979). Modulation of erythrocyte membrane proteins by membrane cholesterol and lipid fluidity. Biochemistry 18, 251-255.

Borochov, H., Zahler, P., Wilbrandt, W. and Shinitzky, M. (1977). The effect of phosphatidylcholine to sphingomyelin mole ratio on the dynamic properties of sheep erythrocyte membrane. Biochem. Biophys. Acta 470, 382-388.

Bowman, R. L. and Chen, R. F. (1965). Fluorescence polarization: Measurement with ultraviolet polarizing filters in a spectrofluorometer. Science 147, 729-732.

Braenden, O. J., Eddy, N. B. and Halbach, H. (1955). Synthetic substances with morphine like effect. Relationship between chemical structure and analgesic action. Bull. W.H.O. 13, 937-998.

Brivio-Hangland, R. P., Louis, S. L., Musch, K., Waldech, N. and Williams, M. A. (1976). Liver plasma membranes from essential fatty acid deficient rats. Isolation, fatty acid composition, and activities of 5'-nucleotidase, ATPase and adenylate cyclase. Biochem. Biophys. Acta 433, 150-163.

Broekhuyse, R. M. (1968). Phospholipids in tissues of the eyes. I. Isolation, characterization and quantitative analysis by two dimensional thin layer chromatography of diacyl and vinyl-ether phospholipids. Biochem. Biophys. Acta 152, 307-315.

Brown, R. E. and Cunningham, C. C. (1982). Negatively charged phospholipid requirement of the oligomycin-sensitive mitochondrial ATPase. Biochem. Biophys. Acta 684, 141-145.

Bruckdorfer, K. R., Demel, R. A., DeGier, J. and Van Deenan, L. L. M. (1969). Effect of partial replacements of membrane cholesterol by other steroids on osmotic fragility and glycerol permeability of erythrocytes. Biochem. Biophys. Acta 183, 334-369.

Bruckdorfer, K. R., Edwards, P. A. and Green, C. (1968). Properties of aqueous dispersion of phospholipid and cholesterol. Eur. J. Biochem. 4, 506-511.

Buscher, H., Hill, R., Romer, D., Cardinaux, F., Closse, A., Hauser, D. and Pless, J. (1976). Evidence for analgesic activity of enkephalin in the mouse. Nature (Lond.) 261, 423-425.

Caron, M. G. and Lefkowitz, R. J. (1976). Solubilization and characteristic of the beta-adrenergic binding sites for frog erythrocytes. J. Biol. Chem. 251, 2374-2384.

Chang, J., Fong, B., Pert, A. and Pert, C. (1976). Opiate receptor affinities and behavioral effects of enkephalin: structure activity relationship of ten synthetic peptide analogues. Life Sci. 18, 1473-1482.

Chang, K. J. and Cuatrecasas, P. (1979). Multiple opiate receptors. Enkephalins and morphine bind to receptors of different specificity. J. Biol. Chem 254, 2610-2618.

Chapman, D. (ed.) (1968). Biological membrane: physical fact and function. (New York Academic), pp. 125-202.

Chen, R. F. and Bowman, R. L. (1965). Fluorescent polarization: Measurement with ultraviolet polarizing filters in a spectrophotofluorometer Science 147, 729-732.

Childers, S. R. and Snyder, S. H. (1980). Differential

regulation by guanine nucleotides of opiate agonist and antagonist receptor interaction. J. Neurochem. 34, 583-593.

Chin. J. H. and Goldstein, D. B. (1980). Membrane disordering action of ethanol, variation with membrane cholesterol content and depth of the spin label probe. Mol. Pharmacol. 19, 425-431.

Cho, T. M., Cho, J. S. and Loh, H. H. (1976a). A model for opiate-receptor interactions: Mechanism of opiate-cerebroside sulfate interaction. Life Sci. 18, 231-244.

Cho, T. M., Cho, J. S. and Loh, H. H. (1976b). ^{3}H-cerebroside sulfate redistribution induced by cation, opiate or phosphatidylserine. Life Sci. 19,117-124.

Cho, T. M., Yamato, C., Cho, J. S. and Loh, H. H. (1981). Solubilization of membrane bound opiate receptor from rat brain. Life Sci. 28, 2651- 2657.

Colard, O., Bard, D., Bereziat, G. and Polonovski, J. (1980). Acylation of endogenous phospholipids and added lysoderivatives by rat liver plasma membranes. Biochem. Biophys. Acta 618, 88-97.

Coleman, R. (1973). Membrane bound enzymes and membrane ultrastructure. Biochem. Biophys. Acta 300, 1-30.

Cooper, R. A.. Arner. E. C., Wiley, J. S. and Shattil, S. J. (1975). Modification of red blood cell membrane structure by cholesterol-rich lipid dispersions. J. Clinic. Invest. 55, 115-126.

Cooper, R. A., Leslie, M. E., Fischkoff, S., Shinitzky, M. and Shattil, S. J. (1978). Factors influencing lipid composition and fluidity of red cell membranes, in vitro production of red cell possessing more than 2 cholesterols per phospholipid. Biochemistry 17, 327-331.

Crain, R. C. and Zilversmit, D. B. (1980). Two nonspecific phospholipid exchange proteins from beef liver. 1: Purification and characterization. Biochemistry 19, 1433-1439.

Crain, R. C. and Zilversmit, D. B. (1980b). Net transfer of phospholipid by the non specific phospholipid transfer proteins from bovine liver. Biochem. Biophys. Acta 620, 37-48.

Craves, F. B., Law, P. Y., Hunt, C. A. and Loh, H. H. (1978). The metabolic disposition of radiolabeled enkephalins in vitro and in situ. J. Pharmacol. Exp.

Therap. 206, 492-506.

Cuatrecasas, P. (1971). Unmasking of insulin receptors in fat cells and fat cell membranes. Perturbation of membrane lipids. J. Biol. Chem. 246, 6532-6542.

Dalziel, A. W., Rollins, E. S. and MacNamee, M. G. (1980). The effect of cholesterol on agonist induced flux in reconstituted acetylcholine receptor vesicles. FEBS Lett. 122, 193-196.

Davies, P. J. A., Davies, D. R., Levitzky, A., Maxfield, F. R., Milhaud, P., Willingham, M. C. and Pastan, I. H. (1980). Transglutaminase is essential in receptor mediated endocytosis of alpha 2-macroglobulin and polypeptide hormones. Nature 283, 162-167.

Dawson, R. M. C. (1973). The exchange of phospholipids between cell membranes. Sub. Cell. Biochem. 2, 69-89.

Day, C. E. and Levy, R. S. (1969). A generalized functional role for phospholipids. J. Theor. Biol. 22, 541-542.

Deamer, D. W. (1973). Isolation and characterization of a lysolecithin- adenosine triphosphatase complex from lobster muscle microsomes. J. Biol. Chem. 248, 5477-5485.

Deamer, D. W. and Boatman, D. E. (1980). An enzymatically driven membrane reconstitution from solubilized components. J. Cell. Biol. 84, 461-467.

Dehlinger, P. J., Jost, P. C. and Griffith, O. H. (1974). Lipid binding to the amphipathic membrane protein cytochrome b_5. Proc. Natl. Acad. Sci. U.S.A. 71, 2280-2284.

Demel, R. A. and Dekruff, B. (1976). Function of sterols in membranes. Biochem. Biophys. Acta 457, 109-132.

Demel, R. A., Jensen, J. W. C. M., Van Dijck, P. W. M. and Van Deenan, L. L. M. (1977). Preferential interaction of cholesterol with different classes of phospholipids. Biochem. Biophys. Acta 465, 1-10.

De Pierre, J. and Dallner, G. (1976). Isolation, subfractionation and characterization of the endoplasmic reticulum. In: Biochemical Analysis of Membranes, A. H. Maddy (ed.) (John Wiley & Sons, Inc., New York). pp. 79-131.

De Plazas, S. F. and De Robertis, E. (1972). Isolation of a proteolipid from spleen capsule binding (plus or minus) (^{3}H) norepinephrine. Biochem. Biophys. Acta 266, 246-254.

.d'Hollander, F. and Chevallier, F. (1972). Movement of cholesterol in vitro in rat blood and quantitation of exchange of free cholesterol between plasma and erythrocytes. J. Lipid Res. 13, 733-744.

Di Corleto, P. E. and Zilversmit, D. B. (1977). Protein catalyzed exchange of phosphatidylcholine between sonicated liposomes and multilamellar vesicles. Biochemistry 16, 2145-2150.

Doherty, F. J. and Rowe, C. E. (1979). Acylation of lysophosphatidyl choline in nervous tissue. J. Neurochem. 33, 819-822.

Dole, V. P. (1956). A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. Clinic. Invest. 35, 150-154.

Draper, N. R. and Smith, H. (1966). Applied Regression Analsyis (Wiley, New York).

Dupont, A., Cusan, L., Caron, M., Alvarado-Urbina, G. and Labri, F. (1977). Extremely rapid degradation of ^{3}H-methionine enkephalin by various rat tissues in vivo and in vitro. Life Sci. 21, 907-914.

Dupont, Y., Harrison, S. C. and Hasselbach, W. (1973). Molecular organization in the sarcoplasmic reticulum membrane studied by X-ray diffraction. Nature 244, 555-558.

Duttera, S. M., Byrne, W. L. and Ganoza, M. C. (1968). Studies on phospholipid requirement of glucose-6-phosphatase. J. Biol. Chem. 243, 2216-2268.

Edidin, M, (1974). Rotational and translational diffusion in membranes. Ann. Rev. Biophys. Bioeng. 3, 179-201.

Eibl, H., Hill, E. E. and Lands, W. E. M. (1969). The subcellular distribution of acyltransferases which catalyze the synthesis of phosphoglycerides. Eur. J. Biochem. 9, 250-258.

Enhnholm, C. and Zilversmit, D. B. (1977). Exchange of various phospholipids and of cholesterol between liposomes in the presence of highly purified phospholipid exchange protein. J. Biol. Chem. 248, 1719-1724.

Erods, E. G. (1977). Angiotensin-1 converting enzyme. Fed. Proc. 36, 1760-1765.

Feldman, H. A. (1972). Mathematical theory of complex ligand-binding systems at equilibrium: Some methods for parameter fitting. Analytical Biochemistry 48, 317-338.

Fiehn, W. and Hasselbach, W. (1970). Effect of phospholipase-A on calcium transport and role of unsaturated fatty acids in ATPase activity of archiplasmic vesicles. Eur. J. Biochem. 13, 510-518.

Fisher, K. and Branton, D. (1974). Application of the freeze fracture technique to natural membranes. Methods Enzymol. 32B, 35-44.

Fleming, J. W. and Ross, E. M. (1980). Reconstitution of beta-adrenergic receptors into phospholipid vesicles: Restoration of [^{125}I]- iodohydroxybenzylpindolol binding to digitonin solubilized receptors. J. of Cyclic Nucleotide Res. 6, 407-419.

Fleming, P. J. and Hajra, A. K. (1977). 1-alkyl-sn-glycero-3-phosphatidic- acyl-CoA acyltransferase in rat brain microsomes. J. Biol. Chem. 252, 1663-1672.

Fletcher, J. E. and Schrager, P. I. (1973). A User's Guide to Least Square Model Fitting. U. S. Department of Health, Education and Welfare, Technical Report No. 1.

Floreani, M., Bonetti, A. C., and Carpanedo, F. (1981). Increase of Na^+/K^+ ATPase activity in intact rat brain synaptosomes after their interaction with phosphatidylserine vesicles. Biochem. Biophys. Res. Commun. 101, 1337-1344.

Folch, J., Lee, M. and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Frederickson, R. C. A. (1977). Enkephalin pentapeptidase - a review of current evidence for a physiological role in vertebrate neurotransmission. Life Sci. 21, 23-42.

Frenk, H., Urca, G. and Liebeskind, J. C. (1978). Epileptic properties of leucine and methionine enkephalin: comparison with morphine and reversibility by naloxone. Brain Res. 147, 327-337.

Giulbault, G. G. (ed.)(1967). Fluorescence Theory, Instrumentation and Practice (Marcel Dekker, Inc., New York).

Goldin, S. M. and Tong, S. W. (1974). Reconstitution of active transport catalyzed by purified sodium and potassium ion stimulated adenosine- triphosphatase from canine renal medulla. J. Biol. Chem. 249, 5907-5915.

Gottlieb, M. H. (1976). The limited depletion of cholesterol from erythrocyte membranes on treatment with incubated

plasma. Biochem. Biophys. Acta 433, 333-343.

Green, D. E. and Tzagoloff, A. (1966). Role of lipids in structure and function of biological membranes. J. Lipid Res. 7, 587-602.

Grisham, C. M. and Barnett, R. E. (1972). Interrelation of membrane and protein structure in functioning of (Na^++Ka+) activated ATPase. Biochem. Biophys. Acta 266, 613-624.

Grisham, C. M. and Barnett, R. E. (1973). The role of lipid phase transition in the regulation of the (sodium and potassium) adenosine triphosphatase. Biochemistry 12, 2635-2637.

Gurr, M. I. and James, A. T. (1971). Lipid biochemistry, An Introduction. (Chapman and Hall, Ltd., London).

Haga, T,, Haga, K. and Gilman, A. G. (1977). Hydrodynamic properties of the beta-adrenergic receptor and adenylate cyclase from wild type and variant S49 lymphoma cells. J. Biol. Chem. 252, 5776-5782.

Hambrook, J, Morgan, B., Rance, M. and Smith, C. (1976). Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. Nature (Lond.) 262, 782-783.

Hancock, A. A., DeLean, A. L., and Lefkowitz, R. J. (1979). Quantitative resolution of beta-adrenergic receptor subtypes by selective ligand binding: Application of a computerized model fitting technique. Mol. Pharmacol. 16, 1-9.

Hanski, E., Rimon, G. and Levitzky, A. (1979). Adenylate cyclase activation by the beta-adrenergic receptor as a diffusion controlled process. Biochemistry 18, 846-853.

Harmon, C. K., and Neiderhiser, D. H. (1978). Lipid metabolism by the gall bladder. The in vitro conversion of lysophosphatidylcholine to phosphatidylcholine. Biochem. Biophys. Acta 530, 217-226.

Harris, R. A., Loh, H. H. and Way, E. L. (1975). Effects of divalent cations, cation chelators and an ionophore on morphine analgesia and tolerance. J. Pharmacol. Exp. Therap. 195, 488-498.

Hauser, H. and Irons, L. (1972). Effect of ultrasonication on chemical and physical properties of aqueous egg yolk lecithin dispersions. Hoppe-Seyler's J. Physiol. Chem. 353, 1579-1590.

Hazum, E., Chang. K. J. and Cuatrecasas, P. (1979a).

Interaction of iodinated human [D-ala^2] beta-endorphin with opiate receptors. J. Biol. Chem. 254, 1765-1767.

Heckman, C. A., Friedman, S. J., Skehan, P. J. et al. (1977). Localization and partial characterization of acyltransferases present during rapid membrane formation in the Drosophila melanogaster embryo. Dev. Biol. 55, 9-32.

Heidmann, T., Sobel, A., Popot, J. L. and Changeux, J. P. (1980). Reconstitution of a functional acetylcholine receptor. Conversions of the conformational and allosteric transitions and recovery of the permeability response: role of lipids. Eur. J. Biochem. 110, 35-55.

Helenius, A. and Simons, K. (1974). Solubilization of membranes by detergents. Biochem. Biophys. Acta 415, 29-79.

Helmkamp, G. M., Harvey, M. S., Writz, K. W. A. and Van Deenan, L. L. M. (1974). Phospholipid exchange between membranes. Purification of brain proteins that preferentially catalyze the transfer of phosphatidylinositol. J. Biol. Chem 249, 6382-6389.

Henderson, G., Hughes, J. and Kosterlitz, H. W. (1978). In vitro release of leu-enkephalin and met-enkephalin from corpus striatum. Nature 271, 677-679.

Heron. D. S., Hershkowitz, M., Shinitzky, M. and Samuel, D. (1980). The lipid fluidity of synaptic membranes and the binding of serotonin and opiate ligands. In: Neurotransmitters and Their Receptors, V. Z. Littauer, Y. Dudai, I. Silman, V. I. Teichberg and Z. Vogel (eds.) (John Wiley & Sons, Ltd.), pp. 125-138.

Hiller, J. M., Angel, L. M. and Simon, E. J. (1981). Multiple opiate receptors: Alcohol selectively inhibits binding to delta receptors. Science 214, 468-469.

Hoffmann, W., Sarzala, M. G., Gomez-Fernandez, J. C., Goni, F. M., Restall, C. J., Chapman, D., Heppeler, G. and Kreutz, W. (1980). Protein rotational diffusion and lipid structure of reconstituted systems of Ca^{++}-activated adenosine triphosphatase. J. Mol. Biol. 141, 119-132.

Holub, B. J. and Piekarski, J. (1979). The formation of phosphatidyl inositol by acylation of 2-acyl-sn-glycerol-3-phosphorylinositol in rat liver microsomes. Lipids 14, 529-532.

Holub, B. J. and Piekarski, J. (1977). The relative utilization of different molecular species of unsaturated 1,2-diacylglycerols by the acyl-CoA:1,2-diacyl-sn-glycerol acyltransferase in rat liver microsomes. Can. J. Biochem.

55, 1186-1190.

Hong, K., and Hubbell, W. L. (1973). Lipids' requirements for rhodopsin regenerability. Biochemistry 12, 4517-23.

Houslay, M. D., Hesketh, T. R., Smith, G. A., Warren, G. B. and Metcalfe, J. C. (1976). The lipid environment of the glucagon receptor regulates adenylate cyclase activity. Biochem. Biophys. Acta 436, 495-504.

Hughes, J. (1975). Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. Brain Res. 88, 295-308.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergrill, L. A., Morgan, B. A. and Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature (Lond.) 258, 577-579.

Hutchison, M., Kosterlitz, H. W., Leslie, M., Watchfield, A. A. and Terenius, L. (1975). Assessment in the guinea pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. Br. J. Pharmacol. 55, 541-546.

Ichida, S., Kuo, C. H., Matsuda, T. and Hiroshi, Y. (1976). Effects of La^{++}, Mn^{++} and ruthenium red on Mg-Ca-ATPase activity and ATP- dependent Ca-binding of synaptic plasma membrane. Japan J. Pharmacol. 26, 39-43.

Insel, P. A. Nirenberg, P., Turnbull, J. and Shattil, S. J. (1978). Relationship between membrane cholesterol, alpa-adrenergic receptors and platelet function. Biochemistry 17, 5269-5274.

James, O. A., MacDonald, G. and Thompson, W. (1979). Acylation of lysophosphatidylserine by rat liver microsomes. J. Neurochem. 33, 1061- 1066.

Jarnefelt, J. (1972). Lipid requirements of functional membrane structure as indicated by reversible inactivation of (Na^{+}-K^{+}) ATPase. Biochem. Biophys. Acta 266, 91-96.

Johnson, L. W., Hughes, M. E. and Zilversmit, D. B. (1975). Use of phospholipid exchange protein to measure inside-outside transposition in phosphatidylcholine liposomes. Biochem. Biophys. Acta 375, 176-185.

Johnson, L. W. and Zilversmit, D. B. (1975). Catalytic properties of phospholipid exchange protein from bovine heart. Biochem. Biophys. Acta 275, 165-175.

Kader, J. C. (1975). Proteins and intracellular exchange of lipids. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. Biochem. Biophys. Acta 380, 31-44.

Kagawa, Y. and Racker, E. (1971). Partial resolution of enzymes catalyzing oxidative phosphorylation, reconstitution of vesicles catalyzing P-32- adenosine triphosphate exchange. J. Biol. Chem. 246, 5477-5487.

Kamp, H. H. and Writz, K. W. A. (1974). Phosphatidylcholine exchange protein from beef liver. Methods Enzymol. 32, 140-146.

Kamp, H. H., Writz, K. W. A. and Van Deenan, L. L. M. (1973). Some properties of phosphatidylcholine exchange of protein purified from beef liver. Biochem. Biophys. Acta 318, 313-325.

Kean, E. L. (1968). Rapid sensitive spectrophotometric method for quantitative determination of sulfatides. J. Lipid Res. 9, 319-327.

Keenan, T. W. and Morre, D. J. (1970). Phospholipid class and fatty acid composition of golgi apparatus isolated from rat liver and comparison with other cell fractions. Biochemistry 9, 19-25.

Kelker, H. C. and Pullman, M. E. (1979). Phospholipid requirement of acyl coenzyme A:sn-glycerol-3-phosphate acyltransferase from rat liver mitochondria. J. Biol. Chem. 245, 5364-5371.

Kimelberg, H. K. (1977). The influence of membrane fluidity on the activity of membrane-bound enzymes. In: Dynamic Aspects of Cell Surface Organization, G. Poste and G. L. Nicholson (eds.)(North Holland Publishing Co., Amsterdam), pp. 205-293.

Kimelberg, H. K. and Papahadjopoulos, D. (1972) Phospholipid requirements for (Na^+-K^+)-ATPase activity: head group specificity and fatty acid fluidity. Biochem. Biophys. Acta 282, 277-292.

King, M. D. and Quinn, P. J. (1980). The use of phospholipid exchange processes to modulate the fluidity of biological membranes. Biochem. Soc. Trans. 8, 322-323.

Kirkpatrick, F. H., Gordesky, S. E. and Marinetti, G. V. (1974). Differential solubilization of proteins, phospholipids and cholesterol of erythrocyte membrane by detergents. Biochem. Biophys. Acta 345, 154-161.

Knowles, A. F. and Racker, E. (1975). Properties of a reconstituted calcium pump. J. Biol. Chem. 250, 3538-3544.

Kornberg, R. D. and McConnell, H. M. (1971b). Inside-outside transitions of phospholipids in vesicle membranes. Biochemistry 10, 1111-1120.

Lands. W. E. (1960). Metabolism of glycerolipids. 2. The enzymatic acylation of lysolecithin. J. Biol. Chem. 235, 2233-2237.

Lands, W. E. M. And Hart, P. (1965). Metabolism of glycerolipids. VI Specificities of acyl coenzyme A: phospholipid acyltransferase. J. Biol. Chem. 240, 1905-1911.

Law, P.Y. and Loh, H. H. (1978). Leu-5'-enkephalin-H-3 specific binding to synaptic membrane. Comparison with dihydromorphine-H-3 and naloxone-H-3 Res. Commun. Chem. Pathol. Pharmaco. 21, 409-434.

Law, P. Y., Loh, H. H. and Li, C. H. (1979a). Properties and localization of beta-endorphin receptors in rat brain. Proc. Natl. Acad. Sci. U.S.A. 76, 5455-5459.

Law, P. Y., Wu, J., Koehler, J. E. and Loh, H. H. (1981). Demonstration and characterization of opiate inhibition of the striatal adenylate cyclase. J. Neurochem. 36, 1834-1846.

Lee, N. M., Leybin, L., Chang, J. K. and Loh, H. H. (1980a). Opiate and peptide interaction. Effect of enkephalins on morphine analgesia. Eur. J. Pharmacol 68, 181-105.

Lee, N. M. and Smith, A. P. (1980). A protein lipid model of the opiate receptor. Life Sci. 26, 1459-1464.

Lenaz, G., Curatola, G. and Masotti, L. (1975). Perturbation of membrane fluidity. J. Bioenergetics 7,223-299.

Levental, M. and Rakic, L. (1980). Na-K, Adenosine triphosphatase activity in myelin, axoplasmic and synaptic plasma membranes isolated from rat brain: effect of ethanol. Subst. Alcohol Action/Misuse 1, 493-506.

Limbird, L. E. and Lefkowitz, R. J. (1976). Adenylate cyclase - coupled beta-adrenergic receptors: Effects of membrane lipid-perturbing agents on receptor binding and enzyme stimulation by catecholamines. Mol. Pharmacol. 12, 559-567.

Lin, D. C., (1980). Involvement of the lipid and protein compounds of (Na^+ + K^+)-adenosine triphosphatase in the

inhibitory action of alcohol. Biochem. Pharmacol. 29, 771-775.

Lin. H. K., Holland, M. J. C., and Simon, E. j. (1981). Characterization of phospholipase A inhibition of stereospecific opiate binding and its reversal by bovine serum albumin. J. Pharmacol. Exp. Therap. 216, 149-155.

Lin, H. K. and Simon, E. J. (1978). Phospholipase A inhibition of opiate receptor binding can be reversed by albumin. Nature (Lond.) 271, 383-384.

Liu, S. C. and Worthington, C. R. (1974). Electron density levels of sarcoplasmic reticulum membranes. Arch. Biochem. Biophys. 163, 332-342.

Loh, H. H., Cho. T. M., Wu. Y. C., Harris, R. A., and Way, E. L. (1975). Opiate binding to cerebroside sulfate: A model system for opiate receptor interaction. Life Sci. 16, 1811-1818.

Loh, H. H., Cho. T. M., Wu. Y. C. and Way, E. L. (1974). Stereospecific binding of narcotics to brain cerebrosides. Life Sci. 14, 2231-2245.

Loh, H. H., Law, P. Y., Oswald, T., Cho, T. M. and Way, E. L. (1978). Possible involvement of cerebroside sulfate in opiate receptor binding. Fed. Proc. 37, 14-152.

Lord, J. A. H., Waterfield, A. A., Hughes, J. and Kosterlitz, H. W. (1976). Multiple receptors. In: Opiates and Endogenous Opioid Peptides, H. W. Kosterlitz (ed.) (Elsevier/North Holland Biomedical Press, Amsterdam), pp. 275-280.

Lord, J. A. H., Waterfield, A. A. , Hughes, J. and Kosterlitz, H. W. (1977). Endogenous opioid peptides: Multiple agonists and receptors. Nature (Lond.) 267, 495-499.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lumh, R. H., Kloosterman, A. D., Writz, K. W. A. and Van Deenan, L. L. M. (1976). Some properties of phospholipid exchange proteins from rat liver. Eur. J. Biochem. 69, 15-22.

MacLennan, D. H., Seeman, P., Iles, G. H. and Yip, C. C. (1971). Membrane formation by the adenosine triphosphatase of sarcoplasmic reticulum. J. Biol. Chem. 246, 2702-2710.

Madden, T. D., King, M. D. and Quinn, P. J. (1981). The modulation of Ca^{++}-ATPase activity of sarcoplasmic reticulum by membrane cholesterol The effect on enzyme coupling. Biochem. Biophys. Acta 641, 265-269.

Maizel, J. V., Jr. (1971). Polyacrylamide gel electrophoresis of viral proteins. In: Methods in Virology, K. Maramorosch and Koprowski, H. (eds.), Vol. 5 (Academic Press, New York), pp. 179-246.

Malfory, B., Swerts, J. P., Guyon, A., Rogues, B. P. and Schwartz, A. (1978). High affinity enkephalin-degrading peptidase in brain is increased after morphine. Nature 276, 523-526.

Marai, L. and Kuksis, A (1973). Molecular species of glycerolipids of adenosine triphosphatase and sarcotubular membranes of rabbit skeletal muscle. Can. J. Biochem. 51, 1248-1261.

Marks, N., Grynbaum, A. and Neidle, A. (1977). On the degradation of enkephalins and endorphins by rat and mouse brain extracts. Biochem. Biophys. Res. Commun. 74, 1552-1559.

Martin, W. R., Fades, C. G., Thompson, J. A., Huppler, R. E. and Gilbert, P. E. (1976). The effects of morphine-nalorphine-like drugs in the non dependent and morphine dependent chronic spinal dog. J. Pharmacol. Exp. Therap. 197, 517-532.

Martonosi, A., Donley, J. and Halpin, R. A. (1968). Sarcoplasmic reticulum. 3. The role of phospholipids in the adenosine triphosphatase activity and Ca^{++} transport. J. Biol. Chem. 243, 61-70.

Martonosi, A., Donley, J. R., Purcell, A. G. and Halpin, R. A. (1971). Sarcoplasmic reticulum. 1. The mode of involvement of phospholipids in the hydrolysis of ATP by sarcoplasmic reticulum membrane. Arch. Biochem. Biophys. 144, 529-540.

Maxfield, F. R., Schlesinger, J.,Schechter, Y., Pastan, I. and Willingham, M. C. (1979). Collection of insulin, EGF and alpha-2- macroglubolin in the same patches on the surface of cultured fibroblasts and common internalization. Cell 14, 805-810.

Maxfield, F. R., Willingham, M. C., Davies, P. G. A., and Pastan, I. (1979). Amines inhibit the clustering of alpha 2-macroglubolin and EGF on the fibroblast cell surface. Nature 227, 661-663.

Meek, J., Yang, H. and Costa, E. (1977). Enkephalin catabolism in vitro and in vivo. Neuropharmacology 16, 151-154.

Meldolesi, M. F., Fishman, P. H., Aloj, S. M., Ledley, F. D., Lee, G., Bradley, R. M., Brady, R. O. and Kohn, L. D. (1977). Separation of the glycoprotein and ganglioside components of thyrotropin receptor activity in plasma membrane. Biochem. Biophys. Res. Comm. 75, 581-588.

Miki, Y., Hosaka, K., Yamashita, S., Honda, H. and Numa, S. (1977). Acyl-acceptor specificities of 1-acylglycerolphosphate acyltransferase and 1-acylglycerophosphorylcholine-acyltransferase resolved from rat liver microsomes. Eur. J. Biochem. 81, 433-441.

Miller, R. J., Chang. K. J., Leighton, J. and Cuatrecasas, P. (1977b). Interaction of iodinated enkephalin analogues with opiate receptors. Life Sci. 22, 379-389.

Miller, R. J., and Cuatrecasas, P. (1979). Neurobiology and neuropharmacology of the enkephalins. Adv. Biochem. Psychopharmacol. 20, 187-225.

Montal, M. (1976). Experimental membranes and mechanisms of bioenergy transduction. Ann. Rev. Biophys. Bioeng. 5, 119-175.

Moore, B. M., Lentz, B. R., Hoechli, M. and Meissner, G. (1981). Effect of lipid membrane structure on the adenosine-5'-triphosphate hydrolyzing activity of the calcium stimulated adenosine triphosphatase of sarcoplasmic reticulum. Biochemistry 20, 6810-6817.

Moore, N. F., Patzer, E. J., Barenholz, Y. and Wagner, R. R. (1977). Effect of phospholipase C and cholesterol oxidase on membrane integrity, microviscosity and infectivity of vesicular somatitis virus. Biochemistry 16, 4708-4715.

Morgan, R. L., Lee, N. M. and Loh, H. H. (1981). In vivo incorporation of a nonperturbing cholesterol-like fluorescent probe into rat brain membranes (submitted for publication).

Morimoto, K., and Kanoh, H. (1978). Acyl chain length dependency of diacylglycerol cholinephosphotransferase and diacylglycerol ethanolaminephosphotransferase. Effect of different saturated fatty acids at the C-1 or C-2 position of diacylglycerol on solubilized rat liver microsomal enzymes. J. Biol. Chem. 253, 5056-5060.

Nelson, G. J. (1967). Lipid composition of erythrocytes in various mammalian species. Biochem. Biophys. Acta 144,

221-232.

Nicholson, G. L., Poste, G. and Ji, T. H. (1977). The dynamics of cell membranes. In: Dynamic Aspects of Cell Surface Organization, G. Poste and G. L. Nicholson (eds.) (North Holland Publishing Co., Amsterdam), pp. 1-73.

Niggli, V., Adunyah, E. S. and Carafoli, E. (1981b). Acidic phospholipids, unsaturated fatty acids and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{++}-ATPase. J. Biol. Chem. 256, 8588-8592.

Niggli, V., Adunyah, E. S., Penniston, J. T. and Carafoli, E. (1981a). Purified (Ca^{++} - Mg^{++})-ATPase of the erythrocyte membrane. Reconstitution and effect of calmodulin and phospholipids. J. Biol. Chem. 256, 395-401.

Nozawa, Y., Kasai, R. and Sekiya, T. (1979). Modification of membrane lipids. Phenethyl alcohol-induced alteration of lipid composition in tetrahymena membranes. Biochem. Biophys. Acta 552, 38-52.

.Ochoa, E., De Plazas, S. F. and De Robertis, E. (1972). The effect of (-)noradrenaline on artificial lipidic membranes containing a proteo lipid with adrenoreceptor properties. J. Pharm. Pharmacol. 24, 75-77.

Pal, B. K., Lowney, L. I. and Goldstein, A. (1973). Further studies on the stereospecific binding of levorphanol by a membrane fraction from mouse brain. In: Agonist and Antagonist Actions of Narcotic Analgesic Drugs, H. Kosterlitz, H. Collier and J. Villarreal (eds.) (University Park Press), pp. 62-69.

Palatini, P., Dabbeni-Sala, F. and Bruni, A. (1972). Reactivation of a phospholipid-depleted sodium-potassium stimulated ATPase. Biochem. Biophys. Acta 288, 413-422.

Papahdjopoulos, D. (1973). In: Form and Function of Phospholipids, G. B. Ansell, R. M. C. Dawson and J. N. Hawthorne (eds.) (2nd Edition, Elsevier, Amsterdam), Chap. 7.

Papahdjopoulos, D., Cowden, M. and Kimbelberg, H. (1973). Role of cholesterol in membrane. Effects on phospholipid-protein interaction, membrane permeability and enzymatic activity. Biochem. Biophys. Acta 330 8-26.

Pasternak, G. W., Goodman, R. and Snyder, S. H. (1975). An endogenous morphine-like factor in mammalian brain. Life Sci. 16, 13-17.

Pasternak, G. W., and Snyder, S. H. (1974). Opiate receptor

binding: Effects of enzymatic treatment. Mol. Pharmacol. 10, 183-193.

Pasternak, G. W. and Snyder, S. H. (1975a). Opiate receptor binding: Enzymatic treatment that discriminates between agonist and antagonist interactions. Mol. Pharmacol. 11, 478-484.

Pasternak, G. W. and Snyder, S. H. (1975b). Identification of novel high affinity opiate receptor binding in rat brain. Nature (Lond.) 253, 563-565.

Pasternak, G. W. and Snyder. S. H. (1975c). Opiate receptor binding: effects of enzymatic treatments. Mol. Pharmacol. 10, 183-193.

Pasternak, G. W., Wilson, H. A. and Snyder, S. H. (1975). Differential effects of protein modifying reagents on receptor binding of opiate agonists and antagonists. Mol. Pharmacol. 11, 34-351.

Pert, C. B. and Snyder, S. H. (1973a). Opiate receptor: Demonstration in nerve tissue. Science 179, 1011-1014.

Pert, C. B. and Snyder, S. H. (1973b). Properties of opiate receptor binding in rat brain. Proc. Natl. Acad. Sci. U.S.A. 70, 2243-2247.

Pert, C. B. and Snyder, S. H. (1974). Opiate receptor binding of agonists and antagonists affected differentially by sodium. Mol. Pharmacol. 10, 868-879.

Peters, W. H. M., Fleuren-Jakobs, A. M. M., DePont, J. J. H. H. M. and Bonting, S. L. (1981). Studies on (Na^+-K^+)-Activated ATPase XLIX. Content and role of cholesterol and other neutral lipids in highly purified rabbit kidney enzyme preparation. Biochem. Biophys. Acta 649, 541-549.

Pohl, S. L., Krans, H. M. J., Kozyreff, V., Birnhaumer, L. and Rodbell, M. (1971). The glucagon-sensitive adenyl cyclase systems in plasma membrane of rat liver. IV. Evidence for a role of membrane lipids. J. Biol. Chem. 246, 4447-4454.

Portius, H. J. and Repke, K. (1963). Versuche zur charakterizierung einer transport-ATPase fur Na^+ und K^+ in der zellmembran des herzmuskels. Arch. Exptl. Pathol. Pharmacol. 245, 62-63.

Post, R. L. and Sen, A. K. (1967). Sodium and potassium stimulated ATPase. Methods Enzymol. 10, 762-768.

Racker, E. (1972). Reconstitution of a calcium pump with phospholipids and a purified Ca^{++}-adenosine triphosphatase from sarcoplasmic reticulum. J. Biol. Chem. 247, 8198-8200.

Racker, E. (1973). A new procedure for the reconstitution of biologically active phospholipid vesicles. Biochem. Biophys. Res. Commun. 55, 224-230.

Racker, E., Knowles, A. F. and Eytan, E. (1975). Resolution and reconstitution of ion transport systems. Ann. N.Y. Acad. Sci. 264, 17-33.

Ragan, C. I. and Racker, E. (1973). Resolution and reconstitution of the mitochondrial transport system. IV. The reconstitution of rotenone sensitive reduced nicotinamide adenine dinucleotide ubiquinone reductase from reduced nicotinamide adenine dinucleotide dehydrogenase and phospholipids. J. Biol. Chem. 248, 6876-6884.

Riordan, J. R., Slavik, M. and Kartner, N. (1977). Nature of the lecithin induced activation of plasma membrane Mg^{++} ATPase. J. Biol. Chem. 252, 5449-5455.

Robson, L. E. and Kosterlitz, H. W. (1979). Specific protection of u and delta binding sites in guinea pig brain against the inhibitory effect of phenoxylbenzamine by agonists preferentially interacting with u or delta sites. In: Endogenous and Exogenous Opiate Agonists and Antagonists, E. L. Way (ed.) (Pergamon Press), pp. 107-108.

Rodbard, D. (1973). Mathematics of hormone-receptor interaction. I. Basic principles. In: Receptors for Reproductive Hormones, B. W. O'Mally and A. R. Means (eds.) (Plenum Press, New York), pp. 289-326.

Rogers, J., Lee, A. G. and Wilton, D. C. (1979). The organization of cholesterol and ergosterol in lipid bilayers based on studies using non-perturbing fluorescent sterol probes. Biochem. Biophys. Acta 552 23-37.

Rosenberg, S. A. and McIntosh, J. R. (1968). Erythrocyte membranes: Effects on sonication. Biochem. Biophys. Acta 163, 285-289.

Ross, E. M. and Gilman, A. G. (1980). Biochemical properties of hormone sensitive adenylate cyclase. Ann. Rev. Biochem. 49, 533-564.

Rothman, J. E. and Dawidowicz, E. A. (1975). Asymmetric exchange of vesicle phospholipids catalyzed by the phosphatidylcholine exchange protein measurement of inside-outside transitions. Biochemistry 14, 2809-2816.

Rothman, J. E. and Engelman, D. M. (1972). Molecular mechanism for the interaction of phospholipid with cholesterol. Nature (New Biol). 232 42-44.

Ruegg, U. T., Hiller, J. M. and Simon, E. J. (1980). Solubilization of an active opiate receptor from Bufo Marinus. Eur. J. Pharmacol. 64, 367-368.

Rydstrom, S., Hoek, J. B. and Hundal, T. (1974). Selective solubilization of nicotinamide nucleotide transhydrogenase from the mitochondrial inner membrane. Biochem. Biophys. Res. Commun. 60, 448-455.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672.

Schiebler, W. and Hucho, F. (1978). Membranes rich in acetylcholine receptor: characterization and reconstitution to excitable membranes from exogenous lipids. Eur. J. Biochem. 85, 55-63.

Schindler, H. and Quast, U. (1980). Functional acetylcholine receptor from Torpedo marmorata in planar membranes. Proc. Natl. Acad. Sci. U.S.A. 77, 3052-3056.

Schwartz, J. C., Malfroy, B. and De La Baume, S. (1981). Biological inactivation of enkephalins and the role of enkephalin dipeptidyl carboxypeptidase ("enkephalinase") as neuropeptidase. Life Sci. 29, 1715-1740.

Sharma, S. F., Nirenberg, M. and Klee, W. A. (1975a). Morphine receptors as regulators of adenylate cyclase activity. Proc. Natl. Acad. Sci. U.S.A.72, 590-594.

Sheats, J. and McConnell, H. M. (1978). A photochemical technique for measuring lateral diffusion of spin labeled phospholipids in membranes. Proc. Natl. Acad. Sci. U.S.A. 75, 4661-4663.

Shinitzky, M. (1979). The concept of passive modulation of membrane responses. In: Physical and Chemical Respects of Cell Surface Events and Cellular Regulation, R. Blumenthal and C. deLisi (eds.) (Elsevier/ North Holland, Amsterdam), pp. 173-181.

Shinitzky, M. and Barenholz, Y. (1974). Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. J. Biol. Chem. 249, 2652-2657.

Shinitzky, M., Dianoux, A. C. , Gittler, C. and Weber, G. (1971). Microviscosity and order in the hydrocarbon region of micelles and membranes determined with fluorescent probe. I. Synthetic micelles. Biochemistry 10, 2106-2113.

Shinitzky, M. and Inbar, M. (1976). Microviscosity parameters and protein mobility in biological membrane. Biochem. Biophys. Acta 433, 133-149.

Shinitzky, M. and Souroujon, M. (1979). Passive modulation of blood group antigen. Proc. Natl. Acad. Sci. U.S.A. 76, 4438-4440.

Simantov, R., Childers, S. R. and Snyder, S. H. (1978). The opiate receptor binding interactions of ^{3}H-methionine enkephalin, an opioid peptide. Eur. J. Pharmacol 47, 319-331.

Simon, E. J., Bonnet, K. A., Grain, S. M., Groth, J., Hiller, J. M. and Smith, S. R. (1980). Recent studies on interaction between opioid peptides and their receptors. Adv. Biochem. Psychopharmacol. 22, 335-346.

Simon, E. J. and Groth, J. (1975). Kinetics of opiate receptor inactivation by sulfhydryl reagents. Evidence for conformational change in the presence of sodium ion. Proc. Natl. Acad. Sci. U.S.A. 72, 2404-2407.

Simon, E. J., Hiller, J. M. and Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic (^{3}H) etorphine to rat brain homogenate. Proc. Natl. Acad. Sci. U.S.A. 70, 1947-1949.

Simon, E. J., Hiller, J. M. and Edelman, I. (1975). Solubilization of a stereospecific opiate macromolecular complex from rat brain. Science 190, 389-390.

Simon, E. J., Hiller, J. M., Edelman, J., Groth, J. and Stahl, K. D. (1975b). Opiate receptors and their interactions with agonists and antagonists. Life Sci. 16, 1795-1800.

Simon, E. J., Hiller, J. M., Groth, J. and Edelman, I. (1975b). Further properties of stereospecific opiate binding sites in rat brain. J. Pharmacol. Exp. Therap. 192, 531-537.

Simond, W. E., Koski, G., Streaty, R. A., Jelmeland, H. and Klee, W. A. (1980). Solubilization of active opiate receptors. Proc. Natl. Acad. Sci. U.S.A. 77, 4623-4627.

Simons, K., Garoff, H., Helenius, A., Kaariainen, L. and Renkonen, O. (1974). Structure and assembly of virus membranes. In: Perspectives in Membrane Biology, O.S. Estrada and C. Gitler (eds.) (Academic Press, New York), pp. 45-70.

Singer, S. J. and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

Skipski, V. P., Peterson, R. F. and Barclay, M. (1964). Quantitative analysis of phospholipids by thin layer chromatography. Biochem. J. 90, 374-378.

Smith, A. P., and Loh, H. H. (1979). Mobile and immobile proteins of synaptosomal plasma membrane. Biochemistry 17, 4343-4347.

Smith, B. A. and McConnell, H. M. (1978). Determination of molecular motion in membranes using periodic pattern photobleaching. Proc. Natl. Acad. Sci. U.S.A. 75, 2759-2763.

Smith, R. J. M. and Green, C. (1979). Fluorescent studies of protein sterol relationship in human plasma lipoproteins. Biochem. J. 137, 413-415.

Smith, T. W., Hughes, J., Kosterlitz, H. W. and Sasa, R. P. (1976). In: Opiates and Endogenous Opioid Peptides, H. W. Kosterlitz (ed.) (Elsevier, Amsterdam), pp. 57-62.

Stahl, K. D., Van Bever, W., Jansson, P. and Simon, E. J. (1977). Receptor affinity and pharmacological potency of a series of narcotic analgesics, antidiarrheal and neuroleptic drugs. Eur. J. Pharmacol. 46, 199-205.

Strittmatter, W. J., Hirata, F., and Axelrod, J. (1979). Phospholipid methylation unmasks cryptic beta-adrenergic receptors in rat reticulocytes. Science 204, 1205-1207.

Subbaiah, P. V. and Bagdade, J. D. (1978). Demonstration of enzymatic conversion of lysolecithin to lecithin in normal human plasma. Life Sci. 22, 1971-1978.

Sun, A. Y. (1972). The effect of lipoxidation on synaptosomal ($Na^+ + K^+$) ATPase isolated from the cerebral cortex of squirrel monkey. Biochem. Biophys. Acta 226, 350-360.

Sun, A. Y., Sun. G. Y. and Samorajski, T. (1971). The effect of phospholipase C on the activity of adenosine triphosphatase and acetylcholinesterase in synaptic membranes isolated from the cerebral cortex of squirrel monkey. J. Neurochem. 18, 1711-1718.

Sun, G. Y., Winniczek, H., Go, J. and Sheng, S. L. (1975). Essential fatty acid deficiency: Metabolism of 20:3(n-9) and 22:3(n-9) of major phosphoglycerides in subcellular fractions of developing and mature mouse brain. Lipids 10,

365-373.

Tamai, Y., Matsukawa, S. and Satake, M. (1971). Lipid composition of nerve cell perikayra. Brain Res. 26, 149-157.

Tanaka, R. and Strickland, K. P. (1965). Role of phospholipid in the activation of Na^+, K^+-activated adenosine triphosphatase of beef brain Arch. Biochem. Biophys. 111, 583-592.

Taniguchi, K. and Tonomura, Y. (1971). Inactivation of Na plus -K plus- dependent ATPase by phospholipase treatment and its reactivation by phospholipids. J. Biochem. (Tokyo). 69, 543-557.

Tateishi, I., Steiner, M. and Baldini, M. G. (1973). Studies on lipid peroxides in platelets. 3. Effect on physiologic platelet functions. J. Lab. Clin. Med. 81, 587-602.

Tatibana, M. (1963). Effect of snake venom upon membrane adenosine triphosphatase of human erythrocyte. J. Biochem. 53, 260-261.

Terenius, L. (1973a). Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Pharmacol. Toxicol. 32, 317-320.

Terenius, L. (1974). Contribution of receptor affinity to analgesic potency. J. Pharm. Pharmacol. 26, 146-148.

Thompson, T. E. (1978). Transmembrane compositional asymmetry of lipids in bilayers and biomembranes. In: Molecular Specialization and Asymmetry in Membrane Function, A. K. Solomon and M. Karnovsky (eds.) (Cambridge, Mass. Harward University Press). pp. 78-79.

Tzagoloff, A. and MacLennan, D. H. (1965). Studies of the electron transfer system. LXIV. Role of phospholipid in cytochrome oxidase. Biochem. Biophys. Acta 99, 476-485.

Tzagoloff, A., McConnell, P. G. and MacLennan, D. A. (1968). Studies on the electron transfer system. LXIX. Solubilization of the mitochondrial inner membrane by sonic oscillation. J. Biol. Chem. 243, 4117-4122.

Urca, G., Frenk, H. and Liebeskind, J. C. (1978). Morphine and enkephalin: analgesic and epileptic properties. Science 197, 83-86.

Vanderkooi, J., Fischkoff, S., Chance, B. and Cooper, R. A. (1974). Fluorescent probe analysis of the lipid architecture

of natural and experimental cholesterol rich membrane. Biochemistry 13, 1589-1595.

Van Holde, K. E. (1971). In: Physical Biochemistry (Prentice-Hall, Inc., Englewood Cliffs, New Jersey).

Vogel, Z. and Altstein, M. (1977). The adsorption of enkephalin to porous polystyrene beads: a simple assay for enkephalin hydrolysis. FEBS Lett. 80, 332-336.

Vogel, Z. and Altstein, M. (1979). The effect of puromycin on the biological activity of leu-enkephalin. FEBS Lett. 98, 44-48.

Wagner, R. (1980). Electron microscopy of membranes. In: Introduction to Biological Membrane, K. J. Mahendra and R. C. Wagner (eds.) (J. Wiley and Sons, New York), pp. 6-24.

Warren, G. B., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974). In: Membrane Proteins in Transport and Phosphorylation, G. F. Azzone, M. E. Klingenberg, E. Quagliariello and N. Siliprandi (eds.) (Amsterdam, North Holland Publishing Co.), pp. 1-12.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974a). Complete control of the lipid environment of membrane-bound proteins: application to calcium transport system. FEBS Lett. 41, 122-124.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974b). Reconstitution of a calcium pump using defined membrane components. Proc. Natl. Acad. Sci. U.S.A. 71, 622-626.

Way, E. L. and Glasgow, C. E. (1978). The endorphins: possible physiologic roles and therapeutic applications. Clin. Ther. 1, 371-386.

Webster, G. R. and Alpern, R. J. (1964). Studies of the acylation of lysolecithin by rat brain. Biochem. J. 90, 35-42.

Weislander, A., Christiansson, A., Rilford, L. and Lindblom, G. (1980). Lipid bilayer stability in membranes regulation of lipid composition in Acholeplasma Laidlawii as governed by molecular shape. Biochemistry 19, 3650-3655.

Weltzien, H. U., Richter, G. and Ferber, E. (1979). Detergent properties of water soluble choline phosphatides: selective solubilization of acyl-CoA lysolecithin acyltransferase from thymocyte plasma membranes. J. Biol. Chem. 254, 2652-3657.

Wheeler, K. P. and Whittam, R. (1970a). The involvement of phosphatidyl serine in adenosine triphosphatase activity of the sodium pump. J. Physol. (London) 207, 303-328.

Wheeler, K. P. and Whittam, R. (1970b). ATPase activity of the sodium pump needs phosphatidylserine. Nature (Lond.) 225, 449-450.

White, D. A., Ansell, G. D., Hawthorne, J. N. and Dawson, R. M. C. (eds.). Form and Function of Phospholipids (Elsevier Publishing Co., New York), 1973.

White, D. A. and Hawthorne, J. N. (1970). Zymogen secretion and phospholipid metabolism in the pancreas. Phospholipids of the zymogen granule. Biochem. J. 120, 533-538.

Whittacker, V. P., Michaelson, I. A., Jeanette, R. and Kirkland, A. (1964). The separation of synaptic vesicles from nerve ending particles "synaptosomes." Biochem. J. 90, 293-303.

Windaus, A. and Linsert, O. (1978). Ultraviolet irradiation of dehydrocholesterol. Justus Liebigs Ann. Chem. 465, 148-166.

Wirtz, K. W. A., Devaux, P. F. and Bievenue, A. (1980). Phosphatidylcholine exchange protein catalyzes the net transfer of phosphatidylcholine to model membranes. Biochemistry 19, 3395-3399.

Wirtz, K. W. A., Vriend, G. and Westerman, J. (1979). Kinetic analysis of the interaction of the phosphatidylcholine exchange protein with unilamellar vesicles and multilamellar liposomes. Eur. J. Biochem. 94, 215-221.

Wirtz, K. W. A. and Zilversmit, D. B. (1968a). Exchange of phospholipids between subcellular particles of liver in vitro. Fed. Proc. 27, 636.

Wirtz, K. W. A. and Zilversmit, D. B. (1968b). Exchange of phospholipids between liver mitochondria and microsomes in vitro. J. Biol. Chem. 243, 3596-3602.

Wirtz, K. W. A. and Zilversmit, D. B. (1970). Partial purification of phospholipid exchange protein from beef hearts. FEBS Lett. 7, 44-46.

Wise, R. W., Sun, G. Y. and MacQuarrie, R. (1980). The kinetic properties of oleoyl-CoA:1-Acyl-sn-glycero-3-Phosphocholine O-acyltransferase from mouse brain microsomes. Eur. J. Biochem. 109, 201-206.

Wu. Y. C., Cho, T. M. and Loh, H. H. (1977). Binding of dimethyl tubocurarine to triphosphoinositide. J. Neurochem. 29, 589-592.

Yedgar, S., Barenholz, Y. and Cooper, V. G. (1974). Molecular weight, shape and structure of mixed micelles of Triton X-100 and sphingomyelin. Biochem. Biophys. Acta 363, 98-111.

Yoshida, S., Orii, Y., Kawato, S. and Ikegami, A. (1979). Effects of temperature on cytochrome oxidase activity in solubilized form and in lipid vesicle systems. J. Biochem. 86, 1443-1450.

Zukin, R. S. and Kream, R. M. (1979). Chemical crosslinking of a solubilized enkephalin macromolecular complex. Proc. Natl. Acad. Sci. U.S.A. 76, 1593-1597.