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INFLUENCE OF SYNAPTIC MEMBRANE CHOLESTEROL
CONTENT ON DOPAMINERGIC FUNCTION

by

Patricia A. Maguire

A Dissertation Submitted to the Faculty of the Graduate
School of Loyola University of Chicago in Partial
Fulfillment of the Requirements of the Degree of
Doctor of Philosophy

January

1988

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VITA

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PUBLICATIONS

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LIST OF ABBREVIATIONS

- ADTN - 2-amino-6,7-dihydroxyl-1,2,3,4-tetrahydronaphthalene
- ANS - 1-anilinonaphthalene-8-sulfonic acid
- cAMP - 3'-5' cyclic adenosine monophosphate
- ATP - adenosine 5'-triphosphate
- ATPase - adenosine 5'-triphosphatase
- Bis - N,N'-methylene-bis-acrylamide
- B_{max} - maximum number of binding sites
- cfg - centrifugation
- Chol/PL - cholesterol to phospholipid molar ratio
- CNPase - 2',3'-cyclic nucleotide phosphohydrolase
- CNS - central nervous system
- DNA - deoxyribonucleic acid
- DPH - 1,6-diphenyl-1,3,5-hexatriene
- EDTA - ethylenediamine-tetraacetic acid
- EGTA - ethyleneglycol-N,N'-tetraacetic acid
- EP - ethanolamine plasmalogen
- ESR - electron spin resonance
- FA - fatty acid, fatty acyl
- GABA - gamma aminobutyric acid
- GDP - guanosine 5'-diphosphate
- G-protein - guanine nucleotide binding protein
- GTP - guanosine 5'-triphosphate
- GTPase - guanosine 5'-triphosphatase

HSA - human serum albumin
I - fluorescence intensity
 K_d - binding dissociation constant
 K_i - inhibition constant
 K_m - Michaelis constant
LTP - lipid transfer protein
NMR - nuclear magnetic resonance
ns-LTP - nonspecific lipid transfer protein
O.D. - optical density
P - fluorescence polarization
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PC - phosphatidylcholine
PE - phosphatidylethanolamine
Pi - inorganic phosphate
PI - phosphatidylinositol
 P_I - isoelectric point
PS - phosphatidylserine
r - fluorescence anisotropy
 R_f - retention factor
RIA - radioimmunoassay
RNA - ribonucleic acid
rpm - revolutions per minute
SCP - sterol carrier protein
SDS - sodium dodecyl sulfate

SET buffer - 0.25 M sucrose, 1 mM EDTA, 50 mM Tris HCl,
0.02% sodium azide, pH 7.4

SP - sphingomyelin

SPM - synaptic plasma membrane

STD - standard

Sucrose buffer - 0.32 M sucrose, 5 mM Tris base, pH 7.0

TEMED - N,N,N',N'-tetramethylethylenediamine

Tris - Tris(hydroxymethyl)aminoethane

V_{max} - maximum velocity

CHAPTER I

INTRODUCTION

The fluidity of synaptic membranes has been shown to affect the activities of several membrane-associated enzymes. These include Na^+K^+ -ATPase (Farias et al., 1975; Alivisatos et al., 1977; Sandermann, 1978; Alivisatos et al., 1981) and adenylate cyclase (Rimon et al., 1978; Whetton et al., 1983). Membrane-associated functions of a number of neurotransmitter systems have also been shown to be modulated by membrane fluidity. The neurotransmitter systems include the serotonergic (Heron et al., 1980a,b; Heron et al., 1981), opioid peptide (Heron et al., 1980a; Heron et al., 1981), GABAergic (Toffano et al., 1981; Chweh & Leslie, 1982; North & Fleischer, 1983b), and cholinergic (Crews et al., 1983a; Freund et al., 1986) systems.

Postnatal development, aging, and alcoholism are accompanied by changes in membrane-associated functions of several neurotransmitter systems (Freund, 1982; Meyer et al., 1984), an increase in the synaptic membrane cholesterol to phospholipid molar ratio, and decreased membrane fluidity (Crews et al., 1983b; Lyon & Goldstein, 1983; Nagy et al., 1983). It is possible that the changes in neural function are due, in part, to the changes in synaptic membrane lipid composition and fluidity.

To study this hypothesis, the influence of cholesterol-induced changes in membrane fluidity on the dopaminergic system was investigated because the dopaminergic system is sensitive to the effects of aging and alcohol (Roth, 1982; Hoffman & Tabakoff, 1985). The cholesterol content of synaptic plasma membranes and synaptosomes was altered using a lipid transfer protein from beef liver, which accelerates the exchange of cholesterol between liposomes and synaptic membranes. The fluidity of control and lipid-modified synaptic plasma membranes (SPM) was assessed by fluorescence polarization techniques, using 1,6-diphenyl-1,3,5-hexatriene, to probe the hydrophobic core of the membrane, and 1-anilinonaphthalene-8-sulfonic acid to probe the membrane surface. Control and lipid-modified synaptosomes were assayed for [³H]-dopamine uptake, binding to the D₁ receptor as assessed by the binding of [³H]-SCH23390, and maximal stimulation of adenylate cyclase activity by dopamine. The results obtained from these studies may provide important information about the influence of synaptic lipid composition and fluidity on receptor-mediated neural function. This data may provide a basis for understanding some of the age- and alcohol-related changes in neural function.

CHAPTER II

REVEIW OF THE RELATED LITERATURE

MEMBRANE COMPOSITION AND FLUIDITY

FACTORS INFLUENCING MEMBRANE FLUIDITY

The fluidity of a biological membrane relates to the dynamic physical state of the fatty acyl chains of the phospholipids comprising that membrane bilayer (Stubbs, 1983). Factors that influence membrane fluidity include: the unsaturation and chain length of fatty acids, the change in frequency of trans-gauche isomerizations of C-C bonds of the methylene groups in phospholipid fatty acyl chains, and the rate and range of motion in phospholipid fatty acyl chains. Also important are the lateral and rotational mobility of phospholipids, changes in phospholipid head group conformations, changes in the interaction of cholesterol with membrane lipids, and the change in the interaction of proteins with lipids (Shinitzky & Barenholz, 1978; Houslay & Stanley, 1982; Stubbs, 1983; Stubbs & Smith, 1984).

The degree of unsaturation and chain length of the fatty acids (FA) in phospholipids are important in membrane fluidity. A decrease in the double bond index:saturated ratio, defined as:

$$\overline{\frac{\text{(mole \% unsaturated FA X \# double bonds)}}{\text{mole \% saturated FA}}} \quad (\text{eq. 1})$$

(Stubbs, 1983) has been used as a chemical measure of membrane fluidity (Farias et al., 1975). This assumption is valid when other parameters, such as cholesterol/phospholipid molar ratio (Chol/PL), remain constant and the FA double bonds have the same cis configuration (Castuma & Brenner, 1983). Studies using biological membranes indicate that a significant change in the degree of unsaturation of FA results in only small changes in the motion of the FA chains as measured using electron spin resonance (ESR) and fluorescence techniques (Stubbs & Smith, 1984).

Phospholipid head groups have a complex effect on membrane fluidity. In model membranes, phospholipid bilayers prepared from individual classes of saturated phospholipids, the transition temperatures vary from 0°C in phosphatidylcholine (PC) membranes to 30°C in sphingomyelin (SP) membranes. It is evident that the head group influences acyl chain motion, but the biological importance is unclear because of the variability in the fatty acid composition between the classes of phospholipids found in biological membranes (Stubbs, 1983).

Membrane-bound proteins have an immobilizing effect on the lipids that surround them (boundary lipids) (Stubbs, 1983). The fluidity of lipids in the region of proteins can be studied using nuclear magnetic resonance (NMR) techniques with deuterated phospholipids. This is

not very efficient, however, because the labelled boundary lipids readily exchange with bulk lipid. Because of the nature of their probes, ESR and fluorescence spectroscopic techniques give no information about the fluidity of this boundary lipid region (Feinstein et al., 1975; Stubbs, 1983).

Cholesterol, a major lipid of plasma membranes of most cells (Stubbs, 1983), is a principal modulator of membrane fluidity. With its rigid, planar, wedge-shaped structure, cholesterol orients perpendicular to the membrane surface, with its hydroxyl group near the carbonyl of FA (Houslay & Stanley, 1982; Stubbs, 1983) (Figure 1). Cholesterol has the ability to condense phospholipid bilayers, making them less permeable to small molecules. Various physical techniques have demonstrated that cholesterol has a small fluidizing effect below the phase transition temperature (gel phase), and a large rigidifying effect above this temperature (Figure 2) (Van Blitterswijk et al., 1981; Stubbs, 1983). Cholesterol diminishes the enthalpy change at phase transition. The presence of large amounts of cholesterol in the membrane removes the phase transition characteristics. Cholesterol appears to act as a buffer against large changes in the bulk physical properties of the membrane produced by other agents such as alcohol (Stubbs, 1983). In most cases, the viscosity (inverse of fluidity) of a biological membrane reflects its

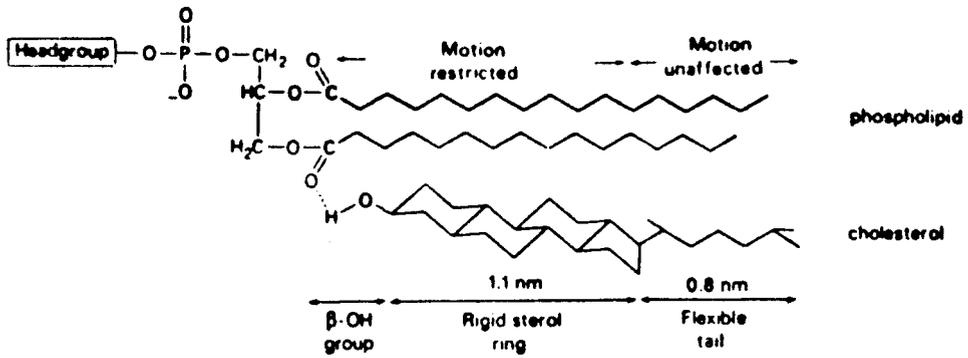


Figure 1. Interaction of Cholesterol with Phospho-
Lipids in a Lipid Bilayer.
(Houslay and Stanley, 1982)

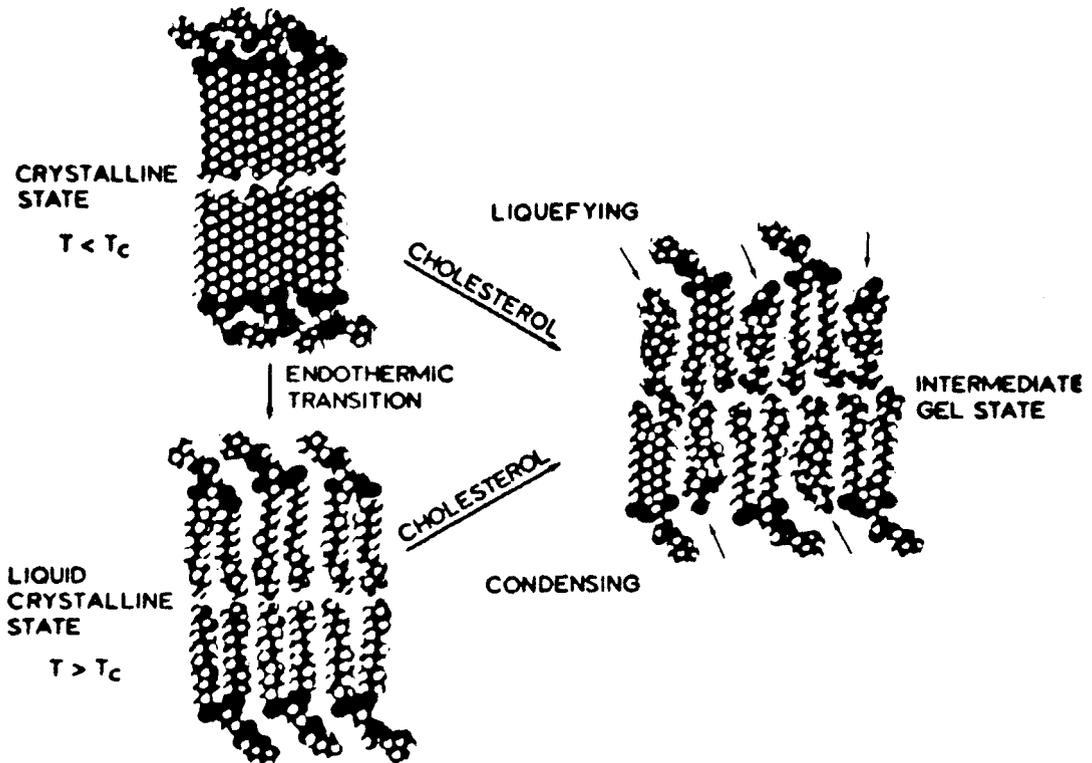


Figure 2: The Effect of Cholesterol on Membrane Phospholipids Above and Below the Transition Temperature.

The arrows point to cholesterol within the bilayer. Above the transition temperature (T_c), cholesterol will condense phospholipid bilayers, reducing their permeability to small molecules. Below the transition temperature, cholesterol liquifies the bilayer, allowing more freedom of movement of the fatty acyl side chains.

(VanBlitterswijk et al., 1981)

Chol/PL, although these cholesterol effects are influenced by the FA composition of phospholipids and charged groups at the membrane surface (Feinstein et al., 1975).

TECHNIQUES FOR MEASURING MEMBRANE FLUIDITY

NMR. In nuclear magnetic resonance (NMR) studies, deuterated lipids are incorporated into the membranes to be studied. Although this method produces the least perturbation of the membrane structure (Stubbs & Smith, 1984), it has several drawbacks. The sensitivity is low, expensive equipment is required, and special deuterated phospholipids must be synthesized in large quantities. Also, the assumptions used to derive the rate of motion and the calculations are very complex and reportedly less reliable than those utilized in fluorescence studies (Stubbs, 1983).

ESR. Electron spin resonance (ESR) of a nitroxide probe can provide information about the mobility of the probe, the orientation of the probe in its environment, the order of the system, and the lateral diffusion of the probe in the plane of the membrane (Jain & Wagner, 1980). The drawbacks of ESR are similar to those of NMR in that the equipment is expensive and the spin-labelled probes must be synthesized. Unlike NMR, ESR probes, because of their unpaired electron, can perturb the membrane.

FLUORESCENCE POLARIZATION. Fluorescence polarization is based on the phenomenon that when a fluorophore is excited by light, it absorbs the light energy and emits

photons of lower energy. This technique can be adapted to give information on the orientation or range and rate of motion of the fluorophore and its adjacent environment (Bentley et al., 1985).

In order to understand the basic principles of fluorescence polarization, several phenomena involved in the fluorescence of a molecule should be described (Bentley et al., 1985). First, an interval of time exists between the absorption and fluorescent emission of light. This interval is the fluorescence lifetime and is usually in the nanosecond range. Second, the emitted light is not released in a random direction, but is oriented in a direction determined by the transition moment of the excited molecules, the direction in which the molecule most easily absorbs light. Light is preferentially absorbed when the electrical vector of the exciting light is parallel to the transition moment of the molecule. The electric vector of the emitted light will also be parallel to the transition moment of the excited molecule.

When a molecule is excited with polarized light, the electric vector of the emitted light will be parallel to the electric vector of the exciting light if the molecule remains stationary during the lifetime of the excited state (Bentley et al., 1985; Shinitzky & Barenholz, 1978). Rotation of a molecule during its excited state will depolarize the emitted light with respect to the exciting

beam. As the molecular motion in a system increases, the emitted light will be increasingly depolarized (Bentley et al., 1985; Shinitzky & Barenholz, 1978).

Fluorescence polarization values (P) are measured by exciting a molecule with polarized light and observing the intensity (I) of the emitted light oriented parallel and perpendicular to the direction of the electric vector of the exciting light. P is calculated by the equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (\text{eq. 2})$$

(Bentley et al., 1985; Shinitzky & Barenholz, 1978).

Experimentally, polarization measurements are made by determining the fluorescence intensity reading with the excitation and emission polarizers in combinations of vertical (v) and horizontal (h) positions. The possible combinations are vv, vh, hh, hv, where the letter order represents the excitation and emission polarizers respectively. I_{\parallel} and I_{\perp} in equation 2 correspond to the settings vv and vh respectively. The hh and hv settings are used to correct for artifacts introduced by the instrument. In practice, P is calculated by the equation:

$$P = \frac{I_{vv} - I_{vh} (I_{hv}/I_{hh})}{I_{vv} + I_{vh} (I_{hv}/I_{hh})} \quad (\text{eq. 3})$$

Polarization data can be expressed as polarization (P) values as described in equations 2 and 3, or as aniso-

tropy (r) values:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (\text{eq. 4})$$

(Shinitzky & Barenholz, 1978).

P values can be converted to r values by the equation:

$$r = \frac{2P}{3-P} \quad (\text{eq. 5})$$

(Shinitzky & Barenholz, 1978).

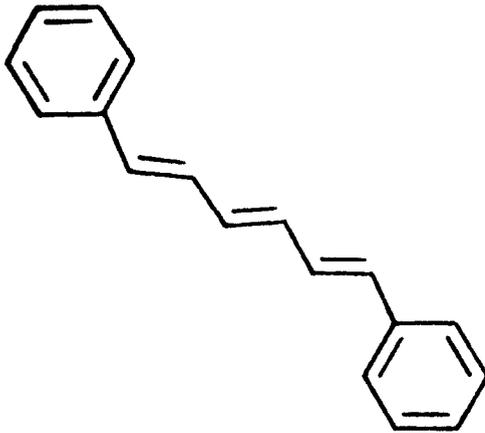
The relative microviscosity of the membrane can be calculated from the r values by the equation:

$$\text{relative microviscosity} = \frac{1}{(r_0/r)-1} \quad (\text{eq. 6})$$

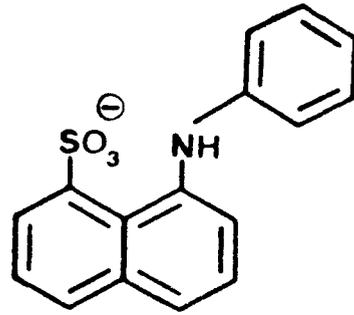
(Shinitzky & Barenholz, 1978). The term r_0 refers to the limiting anisotropy and is experimentally determined for each fluorescent probe. For DPH, $r_0 = 0.362$ (Shinitzky & Barenholz, 1978). For ANS, $r_0 = 0.346$ (Weber & Daniel, 1966).

FLUORESCENT PROBES

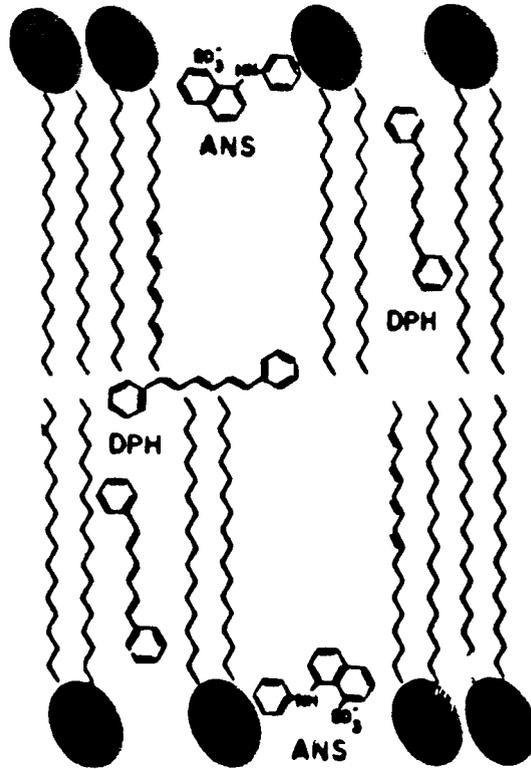
DPH. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Figure 3a) is a polyene hydrocarbon with a stable all-trans configuration of an elongated rectangular shape (Shinitzky & Barenholz, 1978). It partitions easily into membranes, orienting itself perpendicular to the plane of the membrane (Stubbs & Smith, 1984) (Figure 3c). DPH does not fluoresce



a) Structure of DPH



b) Structure of ANS



c) Orientation of DPH and ANS in a Phospho-
Lipid Bilayer.

(Jost and Griffith, 1980)

in an aqueous environment (Stubbs & Smith, 1984). Its quantum yield increases when the environment becomes hydrophobic, and continues to increase as the viscosity increases, approaching 1 (Shinitzky & Barenholz, 1978). The excitation and emission maxima (360 nm and 430 nm, respectively) (Litman & Barenholz, 1982) remain constant, regardless of the phase or composition of the membrane (Shinitzky & Barenholz, 1978). When cholesterol is incorporated into a phospholipid bilayer, the DPH polarization values are greater than those in the absence of cholesterol. The increase in DPH polarization has been correlated to an increase in Chol/PL (Van Blitterswijk et al., 1984). These properties make DPH an almost ideal probe for the hydrophobic region of the membrane (Stubbs & Smith, 1984).

ANS. 1-Anilino-naphthalene-8-sulfonic acid (ANS) (Figure 3b) is a negatively charged molecule that, in the membrane, localizes in the region of the phospholipid polar head, with its aromatic rings at the level of the FA carbonyl, and its sulfonate group directed outward (Figure 3c) (Slavik, 1982). The magnitude of the fluorescence signal from ANS in aqueous solvents is low, but increases as the solvent becomes less polar. The probe prefers to interact with the choline-containing head groups of phosphatidylcholine (PC) and sphingomyelin (SP). Although the positive charge of these groups plays a role in the binding of ANS, the interaction is not entirely electrostatic in nature as

phosphatidylethanolamine (PE), which is also positively charged, does not bind ANS. As the Chol/PL increases within the membrane, the ANS is pushed closer to the surface of the membrane, which makes it more accessible to the aqueous environment, and which decreases the quantum yield (Slavik, 1982).

Although ANS is sensitive to the changes in its environment near the membrane surface, it also binds non-covalently to proteins within the membrane. This can make it difficult to interpret fluorescence polarization data if it is not properly acquired. ANS binding to proteins is of very high affinity, but the number of binding sites is low. The excitation and emission maxima are 380 nm and 480 nm, respectively. The lipid binding sites are of lower affinity, but are more abundant than those on the proteins. The excitation and emission maxima for these sites are 395 nm and 490 nm respectively. When low concentrations of ANS are used (1 μM), ANS binds primarily to proteins. At high concentrations (200 μM), the fluorescence reflects ANS bound predominantly to lipids (Slavik, 1982). Under the appropriate conditions, ANS can be a useful probe of the membrane surface.

MEMBRANE FLUIDITY DURING DEVELOPMENT

As the brain develops, the lipid composition of synaptic plasma membranes (SPM), and hence the fluidity of SPM, have been observed to undergo a series of alter-

ations. Hitzemann (1981) studied the FA in rat cortical SPM phospholipids as a function of postnatal age (7 days, 14 days, and adult). Overall, between day 7 postnatal and adult, there was a trend toward increased polyunsaturated long chain FA and decreased short chain saturated FA. Specific changes are detailed below. The double bond index-saturated ratio (equation 1, page 4) increased from day 7 to day 14, followed by a slight decrease from day 14 to adult. (Hitzemann & Johnson, 1983). In PC, the major phospholipid, decreases were observed in 14:0, 16:1, and 22:5 FA, with increases in 18:0, 22:6, and the largest increase in the ratio of 22:6 to 22:5 ($22:6/22:5$) (281%). In PE, 18:0 increased from day 7 to 14 and decreased from day 14 to adult. Increases were also observed in 22:6, 22:4, and the 22:6/22:5 ratio (104%) associated with PE. Ethanolamine plasmalogen (EP) showed an increase in 22:4, a decrease in 22:5, and an increase in the 22:6/22:5 ratio. SP showed a decrease in 16:0 and an increase in 18:0. In phosphatidylinositol (PI), 16:0 and 18:0 were decreased while 20:4 was increased. Phosphatidylserine (PS) showed the opposite trend. Decreases were observed in 20:4, 22:4, and 22:5. A transient increase in 22:6 was observed along with increases in 16:0 and 18:0. Taken alone, these changes would not be sufficient to produce a significant change in membrane viscosity.

Further studies of age-related changes in synaptic

membrane composition during development (Hitzemann & Johnson, 1983) focused on the observed molar Chol/PL, protein to lipid ratio, SP content, degree of phospholipid methylation, and ganglioside content, along with steady-state anisotropy of DPH fluorescence of the intact SPM as well as bilayers prepared from lipid extracted from these membranes. The results of these studies demonstrated an increase in fluorescence polarization of intact membranes and lipid bilayers during development, and hence a decrease in fluidity (increase in viscosity) with age. The sterol/phospholipid ratio in adult rats was increased as compared to both 7 day and 14 day membranes. These changes would produce an increase in the viscosity of the membrane. The protein/phospholipid ratio increased after day 14. This suggested that the fluidity change observed from day 7 to day 14 was due to changes in the lipid components of the membrane (Hitzemann & Johnson, 1983).

There is a developmental decrease in choline phospholipids, primarily due to a decrease in PC. Ganglioside levels were not changed during development (Hitzemann & Johnson, 1983). Thus, it is seen that during development, the decrease in membrane fluidity is primarily due to increases in Chol/PL and the protein to lipid ratio, as well as a decrease in PC.

MEMBRANE FLUIDITY DURING AGING

In order to maintain the normal function of biological membranes, it is important to maintain the fluidity of those membranes within a narrow range. This is referred to as membrane fluidity homeostasis (Shinitzky, 1984). This is believed to occur through enzymatic processes which change the degree of unsaturation of FA in phospholipids and/or which lead to the esterification of excess membrane cholesterol. Lipid changes observed in aging have been suggested to be due to a weakening of the processes which maintain homeostasis (Shinitzky, 1984).

Early studies of membrane fluidity utilized homogenates of whole brain and brain regions. With increasing age, an increase in viscosity was observed in all brain regions studied (Samuel et al., 1982). Total protein and cholesterol were increased in whole brain homogenate (Calderini et al., 1983) as well as the Chol/PL in homogenates prepared from hippocampus, caudate, and cerebral cortex (Samuel et al., 1982) from 24 month old rats, as compared to 3 month old rats. These studies did not attempt to correlate whole brain changes with particular neural membranes, such as SPM or myelin.

Using DPH (Shinitzky et al., 1983; Calderini et al., 1983) and 16-doxyl stearic acid (Nagy et al., 1983) to probe the hydrophobic core of the membrane, it was found that the viscosity of synaptic membranes in aged mice (24-

28 months) (Shinitzky et al., 1983) and rats (24 months) (Calderini et al., 1983; Nagy et al., 1983) was increased as compared to that of young animals (2-3 months). This increased viscosity was accompanied by an increase in Chol/PL of up to 30% in aged animals (Shinitzky et al., 1983; Armbrecht et al., 1983).

It has been suggested (Hitzemann, 1984) that an incremental increase in cholesterol will have a small effect on membrane viscosity if the membrane normally contains a relatively high amount of cholesterol (Chol/PL = 0.5), as is the case with SPM. Therefore, additional factors must be involved in the observed increase in viscosity found in aging.

MEMBRANE FLUIDITY AND ETHANOL

Ethanol, added in vitro, will increase the fluidity of synaptic membranes as assessed using ESR probes (Chin & Goldstein, 1977a,b; Chin & Goldstein, 1981; Goldstein et al., 1982; Armbrecht et al., 1983; Logan et al., 1983). Many such studies used high, and non-physiological ethanol concentrations (up to 500 mM) (Armbrecht et al., 1983; Logan et al., 1983). However, ethanol has demonstrated a fluidizing effect at concentrations as low as 10 mM ethanol (50 mg%) (Chin & Goldstein, 1977a) and produced a dose-related change in fluidity between 10 mM and 350 mM ethanol (Chin & Goldstein, 1977a).

Chronic ethanol administration produces a type of membrane tolerance in synaptic and other cellular membranes. Although no change in baseline fluidity of the membranes was observed, in studies using DPH (Beauge et al., 1984) and 5-doxy stearic acid (Chin & Goldstein, 1977b) as probes, the membranes from the ethanol-treated animals were resistant to the fluidizing effects of ethanol (Chin & Goldstein, 1977b; Johnson et al., 1979; Beauge et al., 1984). In addition, using a 12-doxy stearic acid probe, which measures the fluidity deeper within the membrane, a decrease in the baseline fluidity was detected in the interior of membranes from ethanol-treated animals (Lyon & Goldstein, 1983). Also, the partition coefficient of ethanol into synaptosomes from ethanol-treated animals was 66% lower than into control membranes (Rottenberg et al., 1981; Rubin & Rottenberg, 1983).

The composition of synaptic membranes from control and ethanol treated rats and mice has been studied by many investigators. The results obtained appear to be dependent on the method of administration of ethanol, as well as the duration and dose administered. Synaptic membranes from Sprague Dawley rats given intragastric doses of 20% aqueous ethanol (Crews et al., 1983b), C57/BL6 (Smith & Gerhart, 1982) and DBA/2J (Chin et al., 1978) mice fed liquid diets containing 7% and 33% ethanol, respectively, were found to have an increase in the Chol/PL as compared to controls.

This was due to an increase in cholesterol content (cholesterol/mg protein) with no change in phospholipids. The magnitude of the increase in Chol/PL was up to 30% over the control, which could be considered the physiological limit of the Chol/PL. The FA composition of the phospholipids was not changed by the chronic ethanol administration (Chin et al., 1978; Smith & Gerhart, 1982; Crews et al., 1983b).

When ethanol was administered to T₀ Swiss mice by inhalation, different results were obtained (Littleton & John, 1977; Littleton et al., 1979). No change in cholesterol content or Chol/PL was observed. However, there was an increase in 18:0 FA and a decrease in 20:4 FA (Littleton & John, 1977; Littleton et al., 1979), producing a decrease in the double bond index:saturated ratio (equation 1, page 4). The discrepancy in the results may be due to the method of administration of ethanol. Inhalation may be a more severe technique, producing and maintaining high ethanol concentrations in the brain. It is possible that alterations in the FA composition can be implemented more quickly than alterations in cholesterol content. Use of liquid diets and intubation produces lower blood and brain ethanol concentrations.

Both an increase in Chol/PL and a decrease in the double bond index:saturated ratio could be responsible for the tolerance observed. To study this further, Johnson et al. (1979) extracted the lipids from synaptic membranes of

control ICR mice and those treated chronically with ethanol by intraperitoneal injection. The lipids were reconstituted and the fluidity determined using DPH as probe. The lipids from the ethanol-treated animals were less fluidized by ethanol and had a lower intrinsic fluidity than controls. When cholesterol was removed from the reconstituted lipids, the change in fluidity in response to in vitro ethanol was indistinguishable in the ethanol-treated and control groups. When cholesterol was added to the reconstituted lipids so that the Chol/PL of the lipids from the control and ethanol-treated groups were equal, the difference in response to in vitro ethanol returned, although there was no difference in the intrinsic fluidity of the reconstituted lipids. This study suggests that cholesterol is involved in tolerance, but is not solely responsible for it (Johnson et al., 1979).

DOPAMINE BINDING

DOPAMINE RECEPTOR SUBTYPES

There appears to be some disagreement in the literature as to the number of dopamine receptor subtypes present in rat brain. Seeman & List (1981) describe four receptor subtypes based on their affinities for dopamine (an agonist) and spiperone (an antagonist). D₁ receptors, localized postsynaptically, have a low affinity for dopamine and spiperone (3000 nM and 2000 nM respectively) and are linked to the stimulation of adenylate cyclase.

D₂ receptors, also localized postsynaptically, have a low affinity for dopamine (5000 nM) but a high affinity for spiperone (0.3 nM). The D₂ receptors are thought to be responsible for the behavioral effects of neuroleptic drugs. D₃ receptors, found both pre- and postsynaptically, have a high affinity for dopamine (3 nM) but a low affinity for spiperone (1500 nM). D₄ receptors, possibly localized postsynaptically, have a high affinity for both dopamine and spiperone (20 nM and 0.1 nM respectively) and are linked to dopamine-inhibition of adenylate cyclase activity. The existence of the D₃ and D₄ receptor subtypes is disputed by Creese et al. (1983), who suggest that the D₃ and D₄ receptors are high agonist-affinity states of the D₁ and D₂ receptors, respectively.

MEASURING DOPAMINE BINDING PARAMETERS

Several ligands are presently available to study dopamine receptors. The most widely used is spiroperidol (spiperone), a dopamine antagonist. Spiperone, at nanomolar concentrations, is known to bind to serotonin as well as dopamine receptors (Creese et al., 1977; Creese & Snyder, 1978). In bovine caudate (Creese et al., 1977) and rat striatum (Hamblin et al., 1984), where the density of serotonin sites is low, spiperone binds primarily to dopamine sites. Because the D₁ receptor has a much lower affinity for spiperone than does the D₂ receptor, binding studies utilizing low spiperone concentrations will detect

primarily the higher affinity D_2 sites (Hamblin et al., 1984). At higher concentrations, binding to both the D_1 and D_2 sites is suggested by nonlinear Scatchard plots.

To quantitate the D_2 sites, the D_2 -specific antagonists, domperidone and sulpiride, have been used (Hamblin et al., 1984). Antagonist/ $[^3\text{H}]$ -domperidone competition curves suggest the presence of a single D_2 dopamine receptor. Whereas, agonist/ $[^3\text{H}]$ -domperidone competition curves suggest the presence of high and low agonist-affinity states of the D_2 receptor (Hamblin et al., 1984).

The partial dopamine agonists, apomorphine and ADTN (2-amino-6,7-dihydroxyl-1,2,3,4-tetrahydronaphthalene) label both D_1 and D_2 receptors in rat striatum. These ligands stimulate adenylate cyclase through the D_1 receptor. They are also displaced by low concentrations of spiperone, characteristic of binding to the D_2 receptor (Creese et al., 1978; Creese & Snyder, 1978b). Both of these ligands exhibit decreased binding in the presence of guanine nucleotides.

The D_1 receptor has been difficult to characterize because of its low affinity for agonists and spiperone (Seeman & List, 1981). The first high affinity D_1 ligand to be characterized was cis-flupenthixol, a dopamine antagonist (Hyttel, 1978; Cross & Owen, 1980; Hyttel, 1980; Leff et al., 1985). In the presence of low concentrations

of spiperone (10 nM) to block binding to D₂ receptors, cis-flupenthixol binding to D₁ receptors was studied in rat striatum (Hyttel, 1978; Hyttel, 1980; Leff et al., 1985) and bovine caudate membranes (Cross & Owen, 1980). However, high nonspecific binding (up to 70%) (Leff et al., 1985), makes cis-flupenthixol a poor choice for a D₁ ligand.

D₁ receptors are specifically labelled with the D₁ antagonist, SCH23390. In vitro binding studies reveal a single class of high affinity sites in rat striatum, with a K_d of 0.14 nM (Anderson & Nielsen, 1986). However, from in vivo studies, the K_d is calculated as 12.3 nM, which is similar to the K_i for SCH23390 inhibition of dopamine-sensitive adenylate cyclase in vitro (Anderson & Nielsen, 1986). Thus, in the in vitro studies using SCH-23390 reflect the kinetics involved in the first step in the activation of dopamine-sensitive adenylate cyclase, namely, the initial binding to the receptor.

As characterized by domperidone and SCH23390 binding studies, there are two types of dopamine binding sites, each of which exhibit high and low agonist affinity states (Hamblin et al., 1984; Hess et al., 1986). In both cases, the high affinity form can be converted to the low affinity form by the interaction with guanine nucleotides (Hamblin et al., 1984; Seeman et al., 1985; Hess et al., 1986). This suggests that both the D₁ and D₂ receptors

may be linked to adenylate cyclase, D₁ in a stimulatory capacity and D₂ in an inhibitory capacity (Creese et al., 1983).

DOPAMINE RECEPTORS IN DEVELOPMENT AND AGING

Much of the work done on dopamine binding during postnatal brain development has used [³H]-spiperone as ligand (Murrin, 1982; Nomura et al., 1982; Hartley & Seeman, 1983). Using autoradiography, following intraperitoneal injection of [³H]-spiperone, Murrin (1982) demonstrated a dramatic increase in dopamine binding sites in the striatum of the rat between 5 and 15 days postnatal. Binding, similar to that found in the adult, was achieved by 15 days of age. In vitro binding studies have demonstrated that [³H]-spiperone binding increases rapidly from day 5 (Hartley and Seeman, 1983) to a peak at 30 days of age (Nomura et al., 1982). Between 30 and 70 days of age, there is reportedly a small decrease in spiperone binding (Nomura et al., 1982).

[³H]-Spiperone has also been used to assess dopamine receptors in studies of aging. In all studies, [³H]-spiperone binding was lower in the aged animals (24 to 28 month old rats, and 5.5 year old rabbits) than in young animals (3 month old rats, and 5 month old rabbits) (Govoni et al., 1978; Govoni et al., 1980; Memo et al., 1980; Misra et al., 1980; Thal et al., 1980; DeBlasi & Mennini, 1982; Trabucchi et al., 1982; Cimino et al., 1983;

Govoni et al., 1983; Missale et al., 1983). In general, the B_{\max} was 30-50% lower in aged animals (Memo et al., 1980; Misra et al., 1980; DeBlasi & Mennini, 1982; Trabucchi et al., 1982; Missale et al., 1983).

Because spiperone binds to both the D_1 and D_2 receptors, there is little information available about which specific receptor subtype is primarily affected by aging. However, limited studies using [^3H]-domperidone (Trabucchi et al., 1982; Missale et al., 1983) and [^3H]-sulpiride, D_2 antagonists, (Memo et al., 1980; Missale et al., 1983) suggest that the decreased spiperone binding in aged animals is due to a decrease in the number of D_1 receptor sites. This decrease may be the result of either a decrease in the number of receptors per cell or a loss of neuronal synapses, or both (Severson & Finch, 1980).

DOPAMINE RECEPTORS AND ETHANOL

There is disagreement as to the effects of chronic ethanol consumption on the binding of [^3H]-spiperone to dopamine receptors. Some investigators find no difference in K_d or B_{\max} between control and ethanol-treated animals (Tabakoff & Hoffman, 1979; Rabin et al., 1980). Others find a small decrease in the K_d for [^3H]-spiperone (Barbaccia et al., 1980; Reggiani et al., 1980; Barbaccia et al., 1982; Lucchi et al., 1983). In summary, it appears that chronic ethanol consumption has little effect on the kinetics of binding to dopamine receptors.

DOPAMINE-SENSITIVE ADENYLATE CYCLASE

BIOCHEMICAL PROPERTIES

Adenylate cyclase is a membrane-associated enzyme which catalyzes the conversion of ATP to 3'-5' cyclic AMP (cAMP) (Spiegel et al., 1985). The cAMP produced then binds to the regulatory subunit of cAMP-dependent protein kinase, causing it to dissociate from the catalytic subunit. The active catalytic subunit of protein kinase subsequently stimulates the phosphorylation of one or more cellular proteins (Figure 4). Adenylate cyclase is activated by specific hormones and neurotransmitters through a two-step process (Spiegel et al. 1985). The unoccupied receptor interacts with the guanine-nucleotide binding protein (G-protein) which enhances the affinity of the receptor for the hormone or neurotransmitter. When dopamine, for example, binds to the D₁ receptor, GTP binds to the G-protein, which results in the activation of the G-protein. The activated G-protein dissociates from the receptor and stimulates the catalytic subunit of adenylate cyclase to form cAMP (Spiegel et al., 1985). The GTP is then hydrolyzed to GDP and P_i by the intrinsic GTPase activity in the G-protein, which causes the G-protein to be deactivated. The deactivated G-protein dissociates from adenylate cyclase and reassociates with the receptor to complete the cycle (Figure 4) (Spiegel et al., 1985).

Dopamine-sensitive adenylate cyclase has a K_m

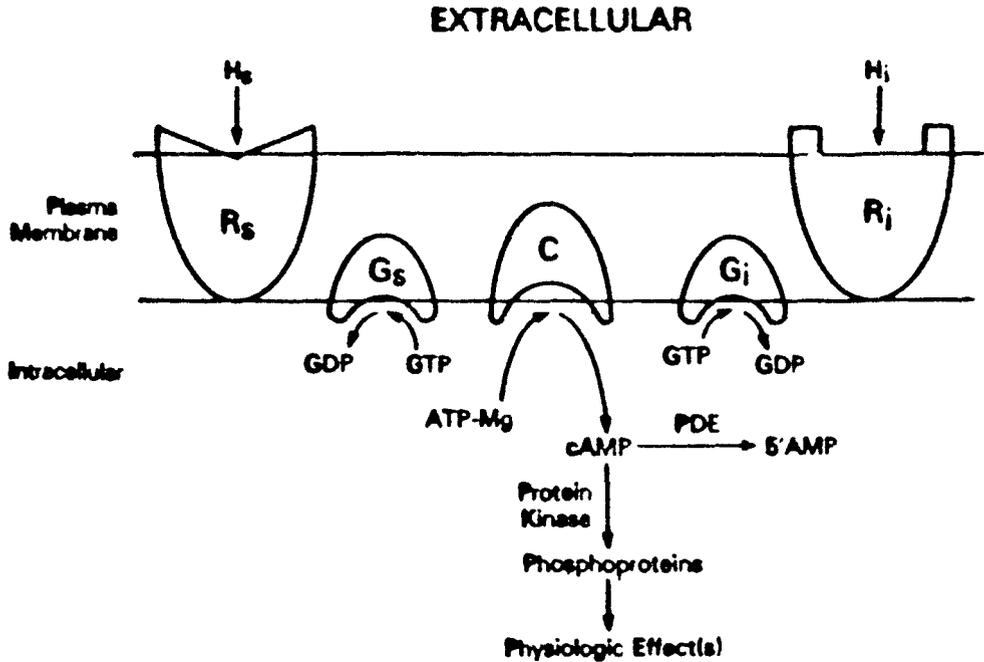


Figure 4. Schematic Representation of Hormone- and Neurotransmitter-Sensitive Adenylate Cyclase.

H, R, and G, represent the hormone, receptor, and guanine nucleotide binding protein, respectively. The subscripts s and i represent stimulatory and inhibitory, respectively.

(Speigel et al., 1985)

for dopamine of 4-5 μM (Nathanson, 1977; Miller & McDermed, 1979), with maximal stimulation above 50 μM dopamine (Miller & McDermed, 1979). Stimulation by dopamine is inhibited well by cis-flupenthixol (Enjalbert et al., 1978; Miller & McDermed, 1979). Haloperidol is a less potent inhibitor of adenylate cyclase. Sulpiride, a D_2 antagonist, does not inhibit dopamine stimulation of adenylate cyclase (Miller & McDermed, 1979).

DOPAMINE-SENSITIVE ADENYLATE CYCLASE DURING DEVELOPMENT AND AGING

During postnatal development in the rat striatum, the basal (unstimulated) activity of adenylate cyclase, as well as the dopamine-stimulated adenylate cyclase activity, increased from days 1 to 23 (Enjalbert et al., 1978). Although the amount of cAMP produced upon stimulation by 100 μM dopamine increased as a function of age, the % stimulation decreased from 350% on day 1 to 180% on day 23 (Enjalbert et al., 1978), because of the age-related rise in the basal activity.

During aging in rabbits (Makman et al., 1980) and rats (Zimmerman & Berg, 1975; Puri & Volicer, 1977; Schmidt & Thornberry, 1978), there was no change in the basal adenylate cyclase activity (Zimmerman & Berg, 1975; Puri & Volicer, 1977; Schmidt & Thornberry, 1978; Makman et al., 1980) or that stimulated by NaF, which acts directly on the

enzyme (Zimmerman & Berg, 1975; Puri & Volicer, 1977). However, the stimulation of adenylate cyclase by 100 μ M dopamine was decreased in aged (24 to 30 month old) rats (Puri & Volicer, 1977; Schmidt & Thornberry, 1978) and (5.5 year old) rabbits (Makman et al., 1980) as compared to adult animals. This decreased stimulation was observed in rats at as early as 12 months of age (Puri & Volicer, 1977; Schmidt & Thornberry, 1978). By 30 months of age, no statistically significant stimulation by dopamine was detected (Puri & Volicer, 1977). Because there was no change in basal adenylate cyclase activity, the total amount of enzyme present was not affected by aging, suggesting that the D_1 receptor-containing cells were not lost. Therefore, the observed decrease in stimulation may be due to changes in membrane composition and fluidity.

DOPAMINE-SENSITIVE ADENYLATE CYCLASE AND ETHANOL

Ethanol, added in vitro to membrane preparations, increased the basal activity of adenylate cyclase (Hoffman et al., 1983), but had no effect on dopamine stimulation of adenylate cyclase activity (Hunt et al., 1979). However, chronic administration of ethanol produced an increase in the basal activity (Kuriyama et al., 1977; Lucchi et al., 1983), with no change in that which was stimulated by NaF (Kuriyama et al., 1977; Tabakoff & Hoffman, 1979; Lucchi et al., 1983). In addition, chronic ethanol treatment produced a decrease in sensitivity to dopamine (Lucchi et al.,

1983), which was most evident upon withdrawal (Tabakoff & Hoffman, 1979). When ethanol was added in physiological concentrations to ethanol-tolerant membranes, the dopamine sensitivity of the adenylate cyclase activity returns to normal values, suggesting that dopamine sensitive adenylate cyclase is adapted to physiological ethanol concentrations in chronic ethanol exposure. The decreased sensitivity of the adenylate cyclase to dopamine stimulation was due to a decrease in the efficiency of coupling between the dopamine receptor and the adenylate cyclase catalytic site (Tabakoff & Hoffman, 1979), which could be due, in part, to a change in membrane composition and fluidity.

DOPAMINE UPTAKE

BIOCHEMICAL PROPERTIES

Dopamine uptake into synaptosomes is an energy dependent, temperature-sensitive process. This process functions to remove released dopamine from the synaptic cleft, and hence, to terminate the biological response (Horn, 1979). The uptake process is dependent on sodium and to a lesser extent potassium. Calcium is not required. At 37°C, the uptake is linear for 5 to 10 minutes and is proportional to the amount of synaptosomal protein in the assay mixture (Horn, 1979). Dopamine uptake is competitively inhibited in vivo (Manias & Taylor, 1983) and in vitro (Hunt et al., 1974; Horn, 1979) by nomifensine (8-amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline)

which inhibits the accumulation of dopamine in synaptosomes without stimulating the release of dopamine (Hunt et al., 1974; Horn, 1979; Manias & Taylor, 1983). When administered in vivo, nomifensine produces a 3-fold increase in the K_m for dopamine (Manias & Taylor, 1983).

DOPAMINE UPTAKE DURING DEVELOPMENT AND AGING

Dopamine uptake has been studied during postnatal development in the rat. The reuptake of dopamine in newborn rats was 10% of that in the adult rats (Horn, 1979). When uptake was determined at 100 nM [^3H]-dopamine, there was a 3-fold increase in uptake (pmole/g wet wt tissue) from day 6 to day 22 postnatal (Slotkin et al., 1980; Deskin et al., 1981). This increase is due to an increase in V_{max} ; the K_m was unchanged (Coyle & Campachiaro, 1976).

The changes in dopamine uptake during aging have been studied in C57BL/6J mice (Jonec & Finch, 1975). Uptake into crude synaptosomes at 10 nM [^3H]-dopamine was 30% lower in 28 month old mice than in mature (2-3 month old) mice. At 120 nM [^3H]-dopamine, no difference was observed. Kinetic analysis revealed a 35% increase in the K_m for dopamine in old mice as compared to controls. The V_{max} was unchanged (Jonec & Finch, 1975). The increase in K_m was suggested to be due to a decreased "native" affinity for dopamine. Alternatively, an endogenous competitive inhibitor may be present in the aged mice, whose inhibition can be overcome at high dopamine concentrations.

MEMBRANE COMPOSITION AND FLUIDITY IN
NEUROTRANSMITTER SYSTEMS

The effect of increasing membrane fluidity on epinephrine- and adenosine-stimulated adenylate cyclase has been studied using cis-vaccenic acid as a fluidizing agent and fluorescence polarization with DPH to determine the membrane microviscosity (Rimon et al., 1978). There are two types of adenylate cyclase-receptor coupling mechanisms, a mobile receptor, and a receptor that is permanently coupled to adenylate cyclase. If the receptor is mobile, requiring lateral diffusion to activate adenylate cyclase, it would be expected that the activation would be increased with increasing fluidity (decreasing the resistance to lateral diffusion). This was observed in the epinephrine-stimulated adenylate cyclase system, known to act through a mobile receptor. The adenosine receptor, on the other hand, is believed to be permanently coupled to adenylate cyclase. As would be expected in this type of system, the activation of adenosine-sensitive adenylate cyclase was independent of membrane fluidity (Rimon et al., 1978).

The influence of membrane fluidity on the binding of serotonin (Heron et al., 1980a,b) and opiates (Heron et al., 1980a; Heron et al., 1981) to their receptors was studied. Crude synaptosomes were incubated with lecithin or linoleic acid, to increase fluidity, or with cholesteryl hemi-succinate or stearic acid, to decrease fluidity. Decreasing membrane fluidity (increasing viscosity) produced

a 5-fold increase in [^3H]-serotonin binding and a 2-fold increase in [^3H]-enkephalin and [^3H]-naloxone binding. As the viscosity was increased further, binding was rapidly lost, possibly due to increased degradation or "shedding" of receptors into the medium (Heron et al., 1980a,b; Heron et al., 1981). Fluidization of the membrane produced a decrease in the binding of all three ligands. The authors suggest that their observations with more viscous membranes have implications in aging and chronic drug treatment.

Dopamine receptors have been studied as a function of membrane fluidity using spiperone as a ligand (Henry & Roth, 1986). The concentrations of spiperone used, 0.25 to 10 nM, would characterize primarily the D_2 receptor. Crude striatal membranes from mature (3-6 months of age) and aged rats (24-25 months of age) were incubated with up to 10% (v/v) ethanol for 15 minutes to fluidize the membranes. Incubation with cholesterol hemisuccinate for 2 hours produced an increase in membrane viscosity. Spiperone binding to membranes from both mature and aged animals showed an increase in the K_d for spiperone as the membrane was made more rigid. However, there was no difference between the response in mature and aged animals that were subjected to identical treatments. The B_{\max} for spiperone binding was lower in the aged animals. But the B_{\max} for membranes from both mature and aged animals was decreased as the membrane viscosity was artificially

increased. Fluidizing the membrane with ethanol had no effect on either the K_d or B_{max} for spiperone binding to membranes from mature or aged animals (Henry & Roth, 1986).

In the above studies (Heron et al., 1980a,b; Heron et al., 1981; Henry & Roth, 1986), incubation times of up to 2 hours at room temperature were utilized to alter the membrane fluidity. In our hands, the incubation of membranes for more than 45 minutes in buffer produced changes in the binding kinetics of the D_1 receptor. Therefore, a method which utilizes shorter incubation times may be the method of choice for studies of this type.

One such method was described by North and Fleischer (1983a,b) who altered the Chol/PL of synaptosomes and SPM using liposomes made of cholesterol and/or PC, and a non-specific lipid transfer protein (ns-LTP) from beef liver (Crain & Zilversmit, 1980). The altered membranes were studied to determine the effect of changes in membrane fluidity on sodium-dependent GABA uptake and GABA binding. This method has advantages over other, non-protein-mediated methods because a large change in Chol/PL can be achieved using short incubation times (30-60 minutes). Using the ns-LTP, the Chol/PL of SPM was varied from 0.21 to 1.19, from the normal value of 0.52. The Chol/PL of synaptosomes was varied from 0.16 to 0.81, from the normal value of 0.38 (North & Fleischer, 1983a,b). It should be noted that the

Chol/PL of synaptosomes is lower than that of SPM. This difference is due to the presence of mitochondria containing little cholesterol within the synaptosomes. The increase in Chol/PL, and accompanying decrease in membrane fluidity as determined by DPH fluorescence polarization, produced no changes in either GABA uptake or binding (North & Fleischer, 1983b). Decreasing the Chol/PL of synaptosomes to 40% of normal produced a 70-100% loss of GABA uptake and a 50% decrease in the B_{\max} for GABA binding.

NONSPECIFIC LIPID TRANSFER PROTEIN

Lipid transfer proteins (LTP) are present in the cytosol of many tissues and vary in their specificity toward the various lipid classes. In beef liver, two types of LTPs have been identified (Zilversmit, 1983), a PC-specific transfer protein with a molecular weight of 28,000 and an isoelectric point, P_I , of 5.8, and two ns-LTPs, CMI and CMII, both with molecular weights of 14,500 and P_I s of 9.55 and 9.75, respectively (Crain & Zilversmit, 1980). The ns-LTPs accelerate the transfer of cholesterol and many phospholipid depending on their relative concentration in the donor and acceptor membranes. These two proteins differ in composition by two amino acids, CMI containing no arginine or histidine, and CMII containing one each of these amino acids. CMI and CMII will transfer cholesterol in preference to PC, even at Chol/PL as low as 0.2 (donor membrane) (Crain & Zilversmit, 1980). Since

these proteins do not transfer cholesterol esters or triglycerides, these lipids can be used as nontransferrable markers. The in vivo function of ns-LTPs is not known, but, based on their amino acid composition, it appears that CMII may be identical to a Sterol Carrier Protein (SCP₂), which shuttles intermediates in cholesterol biosynthesis between the inner and outer mitochondrial membranes (Noland et al., 1980; Crain, 1982).

Ns-LTP has been used to alter the Chol/PL of SPM and synaptosomal membranes as previously described (North & Fleischer, 1983a,b). This method has advantages over previously used methods which required long incubation times. Because a wide range of Chol/PL can be obtained using short incubation times, the integrity and biological activity of SPM and synaptosomes can be preserved.

CHAPTER III

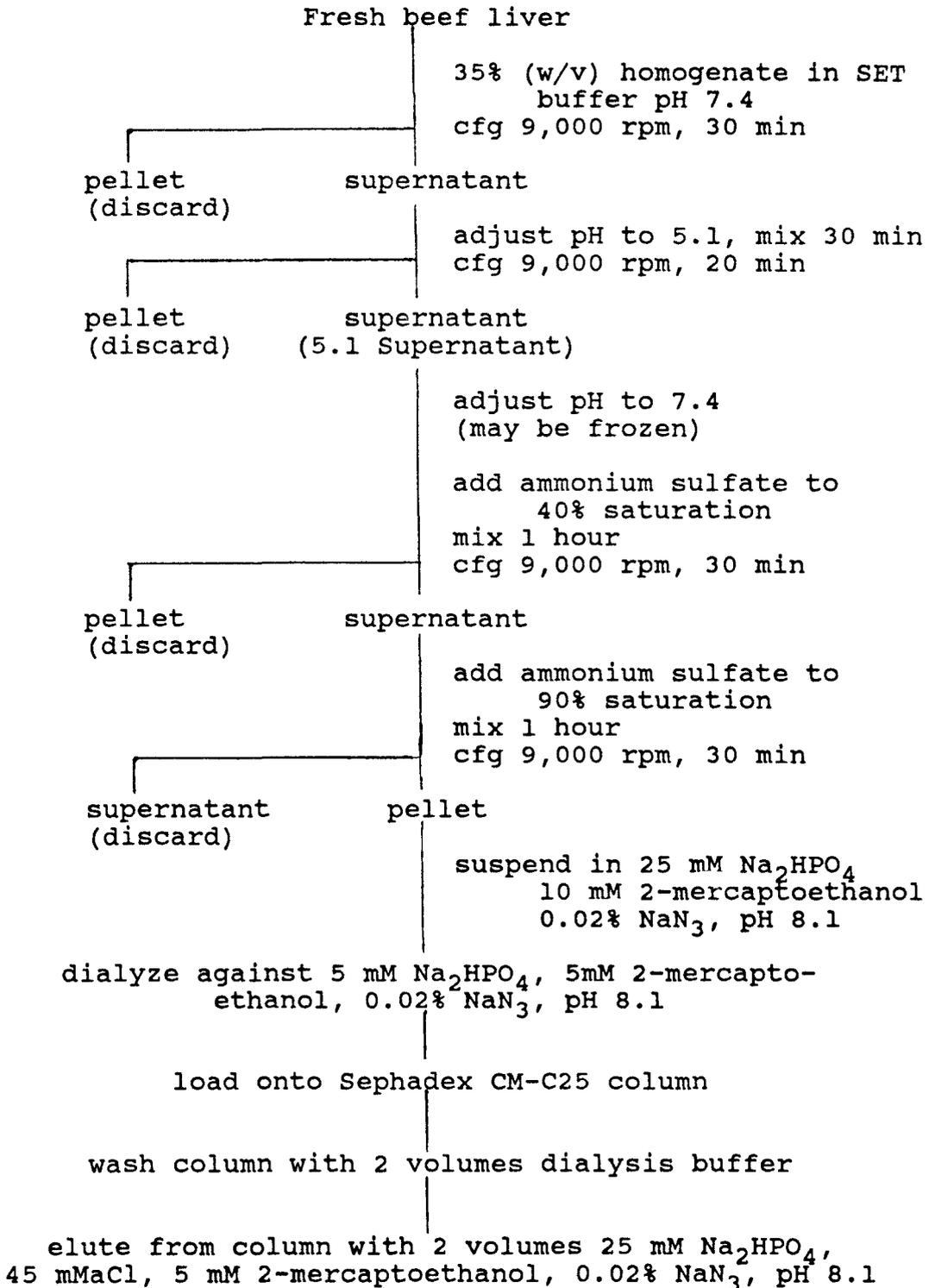
MATERIALS AND METHODS

Purification of Lipid Transfer Protein from Beef Liver

Nonspecific Lipid Transfer Protein (ns-LTP) was partially purified from beef liver by a modification of the methods of Crain and Zilversmit (1980), and North and Fleischer (1983b) (Figure 5). All steps were carried out at 4°C. Fresh beef liver was obtained from Aurora Packing Company, North Aurora, Illinois. A 35% (w/v) homogenate was prepared in a solution containing 0.25 M sucrose, 1 mM EDTA, 50 mM Tris HCl, 0.02% (w/v) sodium azide, pH 7.4 (SET buffer), and centrifuged at 13,000 x g (9,000 rpm in GS3 rotor, Sorvall RC-5B centrifuge) for 30 minutes. The supernatant was decanted and the pH adjusted to 5.1 with 3 N HCl. After mixing for 30 minutes, the solution was centrifuged at 13,000 x g (9,000 rpm) for 20 minutes. The clear supernatant (5.1 Supernatant) was decanted and the pH returned to 7.4 with 3 N NaOH. Aliquots of 900 ml were stored at -20°C for up to 6 months.

Two 900 ml aliquots of 5.1 Supernatant were thawed. Over 1 hour, ammonium sulfate was added to 40% saturation (243 g/liter). After an additional hour of mixing, the solution was centrifuged at 13,000 x g (9,000 rpm) for 30 minutes. The supernatant was decanted and addition-

PREPARATION OF LIPID TRANSFER PROTEIN



al ammonium sulfate was added over 1 hour to 90% saturation (375 g/liter). After 1 hour of mixing, the solution was centrifuged at 13,000 x g (9,000 rpm) for 30 minutes. The supernatant was discarded, and the pellet was suspended in a solution containing 25 mM sodium phosphate, 10 mM 2-mercaptoethanol, 0.02% (w/v) sodium azide, pH 8.1. The suspension was extensively dialyzed against a solution containing 5 mM sodium phosphate, 5 mM 2-mercaptoethanol, 0.02% (w/v) sodium azide, pH 8.1 (dialysis buffer), using Spectra/Por 1 (Spectrum Medical Industries, Inc., Los Angeles) dialysis tubing with a molecular weight cutoff of 6000-8000 daltons.

The dialysate was applied, at a flow rate of 1 ml/min, to a 5 x 60 cm Sephadex CM-C25 (Pharmacia, Inc., Uppsala, Sweden) ion exchange column, which had been pre-equilibrated with dialysis buffer. The column was washed with 2 column volumes of dialysis buffer, and eluted, at a flow rate of 2 ml/min, with 2 column volumes of a solution containing 25 mM sodium phosphate, 45 mM NaCl, 5 mM 2-mercaptoethanol, 0.02% (w/v) sodium azide, pH 8.1 (elution buffer). LTP was eluted following a large peak of yellow material. Transfer activity was stable for up to 1 month when stored at 4°C in elution buffer. Prior to use in lipid transfer experiments, the eluate was concentrated 5- to 10- fold by ultrafiltration through an Amicon stirred ultrafiltration cell, equipped with a Diaflo YM5 membrane,

with a molecular weight cutoff of 5000 daltons (Amicon Corp., Danvers, MA).

SDS-PAGE

To qualitatively determine the purity of the transfer protein preparations, aliquots of fractions from the different purification steps were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Samples were prepared in a solubilizing solution and boiled for 2 minutes at 100°C. The solubilizing solution was prepared from 1 ml of 10% (w/v) SDS, 150 mg dithiothreitol, and 800 mg sucrose in 10 ml of deionized water. Bromothymol blue was added and the samples were centrifuged before application to the gel.

Slab Gel Preparation. Because the expected molecular weight of the transfer protein was approximately 14K, gels of 12.5% (w/v) acrylamide were prepared to assure a good separation in the low molecular weight region. Gels of 3 mm thickness were prepared with 41.7 ml Solution A (below), 12.5 ml Solution B, 1 ml of Solution C, 44.65 ml deionized water, 0.15 ml TEMED (diluted 1:10 with deionized water), and 100 mg ammonium persulfate. After pouring, the gels were overlayed with isobutanol and allowed to polymerize. After polymerization, the gels were rinsed with deionized water and blotted. Spacer gels of 3% (w/v) acrylamide were prepared with 1 ml Solution A, 1.25 ml Solution D, 0.1 ml Solution C, 8.15 ml deionized water, 0.05 ml

TEMED (diluted 1:10 with deionized water) and 10 mg ammonium persulfate, and poured within a few mm of the top of the comb used to form the sample wells. Solution A contained 30% (w/v) acrylamide and 0.8% (w/v) Bis. Solution B contained 36.6% (w/v) Tris base, pH 8.9. Solution C contained 10% (w/v) SDS. Solution D contained 5.89% (w/v) Tris base, pH 6.7.

Samples were applied and overlaid with running buffer. The running buffer contained 0.02% (w/v) SDS, 1.4% (w/v) glycine, 0.3% (w/v) Tris pH 8.4. Low molecular weight standards (Bio-Rad), including lysozyme (14.4K), soybean trypsin inhibitor (21.5K), carbonic anhydrase (31K), ovalbumin (45K), bovine serum albumin (66.2K), and phosphorylase B (92.5K), were run with samples in each gel. Current was applied at 10 amps per gel until the samples reached the stacking gel. Thereafter, the current was maintained at 20 amps per gel. Gels were run until the tracking dye reached the end of the gel. Gels were fixed and stained in a solution containing 0.1% (w/v) Coomassie Blue, 50% (v/v) methanol, and 10% (v/v) acetic acid, followed by destaining in a solution containing 7.5% (v/v) acetic acid and 7.5% (v/v) methanol.

PREPARATION OF MITOCHONDRIA FOR CHOLESTEROL TRANSFER ASSAY

Mitochondria were prepared using an adaptation of the method of Cotman and Matthews (1971). A 10% (w/v) homogenate of beef heart was prepared in 0.32 M sucrose, 5 mM

Tris base, pH 7.0 (sucrose buffer) using a Tekmar tissue-mizer (Tekmar, Cincinnati, OH). The homogenate was centrifuged at $1,100 \times g$ (3,000 rpm in an SS34 rotor) for 5 minutes. The supernatant was decanted into clean centrifuge tubes and centrifuged at $17,000 \times g$ (13,700 rpm) for 10 minutes. The pellet was suspended in 15 ml of sucrose buffer and layered onto 15 ml of 38% (w/v) sucrose, followed by centrifugation at $106,000 \times g$ (24,250 rpm in SW-28 rotor) for 2 hours. The pellet was resuspended in sucrose buffer and stored at -80°C . Prior to use in the cholesterol transfer assay, mitochondria were heated to 80°C for 20 minutes to inactivate endogenous lipases. Heat-treated mitochondria were subsequently washed with 50 mM Tris HCl, pH 7.4.

PREPARATION OF LIPOSOMES

Liposomes that would be used in an assay of cholesterol transfer activity were prepared with 1 mg egg PC per ml, 1 mole of cholesterol per mole of PC, with 0.25 μCi each of [^3H]-triolein and [^{14}C]-cholesterol per ml of liposomes added as nontransferrable and transferrable markers, respectively (North & Fleischer, 1983b). Liposomes, for depletion of cholesterol from synaptosomes or SPM, were prepared using 10 mg of egg phosphatidylcholine (PC) per ml. Those to be used to increase the Chol/PL of synaptosomes and SPM were prepared with 5 mg PC per ml with 1.7 moles of cholesterol per mole of PC (4.4 mg cholesterol per

ml). [^{14}C]-Tripalmitin (0.25 $\mu\text{Ci/ml}$) was included in all liposomes as a nontransferrable marker (North & Fleischer, 1983b). To prepare liposomes, the lipids were mixed in chloroform and dried under nitrogen to a thin film which coated the walls of an acid-washed tube. The lipid layer was dispersed in 50 mM Tris HCl, pH 7.4 (for the liposomes to be used for the cholesterol transfer assay) or 0.32 M sucrose, 5 mM Tris base, pH 7.0 (for the liposomes to be used for cholesterol loading and depletion) by extensive mixing using a vortex mixer, followed by sonication until the lipid mixture was translucent, using a Cole Palmer Ultrasonic Homogenizer 4710 (Cole Palmer Instrument Co., Chicago, IL), equipped with a 1/2 inch tipped horn, at 80% duty cycle. After sonication, the liposomes were centrifuged at 27,000 x g (15,000 rpm, SS34 rotor) for 60 minutes at 25°C to remove titanium fragments from the sonicator tip and undispersed lipid. Liposomes were generally used within 3 hours of preparation.

CHARACTERIZATION OF LIPOSOMES

In order to correct for the sticking of liposomes to the synaptosomes and SPM in the calculation of the Chol/PL, it is necessary to establish that the phospholipid, cholesterol, and nontransferrable marker are uniformly distributed throughout the population of each type of liposome used. To accomplish this, the liposomes were chromatographed on a Sepharose 4B-CL column (Wong et al., 1982),

with 50 mM Tris HCl, pH 7.4 as the running buffer. Before applying the liposomes to be separated, the column was saturated with unlabelled liposomes, to occupy the hydrophobic binding sites, and washed extensively. Fractions of 1 ml were collected and assayed for phosphorus and cholesterol. The radioactivity in each fraction was determined by liquid scintillation counting.

ASSAY OF CHOLESTEROL TRANSFER ACTIVITY

Cholesterol transfer activity was assayed by incubating purified transfer protein (up to 100 μ l) with 800 μ l of liposomes and 100 μ l of heat-treated mitochondria at 32°C for varying times. Blanks (without transfer protein) were included for each time point. The reaction was terminated by vacuum filtration through Whatman GF/C filters, which had been preincubated with unlabelled liposomes, followed by 3 washes with 5 ml of 50 mM Tris HCl, pH 7.4 (North & Fleischer, 1983b). Filters were placed in 10 ml of Ecoscint and the radioactivity determined in a Beckman LS-7500 liquid scintillation counter with data reduction.

The purpose of the assay was to demonstrate a unidirectional transfer of cholesterol from a donor liposome into mitochondria. The mitochondrial membrane is a good acceptor because it contains very little cholesterol. The liposomes used in the assay contained [14 C]-Cholesterol and [3 H]-Triolein as transferrable and nontrans-

ferrable markers, respectively. After the incubation, the mitochondria will have a $^{14}\text{C}/^3\text{H}$ that is greater than the $^{14}\text{C}/^3\text{H}$ of the liposomes, it a net transfer has taken place. The net transfer was calculated as follows:

$$^{14}\text{C} \text{ due to sticking} = (^3\text{H in sample}) \times (^{14}\text{C}/^3\text{H in liposomes})$$

$$\text{net } ^{14}\text{C} = ^{14}\text{C in sample} - ^{14}\text{C due to sticking}$$

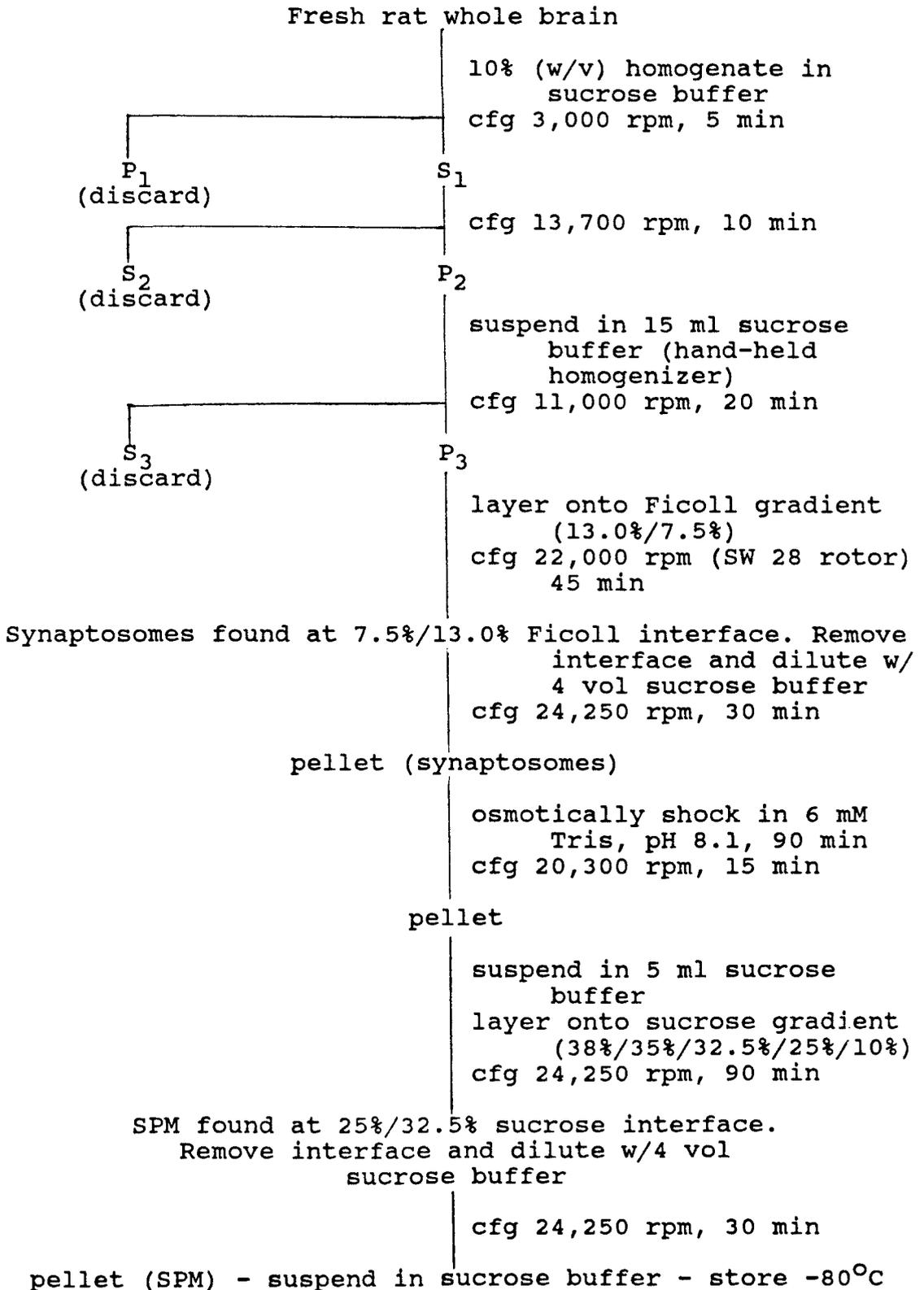
$$\text{nmol chol transferred} = \text{net } ^{14}\text{C} \times \frac{\text{nmole chol in liposomes}}{^{14}\text{C in liposomes}}$$

PREPARATION ON SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES

Synaptosomes and synaptic plasma membranes (SPM) were prepared from whole rat brain by the method of Cotman and Matthews (1971) (Figure 6). All steps were carried out at 4°C.

Male Sprague-Dawley rats (Holtzman, Madison, WI), 40 +/- 5 days old were housed 3 to a cage with a 12 hour light dark cycle. Animals were sacrificed between 7 a.m. and 8 a.m. by decapitation. The brains were removed and placed in ice cold 0.32 M sucrose, 5 mM Tris base, pH 7.0 (sucrose buffer). A 10% (w/v) homogenate was prepared using a glass homogenizer with a teflon pestle. The tissue was homogenized at 1000 rpm, with 4 up and down strokes. The homogenate was centrifuged at 1100 x g (3,000 rpm in SS34 rotor, Sorvall RC-5B centrifuge) for 5 minutes. The supernatant was decanted into clean centrifuge tubes and pelleted at 17,000 x g (13,700 rpm) for 10 minutes. The pellet was resuspended in 15 ml of sucrose buffer using a

RAT SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES



round bottom, hand-held glass homogenizer, prior to centrifugation at 11,000 x g (11,000 rpm) for 20 minutes. The pellet was suspended in 5 ml of sucrose buffer and layered onto a discontinuous Ficoll-sucrose gradient consisting of 13 ml of 13.0% (w/v) Ficoll 400 in sucrose buffer and 13 ml of 7.5% (w/v) Ficoll 400 in sucrose buffer. Following centrifugation at 63,581 x g (22,000 rpm in SW-28 rotor, Beckman L5-65 ultra-centrifuge) for 45 minutes, the synaptosomal material, found at the 7.5%/13.0% Ficoll interface, was removed, diluted with 4 volumes of sucrose buffer, prior to centrifugation at 106,000 x g (24,250 rpm) for 30 minutes. The pellet contained synaptosomes which were used directly for cholesterol transfer and dopamine uptake assays.

To prepare SPM for membrane fluidity studies, the synaptosomal pellet was suspended in a small volume of sucrose buffer, osmotically shocked with 6 volumes of 6 mM Tris base, pH 8.1 for 90 minutes, and centrifuged at 54,500 x g (20,300 rpm) for 15 minutes. The resulting pellet was resuspended in 5 ml of sucrose buffer and layered onto a discontinuous sucrose gradient which consisted of the following concentrations of sucrose solutions with 5 mM Tris base, pH 7.0, in ascending order: 38.0% (w/v) (5 ml), 35.0% (w/v) (5 ml), 32.5% (w/v) (5 ml), and 25.0% (w/v) (10 ml). The gradient was centrifuged at 106,000 x g (24,250 rpm) for 90 minutes. SPM, found at the 32.5%/25.0% inter-

face, were removed, diluted with 4 volumes of sucrose buffer, and pelleted at $106,000 \times g$ (24,250 rpm) for 30 minutes. The pellet was suspended in a small volume of sucrose buffer and stored at -80°C .

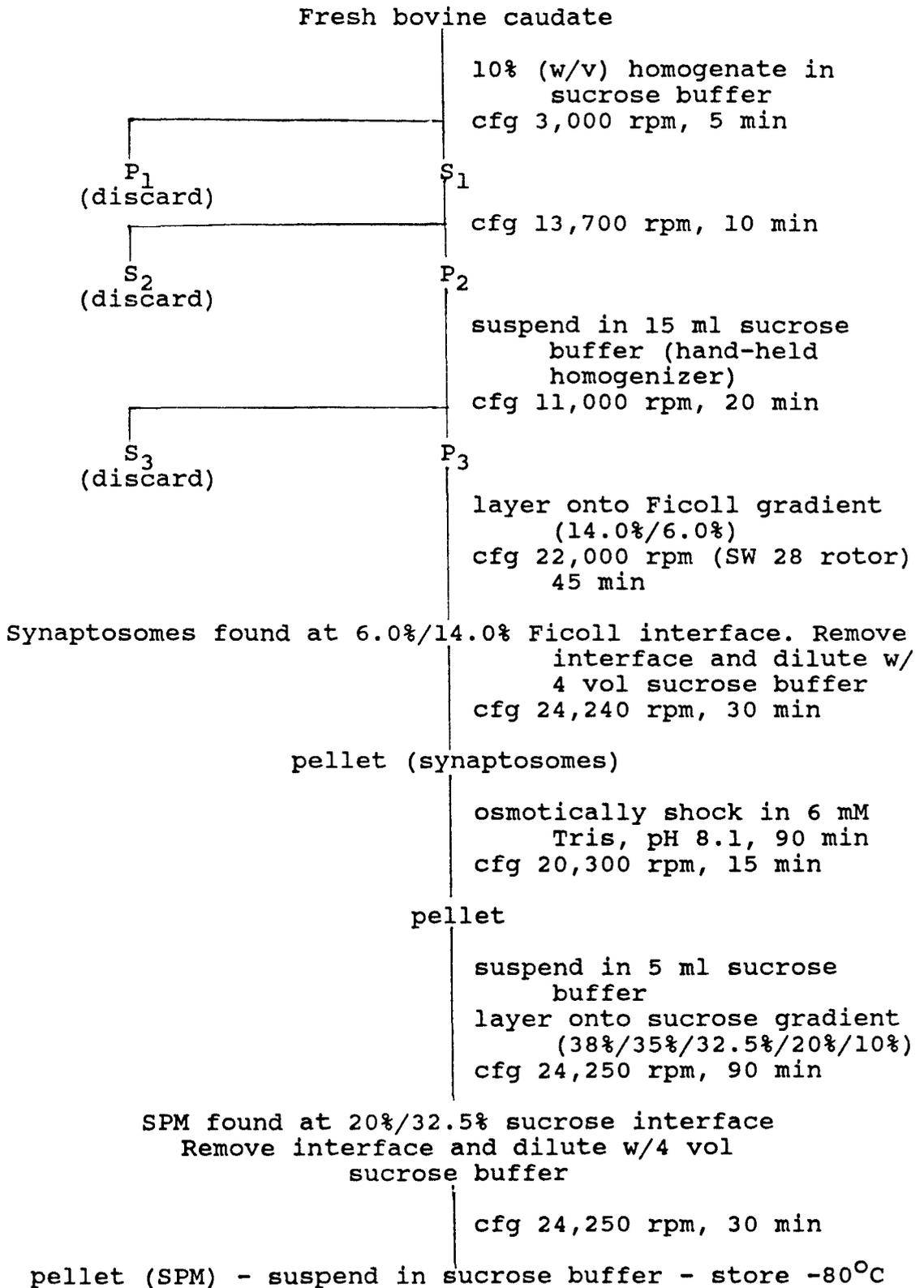
Bovine brain was obtained as a generous gift from Aurora Packing Company, North Aurora, Illinois. Fresh brains were kept ice-cold in 50 mM Tris HCl, pH 7.4, during transportation. The caudate was removed (Yoshikawa, 1968), and homogenized in sucrose buffer as described for rat brain. Because the bovine myelin and synaptosomal fractions were found to have a lower density than those from rat brain, it was necessary to make two modifications of the Cotman and Matthews (1971) method. 1) In the preparation of synaptosomes, the Ficoll-sucrose gradient consisted of 13 ml of 14.0% (w/v) Ficoll and 13 ml of 6.0% (w/v) Ficoll. 2) In the isolation of SPM from synaptosomes, the 25% (w/v) sucrose solution from the sucrose gradient was replaced by 20.0% (w/v) sucrose (Figure 7). Bovine synaptosomes were used directly for cholesterol transfer and D_1 binding and adenylate cyclase assays. SPM were stored at -80°C .

MARKER ENZYME ASSAYS

The relative purity of SPM and synaptosomes prepared from rat whole brain and bovine caudate was assessed by determining the activities of enzymatic markers.

Mg^{2+} -dependent, ouabain-sensitive (Na^+ - K^+)-ATPase (EC

BOVINE SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES



3.6.1.4) (Sweadner, 1978) is found enriched in plasma membranes. Acid phosphatase (EC 3.1.3.2) (Linhardt & Walter, 1963), cytochrome c oxidase (EC 1.9.3.1) (Duncan & Mackler, 1966), and 2',3'-cyclic nucleotide 3'-phosphohydrolase, CNPase (EC 3.1.4.16) (Kurihara & Tsukada, 1967) are enzymatic markers for lysosomal, inner mitochondrial, and myelin membranes respectively.

Na⁺-K⁺ ATPase. Na⁺K⁺-dependent, Mg²⁺-stimulated, ouabain-sensitive ATPase is a marker for plasma membranes. Enzyme activity was assayed by a modification of the method of Abdel-Latif et al. (1970). All samples were preincubated for 2 minutes at room temperature in 0.02% (w/v) SDS. Rat whole brain and bovine caudate homogenates (90-100 ug protein), synaptosomes (50-80 ug protein), or SPM (30-50 ug protein) were incubated in 450 µl of reaction mixture for 10 minutes at 37°C before initiating the reaction with 50 µl of ATP (18 mg ATP per ml - final concentration 3 mM ATP). The final assay buffer contained: 150 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 40 mM Tris base, and 3 mM ATP, pH 7.4. Ouabain sensitivity was determined in the presence of 10 mM ouabain. After 10 minutes, the reaction was terminated by the addition of 150 µl of ice cold 50% (w/v) trichloroacetic acid, and placing the tubes in an ice bath. After centrifugation at low speed, a 500 µl aliquot of the supernatant was removed for quantitation of the inorganic phosphate released in the reaction,

using this laboratory's modification of the Fiske Subbarow phosphorus assay (Fiske & Subbarow, 1925) (assay described on page 63).

CNPase. CNPase, a marker for myelin, was assayed by the method of Kurihara and Tsukada (1967). Tissue samples were prepared in 1% (v/v) Triton X-100 to give a concentration of 0.25 μg protein/ μl . The reaction tubes, containing 50 μl of 0.03 M 2',3'-cAMP, 50 μl of a solution containing 0.2 M Na_2HPO_4 and 0.1 M citrate pH 6.2, and 80 μl deionized water, were preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of 20 μl of tissue in Triton X-100. Tubes without tissue were also included as substrate blanks. After 20 minutes, the reaction was terminated by the addition of 20 μl of glacial acetic acid and the tubes were placed on ice. After centrifugation at low speed, a 20 μl aliquot of the supernatant was removed for separation on Whatman chromatography paper, using isopropanol:ammonia:water, 7:1:2 (v/v/v) (Banik and Davison, 1969) as the solvent system. The paper was dried in air and the nucleotide spots were visualized under ultraviolet light and marked. Spots corresponding to substrate, product, and paper blank were cut into small pieces and placed in test tubes. Four ml of 0.01 N HCl was added to each tube prior to shaking for at least 2 hours at room temperature. The paper was removed by low speed centrifugation and the optical density (O.D.) of the superna-

tant was determined at 260 nm. Calculations were as follows:

$$S_1 = A_{\text{substrate standard}} - A_{\text{paper blank}}$$

$$P_1 = A_{\text{product}} - A_{\text{paper blank}}$$

$$X = S_1/1.5 = \text{Absorbance}/\mu\text{mole/assay}$$

$$Y = 3(P_1/X) = \mu\text{mole}/20 \mu\text{l of assay/hour}$$

$$Z = Y/\text{mg protein in } 20 \mu\text{l} = \mu\text{mole/hour/mg protein}$$

Acid Phosphatase. Acid phosphatase, a marker for lysosomes, was assayed by the method of Linhardt and Walter (1963). The assay buffer (500 μl) containing 50 mM citrate, 5.5 mM p-nitrophenyl phosphate pH 4.8, was preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of 50 μl of homogenate (100-500 μg protein), synaptosome or SPM (50 μg protein), and allowed to proceed for 30 minutes at 37°C. Blanks, without tissue, and standards containing $2.5-10 \times 10^{-5}$ moles of p-nitrophenol were included. The reaction was terminated by the addition of 2 ml of 0.1 N NaOH. The O.D. of blanks, standards, and samples was determined at 405 nm. Calculations were as follows:

$$(A_{405})(2.81)/(.5)(\text{mg protein}) = \text{mmole acid}$$

$$\text{phosphatase units (mmole/hr/mg protein)}$$

Cytochrome c Oxidase. Cytochrome c oxidase, a marker for mitochondria, was assayed by the method of Duncan and Mackler (1966). The assay measures the rate of the decrease in absorbance at 550 nm due to the cytochrome

c oxidase-catalyzed conversion of ferrocytochrome c (Fe^{2+}) to ferricytochrome c (Fe^{3+}). Ferrocytochrome c was prepared by the addition of sodium dithionite to ferricytochrome c. Excess dithionite was removed by aeration. To the assay buffer, containing 0.2 ml of 0.2 M phosphate buffer, pH 7.5, 0.1 ml of 1% (w/v) ferrocytochrome c, and 0.7 ml deionized water, tissue was added to give an absorbance change that was linear for 2-5 minutes. The enzyme activity was calculated from the slope of the linear decrease in absorbance as a function of time as follows:
(change in A_{550}/min)/(total absorbance change/ μmole cytochrome c)(mg protein) = $\mu\text{mole}/\text{min}/\text{mg}$ protein.

CHOLESTEROL TRANSFER

Synaptosomes for the D_1 binding and adenylate cyclase assays and SPM for fluidity studies (1 mg of protein in 50 μl of 0.32 M sucrose) were preincubated for 10 minutes at 32°C with 200 μl of concentrated transfer protein in elution buffer, 80 μl of 1.8 M sucrose, and 170 μl of deionized water to give a final sucrose concentration of 0.32 M. The extent of cholesterol transfer was controlled by the amount of transfer protein added. The reaction was initiated by the addition of 500 μl of liposomes and allowed to continue for 30 minutes (synaptosomes) or 60 minutes (SPM) with gentle shaking. The shorter incubation time was used for the synaptosomes because the viability of the synaptosomes was compromised, and the kinetics of

dopamine binding was altered with longer incubation times. The transfer was terminated by centrifugation through a layer of 0.5 M sucrose at 106,000 x g (24,250 rpm in SW-28 rotor) for 40 minutes (North and Fleischer, 1983b).

Several modifications were made in the cholesterol transfer procedure for the synaptosomes used for the dopamine uptake studies (North & Fleischer, 1983b). 1) Sodium azide, which inhibits uptake processes, was removed prior to use in the cholesterol transfer from the transfer protein solution by dialysis against elution buffer without sodium azide. 2) In addition, 15% (v/v) Krebs-Henseleit buffer (see below) was included in the incubation medium to preserve synaptosome viability without inhibiting the transfer activity. 3) The reaction was terminated by centrifugation at 63,581 x g (22,000 rpm in SW-28 rotor) through 0.5 M sucrose containing 40% (v/v) Krebs-Henseleit buffer.

After cholesterol transfer, the surface of each pellet was rinsed to remove any sedimented lipid. The pellet was then suspended in the assay buffer. Binding, uptake, and adenylate cyclase assays were performed immediately after the cholesterol transfer.

FLUORESCENCE POLARIZATION DETERMINATION OF MEMBRANE FLUIDITY

The fluidity of SPM from rat whole brain and bovine caudate was determined using fluorescence polarization

techniques. The surface and the hydrophobic core of the membrane were probed using 1-anilinonaphthalene-8-sulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. The SPM fraction was used rather than the more crude synaptosomal fraction because the hydrophobic fluorescent probes will partition into all cellular membranes. A semi-crude fraction contains mitochondria, which are low in cholesterol content and thereby very fluid, and myelin, which is high in cholesterol content and, hence, very rigid. Fluorescence polarization analysis of such mixed membrane fractions could confound the interpretation of the data.

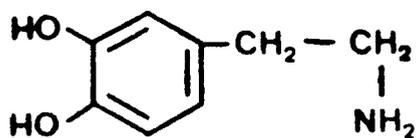
For the DPH studies, SPM (50 ug of SPM protein) were incubated with 1 μ M DPH in phosphate buffered saline (PBS), pH 7.4 (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4) at 37°C for 30 minutes to allow the probe to equilibrate into the membrane. SPM for the ANS study were incubated at room temperature for 15 minutes with 200 μ M ANS in PBS containing 10 mM MgSO_4 . The divalent magnesium cation was used to neutralize surface negative charges and to allow the ANS to interact more efficiently with the membrane, and hence to enhance the fluorescence signal.

The fluorescence of the SPM was determined in a Perkin Elmer MPF-44B Fluorescence Spectrophotometer, equipped with rotating polarizing filters. For the DPH

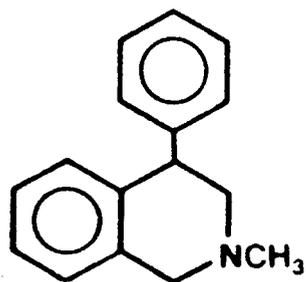
studies, an excitation wavelength of 360 (slit width 3 nm) and emission wavelength of 430 nm (slit width 20 nm) were used (Shinitzky & Barenholz, 1978). For the ANS studies, an excitation wavelength of 395 (slit width 5 nm) and emission wavelength of 490 nm (slit width 20 nm) were used (Slavik, 1982). The fluorescence of the prepared samples was determined at different orientations of the polarizing filters.

DOPAMINE UPTAKE ASSAY

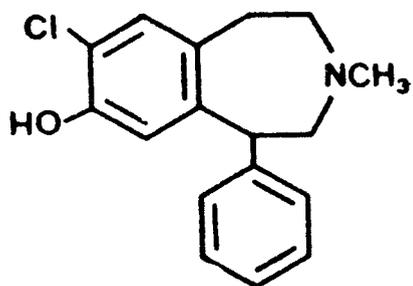
The uptake of dopamine into control and lipid-modified synaptosomes was assayed by a modification of the method of Deskin et al. (1981). After lipid transfer, synaptosomes were gently suspended in modified Krebs-Henseleit buffer (118 mM NaCl, 26 mM Na₂HPO₄, 6 mM NaH₂PO₄, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, 5.6 mM D-glucose, 2 μM ascorbic acid, 10 μM pargyline, pH 7.4) for assay. Triplicate 100 μl aliquots of prepared synaptosomes, containing 200-300 μg of synaptosomal protein, were added to 900 μl of Krebs buffer, containing 10-15 nM of [³H]-dopamine and 0-5,000 nM of unlabelled dopamine (Figure 8a). Nonspecific uptake was determined in the presence of 10 μM nomifensine (Figure 8b) (Hunt et al., 1974). All samples were incubated for 5 minutes at 37°C with gentle shaking. The reaction was terminated by transferring the tubes to ice and adding 2 ml of ice cold Krebs buffer. All samples were vacuum filtered



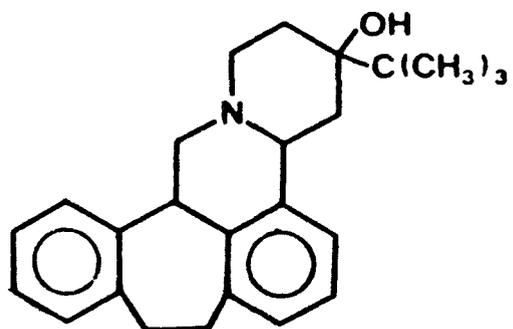
a) Structure of Dopamine



b) Structure of Nomifensine



c) Structure of SCH23390



d) Structure of Butaclamol

through Whatman GF/F filters and washed twice with 3 ml of Krebs buffer. Radioactivity associated with the filters was determined as described previously. The kinetic parameters K_m and V_{max} were calculated for each assay by Hanes plot analysis. The Hanes plot is a linear transformation of the data which plots s/v (the substrate concentration/the observed rate) on the Y-axis and s (the substrate concentration) on the X-axis. From this plot, the K_m is the negative X- intercept, and the V_{max} is $1/\text{slope}$.

DOPAMINE (D_1) BINDING ASSAY

The dopamine D_1 binding sites in control and lipid-modified membranes were assayed using the method of Hess et al. (1986) with SCH23390 (Figure 8c) as a ligand. After cholesterol transfer, synaptosomes were suspended in 50 mM Tris pH 7.4 containing 5 mM $MgSO_4$. Membranes were incubated at 37°C for 15 minutes, followed by centrifugation at 20,000 x g for 20 minutes, to remove endogenous dopamine. The pellets were suspended in a solution containing 50 mM Tris, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.02% (w/v) ascorbic acid, pH 7.4 (assay buffer). Duplicate 100 μ l aliquots of synaptosomes, containing 300-500 μ g of synaptosomal protein were added to 2.9 ml of assay buffer containing 0.2-0.5 nM of [3H]-SCH23390, and 0-5.1 nM of unlabelled SCH23390. Samples were incubated at 37°C for 30 minutes with gentle shaking. Nonspecific binding was

determined in the presence of 1 μM (+)-butaclamol (Figure 8d) (Seeman et al., 1985). The reaction was terminated by vacuum filtration through Whatman GF/C filters, followed by three washes with 5 ml of 50 mM Tris, pH 7.4. The radioactivity associated with the filters was determined as described previously. The kinetic parameters, K_d and B_{max} , were calculated for each assay by Scatchard analysis. The Scatchard plot is a linear transformation of the experimental data in which the ratio of the concentrations of bound ligand and free ligand is plotted on the Y-axis and the concentration of bound ligand is plotted on the X-axis. From the Scatchard plot, the B_{max} is the X-intercept, and the K_d is $-1/\text{slope}$.

DOPAMINE-SENSITIVE ADENYLATE CYCLASE ASSAY

Basal and dopamine-sensitive adenylate cyclase activity was assayed using control and lipid-modified bovine caudate synaptosomal membranes by a modification of the methods of Enjalbert et al. (1978), Makman et al. (1980), and Waldman et al. (1985). Synaptosomes from the dopamine D_1 binding assays were used directly for the adenylate cyclase assays. Synaptosomes (50 μg of protein) were preincubated at 30°C in a solution containing 80 mM Tris-maleate, 2 mM MgSO_4 , 2 mM isobutylmethylxanthine, 0.2 mM EGTA, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 50 μM GTP, pH 7.4, in the presence or absence of 100 μM dopamine. The reaction was initiated by the

addition of 0.5 mM ATP and allowed to proceed for 5 minutes. The reaction was terminated by the addition of 50 mM sodium acetate pH 4.0 and immersion in a boiling water bath for 3 minutes. Precipitated protein was removed by centrifugation at 23,000 x g (15,000 rpm in SE12 rotor) for 15 minutes. The supernatants were stored at -80°C until cyclic AMP radioimmunoassays were performed.

CYCLIC AMP RADIOIMMUNOASSAY

The cAMP radioimmunoassay was a modification of the method of Steiner (1972a,b). Before the RIA was run, all samples and standards were acetylated to more closely duplicate the antigen used to generate the antibody. This acetylation step greatly increases the sensitivity of the assay. To acetylate samples, 20 µl of triethylamine and 10 µl of acetic anhydride were added to 500 µl of standards and samples. Duplicate 100 µl aliquots of standards (containing 0 to 500 fmole of cAMP) and samples were transferred to 12 x 75 mm tubes, and 100 µl of [¹²⁵I]-cAMP, in 50 mM sodium acetate, pH 6.2, was added. To start the assay, 100 µl of anti-cAMP antibody (primary antiserum to cAMP) in acetate buffer was added, and the tubes were incubated in the refrigerator for 12 hours. The assay was stopped by the addition of 50 µl of 1% gamma globulin in acetate buffer and 2 ml of ice-cold 95% isopropanol. The tubes were centrifuged, in a Jouan GR4.11 centrifuge, at 1,623 x g (2800 rpm) for 30 minutes at 4°C and the super-

natant was decanted. The radioactivity associated with the pellets was determined in a gamma counter.

PROTEIN DETERMINATION

Protein was assayed following the method of Lowry et al. (1951). Human serum albumin (HSA), prepared at a concentration of 1 mg/ml in deionized water, was used as a protein standard. The standard curve consisted of 0-50 μ g of protein. Standards and samples were incubated in 0.1 ml of 1 N NaOH for 30 minutes at room temperature to solubilize membrane-bound proteins. To each tube was added 1 ml of a solution freshly prepared with 10 ml of 2% (w/v) sodium carbonate, 0.1 ml of 1% (w/v) cupric sulfate, and 0.1 ml of 2% (w/v) K^+ - Na^+ tartrate. After 10 minutes, 0.1 ml of 1 N Folin phenol reagent was added. After 30 minutes, the O.D. of standards and samples was determined at 700 nm.

CHOLESTEROL ASSAY

Cholesterol was assayed using a diagnostic kit (Sigma Kit 351). The assay, which quantitates both cholesterol esters and cholesterol, combines cholesterol esterase, cholesterol oxidase, and peroxidase in a colorimetric reaction. Cholesterol esters are hydrolyzed to free cholesterol and fatty acids by cholesterol esterase. The cholesterol is then oxidized to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. The hydrogen

peroxide reacts with 4-amino-antipyrine and phenol in the presence of peroxidase to form a quinoneimine dye which has an absorbance maximum at 500 nm. The reagent contains a detergent (sodium cholate) to free membrane bound cholesterol and gives a linear response up to 60 ug of cholesterol. Blanks, aqueous cholesterol standards, and tissue samples (containing up to 60 µg of cholesterol) were incubated with 1 ml of reagent at 37°C for 10 minutes. The O.D. of samples and standards was determined at 500 nm against the blank. Because turbidity in the samples can lead to overestimation of the cholesterol content, the O.D. of each sample and standard was determined after the addition of 2 drops of 0.5 M ascorbate, which will decolorize the reaction mixture. The net absorbance was used to calculate the cholesterol content.

PHOSPHORUS ASSAY

Phospholipid and inorganic phosphate was determined by a modification of the method of Fiske and Subbarow (1925). Before determining the phosphorus concentration of SPM and synaptosomes, the tissue samples were extracted three times with chloroform:methanol (2:1) (Folch et al., 1957). The chloroform layers were combined and dried under nitrogen. To each residue was then added 0.1 ml of 70% perchloric acid. Each tube was digested at 170°C until no yellow color remained. After cooling, 1 ml of deionized water was added. A standard solution of

0.04 mg/ml of KH_2PO_4 was prepared. To standards of 0 to 40 μg of phosphorus in 1 ml of deionized water, 0.1 ml of 70% perchloric acid was added. To each standard and sample, 0.25 ml of 2.5% ammonium molybdate, 0.1 ml of reducer, and deionized water, up to a total volume of 2.5 ml, were added. After 10 minutes at room temperature, the O.D. of the standards and samples was determined at 625 nm. The reducer was prepared by dissolving 3 g of Na_2SO_3 , 60 g of NaHSO_3 , and 1 g of 1-amino-2-naphthalene-4-sulfonic acid in 500 ml of deionized water.

CALCULATION OF CHOL/PL

In order to determine an accurate Chol/PL for the lipid-modified SPM and synaptosomes, it was necessary to correct the cholesterol and phospholipid phosphorus concentrations for the sticking of liposomes. This was made possible by the inclusion of [^{14}C]-tripalmitin in the liposomes as a nontransferrable marker. The radioactivity in the liposomes and tissue samples was determined by liquid scintillation counting. The corrected Chol/PL was calculated as follows:

$$\text{nmole chol due to sticking} = (\text{^{14}C in sample}) \times \left(\frac{\text{nmole chol/^{14}C}}{\text{in liposomes}} \right)$$

$$\text{nmole PL due to sticking} = (\text{^{14}C in sample}) \times \left(\frac{\text{nmol PL/^{14}C}}{\text{in liposomes}} \right)$$

$$\text{net chol} = (\text{total chol} - \text{chol due to sticking})/\text{mg protein}$$

$$\text{net PL} = (\text{total PL} - \text{PL due to sticking})/\text{mg protein}$$

$$\text{corrected Chol/PL} = \text{net chol}/\text{net PL}$$

DATA ANALYSIS

Mean values reported for the marker enzyme activities, adenylate cyclase activities, and Chol/PL of control membranes represent the mean +/- the standard deviation of several samples, where n = the number of determinations. Student's 't' test for significance was used when comparing the marker enzyme activities of synaptosomes and SPM to homogenates. P values of <0.05 were considered to represent significant differences.

Data from the dopamine uptake studies was analyzed by Hanes plot analysis of triplicate determinations. The K_m and V_{max} values for each assay were calculated from the X-intercept and the slope, respectively. Only assays with at least 5 data points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included.

Data from the SCH23390 binding studies was analyzed by Scatchard analysis of duplicate determinations. The K_d and B_{max} were calculated from the slope and X-intercept, respectively. The criteria for inclusion of assays was identical to that used for the dopamine uptake studies.

Linear analysis of standard curves, Hanes and Scatchard plots was by the method of least squares. Unless otherwise noted, the lines describing the relationship between the Chol/PL and the various parameters studied were

estimated from the data to show a general trend, and were not calculated from theoretical equations relating Chol/PL to the experimental parameters.

CHEMICALS

<u>Chemical</u>	<u>Source</u>
Sucrose	J.T. Baker, Phillipsburg, NJ
EDTA	Sigma, St. Louis, MO
Trizma HCl	Sigma
Trizma OH	Sigma
Sodium azide	Sigma
Ammonium sulfate	Sigma
2-Mercaptoethanol	Sigma
Ficoll-400	Pharmacia, Upsala, Sweden
Egg phosphatidyl choline	Sigma
Sodium phosphate, monobasic	Sigma
Sodium phosphate, dibasic	Sigma
Sodium chloride	Sigma
Cholesterol	Sigma
[¹⁴ C]-Cholesterol	Amersham, Arlington Heights, IL
[³ H]-Triolein	ICN Radiochemicals, Irvine, CA
[¹⁴ C]-Tripalmitin	Amersham
Potassium chloride	Mallinckrodt, Paris KY
Calcium chloride	Baker
Magnesium sulfate	Mallinckrodt
D-Glucose	Mallinckrodt
Ascorbic Acid	Sigma
Pargyline	Sigma
Dopamine	Sigma

CHEMICALS (cont.)

<u>Chemical</u>	<u>Source</u>
[³ H]-Dopamine	New England Nuclear, Boston MA
Nomifensine	Hoechst-Roussel, Somerville, NJ
Ecoscint	National Diagnostics, Manville, NJ
SCH23390	Research Biochemicals, Inc., Wayland, MA
[³ H]-SCH23390	New England Nuclear
(+)-Butaclamol	Research Biochemicals, Inc.
Tris-maleate	Sigma
Isobutylmethylxanthine	Sigma
EGTA	Sigma
Creatine phosphate	Sigma
Creatine kinase	Sigma
GTP	Sigma
ATP	Sigma
ANS	Molecular Probes, Inc., Junction City, OR
DPH	Molecular Probes, Inc.
Potassium phosphate, mono- basic	Baker
SDS	Sigma
Magnesium chloride	Sigma
Trichloroacetic acid	Sigma
Triton X-100	Aldrich Chemical, Milwaukee, WI
2',3'-cAMP	Sigma

CHEMICALS (cont.)

<u>Chemical</u>	<u>Source</u>
Sodium citrate	Baker
Isopropanol	Mallinckrodt
Ammonia	Mallinckrodt
p-Nitrophenyl phosphate	Sigma
Ferricytochrome c	Sigma
Sodium dithionite	Fisher Scientific, Fair Lawn, NY
Acrylamide	Sigma
bis-Acrylamide	Eastman Kodak, Rochester, NY
Glycine	Sigma
Bromothymol blue	Sigma
Ammonium persulfate	Sigma
Isobutanol	Eastman Kodak
TEMED	Sigma
Methanol	Baker
Acetic Acid	Mallinckrodt
Coomassie blue	Sigma
anti-cAMP antibody	Research Products Inter- national, Mt. Prospect, IL
cAMP	Sigma
[¹²⁵ I]-cAMP	Meloy Laboratories, Spring- field, VA
Gamma-globulin	Sigma
Triethylamine	Sigma
Acetic anhydride	Sigma

CHEMICALS (cont.)

<u>Chemicals</u>	<u>Source</u>
HSA	Sigma
K ⁺ -Na ⁺ tartrate	Mallinckrodt

CHAPTER IV

RESULTS

NONSPECIFIC LIPID TRANSFER PROTEIN

Purification of Lipid Transfer Protein

The lipid transfer protein was eluted from the Sephadex CM-C25 ion exchange column in a single peak which immediately followed a large peak containing yellow material (Figure 9). It was necessary to extensively wash the column with the dialysis buffer prior to elution in order to achieve a good separation of the LTP peak. The purification of LTP is represented in Table I. After the ammonium sulfate precipitation, there was an increase in the apparent yield of transfer activity. This may have been due to the removal of possible endogenous inhibitors in the 5.1 Supernatant fraction. After the ion exchange chromatography, the LTP had been purified 217-fold over the 5.1 Supernatant. The yield of transfer activity was 53%. Figure 10 depicts the separation of proteins in the LTP fraction, the 5.1 Supernatant, and the ammonium sulfate fractions by SDS-PAGE. As shown in Figure 10, the purification of LTP yielded an enrichment of transfer activity and a reduction of the contaminating proteins. From the R_f of the molecular weight standards and the LTP

Figure 9. The Elution of LTP from Sephadex CM-C25.

After ammonium sulfate precipitation and dialysis, the dialysate was applied to a Sephadex CM-C25 ion exchange column and washed extensively with dialysis buffer. The transfer protein was eluted with 2 volumes of a solution containing 25 mM sodium phosphate, 45 mM NaCl, 5 mM 2-mercaptoethanol, and 0.02% (w/v) sodium azide. The O.D. of individual fractions was determined at 280 nm to follow the elution. The LTP peak was identified by its cholesterol transfer activity.

FIGURE 9

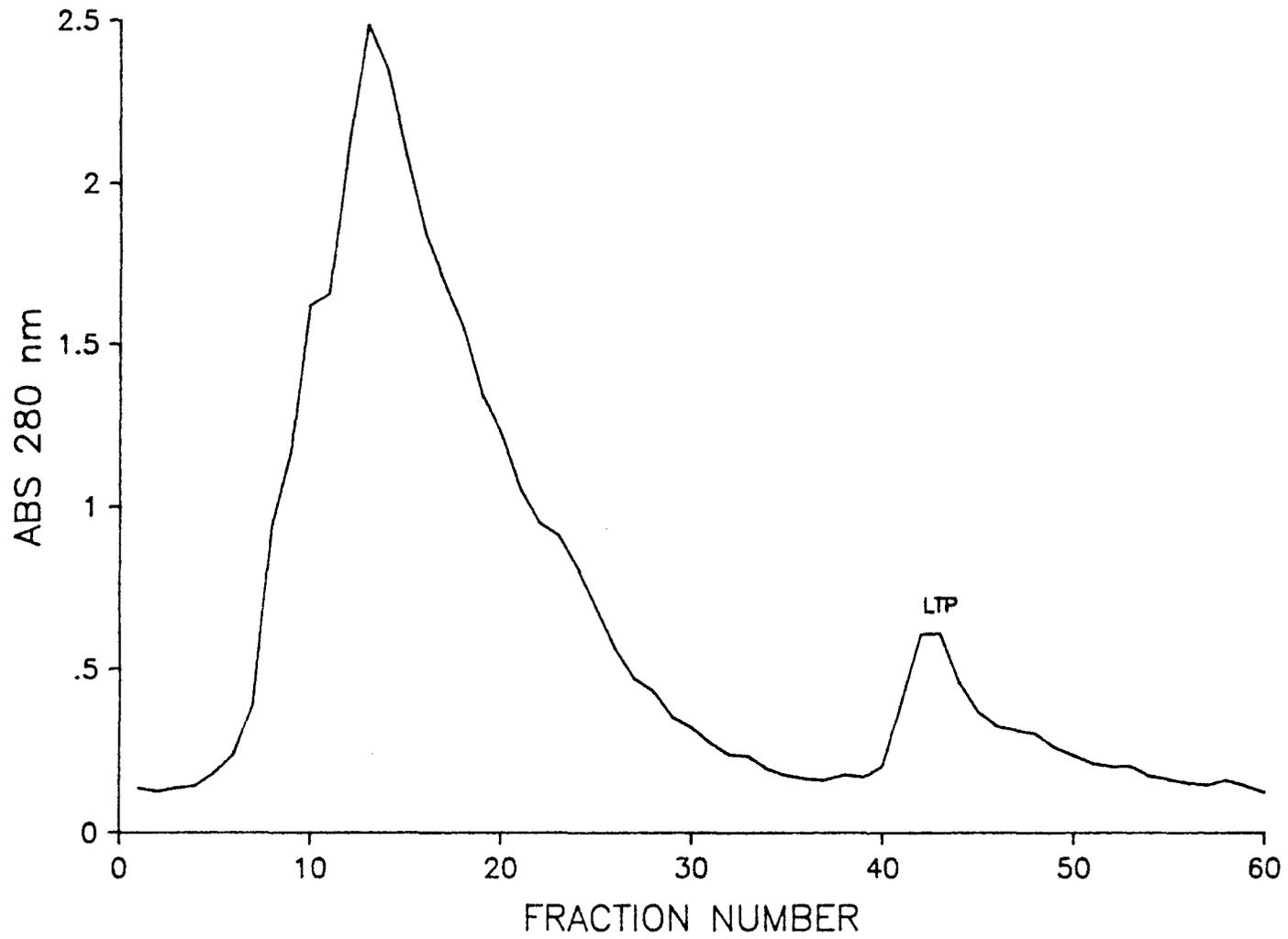


TABLE I
PURIFICATION OF LIPID TRANSFER PROTEIN

FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (nmol chol transferred/ mg protein)	TOTAL ACTIVITY	% YIELD	PURIFICATION
5.1 SUPERNATANT	1390	26.27	36515	0.3	10955	100	1
AMMONIUM SULFATE	750	27.77	20828	0.89	18599	170	3
CM-C25	380	0.23	88.54	65.24	5776	53	217

Lipid Transfer Protein was partially purified from beef liver as described in the Materials and Methods. Aliquots of the 5.1 Supernatant, the ammonium sulfate fraction and the LTP fraction obtained after ion exchange chromatography on Sephadex CM-C25, were assayed for cholesterol transfer activity as described.

Figure 10. SDS-PAGE of Fractions from the Purification of LTP.

The separating gel contained 12.5% (w/v) acrylamide and the stacking gel contained 3% (w/v) acrylamide. The arrows indicate the LTP band in each experimental lane.

Lane 1: 5.1 Supernatant (100 μ g protein)

Lane 2: Ammonium Sulfate fraction (100 μ g protein)

Lane 3: LTP fraction (10 μ g protein)

Lane 4: Low molecular weight standards

Phosphorylase B, 92.5 K

Bovine Serum Albumin, 66.2 K

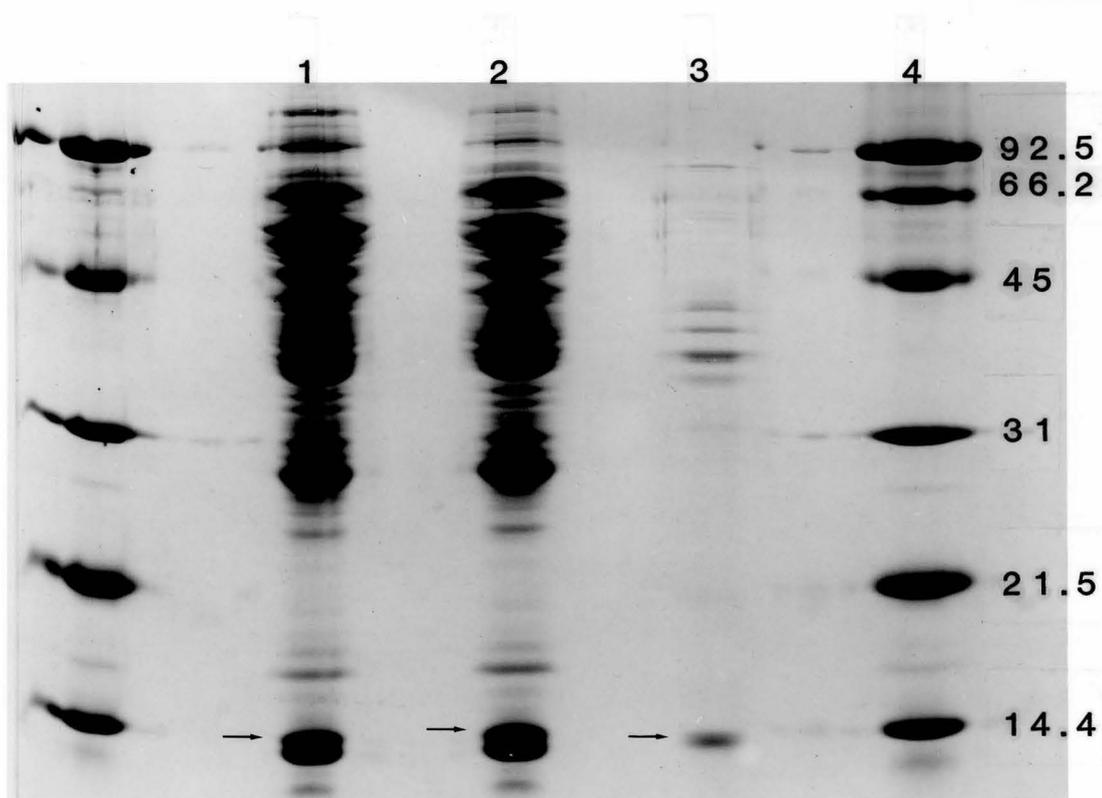
Ovalbumin, 45 K

Carbonic Anhydrase, 31 K

Soybean Trypsin Inhibitor, 21.5 K

Lysozyme, 14.4 K

FIGURE 10



band, the apparent molecular weight of LTP was calculated as 14,300 daltons (Figure 11). It was not necessary to continue the purification to homogeneity because the most important contaminating protein, a PC-specific transfer protein with a P_I of 5.8 (Zilversmit, 1983), would be expected to be removed during the ion exchange chromatography. The molecular weight of this PC-specific transfer protein is 28,000 daltons (Zilversmit, 1983). In the SDS-PAGE of the LTP fraction, no bands appeared in this molecular weight region (Figure 10).

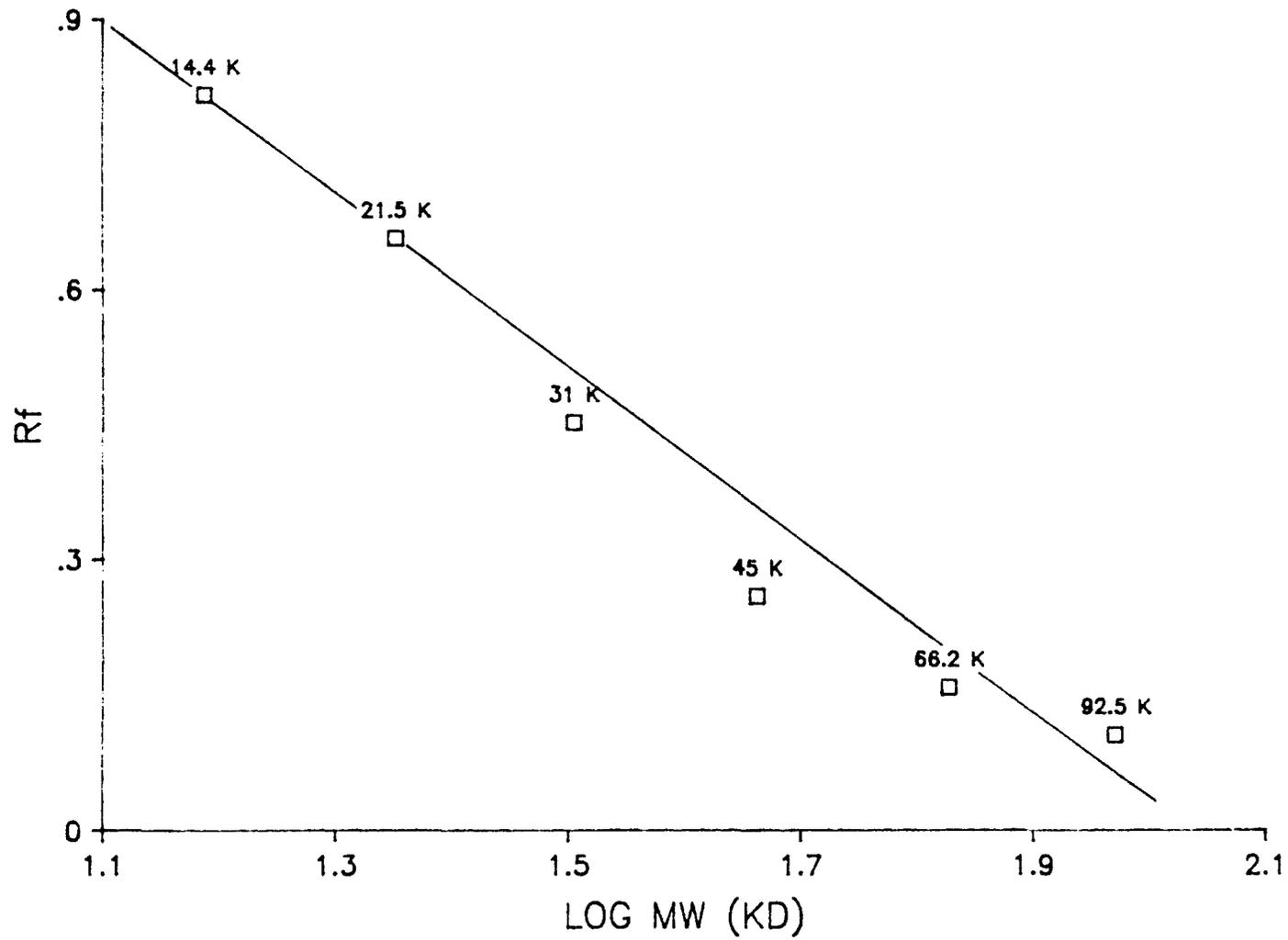
ASSAY OF CHOLESTEROL TRANSFER ACTIVITY

The purpose of the assay was to demonstrate a unidirectional transfer of cholesterol from a donor liposome into mitochondria. The mitochondrial membrane is a good acceptor because it contains very little cholesterol. The amount of cholesterol in the mitochondrial membrane preparation was below the limits of detection for the cholesterol assay. The liposomes used in the assay contained PC and cholesterol (1:1, mole:mole) as well as [^3H]-triolein and [^{14}C]-cholesterol. After the incubation, the mitochondria will have a $^{14}\text{C}/^3\text{H}$ that is greater than the $^{14}\text{C}/^3\text{H}$ of the liposomes, if a net transfer of cholesterol has taken place. An assumption that must be made is that the ^{14}C , ^3H , and cholesterol in these liposomes are uniformly distributed throughout the population of liposomes. In order to show this, the

Figure 11. Sodium Dodecyl Sulfate Polyacrylamide Gel
Electrophoresis of the LTP Sample.

From the R_f of the LTP band after SDS-PAGE on a 12.5% acrylamide gel, the apparent molecular weight of LTP was determined to be 14,300 daltons.

FIGURE 11



liposomes were chromatographed on Sepharose 4B-CL. Each fraction was assayed for phospholipid phosphorus and cholesterol, and the radioactivity was determined by liquid scintillation counting. The distribution of lipid and radioactivity in the liposomes for the transfer assay is shown in Figure 12. One population of liposomes is found. The phosphorus (Pi), cholesterol, ^3H , and ^{14}C coeluted, indicating that the liposomes were uniform in composition.

The results of a typical transfer assay using beef heart mitochondria is shown in Figure 13. The nonspecific transfer increased linearly over the time assayed and accounted for approximately 20% of the total cholesterol transferred. The specific transfer was nearly linear for up to 25 minutes.

RELATIVE PURITY OF SYNAPTOSOMES AND SPM

RAT WHOLE BRAIN MEMBRANES

Rat whole brain synaptosomes and SPM were prepared. The relative purity of these fractions, as compared to homogenates, is shown in Table II. Each determination is the average of tissue from four preparations. Na^+ - K^+ -ATPase, found primarily in plasma membranes, was enriched in both the synaptosomal and SPM fractions, 1.7- and 4.7-fold, respectively. The relative specific activities of acid phosphatase, cytochrome c oxidase, and CNPase demonstrate a significant reduction in the contamination of the synaptosomal and SPM preparations by lysosomal, mito-

Figure 12. Sepharose 4B-CL Chromatography of Liposomes
used for Cholesterol Transfer Assay.

Liposomes were prepared containing 1 mg PC/ml and 1 mole of cholesterol per mole of PC. [^{14}C]-Cholesterol was included as a transferrable marker. [^3H]-Triolein was included as a nontransferrable marker. One ml of liposomes was loaded onto the column (1 x 15 cm) and was eluted with 20 ml of 50 mM Tris HCl, pH 7.4. One ml fractions were collected and assayed for cholesterol and phospholipid phosphorus. The radioactivity in each fraction was determined by liquid scintillation counting.

FIGURE 12

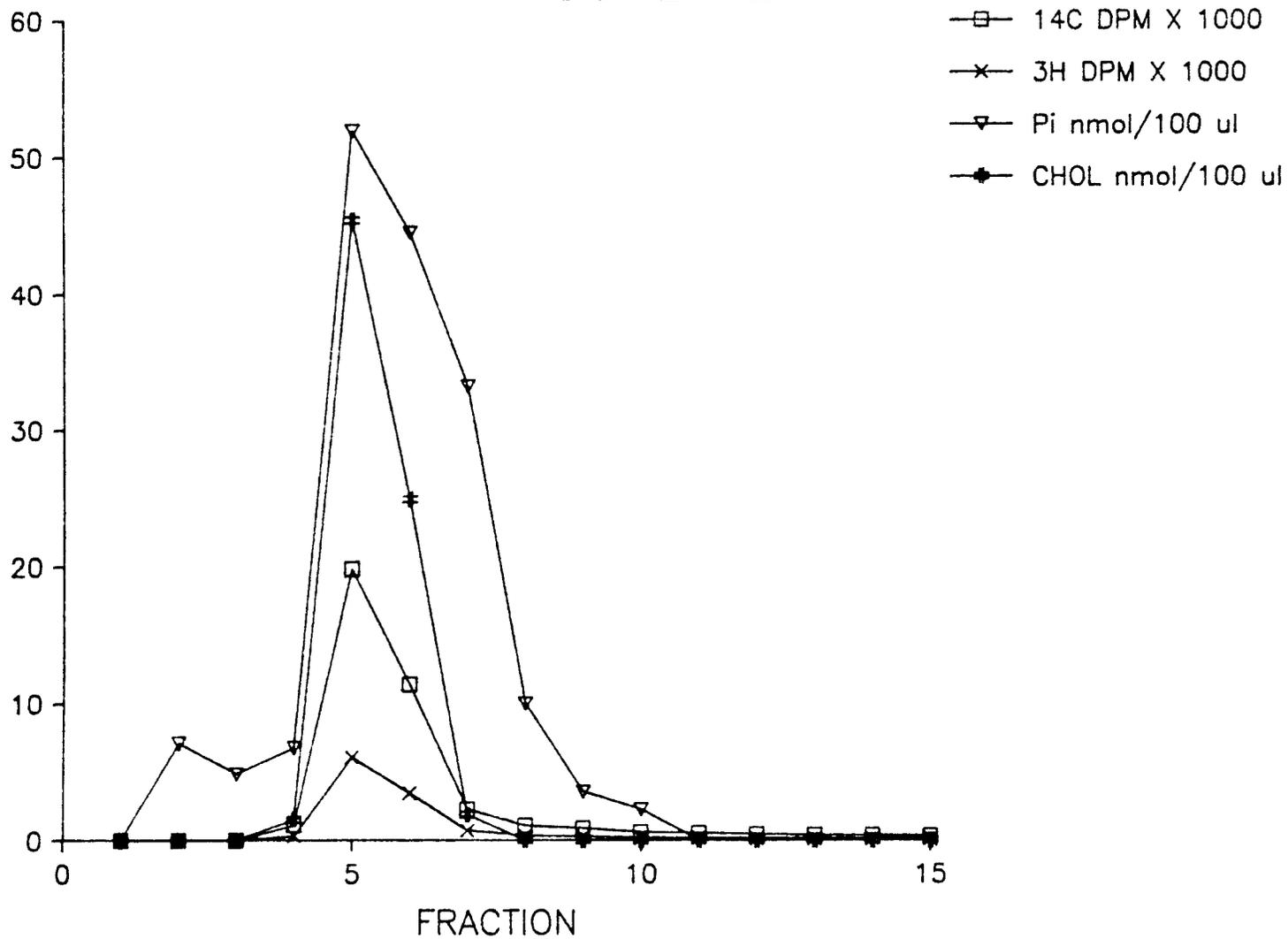


Figure 13. Cholesterol Transfer Activity of Purified LTP.

Cholesterol transfer activity was assayed by incubating heat-treated mitochondria with LTP and [^{14}C]- and [^3H]-labelled liposomes for varying time periods at 32°C . The reaction was terminated by filtration through GF/C filters, followed by 3 washes with 5 ml of 50 mM Tris HCl, pH 7.4. Nonspecific transfer was determined in the absence of LTP. The radioactivity associated with the filters was determined by liquid scintillation counting.

FIGURE 13

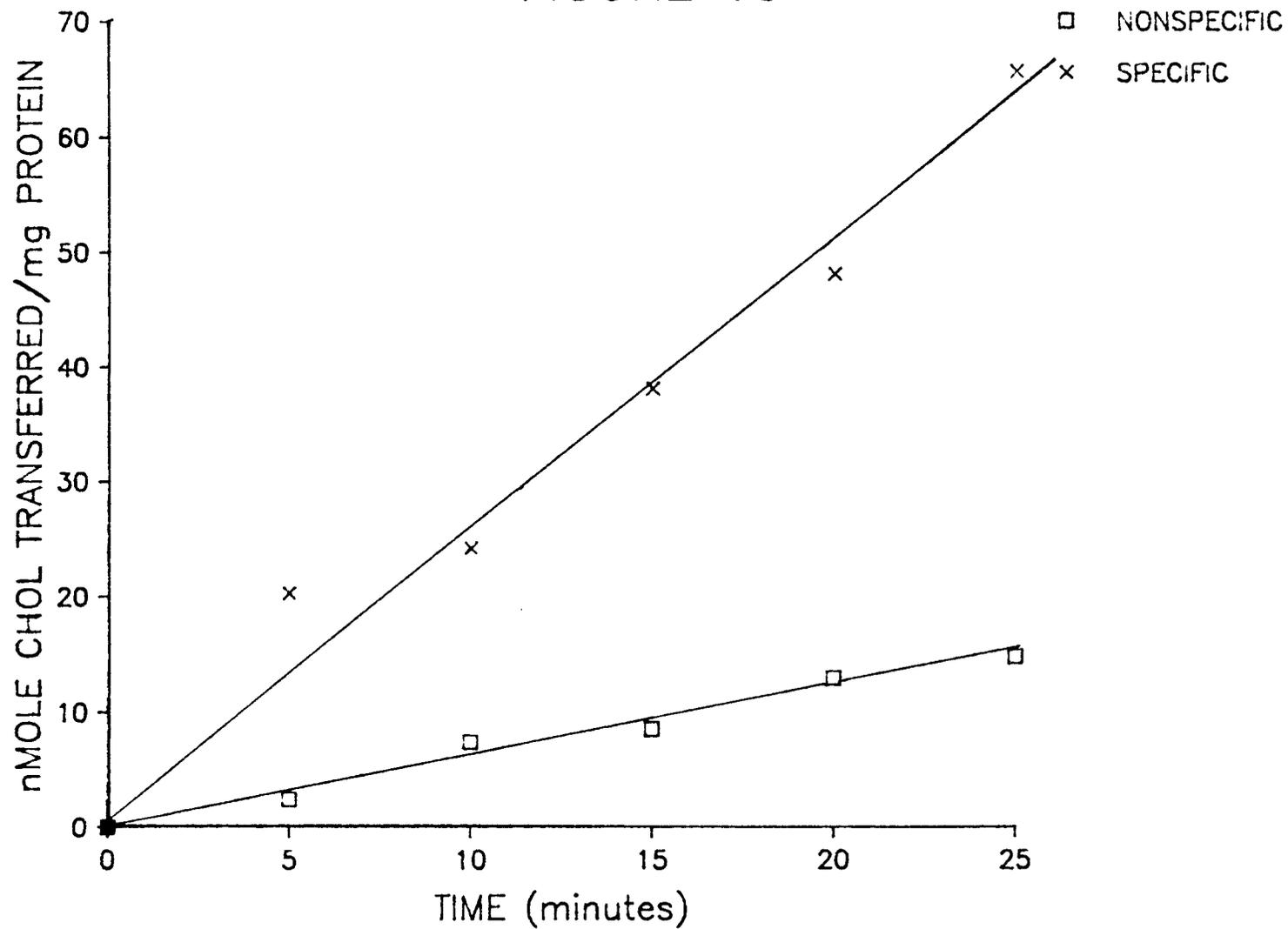


TABLE III

ACTIVITY OF ENZYME MARKERS IN BOVINE BRAIN SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES

	HOMOGENATE	SYNAPTOSOME	SPM
Na ⁺ K ⁺ ATPase µg P _i released/hr/ mg protein	2.89 +/- 0.79	4.91 +/- 0.49*	12.73 +/- 4.73**
Relative specific activity	1	1.70	4.40
Acid Phosphatase nmole substrate hydrolyzed/ hr/mg protein	13.49 +/- 1.85	12.65 +/- 1.95	10.34 +/- 3.67
Relative specific activity	1	0.94	0.77
Cytochrome c Oxidase µmole substrate hydrolyzed/ min/mg protein	0.555 +/- 0.017	0.260 +/- 0.080*	0.100 +/- 0.030**
Relative specific activity	1	0.47	0.18
CNPase µmole product formed/hr/ mg protein	1665.3 +/- 120.6	1049.5 +/- 110.9**	818.8 +/- 193.4**
Relative specific activity	1	0.63	0.49

Na⁺K⁺ATPase (plasma membranes), acid phosphatase (lysosomes), cytochrome c oxidase (mitochondria), and CNPase (myelin) assays were performed on samples from 4 individual membrane preparations. The relative specific activity is used to compare the specific activities of synaptosomes and SPM to that of the homogenates. Values of greater than one represent enrichment of the enzyme activity.

* Values are statistically different from the homogenates at P<.05.

** Values are statistically different from the homogenates at P<.01.

chondrial, and myelin membranes, respectively.

BOVINE CAUDATE MEMBRANES

Bovine caudate synaptosomes and SPM were initially prepared following the method of Cotman and Matthews (1971) (Figure 6). The myelin fraction was not well separated in the Ficoll gradient or the sucrose gradient. Because of the high cholesterol content and microviscosity of the myelin membrane, it is desirable to minimize the contamination of the synaptosomal and SPM preparations with myelin. Thus, the gradients used to isolate synaptosomes and SPM were modified as described in the Materials and Methods. This allowed the isolation of relatively pure fractions.

The relative purity of synaptosomes and SPM prepared using the modified method is compared to membrane homogenates (Table III). Each determination is the average of four separate preparations. $\text{Na}^+\text{-K}^+\text{-ATPase}$ was enriched in both the synaptosomal and SPM fractions, 1.7- and 4.4-fold, respectively. The relative specific activities of cytochrome c oxidase and CNPase demonstrate a significant reduction in the contamination of synaptosomal and SPM preparations by mitochondrial and myelin membranes, respectively. However, the CNPase activity in each of the bovine caudate fractions was approximately 6-fold higher than that found in rat whole brain (Table II). The contamination by lysosomal membranes, as identified by the activity of acid phosphatase, was not significantly

TABLE II

ACTIVITY OF ENZYME MARKERS IN RAT BRAIN SYNAPTOSOMES AND SYNAPTIC PLASMA MEMBRANES

	HOMOGENATE	SYNAPTOSOME	SPM
Na ⁺ K ⁺ ATPase µg P _i released/hr/ mg protein	4.64 +/- 1.15	7.97 +/- 1.92*	21.87 +/- 3.14**
Relative specific activity	1	1.72	4.74
Acid Phosphatase nmole substrate hydrolyzed/ hr/mg protein	18.18 +/- 2.12	15.23 +/- 2.10*	8.95 +/- 0.76**
Relative specific activity	1	0.84	0.49
Cytochrome c Oxidase µmole substrate hydrolyzed/ min/mg protein	0.675 +/- 0.064	1.195 +/- 0.040*	0.192 +/- 0.031**
Relative specific activity	1	0.29	0.18
CNPase µmole product formed/hr/ mg protein	291.0 +/- 37.8	135.2 +/- 32.2*	128.0 +/- 17.3**
Relative specific activity	1	0.46	0.44

Na⁺K⁺ATPase (plasma membranes), acid phosphatase (lysosomes), cytochrome c oxidase (mitochondria), and CNPase (myelin) assays were performed on samples from 4 individual membrane preparations. The relative specific activity is used to compare the specific activities of synaptosomes and SPM to that of the homogenates. Values of greater than one represent enrichment of the enzyme activity.

* Values are statistically different from the homogenates at P<.05.

** Values are statistically different from the homogenates at P<.01.

different from the homogenates.

CHOLESTEROL TRANSFER

RAT WHOLE BRAIN MEMBRANES

Accurate calculation of the Chol/PL in all experimental membranes required the correction of cholesterol and phosphorus content for the sticking of liposomes. The inclusion of [^{14}C]-tripalmitin in the liposomes made this correction possible. However, in order for this correction to be accurate, the cholesterol, PL, and ^{14}C label must be uniformly distributed throughout the liposome population. The analysis of the fractions from Sepharose 4B-CL chromatography of PC and PC-Chol liposomes is shown in Figures 14 and 15, respectively. Two populations of PC liposomes were found, due to the high concentration of PC (10 mg/ml). The radioactivity eluted with the phosphorus. One population of PC-Chol liposomes was found. Phosphorus, cholesterol, and radioactivity were coeluted from the column. These results suggest that, in both the PC and PC-Chol liposomes, the radioactivity was associated with the liposomes and the correction for liposome sticking was accurate.

The analysis of rat whole brain SPM from a typical lipid transfer experiment is shown in Table IV. The change in Chol/PL was paralleled by a change in cholesterol/protein. Although the PL/protein was slightly increased, this change, which was up to 10.55% of control, was small when

Figure 14. Sepharose 4B-CL Chromatography of PC Liposomes
used for Cholesterol Transfer.

PC liposomes, prepared in sucrose buffer, contained 10 ml PC/ml. One ml of liposomes was loaded onto the column (1 x 15 cm) and eluted with 20 ml of sucrose buffer. One ml fractions were collected and assayed for phospholipid phosphorus. The radioactivity in each fraction was determined by liquid scintillation counting.

Figure 15. Sepharose 4B-CL Chromatography of PC-Chol Liposomes used for Cholesterol Transfer

PC-chol liposomes, containing 5 ml PC/ml and 1.7 mole of cholesterol per mole of PC, were chromatographed as described in Figure 14. The fractions were assayed for phospholipid phosphorus and cholesterol. The radioactivity in each fraction was determined by liquid scintillation counting.

FIGURE 14

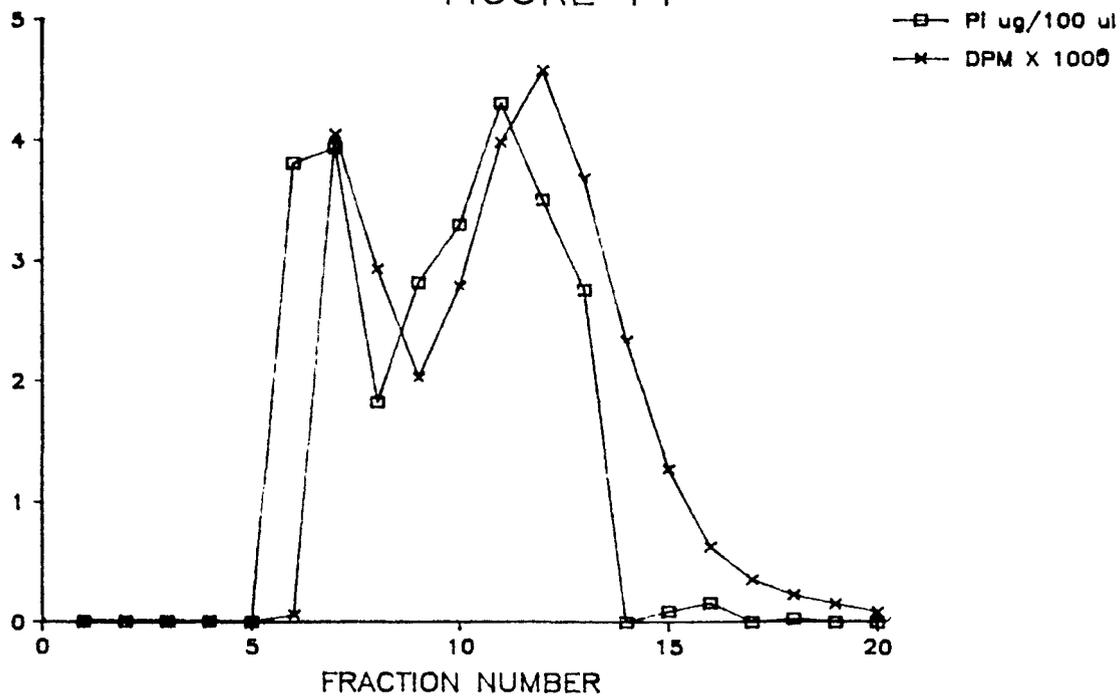


FIGURE 15

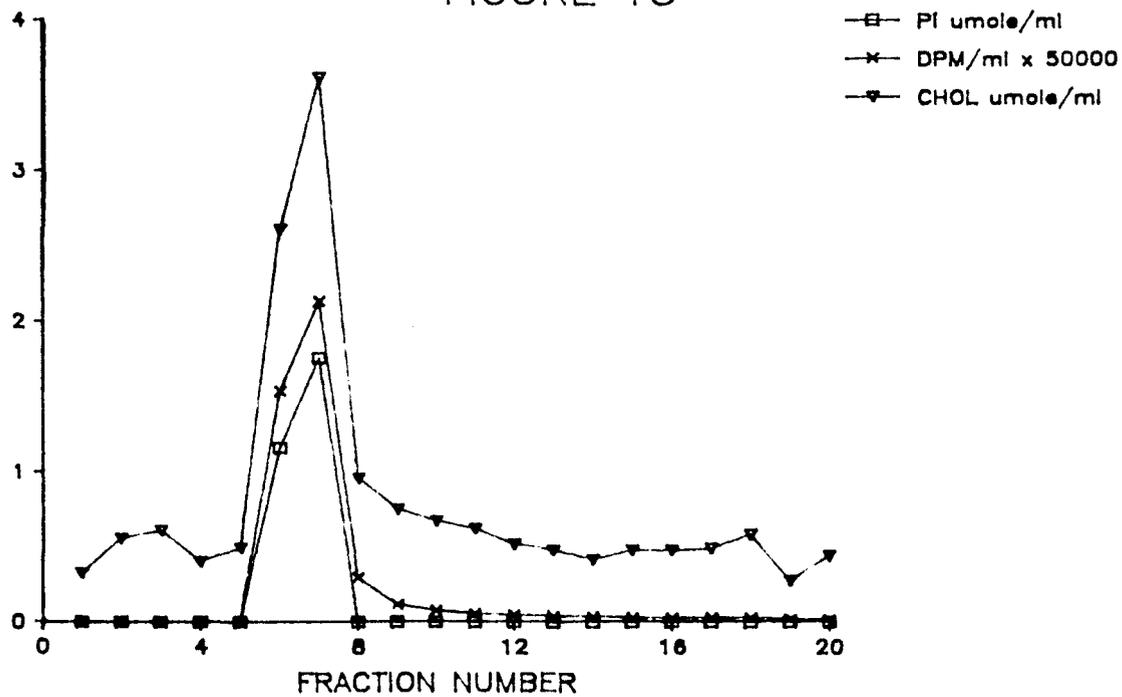


TABLE IV
 CHOLESTEROL DEPLETION AND LOADING OF RAT BRAIN SPM
 ANALYSIS OF LIPID-MODIFIED SPM

TYPE OF LIPOSOME USED	<u>CHOL</u> <u>PROTEIN</u> <u>nmole</u> mg	<u>PL</u> <u>PROTEIN</u> <u>nmole</u> mg	<u>CHOL</u> <u>PL</u> <u>mole</u> <u>mole</u>	CHANGE IN CHOL <u>nmole</u> mg protein	% CHANGE	CHANGE IN PL <u>nmole</u> mg protein	% CHANGE
PC	378	985	.384	-137	-26.60	+18	+1.86
PC	410	967	.424	-105	-20.39	0	0
PC	465	1056	.440	-50	-9.71	+89	+9.20
NONE *	515	967	.533	0	0	0	0
PC-CHOL	633	975	.649	+118	+22.91	+8	+0.82
PC-CHOL	744	1032	.720	+229	+44.47	+65	+6.72
PC-CHOL	938	1069	.878	+423	+82.14	+102	+10.55

The results presented are from a typical cholesterol transfer experiment using SPM. Similar results were obtained using synaptosomes. SPM were incubated with liposomes and varying amounts of transfer protein, from 0.1 to 0.4 mg per ml of incubation mixture. PC liposomes were used for cholesterol depletion. PC-CHOL liposomes were used for cholesterol loading. All membranes were assayed for protein, cholesterol, and phospholipid phosphorus. Also, the sticking of liposomes to the membranes was corrected for by the inclusion of [¹⁴C]-tripalmitin in the liposomes. All values presented are after the correction for the sticking of liposomes. The changes in cholesterol and phospholipid are as compared to the control (*), which was incubated without liposomes.

compared to the change in cholesterol/protein, which is up to 82.14%. As shown in Table IV, the alteration of the cholesterol content of SPM using LTP was effective with little change in the phospholipid composition of the membranes.

BOVINE CAUDATE MEMBRANES

The analysis of bovine caudate SPM from a typical lipid transfer experiment is shown in Table V. The change in Chol/PL was paralleled by the change in Chol/protein. The PL/protein was slightly altered.

FLUORESCENCE POLARIZATION

RAT WHOLE BRAIN MEMBRANES

The hydrophobic core of the membrane was probed with DPH. When incorporated into SPM, the magnitude of the DPH fluorescence signal was proportional to the amount of SPM lipid present. The polarization (P) value was independent of the lipid and protein concentration. The most reproducible P values were obtained at a membrane protein concentration of 50 μg per 3 ml. Fluorescence polarization and anisotropy (r) values decreased with increasing temperature (Figure 16). A decrease in P or r indicates a decrease in the relative microviscosity (Figure 17). Arrhenius plots of the DPH anisotropy (Figure 16) of cholesterol-depleted and cholesterol-loaded membranes, having Chol/PL of 0.366 and 0.878, respectively, showed no discontinuities

TABLE V
 CHOLESTEROL DEPLETION AND LOADING OF BOVINE BRAIN SPM
 ANALYSIS OF LIPID-MODIFIED SPM

TYPE OF LIPOSOME USED	<u>CHOL</u> <u>PROTEIN</u> <u>nmole</u> mg	<u>PL</u> <u>PROTEIN</u> <u>nmole</u> mg	<u>CHOL</u> <u>PL</u> <u>mole</u> mole	CHANGE IN CHOL <u>nmole</u> mg protein	% CHANGE	CHANGE IN PL <u>nmole</u> mg protein	% CHANGE
PC	386	1031	.375	-166	-30.07	+23	+2.28
PC	430	999	.430	-122	-22.10	-9	-0.89
PC	495	1020	.485	-57	-10.33	+12	+1.19
NONE *	552	1008	.547	0	0	0	0
PC-CHOL	625	1009	.619	+73	+13.22	+1	+0.10
PC-CHOL	713	1023	.697	+161	+29.17	+15	+1.49
PC-CHOL	844	1012	.834	+292	+52.90	+4	+0.40

The results presented are from a typical cholesterol transfer experiment using SPM. Similar results were obtained using synaptosomes. SPM were incubated with liposomes and varying amounts of transfer protein, from 0.1 to 0.4 mg per ml of incubation mixture. PC liposomes were used for cholesterol depletion. PC-CHOL liposomes were used for cholesterol loading. All membranes were assayed for protein, cholesterol, and phospholipid phosphorus. Also, the sticking of liposomes to the membranes was corrected for by the inclusion of [¹⁴C]-tripalmitin in the liposomes. All values presented are after the correction for the sticking of liposomes. The changes in cholesterol and phospholipid are as compared to the control (*), which was incubated without liposomes.

Figure 16. Arrhenius Plots of DPH Anisotropy in Rat
Brain SPM

DPH anisotropy (r) was calculated from polarization (P) values measured at 4°C , 15°C , 25°C , 37°C , and 50°C . Representative plots for cholesterol-depleted (Chol/PL = 0.366, \square), control (Chol/PL = 0.553, \times), and cholesterol-loaded (Chol/PL = 0.878, ∇) membranes are shown.

FIGURE 16

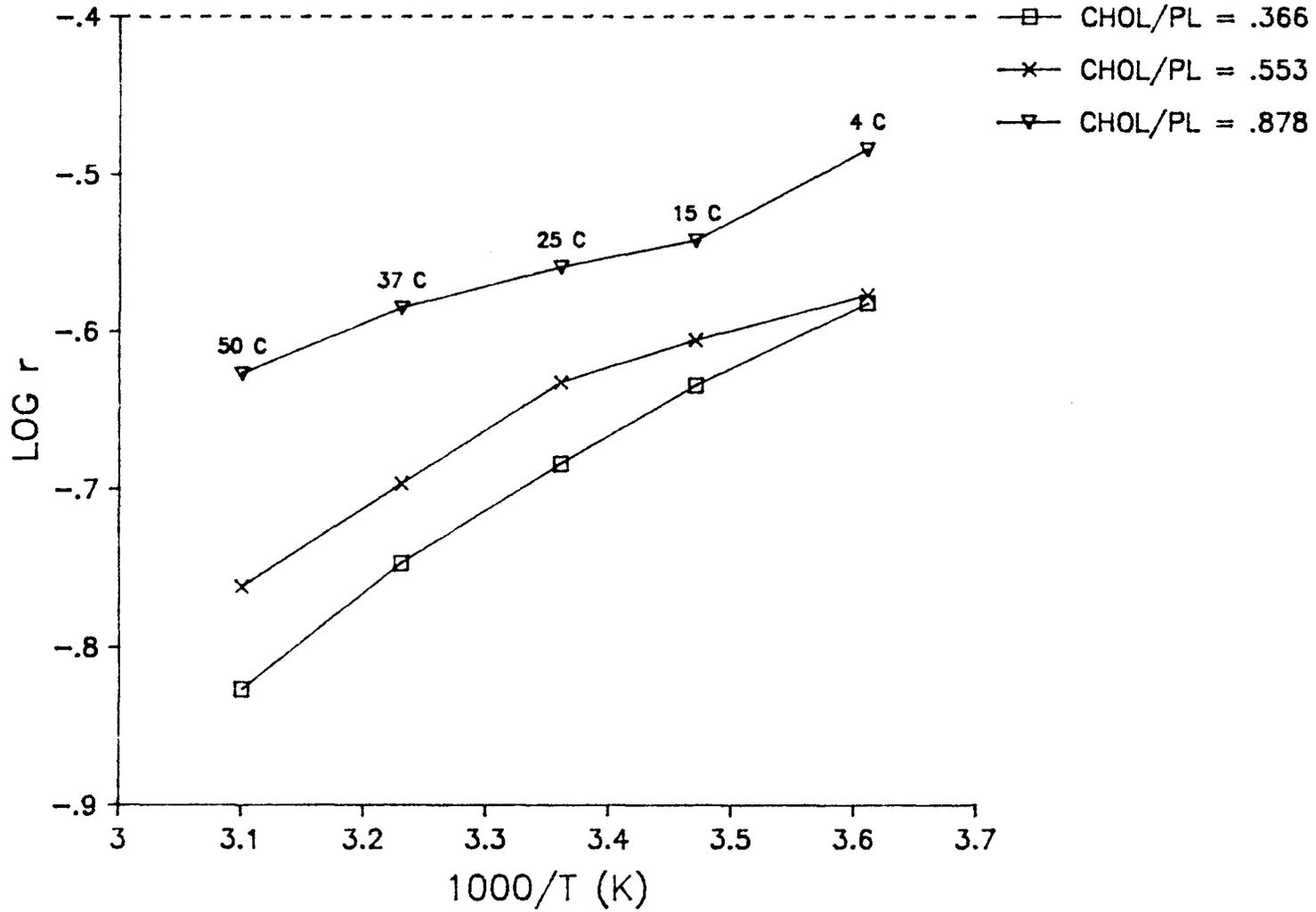
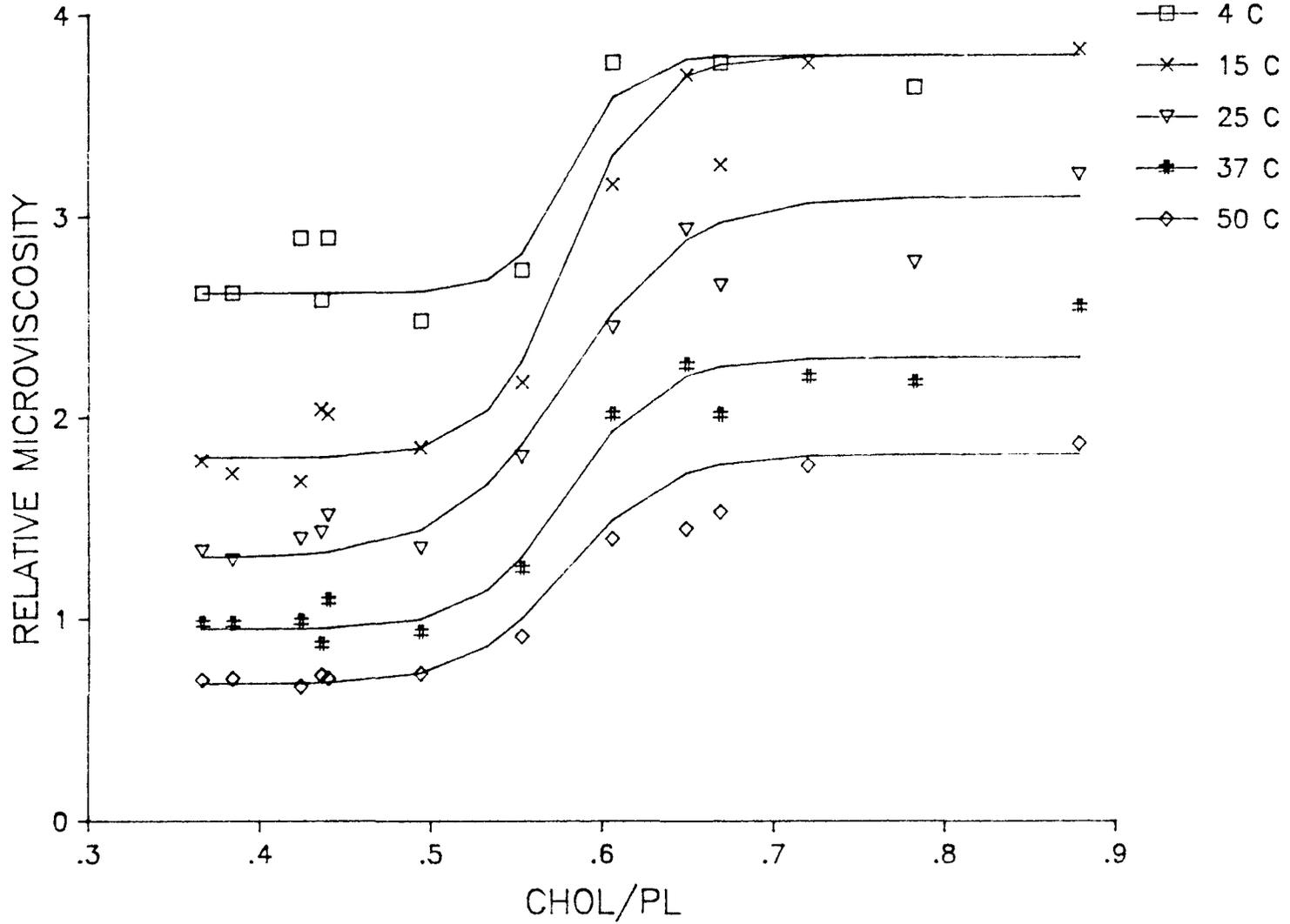


Figure 17. The Relative Microviscosity of the Hydrophobic
Core of Rat Brain SPM as a Function of Chol/PL.

The relative microviscosity was calculated from DPH r values measured at 4°C (\square), 15°C (\times), 25°C (∇), 37°C ($\#$), and 50°C (\diamond). The Chol/PL of control SPM is 0.543 \pm 0.014 ($n = 4$). Chol/PL of 0.35 to 0.706 could be considered physiological. The lines were generated to fit a theoretical sigmoidal curve that approximated the experimental data.

FIGURE 17



suggestive of a sharp phase transition over the temperature range of 4°C to 50°C. In contrast, the Arrhenius plot of the DPH anisotropy of the control membrane was curvilinear with limiting slopes at the low and high temperatures similar to those of the high and low cholesterol-containing membranes respectively.

The relationship between the relative microviscosity of the hydrophobic core of rat whole brain SPM and Chol/PL is shown in Figure 17. The DPH data appeared to fit a sigmoidal relationship, with an inflection point at a Chol/PL of 0.58. In the range of Chol/PL of 0.5 to 0.65, there was an increase in the relative microviscosity at each temperature studied. At Chol/PL above and below this range, the relative microviscosity was relatively independent of Chol/PL. At 37°C, a 10% increase in Chol/PL produced a 75% increase in the relative microviscosity. A further increase in Chol/PL produced a smaller effect. A similar decrease in Chol/PL produced a change in the relative microviscosity of 30%. The greatest change in the relative microviscosity occurred in the range of Chol/PL of 0.5 to 0.6. The Chol/PL of control rat whole brain SPM was 0.543 +/- 0.014 (n = 4).

The membrane surface was probed with ANS. Because ANS binds to proteins as well as to lipids in the membrane, it was important to establish the assay conditions which would optimize the signal from the lipid-bound ANS. At

lower concentrations of ANS, up to 100 μM , the wavelength of the emission maximum was 480 nm. This wavelength is characteristic of ANS bound to proteins (Slavik, 1982). Above 200 μM ANS, the wavelength of the emission maximum was 495 nm. At this concentration, the fluorescence signal would be expected to be due to ANS bound primarily to lipids (Slavik, 1982). This was further verified by the wavelength of the emission maximum of ANS bound to liposomes, 495 nm. A membrane protein concentration of 100 μg per 3 ml was found to produce the most reproducible P values.

As seen with the DPH probe, the anisotropy (r) values decreased with increasing temperature (Figure 18). This indicates an increase in relative microviscosity (Figure 19). Arrhenius plots of ANS anisotropy (Figure 18) of control, cholesterol-depleted, and cholesterol-loaded membranes, having Chol/PL of 0.384, 0.553, and 0.782, respectively, showed a response to temperature similar to that observed with the control membranes using DPH over the temperature range of 4°C to 37°C. Also, the plots for membranes with Chol/PL of 0.553 and 0.782 are nearly superimposable, suggesting that increasing the Chol/PL had little effect on the relative microviscosity at the membrane surface (Figure 19). A small temperature range was studied because as the temperature was increased, the magnitude of the fluorescence signal decreased sharply.

Figure 18. Arrhenius Plots of ANS Anisotropy in Rat
Brain SPM.

ANS anisotropy (r) was calculated from P values measured at 4°C , 25°C , and 37°C . Representative plots for cholesterol-depleted (Chol/PL = 0.384, \square), control (Chol/PL = 0.553, \times), and cholesterol-loaded (Chol/PL = 0.782, ∇) membranes are shown.

FIGURE 18

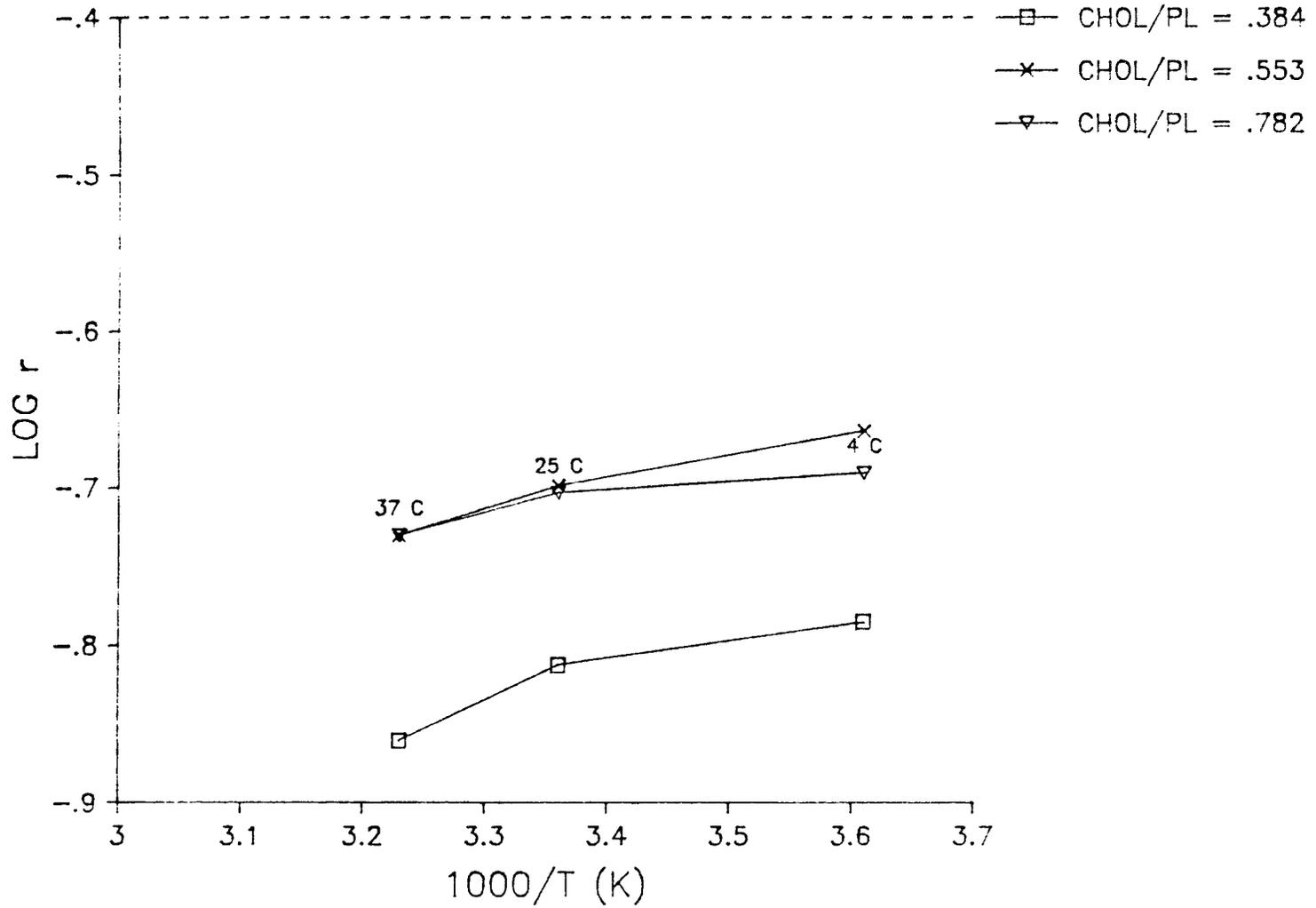
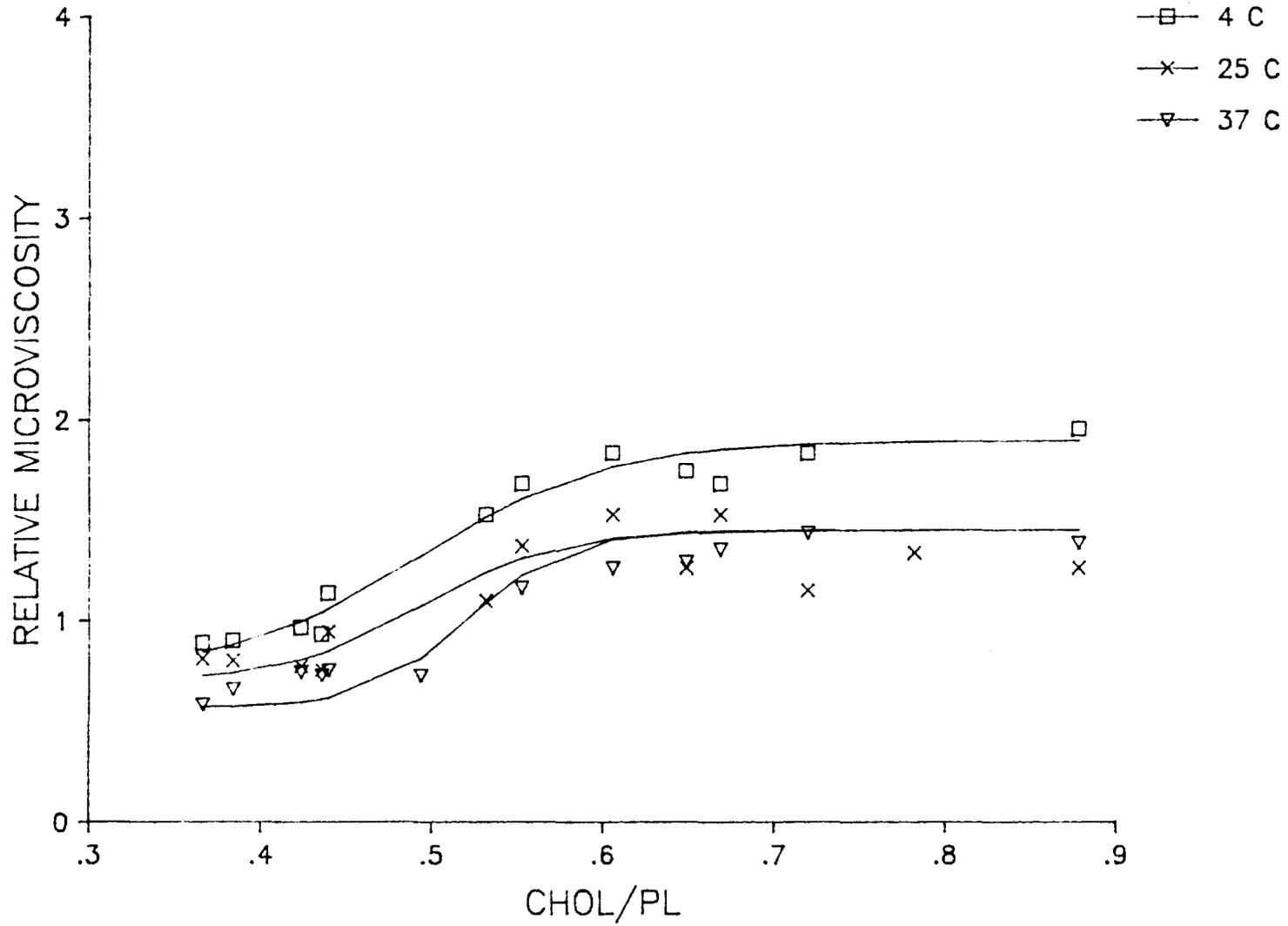


Figure 19. The Relative Microviscosity of the Surface of
Rat Brain SPM as a Function of Chol/PL.

The relative microviscosity was calculated from ANS r values measured at 4°C (\square), 25°C (\times), and 37°C (∇). The Chol/PL of control SPM is 0.543 \pm 0.014 (n = 4). Chol/PL of 0.35 to 0.706 could be considered physiological. The lines were generated to fit a theoretical sigmoidal curve that approximated the experimental data.

FIGURE 19



The relationship between the relative microviscosity at the membrane surface and Chol/PL is shown in Figure 19. The ANS data also appeared to fit a sigmoidal relationship. However, the inflection point was at a Chol/PL of 0.50. In the range of Chol/PL of 0.45 to 0.6, there was an increase in relative microviscosity at each temperature studied. At Chol/PL above and below this range, the relative microviscosity was independent of Chol/PL. At 37°C, the overall change was less dramatic than was seen with DPH. The greatest change occurred in the range of Chol/PL of 0.5 to 0.6. The Chol/PL of control SPM was 0.543 +/- 0.014 (n = 4).

Comparing the hydrophobic core (Figure 17) and the membrane surface (Figure 19), at each Chol/PL, the relative microviscosity of the hydrophobic core of the membrane was greater than that at the surface. However, above a Chol/PL of 0.6, the difference was much greater. The core of the membrane was much more rigid than the surface at high Chol/PL. As seen in Figures 19 and 17, respectively, the membrane surface was more sensitive to decreases in Chol/PL, while the hydrophobic core was more sensitive to increases in Chol/PL.

BOVINE CAUDATE MEMBRANES

Bovine caudate SPM were assayed in the fluorescence studies as described for rat brain SPM. The hydrophobic core of the membrane was probed with DPH. Polariza-

tion (P) and anisotropy (r) values decreased with increasing temperature (Figure 20), indicating a decrease in microviscosity (Figure 21). Arrhenius plots of the DPH anisotropy (Figure 20) of control, cholesterol-depleted, and cholesterol-loaded membranes, having Chol/PL of 0.547, 0.345, and 0.881, respectively, were curvilinear, with the greatest curvature evident at the lowest Chol/PL, suggesting that any change in state of the interior of the bovine caudate SPM would be most evident at lower Chol/PL.

The relationship between the relative microviscosity of the hydrophobic core of bovine caudate SPM and Chol/PL is shown in Figure 21. The bovine SPM did not exhibit the same sigmoidal relationship as did the SPM from rat brain. This may have been due to the higher amounts of cholesterol-rich myelin that were present in the preparations (Table III). As the Chol/PL was decreased from 0.6, there was a decrease in microviscosity at each temperature studied. The greatest decrease occurred at lower temperatures (4°C and 15°C) and smallest at 50°C . Furthermore, at the lower temperatures, the midpoint of the transition in microviscosity occurred at Chol/PL below the physiological, as expected from the Arrhenius plot. At 37°C , increasing the Chol/PL produced only a gradual increase in microviscosity with a maximal change over the range studied of 170%. Furthermore, no dramatic changes in the relative microviscosity are seen in the region of the

Figure 20. Arrhenius Plots of DPH Anisotropy in Bovine
Caudate SPM.

DPH anisotropy (r) was calculated from P values measured at 4°C , 15°C , 25°C , 37°C , and 50°C . Representative plots for cholesterol-depleted ($\text{Chol/PL} = 0.345, \square$), control ($\text{Chol/PL} = 0.547, \times$) and cholesterol-loaded ($\text{Chol/PL} = 0.881, \nabla$) membranes are shown.

FIGURE 20

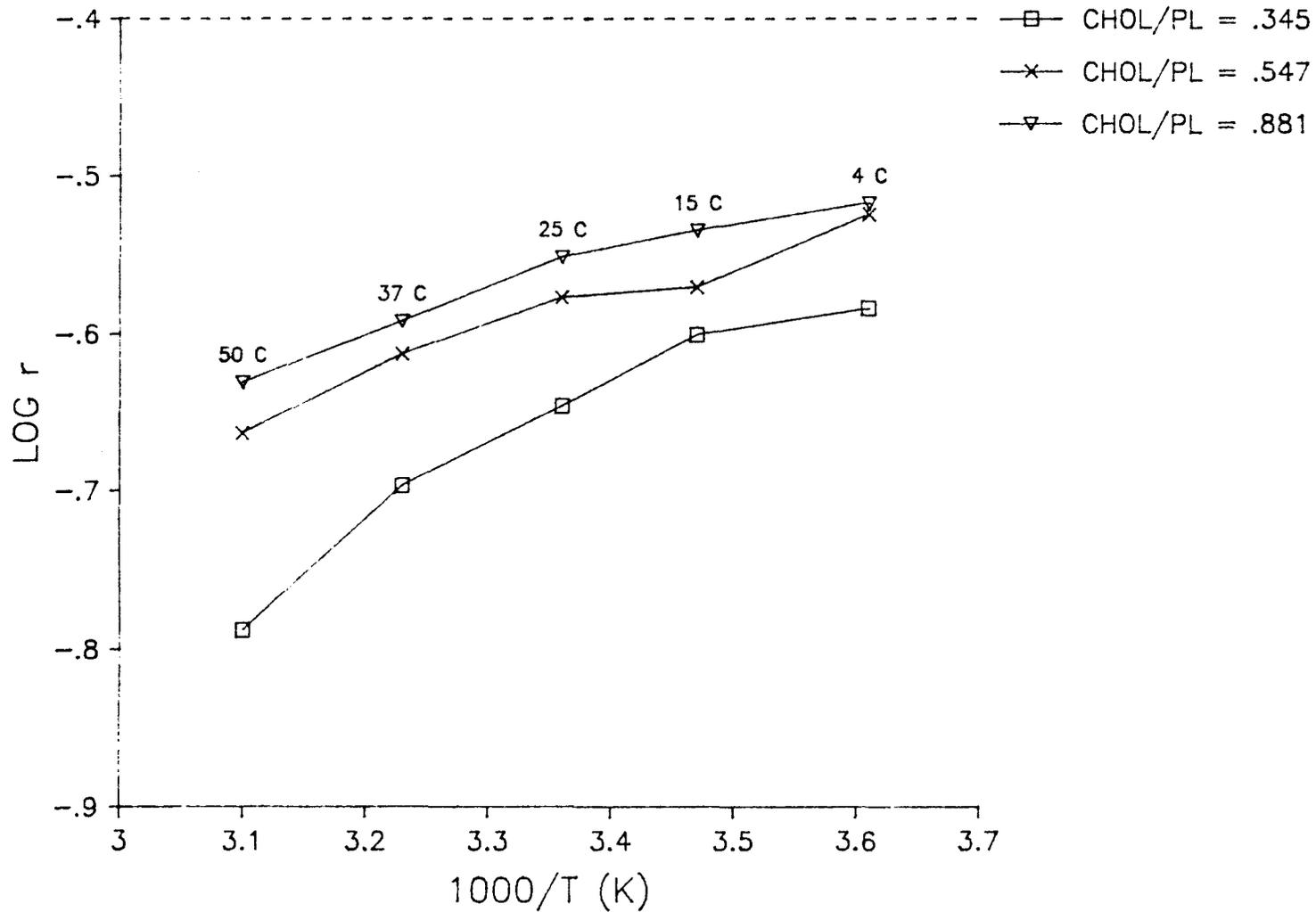
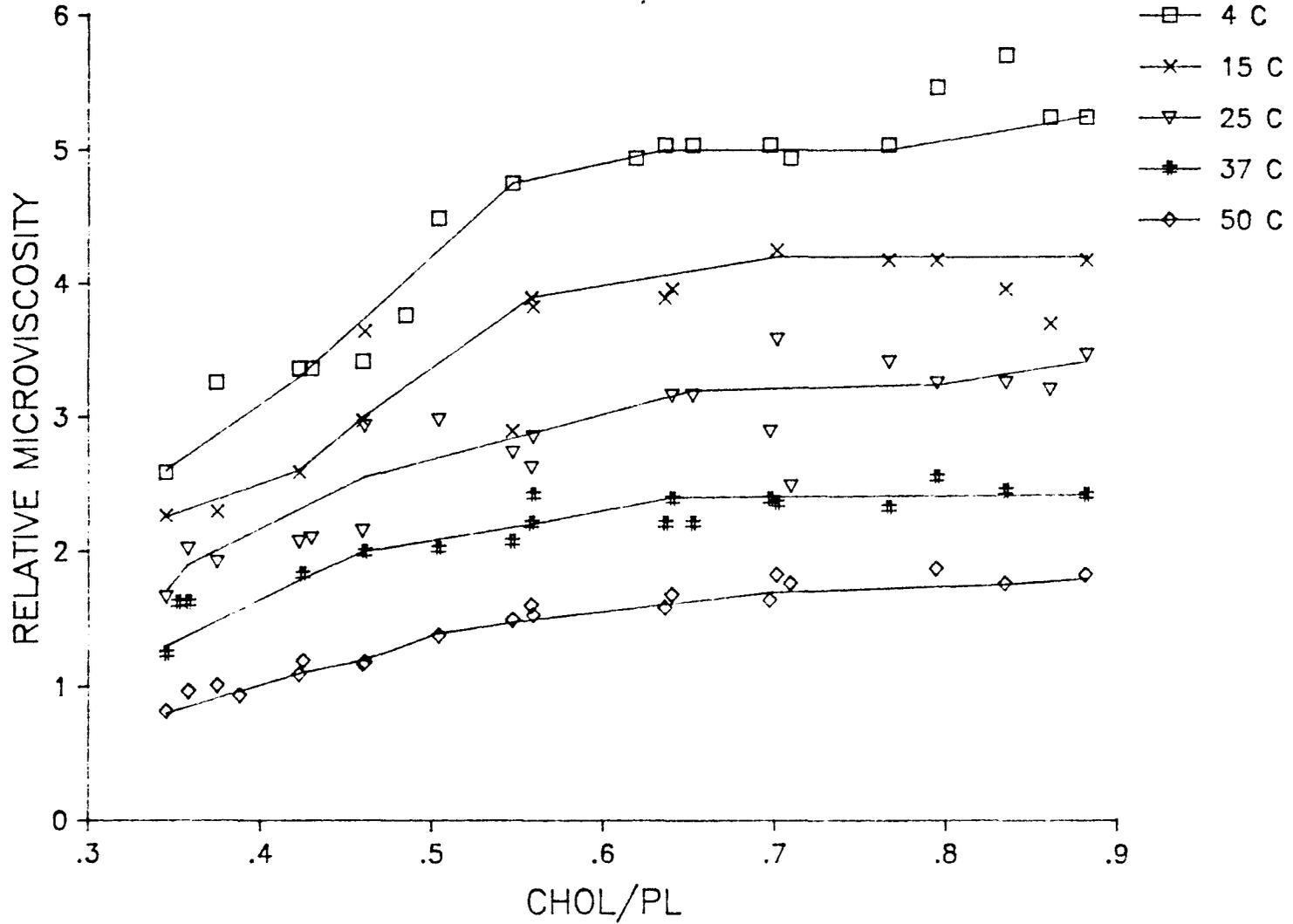


Figure 21. The Relative Microviscosity of the Hydrophobic Core of Bovine Caudate SPM as a Function of Chol/PL.

The relative microviscosity was calculated from DPH r values measured at 4°C (\square), 15°C (\times), 25°C (∇), 37°C ($\#$), and 50°C (\diamond). The Chol/PL of control SPM was 0.542 \pm 0.026 ($n = 4$). Chol/PL of 0.30 to 0.705 could be considered physiological.

FIGURE 21



physiological Chol/PL, which had a value of 0.542 ± 0.026 ($n = 4$).

The membrane surface was probed with ANS. Bovine caudate SPM were assayed under conditions identical to those used to study the rat whole brain SPM. As with DPH, the ANS anisotropy (r) values decreased with increasing temperature (Figure 22), corresponding to a decrease in relative microviscosity (Figure 23). Arrhenius plots of ANS anisotropy (Figure 22) of control, cholesterol-depleted, and cholesterol-loaded SPM, having Chol/PL of 0.504, 0.351, and 0.834, respectively, were similar to those observed for the rat membranes.

The relationship between the relative microviscosity at the membrane surface and Chol/PL is shown in Figure 23. As with DPH, there was a decrease in the relative microviscosity as the Chol/PL decreased from 0.6. At Chol/PL above this point, no further change in microviscosity was detected. At 37°C , increasing the Chol/PL produced only a small increase in the relative microviscosity, up to 20% over the control. However, decreasing the Chol/PL to 0.46 produced a 52% decrease in the relative microviscosity. Again, the midpoint of the change in the surface microviscosity occurred at a Chol/PL below the physiological value of 0.542 ± 0.026 ($n = 4$).

Comparing the hydrophobic core (Figure 21) and the surface (Figure 23) of bovine caudate SPM, at each Chol/PL,

Figure 22. Arrhenius Plots of ANS Anisotropy in Bovine
Caudate SPM.

ANS anisotropy (r) was calculated from P values measured at 4°C , 25°C , and 37°C . Representative plots for cholesterol-depleted (Chol/PL = 0.351, \square), control (Chol/PL = 0.504, \times), and cholesterol-loaded (Chol/PL = 0.834, ∇) membranes are shown.

FIGURE 22

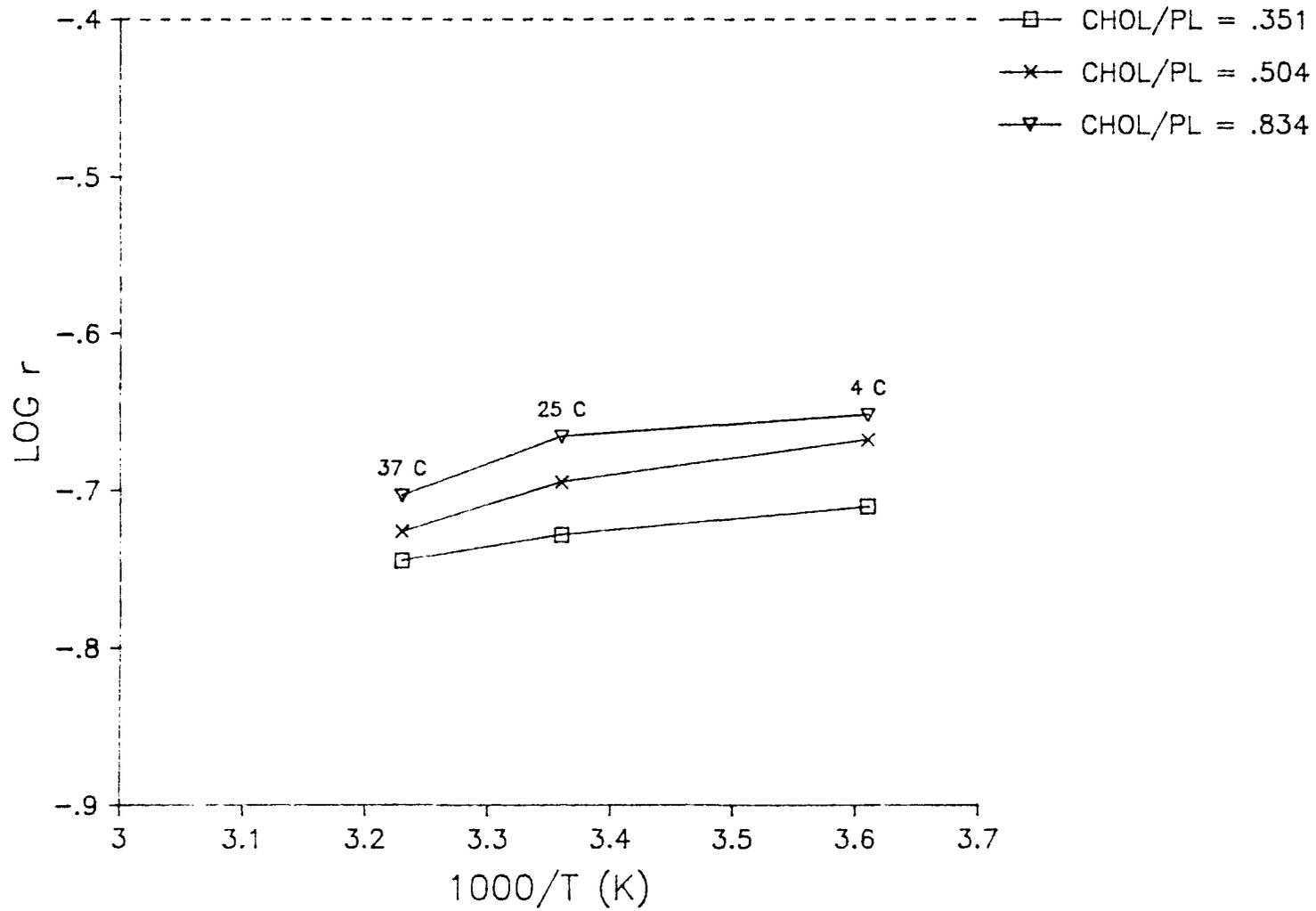
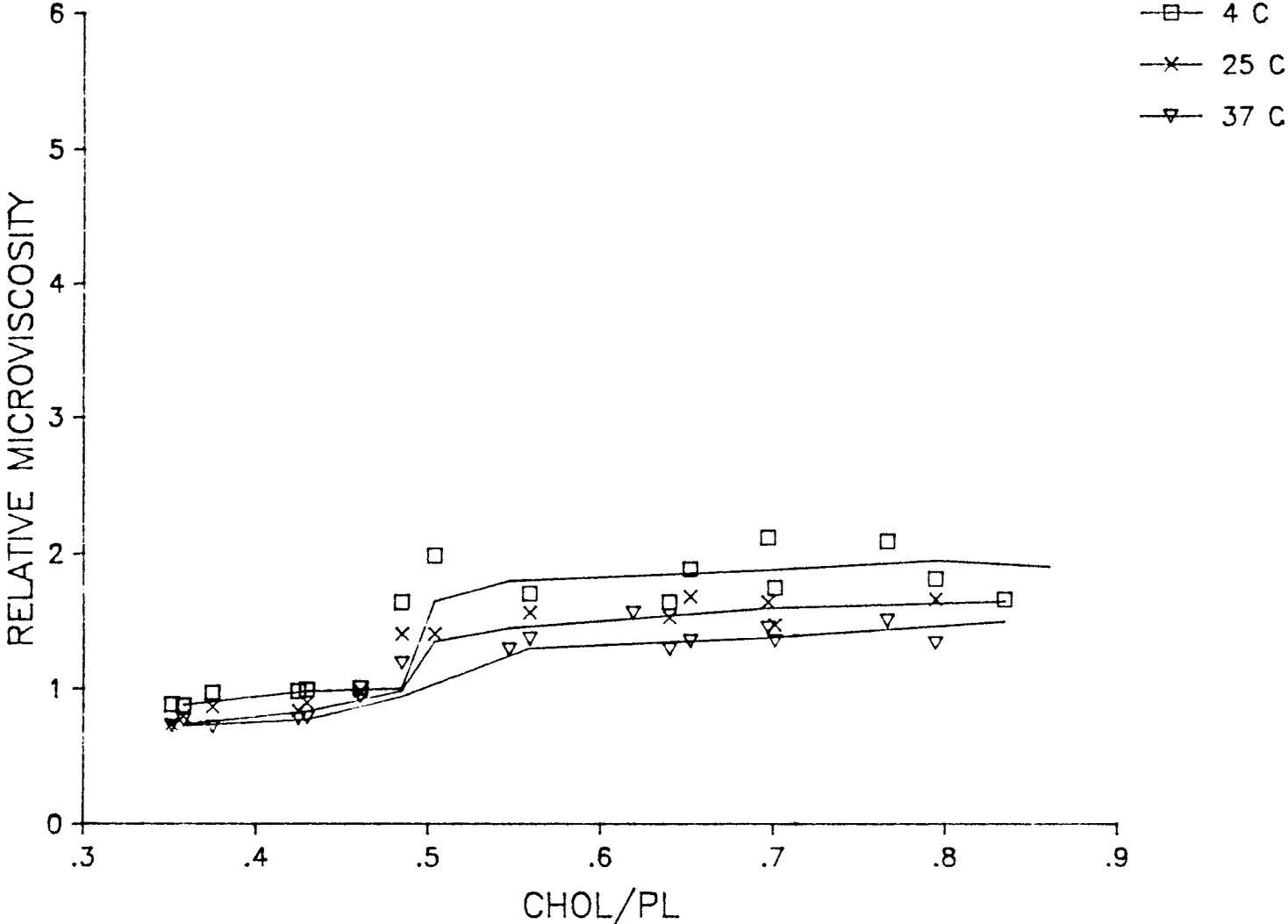


Figure 23. The Relative Microviscosity of the Surface of
Bovine Caudate SPM as a Function of Chol/PL.

The relative microviscosity was calculated from ANS r values measured at 4°C (\square), 25°C (\times), and 37°C (∇). The Chol/PL of control SPM was 0.542 \pm 0.026 (n = 4). Chol/PL of 0.30 to 0.705 could be considered physiological.

FIGURE 23



the relative microviscosity of the hydrophobic core of the membrane was greater than that at the membrane surface. In contrast to rat whole brain SPM (Figures 17 and 19), the surface and hydrophobic core of bovine caudate SPM were affected by the presence of cholesterol in a similar way. Both regions of the membrane were more sensitive to decreases in Chol/PL than to increases in the Chol/PL at values near that of the control (Figures 21 and 23).

DOPAMINE UPTAKE STUDIES USING RAT WHOLE BRAIN MEMBRANES

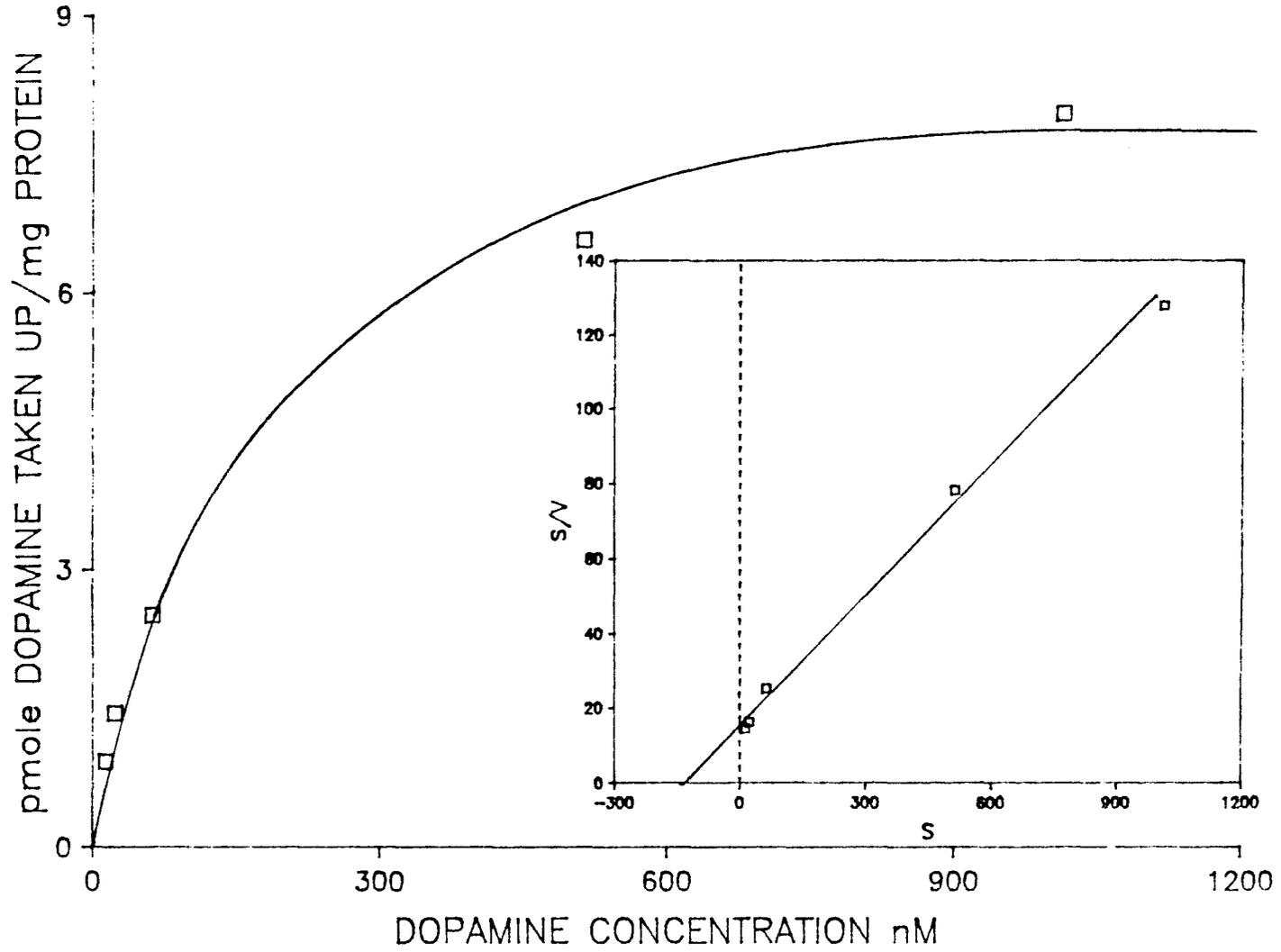
The uptake of [^3H]-dopamine into control, cholesterol-depleted, and cholesterol-loaded rat whole brain synaptosomes was studied. Each experimental set of five samples included synaptosomes which had been incubated with PC liposomes or PC-Chol liposomes, using two concentrations of LTP for each type of liposome. A control was included in each set, which was incubated under standard conditions, except that sucrose buffer was substituted for the liposomes.

A typical assay for control synaptosomes is shown in Figure 24. For control samples, a concentration range of 10-1000 nM dopamine was sufficient to show saturation. Nonspecific uptake, determined in the presence of 10 μM nomifensine, typically accounted for 15-25% of the total radioactivity bound. The K_m was calculated from the X-intercept and the V_{max} from the slope of the Hanes plot (s/v vs. s) (Figure 24, insert). For the typical control

Figure 24. Kinetics of Dopamine Uptake into Control Rat
Brain Synaptosomes.

The uptake of dopamine into control synaptosomes was assayed in triplicate using 10-15 nM [³H]-dopamine and 7 concentrations of unlabelled dopamine ranging from 0-1000 nM . Nonspecific binding, determined in the presence of 10 μM nomifensine, accounted for 15-25% of the total uptake detected. A typical assay is shown. From the Hanes plot, which is the substrate concentration/the observed rate versus the substrate concentration (s/v vs. s) (insert), the control K_m (135.7 nM) and the V_{max} (8.81 pmole/mg protein/5 min assay) were determined. The Chol/PL of control synaptosomes is 0.440 +/- 0.040 (n = 11).

FIGURE 24



assay shown, the K_m was 135.7 nM, and the V_{max} was 8.81 pmole/mg protein/5 minute assay. The Chol/PL of control synaptosomes was 0.440 +/- 0.040 (n = 11).

The effect of cholesterol loading on the K_m for dopamine uptake is illustrated in Figure 25. Each point represents an individual uptake experiment. Only assays with at least 5 data points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. As shown in Figure 25, the K_m began to sharply increase as the Chol/PL was increased above 0.5. Chol/PL above 0.6 are not physiological. But within the physiological range of Chol/PL, the increase in K_m was 3-fold. Decreasing the Chol/PL from the control value produced an approximately 50% decrease in the K_m .

The V_{max} for uptake showed a similar relationship to Chol/PL (Figure 26). Each point represents an individual uptake experiment. Only assays with at least 5 data points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. At Chol/PL above 0.5, there was a sharp increase in the V_{max} , reaching a plateau above Chol/PL of 0.6. Decreasing the Chol/PL produced an approximately 35% decrease in the V_{max} .

The V_{max}/K_m , a first order rate constant for the uptake transporter at low substrate concentrations (Cornish-Bowden, 1979), was calculated for each assay in-

Figure 25. The K_m for Dopamine Uptake as a Function of Chol/PL.

Dopamine uptake was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Each assay consisted of triplicate determinations using 10-15 nM [3 H]-dopamine and 7 concentrations of unlabelled dopamine, ranging from 0-1000 nM for control and cholesterol depleted membranes, and 0-5000 nM for cholesterol-loaded membranes. The K_m was calculated from the x-intercept of the Hanes plot (s/v vs. s). Each point represents an individual uptake assay. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. The Chol/PL of control synaptosomes was 0.440 +/- 0.040 (n = 11). Chol/PL of 0.29 to 0.572 could be considered physiological. The error bar represents the mean +/- standard deviation of K_m values at control Chol/PL.

FIGURE 25

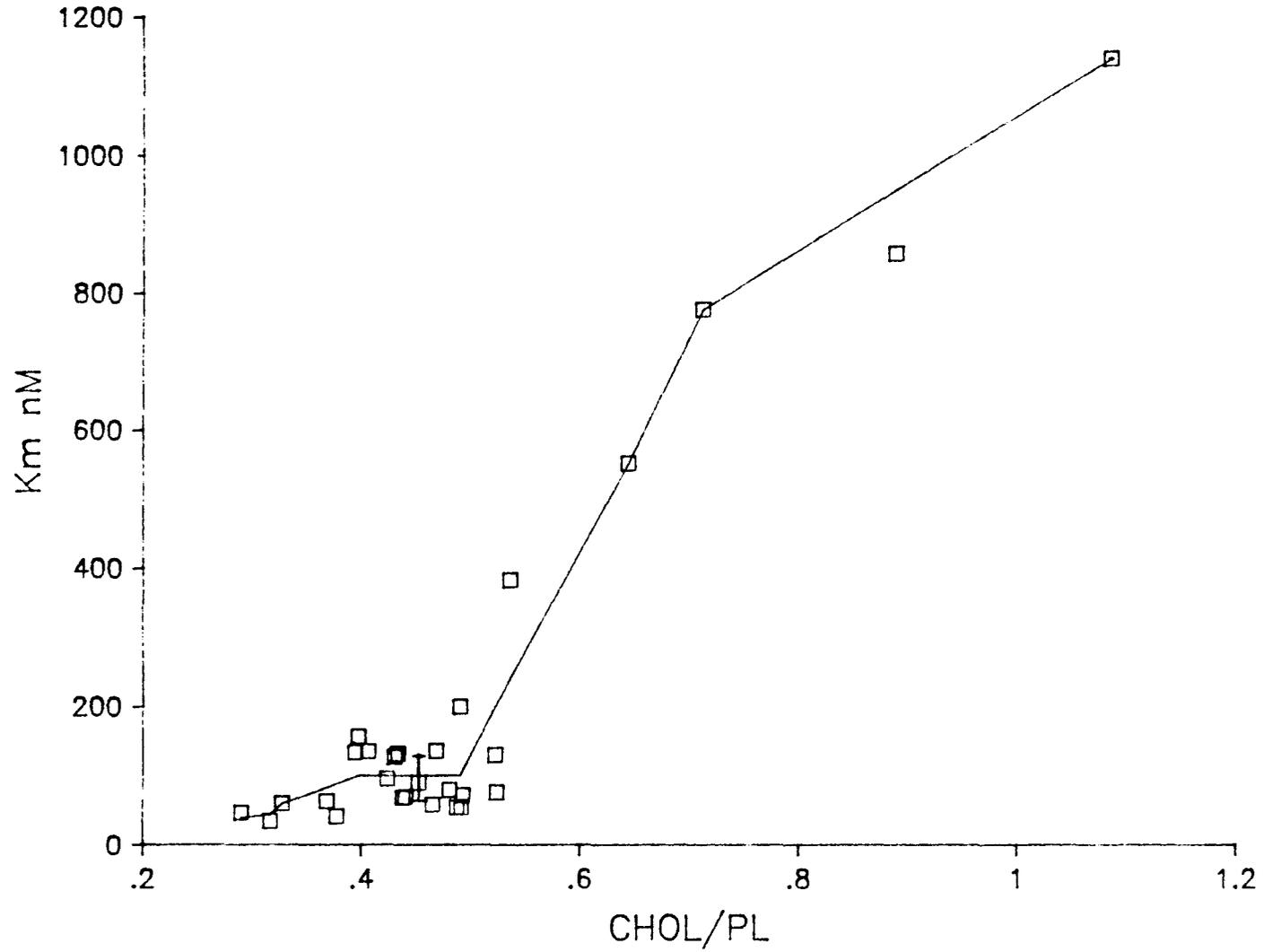
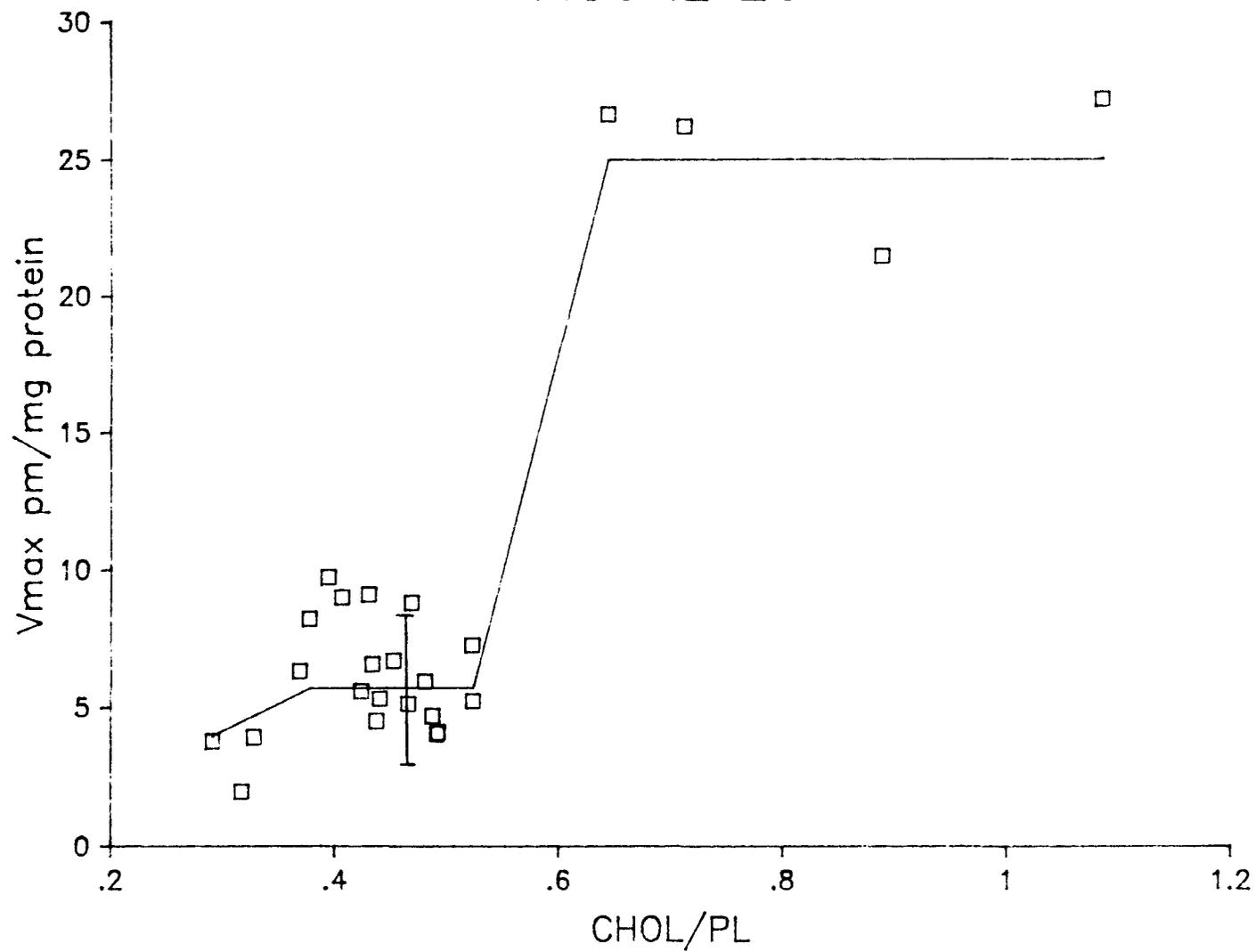


Figure 26. The V_{\max} for Dopamine Uptake as a Function of Chol/PL.

Dopamine uptake was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Each assay consisted of triplicate determinations using 10-15 nM [^3H]-dopamine and 7 concentrations of unlabelled dopamine ranging from 0-1000 nM for control and cholesterol-depleted membranes, and 0-5000 nM for cholesterol-loaded membranes. The V_{\max} was calculated from the slope of the Hanes plot (s/v vs. s). Each point represents an individual uptake assay. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. The Chol/PL of control synaptosomes is 0.440 ± 0.040 (n = 11). Chol/PL of 0.29 to 0.572 could be considered physiological. The error bar represents the mean \pm standard deviation of V_{\max} values at control Chol/PL.

FIGURE 26



cluded in Figures 25 and 26. The relationship between V_{\max}/K_m and Chol/PL is shown in Figure 27. For Chol/PL between 0.5 and 0.7, the V_{\max}/K_m decreased with increasing Chol/PL. At Chol/PL below 0.5 and above 0.7, there was no change in V_{\max}/K_m with changes in Chol/PL.

Because the Chol/PL of control SPM and synaptosomes were different, 0.543 ± 0.014 ($n = 4$) and 0.440 ± 0.040 ($n = 11$), respectively, it may be helpful to use the percent change in Chol/PL to compare the relative microviscosity with the biological effect. The relative microviscosity of the hydrophobic core increased sharply at a Chol/PL 10-15% above the control (Figure 28). As the Chol/PL was increased 30%, which can be considered the upper limit of the physiological range of Chol/PL, the relative microviscosity of the membrane core was about 2-fold greater than control, while that at the membrane surface was increased 20%. Over this same range of Chol/PL, the V_{\max}/K_m decreased 33% (Figure 29). As the Chol/PL was decreased 40%, the relative microviscosity of the membrane core decreased 20% while that at the surface decreased 50%. Little change in the V_{\max}/K_m was observed over this range of Chol/PL.

SUMMARY OF STUDIES WITH RAT WHOLE BRAIN MEMBRANES

Decreasing the Chol/PL produced a decrease in the relative microviscosity at the surface as well as in the hydrophobic core. Because of the sigmoidal relationship

Figure 27. V_{\max}/K_m for Dopamine Uptake as a Function of
Chol/PL

Each point represents the V_{\max}/K_m for dopamine uptake. The K_m and V_{\max} were calculated from the X-intercept and slope, respectively, of the Hanes plot (s/v vs. s). Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. The Chol/PL of control synaptosomes was 0.440 ± 0.040 (n = 11). Chol/PL of 0.29 to 0.572 could be considered physiological. The error bar represents the mean \pm standard deviation of V_{\max}/K_m values at control Chol/PL.

FIGURE 27

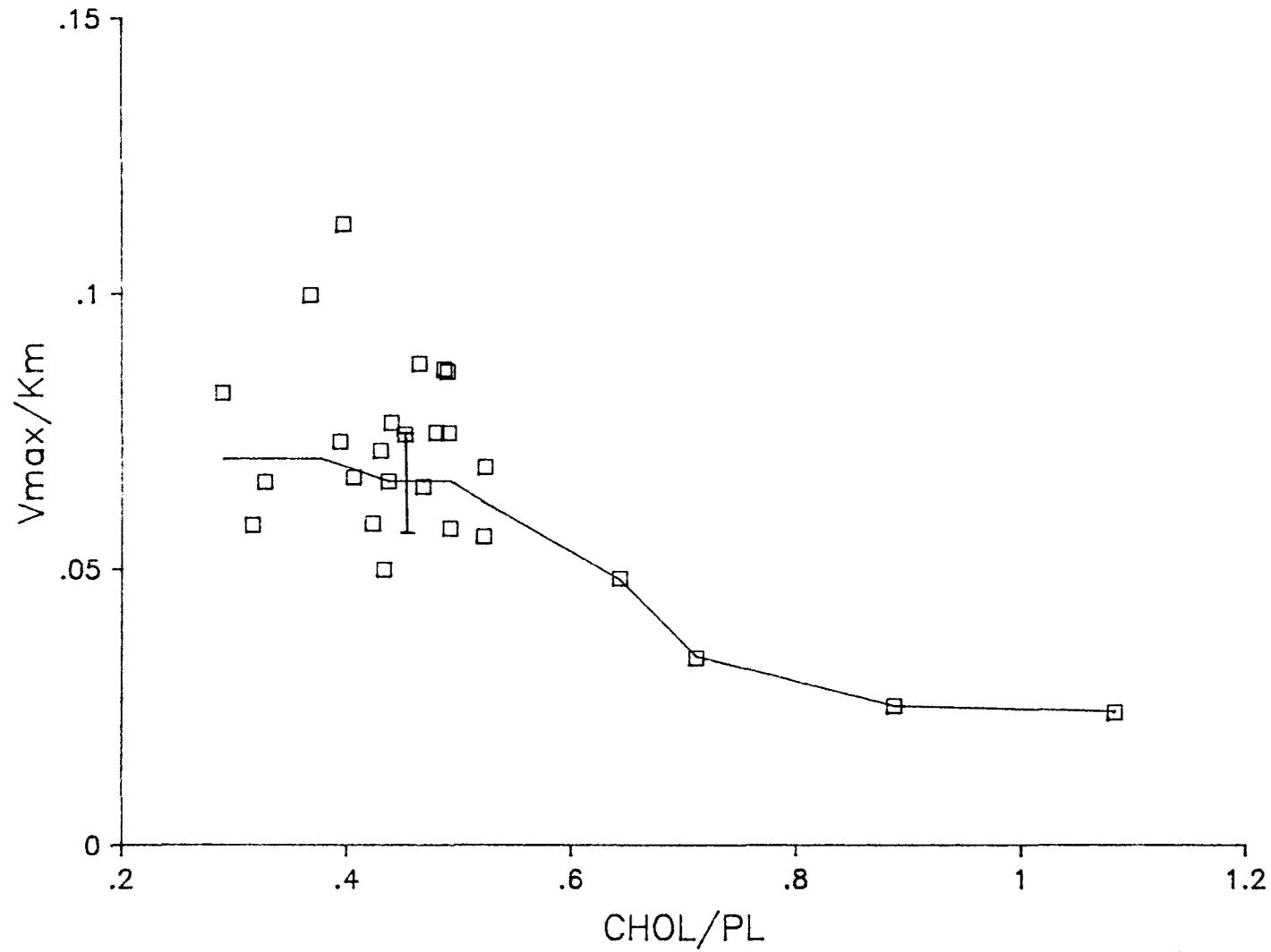


Figure 28. The Relative Microviscosity of the Membrane Surface and Hydrophobic Core as a Function of the Percent Change in Chol/PL.

The relative microviscosity of the membrane surface (ANS, \square) and core (DPH, \times) were calculated from r values determined at 37°C. The Chol/PL of control SPM was 0.543 \pm 0.014 ($n = 4$). A change in Chol/PL of -50% to 30% could be considered physiological. The lines were generated to fit a theoretical sigmoidal curve that approximated the experimental data.

Figure 29. V_{\max}/K_m for Dopamine Uptake as a Function of the Percent Change in Chol/PL.

Each point represents the V_{\max}/K_m for an individual dopamine uptake assay. The K_m and V_{\max} were calculated from the X-intercept and slope, respectively, of the Hanes plot (s/v vs. s). Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Hanes plot were included. The Chol/PL of control synaptosomes was 0.440 \pm 0.040 ($n = 11$). A change in Chol/PL of -50% to 30% could be considered physiological.

FIGURE 28

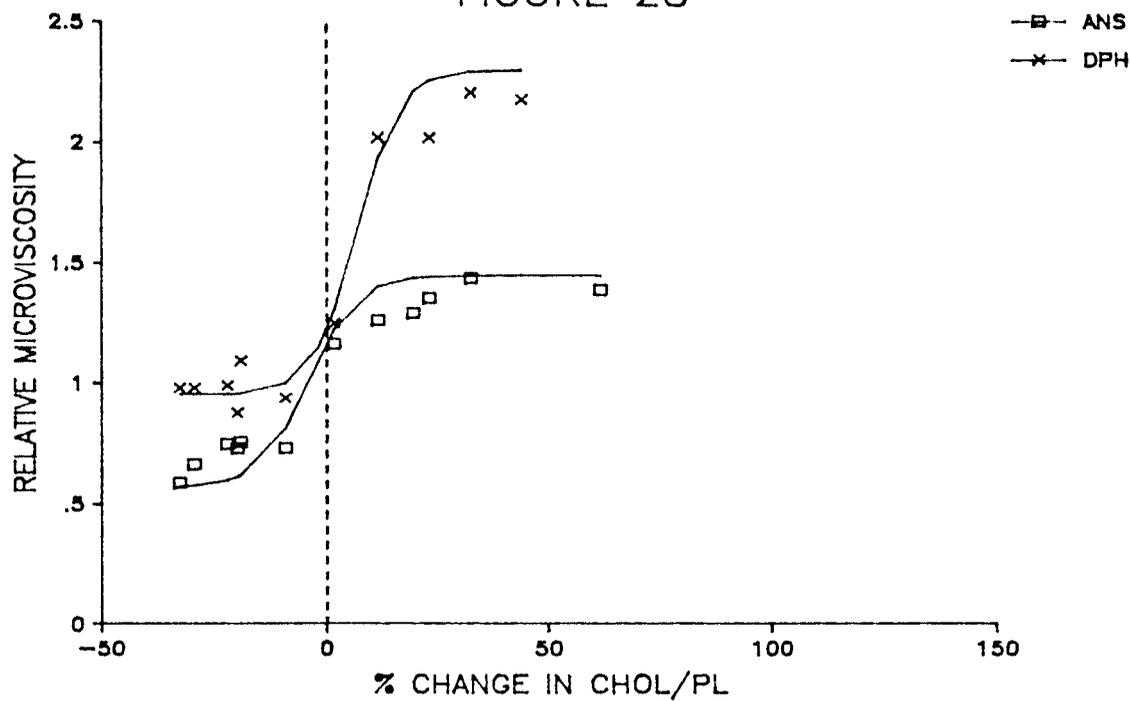
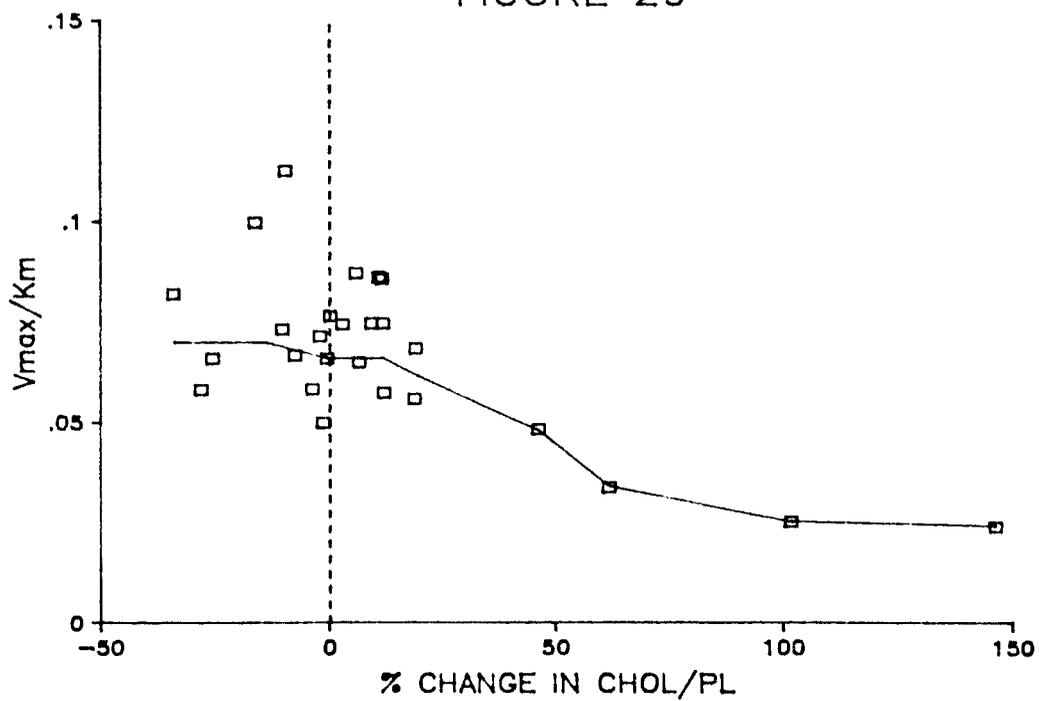


FIGURE 29



observed, the range of Chol/PL that correlated with the greatest change in relative microviscosity was near the physiological Chol/PL. Decreases in Chol/PL that were outside the physiological range produced little additional effect on the relative microviscosity. An increase in Chol/PL produced an increase in the relative microviscosity of both regions of the membrane, although the magnitude of the increase was greater in the hydrophobic core than at the membrane surface (Figure 28).

The K_m for dopamine uptake began to increase sharply as the Chol/PL was increased, producing a 3-fold increase within the physiological range of Chol/PL. At higher, nonphysiological Chol/PL, the increase was 8- to 10-fold. Decreasing the Chol/PL, within the physiological range, produced a 50% decrease in the K_m (Figure 25). The V_{max} showed a similar relationship to Chol/PL (Figure 26), increasing 3.5-fold as the Chol/PL was increased and decreasing 35% as the Chol/PL was decreased. The V_{max}/K_m was unaffected as the Chol/PL was decreased, but decreasee about 33% as the Chol/PL was increased (Figure 27). At Chol/PL above 0.8, no additional effect was observed in K_m , V_{max} , or V_{max}/K_m .

DOPAMINE (D₁) BINDING STUDIES USING
BOVINE CAUDATE MEMBRANES

The binding of [³H]-SCH23390 to control, cholesterol-depleted, and cholesterol-loaded bovine caudate synaptosomes was studied. Each experimental set of seven samples included membranes incubated with PC liposomes or PC-Chol liposomes, using three concentrations of LTP for each type of liposome. A control, incubated with sucrose buffer instead of liposomes, was included with each set.

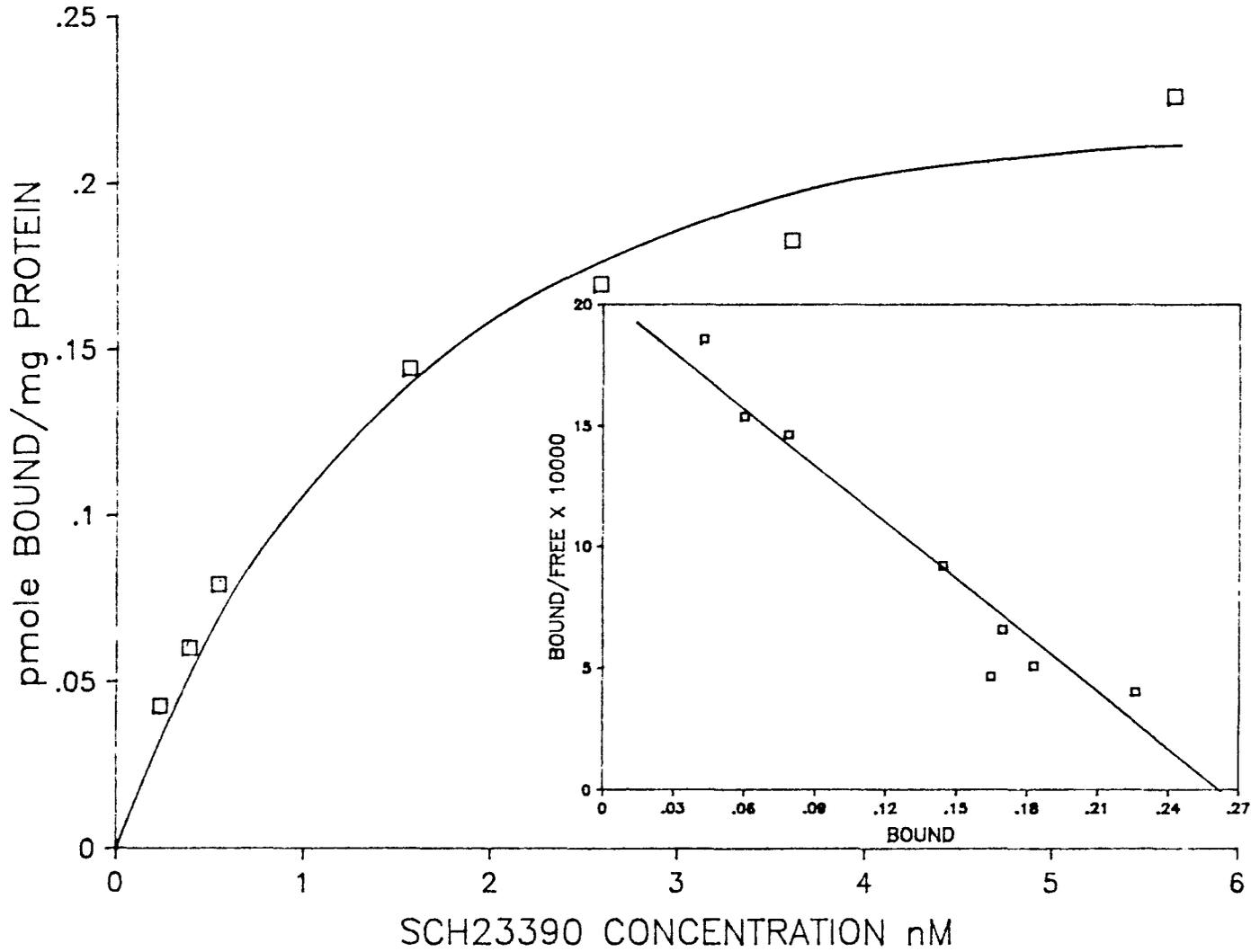
Before beginning the binding studies, the SCH-23390 binding assay was optimized in this laboratory. Because preliminary experiments found the K_d to be in the range of 1 nM, a concentration range of 0.2 to 5.1 nM was chosen for the kinetic assays. The optimal membrane protein concentration was determined to be 300-400 ug protein per 3 ml assay volume.

A typical binding assay, using control membranes, is shown in Figure 30. For control assays, a concentration range of 0.2 to 5.1 nM SCH23390 was sufficient to show saturation (Figure 30). Nonspecific binding, determined in the presence of 1 μM (+)-butaclamol, typically accounted for 10-15% of the total radioactivity bound. The K_d was calculated from the slope, and the B_{max} from the X-intercept of the Scatchard plot (bound/free vs. bound) (Figure 30, insert). For the typical control assay shown, the K_d was 1.242 nM and the B_{max} was 0.261 pmole/mg protein.

Figure 30. Kinetics of SCH23390 Binding to Control Bovine
Caudate Synaptosomes.

The binding of SCH23390 to control synaptosomes was assayed in duplicate using 0.2-0.5 nM of [³H]-SCH23390 and 7 concentrations of unlabelled SCH23390 ranging from 0-5.1 nM. Nonspecific binding, determined in the presence of 1 μM (+)-butaclamol, accounted for 10-15% of the total binding. From the Scatchard plot, bound/free vs. bound (insert), the control K_D (1.242 nM) and the B_{max} (0.261 pmole/mg protein) were determined. The Chol/PL of control synaptosomes was 0.556 +/- 0.013 (n = 8).

FIGURE 30



The effect of altering the membrane Chol/PL on the K_d of SCH23390 binding is illustrated in Figure 31. Each point represents an individual binding experiment. Only assays with at least 5 data points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. There appeared to be a trend toward a decreased K_d as the Chol/PL was increased. The change in K_d over the entire range of Chol/PL appeared to be about 3-fold.

The B_{max} for SCH23390 binding, as a function of Chol/PL, is shown in Figure 32. Each point represents an individual binding experiment. Only assays with at least 5 data points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. The B_{max} appeared to be independent of Chol/PL.

The B_{max}/K_d was calculated for each assay included in Figures 31 and 32. Its relationship to Chol/PL is illustrated in Figure 33. Over the entire range of Chol/PL, there appeared to be a trend toward an increasing B_{max}/K_d as the Chol/PL was increased. The magnitude of this change was about 2-fold.

Although the Chol/PL of control SPM and synaptosomes were not statistically different, 0.542 ± 0.026 ($n = 4$) and 0.556 ± 0.013 ($n = 8$), respectively, it may be helpful to use the percent change in Chol/PL to compare the relative microviscosity and the biological effect, in this

Figure 31. The K_d for SCH23390 Binding to Bovine Caudate Synaptosomes as a Function of Chol/PL.

SCH23390 binding was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Each assay consisted of duplicate determinations using 0.2-0.5 nM [^3H]-SCH23390 and 7 concentrations of unlabelled SCH23390 ranging from 0-5.1 nM. The K_d was calculated from the slope of the Scatchard plot. Each point represents an individual binding assay. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. The Chol/PL of control synaptosomes was 0.556 \pm 0.013 (n = 8). Chol/PL of 0.30 to 0.723 could be considered physiological. The error bar represents the mean \pm standard deviation of K_d values at control Chol/PL.

FIGURE 31

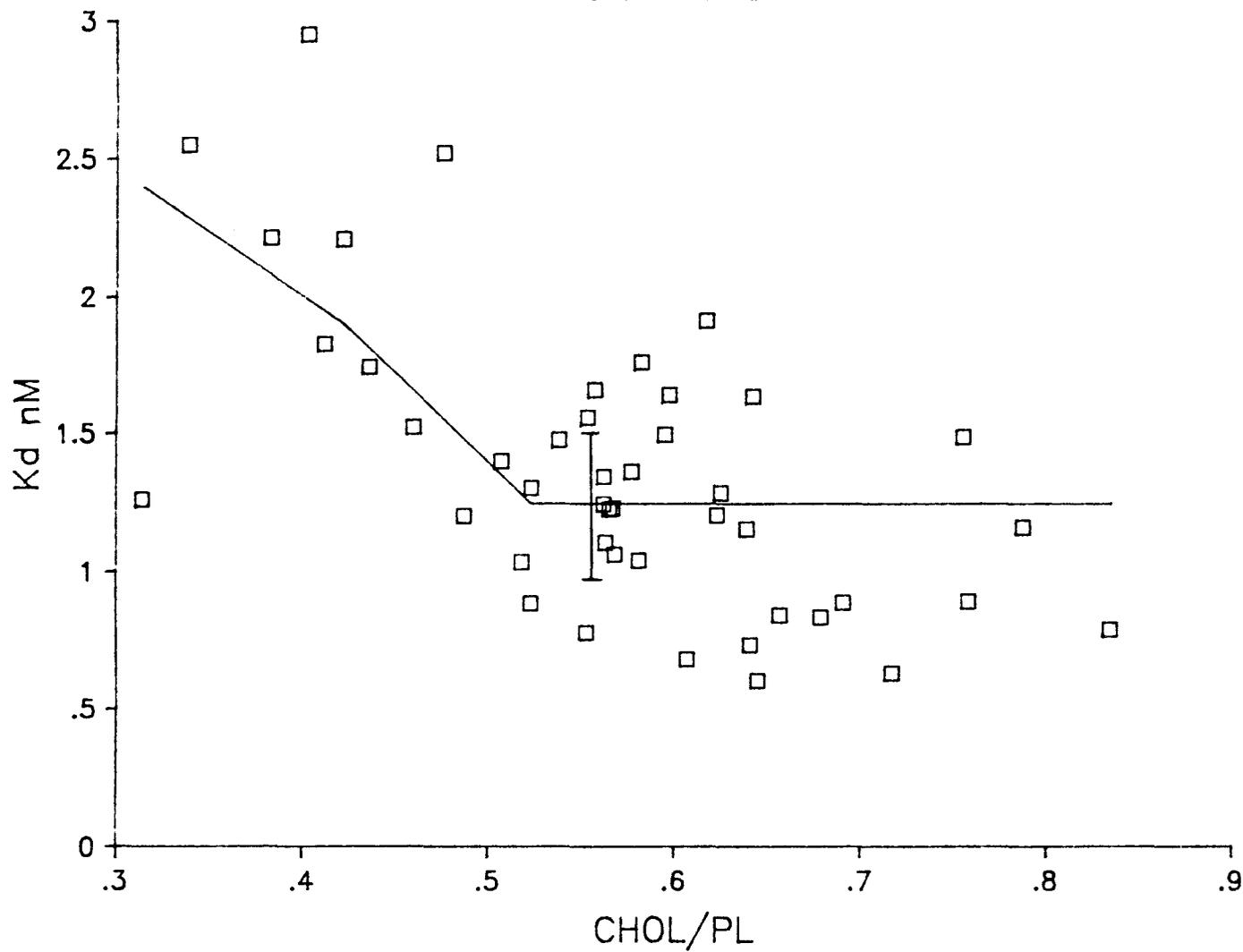


Figure 32. The B_{\max} for SCH23390 Binding to Bovine
Caudate Synaptosomes as a Function of Chol/PL.

SCH23390 binding was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Each assay consisted of duplicate determinations using 0.2-0.5 nM [^3H]-SCH23390 and 7 concentrations of unlabelled SCH23390 ranging from 0-5.1 nM. The B_{\max} was calculated from the X-intercept of the Scatchard plot. Each point represents an individual binding assay. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. The Chol/PL of control synaptosomes was 0.556 \pm 0.013 (n = 8). Chol/PL of 0.30 to 0.723 could be considered physiological. The error bar represents the mean \pm standard deviation of B_{\max} values at control Chol/PL.

FIGURE 32

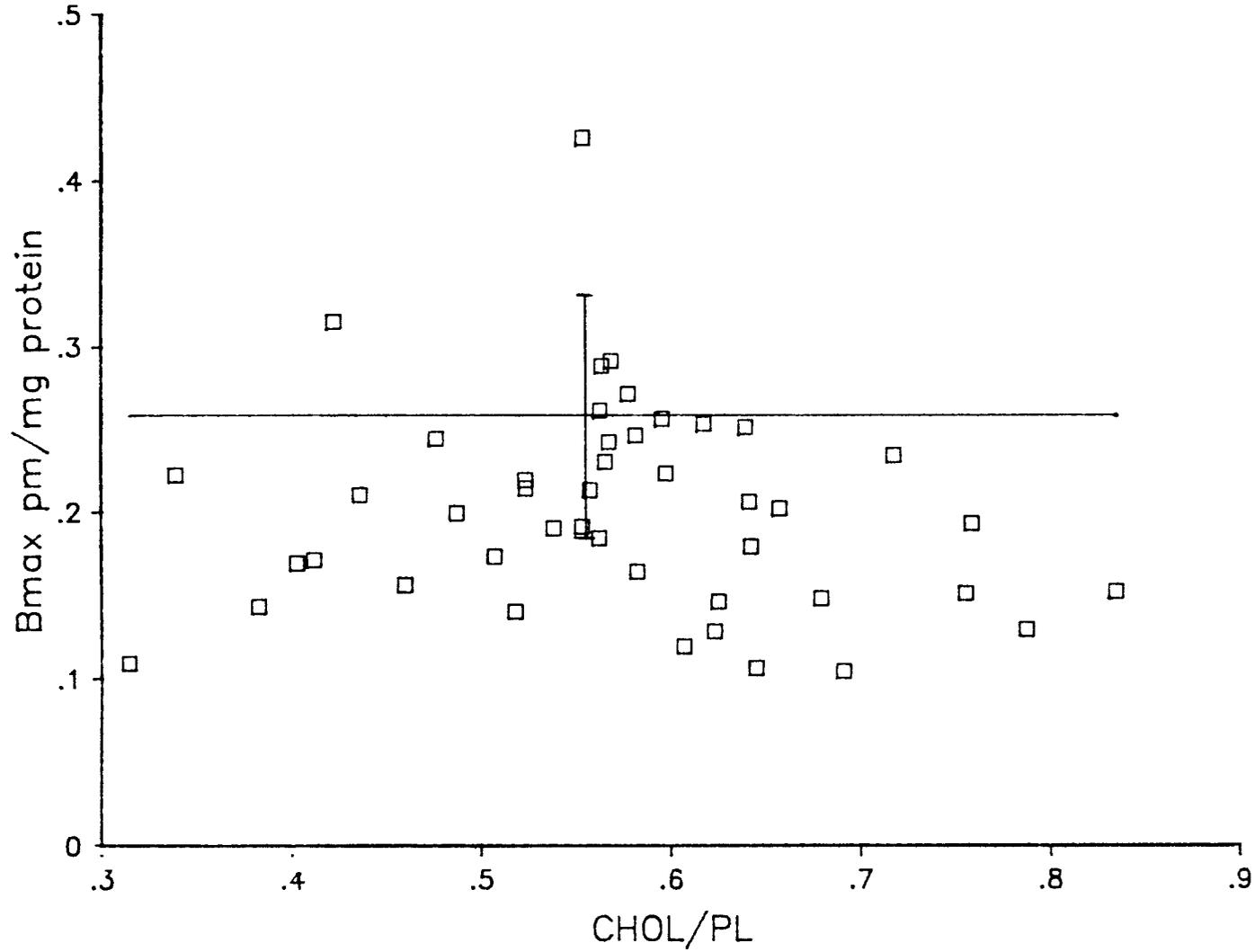
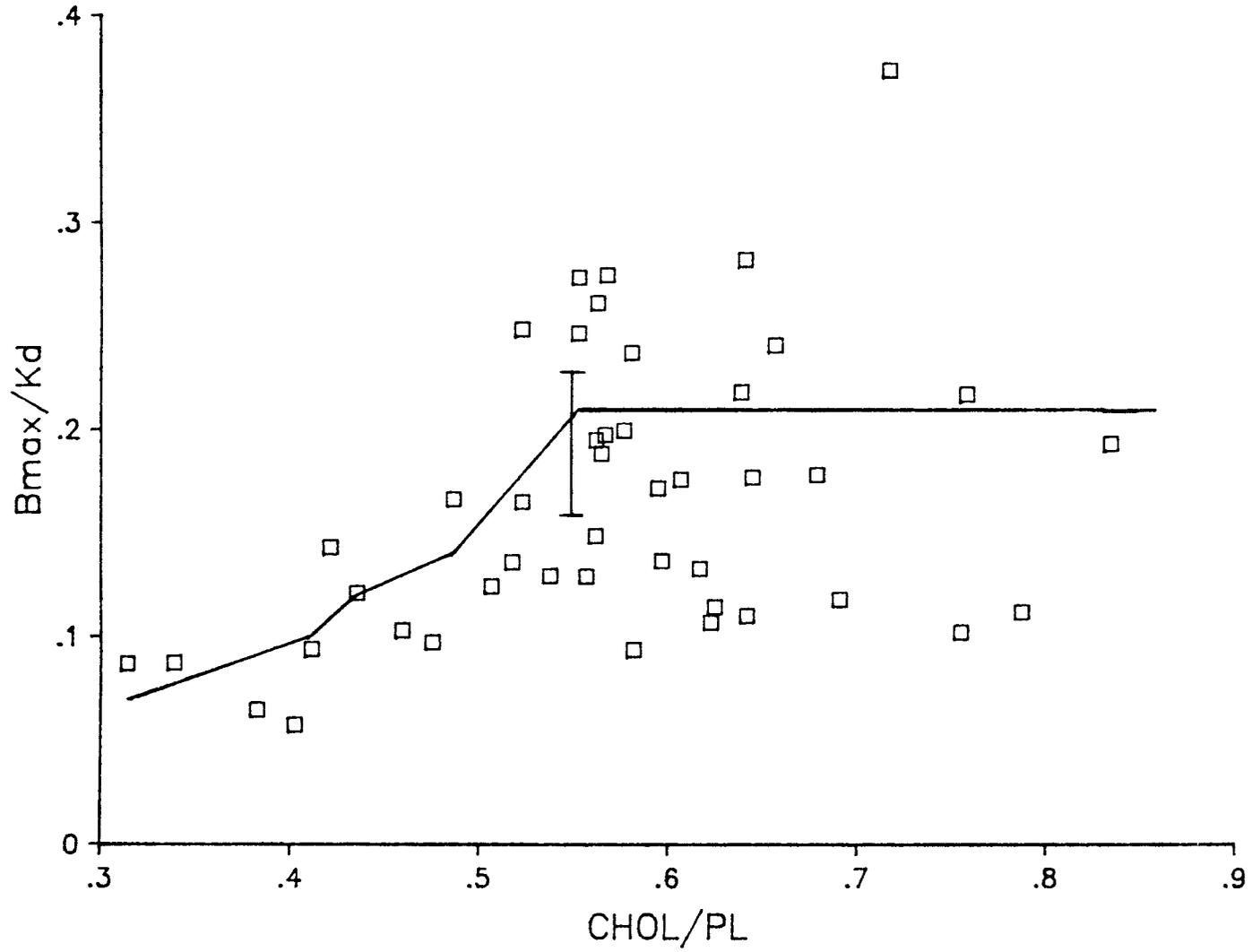


Figure 33. B_{\max}/K_d for SCH23390 Binding to Bovine

Caudate Synaptosomes as a Function of Chol/PL.

Each point represents the B_{\max}/K_d from an individual binding assay. The K_d and B_{\max} were calculated from the slope and X-intercept, respectively, of the Scatchard plot. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. The Chol/PL of control synaptosomes was 0.556 ± 0.013 ($n = 8$). Chol/PL of 0.30 to 0.723 could be considered physiological. The error bar represents the mean \pm standard deviation of B_{\max}/K_d values at control Chol/PL.

FIGURE 33



case the B_{\max}/K_d for SCH23390 binding. As the Chol/PL of the SPM was decreased 30%, there was a decrease in the relative microviscosity at both the membrane surface and in the core of the membrane (Figure 34). This was accompanied by a trend toward a decreased B_{\max}/K_d (Figure 35). As the Chol/PL was increased 30%, there was little change in the relative microviscosity of either region of the membrane (Figure 34). Also, there was little effect observed in B_{\max}/K_d (Figure 35).

DOPAMINE-SENSITIVE ADENYLATE CYCLASE USING BOVINE CAUDATE MEMBRANES

Additional aliquots of the membranes that were used to assess the binding of [^3H]-SCH23390 were assayed for dopamine stimulation of adenylylase activity. Four experimental sets, each including a control, three cholesterol-depleted, and three cholesterol-loaded membranes, were assayed. For control membranes, the basal activity was 120.35 ± 6.45 ($n = 4$) fmole cAMP formed per mg protein per 5 minute assay. The activity stimulated by $100 \mu\text{M}$ dopamine was 228.57 ± 4.58 ($n = 4$) fmole cAMP/mg protein/5 min.

The basal activity was not strongly affected by changes in the Chol/PL (Figure 36). The dopamine-stimulated activity was not affected when the Chol/PL was decreased (control Chol/PL = 0.556 ± 0.013 , $n = 8$). However, increasing the Chol/PL produced a decline in the

Figure 34. The Relative Microviscosity of the Surface and Hydrophobic Core of Bovine Caudate SPM as a Function of the Percent Change in Chol/PL.

The relative microviscosity of the membrane surface (ANS, \square) and core (DPH, \times) were calculated from r values determined at 37°C. The Chol/PL for control SPM was 0.542 +/- 0.026 (n = 4). A change in Chol/PL of -50% to 30% could be considered physiological.

Figure 35. B_{\max}/K_d for SCH23390 Binding to Bovine Caudate Synaptosomes as a Function of the Percent Change in Chol/PL

Each point represents the B_{\max}/K_d for an individual binding experiment. The K_d and B_{\max} were calculated from the slope and X-intercept, respectively, of the Scatchard plot. Only assays with at least 5 concentration points and a correlation coefficient of 0.9 or better for the linear fit of the Scatchard plot were included. The Chol/PL for control synaptosomes was 0.556 +/- 0.013 (n = 8). A change in Chol/PL of -50% to 30% could be considered physiological.

FIGURE 34

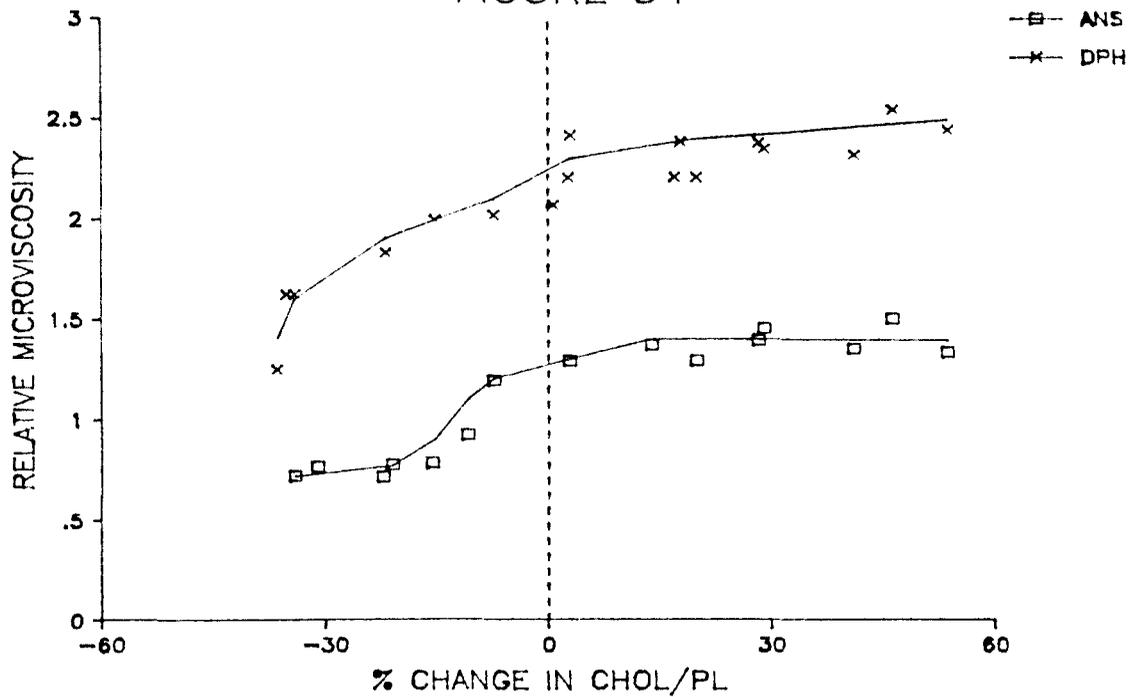


FIGURE 35

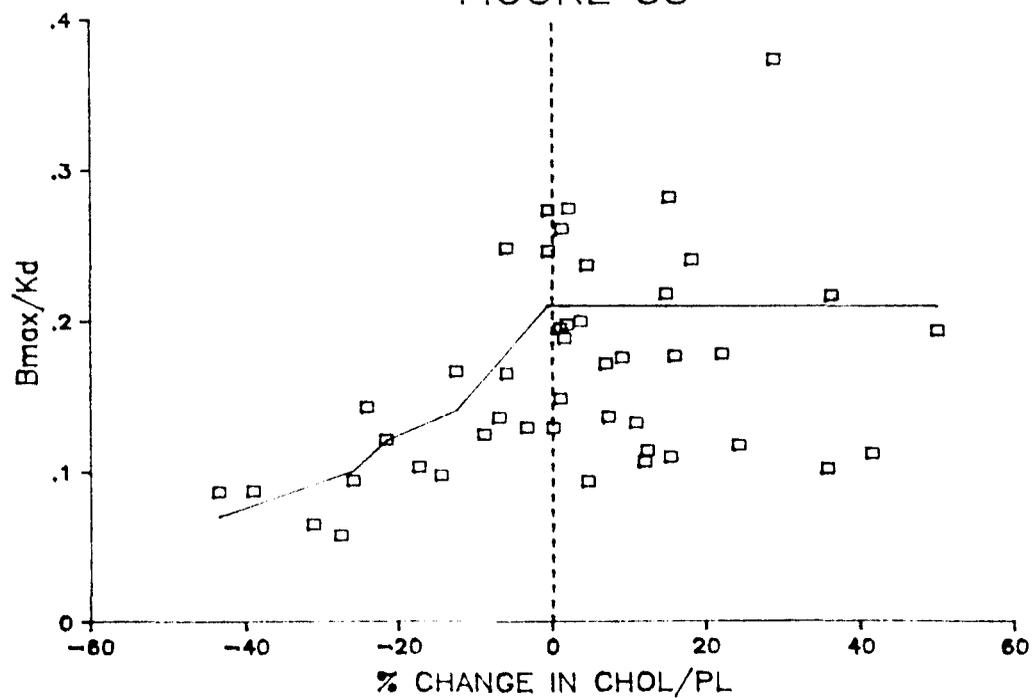


Figure 36. Adenylate Cyclase Activity of Bovine Caudate Synaptosomes as a Function of Chol/PL

Adenylate cyclase activity was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Cyclic AMP was detected by radioimmunoassay. The stimulated activity was assayed in the presence of 100 μ M dopamine. Each point represents the mean and standard deviation of 4 determinations. The Chol/PL of control synaptosomes was 0.556 \pm 0.013 (n = 8). Chol/PL of 0.30 to 0.723 could be considered physiological.

Figure 37. Percent Stimulation of Adenylate Cyclase Activity of Bovine Caudate Synaptosomes by 100 μ M Dopamine as a Function of Chol/PL.

Adenylate cyclase was assayed as described in Figure 36. The % stimulation is over the basal activity. The Chol/PL of control synaptosomes was 0.556 \pm 0.013 (n = 8). Chol/PL of 0.30 to 0.723 could be considered physiological.

FIGURE 36

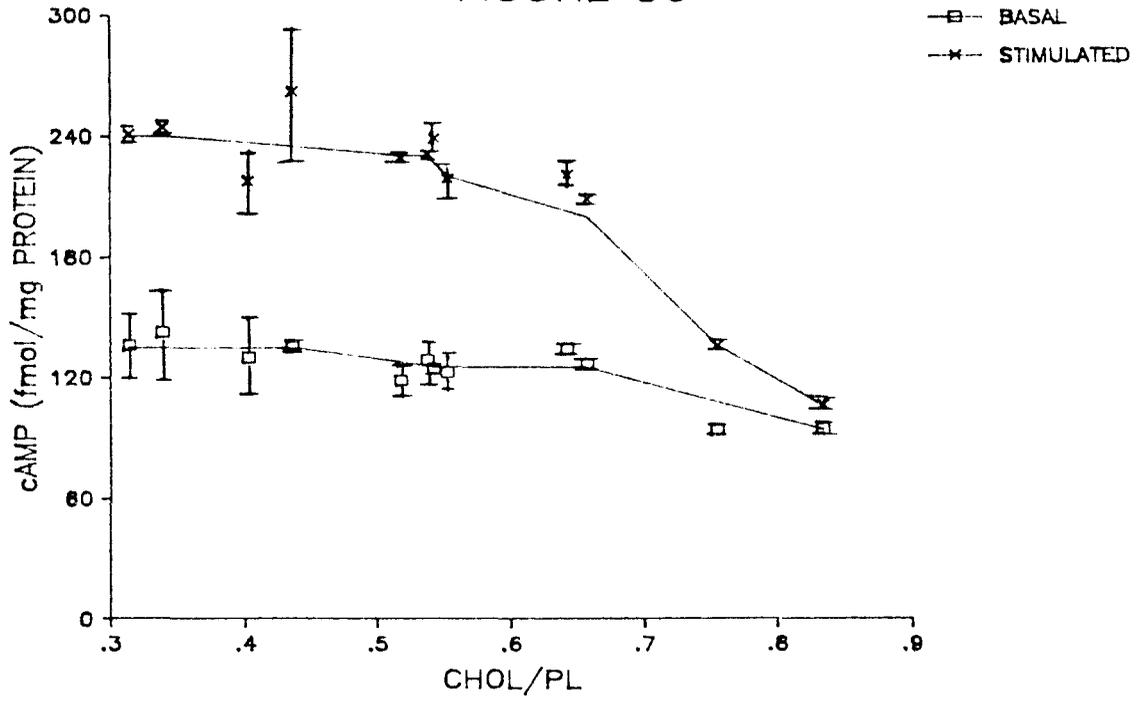
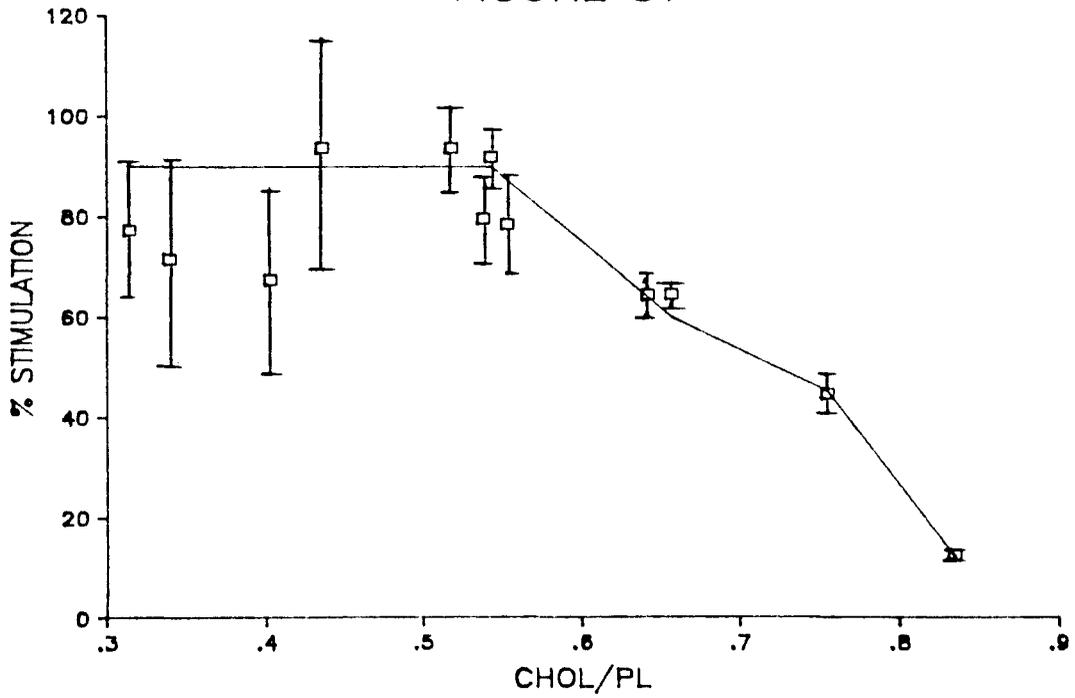


FIGURE 37



cAMP formed in the presence of dopamine to a value similar to the basal level in the absence of dopamine (Figure 36). Because the basal activity was largely unchanged, the percent stimulation by 100 μ M dopamine declined as the Chol/PL was increased to greater than 0.6 (Figure 37).

The percent stimulation of adenylate cyclase activity by 100 μ M dopamine as a function of the percent change in Chol/PL is shown in Figure 38. Figure 39 shows the relative microviscosity of the surface (ANS) and hydrophobic core (DPH) of the membrane as a function of the percent change in Chol/PL. Although the relative microviscosity decreased as the Chol/PL was decreased 30% (Figure 39), no effect on dopamine stimulation of adenylate cyclase activity was detected (Figure 38). As the Chol/PL was increased from the control, the very small changes in relative microviscosity of the membrane (Figure 35) were accompanied by a dramatic decrease in the stimulation of adenylate cyclase activity by 100 μ M dopamine (Figure 38).

SUMMARY OF STUDIES USING BOVINE CAUDATE MEMBRANES

Decreasing the Chol/PL produced a steady decrease in the relative microviscosity of the membrane surface and hydrophobic core. Only a small increase in microviscosity was detected as the Chol/PL was increased. There appeared to be a trend toward a higher K_d for SCH23390 binding as the Chol/PL was increased (Figure 31). No change in the B_{max} was detected (Figure 32). The B_{max}/K_d appeared

Figure 38. The Percent Stimulation of Adenylate Cyclase Activity in Bovine Caudate Synaptosomes by 100 μ M Dopamine as a Function of the Percent Change in Chol/PL.

Adenylate cyclase activity was assayed in cholesterol-depleted, control, and cholesterol-loaded synaptosomes. Cyclic AMP was detected by radioimmunoassay. The stimulated activity was assayed in the presence of 100 μ M dopamine. Each point represents the mean and standard deviation of 4 determinations. The Chol/PL of control synaptosomes was 0.556 \pm 0.013 (n = 8). A change in Chol/PL of -50% to 30% could be considered physiological.

Figure 39. The Relative Microviscosity of the Surface and Hydrophobic Core of Bovine Caudate SPM as a Function of the Percent Change in Chol/PL.

The relative microviscosity was of the membrane surface (ANS, \square) and core (DPH, \times) were calculated from r values determined at 37°C. The Chol/PL of control SPM was 0.542 \pm 0.026 (n = 4). A change in Chol/PL of -50% to 30% could be considered physiological.

FIGURE 38

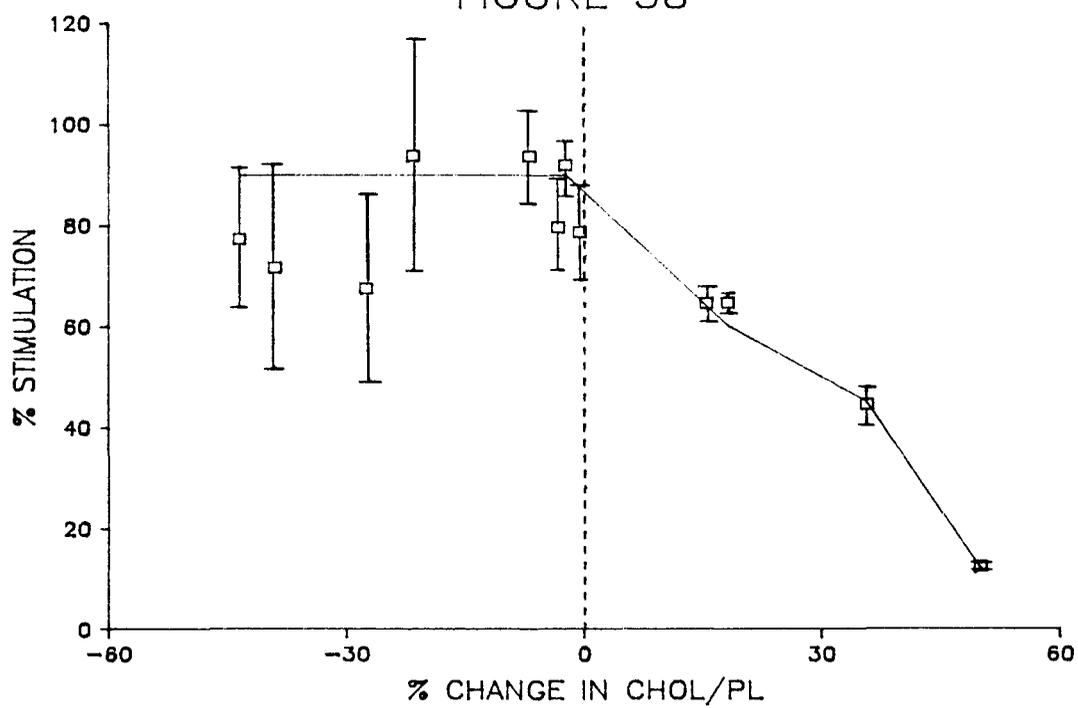
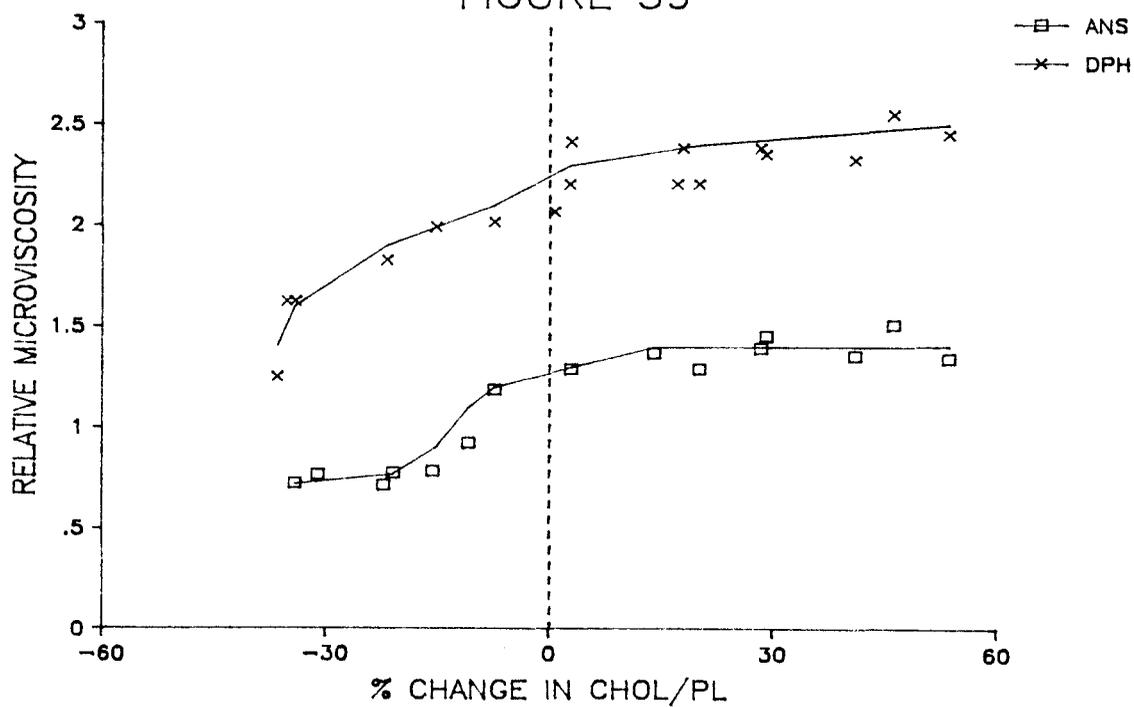


FIGURE 39



to decrease as the Chol/PL was decreased, and to increase as the Chol/PL was increased (Figure 33). Basal adenylate cyclase activity was unaffected by the experimentally produced changes in Chol/PL (Figure 36). However, that stimulated by 100 μ M dopamine decreased as the Chol/PL was increased (Figure 36). This resulted in a decrease in the percent stimulation by dopamine as the Chol/PL was increased (Figure 37).

CHAPTER V

DISCUSSION

The research described in this dissertation examined the influence of the molar ratio of cholesterol to phospholipid (Chol/PL) on synaptic membrane fluidity, and on selected membrane-associated functions of the dopaminergic system. Modification of the synaptic ratio of cholesterol to phospholipid was facilitated by a lipid transfer protein. Alterations in membrane fluidity, dopamine uptake, D₁ binding, and dopamine-stimulation of adenylate cyclase activity were observed over a wide range of Chol/PL, some of which were beyond the physiological range. However, significant changes in the dopaminergic system were also detected within the physiological range of Chol/PL.

The following discussion will address: 1) the impact of changes in the synaptic Chol/PL on synaptic membrane fluidity at the surface and core of the membrane; 2) the impact of changes in the synaptic Chol/PL on the dopaminergic system; and 3) the potential relevance of such changes within the physiological range to age- and/or alcohol-related changes in the dopaminergic system.

In the present studies, the relative microviscosity of both the hydrophobic core and the surface of

rat whole brain SPM was affected by changes in Chol/PL in an unexpected manner. The relationship appeared to be sigmoidal, producing the greatest change in the relative microviscosity within the physiological range (Figures 17 and 19). These results suggest that synaptic membranes from rat exist in two distinct states of microviscosity which is a function of the Chol/PL. The transition between these states occurs over a relatively narrow range of Chol/PL within the physiological range. This is supported by Arrhenius plots of DPH anisotropy (Figure 16) which show that at the physiological Chol/PL, the membranes appear to undergo a transition between these states such that at low temperature, the slope of the plot approaches that obtained at high Chol/PL, and at high temperature, the slope of the Arrhenius plot approaches that obtained at low Chol/PL.

The presence of cholesterol-rich domains within the membrane may be responsible for the mentioned sigmoidal relationship. In model membranes, at low Chol/PL (0.25, 20 mole %), there are pure phospholipid domains, and a cholesterol-phospholipid phase. These domains would be readily detected by the ANS surface probe due to its preference for interaction with phosphatidylcholine (Slavik, 1982). The DPH probe, in theory, should not detect these two domains because of its presumed uniform distribution throughout the membrane. However, at low Chol/PL, the contribution of proteins to the DPH anisotropy would be greater than at

high Chol/PL. This could account for the independence of microviscosity on Chol/PL at low values.

As the Chol/PL increases (0.43 to 0.54, 30 to 35 mole %), the pure phospholipid phase is abolished (Schroeder, 1984). This is accompanied by an inhibition of the lateral diffusion of cholesterol (Alecio et al., 1982), which may indicate a marked decrease in the fluidity of the membrane. Also, this transition to the entirely PL-chol membrane may be accompanied by an overall change in the packing of the phospholipids within the membrane. The range of Chol/PL over which this phenomenon occurs in model membranes is similar to the Chol/PL at which a marked change in the relative microviscosity was observed in the experimental membranes used in the present studies. At high Chol/PL (1.0, 50 mole %), the cholesterol would begin to aggregate, forming irregular structures, such as inverted micelles, in the membrane. These structures may exclude both ANS and DPH. This may explain the independence of microviscosity on Chol/PL at high values.

Many membrane-associated synaptic functions are markedly affected by changes in Chol/PL or changes in fluidity. For example, it is known that the activity of such key synaptic enzymes as Na^+ - K^+ -ATPase and ligand-stimulated adenylate cyclase are altered by changes in the fluidity of the membrane (Farias et al., 1975; Alivisatos et al., 1977; Rimon et al., 1978; Alivisatos et al., 1981;

Whetton et al., 1983). In addition, the binding to cholinergic, seritonergeric and opiate receptors, and to the GABA transporter are influenced markedly by membrane cholesterol (Heron et al., 1980a,b; Heron et al., 1981; Chweh & Leslie, 1982; Crews et al., 1983a; North & Fleischer, 1983b).

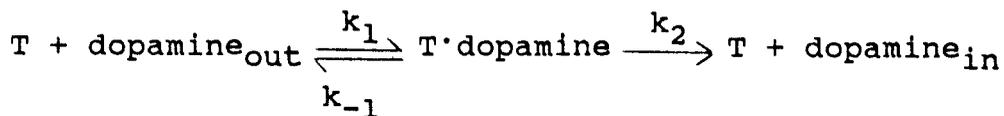
Presumably, there is a mechanism by which the central nervous system (CNS) regulates the Chol/PL within a narrow range in order to minimize the influence of such changes on neural function. The mechanism of regulation of the Chol/PL is unknown. However, it is possible that such regulation involves the synthesis of membrane precursors and the axonal transport of these precursors between the cell body and the nerve terminal. Since there is a blood brain barrier to plasma lipoproteins which transport cholesterol and phospholipids throughout the rest of the body (concepts reviewed by Ochs, 1981, and Katzman, 1981, in Basic Neurochemistry, Chapters 22 and 25, respectively), such a barrier may serve to protect the brain and synaptic membranes from marked changes in plasma lipid levels.

It is interesting that more marked changes in Chol/PL are seen in membranes outside the blood brain barrier in response to changes in serum levels of cholesterol and phospholipids. For example, the lipid composition of erythrocyte membranes is more susceptible to changes in serum cholesterol levels than is the lipid composition of synaptic membranes (Chin et al., 1978).

Although the functions associated with peripheral membranes are important, it may be more essential to the short-term survival of the animal to more finely control the environment of those functions associated with CNS neural membranes.

In the present studies, the modification of synaptic Chol/PL was found to have a marked effect on the uptake of dopamine. The K_m for dopamine uptake into rat whole brain synaptosomes increased sharply as the Chol/PL was increased (Figure 25). An increase in Chol/PL of 30% produced a 3-fold increase in the K_m (Figure 25). Over the same range of Chol/PL, there was a small increase in the V_{max} (Figure 26). These results suggest that as the membrane becomes more rigid, the affinity of the uptake transporter for dopamine decreases. At the same time, there is a partially compensatory increase in the maximum velocity. However, although the V_{max} was increased, a higher concentration of dopamine would be required to achieve this maximum rate, due to the increase in K_m . It should be noted that the increase in K_m occurs within the range of Chol/PL that produced the dramatic change in the relative microviscosity.

At first glance, the increase in both the K_m and the V_{max} appears unusual. However, this is explained easily in kinetic terms. For the simplest scheme for the uptake process:



the $K_m = \frac{k_2 + k_{-1}}{k_1}$, and the $V_{\text{max}} = k_2 [T]$,

with T representing the uptake transporter. Because in the in vitro assay the concentration of the transporter is constant, the increase in the V_{max} would be due to an increase in k_2 . This increase in k_2 will also produce an increase in K_m .

Perhaps a more useful index of dopamine uptake is the V_{max}/K_m (Figure 27), a first order rate constant for the uptake transporter at low substrate concentrations. At synaptic concentrations of dopamine that are less than the K_m , the rate of uptake will be determined by the V_{max}/K_m . At concentrations that are greater than the K_m , the rate of uptake will be determined by the V_{max} (Cornish-Bowden, 1979).

As the Chol/PL was increased 30% (within the physiological range), there was a 33% decrease in the V_{max}/K_m . This suggests that as the membrane becomes more rigid, the rate of uptake will decrease if the synaptic concentration of dopamine is less than the K_m . Because the concentration of dopamine in the synapse is not known, an absolute statement as to the expected effect of changes in Chol/PL on in vivo dopamine uptake are not possible.

Since synaptic uptake of dopamine must, by definition, involve a transmembrane process, it is not surprising that the uptake would be sensitive to changes in the membrane environment of the transporter. Although the mechanism of the dopamine uptake process into synaptosomes is not known, it is presumably similar to the uptake of the other biogenic amines. Kinetic studies suggest that the transport of norepinephrine into synaptosomes is protein-mediated and coupled to the transport of Na^+ (Trendelenburg, 1981). Such a transmembrane transport protein must have certain structural features in order to function efficiently (Carruthers & Melchior, 1986). The protein must contain domains that catalyze the unidirectional transfer of substrates across the lipid bilayer. These domains could be a series of gates that are triggered as the substrate moves through the protein. Alternately, the domains may form a channel through the protein, through which substrates may pass. The transporter must also contain substrate recognition sites. These sites would initiate the gating mechanism or open the transport channel when the substrate is bound. Also, regulatory domains might be present. Each of these domains, and the coupling between these domains could be modulated by changes in the lipid environment (Carruthers & Melchior, 1986). It is possible that the dopamine uptake transporter is within one of the pure phospholipid regions of the membrane. As the Chol/PL

increases, and the phospholipid domain is abolished, the uptake may be inhibited, due to the accompanying increase in the membrane microviscosity. Also, the rate of dopamine uptake would be dependent on the sodium gradient across the synaptic membrane. At low Chol/PL, the membrane may be more permeable to sodium ions, dissipating the gradient and slowing the rate of uptake. At high Chol/PL, the membrane may become less permeable to sodium ions, resulting in a greater potential difference across the membrane, increasing the uptake rate.

The present studies suggest that there is a small influence of Chol/PL and membrane fluidity on the binding of SCH23390 to the D_1 receptor in bovine caudate membranes. Over the range of Chol/PL of 0.3 to 0.5, there was an decrease in the K_d for SCH23390 of about 50% (Figure 31). Because the B_{max} was unchanged (Figure 32), the B_{max}/K_d tended to decrease as the Chol/PL decreased over this range. At Chol/PL above 0.5, the D_1 receptor appears to be unaffected.

Dopamine-stimulated adenylate cyclase is a membrane-associated enzyme whose activity is stimulated through a multi-step process, initiated by the binding of dopamine to the D_1 receptor. The activation of adenylate cyclase requires the lateral diffusion of the receptor, as well as the regulatory G-protein (Rimon et al., 1978). This type of mechanism would be expected to be affected by

changes in membrane fluidity.

The present studies examined the effects of altering the Chol/PL of bovine caudate synaptosomal membranes on basal and dopamine-stimulated adenylate cyclase activity. These studies demonstrated that the activity of dopamine-stimulated adenylated cyclase was modulated by the synaptic Chol/PL. If the dopamine receptor system is coupled to adenylate cyclase through a mobile receptor, as is epinephrine (Rimon et al., 1978), decreasing the relative microviscosity of the membrane would be expected to enhance the stimulatory effect of the ligand (Rimon et al., 1978). The dopamine stimulation of adenylate cyclase activity was not affected by decreasing the membrane fluidity via decreasing the Chol/PL (Figures 38 and 39). The discrepancy of the present results with those expected of a mobile receptor may be due to the masking of the effect by maximally stimulating the enzyme. Cholesterol loading of the synaptic membranes produced a marked inhibition of maximal dopamine stimulation of adenylate cyclase activity (Figure 36). Because of the lateral movement of the G-protein required for activation of the enzyme, the dopamine-stimulated adenylate cyclase system may require a more fluid phospholipid domain for optimal stimulation. When the phospholipid domain is abolished by increasing the Chol/PL, the stimulated activity may be expected to decrease. The basal activity was unaffected by changes in membrane composition

and fluidity (Figure 36).

The results of the present studies have demonstrated that the transmembrane processes involved with the dopaminergic system, namely, the uptake of dopamine and dopamine stimulation of adenylate cyclase, are inhibited by an increase in Chol/PL. Since aging and alcohol are known to be associated with an increase in synaptic Chol/PL (Chin et al., 1978; Smith & Gerhart, 1982; Armbrecht et al., 1983; Crews et al., 1983b; Shinitzky et al., 1983), some of the dopaminergic changes which are found in these conditions may be due to increases in Chol/PL. Other changes appear to be unrelated. Specific examples of the potential contribution of the change in Chol/PL to age- and/or alcohol-associated changes in dopaminergic function are discussed.

The change in dopamine uptake with normal postnatal development as well as with aging may be explained, in part, by normal physiological changes in Chol/PL. During postnatal development, there is an increase in dopamine uptake, due to an increase in the V_{\max} (Coyle & Campachiaro, 1976). In the present studies, as the Chol/PL was decreased from the normal adult ratio to that found in the synaptic membranes from young animals, the V_{\max} decreased (Figure 26). This suggests that the increased V_{\max} for dopamine uptake, which occurs during development may be due to an increase in the number of uptake trans-

porters per cell rather than to changes in membrane composition and fluidity.

Studies of the effects of aging on dopamine uptake (Jones & Finch, 1975) showed a 30% decrease in dopamine uptake at a dopamine concentration of 10 nM, which is below the K_m (40 nM). This decrease was attributed to a 35% increase in the K_m . Because no change in V_{max} was observed, the V_{max}/K_m was decreased with aging. As would be expected, at 120 nM, which is above the K_m , no difference was observed between the young and old animals. The increased K_m for dopamine uptake observed in the present studies as the Chol/PL was increased suggests that the changes in Chol/PL and membrane fluidity which occur during aging may be responsible, in part, for the observed changes in dopamine uptake.

In addition, it appears that the age- and/or alcohol-related changes in dopamine stimulation of adenylate cyclase activity may be related to changes in the Chol/PL. During aging (Puri & Volicer, 1977; Makman et al., 1980; Schmidt & Thornberry, 1981) and with chronic ethanol treatment (Tabakoff & Hoffman, 1979; Lucchi et al., 1983), there was a decreased sensitivity of adenylate cyclase for dopamine, due to a decreased efficiency of coupling between the receptor and the catalytic site of adenylate cyclase (Tabakoff & Hoffman, 1979). The present studies suggest that this decreased sensitivity could be

due to age- and/or alcohol-related membrane fluidity changes.

It is difficult to correlate the present studies with the current literature on dopamine receptors in developing, aging, and ethanol-treated animals. Past studies have generally used the radioactive ligand, [³H]-spiperone, to label dopamine receptors. However, depending on the concentration of spiperone, the assay conditions, and the brain region analyzed, spiperone can label D₁, D₂, and serotonin receptors. There is indirect evidence, from studies using spiperone and a D₂ specific ligand, that the decrease in the B_{max} for spiperone binding detected in aged animals, may be due to the D₁ receptors (Memo et al., 1980; Severson & Finch, 1980; Trabucchi et al., 1982; Missale et al., 1983). In the present studies, the B_{max} of the D₁ receptor, as assessed using SCH23390, was unaffected by changes in membrane fluidity. Therefore, if the decrease in the B_{max} detected in aged animals is due to the D₁ receptors, it is unlikely that this decrease is due to membrane fluidity effects.

SUMMARY

The research described in this dissertation examined the influence of the cholesterol to phospholipid molar ratio on synaptic membrane fluidity, and on selected membrane-associated functions of the dopaminergic system. Modifications of the synaptic cholesterol to phospholipid molar ratio was facilitated by a lipid transfer protein. Synaptic membrane fluidity was assessed using 1-anilino-naphthalene-8-sulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) as probes of membrane surface and core fluidity, respectively. The components of dopaminergic function which were assessed include dopamine uptake, D₁ binding, and D₁-stimulated adenylate cyclase activity. Alterations in membrane fluidity, dopamine uptake, D₁ binding and dopamine stimulation of adenylate cyclase activity were observed over a wide range of Chol/PL, some of which were beyond the physiological range. However, significant changes in the dopaminergic system were also detected within the physiological range of Chol/PL.

The results of the studies described in this dissertation suggest that the transmembrane processes of the dopaminergic system, dopamine uptake and dopamine-stimulated adenylate cyclase activity, are highly sensitive to changes in the Chol/PL of synaptic and synaptosomal membranes within the physiological range of Chol/PL. For example, as the fluidity of the membrane was decreased by

increasing the Chol/PL 30% above the normal value, the K_m for dopamine uptake was increased 3-fold. Such an increase also caused a 33% decrease in the V_{max}/K_m , the first order rate constant for dopamine uptake at low concentrations of dopamine. In addition, cholesterol loading of the synaptic membranes produced a marked inhibition (80-90%) of the maximal stimulation of adenylate cyclase by dopamine. In contrast to the highly sensitive transmembrane processes of the dopaminergic system, D_1 binding was less sensitive to the effects of changes in membrane Chol/PL and fluidity. As the Chol/PL was increased 33% above the control value, the binding of SCH23390 to the D_1 site was unaffected.

In light of the findings presented here, it appears likely that some of the changes in dopaminergic function which are known to accompany aging and chronic alcohol consumption can be explained by the age- and alcohol-associated changes in the synaptic Chol/PL and fluidity. In particular, it seems likely that the age-associated decrease in V_{max}/K_m for uptake and decreased sensitivity of adenylate cyclase for dopamine could be related to the increased Chol/PL in those animals. The decreased efficiency of coupling of the dopamine receptor with the catalytic site of adenylate cyclase in alcohol-treated animals might similarly be explained. These observations are consistent with reports from other lab-

oratories of cholesterol inhibition of diffusion-controlled enzyme systems. In contrast, it does not seem likely that altered synaptic Chol/PL and fluidity contribute significantly to physiological changes in D_1 binding.

The results presented here provide significant new information about how synaptic membrane composition and fluidity affect synaptic function. In addition, this new information may contribute to our understanding of the causes of age- and alcohol-associated changes in CNS function.

BIBLIOGRAPHY

- Abdel-Latif, A.A., J.P. Smith, and N. Hedrick (1970) Adenosine-Triphosphate and Nucleotide Metabolism in Synaptosomes of Rat Brain. J. Neurochem. 17: 391-401.
- Alecio, M.R., D.E. Golan, W.R. Veatch, and R.R. Rando (1982) Use of Fluorescent Cholesterol Derivative to Measure Lateral Mobility of Cholesterol in Membranes. Proc. Natl. Acad. Sci. 79: 5171-5174.
- Alivisatos, S.G.A., C. Papastavrou, E. Drouka-Liapati, A.P. Molyvdas, and G. Nikitopoulou (1977) Enzymatic and Electrophysiological Changes of the Function of Membrane Proteins by Cholesterol. Biochem. Biophys. Res. Commun. 79: 677-683.
- Alivisatos, S.G.A., G. Deliconstantinos, and G.P. Theodosiadis (1981) Specificity of Binding of Cholesterol, Steroid Hormones, and Other Compounds in Synaptosomal Plasma Membranes, and Their Effect on Ouabian-Sensitive ATPase. Biochim. Biophys. Acta 643: 650-658.
- Anderson, P.H., and E.B. Nielsen (1986) The Dopamine D₁ Receptor: Biochemical and Behavioral Aspects. In Neurobiology of Central D₁-Dopamine Receptors. G.R. Breese and I. Creese, (ed.), Plenum Press, New York.
- Armbrecht, H.J., W.G. Wood, R.W. Wise, J.B. Walsh, B.N. Thomal, and R. Strong (1983) Ethanol-Induced Disordering of Membranes from Different Age Groups of C57BL/6NNIA Mice. J. Pharmacol. Exp. Ther. 226: 387-391.
- Banik, N.L., and A.N. Davison (1969) Enzyme Activity and Composition of Myelin and Subcellular Fractions in the Developing Rat Brain. Biochem. J. 115: 1051-1062.
- Barbaccia, M.L., A. Bosio, L. Lucchi, P.F. Spano, and M. Trabucchi (1982) Neuronal Mechanisms Regulating Ethanol Effects on the Dopaminergic System. Life Sci. 30: 2163-2170.

- Barbaccia, M.L., A. Reggiani, P.F. Spano, and M. Trabucchi (1980) Ethanol Effects on Dopaminergic Function: Modulation by the Endogenous Opioid System. Pharmacol. Biochem. Behav. 13 (Suppl. 1): 303-306.
- Bentley, K.L., L.K. Thompson, R.J. Klebe, and P.M. Horowitz (1985) Fluorescence Polarization: A General Method for Measuring Ligand Binding and Membrane Microviscosity. BioTechniques 3: 356-366.
- Beauge, F., C. Fleuret-Balter, J. Nordmann, and R. Nordmann (1984) Brain Membrane Sensitivity to Ethanol During Development of Functional Tolerance to Ethanol in Rats. Alcohol. Clin. Exp. Res. 8: 167-171.
- Calderini, G., A.C. Bonetti, A. Battistella, F.T. Crews, and G. Toffano (1983) Biochemical Changes of Rat Brain Membranes with Aging. Neurochem. Res. 8: 483-492.
- Carruthers, A., and D.L. Melchior (1986) How Bilayer Lipids Affect Membrane Protein Activity. Trends Biol. Sci. 11: 331-335.
- Castuma, C.E., and R.R. Brenner (1983) Effect of Fatty Acid Deficiency on Microsomal Membrane Fluidity and Cooperativity of the UDP-Glucuronyltransferase. Biochim. Biophys. Acta 729: 9-16.
- Chin, J.H., and D.B. Goldstein (1977a) Effects of Low Concentrations of Ethanol on the Fluidity of Spin-Labeled Erythrocytes and Brain Membranes. Mol. Pharmacol. 13: 435-441.
- Chin, J.H., and D.B. Goldstein (1977b) Drug Tolerance in Biomembranes: A Spin Label Study of the Effects of Ethanol. Science 196: 684-685.
- Chin J.H., and D.B. Goldstein (1981) Membrane-Disordering Action of Ethanol. Variation with Membrane Cholesterol Content and Depth of the Spin Label Probe. Mol. Pharmacol. 19: 425-431.
- Chin, J.H., D.B. Goldstein, and L.M. Parsons (1979) Fluidity and Lipid Composition of Mouse Biomembranes During Adaptation to Ethanol. Alcoholism (NY) 3: 47-49.

- Chin, J.H., L.M. Parsons, and D.B. Goldstein (1978) Increased Cholesterol Content of Erythrocyte and Brain Membranes in Ethanol-Tolerant Mice. Biochim. Biophys. Acta 513: 358-363.
- Christiansson, A., F.A. Kuypers, B. Roelofsen, K.W.A. Wirtz, and J.A.F. Op den Kamp (1984) A Comparative Fluorescence Polarization Study of cis-Parinaroyl-Phosphatidylcholine and Diphenylhexatriene in Membranes Containing Different Amounts of Cholesterol. Chem. Phys. Lip. 35: 247-258.
- Chweh, A.Y., and S.W. Leslie (1982) Phosphatidylserine Enhancement of [³H]-Gamma-Aminobutyric Acid Uptake by Rat Whole Brain Synaptosomes. J. Neurochem. 38: 691-695.
- Cimino, M., G. Curatola, C. Pezzoli, G. Stramentinoli, G. Vantini, and S. Algeri (1983) Age-related Modification of Dopaminergic and Alpha-Adrenergic Receptor System: Restoration of Normal Activity by Modifying Membrane Fluidity with S-Adenosyl Methionine. In Aging Brain and Ergot Alkaloids, A. Agnoli et al. (ed.), Raven Press, New York.
- Cornish-Bowden, A. (1979) Fundamentals of Enzyme Kinetics. Butterworth Inc. Woburn, MA.
- Cotman, C.W., and D.A. Matthews (1971) Synaptic Plasma Membranes from Rat Brain Synaptosomes: Isolation and Partial Characterization. Biochim. Biophys. Acta 249: 380-394.
- Coyle, J.T., and P. Campachiaro (1976) Ontogenesis of Dopaminergic-Cholinergic Interactions in the Rat Striatum: A Neurochemical Study. J. Neurochem. 27: 673-678.
- Crain, R.C. (1982) Nonspecific Lipid Transfer Proteins as Probes of Membrane Structure and Function. Lipids 17: 935-943.
- Crain, R.C., and D.B. Zilversmit (1980) Two Nonspecific Phospholipid Exchange Proteins from Beef Liver. 1. Purification and Characterization. Biochemistry 19: 1433-1439.
- Creese, I., and A. Chen (1985) Selective D-1 Dopamine Receptor Increase Following Chronic Treatment with SCH23390. Eur. J. Pharmacol. 109: 127-128.

- Creese, I., T. Prosser, and S.H. Snyder (1978) Dopamine Receptor Binding: Specificity, Localization and Regulation by Ions and Guanyl Nucleotides. Life Sci. 23: 495-500.
- Creese, I., R. Schneider, and S.H. Snyder (1977) ³H-Spiroperidol Labels Dopamine Receptors in Pituitary and Brain. Eur. J. Pharmacol. 46: 377-381.
- Creese, I., D.R. Sibley, M.W. Hamblin, and S.E. Leff (1983) The Classification of Dopamine Receptors: Relationship to Radioligand Binding. Ann. Rev. Neurosci. 6: 43-71.
- Creese, I., and S.H. Snyder (1978a) ³H-Spiroperidol Labels Serotonin Receptors in Rat Cerebral Cortex and Hippocampus. Eur. J. Pharmacol. 49: 201-202.
- Creese, I., and S.H. Snyder (1978b) Dopamine Receptor Binding of ³H-ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) Regulated by Guanyl Nucleotides. Eur. J. Pharmacol. 50: 459-461.
- Crews, F.T., A. Camacho, and M.I. Phillips (1983a) Cholinergic Stimulation of Hippocampal Pyramidal Cells is Inhibited by Increasing Membrane Cholesterol. Brain Res. 261: 155-158.
- Crews, F.T., E. Majchrowicz, and R. Meeks (1983b) Changes in Cortical Synaptosomal Plasma Membrane Fluidity and Composition in Ethanol-Dependent Rats. Psychopharmacology 81: 208-213.
- Cross, A.J., and F. Owen (1980) Characteristics of ³H-cis-Flupenthixol Binding to Calf Brain Membranes. Eur. J. Pharmacol. 65: 341-347.
- De Blasi, A., and T. Mennini (1982) Selective Reduction of One Class of Dopamine Receptor Binding Sites in the Corpus Striatum of Aged Rats. Brain Res. 242: 361-364.
- Demel, R.A., and B. De Kruyff (1976) The Function of Sterols in Membranes. Biochim. Biophys. Acta 457: 109-132.
- Deskin, R., F.J. Seidler, W.L. Whitmore, and T.A. Slotkin (1981) Development of Beta-Noradrenergic and Dopaminergic Receptor Systems Depends on Maturation of Their Presynaptic Nerve Terminals in the Rat Brain. J. Neurochem. 36: 1683-1690.

- Duncan, H.M., and B. Mackler (1966) Electron Transport Systems of Yeast. III. Preparation and Properties of Cytochrome Oxidase. J. Biol. Chem. 241: 1694-1697.
- Enjalbert, A., M. Hamon, S. Bourgoïn, and J. Bockaert (1978) Post-Synaptic Serotonin-Sensitive Adenylate Cyclase in the Central Nervous System. II. Comparison with Dopamine- and Isoproterenol-Sensitive Adenylate Cyclases in Rat Brain. Mol. Pharmacol. 14: 11-23.
- Farias, R.N., B. Bloj, R.D. Morero, F. Sineriz, and R.E. Trucco (1975) Regulation of Allosteric Membrane-Bound Enzymes Through Changes in Membrane Lipid Composition. Biochim. Biophys. Acta 415: 231-251.
- Feinstein, M.B., S.M. Fernandez, and R.I. Sha'afi (1975) Fluidity of Natural Membranes and Phosphatidylserine and Ganglioside Dispersions. Effects of Local Anesthetics, Cholesterol, and Protein. Biochim. Biophys. Acta 413: 354-370.
- Fiske, C.H., and Y. Subbarow (1925) The Colorimetric Determination of Phosphorus. J. Biol. Chem. 66: 375-400.
- Folch, J., M. Lees, and G. Sloane-Stanley (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem. 226: 497-509.
- Freund, G. (1982) The Interaction of Chronic Alcohol Consumption and Aging on Brain Structure and Function. Alcoholism (NY) 6: 13-21.
- Freund, G., T.R. Brophy, and J.D. Scott (1986) Membrane Fluidization Increases Low-Affinity Muscarinic Receptor Binding in Brain: Changes with Aging. Exp. Gerontol. 21: 37-44.
- Goldstein, D.B., J.H. Chin, and R.C. Lyon (1982) Ethanol Disordering of Spin-Labeled Mouse Brain Membranes: Correlation with Genetically Determined Ethanol Sensitivity of Mice. Proc. Natl. Acad. Sci. 79: 4231-4233.
- Govoni, S., M. Memo, L. Saiani, P.F. Spano, and M. Trabucchi (1980) Impairment of Brain Neurotransmitter Receptors in Aged Rats. Mech. Ageing Dev. 12: 39-46.

- Govoni, S., P.F. Spano, and M Trabucchi (1978) [³H]-Haloperidol and [³H]Spiroperidol Binding in Rat Striatum During Ageing. J. Pharm. Pharmacol. 30: 448-449.
- Govoni, S., M. Trabucchi, A. Bosio, and P.F. Spano (1983) Functional Changes in Neurotransmitter Receptors in the Brains of Aged Rats. In Brain Aging: Neuropathology and Neuropharmacology, J. Cervos-Navarro and H.I. Sarkander (ed.), Raven Press, New York.
- Hamblin, M.W., S.E. Leff, and I. Creese (1984) Interactions of Agonists with D-2 Dopamine Receptors: Evidence for a Single Receptor Population Existing in Multiple Agonist Affinity States in Rat Striatal Membranes. Biochem. Pharmacol. 33: 877-887.
- Harris, R.A., and R.J. Hitzemann (1981) Membrane Fluidity and Alcohol Actions. Curr. Alc. VIII: 379-404.
- Hartley, E.J., and P. Seeman (1983) Development of Receptors for Dopamine and Noradrenaline in Rat Brain. Eur. J. Pharmacol. 91: 391-397.
- Henry, J.M., and G.S. Roth (1986) Modulation of Rat Striatal Membrane Fluidity: Effects on Age Related Differences in Dopamine Receptor Concentrations. Life Sci. 39: 1223-1229.
- Heron, D.S., M. Hershkowitz, M. Shinitzky, and D. Samuel (1980a) The Lipid Fluidity of Synaptic Membranes and the Binding of Serotonin and Opiate Ligands. In Neurotransmitters and Their Receptors, U.Z. Littauer et al. (ed.) John Wiley & Sons, Ltd.
- Heron, D.S., M. Shinitzky, M. Hershkowitz, and D.S. Samuel (1980b) Lipid Fluidity Markedly Modulates the Binding of Serotonin to Mouse Brain Membranes. Proc. Natl. Acad. Sci. 77: 7463-7467.
- Heron, D., M. Israeli, M. Hershkowitz, D. Samuel, and M. Shinitzky (1981) Lipid-Induced Modulation of Opiate Receptors in Mouse Brain Membranes. Eur. J. Pharmacol. 72: 361-364.
- Hess, E.J., G. Battaglia, A.B. Norman, L.C. Iorio, and I. Creese (1986) Guanine Nucleotide Regulation of Agonist Interactions at [³H]SCH23390-Labelled D₁ Dopamine Receptors in Rat Striatum. Eur. J. Pharmacol. 121: 31-38.

- Hitzemann, R.J., (1981) Developmental Changes in the Fatty Acids of Synaptic Membrane Phospholipids: Effect of Protein Malnutrition. Neurochem. Res. 6: 935-947.
- Hitzemann, R.J. (1984) Age Related Changes in Nerve Membrane Structure. Neurobiol. Aging 5: 346-348.
- Hitzemann, R.J., and D.A. Johnson (1983) Developmental Changes in Synaptic Membrane Lipid Composition and Fluidity. Neurochem. Res. 8: 121-131.
- Hoffman, P.L., G.R. Luthin, D. Theodoropoulos, P. Cordopatis, and B. Tabakoff (1983) Ethanol Effects on Striatal Dopamine Receptor-Coupled Adenylate Cyclase and on Striatal Opiate Receptors. Pharmacol. Biochem. Behav. 18 (Suppl. 1): 355-359.
- Hoffman, P.L., and B. Tabakoff (1985) Ethanol's Action on Brain Biochemistry. In Alcohol and the Brain. Chronic Effects, R.E. Tarten and D.H. Van Thiel (ed.), Plenum Medical Book Co., New York.
- Hollenberg, M.D. (1978) Receptor Models and the Action of Neurotransmitters and Hormones. In Neurotransmitter Receptor Binding, H.I. Yamamura (ed.), Raven Press, New York.
- Horn, A.S. (1979) Characteristics of Dopamine Uptake. In The Neurobiology of Dopamine. A.S. Horn et al. (ed.), Academic Press, New York.
- Houslay, M.D., and K.K. Stanley (1982) Influence on Synthesis, Structure and Function. In Dynamics of Biological Membranes. John Wiley and Sons, New York.
- Hunt, P., M. Kannengiesser, and J. Raynaud (1974) Nomifensine: A New Potent Inhibitor of Dopamine Uptake into Synaptosomes from Rat Brain Corpus Striatum. J. Pharm. Pharmacol. 26: 370-371.
- Hunt, W.A., E. Majchrowicz, T.K. Dalton, H.S. Swartzwelder, and H. Wixon (1979) Alterations in Neurotransmitter Activity after Acute and Chronic Ethanol Treatment: Studies of Transmitter Interactions. Alcohol. Clin. Exp. Res. 3: 359-363.
- Hyttel, J. (1978) Effects of Neuroleptics on ³H-Haloperidol and ³H-cis(Z)-Flupenthixol Binding and on Adenylate Cyclase Activity in Vitro. Life Sci. 23: 551-556.

- Hyttel, J. (1980) Further Evidence That ^3H -cis(Z)-Flupenthixol Binds to the Adenylate Cyclase-Associated Dopamine Receptor (D-1) in Rat Corpus Striatum. Psychopharmacology 67: 107-109.
- Jain, M.K., and R.C. Wagner (1980) Introduction to Biological Membranes. John Wiley and Sons, New York.
- Johnson, D.A., N.M. Lee, R. Cooke, and H.H. Loh (1979) Ethanol-Induced Fluidization of Brain Lipid Bilayers: Required Presence of Cholesterol in Membranes for the Expression of Tolerance. Mol. Pharmacol. 15: 739-746.
- Jonec, V., and C.E. Finch (1975) Ageing and Dopamine Uptake by Subcellular Fractions of the C57BL/6J Male Mouse Brain. Brain Res. 91: 197-215.
- Jost, P.C., and O.H. Griffith (1980) Lipid-Lipid and Lipid-Protein Interactions in Membranes. Pharmacol. Biochem. Behav. 13 (Suppl. 1): 155-165.
- Katzman, R. (1981) Blood-Brain-CSF Barriers. In Basic Neurochemistry, G.J. Siegel et al., (ed.), Little, Brown and Company, Boston, pp. 497-512.
- Kurihara, T., and Y. Tsukada (1967) The Regional and Subcellular Distribution of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase in the Central Nervous System. J. Neurochem. 14: 1167-1174.
- Kuriyama, K., K. Nakagawa, and N. Miki (1977) Alterations of Cyclic Nucleotide Metabolism in Brain Following Ethanol Intoxication and Withdrawl. In Alcohol Intoxication and Withdrawl - IIIa. M.M. Gross (ed.) Plenum Press, New York.
- Leff, S.E., M.W. Hamblin, and I. Creese (1985) Interactions of Dopamine Agonists with Brain D_1 Receptors Labeled by ^3H -Antagonists: Evidence for the Presence of High and Low Affinity Agonist-Binding States. Mol. Pharmacol. 27: 171-183.
- Linhardt, K., and K. Walter (1963) Phosphatases (Phosphomonoesterases). In Methods of Enzymatic Analysis, Hans Ulrich Bergmeyer (ed.), Academic Press, New York.
- Litman, B.J., and Y. Barenholz (1982) Fluorescent Probe: Diphenylhexatriene. Methods Enzymol. 81: 678-685.

- Littleton, J.M., and G. John (1977) Synaptosomal Membrane Lipids of Mice During Continuous Exposure to Ethanol. J. Pharm. Pharmacol. 29: 579-580.
- Littleton, J.M., G.R. John, and S.J. Grieve (1979) Alterations in Phospholipid Composition in Ethanol Tolerance and Dependence. Alcohol. Clin. Exp. Res. 3: 50-56.
- Logan, B.J., R. Laverty, and B.M. Peake (1983) ESR Measurements on the Effects of Ethanol on the Lipid and Protein Conformation in Biological Membranes. Pharmacol. Biochem. Behav. 18 (Suppl. 1): 31-35.
- Lowry, O.H., N.H. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein Measurement with Folin Phenol Reagent. J. Biol. Chem. 193: 265-275.
- Lucchi, L., M. Lupini, S. Govoni, V. Covelli, P.F. Spano, and M. Trabucchi (1983) Ethanol and Dopaminergic Systems. Pharmacol. Biochem. Behav. 18 (Suppl. 1): 379-382.
- Lyon, R.C., and D.B. Goldstein (1983) Changes in Synaptic Membrane Order Associated with Chronic Ethanol Treatment in Mice. Mol. Pharmacol. 23: 86-91.
- Makman, M.H., H.S. Ahn, L.J. Thal, N.S. Sharpless, B. Dvorkin, S.G. Horowitz, and M. Rosenfeld (1980) Evidence for Selective Loss of Brain Dopamine- and Histamine-Stimulated Adenylate Cyclase Activities in Rabbits with Aging. Brain Res. 192: 177-183.
- Manias, B., and D.A. Taylor (1983) Inhibition of In Vitro Amine Uptake into Rat Brain Synaptosomes After In Vivo Administration of Antidepressants. Eur. J. Pharmacol. 95: 305-309.
- Memo, M., L. Lucchi, P.F. Spano, and M. Trabucchi (1980) Aging Process Affects a Single Class of Dopamine Receptors. Brain Res. 202: 488-492.
- Meyer, J.S., J.W., Largen, T. Shaw, K.F., Mortel, and R. Rogers (1984) Interaction of Normal, Aging, Senile Demential, Multi-Infarct Dementia, and Alcoholism in the Elderly. In Alcoholism in the Elderly, J.T. Hartford and T. Samorajski (ed.), Raven Press, New York.

- Miller, R.J., and J. McDermed (1979) Dopamine Sensitive Adenylate Cyclase. In The Neurobiology of Dopamine, A.S. Horn et al. (ed.), Academic Press, New York.
- Missale, C., S. Govoni, L. Castelletti, P.F. Spano, and M. Trabucchi (1983) Brain Neurotransmitter Changes in Brains of Aged Rats. In Aging Brain and Ergot Alkaloids, A. Agnoli et al. (ed.), Raven Press, New York.
- Misra, C.H., H.S. Shelat, and R.C. Smith (1980) Effect on Age on Adrenergic and Dopaminergic Receptor Binding in Rat Brain. Life Sci. 27: 521-526.
- Murrin, L.C. (1982) In Vivo Studies of Dopamine Receptor Ontogeny. Life Sci. 31: 971-980.
- Nagy, K., P. Simon, and I. Zs.-Nagy (1983) Spin Label Studies on Synaptosomal Membranes of Rat Brain Cortex During Aging. Biochem. Biophys. Res. Commun. 117: 688-694.
- Nathanson, J.A. (1977) Cyclic Nucleotides and Nervous System Function. Physiol. Rev. 57: 157-256.
- Noland, B.J., R.E. Arebalo, E. Hansbury, and T.J. Scallen (1980) Purification and Properties of Sterol Carrier Protein. J. Biol. Chem. 255: 4282-4289.
- Nomura, Y., K. Oki, I. Yotsumoto, and T. Segawa (1982) Age-Related Change of Binding Regulation in Dopamine and Alpha₂-Adrenergic Receptors in the Brain. In The Aging Brain: Cellular and Molecular Mechanisms of Aging in the Nervous System, E. Giacobini et al. (ed.), Raven Press, New York, 185-194.
- North, P., and S. Fleischer (1983a) Use of a Nonspecific Lipid Transfer Protein to Modify the Cholesterol Content of Synaptic Membranes. Methods Enzymol. 98: 599-613.
- North, P., and S. Fleischer (1983b) Alteration of Synaptic Membrane Cholesterol/Phospholipid Ratio Using a Lipid Transfer Protein: Effect on Gamma-Aminobutyric Acid Uptake. J. Biol. Chem. 258: 1242-1253.
- Ochs, S. (1981) Axoplasmic Transport. In Basic Neurochemistry, G.J. Siegel et al. (ed.), Little, Brown and Company, Boston, pp. 425-444.

- Puri, S.K., and L. Volicer (1977) Effect of Aging on Cyclic AMP Levels and Adenylate Cyclase and Phosphodiesterase Activities in the Rat Corpus Striatum. Mech. Ageing Dev. 6: 53-58.
- Rabin, R.A., and P.B. Molinoff (1983) Multiple Sites of Action of Ethanol on Adenylate Cyclase. J. Pharmacol. Exp. Ther. 227: 551-556.
- Rabin, R.A., B.B. Wolfe, M.D. Dibner, N.R. Zahniser, C. Melchior, and P.B. Molinoff (1980) Effects of Ethanol Administration and Withdrawal on Neurotransmitter Receptor Systems in C57 Mice. J. Pharmacol. Exp. Ther. 213: 491-496.
- Reggiani, A., M.L. Barbaccia, P.F. Spano, and M. Trabucchi (1980) Dopamine Metabolism and Receptor Function After Acute and Chronic Ethanol. J. Neurochem. 35: 34-37.
- Rimon, G., E. Hanski, S. Braun, and A. Levitzki (1978) Mode of Coupling Between Hormone Receptors and Adenylate Cyclase Elucidated by Modulation of Membrane Fluidity. Nature 276: 394-396.
- Roth, G.S. (1982) Altered Mechanisms of Opiate and Dopaminergic Action During Aging. In Mechanisms of Aging in the Nervous System, E. Giacobini et al. (ed.), Raven Press, New York.
- Rottenberg, H., A. Waringa and E. Rubin (1981) Tolerance and Cross-Tolerance in Chronic Alcoholics: Reduced Membrane Binding of Ethanol and Other Drugs. Science 213: 583-585.
- Rubin, E., and H. Rottenberg (1983) Ethanol and Biological Membranes: Injury and Adaptation. Pharmacol. Biochem. Behav. 18 (Suppl. 1): 7-13.
- Samuel, D., D.S. Heron, M. Hershkowitz, and M. Shinitzky (1982) Aging, Receptor Binding, and Membrane Microviscosity. In The Aging Brain: Cellular and Molecular Mechanisms of Aging in the Nervous System, E. Giacobini et al. (ed.) Raven Press, New York, 93-97.
- Sandermann, H. (1978) Regulation of Membrane Enzymes by Lipids. Biochim. Biophys. Acta 515: 209-237.

- Schmidt, M.J., and J.F. Thornberry (1978) Cyclic AMP and Cyclic GMP Accumulation In Vitro in Brain Regions of Young, Old, and Aged Rats. Brain Res. 139: 169-177.
- Schroeder, F. (1984) Fluorescent Sterols: Probe Molecules of Membrane Structure and Function. Prog. Lipid Res. 23: 97-113.
- Seeman, P., and S. List (1981) Dopamine Receptors and Dopaminergic Supersensitivity. In Advances in Pharmacology and Therapeutics II, Vol. 2, H. Yoshida, et al. (ed.), Pergamon Press, New York, 41-49.
- Seeman, P., C. Ulpian, D. Grigoriadis, I. Pri-Bar, and O. Buchman (1985) Conversion of Dopamine D₁ Receptors from High to Low Affinity for Dopamine. Biochem. Pharmacol. 34: 151-154.
- Severson, J.A., and C.E. Finch (1980) Reduced Dopaminergic Binding During Aging in the Rodent Striatum. Brain Res. 192: 147-162.
- Shinitzky, M. (1984) Peer Commentary on Schroeder's Review: Physiological Consequences of Membrane Ageing. Neurobiol. Aging 5: 334-336.
- Shinitzky, M., and Y. Barenholz (1978) Fluidity Parameters of Lipid Regions Determined by Fluorescence Polarization. Biochim. Biophys. Acta 515: 367-394.
- Shinitzky, M., D.S. Heron, and D. Samuel (1983) Restoration of Membrane Fluidity and Serotonin Receptors in the Aged Mouse Brain. In Aging of the Brain D. Samuel et al. (ed.), Raven Press, New York, 329-336.
- Slavik, J. (1982) Anilinonaphthalene Sulfonate as a Probe of Membrane Composition and Function. Biochim. Biophys. Acta 694: 1-25.
- Slotkin, T.A., S.M. Schanberg, and C.M. Kuhn (1980) Synaptic Development in Brains of Rats Exposed Perinatally to Ethanol. Experientia 36: 1005-1006.
- Smith, R.L., and E. Oldfield (1984) Dynamic Structure of Membranes by Deuterium NMR. Science 225: 280-288.
- Smith, T.L., and M.J. Gerhart (1982) Alterations in Brain Lipid Composition of Mice Made Physically Dependent to Ethanol. Life Sci. 31: 1419-1425.

- Spiegel, A.M., P. Gierschik, M.A. Levine, and R.W. Downs (1985) Clinical Implication of Guanine Nucleotide-Binding Proteins as Receptor-Effector Couplers. New Engl. J. Med. 312: 26-33.
- Steiner A.L., C.W. Parter, and D.M. Kipnis (1972) Radio-immunoassay for Cyclic Nucleotides. I. Preparation of Antibodies and Iodinated Cyclic Nucleotides. J. Biol. Chem. 247: 1106-1113.
- Steiner, A.L., A.S. Pagliara, L.R. Chase, D.M. Kipnis (1972) Radioimmunoassay for Cyclic Nucleotides. II. Adenosine 3',5'-Monophosphate and Guanosine 3',5'-Monophosphate in Mammalian Tissue and Body Fluids. J. Biol. Chem. 247: 1114-1120.
- Stubbs, C.D. (1983) Membrane Fluidity: Structure and Dynamics of Membrane Lipids. Essays in Biochemistry 19: 1-39.
- Stubbs, C.D., and A.D. Smith (1984) The Modification of Mammalian Membrane Polyunsaturated Fatty Acid Composition in Relation to Membrane Fluidity and Function. Biochim. Biophys. Acta 779: 89-137.
- Sweadner, K.J. (1979) Two Molecular Forms of (Na^+K^+) -Stimulated ATPase in Brain. Separation and Difference in Affinity for Strophanthidin. J. Biol. Chem. 254: 6060-6067.
- Tabakoff, B., and P.L. Hoffman (1979) Development of Functional Dependence on Ethanol in Dopaminergic Systems. J. Pharmacol. Exp. Ther. 208: 216-222.
- Thal, L.J., S.G. Horowitz, B. Dvorkin, and M.H. Makman (1980) Evidence for Loss of Brain [3H]-Spiroperidol and [3H]-ADTN Binding Sites in Rabbit Brain with Aging. Brain Res. 192: 185-194.
- Toffano, G., C. Aldinio, M. Balzano, A. Leon, and G. Savoini (1981) Regulation of GABA Receptor Binding to Synaptic Plasma Membrane of Rat Cerebral Cortex: The Role of Endogenous Phospholipids. Brain Res. 222: 95-102.
- Trabucchi, M., P.F. Spano, S. Govoni, F. Riccardi, and A. Bosio (1982) Dopaminergic Function During Aging in Rat Brain. In The Aging Brain: Cellular and Molecular Mechanisms of Aging in the Nervous System, E. Giacobini et al. (ed.), Raven Press, New York, 195-201.

- Trendelenburg, U. (1981) Kinetic Analysis of the Neuronal and Extraneuronal Uptake and Metabolism of Catecholamines. In Neurotransmitters Receptors, H. Yoshida et al. (ed.), Pergamon Press, New York.
- Van Blitterswijk, W.J., R.P. Van Hoeven, and B.W. Van der Meer (1981) Lipid Structural Order Parameters (Reciprocal of Fluidity) in Biomembranes Derived from Steady-State Fluorescence Polarization Measurements. Biochim. Biophys. Acta 644: 323-332.
- Waldman, S.A., R.M. Rapaport, R.R. Fiscus, and F. Murad (1985) Effects of Atriopeptin on Particulate Guanylate Cyclase from Rat Adrenal. Biochim. Biophys. Acta 845: 298-303.
- Weber, G., and E. Daniel (1966) Cooperative Effects in Binding to Bovine Serum Albumin. II. The Binding of 1-Anilino-8-naphthalenesulfonate. Polarization of the Ligand Fluorescence and Quenching of the Protein Fluorescence. Biochemistry 5: 1900-1907.
- Whetton, A.D., L.M. Gordon, and M.D. Houslay (1983) Elevated Membrane Cholesterol Concentrations Inhibit Glucagon-Stimulated Adenylate Cyclase. Biochem. J. 210: 437-449.
- Wong, M., F.H. Anthony, T.W. Tillack, and T.E. Thompson (1982) Fusion of Dipalmitoylphosphatidylcholine Vesicles at 4°C. Biochemistry 21: 4126-4132.
- Yoshikawa, T. (1968) The Brain of the Cattle. In Atlas of the Brains of Domestic Animals, University of Tokyo Press, Tokyo.
- Zilversmit, D.B. (1983) Lipid Transfer Proteins: Overview and Applications. Methods Enzymol. 98: 565-573.
- Zimmerman, I.D., and A.P. Berg (1975) Phosphodiesterase and Adenyl Cyclase Activities in the Cerebral Cortex of the Aging Rat. Mech. Ageing Dev. 4: 89-96.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 11, 1987

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