TRICYCLIC ANTIDEPRESSANT AND BETA-ADRENERGIC ANTAGONIST INTERACTIONS WITH BIOLOGICAL AND MODEL MEMBRANES

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by

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ABSTRACT

Tricyclic antidepressants and beta-adrenergic antagonists have been shown to alter lymphocyte function in vitro and in vivo. These studies were designed to characterize tricyclic antidepressant and beta-adrenergic drug:cell membrane interactions that may be related to drug-induced alterations of lymphocyte function and growth.

A single population of specific, high affinity tricyclic antidepressant (desipramine) binding sites has been identified on murine splenic lymphocyte membrane fragments. The binding is saturable, competitive, reversible, and does not display interactions characteristic of cooperativity. There are approximately 300 binding sites per cell and the apparent affinity constant (K_d) for the binding site is approximately 0.4 nM. The presence of a specific tricyclic antidepressant binding site on the lymphocyte suggests a possible mechanism through which these compounds may alter lymphocyte function.

The tricyclic antidepressants (desipramine, imipramine, nortriptyline, and protriptyline) have been shown to inhibit lymphocyte mitogenesis in a concentration- and time- dependent manner. Concentrations of tricyclic antidepressants which inhibit lymphocyte mitogenesis half maximally (approximately 10 μM), however, are much greater than the apparent K_d for the specific tricyclic antidepressant binding site. These results indicate that tricyclic antidepressant induced inhibition of lymphocyte mitogenesis is not mediated by the specific tricyclic antidepressant binding site.

In a manner similar to the tricyclic antidepressants, the beta-adrenergic antagonists (propranolol, alprenolol, metoprolol, and sotalol) inhibit lymphocyte mitogenesis at concentrations much greater than the apparent K_d for the specific beta-adrenergic binding site on the lymphocyte. The beta-adrenergic antagonists alter (as determined by fluorescence spectroscopy in this study) lymphocyte membrane structure at concentrations that are similar to beta-adrenergic antagonist concentrations that inhibit lymphocyte mitogenesis. In addition, beta-adrenergic antagonist induced alterations of membrane structure and inhibition of lymphocyte mitogenesis parallel the apparent lipophilicity of beta-adrenergic antagonists. Beta-adrenergic antagonist-induced alterations of membrane structure were found to occur without apparent alterations in cell surface receptor mobility or membrane permeability changes.

Fluorescence spectroscopic techniques have also been used to demonstrate tricyclic antidepressant-induced perturbations of lymphocyte membranes in a concentration-, time-, and temperature-dependent manner. The concentrations of tricyclic antidepressants that perturb lymphocyte membranes are similar to tricyclic antidepressant concentrations that inhibit lymphocyte mitogenesis. Since the membrane perturbations were suspected to originate in the phospholipid bilayer, liposomes were used to characterize tricyclic antidepressant drug interactions with single and double component phospholipid bilayers. Tricyclic antidepressant-induced alterations in two component liposomes can be produced at tricyclic antidepressant concentrations that inhibit lymphocyte mitogenesis

and perturb lymphocyte membranes.

In conclusion, tricyclic antidepressants have been shown to interact with specific tricyclic antidepressant binding sites on the murine splenic lymphocyte. The tricyclic antidepressants also inhibit lymphocyte mitogenesis but at concentrations much greater than the K_d for the specific binding site. These results suggest that tricyclic antidepressant-induced inhibition of lymphocyte mitogenesis is not mediated by the specific binding site. Results from fluorescent spectroscopic studies indicate that the beta-adrenergic antagonist and tricyclic antidepressant concentrations that perturb lymphocyte membrane structure are similar to the beta-adrenergic antagonist and tricyclic antidepressant concentrations that inhibit lymphocyte mitogenesis. Further, beta-adrenergic antagonist lipophilicity is related to beta-adrenergic antagonist alterations of lymphocyte mitogenesis and lymphocyte membrane structure. Also perturbations of the phospholipid bilayer of liposomal membranes by tricyclic antidepressants occur at tricyclic antidepressant concentrations that alter lymphocyte mitogenesis and lymphocyte membrane structure. The results of these studies demonstrates the importance of nonspecific drug:cell membrane interactions which may be important in the determination of the biological activity of these two classes of compounds.

INTRODUCTION AND STATEMENT OF PURPOSE

INTRODUCTION

Understanding the basic interactions between drugs and cell membranes is of considerable interest and importance in pharmacology. While many drugs have specific sites of action on the cell membrane, receptors, many drugs are capable of altering membrane properties or function through non-specific or non-receptor mediated mechanisms. In this regard, it is relevant to determine how a cell recognizes the wide variety of drugs to which it may be exposed, and how membrane-dependent interactions might result in the alteration of cell function.

The tricyclic antidepressants and the beta-adrenergic antagonists are frequently used therapeutic agents. In general, agents within these drug classes interact with cell membranes through both specific binding sites and non-specific mechanisms. The pharmacology of the specific and non-specific tricyclic antidepressant drug:cell membrane interactions is not clear. In contrast, the specific mechanism of beta-adrenergic antagonist:cell membrane interactions (beta-adrenergic receptor blockade) has been clearly defined. Yet, like the tricyclic antidepressants, the nature of the non-specific beta-adrenergic antagonist:cell membrane interactions remain poorly characterized. The fact that both tricyclic antidepressants and beta-adrenergic antagonists alter lymphocyte responsiveness *in vivo* and *in vitro* is relevant to pharmacology and immunology. It is of interest, then, to characterize previously undefined specific and non-specific tricyclic antidepressant and beta-adrenergic antagonist interactions

with the cell membrane of the lymphocyte.

To provide the background upon which this dissertation is based, the remainder of the introduction will include general overviews of the following subjects: 1) pharmacology of the tricyclic antidepressants; 2) pharmacology of the beta-adrenergic antagonists; 3) the lymphocyte and neurohumoral receptors; 4) model membranes; and 5) probes for investigating drug-membrane interactions. The statement of purpose concludes the introductory portion of this dissertation.

Pharmacology of the Tricyclic Antidepressants

The first tricyclic antidepressant introduced as a clinically useful antidepressant was imipramine (Kuhn, 1958). Development of the tricyclic antidepressants as antidepressants emerged from studies in the 195Ø's demonstrating that reserpine could produce clinical depression in normal persons (Hollister, 1983). Since reserpine depleted biogenic amines in nerve terminals, it was postulated that depleted amine levels in the brain might be related to depression. Clinical research was then directed at developing drugs that increased amine levels in the brain. Subsequently, Kuhn (1958) discovered that a structural derivative of the phenothiazine, promazine, and which is now known as imipramine, was effective for treatment of endogenous depression.

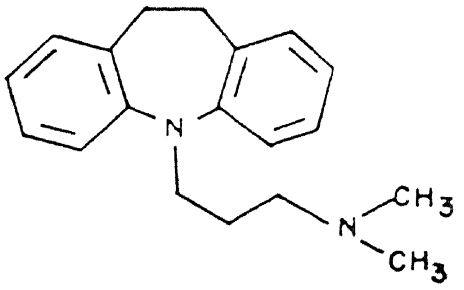
The therapeutic effectiveness of imipramine for depression has led to the synthesis of chemically related drugs that are commonly used clinically in the United States. Imipramine, a dibenzazepine, its major metabolite, desipramine, nortriptyline (an active

metabolite of amitriptyline), a dibenzocycloheptadiene, and protriptyline, a dibenzoxepine, have been selected as representative tricyclic antidepressants for use in the experimental studies of this dissertation (Table I). Imipramine possesses a tertiary amine group on its aliphatic side chain, while desipramine, nortriptyline, and protriptyline possess secondary amine groups on the aliphatic side chain. Geometric isomers for these compounds do not exist because of the abscence of a center of asymmetry in their chemical structures (Baldessarini, 1980).

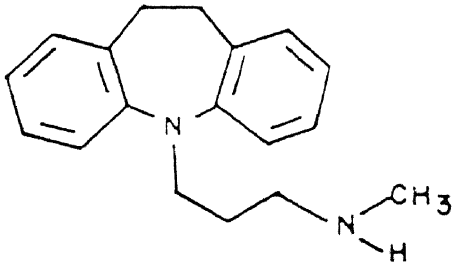
Molecular models of the tricyclic antidepressants reveal that the most efficacious compounds for treating endogenous depression are those compounds with a large angle between the planes of the aromatic rings (Maxwell et al., 1969). For example, the angle between the planes of the phenyl rings or angle of flexure for imipramine is approximately 55°, in contrast to 25° for chlorpromazine, a structurally similar compound but of little use clinically for treatment of endogenous depression (Finch, 1975). Also in contrast to phenothiazine related compounds, the tricyclic antidepressants retain biological activity when the N-termninal is separated from the ring nucleus by only two carbons (Baldessarini, 1980). Due to the pK_a of the amine group in the aliphatic side chains of these compounds (9.5 to 10.5), at pH 7.4, >90% of the drugs exist in a protonated, positively charged form. The charged form of the tricyclic antidepressants is probably the active chemical species in the blockade of amine uptake mechanisms (Maxwell et al., 1969). Substitution in the aromatic rings reduces

TABLE I

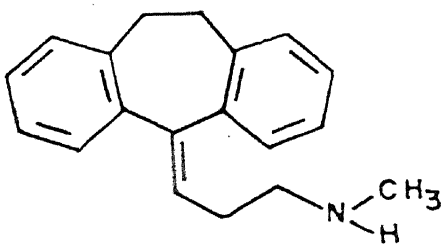
Tricyclic Antidepressant Structures.



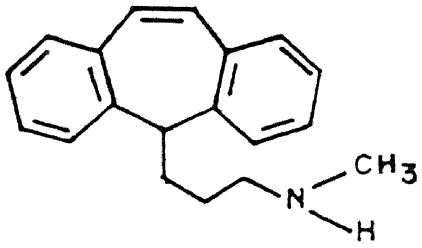
Imipramine



Desipramine



Nortriptyline



Protriptyline

biological activity (Finch, 1975). Unfortunately, the structure-activity relationships of the tricyclic antidepressants are not well understood and the above generalizations may not be applicable for a given compound (Baldessarini, 1980; Finch, 1975).

The tricyclic antidepressants have strong lipophilic properties which have been characterized by partitioning in immiscible organic solvent-water systems. Imipramine, desipramine, and nortriptyline have log octanol-water partition coefficients ranging from 4.6 to 4.9 (Frisk-Holmberg and van der Kleijn, 1972; Leo et al., 1971). The log octanol-buffer (pH 7.0) partition coefficients for imipramine, desipramine, nortriptyline, and protriptyline are also quite similar, ranging from about 1.5 to 2.3 (Frisk-Holmberg and van der Kleijn, 1972). This lipophilic character may contribute to the biological properties of the tricyclic antidepressants. For instance, a significant correlation between tricyclic antidepressant drug lipophilicity, based upon partition coefficients, and histamine release from rat mast cells has been reported (Frisk-Holmberg and van der Kleijn, 1972).

The amine hypothesis of depression is based on the association of depression with decreased functional aminergic transmission (Garver and Davis, 1979). For a tricyclic antidepressant to be effective in treatment of depression, it is considered essential that neuronal uptake of monoamines is inhibited (Kostowski, 1981). Some pharmacological evidence supports these statements. In fact, at least two types of specific tricyclic antidepressant binding sites have been associated with the inhibition of the neuronal

uptake of norepinephrine and serotonin in the brain and periperal tissues (Langer and Raisman, 1983).

Recently, the hypothesis that the tricyclic antidepressant binding site may be related to the therapeutic benefit of these compounds has been subjected to some criticism. Sulser (1982) has outlined a number of reasons why the amine hypothesis is inadequate for describing affective disorders. These reasons include: 1) inhibition of neuronal amine uptake by tricyclic antidepressants occurs immediately, yet, therapeutic effects are not achieved for days or weeks; 2) clinical responsiveness to tricyclic antidepressants is seldom uniform despite a uniform inhibition of neuronal amine uptake; 3) other effective antidepressants (iprindole and mianserin) do not block neuronal amine uptake; 4) cocaine, which also blocks norepinephrine uptake, is not an effective antidepressant; 5) the failure of other compounds such as amphetamine, which possesses properties similar to both MAO inhibitors and tricyclic antidepressants, to reverse depression; and 6) some treatments effective in depression also decrease amine levels in the nerve synapses, a finding that is contradictory to the amine hypothesis (Sulser, 1982). Results from recent experimental studies also indicate that there is no correlation between tricyclic antidepressant binding to the specific tricyclic antidepressant binding site and the blockade of neuronal amine uptake (Wood et al., 1983; Laduron et al., 1982). A more appealing mode of action for the tricyclic antidepressants may be the down-regulation of certain neurotransmitter receptors (Sulser, 1982).

Further studies are required to elucidate the true significance of the tricyclic antidepressant binding site.

Despite the lack of knowledge about the mechanism of action of the tricyclic antidepressants, they have an established clinical usefulness in the treatment of endogenous depression, enuresis, chronic pain, and less frequently in obsessive-compulsive-phobic states, school phobia in children, minimal brain damage and hyperkinesis in children, cataplexy associated with narcolepsy, and acute panic attacks (Hollister, 1983). In general, among the tricyclic antidepressants, there is little difference in efficacy although individual differences may occur. Major adverse effects are related to the anticholinergic activity of the tricyclic antidepressants and include classical anticholinergic signs such as dry mouth, constipation, loss of visual accommodation, and urinary hesitancy. Cardiovascular effects are common and include palpitation, tachycardia, arrhythmias, orthostatic hypotension, and sudden death. Other adverse effects include sedation, confusion, weight gain, sexual disturbances, agranulocytosis, and seizures (Hollister, 1983).

Plasma levels of tricyclic antidepressants approach, for example, 2.5 μM for imipramine (Perel et al., 1976). These drugs are, however, are extensively bound to plasma protein (>90%) and to the constituents of tissues and therefore may accumulate in the tissues (Baldessarini, 1980; Borga et al., 1969). A more recent report demonstrates that concentrations of tricyclic antidepressants in the red blood cells of depressed individuals undergoing tricyclic

antidepressant therapy are up to six times higher than the plasma concentration of tricyclic antidepressants (Linnoila et al., 1978). Similarly, a relationship between the tricyclic antidepressant levels in the brain tissues and tricyclic antidepressant plasma levels exists. The ratio of brain tissue tricyclic antidepressant concentration to plasma tricyclic antidepressant concentration is 40:1 to 8:1 with increasing plasma tricyclic antidepressant concentration (Glotzbach and Preskorn, 1982).

Tricyclic antidepressants are metabolized by two major routes: 1) transformation of the ring nucleus and 2) alteration of the aliphatic side chain. Hydroxylation of the tricyclic ring nucleus (at the two or ten position depending upon the compound) followed by conjugation with glucuronic acid occurs by the first route, and monodemethylation of tertiary amines on the aliphatic side chain occurs by the second route. Active metabolites from both routes have been described (Hollister, 1983). The half-lifes of these compounds range from about 9-24 hours for imipramine to an extreme of 54-198 hours for protriptyline (Hollister, 1983).

Pharmacology of the Beta-Adrenergic Antagonists

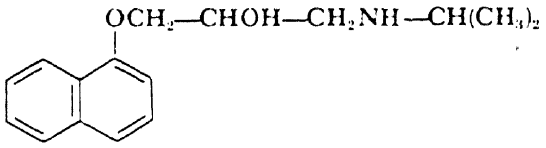
The clinical importance of the beta-adrenergic receptor in the cardiovascular disorders such as angina pectoris and arrhythmias has resulted in the development of a number of beta-adrenergic receptor antagonists. Although dichloroisoproterenol in 1957 (Powell and Slater, 1958), was the first beta-adrenergic antagonist discovered, propranolol, first introduced and approved for use in 1968, has become the most important beta-adrenergic antagonist in clinical

medicine (Goth, 1981; Weiner, 1980). A number of beta-adrenergic antagonists have since been introduced for clinical use and vary from propranolol in their relative effects on cardiac and bronchial beta receptors, intrinsic sympathomimetic activity, and membrane depressant activity (Goth, 1981; Weiner, 1980). The following beta-adrenergic antagonists were selected for use in the experimental studies of this dissertation: propranolol, alprenolol, metoprolol, and sotalol (Table II).

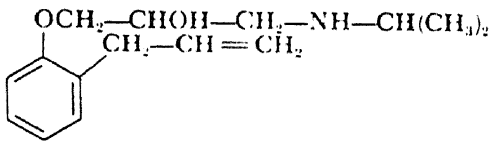
The most effective beta-adrenergic antagonists resemble in chemical structure, the beta-adrenergic agonist isoproterenol. An aliphatic side chain with an isopropyl or bulkier substituent on the amine group favors interactions with beta adrenergic receptors. The type of substituents on the aromatic ring determines whether or not the compound will be an antagonist or agonist, and the cardioselective properties of the agent (Weiner, 1980). For example, propranolol, alprenolol, and sotalol are non-selective beta-adrenergic antagonists while metoprolol is selective for beta receptors of the heart (beta-1 receptors). An aliphatic hydroxyl group which is required for activity, also gives the drug molecule optical activity. The levorotatory (l) form of both beta-adrenergic agonists and antagonists is much more potent than the dextrorotatory (d) form with regard to drug interactions with the beta receptor (Weiner, 1980). The optical activity of the beta-adrenergic agents can be used to distinguish between receptor mediated effects and non-specific effects of these compounds. The d- and l- forms of propranolol for instance, are approximately equal in potency as

TABLE II

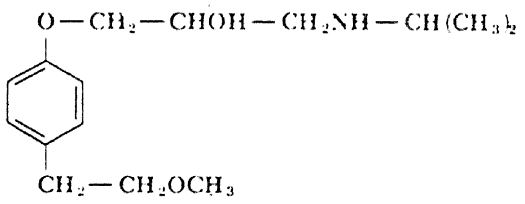
Beta-Adrenergic Antagonist Structures.



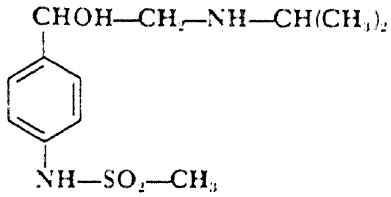
Propranolol



Alprenolol



Metoprolol



Sotalol

local anesthetics, however, d-propranolol is only about 1% as effective as l-propranolol in blocking beta-adrenergic receptors (Barrett and Cullum, 1968).

Beta-adrenergic antagonists competitively antagonize the interactions of catecholamines released from adrenergic neurons or the adrenal medulla with beta receptors. As a result of beta-receptor blockade, these drugs exert negative chronotropic and inotropic effects on the heart, slow atrioventricular conduction, promote bronchoconstriction, lower plasma renin activity, and may produce hypoglycemia (Goth, 1981). Some of these drugs may also have a quinidine-like or depressant effect on the heart. More specifically, the therapeutic effectiveness of propranolol in some arrhythmias, for example, may be the result of both beta-adrenergic receptor blockade and non-specific membrane depressant activity (Singh and Jewitt, 1974; Kelliher and Roberts, 1974).

The non-specific membrane depressant effects of the beta-adrenergic antagonists are positively correlated to liposolubility (Dax and Partilla, 1982; Woods and Robinson, 1981; Hellenbrecht et al., 1973). Characteristic log octanol-buffer (pH 7.0) partition coefficients for propranolol, 5.4, alprenolol, 3.27, sotalol, 0.011 (Hellenbrecht et al., 1973), and metoprolol, 0.15 (Woods and Robinson, 1981), agree favorably with the relative local anesthetic or membrane depressant effects of these compounds (Hellenbrecht et al., 1973). Further, propranolol and lidocaine are equipotent as local anesthetics (Weiner, 1980). Expressed relative to propranolol (1.0), the potency of the beta-adrenergic receptor antagonism ranges

from Ø.5 - 2.Ø for metoprolol, Ø.3 - 1.Ø for alprenolol, and Ø.3 for sotalol. Therefore, agents possessing less lipophilicity are not less effective as beta-adrenergic receptor antagonists (Weiner, 198Ø).

Common adverse effects of the beta-adrenergic antagonists are referrable to the cardiovascular and respiratory systems, and alterations in metabolism (Weiner, 198Ø). When contractility of the heart is impaired, beta-adrenergic antagonists may produce bradycardia and reduced cardiac output precipitating congestive heart failure. Beta-adrenergic antagonists may also produce sensitivity to insulin and oral hypoglycemics. This is due to the presence of beta-adrenergic receptors which mediate glycogenolysis in muscle and, in some species, glycogenolysis in the liver. In patients with comprimised pulmonary function, bronchoconstriction is a common side effect of beta-2 and non-selective beta-adrenergic antagonists (Weiner, 198Ø). The central nervous system effects of beta-adrenergic antagonists are not common but include dizziness, depression, hallucinations, nightmares, lassitude, and insomnia (Weiner, 198Ø).

The plasma concentrations of beta-adrenergic antagonists required for control of certain arrhythmias may be quite high. Concentrations of d- and l- propranolol, and alprenolol necessary to control ventricular arrhythmias, for instance, may approach 5 μM (Courtney, 198Ø; Woosley et al., 1977) and 2Ø μM (Courtney, 198Ø), respectively. Similar to other lipophilic compounds that are also extensively bound to protein, propranolol, for example may

accumulate in tissues such as the heart (Pruett et al., 1980) and brain (Meyers et al., 1975). In the heart, a 40-fold accumulation of propranolol has been reported (Pruett et al., 1980), while a 5 to 30-fold accumulation in the brain has been reported (Meyers et al., 1975). A positive correlation, however, exists between the logarithm of the plasma concentration of beta-adrenergic antagonists and beta-adrenergic receptor blockade. A correlation between beta-adrenergic antagonist plasma concentration and therapeutic effects is harder to establish and may be attributable to interindividual variation in plasma protein binding and metabolism (Johnsson and Regardh, 1976).

To some extent, the lipophilicity of the beta-adrenergic antagonists is correlated with the extent to which they are metabolized. Highly lipophilic beta-adrenergic antagonists like propranolol are almost completely metabolized. In contrast, a less lipophilic beta-adrenergic antagonist like sotalol is relatively unsusceptible to liver metabolism (Johnsson and Regardh, 1976). The protein binding of the beta-adrenergic antagonists (propranolol, 93%; alprenolol, 85%; metoprolol, 12%; and sotalol, little or none) also follows the same order as the relative lipophilicity of these agents (see above). The elimination half-lifes for these compounds are approximately the same ranging from two to four hours for propranolol, alprenolol, and metoprolol, and from five to six hours for sotalol. These pharmacokinetic parameters will vary in hepatic and renal disease states (Johnsson and Regardh, 1976).

Oxidative deamination and, O-dealkylation and oxidation of the

side chain, are two common pathways for metabolic transformation of beta-adrenergic antagonists. In addition, aromatic and aliphatic hydroxylations, and the formation of glucuronic acid conjugates with the secondary alcohol group of the aliphatic side chain, are not uncommon. Generally three to six metabolites for each beta-adrenergic antagonist can be identified, but can include as many as 18 different metabolites, as has been found for propranolol (Johnsson and Regardh, 1976).

The Lymphocyte and Neurohumoral Receptors

The immune system is regulated by a complex interplay of a number of mechanisms including cell-cell interactions, antibodies, lymphokines (Jegosthy, 1978), factors influencing the lymphatic microenvironment (Jankovic and Isakovic, 1973), the complement system, extracellular electrolytes (Freedman, 1979), and a number of other factors such as neurohormones (Mertin and Thompson, 1981; Strom, 1980). Until recently, the role of the central nervous system in the regulation of the immune response has been overlooked. It has been demonstrated that immunocompetence of man and animals is altered in the presence of stress (Riley, 1981) and affective disorders (Kronfol et al., 1983). Studies have also shown that there are alpha-adrenergic (Hadden et al., 1970), beta-adrenergic (Johnson and Gordon, 1980), muscarinic-cholinergic (Gordon et al., 1978), nicotinic-cholinergic (Richman and Arnason, 1979), histaminergic (Rocklin, 1976), and opiate (Hazum et al., 1979) type binding sites on the lymphocyte. Results from experimental studies in in vitro systems would suggest that some of these binding sites

may be considered pharmacological receptors (Gilman et al., 1982; Johnson et al., 1981; Dulis et al., 1979; Hadden et al., 1970). In addition, studies with brain-lesioned animals have demonstrated an apparent relationship between activity of certain structures of the brain, particularly the hypothalamus, and immune function (Mertin and Thompson, 1981; Jankovic and Isakovic, 1973). Collectively, this evidence indicates that there may be a functional relationship between the central nervous system and the immune system. Further, that neurohormones may function as regulators of the immune response through specific neuro-type receptors on the lymphocyte. Based on similar evidence, Golub (1982) and Mertin and Thompson (1981), also proposed the existence of a functional connection between the nervous system and the immune system .

The presence of a specific tricyclic antidepressant binding site in various brain regions (Langer et al., 1981), like the neuroreceptors discussed above, and on non-neuronal cells such as platelets (Briley et al., 1979) and lung (Raisman and Langer, 1983), presents the possibility that there may tricyclic antidepressant binding sites on other non-neuronal cell types such as lymphocytes. A lymphocyte tricyclic antidepressant binding site might then be implicated in reports that imipramine has been observed to produce leukopenia (Leyberg and Denmark, 1959) and reduce elevated rheumatoid factor levels (Haydu et al., 1974) in vivo, and inhibit mitogenesis (Nahas et al., 1979; Smith et al., 1978; Waterfield et al., 1976) and the number of plaque-forming cells (Smith et al., 1978) in vitro. Tricyclic antidepressant-induced alterations of

lymphocyte reponsiveness in in vitro systems have been attributed to the non-specific effects of these drugs although direct experimental evidence supporting this assumption has not been reported. In in vivo studies the possible mechanisms involved in alteration of lymphocyte reponsiveness and function by tricyclic antidepressants have been suggested to be related to membrane ATPase function (Haydu et al., 1974). Direct in vitro studies of lymphocyte ecto-ATPase activity indicate that the tricyclic antidepressants selectively alter the enzyme's activity at high concentrations (.1 to 1 mM) (Medzihradsky et al, 1980).

A beta-adrenergic binding site on the lymphocyte has been identified. Propranolol, for example, binds to the lymphocyte beta-adrenergic receptor with an apparent affinity constant (K_d) of 10 nM (Johnson and Gordon, 1980). The concentrations of propranolol, however, that alter lymphocyte reponsiveness, in vitro, are at least 1000-fold higher (Johnson et al., 1981; Anderton et al., 1981; Montecucco et al., 1981; Moore et al., 1978; Dunne et al., 1978) than the apparent K_d for the beta-adrenergic receptor on the lymphocyte. It is probable, then, that the propranolol binding site is not involved in the alteration of lymphocyte responsiveness at high concentrations. Propranolol's non-specific or local anesthetic properties have been implicated in altering lymphocyte responsiveness and function, although direct evidence in the lymphocyte has not been reported.

The identification of neuro-type binding sites on the lymphocyte is important in the pursuit of an understanding of the

cell:soluble ligand interactions that may be important in altering the immune response. In addition, it is important to consider other cell:soluble ligand interactions, such as non-specific mechanisms, that may provide information about how the lymphocyte recognizes and responds to different pharmacological agents. Furthermore, an understanding of cell:soluble ligand interactions relevant to the lymphocyte may be applicable to cell:soluble ligand interactions in general. Cell biologists have long recognized the lymphocyte as a useful model cell for investigations of membrane phenomena that extends beyond the scope of immunology (Nossal, 1978). It is one of the few mammalian cells which can be isolated easily, incurring little damage in the isolation, do well in culture, and for which a significant amount of information about the cell membrane structure exists (Nossal, 1978; Resch, 1976; Zuckerman and Douglas, 1976). It is for all the reasons above that the lymphocyte was chosen as a suitable cell type for the experimental studies of this dissertation.

Model Membranes

The complex composition of the biological membrane complicates the interpretation of drug-membrane interactions. Specific molecular interactions of drug with the phospholipid bilayer, for example, can not be precisely defined. Phospholipid vesicles, or liposomes, provide a relatively simple model membrane system which one can use to study drug:phospholipid bilayer interactions (Bangham, 1978; Tritton et al., 1977). Liposomes are easy to prepare, the composition in terms of polar head group and lipid is

known and can be selected, and more importantly, the liposome has the same basic phospholipid bilayer structure found in the biological membrane (Bangham, 1978; Tritton et al., 1977; Tyrrell et al., 1976). Liposomes have been used characterize local anesthetic (Papahadjopoulos et al., 1975), opiate (Johnson et al., 1979), tricyclic antidepressant (Romer and Bickel, 1979; Bermejo et al., 1975; Cater et al., 1974; Bermejo et al., 1974), quinidine (Surewicz, 1982), and propranolol (Surewicz et al., 1981) interactions with phospholipid bilayers.

Calorimetry, electron spin resonsance, fluorescence spectroscopy, and nuclear magnetic resonance techniques have been used to study tricyclic antidepressant drug interactions with dipalmitoylphosphatidylcholine bilayers (Romer and Bickel, 1979; Bermejo et al., 1975; Cater et al., 1974; Bermejo et al., 1974). The results of these studies have been interpreted in terms of drug interactions with the surface or polar head groups of the phospholipid bilayers. Fluorescence spectroscopic studies of the ability of these drugs to perturb the structural order of the fatty acid chains of the phospholipid bilayer of liposomes have not been reported. Therefore, large multilamellar liposomes will be used in an experimental study to characterize tricyclic antidepressant drug interactions with the lipid domains of liposomes. The phospholipids chosen for preparation of liposomes in this study will be phosphatidylcholines or lecithins, the most abundant phosphoglycerides in animal cell membranes (Chapman, 1968). Liposomes prepared from these phospholipids are well characterized

(Stubbs et al., 1981; Lentz et al., 1976a; Lentz et al., 1976b; Andrich and Vanderkooi, 1976).

Probes for Investigating of Drug-Membrane Interactions

Presently, the pharmacologist has a number of membrane probes available to explore drug-cell membrane interactions. Obviously, the choice of probe depends upon the nature of the drug-cell membrane interaction to be studied. It is appropriate at this time to discuss the membrane probes applicable to this study that will be used to elicit information about the specific and non-specific tricyclic antidepressant and beta-adrenergic antagonist:cell membrane interactions.

A large contribution to the current understanding of the receptor mediated mechanisms of a wide variety of drugs, hormones, and toxins is due in part to the development of reliable ligand binding assays (Hollenberg and Nexo, 1981). Providing certain criteria are met, precise quantitative data relating to drug-receptor interactions may be obtained. The radio-labeled ligand must: have a high specific radioactivity, be biologically active, be used at a temperature that minimizes ligand and receptor degradation and permits convenient binding equilibration times, be used to study appropriate ligand concentration ranges, and be used in a study utilizing a method of separating free and receptor bound ligand within a time period appropriate for the ligand off-rate (Hollenberg and Nexo, 1981). Two tritium-labeled ligands, desipramine and imipramine, are available and considered appropriate, under the conditions above, for studying tricyclic

antidepressant binding sites (Langer and Raisman, 1983). ^{3}H-Desipramine has been chosen as the radio-labeled ligand for detecting the presence of specific tricyclic antidepressant binding sites on the murine lymphocyte.

The non-specific interactions of tricyclic antidepressants and beta-adrenergic antagonists, similar to those exhibited by local anesthetics, have been attributed to the ability of these compounds to perturb structural integrity of the biological membrane (Roth, 1979; Lee, 1976; Seeman, 1972). More specifically, by perturbing the structural characteristics of the phospholipid bilayer of the cell membrane, these compounds may alter the function of components (receptors, enzymes, and ionophores, for example) residing in the phospholipid bilayer (Maher and Singer, 1984; Roth, 1979; Metcalfe et al., 1974; Sheetz and Singer, 1974; Seeman, 1972). The nature of the mechanism involved in drug-induced membrane perturbations, however, is not clear. At least four possible drug-bilayer interactions have been suggested as possible mechanisms of action for the nonspecific effects of these compounds and include: 1) intercalation of the drugs into the phospholipid bilayer (Seeman, 1972); 2) selective intercalation of the drugs into the two halves of the phospholipid bilayer (Sheetz and Singer, 1974); 3) binding of the drugs to lipid-protein interfaces within the phospholipid bilayer (Metcalfe et al., 1974); and 4) drug-induced rearrangement of lipids and proteins in the plane of the membrane (Maher and Singer, 1984). Direct experimental evidence for tricyclic antidepressant or beta-adrenergic antagonist drug-induced membrane

perturbations, for the most part, have not been reported.

A number of techniques are available for studying membranes including direct techniques such as nuclear magnetic resonance analysis and x-ray diffraction analysis, and indirect techniques such as electron spin resonance and fluorescence spectroscopy (Levine, 1972). The direct techniques are perhaps potentially more useful, however, due to the complexity of biological membrane interpretation of results is difficult. Indirect techniques can provide information that is supplemental to direct techniques (Vanderkooi and McLaughlin, 1976).

Fluorescent probes are useful in the study of membranes because of the selectivity of the probe for certain membrane environments and the sensitivity of the probe to changes in the surrounding microenvironment. When a fluorophore is exposed to radiation of proper energy, quanta of energy are absorbed by the fluorophore causing the electrons to be excited to a higher energy state. The equilibrium excited state lasts approximately Ø.1 to 1 nsec. During the excited state, interactions between the fluorophore and other molecules in the fluorophore's microenvironment affect the characteristic fluorescence parameters of the fluorophore. Fluorescence parameters that are altered by fluorophore-environmental molecule interactions include the spectra of excitation and emission, the lifetime of the excited state, the quantum yield, and the polarization or anisotropy of the fluorescence emission (Vanderkooi and McLaughlin, 1976).

Diphenylhexatriene (Shinitzky and Barenholz, 1974), a highly

lipophilic fluorescent probe has been used to characterize alterations of membrane hydrophobic or lipid domains in the presence of disease (French et al, 1983; Shinitzky and Inbar, 1974; Inbar, 1976), and a number of pharmacologically active agents (Kutchai et al., 1980; Johnston and Melnkovych, 1980; Luly and Shinitzky, 1979; Johnson et al., 1979). Diphenylhexatriene aligns itself parallel to the fatty acid chains in the phospholipid bilayer (Van Blitterswijk et al., 1981; Andrich and Vanderkooi, 1976), but does not, itself, alter membrane structure (Andrich and Vanderkooi, 1976). Deviation of the diphenylhexatriene molecule from the preferential alignment parallel to the fatty acid chains can be determined by change in the fluorescence emission in vertical and horizontal planes. Fluorescence anisotropy is a relative measure of the deviation in the alignment of the diphenylhexatriene molecule relative to the fatty acid chains of the phospholipid bilayer (Lackowicz, 1983; Van Blitterswijk et al., 1981; Andrich and Vanderkooi, 1976). Measurement of the fluorescence anisotropy or polarization of diphenylhexatriene provides information then, about the structural order of the phospholipid bilayer (Lackowicz, 1983; Van Blitterswijk et al, 1981). Further, the fluorescence lifetime of diphenylhexatriene is used to interpret fluorescence anisotropy or polarization changes as alterations of the structural order of the membrane, or as quenching when the fluorescence lifetime is decreased (Lackowicz, 1983).

Diphenylhexatriene distributes preferentially into the phospholipids of the lymphocyte with at least 60% of the

diphenylhexatriene found in the plasma membrane (Johnson and Nicolau, 1977). The fluorescence emission of diphenylhexatriene is greatly enhanced in a hydrophobic environment and there are little, if any, differences in the partitioning of diphenylhexatriene between different lipid phases (Andrich and Vanderkooi, 1976; Lentz et al., 1976b). Based on fluorescence and distribution characteristics, diphenylhexatriene was chosen as an appropriate membrane probe of tricyclic antidepressant and beta-adrenergic antagonist interactions with lymphocyte membranes.

Fluorescein-labeled concanavalin A binds to concanavalin A receptors on the cell surface of lymphocytes (Inbar and Sachs, 1969). The fluorescence polarization of fluorescein-labeled concanavalin A has been used to detect differences in the mobility of cell surface receptors on normal and malignant lymphocytes (Ben-Bassat et al., 1977; Shinitzky and Inbar, 1974; Inbar et al., 1973). Since the mobility of cell surface receptors is probably associated with changes in the dynamic nature of the phospholipid bilayer (Ben-Bassat et al., 1977), the use of fluorescein-labeled concanavalin A should provide information that is complementary to studies of diphenylhexatriene reported membrane perturbations (Shinitzky and Inbar, 1974). It is noted that perturbations of the phospholipid bilayer by pharmacological agents must apparently be quite large before alterations of the mobility of cell surface concanavalin A receptors are observed. For example, the movement of concanavalin receptors to one pole of the lymphocyte (capping) is inhibited by concentrations of propranolol 100-fold higher than propranolol

concentrations that inhibit lymphocyte transformation presumably through membrane perturbations (Anderton et al., 1981).

Dithio-diisopropyl-oxacarbocyanine iodide, a cyanine dye, is a fluorescent probe that useful for monitoring changes in the transmembrane potential of cells (Waggoner, 1979). This cyanine dye molecule possesses a delocalized positive charge and, depending upon the transmembrane potential, partitions between extra- and intracellular compartments and the plasma membrane. The exact mechanism of action is unknown, though it has been suggested that non-fluorescent aggregates form when the dye associates with the plasma membrane. Association of the cyanine dye with the plasma membrane is promoted by hyperpolarization of the transmembrane potential resulting in decreased fluorescence emission. Dissociation of the cyanine dye from the plasma membrane is promoted by depolarization of the transmembrane potential resulting in increased fluorescence emission (Bramhall et al., 1976). The fluorescence emission of this cyanine dye will be used to monitor the resting transmembrane potential of the murine lymphocyte in the presence of different pharmacological agents.

Lectins, proteins or glycoproteins, which stimulate lymphocytes to undergo mitosis and transformation through oligosaccharide receptors on the lymphocyte membranes, can be used as probes of cellular responsiveness (Zuckerman and Douglas, 1976). The ability of lectins or "mitogens" (agents that promote mitosis) to induce normal lymphocyte transformation is considered to be an <u>in vitro</u> correlate of cell mediated immunity (Oppenheim and

Rosenstreich, 1976). Mitogen stimulated lymphocytes in a short-term culture system have also been used as a model system for characterizing the effects of pharmacological agents on the immune response (Strom, 1979). To assess the physiological activity of lymphocyte cell surface-binding sites, for example, the normal mitogen response is determined in the presence of relevant concentrations of the corresponding ligand. This system has been used sucessfully to demonstrate the physiological relevance of opiate (Gilman et al., 1982), nicotinic-cholinergic (Mizuno et al., 1982), beta-adrenergic (Johnson et al., 1981), muscarinic-cholinergic (Dulis et al., 1979), and alpha-adrenergic (Hadden et al., 1970) binding sites.

Mitogen stimulated lymphocytes are of interest in studying the effects of compounds that perturb cell membranes through non-receptor mediated mechanisms. Upon interaction with a mitogen, the lymphocyte undergoes extensive biochemical and morphological alterations. In addition to changes in DNA, RNA, lipid, and protein synthesis, complex changes in the plasma membrane occur. Some of these changes include the stimulation of glycoprotein and phospholipid synthesis and metabolism, uptake of amino acids, sugars, cations, phosphate, and an overall increase in the permeability of the plasma membrane (Zuckerman and Douglas, 1976). Lipophilic agents such as local anesthetics, beta-adrenergic antagonists, and psychotropic drugs, for instance, probably alter lymphocytes responsiveness to mitogen stimulation by perturbing the phospholipid bilayer of the lymphocyte plasma membrane (Montecucco

et al., 1981; Anderton et al., 1981; Nahas et al., 1979). An altered lipid bilayer presumably alters the environment of components (receptors, enzymes, and ionophores) dissolved in that phase to a degree which is incompatible with normal cell activity (Maher and Singer, 1984; Roth, 1979; Metcalfe et al., 1974; Sheetz and Singer, 1974; Seeman, 1972). Experimental evidence from biological membranes to support these assumptions, specifically with regard to tricyclic antidepressants and beta-adrenergic antagonists, has not been reported.

The mitogens, concanavalin A, a polyclonal activator of T lymphocytes, and lipopolysaccharide B, a polyclonal activator of B lymphocytes, have been selected for use in the experimental studies of this dissertation.

Statement of Purpose

The specific and non-specific mechanisms through which tricyclic antidepressants may alter lymphocyte responsiveness in vitro and in vivo have not been determined. Clearly, imipramine is capable of altering lymphocyte responsiveness in vitro (Nahas et al., 1979; Smith et al., 1978; Waterfield et al., 1976) and in vivo (Haydu et al., 1974; Leyberg and Denmark, 1959). Although tricyclic antidepressant binding sites appear to be ubiquitously distributed throughout the body on neuronal and non-neuronal cells, the identification and characterization of a lymphocyte tricyclic antidepressant binding site has not been reported. The presence of a specific tricyclic antidepressant binding site on the lymphocyte could be implicated as a mechanism through which tricyclic

antidepressant drugs might alter the lymphocyte responsiveness. In the first experimental study, the presence of a specific tricyclic antidepressant binding site on the murine lymphocyte will be determined.

In the second experimental study, the ability of four tricyclic antidepressants to alter the normal mitogen response of murine lymphocytes will be assayed. Murine lymphocytes will be stimulated to undergo mitosis by either concanavalin A or lipopolysaccharide B and the concentration- and time- dependency of drug action on lymphocyte mitogenesis determined. The results will be compared to results in the first experimental study to determine if the tricyclic antidepressant binding site on the lymphocyte may have physiological activity.

It is presumed that high liposolubility is associated with the ability of a drug to perturb the phospholipid bilayer's structural order. Therefore it is appropriate to consider the beta-adrenergic antagonists as part of this study based on the large differences in apparent the liposolubility of one beta-adrenergic antagonist as compared to another. The tricyclic antidepressants, in contrast, all have similar apparent liposolubilities. The intent of the third and fourth experimental studies is to relate drug-induced membrane perturbations to drug liposolubility, and to compare the drug-induced membrane perturbations to drug-induced alterations of lymphocyte responsiveness. The two classes of compounds provide a range of different apparent liposolubities.

The third experimental study will involve investigation of the

the ability of beta-adrenergic antagonists to perturb the membranes of the murine lymphocyte. Fluorescent probes will be used to monitor the concentration-, time-, and temperature- dependency of beta-adrenergic antagonist effects on membrane structural order. These experiments will provide experimental support for the assumption that beta-adrenergic antagonist-induced inhibtion of lymphocyte responsiveness may be associated with drug-induced membrane perturbations.

In the fourth experimental study, fluorescent probes will be used to determine the ability of the tricyclic antidepressants to perturb murine lymphocyte membranes. The concentration-, time-, and temperature- dependency of tricyclic antidepressant-induced alterations of membrane structural order will be assayed. These experiments will provide experimental support for the assumption that tricyclic antidepressant effects on membrane structural order can be implicated as a mechanism for tricyclic antidepressant-induced alterations of lymphocyte responsiveness.

Tricyclic antidepressant drug interactions with the liposomal membranes will be characterized in the fifth experimental study. Previous studies, involving a variety of techniques, have examined only desipramine or imipramine effects on dipalmitoyl-phosphatidylcholine or egg-phosphatidylcholine liposomes (Romer and Bickel, 1979; Bermejo et al, 1975; Cater et al., 1974; Bermejo, et al., 1974). The emphasis of these studies was on drug:polar head group interactions. In this study drug:lipid interactions will be characterized using the fluorescent probe diphenylhexatriene.

Electron microscopy will be used to determine the size of the liposomes.

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EXPERIMENTAL STUDIES

PART I

CHARACTERISTICS OF TRICYCLIC ANTIDEPRESSANT BINDING SITES ASSOCIATED WITH MURINE LYMPHOCYTES FROM SPLEEN

ABSTRACT

A single population of high affinity, saturable, tricyclic antidepressant (^{3}H-desipramine) binding sites has been identified on the membranes of murine lymphocytes. There are approximately 300 binding sites per cell and the apparent dissociation constant (K_d) for these binding sites is about 0.4 nM. Competition studies between the radiolabeled ligand in concentrations near the K_d and other members of the class of tricyclic antidepressants indicate that binding is competitive, reversible, and does not display interactions characteristic of cooperativity.

INTRODUCTION

Depression is one of the most common emotional disorders presented to today's physician. Often this disorder consists of one or more syndromes varying in degree of severity and characterized by abnormal, sometimes enduring, changes of affect, and physical alterations (Linn, 1980). Because of their widespread occurrence, the availability and utility of mood elevating drugs in therapy has become quite important. The drugs of choice for treatment of depressive syndromes of moderate severity are members of a class of compounds known as the tricyclic antidepressants (Frazer, 1980). While an exact mechanism of action for members of this class has not yet been resolved, it is currently presumed that the therapeutic action is at least in part a result of the blocking of neuronal uptake of norepinephrine and/or serotonin (Kostowski, 1981). Recently methods which successfully led to the identification of opiate (Pert and Snyder, 1973), benzodiazepine (Braestrup and Squires, 1977; Mohler and Okada, 1977), and neuroleptic (Burt et al., 1976) binding sites, have been employed to demonstrate that specific saturable tricyclic antidepressant binding sites are present on the cellular membranes of rat brain (Raisman et al., 1979; Rehavi et al., 1981; Sette et al., 1981), human brain (Rehavi et al., 1980), and human platelets (Briley et al., 1979; Paul et al., 1980).

This study demonstrates the presence of a single population of tricyclic antidepressant (^{3}H-desipramine) binding sites on the cellular membranes of murine lymphocytes. Johnson et al. (1981)

have shown the murine lymphocyte to be a useful model of cellular responsiveness. Interestingly, the ability of the tricyclic antidepressants to alter lymphocyte responsiveness has not been reported. One clinical study has reported a correlation of autoantibody levels and simultaneous treatment of depression with imipramine (Haydu et al., 1974); however, confirming studies of this nature have not been reported. The presence of tricyclic antidepressant binding sites on murine lymphocytes would provide a possible mechanism by which these drugs might alter the immune response.

MATERIALS AND METHODS

Desmethylimipramine hydrochloride, (2,4,6,8-^{3}H)-, (lot no. 1433-170, 59.6 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. Desipramine hydrochloride was a gift from Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH. Nortriptyline hydrochloride was a gift from Lilly Research Laboratories, Indianapolis IN. Imipramine hydrochloride (lot no. 121F-0086) was obtained from Sigma Chemical Co., St. Louis, MO. All other compounds used were of the highest grade commercially available. Swiss-Webster, Lai:Cox, outbred male mice were purchased from Laboratory Supply Company, Inc., Indianapolis, IN.

Lymphocyte Isolation. This procedure has been described by Johnson and Gordon (1980). Briefly, adult male mice (25-30 gm) were sacrificed, spleens removed and lymphocytes isolated by centrifugation. Erythrocytes were lysed with ammonium chloride and the final pellet resuspended in distilled water. Lymphocyte

membrane fragments were then frozen until used (overnight at -70°C).

Binding Assay. Membrane fragment suspensions were thawed at 37°C and then centrifuged at 250 * g for 15 min (18°C). The resultant pellet was resuspended in 0.05 M Tris-Cl, pH 7.4. A homogenous suspension was prepared by homogenizing with a motor driven teflon pestle (8-10) passes. Absence of intact cells was ascertained by the lack of trypan blue exclusion. Protocol for the binding assay was essentially the same as that used by Johnson and Gordon (1980) and is as follows: 0.25 ml aliquots of the membrane fragment mixture (0.1 - 0.3 mg protein) were placed in 13 x 100 mm test tubes along with appropriate concentrations of competing ligands to give a final volume in each assay tube of 0.3 ml. The assay was initiated by the addition of the radiolabeled ligand to the assay tube. The assay tube was immediately placed in a 37°C water bath with gentle shaking for 15 min. After addition of 1 ml of cold incubation buffer (0.05 M Tris-Cl buffer, pH 7.4) to terminate the assay, contents of the assay tube were rapidly filtered through pre-wetted (with 10^{-5} M desipramine) Whatman GF/B filters. The assay tube was rinsed once with 5 ml of wash buffer (0.01 M phosphate buffered saline, pH 7.4) and each filter washed with 4 x 5 ml of wash buffer. Rapid filtration and washing procedures required approximately 15-20 seconds. Filters were air dryed then placed in 3.5 ml of scintillation fluid (160 ml of Biosolv per 1 liter of LSC Complete, Yorktown Research, Miami, FL) and the tritium assayed in a Packard Tri-Carb scintillation

spectrophotometer (approximately 58% tritium efficiency) to 0.5% accuracy.

Desipramine binding to lymphocyte membrane fragments was determined by addition of $7 * 10^{-11}$ to $9 * 10^{-8}$ M desipramine to assay tubes either containing no competitor or a competitor of a concentration of 10^{-5} M competitor. Specific binding is the difference between total bound and that bound in the presence of 10^{-5} M competitor (nonspecifically bound).

Dissociation Kinetics. Dissociation of desipramine bound to lymphocyte membrane fragments was studied by a dilution technique described by Krawietz et al. (1980), and Johnson and Gordon (1980). A 4 ml aliquot of homogenous mixture of lymphocyte membrane fragments was incubated at 37°C for 15 min in the presence of $5 * 10^{-9}$ M ^{3}H-desmethylimipramine. At times specified after the 15 min incubation, 0.01 ml of the mixture was added to each of duplicate test tubes containing either 10 ml of incubation buffer containing no competitor or 10 ml of incubation buffer that was 10^{-5} M in desipramine. The contents of the dissociation assay tubes were rapidly filtered, washed, and counted in the same manner as the binding assay tubes. A plot of both dilution displacement and dilution displacement in the presence of a competitor was generated. Data points were calculated as a percentage of the total bound in each set and the results plotted as % Bound (log scale) versus Time.

All experiments were performed in duplicate or triplicate. All protein concentrations were determined by a modified Lowry method which included the use of 1% deoxycholate.

RESULTS

The binding of ^{3}H-desipramine to murine lymphocyte membrane fragments is saturable and of high affinity (Figure 1). The concentration of ^{3}H-desipramine required to achieve saturation is approximately 4 nM. Scatchard analysis (Figure 2) indicates a single population of binding sites with an apparent K_d of about .4 nM. The maximal concentration of binding sites is about 50 fmol/mg protein which corresponds to approximately 300 binding sites per cell. Specific binding as defined earlier in the text was usually 10-40% of the total bound.

Competition studies between ^{3}H-desipramine and other unlabeled members of the tricyclic antidepressant class were conducted in the presence of approximately 3 nM ^{3}H-desipramine. The ability to compete for ^{3}H-desipramine binding sites was greatest for desipramine (Figure 3), followed by imipramine (not shown), and nortriptyline (Figure 3). Propranolol (not shown) failed to compete for these binding sites over the same concentration range (10^{-10} to 10^{-6} M). The ability of various tricyclic antidepressants to compete with ^{3}H-tricyclic antidepressants at low concentrations has been observed previously (Table I) by Rehavi et al. (1981).

The reversibility and absence of cooperativity for the binding of ^{3}H-desipramine to murine lymphocyte membrane fragments is shown by the dissociation curves (Figure 4). The dissociation rate constant as determined from the slope is .02 min^{-1}; the calculated association rate constant is $4 * 10^{7}$ M^{-1} min^{-1}.

Figure 1. Specific binding of ^{3}H-desipramine to murine lymphocyte fragments. Approximately Ø.3 mg of membrane protein was incubated with increasing concentrations of ^{3}H-desipramine in a total volume of Ø.3 ml for 15 min at 37°C. Specific binding was determined as described in Materials and Methods. Each point is the mean of duplicate determinations.

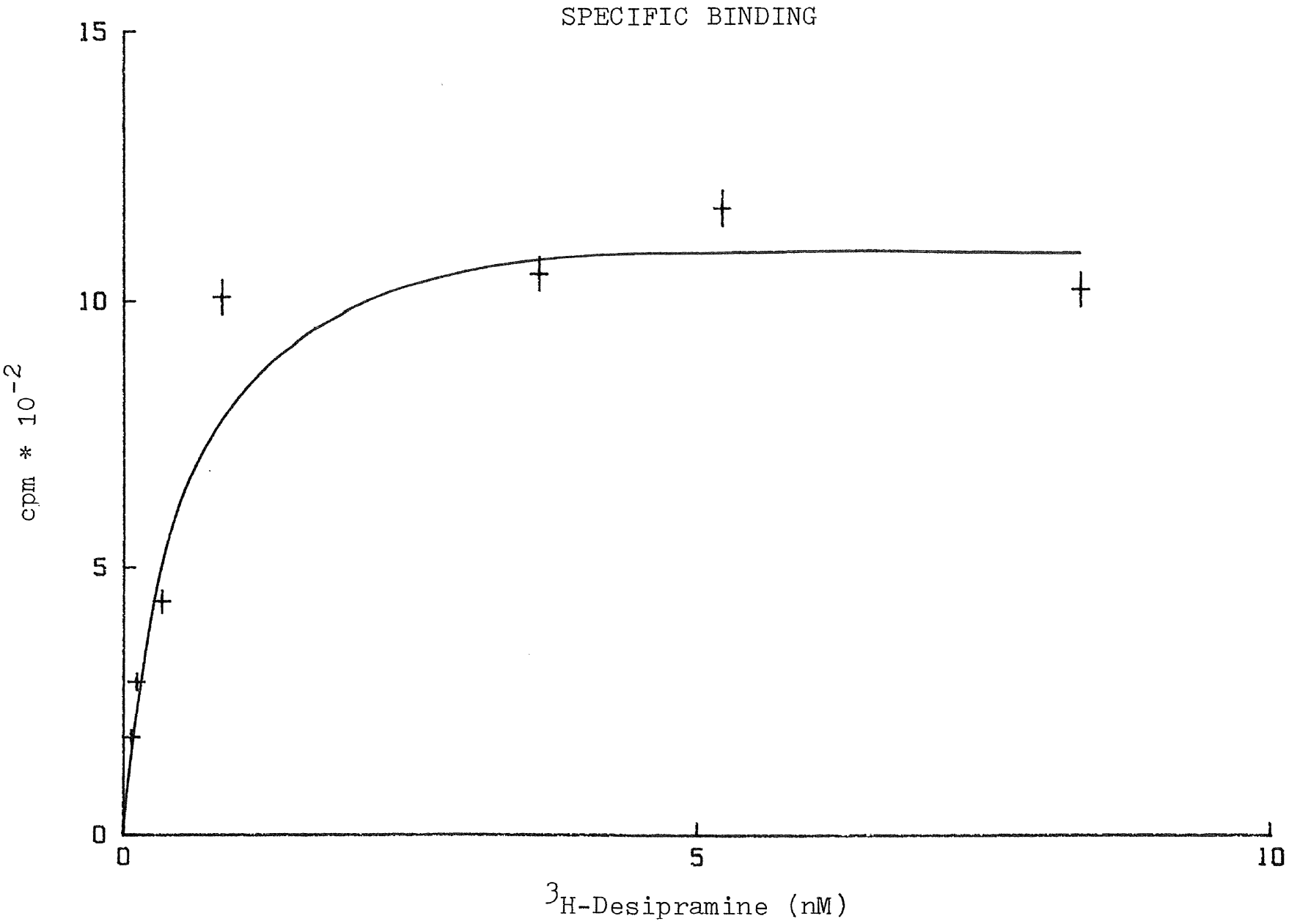

SPECIFIC BINDING
15
10
5
0
cpm * 10^{-2}
0
5
10
^{3}H-Desipramine (nM)

Figure 2. Scatchard Analysis of the specific binding of ^{3}H-desipramine to murine lymphocyte membrane fragments (about Ø.3 mg membrane protein).

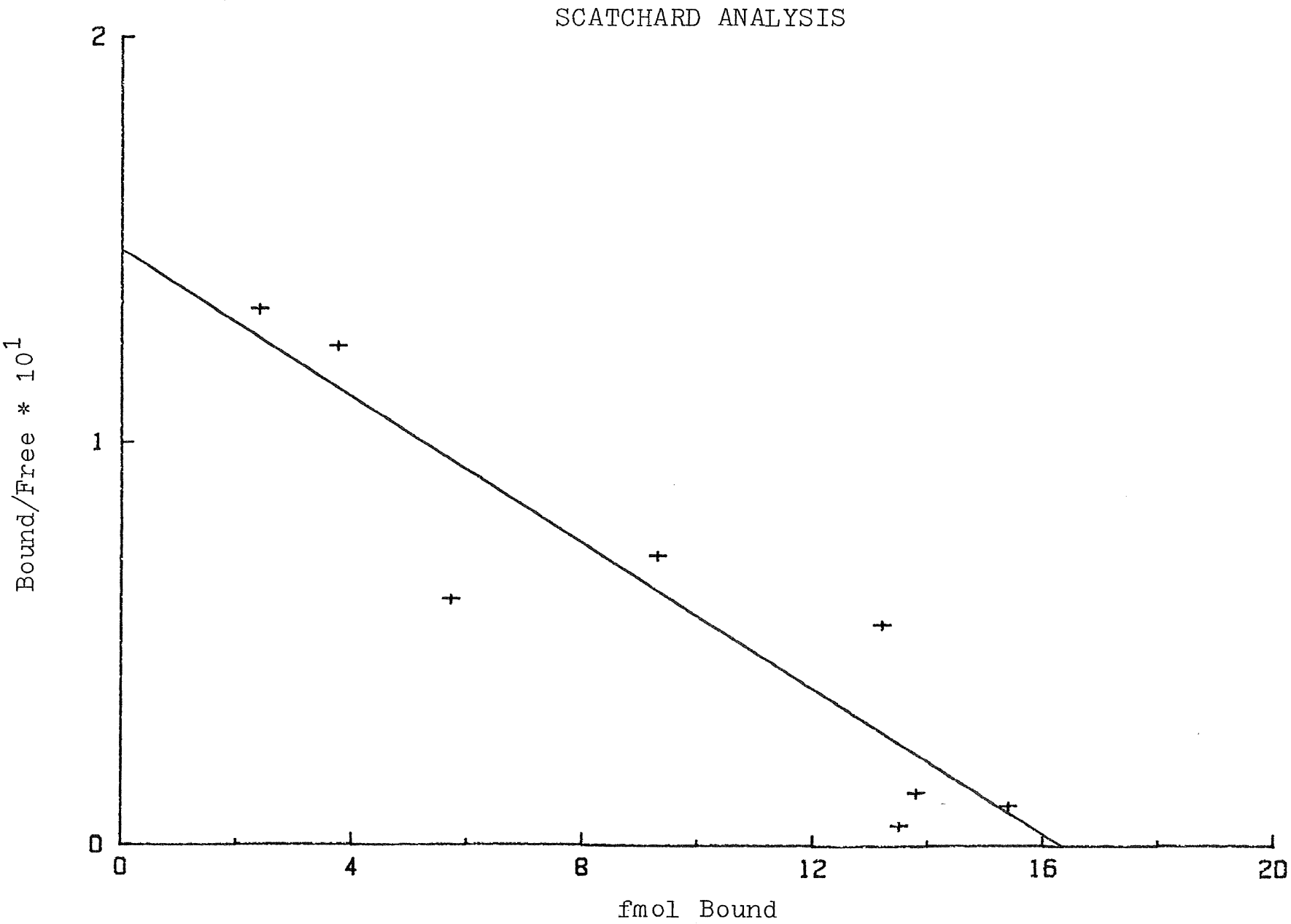

SCATCHARD ANALYSIS
Bound/Free * 10^1
2
1
0
0
4
8
12
16
20
fmol Bound

Figure 3. Effect of desipramine (+) and nortriptyline (o) on the specific binding of ^{3}H-desipramine to murine lymphocyte membrane fragments.

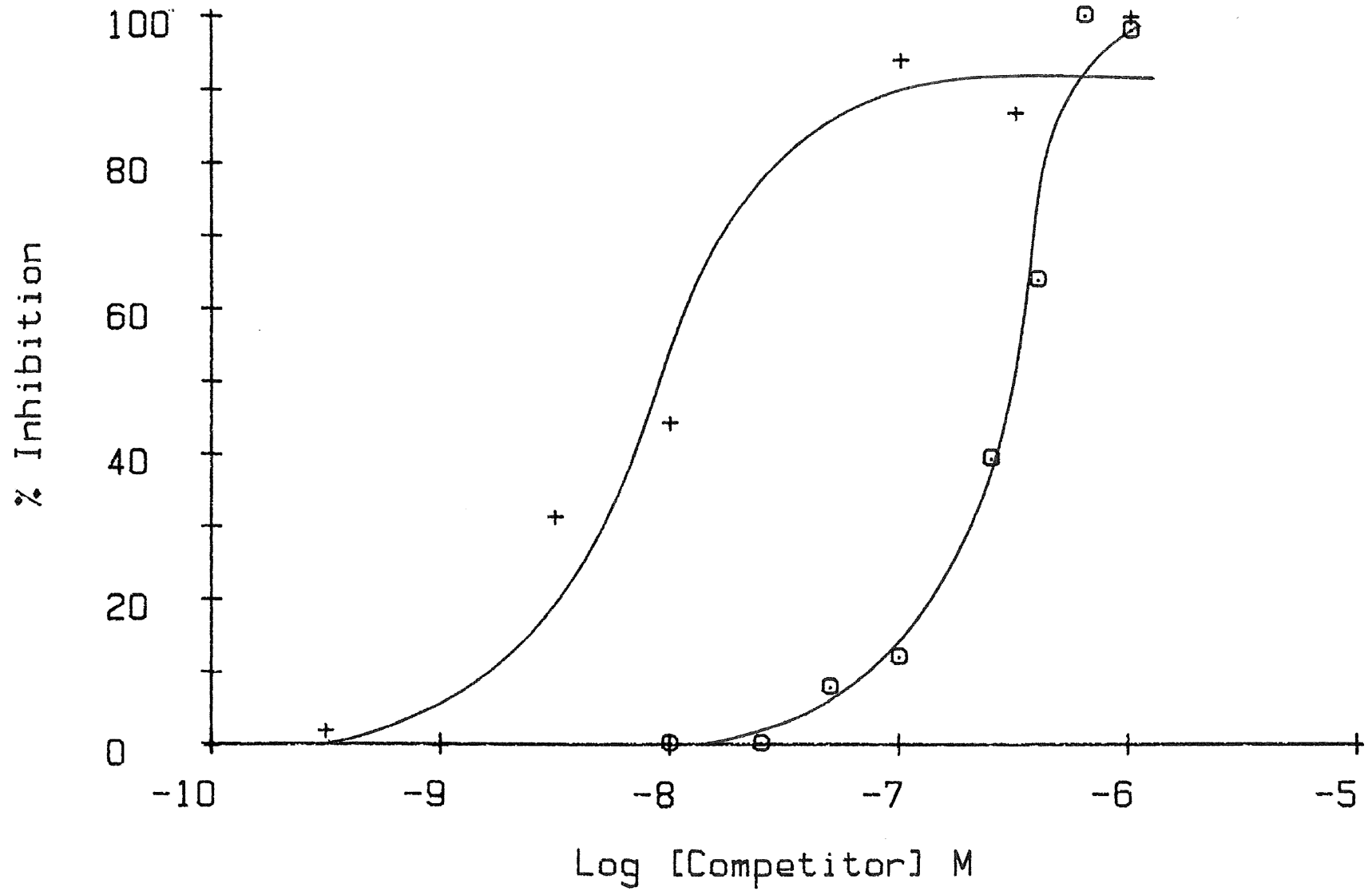

100
80
60
40
20
0
% Inhibition
-10
-9
-8
-7
-6
-5
Log [Competitor] M

TABLE I

Comparison of ^{3}H-Desipramine Binding Site Characteristics

	K_d (nM)
Murine Lymphocyte	Ø.4
Rat Cerebral Cortex *	1.4

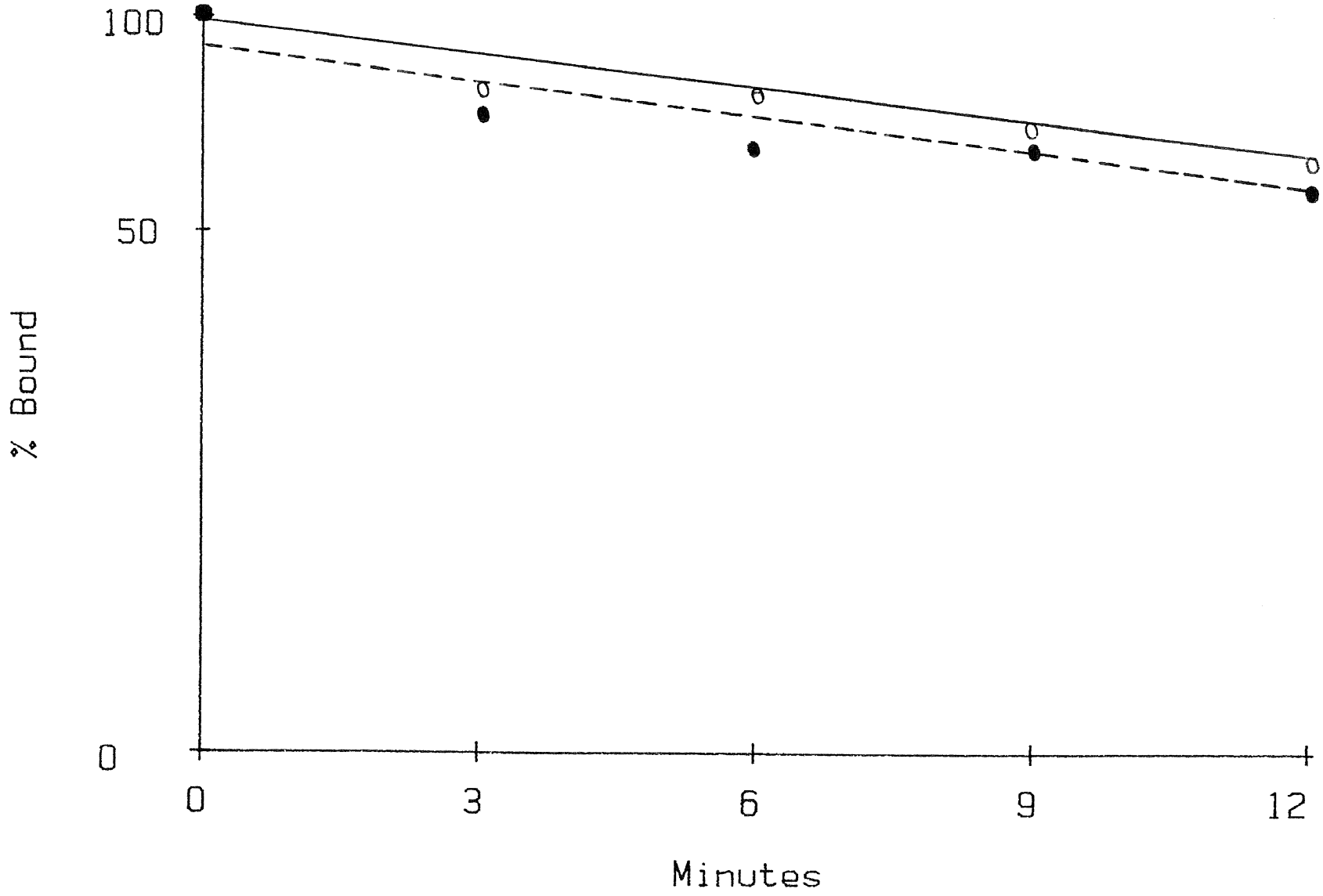
	IC_{50} (nM)** Murine Lymphocyte	IC_{50} (nM)*** Rat Cerebral Cortex*
Desipramine	7	3
Imipramine	54	62
Nortriptyline	375	6

* Rehavi et al., 1981.

** IC_{50} represents the concentration required to inhibit 5Ø% of the specific binding of ^{3}H-Desipramine (3 nM) to murine lymphocyte membranes.

*** IC_{50} represents the concentration required to inhibit 5Ø% of the specific binding of ^{3}H-Desipramine (1 nM) to rat cerebral cortex membranes (Rehavi et al., 1981).

Figure 4. Dissociation of ^{3}H-desipramine binding to murine lymphocyte membrane fragments. ●——● represents displacement in the absence of a competitor. o——o represents displacement in the presence of a competitor.


100
50
0
% Bound
0
3
6
9
12
Minutes

DISCUSSION

Binding sites for a radiolabeled tricyclic antidepressant (^{3}H-desipramine) with the characteristics of high affinity, saturability, and which can be competed for by other pharmacologically active tricyclic antidepressants have been demonstrated on the murine splenic lymphocyte. The results reported here are similar to those observed in previous studies using ^{3}H-desipramine and ^{3}H-imipramine to identify binding sites on the membranes of various brain tissues and platelets (Rehavi et al., 1981; Sette et al., 1981; Rehavi et al., 1980; Paul et al., 1980; Raisman et al. 1979; Briley et al., 1979).

It is well known that the tricyclic antidepressants interact with a number of neurotransmitter binding sites including, α-adrenergic (U'Prichard et al., 1978), muscarinic-cholinergic (Rehavi and Sokolovsky, 1978), and histaminergic (Green and Maayani, 1977), with high affinity, and are also known to alter β-adrenergic receptor function (Kinnier et al., 1980). These interactions correlate well with the known side-effects associated with tricyclic antidepressant therapy. It also known that murine lymphocytes possess β-adrenergic (Johnson and Gordon, 1980), and muscarinic-cholinergic (Gordon et al., 1978) binding sites. Further studies will elucidate whether or not the ^{3}H-desipramine is interacting with the muscarinic-cholinergic binding site on the murine lymphocyte.

The absence of cooperativity is suggested by the linear Scatchard plot (Figure 2), and the dissociation curves (Figure 4). A 100-fold dilution in buffer of ^{3}H-desipramine bound to membrane

fragments gave an off-rate for ^{3}H-desipramine that was equal to an off-rate determined from a 100-fold dilution into a buffer 10^{-5} M in desipramine. The rationale for this experiment is that a 100-fold dilution of ^{3}H-desipramine lowers the ligand concentration two orders of magnitude below the K_d for the ligand resulting in a minimal reassociation of ligand to binding site. Cooperative effects could be suggested if the off-rate for the dilution in the presence of the competitor was different. That is, the latter off-rate might be slower if cooperativity were involved. The interpretation of these results the cooperative interactions are not occurring is consistent with the linear Scatchard plot.

Many of the reports demonstrating the presence of tricyclic antidepressant binding sites correlate these findings with the physiological action most often attributed to the tricyclic antidepressants. That action is the blocking of the uptake of norepinephrine and/or serotonin by neurons or platelets (Rehavi et al., 1981; Sette et al., 1981; Rehavi et al., 1980; Paul et al., 1980; Raisman et al., 1979; Briley et al., 1979). Since the murine lymphocytes do not appear to be involved in the uptake of either of these monoamines, the presence of tricyclic antidepressant binding site seems perplexing. However, it is important to recognize that not all of the known antidepressants produce their effects through this monoamine uptake mechanism (Kostowski, 1981). Although it is one of the prominent effects of the tricyclic antidepressants, it is probable that not all of the facets of the biochemical events occurring in the mechanism of action for the tricyclic

antidepressants have been described. Therefore it is possible that the tricyclic antidepressant binding site on the murine lymphocyte may perform some physiological function that has not been delineated.

The detection of tricyclic antidepressant binding sites on the murine lymphocyte presents the possibility that these drugs might possess some selective action on the overall immune response. This report forms the foundation of future studies to determine if the tricyclic antidepressant action on the immune response may manifest itself by suppressing or enhancing that response.

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EXPERIMENTAL STUDIES

PART II

TRICYCLIC ANTIDEPRESSANT EFFECTS ON THE MURINE LYMPHOCYTE MITOGEN RESPONSE

ABSTRACT

Tricyclic antidepressant binding sites have recently been detected on the membranes of murine splenic lymphocytes (Audus and Gordon, 1982). It is reported here that the mitogen response of murine lymphocytes is altered in the presence of tricyclic antidepressants at concentrations of 10^{-5}M or greater. The time-dependent effects of these drugs, when added to the cultures at various times up to 24 hours subsequent to addition of a mitogen (either concanavalin A or lipopolysaccharide B), are also reported.

INTRODUCTION

The tricyclic antidepressants are a class of drugs used to treat depressive syndromes of moderate severity (Frazer, 1980). The exact mechanism of action for these drugs has not been precisely defined; however, the current literature supports a hypothesis that the tricyclic antidepressants act in part by blocking catecholamine and/or serotonin uptake (Kostowski, 1981).

Recently, a single population of saturable, high affinity binding sites were identified on membrane fragments of murine splenic lymphocytes (Audus and Gordon, 1982). The presence of the tricyclic antidepressant binding site on lymphocytes could provide a mechanism through which these drugs might alter the immune response. To verify physiological activity of the binding site, a short-term culture system in which the physiological response measured is a lymphocyte response to a particular mitogen was established. The murine lymphocyte system has been used to further characterize the immune response, as modified by a variety of pharmacologic agents (Strom et al., 1980).

Previous reports have identified muscarinic cholinergic (Gordon et al., 1978), nicotinic (Richman and Arnason, 1979), alpha-adrenergic (Hadden et al., 1970), and beta-adrenergic (Johnson and Gordon, 1980) binding sites on both human and murine lymphocytes. The relative importance of identifying these binding sites may be illustrated by the recent reports of Davis et al. (1980), and Johnson and Gordon (1980), whose observations show that alterations of beta-adrenergic binding site sensitivity by chronic

administration of beta-adrenergic agents can result in changes of human lymphocyte regulation. The implications of these changes in the immune response may be of importance to individuals undergoing catecholamine treatment for cystic fibrosis and asthma. The detection of a tricyclic antidepressant binding site likewise establishes the importance of determining whether or not tricyclic antidepressants alter the immune system.

MATERIALS AND METHODS

Thymidine (6-^{3}H)-, was purchased from New England Nuclear, Boston, MA. Concanavalin A (lot no. 910026) was obtained from Calbiochem-Behring Corp., La Jolla, CA. Lipopolysaccharide B, serotype no. 0111:B4 (lot no. 98C-0407) and imipramine HCl (lot no. 121F-0086) were from Sigma Chemical Co., St. Louis, MO. Desipramine HCl was a gift from Merrell Dow Pharmaceutical Inc., Cincinnati, OH. Nortriptyline HCl was a gift from Lilly Research Laboratories, Indianapolis, IN. Protriptyline HCl was a gift from Merck Sharp & Dohme Research Laboratories, West Point, PA. All other compounds used in this study were of the highest grade commercially available. Lai:Cox, Swiss-Webster, outbred male mice were purchased from Laboratory Supply, Co., Inc., Indianapolis, IN.

Lymphocyte Isolation. This procedure has been described in detail by Johnson and Gordon (1980). Briefly, adult mice (25-30 g) were sacrificed, spleens were removed aseptically through a flank incision, and the lymphocytes isolated by centrifugation. Erythrocytes were removed by lysis with ammonium chloride. Lymphocytes were suspended in a final concentration of approximately

5 * 10^6 cells/ml in RPMI 1640 (supplemented with 10% bovine serum, 100 U penicillin, and 100 µg streptomycin). Lymphocyte viability, as assessed by trypan blue exclusion, was always greater than 92% following the isolation procedure.

Short-Term Lymphocyte Cultures. 0.05 ml of the mitogen (either Concanavalin A or Lipopolysaccharide B) in concentrations of 0 to 90 µg/ml, and 0.02 ml of a tricyclic antidepressant solution were added to each of 96 wells of a microtiter plate (Model 3040, Falcon Division, Becton-Dickinson, Oxnard, CA). 0.1 ml of cell suspension was added to each well as the last addition. Imipramine and desipramine were dissolved in sterile water (sterile water served as the corresponding control), and protriptyline and nortriptyline in sterile 0.01 M phosphate buffered saline (PBS), pH 7.4 (PBS served as the corresponding control). All solutions added to the culture plates were first filtered through 0.22 micron filters (type GS, Millipore Corp., Bedford, MA). Incubations were carried out at 37°C, with 5% CO_2 and 95% air. After a 24 h incubation period, each well was pulsed with 0.03 ml tritiated thymidine (0.5 µCi), bringing the total well volume to 0.2 ml. Cells were collected on Whatman glass fiber filters with a cell harvester (Model 24V, Brandel, Gaithersburg, MD). Filters were allowed to air dry, then were placed in scintillation vials with 3 ml of LSC Complete (Yorktown Research, Miami, FL) and assayed for radioactivity to an accuracy of 0.05% in a Beckman Tri-Carb Scintillation Spectrophotometer (approximately 58% tritium efficiency).

Procedure to Determine the Effects of Tricyclic Antidepressants on the Response to Mitogen. Typically, the microtiter plates were divided in half, in order that the top half of the plate (4 rows by 12 columns) could serve as control wells for the remaining wells of the plate. The control wells could contain mitogen, cells, and either sterile water or PBS only. The remainder of the plate received, in addition to the above, various concentrations of drug. In some experiments, the concentrations of mitogen was varied over the 12 columns. In other experiments, the variable was the drug concentration or time of addition of the drug. In the latter case, these drug additions occurred at times Ø, 1, 4, 8, 12, and 24 h. The addition of the mitogen and cells to the plate defines time Ø. Results from the bottom portion of the plate were compared with the corresponding controls obtained from the top half of the plate. On occasion, the relationship was expressed as a ratio of incorporated counts (Modified Stimulation Index: MSI).

Statistical Analysis. Results of the mitogen dose-response curves, as altered by tricyclic antidepressants, are expressed as means + or - the standard deviation of quadruplicate samples. The statistical method of error propagation was applied to results where the MSI was calculated (Bevington, 1969).

RESULTS

The four tricyclic antidepressants tested suppressed the normal mitogen response of the murine splenic lymphocytes when the drug concentration was 10^{-5} M or greater. Figures 1 and 2 are representative of the typical response observed for each of the

tricyclic antidepressants when the final drug concentration was 10^{-5} M and 10^{-6}M, respectively. Greater than a 90% suppression of the mitogenic response was observed at 10^{-4}M for all four compounds, while only partial suppression was observed when the concentration was 10^{-5} M. Any suppression or enhancement of the response is absent at concentrations less than or equivalent to 10^{-6} M.

The suppressive effects of the tricyclic antidepressants were comparable when lipopolysaccharide B (LPS B; a B-lymphocyte specific mitogen) was introduced rather than concanavalin A (a T-lymphocyte specific mitogen). Each of the four tricyclic antidepressants produced greater than 90% suppression of the mitogen dose-response at a final concentration of 10^{-4} M while only partial suppression is observed at 10^{-5} M. Little or no suppression of the mitogenic response at a final concentration of 10^{-6} M is observed for any of the tricyclic antidepressants tested. Figures 3 and 4 are representative of the effects of the tricyclic antidepressants on the LPS B dose-response curve.

In the above experiments the time of addition of the drug was time 0. Drug addition subsequent to time 0 resulted in an attenuation of the observed suppression. Figure 5 represents a typical time dependent decrease of the suppression observed for all four compounds in the presence of an optimal doese of concanavalin A (2.5 μg/ml). In another series of timed addition experiments, a significant change in the degree of suppression was observed when the concentration of the mitogen was greater than optimal. Figures

Figure 1. Modulation of the Concanavalin A dose-response by 10^{-5}M desipramine. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-5}M desipramine. A resultant point is the mean of four determinations + or - the standard deviation.

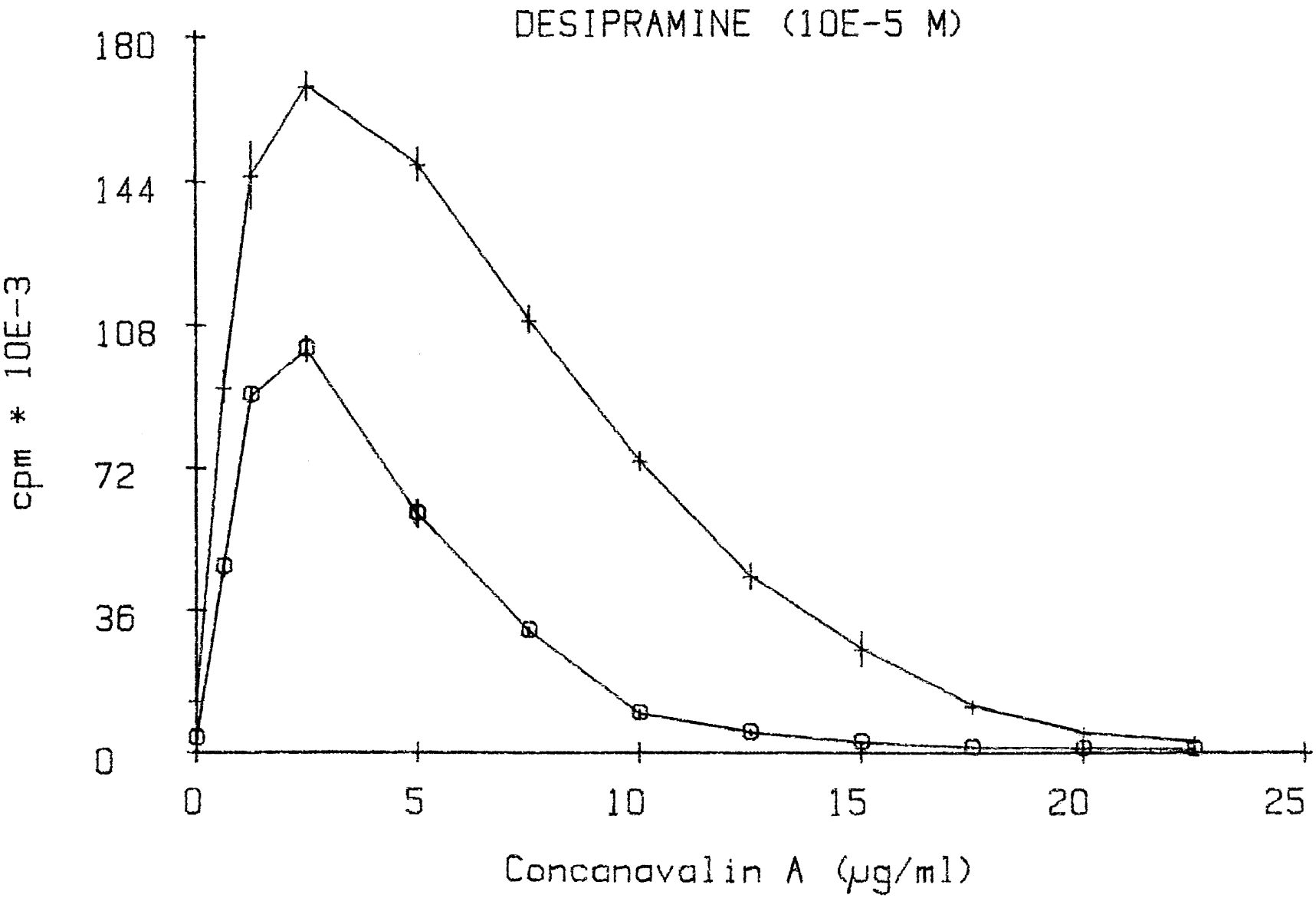

DESIPRAMINE (10E-5 M)
180
144
108
72
36
0
cpm * 10E-3
0
5
10
15
20
25
Concanavalin A (µg/ml)

Figure 2. Modulation of Concanavalin A dose-response by 10^{-6} M desipramine. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-6} M desipramine. A resultant point is the mean of four determinations + or - the standard deviation.

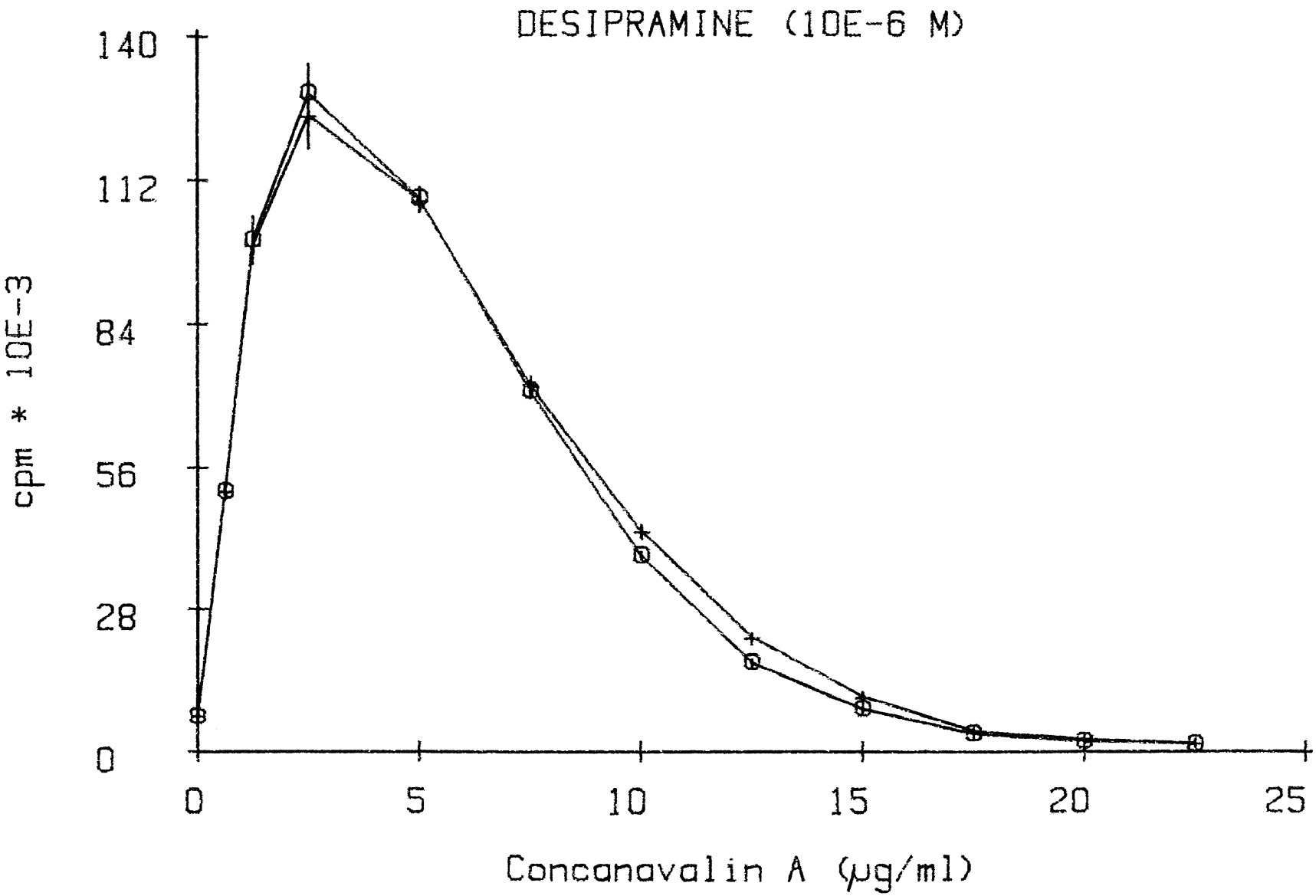

DESIPRAMINE (10E-6 M)
cpm * 10E-3
140
112
84
56
28
0
0
5
10
15
20
25
Concanavalin A (µg/ml)

Figure 3. Modulation of Lipopolysaccharide B dose-response by 10^{-5} M nortriptyline. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-5} M nortriptyline. A resultant point is the mean of four determinations + or - the standard deviation.

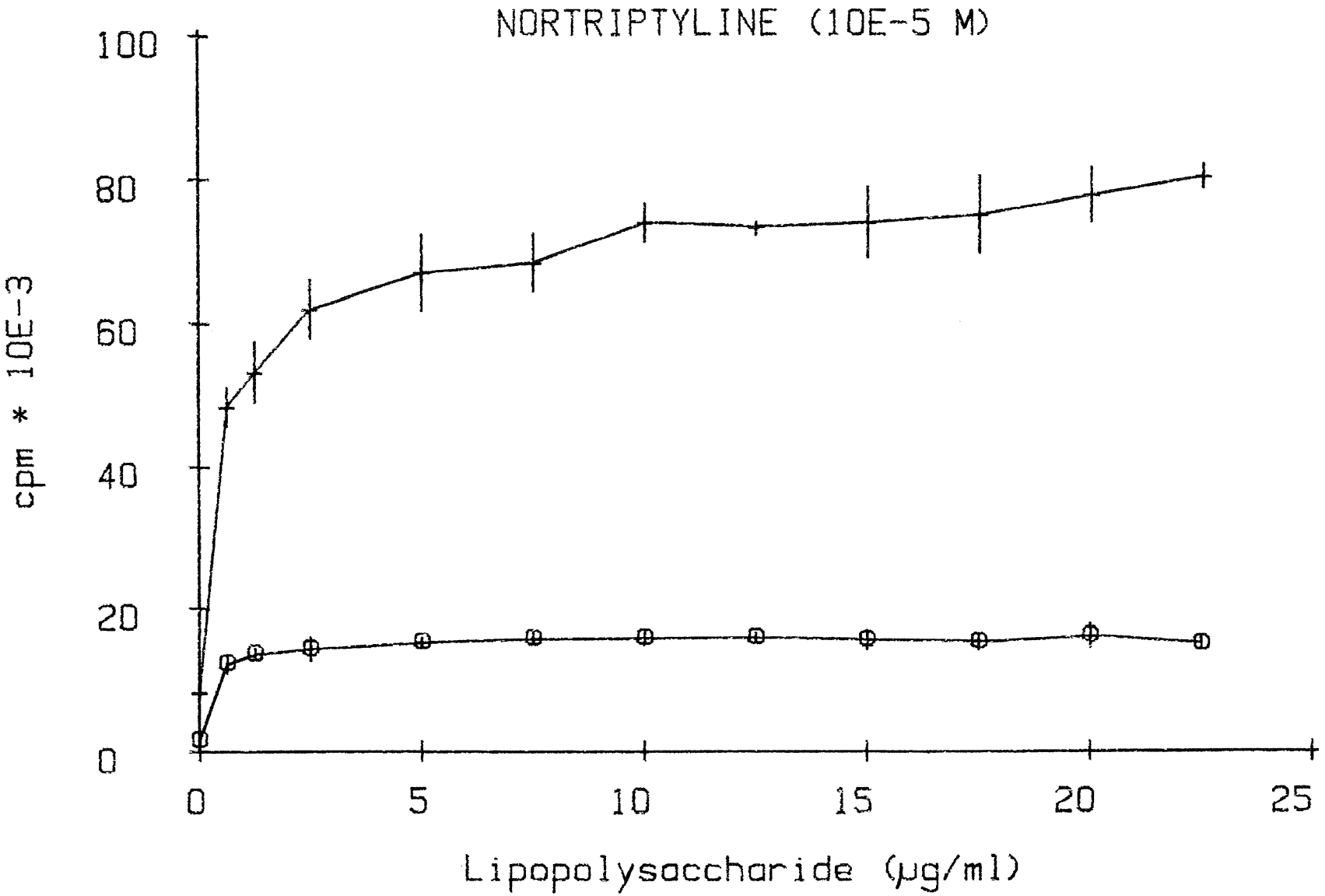
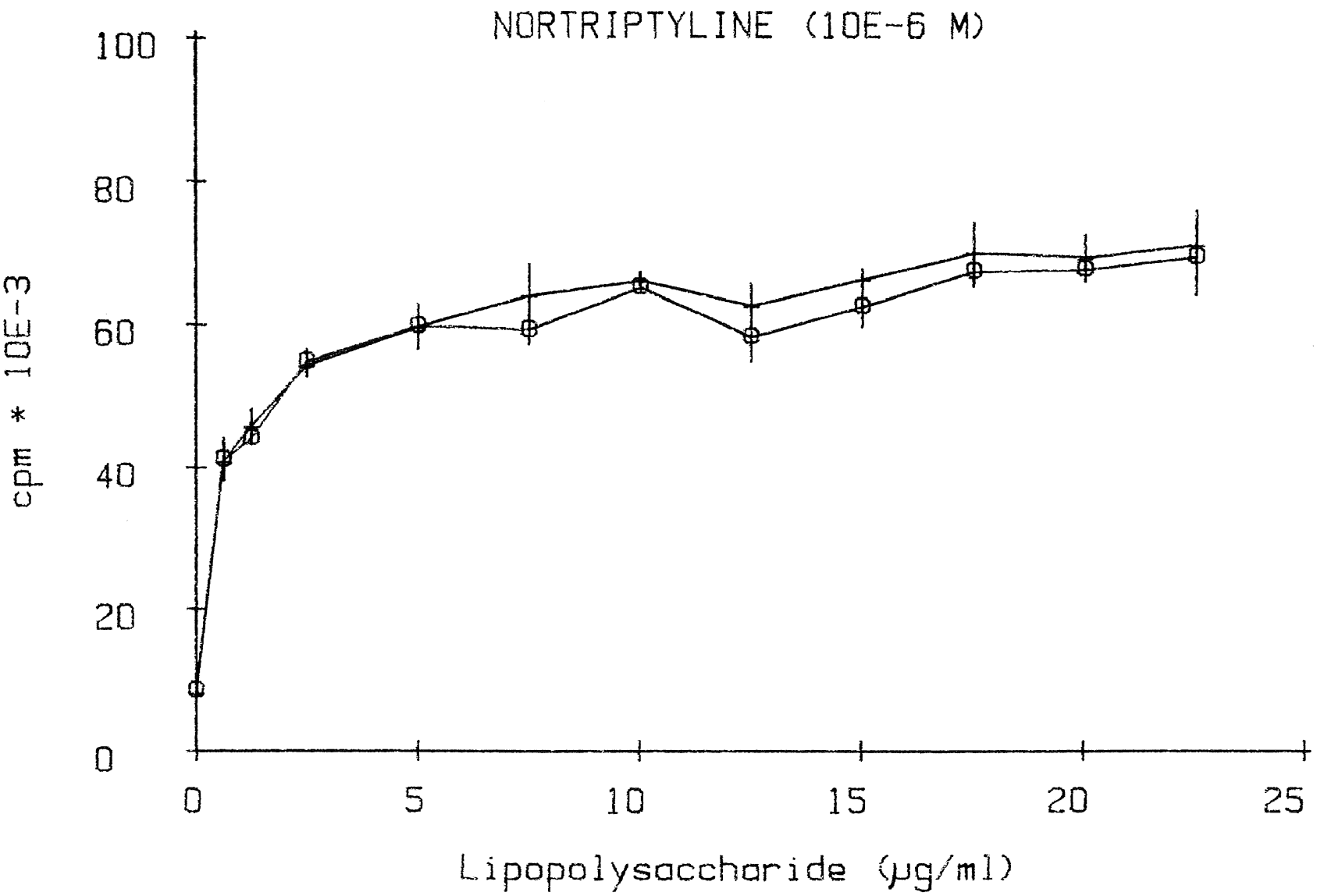

NORTRIPTYLINE (10E-5 M)
cpm * 10E-3
100
80
60
40
20
0
0
5
10
15
20
25
Lipopolysaccharide (µg/ml)

Figure 4. Modulation of the Lipopolysaccharide B dose-response by 10^{-6} M nortriptyline. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-6}M nortriptyline. A resultant point is the mean of four determinations + or - the standard deviation.


NORTRIPTYLINE (10E-6 M)
cpm * 10E-3
100
80
60
40
20
0
0
5
10
15
20
25
Lipopolysaccharide (µg/ml)

6 and 7 compare mitogenic suppression observed at drug addition time Ø with that observed drugs addition at 12 h and 24 h in the presence of $1Ø^{-5}$M nortriptyline.

DISCUSSION

This study was designed to determine if the recently identified tricyclic antidepressant binding site on murine splenic lymphocytes might provide a mechanism through which tricyclic antidepressants could alter the immune response. To investigate the possiblity that the binding site may possess this physiological function, a series of short-term culture experiments were conducted using four common tricyclic antidepressants (desipramine, imipramine, nortriptyline, and protriptyline). The murine lymphocyte mitogen response in a short-term culture system has been shown to be a useful model of the immune response (Wedner and Parker, 1976). Each drug was subsequently tested for the ability to alter the normal lymphocyte mitogen response.

Results of the culture experiments indicate that all four tricyclic antidepressants have similar effects on the normal mitogen response. A concentration of $1Ø^{-4}$ M effects almost complete suppression of the mitogen response. While $1Ø^{-5}$M drug results in partial suppression, a ten-fold lower concentration failed to effect suppression. This observation, when considered in view of the apparent K_d of $4 * 1Ø^{-10}$M for the tricyclic antidepressant binding site, suggests the possibility that the effects of concentrations of tricyclic antidepressants greater than $1Ø^{-5}$ M may not be mediated by this high affinity binding site.

Figure 5. Modulation of the optimal dose of Concanavalin A by additions of 10^{-4}M and 10^{-5}M imipramine at various times up to 24 h after time 0. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. The MSI for results of the 10^{-4}M imipramine addition (o) are compared to the results of the 10^{-5}imipramine addition (+). A resultant point is the ration of mean incorporated counts with drug present/mean incorporated counts without drug (control). A mean is calculated from quadruplicate wells. The method of error propagation has been applied in determination of the standard deviation for the MSI.

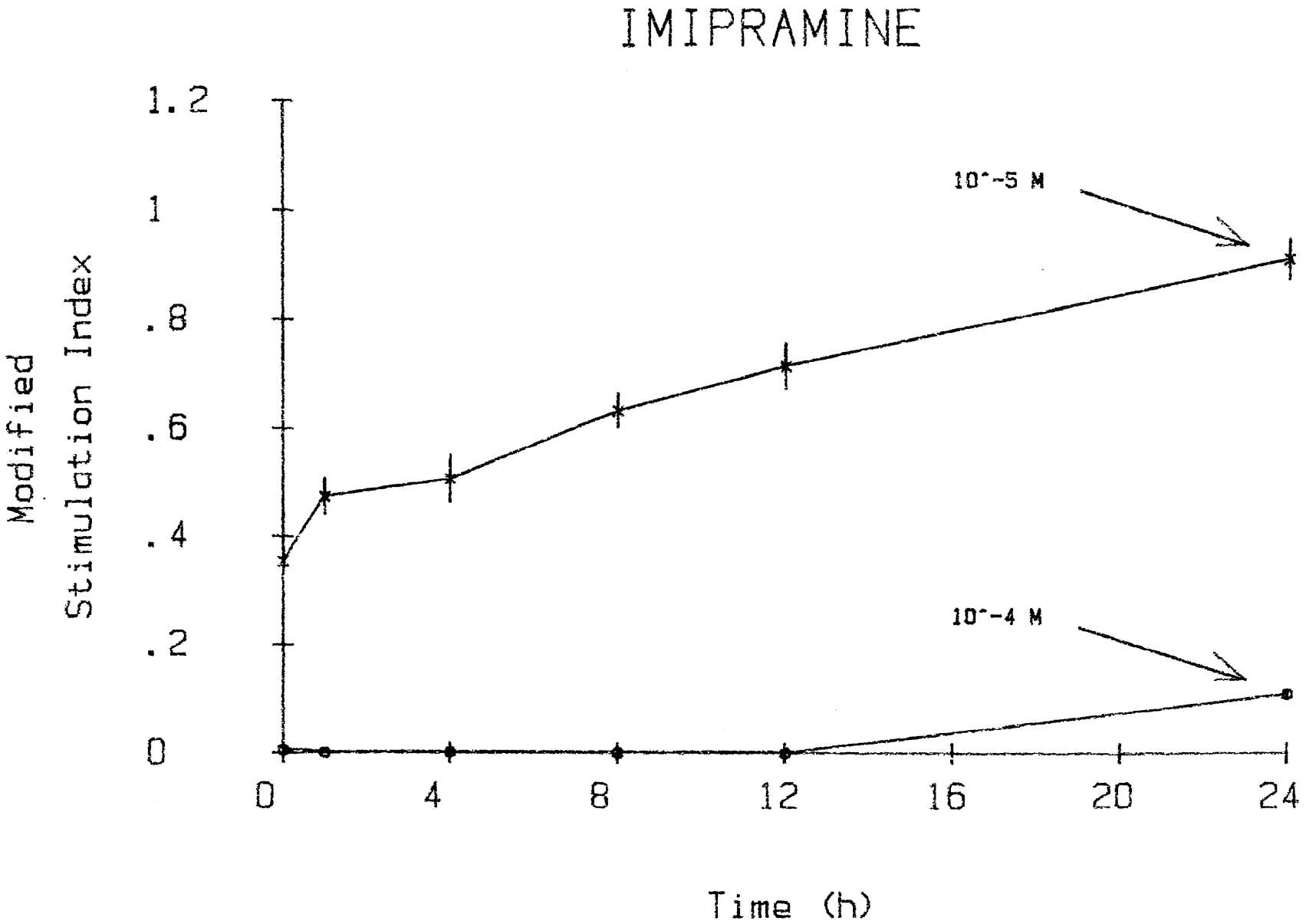

IMIPRAMINE
Modified
Stimulation Index
1.2
1
.8
.6
.4
.2
0
10^-5 M
10^-4 M
0
4
8
12
16
20
24
Time (h)

Figure 6. Modulation of the Concanavalin A dose-response by 10^{-5}M nortriptyline added 12 hours after time 0. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-5} M nortriptyline 12 hours after time 0. A resultant point is the mean of four determinations + or - the standard deviation.

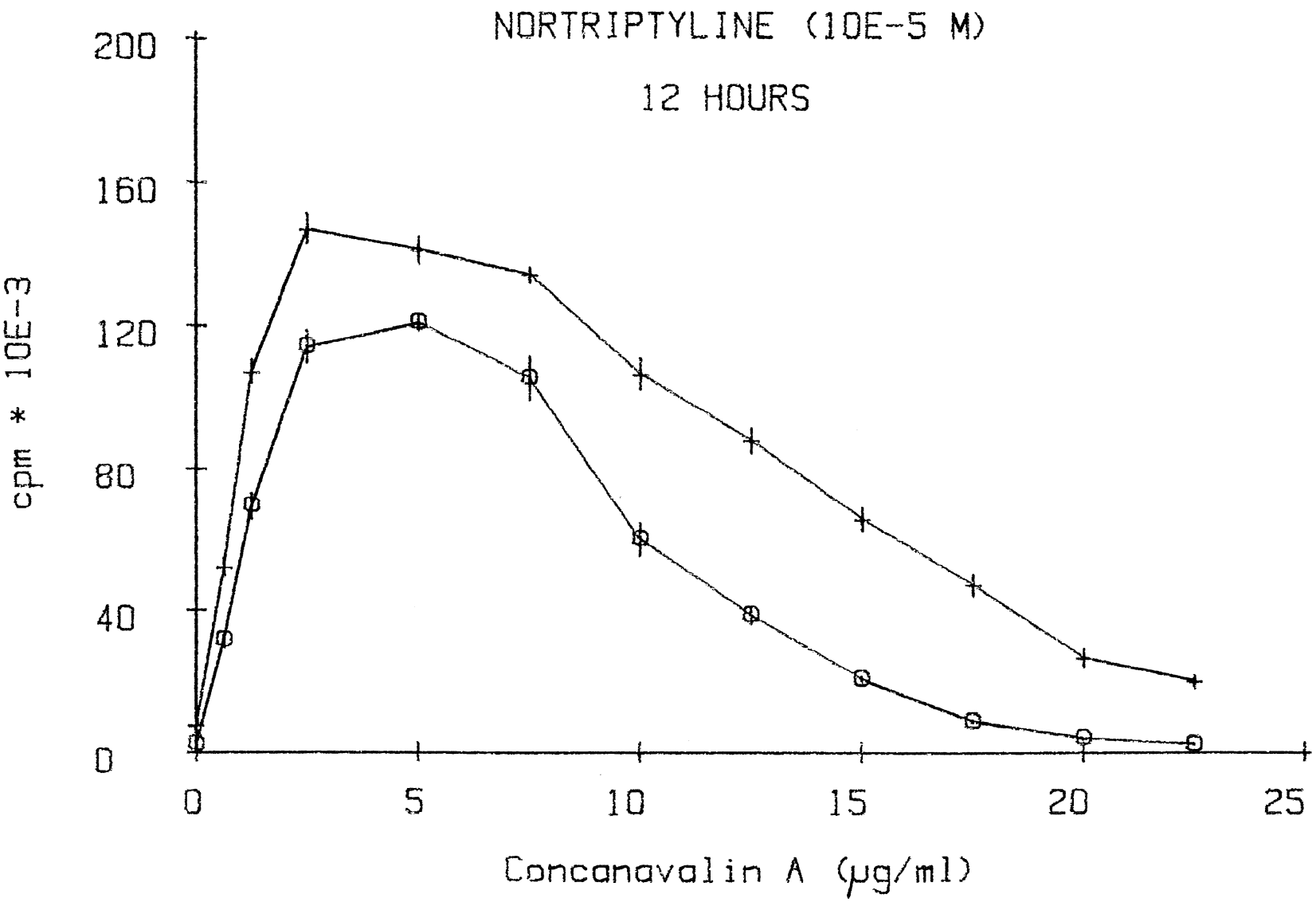
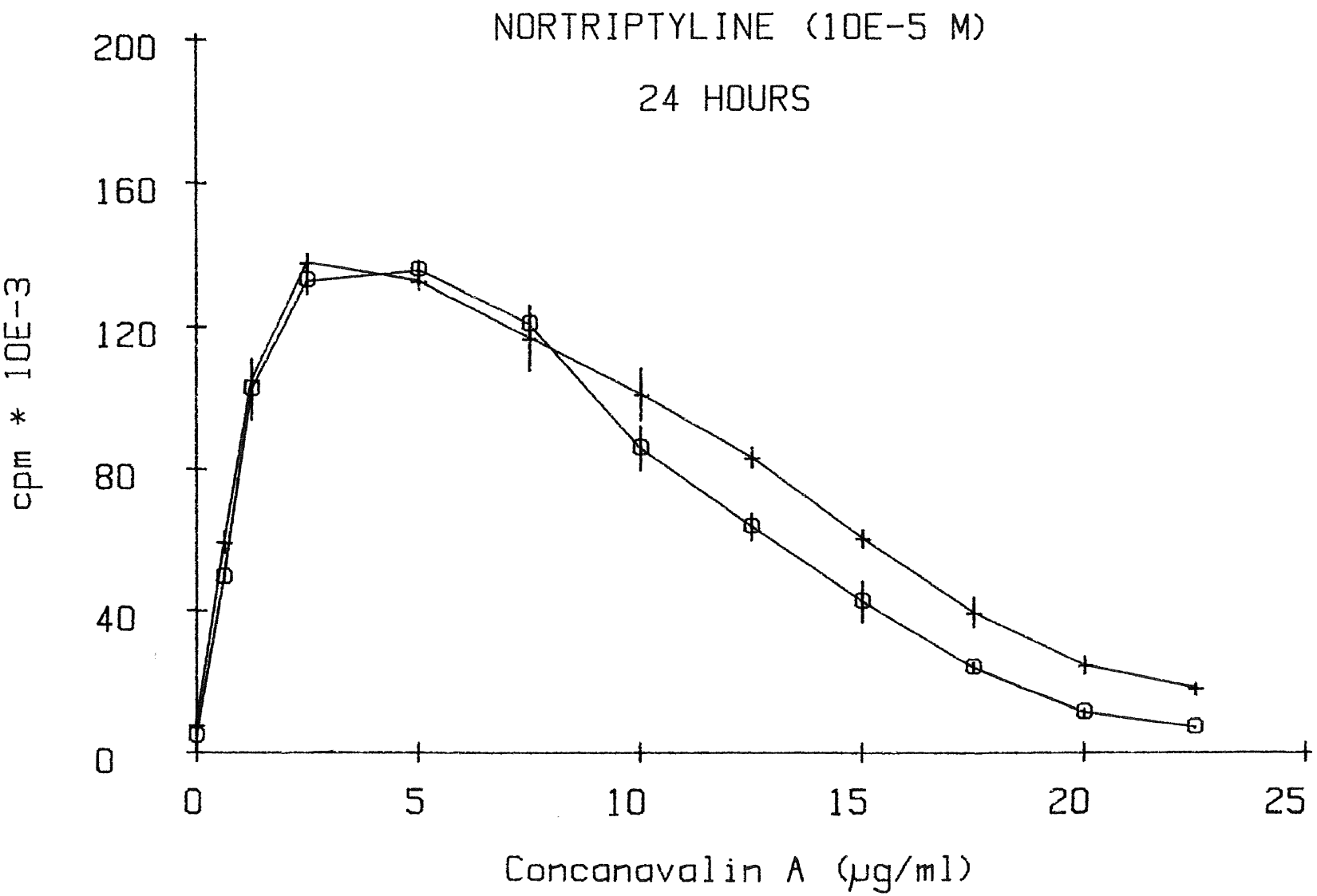

NORTRIPTYLINE (10E-5 M)
12 HOURS
cpm * 10E-3
200
160
120
80
40
0
0
5
10
15
20
25
Concanavalin A (µg/ml)

Figure 7. Modulation of the Concanavalin A dose-response by 10^{-5} M nortriptyline added 24 hours after time Ø. Murine splenic lymphocytes were added to each well of a microtiter plate set up as detailed in Materials and Methods. Control wells (+) are compared to experimental wells (o) which received 10^{-5}M nortriptyline 24 hours after time Ø. A resultant point is the mean of four determinations + or - the standard deviations.


NORTRIPTYLINE (10E-5 M)
24 HOURS
cpm * 10E-3
200
160
120
80
40
0
0
5
10
15
20
25
Concanavalin A (µg/ml)

In contrast, the recently identified beta-adrenergic binding site on the murine lymphocyte possesses an apparent K_d which correlates well with the concentrations of beta-adrenergic agonists necessary to suppress the mitogen response (Johnson et al., 1981; Johnson and Gordon, 1980). Similarly, the muscarinic binding site exhibits an apparent K_d as determined by direct binding studies which correlate with cholinergic agonist concentrations known to be physiologically active (Dulis et al., 1979).

The physiological function most often associated with the tricyclic antidepressant binding site of the CNS and platelet is the blocking of catecholamine and/or serotonin uptake (Lee and Snyder, 1981; Rehavi et al., 1981; Sette et al., 1981; Rehavi et al., 1980; Paul et al., 1980; Briley et al., 1979; Raisman et al., 1979). While such a function has not been ascribed to lymphocytes, this does not preclude the possibility that the tricyclic antidepressant binding site may be involved in other biochemical or physiological activities on the lymphocyte, platelet, and in the CNS.

Results of the time addition of tricyclic antidepressants to the short-term cultures indicate that the drugs are effective in suppressing the concanavalin A response when added up through 12 h after time 0. All four of the drugs tested were equally effective in concentrations of 10^{-4} M and 10^{-5} M. Studies of the mouse splenic lymphocyte response to concanavalin A have indicated that there may be two crucial time periods (the first 0-3 h and a second 15-18 h after time 0) during which it is necessary for the concanavalin A to interact will cell surface receptors and deliver

signals initiating mitogenic events. Providing both signals are received, mitosis will occur about 48 h after time Ø (Toyoshima et al., 1976). These signals can be interrupted by certain drugs resulting in suppression or prevention of mitosis. Beta-adrenergic agonists when added to the lymphocyte cultures are effective only if added within 1 h post time Ø (Johnson et al., 1981). Later additions of beta-adrenergic agonists to the cultures have no effect, suggesting that the beta-adrenergic may interfere with events involved in the first signal period. The tricyclic antidepressants appear capable of altering mitotic events at the time of, and after, the first signal period (Ø-3 h after time Ø). The tricyclic antidepressants are not apparently able to effect a large suppression, however, if added after the second signal period, 15-18 h after time Ø. From these observations it may be concluded that the mitotic events occurring after the second signal period are relatively unsusceptible to the disrupting effects of the tricyclic antidepressants.

The reduced mitogen response of lymphocytes exposed to high concentrations of concanavalin A has been assumed to be analogous to high antigen dose tolerance (Wedner and Parker, 1976). The lymphocytes exposed to high concentrations of concanavalin A enter into an entirely reversible, unresponsive state for the duration of the 48 h culture (Andersson et al., 1972). In our experiments the concentrations of tricyclic antidepressants of 10^{-5} M or greater sharpened the concanavalin A dose-response curve. The degree of sensitivity of the mitogen dose-response curve to different

concentrations of tricyclic antidepressants appeared greater at higher concentrations of concanavalin A. The significance of this observation is that the tricyclic antidepressants appear to accentuate the high antigen dose attenuation of the mitogen response.

In summary, the tricyclic antidepressants are capable of suppressing the mitogen dose-response at concentrations of 10^{-5} M or greater in a time-dependent manner that may be related to the two signal period hypothesis. In addition, the tricyclic antidepressants may magnify the effects of higher concentrations of concanavalin A on lymphocyte response according to the suggested high dose tolerance model. Finally, the results indicate that the inhibitory effects of the tricyclic antidepressants on the immune response is probably not mediated by the recently identified high affinity binding site.

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EXPERIMENTAL STUDIES

PART III

USE OF FLUORESCENT PROBES TO MONITOR PROPRANOLOL EFFECTS ON THE MURINE SPLENIC LYMPHOCYTE

ABSTRACT

Recent studies indicate that propranolol suppresses the normal murine lymphocyte mitogen dose-response (Johnson et al., 1981) and that propranolol has a high affinity for the beta-adrenergic binding site on the murine lymphocyte (Johnson and Gordon, 1980). Since concentrations which suppress the lymphocyte mitogen dose-response are much greater than the K_d for the binding site, it may be suggested that the suppression of the mitogen dose-response is a result of nonspecific effects of propranolol. Three fluorescent probes (1,6-diphenyl-1,3,5-hexatriene (DPH), fluorescein conjugated concanavalin A (F-Con A), and 3,3'-dithio-diisopropyl-oxacarbocyanine iodide (diS-C3-(5)) were used to investigate possible mechanisms of the nonspecific effects of propranolol on the murine splenic lymphocyte.

This report presents data which suggests a possible relationship between propranolol concentrations that alter lipid membrane dynamics, as monitored by the fluorescence polarization of DPH, and suppression of the mitogen dose-response in short term cultures as effected by propranolol. Also reported are effects of propranolol on the relative mobility of surface membrane lectin receptors as monitored by the fluorescence polarization of F-Con A, effects of propranolol on the transmembrane potential as monitored by the fluorescence emission of diS-C3-(5), and the propranolol effect on the fluorescence polarization temperature-dependence of

DPH and F-Con A. Studies of the effects of sotalol, alprenolol, and metoprolol are included and used for comparison of different drug effects.

INTRODUCTION

Propranolol is a nonspecific beta-adrenergic antagonist that has found clinical utility in the treatment of certain cardiac disease states. Recently, propranolol, at concentrations of 10 μM or greater, was shown to suppress the normal mitogen dose-response of murine lymphocytes in a short term culture system (Johnson et al., 1981). Additionally, propranolol appears to prolong the time to rejection of heart-lung allografts in the rat (Larson et al., 1982). It has also been demonstrated that propranolol binds with high affinity (K_d = 10 nM) in a competitive manner to the murine lymphocyte beta-adrenergic binding site (Johnson and Gordon, 1980). Since concentrations that resulted in suppression of the normal mitogen dose-response are significantly greater than the K_d, it is probable that suppression of the mitogen dose-response is due to non-receptor mediated (nonspecific) effects of propranolol. Further evidence for suppression of the mitogen dose-response is derived from the observation that d- and l- isomers of propranolol are equally effective, at concentrations of 10 μM or greater, in suppressing the mitogen dose-response (Johnson et al., 1981).

Three fluorescent probes, DPH, F-Con A, and DiS-C3-(5), have been used in this study to investigate the interactions of propranolol with the murine lymphocyte. DPH is a lipophilic fluorescent probe used to measure relative changes in the dynamic nature of the hydrophobic regions of cellular membrane. Monitoring the polarization of DPH has been used for investigation of the effects of a number of drugs including anesthetics (Pang et al.,

1979), steroids (Shinitzky and Inbar, 1974; Johnston and Melnykovych, 1980), alcohols (Kutchai et al, 1980), tumor promoting agents (Fisher et al., 1979), hormones (Luly and Shinitzky, 1979), and opiates (Johnson et al., 1979) on changes in fluidity of live cells or artificial membranes. DPH has also been used to differentiate between normal and abnormal cells (Inbar, 1976; Shinitzky and Inbar, 1974). The polarization of F-Con A bound to cell surface Concanavalin A (Con A) binding sites has been used a monitor of the mobility of the Concanavalin A binding site and the dynamic characteristics of the cell membrane surface of normal and abnormal cells (Ben-Bassat et al., 1977; Inbar et al., 1973). DiS-C3-(5) is a fluorescent dye that exhibits differences in fluorescence emission as it partitions between intra- and extracellular compartments. The partition of diS-C3-(5) between intra- and extra- cellular compartments is altered by changes in the transmembrane potential (Waggoner, 1979) of the lymphocyte. Results with other beta-adrenergic antagonists, sotalol and alprenolol, and a selective beta-1-adrenergic antagonist, metoprolol, will be used for comparison with the results observed for propranolol.

This report demonstrates the ability of propranolol, which is much more lipid soluble than most beta-adrenergic compounds (Dax and Partilla, 1982; Woods and Robinson, 1981), alter the relative dynamics of the hydrophobic regions of the murine lymphocyte cell membrane. The alteration of the relative hydrophobic dynamics by propranolol occurs in a concentration range that is similar to the concentration range required for its suppressive effect on the

normal lymphocyte mitogen dose-response. These observations along with those for the other beta-adrenergic compounds tested, appear to be consistent with recent reports assessing the liposolubility of various beta-adrenergic compounds (Dax and Partilla, 1982; Woods and Robinson, 1981).

MATERIALS AND METHODS

1,6-Diphenyl-1,3,5-hexatriene was purchased from Molecular Probes, Inc., Plano, TX. Fluorescein conjugated Concanavalin A (F-Con A; fluorescein/protein (molar): 4.9) and Concanavalin A (Con A) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Dithio-diisopropyl-oxacarbocyanine iodide (diS-C3-(5)) was a gift from Professor Alan Waggoner, Amherst College, Amherst, MA. Propranolol HCl and Histopaque-1077 were purchased from Sigma Chemical Co., St. Louis, MO. Sotalol HCl was a gift from AB Hassle, Fack, S-431 20 Molndal, Sweden. Metoprolol was a gift form CIBA Pharmaceutical Co., Summit, NJ. RPMI1640 was obtained from Grand Island Biological Company (Gibco), Grand Island, NY. Swiss-Webster, Lai:Cox, outbred white male mice were purchased from Laboratory Supply Company, Inc., Indianapolis, IN. All other compounds were of the highest grade commercially available.

Lymphocyte Isolation. Briefly, adult male mice (25-30 g) were sacrificed by cervical dislocation and the mouse spleens were removed through a flank incision. The spleens were macerated in phosphate buffered saline (PBS), pH 7.4. The lymphocytes were isolated by Histopaque gradient centrifugation and the cells were then washed in PBS (Boyum, 1968). The final lymphocyte pellet was

suspended in an appropriate concentration in PBS for fluorometric experiments, or in RPMI1640 (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco)) for short term lymphocyte cultures. Viability of the isolated lymphocytes, as determined by trypan blue exclusion was always greater than 90%.

Short Term Lymphocyte Cultures. 0.10 ml of cells suspended in RPMI1640 in a concentration of $5 * 10^6$ cells/ml were added to each of 96 wells of a microtiter plate (Model 3040, Falcon Division, Becton-Dickinson, Oxnard, CA). In addition 0.05 ml of Con A in concentrations of 0 to 90 μg/ml, and 0.02 ml of a beta-adrenergic antagonist solution were added to each well. Solutions of beta-adrenergic antagonists were prepared by dissolving the appropriate compound in PBS, pH 7.4, PBS therefore served as a control when added to a microtiter plate well alone. Solutions added to the microtiter plate wells were first filtered through 0.22 micron filters (type GS, Millipore Corp., Bedford, MA). After a 24 h incubation period (at 37°C, with 5% CO_2 and 95% air) each well was pulsed with 0.03 ml of tritiated thymidine (0.5 μCi). Total well volume was 0.20 ml. Incorporated thymidine was harvested 24 h after pulsing on Whatman glass fiber filters with a cell harvestor (Model 24V, Brandel, Gaithersburg, MD). Filters were air dried, placed in scintillation vials with 3 ml of LSC Complete (Yorktown Research, Miami, FL) and assayed for radioactivity to an accuracy of 0.05% in a Beckman Tri-Carb Scintillation Spectrophotometer (approximately 58% tritium efficiency).

Beta-Adrenergic Antagonist Effects on the Normal Mitogen Dose-Response. Microtiter plates were divided in half such that the top portion of the plate (4 rows x 12 columns) served as controls for the remaining wells of the plate. Wells in the top half of the plate each received mitogen, PBS, and cells. Remaining wells received in addition, an appropriate concentration of beta-adrenergic antagonist in PBS.

Steady-State Polarization Procedures. Cells were labeled with DPH following a procedure used by Kyoizumi et al. (1981). A 2 mM stock solution of DPH in tetrahydrofuran was used to prepare a 2 μM DPH solution in PBS. 2 ml of the 2 μM DPH solution was mixed with 2 ml of a 2 * 10^6 cells per ml suspension and allowed to incubate at 37°C for 45 min. After the incubation period, 3 ml of the DPH labeled cell mixture was placed in a quartz cuvette and polarization experiments conducted immediately.

F-Con A bound cells were prepared in the following manner: Approximately 0.390 ml of the 8.0 mg/ml (protein) F-Con A stock was incubated with 5 * 10^6 cells/ml suspended in PBS at 37°C for 15 min. Following the incubation, the cell suspension was centrifuged at 150 * g for 10 min and the pellet resuspended in PBS to a concentration of aout 1 * 10^6 cells/ml. F-Con A bound cells were used immediately for polarization experiments.

All drugs were first dissolved in PBS and then added to the cuvette in quantities of .3 - 40 μl with Hamilton microliter syringes. Each drug addition was followed by a 15 minute incubation period before a polarization measurement was made. PBS added alone

in equal amounts and at similar times, as for drug addition, served as a control.

Polarization was measured with an SLM 8000 spectrofluorometer interfaced with a Hewlett-Packard (HP) 85 computer for data analysis. Photomultiplier tubes were placed to the right and left of the sample cell with polarizers inserted in the emission and excitation beams. A Schott cut-on filter was placed in the horizontally polarized emission beam and an SLM MC320 monochromator placed in the perpendicularly polarized emission beam. Emission with the MC320 monochromator was monitored at 430 nm for DPH experiments and at 519 nm for F-Con A experiments. Fluorescence intensity was first measured with the sample excited (source, SLM Xenon arc lamp) with a parallel polarized beam, and then measured a second time with the sample excited with a perpendicularly polarized beam. For DPH experiments, the sample was excited with 360 nm light, and for the F-Con A experiments the sample was excited with 492 nm light. The fluorometer was operated in the polarization mode

$$P = A - B / A + B$$

where P is the polarization, A is the fluorescence intensity parallel to and B the fluorescence intensity perpendicular to the plane of the polarizarized excitation beam.

Background subtractions of cell-drug and fluorophore-drug contributions to polarizations measurements were made but were found not to be significant. This concentration of cells was not turbid

enough to cause significant light scattering. Temperature of the sample cell was controlled by a circulating water bath and the contents stirred with a magnetic stirrer.

Membrane Potential Determination. A stock solution of diS-C3-(5) was prepared by dissolving the dye in absolute ethanol. An aliquot of the stock solution was added to 3 ml of PBS or cells suspended in PBS in a quartz cuvette. The final concentration of the dye was 10^{-8} M. The mixture was allowed to incubate at 37°C until the fluorescence emission was constant. Aliquots of potassium chloride were then added to the solution in the cuvette to obtain desired extracellular concentrations of potassium. Fluorescence emission was recorded following an addition of potassium. From the Goldman Field equation and the Nernst equation (assuming intracellular potassium concentration is .132 M (Deutsch et al., 1979)), a change in membrane potential as a function of extracellular potassium was calculated (Deutsch et al., 1979). A membrane potential vs. % fluorescence curve was generated. In additional experiments valinomycin and propranolol were added to cuvettes conctaining diS-C3-(5) and cells in PBS to test for the possibility of dilutional or solute effects.

The SLM instrument interfaced with a HP-85 as described above was used to determine the transmembrane potential of lymphocytes. The fluorometer was set-up for observing changes in fluorescence emission as a function of time in the single photon counting mode. The sample cell solution was excited with 622 nm light and fluorescent emission recorded at 670 nm.

From the changes in fluorescence emission recorded upon addition of potassium and valinomycin, a fluorescence-membrane potential calibration curve was generated. The limit of detectability, as calculated from the slope of the line multiplied by the mean counting error, was approximately 3% and corresponds to about 3 mV.

Statistical Analysis. Data points (expressed as a % of Control) for the short term cultures in the figures represent the ratio of mean counts/min ^{3}H-thymidine incorporated in the presence of the indicated concentration of beta-adrenergic antagonist (sample) divided by the mean counts/min ^{3}H-thymidine incorporated in the absence of drug (control). This ratio is multiplied by 100. The mean response (counts/min ^{3}H-thymidine incorporated) was calculated from quadruplicate samples or controls (standard deviations were less than 10%) in the presence of an optimal concentration of Con A (2.5 μg/ml). For polarization experiments, data points are the means of at least ten samples (standard deviations were less than 3%) and are expressed as the difference from the control. The statistical method of error propagation was applied as appropriate (Bevington, 1969).

RESULTS

Figures 1-4 show relationships between the short term cell culture results and the fluorescence polarization experiments with DPH and F-Con A.

Propranolol, in Figure 1, suppresses the murine lymphocyte mitogen dose-response at concentrations of 10 μM or greater. In

comparison, the effects of propranolol on the hydrophobic regions of the cell membrane appear to begin at a comparable concentration, as measured by the change in fluorescence polarization of DPH. In contrast, the effects of propranolol on cell surface dynamics as reported by the fluorescence polarization of F-Con A begin at a much higher concentration (approximately 500 μM). Isomers of propranolol, Figure 2, produced similar results in the short term cultures and in the fluorescence polarization studies (DPH only) as were observed for the racemic propranolol. Alprenolol, in Figure 3, suppresses the mitogen dose-response at concentrations greater than 10 μM. Similar concentrations were required to alter the fluorescence polarization of DPH in the cell membrane. Alprenolol did not effect changes in the fluorescence polarization of F-Con A on the cell surface at any concentration tested. Metoprolol, Figure 4, did not have a significant effect on the mitogen dose-response of lymphocytes in the short term culture system. There was little, if any, effect of metoprolol on the fluorescence polarization of DPH in the cell membrane and F-Con A on the cell surface. Sotalol, not shown, was not used in the cell culture system and fluorescence polarization experiments do not indicate the presence of significant sotalol effects (at any concentration) on the fluorescence polarization of either DPH in the cell membrane or F-Con A on the cell surface.

The temperature-dependence of the fluorescence polarization of DPH in cell membranes and F-Con A on the surface of cell membranes was determined in the presence and absence of propranolol. Results

summarized in Figure 5 show that 1000 μM propranolol appears to affect the fluorescence polarization of DPH at different temperatures to differing degrees. At higher temperatures the polarization of DPH is altered to a larger degree than at lower temperatures. The appearance of a distinct transition temperature is not evident due to the heterogeneity of the membrane components of the lymphocyte. 1 μM propranolol had little effect on the temperature-dependence of DPH in cell membranes. Concentrations of propranolol of 1000 μM produced a fairly parallel shift of the observed fluorescence polarization of F-Con A. Concentrations below 100 μM have no effect.

Figure 6 shows the calculated % membrane potential vs. % fluorescence change curve. The resting membrane potential of the lymphocyte was estimated to be approximately -65 mV. Elevation of extracellular potassium to about 200 mM results in an absolute decrease of the membrane potential to about -10 mV. Figure 7 is representative of the result of additions of propranolol up to 30 μM in concentration. In the concentration range tested (30 nM - 30 μM) propranolol did not effect a change in the resting membrane potential. Following additions of propranolol, either valinomycin, a potassium ionophore, or potassium was added to the sample to test for the presence of a membrane potential. Valinomycin's presence results in decreased fluorescence emission (hyperpolarization) and addition of external potassium results in an increased fluorescence

Figure 1. Effect of dl-propranolol on the normal murine lymphocyte Con A dose-response and the fluorescence polarization of DPH and F-Con A labeled murine lymphocytes. A) Con A dose-response in the presence of indicated concentrations of propranolol (+) expressed as a percent of the control (right ordinate). B) Fluorescence polarization of F-Con A in the presence of indicated concentrations of propranolol (●). C) Fluorescence polarization (left ordinate) of DPH in the presence of indicated concentrations of propranolol (o). Experiments were conducted as detailed in Materials and Methods.

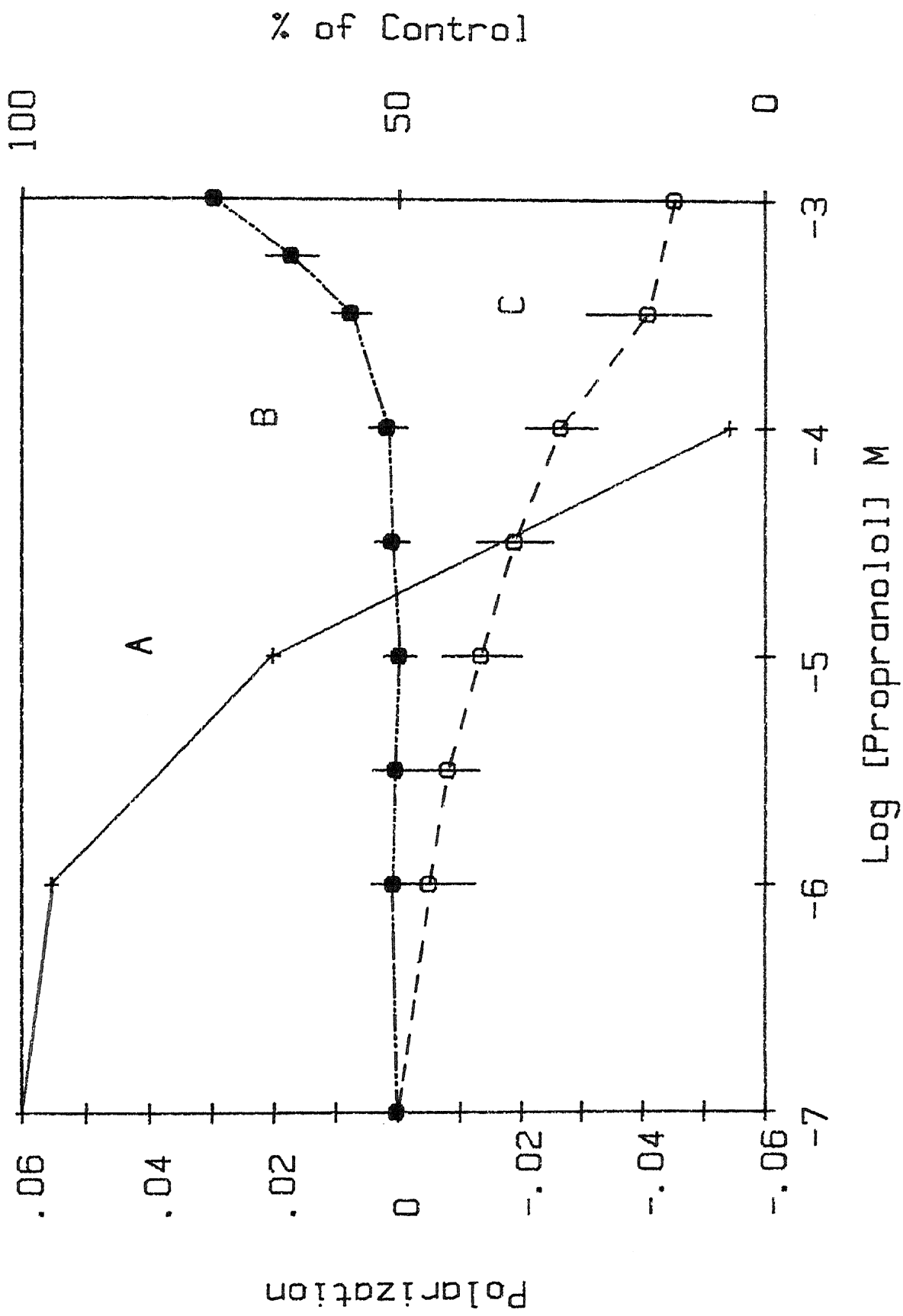

% of Control
100
50
0
A
B
C
-3
-4
-5
-6
-7
Log [Propranolol] M
.06
.04
.02
0
-.02
-.04
-.06
Polarization

Figure 2. Effect of d- and l- isomers of propranolol on the normal murine lymphocyte Con A dose-response and the fluorescence polarization of DPH labeled murine lymphocytes. A) Con A dose-response in presence of indicated concentrations of d-propranolol (+) expressed as a percent of the control (right ordinate). B) Con A dose-response in the presence of indicated concentration of l-propranolol (o) expressed as a percent of the control (right ordinate). C) Fluorescence polarization of DPH in the presence of indicated concentrations of l-propranolol (o). D) Fluorescence polarization of DPH in the presence of indicated concentrations of d-propranolol (+). Experiments were conducted as detailed in Materials and Methods.

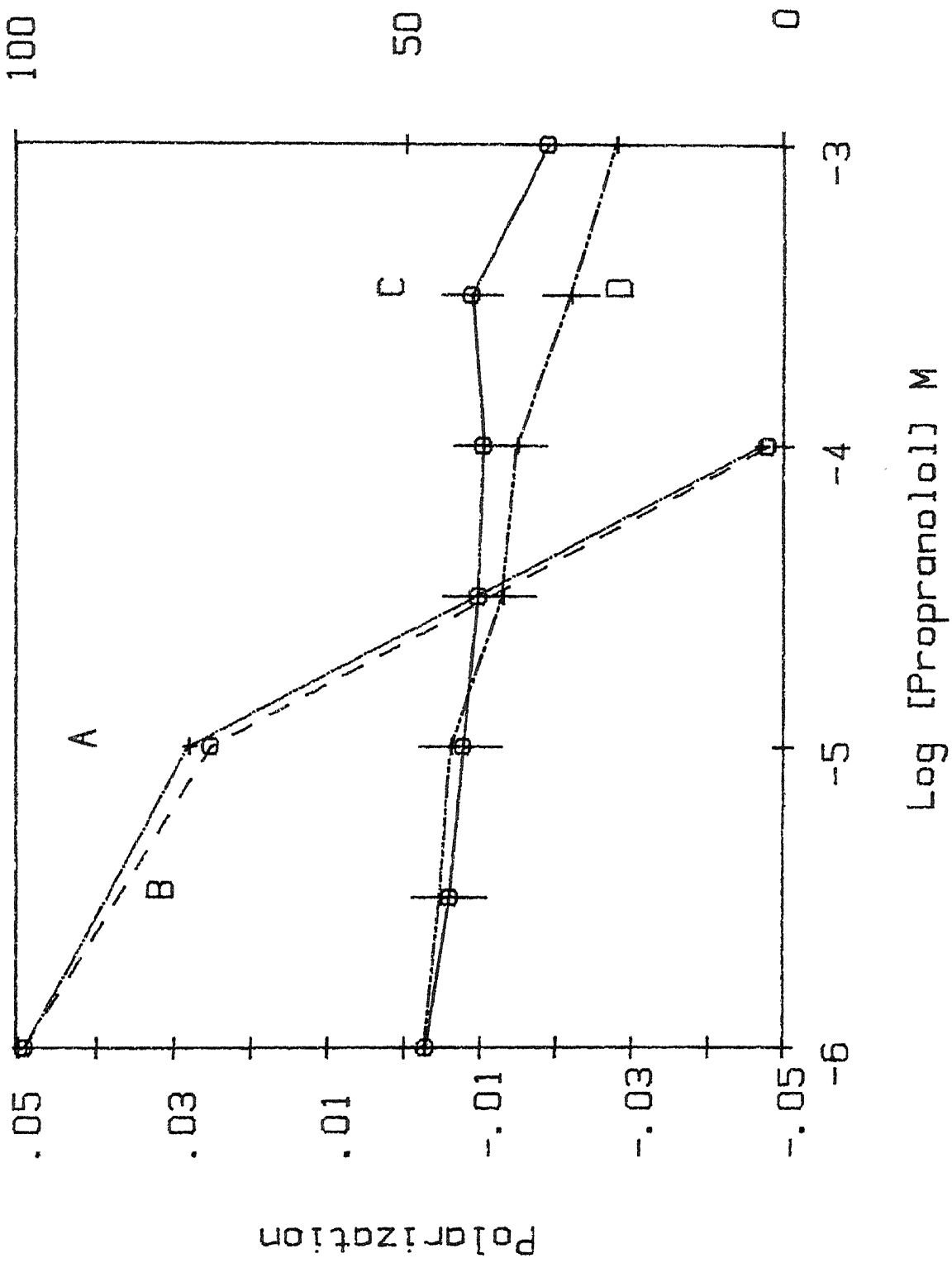

% of Control
100
50
0
-3
-4
-5
-6
Log [Propranolol] M
A
B
C
D
.05
.03
.01
.01
.03
.05
Polarization

Figure 3. Effect of alprenolol on the normal murine lymphocyte Con A dose-response and the fluorescence polarization of DPH and F-Con A labeled murine lymphocytes. A) Con A dose-response in the presence of indicated concentrations of alprenolol (+) expressed as a percent of the control (right ordinate). B) Fluorescence polarization of F-Con A in the presence of indicated concentrations of alprenolol (●). C) Fluorescence polarization (left ordinate) of DPH in the presence of indicated concentrations of alprenolol (o). Experiments were conducted as detailed in Materials and Methods.

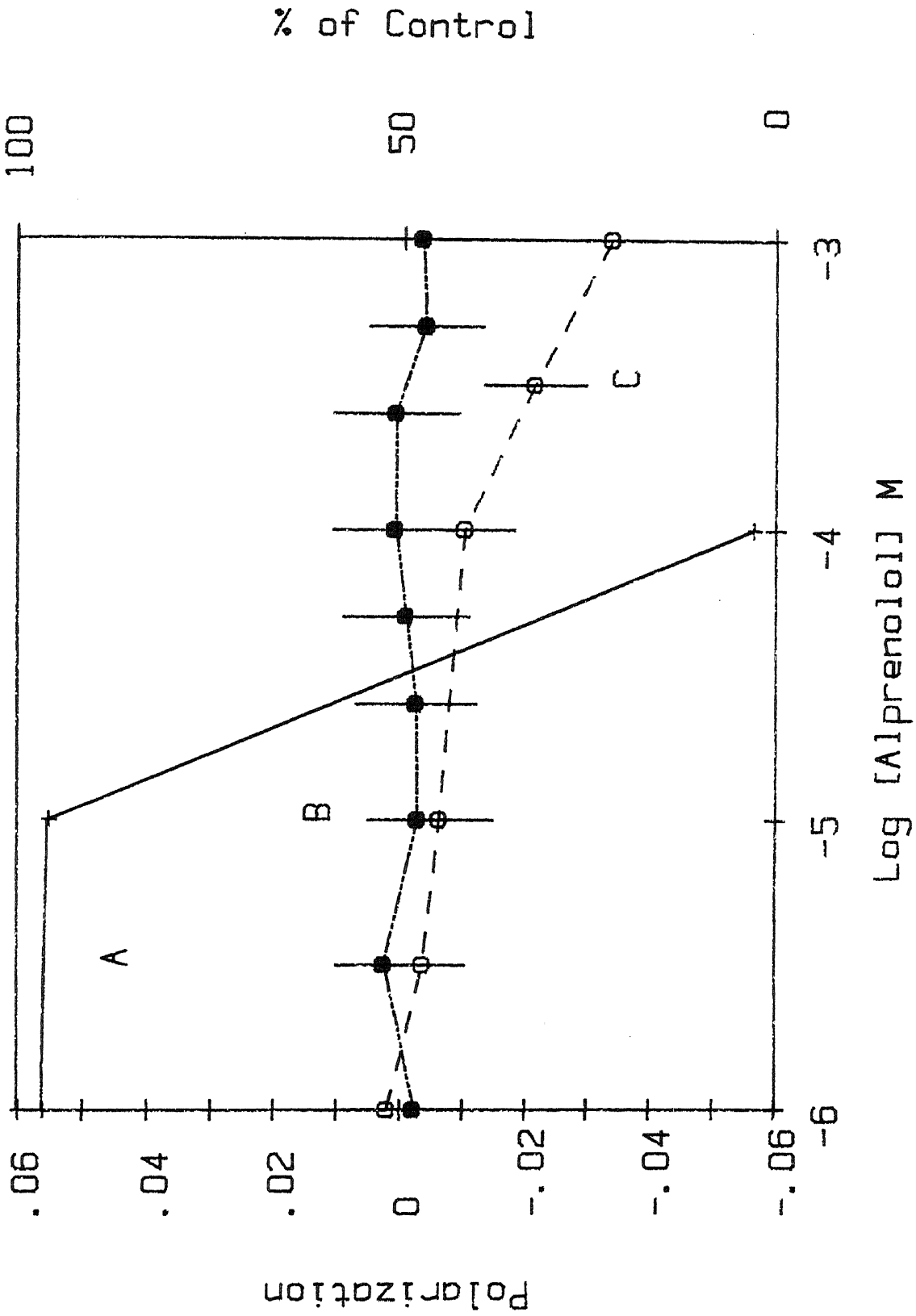

% of Control
100
50
0
-3
-4
-5
-6
Log [Alprenolol] M
A
B
C
.06
.04
.02
0
-.02
-.04
-.06
Polarization

Figure 4. Effect of metoprolol on the normal murine lymphocyte Con A dose-response and the fluorescence polarization of DPH and F-Con A labeled murine lymphocytes. A) Con A dose-response in the presence of indicated concentrations of metoprolol (+) expressed as a percent of the control (right ordinate). B) Fluorescence polarization of F-Con A in the presence of indicated concentrations of metoprolol (●). C) Fluorescence polarization (left ordinate) of DPH in the presence of indicated concentrations of metoprolol (o). Experiments were conducted as detailed in Materials and Methods.

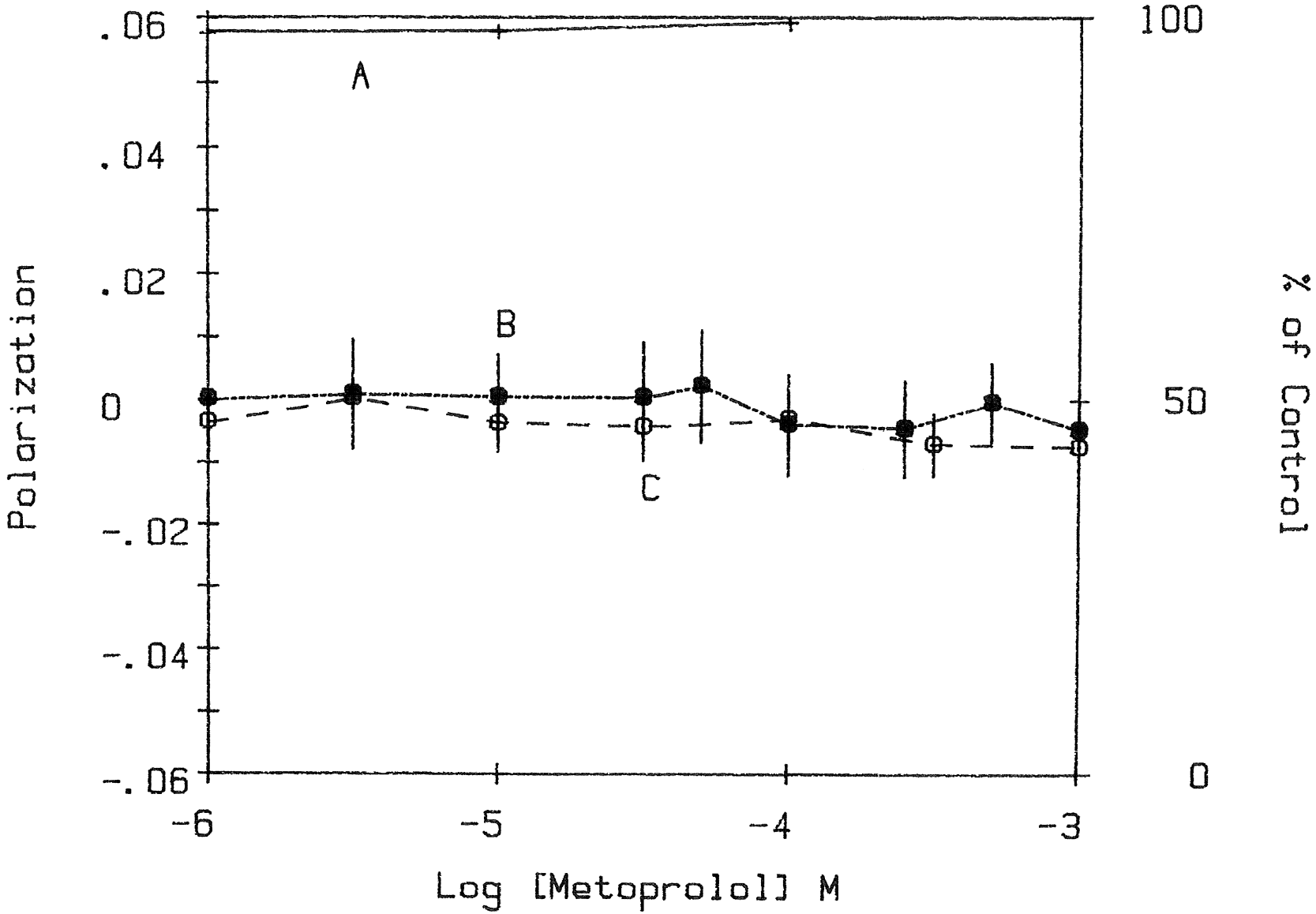

.06
.04
.02
0
-.02
-.04
-.06
Polarization
100
50
0
% of Control
A
B
C
-6
-5
-4
-3
Log [Metoprolol] M

Figure 5. Effect of propranolol on the temperature dependency of DPH and F-Con A. A) represents the fluorescence polarization of DPH and B), the effect of 1 mM propranolol on the fluorescence polarization of DPH, relative to changes in temperature. C) represents the effect of 1 mM propranolol on the fluorescence polarization of F-Con A and D), the fluorescence polarization of F-Con A, relative to changes in temperature. At 20°C, C) (sample) is statistically different from D) (control), $p < .005$ (Student's t-test), and at 25°, 30°, 35°, 40°, and 45°, C) (sample) is statistically different from D) (control), $p < .001$ (Student's t-test). Experiments were conducted as detailed in Materials and Methods.

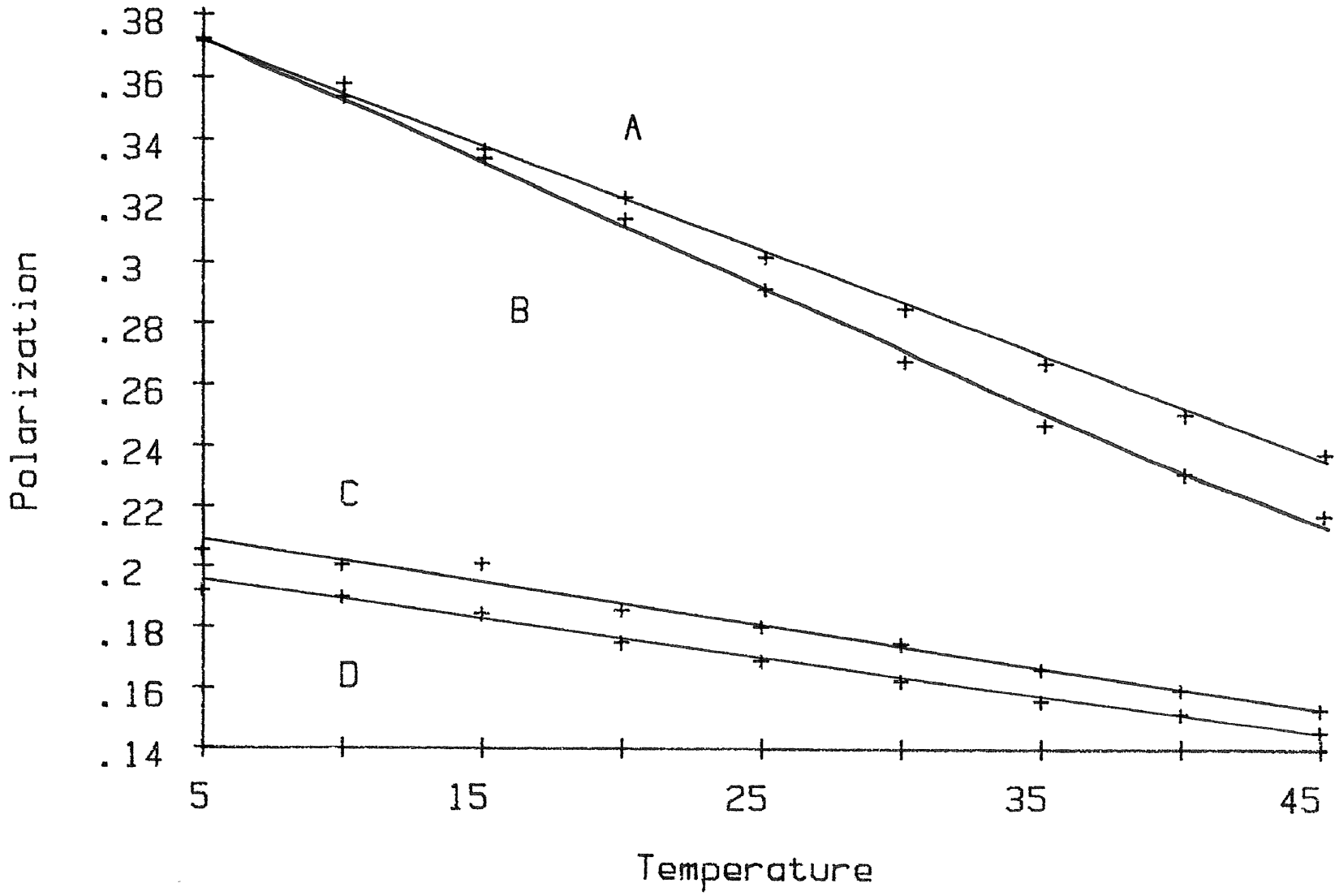

Polarization
.38
.36
.34
.32
.3
.28
.26
.24
.22
.2
.18
.16
.14
A
B
C
D
5
15
25
35
45
Temperature

Figure 6. % changes of membrane potential relative to % fluorescence changes. See Materials and Methods for curve generation.

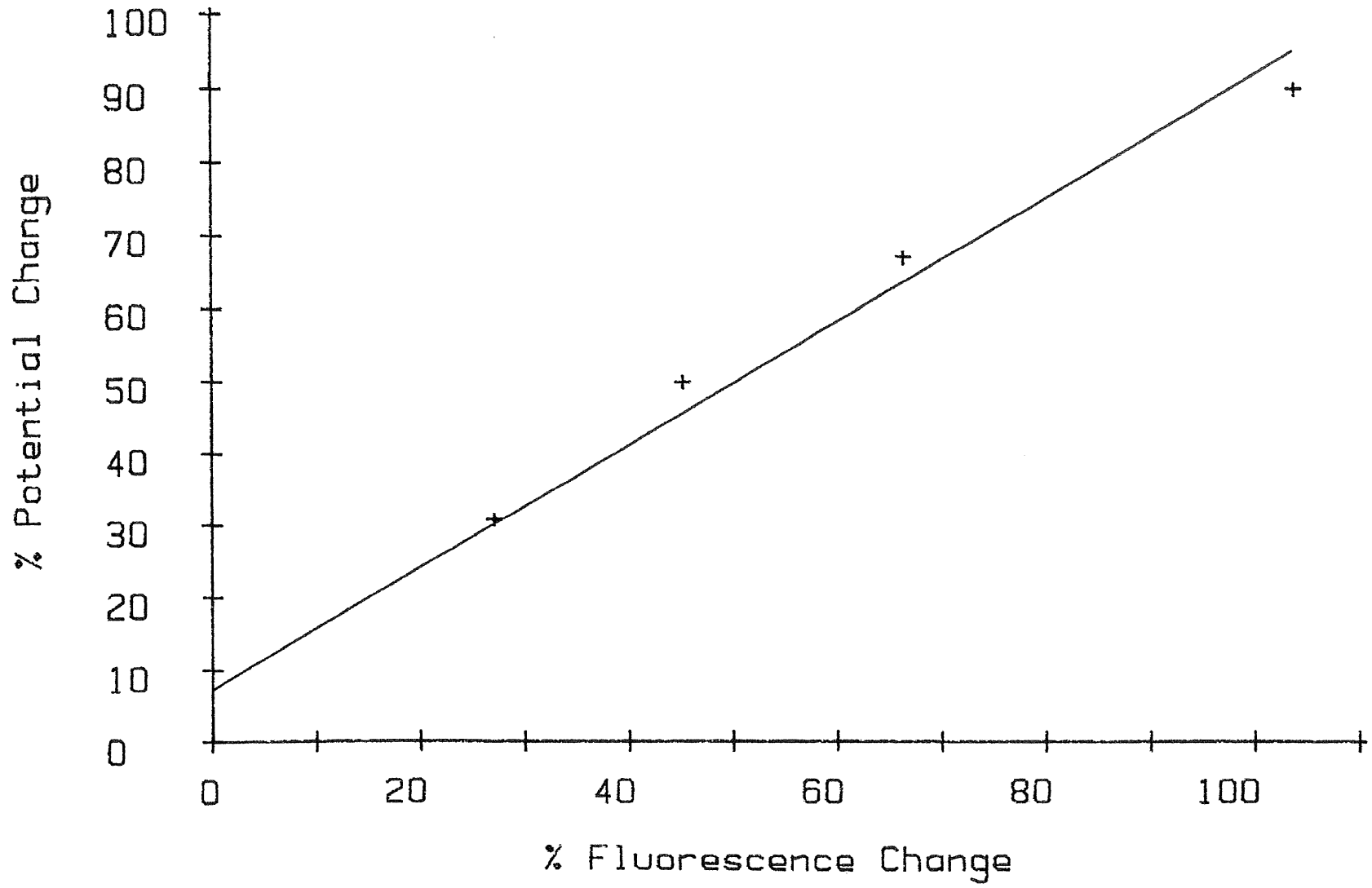
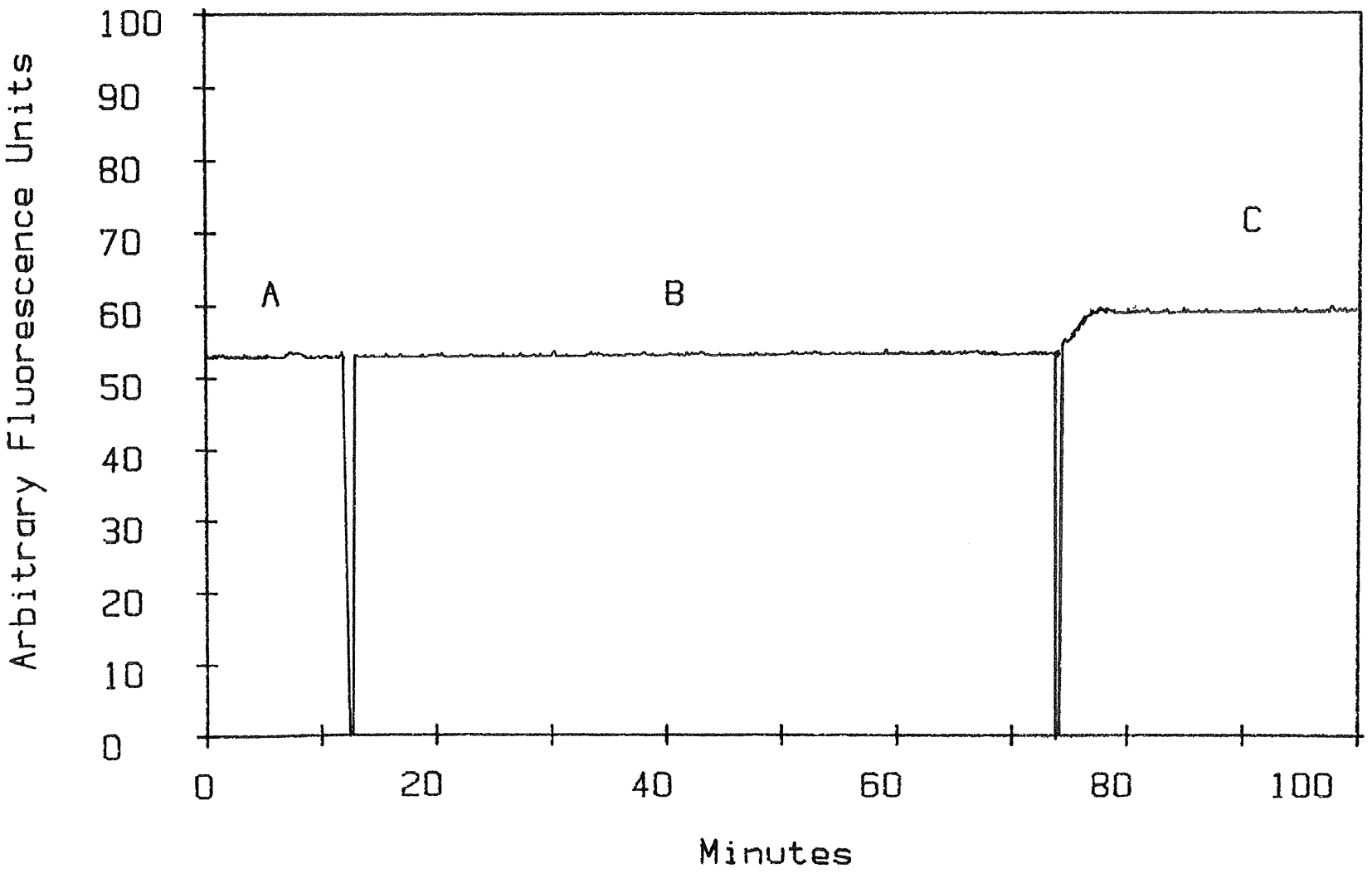

100
90
80
70
60
50
40
30
20
10
0
% Potential Change
0
20
40
60
80
100
% Fluorescence Change

Figure 7. Fluorescence emission of DiS-C3-(5) labeled murine lymphocytes. A) is the fluorescence emission of diS-C3-(5) labeled murine lymphocytes indicating the resting membrane potential of the cell. B) effect of 30 μM propranolol on the fluorescence emission of diS-C3-(5) labeled cells. Experiments were conducted as detailed in Materials and Methods.


Arbitrary Fluorescence Units
100
90
80
70
60
50
40
30
20
10
0
A
B
C
0
20
40
60
80
100
Minutes

emission (depolarization). The resting membrane potential was not altered by addition of either sodium chloride or calcium chloride (not shown) before or after addition of propranolol.

DISCUSSION

Three fluorescent probes (DPH, F-Con A, and diS-C3-(5)) have been used in an attempt to characterize the nonspecific (non-receptor mediated) effects of propranolol observed in short term cell cultures on the normal murine splenic lymphocyte mitogen dose-response. Two other beta-adrenergic antagonists, sotalol and alprenolol, and a selective beta-1-adrenergic antagonist, metoprolol, were also included in experiments and the results used for comparison to results obtained for propranolol.

Alterations of the fluorescence polarization of DPH have been interpreted as changes in the dynamic nature or "fluidity" of the hydrocarbon regions of live cells and artificial membranes (Johnston and Melnykovych, 1980; Kutchai et al., 1980; Pang et al., 1979; Fisher et al., 1979; Luly and Shinitzky, 1979; Johnson et al., 1979; Andrich and Vanderkooi, 1976; Shinitzky and Inbar, 1974; Shinitzky and Barenholz, 1974). For example, a decrease in the fluorescence polarization of DPH would be indicative of relative increases in the dynamic nature of the hydrocarbon regions around the probe.

Results here show that propranolol concentrations in the ten micromolar range or greater produced a significant decrease in the fluorescence polarization of DPH in the cell membranes of lymphocytes. By extension, these observations would suggest that propranolol is increasing the dynamic nature of hydrophobic regions

of the lymphocyte cell membrane. Suppression of the normal mitogen dose-response in short-term cultures is observed over a similar concentration range of propranolol. In experiments with d- and l-propranolol isomers, similar results were observed. By comparison, alprenolol effected a decrease in the fluorescence polarization at slightly higher concentrations, greater than 10 μM. In the short term cultures, alprenolol suppressed the normal mitogen dose-response at concentrations greater than that observed for propranolol. Alprenolol, therefore, also appears to increase the relative dynamic nature of the hydrophobic regions of the lymphocyte membranes at drug concentrations similar to those observed as effective in suppression of the normal lymphocyte mitogen dose-response.

Metoprolol and sotalol did not alter the fluorescence polarization of DPH to a significant degree. Of these two compounds, only metoprolol was tested in the cell culture system and this compound did not alter the normal mitogen-dose response. On the basis of the fluorescence polarization of DPH, sotalol and metoprolol do not change to a significant degree the relative fluidity of the hydrophobic regions of the lymphocyte cell membrane. This observation might be predicted, since sotalol and metoprolol, compared to propranolol and alprenolol, have been shown to have relatively poor liposolubility characteristics (Dax and Partilla, 1982; Woods and Robinson, 1981). Our results would suggest that very small changes in the hydrophobic regions around the probe have a significant affect on the responsiveness of the murine lymphocyte

to mitogenic stimulation. It has been suggested that the mobility of membrane components plays an important role in the immune response, possibly by modulation of specific ion permeabilities and uncoupling of transmembrane proteins (McConnell, 1978).

Courtney (1980) indicated that various drugs could block frequency-dependent myocardial arrhythmias (Courtney, 1980). Concentrations of d- and l- propranolol, and alprenolol necessary to produce frequency-dependent block were 5 μM and 20 μM, respectively (Courtney, 1980). Woosley et al. (1977), have described similar propranolol concentrations as being required for control of ventricular arrhythmias. The above references to the use of propranolol or alprenolol for treatment of cardiac arrhythmia is of interest here based on the ability of propranolol and alprenolol to suppress the normal mitogen dose-response of lymphocytes over similar concentration ranges. In addition, relative changes in the hydrophobic regions of the lymphocyte membrane are noted at similar concentrations of propranolol and alprenolol.

The concentrations of propranolol and alprenolol required for treatment of the cardiac arrhythmias noted above are much greater than for receptor mediated beta-adrenergic processes. These effects are found to be non-receptor mediated effects (non-specific effects) and are generally referred to as quinidine-like or local anesthetic effects. In theory, local anesthetics cause an increase in the relative dynamic nature of hydrophobic regions of cell membranes (Roth, 1979). From our results, it could be suggested that concentrations of propranolol and alprenolol that have local

anesthetic effects on the heart may also suppress the normal lymphocyte mitogen dose-response through a similar mechanism (local anesthetic effect or increased fluidity of the lymphocyte cell membrane).

The fluorescence polarization of F-Con A has been used to study differences in cell surface dynamics and mobility of the Con A receptor on normal and malignant cells (Ben-Bassat et al., 1977; Inbar et al., 1973). In the lymphoid system, increases in the fluorescent polarization are interpreted as decreases in mobility of the Con A receptor and a decreased exposure of the Con A receptor to the aqueous environment. Decreased fluorescence polarization would be interpreted as the opposite, increased Con A receptor mobility and an increased exposure of Con A receptors to the aqueous environment (Ben-Bassat et al., 1977). Also increases in the dynamic nature of the hydrophobic regions of the lymphocyte membrane are consistent with a decreased mobility of the Con A receptors on the cell surface. The opposite conditions have also been suggested for cell surface Con A receptors and the hydrophobic regions of the cell membrane (Ben-Bassat et al., 1977).

Of the four beta-adrenergic compounds that were tested, only propranolol altered the fluorescent polarization of F-Con A. Concentrations greater than 100 μM propranolol were required to increase the polarization of F-Con A. This increase in the fluorescence polarization of F-Con A is consistent with a decrease in lymphocyte surface Con A receptor mobility. Since propranolol increases the dynamic nature of the hydrophobic regions of the

lymphocyte membrane at these concentrations, the decreased mobility of the cell surface Con A receptor observation is consistent with the idea of increased fluidity of hydrocarbon regions paralleling decreased cell surface dynamics (Ben-Bassat et al., 1977). However, there is a significant difference between the concentrations at which there is an alteration of cell surface component dynamics and the alteration of the hydrophobic regions of the cell membrane. This difference in concentration dependency suggests that alterations of the hydrophobic regions of the cell membrane must be significantly large before cell surface Con A receptors are affected to a significant degree.

The temperature-dependent fluorescence polarization of DPH, and of F-Con A was completed both in the presence and absence of propranolol. At higher temperatures there is a greater fluidity of the lymphocyte membrane in the presence of propranolol than at lower temperatures where the effect of propranolol diminishes. The effect of propranolol on the DPH curve may be indicative of selectivity of propranolol for certain hydrophobic regions of the lipid matrix of the cell membrane or hydrophobic regions of some proteins. In contrast, propranolol causes an upward shift of the fluorescence polarization of F-Con A. Therefore, the presence of propranolol decreases the mobility of the Con A receptor uniformly at all temperatures. Given that the relative fluidity of the cell membrane is increased, the decrease in mobility of the F-Con A receptors is consistent.

The membrane potential of the murine splenic lymphocyte has

been determined to be approximately -65 mV. This is in agreement with the -65 mV determined by Kiefer et al. (1980) (using ^{3}H-tetraphenylphosphonium). DiS-C3-(5) provides a useful monitor of the resting membrane potential and reflects changes of the membrane potential (hyperpolarization or depolarization) that may occur as a result of a stimulus (Waggoner, 1979). With diS-C3-(5) the lymphocyte resting membrane potential was monitored for alterations induced by various concentrations of propranolol. A significant change could not be distinguished at any concentration of propranolol tested. Therefore propranolol does not appear to influence the resting membrane potential. It is important to note that concentrations of propranolol which resulted in a significant change in the fluidity of the hydrophobic regions of the cell membrane (30 μM) did not appear to alter membrane permeability to potassium. Addition of external potassium to cell suspensions resulted in depolarization regardless if added before or after propranolol. It is difficult to determine whether or not sodium or calcium permeability is altered relative to the lymphocyte membrane since neither alters the membrane potential when external levels are elevated in the absence of propranolol.

In recent years it has become popular to describe the actions of a xenobiotic as occurring through receptor mediated processes. From data presented here, and in other recent studies (Dax and Partilla, 1982; Woods and Robinson, 1981; Deacon et al., 1981), it is apparent that the non-receptor mediated activity of a xenobiotic such as propranolol may be of substantial significance, along with

the traditional beta-receptor mediated processes. Therefore, it remains important to characterize non-receptor mediated mechanisms that may or may not produce significant alterations in biological systems.

In summary, the data suggests that concentrations of propranolol and alprenolol (non-selective beta-adrenergic antagonists) that suppress that normal lymphocyte Con A dose-response are similar to the concentrations of propranolol and alprenolol that increase the relative fluidity of the hydrophobic regions of the lymphocyte cell membrane. Also, metoprolol, a beta-1-adrenergic antagonist, does not alter either the normal lymphocyte mitogen dose-response or the relative dynamic nature of the hydrophobic regions of the cell membrane. Sotalol, another non-selective beta-adrenergic antagonist, does not alter the relative fluidity of the cell membrane. Propranolol alone was also shown to alter the mobility of the Con A receptor on the lymphocyte cell surfaces but at concentrations significantly higher than those altering the fluidity of the hydrophobic regions of the lymphocyte cell membrane. In temperature-dependent polarization experiments with DPH, propranolol may affect different hydrophobic regions of the lymphocyte cell membrane to differing degrees. At low temperatures propranolol had little effect on the fluidity of the lymphocyte cell membrane but, at high temperatures, propranolol significantly increased the relative dynamic nature of the lymphocyte cell membrane. In temperature-dependent polarization experiments with F-Con A, propranolol effected a parallel decrease

in cell surface Con A receptor mobility at all temperatures. Propranolol does not alter the resting membrane potential of the murine lymphocyte or the lymphocyte's permeability to potassium.

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EXPERIMENTAL STUDIES

PART IV

EFFECT OF TRICYCLIC ANTIDEPRESSANT DRUGS ON LYMPHOCYTE MEMBRANE STRUCTURE

ABSTRACT

Tricyclic antidepressant-induced perturbations of murine splenic lymphocyte membranes and cell surface concanavalin A receptor mobility have been investigated using the fluorescent probes diphenylhexatriene and fluorescein-conjugated concanavalin A. Results of these studies illustrate the possible relationship between tricyclic antidepressant-induced membrane perturbations and tricyclic antidepressant-induced suppression of the normal murine lymphocyte mitogen response. Tricyclic antidepressant effects on murine splenic lymphocyte membranes are dose-, time- and temperature- dependent. Murine lymphocyte concanavalin A cell surface receptor mobility is not apparently altered by the tricyclic antidepressants.

INTRODUCTION

The therapeutic application of tricyclic antidepressant drugs in the management of affective disorders is well known. The precise mechanism of action for these drugs, however, remains unresolved (Sulser, 1982). Specific binding sites for tricyclic antidepressants have been identified on membrane preparations of brain (Dumbrille et al., 1981; Kinnier et al., 1981; Rehavi et al., 1981; and Raisman et al., 1979), platelets (Langer et al., 1981; and Wennogle et al., 1981), lymphocytes (Audus and Gordon, 1982a), and lung (Raisman and Langer, 1983). Since the role of these binding sites in tricyclic antidepressant therapy is not clearly established (Sulser, 1982), it is of interest to investigate other tricyclic antidepressant drug-membrane interactions that may contribute to an understanding of the pharmacology of the tricyclic antidepressants.

It has been demonstrated that the tricyclic antidepressants suppress the normal mitogenic response (Audus and Gordon, 1982b; Nahas et al., 1979, Smith et al., 1978; and Waterfield et al., 1976) and the number of plaque forming cells (Smith et al., 1978) in short term cultures of murine splenic lymphocytes. The concentrations (10 μM or greater) of the tricyclic antidepressants that suppress the normal mitogenic response and the number of plaque forming cells in culture, are much greater than the apparent K_d (.4 nM) for the tricyclic antidepressant binding site (Audus and Gordon, 1982b). Therefore, it is probable that the suppression of the mitogenic response and the reduction in the numbers of plaque-forming cells in the short term cultures of murine splenic lymphocytes is not

mediated by the specific high affinity binding site. Moreover, concentrations at which the tricyclic antidepressants depress murine lymphocyte responses in the short term cultures described above, are similar to those concentrations of tricyclic antidepressants which are known to alter the phase-transition of lipids in artificial cell membranes (Romer and Bickel, 1979; Bermejo et al., 1975; Cater et al., 1974; and Bermejo et al., 1974).

In this report, we present data that illustrates the relationship between the concentrations of tricyclic antidepressants that alter the lipid or hydrophobic domains of murine splenic lymphocyte membranes, as monitored by a fluorescent probe, and the concentrations of tricyclic antidepressants that alter murine splenic lymphocyte mitogenesis, a cellular process in which the cell membrane lipids play a significant role (Gurr, 1983). In addition, tricyclic antidepressant effects on the mobility of the Concanavalin A receptor, as monitored by a fluorescent probe, and the time and temperature dependency of the tricyclic antidepressant effects on lymphocyte membranes, will be described.

The fluorescent probes used in this study are 1,6-diphenyl-1,3,5-hexatriene (DPH), and fluorescein conjugated Concanavalin A (F-Con A). DPH, a highly lipophilic fluorescent probe, has been used to characterize structural perturbations in the lipid or hydrophobic domains of live and artificial cell membranes in response to temperature (Andrich and Vanderkooi, 1976) and pressure changes (Lackowicz and Thompson, 1983), disease (Ben-Bassat et al., 1977: Inbar, 1976; Shinitzky and Inbar, 1974; and Inbar et al.,

1973), and various pharmacologically active compounds (Audus et al., 1983; Kutchai et al., 1980; Johnston and Melnykovych, 1980; and Luly and Shinitzky, 1979). F-Con A has been used to study alterations of cell surface Concanavalin A receptor mobility in the presence of disease and pharmacologically active compounds (Audus et al., 1983; Ben-Bassat et al., 1977; Shinitzky and Inbar, 1974; and Inbar et al., 1973).

MATERIALS AND METHODS

1,6-Diphenyl-1,3,5-hexatriene (lot no. 2021A) was purchased from Molecular Probes, Inc., Junction City, OR. Fluorescein conjugated Concanavalin A (lot no. 00387; fluorescein/protein (molar): 4.9) and Concanavalin A (lot no. 910026) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Desipramine HCl was a gift from Merrell Dow Pharmaceutical Inc., Cincinnati, OH. Nortriptyline HCl was a gift from Lilly Research Laboratories, Indianapolis, IN. Protriptyline HCl was a gift from Merck, Sharp & Dohme Research Laboratories, West Point, PA. Phosopholipids and imipramine HCl (lot no. 121F-0086) was purchased from Sigma Chemical Co., St. Louis, MO. Thymidine, (6-^{3}H)-, was purchased from New England Nuclear, Boston, MA. All other compounds used were of the highest grade commercially available.

Lymphocyte Isolation. Outbred, adult male mice (25-30 g) were sacrificed by cervical dislocation and spleens removed. The spleens were macerated in phosphate buffered saline (PBS), pH 7.4, lymphocytes isolated by Histopaque gradient centrifugation and washed in PBS (Boyum, 1968). The final lymphocyte pellet was

suspended in an appropriate concentration in PBS for fluorometric experiments, or in RPMI 1640 (supplemented with 10% bovine serum, 100 U penicillin, and 100 μg streptomycin) for short-term lymphocyte cultures. Viability of the isolated lymphocytes, as determined by trypan blue exclusion was always greater than 90%.

Short Term Lymphocyte Cultures. Methodology for conducting the short-term cultures used in this study have been described in detail in previous work (Audus and Gordon, 1982b). Briefly, 0.01 ml aliquots of lymphocytes suspended in RPMI 1640 were added to microtiter plates (Model 3040, Falcon Division, Becton-Dickinson, Oxnard, CA). 0.05 ml of Con A in concentrations of 0 to 90 μg/ml, and 0.02 ml of an appropriate concentration of tricyclic antidepressant or PBS were also added to each of the 96 wells. The cultures were incubated at 37 $^{\circ}$C, with 5% CO_2 and 95% air. Each well was pulsed with 0.03 ml tritiated thymidine (0.5 μCi), bringing the total well volume to 0.2 ml. Incorporated labeled thymidine was collected on Whatman glass fiber filters with a cell harvester (Model 24V, Brandel, Gaithersberg, MD). Filters were air dryed, then placed in scintillation vials with 3 ml of LSC Complete (Yorktown Research, Miami, FL) and radioactivity assayed to an accuracy of 0.5% in a Beckman Tri-Carb Scintillation Spectometer (approximately 58% tritium efficiency).

Tricyclic Antidepressant Effects on the Normal Mitogen Dose-Response. Microtiter plates were divided in half such that the top half of the plate (4 rows x 12 columns) served as controls for the remaining wells of the plate. Control wells received aliquots of

PBS not containing a tricyclic antidepressant in addition to mitogen and cells. Remaining wells received in addition, an appropriate concentration of a tricyclic antidepressant.

Cell Labeling Procedure. Cells were labeled with DPH as described previously (Audus et al., 1983). $2 * 10^6$ cells/ml in PBS were mixed (1:1; v/v) with a 2 μM solution of DPH in PBS, incubated at 37° C for 45 min, and washed with PBS. 3 ml of DPH labeled cell solution was placed in a quartz cuvette and fluorescence anisotropy and lifetimes measured immediately. The final concentration of DPH was 1 μM and the final concentration of cells was approximately $1 * 10^6$ cells/ml.

The procedure for labeling cells with F-Con A has been described in a prior report (Audus et al., 1983). Approximately .390 ml of 8.0 mg/ml (protein) F-Con A stock was incubated with $5 * 10^6$ cells/ml suspended in PBS at 37° C for 15 min. Following incubation, the cell suspension was centrifuged at 150 * g for 15 min and the pellet resuspended in PBS to a final concentration of about $1 * 10^6$ cells/ml. F-Con A labeled cells were used immediately in fluorometric measurements.

All drugs were dissolved in distilled water or PBS and added to the fluorometer cuvettes in aliquots of 1-40 μl with a Hamilton microliter syringe. Each drug addition to the cuvette was followed by a 5 min (unless otherwise stated) incubation period before a fluorescence polarization or lifetime measurement was made. Aliquots of PBS or distilled water containing no drug, added in

equal amounts and at similar times, as for drug addition, served as a control.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were made with an SLM 8000 interfaced with an Hewlett-Packard 87 computer for on-line data acquisition and analysis as described previously (Audus et al., 1983). Photomultiplier tubes were placed to the right and left of the sample cell with polarizers inserted in the emission and excitation beams. A Schott KV-389 cut on filter was placed in the horizontally polarized emission beam and an SLM MC320 monochromator placed in the vertically polarized emission beam. Emission was monitored at 430 nm for DPH experiments and at 519 nm for F-Con A experiments. Fluorescence intensity was first measured with the sample excited (source, 450 W Xenon arc lamp) with a horizontally polarized beam, and then measured a second time with the sample excited with a vertically polarized beam. For DPH experiments the sample was excited with 360 nm light, and for the F-Con A experiments the sample was excited with 492 nm light. The fluorometer was set up to measure fluorescence anisotropy

$$r = A - B / A + 2B$$

where r is anisotropy. A is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the vertical position. B is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the horizontal position.

Background subtractions of cell + drug and fluorophore + drug

contributions were made but were not found to be significant (exception tricyclic antidepressant-F-Con A interactions see Results and Discussion sections). Light scattering due to turbidity of the cell suspension was also insignificant. The tricyclic antidepressants absorb in the UV region (maximally around 250 nm) well below the excitation wavelength for DPH (360 nm) and did not significantly contribute to the fluorescence of the labeled lymphocytes. The temperature of the sample cell was controlled by a circulating water bath and the contents of the sample cuvette stirred with a magnetic stirrer.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were calculated from phase and modulation measurements made with an SLM 4800 subnanosecond fluorometer interfaced with a Hewlwett Packard 87 computer for on-line acquisition and analysis. Excitation light was passed through a MC320 monochromator set at the excitation wavelength of DPH (360 nm with a bandpass of .5 nm) and the intensity was modulated at 30 MHz by a Debye-Sears light modulator. Emission wavelengths were selected by Schott cut-on filters, RG630 for the reference channel, and a KV389 filter for the sample chamber. A rhodamine quantum counter solution in a triangular cell was placed in the reference chamber. In the sample chamber, a sample solution (DPH labeled cells) and a reference solution (dimethyl POPOP, a fluorophore with a known lifetime of 1.45 nsec dissolved in ethanol) was placed in the two sample turret. Fluorescence emission from the two solutions was balanced by diluting the dimethyl POPOP solution.

The contents of the cuvettes in the sample chamber were stirred continuously with a magnetic stirrer and the temperature of the sample chamber controlled by a circulating water bath. A polarizer set at approximately 55° to the vertical was placed in the emission beam to eliminate Brownian rotation effects (Spencer and Weber, 1970).

The phase shift and the modulation of each sample was measured alternately 4 times, with the time interval between each measurement of about 10 sec. Data was acquired by the interfaced computer, averaged and the lifetimes calculated from the following basic relationships:

$$T_{phase} = 1/w \tan \theta$$

$$T_{mod.} = (1/w)\ (1/D^2 - 1)^{1/2}$$

where T_{phase} is the phase lifetime, θ the phase shift in degrees caused by a sample of lifetime T, w is the angular frequency of excitation which is 2 * pi * modulation frequency (30 MHz), $T_{mod.}$ is the modulation lifetime, and D is the ratio $M_{fluorophore}/M_{reference}$, where M represents the relative modulation of a reference solution.

The basic theory of fluorescence lifetime calculations from phase-modulation measurements follows from the fact that emission from fluorescent species, excited by sinusoidally modulated light, will be phase shifted and demodulated relative to the exciting light by an amount dependent on the fluorophore lifetime. (Characteristically, the modulation lifetime will always be greater than the phase lifetime in a system with a heterogenous distribution

of emitting fluorophores.) Detailed methodology and discussion of the theory of fluorescence lifetime calculations from phase-modulation measurements is described elsewhere (Lackowicz, 1983).

RESULTS

Figure 1 illustrates the dose-dependent effects of protriptyline and nortriptyline on the fluorescence anisotropy of DPH in murine splenic lymphocytes. With increasing concentrations of protriptyline and nortriptyline, there is a corresponding decrease in the fluorescence anisotropy of DPH. Additions of equal aliquots of PBS at similar times, as for nortriptyline and protriptyline additions, did not alter the fluorescence anisotropy of DPH. Reproducible tricyclic antidepressant effects on the fluorescence anisotropy of DPH begin at a concentration of about 30 μM. Protriptyline and nortriptyline do not quench DPH fluorescence to a significant degree at concentrations less than 1 mM (results not shown).

Figure 2 shows the dose-dependent decrease in the fluorescence lifetime of DPH in the presence of increasing concentrations of either desipramine or imipramine. Reproducible quenching of DPH fluorescence by desipramine and imipramine begins at a concentration of approximately 10 μM for desipramine and 30 μM for imipramine. Additions of equal aliquots of water at similar times, as for desipramine and imipramine additions, did not alter the fluorescence lifetime of DPH. Desipramine produces a dose-dependent increase in the fluorescence anisotropy of DPH that parallels effects on the fluorescence lifetime of DPH (not shown). In contrast, the

fluorescence anisotropy of DPH in murine lymphocyte membranes does not consistently increase or decrease in the presence of increasing concentrations of imipramine.

Data in Figure 3 illustrates tricyclic antidepressant effects on the murine lymphocyte mitogen response. Note that at a concentration of 1 μM, for each of the four tricyclic antidepressants, little supression of the mitogen response is observed while at a concentration of 10 μM, for each of the four tricyclic antidepressants, the mitogen response is suppressed to 50-60% of the control. At a concentration of 100 μM, for each of the four tricyclic antidepressants, almost complete suppression of the mitogen is observed.

In figures 4 and 5, DPH fluorescence anisotropy or fluorescence lifetime is plotted versus time (min) in the presence of the indicated concentration of tricyclic antidepressant. All of the tricyclic antidepressants produced maximal effects on either fluorescence anisotropy or fluorescence lifetime within five minutes after injection into a suspension of DPH labeled lymphocytes.

DPH fluorescence anisotropy or fluorescence lifetime is plotted versus temperature in figures 6 and 7. Nortriptyline and protriptyline at a concentration of 100 μM produce a decrease in the fluorescence anisotropy that is reversible with decreasing temperatures. Desipramine and imipramine at a concentration of 100 M produce a decrease in the fluorescence lifetime of DPH that is also reversible with decreasing temperatures.

The fluorescence anisotropy of F-Con A is not altered in the

Figure 1. Effect of Nortriptyline and Protriptyline on the DPH Fluorescence Anisotropy in Murine Splenic Lymphocytes. A) Initial value of DPH fluorescence anisotropy was .1842 +/- .002 for the sample receiving indicated concentrations of nortriptyline (o). B) Initial value of DPH fluorescence anisotropy was .1753 +/- .003 for the sample receiving indicated concentrations of protriptyline (+).

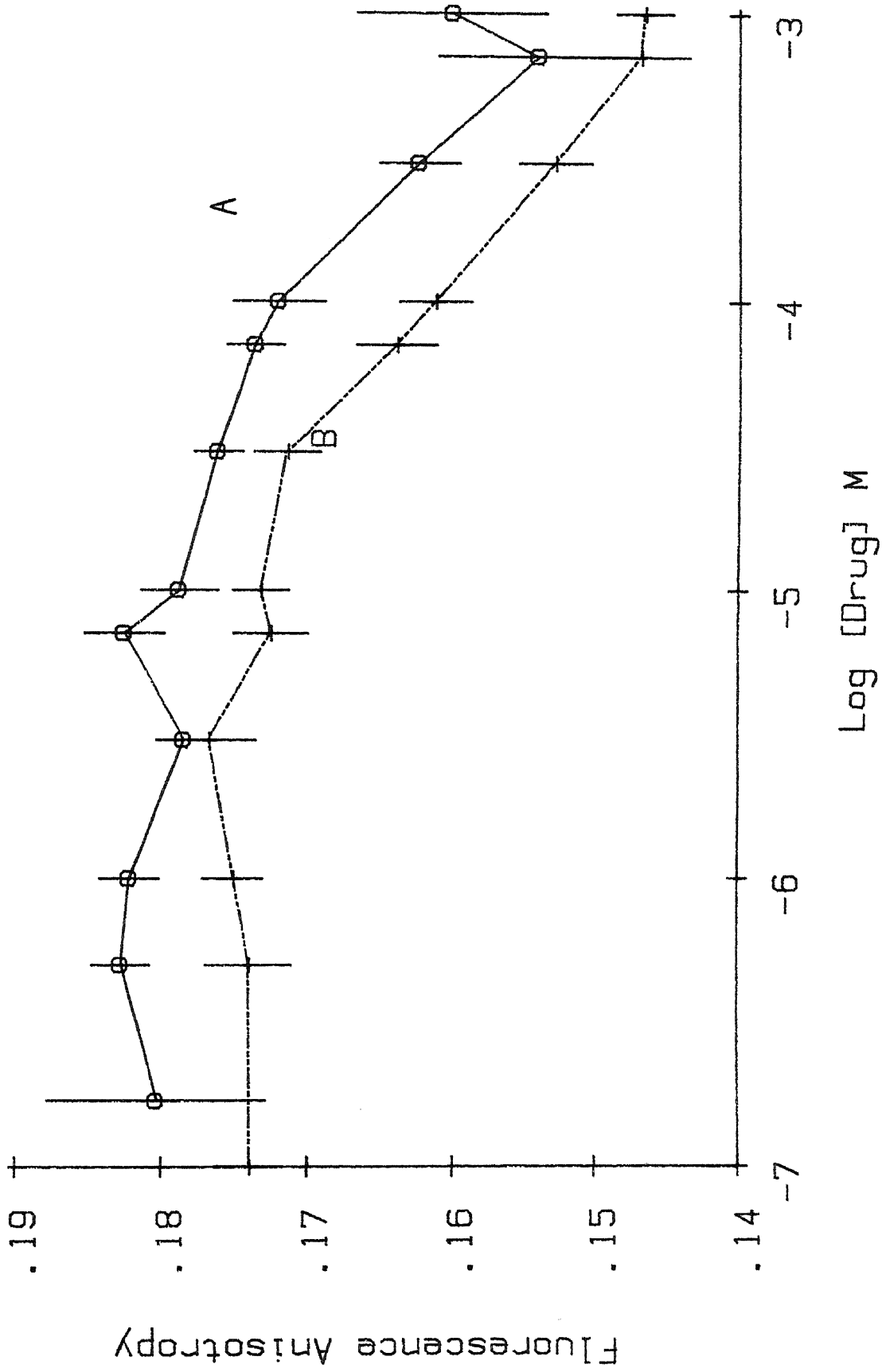
Fluorescence Anisotropy
.19
.18
.17
.16
.15
.14
-7
-6
-5
-4
-3
Log [Drug] M
A
B

Figure 2. Effect of Desipramine and Imipramine on the DPH Fluorescence Phase Lifetime in Murine Splenic Lymphocytes. A) Initial value of the DPH fluorescence phase lifetime was 7.961 +/- .Ø48 for the sample receiving indicated concentrations of desipramine (o). B) Initial value of the DPH fluorescence phase lifetime was 8.166 +/- .Ø96 for the sample receiving indicated concentrations of imipramine (+).

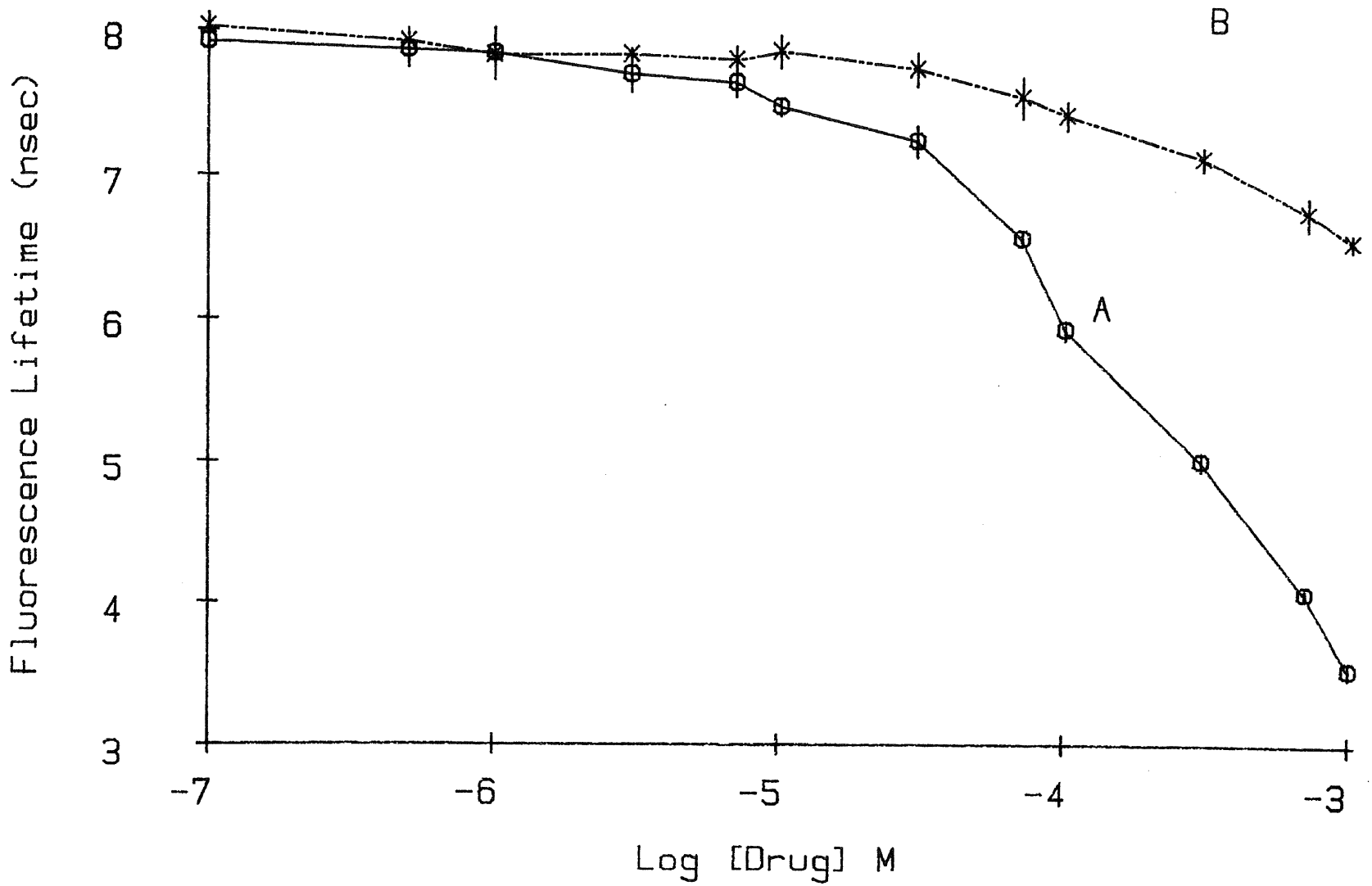
Fluorescence Lifetime (nsec)
8
7
6
5
4
3
-7
-6
-5
-4
-3
Log [Drug] M
A
B

Figure 3. Effect of the Tricyclic Antidepressants on the Normal Mitogenic Response of the Murine Splenic Lymphocyte. Each data point, expressed as a percent of a control, represents the mean counts of tritiated thymidine incorporated in the presence of the indicated concentrations of tricyclic antidepressant divided by the mean counts of tritiated thymidine incorporated without a tricyclic antidepressant present (control) times 100%. The mean counts of tritiated thymidine were calculated from quadruplicate samples in the presence of an optimal concentration of concanavalin A (10 μg/ml). (nortriptyline (o); protriptyline (+); desipramine (●); imipramine (■).

Percent of Control
100
80
60
40
20
0
-8
-7
-6
-5
-4
Log [Drug] M

Figure 4. Time Course of Nortriptyline and Protriptyline Effects on the DPH Fluorescence Anisotropy in Murine Splenic Lymphocytes. A) 300 μM nortriptyline (o) was added to DPH labeled murine lymphocytes and the fluorescence anisotropy recorded at one minute intervals. B) 100 μM protriptyline (+) was added to DPH labeled murine lymphocytes and the fluorescence anisotropy recorded at one minute intervals.

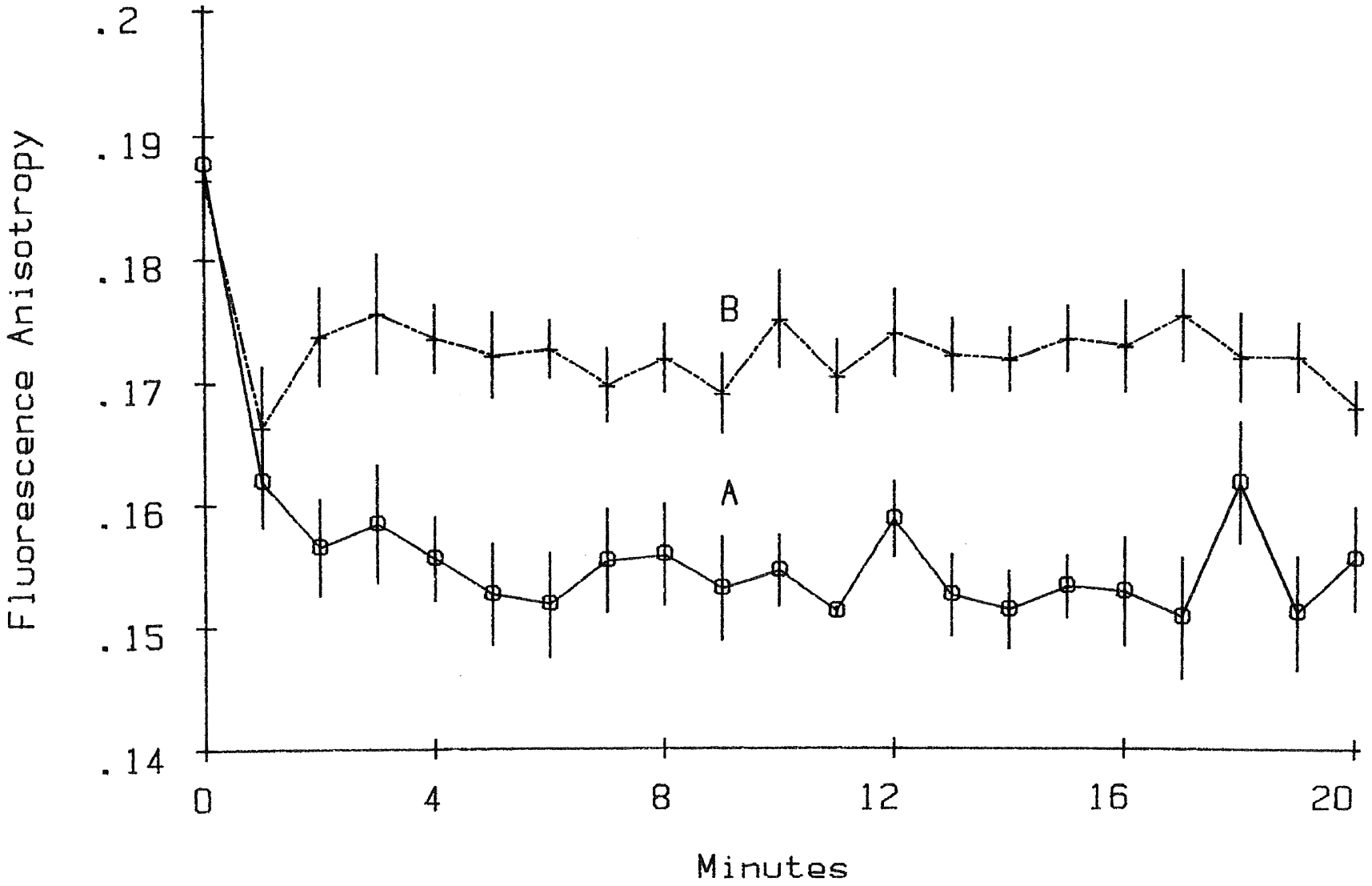
Fluorescence Anisotropy
.2
.19
.18
.17
.16
.15
.14
0
4
8
12
16
20
Minutes
B
A

Figure 5. Time Course of Desipramine and Imipramine Effects on the DPH Fluorescence Phase Lifetime in Murine Splenic Lymphocytes. A) 100 μM desipramine (o) was added to DPH labeled murine lymphocytes and the fluorescence phase lifetime recorded at one minute intervals. B) 100 μM imipramine was added to DPH labeled murine lymphocytes and the fluorescence phase lifetime recorded at one minute intervals.

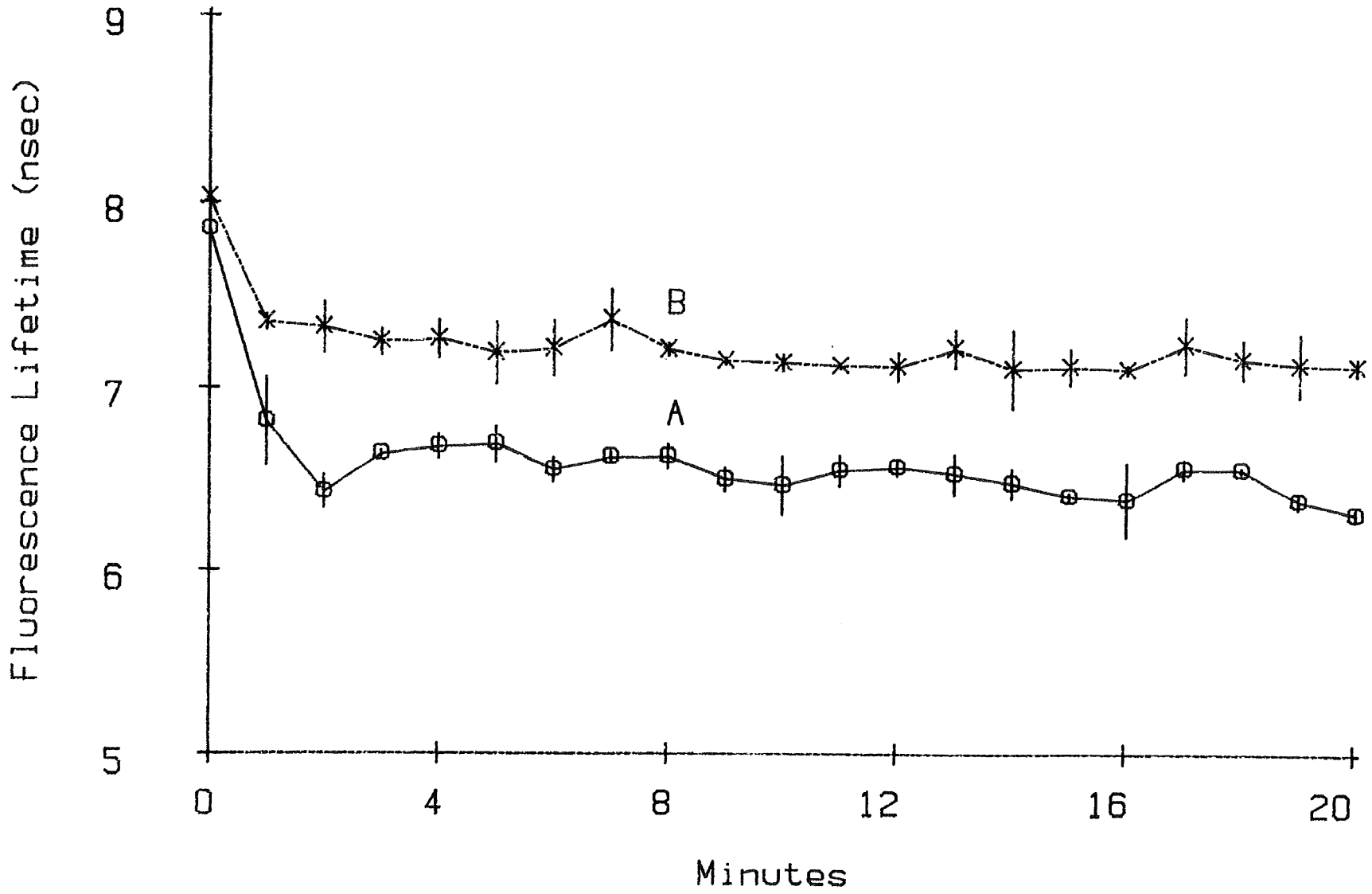
Fluorescence Lifetime (nsec)
9
8
7
6
5
0
4
8
12
16
20
Minutes
B
A

Figure 6. Temperature Dependency of the Effects of Nortriptyline and Protriptyline on the DPH Fluorescence Anisotropy in Murine Splenic Lymphocytes. A) 100 μM nortriptyline (+), B) 100 μM protriptyline (o), and C) an equal amount of PBS (control, x), was added to DPH labeled lymphocytes and the fluorescence anisotropy recorded at five degree intervals.

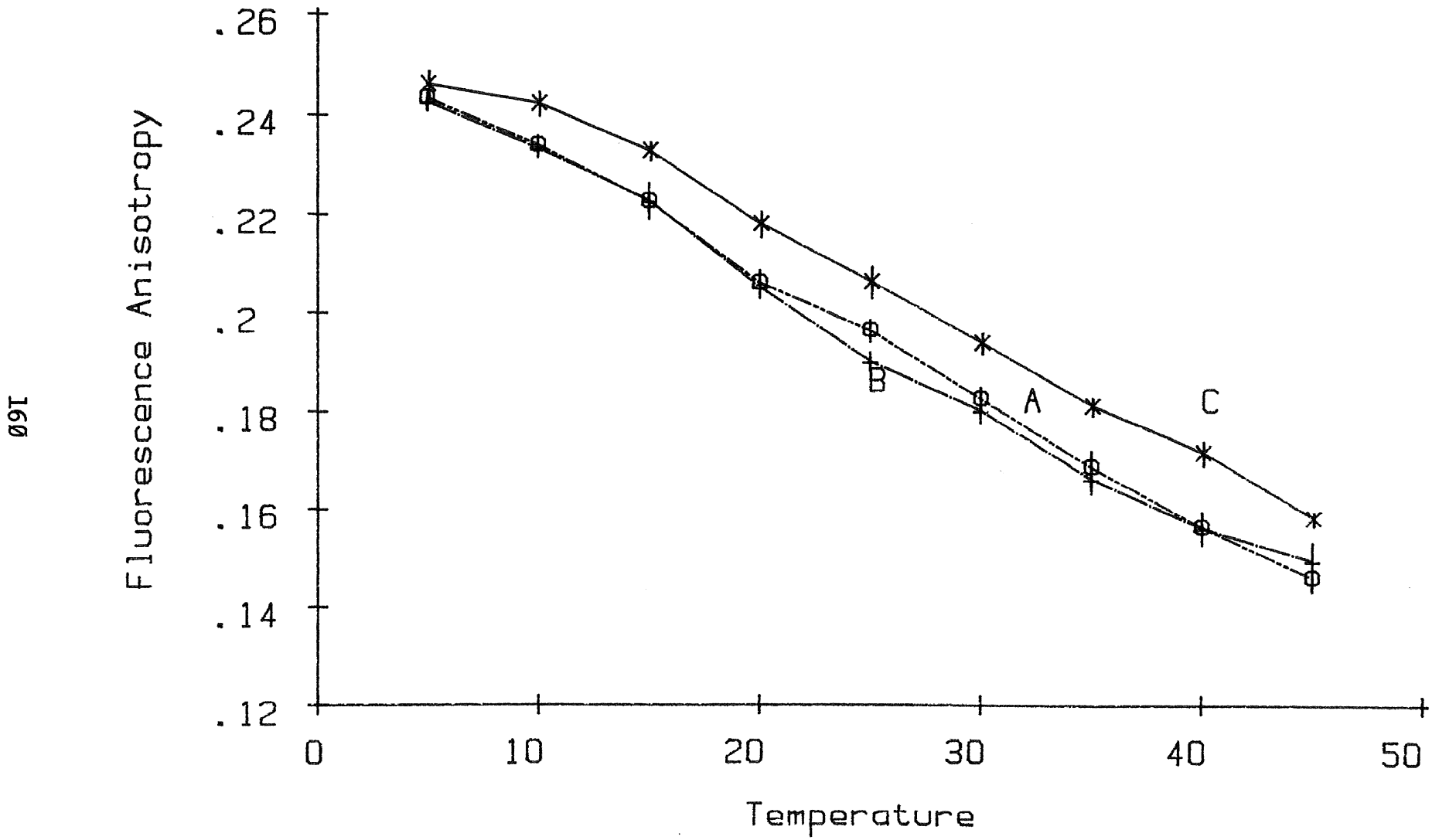
160
Fluorescence Anisotropy
.26
.24
.22
.2
.18
.16
.14
.12
0
10
20
30
40
50
Temperature
A
B
C

Figure 7. Temperature Dependency of the Effects of Desipramine and Imipramine on the DPH Fluorescence Phase Lifetime in Murine Splenic Lymphocytes. A) 100 μM desipramine (+), B) 100 μM imipramine (o), and C) an equal amount of PBS (control, x), was added to DPH labeled lymphocytes and the fluorescence phase lifetime recorded at 5 degree intervals.

Fluorescence Lifetime (nsec)
9
8
7
6
0
10
20
30
40
50
Temperature
A
B
C

presence of any of the four tricyclic antidepressants (results not shown). Although changes in the fluorescence anisotropy of F-Con A can be observed at concentrations of about 1 mM, these effects appear to be due to a direct interaction of the tricyclic antidepressants with the F-Con A since results in the presence and absence of cells are similar.

DISCUSSION

This study was conducted to compare directly tricyclic antidepressant induced effects on the lipid or hydrophobic domains of lymphocyte membranes and lymphocyte cell surface Con A receptors, with the tricyclic antidepressant induced effects on lymphocyte mitogenesis in short term cultures.

DPH fluorescence anisotropy has been used in a number of studies to characterize changes in membrane "fluidity" or to estimate the viscosity of live (Kutchai et al., 1980; Johnston et al., 1980; Luly and Shinitzky, 1979; and Inbar 1976) and artificial (Lackowicz and Thompson, 1983; Johnson et al., 1979; Pang et al. 1979; and Andrich and Vanderkooi, 1976) cell membranes. However, in a biomembrane, DPH experiences hindered rotations due to structural constraints in the hydrophobic microenvironment of the DPH molecule and therefore, the fluorescence anisotropy of DPH in biomembranes is representative of the degree of structural order in the hydrophobic or lipid microenvironment of the DPH molecule (Lackowicz et al., 1979; Lackowicz and Prendergast, 1978a; and Lackowicz and Prendergast, 1978b). For example, the fluorescence anisotropy of DPH in a biomembrane decreases with increasing temperatures (Andrich

and Vanderkooi, 1976). The decrease fluorescence anisotropy may be appropriately interpreted as a decrease in the structural order of the lipid microenvironment of the DPH molecule. In contrast, an increase in the fluorescence anisotropy of DPH in a biomembrane, observed in the presence of decreasing temperatures (Andrich and Vanderkooi) or increased hydrostatic pressure (Lackowicz and Thompson, 1983), could be interpreted as an increased ordering of the lipid microenvironment of the DPH molecule. An increased fluorescence anisotropy can also result from the quenching of DPH fluorescence emission. Therefore, the fluorescence lifetime, which decreases as a result of some types of quenching, is used to properly interpret fluorescence anisotropy measurements (Lackowicz, 1983).

Desipramine, imipramine, nortriptyline, and protriptyline can be placed into two groups based on their effects on the fluorescence anisotropy and lifetime of DPH. Nortriptyline and protriptyline produce a dose-dependent decrease in DPH fluorescence anisotropy and have no significant effect on the fluorescence lifetime of DPH except at very high concentrations (mM). These results are interpreted as drug-induced perturbations of the structural order of the lipid microenvironment of the DPH molecule in the murine lymphocyte membranes. Desipramine, on the other hand, produces an increase in DPH fluorescence anisotropy and a corresponding decrease in the fluorescence lifetime of DPH in murine lymphocyte membranes. Imipramine, like desipramine, produces a dose-dependent decrease in the fluorescence lifetime of DPH and produces neither a consistent

increase nor decrease in the fluorescence anisotropy of DPH. The drug-induced effects of desipramine and imipramine on the fluorescence of DPH are interpreted as quenching effects. The results of the fluorescence experiments, involving desipramine and imipramine, indicate that the respective drugs can diffuse into murine lymphocyte membranes and directly interact with the DPH molecule during the excited state resulting in a quenching of the fluorescence emission of DPH. The drug-induced quenching of DPH fluorescence by imipramine and desipramine is probably collisional, since, spectral overlaps between DPH, and desipramine and imipramine are not observed.

The concentrations at which desipramine and imipramine quench DPH fluorescence are similar to those concentrations of nortriptyline and protriptyline that induce perturbations of the structural order of the lipid domains of the murine lymphocyte. Other indirect and direct techniques, ESR and NMR, however, show that concentrations of desipramine and imipramine that produce structural perturbations in model membranes are similar to those concentrations of desipramine and imipramine that quench DPH fluorescence (Romer and Bickel, 1979; Bermejo et al., 1975; and Bermejo et al., 1974). Therefore, the evidence suggests that nortriptyline and protriptyline, and most likely, desipramine and imipramine, produce structural perturbations in murine splenic lymphocyte membranes that are probably consistent with membrane perturbations produced by local anesthetics.

The partition coefficients of the tricyclic antidepressants

used in this study are similar (Frisk-Holmberg and van der Kleijn, 1972). It is not surprising then, that the concentrations (>1 μM) of tricyclic antidepressant necessary to alter DPH fluorescence anisotropy or lifetime in the lipid domains of the murine lymphocyte, are similar for one tricyclic antidepressant as compared to another.

In the short term cultures, the tricyclic antidepressants, at concentrations that induce membrane structural perturbations, as monitored by DPH, inhibited mitogenesis. Nortriptyline, for example, began to perturb the murine lymphocyte membrane at a final concentration of about 30 μM, and depress mitogenesis at a final concentration of 10 μM. By comparison, desipramine began to quench DPH fluorescence at a concentration of about 10 μM, and depress mitogenesis at a final concentration of 10 μM. Studies with protriptyline produced experimental results similar to those described for nortriptyline and for imipramine, experimental results were similar to those for desipramine. These results suggest that there may be a relationship between tricyclic antidepressant induced membrane perturbations in the murine lymphocyte, and the inhibition of murine lymphocyte mitogenesis.

The peak therapeutic plasma concentrations of tricyclic antidepressants approach, for example, 2.5 μM for imipramine (Perel et al., 1976). However, tricyclic antidepressants can accumulate in cells and tissues in greater concentrations than observed in the plasma. Concentrations of tricyclic antidepressants in brain tissue, for instance, in the presence of therapeutic plasma levels,

are 1 to 10 μM. The ratio of the brain tissue concentration of tricyclic antidepressants to plasma concentration concentration of tricyclic antidepressants, is a function of plasma concentration, ranging from 40:1 to 8:1 with increasing plasma concentration (Glotzbach and Preskorn, 1982). Tricyclic antidepressants can also accumulate in erythrocytes. The concentration of tricyclic antidepressants in the erythrocyte is up to 6-fold higher than the plasma concentration in patients treated for depression. It has been suggested that the ratio of tricyclic antidepressant concentration in the erythrocyte to the plasma tricyclic antidepressant concentration is similar to the ratio of tricyclic antidepressant concentration in brain tissue to the plasma tricyclic antidepressant concentration (Matuzas et al., 1983; and Linnoila et al., 1978). We are not aware of any reports demonstrating tricyclic antidepressant accumulation in the lymphocyte, although, lymphocytes have been reported to uptake other liposoluble compounds such as antiarrhythmic agents (Somani et al., 1983).

It is probable that after long chronic treatment, tricyclic antidepressants may accumulate in sufficiently high enough concentrations in the lymphocyte in vivo to induce membrane structural perturbations that may result in altered lymphocyte function or lymphocyte numbers. At least one report describes the effectiveness of imipramine in reducing elevated rheumatoid antibody titers in patients with or without depression (Haydu et al., 1974). Moreover, alterations of lymphocyte populations in patients have been described following long-term therapy with imipramine (Leyberg

and Denmark, 1959), and other highly liposoluble agents such as phenothiazines (Enake et al., 1978). This study, then, provides information about tricyclic antidepressant-cell membrane interactions that may be representative of drug effects occuring after long-term therapy with tricyclic antidepressants.

Tricyclic antidepressant effects on the murine lymphocyte are time-dependent. All of the tricyclic antidepressants used in this study produce maximal membrane perturbations, as represented by alterations of DPH fluorescence anisotropy or lifetime, within 5 min at 37° C. It is apparent from the time course studies of the tricyclic antidepressants in the short term cultures (Audus and Gordon, 1982b), that these compounds produce the greatest inhibition of the mitogenic response if present in the cultures during the first 24 h of the culture. This initial 24 h period of the culture period represents a time frame during which cellular events take place that determine whether the lymphocyte will undergo mitogenesis. These results suggest that the tricyclic antidepressants apparently reach a rapid equilibrium distribution in the lymphocyte and that these drugs are most effective in inhibting the mitogenic response during the time period in which the cell experiences the dynamic membrane and cytoplasmic events that lead to mitogenesis.

The tricyclic antidepressant-induced membrane perturbations can be reversed in the presence of decreasing temperatures. The membrane perturbations produced by protriptyline and nortriptyline, as monitored by DPH fluorescence anisotropy, indicate that

nortriptyline and protriptyline may preferentially dissolve in different lipid phases or that the increasing structural order of the membrane lipids, with decreasing temperatures, may force these drugs out of the membrane. Experimental results with nortriptyline and protriptyline are consistent with observations of the temperature-dependency of other drug-induced membrane perturbations in the murine lymphocyte (Audus et al., 1983).

Desipramine and imipramine quenching of the DPH fluorescence lifetime was also reversed in the presence of decreasing temperatures. These results indicate that the quenching of DPH fluorescence is diffusion dependent and that the quenching is probably due to collisions (dynamic quenching) between DPH, and desipramine and imipramine. If the quenching of the DPH fluorescence was due to the formation of drug-fluorophore complexes (static quenching) one would not have observed a reversal of the quenching with decreasing temperature (Lackowicz, 1983). The reversal of tricyclic antidepressant induced membrane perturbations with decreasing temperatures is a characteristic shared by local anesthetics or anesthetics (Roth, 1979).

Alterations of the fluorescence anisotropy of F-Con A have been interpreted as changes in cells surface dynamics and changes in the mobility of the Con A receptor (Ben-Bassat et al., 1977; Shinitzky and Inbar, 1974; and Inbar et al., 1973). Increased fluorescence polarization is interpreted as decreases in the mobility of the Con A receptor and decreased exposure of the Con A receptor to the aqueous environment (Shinitzky and Inbar, 1976).

The opposite conditions can also exist. An increase in the mobility of the Con A receptor in normally associated with decreases in the "fluidity" of the lymphocyte cell membrane (Shinitzky and Inbar, 1976).

The tricyclic antidepressants do not alter the mobility of the Con A receptor as monitored by the fluorescence polarization of F-Con A. Observations of any fluorescence polarization changes of F-Con A in these experiments could be duplicated in aqueous solutions of F-Con A only, suggesting a significant interaction between tricyclic antidepressants and F-Con A. It should be noted that the tricyclic antidepressant concentrations that alter the polarization of F-Con A were 100-1000 fold greater than those concentrations of tricyclic antidepressants that altered the fluorescence anisotropy and lifetime of DPH, and inhibited lymphocyte mitogenesis. These results suggest that the tricyclic antidepressants do not alter the mobility or exposure of the Con A receptor of the murine splenic lymphocyte.

The absence of a relationship between the tricyclic antidepressant binding site apparent K_d and concentrations which inhibit lymphocyte mitogenesis is not unique. The murine lymphocyte also possesses a propranolol binding site of high affinity (10 nM) but propranolol concentrations that inhibit lymphocyte mitogenesis are 1000-fold higher (Audus et al., 1983). The effects of propranolol at the concentrations higher than the apparent K_d for the binding site are probably attributable to the local anesthetic properties of propranolol (Audus et al., 1983). Similarly, the

tricyclic antidepressant effects on the murine lymphocyte at concentrations much higher than the K_d for the binding site, could be attributable, at least in part, to local anesthetic properties of the tricyclic antidepressants. Concentrations of tricyclic antidepressants that produce membrane perturbations, as monitored by DPH fluorescence, can also alter the function of membrane associated proteins in a manner that is partially reversible. The ecto-ATPase of leukocytes which may play a role in regulating membrane permeability is inhibited by Ø.1 to 1 mM concentrations of tricyclic antidepressants and related phenothiazines (Medzihradsky et al., 198Ø). Further, this ecto-ATPase is not sensitive to other pharmacologically active agents such as phenobarbital, morphine, amphetamine, and phenelzine (Medzihradsky et al., 198Ø.), suggesting that there are, possibly, specific drug-protein or drug-membrane interactions required to produce inhibition of ecto-ATPase. This evidence, along with the results of our study, would suggest that the tricyclic antidepressants may alter normal cell processes by not only perturbing the structural integrity of the cell membranes but also perhaps by altering the function of membrane associated proteins.

In summary, the results suggest a possible relationship between the concentrations of tricyclic antidepressants that inhibit murine lymphocyte mitogenesis, and the concentrations of tricyclic antidepressants that perturb the structural order of the murine lymphocyte membranes. As determined by fluorescence spectroscopy, tricyclic antidepressants perturb the lymphocyte membrane in a

dose-, time- and temperature- dependent manner that is consistent with membrane effects produced by agents with known local anesthetic properties. In other experiments using a fluorescent probe for Con A receptors, results suggest that the tricyclic antidepressants do not alter the mobility or exposure of the Con A receptor.

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EXPERIMENTAL STUDIES

PART V

TRICYCLIC ANTIDEPRESSANT DRUG EFFECTS ON LIPOSOMAL MEMBRANES

ABSTRACT

Tricyclic antidepressant drug:liposomal membrane interactions have been characterized by using a fluorescent probe, diphenylhexatriene (DPH). The tricyclic antidepressants used in this study (nortriptyline, protriptyline, imipramine, and desipramine) can be placed into two groups based on their interactions with DPH labeled liposomes. 1) Nortriptyline and protriptyline were found to induce lipid bilayer perturbations in liposomes composed of 67 mol% dipalmitoyl-phosphatidylcholine (DPPC) and 33 mol% dioleoyl-phosphatidylcholine (DOPC). Perturbations of DPPC + DOPC liposomal membranes, as monitored by the fluorescence anisotropy and lifetime of DPH, are concentration- and temperature-dependent. Effects of nortriptyline and protriptyline on DPH fluorescence anisotropy or lifetime in DPPC, DOPC, or egg yolk-phosphatidylcholine (egg PC) liposomes could not be established. 2) Desipramine and imipramine quench the fluorescence emission of DPH in DPPC + DOPC and egg PC liposomes. The concentrations of imipramine and desipramine that quench DPH fluorescence emission in DPPC + DOPC liposomes are similar to the concentrations nortriptyline and protriptyline that perturb the lipid bilayers of DPPC + DOPC liposomes. Desipramine and imipramine do not alter the fluorescence anisotropy or lifetime of DPH in DPPC liposomes, but do quench the fluorescence emission of DPH in egg PC liposomes.

The size of the liposomes was assessed by electron microscopy and range from about .1 to .8 μm in diameter.

INTRODUCTION

A growing body of evidence suggests that alterations of membrane lipid structure may be an important mechanism by which hormones and other small molecules regulate cell function (Rasmussen et al., 1981; Hirata and Axelrod, 1980; Pilch et al., 1980; Goodman et al., 1975). The tricyclic antidepressants inhibit lymphocyte mitogenesis (Audus and Gordon, 1982; Nahas et al., 1979; Smith et al., 1978; Waterfield et al., 1978), reduce numbers of plaque forming cells (Smith et al., 1978), in vitro, reduce elevated rheumatoid autoantibody levels (Haydu et al., 1974) and produce leukopenia (Leyberg and Denmark, 1959) in vivo. Recent experimental evidence suggests that tricyclic antidepressant drug-induced perturbations of membrane lipid structure may be related to tricyclic antidepressant-induced inhibition of lymphocyte mitogenesis (Audus and Gordon, 1984).

Biological membranes are enormously complex, and therefore, it is very difficult to specifically characterize detailed molecular interactions between drugs and the phospholipid bilayer of biological membranes. Phospholipid vesicles or liposomes, represent relatively simple models of the phospholipid bilayer of the biological membrane that can be used to characterize lipophilic drug:membrane interactions (Bangham, 1978: Tritton et al., 1977).

In this paper, we describe tricyclic antidepressant drug effects on the structure of large multilamellar liposomes prepared from DPPC, DOPC, and egg PC. 1,6-Diphenyl-1,3,5-hexatriene (DPH), a highly lipophilic fluorescent probe (Shinitzky and Barenholz, 1974),

has been used to characterize the concentration and temperature dependence of drug induced alterations of liposomal membrane structure. The size of the multilamellar liposomes was determined by electron microscopy.

MATERIALS AND METHODS

1,6-Diphenyl-1,3,5-hexatriene (DPH; lot no. 2021A) was purchased from Molecular Probes, Inc., Junction City, OR. Imipramine hydrochloride (lot no. 121F-0086), L-α-phosphatidylcholine dipalmitoyl (DPPC), and L-α-phosphatidylcholine dioleoyl (DOPC) were obtained from Sigma Chemical Co., St. Louis, MO. Egg L-α-Lecithin (egg PC) was obtained from Avanti Polar-Lipids, Inc., Birmingham, AL. Desipramine hydrochloride was a gift from Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH. Nortriptyline hydrochloride was a gift from Lilly Research Laboratories, Indianapolis, IN. Protriptyline hydrochloride was a gift from Merck Sharp & Dohme Research Laboratories, West Point, PA. All other compounds used in this study were of the highest grade commercially available.

Liposome Preparation. An appropriate aliquot of phospholipid, from a 20 mg/ml stock in chloroform, was placed in round bottomed nitrogen purged, stoppered-test tube. The chloroform was evaporated off with a gentle stream of nitrogen, and the phospholipid resuspended in phosphate buffered saline (PBS; pH 7.4). The phospholipid mixture was then heated to at least ten degrees above the transition temperature for the respective lipid and shaken by means of a vortex rotamixer for 5 minutes. To the resulting

suspension of large multilamellar liposomes, an equal volume of PBS containing dispersed DPH was added to a final phospholipid to DPH ratio of about 500:1. The final concentration of phospholipid is approximately 0.1 μg/ml. The liposome mixture was incubated at room temperature for 45 minutes and used immediately for spectroscopic measurements at 37°C unless otherwise indicated.

DPPC(67 mol%) + DOPC(33 mol%) were prepared by adding appropriate amounts of each phospholipid to the same round bottomed test tube. The remainder of the preparatory procedures for DPPC + DOPC liposomes were as described above.

Drugs were dissolved in either PBS or distilled water and added to liposome suspensions (sample) in aliquots of 1 to 30 μl with Hamilton microliter syringes. Addition of either PBS or distilled water in equal amounts, and at similar times, to a liposome suspension served as the corresponding control. Unless otherwise indicated, sample and control liposome suspensions were incubated at 37°C for 5 minutes prior to measurement of fluorescence anisotropy or lifetimes.

Following the protocol of Bangham and Horne (1964), liposomes for electron microscopy were prepared in a similar manner, except that the liposomes were prepared in distilled water to a final concentration of approximately 5 mg/ml. Equal volumes of the liposome suspension and 2% potassium phosphotungstate in water were mixed for 30 sec. A drop of the stained liposome mixture was placed on a cellulose carbon grid, the drop drained, and after drying,

examined with a JEOL-100S electron microscope at instrumental magnifications of 40,000 to 100,000.

Steady-State Fluorescence Anisotropy Measurements. Instrumentation has been described in detail previously (Audus et al., 1983; Audus and Gordon, 1984). Briefly, measurements were made with an SLM 8000 interfaced with a Hewlett-Packard 87 computer for on-line data acquisition and analysis. The fluorometer was set up to measure fluorescence anisotropy

$$r = (A - B) / (A + 2B)$$

where r is anisotropy. A is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the vertical position. B is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the horizontal position. The sample was excited with 360 nm light and emission monitored at 430 nm.

Light scattering due to turbidity of the liposome suspension was found not to be significant at the phospholipid concentrations used in these experiments. The tricyclic antidepressants absorb UV light maximally around 250 nm, and therefore the fluorescence of the tricyclic antidepressants did not contribute significantly to the fluorescence of DPH labeled liposomes. The temperature of the sample cell was controlled by a circulating water bath and the contents of the sample cuvettes stirred with a magnetic stirrer.

Fluorescence Lifetime Measurements. Methodology and instrumentation for determination of fluorescence lifetimes has been

described elsewhere (Audus and Gordon, 1984; Lackowicz, 1983). Briefly, fluorescence lifetimes were calculated from phase and modulation measurements made with an SLM 4800 subnanosecond fluorometer interfaced with a Hewlett Packard 87 for on-line acquisition and analysis. The phase shift and the modulation of each sample was measured alternately 4 times, with the time interval between each measurement of about 10 sec. Data was acquired by the interfaced computer, averaged and the lifetimes calculated from the following basic relationships:

$$T_{phase} = 1/w \tan \theta$$

$$T_{mod.} = (1/w)(1/D^2 - 1)^{1/2}$$

where T_{phase} is the phase lifetime, θ the phase shift in degrees caused by a sample of lifetime T, w is the angular frequency of excitation which is 2 * pi * modulation frequency (30 MHz), $T_{mod.}$ is the modulation lifetime, and D is the ratio of $M_{fluorophore}/M_{reference}$, where M represents the relative modulation of a reference solution.

The sample was excited with 360 nm light and emission from the sample monitored through a KV-389 cut on filter. Lifetimes were determined in the presence of a reference fluorophore with a known lifetime (dimethyl-POPOP, 1.45 nsec). A rhodamine quantum counter solution was placed in the reference chamber and emission monitored through a RG630 filter. The contents of the sample cuvettes were stirred continuously with a magnetic stirrer and the temperature of the sample chamber controlled by a circulating water bath.

RESULTS

Figure 1 illustrates the nortriptyline and protriptyline induced decreases of DPH fluorescence anisotropy of DPH labeled DPPC(67 mol%) + DOPC(33 mol%) liposomes. Nortriptyline and protriptyline do not alter the fluorescence lifetime of DPH in DPH labeled DPPC + DOPC liposomes except at very high concentrations (1 mM). The fluorescence anisotropy of DPH in the DPPC + DOPC liposome suspension receiving only PBS (control) remained at Ø.151 +/- .ØØ4 during these experiments (data not shown). Nortriptyline and protriptyline did not alter the fluorescence anisotropy or lifetime of DPH in egg PC liposomes at concentrations of $1Ø^{-7}$ to $1Ø^{-3}$ M (not shown), or DOPC and DPPC liposomes at $1Ø^{-4}$ M as shown in Table I.

The four tricyclic antidepressants used in these experiments do not induce concentration-dependent alterations of either the fluorescence anisotropy or the fluorescence lifetime of DPH in DPPC liposomes. Figure 2 illustrates the inability of desipramine or protriptyline to alter the fluorescence anisotropy of DPH in DPPC liposome suspensions. The fluorescence anisotropy of DPH in a control suspension of DPPC liposomes remained at .337 +/- .ØØ1 during these experiments.

In Figure 3, data are presented which demonstrates desipramine and imipramine induced decreases of the fluorescence lifetime (phase) of DPH in DPPC(67 mol%) + DOPC(33 mol%) liposomes. The DPH fluorescence phase lifetime of a control liposome suspension remained at 7.75 +/- Ø.Ø7 nsec during these experiments. Desipramine

and imipramine concentrations of 10^{-7} to 10^{-3} M do not alter the fluorescence lifetime or anisotropy of DPH in DPPC liposomes (not shown). The fluorescence lifetime is decreases and the fluorescence anisotropy of DPH in egg PC liposomes with increasing concentrations of either desipramine or imipramine. Figure 4 illustrates the effect of increasing concentrations of imipramine, after a 3 h incubation period at 37°C, on the fluorescence anisotropy of DPH in egg PC liposomes. The DPH fluorescence anisotropy of a control liposome suspension remained at .0648 +/- .001 during these experiments.

100 μM (50 mol %) of either nortriptyline or protriptyline shifts the temperature dependent changes of the DPH fluorescence anisotropy in DPPC + DOPC liposomes to lower temperatures. Figure 5 presents the typical temperature transition profile of DPPC + DOPC liposomes in the absence and presence of either protriptyline or nortriptyline. The fluorescence anisotropy of DPH in these liposomes is decreased by an average of approximately .025 anisotropy units throughout the transition phase (20 °C to 40°C) of the liposomes.

Figure 6 is an electron photomicrograph of a representative sample of DPPC(67 mol%) + DOPC(33 mol%) liposomes. The multilamellar liposomes range from .1 to .8 μm in diameter.

DISCUSSION

Understanding the mechanisms by which small molecules such as drugs alter cell function through interactions with the cell membrane is a fundamental problem in pharmacology. The liposome

Figure 1. Effect of Nortriptyline and Protriptyline on the fluorescence anisotropy of DPH in DPPC (67 mol%) + DOPC (33 mol%) multilamellar liposomes. The fluorescence anisotropy value of DPH in a control liposome suspension remained at .151 +/- .004.

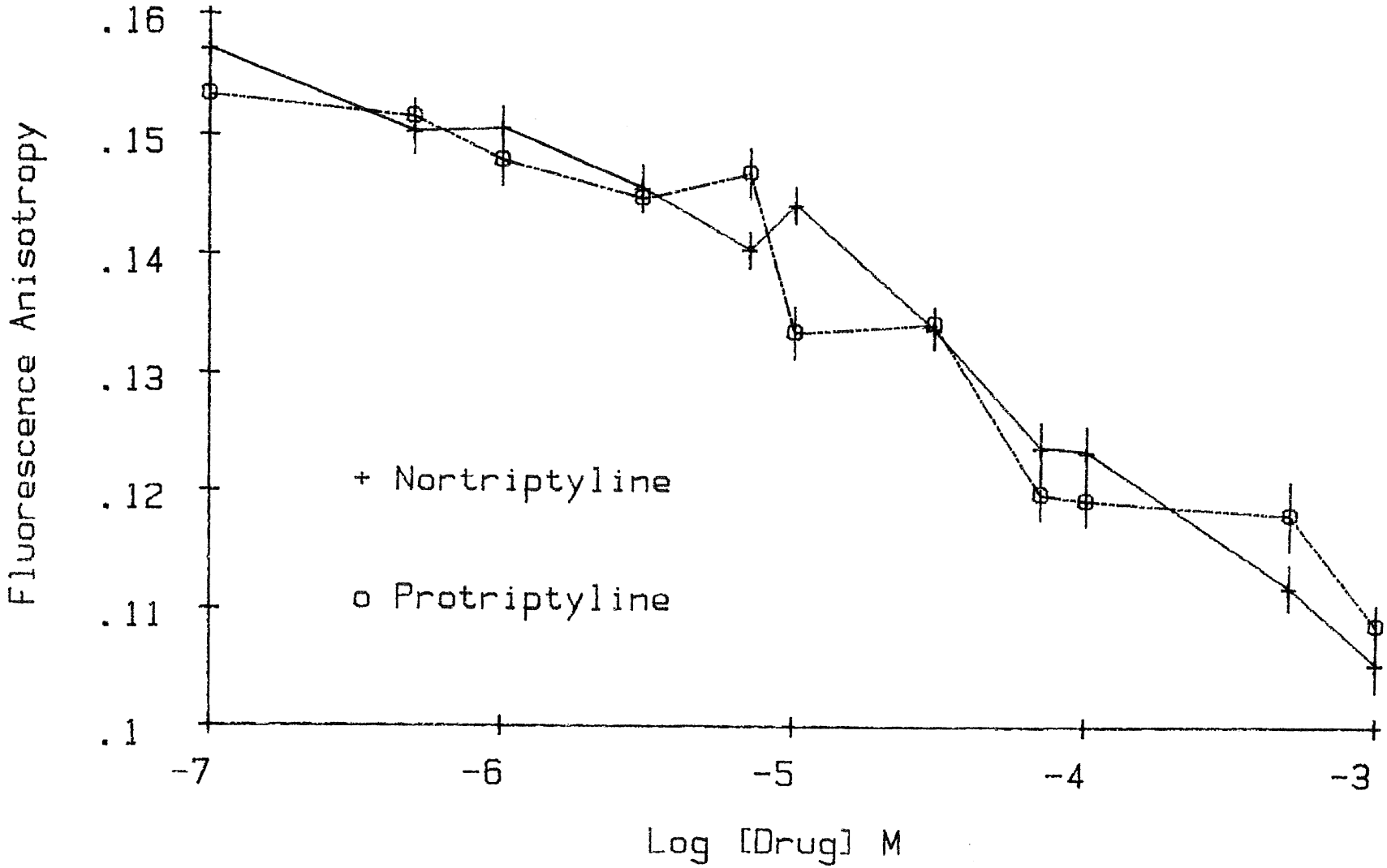

.16
.15
.14
.13
.12
.11
.1
Fluorescence Anisotropy
+ Nortriptyline
o Protriptyline
-7
-6
-5
-4
-3
Log [Drug] M

TABLE I

Temperature Dependence of the Fluorescence Anisotropy of DPH in DPPC or DOPC Liposomes.

DPPC Liposomes

Additions	25°C	37°C	50°C
+PBS	.342 +/- .001	.317 +/- .001	.089 +/- .002
$+10^{-4}$ M Protriptyline	.340 +/- .001	.307 +/- .001	.089 +/- .001
$+10^{-4}$ M Nortriptyline	.343 +/- .001	.312 +/- .001	.088 +/- .001

DOPC Liposomes

Additions	25°C	37°C	50°C
+PBS	.097 +/- .001	.071 +/- .001	.053 +/- .002
$+10^{-4}$ M Protriptyline	.097 +/- .001	.073 +/- .001	.053 +/- .002
$+10^{-4}$ M Nortriptyline	.094 +/- .001	.071 +/- .001	.054 +/- .001

Figure 2. Effect of Desipramine and Protriptyline on the fluorescence anisotropy of DPH in DPPC (67 mol%) + DOPC (33 mol%) multilamellar liposomes. The fluorescence anisotropy value of DPH in a control liposome suspension remained at .337 +/- .ØØ1.

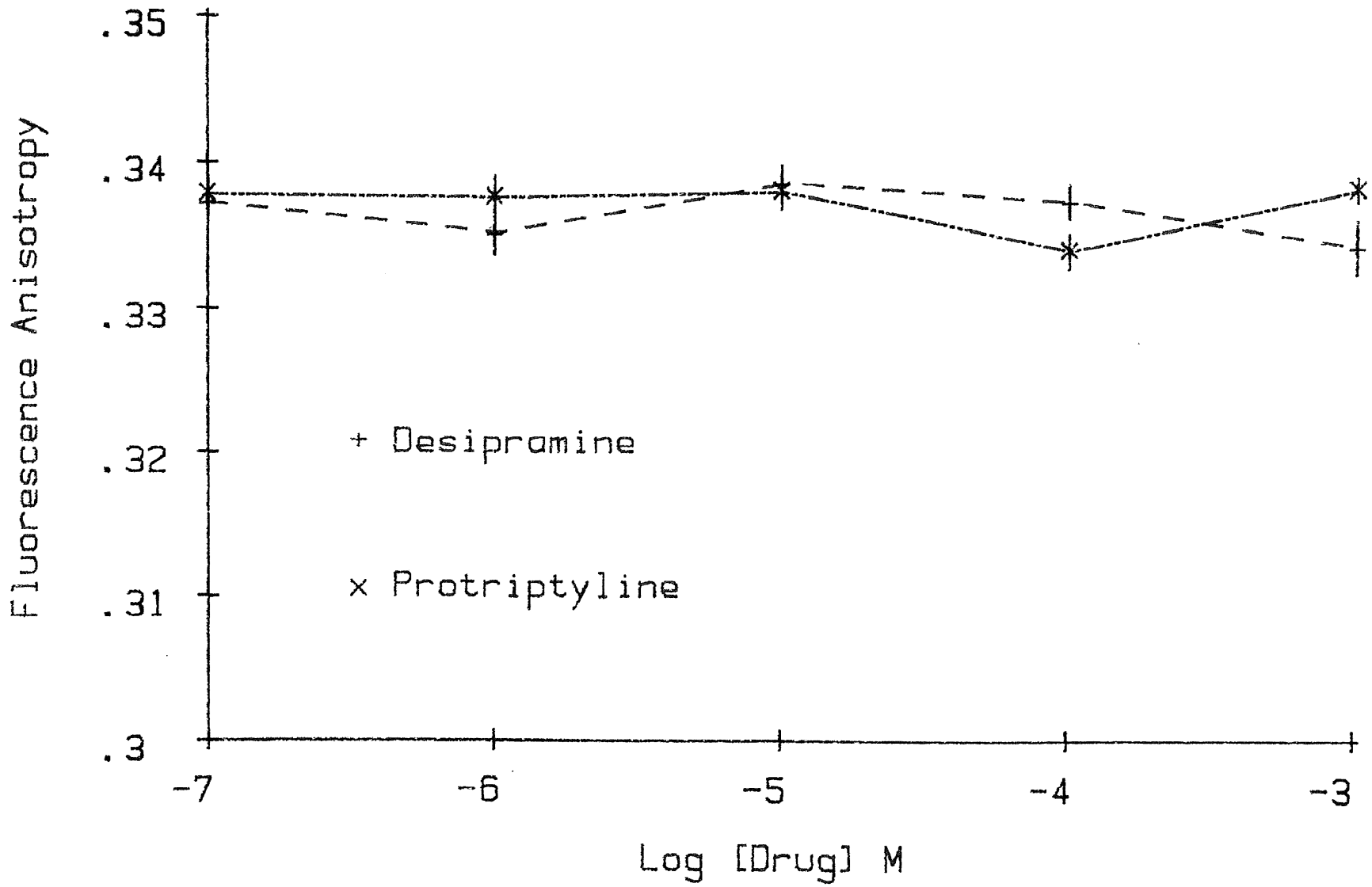

.35
.34
.33
.32
.31
.3
Fluorescence Anisotropy
+ Desipramine
x Protriptyline
-7
-6
-5
-4
-3
Log [Drug] M

Figure 3. Effect of Desipramine and Imipramine on the fluorescence phase lifetime of DPH in DPPC (67 mol%) + DOPC (33 mol%) multilamellar liposomes. The fluorescence lifetime of DPH in a control suspension of liposomes remained at 7.75 +/- Ø.Ø7 nsec.

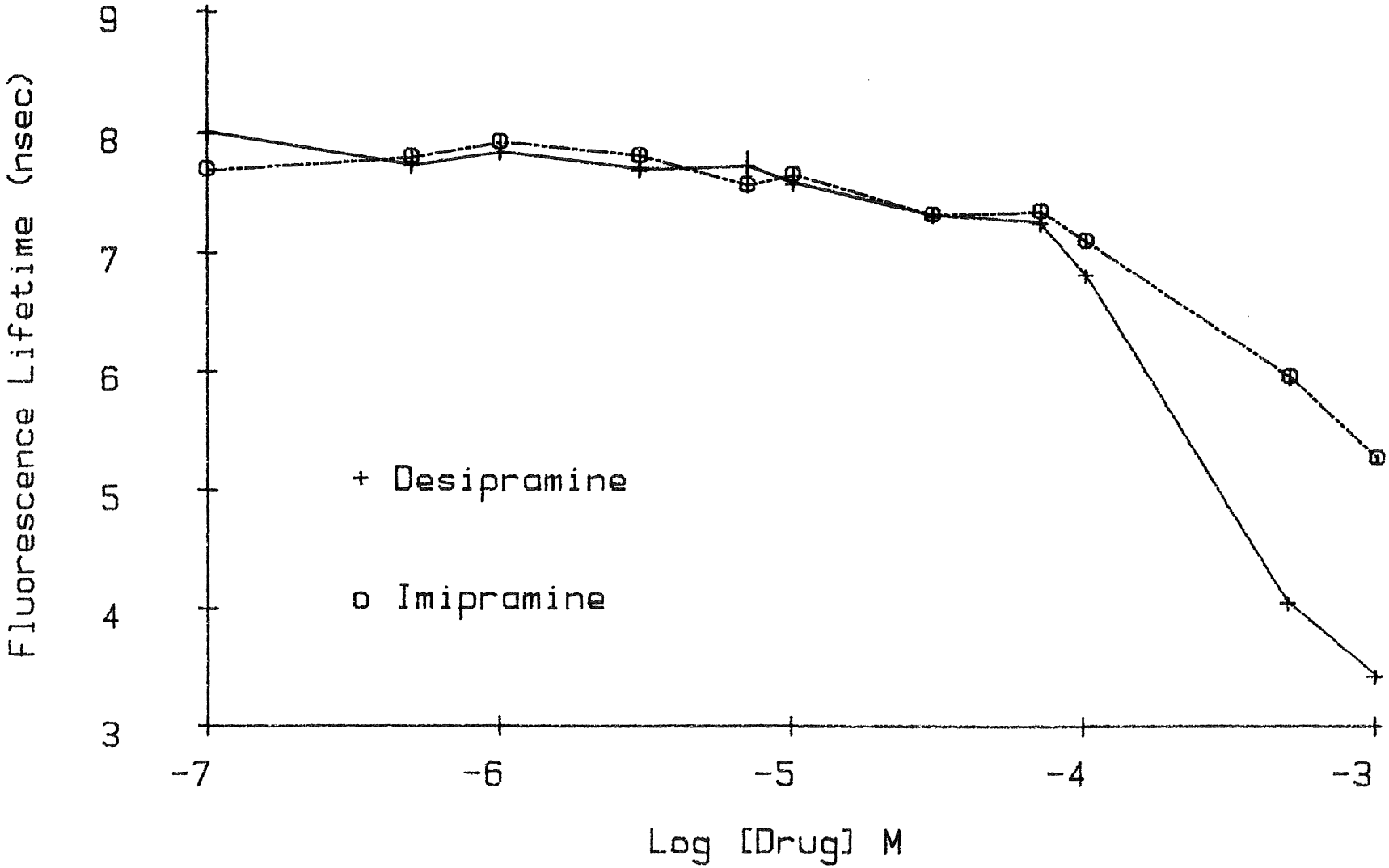

Fluorescence Lifetime (nsec)
9
8
7
6
5
4
3
+ Desipramine
o Imipramine
-7
-6
-5
-4
-3
Log [Drug] M

Figure 4. Effect of Imipramine on the Fluorescence Anisotropy of DPH in Egg PC Multilamellar Liposomes. The fluorescence anisotropy of DPH in a control suspension of egg PC liposomes remained at .0648 +/- .001.

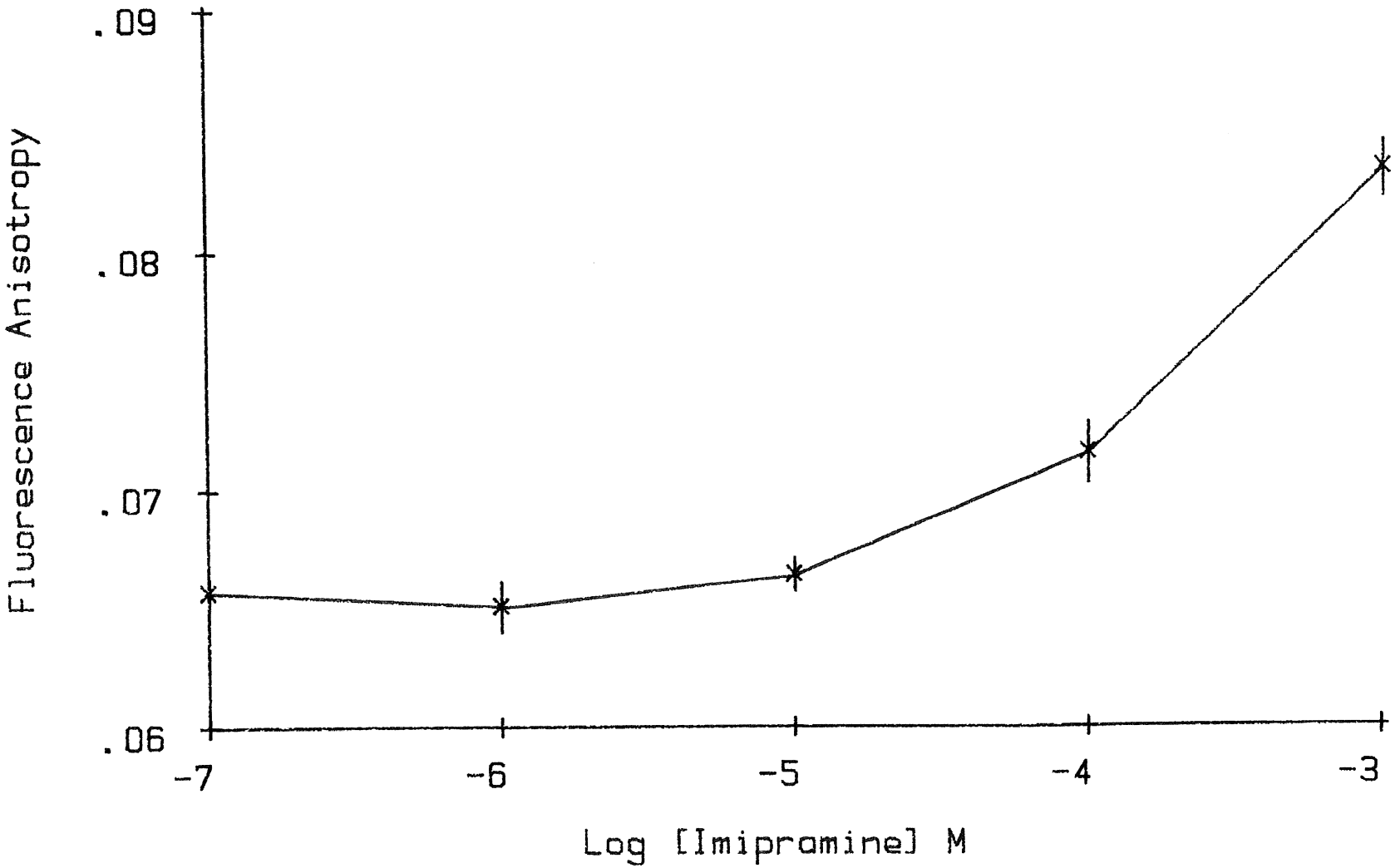

.09
.08
.07
.06
Fluorescence Anisotropy
-7
-6
-5
-4
-3
Log [Imipramine] M

Figure 5. Temperature Dependency of the Effects of Nortriptyline and Protriptyline on the Fluorescence Anisotropy of DPH in DPPC (67 mol%) + DOPC (33 mol%) Multilamellar Liposomes. 100 μM nortriptyline (+), 100 μM protriptyline (o), and an equal amount of PBS (control, x), was added to DPH labeled multilamellar suspensions and the fluorescence anisotropy recorded at 2.5 degree intervals.

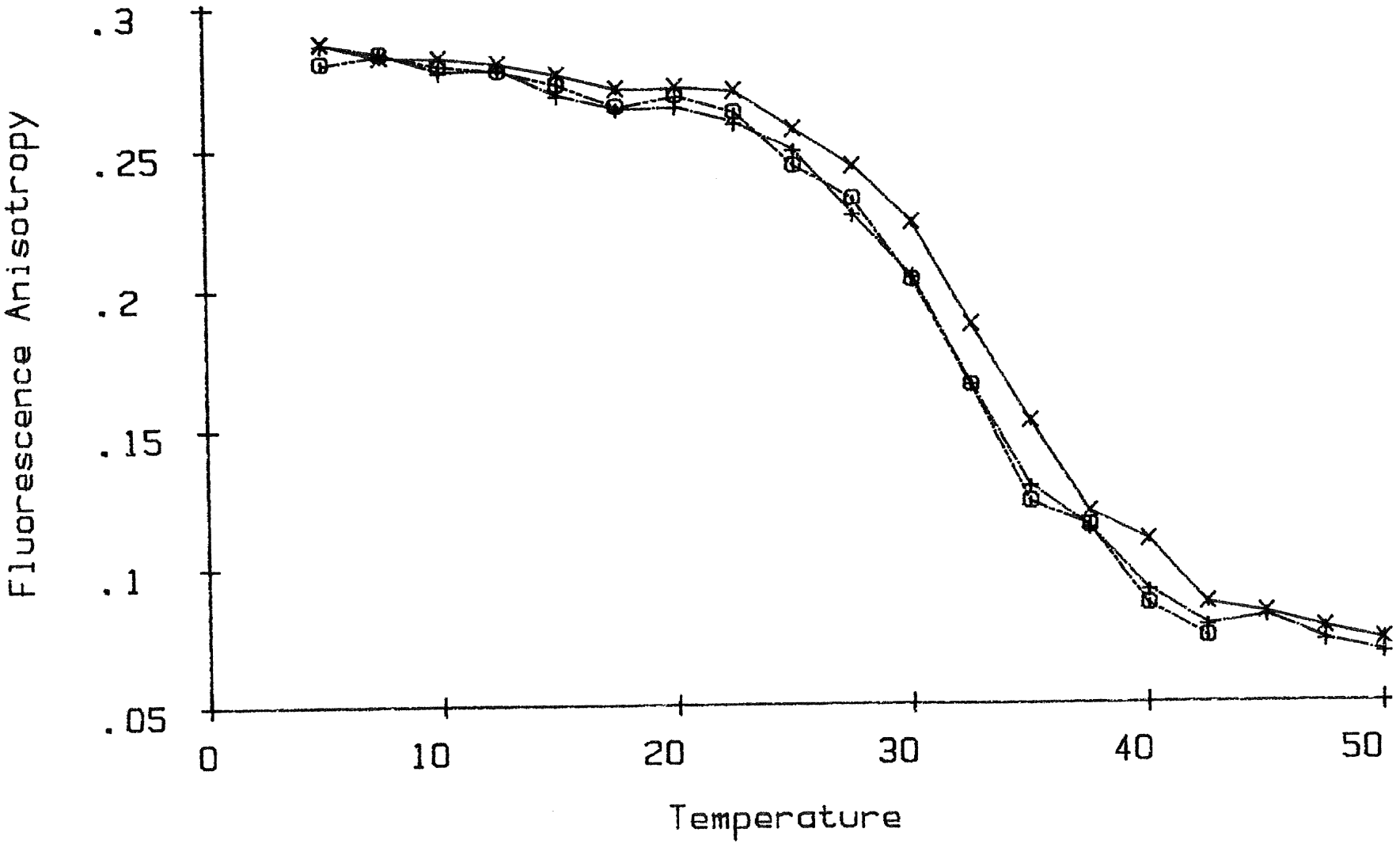
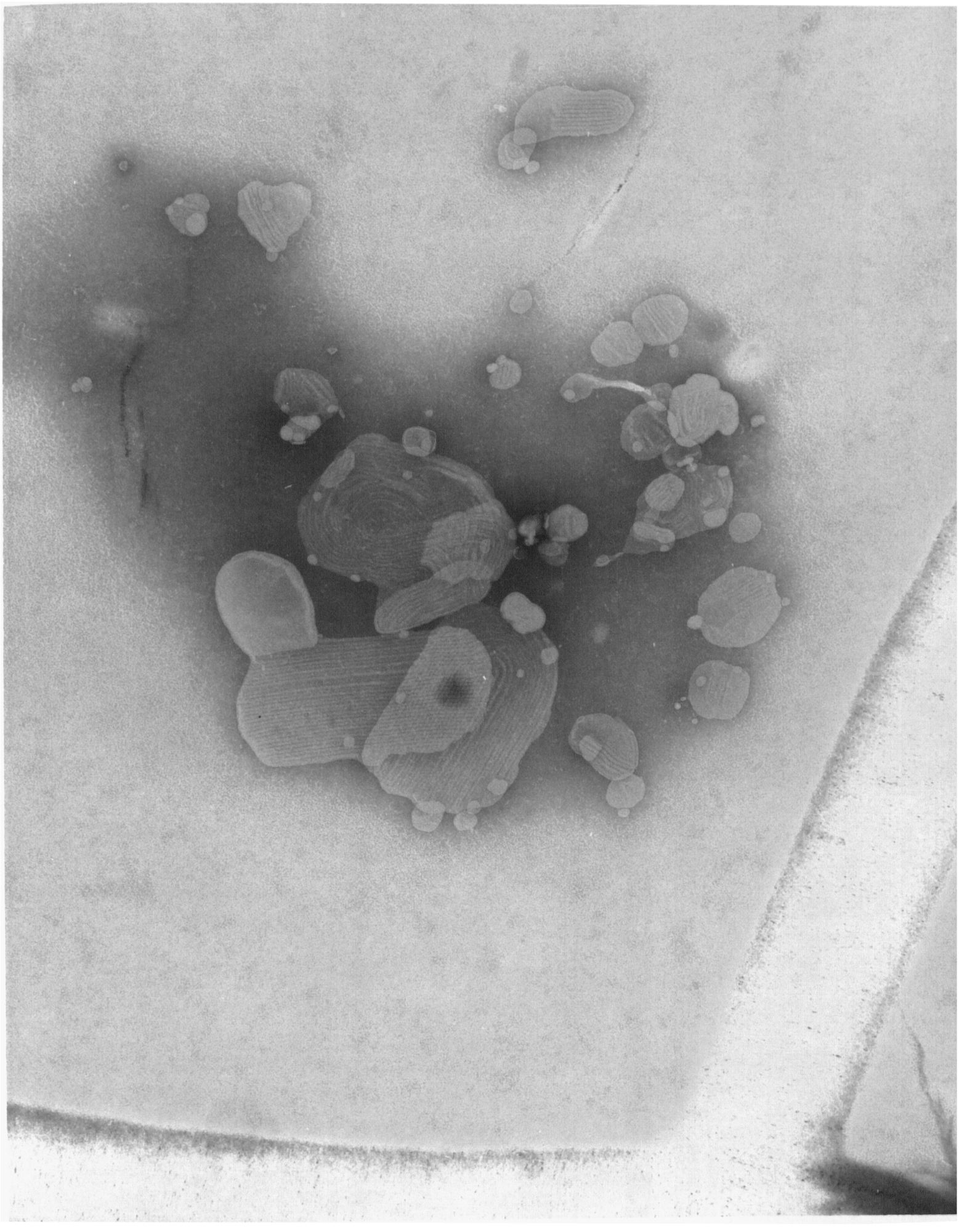

Fluorescence Anisotropy
.3
.25
.2
.15
.1
.05
0
10
20
30
40
50
Temperature

Figure 6. Electron Micrograph of DPPC (67 mol%) + DOPC (33 mol%) Liposomes. Liposomes were prepared as described in Materials and Methods section and observed at an instrument magnification of 50,000x.



provides a useful model of the biomembrane's phospholipid bilayer, enabling one to study, more directly, drug:phospholipid bilayer interactions (Bangham, 1978; Tritton et al., 1977). This study was conducted to determine the ability of the tricyclic antidepressants to alter the lipid structure of liposomes as monitored by the fluorescence lifetime and anisotropy of DPH.

The fluorescence anisotropy of DPH in liposomes is interpreted in terms of changes in the structural order of the phospholipid bilayer (Lackowicz et al., 1979; Lackowicz and Prendergast, 1978a; Lackowicz and Prendergast, 1978b). Increasing temperatures, for example, result in a decrease of the DPH fluorescence anisotropy of DPH labeled liposomes (Andrich and Vanderkooi, 1976). The decrease in the DPH fluorescence anisotropy may be interpreted as a decrease in the structural order of the lipid microenvironment of the DPH molecule. In the presence of decreasing temperatures (Andrich and Vanderkooi, 1976) or increasing hydrostatic pressure (Lackowicz and Thompson, 1983), DPH fluorescence anisotropy in the liposome, increases. This may be interpreted as an increased ordering of the lipid microenvironment of the DPH molecule. An increase in the DPH fluorescence anisotropy may also be due to quenching, and therefore, measurement of fluorescence lifetimes, which decrease as a result of some types of quenching, is used to interpret fluorescence anisotropy measurements (Lackowicz, 1983).

As observed in the lymphocyte membrane (Audus and Gordon, 1984), desipramine, imipramine, nortriptyline, and protriptyline can be placed into two groups based on their effects on the fluorescence

anisotropy and lifetime of DPH in the DPPC (67 mol%) + DOPC (33 mol%) liposome. Nortriptyline and protriptyline effect concentration-dependent decreases in the DPH fluorescence anisotropy of DPH labeled DPPC (67 mol%) + DOPC (33 mol%) liposomes and have no significant effect on the fluorescent lifetime of DPH. In contrast, desipramine and imipramine produce a concentration-dependent increase in the fluorescence anisotropy of DPH in DPH labeled DPPC (67 mol%) + DOPC (33 mol%) liposomes. However, the increase in fluorescence anisotropy of DPH caused by desipramine and imipramine can be explained in terms of quenching, since both imipramine and desipramine produce a concentration-dependent decrease in the fluorescence lifetime of DPH. The quenching of DPH fluorescence by desipramine and imipramine is probably due to intermolecular collisions (dynamic quenching) because 1) there is no spectral overlap between DPH and desipramine and imipramine, and 2) the quenching is temperature dependent (greater at higher temperatures), suggesting dynamic diffusion-dependent mechanisms.

Protriptyline and nortriptyline induced decreases in DPH fluorescence anisotropy in DPH labeled DPPC (67 mol%) + DOPC (33 mol%) liposomes are interpreted as drug-induced perturbations of the phospholipid bilayer. Desipramine and imipramine, on the other hand, do penetrate the DPPC (67 mol%) + DOPC (67 mol%) phospholipid bilayer and perhaps localize in the proximity of the excited DPH molecule resulting in the quenching of DPH fluorescence emission. It is probable that desipramine and imipramine, like protriptyline and nortriptyline, also perturb the phospholipid bilayer of DPPC (67

mol%) + DOPC (33 mol%) liposomes. Calorimetric, ESR, and NMR studies have demonstrated that desipramine and imipramine perturb the phospholipid bilayer of DPPC liposomes (Bermejo et al., 1975; Cater et al., 1974; Bermejo et al., 1974).

In order to characterize the effect of the tricyclic antidepressants on the temperature transition profile of DPPC (67 mol%) + DOPC (33 mol%) liposomes, 100 μM of either nortriptyline or protriptyline was added to DPPC (67 mol%) + DOPC (33 mol%) liposome suspensions. The typical temperature transition profile of DPH labeled DPPC+DOPC liposomes in the absence of drug is consistent with the findings of Lentz et al. (1976b). However, addition of nortriptyline or protriptyline to DPPC (67 mol%) + DOPC (33 mol%) liposomes shifts the temperature transition profile to the right. These results are consistent with the understanding that molecules which perturb the lipid bilayers of liposomal and biological membranes, lower the transition temperature of the lipid bilayer (Cater et al., 1974).

In our studies we were unable to demonstrate lipid structure perturbations in DPPC liposomes at a concentration of 100 μM (50 mol%) of any of the tricyclic antidepressants. This in contrast to calorimetric studies where desipramine produced lipid structural alterations in DPPC liposomes at 2 mol% (Cater et al., 1974). Cater et al. (1974) studies indicated that the lipid structural perturbations originated at the polar-head groups of the phospholipid bilayer. In such case, DPH may not be sensitive to changes in the fatty acid chain adjacent to the polar-head groups.

Moreover, desipramine and other tricyclic antidepressants may not readily penetrate to the fatty acids regions of DPPC liposomes due to the physical state of the lipid (gel) below the transition temperature (T_m 41°C).

Lower concentrations (<100μM or 50 mol%) of desipramine and imipramine produced an increase in the fluorescence anisotropy and a corresponding decrease in the fluorescence lifetime of DPH in egg PC liposomes. Nortriptyline and protriptyline, by comparison had no effect on the fluorescence lifetime or anisotropy of DPH in egg PC or DOPC liposomes. Since the quenching of DPH requires that desipramine and imipramine localize in close proximity to the excited DPH molecule, these results suggest that desipramine and imipramine must diffuse into the fatty acid domains of egg PC liposomes. The fatty acid chains of DOPC (T_m -20°) and egg PC liposomes are less tightly packed due to the presence of double bonds. Consequently, the lecithin bilayers of the DOPC and egg PC liposmes are much less ordered than DPPC bilayers. In this study high concentrations of (>50 mol%) nortriptyline and protriptyline are not apparently capable of perturbing the bilayers of the less ordered DOPC and egg PC liposome bilayers to a greater degree than they already are.

DPPC, DOPC, and DPPC + DOPC liposomes have been well characterized with DPH fluorescence (Stubbs et al., 1981; Lentz et al., 1976a; Lentz et al., 1976b). It is interesting that concentrations (<50 mol%) of tricyclic antidepressants that perturb DPH labeled bilayers of DPPC (67 mol%) + DOPC (33 mol%) liposomes

did not perturb DPH labeled bilayers of either DPPC or DOPC liposomes. Since DPPC and DOPC are immiscible in both small vesicles and large multilamellar liposomes (Lentz et al., 1976b), it is possible that the tricyclic antidepressants perturb DPPC (67 mol%) + DOPC (33 mol%) bilayers at interfaces between lipid phases. An alternative explanation might be that the tricyclic antidepressants may access DPH labeled-DPPC (67 mol%) + DOPC (33 mol%) bilayers through less ordered DOPC domains where permeation of the drug is less restricted. Concerning this latter point, there is a correlation between liposome permeability and the presence of unsaturated hydrocarbon chains (Demel et al., 1972).

The DPPC (67 mol%) + DOPC (33 mol%) liposome is probably not an adequate model of a more complex biological bilayer. However, the formation of separate lipid domains, as in the DPPC (67 mol%) + DOPC (33 mol%) liposomes, may represent lipid-lipid relationships characteristic of some biomembranes (Klausner et al., 1980; Cullis and DeKruijff, 1979; Jain and White, 1977; Lentz et al., 1976b). The fact that tricyclic antidepressants interact differently with DPPC (67 mol%) + DOPC (33 mol%) liposomes (Fig. 1, 3, and 5) than with DPPC or DOPC liposomes (Fig. 2 and Table I) may indicate how lipid-lipid relationships affect drug:cell membrane interactions.

The apparent octanol/water and octanol/buffer partition coefficients of the tricyclic antidepressants used in this study are significant and quite similar from one compound to another (Frisk-Holmberg and Van der Kleijn, 1972; Leo et al., 1971). Therefore, similarity of the concentration- and temperature- dependent

alterations in the fluorescence lifetime and anisotropy of DPH labeled bilayers is not considered unusual.

Recent studies indicate that tricyclic antidepressant-drug induced down-regulation of central beta-adrenergic receptors may be related to the clinical effects of the tricyclic antidepressants (Richardson and Hertz, 1983; Sulser, 1982). Effects, in vitro, of tricyclic antidepressants on beta-adrenergic ligand binding to murine astrocytes are observed at micromolar tricyclic antidepressant concentrations (Richardson and Hertz, 1983). Since micromolar amounts of tricyclic antidepressants perturb the liposomal membrane, similar tricyclic antidepressant drug-induced perturbations of the murine astrocyte membrane may be relevant to beta-adrenergic receptor subsensitivity. Liponomic control of proteins has been suggested as a possible mode of action for the action of some small molecules on biomembranes (Rasmussen and Matsumoto, 1982).

The results of our study indicate that the tricyclic antidepressants, nortriptyline and protriptyline, perturb the DPH labeled bilayers of DPPC (67 mol%) + DOPC (33 mol%) liposomes but do not perturb the DPH labeled bilayers of DPPC, DOPC, or egg PC liposomes. Nortriptyline and protriptyline induced perturbations of the bilayers of DPPC + DOPC liposomes occur at concentrations that are similar to concentrations of nortriptyline and protriptyline perturbed lymphocyte membranes (Audus and Gordon, 1984). In addition, nortriptyline and protriptyline decrease the transition

temperature of the lipids in DPPC (67 mol%) + DOPC (33 mol%) liposomes.

In contrast, desipramine and imipramine quench the fluorescence emission of DPH in DPPC + DOPC and egg PC Liposomes at concentrations similar to those concentrations of desipramine and imipramine that quenched DPH fluorescence emission in murine lymphocytes. The concentrations of desipramine and imipramine that quench DPH fluorescence are also similar to the concentrations of nortriptyline and protriptyline that perturb the phospholipid bilayers of liposomes and lymphocytes. Other methods will be required to determine the presence of desipramine and imipramine effects on the lipids of DPPC (67 mol%) + DOPC (33 mol%) liposome and lymphocyte membranes. Finally, desipramine and imipramine do not alter the fluorescence anisotropy or lifetime of DPH in DPPC liposomes. This finding is not consistent with previous studies of the effect of desipramine and imipramine on liposomal membrane structure.

The large multilamellar liposomes used in this study were sized by electron microscopy and range from about .1 to .8 μm in diameter.

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SUMMARY AND CONCLUSIONS

Initial experiments were conducted to determine if a specific tricyclic antidepressant binding site was present on the lymphocyte membrane. Prior studies had indicated that the tricyclic antidepressants were capable of altering lymphocyte function and growth both *in vivo* and *in vitro*. The mechanisms involved in alteration of lymphocyte function and growth, though, are poorly understood. Results from the experimental study herein describe a specific, saturable, high affinity tricyclic antidepressant binding site on the lymphocyte. The binding of tricyclic antidepressants to the high affinity binding site was also competitive, reversible, and does not display characteristics of cooperativity. The existence of the high affinity binding site suggested a possible mechanism by which tricyclic antidepressants might alter lymphocyte function.

The second experimental study was conducted to determine if the high affinity binding site possessed biological activity. The ability of the tricyclic antidepressants to modulate mitogen stimulated murine lymphocytes was assayed. Concentrations of tricyclic antidepressants that inhibited mitogenesis were about 1000 fold greater than the apparent affinity constant for the tricyclic antidepressant binding site. These results suggested that the specific tricyclic antidepressant binding site is not involved in the inhibition of mitogenesis. This does not preclude the possibility that the binding site may be involved in a biological function not associated with mitogenesis. Results from additional experiments demonstrated that the tricyclic antidepressants inhibit

mitogenesis when added to mitogen stimulated lymphocytes at various times up to 24 hours after initiating the cultures.

Other drugs such as beta-adrenergic antagonists have high affinity binding sites on the murine lymphocyte and also inhibit mitogenesis at concentrations much higher than the apparent affinity constant of the binding site. The inhibition of mitogenesis by these drugs at high concentrations have been attributed to the ability of the compounds to perturb the lymphocyte's membrane structure. These perturbations are often referred to as "quinidine-like" or local anesthetic effects. It has been presumed that the high concentrations of tricyclic antidepressants perturb membrane structure in a similar fashion. However, experimental evidence in biological membranes to support these assumptions has not been reported.

The third and fourth experimental studies were conducted to provide experimental evidence that membrane perturbations at high concentrations of either beta-adrenergic antagonists or tricyclic antidepressants are involved in the inhibition of lymphocyte mitogenesis. The ability of a compound to perturb biomembranes is associated with lipophilicity of the respective compound. Since the tricyclic antidepressants possess very similar apparent organic solvent/aqueous partition coefficients, it was desirable to include in this study a class of compounds with a range of different apparent organic solvent/aqueous partition coefficients. The beta-adrenergic antagonists were found to be suitable for this investigation based on the range of apparent partition coefficients.

Results of the third experimental study indicate that concentrations of beta-adrenergic antagonists that perturb lymphocyte membranes are similar to those concentrations of beta-adrenergic antagonists that inhibit lymphocyte mitogenesis. Further, beta-adrenergic antagonists with high partition coefficients perturbed lymphocyte membranes, while beta-adrenergic antagonists with very low partition coefficients did not alter lymphocyte membrane structure. By comparison, beta-adrenergic antagonists with high partition coefficients inhibited lymphocyte mitogenesis in a concentration-, time-, and temperature- dependent manner, and beta-adrenergic antagonists with low partition coefficients had little or no effect on lymphocyte mitogenesis.

Results of the fourth experimental study demonstrate that, like the beta-adrenergic antagonists, concentrations of tricyclic antidepressants that perturb lymphocyte membranes are similar to those concentrations of tricyclic antidepressants that perturb lymphocyte membranes. Perturbations of the lymphocyte membrane, by tricyclic antidepressants are concentration-, time-, and temperature- dependent. The concentrations of each of the tricyclic antidepressants necessary to perturb lymphocyte membranes and inhibit lymphocyte mitogenesis were similar and consistent with the fact that the apparent partition coefficients for these tricyclic antidepressants are also similar.

In a final series of experiments, the ability of the tricyclic antidepressants to alter liposomal membranes was determined. Since the biological membrane is extremely complex,

specific information about drug:phospholipid bilayer interactions can not be determined. The liposome provides a simpler model of the biological membrane phospholipid bilayer which can be used to investigate specific drug:phospholipid bilayer interactions. In previous studies, low concentrations of tricyclic antidepressants were shown to perturb the polar-head groups of dipalmitoyl-phosphatidylcholine (DPPC) liposomes. Interestingly, the specific fluorescent probe diphenylhexatriene, used in the studies herein, was not sensitive to DPPC or DOPC bilayer perturbations induced by lower concentrations of tricyclic antidepressants. However, low concentrations of tricyclic antidepressants that were effective in perturbing the bilayers of DPPC + dioleic-phosphatidylcholine (DOPC) (2:1) liposomes were similar to the concentrations of tricyclic antidepressants that perturb lymphocyte membranes. The results also indicate that lipid:lipid interactions may be important in how a drug affects the phospholipid bilayer.

To conclude, these studies demonstrate tricyclic antidepressant drug:lymphocyte membrane interactions at very low concentrations with a specific tricyclic antidepressant binding site, and at higher concentrations with, presumably a non-specific binding site, the phospholipid bilayer. Lymphocyte responsiveness, as monitored by the lymphocyte mitogen response in vitro, was found to be unaffected by tricyclic antidepressant interactions with the specific tricyclic antidepressant binding site. Other studies, however, demonstrated that inhibition of the lymphocyte mitogen reponse may be related to the ability of these drugs to perturb the lymphocyte membrane

structure. Additional studies in model membranes support the assumption that lymphocyte membrane perturbations by the tricyclic antidepressants originate in the lipid domains of biological and liposomal membranes. Studies of the non-specific effects of the beta-adrenergic antagonists showed that inhibition of the mitogen response of lymphocytes may related to beta-adrenergic antagonist induced perturbations of lymphocyte membranes. Concentrations of beta-adrenergic antagonists that interact with the specific beta-adrenergic bindings site do not alter the lymphocyte mitogen response. Finally, evidence from these studies shows the importance of non-specific drug:membrane-dependent interactions that may contribute to the understanding of the biological activity of these two classes of drugs.