BIOLOGICAL MARKERS FOR MAJOR DEPRESSIVE DISORDER IN CHILDREN AND ADOLESCENTS.

by

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AND BIOLOGICAL SCIENCES AT

THE CAPE TECHNIKON.

I HEREBY DECLARE THAT THE CONTENT

OF THIS THESIS IS MY OWN WORK AND

ALL VIEWS EXPRESSED ARE MY OWN

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A.H. ENGELBRECHT

TO

MY

PARENTS

PREFACE.

Child psychiatrists have become increasingly aware of the existence of affective disorders in prepubertal and pubertal patients. This has led to the investigation of possible biological factors contributing to the disorders.

Due to the lack of availability of human brain material, different parameters have been investigated in the periphery in order to obtain information regarding the aetiology of major depressive disorder. The neurotransmitters, NA, 5-HT and DA have been implicated in depression. Levels of the metabolites of these transmitters have been measured in plasma, urine and CSF of adult depressed patients.

Two other peripheral "tools" used in the study of major depressive disorder are blood platelets and lymphocytes. The former contain α_2-adrenoceptors and imipramine binding sites (indicative of 5-HT uptake into the platelet) and the latter β-adrenoceptors. Platelets have been widely used as a model for indirectly evaluating changes in central α_2-adrenoceptor and imipramine binding whereas lymphocytes have been used to measure changes in β-adrenoceptor binding and activity in adults with major depressive disorder.

Except for one group, who investigated imipramine binding sites on platelets of children with major depressive disorder, no other studies have been carried out to measure α_2-and β-adrenoceptor levels in children and adolescents

with major depressive disorder. Therefore the present study was undertaken to establish possible markers for juvenile major depressive disorder.

PUBLICATIONS

Of this work, the following have been submitted for publication:

Carstens, M.E., Engelbrecht, A.H., Russell, V.A., Van Zyl, A.M. and Taljaard, J.J.F. (1986). α_2-Adrenoceptor levels on platelets of children and adolescents with major depressive disorder. Psychiatry Research. Submitted for publication.

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Van Zyl, A.M., Taljaard, J.J.F., Carstens, M.E., Russell, V.A. and Engelbrecht, A.H. (1986). Depression in children and adolescents and the role of biological markers.
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ABBREVIATIONS

In addition to the conventional atomic symbolism and S.I. units the following abbreviations are used throughout this study:

ACD	Acid-citrate-dextrose
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Bmax	Maximum number of binding sites
c-AMP	Adenosine 3':5'-cyclic monophosphate
Ci	Curie
COMT	Catechol-o-methyltransferase
C.S.F.	Cerebrospinal fluid
DA	Dopamine
DHA	Dihydroalprenolol
DNA	De-oxyribonucleic acid
DOPAC	Dihydroxyphenylacetic acid
DST	Dexamethasone suppression test
fmol	Femtomole
g	Gravitational force
GABA	γ-Aminobutyric acid
GDP	Guanosine-5-diphosphate
GTP	Guanosine-5-triphosphate
5-HIAA	5-Hydroxyindole-acetic acid
5-HT	Serotonin/5-Hydroxytryptamine
HVA	3-Methoxy-4-hydroxyphenylacetic acid/Homovanillic acid
Kd	Equilibrium binding constant
MAO	Monoamine oxidase
MHPG	3-Methoxy-4-hydroxyphenylethyl glycol

mM	Millimolar
µg	Microgram
µl	Microlitre
µm	Micromolar
NA	Noradrenaline
nM	Nanomolar
RNA	Ribonucleic acid
S.D.	Standard deviation
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin-stimulating hormone
V.M.A.	3-Methoxy-4-hydroxy mandelic acid

CONTENTS

CHAPTER 1

MONOAMINE NEUROTRANSMITTERS

1.1 INTRODUCTION

The important monoamines are the catecholamines: noradrenaline (NA) and dopamine (DA), and the indoleamine, serotonin or 5-hydroxytryptamine (5-HT). Similarities in their metabolic pathways exist, in both cases the precursors are amino acids and their hydroxylated derivatives. DA, NA and 5-HT serve as neurotransmitters in various parts of the brain (Lader, 1980).

1.1.1 Synthesis and Storage of Catecholamines

The precursor of the catecholamines is the amino acid tyrosine (monohydroxyphenylalanine), which is taken up into the nerve ending where it is hydroxylated to dihydroxyphenylalanine (L-dopa) by the enzyme tyrosine hydroxylase (Fig. 1.1). Tyrosine hydroxylase is located in the cytoplasm and on cell membranes and contains iron and utilizes tetrahydrobiopterin as its co-factor. The next step is the conversion of L-dopa to DA by the soluble enzyme L-aromatic amino acid decarboxylase (sometimes called dopa decarboxylase), which needs pyridoxal phosphate as its cofactor.

DA is then taken up into vesicles, which in noradrenergic neurons contain dopamine-β-hydroxylase, an enzyme that adds a hydroxyl group to the side chain. This enzyme contains copper, and its cofactor is ascorbic acid. Finally, in the adrenal medulla and

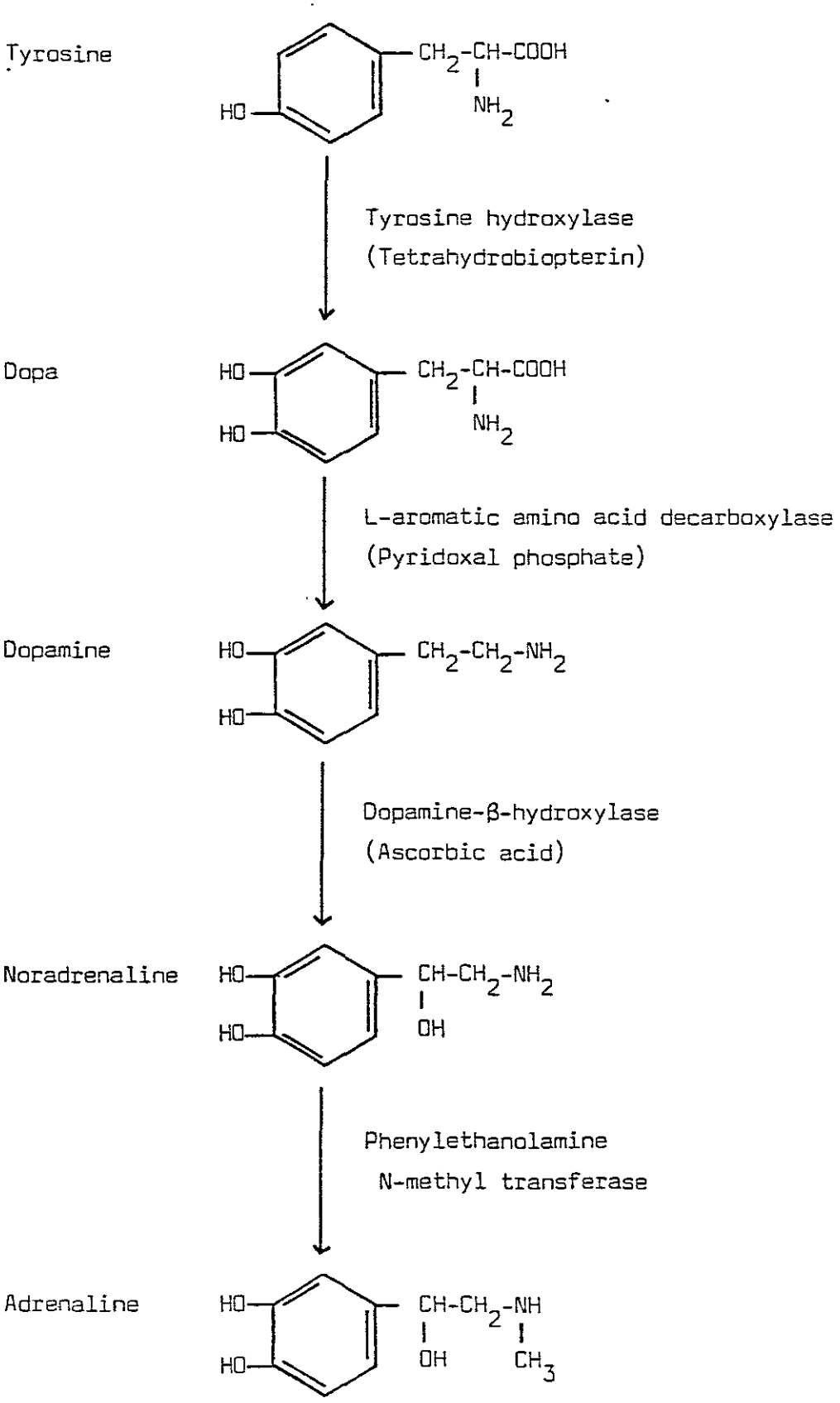


Fig. 1.1 Synthesis of catecholamines (from Lader, M. "Introduction to Psychopharmacology", Upjohn (Pty) Ltd, 1980)

in certain parts of the brain NA is methylated to adrenaline by the cytoplasmic enzyme, phenylethanolamine N-methyltransferase, using S-adenosylmethionine as the methyl donor. The rate of synthesis of catecholamines depends on the amount of available tyrosine hydroxylase, i.e. tyrosine hydroxylase is the rate limiting enzyme in this pathway.

The catecholamines are stored in granules which contain high concentrations (up to a fifth of the total concentration) of the catecholamines - probably stored as a complex with adenosine triphosphate (ATP), four molecules of catecholamine to one of ATP. Chromogranin (a specific protein) and dopamine-β-hydroxylase are also present in the granules. Catecholamines are also found free in the cytoplasmic fluid and in the granules, thus forming two mobile pools as well as the intragranular reserve pool. Catecholamines move by active uptake from the cytoplasmic mobile pool into the granules.

1.1.2 Release and Metabolism of Catecholamines

When the neuronal membrane is depolarized by the nerve action potential, major fluxes of sodium, potassium and calcium occur. The last in particular is essential in the activation of the storage vesicles, which then migrate to the cell boundary, where they fuse to the membrane. The vesicle contents are extruded into the synaptic cleft by a process of exocytosis (Fig. 1.2). As well as the neurotransmitter, other substances contained in the synaptic vesicles may be released. For example NA, ATP and dopamine-β-hydroxylase are released together from peripheral

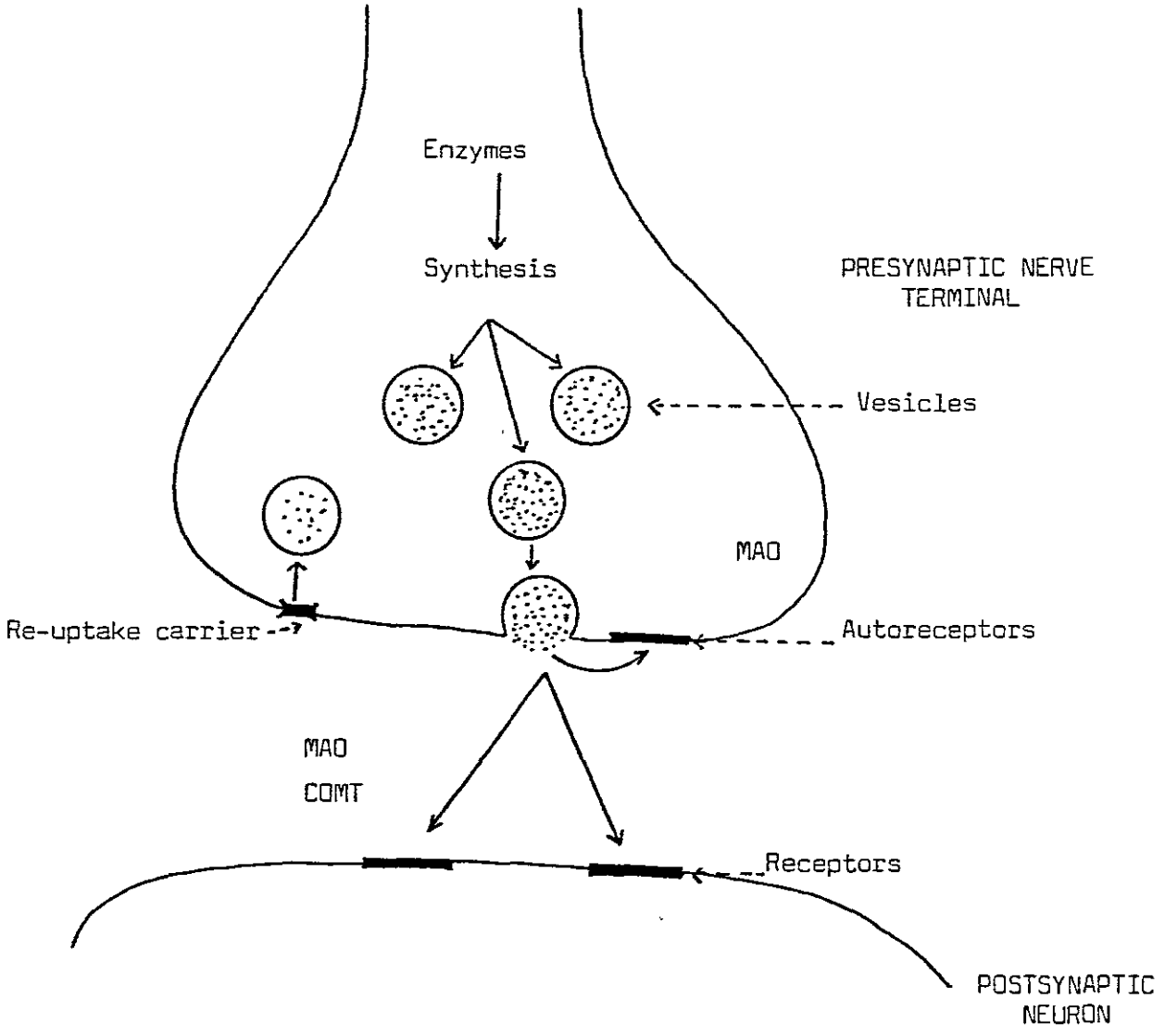


Fig. 1.2 Release and metabolism of catecholamines at the synapse (from Lader, M. "Introduction to Psychopharmacology", Upjohn (Pty) Ltd, 1980)

adrenergic nerves.

The most important mechanism whereby DA and NA are removed from the synaptic cleft and their influence on receptors terminated is by re-uptake, first across the presynaptic membrane into the cytoplasm and then into the storage vesicles. Simple diffusion also accounts for some of the transmitter inactivation. Enzymatic breakdown requires several enzymes, both intracellular and extracellular (Figs. 1.3 and 1.4). The two enzymes of major importance are monoamine oxidase (MAO) and catechol-o-methyl-transferase (COMT). Both enzymes are widespread throughout the body. MAO is a mitochondrial enzyme that converts the catecholamine to its corresponding aldehyde by oxidative deamination. Thus, DA forms dihydroxyphenylacetaldehyde (Fig. 1.3), and NA and adrenaline form 3,4-dihydroxyphenylglycolaldehyde (Fig. 1.4). COMT which is found in the synaptic cleft is closely associated with the postsynaptic membrane. It uses S-adenosylmethionine as a methyl donor to convert DA into 3-methoxy-4-hydroxyphenylethylamine and NA into normetanephrine; i.e. COMT changes one of the hydroxyl groups into a methoxy (CH_3O) group.

Further breakdown takes place involving MAO, COMT and aldehyde reductase and dehydrogenase. The outcome of all these complex processes is that DA is converted mainly to its acidic derivative 3-methoxy-4-hydroxyphenylacetic acid (also called homovanillic acid (HVA), and to a minor extent to dihydroxyphenylacetic acid (DOPAC; Fig. 1.3).

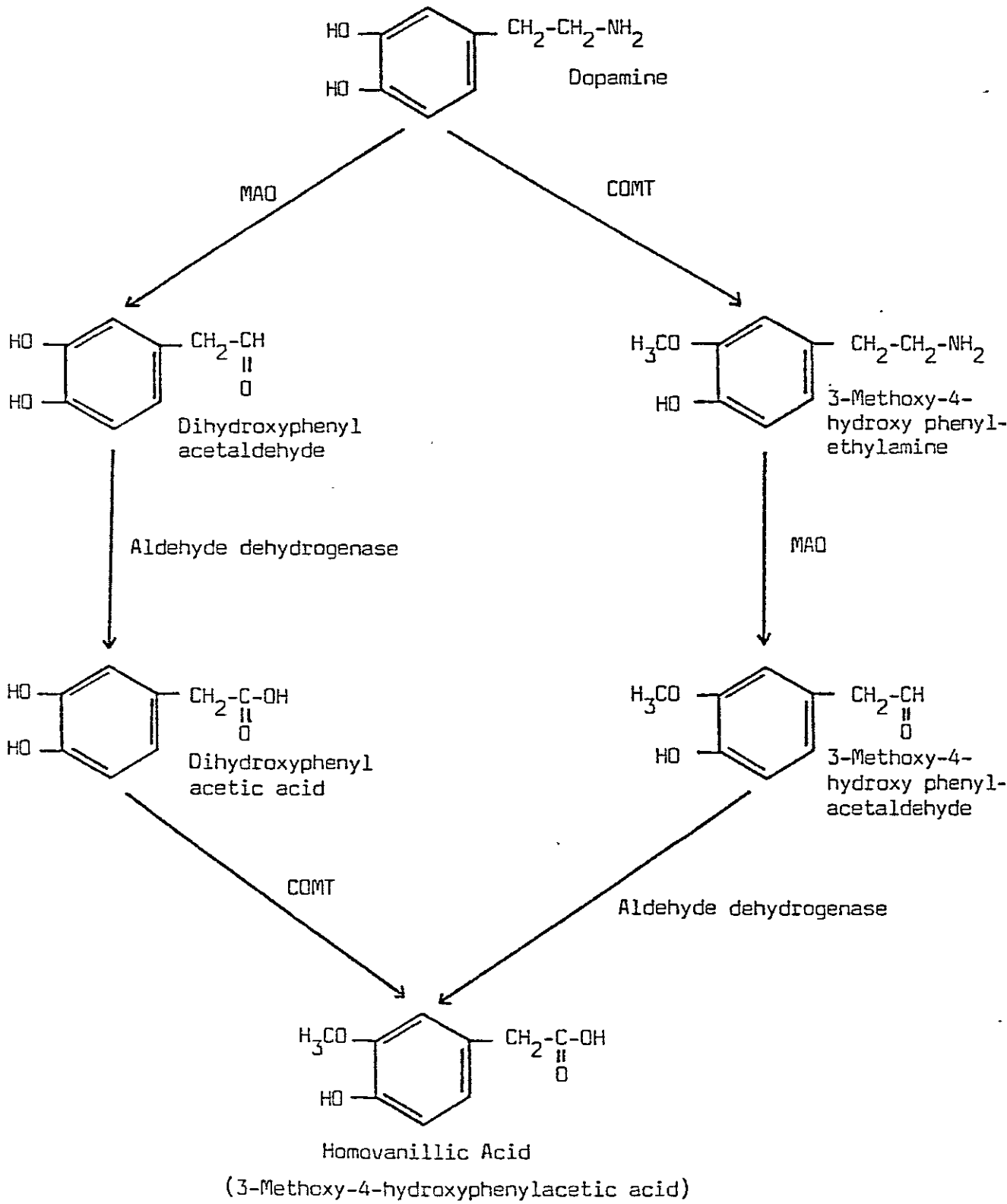


Fig. 1.3 Catabolism of dopamine (from Lader, M. "Introduction to Psychopharmacology", Upjohn (Pty) Ltd, 1980)

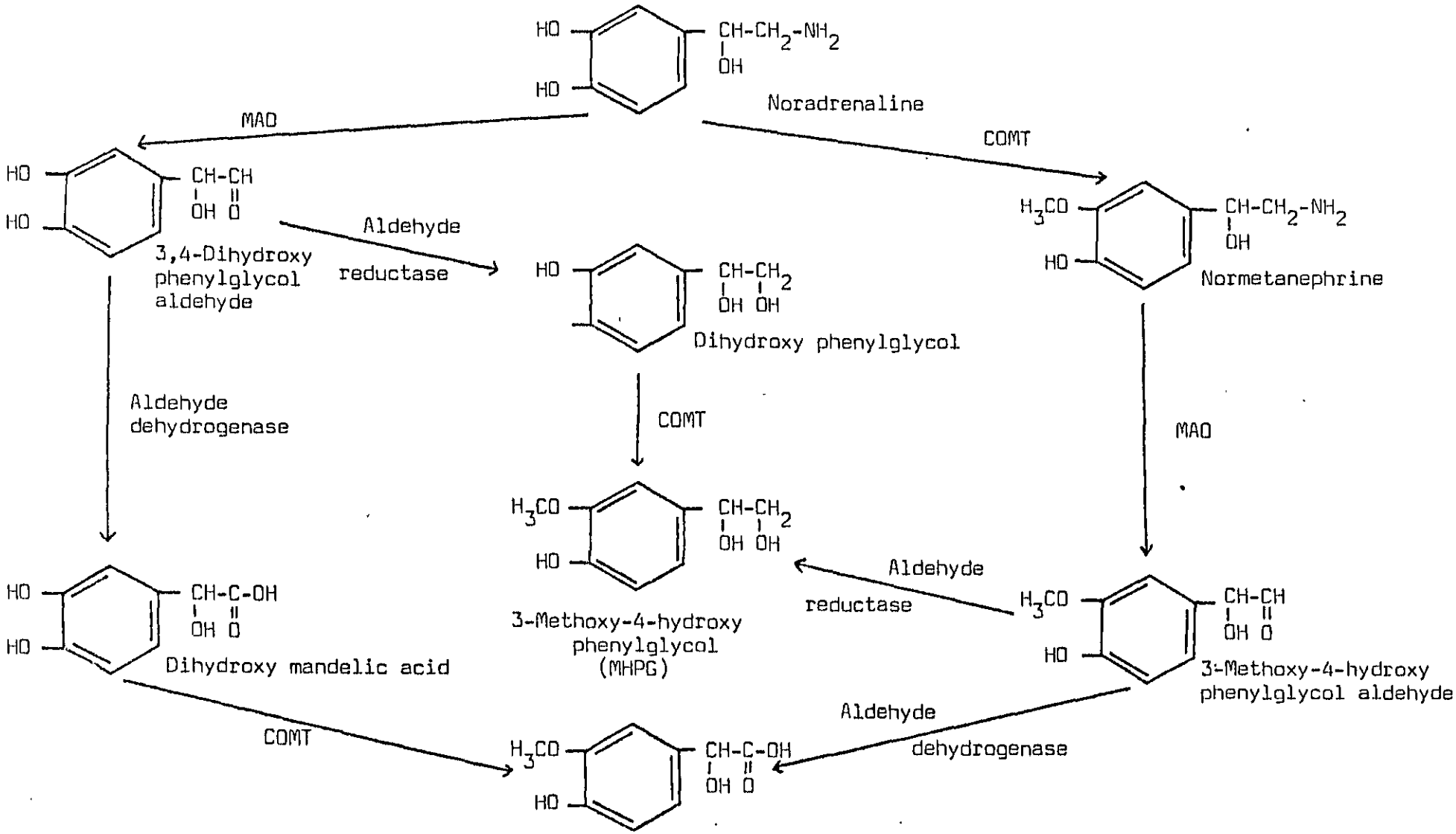


Fig. 1.4 Catabolism of Noradrenaline
(from Lader, M. "Introduction to Psychopharmacology", Upjohn (Pty) Ltd, 1980)

The metabolites of NA are more complex. The main acidic metabolite is 3-methoxy-4-hydroxy mandelic acid (also called vanillyl-mandelic acid, VMA). In the rat and possibly in the human brain, the main metabolite is the alcohol derivative formed from the intermediate aldehydes by aldehyde reductase. This substance is 3-methoxy-4-hydroxy phenylethylglycol (MHPG), which is finally conjugated as the sulphate or glucoronide and is excreted (Fig. 1.4). Adrenaline follows mostly the same metabolic pathways as NA, inasmuch as the terminal methyl group does not affect the process.

Both the alcoholic and acidic breakdown products can be detected in the cerebrospinal fluid (CSF) as well as in the urine. The acidic products move out of the CSF by means of an active transport mechanism that can be blocked by the drug probenecid. Thus, measurement of CSF metabolite concentrations before and after administration of probenecid can give a gross index of amine turnover in the brain.

1.1.3 Synthesis and Storage of Indolamines

The precursor of 5-HT is the essential amino acid tryptophan, an indolic compound. Tryptophan is the only amino acid bound largely to plasma albumin, and it is taken up into the brain by an active transport process. Hydroxylation to 5-hydroxytryptophan then takes place, by means of the enzyme tryptophan hydroxylase which uses tetrahydrobiopterin as cofactor (Fig. 1.5). This is the rate limiting step, and concentrations of tryptophan are normally below maximal so that the availability of trypto-

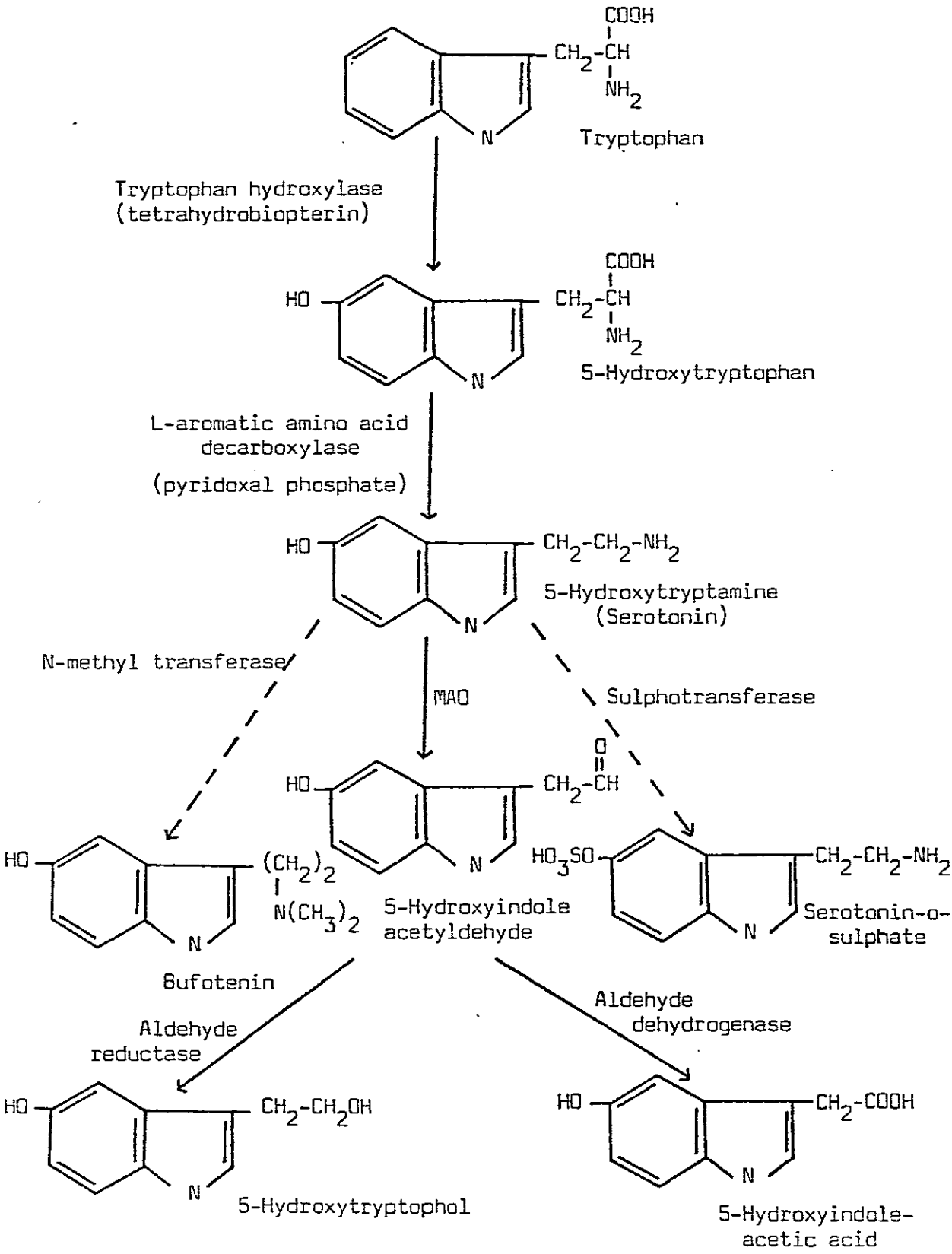


Fig. 1.5 Metabolism of Serotonin (from Lader, M. "Introduction to Psychopharmacology", Upjohn (Pty) Ltd, 1980)

phan and the extent of its binding to plasma proteins govern the amount of neurotransmitter synthesized. The 5-hydroxy-tryptophan is decarboxylated by L-aromatic amino acid decarboxy-lase to 5-HT.

Like the catecholamines, 5-HT is taken up and stored in granules at the presynaptic nerve ending. The storage is in association with adenine nucleotides, mainly ATP. Some 5-HT probably exists in a mobile extragranular pool. Other tissues such as blood platelets can take up and store 5-HT.

1.1.4 Release and Metabolism of Indoleamines

The release of 5-HT into the synaptic cleft is by ionic activation and exocytosis as described in section 1.1.2.

Re-uptake into nerve terminals is the primary route of inactiva-tion of 5-HT. The process is energy dependent and can work against a considerable concentration gradient. Similar uptake mechanisms exist in the blood platelet, which has been proposed as an accessible model in man of central serotonergic processes.

Intracytoplasmic 5-HT can form a substrate for MAO, type A es-pecially. The enzyme converts 5-HT into 5-hydroxyindoleacetal-dehyde, which can then be oxidized by aldehyde dehydrogenase to the acidic metabolite 5-hydroxyindole-acetic acid (5-HIAA; Fig. 1.5). Like HVA and VMA, the egress of 5-HIAA from the CSF can be blocked by probenecid, thus giving a rough measure of 5-HT turnover.

Under certain conditions, 5-HIAA can be reduced to the alcoholic derivative 5-hydroxytryptophol (Fig. 1.5). Other minor metabolic pathways include conjugation to a sulphate derivative and perhaps action by N-methyl transferase to methylated compounds, which are believed to be hallucinogenic (e.g. 5-hydroxy-N-dimethyltryptamine, or bufotenin, and dimethyltryptamine.

1.2 SYNAPTIC MECHANISMS

Chemical synaptic transmission takes place when an influx of calcium ions during a presynaptic nerve impulse triggers exocytosis of neurotransmitter substance from synaptic vesicles. The neurotransmitter diffuses across the narrow synaptic cleft (10-50 nm) and occupies receptors embedded in the postsynaptic membrane. This interaction operates on characteristic ion channels and produces an increase in the postsynaptic membrane permeability to particular ions. Depending on the ionic species to which the postsynaptic membrane becomes more permeable, the physiological response will be an excitatory or an inhibitory postsynaptic potential. The action of neurotransmitters may be terminated either by enzymic inactivation or by cellular uptake mechanisms.

Neurotransmitters are stored in synaptic vesicles and are released by fusion of these vesicles to the plasma membrane. Vesicle fusion is triggered by Ca^{2+}-influx through specific Ca^{2+} channels that open in response to depolarization of the plasma membrane and is terminated by the disappearance of Ca^{2+} from the vicinities of the active zones. The voltage signal

that opens the Ca^{2+} gates is not constant, but also subject to regulation. The key elements are the Na^{+} and K^{+} channels in the nerve terminal. The voltage sensitive Na^{+} channel is responsible for depolarizing the membrane. K^{+} channels are responsible for repolarizing the membrane. Nerve terminal functions are regulated by changes in cyclic nucleotide and Ca^{2+} levels in response to membrane depolarization or the binding of transmitters to receptors. Nerve terminals contain high levels of calmodulin and adenylate cyclase.

1.3 RECEPTORS

Neurotransmission depends on the release of neurotransmitters from axon terminals, diffusion of the neurotransmitter across the synaptic cleft and activation of specific recognition sites on the postsynaptic cell membrane called receptors. These receptors are proteins located in the membranes of presynaptic and postsynaptic neurones and often also on glial cells (Snyder 1984).

1.3.1 Noradrenergic Receptors

The NA-producing cells of the brain are almost exclusively confined to the medulla oblongata and pons. Topographically they can be divided into three major cell systems (Dahlström and Fuxe 1964, Lindvall and Björklund 1983): the locus ceruleus - subceruleus complex, the lateral tegmental cell system (which has a medullary and a pontine component), and the dorsal medullary cell group.

The clearest cell-body grouping is the locus ceruleus, the "blue site", situated in the floor of the fourth ventricle. It constitutes A6 cells, with A7 cells located ventrolaterally to it and A4 cells just caudally (Fig. 1.6). The nucleus locus ceruleus is usually divided into a dorsal part, composed of densely packed fusiform cells, and a ventral part containing somewhat larger multipolar neurones (Swanson 1976). These latter neurones are morphologically similar to the NA neurones in the subceruleus area; they are topographically continuous with the more ventrally located subceruleus cells and have similar projection patterns.

The cells of the lateral tegmental cell system are located in the ventrolateral tegmentum, from the caudal pole of the medulla oblongata up to the level of the motor nucleus of the trigeminal nerve in the pons (Dahlström and Fuxe 1964; Palkovits and Jacobowitz 1974; Swanson and Hartman 1975). The cells in the medullary part of the lateral tegmental cell system (designated A1 and A3; Fig. 1.6) extend from the pyramidal decussation up to the rostral part of the inferior olivary nucleus. They occur mainly scattered around and partly within the lateral reticular nucleus (Dahlström and Fuxe 1964). In the rat, the A3 cells are difficult to distinguish from those of the A1 group. The cells in the pontine part of the lateral tegmental cell system (designated A5 and A7; Fig. 1.6) are distributed from the level of the rostral part of the facial nucleus up to the level of the trigeminal motor nucleus.

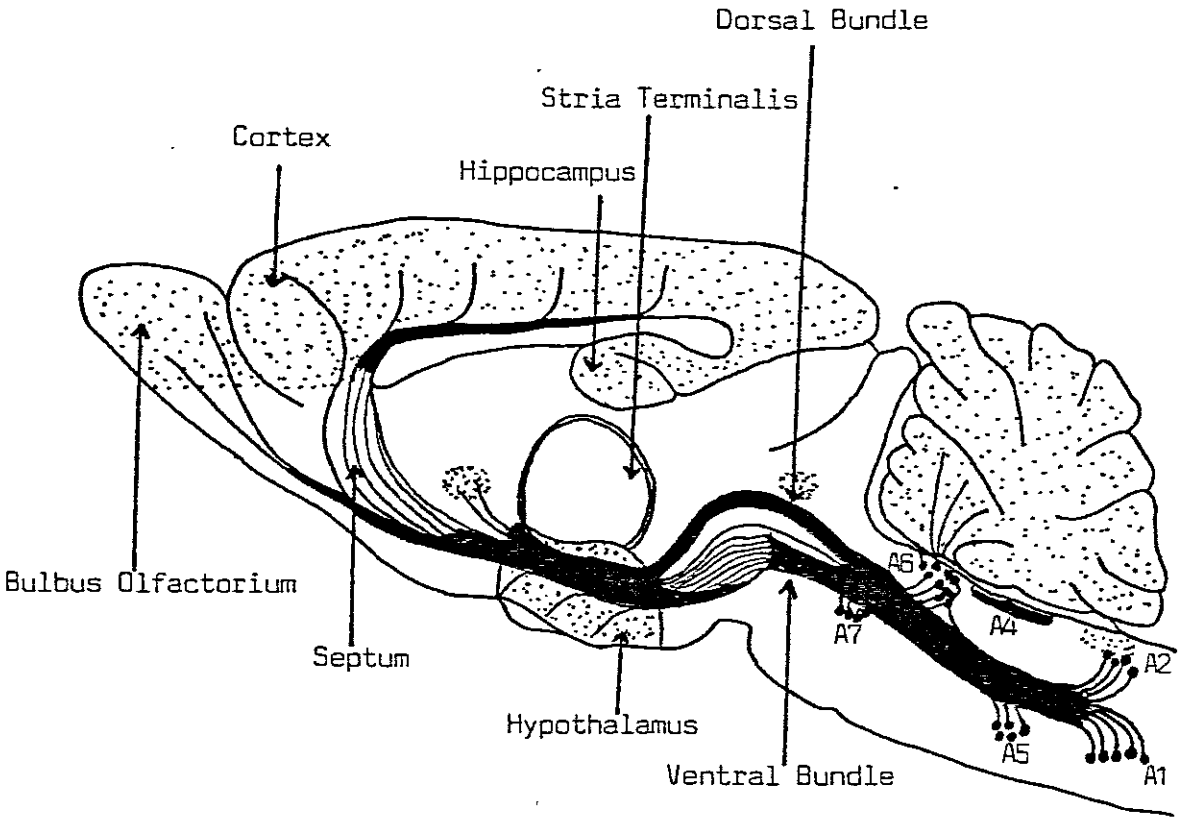


Fig. 1.6 Schematic representation of the organisation of NA containing neural systems in the rat brain. Fibres (black lines) arise from groups of cell bodies in the brainstem (A1-A7) and project widely to terminal fields (dotted areas) in various forebrain regions

The cells of the dorsal medullary cell group (designated A2; Fig. 1.6), occur in the nucleus of the solitary tract and the commissural nucleus, with some cells also in the dorsal motor nucleus of the vagus (Dahlström and Fuxe, 1964). A1, A2 and A5 cells (Fig. 1.6) project both to the spinal cord and rostrally. The rostral projection pathways are the dorsal and the ventral. The dorsal pathway arises in the locus ceruleus and projects ventrolaterally to the central grey area. Its main component runs in the medial forebrain bundle to innervate all the cortices, the thalamus, geniculate bodies, colliculi, habenula, some hypothalamic nuclei, the amygdala and the olfactory bulb. The ventral pathway is formed from A1, A5 and A7 cell bodies (Fig. 1.6) and runs through the medullary reticular formation, the pons and mesencephalon, gradually overlapping the dorsal bundle. It extends through the cuneate nucleus and the A8 cell region (Fig. 1.6) to innervate the septal area, amygdala, preoptic area, hypothalamus, periventricular area, mammillary bodies and substantia nigra. The locus ceruleus complex has a dorsolateral extension of cells along the medial aspect of the superior cerebellar peduncle into the roof of the fourth ventricle (designated A4, Dahlström and Fuxe, 1965; Fig. 1.6).

1.3.1.1 α-Adrenoceptors

Noradrenergic receptors are found in nearly all areas of the central and peripheral nervous systems. The subdivision of the α-adrenoceptors into α_1/α_2 and pre/postjunctional subtypes, respectively, has led to a logical although complex classification. The introduction of highly selective agonists and anta-

gonists towards α_1/α_2-adrenoceptors as well as sophisticated radioligand binding studies have substantiated the concept of the existance of two types of α-adrenoceptors with rather different structural demands (Van Zwieten and Timmermans, 1984).

Initially α_1-adrenoceptors were thought to be postsynaptic and α_2-adrenoceptors presynaptic (Langer, 1974). However, the discovery of receptors with a preference for agonists and antagonists identical with that of presynaptic α_2-adrenoceptors at postsynaptic sites suggested that a revised classification was required. Prejunctional α-adrenoceptors are however predominantly of the α_2-subtype, as concluded from their preference for selective α_2-adrenoceptor agonists and antagonists, although Kobinger and Pichler (1980, 1982) have suggested that a minor proportion of the presynaptic α-adrenoceptors display α_1-characteristics. Receptors with characteristics of α_2-adrenoceptors at prejunctional sites have been identified at cholinergic nerve endings (Drew, 1978; Wikberg, 1979; Starke, 1977, 1981a,b), at serotonergic nerve endings (Göthert and Huth, 1980) and on the cell bodies of noradrenergic neurones(Brown and Caufield, 1979). Presynaptic α_2-adrenoceptors appear to be involved in the regulation of NA release from noradrenergic nerve endings. α_2-Adrenoceptor action has been suggested to involve inhibition of the influx of extracellular calcium ions (Göthert, 1977, 1979; Göthert et al, 1979; De Langen and Mulder, 1980), hyperpolarization (Stjärne 1978, 1979), increased Na^+/K^+-ATPase activity (Vizi, 1977, 1979) and inhibition of adenylate cyclase activity (Fain and Garcia-Sainz, 1980; Schultz et al, 1980; Jakobs and Schultz, 1982).

At postsynaptic sites, both α_1-and α_2-adrenoceptors are present. Postjunctional α_1- and α_2-adrenoceptors have been shown to display subtle but relevant differences concerning their anatomical position with respect to the synapse. Postjunctional α_1-adrenoceptors appear to be located intrasynaptically and thus to be readily accessible to the endogenous neurotransmitter, NA, whereas postjunctional α_2-adrenoceptors appear to be located extrasynaptically (Langer et al, 1980b, 1981a, 1981b; Yamaguchi and Kopin, 1980; Wilfert et al, 1982a, 1982b). Accordingly, they are less accessible to intrasynaptically released NA and peripheral α_2-adrenoceptors will rather react with circulating catecholamines, such as NA and adrenaline.

Differences have been demonstrated between the events which follow the stimulation of postsynaptic α_1-and α_2-adrenoceptors. These differences were particularly evident in the vasoconstrictor responses to α_1-and α_2-adrenoceptor stimulation. Whereas the α_2-adrenoceptor response appeared to be sensitive to impairment of calcium entry, the α_1-adrenoceptor response was not (Van Meel et al 1981a, 1981b, 1982). Other workers however have found that α_1-adrenoceptor stimulation is accompanied by calcium influx which is sensitive to blockade by calcium antagonists (Vanhoutte, 1982; Vanhoutte and Rimele, 1982). Godfraind et al (1982) suggested that α_2-adrenoceptor stimulation will open up calcium channels whereas the stimulation of α_1-adrenoceptors will induce depolarization and subsequently the release of calcium ions from intracellular stores.

Non-neuronal α_2-adrenoceptors are found too. In platelets they are linked to aggregation, in fat cells they are involved in the inhibition of lipolysis. In pancreatic islets they mediate inhibition of insulin secretion and in vascular smooth muscle they mediate contraction (Langer and Pimoule, 1982).

1.3.1.2 β-Adrenoceptors

At least two major subtypes of β-adrenoceptors, the β_1- and β_2-adrenoceptors, can be distinguished by a variety of pharmacological criteria (Lands et al, 1967). Both β_1- and β_2-adrenoceptors stimulate the enzyme, adenylate cyclase. This action leads to the intracellular accumulation of adenosine 3':5'-cyclic monophosphate (cAMP), the second messenger of β-adrenergic action in virtually all tissues examined to date (Sutherland and Rall, 1960).

Catecholamine receptor-coupled adenylate cyclase systems consist of three distinct protein components in the phospholipid membrane: the receptor with its specific recognition site for the neurotransmitter, the catalytic unit (C) of the adenylate cyclase and the nucleotide regulatory protein (N) with its binding site for guanine nucleotides (Fig. 1.7). In the case of the β-adrenoceptors, binding of the catecholamines, NA and adrenaline to the receptors results in the formation of a high affinity catecholamine-receptor complex, which can bind the nucleotide regulatory protein (N). Guanosine-5-triphosphate (GTP), binds to the latter component, releasing the β-adrenceptor and the catecholamine. The catalytic unit (C) of adenylate cyclase is activated by binding to the nucleotide regulatory protein (N) - GTP complex. After activation,

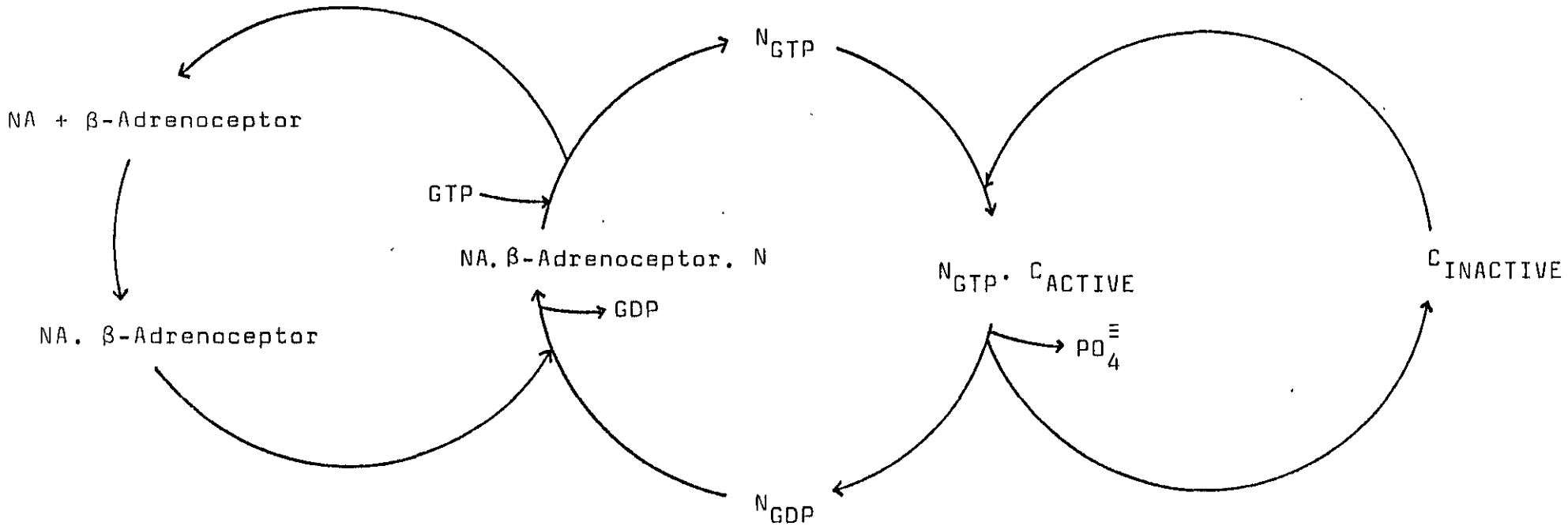


Fig. 1.7 Working model for adenylate cyclase regulation by β-adrenergic agonists and GTP

GTP-ase converts GTP to guanosine-5-diphosphate (GDP) and the nucleotide regulatory protein (N) is released from the catalytic moiety (Stiles et al, 1984). A schematic representation of the events is shown in Fig. 1.7.

The two β-adrenoceptor subtypes were originally defined by their relative affinities for adrenaline and NA. β_1-Adrenoceptors (e.g. those found in mammalian cardiac and adipose tissue) displayed approximately equal affinities for adrenaline and NA. On the other hand, β_2-adrenoceptors (found in tracheal and vascular smooth muscle tissue) had considerably greater affinity for adrenaline than for NA (Stiles et al, 1984). Whereas rat and guinea pig atria contain exclusively β_1-adrenoceptors, the right atria of cats, dogs and humans contain mixed populations of β_1-and β_2-adrenoceptors (Ablad et al, 1974; Carlsson et al, 1972, 1977; O'Donnell and Wanstall, 1979a, b). Other tissues also show heterogeneity of β-adrenoceptors. These include guinea-pig trachea (Furchgot, 1975; O'Donnell and Wanstall, 1979a, canine gastric mucosa (Daly et al, 1978), rat jugular veins (Cohen et al, 1980) and mammalian brain (Dolphin et al, 1979; Ebersolt et al, 1981). In the cerebral cortex, limbic forebrain and striatum of the rat, β_1-adrenoceptors are predominant while the cerebellum contains exclusively β_2-adrenoceptors (Minnerman et al, 1979; Nahorski, 1981). The β_1-adrenoceptors are primarily involved in neuronal function (Minnerman et al, 1979). Both β-adrenoceptor subtypes are not necessarily present on a single cell, because any mammalian organ is composed of a heterogeneous population of cells.

Lymphocytes have also been shown to contain a β-adrenoceptor coupled adenylate cyclase system (Bourne and Melmon, 1971; Pandy et al, 1979) and in 1981, Brodde et al (Brodde et al, 1981) identified the β-adrenoceptors on human lymphocytes as a homogeneous population of the β_2-subtype.

Different labelled ligands have been employed in studies to identify and characterise the β-adrenoceptors in central and peripheral tissues. Dihydroalprenolol (DHA), a β-adrenoceptor antagonist has been widely used (Sugrue, 1983), but this labelled compound has a rather low specific activity. Another widely used β-adrenoceptor ligand ^{125}I-iodohydroxybenzylpindolol, has a very high specific activity, but also labels α_1-adrenoceptors and 5-HT receptors (Engel et al, 1981). A new high-affinity β-ligand, ^{125}I-iodocyanopindolol, is currently used to evaluate β-adrenoceptor subtypes, employing selective competitors (Petrovic et al, 1983).

1.3.2 Serotonergic Receptors

Azmitia and Henriksen (1978) reviewed the serotonergic pathways of the brain in great depth. 5-HT cell bodies were found to be located in the raphe of the midbrain region (Fig. 1.8). Nine nuclear groups have been identified by histochemical fluorescence techniques and arbitrarily designated B1-B9 (Fig. 1.8). The dorsal (B7) and median (B8) raphe nuclei were shown to contain most of the 5-HT-producing neurons of the midbrain.

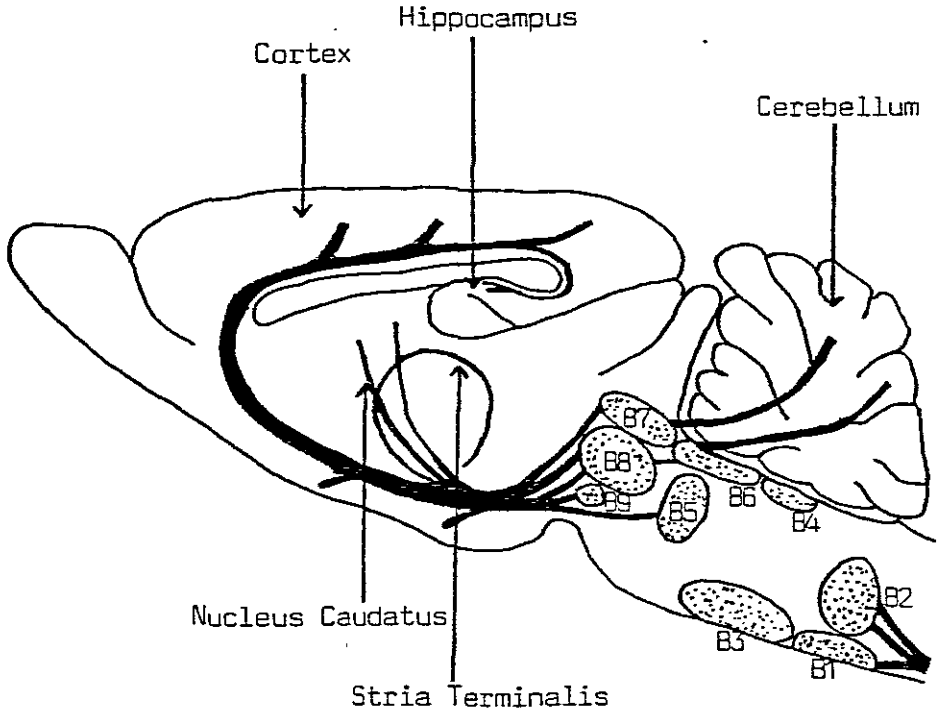


Fig. 1.8 Schematic diagram of the central serotonergic cell groups and projections in a sagittal section from rat brain

The ascending dorsal raphe forebrain tract runs in the ventrolateral aspect of the medial forebrain bundle and innervates mainly lateral forebrain structures including the basal ganglia, amygdala, nucleus accumbens and piriform cortex (Fig. 1.8). The median raphe forebrain tract runs in the ventromedial aspect of the medial forebrain bundle and innervates mainly medial forebrain structures including the cingulate cortex, septum and hippocampus.

The serotonergic projection to the thalamus has been shown to modulate nonspecific nuclei, which in turn modulate large areas of CNS tissue. Furthermore, in addition to their effects in the thalamus and cortex, 5-HT fibres also project to the hypothalamus (Bodian, 1940), the globus pallidus (Nauta and Mehler, 1966), the striatum (Powell and Cowan, 1956) and the brainstem reticular nuclei (Scheibel and Scheibel, 1967). The serotonergic projections to the basal ganglia arise mainly from the dorsal raphe nucleus and also from the median raphe forebrain tract.

1.3.2.1 5-HT_1 and 5-HT_2 Receptors

The multiplicity of pharmacological and physiological effects of 5-HT suggests the existance of multiple types of 5-HT receptors and has inspired extensive research into their biochemical characterization. Two distinct types of purported 5-HT receptors have thus far accrued from in vitro receptor binding studies (Leysen, 1983). The terminology of S_1 or 5-HT_1 binding sites was introduced by Peroutka and Snyder (1979). These are saturable binding sites on brain membrane preparations which are labelled with high

affinity i.e. nanomolar concentrations, by ^{3}H -5-HT. 5-HT_1 binding sites occur in highest density in the hippocampus and the striatum followed by the cortex. Known 5-HT antagonists such as cyproheptadine, cinanserin, mianserin and ketanserin (Leysen et al, 1981) bind very poorly or not at all to these sites (Leysen, 1981).

The S2 or 5-HT_2 binding site was first detected in rat frontal cortex membrane preparations. All known serotonin antagonists including cyproheptadine, cinanserin, mianserin, ketanserin, methysergide and metergoline bind with nanomolar affinity to these sites. 5-HT and various 5-HT agonists (bufotenine, quipazine) reveal micromolar binding affinities whereas other neurotransmitters such as DA, NA, histamine, acetylcholine and γ-aminobutyric acid (GABA), virtually do not bind to these sites. Mammalian brain areas enriched in 5-HT_2 binding sites are the frontal cortical areas followed by the nucleus accumbens, tuberculum olfactorium and striatum (Leysen et al, 1982). Various types of neuronal lesions were used to demonstrate that the 5-HT_2 binding sites were not localized on terminals of dopaminergic, noradrenergic or serotonergic neurones (Leysen et al, 1982). 5-HT_2 sites were shown to be localized on post-synaptic cells in the frontal cortex. 5-HT_2 receptors have been shown to be involved in 5-HT induced behavioural excitation (Leysen et al, 1978, 1982), inflammation (Ortmann et al, 1982) and smooth muscle contraction (Leysen et al, 1981, 1982; Van Nueten et al, 1981, 1982) 5-HT_2 receptors have been identified on cat blood platelets (Leysen et al, 1983) where they appeared to play a role in vasoconstriction and platelet aggregation.

1.3.2.2 5-HT reuptake sites

The association of ^{3}H - imipramine binding with the transporter for the serotonin reuptake mechanism in serotonergic nerve endings (Section 1.1.4) has been clearly established (Langer et al, 1980c; Sette et al, 1981; Gross et al, 1981; Brunello et al, 1982). It is likely that ^{3}H - imipramine binding labels a physiologically relevant site that modulates serotonin reuptake (Langer et al, 1983) rather than a simple tricyclic recognition site. In support of this view, it was shown that tritiated non-tricyclic inhibitors of serotonin reuptake like ^{3}H - norzimelidine (Hall et al, 1982) and ^{3}H -paroxetine (Mellerup et al, 1982) label with high affinity the same site labelled with ^{3}H - imipramine, which is associated with the neuronal reuptake of serotonin.

Results suggest that ^{3}H - imipramine binds with high affinity to sites associated with the serotonin transport system which may be different from the substrate recognition site for serotonin (Fig. 1.9; Langer et al, 1983). It is possible that ^{3}H - imipramine binds with high affinity to a presynaptic site that modulates neuronal reuptake of serotonin, as presynaptic autoreceptors modulate the release of their neurotransmitter (Langer, 1980b). It should be pointed out, however, that the release-modulating presynaptic autoreceptors are acted upon by the neurotransmitter itself (Langer, 1980a), while the presynaptic sites involved in the modulation of serotonin reuptake may be acted upon by a known co-transmitter or a novel endogenous substance present in the serotonergic or in adjacent nerve terminals or in the circulation.

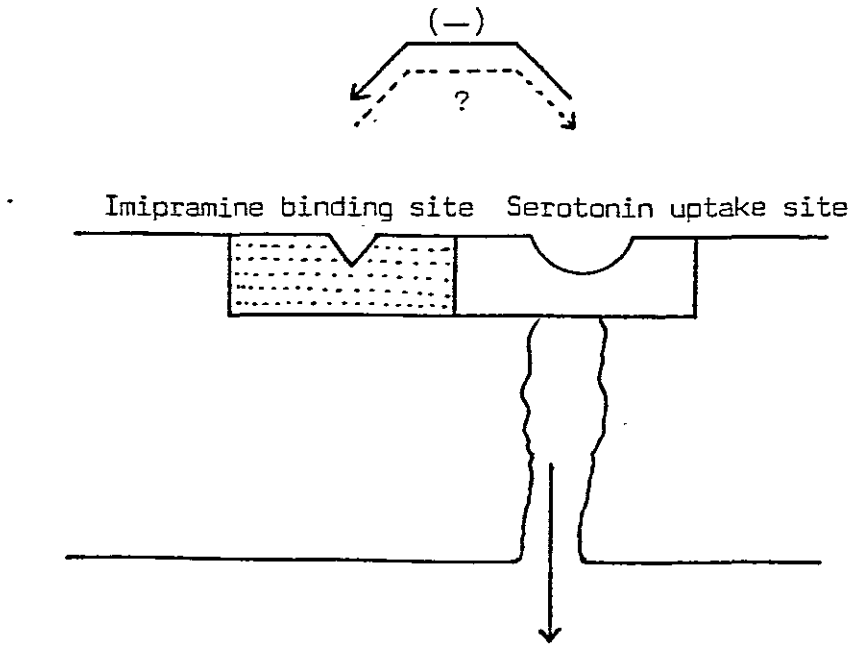


Fig. 1.9

A schematic representation of the possible relationship between the ^{3}H - imipramine binding site and the serotonin uptake mechanism. Two different recognition sites appear to be present for the transporter of serotonin at the level of the nerve terminals. One is the substrate recognition site for serotonin and the second, where ^{3}H - imipramine binds, may be a modulator unit for the uptake of serotonin. Serotonin and other tricyclic uptake blockers, change the affinity of this site for imipramine. The ^{3}H - imipramine binding site has been suggested to be activated by an endogenous ligand which is different from serotonin (Langer and Raisman, 1983).

Rehavi et al (1985) described the extraction and partial purification of an endogenous "imipramine-like" substance from rat brain. The endogenous factor obtained after gel filtration and silica chromatography inhibits specific ^{3}H - imipramine binding and mimics the inhibitory effect of imipramine on ^{3}H - serotonin reuptake in both brain and platelet preparations. The effects of the endogenous material are dose-dependent and it inhibits ^{3}H - imipramine binding in a competitive fashion. The factor is unevenly distributed in the brain with high concentrations in the hypothalamus and low concentration in the cerebellum.

It is of interest to note that the reuptake of serotonin in the hypothalamus and suprachiasmatic nuclear region of the rat undergoes important 24 hour changes with a circadian pattern (Meyer and Quay, 1976). The peak for ^{3}H-5-HT reuptake is observed near the onset of darkness and the trough or minimum near the onset of light (Meyer and Quay, 1976). It is tempting to speculate that changes in the local concentration of an endogenous modulator may be related to the circadian rhythm of ^{3}H - 5-HT reuptake reported in the hypothalamus and suprachiasmatic nucleus of the rat (Langer and Raisman, 1983). Of interest is the fact that the suprachiasmatic nucleus has a very rich serotonergic innervation and plays a major role in the occurrence and maintenance of normal circadian rhythms (Rusak and Zucker, 1979). On the other hand, there is a clear circadian rhythm for both ^{3}H-5-HT reuptake and ^{3}H - imipramine binding in the suprachiasmatic nucleus and this phenomenon may be of considerable significance in view of the hypothesis that internal desynchronization of circadian rhythms might be causally related to depression (Wehr and Goodwin, 1981).

CHAPTER 2

MAJOR DEPRESSIVE DISORDER IN CHILDREN AND ADOLESCENTS

2.1 Clinical Features

During the past decade, childhood depression has become recognized as a major clinical entity. Work has begun to identify and treat children manifesting depression, but instrumentation and measurement are still at a rudimentary level. Although there have been numerous anecdotal reports, major depressive disorders in children have only recently been the subject of systematic research. (Carlson and Cantwell, 1980; Puig-Antich et al, 1978; Weinberg et al, 1973).

According to DSM III, the criteria for a major depressive episode are the same for children and adults - dysphoric mood or loss of interest/pleasure lasting at least two weeks, and at least four of the following symptoms: appetite disturbance, sleep disturbance, psychomotor agitation or retardation, loss of energy, feelings of worthlessness or guilt, diminished ability to think, and thoughts of death and suicide. (Preskorn et al, 1982). Without effective treatment, these depressive episodes can last for months and lead to impaired school performance, poor peer and family relationships, and suicide. There are, at present several objective approaches to the diagnosis of childhood depression, which, though different in specific content, have all accepted models of adult depression as appropriate to the phenomenology of childhood psychopatho-

logy. Thus, sleep, endocrine and genetic factors associated with adult depression are the most crucial and strongest criteria for the diagnostic validity of childhood depression. (Puig-Antich and Gittelman, 1982).

With children, two sources of information beside the patient can be used routinely in clinical practice and research: the parent and the school. These are necessary because of the cognitive limitations inherent to the developmental stage of the prepubertal child, which make it usually impossible for him to provide an accurate chronological structure for the present episode of illness. (Puig-Antich and Gittelman, 1982). Several diagnostic instruments are used today in a polydiagnostic approach to identify the depressive episode. These instruments include the Diagnostic and Statistical Manual of Mental Disorders III (1980), the Research Diagnostic Criteria (Spitzer et al, 1978), and are assessed by the Kiddie - Schedule for Affective Disorders and Schizophrenia (K-SADS; Puig-Antich and Chambers 1982).

In addition, each child and parent are assessed with the Interview Schedule for Children (ISC; Kovacs, 1983) and Rutter's Parent and Teacher questionnaires are also completed (Rutter et al, 1970). Furthermore, the Birleson Self-rating Scale for Depression (Birleson, 1981) and the Visual Analogue Scale (Aitken, 1969) are completed by each child. Children between the ages 12 and 18 years have to complete the Health and Daily Living Youth form (Billings and Moos, 1982) and the Family Assessment Device (Epstein et al, 1983). The latter is also completed by the parents.

2.2 Biochemical Findings

In adults the noradrenergic system has been implicated by biochemical evidence as playing an important role in the psychobiology of depression. It has been shown in several studies (Siever and Uhde, 1984) that growth hormone responses to clonidine are blunted in depressed patients. While these responses may be mediated by hypothalamic post-synaptic α_2-adrenoceptors, blunted responses to clonidine of plasma MHPG observed in depressed patients, reflect effects of clonidine on inhibitory α_2-adrenoceptors which play a role in modulating presynaptic noradrenergic activity. (Siever and Davis, 1984).

There are also several clinical signs and symptoms in the typical endogenous depression that seem to indicate endocrine disturbances. Although the basal levels of the hormones may be within normal limits, there is often a reduced reactivity in hormonal systems, probably because of a deficiency in the regulatory mechanisms in the hypothalamus-pituitary axis.
Two tests have emerged as especially important and revealing in this connection: the dexamethasone suppression test (DST) and the thyrotropin-releasing hormone (TRH)- thyrotropin (TSH)-stimulation test.

There is considerable evidence that resistance to suppression of the hypothalamic-pituitary-adrenal axis by dexamethasone is a specific marker of endogenous depression in adults. Studies by Carroll et al (1981) have shown that the DST iden-

tifies up to 65% or more of patients with endogenous depression with melancholia at high levels of confidence. Adult depressed patients with abnormal DST results are diagnosed clinically as having either endogenous depression (Carroll et al, 1981; and Brown et al 1979) or primary depression (Schlesser et al 1980; Brown and Shuey 1980) Patients with clinical diagnoses of non-endogenous or secondary depression have normal DST results, as do normal subjects and patients with nondepressive psychiatric diagnoses (Carroll et al, 1980a, 1980b, 1981; Brown et al 1979, Schlesser et al 1980, Brown and Shuey 1980). The DST shows promise of predicting response to biological treatment (Brown et al, 1979), demonstrating when biological treatment can be discontinued without relapse (Carroll 1982b), of helping to clarify difficult diagnostic problems (Carroll 1982b), and, possibly, in anticipating which patients are at greatest risk for suicide (Carroll 1982a; 1982b).

The DST has been used by pediatricians for the study of endocrine function for years (Pavlatos et al 1965), and it may be applicable to the study of depression in adolescents as well. While there are reports of depressed adolescents (Carroll 1982a; De la Fuente and Rosenbaum 1980) and prepubertal children (Poznanski et al, 1982) with abnormal DST results, there has been no systematic study of the specificity and sensitivity of the marker in adolescence. For depressed children, Poznanski et al (1982) used the DST procedure described by Carroll et al (1981). The dose of dexamethasone for the study of children was 0,5 mg (one tablet orally given at 23h00. The next day a single blood sample was drawn at 16h00. The plasma cortisol concentrations were determined by a competitive protein-binding method (Carroll

et al 1981). Poznanski et al (1982) found that, of the 9 children with major depressive disorder, 5 had abnormal DST results; thus the sensitivity of the DST for major depressive disorder was at least 56%.

In a preliminary report Robins et al (1982) reported a DST sensitivity of 50% in 4 children with major depressive disorder. These results support the hypothesis that the DST may be as clinically useful as a biological marker of endogenous depression in adolescents, as it is in adults. In addition, a positive (abnormal) DST result may support the clinical suspicion of this syndrome with high confidence, but a negative (normal) test result will not exclude it. The DST may be useful in helping clinicians discriminate which adolescents should receive medication and when medication can be discontinued without relapse.

The TRH-TSH-stimulation test is a test of serum TSH response to a test-dose of TRH; it can be seen as a test of central regulation of the pituitary-thyroid axis. The test is carried out by giving an intravenous injection of TRH (250µg) in 1 min. Blood samples are collected for the determination of serum TSH levels before and 20 min., 60 min. and 90 min. after injection of TRH (Loosen and Prange, 1982). Normally there is a marked increase in TSH, with levels peaking at about 30 min. after the TRH injection. A "blunted response" is said to be present if the maximum increase in serum TSH (ΔTSH) is lower than 7µU/ml (Kirstein et

al, 1981). Studies have shown that a large proportion of patients with endogenous depression have a blunted response in this test (Loosen and Prange, 1982). On the average, clinical studies have found blunted responses to TRH in about 25% of patients with endogenous depression. This decreased response is therefore not as frequent as the abnormal DST response in depression. Like the DST, the TRH-TSH test does not differentiate between unipolar and bipolar endogenous depression. Unlike the non-suppression of the DST, a blunted TSH response, when present in depression, sometimes persists into remission, although in some cases (usually after prolonged remission) the response normalizes. What the mechanisms are underlying the abnormal response to these two tests in endogenous depression is not known for certain. It has been hypothesized that a central hyperactivity in cholinergic systems may be responsible for non-suppression in the DST (Carroll, 1982a), whereas excess dopaminergic activity might account for the blunted TSH response which might, in fact be due to hypersecretion of TRH in endogenous depression (Loosen and Prange, 1982).

As far as the TRH-TSH stimulation test in children and adolescents is concerned, however no reports have been published to our knowledge.

2.3 AETIOLOGY OF MAJOR DEPRESSIVE DISORDER

The first plausible hypothesis concerning the nature of the biological basis of the affective disorders was the monoamine hypothesis. This hypothesis was proposed as a result of stu-

dies on the actions of various drugs in animals and in adults in the 1950's and 1960's. The hypothesis was first put forward by Pare and Sandler (1959) and Jacobsen (1959). The first drug to be considered in the aetiology of the depressive disorder was rauwolfia serpentina. This substance had been known for several centuries to have sedative properties (Bein, 1956). In the 1950's it began to be used in the treatment of hypertension and it was found that its active hypotensive principle was reserpine (Wilkins, 1954). Reserpine was widely used and severe depressive illness occurred in some hypertensive patients treated with this drug (Freis, 1954; Achor et al, 1955; Müller et al, 1955; Lemieux et al, 1956). The synthetic analogue of reserpine, tetrabenazine, was also shown to produce severe depressive effects in man (Ashcroft et al, 1961; Lingjaerde, 1963). Studies on the use of reserpine and tetrabenazine in animals suggested a possible mechanism for these depressant effects in man. Reserpine and tetrabenazine produced a state of sedation and withdrawal in animals. This state was shown to be associated with depletion from the brain of 5-HT (Pletscher et al, 1956); NA (Holzbauer, M. and Vogt, M., 1956; Quinn et al, 1959) and DA (Carlsson, et al, 1957). These drugs were shown to inhibit uptake of the neurotransmitters into vesicles in the presynaptic nerve terminal, probably by a modificaiton of the Na^+, K^+-ATPase uptake pump mechanism (Stitzel, 1977).

The second group of drugs relevant to the development of the monoamine hypothesis is the MAO inhibitors. Iproniazid has been shown to inhibit MAO (Zeller and Barsky, 1952) and to increase

brain levels of NA and 5-HT in animals (Spector et al, 1958). Pretreatment of mice with iproniazid was found to cause them to respond to reserpine administration with marked excitation instead of the usual withdrawal and sedation (Chessin et al, 1957), suggesting that the depressive symptoms could be prevented by elevation of brain NA and 5-HT levels. In 1957, the antidepressant efficacy of iproniazid in man was demonstrated (Loomer et al, 1957) and this drug was later shown to increase the levels of NA, DA and 5-HT in human brain (Ganrot et al, 1962; Maclean et al, 1965). These observations gave rise to the hypothesis that in depression there is a functional deficit of monoamine neurotransmitters at certain synaptic sites in the brain. The hypothesis was extended by the suggestion that in mania there is an excess of neurotransmitters at these sites. A single hypothesis concerning all three monoamines is not the only possibility. Separate hypotheses concerning each monoamine may be formulated and the evidence for each considered individually. Some authors have stressed the importance of catecholamines (Bunny and Davis, 1965; Schildkraut, 1965) whereas others have emphasized 5-HT (Lapin and Oxenkrug, 1969; Coppen et al, 1972). The monoamine deficiency hypothesis was further based on the acute action of antidepressant drugs. Classical tricyclic antidepressants were shown to inhibit the reuptake of NA and 5-HT from the synaptic cleft and thus to cause an increased availability of the neurotransmitter in the synaptic cleft and hence stimulation of neurotransmission.

There were however drugs whose actions could not be explained by the monoamine deficiency hypothesis. These drugs included iprindole and mianserin which did not appear to alter either the re-

uptake or metabolism of NA (Zis and Goodwin, 1979; Goodlet et al, 1977) but were effective antidepressants. Furthermore, known effective amine uptake inhibitors such as amphetamine (Overall et al, 1962) and cocaine (Post et al, 1974) did not appear to be useful in the treatment of depression. The time-course of the acute drug effects on amine availability was also not consistent with that of clinical improvement. Blockade of neurotransmitter reuptake and MAO inhibition of amine catabolism occur within minutes to hours after a single dose of the drug, yet clinical response to these agents usually requires two or more weeks to become evident (Oswald et al, 1972). The inhibition of NA reuptake processes and metabolism by tricyclic antidepressants and MAO inhibitors therefore did not adequately explain their clinical effectiveness.

Complex presynaptic and postsynaptic monoamine receptor changes have been shown to occur in animals after long-term administration of antidepressant drugs. In 1977, Banerjee et al reported that long-term tricyclic antidepressant administration reduced the density of β-adrenoceptors in homogenates of whole rat brain. A number of studies have replicated this finding in several brain areas and with a variety of antidepressants (Sellinger et al, 1978; Bergstrom and Kellar, 1979). In addition it has been reported that chronic administration of antidepressant drugs causes a decrease in NA stimulated cAMP accumulation in rat brain (Sulser et al, 1978). Since β-adrenoceptors are closely coupled to adenylate cyclase, (Limbird, 1981), this decrease in cAMP accumulation was partially attributed to a decrease in β-adrenoceptor density. No change in the Kd was observed (Pandey and Davis, 1983). Long term antidepressant treatment was also found to

decrease 5-HT_2 receptor density in rat cerebral cortex (Peroutka and Snyder, 1979, 1980). This apparent down-regulation of β-adrenoceptors and 5-HT receptors gave rise to a second hypothesis postulating monoaminergic hyperfunction as the primary defect in depression with subsequent down-regulation of post-synaptic monoamine receptors (Sulser, 1979) occurring after antidepressant treatment.

In 1984, Siever and Uhde suggested that adult depressed patients may vary along a spectrum of dysregulation of noradrenergic activity. Particularly highly anxious depressed patients were suggested to be characterized by increased presynaptic output of NA associated with depressed receptor responsiveness. This hypothesis was also based on the finding that many unipolar depressed patients have increased NA or MHPG in their urine, plasma and CSF and that these increases are often correlated with the levels of anxiety of the patients (Post et al, 1978; Uhde et al, 1982 and Lake et al, 1982). In contrast, decreased presynaptic noradrenergic activity has been observed primarily in bipolar patients (Siever and Uhde, 1984). Schildkraut et al (1978a; 1978b) also observed increased excretion of urinary MHPG in unipolar depressives and decreased excretion of urinary MHPG in bipolar depressives.

The biochemical model of childhood depression has scarcely been explored (Kashani et al, 1981). The pioneer work of Cytryn et al (1974) comprised one of the first biological studies of childhood depression. These authors concluded that changes in the

excretion of urinary metabolites do occur in affectively disturbed children and that these changes are more pronounced in children with chronic affective disorder. However, these biochemical differences, especially in MHPG were not consistent and tended to vary with age. The variation of MHPG with age was also observed by Shekim et al (1977, 1978) In 1979, Mc Knew and Cytryn investigated urinary excretion of MHPG, NA, and 4-hydroxy-3-methoxymandelic acid (VMA) in 9 children with chronic depressive reaction and 18 normal control subjects. The depressed children excreted significantly less MHPG than the control subjects, but there were no significant differences in NA or VMA excretion.

Tricyclic antidepressants have been found to be effective in treatment of children and adolescents with major depressive disorder. These drugs include imipramine and desmethylimipramine. Several studies showed that an apparent relationship exists between plasma drug concentration and response. (Puig-Antich et al, 1979; Weller et al, 1983a, 1983b; Petti and Conners, 1983). These authors reported drug plasma levels between 125 - 225 ng/ml to be desirable, since recovery of the young depressed patient occurred within six weeks and no side effects were observed. The lower limit described above for total tricyclic antidepressant plasma concentration (125 ng/ml) is similar to the minimum effective concentration (120ng/ml) reported for adults (Gram et al, 1976).

Although biochemical research regarding the aetiology of depression in childhood needs much more investigation, it appears that the underlying mechanisms may be similar in children and

adults with major depressive disorder. Studies aimed at identifying possible biological markers for the disorder, could therefore be of great benefit.

CHAPTER 3

RECEPTOR BINDING IN NON-NEURAL TISSUE

3.1 PLATELETS

Every cubic millimetre of blood contains between 200,000 and 400,000 platelets, which are smooth, roughly disc-shaped cells, measuring approximately 1-2μ in diameter. Blood platelets are produced in the bone marrow by fragmentation of large cells known as megakaryocytes. Under normal conditions, platelets circulate in the blood for seven to ten days and do not adhere to each other or to normal vascular endothelial surfaces.

Platelets are non-nucleated cells which do not contain DNA, but do contain a small amount of RNA and structures resembling ribosomes. Platelets possess little ability to synthesize proteins, but they do, however, have an active metabolism which takes place both in the cytoplasm and in the mitochondria. The active metabolism supplies the energy which is required for platelet function. In addition to the mitochondria and small accumulations of glycogen, several types of granules are visible in the platelet cytoplasm. Dense granules appear in small numbers in human platelets and contain a concentrated mixture of serotonin, calcium and two adenine nucleotides; adenosine diphosphate (ADP) and ATP.

Under normal conditions, platelets do not aggregate or adhere to vascular endothelium. They can, however, adhere to non-endothelial surfaces, aggregate in response to various stimuli and

release certain substances such as ADP and Thromboxane A2 which cause further platelet aggregation. Platelets can also accelerate the process of blood coagulation. Various substances released from platelets, such as thrombin, 5-HT, histamine, prostaglandins, permeability factors and mitogens mediate other biological reactions (Lindberg and Nilsson, 1984). Stimulation of human platelets by adrenaline and NA causes both inhibition of adenylate cyclase (Mills, 1975) and induction of aggregation (O'Brien, 1964). Although both these responses are mediated by α_2-adrenoceptors (Grant and Scrutton, 1979; Hsu et al, 1979) the evidence which is now available suggests that the aggregatory response is not initiated in resting platelets by a decrease in platelet cAMP concentration. Thus addition of adrenaline has no detectable effect on the cAMP content of the resting platelet, although it inhibits the increase in the level of this second messenger induced by prostaglandin E1 (Haslam, 1975). The aggregatory response to adrenaline cannot be enhanced by addition of an inhibitor of adenylate cyclase (Haslam et al, 1978).

5-HT activates blood platelets of various species including humans. In contrast to cat, pig and sheep where platelets respond to 5-HT with irreversible aggregation (De Clerck and Herman, 1983), human blood platelets respond to 5-HT mainly with a shape change and reversible aggregation only. However, depending on the concentration and the time interval between its addition and that of another agonist, 5-HT amplifies the human platelet aggregation induced by ADP, collagen, adrenaline and NA. The monoamine itself induces strong aggregation of platelets presensitised with NA, lysolecithin, or Thrombofax. Prolonged exposure of platelets to

5-HT results in transient tachyphylaxis. Pharmacodissection and receptor binding studies suggest the presence of functional receptors, possibly of the 5-HT_2 (S2) type and different from the active uptake sites of the monoamine by the platelets. As a modulator of platelet reactions, 5-HT may be involved in secondary platelet aggregation, hemostasis and thrombus formation (De Clerck and Herman, 1983).

α_2-Adrenoceptors

Recent investigations into abnormalities in noradrenergic function in the affective disorders in adults have examined possible alterations in noradrenergic receptor sensitivity in these disorders (Garcia-Sevilla et al, 1981a; Siever et al, 1981a; Charney et al, 1982; Siever et al, 1984). One approach has been the study of adrenergic receptors on blood elements. While central adrenergic receptors are more likely to play a role in the regulation of mood than comparable peripheral receptors, alterations in peripheral adrenergic receptor systems may still represent legitimate markers for psychiatric disorders and might provide clues as to possible abnormalities in central adrenergic receptor function. α_2-Adrenoceptors are located on platelets and their number can be determined by the amount of specific binding of radiolabelled α_2-adrenergic agonists or antagonists to the platelet membrane (Kafka et al, 1977). Studies of platelet α_2-adrenoceptors in adults have reported conflicting results and are reviewed by Carstens et al (1986 a). The discrepancy between the results reported from different laboratories is thought to lie in the different labelled ligands used to determine α_2-

adrenoceptor binding.

The α_2-adrenergic receptor appears to exist in high and low affinity conformations (U'Prichard et al, 1982; Bylund and U'Prichard, 1983). ^{3}H-Clonidine used as a ligand by Garcia-Sevilla et al (1981b), preferentially labels the high affinity sites (U'Prichard et al, 1982; Bylund and U'Prichard, 1983). ^{3}H-Yohimbine used by Daiguji et al (1981a) and Stahl et al (1983) and ^{3}H-rauwolscine, used by Pimoule et al (1983), label both high and low affinity sites (Bylund and U'Prichard, 1983). ^{3}H-DHE also labels both high and low affinity sites (Bylund and U'Prichard, 1983). Since the high affinity sites seem to be the physiologically active sites (Bylund and U'Prichard, 1983), measurement of these binding sites with ^{3}H-clonidine would seem most appropriate. To our knowledge, no such study has been performed in children and adolescents with major depressive disorder.

Imipramine Binding Sites

The use of the platelet as a model for central 5-HT activity is based largely on similarities in the uptake, storage and metabolism of 5-HT in these two tissues (Sneddon, 1973; Stahl, 1977; Pletscher, 1978). Following the identification of a saturable, high-affinity binding site for ^{3}H-imipramine in rat brain (Raisman et al, 1979, 1980) and human platelets (Langer et al, 1980a; Paul et al, 1980), inhibition studies demonstrated a close correspondence between the affinity of compounds for the ^{3}H-imipramine binding site and for the active uptake of 5-HT (Paul et al, 1981a). The relative potencies of a series of tricyclic antidepressants in inhibiting serotonin uptake correlated

well with inhibition of high affinity ^{3}H-imipramine binding in platelets (Paul et al, 1981a). The potencies of the various antidepressants in inhibiting both uptake and binding were also within the same (nanomolar) concentration range. Studies using a large series of antidepressants have revealed an identical pharmacological profile for the ^{3}H-imipramine binding sites in human (Rehavi et al, 1980) as well as cat brain and platelets (Briley et al, 1982). When imipramine was administered chronically to cats, it was possible to measure a concomitant decrease in the Bmax values of ^{3}H-imipramine binding in the hypothalamus and platelets. The K_D of ^{3}H-imipramine binding was significantly increased in the hypothalamus, but unchanged in platelets. This discrepancy was ascribed to unlabelled imipramine in the hypothalamic membrane preparation, since the preparation of platelet membranes involved a more thorough washing procedure. Studies of ^{3}H-imipramine binding in human brain tissue suggest a close similarity to binding characteristics in the platelet although the affinity of ^{3}H-imipramine binding in brain appears to be slightly lower (Rehavi et al, 1980; Langer et al, 1981c).

Clearly, there are a number of limitations to the platelet as a neuronal model for receptor binding studies, but recent data suggest that the platelet model may prove useful as a biochemical marker and subsequently as a tool for elucidation of biochemical dysfunction in affective disorders.

3.2 LYMPHOCYTES

In blood smears, lymphocytes are recognized as a reasonably homogenous population of mononuclear cells with a small amount of

cytoplasm containing a few granules. Through the analysis of antigens and receptors on the surface of these cells and their responses to culture with various antigenic and mitogenic stimuli in vitro, it has been established that there are two main types of circulating lymphocytes namely T and B cells. Characteristically, T cells form rosettes when incubated with sheep erythrocytes whereas B cells do not. This is the principal manner in which these populations can be distinguished. Approximately 80 percent of blood lymphocytes are T cells and 12 to 15 percent are B cells. The remaining small percentage of lymphocytes lack the characteristic surface receptors of T and B cells and are called "null" cells. Both T and B cells originate from stem cells of hematopoietic tissues (Dale, 1983). The absolute numbers of T and B cells are altered by many disease states.

T cells

Approximately 70 to 80 percent of normal peripheral blood lymphocytes and 90 percent of lymphocytes in thoracic duct fluid are T cells. They circulate primarily as long lived small lymphocytes. These cells are the principal lymphocytes in the deep cortical areas of lymph nodes and in the periarteriolar areas of the splenic white pulp. The T cells are the main effectors of cell-mediated immunity and also are involved as helper or suppressor cells in modulating the immune response. T cells possess cell surface antigens that are identified by a series of monoclonal antibodies to surface antigens. T cells with surface antigens $T1^+$, $T3^+$, $T4^+$ function as helper inducer cells and those

with $T1^+$, $T3^+$, $T5^+$ as cytotoxic-suppressor cells. These cells migrate to various lymphoid tissues such as spleen, lymph nodes and bone marrow (Gilliland, 1983).

B cells

B cells represent approximately 12 to 15 percent of the normal peripheral blood lymphocytes, 50 percent of the splenic lymphocytes and 75 percent of the lymphocytes in the bone marrow in normal individuals. They are the principal cells in the cortical germinal centers and medullary cords of lymph nodes. Their main role is the production of antibodies. The B cells carry membrane-bound immunoglobulins as demonstrated by immunofluorescence staining with anti-immuno-globulin antiserum. The main immunoglobulin classes on the surface of peripheral blood B cells are IgM and IgD (Gilliland, 1983).

β-adrenoceptors

Lymphocytes have been employed as a model for investigations of β-adrenoceptor activity, since these cells exhibit adenylate cyclase activity which responds to catechoalmines with a typical β-adrenergic specificity (Bourne and Melman, 1971; Williams et al, 1976). Brodde et al (1981), using ^{125}I-cyanopindolol, identified the β-adrenoceptors on human lymphocytes as a homogeneous population of the β_2-subtype.

Several groups (Pandey et al, 1979; Kronfol et al, 1983; Wright et al, 1984) have investigated lymphocyte β-adrenoceptor responses in psychiatric disorders in adults. For example, it was

shown (Pandey et al, 1979) that in affective illness, the β-adrenoceptor-coupled adenylate cyclase activity was impaired. No such study has been performed in children and adolescents.

3.3 RECEPTOR BINDING STUDIES

The vast majority of receptor-labelling studies involve the binding of a radioactive form of either the neurotransmitter/hormone itself or a biologically active analog (agonist), or an appropriate antagonist to membrane preparations of target tissues (Bennett, 1978). The first and most critical step in any receptor study is to acquire a radiolabelled ligand of sufficient radiochemical specific activity, purity, stability and biological activity. In addition most receptor sites have equilibrium dissociation constants for ligands in the nanomolar range and below, and therefore the particular radioisotope utilized must have a specific activity sufficient to allow accurate measurement of low concentrations. The most commonly used isotope is tritium.

Receptor-binding studies usually follow kinetics very similar to those of classic enzyme-substrate interactions. For reversible ligand-receptor interactions where (R) = concentration of unoccupied receptor sites, (L) = concentration of free ligand, (RL) = concentration of receptor-ligand complex,

$$a\,(R) + b\,(L) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} c\,(RL) \qquad 1$$

describes a general reversible binding phenomenon with a, b and c representing the stoichiometry of the reaction. At equilibrium,

or "steady state", the rate of the forward reaction equals the rate of the reverse reaction.

$$k_1 (R)^a (L)^b = k_{-1} (RL)^c \qquad 2$$

The equilibrium binding constant may then be defined either as an association binding constant (K_A).

$$K_A = \frac{k_1}{k_{-1}} = \frac{(RL)^c}{(R)^a(L)^b} \qquad 3$$

or as a dissociation binding constant (K_D)

$$K_D = \frac{k_{-1}}{k_1} = \frac{(R)^a(L)^b}{(RL)^c} \qquad 4$$

Thus experimental determination of equilibrium binding affinity constants for reversible reactions requires that experiments be performed under steady-state conditions.

Another property of ligand-receptor interactions is saturability; that is, only a finite number of specific receptor sites exist per unit tissue. This maximum number of specific receptor sites is usually disignated as B_{max}.

$$(RL) + (R) = B_{max}$$

multiply by (L)

$$(RL)(L) + (R)(L) = B_{max} (L)$$

$$(RL)(L) + \frac{(RL)}{(RL)} (R)(L) = B_{max} (L)$$

substitute Eq (4) with a = b = c = 1

$$(RL)(L) + (RL)\,K_D = B_{max}\,(L)$$

$$(RL)\,[(L) + K_D] = B_{max}\,(L)$$

$$(RL) = \frac{B_{max}\,(L)}{(L) + K_D} \qquad 5$$

which is the classic law of mass action for enzyme-substrate interactions adapted to receptor-ligand interactions. If we now define RL as bound ligand = B, and L as free ligand = F, from Eq (5)

$$B = \frac{B_{max}\,F}{F + K_D}$$

$$BF + BK_D = B_{max}\,F$$

Dividing by F

$$B + \frac{B}{F}\,K_D = Bmax$$

transferring fields

$$B/F = \frac{B_{max} - B}{K_D}$$

which is the Scatchard (1949) equation. Thus knowing the concentrations of ligand bound and free at equilibrium allows the determination of both the equilibrium binding constant (K_D) and the maximum number of binding sites (B_{max}).

The next step is to choose an appropriate separation technique. The choice of a separation technique depends on whether the receptor under study is in a particulate or a soluble form. For particulate preparations the choice is usually between some form of centrifugation or filtration. Filtration techniques, which are unsurpassed for speed and efficiency, are the most widely used in particulate receptor studies. Total elapsed time for filtration and washing (separation time) is in the order of approximately 15 to 20 sec. per sample. Radiolabelled chemicals used as ligands in receptor-binding studies possess remarkable abilities to bind non-specifically to both biological and non-biological substances (Hollenberg and Cuatrecasas, 1975). Care and caution are thus needed in establishing the criteria for non-specific binding in a receptor-ligand assay. In general, specific binding is taken as the difference between total binding and binding that occurs in the presence of an excess concentration of unlabelled ligand. Many drugs with structures markedly different from that of the radioligand used in receptor binding studies, interact potently at the receptor site, and a 1000-fold excess concentration of a suitable unlabelled drug can be used to determine non-specific radioligand binding. When possible it is always best to use a displacing ligand that is chemically different from the radioligand to increase the probability of obtaining receptor-specific binding.

Scatchard Plots

Determination of the K_d and B_{max} for a given radioligand and

tissue receptor, involves incubating various concentrations of the radioligand with a fixed concentration of tissue, measuring the amount of ligand bound, and analyzing the data according to the Scatchard (1949) equation. With radioligand saturation studies the concentration of radio-activity is increased in the incubation medium while the specific activity of the radioligand is held constant. The amount of radioligand specifically bound at each radioligand concentration is then determined, radioligand bound is converted to moles of radioligand bound per unit weight of tissue or tissue protein.

The results are plotted as B/F (y-axis) versus B (x-axis) calculated for each radioligand concentration. After the line of best fit for the data is obtained, the K_d is determined as the negative reciprocal of the slope and the B_{max} is estimated by the abscissa intercept of the line.

An example of a Scatchard plot will be shown in Section 5.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

All chemicals and solvents used were of the purest grade commercially available.

Instagel and counting vials were obtained from Packard Instrument Co., Inc., Illinois, U.S.A. and bovine serum albumin from Calbiochem., San Diego California.

Noradrenaline (L-arterenol bitartrate) and L-isoproterenol HCl were purchased from Sigma Chemical Co., St. Louis, U.S.A.

Chlorimipramine was kindly donated by Ciba-Geigy (Pty) Ltd Kempton Park. S.A. and Lymphoprep TM purchased from Nyegaard and Co. AS, Oslo, Norway (density : 1.077 ± 0.001 g/ml; 20°C) SABAX wing infusing sets were obtained from SABAX Pty Ltd Johannesburg and Falcon 2099 tubes from Becton Dickinson and Co. U.S.A. Filters were supplied by Schliecher and Schüll, W- Germany.

Radiochemicals used were: p-aminoclonidine [3,5 - ^{3}H] - (40,0 Ci/mmol), imipramine HCl [benzene ring - $^{3}H(N)$] (51.3 Ci/mmol) and dihydroalprenolol HCl, Levo - [propyl-1,2,3 - ^{3}H] (35.6 Ci/mmol) and were obtained from New-England Nucleur, Boston Massachusetts.

4.2 Patient Selection

All patients were examined by the psychiatrist and treated in the Department of Child Psychiatry, Tygerberg Hospital. Each child and parent were assessed with the Interview Schedule for Children (ISC, Kovacs, 1983). The ISC symptoms and signs were evaluated towards a particular diagnosis, only if they met the operationally defined level of clinical severity, for example a rating of 5 or above on a 0- to 8-point scale (Kovacs, 1983). The diagnosis of major depressive disorder was made according to the DSM III (1980) criteria. All children presenting with the symptom of depression or a suicide attempt were evaluated. None of these children had received prior antidepressant treatment. The exclusion criteria were an organic deficit, mental retardation and childhood schizophrenia or autism. A volunteer control group of normal, healthy school-going children was also evaluated.

Patients and controls were kept drug free for three weeks prior to the receptor binding studies. Blood samples were collected between 08h00 and 08h30 to avoid possible circadian variation.

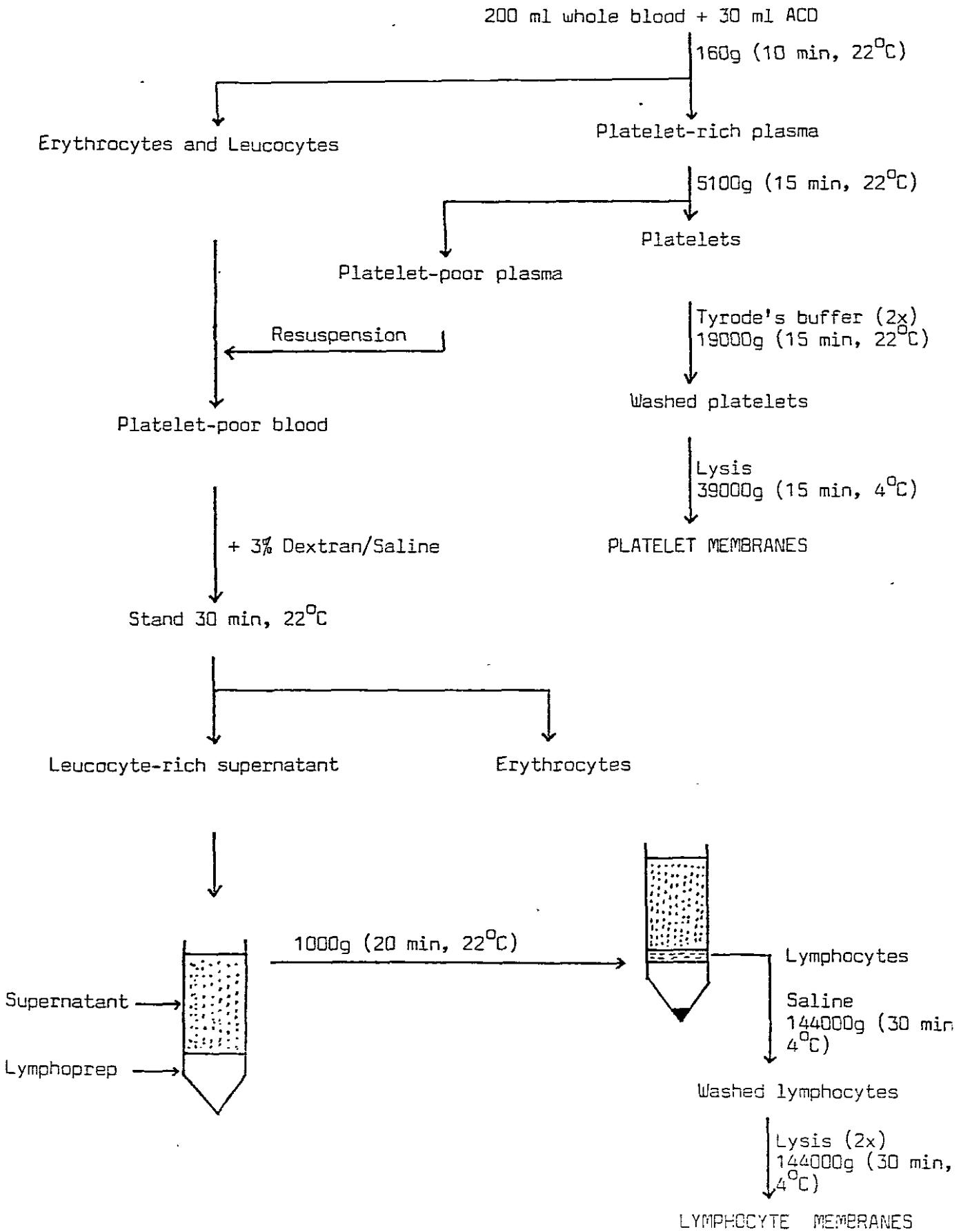
4.3 PREPARATION OF PLATELET MEMBRANES

The method used for the preparation of platelet membranes was essentially that of Garcia-Sevilla et al (1981b).

Initially a two hundred ml sample of blood was collected using two single 20-ml plastic syringes consecutively connected to a no. 19 gauge SABAX wing infusion set and transferred to a plastic beaker containing 30-ml acid-citrate-dextrose (ACD) solution as anticoagulant (National Institutes of Health formula A: 0.8% citric acid; C: 2.2% trisodium citrate; D: 2.4% dextrose). This procedure proved inadequate, since the blood frequently coagulated. In order to prevent blood coagulation, the procedure was modified (Fig. 4.1) to allow the blood to be collected in ten 20-ml plastic syringes each containing 3-ml of ACD solution as anticoagulant.

The blood was centrifuged at 160 x g for 10 min. (22°C). The resulting platelet-rich plasma was titrated to pH 6.5 with ACD solution and centrifuged at 5100 x g for 15 min. (22°C) to sediment the platelets. The platelet pellet was washed and homogenised twice with 10 ml of Tyrode's buffer solution (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 0.36 mM; $MgCl_2$, 0.1 mM; $NaHCO_3$, 12 mM and dextrose, 0.56 mM; pH 8.0) and centrifuged again at 19000 x g for 15 min. The washed pellet was lysed by homogenisation in 10- ml of ice-cold hypotonic buffer (Tris - EDTA, 5 mM; pH 7.5) and divided into two tubes. After centrifugation at 39000 x g for 15 min. (4°C), the one platelet membrane pellet was resuspended in incubation buffer containing 50 mM Tris HCl, 10 mM $MgCl_2$ pH 7.7 (22°C), and the other in an incu-

FIG. 4.1 Preparation of platelet and lymphocyte membranes



bation buffer containing 50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, pH 7.4 (4°C). Electron microscopy was employed to establish the presence of intact platelets (Fig. 4.2).

4.4 PREPARATION OF LYMPHOCYTE MEMBRANES

A modification of the method of Davies and Lefkowitz (1980) was used to prepare lymphocyte membranes (Fig. 4.1). The original method could not be used for whole blood, since platelet contamination of the band containing lymphocytes could not be avoided.

After separation of the platelets from the blood sample, 3% dextran (MW 500,000) in saline was added (1:3, v/v) to sediment erythrocytes. The mixture was stirred slowly for 15 sec. and left for 30 min. at room temperature. The supernatant plasma-dextran solution was layered onto 15 ml Lymphoprep in 50 ml plastic conical tubes and centrifuged at 1000 x g for 20 min. (22°C). The yellow top layer was removed and discarded down to within 3 mm of the interface layer containing the lymphocytes. This layer was aspirated off and diluted with saline (1:1, v/v). Cell counts were performed on whole blood and on each of the fractions obtained after centrifugation in order to establish the relative recovery of the different fractions (Table 4.1).

After centrifugation of the diluted interface layer at 144 000 x g for 30 min. (4°C), the lymphocytes were lysed in ice-cold water, using a Polytron homogeniser. The lymphocyte membranes were spun down at 144 000 x g (30 min.; 4°C) and lysis repeated. The final pellet was resuspended in a buffer containing

TABLE 4.1

COULTER COUNTS OF THE DIFFERENT FRACTIONS OBTAINED DURING THE PREPARATION OF PLATELETS AND LYMPHOCYTES FROM WHOLE BLOOD

FRACTION	PLATELETS ($\times 10^9/1$)	LYMPHOCYTES (%)
Whole blood	303	36
Platelet-rich plasma	248	0
Platelet-poor plasma	7	0
Plasma/Dextran before centrifugation	140	34
Plasma/Dextran after centrifugation	6	0
Lymphocyte layer	0	82

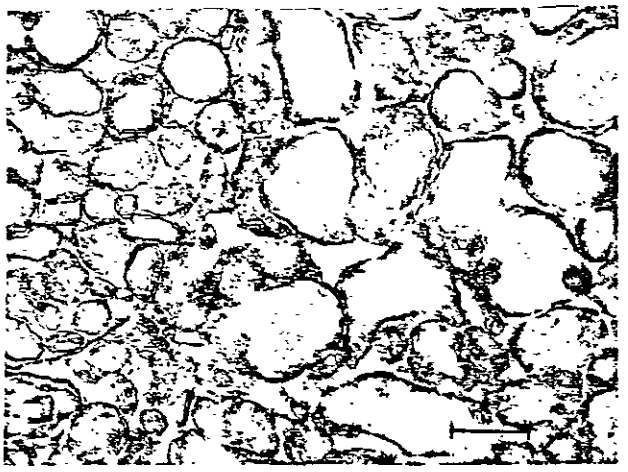


Fig 4.2 An electron micrograph of platelet membranes in incubation buffer (X 8000). Scale bar = 1000 nm.

50 mM Tris HCl and 10 mM $MgCl_2$, pH 7.7 (22°C).

Electron microscopy was employed to establish the presence of intact lymphocytes (Fig. 4.3).

4.5 PROTEIN DETERMINATION

The protein content of samples was assayed by the colorimetric method of Lowry et al (1951), as modified by Miller (1959). The phenolic-OH groups of amino acid side chains are estimated by the Folin-Ciocalteus colour reaction. Crystalline bovine serum albumin (Merck, 99% pure) was used as standard.

A standard curve was initially constructed over the linear range (0 - 100µg protein), but a single 20µg protein/20µl standard was routinely used for the calculation of protein concentration. One ml of a freshly prepared solution of 10% Na_2CO_3 in 0.5 M NaOH containing 1% potassium tartrate and 5% $CuSO_4$ (10:1:0,1) was added to sample or standard (20µl diluted to 1 ml) and allowed to stand for 10 min. at room temperature. Three ml of Folin-Ciocalteus phenol reagent (1:10 dilution with water) was added to the tubes, heated for 10 min. at 50°C, cooled to room temperature and the absorbance read at 650 nm in a Gilford Stasar III spectrophotometer. To correct for the effects of the incubation buffer used as suspending medium for the membrane preparations, the same volume (20µl) of buffer was added to blank tubes and treated as the samples.

4.6 α_2-ADRENOCEPTOR BINDING ASSAY

The assay used for measuring α_2-adrenoceptor levels on platelet

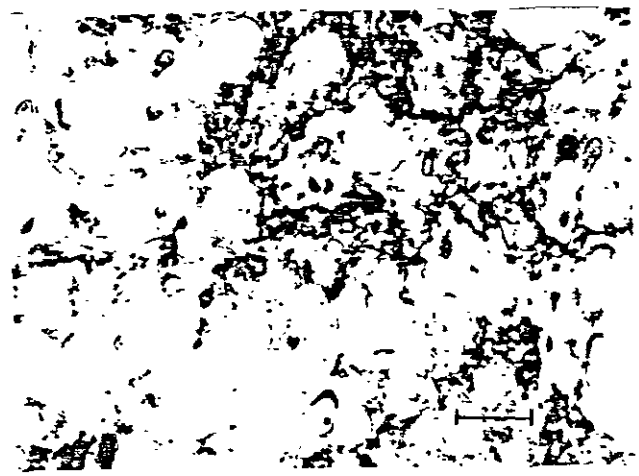


Fig 4.3 An electron micrograph of lymphocyte membranes in incubation buffer (X 8000). Scale bar = 1000 nm.

membranes was that described by Garcia-Sevilla et al (1981b).

The assay was initially optimised according to pH and Mg^{2+} requirement. Temperature, incubation time, protein concentration and NA concentration required for the determination of non-specific binding were also characterised.

Aliquots of platelet membrane preparations (100µg protein) were incubated with 4 different concentrations of the radioactively labelled α_2-adrenoceptor agonist, p-aminoclonidine (p-aminoclonidine 3,5 - ^3H, specific radioactivity 40 Ci/mmol) ranging from 1 nM, to 4 nM, in buffer (50 mM Tris HCl, 10 mM $MgCl_2$, pH 7.7) to obtain an estimate of total binding. Non-specific binding was determined by incubating platelet membranes with ^3H-p-aminoclonidine in the presence of 100µM NA. Specific binding was defined as the difference between the total and non-specific binding.

The binding reaction was allowed to proceed for 25 min. at 25°C and was stopped by the addition of excess (5 ml) incubation buffer and filtration through Whatman GF/C glass microfibre filters in a 10 - well filtration apparatus. After washing the filters three times with 5 ml incubation buffer, the filters were removed from the filtration apparatus, air-dried and placed in counting vials. Ten ml of Instagel was added and the radioactivity on the filters determined in a Beckman LS 9000 liquid scintillation counter. A series of quenched tritium standards was used to calibrate the LS 9000 in order to correct for quenching present in the samples and to convert the measured counts per minute to actual degradations per minute (Section 5.1). Samples were counted at an efficiency of 41%.

4.7 IMIPRAMINE BINDING ASSAY

The method used for measuring imipramine binding sites on platelet membranes was that of Paul et al (1981a).

Initially the assay was optimised according to pH, Mg^{2+} requirement, $Na^{+}$and K^{+} requirement, incubation time, protein concentration and the chlorimipramine, serotonin or amitriptyline concentration required for the determination of non-specific binding. Aliquots of platelet membrane preparations (100µg protein) were incubated with 4 different concentrations of the radioactively labelled 5-HT reuptake site marker, imipramine (imipramine HCl [benzene ring - $^{3}H(N)$] specific radioactivity 51.3 Ci/mmol) over a concentration range of 1 nM to 6 nM in buffer (50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, pH 7.4). The binding reaction was allowed to proceed for 60 min. at 4°C, after which time it was terminated by the addition of excess (5 ml) ice-cold incubation buffer and filtration through Whatman GF-B glass microfibre filters in a 10 - well filtration apparatus. The filters were washed, air-dried and the radioactivity determined as described in section 4.6 Non-specific binding was determined by inclusion of 100µM chlorimipramine in the incubation buffer.

4.8 β-ADRENOCEPTOR BINDING ASSAY

The assay used for measuring the β-adrenoceptor binding parameters was that described by Davies and Lefkowitz (1980).

The assay was optimised according to pH and Mg^{2+} requirement. Temperature, incubation time, protein concentration and isoproterenol concentration required for the determination of non-spe-

cific binding were also characterised.

Aliquots of platelet membrane preparations (100µg protein) were incubated with 4 different concentrations of the β-adrenoceptor agonist DHA (dihydroalprenolol HCl, levo-(propyl-1,2,3-^{3}H-, specific activity 35.6 Ci/mmol) and 5 mM ascorbic acid over a concentration range of 0.5 nM to 3.0 nM in 50 mM Tris HCL, 10 mM $MgCl_2$ (pH 7.7 at 22°C). The binding reaction was allowed to proceed for 15 min. at 37°C and was terminated by the addition of excess (5 ml) ice-cold incubation buffer solution and filtration under vacuum through Whatman GF/C glass microfibre filters in a 10 - well filtration apparatus. The filters were washed, air-dried and the radioactivity determined as described in section 4.6.

Non-specific binding was determined by incubating lymphocyte membranes with ^{3}H-DHA in the presence of 5 mM isoproterenol. From the results obtained in the different binding assays (sections 4.6, 4.7 and 4.8), Scatchard plots were constructed to determine the K_d and B_{max} values (sections 5.1, 5.2 and 5.3).

CHAPTER 5

RESULTS

5.1 α_2-ADRENOCEPTOR BINDING TO PLATELET MEMBRANES

5.1.1 Characterisation of the ^{3}H-p-aminoclonidine binding assay

The method upon which the characterisation of ^{3}H-p-aminoclonidine binding to α_2-adrenoceptors on platelet membranes was based, was that of Garcia-Sevilla et al (1981b). Blood was obtained from adults, since difficulties were experienced in the availability of children for research purposes. Initially ^{3}H-clonidine was used, but since the specific activity of the labelled ligand was very low (about 22 Ci/mmol), ^{3}H-p-aminoclonidine was used instead (Rouot and Snyder, 1979). A concentration of 3nM ^{3}H-p-aminoclonidine was used to characterise the binding assay which is described in Section 4.6.

5.1.1.1 pH and Mg^{2+} requirement

α_2-Adrenoceptor binding was investigated over a pH range of 7.1 to 7.9 in the presence and absence of 10mM $MgCl_2$ in order to establish the pH optimum for the binding assay. Samples of platelet membranes (100µg protein) were incubated with ^{3}H-p-aminoclonidine (3nM) for 25 min. at 25°C. Nonspecific binding was determined by addition of 100µM NA as described in Section 4.6. Figure 5.1.1 shows the reaction to take

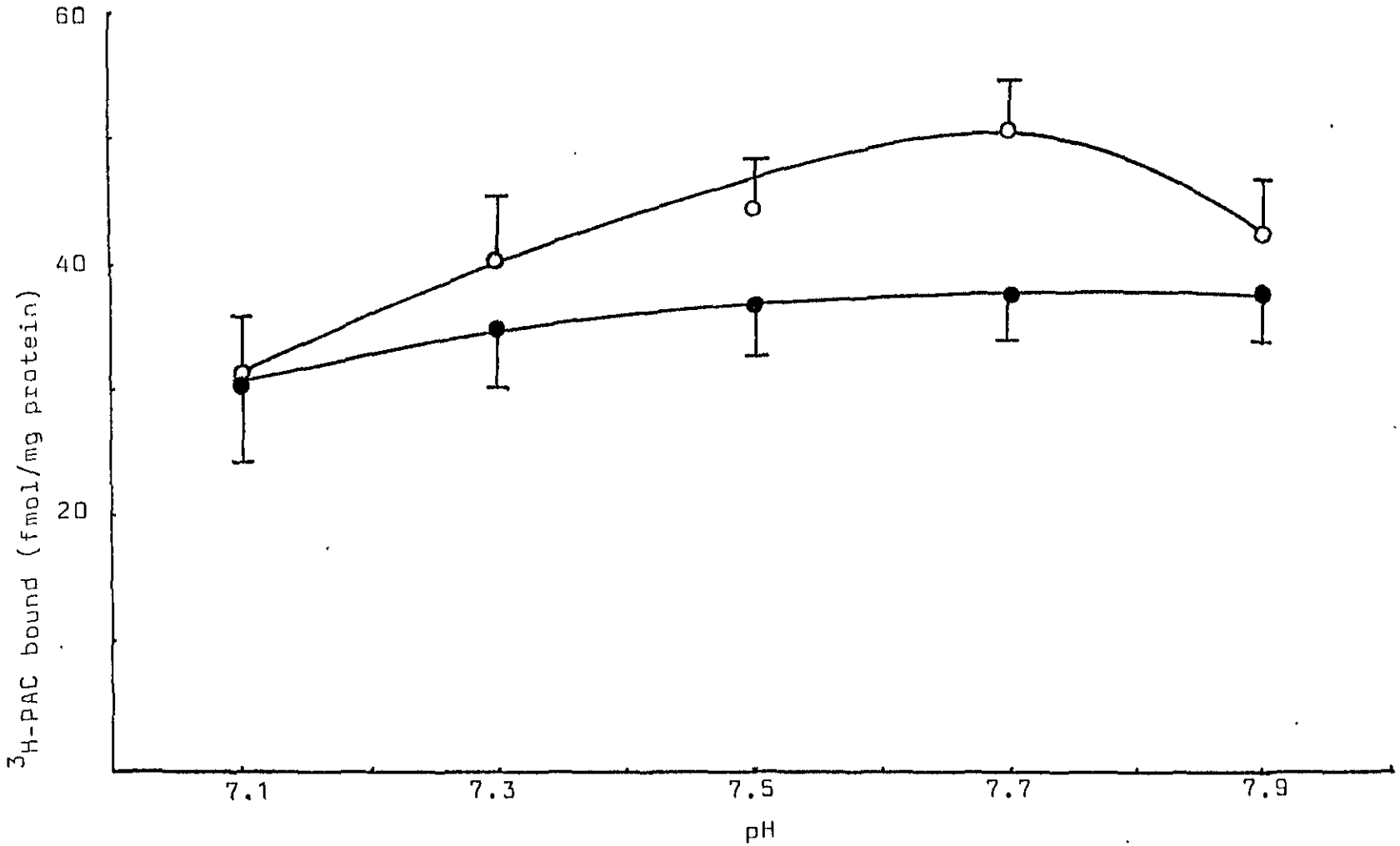


Fig 5.1.1 pH-Dependence of α_2-adrenoceptor binding on platelet membranes at 25°C. Results are expressed as the mean ± SD of the specific amount of ^{3}H-p-aminoclonidine (^{3}H-PAC) bound in the presence (—o—) and absence (—•—) of Mg^{2+}. (n = 12)

place optimally at pH 7.7 in the presence of Mg^{2+}.

5.1.1.2 Temperature

All binding reactions require an optimal temperature for maximum activity. Platelet membranes (100µg protein) were incubated with ^{3}H-p-aminoclonidine (3nM) in buffer (Tris HCl, 50 mM; $MgCl_2$ 10mM; pH 7.7) for 25 min. at 25°C. NA (100µM) was used to determine non-specific binding (Section 4.6). Maximum binding of ^{3}H-p-aminoclonidine was found to occur at 25°C (Fig. 5.1.2).

5.1.1.3 Time of incubation

Since most binding reactions show saturability within a specific time limit, it was essential to establish the time within which the specific binding of ^{3}H-p-aminoclonidine to platelet membranes reached saturation. Platelet membranes were incubated with the labelled ligand over time periods of 10 to 30 min. and it was found that saturation of specific binding was reached within 25 min. (fig. 5.1.3).

5.1.1.4 Non-specific displacer requirement

In order to determine non-specific binding, samples of platelet membranes were incubated with 3nM ^{3}H-p-aminoclonidine in the presence of either unlabelled clonidine or unlabelled NA, at final concentrations ranging from 0,1 to 100µM (fig. 5.1.4).

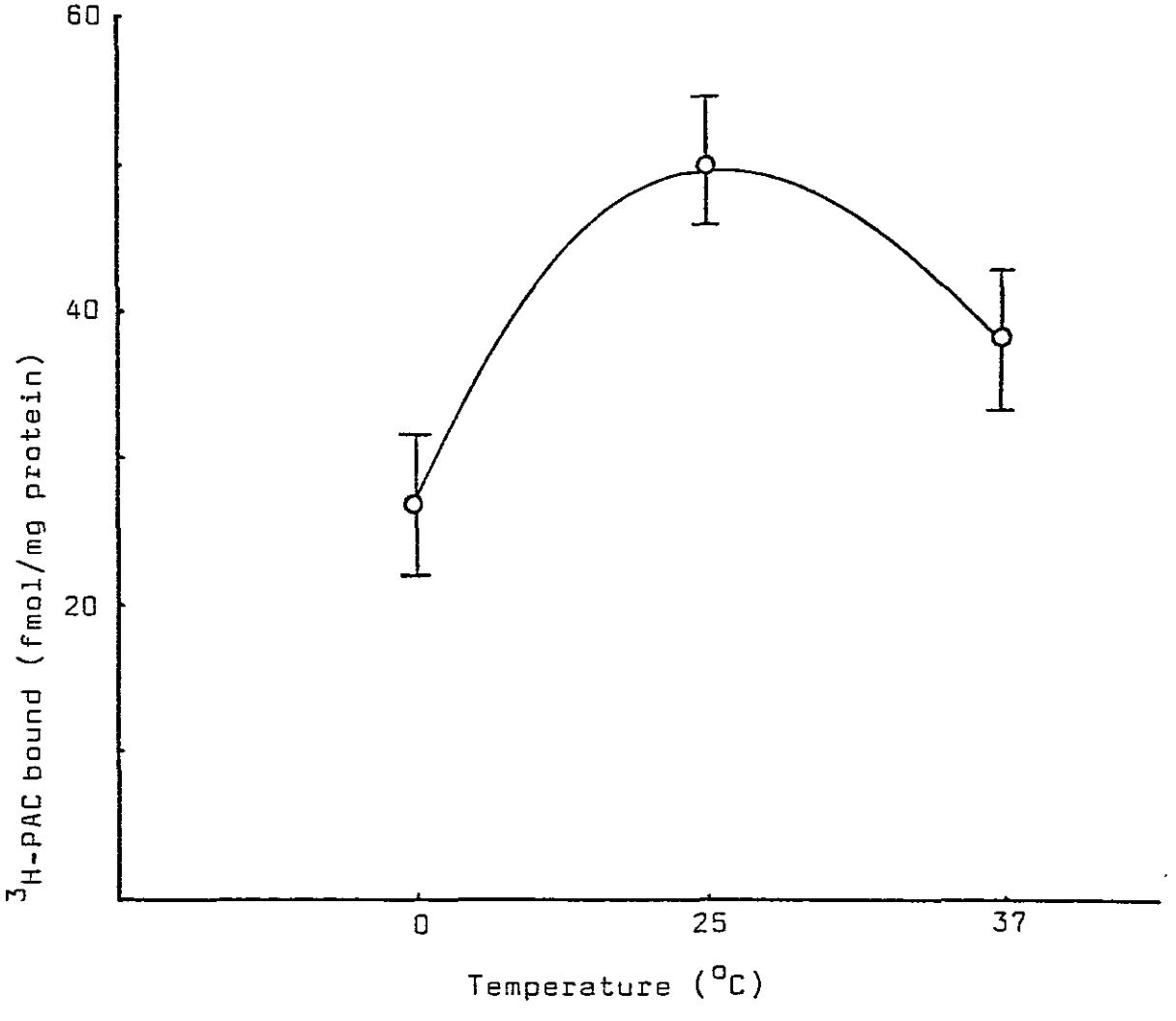


Fig 5.1.2 Temperature-dependence of α_2-adrenoceptor binding on platelet membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-p-aminoclonidine (^{3}H-PAC) bound. (n = 12)

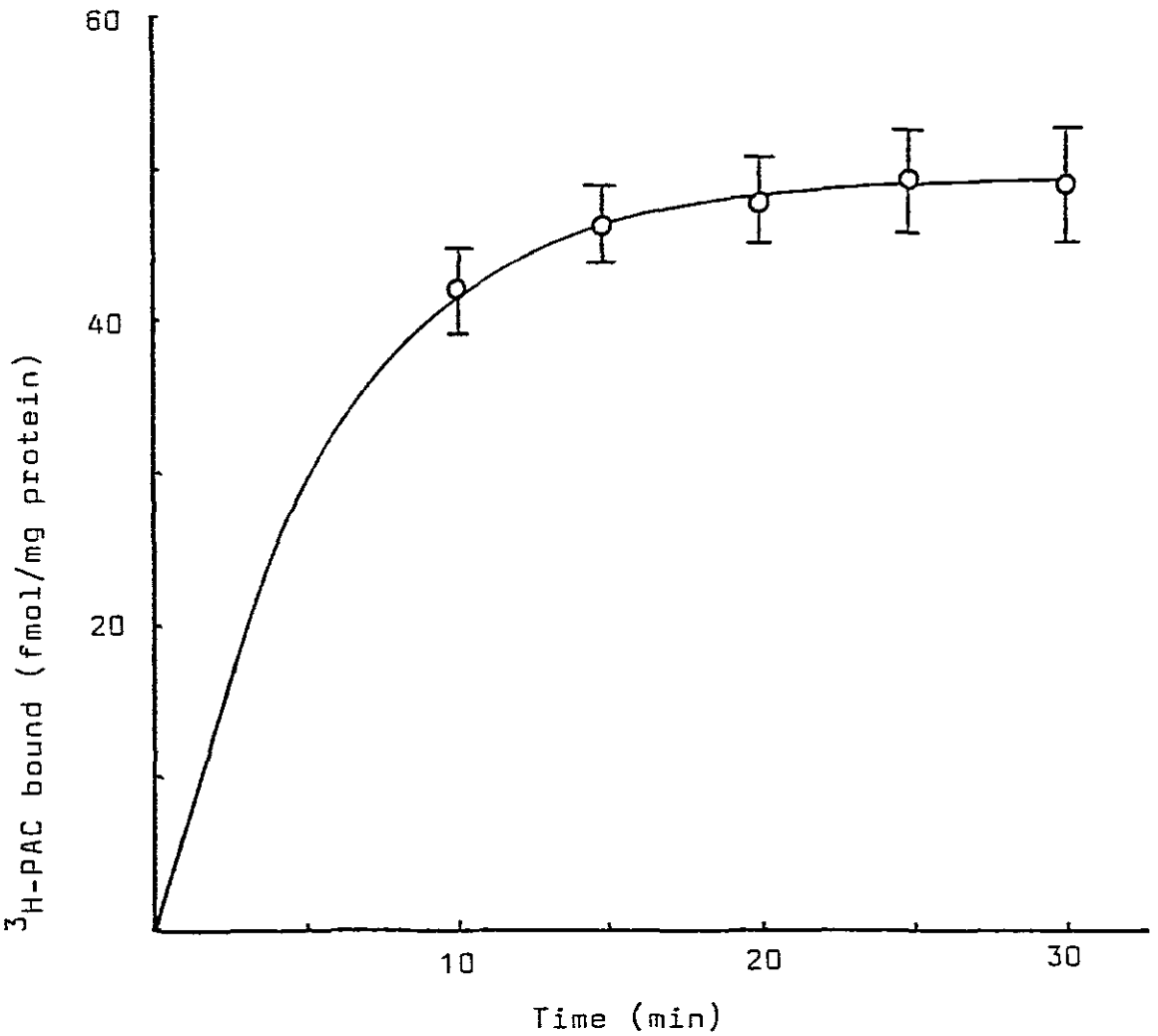


Fig 5.1.3 Time-course of incubation of ^{3}H-p-amino-clonidine with platelet membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-p-amino-clonidine (^{3}H-PAC) bound. (n = 11)

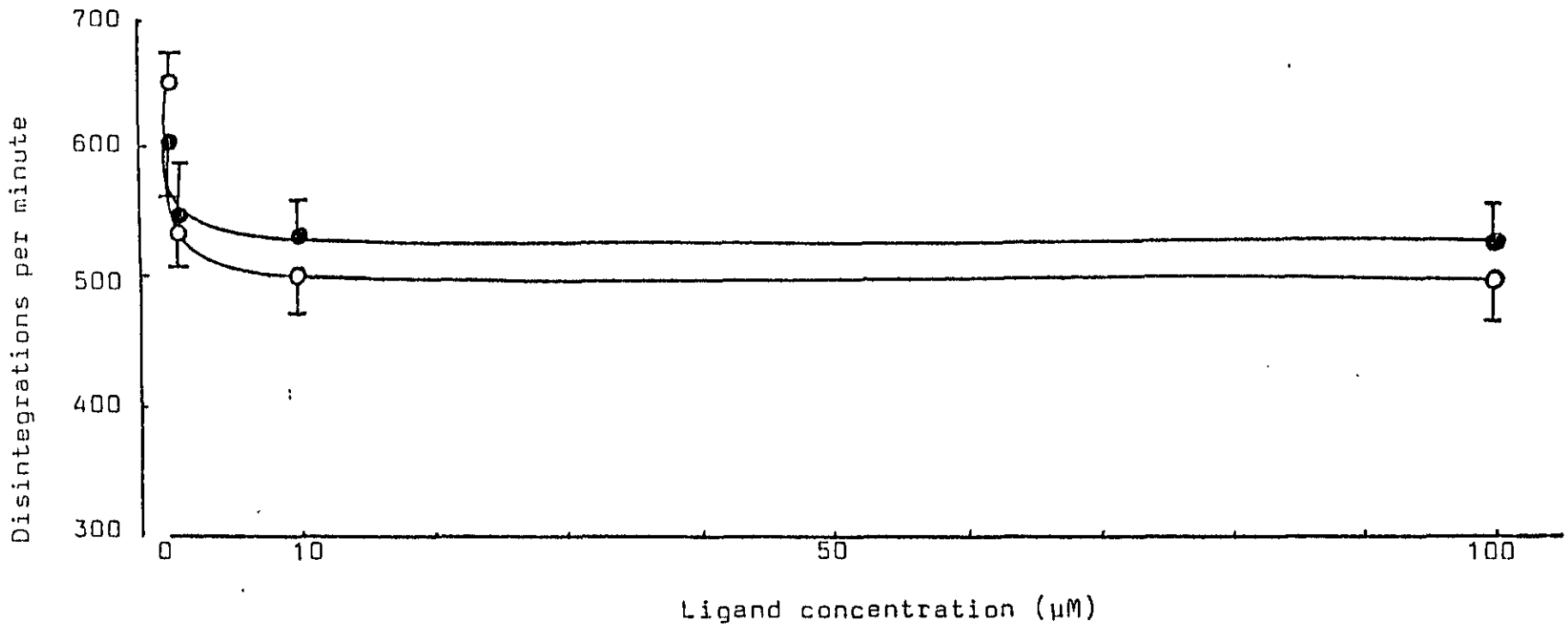


Fig 5.1.4 Displacement of ^{3}H-p-aminoclonidine binding from platelet membranes by clonidine (●) and NA (o). Results are expressed as the mean ± SD of the amount of radioactivity after displacement by the two unlabelled ligands. (n = 9)

NA and clonidine caused 50% and 46% displacement, respectively, at concentrations of 10 and 100μM. The latter concentration of NA was used in the present study.

5.1.1.5 Protein concentration

Platelet membranes were incubated with 3nM ^{3}H-p-aminoclonidine over a protein concentration range of 50 to 100μg protein per assay. Fig. 5.1.5 shows specific binding to be linear over this concentration range and 100μg protein was used in all subsequent studies.

5.1.6 Scatchard analysis of the binding data

Once the binding of ^{3}H-p-aminoclonidine to α_2-adrenoceptors on platelet membranes had been characterised, samples of platelet membranes (100μg protein) were incubated with ^{3}H-p-aminoclonidine over a concentration range of 1 to 10nM (Section 4.6, fig. 5.1.6). Scatchard analyses of the binding data, however, suggested the existence of two binding sites with different Kd and Bmax values (fig. 5.1.7). This finding was in agreement with results published by Garcia-Sevilla et al (1981b), using ^{3}H-clonidine. The present study was subsequently carried out, using four concentrations of ^{3}H-p-aminoclonidine, ranging from 1nM to 4nM.

5.1.2 ^{3}H-p-Aminoclonidine binding to platelet membranes of depressed children and controls

The aim of the present study was to investigate ^{3}H-p-amino-

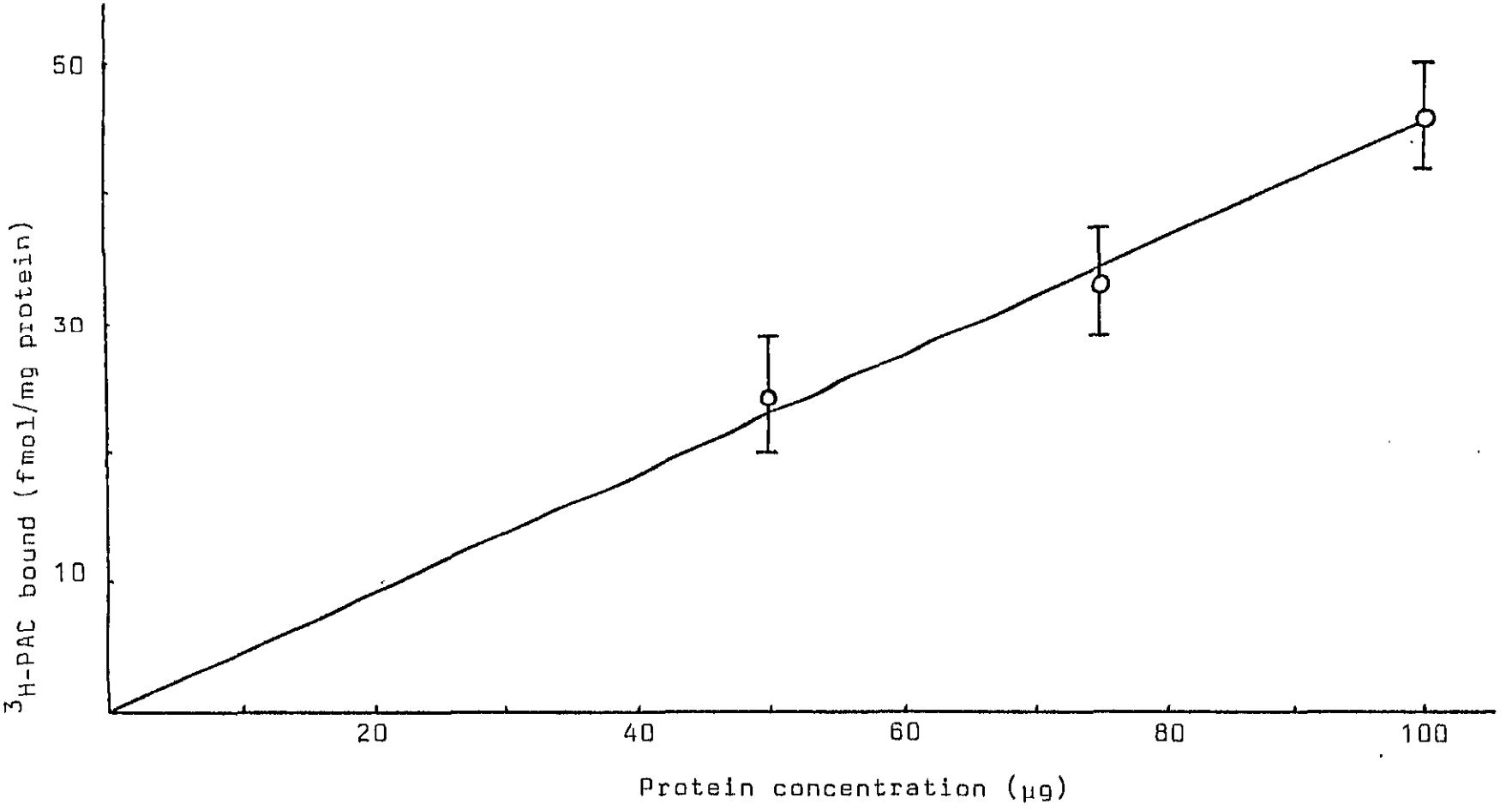


Fig 5.1.5 Linearity of ^{3}H-p-aminoclonidine (^{3}H-PAC) binding to platelet membranes over a protein concentration range. Results are expressed as the mean ± SD of the specific amount of ^{3}H-PAC bound. (n = 12)

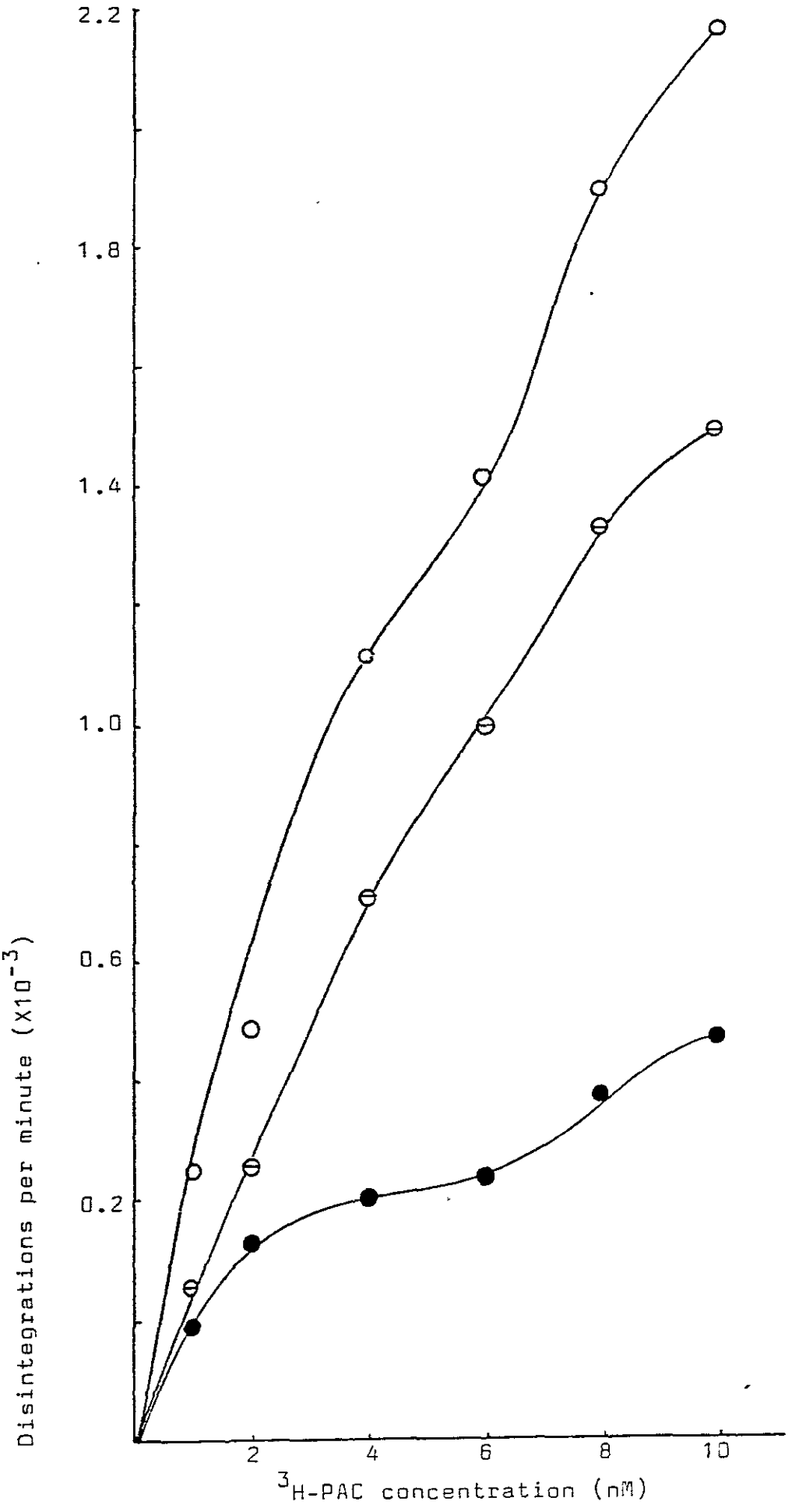


Fig 5.1.6 A representation of ^{3}H-p-aminoclonidine (^{3}H-PAC) binding to platelet membranes (total binding —o—; non-specific binding (—▪—; specific binding —s—).

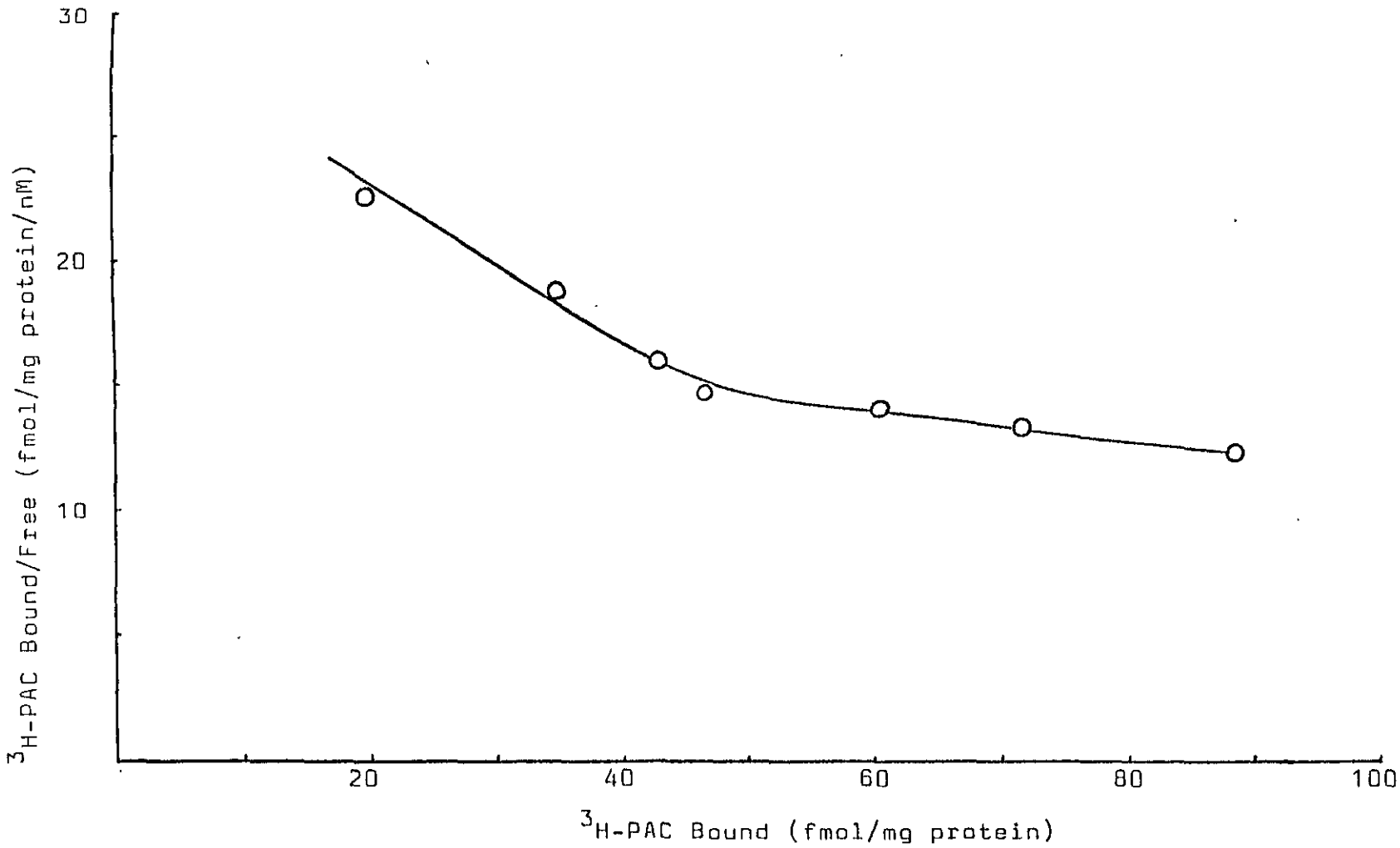


Fig 5.1.7 A representative Scatchard plot of ^{3}H-p-aminoclonidine (^{3}H-PAC) binding to platelet membranes, showing the presence of two conformations of the α_2-adrenoceptors.

clonidine binding to platelet membranes of children and adolescents with major depressive disorder. A large number of these children had a history of suicide attempts. The α_2-adrenoceptor binding parameters of patients were compared to those of 16 normal, healthy controls (mean age 16.1 ± 1.0 yrs). No significant difference could be demonstrated between control male and female α_2-adrenoceptor Kd or Bmax values (6 males: age = 15.8 ± 1.5 yrs, mean Kd = 2.3 ± 1.55nM, median = 1.9nM, mean Bmax = 67.6 ± 15.7 fmol-mg protein, median = 65,3 fmol/mg protein; 10 females: age 16.3 ± 0.51 yrs, mean Kd = 2.0 ± 0.73 nM, median = 2.1 nM, mean Bmax = 58.5 ± 15.5 fmol/mg protein, median = 55.4 fmol/mg protein, Mann-Whitney U-test).

Table 5.1.1 shows the data for the two psychiatric populations and the controls. The α_2-adrenoceptor Kd values of children with major depressive disorder with a suicide attempt were found to be significantly higher than control values ($p < 0.05$) as were those of the total population of children with major depressive disorder ($p < 0.01$, Mann-Whitney U-test). Significantly higher α_2-adrenoceptor Bmax values were observed in the total population of children with major depressive disorder ($p < 0.05$, Mann-Whitney U-test), when compared to controls.

Scattergrams of the Kd and Bmax values of the control and patient populations are shown in figures 5.1.8 and 5.1.9, respectively. As can be seen, these values varied markedly in both controls and patients. Significantly greater variance in α_2-adrenoceptor Bmax values was observed in patients with major depressive disorder with a suicide attempt ($p < 0.01$, F test), as well as the total population of children with major depressive disorder ($p < 0.05$, F test), than in the control group. Representative

TABLE 5.1.1.

Kd and Bmax values of ^{3}H-p-aminoclonidine binding to platelet membranes of normal healthy controls and children with major depressive disorder (numbers and ages included).

Controls	n	Age(yrs)	Kd (nM)		Bmax(fmol/mg protein)	
		mean ± SD	mean ± SD	median	mean ± SD	median
Controls	16	16.1 ± 1.0	2.1 ± 1.1	2.0	62.1 ± 15.7	58.5
MDD	12	13.4 ± 2.5	2.8 ± 1.1	2.7	71.5 ± 18.7	75.3
MDDS	7	15.7 ± 2.9	3.1 ± 0.91[a]	3.2	87.1 ± 35.1	89.3
MDD + MDDS	19	14.3 ± 2.8	2.9 ± 1.1 [a]	3.0	77.3 ± 26.2[a]	76.8

MDD = major depressive disorder

MDDS = major depressive disorder with suicide attempt

[a] Significantly different from controls

($p < 0.05$)

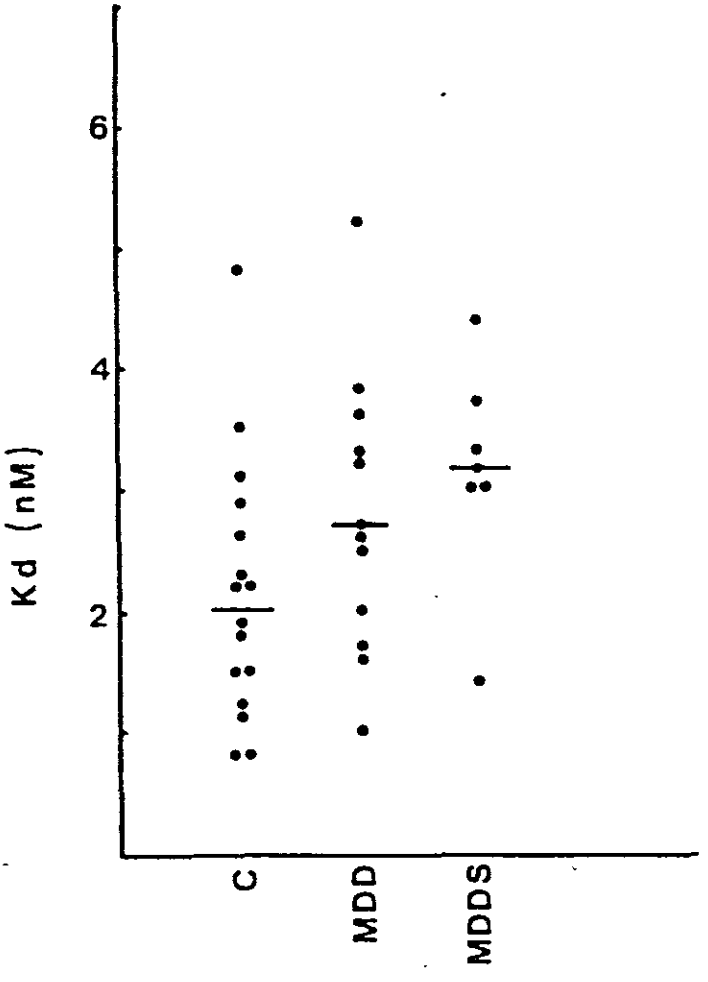


Fig 5.1.8 Scattergrams of platelet α_2-adrenoceptor Kd values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder
MDDS : major depressive disorder and a suicide attempt.

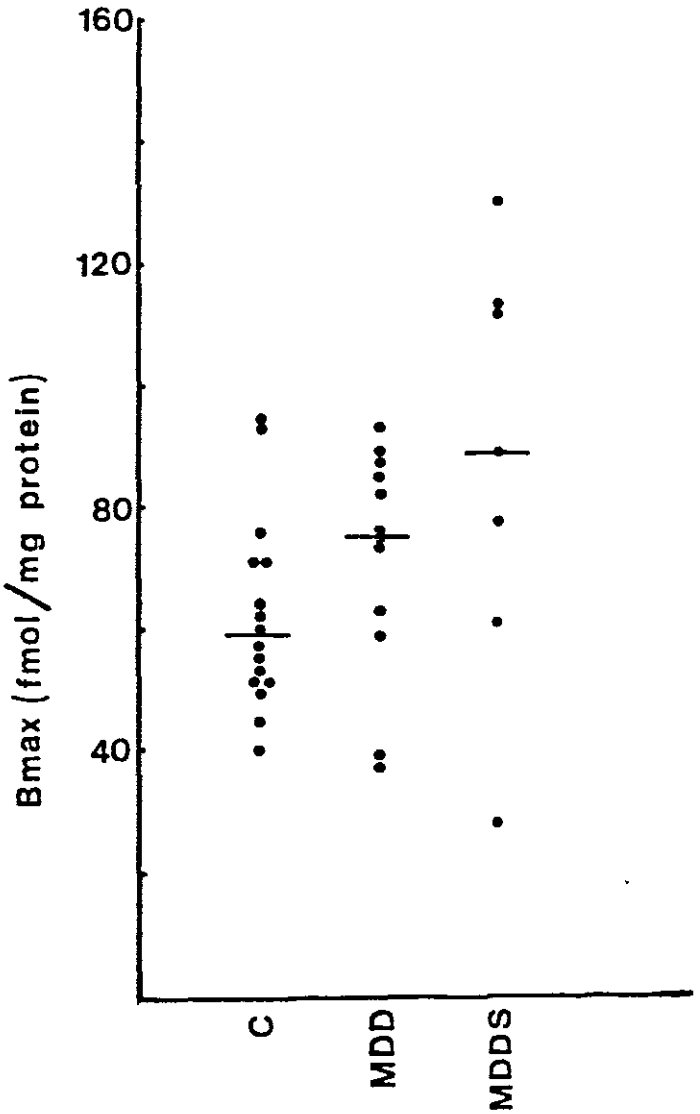


Fig 5.1.9 Scattergrams of platelet α_2-adrenoceptor Bmax values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder

MDDS : major depressive disorder and a suicide attempt.

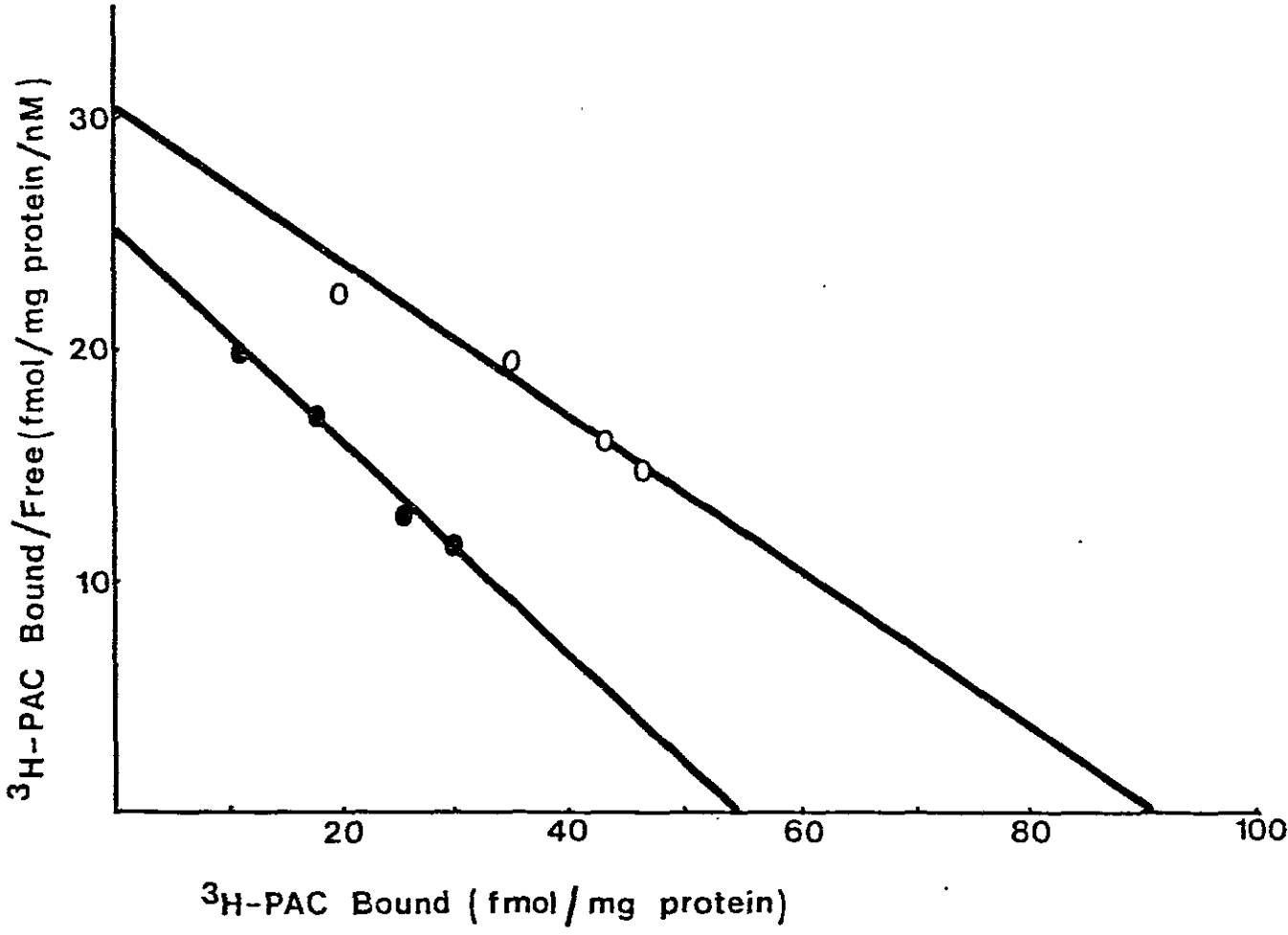


Fig 5.1.10 Representative Scatchard plots of platelet ^{3}H-p-aminoclonidine binding data of controls (-■-■-) and patients with major depressive disorder (-O-O-).

Scatchard plots of the data are shown in figure 5.1.10.

Neither a seasonal, nor an age-dependent variation was observed for either the α_2-adrenoceptor Kd or Bmax values of both the control and patient populations (result not shown).

5.2 IMIPRAMINE BINDING TO PLATELET MEMBRANES

5.2.1 Characterisation of the ^{3}H-imipramine binding assay

The method upon which the characterisation of ^{3}H-imipramine binding to platelet membranes was based, was that of Paul et al (1981a). Blood used in these studies was obtained from adults, because of difficulties in the availability of children. ^{3}H-Imipramine was used at a concentration of 1nM for the characterisation of the imipramine binding assay as described in Section 4.7.

5.2.1.1 pH and Na^+ and K^+ requirement

^{3}H-Imipramine binding was measured over a pH range of 7.1 to 7.6 in the presence and absence of 120nM NaCl and 5mM KCl in order to establish the pH optimum for the binding assay. Samples of platelet membranes (100µg protein) were incubated with 1nM ^{3}H-imipramine for 60 min. at 0°C. Non-specific binding was determined by the addition of 100µM chlorimipramine (Section 4.7). In the absence of the two salts, no binding was detectable (results not shown). Figure 5.2.1 shows the reaction to take place optimally at pH 7.4 in the presence of both Na^+ and K^+.

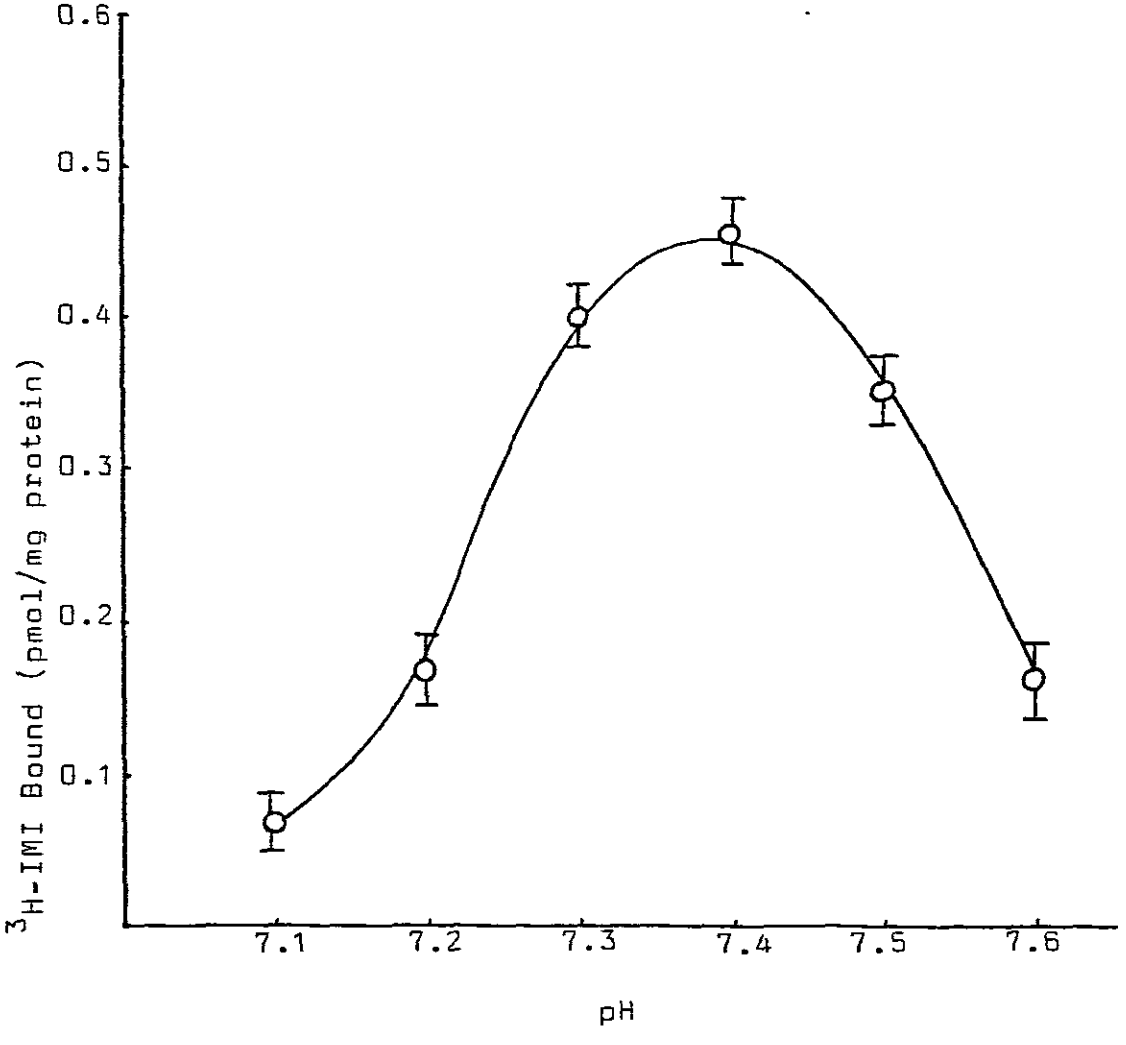


Fig 5.2.1 pH-dependence of imipramine binding to platelet membranes at 0°C. Results are expressed as the mean ± SD of the specific amount of ^{3}H-imipramine (^{3}H-IMI) bound in the presence of Na^+ and K^+. (n = 9)

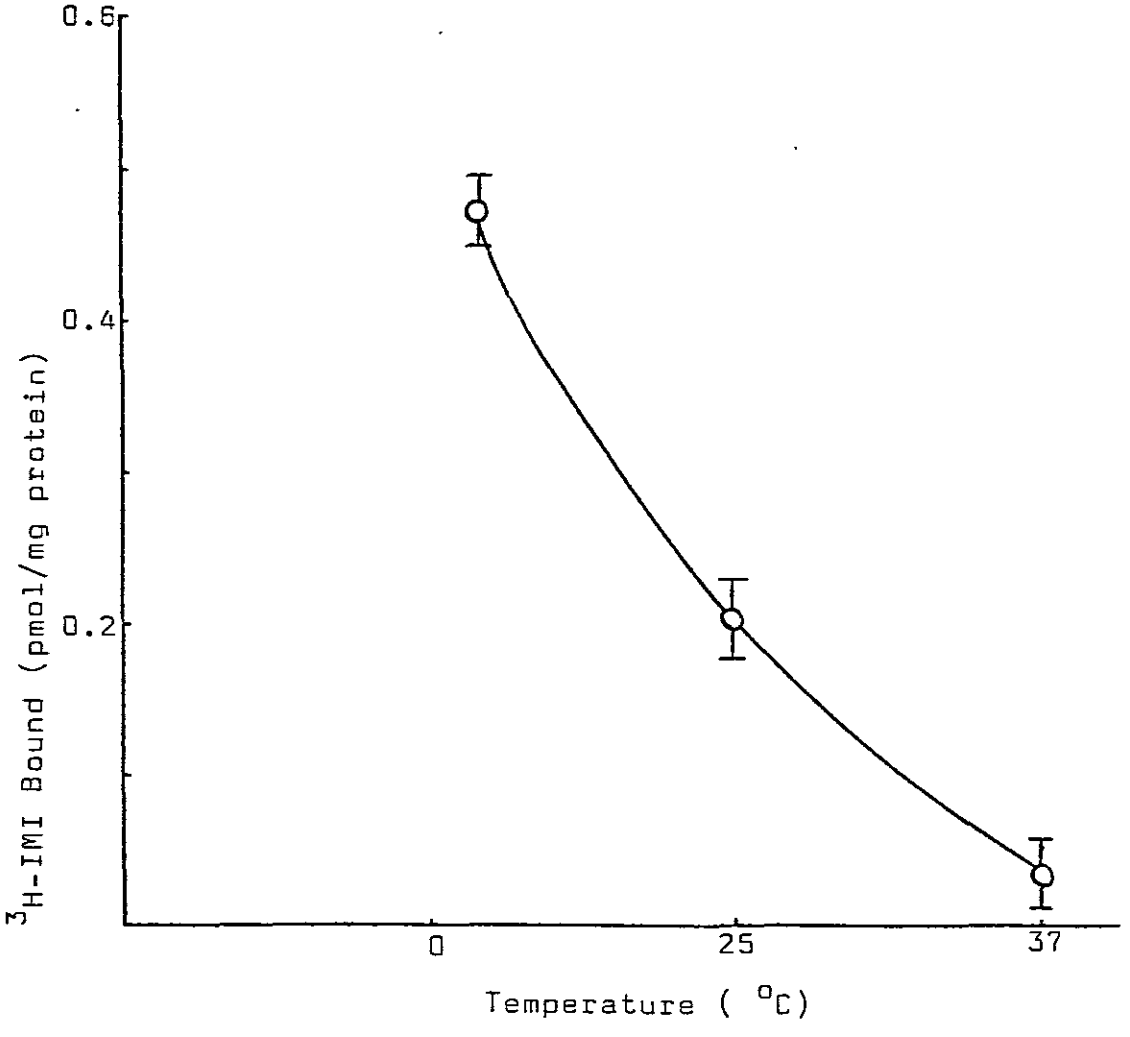


Fig 5.2.2 Temperature-dependence of imipramine binding to platelet membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-imipramine (^{3}H-IMI) bound. (n = 9)

5.2.1.2 Temperature

In order to establish the temperature optimum for ^{3}H-imipramine binding, platelet membranes were incubated with the labelled ligand for 60 min. at 0°C, 25°C and 37°C. From figure 5.2.2 it can be seen that maximum specific binding was achieved at 0°C.

5.2.1.3 Time of incubation

Platelet membranes were incubated in the presence of ^{3}H-imipramine over time periods of 10 to 60 min. This was done to establish the time required for specific binding to reach saturation. As can be seen from figure 5.2.3, the reaction reached saturation within 60 min.

5.2.1.4 Non-specific displacer requirement

Samples of platelet membranes were incubated with ^{3}H-imipramine in the presence of either 5HT, amitriptyline or chlorimipramine in order to determine non-specific binding. The drugs were incubated at final concentrations ranging from 0.1 to 100µM. Figure 5.2.4 shows chlorimipramine to be the most suitable drug to use as displacer at a concentration of either 10 or 100µM. The latter concentration was subsequently used in the present study.

5.2.1.5 Protein concentration

^{3}H-Imipramine binding to platelet membranes was determined over a platelet protein concentration range of 50 to 100µg.

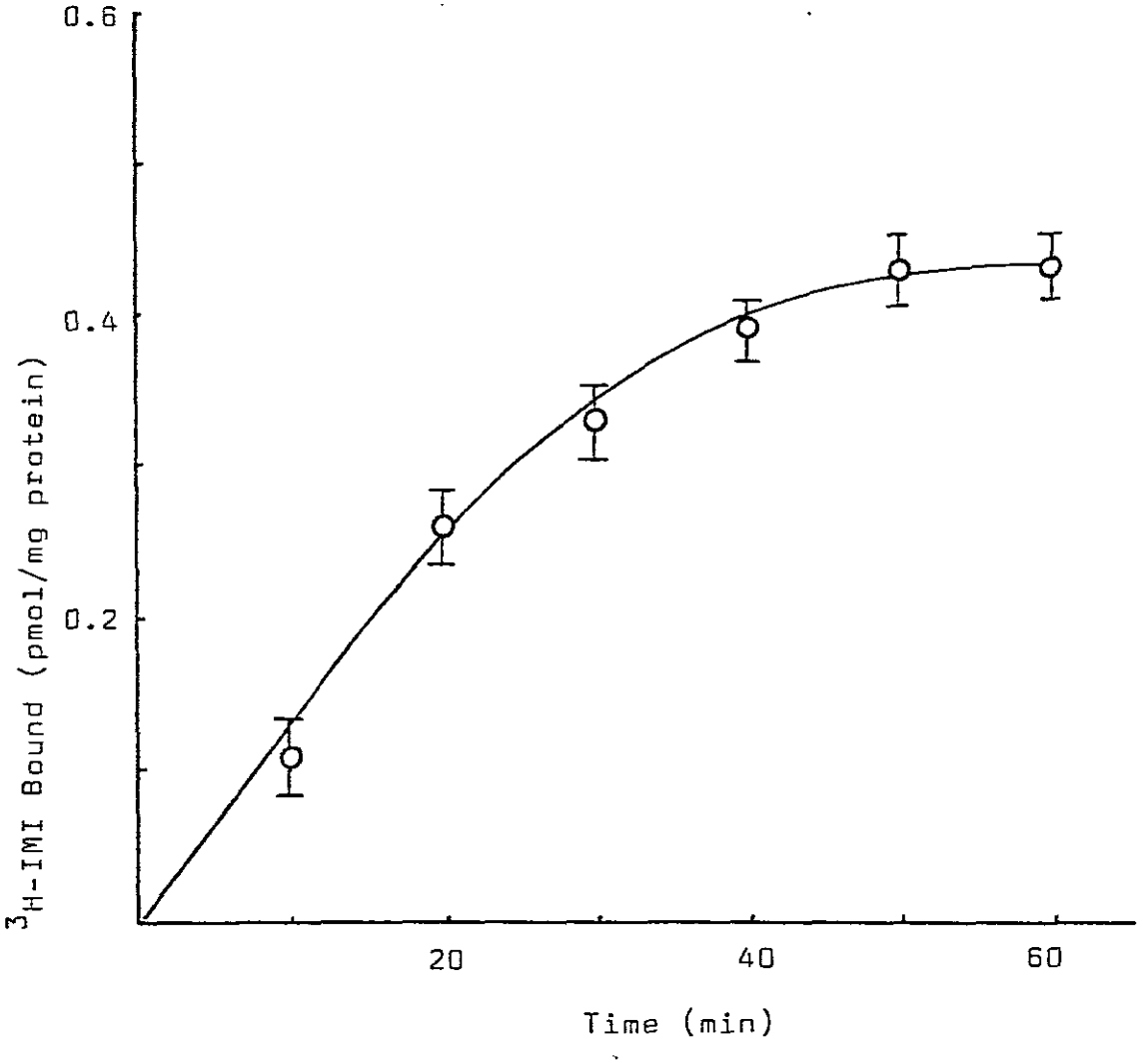


Fig 5.2.3 Time-course of incubation of ^{3}H-imipramine (^{3}H-IMI) with platelet membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-IMI bound. (n = 9)

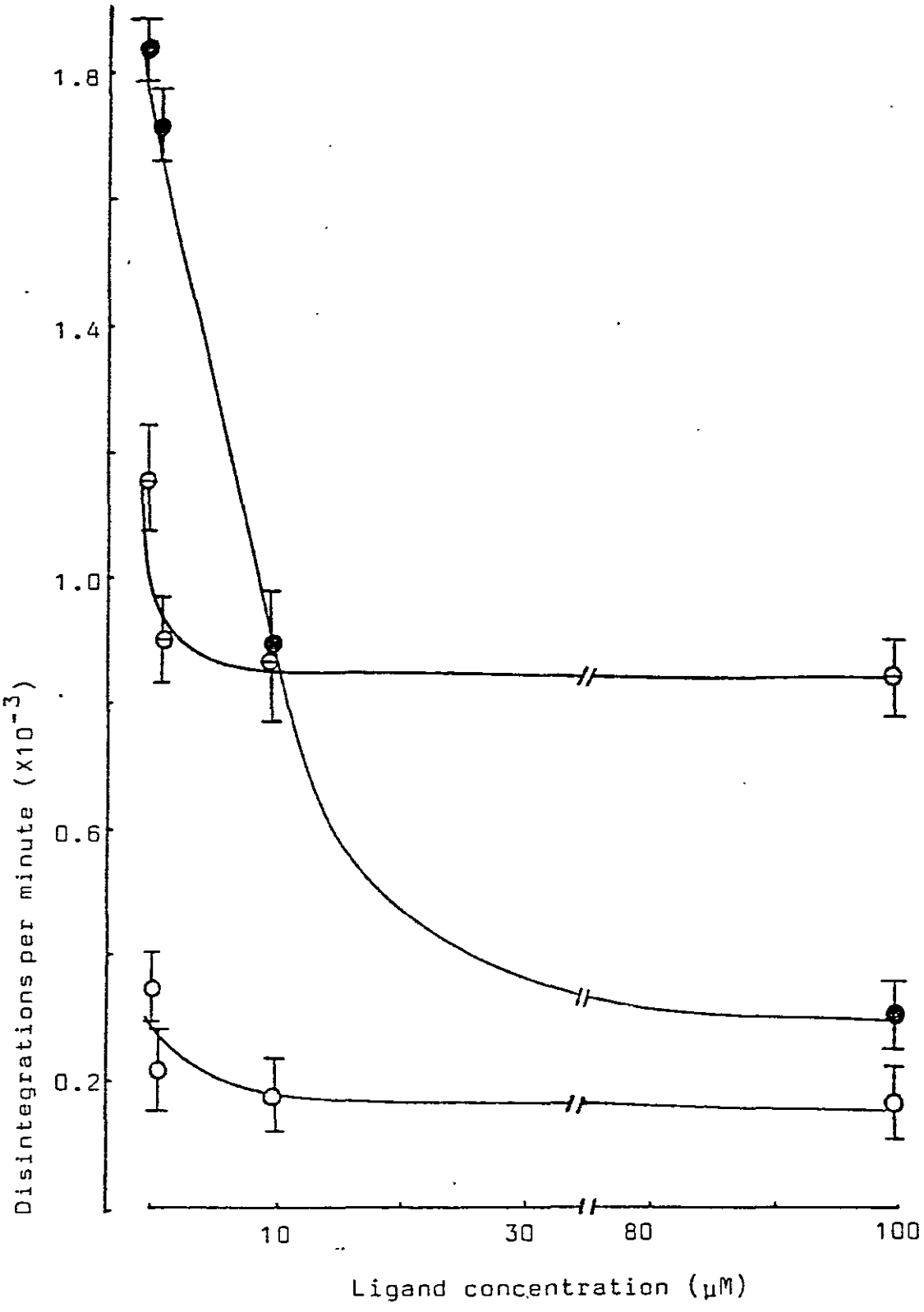


Fig 5.2.4 Displacement of ^{3}H-imipramine binding from platelet membranes by 5-HT (—•—), chlorimipramine (—e—) and amitriptyline (—o—) Results are expressed as the mean ± SD of the amount of radioactivity after displacement by the three unlabelled ligands. (n = 9)

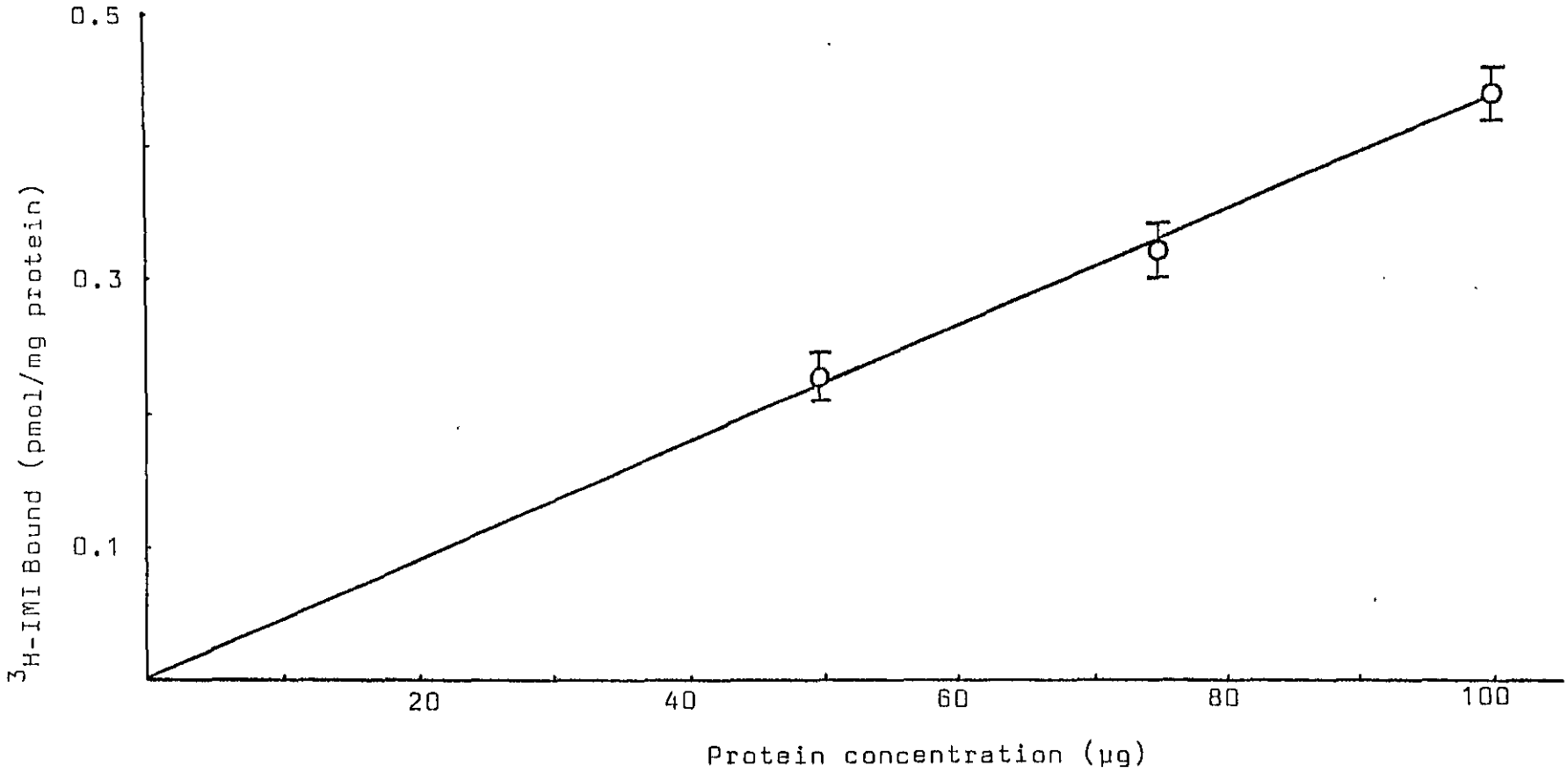


Fig 5.2.5 Linearity of ^{3}H-imipramine (^{3}H-IMI) binding to platelet membranes over a protein concentration range. Results are expressed as the mean ± SD of the specific amount of ^{3}H-IMI bound. (n = 9)

Binding was found to be linear over this concentration range (fig. 5.2.5) and 100µg protein was used in all subsequent studies.

5.2.1.6 Scatchard analysis of the binding data

After characterisation of the binding of ^{3}H-imipramine to imipramine binding sites on platelet membranes, samples of platelet membranes (100µg protein) were incubated with ^{3}H-imipramine over a concentration range of 1nM to 8nM (Section 4.7, fig. 5.2.6). Scatchard analyses of the binding data suggested the existence of two binding sites with different Kd and Bmax values (fig. 5.2.7). This was in agreement with findings reported by Wägner et al (1985). The present study was subsequently carried out, using four concentrations of ^{3}H-imipramine, ranging from 1nM to 6nM.

5.2.2 ^{3}H-Imipramine binding to platelet membranes of depressed children and controls

The aim of the present study was to investigate ^{3}H-imipramine binding to platelet membranes of children and adolescents with major depressive disorder. A large number of these children had a history of suicide attempts. The imipramine binding parameters of the patients were compared to those of 21 normal, healthy controls (mean age 16.5 ± 1.1 yrs). No significant difference could be demonstrated between control male and female imipramine Kd or Bmax values (11 males: age = 16.5 ± 1.4 yrs, mean Kd = 1.26 ± 0.31 nM, median = 1.11 nM, mean Bmax = 1.16 ± 0.27 pmol/mg protein, median = 1.13 pmol/mg protein; 10 females: age = 16.4 ± 0.51 yrs, mean Kd = 1.46 ± 0.28 nM,

median = 1.44 nM, mean Bmax = 1.22 ± 0.20 pmol/mg protein, median = 1.16 pmol/mg protein, Mann-Whitney U test).

Table 5.2.1 shows the data for the two psychiatric populations and the controls. The imipramine Kd values of children with major depressive disorder with a suicide attempt were found to be significantly higher than control values ($p < 0.0005$), as were the Kd values of the total population of children with major depressive disorder ($p < 0.03$, Mann-Whitney U test). Significantly higher imipramine Bmax values were observed for platelets of children with major depressive disorder ($p < 0.0005$), children with major depressive disorder with a suicide attempt ($p < 0.0005$), and a combination of these two groups ($p < 0.0005$, Mann-Whitney U test), when compared to controls.

Scattergrams of imipramine Kd and Bmax values of the control and patient populations are shown in figures 5.2.8 and 5.2.9, respectively. Representative Scatchard plots of the data are shown in figure 5.2.10.

Neither a seasonal, nor an age variation was observed for either the imipramine Kd or Bmax values of controls and patients (results not shown).

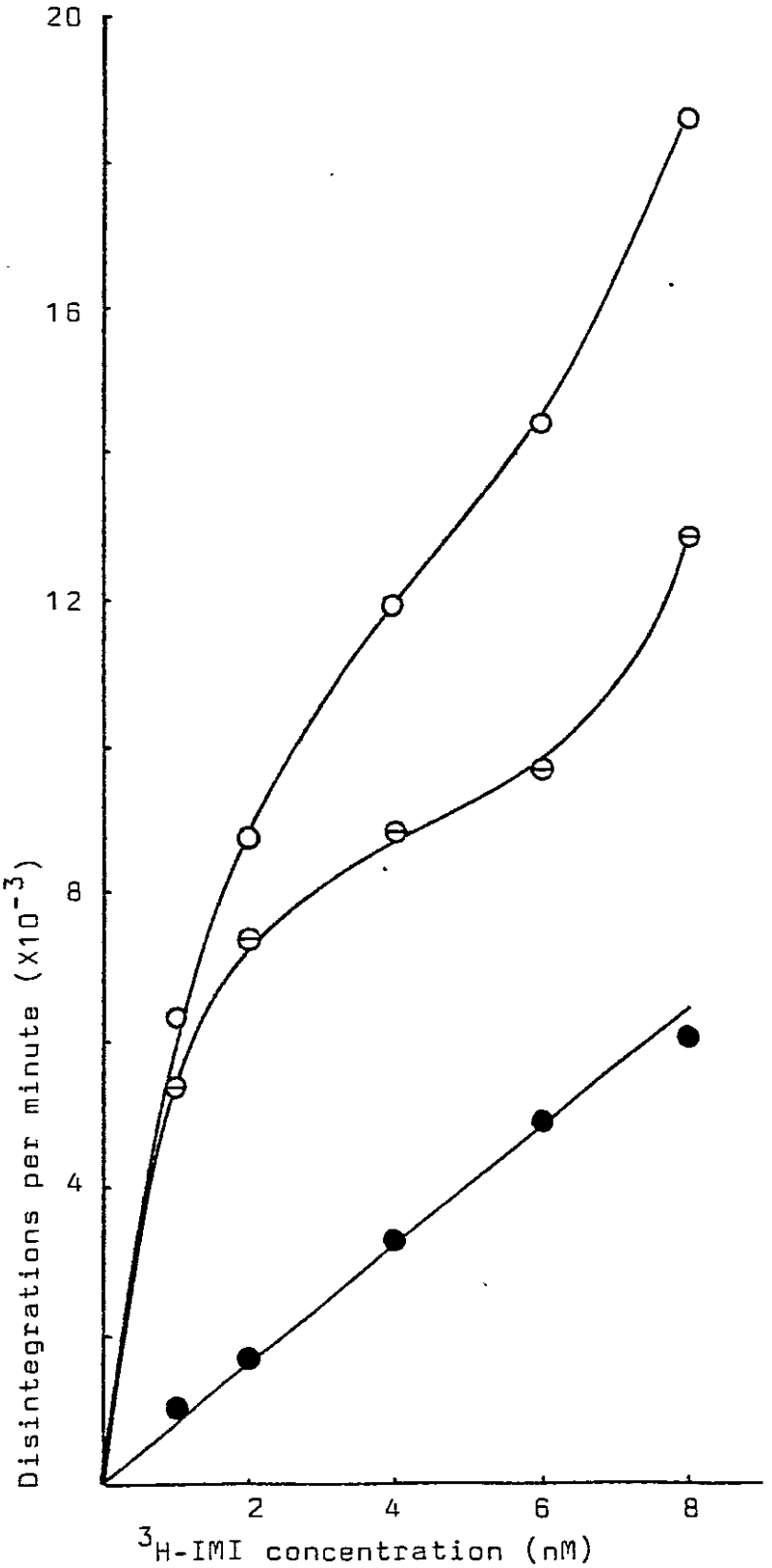


Fig 5.2.6 A representation of ^{3}H-imipramine (^{3}H-IMI) binding to platelet membranes (total binding —o—; non-specific binding —▪—; specific binding —⊖—).

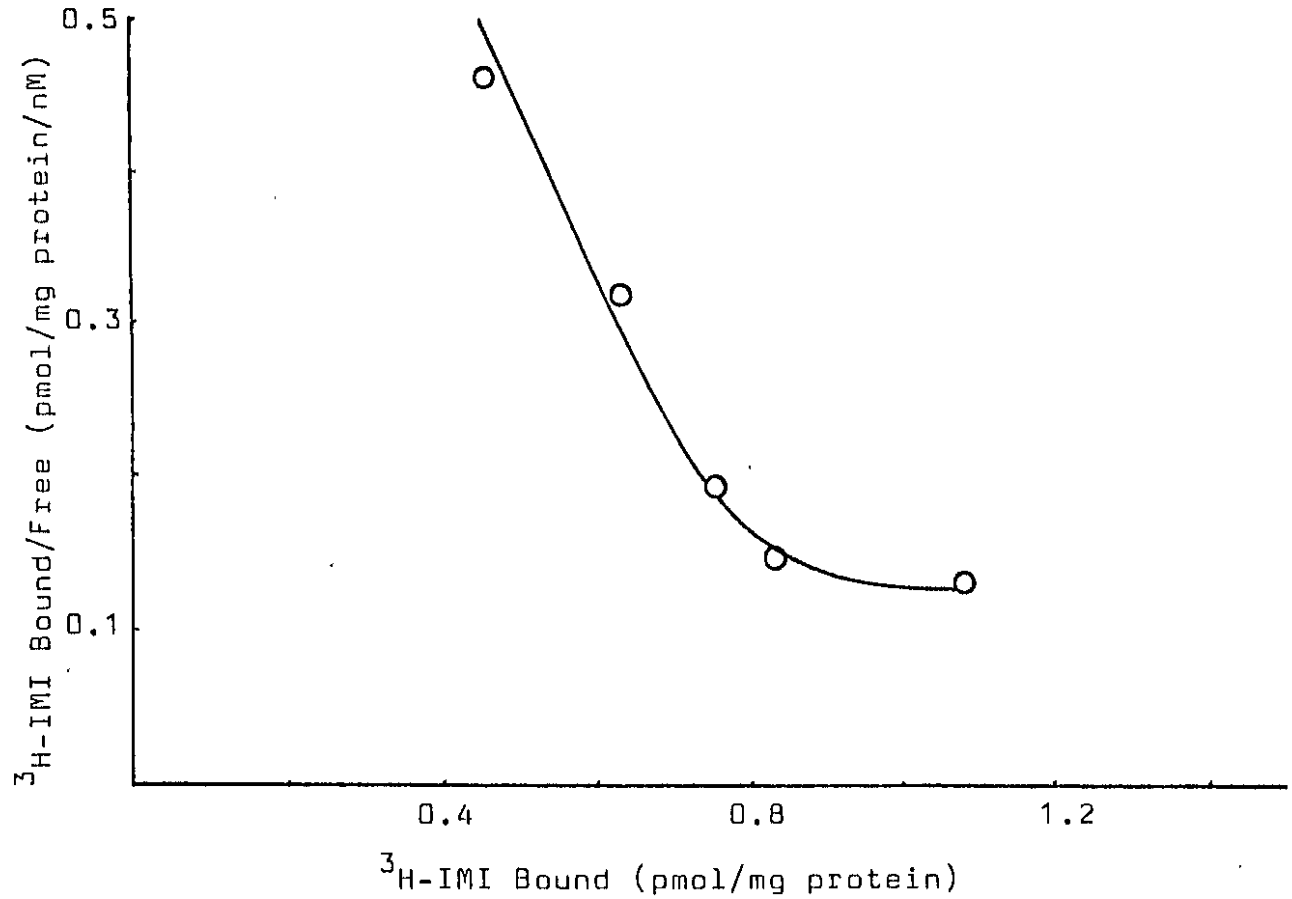


Fig 5.2.7 A representative Scatchard plot of ^{3}H-imipramine (^{3}H-IMI) binding to platelet membranes, showing the presence of two conformations of the imipramine binding sites.

TABLE 5.2.1.

Kd and Bmax values of ^{3}H-imipramine binding to platelet membranes of normal, healthy controls and children with major depressive disorder (numbers and ages included).

Population	n	Age(yrs)	Kd(nM)		Bmax(pmol/mg protein	
		mean ± SD	mean ± SD	median	mean ± SD	median
Controls	21	16.5 ± 1.1	1.35 ± 0.31	1.35	1.19 ± 0.23	1.15
MDD	12	13.4 ± 2.5	1.51 ± 0.37	1.48	1.63 ± 0.39[a]	1.49
MDDS	10	16.1 ± 2.5	1.78 ± 0.41[a]	1.71	1.67 ± 0.34[a]	1.60
MDD+MDDS	22	14.6 ± 2.8	1.63 ± 0.41[ℓ]	1.59	1.65 ± 0.36[a]	1.53

MDD = major depressive disorder

MDDS = major depressive disorder with suicide attempt

[a]Significantly different from controls ($p < 0.0005$); [ℓ]$p < 0.03$

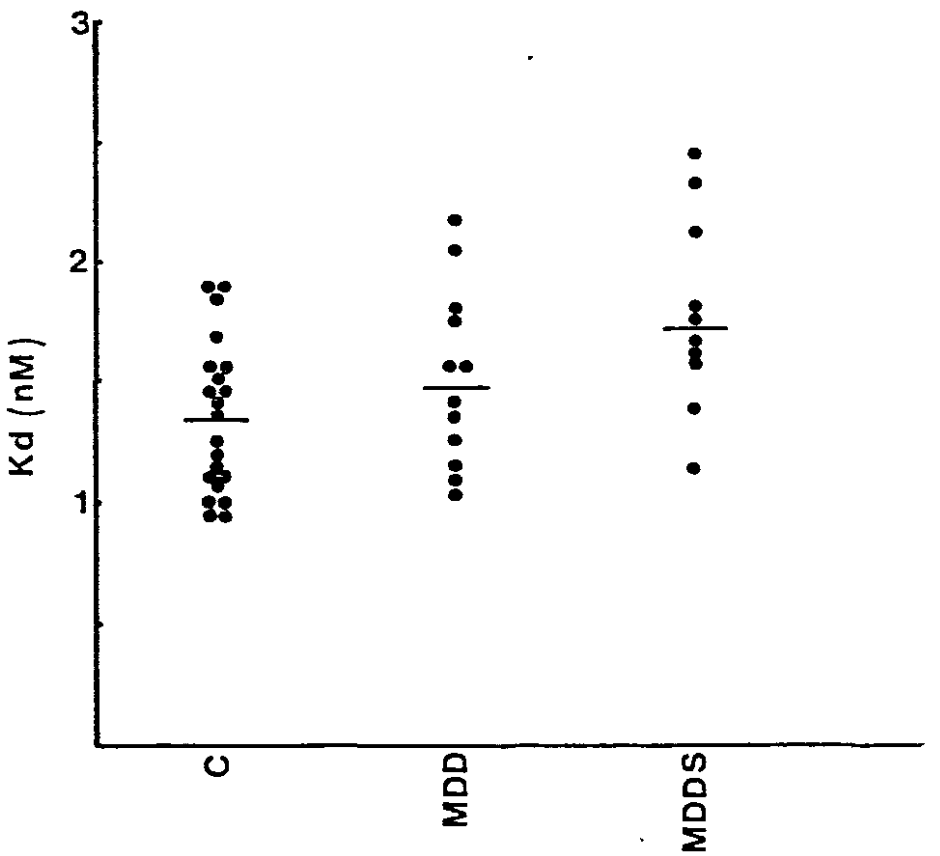


Fig 5.2.8 Scattergrams of platelet imipramine Kd values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder

MDDS : major depressive disorder and a suicide attempt.

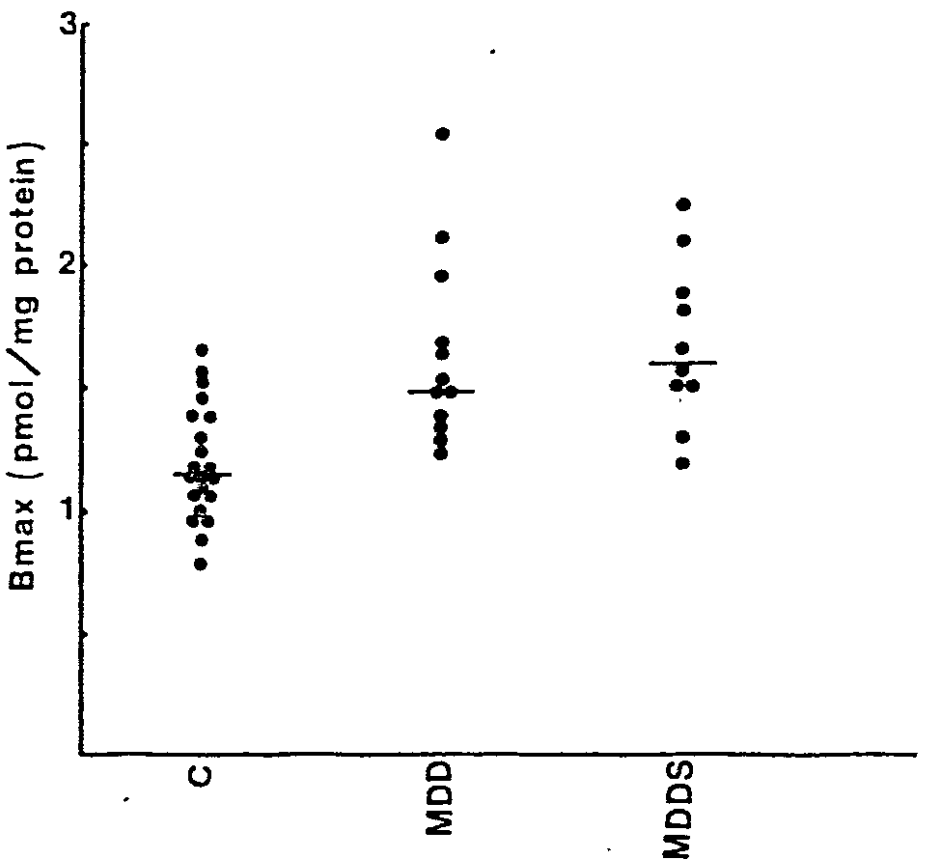


Fig 5.2.9. Scattergrams of platelet imipramine Bmax values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder
MDDS : major depressive disorder and a suicide attempt.

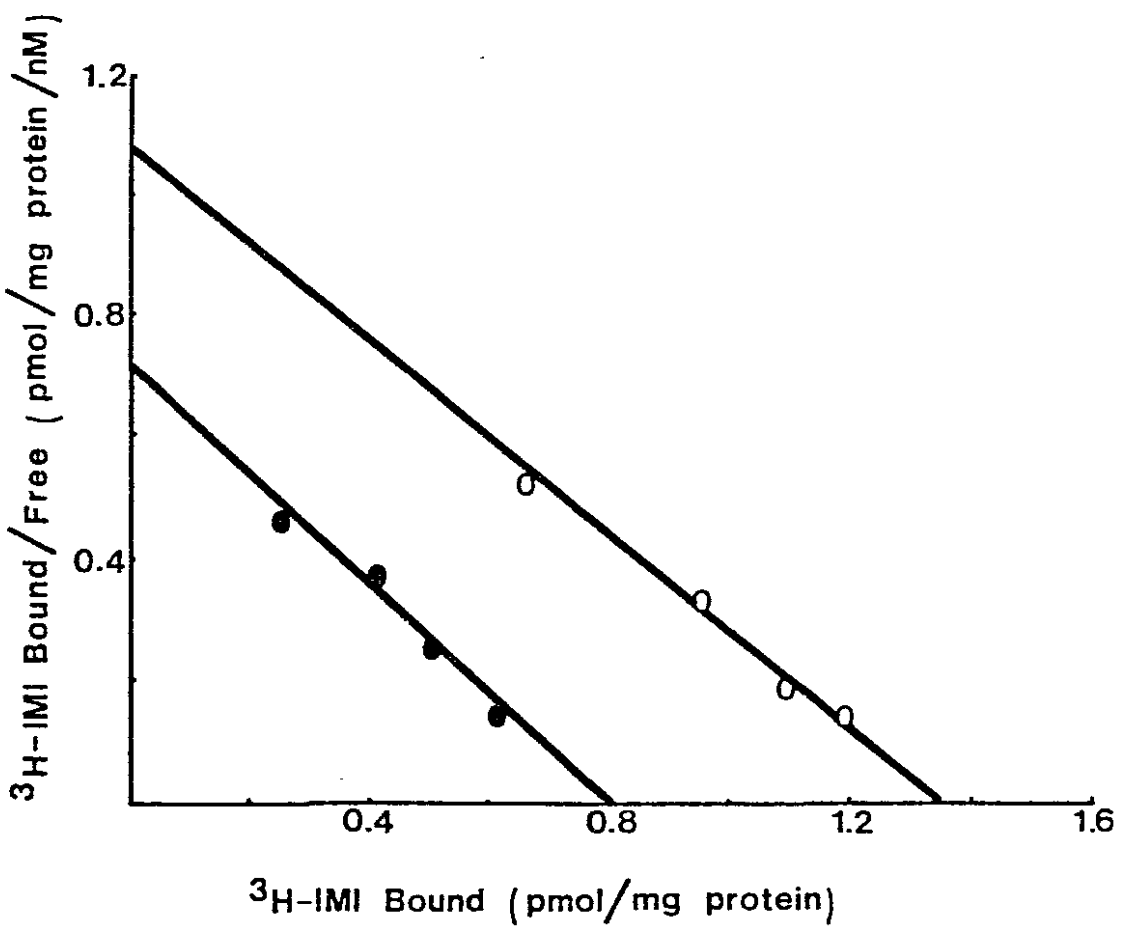


Fig 5.2.10 Representative Scatchard plots of platelet ^{3}H-imipramine binding data of controls (-■-■-) and patients with major depressive disorder (-O-O-).

5.3 β-ADRENOCEPTOR BINDING TO LYMPHOCYTE MEMBRANES

5.3.1 Characterisation of the ^{3}H-DHA binding assay

The method upon which the characterisation of ^{3}H-DHA binding to β-adrenoceptors on lymphocyte membranes was based, was that of Davies and Lefkowitz (1980). Blood for these studies was obtained from adults, because of difficulties in the availability of children. The concentration of ^{3}H-DHA used, for initial characterisation of the assay (Section 4.8) was 1nM in the presence of 5mM ascorbic acid.

5.3.1.1 pH and Mg^{2+} requirement

^{3}H-DHA binding to lymphocyte membranes was investigated over a pH range of 7.5 to 8.1 in the presence and absence of 10mM $MgCl_2$. Lymphocyte membranes (100µg protein) were incubated with 1nM ^{3}H-DHA for 15 min. at 37°C. Non-specific binding was determined by the addition of 5mM isoproterenol (Section 4.8). Figure 5.3.1 shows the optimum pH to be 7.7 in the presence of Mg^{2+}. The data of ^{3}H-DHA binding performed in the absence of Mg^{2+} were too scattered to obtain any result (results not shown).

5.3.1.2 Temperature

Since binding reactions require a specific temperature to occur optimally, lymphocyte membranes (100µg protein) were incubated with ^{3}H-DHA for 15 min. at 0°C, 25°C and 37°C (Section 4.8). From figure 5.3.2 it can be seen that this reaction has an optimum temperature of 37°C.

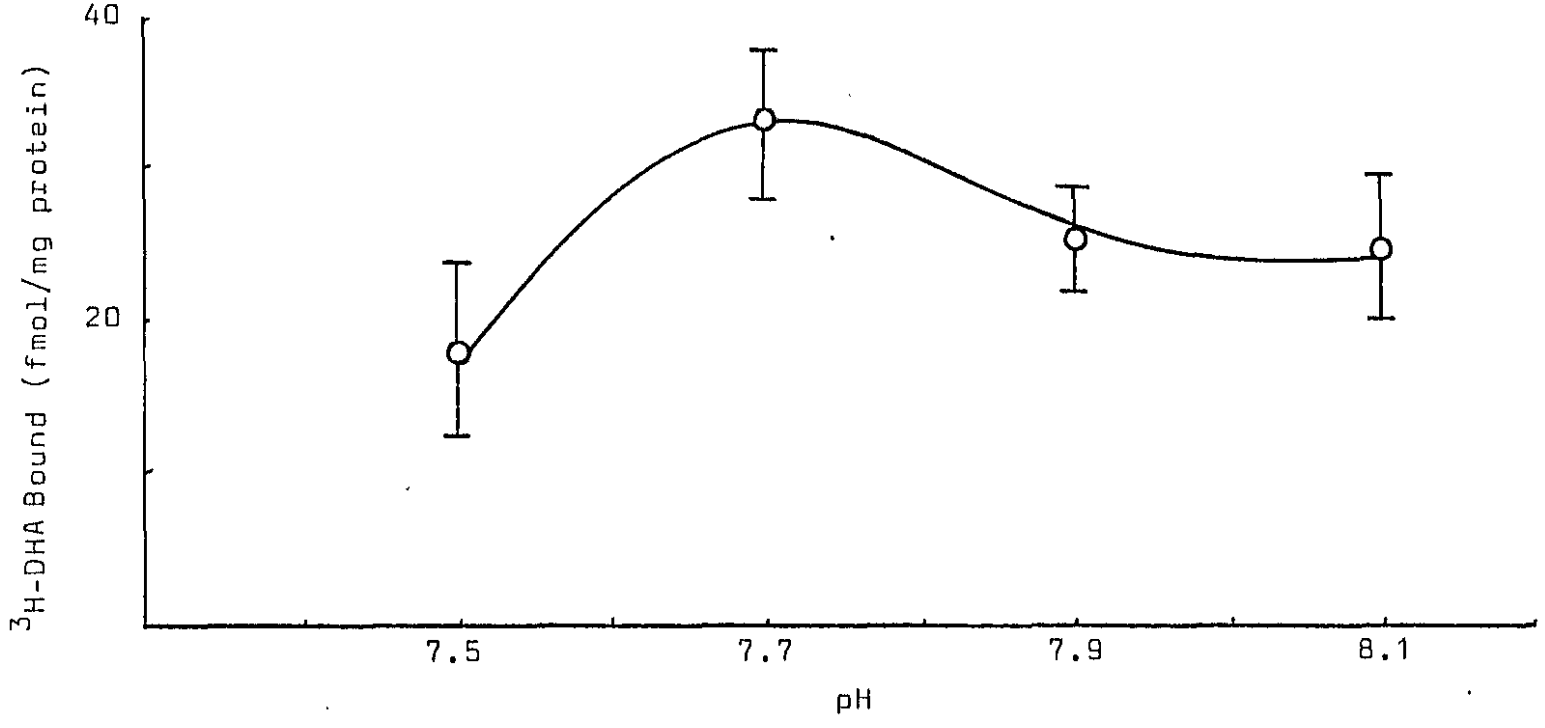


Fig 5.3.1 pH-dependence of ^{3}H-DHA binding to lymphocyte membranes at 37^{o}C. Results are expressed as the mean $\pm$ SD of the specific amount of ^{3}H-DHA bound in the presence of Mg^{2+}. (n = 9)

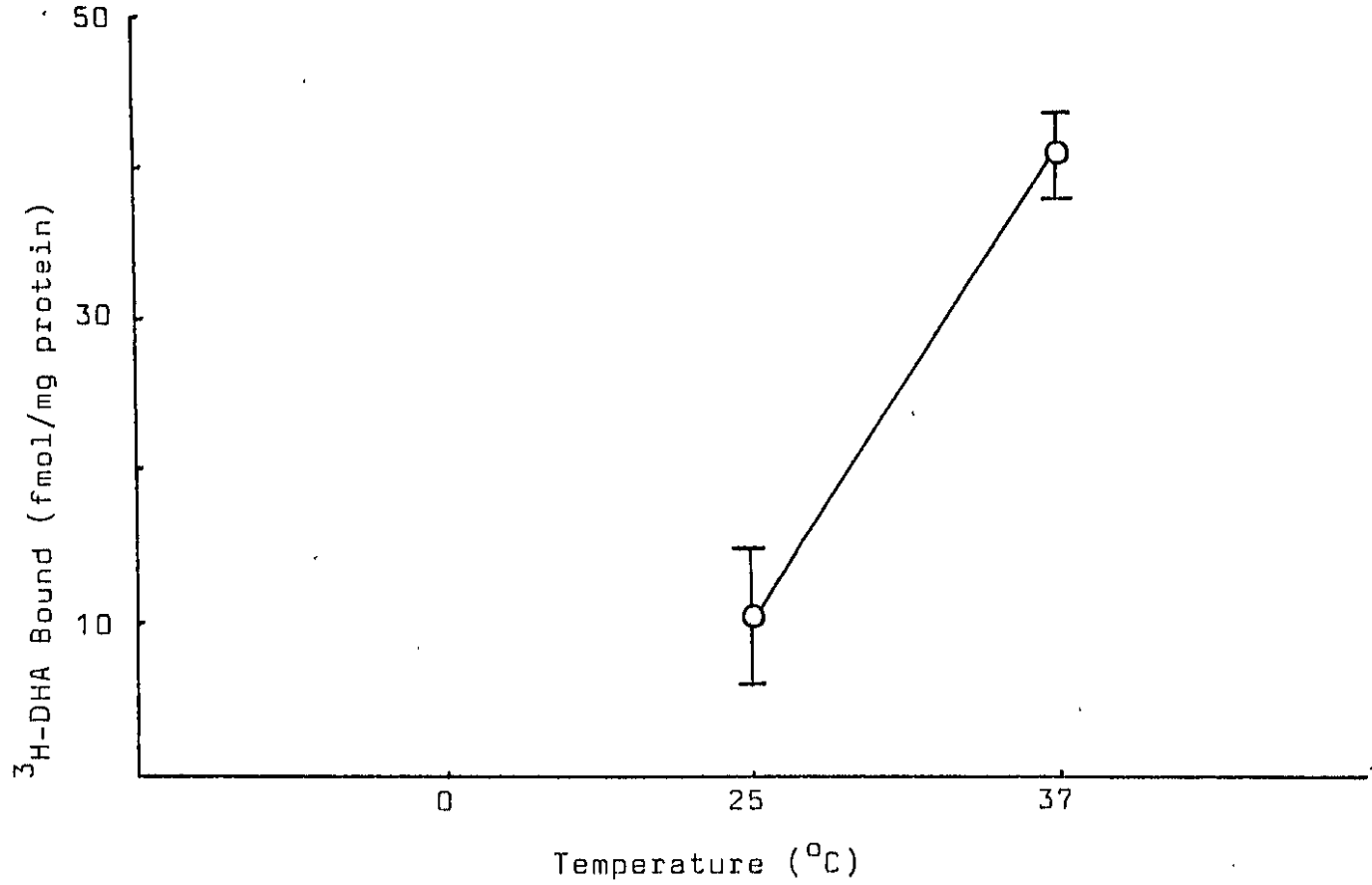


Fig 5.3.2 Temperature-dependence of ^{3}H-DHA binding to lymphocyte membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-DHA bound. (n = 9)

5.3.1.3 Time of incubation

In order to establish the time required for saturation of ^{3}H-DHA binding to lymphocyte membranes, incubations were carried out in the presence of the labelled ligand over time periods of 5 to 25 min. Saturation of specific binding was reached within 15 min. (Fig. 5.3.3).

5.3.1.4 Non-specific displacer requirement

Lymphocyte membranes were incubated with ^{3}H-DHA in the presence of 0,01mM to 5mM isoproterenol in 5mM ascorbic acid in order to establish the optimal concentration of the unlabelled ligand required for the determination of non-specific binding (Section 4.8). Figure 5.3.4 shows 1mM and 5mM isoproterenol to be equipotent in displacing ^{3}H-DHA and the latter concentration was used in all subsequent binding assays.

5.3.1.5 Protein concentration

^{3}H-DHA (1nM) was incubated with lymphocyte membranes over a protein concentration range of 50µg to 100µg protein per assay. Figure 5.3.5 shows specific binding to be linear over this concentration range and 100µg protein was used in all subsequent studies.

5.3.1.6 Scatchard analysis of the binding data

After characterisation of the binding of ^{3}H-DHA to the β-adrenoceptor on lymphocyte membranes, samples of the membranes (100µg

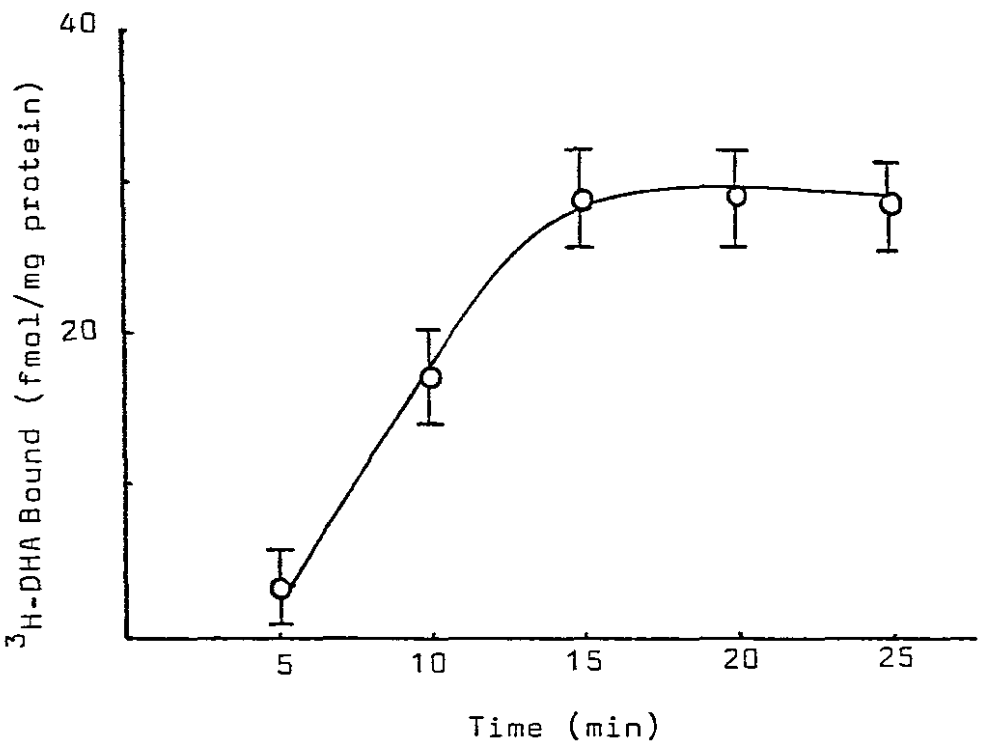


Fig 5.3.3 Time-course of incubation of ^{3}H-DHA with lymphocyte membranes. Results are expressed as the mean ± SD of the specific amount of ^{3}H-DHA bound. (n = 9)

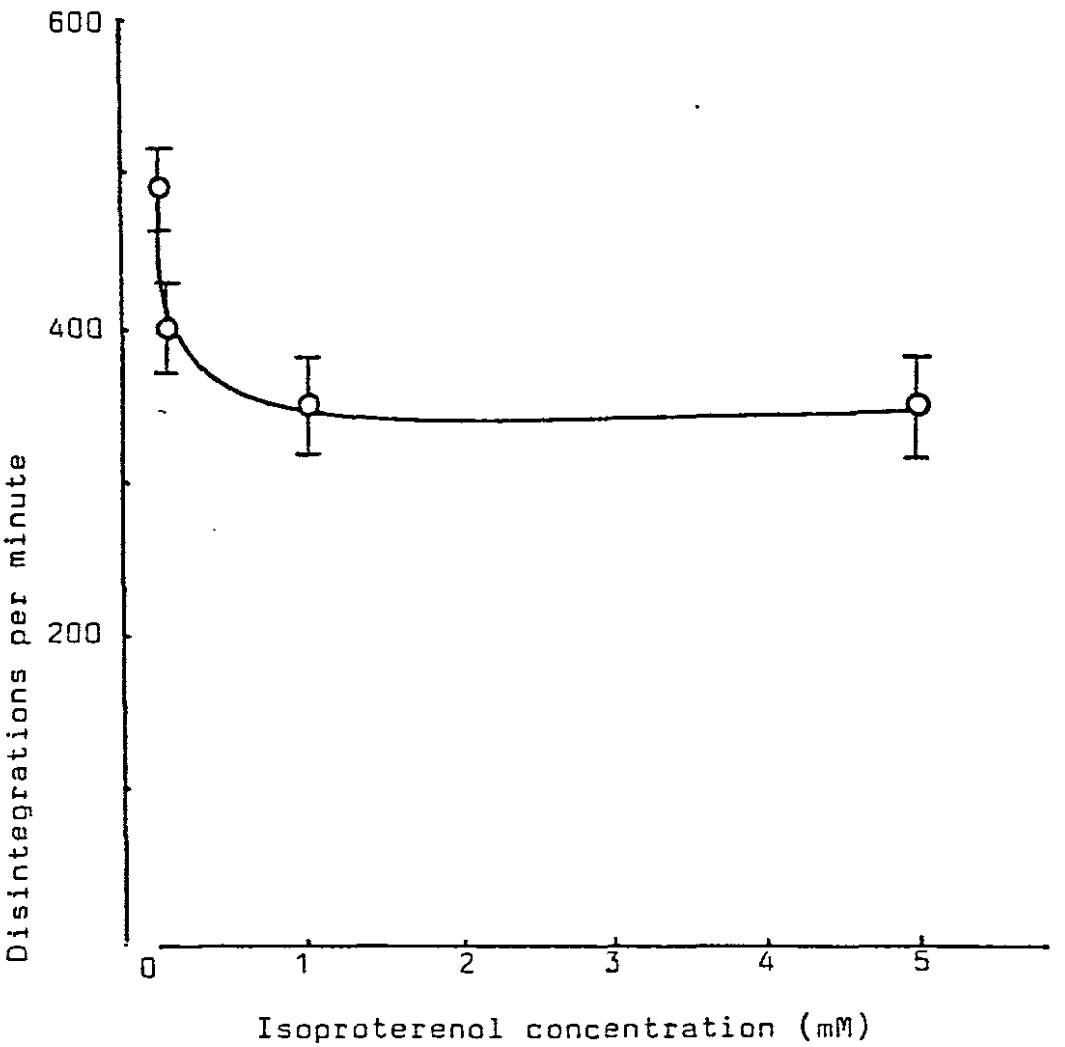


Fig 5.3.4 Displacement of ^{3}H-DHA binding from lymphocyte membranes by isoproterenol. Results are expressed as the mean ± SD of the amount of radioactivity after displacement by the unlabelled ligand. (n = 9)

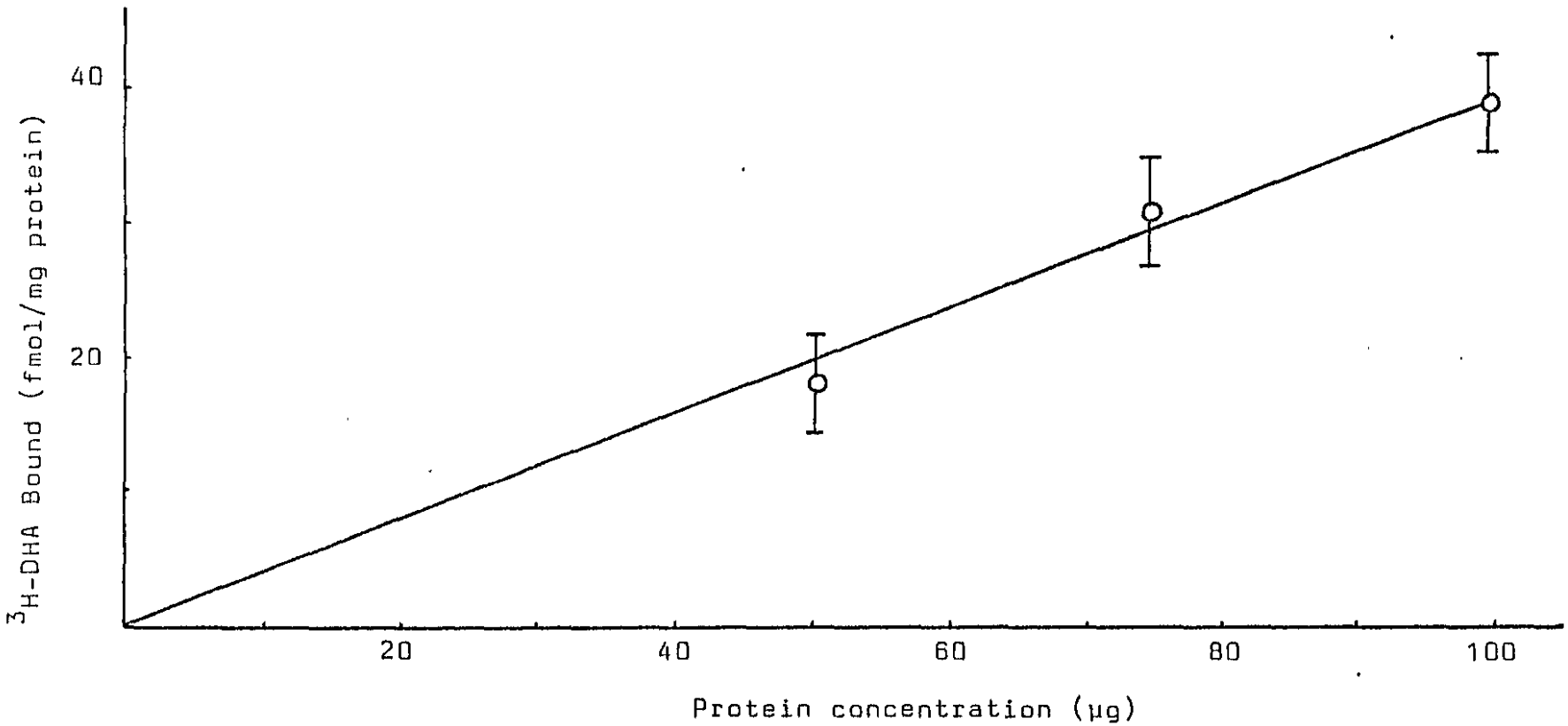


Fig 5.3.5 Linearity of ^{3}H-DHA binding to lymphocyte membranes over a protein concentration range. Results are expressed as the mean ± SD of the specific amount of ^{3}H-DHA bound. (n = 9)

protein) were incubated with 4 concentrations of ^{3}H-DHA over a concentration range of 0.5nM to 3nM (Section 4.8, fig. 5.3.6). Scatchard analyses of the binding data suggested a single population of high-affinity β-adrenoceptor sites (fig. 5.3.7).

5.3.2 ^{3}H-DHA binding to lymphocyte membranes of depressed children and controls

The aim of the present study was to investigate ^{3}H-DHA binding to lymphocyte membranes of children and adolescents with major depressive disorder. A large number of these children had histories of suicide attempts. The β-adrenoceptor binding parameters of patients were compared to those of 23 normal, healthy controls (mean age 16.4 ± 1.0 yrs). A significant difference was observed between control male and female β-adrenoceptor Kd values (11 males: age = 16.5 ± 1.4 yrs, mean Kd = 0.6 ± 0.27 nM, median = 0.61 nM; 12 females: age = 16.3 ± 0.5 yrs, mean Kd = 1.03 ± 0.44 nM, median = 1.03 nM, $p < 0.02$, Mann-Whitney U test). No significant difference, however, could be demonstrated between control male and female β-adrenoceptor Bmax values (11 males: mean Bmax = 40.8 ± 11.8 fmol/mg protein, median = 37.0 fmol/mg protein; 12 females: mean Bmax = 44.0 ± 7.9 fmol/,g protein, median = 40.2 fmol/mg protein, Mann-Whitney U test).

Table 5.3.1 shows the β-adrenoceptor Kd values of the two psychiatric populations and the controls. No difference was found between control males and males with major depressive disorder. Control females, however, had significantly greater β-adrenoceptor Kd values when compared to female patients with major depressive disorder ($p < 0.05$), major depressive disor-

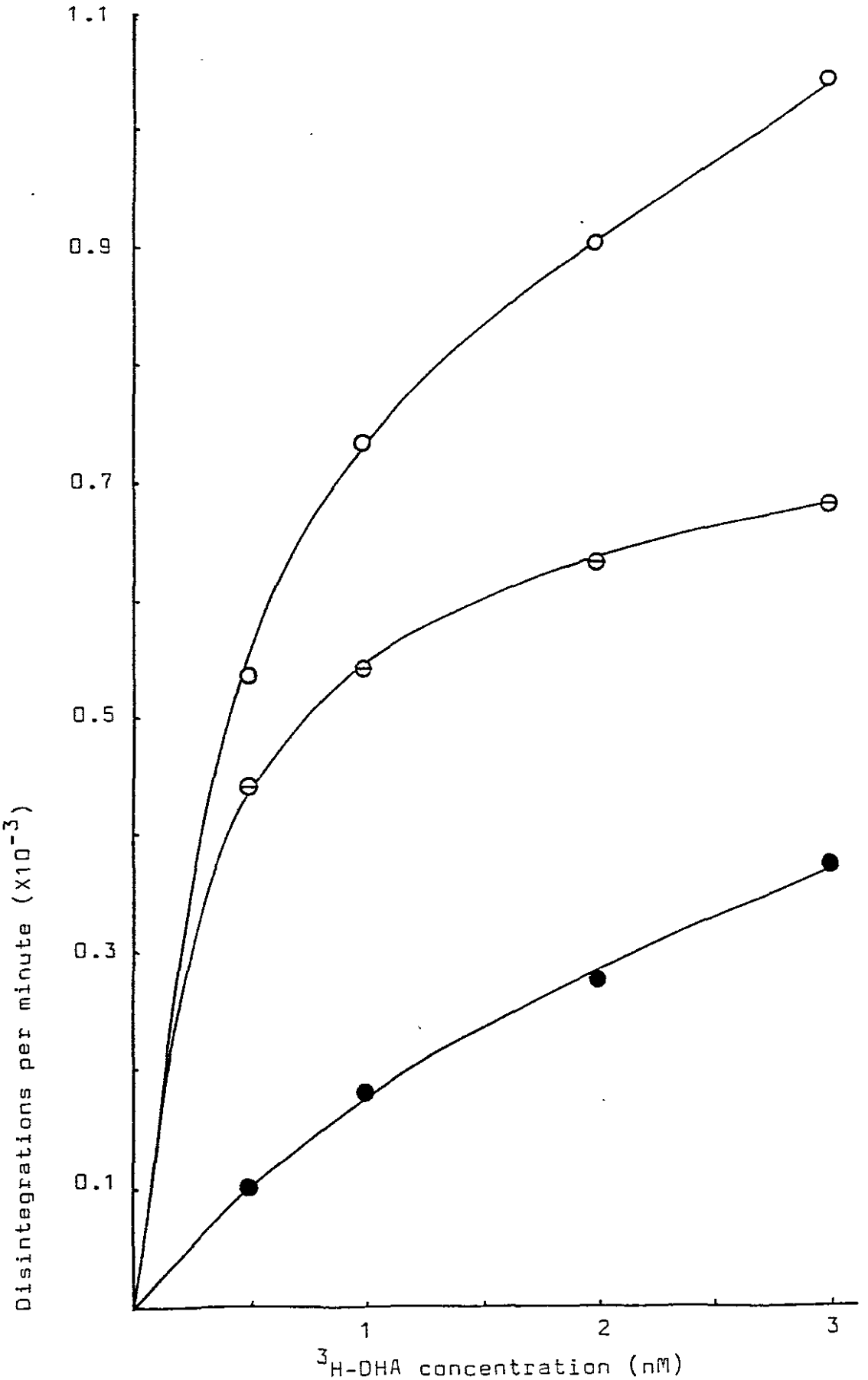


Fig 5.3.6 A representation of ^{3}H-DHA binding to lymphocyte membranes (total binding —o—; non-specific binding —•—; specific binding —e—).

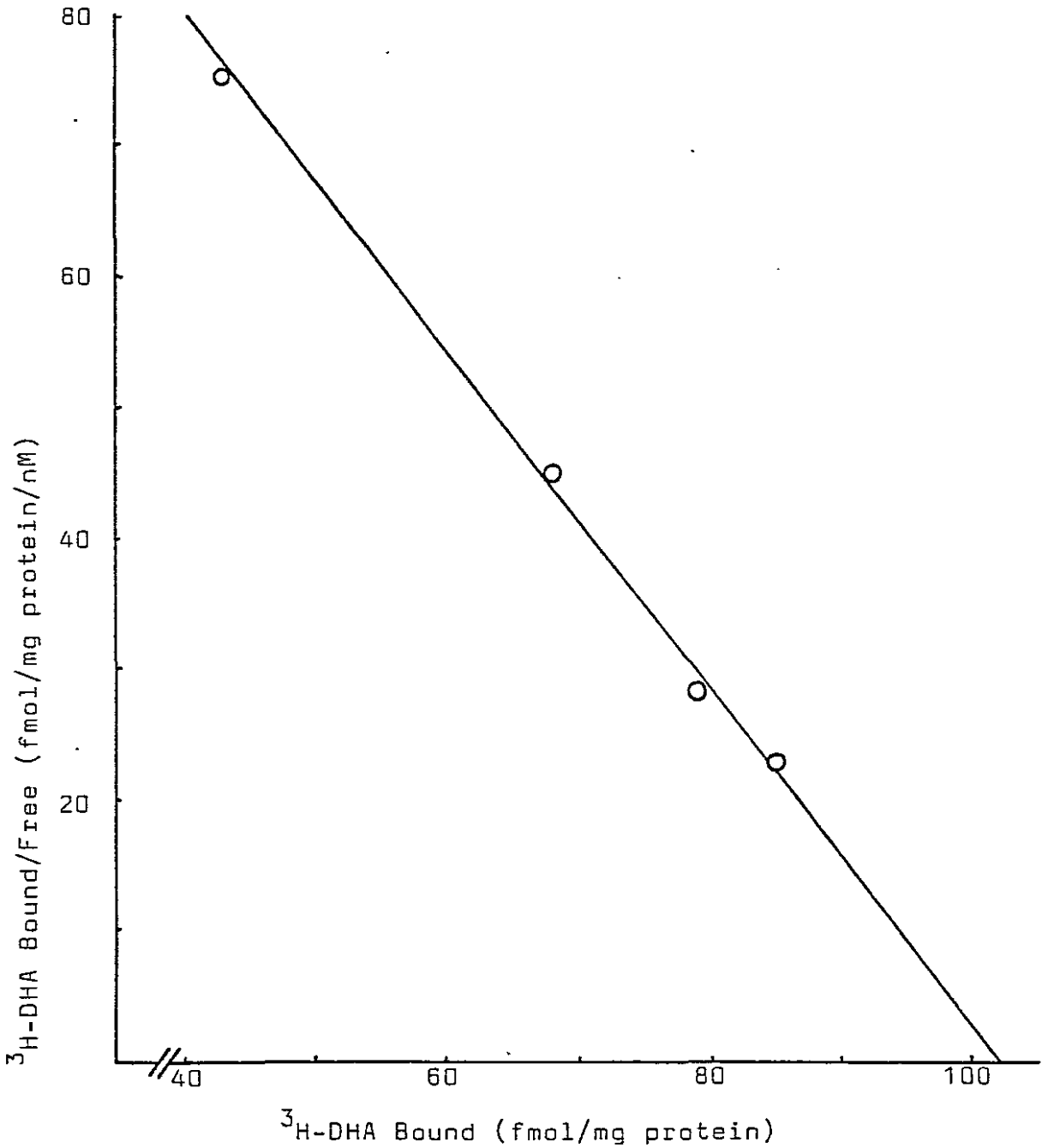


Fig 5.3.7 A representative Scatchard plot of ^{3}H-DHA binding to lymphocyte membranes.

TABLE 5.3.1

Kd values of ^{3}H-DHA binding to lymphocyte membranes of normal, healthy controls and children with major depressive disorder (numbers and ages included).

Population	Males			Females			
	n	Age mean±SD	Kd (nM) mean±SD	median	n	Age mean±SD	Kd (nM) mean±SD	median
Controls	11	16.5±1.4	0.60±0.27	0.61	12	16.3±0.5	1.03±0.4 [a]	1.03
MDD	5	13.2±1.9	0.84±0.32	0.85	7	13.6±2.9	0.56±0.13 [b]	0.56
MDDS	2	13.0±4.2	0.51±0.24	0.51	11	16.3±2.5	0.67±0.58 [b]	0.44
MDD+MDDS	7	13.1±2.3	0.75±0.32	0.68	18	15.2±2.9	0.62±0.46 [c]	0.52

MDD: major depressive disorder

MDDS: major depressive disorder and a suicide attempt.

[a] Significantly different from male controls ($p < 0.02$)

[b] Significantly different from female controls ($p < 0.05$);

[c] $p < 0.02$.

der and a suicide attempt ($p < 0.05$), as well as a combination of the two subgroups ($p < 0.02$, Mann-Whitney U test).

Table 5.3.2 shows the β-adrenoceptor Bmax values for the different psychiatric populations and the controls. Patients with major depressive disorder had significantly higher Bmax values than controls ($p < 0.02$), as did a combination of patients with major depressive disorder with and without a suicide attempt ($p < 0.05$, Mann-Whitney U test).

Scattergrams of the β-adrenoceptor Kd and Bmax values of the control and patient populations are depicted in figures 5.2.8 and 5.2.9, respectively. Representative Scatchard plots of the data are shown in figure 5.2.10.

Neither seasonal, nor age variation was observed for either the β-adrenoceptor Kd or Bmax values of controls and patients (results not shown).

TABLE 5.3.2

Bmax values of ^{3}H-DHA binding to lymphocyte membranes of normal, healthy controls and children with major depressive disorder (numbers and ages included).

Population	n	Age (yrs) mean±SD	Bmax (fmol/mg protein) mean±SD	median
Controls	23	16.4±1.0	42.5±9.8	38.0
MDD	12	13.4±2.5	50.8±10.9[a]	51.3
MDDS	13	15.8±2.8	47.1±9.61	48.5
MDD+MDDS	25	14.6±2.9	48.8±10.24[b]	49.1

MDD: major depressive disorder

MDDS: major depressive disorder and a suicide attempt

[a]Significantly different from controls ($p < 0.02$); [b]$p < 0.05$.

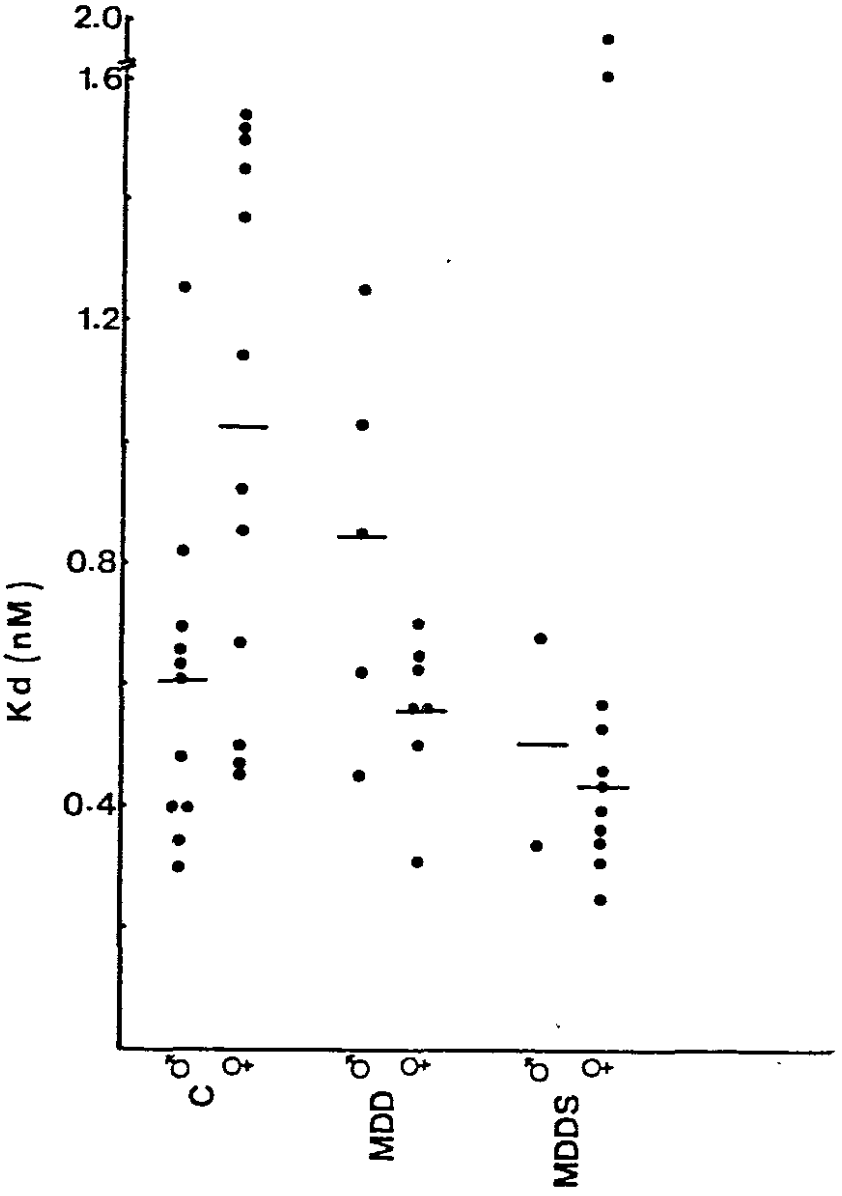


Fig 5.3.8 Scattergrams of lymphocyte β-adrenoceptor Kd values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder

MDDS : major depressive disorder and a suicide attempt.

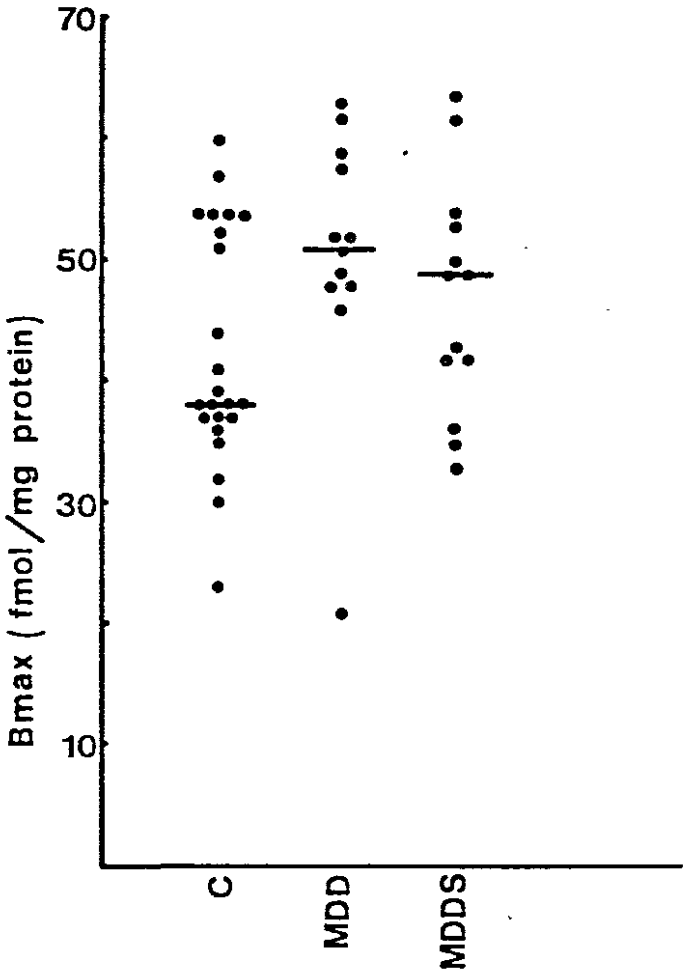


Fig 5.3.9 Scattergrams of lymphocyte β-adrenoceptor Bmax values of controls (c) and patients with the psychiatric conditions indicated below:

MDD : major depressive disorder
MDDS : major depressive disorder and a suicide attempt.

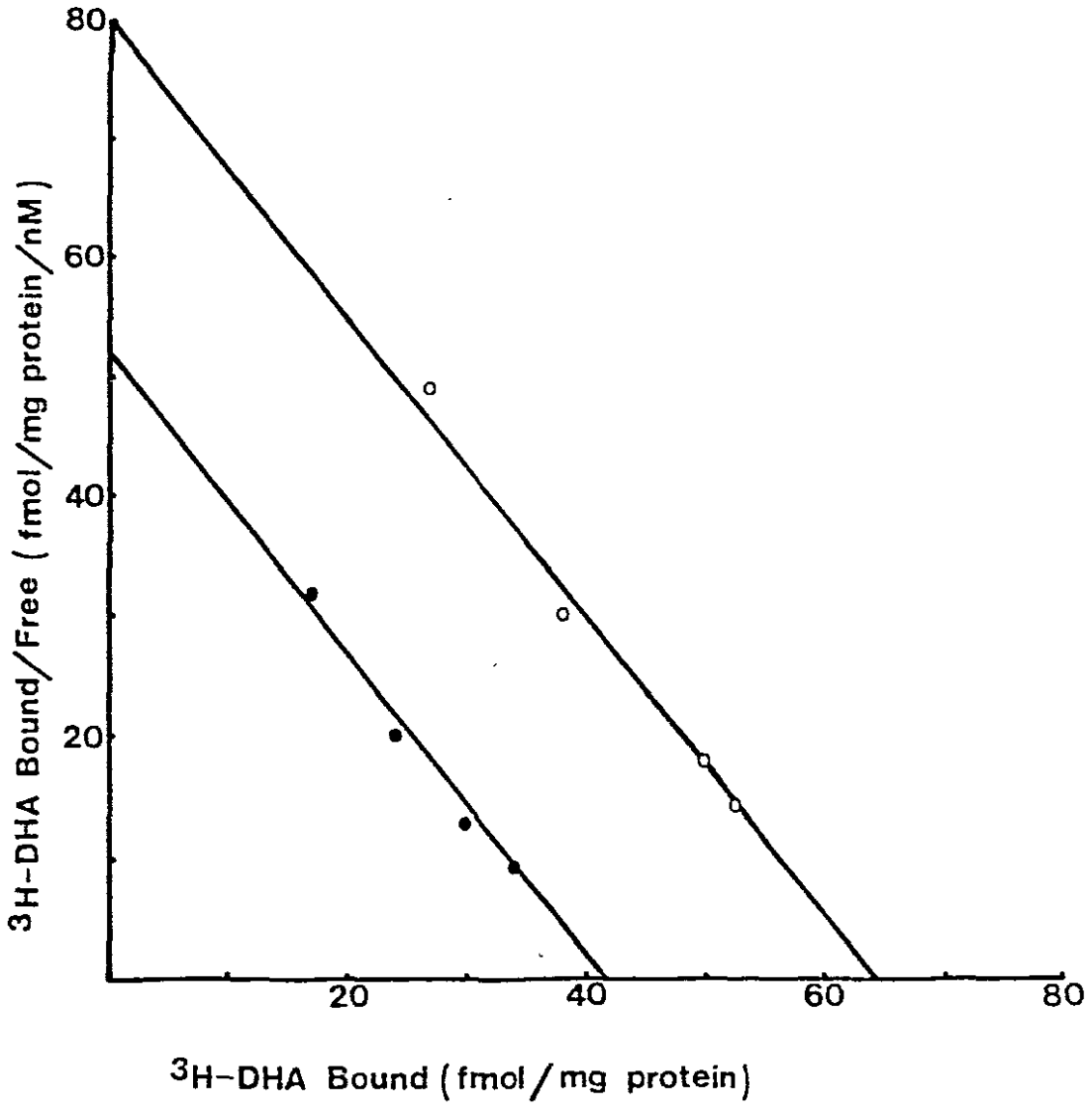


Fig 5.3.10 Representative Scatchard plots of lymphocyte ^{3}H-DHA binding data of controls (-■-■-) and patients with major depressive disorder (-O-O-).

CHAPTER 6

DISCUSSION

6.1 α_2-ADRENOCEPTOR BINDING TO PLATELETS OF CHILDREN AND ADOLESCENTS WITH MAJOR DEPRESSIVE DISORDER.

The agonist, p-aminoclonidine, was chosen to measure platelet α_2-adrenoceptor binding, since it has a greater affinity for the α_2-adrenoceptor than clonidine. This is attributable mainly to a slower dissociation of the analogue from the binding site (Rouot and Snyder, 1979). In addition, the commercially available labelled p-aminoclonidine has a higher specific activity than clonidine. A concentration of 3nM ^{3}H-p-aminoclonidine was used in all characterisation assays, since this concentration was very close to the Kd of the α_2-adrenoceptor for this agonist. Despite the higher specific activity of p-aminoclonidine, only four concentrations of ^{3}H-p-aminoclonidine were used in each α_2-adrenoceptor binding assay, since the binding to platelet membranes was not sufficient to allow the use of concentrations lower than 1nM. On the other hand, concentrations higher than 4nM revealed the presence of the low affinity component of the α_2-adrenoceptor population. (U'Prichard et al, 1983).

The young patients used in this study were selected according to the DSM III criteria for the classification of major depressive disorder (Section 4.2). These patients were kept drug-free for at least three weeks prior to receptor binding studies.

An interesting observation in the present study is the elevated α_2-adrenoceptor Kd values observed in the subgroup of children and adolescents suffering from major depressive disorder with a suicide attempt. The platelet α_2-adrenoceptor Kd values of the total population of children with major depressive disorder were also significantly elevated. We would like to propose that these raised α_2-adrenoceptor Kd values may possibly serve as a trait marker for suicidality in these young patients.

In addition to the finding mentioned above, it was also noted that the α_2-adrenoceptor Bmax values of the total population of children and adolescents with major depressive disorder were significantly greater than those of controls. Once again there was a greater tendency for patients with major depressive disorder with a suicide attempt to have higher α_2-adrenoceptor Bmax values than those without a suicide attempt. These elevated α_2-adrenoceptor Bmax values may, in conjunction with the elevated Kd values, serve as a biological marker for depression in children and adolescents and may possibly give an indication of the severity of the disorder and a tendency towards suicidal behaviour. Suicidal behaviour has been associated with monoaminergic dysfunction in adults (Agren, 1983).

The strikingly greater variance in patient Bmax values, seems to stress the noradrenergic abnormality in these children. Increased variances in the platelet α_2-adrenoceptor Kd and Bmax values of adults with major depressive disorder were recently reported (Carstens et al, 1986a). It was also shown in depressed adults (Halaris et al, 1984) that the noradrenergic output was variable and unstable over hours and days.

These authors measured plasma MHPG levels at 3 hour intervals over a 24 hour period in the depressed patients and observed not only a phase advance in plasma MHPG concentrations in these patients, but also multiple erratic unexplained MHPG peaks during the course of the 24 hours. This suggested that noradrenaline release was dysregulated (Siever and Davis, 1984). The platelet α_2-adrenoceptor variability may reflect the individual's attempts to cope with a poorly regulated noradrenergic system. The increased α_2-adrenoceptor Bmax values observed in children and adolescents in the present study contrasts with the finding that, in adults with major depressive disorder, the α_2-adrenoceptor Bmax values were significantly lower than control values (Carstens et al, 1986a). This discrepancy may be due to the short duration of the disorder in the younger patient population.

In conclusion, we would like to propose that elevated levels of platelet α_2-adrenoceptor Kd and Bmax values may serve as possible biological markers for children and adolescents with major depressive disorder and a tendency towards suicidality.

6.2 IMIPRAMINE BINDING TO PLATELETS OF CHILDREN AND ADOLESCENTS WITH MAJOR DEPRESSIVE DISORDER

^{3}H-Imipramine binding has frequently been used to provide information concerning 5-HT reuptake sites in platelet membranes of depressed patients (Paul et al, 1981 ; Langer and Raisman, 1983). Characterisation of the binding of imipramine to platelet membranes was carried out in the presence of 1nM ^{3}H-imipramine, since this concentration was in the vicinity of the Kd

value. It was soon noticed that a high and low affinity imipramine binding site existed, and all studies were subsequently carried out in the presence of 0.5nM to 4nM ^{3}H-imipramine only, in order to label the high affinity binding site. This was in agreement with findings reported by Wägner et al (1985). In addition the concentrations of ^{3}H-imipramine used in the present study were limited by the amount of platelet membranes obtained from the patients. All Scatchard plots, however, yielded reasonably straight lines.

The young patients used in this study were selected according to the DSM III criteria for the classification of major depressive disorder. All patients were kept drug-free for at least three weeks prior to receptor binding studies.

No difference was observed between control male and female Kd or Bmax values. Interestingly, significantly elevated imipramine Kd values were observed in children and adolescents with major depressive disorder and a suicide attempt, as well as a combination of these and patients with major depressive disorder without a suicide attempt. The significance of the elevated Kd values in the latter subgroup appears to result from the contribution of the elevated Kd values of the patients with major depressive disorder and a suicide attempt. We would like to propose that raised platelet imipramine Kd values may possibly serve as a trait marker for suicidality in young patients with major depressive disorder.

In addition to the findings mentioned above it was also noted that the imipramine Bmax values of patients with major depressive disorder (with and without a suicide attempt) were signi-

ficantly higher than control values. The present results suggest that elevated imipramine Bmax values may serve as a biological marker for depression in children and adolescents and may possibly indicate the severity of the disorder and a tendency towards suicidal behaviour. Suicidality in depressed adults has been shown to be marked by disturbances in 5-HT activity, as indicated by changes in CSF 5-HIAA levels (Agren, 1983).

The results of the present study are not in agreement with those of Rehavi et al (1984) who found no difference between imipramine Kd or Bmax values of controls and 12 patients with major depressive disorder. Five of these patients were described as having bipolar disorder, depressed type. The discrepant findings could perhaps be due to differences in methodology or the composition of the patient population.

In addition, the present findings are not in agreement with results previously reported for adult patients with primary unipolar major depressive disorder (Carstens et al, 1986b). It is possible, however, that chronic antidepressant treatment may have a long - term effect on the number of platelet imipramine binding sites in adults (Briley et al, 1982). The short duration of the illness in children and adolescents may also account for different responses of the imipramine binding site to serotonergic dysfunction in depression.

In conclusion, we propose that elevated platelet imipramine Kd values may possibly serve as biological markers for suicidality in juvenile major depressive disorder, whereas elevated imipramine Bmax values may serve as biological markers for children and adolescents with major depressive disorder with a tendency towards suicidality.

6.3 β-ADRENOCEPTOR BINDING TO LYMPHOCYTES OF CHILDREN AND ADOLESCENTS WITH MAJOR DEPRESSIVE DISORDER.

Binding of DHA to lymphocyte membranes was characterised in the presence of 1nM ^{3}H-DHA, a concentration similar to the Kd of the β-adrenoceptor. After characterisation of the binding reaction, ^{3}H-DHA binding to lymphocyte membranes of children and adolescents, selected as described in section 6.1, was compared to controls. Employing ^{3}H-DHA concentrations ranging from 0,5 nM to 3nM the study revealed the presence of only one high-affinity β-adrenoceptor binding site.

Lymphocytes have frequently been employed as a model for investigation of β-adrenoceptor activity, since these cells exhibit adenylate cyclase activity which responds to catecholamines with a typical β-adrenergic specificity (Bourne and Melmon, 1971; Williams et al, 1976). In the present study, lymphocyte membranes were used to compare β-adrenoceptor Kd and Bmax values of children and adolescents with major depressive disorder with those of a normal, healthy control group, using ^{3}H-DHA. DHA, a β-adrenoceptor antagonist, has been widely used in its labelled form to measure β-adrenoceptor densities in different tissues (Sugrue, 1983). The maximum DHA concentration used in this study was 3nM, because of increased irreproducibility caused at higher concentrations (Davies and Lefkowitz, 1980). In addition, only four concentrations of the labelled ligand were employed, because of the low lymphocyte membrane yield, as well as the low specific activity of the ligand.

An interesting observation in the present study was the diffe-

rence between the β-adrenoceptor Kd values of control males and females. These values, however, correspond to those determined in a similar study in adults (males: 0.8 ± 0.2nM; females: 1.1 ± 0.6nM; Carstens et al, in press). Comparison of the control male Kd values with those of male patients with major depressive disorder, revealed no significant difference. As far as the female patients are concerned, however, significantly lower β-adrenoceptor Kd values were found in females with major depressive disorder, females with major depressive disorder and a suicide attempt as well as a combination of these two psychiatric subgroups, compared to controls. We would like to propose that the decreased β-adrenoceptor Kd values may possibly serve as a biological marker for depression in young female patients.

In addition to the findings mentioned above, it was also noted that children and adolescents with major depressive disorder, as well as a combination of those with major depressive disorder and major depressive disorder with a suicide attempt had significantly higher β-adrenoceptor Bmax values than controls. The elevated β-adrenoceptor Bmax values observed in patients with major depressive disorder may serve as a biological marker for major depression in children and adolescents. The present observations stress the noradrenergic abnormality in depression as proposed by Siever and Davis (1984). The increased β-adrenoceptor Bmax values found in children and adolescents with major depressive disorder contrast with the finding that, in adults with the same psychiatric disorder, the β-adrenoceptor Bmax values were significantly lower than control values (Carstens et al, in press). This apparent discre-

pancy may be due to the short duration of the illness in the younger patient population.

In conclusion, we would like to propose that decreased β-adrenoceptor Kd values may possibly serve as a biological marker for depression in young females, whereas increased β-adrenoceptor Bmax values may possibly serve as a biological marker for major depressive disorder in children and adolescents.

CHAPTER 7

SUMMARY

In this study, possible peripheral biological markers for major depressive disorder in children and adolescents were investigated. For this purpose, the levels and binding affinities of the α_2-adrenoceptor and imipramine binding site were measured on blood platelets and the β-adrenoceptor on lymphocytes of children and adolescents with major depressive disorder.

Initially the binding reactions were characterised. ^{3}H-p-Aminoclonidine was used instead of ^{3}H-clonidine to determine the α_2-adrenoceptor binding parameters, because of the higher specific activity and higher affinity of this radiolabelled ligand. ^{3}H-Imipramine was used to determine the binding parameters of the imipramine binding site and ^{3}H-dihydroalprenolol for the β-adrenoceptor binding parameters. Subsequently the binding parameters of the three sites were measured in blood samples of patients with major depressive disorder and compared to those of normal, healthy controls.

Diagnosis of depression was based on the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM III, 1980), as well as three rating scales for depression: the Montgomery and Asberg Depression Scale (1979), the Children's Depression Rating Scale (Poznanski et al, 1979) and the Children's Global Assessment Scale (Shaffer et al, 1983) were used.

The α_2-adrenoceptor Kd values of children with major depressive disorder with a suicide attempt were found to be significantly higher than control values, as were those of the total population of children with major depressive disorder. Significantly higher α_2-adrenoceptor Bmax values were observed in the total population of children with major depressive disorder, when compared to controls.

The results obtained for the imipramine Kd determinations were similar to those of the α_2-adrenoceptor Kd determinations. Significantly higher imipramine Bmax values were, however, observed for platelets of children with major depressive disorder, children with major depressive disorder with a suicide attempt and a combination of these two groups.

In the case of the β-adrenoceptor a significant difference was observed between control male and female Kd values. No Kd difference was found between male controls and patients. Control females, however, had significantly greater β-adrenoceptor Kd values when compared to female patients with major depressive disorder, major depressive disorder and a suicide attempt, as well as a combination of the two subgroups. Patients with major depressive disorder had significantly higher β-adrenoceptor Bmax values than controls, as did a combination of patients with major depressive disorder with and without a suicide attempt.

We would like to propose that elevated levels of platelet α_2-adrenoceptor Kd and Bmax values, as well as platelet imipramine Bmax values may serve as possible biological markers

for children and adolescents with major depressive disorder and a tendency towards suicidality. Elevated platelet imipramine Kd values may possibly serve as biological markers for suicidality in juvenile major depressive disorder. Decreased β-adrenoceptor Kd values may serve as a biological marker for depression in young females, whereas increased β-adrenoceptor Bmax values may serve as a biological marker for major depressive disorder in children and adolescents.

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APPENDIX

INSTRUMENTATION

The following instruments were used:

Beckman Model LS-9000 Liquid Scintillation Counter

Beckman pHI 71 pH meter

Gilford Stasar III Spectrophotometer

Heidolph Stirrer

Polytron PCU-2 Homogeniser

Sartorius Top-Loading balance

Sorvall RC-5B Refrigerated Superspeed Centrifuge

Sorvall RC-2B Superspeed Centrifuge

Techne Waterbath

Vortex mixer

HOOFSTUK 7

OPSOMMING:

In hierdie studie is moontlike perifêre biologiese merkers vir major depressiewe siekte in kinders en adolesente ondersoek a.g.v. die onbeskikbaarheid van brein materiaal. Vir hierdie doel is die vlakke en bindings-affiniteite van die α_2-adrenerge reseptor en imipramien bindingsetels gemeet op bloedplaatjies asook die β-adrenerge reseptore op limfosiete van kinders en adolesente met major depressiewe siekte.

Aanvanklik is die bindingsreaksies gekarakteriseer. ^{3}H-p-Aminoclonidine is instede van ^{3}H-clonidine gebruik om die α_2-adrenerge reseptor binding-parameters te bepaal, omdat eersgenoemde 'n hoër spesifieke aktiwiteit en 'n hoër affiniteit het as laasgenoemde. ^{3}H-Imipramien is gebruik om die bindingparameters van die imipramien bindingsetel te meet en ^{3}H-dihidroalprenolol vir die β-adrenerge reseptor bindingparameters. Die bindingparameters van die drie setels is bepaal op bloed monsters van pasiënte met major depressiewe siekte en vergelyk met dié van normale gesonder kontroles.

Depressie is gediagnoseer op grond van die kriteria in die Diagnostiese en Statistiese Handleiding vir Geestessiektes (DSM III, 1980), en drie beoordelings-skale vir depressie nl.: die Montgomery en Asberg Depressieskaal (1979), die Kinder Depressie Beoordeling Skaal (Poznanski et al, 1979) en die Kinder; Wêreld Skattings Skaal (Shaffer et al, 1983).

Kd waardes van α_2-adrenergereseptor by kinders met major depressiewe siekte met 'n selfmoordpoging was betekenisvol hoër as kontrole waardes, en dit was ook hoër as by kinders met major depressiewe siekte in geheel geneem. Betekenisvolle hoër α_2-adrenerge reseptor Bmaks waardes is ook gevind in die totale bevolking van kinders met major depressiewe siekte wanneer vergelyk met kontrole groepe.

Die resultate wat verkry is vir imipramien Kd bepalings was dieselfde as die van α_2-adrenerge reseptor Kd bepalings. Betekenisvolle hoër imipramien Bmaks waardes is egter gevind in bloedplaatjies van kinders met major depressiewe siekte met 'n selfmoorpoging asook 'n kombinasie van hierdie twee groepe.

Met betrekking tot β-adrenerge reseptore is 'n betekenisvolle verskil waargeneem tussen Kd waardes van kontrole seuns en meisies. Geen verskil is tussen Kd waardes van manlike kontroles en pasiënte gevind nie. Vroulike kontroles het egter betekenisvolle groter β-adrenoseptor Kd waardes getoon as vroulike pasiënte met major depressiewe siekte, major depressiewe siekte met 'n selfmoord-poging, asook 'n kombinasie van hierdie twee subgroepe.

Pasiënte met major depressiewe siekte het betekenisvolle hoër β-adrenerge reseptor Bmaks waardes getoon as kontrole-groepe, asook ten opsigte van 'n kombinasie van pasiënte met major depressiewe siekte met en sonder 'n selfmoordpoging.

Ons stel voor dat verhoogde bloedplaatjie α_2-adrenerge reseptor Kd en Bmaks waardes, asook plaatjie imipramien Bmaks waardes

moontlik gebruik kan word as 'n biologiese merker vir major depressiewe siekte en 'n neiging tot selfmoord in kinders en adolessente. Verhoogde plaatjie imipramien Kd waardes kan moontlik dien as 'n biologiese merker vir selfmoord neiging onder jeugdiges met major depressiewe siekte. Verlaagde β-adrenerge reseptor Kd waardes kan moontlik dien as 'n biologiese merker vir depressie by jong meisies, terwyl verhoogde β-adrenerge reseptor Bmaks waardes gebruik kan word as 'n biologiese merker vir major depressiewe siekte by kinders en adolessente.

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