THE UNIVERSITY OF SOUTHAMPTON

PHARMACOLOGICAL CHARACTERISATION

OF

RENAL DOPAMINE RECEPTORS

by

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A thesis presented for the degree of Doctor of Philosophy

in the Faculty of Science of the

University of Southampton.

Dedicated to Mum, Dad, Mark and Chris.

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ABSTRACT

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The population of specific vascular dopamine receptors thought to mediate renal vasodilatation has been studied in the prazosin pre-treated guinea pig. The increase of cortical renal blood flow produced by dopamine was compared with the ability of the catecholamine to stimulate cAMP production in renal particulate preparations. In addition, radioligand binding studies have been used to investigate the interaction of [^{3}H] sulpiride, a novel dopamine receptor antagonist of the substituted benzamide group, with renal cortical membranes.

Renal vascular responses to dopamine, ADTN and SKF 38393 were (±)-propranolol resistant but were specifically inhibited by (±)-sulpiride. Similarly the elevation of cAMP content produced by these agonists was unaffected by (±)-propranolol and atenolol but was powerfully inhibited by the classical neuroleptic fluphenazine. In contrast (±)-sulpiride, the highly specific antagonist of renal vasodilator responses to agonists was completely ineffective at inhibiting dopamine-sensitive adenylate cyclase activity. Analogous results obtained in the study of dopamine receptors in the central nervous system have been interpreted as evidence for the existence of two subtypes of dopamine receptors (Kebabian and Calne, 1979); one linked to a stimulatory adenylate cyclase and unaffected by sulpiride and the other non-cyclase linked and selectively inhibited by sulpiride. In order to clarify these anomolous results the possibility that (±)-sulpiride might function as an antagonist by exerting its effects on a subclass of adenylate cyclase-independent dopamine receptors was investigated using radioligand labelling techniques. [^{3}H] sulpiride bound selectively and with high affinity to membranes prepared from dopaminergic regions of the kidney in a stereospecific, saturable manner. The pharmacological profile of this binding component provided corroborative evidence of a highly specific interaction between sulpiride and dopaminergic sites but failed to vindicate sulpiride as a selective antagonist of the non-cyclase linked receptor. Possible modes of action of sulpiride at the basic receptor level are discussed as are the advantages of combining the measurement of physiological responses with direct biochemical models in the study of neurotransmitter receptors.

CONTENTS

ABBREVIATIONS

ADTN	2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene
isoADTN	2-amino-5-6-dihydroxy-1,2,3,4-tetrahydronaphthalene
cAMP	adenosine 3':5' cyclic monophosphate
ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
Bmax	maximum binding capacity
CNS	central nervous system
DA	dopamine
DPM	disintegrations per minute
EC_{50}	concentration stimulating by 50%
ED_{50}	dose stimulating by 50%
GABA	gamma-aminobutyric acid
GTP	guanosine 5'-triphosphate
$[^3H]$	tritium
H_2	hydrogen gas
hr	hour
5HT	5-hydroxytryptamine
IC_{50}	concentration inhibiting by 50%
i.p.	intra-peritoneal
i.v.	intra-venous
Kd	dissociation constant
Ki	inhibition constant
kg	kilogram
ml	millilitre
min	minute
%max	percentage of maximum
MABP	mean arterial blood pressure
NA	noradrenaline
NS	not statistically significant
p	statistical significance
RBF	renal blood flow
SKF 38393	2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine
SEM	standard error of mean
tris	2-amino-2(hydroxymethyl)-propane-1,3-diol
w/v	weight to volume

CHAPTER I

INTRODUCTION

1-1 Dopaminergic neurotransmission

1-1-1 Dopamine as a neurotransmitter

Dopamine (DA) is an established neurotransmitter in the mammalian central nervous system (CNS). It also has important peripheral physiological actions especially in the kidney where DA appears to be involved in the regulation of renal blood flow (RBF) and possibly renal sodium, water and renin excretion.

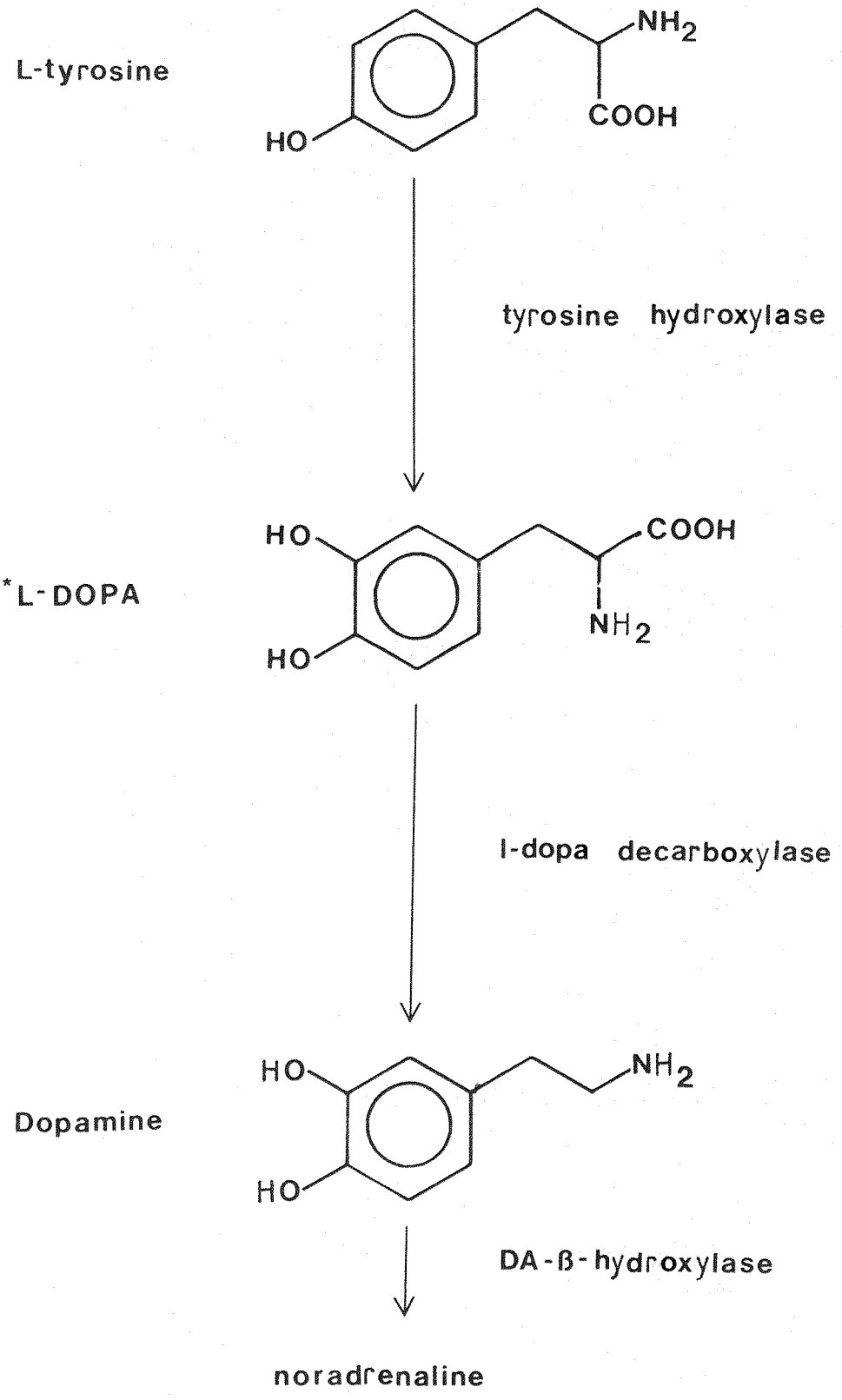
1-1-2 Dopamine synthesis

Of the catecholamines present in sympathetic nerve endings, DA, which was originally thought to be important only as a precursor for noradrenaline (NA), has now been shown to have major involvement in both central and peripheral neural transmission (see reviews by Ungerstedt, 1978; Woodruff, 1978; Goldberg et al, 1978a). The precursor for catecholamine synthesis is tyrosine, a naturally occurring amino acid found in neurones, the location of DA synthesis. Tyrosine is hydroxylated in the 3 position to give dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, the rate limiting enzyme of catecholamine synthesis (Figure 1.1). Tyrosine hydroxylase requires tyrosine, molecular oxygen and a reduced pterin co-factor for activity (Waggoner et al, 1980). End product feedback regulation of tyrosine hydroxylase by catecholamines is considered a major mechanism for control of DA synthesis (Levitt et al, 1965; Nagatsu et al, 1964). DA is thought to compete with the pterin co-factor for binding to the enzyme; activation of certain DA receptors may produce allosteric changes in tyrosine hydroxylase activity (Zivkovic et al, 1974) possibly by phosphorylation by an adenosine-3':5'-cyclic monophosphate (cAMP) dependent protein kinase (Joh et al, 1978; Yamauch and Fujisawa, 1979). L-DOPA is then decarboxylated to DA by L-aromatic acid decarboxylase (L-DOPA decarboxylase) and stored in vescicles or granules. In neurones using NA as their neurotransmitter DA is further hydroxylated by dopamine β-hydroxylase (see review by McGeer and McGeer, 1973).

1-1-3 Release and inactivation of dopamine

Depolarisation of the neural membrane releases DA which diffuses across the synaptic cleft to interact with receptors located on the postsynaptic membrane. DA is subsequently inactivated by several means.

Figure 1-1 Biosynthesis of dopamine



* L-DOPA (dihydroxyphenylalanine)

Most important is active re-uptake back into presynaptic storage vessels. Alternatively, DA can be metabolised by either of two major degradative pathways, both resulting in the acid metabolite homovanillic acid (HVA) (Figure 1.2). Either mitochondrial momo-amine oxidase (MAO) oxidises the ethylamine side chain of DA to give 3,4-dihydroxyphenylacetaldehyde followed by conversion to 3-4-dihydroxyphenylacetic acid (DOPAC), or the predominantly extraneuronal DA metabolising enzyme, catechol-o-methyl transferase (COMT), methylates the 3-hydroxyl group on the phenolic ring of DA to produce O-methyl DA. DOPAC and O-methyl DA are broken down by COMT and MAO respectively to produce HVA, the latter reaction again operating via an aldehyde oxidation step.

Precise regulation of homeostatic mechanisms controlling the amount of neurotransmitter released into the synapse is crucial since the functional activity of a nervous system depends upon the frequency and propogation of nerve impulses from neurone to neurone. Firstly, dopaminergic activity is controlled by 'long loop' neuronal feedback, for example in the basal ganglia an inhibitory mechanism probably operates via postsynaptic GABAminergic neurones. Secondly, 'short loop' mechanisms control DA synthesis and release more locally at the level of the synaptic cleft. It seems well established that DA synthesis is regulated at the level of tyrosine hydroxylase (Kehr et al, 1972; Iversen et al, 1976; Westfall et al, 1976), this seems a logical arrangement for the rate limiting enzyme of catecholamine synthesis. However, authors disagree about how physiological control of DA release via changes in tyrosine hydroxylase activity is achieved. The existence of presynaptic DA receptors (autoreceptors), activated by DA, present in excess in the synaptic cleft, to depress its own additional release has been postulated (see review by Langer, 1977). Furthermore, autoreceptor mediated inhibition of DA release is mimicked by exogenous agonists and diminished by antagonists (Farnebo and Hamberger, 1971; Westfall et al, 1979). However, this concept is opposed by a hypothesis suggesting that intraneuronal DA re-uptake is the predominant mechanism modulating a local negative feedback inhibition of DA release (Seeman and Lee, 1975; Raiteri et al, 1978) because DA release was depressed rather than augmented by neuroleptics.

In addition to autoreceptors located upon cell bodies or presynaptic membranes, presynaptic DA receptors may also be present in noradrenergic nerve terminals (Langer, 1973) which serve to inhibit NA

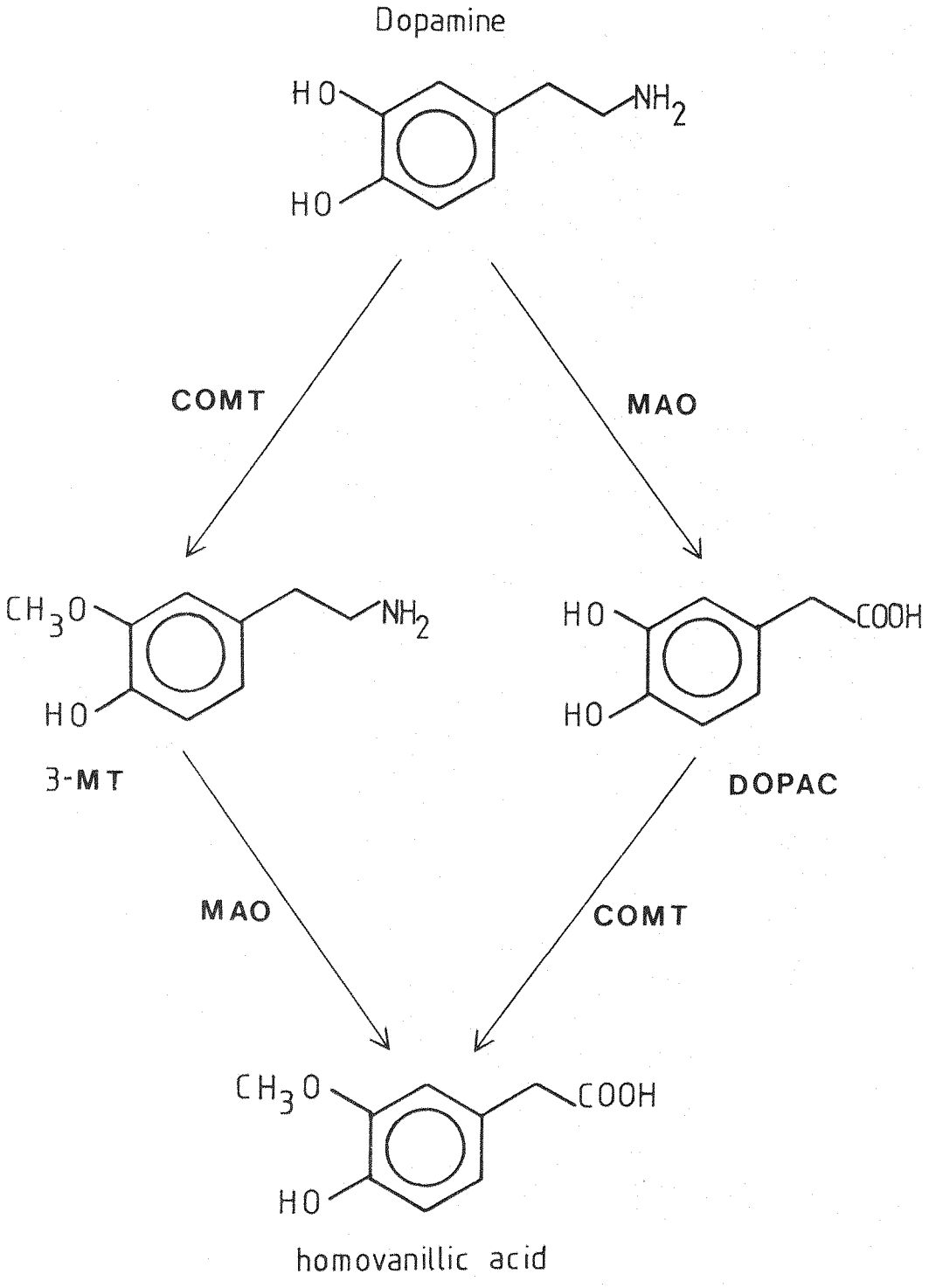


Figure 1.2 Degradation of dopamine.

3-MT, 3-methoxy tyramine; DOPAC, 3,4-dihydroxyphenyl-acetic acid; MAO, monoamine oxidase; COMT, catechol-O-methyl transferase.

TABLE 1.1: Summary of the physiological functions of each dopaminergic pathway together with the conditions thought to arise in man from imbalances of dopaminergic neurotransmission. The table is simplified for clarity, however, it should be appreciated that there is a complex interdependence between dopaminergic systems.

	NIGROSTRIATAL	MESOLIMBIC	MESOCORTICAL	TUBEROINFUNDIBULAR
CELL BODIES	pars compacta of substantia nigra	ventral tegmentum	ventral tegmentum regions	arcuate nucleus
TERMINALS	caudate nucleus of neostriatum	nucleus accumbens and stria terminalis olfactory tubercle	limbic cortex	median eminence
PHYSIOLOGICAL FUNCTION	regulates extra-pyramidal system	regulates some behaviour	?	regulates prolactin secretion
OVERACTIVITY	tardive dyskinesia ?	? psychosis with stereotypes	? psychosis	prolactin secretion falls
UNDERACTIVITY	Parkinson's disease	? antipsychotic	? antipsychotic	prolactin rises

? = uncertain in the literature

release (see review by Langer, 1981).

1-2 Role of dopamine in the CNS

1-2-1 The organisation and physiology of central dopaminergic neurones in the mammalian brain

DA has now justified its proposed function as a central neurotransmitter by fulfilling most of the physiological and pharmacological criteria demanded of such a role. DA is localised intraneuronally, the pertinent neuronal tracts being coincidentally distributed throughout the CNS (Carlsson, 1959) as demonstrated by a combination of highly sensitive biochemical and mechanical or chemical lesioning studies (Ungerstedt, 1971a; Dahlström and Fuxe, 1965; Lindvall and Björkland, 1974; 1978). The visualisation of afferent and efferent pathways has been achieved by retrograde axoplasmic transport of injected proteins such as horseradish peroxidase or orthograde transport of amino acids. The enzymes necessary for metabolism regulation of DA are locationally coincidental with these discrete DA neurones (Axelrod, 1966; Hornykiewicz, 1966). Also the fundamental homeostatic principles of the DA synapse are relatively well understood. DA, but not NA, is released into the synaptic cleft in response to depolarising stimuli and elicits a physiological response after binding to a postsynaptic receptor. Excess DA subsequently undergoes inactivation via re-uptake or enzymic degradation (see section 1-1-3). Specific drugs (DA receptor agonists or neuroleptics) directed at the basic receptor level will either mimic or antagonise the actions of DA (for review see Langer, 1981). The origins and projections of central dopaminergic tracts in the rat brain are shown in figure 1.3.

1-2-2 The involvement of dopamine in CNS diseased states

Dopamine has an important role in the aetiology of diseased states in the CNS such as Parkinson's disease and schizophrenia. Parkinson's disease is associated with a degeneration of the nigrostriatal tract, resulting in a loss of DA from the striatum (Table 1.1). The consequent cholinergic dominance in this area causes the classic symptoms of akinesia, tremor and rigidity. In contrast to Parkinson's disease, schizophrenia is a mental disorder, characterised by hallucinations, delusions, a disturbance of emotions, affective responses to the environment and a withdrawal from contacts. Schizophrenia might be due to

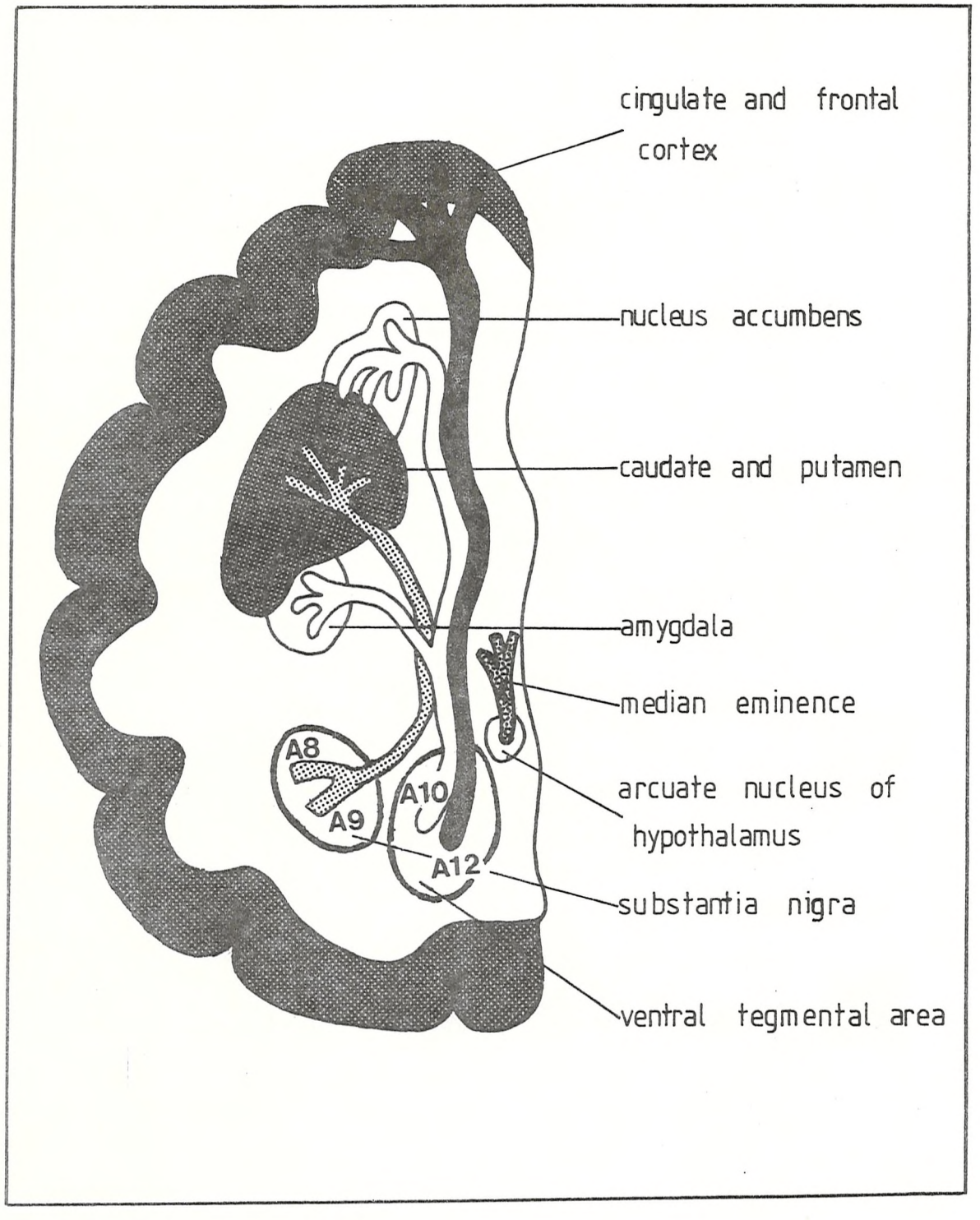
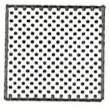
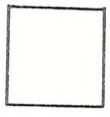

cingulate and frontal cortex
nucleus accumbens
caudate and putamen
amygdala
median eminence
arcuate nucleus of hypothalamus
substantia nigra
ventral tegmental area
A8
A9
A10
A12

FIGURE 1.3 Origins and projections of central dopaminergic tracts in the rat brain (Modified from Lindvall and Björkland, 1978; Ungerstedt, 1971a; 1978).



The nigrostriatal pathway has duel origins in the A8 and A9 cell bodies in the area of the zona compacta of the substantia nigra. The tract ascends through the lateral hypothalamus and fans out into the caudate and putamen of the neostriatum and probably also the globus pallidus and to the central portion of the amygd aloid nucleus. These dopaminergic neurones terminate on small cholinergic interneurones. The nigrostriatal pathway is thought to regulate the extrapyramidal nervous system's control of motor movements.



The mesolimbic pathway arises from A10 cell bodies in the ventral tegmental area (VTA) and innervates the amygdala, nucleus accumbens just anterior to the caudate nucleus, stria terminalis and olfactory tubercule.

The tuberohypophyseal pathway is short; it arises from cell bodies in the A12 area of the arcuate nucleus and in the part of the periventricular nucleus lying dorsal to the arcuate nucleus. These neurones mainly innervate the median emminence, the stalk, neural lobe and pars intermedia of the adenohypophysis. This pathway exerts an inhibitory effect on the release of prolactin from the anterior pituitary.

The mesocortical pathway originates from cell bodies in the substantia nigra and VTA and its projections are subdivided into two. Firstly, the allocortical pathway innervates the olfactory tubercule, septum, interstitial nucleus of the stria terminalis and the amygdala. Secondly, the neocortical subdivision innervates the suprahinal cortex, pregenual and supragenual anteriomedial cortex and ventral entorhinal cortex. This pathway and the mesolimbic tract are not fully understood but indirect evidence suggests an involvement in autonomic, affective, memory and learning functions (see review by Hornykiewicz, 1978).

There are also smaller dopaminergic tracts in the retina and olfactory lobe, providing localised innervation.

dopaminergic overactivity in the limbic system.

The discovery that neuroleptic drugs such as chlorpromazine or haloperidol alleviate psychotic symptoms in schizophrenic patients (Carlsson and Lindqvist, 1963; Carlsson, 1978), whereas L-DOPA, amphetamine or methylphenidate enhance the symptoms of schizophrenia (van Rossum, 1966) and elicit hallucinations in Parkinsonian patients (Goodwin, 1972), provided early circumstantial evidence that neuroleptic drugs block DA receptors. Drugs used in the treatment of these mental disorders have been aimed at, in the one case, raising DA levels by the use of DA agonists and L-DOPA or, in the other, by reducing DA content with the aid of neuroleptics directed at the basic postsynaptic receptor level. This topic is extensively reviewed by van Rossum (1967) and Hornykiewicz (1975). Neuroleptics selectively accelerated DA synthesis and turnover (Da Prada and Pletscher, 1966), as reflected by raised levels of the DA metabolites HVA and DOPAC (Westerink, 1978; Matthysse, 1973). This stimulation was originally attributed to feedback augmentation of DA turnover (Carlsson, 1965) to overcome DA receptor blockade by neuroleptics but is currently assigned to a presynaptic action of neuroleptics. Consequently low doses of DA agonists might be beneficial to schizophrenic patients by preferentially activating DA autoreceptors, thereby promoting a reduction of DA synthesis and release (Clow et al, 1980; Langer and Dubocovich, 1979). In support of this view, it was recently reported that administration of low doses of apomorphine to chronic schizophrenic patients resulted in a significant improvement in their psychotic symptoms (Tamminga et al, 1978; Di Chiara et al, 1978; Serra et al, 1979).

1-3 Role of dopamine in the periphery

1-3-1 Dopamine receptors in peripheral vascular beds (other than the kidney)

There is considerable evidence for the presence of DA receptors in mesenteric, coronary, cerebral, femoral, hepatic and splenic vascular beds (Goldberg, 1972; Edvinsson et al, 1978; Pendleton and Setler, 1977; Toda, 1976; Day and Blower, 1975; McCulloch and Harper, 1977; Kitzen et al, 1978; Richardson and Withrington, 1978; Clark and Menninger, 1980; Bell and Lang, 1973; Bell and Stubbs, 1978; Kullman et al, 1979).

However, the presence of a DA receptor in vascular smooth muscle

does not necessarily mean that DA serves it as a transmitter (see Section 1-2). Apart from in the kidney direct histochemical evidence substantiating dopaminergic innervation has been shown only in vascular structures of the canine paw pad (Bell, Lang and Laska, 1978b). The actions of DA in the periphery are complicated by its ability to stimulate other receptors such as α- and β-adrenoceptors, as described in Section 1-5-2. Small doses of DA when injected into some species cause a fall in mean arterial blood pressure (MABP) (Hornykiewicz, 1958; Gründ et al, 1978). This vasodilatory effect of DA on the circulation is predominantly due to its effects on specific vascular receptors for DA (Eble, 1964; McNay et al, 1965) and to a lesser extent on presynaptic feedback inhibition of NA release at the sympathetic nerve endings (Enero and Langer, 1975; Rand et al, 1975)

1-3-2 Sympathetic nervous system involvement in vascular responses to dopamine

The overall fall in MABP observed in many species can be further subdivided as resulting from the actions of DA upon:-

1) Specific postsynaptic vascular receptors for DA (see Section 1-5-2) and β-adrenoceptors.

2) Presynaptic feedback inhibition of NA release at sympathetic nerve endings.

3) Sympathetic ganglia (Libet and Tosaka, 1970).

4) Inotropic cardiac stimulation by direct and indirectly mediated sympathetic effects (Einstein and Barrett, 1977; Gorczynski et al, 1979).

The strongest evidence to date is in support of the postsynpatic vascular DA receptor (see Section 1-5-5 and reviews by Goldberg, et al, 1979a;b; Pendleton and Setler, 1977). The presence of sites resembling presynaptic DA receptors which constitute a presynpatic inhibitory system for noradrenergic transmitter release is generally accepted. The presence of such a mechanism has been demonstrated both *in vivo* and *in vitro*. Activation of presynaptic DA receptors in sympathetic nerves innervating arteries and veins of several species *in vitro*, inhibits NA release and vasoconstrictory responses to sympathetic nerve stimulation (Buylaert and Willems, 1976; Ziegler et al, 1979). *In vivo* DA induced neurogenic vasodilation is the manifestation of a passive withdrawal of tonic sympathetic discharge to the vasculature (Lokhandwala and

Jandhyala, 1979; Bogaert et al, 1978; Sanders and Ross, 1975; Bell, 1979). In addition, it has been proposed that certain mammalian sympathetic ganglia contain dopaminergic interneurones (Libet & Tosaka, 1970). *In vivo* DA produces reversible inhibition of synaptic transmission in lumbar ganglia and the inferior mesenteric ganglion of the dog, an effect which is selectively inhibited by the dopaminergic antagonist, haloperidol (Lins and Willems, 1974; Bogaert et al, 1977).

Cardiac effects

Although the net effect of peripheral DA receptor stimulation is a reduction of vascular smooth muscle tone, a principle feature of DA induced fall in blood pressure is that it does not evoke reflex tachycardia. The reason for this lack of cardiac acceleration is not immediately apparent, since the mechanism of action of DA upon the heart is complex and has been shown to involve both direct stimulation of postsynaptic β-adrenoceptors and indirect release of NA.

a) Indirect effects

DA is an inotropic selective cardiac stimulant which means that it increases cardiac contractility at doses which produce little or no change in heart rate (Clark et al, 1978; Mugelli et al, 1977; Chiba, 1978; Goldberg, 1972). DA also inhibits the sympathetic nervous supply to the heart by its actions on both presynaptic α-adrenoceptors and dopaminergic receptors (see Section I-5-2). Therefore, it was suggested that DA's selective action on cardiac contractility was due to preferential release by DA of NA from adrenergic nerves located in the ventricular or contractile myocardium (Tuttle and Mills, 1975; Gorczynski et al, 1979). Desmethylimipramine (DMI), which blocks amine uptake at adrenergic nerve endings (Thoenen et al, 1964), diminished the inotropic selectivity of DA.

b) Direct effects

The chronotropic effect of DA is manifested at higher doses and is probably due to direct stimulation of postsynaptic β-adrenoceptors located in the sino-atrial or pacemaker region of the heart.

However, the mechanism responsible for the differential effect of

DA on the heart has not been conclusively identified. Two theories have been postulated to explain DA's inotropic selectivity. The first is based upon differences in NA stores in the cardiac adrenergic nerves and the second is based upon differences in presynpatic DA receptor distribution on sympathetic nerve terminals in the heart.

Firstly, on the basis of there being more NA in nerves supplying ventricular muscle than the sino-atrial node (Angelakos et al, 1969), Tuttle and Mills (1975) have speculated that there is insufficient NA available for release in the pacemaker region to mediate an indirect chronotropic effect by DA. According to this view, DA is readily taken up by all cardiac adrenergic nerves, but can only release substantial amounts of NA from nerves in the ventricles. This results in an increased cardiac contractility, without an accompanying increase in heart rate.

The second theory is based upon the ineffectiveness of the DA receptor stimulant, bromocriptine, upon contractile force but not heart rate (after division of cervical vagi and stellate ganglia to prevent reflex changes in resting rate and contractility in dog) (Clark and Menninger, 1980). Bradycardia is not affected by vagotomy but is absent after blockade of DA receptors or β-adrenoceptors suggesting depression of noradrenaline release from cardiac sympathetic nerves. These authors suggested that presynpatic DA receptors are present in nerves innervating the sino-atrial node but not the ventricles, thus possibly explaining the lack of effect of DA upon cardiac contractile force.

1-4 Physiology of the kidney

1-4-1 Function

The kidney plays a crucial role in the regulation of body water content with the aid of anti-diuretic hormone (ADH) secreted from the hypothalamo-neurohypophysial system, the renin-angiotensin system, prostaglandins, aldosterone and other adrenocorticoids. This regulation is closely integrated with waste excretion, and electrolyte and acid-base balance. Acid metabolites are excreted by an ion exchange mechanism where a hydrogen ion is secreted in exchange for a sodium ion and ammonia is secreted by tubular cells to replace base. The

kidney excretes wastes such as urea, uric acid, creatinine and creatine, whilst selectively reabsorbing glucose and amino acids. Other metabolic functions such as amino acid oxidation and deamination, conjugation of glycocol and benzoic acid to hippuric acid also occur in tubular cells. The functional unit of the kidney is the nephron which consists of a Bowman's capsule in close contact with capillary loop networks (glomeruli) important in filtration (Figure 1.6).

1-4-2 Anatomy

Each kidney is compartmentalised; the three main divisions being the cortex, inner and outer medulla and pelvis which are supplied with blood from the renal artery (Figure 1.4). Each renal artery divides into interlobar arteries, which subdivide along consecutive vascular segments into arcuate arteries and then interlobular arteries (Figure 1.5). Afferent arterioles arise from these to supply blood to the glomeruli. The glomerular capillaries converge in efferent arterioles, these in turn form a second capillary bed around the convoluted tubules, which drain into interlobular, arcuate and interlobar veins. Figure 1.6 compares the blood supply of cortical and juxtamedullary nephrons. It has been suggested that nephron segments in the outer cortex have a higher blood flow than those in the inner and juxtamedullary regions, and that this is related to differences in function.

The distribution of RBF among outer and inner cortical layers has been the subject of many studies (see reviews by Aukland, 1976; 1980a; Lameire et al, 1977). The increase in sodium excretion following infusion of isotonic saline has been attributed to redistribution of renal cortical blood flow from 'salt retaining' nephrons in the deep layer to 'salt losing' nephrons in the superficial cortical layer (Barger, 1966; Barton et al, 1968). This observation has been disputed by Munck et al (1970) and Løyning (1974), who found total RBF is increased during saline infusion but no difference in distribution of local blood flow in these two cortical regions after saline loading. This latter observation suggests that inner and outer cortical arteriolar vasodilation is equally sensitive to variations in perfusion pressure (Aukland, 1980a; Clausen et al, 1980).

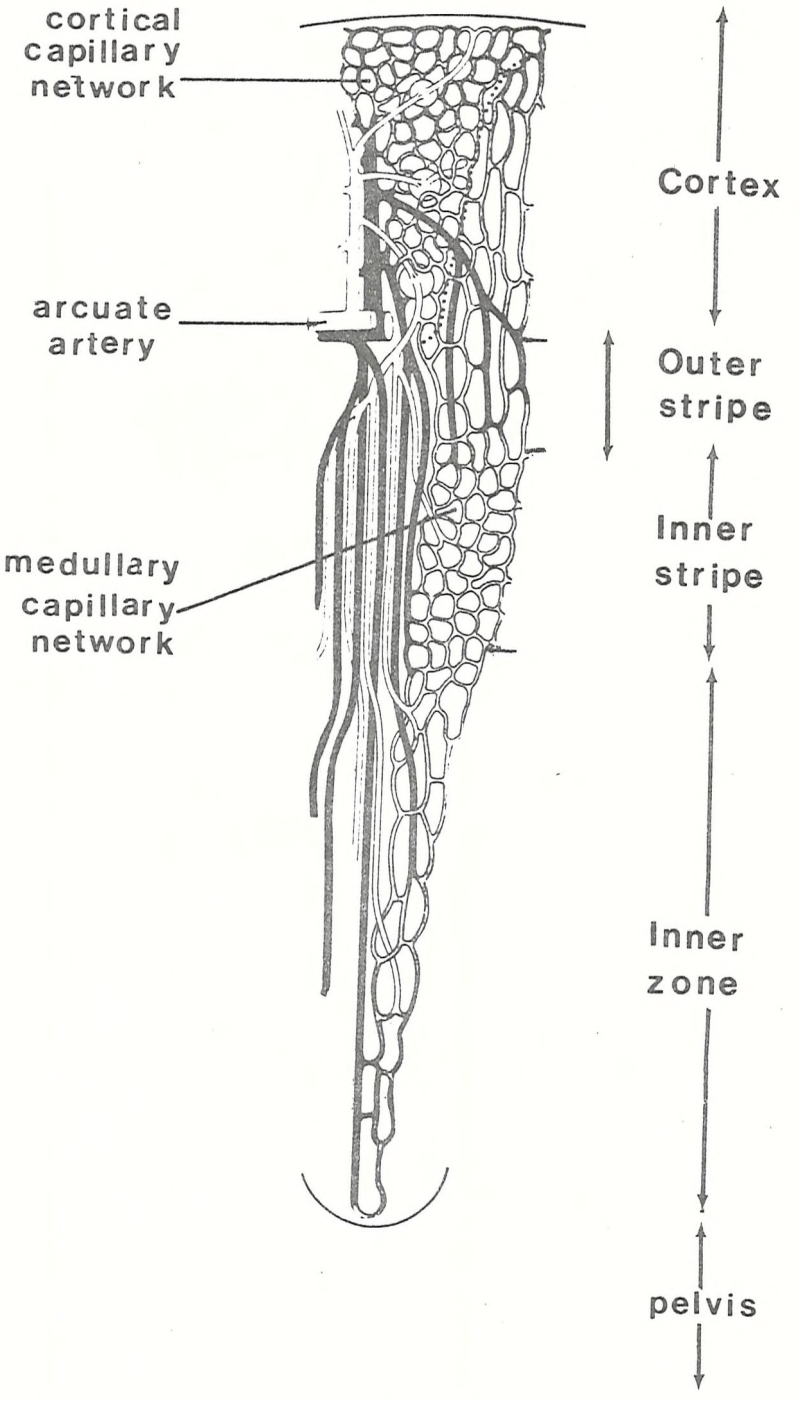


FIGURE 1.4 Schematic representation of the basic pattern of the vessels of the mammalian kidney (not drawn to scale). Arteries and arterioles are drawn white, capillaries are stipled, and the venous vessels are black. The representation of the intrarenal vasculature starts at an arcuate artery and ends at an arcuate vein, both lying at the corticomedullary border. (From Kriz, Barrett and Peter, The renal vasculature: anatomical-functional aspects. International Review of Physiology, vol. II, Kidney and Urinary Tract Physiology II, University Park Press, 1976).

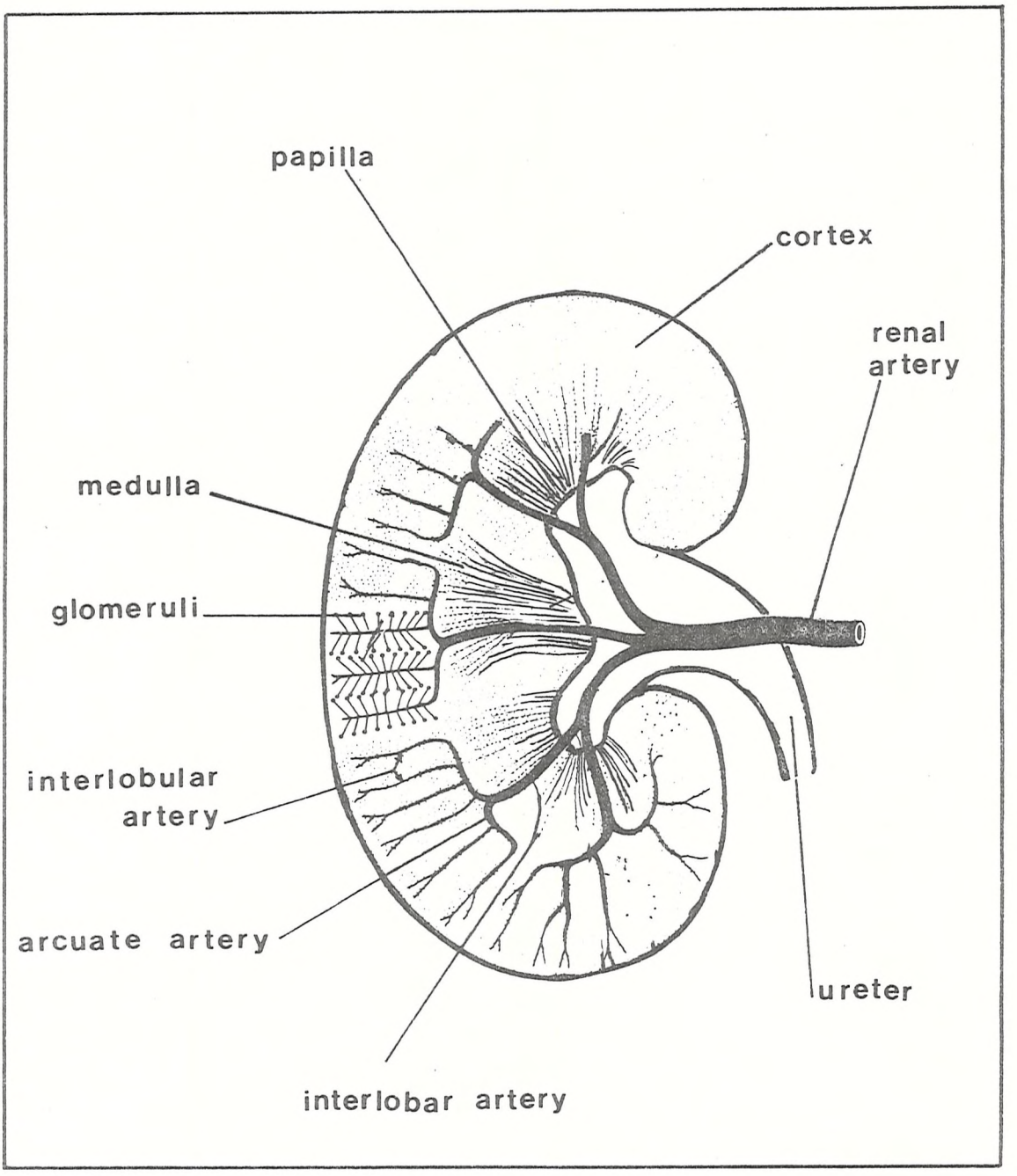


FIGURE 1.5 Schematic representation of arterial blood supply to guinea pig kidney. Modified from Leaf and Cotran, Renal Patho-Physiology, Oxford University Press, 1976.

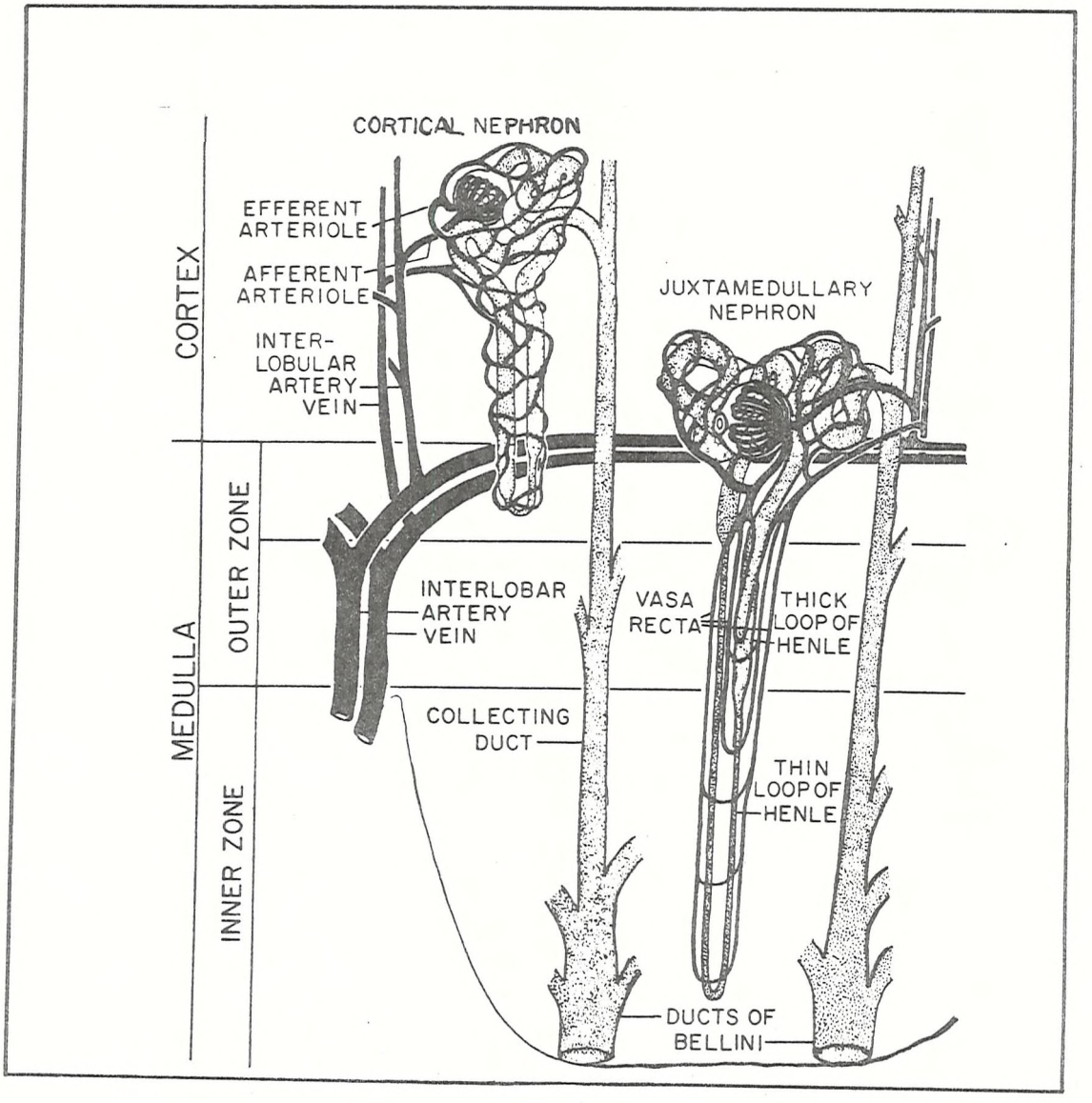


FIGURE 1.6 Comparison of the blood supplies of cortical and juxtamedullary nephrons. (From Pitts, Physiology of the kidney and body fluids, Year Book Medical Publishers Inc., 1976)

1-4-3 Innervation of the kidney

a) Morphological studies

It is nearly a century since renal nerves were first described histologically (Bradford, 1889), yet controversy still exists concerning their anatomical distribution in the renal parenchyma. This confusion arose from methodological limitations of hitherto available histological techniques. However, the development of electron microscopy has shed light on the precise relationship between renal nerves and other structures in the renal cortex. Furthermore, fluorescence histochemistry (Falck et al, 1962) and the thiocholine method of Koelle and Friedenwald (1949), as modified by Karnovsky and Roots (1964), has permitted the demonstration of adrenergic and acetylcholinesterase-containing renal nerves, respectively, in many species (Müller and Barajas, 1972; Norvell, 1969).

b) Adrenergic innervation of the kidney

Fluorescence, characteristic of adrenergic innervation, was detected entering the kidney along the renal artery and followed as it passed between blood vessels and cortical tubules in monkey. Adrenergic nerves appear in the space between afferent and efferent arterioles surrounding the renal tubules (Barajas, 1978). Selective destruction of adrenergic neurones with 6-hydroxydopamine (6-OHDA) resulted in the disappearance of fluorescence from almost all glomerular arterioles.

After histochemical detection of catecholamine containing neurones in the kidney, the logical progression was to differentiate between DA and NA containing renal nerve fibres. In the CNS these differences were relatively simple to demonstrate because dopaminergic and noradrenergic neurones are independently located within discrete anatomical areas. However, renal DA- and NA-containing neurones are extremely diffuse, moreover, they are difficult to distinguish by fluorescence histochemistry because fluorophores formed by each catecholamine are spectrally very similar (Corrodi and Jonsson, 1965). One approach to this problem was pharmacological manipulation of catecholamine levels. In dogs, formaldehyde induced fluorescence and endogenous DA and NA content associated with arcuate nucleus, interlobular cortical arteries and juxtaglomerular arterioles were diminished following pretreatment

with 6-OHDA and reserpine, hence establishing that the fluorescence was due to the presence of intra-axonal catecholamines (Bell et al, 1978a). Moreover, in reserpine pretreated animals, administration of L-DOPA led to a selective restoration of fluorescence in some axons of the renal cortex but not noradrenergic axons. Guanethidine caused a depletion of NA levels in the renal cortex but DA levels and associated fluorescence were unimpaired. Guanethidine is thought to selectively deplete noradrenergic nerves, possibly after gaining entry into the axon via a membrane uptake process which is specific for NA but not DA. The resistance to guanethidine shown by renal cortical axons might indicate the presence of discrete dopaminergic fibres (Fuxe et al, 1967).

A second approach for differentiating between these two primary catecholamines was to shift the excitation spectrum of catecholamine-specific histofluorescence by exposure to gaseous hydrochloric acid and thereby produce two separate spectra, each one synonymous with either DA or NA. This microspectrofluorometric technique (Björklund, Ehinger and Falck, 1968) takes advantage of the dehydration of the NA fluorophore which occurs at acid pH (Corrodi and Jonsson, 1965). In dog, DA containing neurones were thus located at the glomerular vascular poles (Dinerstein et al, 1979). In contrast NA fluorescence was located in the periadventitial layer of the arcuate arteries. These studies presented evidence that both intracortical arteries and juxtaglomerular arteries have DA-containing axons in close proximity, but not that dopaminergic axons influence any particular segment of the cortical vasculature.

To achieve status as a peripheral neurotransmitter, DA must not only be present within neurones but must also be stored and synthesised therein (see Section 1-2-1). Indirect evidence for such a role is invariably useful. In the dog, for example, the level of DA found in the renal cortex is 0.081 ± 0.006 µg/g ($n = 5$) (Bell et al, 1978a). The proportion of DA to NA in renal cortex was 24% which is several times greater than would be anticipated to be present in purely noradrenergic axons (Bell et al, 1978a; Bell and Gillespie, 1981). This excess DA was predominantly detected in the outer cortical layer and reinforces histochemical evidence for the existence of dopaminergic axons. Collectively, these observations offer strong evidence that the blood vessels of renal cortex receive dilator innervation by dopaminergic nerves (Bell, 1979).

1-4-4 Effect of catecholamines on sodium transport in the kidney

The presence of α- and β-adrenoceptors and dopaminergic receptors in the kidney has been reported in many studies (see Table 1.2; reviews by Goldberg, 1972; 1978a; Kim et al, 1980). Alterations in adrenergic neural activity may alter sodium reabsorption by one of several mechanisms, i) systemic haemodynamics which may in turn influence renal function and sodium excretion, ii) renal adrenergic neural tone which can directly influence renal epithelial cell sodium transport or indirectly alter RBF (Cadnapaphornchai et al, 1977; Besarab et al, 1977). Evidence derived from renal nerve stimulation and denervation studies, specific receptor agonist and antagonist studies and from pathological states suggests that an increase or decrease in renal sympathetic nerve activity decreases and increases renal sodium excretion respectively. These effects are probably mediated by a direct effect of catecholamines upon renal proximal tubule epithelial cells (Bell and Lang, 1973; Barton et al, 1968; Greven and Klein, 1977; Bell, 1979).

1-4-5 Therapeutic potential of dopamine in cardiovascular diseased states

Drugs used in the treatment of congestive heart failure have been aimed at elevating RBF with only a minimal accompanying increase in myocardial oxygen consumption. A characteristic symptom of patients with congestive heart failure is the inability to excrete an intravenous saline load as rapidly as normal, resulting in raised sodium and water retention with associated hypertension. It was suggested that sodium retention occurs when intrarenal blood flow is redistributed so that inner cortical and outer medullary regions, the location of 'salt retaining' nephron populations, receive a proportionally greater volume of blood.

Several studies have demonstrated that DA increases renal plasma flow, glomerular flow rate and sodium excretion in such patients (McDonald et al, 1964; Goldberg et al, 1963). The sodium diuresis may be haemodynamic in source (Barger, 1966; McDonald et al, 1964), due to vasodilation mediated by specific DA receptors, or could be due to the direct action of DA upon renal tubular cells.

The cardiac effects of DA make it suitable for the treatment of congestive heart failure (Goldberg et al, 1977) because heart disease is often accompanied by ischaemia. Therefore a pharmacological agent used in the therapy of myocardial power failure in patients with coronary insufficiency, should possess the haemodynamic property of stimulating stroke volume without elevating arterial pressure and heart rate so that there is the smallest possible increase in myocardial oxygen consumption and no hypoxia induced. DA increases cardiac contractility, cardiac output and RBF without changing heart rate, whilst MABP is either unchanged or slightly decreased.

In addition to DA's postsynaptic, tubular and cardiac actions, presynaptic inhibitory DA receptors can be considered as targets for the development of selective agonists that might be useful antihypertensive agents. The stimulation of presynaptic inhibitory DA receptors by agonists like DA, apomorphine, bromocryptine or di-n-propylDA reduces the stimulation evoked release of NA (see review by Langer,1981) and thus a therapeutic effect would be derived from a decrease in sympathetic tone (Langer and Dubocovich, 1979; Dubocovich and Langer, 1980; Lokhandwala and Jandhyala, 1979; Massingham et al, 1980).

I-5 Dopamine receptor model systems

I-5-1 Model systems in general

The molecular pharmacological characterisation of DA receptors has been accelerated by the development of experimental models in the CNS and in the periphery. A major criticism of these models has been that many conclusions regarding the actions of drugs at DA receptors are based upon indirect studies such as behavioural effects or changes in CNS metabolism and are therefore disregarding the complexity of drug-receptor interactions.

The most important criterion for a good DA receptor model is that it represents a distinct physiological response which qualitatively and quantitatively distinguishes DA receptors from other receptors such as α- or β-adrenoceptors or 5HT receptors. Well defined drug-receptor interaction at the molecular level can be established if the majority of DA receptor agonists tested are full agonists and the antagonists are truly competitive.

The two earliest models developed demonstrated the existence of specific DA receptors in the dog renal artery (Goldberg et al, 1968; 1978a) and the isolated brain of the snail, *Helix aspersa* (Woodruff and Walker, 1969; Woodruff, 1978).

1-5-2 Active conformation of dopamine

The ability of DA to activate many receptor types may be related to its structure which permits flexibility of the side chain and freedom of rotation of the benzene ring. Although the DA side chain possesses unlimited flexibility about the β-carbon-phenyl bond, in solution it probably assumes an anti (extended or trans) or gauche (folded or cis) form (Bustard and Egan, 1971) depending upon the orientation of the ethylamine chain with respect to the catechol nucleus. When extended there is the additional possibility of rotation about the phenyl-carbon bond, which leads to the existence of two rotameric extremes designated α and β (Figure 1.7). Since Woodruff (1971) first suggested that the β-rotamer is the preferred conformation for activation of DA receptors, the development of semirigid analogues, such as 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) and other DA-related compounds, each containing essential components of the DA molecule locked in a particular orientation, has enabled detailed structure-activity analyses to determine the structural and conformational requirements for DA agonist activity in a variety of dopaminergic responses (Volkman et al, 1977; Woodruff, 1978; Goldberg et al, 1968; 1978a; Seeman, 1980).

DA may activate α- or β-adrenoceptors and dopaminergic receptors (Table 1.2). Therefore, in order to establish a selective DA receptor model system, it is important to distinguish between effects mediated by specific DA receptors and actions of DA upon other receptors which may be present in the particular tissue under investigation. This is achieved by the use of specific antagonist drugs.

1-5-3 The neuroleptics

As a consequence of the discovery that the principle target for neuroleptic drugs was the DA receptor, many compounds with similar properties have been synthesised in an attempt to develop more specific drugs for the treatment of mental disorders and subsequently classified into major groups on the basis of their chemical structure. These are

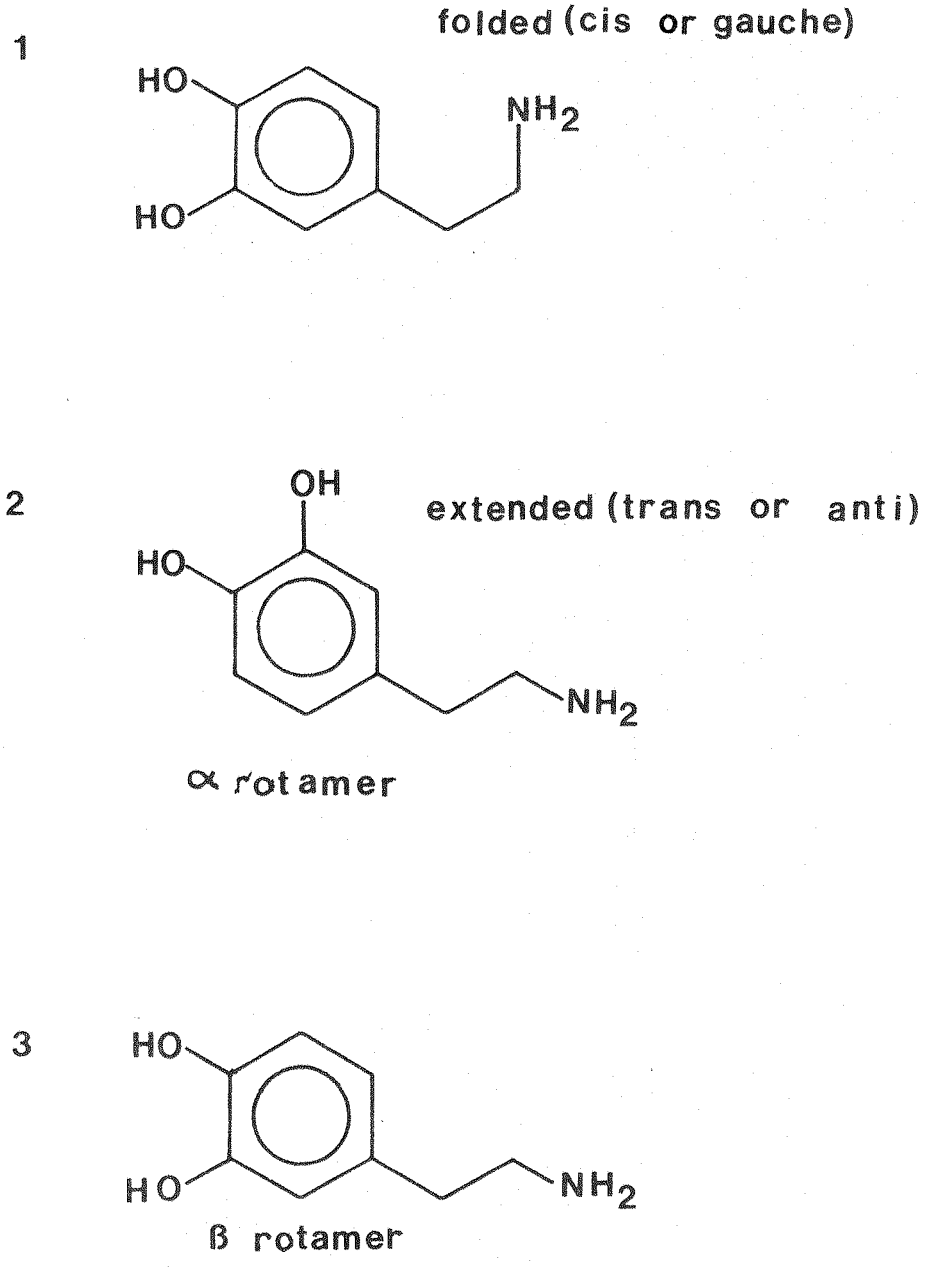


FIGURE I.7 Conformation of dopamine in its folded (1) and extended (2 and 3) forms. The dopamine molecule in its extended conformation can exist as two rotameric extremes, α (2) and β (3). The β-rotameric form of dopamine is generally accepted as the preferred conformation of dopamine at postsynaptic receptor sites.

TABLE 1.2 Actions of dopamine upon peripheral catecholamine receptors (from Goldberg and Kohli, 1979).

β_1-adrenergic	postsynaptic cardiac stimulant (direct and indirect).
β_2-adrenergic	postsynaptic vasodilation.
α_1-adrenergic	postsynaptic vasoconstriction.
α_2-neuronal	presynaptic inhibition of NA release.
DA vascular	postsynaptic vasodilation
DA neuronal	presynaptic inhibition of NA release.

the butyrophenones, thioxanthenes, phenothiazines, benzamides and a few others which do not fall into any particular category. Figure 1.8 summarises the general structure for each class of neuroleptic compound.

Discrepancies such as a lack of correlation between classical neuroleptics like haloperidol, spiroperidol (butyrophenones), fluphenazine (thioxanthene) and the substituted benzamide, sulpiride upon clinical potency and upon several DA receptor models (see Section 1-5-1) form the basis of a current controversy about the existence of multiple receptor types for DA in the CNS.

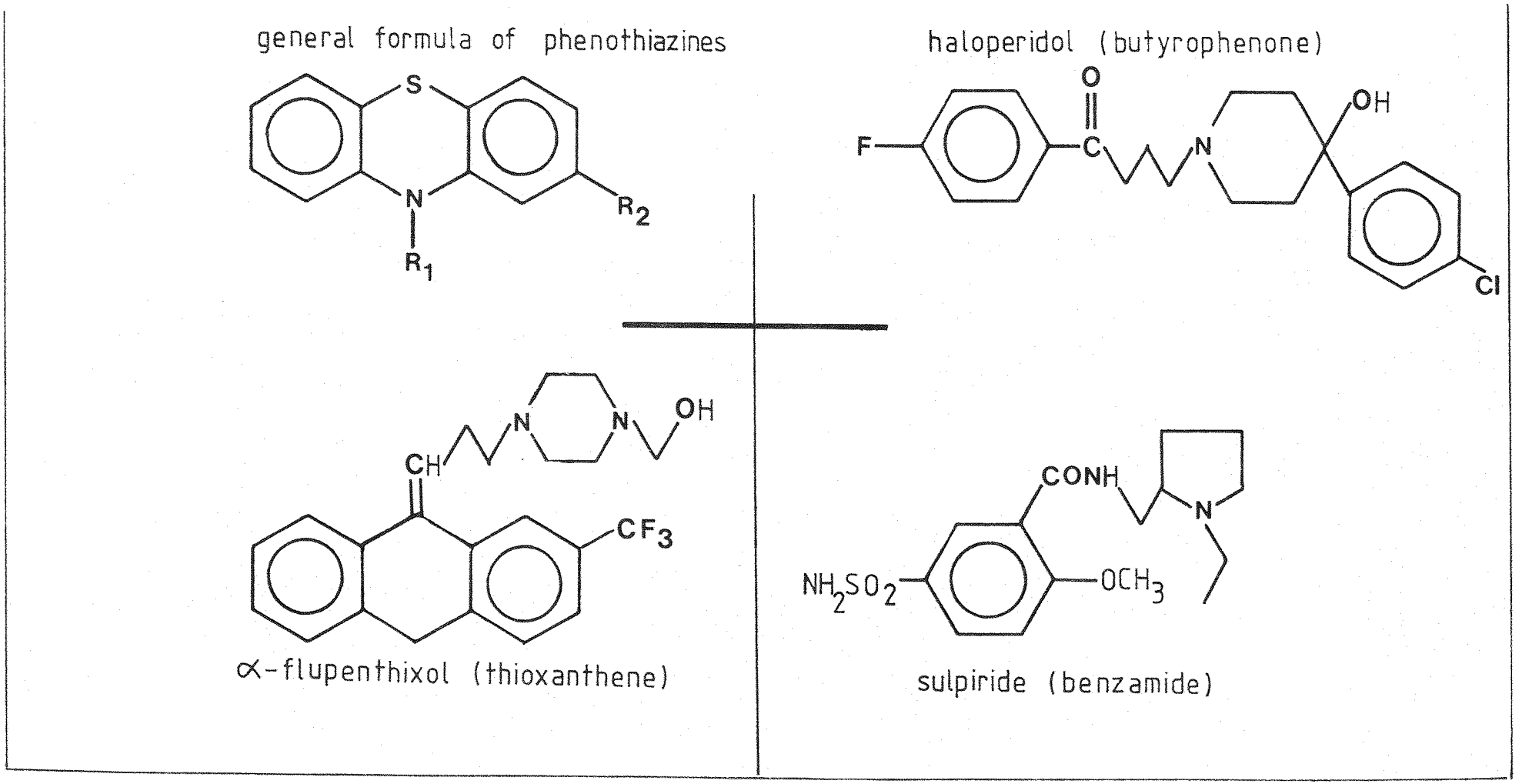
1-5-4 *Helix aspersa*

The spontaneous firing of APs from cells of the right parietal ganglion of isolated snail brain, *Helix aspersa*, is inhibited by DA (Kerkut and Walker, 1961). Subsequent structure-activity studies demonstrated that the DA-mediated inhibition was distinct from α- and β-adrenoceptors or serotonergic (5HT) receptor mediated responses and moreover was extremely specific (Woodruff and Walker, 1969). The absolute requirements for activation of this DA receptor were deduced from a series of phenylethylamine derivatives. Woodruff and Walker (1969) proposed that two hydroxyl groups, one on each of positions 3 and 4 of the benzene ring, and a terminal nitrogen, either unsubstituted or substituted with one methyl group were prerequisites for DA-induced inhibition of neuronal activity. From this analysis, it was reasoned that DA is active in the extended or β-conformation (see Section 1-5-2) since norsalsolinol and salsolinol, which contain the DA skeleton fixed in a folded or α-conformation, are only weak agonists on DA receptors (Woodruff, 1971; Iversen, 1975) and it was predicted that a compound which held DA rigidly in the extended form would be a potent dopaminergic agonist. This was ADTN. The development of several other DA receptor models (see Sections 1-5-5 to 1-5-10) has since confirmed that ADTN is an extremely potent agonist of DA receptors (Miller et al, 1974; Munday et al, 1974; Woodruff, 1978; Crumley et al, 1976; Seeman, 1980).

1-5-5 The vascular dopamine receptor model

At about the same time, Goldberg and his colleagues developed an alternative model for specific DA receptors, based upon another physiologically distinct response to DA, the canine RBF model. DA

FIGURE 1.8 Major classes of neuroleptics.



caused dose related vasodilation when injected into the renal artery of phenoxybenzamine (POB) pretreated dogs (McNay, McDonald and Goldberg, 1965). In order to differentiate between the actions of DA on its own receptor and its action on any other receptors present in the renal artery or on neuronal elements of the kidney, several criteria were imposed for the identification of drugs as true DA receptor agonists.

Firstly, the physiological response should be mimicked; drugs injected into the renal artery should produce a dose-related increase in RBF. Secondly, pharmacological characteristics must be dopaminergic, the vasodilator response should be selectively blocked by specific DA antagonists, but not β-adrenergic blocking agents, atropine or anti-histamines. Thirdly, to eliminate the possibility of a neuronally mediated response, the effect should not be attenuated by reserpine pretreatment, administration of the ganglionic blocking agent hexamethonium or denervation. Fourthly, to differentiate DA from non-specific vasodilating agents, such as nitroglycerin or bradykinin, there should be a qualitatively different vasodilation or no vasodilation produced at all, after injection into the POB pretreated, denervated femoral vascular bed. Obviously α-adrenoceptor or 5HT receptor mediated vascular responses could be eliminated immediately, since agonists of these receptors cause vasoconstriction (for reviews see Goldberg et al, 1978 a and b).

Collectively these studies suggested that DA was acting upon a specific postsynaptic DA receptor located in the renal vascular bed which, when activated, would produce a renal vasodilation. Subsequent structure-activity studies with phenylethylamine derivatives emphasised the highly specific nature of this receptor and provided supporting evidence for the previously proposed molecular organisation of DA at its own receptor in the brain of *Helix aspersa*.

a) Agonist studies

Active DA vascular agonists required two hydroxyl groups on positions 3 and 4 of the catechol nucleus and an unsubstituted α or β carbon (Goldberg, Sonneville and McNay, 1968). Substitution of the amino group is limited to 1 methyl in mono-substituted derivatives, since n-methyl DA (epinine) was equipotent with DA, and to a propyl or butyl derivative as one of the groups in di-N-substituted compounds

(Ginos et al, 1978; Kohli et al, 1980) but the latter are 30-60 times less active than DA. Having established that the structural requirements for activation of the vascular DA receptor were highly restrictive, the logical progression was to investigate its conformational requirements. At this time ADTN, the compound which Woodruff (1971) had suggested would possess dopaminergic activity, was synthesised (Miller et al, 1974) and found to be approximately equipotent with DA on the RBF model (Crumly et al, 1976; Volkman et al, 1977). Studies with derivatives of the 2-aminotetralin series were in agreement with the original *Helix aspersa* studies, since they supported the hypothesis that the most potent conformation for activation of the vascular DA receptor is the β-rotamer of DA (Kohli et al, 1979) as contained by ADTN. The α-rotameric form of DA corresponds to the dihydroxyphenylethylamine moiety contained in 2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene (isoADTN) and is inactive on the RBF model, but is active as a β_2-adrenoceptor agonist (Volkman et al, 1977). Substitutions of the ADTN molecule reveal a similar structure-activity relationship to that for DA. N-methyl-ADTN is approximately equipotent to ADTN; however N,N-di-n-propyl ADTN and N-n-propyl-N-n-butyl ADTN were more potent than their respective DA derivatives. Increasing the size of the substituted group, for example N,N-dimethyl-,N-diethyl-,N-propyl-ADTN produced active compounds, as was the case with corresponding DA analogues. Table 1.3 lists the potency order of phenylethylamine, 2-aminotetralin, apomorphine and nomifensine derivatives on the DA vascular receptor.

Interestingly apomorphine, a relatively rigid molecule which contains the structure of n-methyl DA with a fully extended side chain and amino group trans to the benzene ring in an α-rotameric conformation, was a partial agonist on RBF (Crumly et al, 1976) whereas it was inactive on the *Helix aspersa* model system (Woodruff, 1971). Moreover its substituted counterpart N-n-propylnorapomorphine was a full agonist of the postsynaptic DA receptor with 1/30-1/50th of the potency of DA. In contrast to N-n-propylnorapomorphine, isoapomorphine, which contains N-methyl DA in a β-conformation, was inactive (Goldberg and Kohli, 1979). Figure 1.9 shows the conformational relationship between DA and its apomorphine and aminotetralin analogues.

TABLE 1.3 Agonists of the dopamine vascular receptor with relative potencies.

Drug	Relative potency	Authors
DA	1	Goldberg et al, 1968
epinine (n-methyl DA)	1	Goldberg et al, 1968
ADTN	1	Crumley et al, 1976
N-methyl-ADTN	0.5-1	Volkman et al, 1977
N-N-di-n-propyl ADTN	0.25	Kohli et al, 1979
N-N-propyl-N-n-butyl ADTN	0.25	Kohli et al, 1979
N-N-di-n-propyl DA	0.03	Kohli et al, 1978a
N-n-propyl-N-n-butyl DA	0.03	Ginos et al, 1978
N-n-propyl-N-n-pentyl DA	0.03	Ginos et al, 1978
6-propylnorapomorphine	0.02	Crumley et al, 1976
apomorphine (partial agonist)	0.01	Crumley et al, 1976
N-n-propyl-N-ethyl DA	0.008	Kohli et al, 1980
N-n-propyl-N-isobutyl DA	0.008	Kohli et al, 1980
N-n-propyl-N-phenethyl DA	0.008	Kohli et al, 1980
3',4'-dihydroxynomifensine	N.P.	Goldberg & Kohli, 1979

N.P. Not published.

FIGURE 1.9 Structures of four dopamine related compounds. In its extended form dopamine can exist as two rotameric extremes (see Figure 1.7) designated α and β. Dopamine in its α-form is contained within the molecule 2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene (isoADTN) and is structurally very similar to apomorphine. In its β-form dopamine is contained within the molecule 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, and is structurally very similar to isoapomorphine.

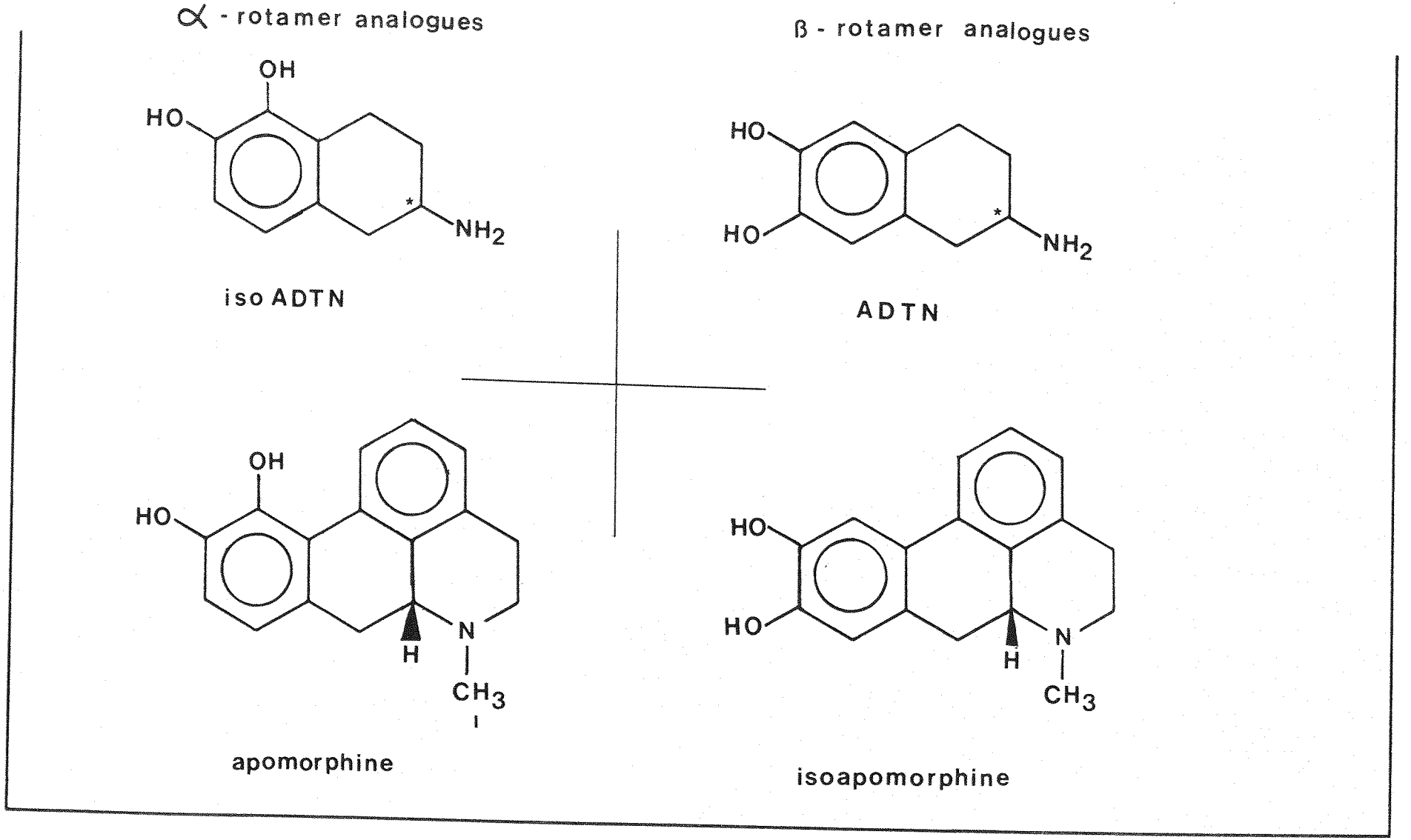


Figure 1.9 * asymmetric carbon

TABLE 1.4 Antagonists of the DA vascular receptor with relative potency and ranges of specificity (Goldberg, Volkman and Kohli, 1978b).

Antagonist	Relative potency (mol)	Range of specificity*	Author
Sulpiride	2.0×10^{-8}	> 10	Kohli et al, 1978b
Metoclopramide	1.5×10^{-6}	> 10	Kohli et al, 1978b
Bulbocapnine	4.7×10^{-8}	∿ 8	Goldberg and Musgrave, 1971
Thioridazine	5.0×10^{-7}	< 2	Goldberg and Yeh, 1971
Fluphenazine	2.5×10^{-7}	< 2	Goldberg and Yeh, 1971
Trifluoroperazine	2.5×10^{-7}	< 2	Goldberg and Yeh, 1971
Prochlorperazine	2.5×10^{-7}	< 2	Goldberg and Yeh, 1971
Chlorpromazine	2.5×10^{-7}	< 2	Goldberg and Yeh, 1971
Haloperidol	1.4×10^{-7}	< 2	Yeh et al, 1969

* $\frac{\text{minimum dose required to inhibit vasodilation produced by bradykinin or isoprenaline}}{\text{minimum dose required to inhibit vasodilation produced by DA}}$

b) Antagonist studies

Potency and specificity are the two most important parameters that must be qualitatively evaluated in the assessment of antagonist drugs. Obviously the difficulty here lies in establishing that the blocking agent produces an inhibition of DA induced vasodilation by acting on DA receptors and not by any other mechanism.

In Goldberg's RBF model, the range of specificity of an agonist has been defined as the ratio between the minimum dose needed to block the response to isoprenaline (β-adrenoceptor agonist) or bradykinin (non-specific) divided by the minimum dose needed to block the response to DA. Table 1.4 lists the potencies and specificities of antagonists of the DA vascular receptor (Goldberg et al, 1978a). It is important to note here that the clinically active benzamide derivative, sulpiride, is more potent and specific at antagonising renal vascular responses to DA (Goldberg, Musgrave and Kohli, 1979), than classical neuroleptic drugs such as haloperidol, fluphenazine or chlorpromazine (Goldberg and Yeh, 1971; Yeh et al, 1969). This observation is discussed in greater detail (Section 1-5-11) in the light of work originating from other DA receptor models described in the following sections.

c) *In vitro* studies

Supporting evidence for the existence of a specific vascular DA receptor has come from *in vitro* studies with an isolated renal arterial vessel preparation (Goldberg and Toda, 1975). DA caused a dose-dependent relaxation of small helical arterial strips pretreated with POB for one hour and subsequently contracted with prostaglandin $F_{2\alpha}$. Responses to DA, n-methyl DA and ADTN were unaffected by propranolol, a specific β-adrenoceptor antagonist, however, specific inhibition by neuroleptics was not convincingly demonstrated.

d) Peripheral multiple dopamine receptors - DA_1 and DA_2?

Recently it has been suggested that the population of peripheral DA receptors concerned with renal vasodilation can be further subdivided into pre- and postsynaptic receptors (Goldberg and Kohli,

1979). This proposal implies that the classical postsynaptic DA receptor situated on vascular smooth muscle can be differentiated from a neuronal, DA-sensitive, presynaptic inhibitory receptor in peripheral noradrenergic nerve terminals. However, presynaptic α_2-adrenoceptors are also located on these same terminals and may be stimulated by DA to result in a similar inhibition of NA release and a loss of vascular tone (see Table 1.2 and Section 1-3-2). Therefore it is vital to distinguish between presynaptic inhibitory DA receptors and α_2-adrenoceptors to prove that DA is acting upon a specific receptor.

The inhibition by DA of the stimulation evoked release of [^{3}H]NA in the perfused cat spleen was first reported in 1973 by Langer. Since then several authors have reported such a phenomenon under *in vitro* conditions in rabbit heart (Fuder and Muscholl, 1978), cat nictitating membrane (Enero and Langer, 1975), rabbit ear artery (Hope et al, 1976) and under *in vivo* experimental conditions in dog heart (Lokhandwala and Buckley, 1977; Lokhandwala and Jandhyala, 1979) and cat heart (Massingham, Dubocovich and Langer, 1980).

Stimulus evoked release of NA is reduced by DA receptor agonists such as DA, apomorphine, bromocryptine, pergalide or dipropyl DA. The inhibition of noradrenergic neurotransmission is unaffected by α-adrenergic blocking agents (Enero and Langer, 1975; Dubocovich and Langer, 1978; 1980), in contrast it is selectively antagonised by neuroleptics such as chlorpromazine, pimozide or sulpiride. Bearing in mind that the role of DA as a peripheral neurotransmitter has not been conclusively demonstrated, but having established that peripheral DA receptors probably exist, Goldberg and Kohli (1979) proposed a classification o D_1 postsynaptic vascular and D_2 presynaptic neuronal receptors on the basis of pharmacological studies in the renal and femoral vascular beds.

The model which these authors claim to differentiate between D_1 and D_2 is based upon DA induced inhibition of NA release, from the sympathetic nerves supplying the canine femoral vasculature, by activating a specific presynaptic or neuronal DA receptor (Langer, 1974), resulting in a reduction of femoral vascular tone viz. vasodilation (Kohli et al, 1978a). Structural criteria demanded for stimulation of the D_2 receptor are less rigorous than D_1. In contrast to D_1, dimethyl,

diethyl and dipropyl substituted analogues of DA and ADTN were active on the neuronal receptor. Dipropyl DA was more active than DA in causing femoral vasodilation (Goldberg, 1978c; Kohli et al, 1979). Moreover N-n-propyl-N-substituted analogues of DA ranging in size from butyl to phenethyl substituents were all equiactive on the neural model but propylbutyl- and propylpentyl-DA were more potent than their larger counterparts, propylisobutyl- and propylphenethyl-DA, on DA vascular receptors. Rotameric specificity was less rigorous for DA induced vasodilation in femoral than renal vascular beds (Goldberg and Kohli, 1979). Both α and β rotameric conformations of DA, presented to the receptor as isoADTN and ADTN respectively, were active as presynaptic receptor agonists. Finally apomorphine, a partial agonist on the vascular DA receptor was transformed to a full agonist on the putative D_2 receptor.

1-5-6 Peripheral dopamine receptor models

To define a receptor model system, specific antagonism must be demonstrated both *in vivo* and in isolated organ systems because in the intact animal non-receptor mechanisms might be involved in the vasodilating actions of DA. In the earliest *in vitro* investigations on isolated canine renal, mesenteric, femoral (Goldberg and Toda, 1975) and coronary arteries (Toda et al, 1975) DA-induced relaxations could not be antagonised by haloperidol or chlorpromazine because these agents relaxed the arterial strips themselves. More recently, specific antagonism to DA induced relaxations were demonstrated by metaclopramide (a substituted benzamide) on isolated rabbit mesenteric arteries (Brodde et al, 1979) and by droperidol on isolated canine renal, mesenteric and coronary arteries (Toda, 1979). Table 1.5 lists *in vivo* and *in vitro* preparations used in the study of of peripheral DA receptors.

1-5-7 Behavioural models

Behavioural studies have provided a valuable tool for studying the functional role of DA within the central dopaminergic tracts described in Section 1-2. However, many criteria used for the evaluation of dopaminergic agents in behavioural models were based upon indirect physiological responses, which were assumed to be a result of activation of a specific population of DA receptors. If the information yielded by behavioural studies is reconsidered in the light of the

TABLE 1.5: A summary of *in vivo* and *in vitro* preparations used to study DA receptors in vascular beds. Abbreviations: renal blood flow (RBF), renal vascular resistance (RVR), mean arterial blood pressure (MABP), glomerular filtration rate (GFR), prostaglandin ($PGF_{2\alpha}$). * Preparations pretreated with i) phenoxybenzamine to minimise α-adrenoceptor mediated vasoconstrictor responses to DA, ii) $PGF_{2\alpha}$ to contract *in vitro* blood vessel preparations, since these were lacking sympathetic tone, so that clear-cut vasodilator responses to DA could be recorded.

PREPARATION	SPECIES	CONDITIONS	PARAMETERS MEASURED	DA RECEPTOR TYPE INVESTIGATED	AUTHORS
Kidney					Goldberg et al, 1968; 1978a;b
renal artery	dog	POB pretreatment *in vivo*	RBF	vascular	Pendleton et al, 1978 Bell, Conway & Lang, 1974
isolated renal artery	dog	*in vitro**	relaxation after $PGF_{2\alpha}$ contraction	vascular	Goldberg & Toda, 1975 Toda, 1979
isolated perfused kidney	rat	*in situ*: perfusion pretreatment with POB and sotalol	RBF, RVR, GFR, perfusion pressure, urine output, fractional tubular Na reabsorption	integrated system + vascular	Schmidt & Imbs 1980
vena saphrena lateralis stips	dog	*in vitro**	relaxation	vascular	Buylaert & Willems, 1976
Cerebral					Toda et al, 1975
cerebral artery	dog	*in vitro**	relaxation	vascular	Toda, 1976
middle cerebral arteries	cat	*in vitro**	relaxation	vascular	Edvinsson et al, 1977; 1978.
pial arteries	man	*in situ*			
pial arteries	rat	*in situ*	vasodilation and vasoconstriction examined by high-resolution closed-circuit television microscope	vascular	Altura et al, 1980
Coronary					Toda et al, 1975
coronary artery	dog	*in vitro**	relaxation	vascular	Toda, 1979
integrated cardiovascular function	guinea pig	*in vivo*	↓ MABP	vascular and neural integrated	Woodruff & Summers, 1979
	cat	*in vivo*	↓ MABP	"	Pendleton et al, 1975
	dog	*in vivo*	cardiac rate, RBF, RVR	"	Hahn & Wardell, 1980
	dog	*in vivo*	heart rate, cardiac contractility	neuronal	Clark & Menninger, 1980
			superfused superior mesenteric artery	vascular	
	rat	*in vivo*	↓ MABP	integrated	Day & Blower, 1975
Mesenteric					
isolated splenic artery	rabbit	*in vitro**	relaxation	vascular	Hilditch & Drew, 1981
isolated mesenteric artery	rabbit	*in vitro**	relaxation	vascular	Brodde et al, 1981
	dog	*in vitro**	relaxation	vascular	Goldberg & Toda, 1975
hepatic & portal vascular bed	dog	*in vivo*	vasodilation	vascular	Richardson & Withrington, 1978
superior mesenteric artery	rabbit & cat	*in vivo*	intestinal blood flow, cardiac output, integrated activity of greater splanchnic & postganglionic mesenteric nerves	vascular	Kullmann et al, 1979
Femoral					
intact hindleg	dog	*in vivo*	femoral blood flow	neurogenic, vasodilator	Bogaert et al, 1979
1) mesenteric vascular bed & 2) femoral neuronal bed	dog	*in vivo*	mesenteric & femoral blood flows	1) vascular 2) neurogenic	Drew & Hilditch, 1980
1) renal vascular bed 2) femoral occluded circulation	dog	*in vivo*	renal & femoral blood flows	1) postsynaptic vascular 2) neuronal vasodilation	Goldberg et al, 1978d

prerequisites for a specific DA receptor model, as described in Section 1-5-1, it is evident that neither absolute quantitative nor qualitative analysis of drug-receptor interaction at the molecular level is possible. Many unexplained observations (see Cools and, van Rossum, 1980) hint that the DA receptor concept has been over-simplified and that inadequate consideration has been given to the possibility of agents under investigation producing a DA-like response merely by modification of one or more synaptic components and consequently changing the prevailing conditions within the synaptic cleft.

Potential dopaminergic substances like apomorphine might interact with either presynaptic or postsynaptic DA receptors, or other receptors (Seeman, 1978), deplete transmitter stores, cause amphetamine-like release of endogenous neurotransmitter substances or even block transmitter release. Alternatively, drugs might modify inactivation processes, for instance they could inhibit degradative enzymes like MAO and COMT or block re-uptake processes. Exogenous substances might undergo conversion to become an active metaboliteswhich possess any of the aforementioned properties. Finally, DA receptors themselves might undergo adaptational changes following pretreatment with drugs to produce phenomena such as hypersensitivity and desensitisation.

In order to differentiate between various dopaminergic tracts and their associated physiological functions (see Section 1-2-1), DA model test symptoms should ideally produce qualitatively different behavioural responses characteristic of the dopaminergic brain tract under investigation. Apomorphine and neuroleptics are used as tools to evaluate brain DA receptors and therefore agents mimicking them are labelled as DA agonists and antagonists respectively. However, models of these drug-induced behavioural changes might only reflect a net disturbance in overall DA balance rather than the effect of DA on individual dopaminergic systems. For instance, increased locomotor activity can be induced by either stimulation of neostriatal DA receptors or inhibition of DA receptors in the nucleus accumbens. Consequently, locationally and functionally distinct dopaminergic tracts might be separated if different types of DA receptors were also located within these structures. Behavioural and other studies do, in fact, provide good evidence for the existence of presynaptic DA receptors (Carlsson, 1975; Bunney and Aghajanian, 1975), and much

controversial evidence for the existence of possibly two or more types of postsynaptic receptors.

Many behavioural techniques have been used to study brain DA receptors; three extensively used models are described here and reviewed in greater detail by Ungerstedt et al (1978) and Cools and van Rossum (1980). These are critically assessed for their representation of specific DA receptors.

a) 6-hydroxydopamine (6-OHDA) rotation model

Unilateral degeneration of dopaminergic innervation to the corpus striatum is achieved by intranigral injection of 6-OHDA and results in a compensatory supersensitivity of DA receptors in the lesioned striatum due to nerve terminal degeneration (Ungerstedt, 1971b). Subsequent injection of apomorphine or ADTN, DA receptor agonists which act directly on these hypersensitive receptors ipsilateral to the lesion, produces contralateral rotation. Indirectly acting dopaminergic agents, like amphetamine, induce rotation towards the lesion in such animals. This turning behaviour is assumed to be caused by the release of endogenous DA from intact neurones innervating the contralateral striatum (Ungerstedt, 1971c; Ungerstedt et al, 1978).

Subsequent studies have shown that these original interpretations underestimated the complexity of interdependence between different dopaminergic mechanisms in the brain. Not only did degeneration of presynaptic nerve terminals indicate that DA receptors may exist in different states of supersensitivity (Ungerstedt, 1971c), but studies with agonists and antagonists led Cools and van Rossum (1976) to postulate the existence of different postsynaptic receptors, labelling them neostriatal DAe (excitatory) and mesolimbic DAi (inhibitory) receptors. They suggested mesolimbic α-adrenoceptors could be involved in the regulation of mesolimbic DAi receptors and have been implicated in the development of a supersensitivity to apomorphine (see review by Cools and van Rossum, 1980). Contralateral rotation elicited by apomorphine might then be due to direct i) stimulation of hypersensitive nigrostriatal DAe receptors, ii) inhibition of unaffected mesolimbic DAi receptors or iii) stimulation of unaffected mesolimbic α-like NA receptors which in turn inhibit DAi receptors in the same region.

Low doses of apomorphine stimulate neostriatal denervated DA receptors to produce a short-lasting response (½-1 hour) which is inhibited by haloperidol. In contrast, high doses of apomorphine produce a long-lasting contralateral rotation (1-4 hours), characteristic of inhibition of mesolimbic intact DA receptors, which is unaffected by haloperidol (Nakamura et al, 1978; Cools, 1978). This behavioural and other evidence form the basis of a multiple receptor concept for DA in behavioural regulation (Cools and van Rossum, 1980). However, there is no good evidence to correlate DAe and DAi receptors with any other putative subclasses of DA receptors such as D_1 and D_2 or stereoselective binding sites for DA which have been proposed on the basis of work in other model systems. The concept of multiple DA receptors is discussed in Chapter 4.

b) Stereotype behaviour

Stereotype behavioural patterns in rats and mice, consisting of repetitive twitching of the head, sniffing, gnawing and licking or emesis in dogs, cats and pigeons, are thought to be responses mediated by a dopaminergic mechanism (van Rossum 1970; Randrup and Munkvad, 1974) in the brain. The assessment of drugs active as DA receptor agonists or antagonists is made upon indirect evidence; agents inhibiting apomorphine induced stereotypy are classified as antagonists and agents mimicking apomorphine responses are agonists. These DA receptor models are crude, non-specific and difficult to quantify. Summing scores given to qualitatively different responses are inadequate because they preclude the possibility of distinguishing between the actions of drugs on different dopaminergic mechanisms.

c) Hyperactivity

The involvement of a dopaminergic mechanism in locomotor activity provided a third behavioural model on which to assess DA receptor agonist and antagonist activity when Pijnenburg, Woodruff and van Rossum (1973) found that bilateral injection of ergometrine into rat nucleus accumbens produced vigorous hyperactivity. The induction of hyperactivity is a more appropriate DA receptor model than other behavioural alternatives. Firstly, it is more specific, as many studies have pin-pointed the nucleus accumbens as a primary target for

dopaminergic agonists stimulating locomotion (Elkhawad and Woodruff, 1975; Woodruff et al, 1977; Pijnenburg et al, 1976; Costall et al, 1977). Secondly, hyperactivity is readily quantified by placing the animal into an activity cage. Thirdly, the response clearly shows dose-dependency to dopaminergic agonists like ADTN which are specifically antagonised by fluphenazine, sulpiride and cis(Z)-flupenthixol but not the α-adrenergic blocker aceperone, the β-adrenergic antagonist propranolol, or the opiate receptor antagonist naloxone.

1-5-8 Electrophysiological models

Better directed approaches for the study of dopaminergic function in the brain have used electrophysiological techniques. These have the advantage of measuring direct changes in membrane ion permeability, the primary event at the cellular level during drug-receptor interaction. In iontophoretic studies the microenvironment of a single neurone is the target of action for picomolar concentrations of pharmacological agents and the resultant alterations in neurotransmission are recorded as alterations in frequency and magnitude of nerve action potentials by either intracellular or extracellular electrodes (see reviews by Aghajanian and Bunney, 1974; Dray, 1979 for details of electrophysiological investigations into dopaminergic systems). An example would better illustrate how the technique specifically identifies DA sensitive neurones.

The nigrostriatal dopaminergic tract originates in the substantia nigra and projects to the striatum (caudate and putamen) and is

involved in the control of locomotion and posture (see Section 1-2-1). Direct evidence for DA as a transmitter in this pathway should come from comparing DA's action on target cells in the caudate, when applied iontophoretically, with electrically stimulating this pathway and recording physiological responses from cells in the striatum. Thus a cell responding to DA may be electrophysiologically and pharmacologically characterised. DA receptor agonists and antagonists housed separately in a multibarrelled electrode complex may be ejected directly on to the same cell, either individually or simultaneously within seconds of characterising a target cell. In this way the nigrostriatal pathway was found to have distinct mixed inhibitory and excitatory fibres (Connor, 1970; Gonzalez-Vegas, 1974) impinging on to target cells.

Microiontophoretic techniques have also provided evidence for the existence of pharmacologically distinct presynaptic DA receptors (autoreceptors) on the soma of DA neurones in the zona compacta of the substantia nigra and ventral tegmental area (A9 and A10 neurones respectively, see Figure 1.4). The firing of DA neurones was depressed by DA and apomorphine; this effect was inhibited by the DA antagonist trifluoroperazine. α- and β-adrenergic agonists did not mimic the response to DA whilst α- and β-adrenergic antagonists were ineffective at blocking the depressant action of DA (Aghajanian and Bunney, 1977).

Iontophoresis has several disadvantages. Firstly, absolute drug concentrations ejected at the cellular level are impossible to quantify; instead the drug ejection current provides a proportionate estimate of the amount of drug released. Secondly, results are often confusing because although gross positioning of recording electrodes within a dopaminergic nucleus is achieved stereotaxically, these electrodes may nevertheless be at some distance from synaptic areas. Thirdly, pharmacological studies designed to test dopaminergic antagonists are non-specific whilst drugs are applied systemically and even the administration of drugs directly on to cells by iontophoresis cannot overcome this specificity problem whilst the selectivity of antagonist drugs remains poor. Finally, the inhibitory nature of DA may be affected by presynaptic receptors or interneurones.

1-5-9 Dopamine-sensitive adenylate cyclase model

During the last decade, more direct DA receptor models have been

developed which are aimed at elucidating the fundamental chain of events beginning with DA interacting with its membrane receptor and terminating in a physiological response. A DA stimulated accumulation of adenosine-3':5'-cyclic monophosphate (cAMP) has been reported in CNS preparations - calf and rat retina (Brown and Makman, 1972), rat caudate nucleus (Kebabian et al, 1972), nucleus accumbens and olfactory tubercle (Horn, Cuello and Miller, 1974; Clement-Cormier et al, 1974) and to a lesser extent peripheral preparations - renal artery (Kotake et al, 1979) and rat kidney (Nakajima et al, 1977).

In 1972 Kebabian, Petzold and Greengard proposed that DA-sensitive adenylate cyclase and the postsynaptic DA receptor were intimately related and that the enzymic product of ATP breakdown, cAMP could act in the classical fashion of a secondary messenger to mediate the physiological effects of DA. The mechanism of action of cAMP was reviewed by Gill (1977). Thereafter followed a tremendous interest in uncovering a biological role for cAMP; although the adenylate cyclase system has been very useful for characterising the DA receptor (see review by Iversen, 1975) many discrepancies have arisen which cast doubt on whether the role of adenylate cyclase is truly as a physiological link between the DA receptor and its response (Laduron, 1980; Woodruff et al, 1979). However, DA sensitive adenylate cyclase has fulfilled most criteria for acceptance as a DA receptor model.

Firstly, the enzyme is located postsynaptically (Von Voightlander et al, 1973; Mishra et al, 1974; Kreuger et al, 1976) in some anatomically dopaminergic areas of the brain, but not in the dopaminergic region of the median eminence (Rappaport and Grant, 1974), where DA inhibits the release of prolactin from the anterior pituitary (see review by MacLeod, 1976) or in DA autoreceptors upon cell bodies of the substantia nigra and upon terminals of the striatum (Kebabian and Calne, 1979). This observation was interpreted to indicate that DA receptors not associated with adenylate cyclase fell into two categories: i) autoreceptors - the adenylate cyclase linkage being purely a postsynaptic phenomenon, ii) postsynaptic DA receptors functioning without an adenylate cyclase linkage.

Secondly, striatal and nucleus accumbens adenylate cyclase activity show dose-dependency and qualitative specificity for DA. DA is the most potent catecholamine at raising cAMP levels, a response which is

unaffected by ∝-and β-adrenergic antagonists (Kebabian et al, 1972).

Thirdly, structure-activity studies with DA and related analogues correlate well with other DA receptor models such as canine RBF, the brain of *Helix aspersa*, behavioural and iontophoretic studies. Epinine was equipotent with DA and substitutions of the 3 and 4 hydroxyl positions of the benzene nucleus resulted in a loss of agonist activity (Miller et al, 1974; Ginos et al, 1975; Watling et al, 1979). ADTN was equipotent with DA (Munday et al, 1974) and 50-350 times more potent than isoADTN (Woodruff et al, 1979) indicating that the DA receptor associated with adenylate cyclase is preferentially activated by the β-rotamer of DA (see Section 1-5-5a). Moreover, this DA receptor model exhibited stereoselectively for R-(+)-ADTN: (+)-ADTN was 100 times more active than S-(-)-ADTN at stimulating the DA-sensitive adenylate cyclase (Woodruff et al, 1979). Interestingly, (-)-apomorphine, a very potent agonist of behavioural models (see Section 1-5-7), was a partial agonist producing only a 50% increase in cAMP compared to DA; at high doses it antagonised DA-sensitive adenylate cyclase both in cell free (Kebabian et al, 1972) and intact cell (Brown and Makman, 1972) preparations.

In contrast, studies with DA receptor antagonists yielded many results which were incompatible with those from other DA receptor model systems. Classical neuroleptic drugs of the phenothiazine and thioxanthene groups and butaclamol were potent inhibitors of the DA-sensitive adenylate cyclase (Clement-Cormier et al, 1974; Miller et al, 1974). However, butyrophenones like haloperidol were much less potent and benzamides like sulpiride (Trabucchi et al, 1975) were totally ineffective (see Section 1-5-11).

After the advent of a binding assay for DA receptors was reported (Seeman et al, 1975; Burt et al, 1975) it was obvious that the anti-psychotic potencies of neuroleptics correlated far better with neuroleptic binding site IC_{50} values, than DA-sensitive adenylate cyclase IC_{50} values. Many dopaminergic antagonists were extremely potent at competing in binding assays but poorly active on the adenylate cyclase; only the phenothiazines and thioxanthenes were equiactive on both. At that time, subcellular fractionation studies indicated that the DA receptor binding site and the DA-sensitive adenylate cyclase might be separate entities (Leyson and Laduron, 1977b). Adenylate cyclase was

located in the mitochondrial fraction of rat striatum (Laduron et al, 1976) whereas neuroleptic binding sites were located in the microsomal fraction. The concept of multiple receptor sites for DA in the CNS was subsequently proposed by Kebabian and Calne (1979) on the basis of these observations, those reported in Chapter 4 and evidence from radioligand binding assays for DA receptors.

1-5-10 Radioligand binding studies

The development of receptor binding techniques has allowed accurate quantitative analysis of the molecular characteristics of membrane binding sites for neuroleptics at very low ligand concentrations similar to those found in patients (Seeman, 1977). Radioligands are employed to label DA receptor binding sites *in vitro* enriched homogenates from dopaminergic tissue.

The purification of [^{3}H] haloperidol and [^{3}H] DA with high specific activity (10 Ci/mmol) and separation of butaclamol into its highly stereoselective enantiomers allowed Seeman et al (1975) and Burt et al (1975) to demonstrate the first dopaminergic ligand binding assays for calf and rat striata. They provided evidence that neuroleptics compete with DA for stereoselective binding sites in proportion to their clinical potency as antischizophrenic agents and that the concentration of drug bound to membrane preparations was in the nanomolar range and therefore similar to that measured in patients undergoing neuroleptic treatment (Seeman, 1977). The technique is essentially a competitive radioligand binding assay and drug potencies are determined by their IC_{50} values which are taken as the concentration of drug required to displace 50% of the specific radioligand from its binding site.

a) Criteria for specificity

Substantial evidence exists that dopaminergic binding studies are truly representative of the physiological and pharmacological events occurring at the receptor level, however, experimental data must be analysed very carefully to preclude the possibility that information might be misinterpreted due to methodological, or other artifacts. Careful analysis requires the following criteria for specificity to be satisfied:-

i) The ligand: The radioligand must have high specific activity

to allow accurate measurement of the receptor site equilibrium constant for the ligand in a nanomolar concentration range. Radioligands (agonist or antagonist) must possess high specific biological activity for the DA receptor, a low dissociation rate from the DA receptor binding site and low specificity for non-dopaminergic binding sites.

ii) Stereoselectivity: Many neuroleptics are highly lipid soluble and can accumulate in extremely high concentrations within membranes to produce disturbances in membrane structure and function, such as altering transmembrane transport mechanisms, exerting local anaesthetic action by retarding membrane excitability in small axons and causing expansion or fluidisation of the membrane. Consequently, neuroleptic binding to membranes alone is insufficient evidence to warrant the designation of specific binding; such identification is verified by the use of drugs with optical or geometric isomers, possessing differences in potency but the same molecular structure. For instance, the stereoselective action of (+)-butaclamol is evident at neuroleptic binding sites in the concentration range 0.1-50 nM, whereas its non-specific solubility into membranes occurs between 100-1,000 nM.

iii) Binding characteristics: The radioligand-membrane receptor interaction should display specificity and saturability within nanomolar concentrations, to permit the determination of an equilibrium binding constant (K_D) and apparent maximum number of binding sites (Bmax). Analysis of experimental data is usually by Scatchard or Hill plots (see Bennett, 1978 for details); kinetic analysis of drug-receptor interactions is reviewed by Weiland and Molinoff (1981). Specific radioligand binding should be regionally distributed throughout dopaminergic brain areas.

iv) Non-specific binding: Total radioligand specific binding to a dopaminergic site is determined by the amount of e.g. [^{3}H] spiroperidol displaceable from the binding site by an excess concentration of a highly specific unlabelled ligand, e.g. (+)-butaclamol. The remaining non-specific radioligand binding could be composed of several components: (1) non-specific binding to the tissue, e.g. albumen, erythrocyte membranes or myelin, (2) free radioligand not effectively washed away or trapped within synaptosomes, (3) non-specific binding to separation materials, e.g. filters and glassware (see Section 3-3-1).

Thus, the criteria for non-specific binding must be cautiously established, with the vital inclusion of an experimental blank, because radiolabelled chemicals can bind to non-biological substances with nanomolar affinities, similar to their affinities for the receptor.

b) Ligands used for dopaminergic binding

Specific binding sites on purified membranes from dopaminergic brain areas have now been demonstrated with a variety of neuroleptic and DA agonist radioligands. The striatum, nucleus accumbens, olfactory tubercle and septum have been labelled by agonists such as [^{3}H] apomorphine (Seeman et al, 1976), [^{3}H] ADTN (Seeman, Woodruff and Poat, 1979; Davis et al, 1980; Woodruff et al, 1979), [^{3}H] DA (Seeman et al, 1975; Burt et al, 1975) and antagonists such as [^{3}H] spiroperidol (Laduron and Leyson, 1977; Creese et al, 1977), [^{3}H] haloperidol (Seeman et al, 1975), [^{3}H] cis(Z)-flupenthixol (Hyttel, 1978), [^{14}C] fluphenazine (Taylor, 1974), [^{3}H] domperidone (Martres et al, 1978), [^{3}H] bromocriptine (Closse et al, 1980) and [^{3}H]-(±)-sulpiride (Woodruff and Freedman, 1981; Theodorou et al, 1979).

Although regional [^{3}H] ligand binding correlated well with brain areas containing the highest density of DA receptor sites, confusion has sometimes arisen because the pattern of binding in various CNS regions has varied with different radioligands used in different species (see review Seeman, 1980).

c) Pharmacological characterisation of DA receptors

Dopaminergic binding sites were qualitatively distinguished from α- or β-adrenoceptor or 5HT receptor binding sites present by displacement studies where the potency of large series of compounds belonging to agonist, neuroleptic and other classes was determined by their efficiency at inhibiting the specific binding of radioligand to membrane receptor. DA was the most potent catecholamine at displacing a variety of [^{3}H] ligands (Burt et al, 1975; Seeman et al, 1975) except for the cerebral cortex and hippocampus where 5HT was more effective than DA in inhibiting [^{3}H] spiroperidol (Leyson and Laduron, 1977a). DA and 5HT receptors are now known to bind similar ligands like (+)-butaclamol and d-lysergic acid diethylamide (LSD) in some regions. α- and β-

adrenoceptor agonists and antagonists lacked such potency.

An important discrepancy was that DA receptor agonists like apomorphine, DA and ADTN displaced the natural ligand [^{3}H] DA (Seeman et al, 1975; Burt et al, 1975) and other agonists (Seeman, Woodruff and Poat, 1979; Seeman et al, 1976) more effectively than antagonists and vice-versa (Creese, Burt and Snyder, 1976). Several interpretations of this observation are briefly listed below.

i) The receptor molecule contained two receptor binding sites, one for agonists and one for antagonists.

ii) The receptor existed in two forms, an agonist state and an antagonist state in rapid equilibrium with each other (Creese et al, 1976).

iii) The two ligand types were labelling different binding sites, either D_1 adenylate cyclase-linked receptors or D_2 non cyclase-linked receptors (Kebabian and Calne, 1979).

iv) The agonist ligands were binding to a presynaptic site and the antagonists to a postsynaptic adenylate cyclase-linked receptor (Leyson, 1979).

v) Both agonists and antagonist ligands were binding to different subunits of a multicomponent receptor complex (Laduron, 1980).

e) Structural-activity requirements for dopaminergic binding

The recent synthesis of a wide variety of semi-rigid DA analogues has permitted detailed structure-activity analysis of the conformation adopted by DA at its binding site and has yielded some information about the topography of the receptor itself. Potential dopaminergic agonists extend across a broad spectrum of chemical compounds including phenylethylamine derivatives, aminotetralins, aporphines, apomorphines, isoapomorphines and octahydrobenzoquinolines. Each group possesses a series of extensively substituted derivatives, however, only those compounds illustrating the essential requirements for dopaminergic potency will be discussed.

i) Conformational requirements: DA receptor binding conformational studies are consistent with interpretations derived from adenylate

cyclase (Woodruff et al, 1977), *Helix aspersa* neurones (Munday et al, 1976) and dog RBF (Volkman et al, 1977) models. All models are in agreement that the active conformation of DA at postsynaptic DA receptors is the β-rotamer (Woodruff et al, 1979). [^{3}H] ADTN binding to rat striatal (Woodruff et al, 1979) and [^{3}H] apomorphine binding to calf caudate (Seeman et al, 1978) preparations were inhibited to a greater extent by ADTN (β-rotameric form of DA) than by isoADTN (α-rotameric form of DA). Moreover the binding site displayed stereospecificity; R-(+)-ADTN was 300 times more potent than S-(-)-ADTN at inhibiting [^{3}H] ADTN binding (Davis et al, 1980), a stereoselective effect in agreement with studies on DA-sensitive adenylate cyclase and locomotor-stimulant activity in the rat nucleus accumbens (Freedman, Wait and Woodruff, 1979).

Surprisingly, apomorphine which is similar to the α-rotameric form of DA, was equipotent or more potent than DA at displacing [^{3}H] apomorphine (Seeman et al, 1978) and [^{3}H] ADTN (Davis et al, 1980) but (±) isoapomorphine, which contains N-methyl DA in a β-conformation, was inactive (Seeman et al, 1978). The lack of biological activity of (±) isoapomorphine both peripherally on RBF (Goldberg and Kohli, 1979) and centrally on the 6-OH DA rotational model (Neumeyer et al, 1974) might possibly be explained by Seeman's (1980) hypothetical model of the DA receptor recognition site. He postulated that an 'obstacle', in the vicinity of the binding site for the N atom of the phenylethylamine of DA, might sterically hinder the approach of the tilted ring in the molecular structure of isoapomorphine.

ii) Absolute requirements for binding: Potential DA receptor agonist compounds must have:-

(1) A hydrogen bonding group equivalent to the 3 position of DA. In DA and most agonists, hydrogen bonding to the receptor site is achieved by an OH group, in ergot alkaloids, e.g. lisuride, hydrogen bonding is by an -NH- group. A second hydrogen bonding group equivalent to the 4'OH group of DA increased potency but was not essential for binding. For instance, the monohydroxylated compound (±)-11-hydroxy-N-n-propylnoraporphine inhibited [^{3}H]spiroperidol binding with 1/40th the potency of (-)-apomorphine (Neumeyer et al, 1974) and (-)5-hydroxy-N-N-dipropyl-2-aminotetralin [(-)-5-OH-DPAT], which represents DA in an α-rotamer conformation, its OH group being equivalent to position 3 on

DA, was more potent than R-(-)-apomorphine at inhibiting [^{3}H] spiroperidol binding (Tedesco et al, 1979) and at induction of stereotypy in rats and emesis in dogs (McDermed et al, 1976). Addition of a second OH group, equivalent to position 4 of DA, enhanced potency slightly in binding experiments. Surprisingly, the affinity of the monohydroxylated β-rotamer DA analogue, 7-hydroxy-N,N-di-n-propyl-2-aminotetralin was 5-22 times weaker than the α-rotamer analogue (Tedesco et al, 1979). Substitution of an OH group into position 6 of the aminotetralin molecule, analogous to p-tyramine, resulted in the lowest affinity for these binding sites.

(2) Optimal positioning of the nitrogen atom: The resolution of dopaminergic structural analogues into their (+) and (-) enantiomers provided evidence that compounds containing a N atom positioned approximately 0.6Å from the place of the ring had the highest potency at inhibiting [^{3}H] spiroperidol, [^{3}H] DA and [^{3}H] apomorphine binding (Tedesco et al, 1979). For instance R-(-)-apomorphine was more potent than S-(+)-apomorphine, and (-)-5-OH-DPAT was 20 times more potent than (+)-5-OH-DPAT (Tedesco et al, 1979; and Figure 1.10). This positional requirement might explain how structural analogues of DA such as R-6a-(-)apomorphine and 5-(+)-ADTN are active despite possessing different configurations about their assymetric carbon atom. Perhaps there is no need for the absolute configuration to be identical so long as the nitrogen atom is positioned away from the plane of the phenyl ring holding the -OH or -NH group.

(3) Distance between -OH and -N groups: Compounds containing a distance of 7.3Å or less between the -OH group and the N atom had the highest potency. For instance, (-)-5-hydroxy-N,N-dipropyl-2-aminotetralin (6.5Å) was more potent than (±)-7-hydroxy-N,N-dipropyl-2-aminotetralin (7.3Å) at displacing [^{3}H] spiroperidol binding (McDermed et al, 1976). If this interpretation is correct, it might explain the potency differences between (+) and (-) ADTN (Figure 1.10 and 1.11). In (+)-ADTN the distance between the N atom and the 7' OH group is 7.3Å in comparison to 7.8Å for (-)-ADTN; also only the (+)-ADTN molecule possesses the N atom positioned below the ring plane.

f) Distribution of binding sites; regional and subcellular localisation: Neuroleptic binding sites and DA-sensitive adenylate cyclase are located predominantly in dopaminergic areas of the brain, especially the striatum and tubercular olfactorum. Their distribution

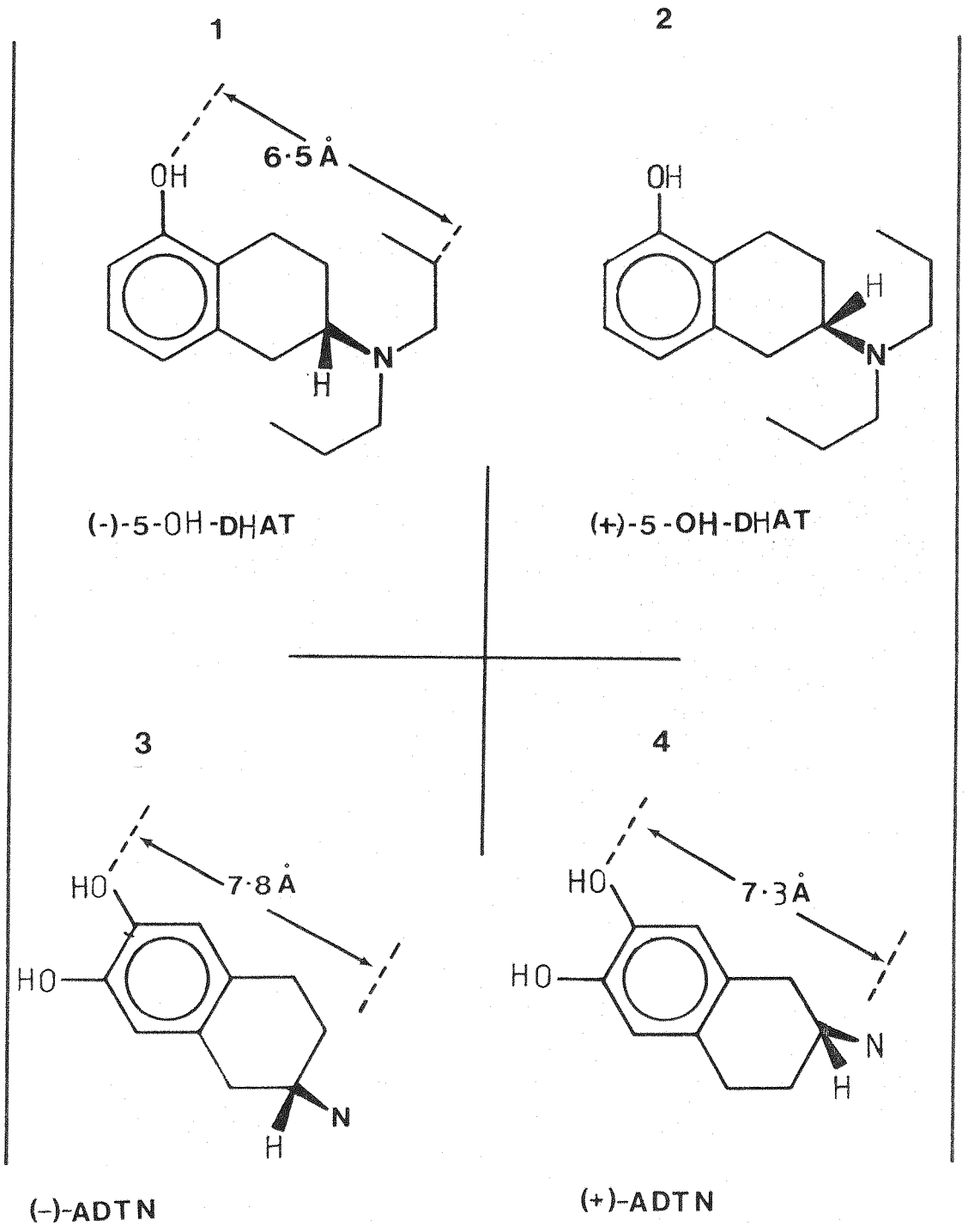


FIGURE 1.10 Isomers of aminotetralin derivatives. 1, (-)-5-OH-DHAT and 2, (+)-5-OH-DHAT are (-) and (+)-5-hydroxy-N,N-dipropyl-2-aminotetralin respectively. 3, (-)-ADTN and 4, (+)-ADTN are (-) and (+)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene respectively. The forward positioning of the N atom in the (+)-isomers reduces potency.

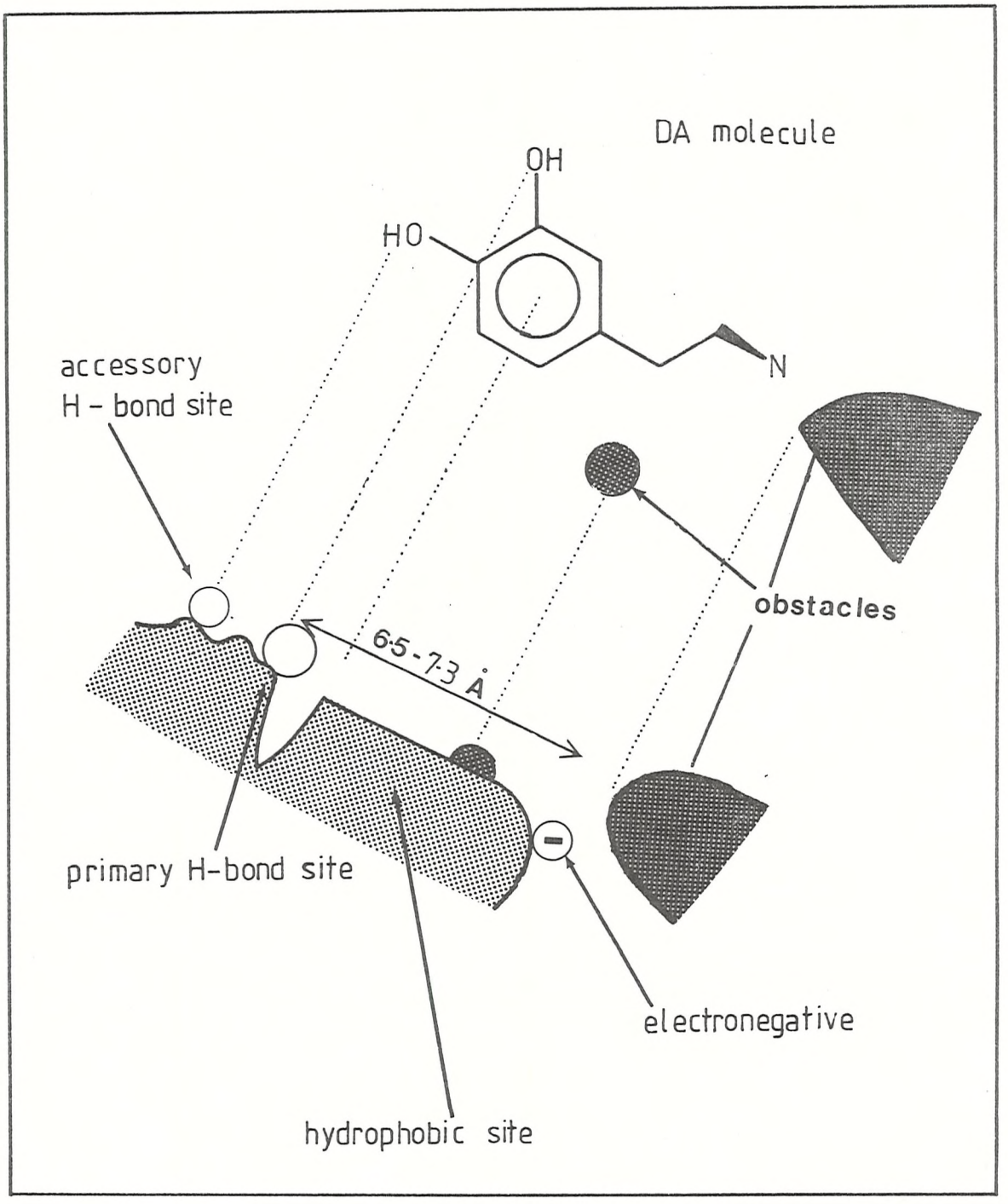


FIGURE 1.11 Diagrammatic representation of the primary binding sites in the vicinity of the dopamine receptor (Seeman, 1980).

is compatible with the regional distribution of labelled neuroleptics in rat and rabbit brain after administration of the drug *in vivo* (Laduron and Leyson, 1977). However, subcellular fractionation studies gave one of the earliest indications that neuroleptic binding sites and the DA-sensitive adenylate cyclase might exist separately (Leyson and Laduron, 1977b). After tissue fractionation [^{3}H] haloperidol binding was greater in the microsomal fraction but DA-sensitive adenylate cyclase was predominantly localised in the heavy mitochondrial fraction (see Section 1-5-9).

1-5-11 Sulpiride

Sulpiride belongs to the class of substituted benzamides which includes drugs such as metaclopramide, sultopride and tiapride. Its efficacy as a DA antagonist typifies classical neuroleptic action in some model systems but bears no resemblance in others.

Centrally, sulpiride is an extremely potent antagonist of ADTN induced hypermotility (Woodruff and Andrews, 1979), an antiemetic (Laville and Margarit, 1968), increases serum prolactin levels in man (Mancini et al, 1976), inhibits apomorphine induced circling behaviour (Elliot et al, 1977), antagonises neuronal inhibitory responses to DA in the substantia nigra (Pinnock et al, 1979) and increases cerebral DA turnover (Tagliamonte et al, 1975). Peripherally sulpiride is the most potent and most specific antagonist of DA induced renal vasodilation in dog (Kohli et al, 1978b; Goldberg et al, 1979) and counteracts DA induced salt and water excretion in patients with salt retention (Agnoli et al, 1978). However, it does not induce catalepsy (Laville, 1972), is relatively weak in displacing [^{3}H] haloperidol binding in the brain (Spano et al, 1978) and is totally ineffective at antagonising DA stimulated adenylate cyclase in the brain (Trabucchi et al, 1975).

Sulpiride's ineffectiveness at inhibiting the adenylate cyclase model is a key piece of evidence in Kebabian and Calne's (1979) interpretation of these observations; namely the existence of two classes of DA receptor, D_1 receptors linked to an adenylate cyclase molecule and unaffected by sulpiride, and D_2 receptors not cyclase linked and inhibited by sulpiride. Moreover Goldberg, Musgrave and Kohli (1979) recently suggested that the DA vascular receptor in the

periphery and the receptor responsible for increase in brain DA sensitive adenylate cyclase might be different because of the extraordinary potency differences of sulpiride on these two receptor models.

1-5-12 Aim of study

Pharmacological characterisation of peripheral DA receptors has been based almost entirely upon studies with the canine renal blood flow model originally developed by Goldberg and his colleagues almost 20 years ago. Today this still remains the most direct model available for the study of peripheral DA receptors, despite the introduction of direct biochemical models which have been used extensively for the investigation of central DA receptors.

The aim of the present study was to establish a model test system for renal DA receptors in a convenient, small, laboratory animal, the guinea pig, to measure direct physiological responses to dopaminergic agents. This was combined with more direct methods for the assessment of DA receptor activity by examining the ability of dopaminergic agents to effect the production of cAMP and [^{3}H] sulpiride binding in this animal. Before undertaking the study, it was known that DA depressed MABP in the anaesthetised guinea pig (Hornykiewicz, 1953) in the absence of pharmacological blockade of α-adrenoceptors, in contrast to its actions in the dog (Goldberg et al, 1968) and rat (Chapman et al, 1980), where DA elevates MABP probably by activation of α-adrenoceptors. Guinea pig vasodepressor responses to DA had previously been employed in our laboratories as a simple model for measuring DA-like activity on specific DA receptors (Woodruff and Sumners, 1979). Therefore we investigated renal responses to DA in the guinea pig.

CHAPTER 2

METHODS AND MATERIALS

2-1 Renal blood flow

2-1-1 Animal preparation

Guinea pigs of either sex (350-450 g), were anaesthetised with 1.2 mg/kg i.p. urethane (ethyl carbamate) and were surgically equipped with a tracheal cannula (Portex, PP 205) and left jugular venous cannula (Portex, PP 25). The latter was connected to a Braun (Melsungen) AG constant infusion pump for administration of drugs (Figure 2.1). Mean arterial blood pressure (MABP) was monitored from the right carotid artery by an Elcomatic EM750 transducer. Intra-renal artery drug infusions or injections were given via finely drawn out cannulation tubing (Portex, PP 100) which was inserted at the level of the left femoral artery and advanced along the aorta so that the tip was situated at the bifurcation of the aorta and left renal artery. This operation allowed drugs to be administered selectively into the circulation of the left kidney, either as continuous infusions or as injections in 0.1 ml/kg vehicle, from a high pressure screw syringe device which was necessary to overcome tubular resistance to injected volumes. The right femoral artery was ligatured to prevent drug escape, down the aorta and away from the kidney, during i.a. injections. Blood flow through the left kidney was maintained by a slow infusion (1.2 ml/hr) of 0.9% heparinised saline, throughout the experiment.

2-1-2 The measurement of renal blood flow by the hydrogen washout technique

The left kidney was exposed by abdominal incision followed by peritoneal cauterisation to minimise blood loss. A platinum polarography electrode was inserted to a known depth (0.5-1.0 mm) into the renal cortex and an Ag/AgCl reference electrode clamped rigidly against the exposed abdominal tissue completed the electrical circuit. The entire kidney was insulated with a thin layer of liquid paraffin to prevent moisture loss from the surface and to minimise electrical interference around the platinum electrode. Both electrodes were connected to a Vitatron pen recorder and integrated to produce a logarithmic response. Cortical RBF was measured by the H_2 washout technique originally described by Aukland *et al* (1964). Briefly, H_2 (2-5ml) inspired by the animal via the tracheal cannula, rapidly equilibrated with blood and body tissue. Local H_2 activity was measured as the

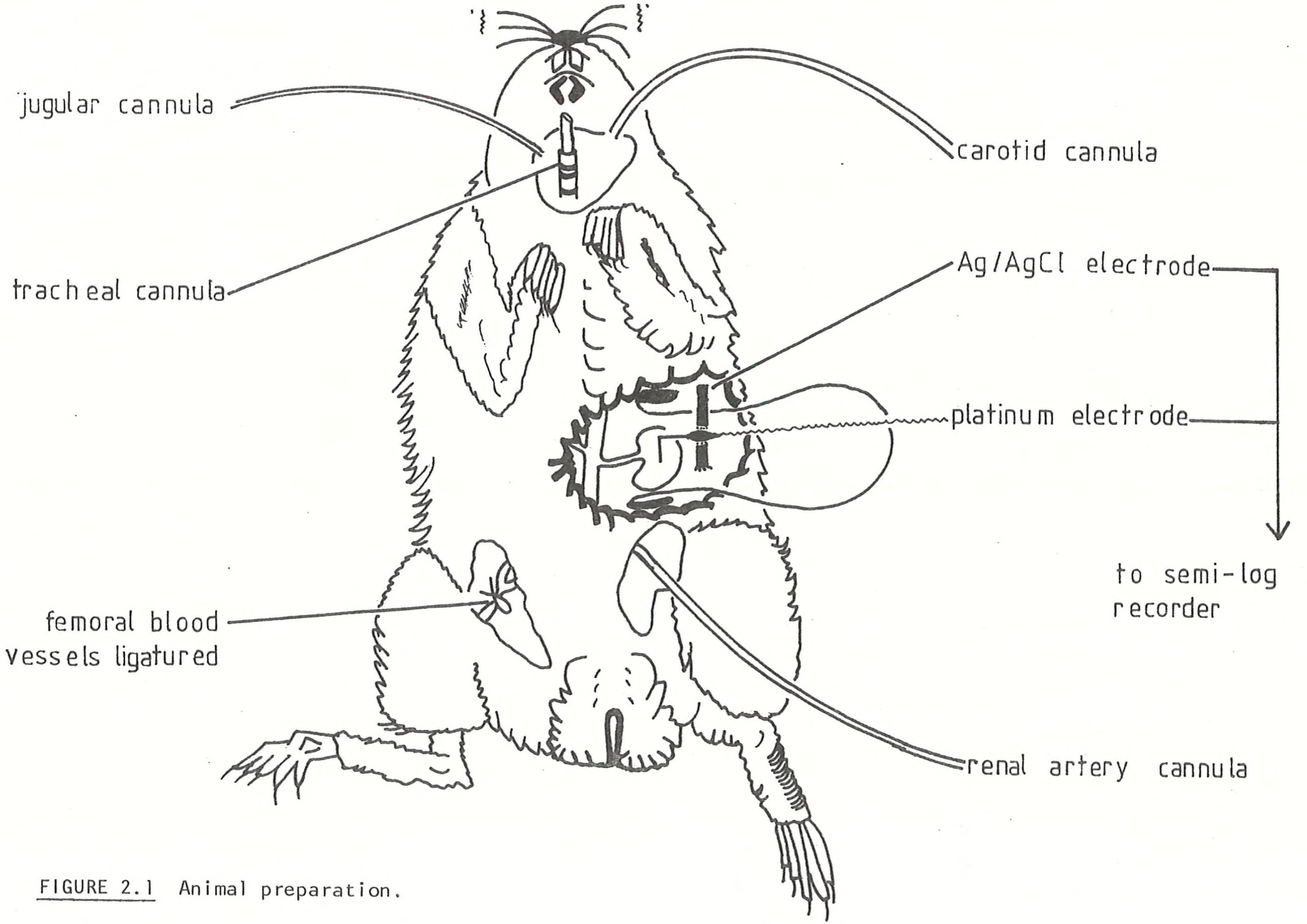


FIGURE 2.1 Animal preparation.

current flowing between the reference and platinum electrodes. As H_2 concentration increased in the kidney an H_2 saturation peak was observed and its subsequent washout was proportional to the rate of RBF. The exponential decay of the H_2 washout curve was displayed on a pen recorder.

The half-life of H_2 concentration is indicative of the rate of change of $[H_2]$ in the kidney. RBF was calculated from the slope of individual desaturation curves, according to an equation derived from the Fick principle:-

$$K = \frac{F}{\lambda W} = \frac{0.693}{t_{\frac{1}{2}}}$$

K = slope of the graph of $\frac{\log [H_2]}{\text{time}}$

F = blood flow

W = volume of tissue

λ = tissue:blood partition coefficient of the gas (assumed to be unity for the kidney)

$t_{\frac{1}{2}}$ = half-life of $[H_2]$ in venous blood

therefore blood flow = $\frac{69.3}{t_{\frac{1}{2}}}$ ml/100 g/min

Renal vascular changes were only attributed to a direct vascular action if changes were in an opposite direction to, or proportionally greater than, those changes expected during normal autoregulation.

To ensure that renal vascular responses were directly due to DA, but not due to compensatory changes in blood pressure the pen recorder was carefully synchronised to measure RBF and MABP simultaneously. A further parameter of renal vascular function, the renal vascular resistance (RVR), was deduced by incorporation of coincident values of RBF and MABP into the equation:-

$$RVR = \frac{MABP}{RBF} \text{ mmHg/ml min}^{-1} \text{ 100 g tissue}^{-1}.$$

2-1-3 Guinea pig RBF measurements as a model for peripheral dopamine receptors

Preliminary experiments revealed that intra-renal artery injections of DA (0.5-1,600 nmol/kg) resulted in dose-related increases in RBF at

low doses (0.5-30 nmol/kg) but vasoconstriction at high doses (100-1,600 nmol/kg). DA is known to constrict the renal vasculature in dog (Goldberg et al, 1968) and rat (Chapman et al, 1980) by activation of α-adrenoceptors (Goldberg et al, 1978a). In guinea pigs receiving α-adrenoceptor antagonist pretreatment, the vasoconstriction response, previously obtained with high doses of DA, was abolished (see Section 3-1-3). Thus, an α-adrenergic blocking agent was incorporated into all subsequent experiments. Various agents were tested for their suitability as α-adrenoceptor antagonists in this model. Phenoxybenzamine (POB) (0.3 mg/kg) and phentolamine (0.1 mg/kg) were lacking in specificity and duration, of α-adrenoceptor blockade, respectively. In contrast, prazosin (0.1 mg/kg), a highly specific α_1 antagonist had prolonged action (2-4 hours). Therefore in all subsequent experiments prazosin was administered, diluted in 2-3 ml of saline, during a 30 minute infusion period prior to experimentation. Supplementary doses of prazosin were administered as required to maintain a complete inhibition of MABP responses to the specific α-agonist phenylephrine (50 nmol/kg). Measurements were begun 30-40 minutes after completion of surgical procedures. Results were expressed as ml blood flow/100 g tissue/min or as the percentage increase of RBF above that of basal flow values during saline infusions.

2-1-4 Evaluation of methodology

1) Efficiency of α-adrenergic blockade

Following prazosin (0.1 mg/kg) treatment the resting blood pressure of anaesthetised guinea pigs used in these studies fell from 60.6 ± 2.5 mmHg (n = 9) to 26.3 ± 0.5 mmHg (n = 6). To test the duration of α-adrenergic blockade single doses of the α-adrenergic agonist phenylephrine (50 nmol/kg) were administered at 15 minute intervals for 3.5 hours post-prazosin. Complete inhibition of MABP responses to phenylephrine by prazosin for two hours are shown in Figure 2.2. A small amount of vascular tone slowly returned approximately two hours after prazosin administration.

2) Verification of positioning of intra-renal artery cannula

Guinea pig renal arteries are extremely delicate and susceptible to damage caused by the mechanics of cannulation. Moreover, insertion of

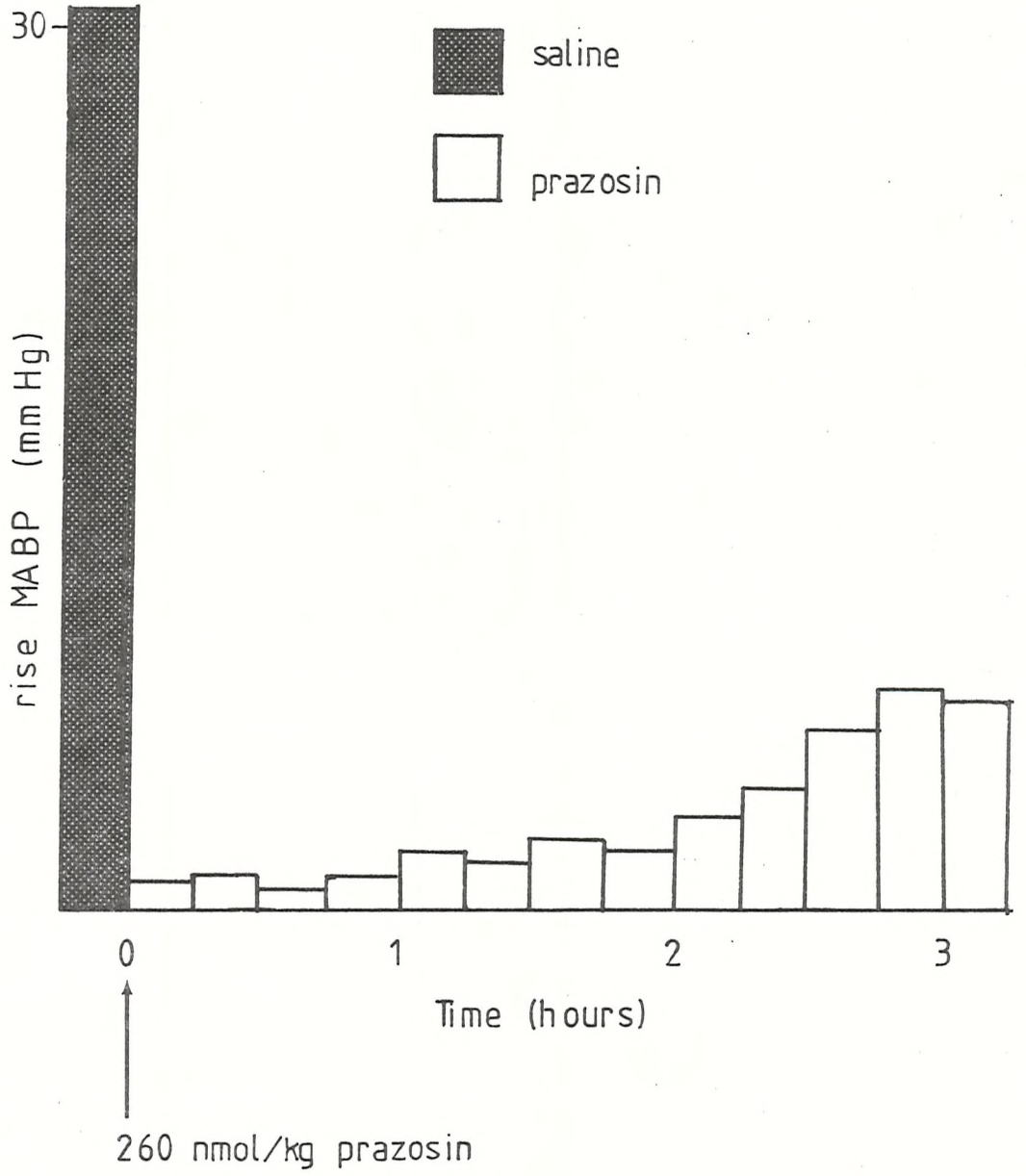


FIGURE 2.2 Blockade of α-adrenergic tone in guinea pigs by prazosin. Mean arterial blood pressure responses to phenylephrine (50 nmol/kg) at time 0 during saline infusion and at 15 minute intervals following prazosin (260 nmol/kg) administration. Ordinate responses are expressed as the increase in blood pressure (mmHg).

cannulation tubing into the renal artery frequently resulted in irreversible, chronic renal vasoconstriction. To overcome this problem cannulae were manoeuvred over the bifurcation point of the aorta and left renal artery, and drugs were administered from this location. Cannulae tips were blackened with a pen to facilitate positioning as they were visible through the translucent blood vessels. Further confirmation that solutions entered the left renal circulation, directly after i.a. injection, was provided by methylene blue dye. After i.a. injection of the dye the left kidney immediately took up a blue colouration and after subsequent removal by the renal venous circulation other peripheral organs went blue. A schematic representation of the cannula positioning is shown in Figure 2.3. Also there was a good correlation between animal body weight and the length of i.a. cannulation tubing required to cover the distance from its insertion point at the left femoral artery to the left renal artery. A graph of animal body weight against length of i.a. cannulation tubing is shown in Figure 2.4, mean animal body weight was 420 ± 20 g (n = 15) and therefore cannulae lengths were between 6.3-6.4 cm.

3) The platinum polarography electrode

a) Manufacture of platinum tissue electrodes: Electrodes were made after modification of the method of Aukland et al (1964), plate 1, 10-15 mm long pieces of L-shaped platinum wire (0.2 mm diameter) were soldered to thin copper wire 40 s.w.g. and the tips were tapered by filing. The electrode-wire junctions were reinforced with epoxy resin before insulating both electrode shafts and junctions with 2-3 layers of Sterling thermoxy varnish, followed by baking in an oven at 100-150°C. Varnish was then scraped from each electrode, leaving a highly localised bare area measuring 0.5 mm at the tip, as shown in plate 2, and about 0.2 mm largest diameter. Further reduction of electrode diameter did not show an improvement of average local blood flow values (Tyssebotn et al, 1979). One mm graduations were made on each electrode shaft to allow accurate positioning of electrodes to a known depth in the renal cortex. At the end of experiments kidneys were removed to check the positions of the electrodes. Results were discarded if electrode tips were inserted more than 1.0-1.5 mm deep into the kidneys or were in contact with calyces or connective tissue.

b) Tissue trauma: Observations made by Løyning (1971; 1974) and

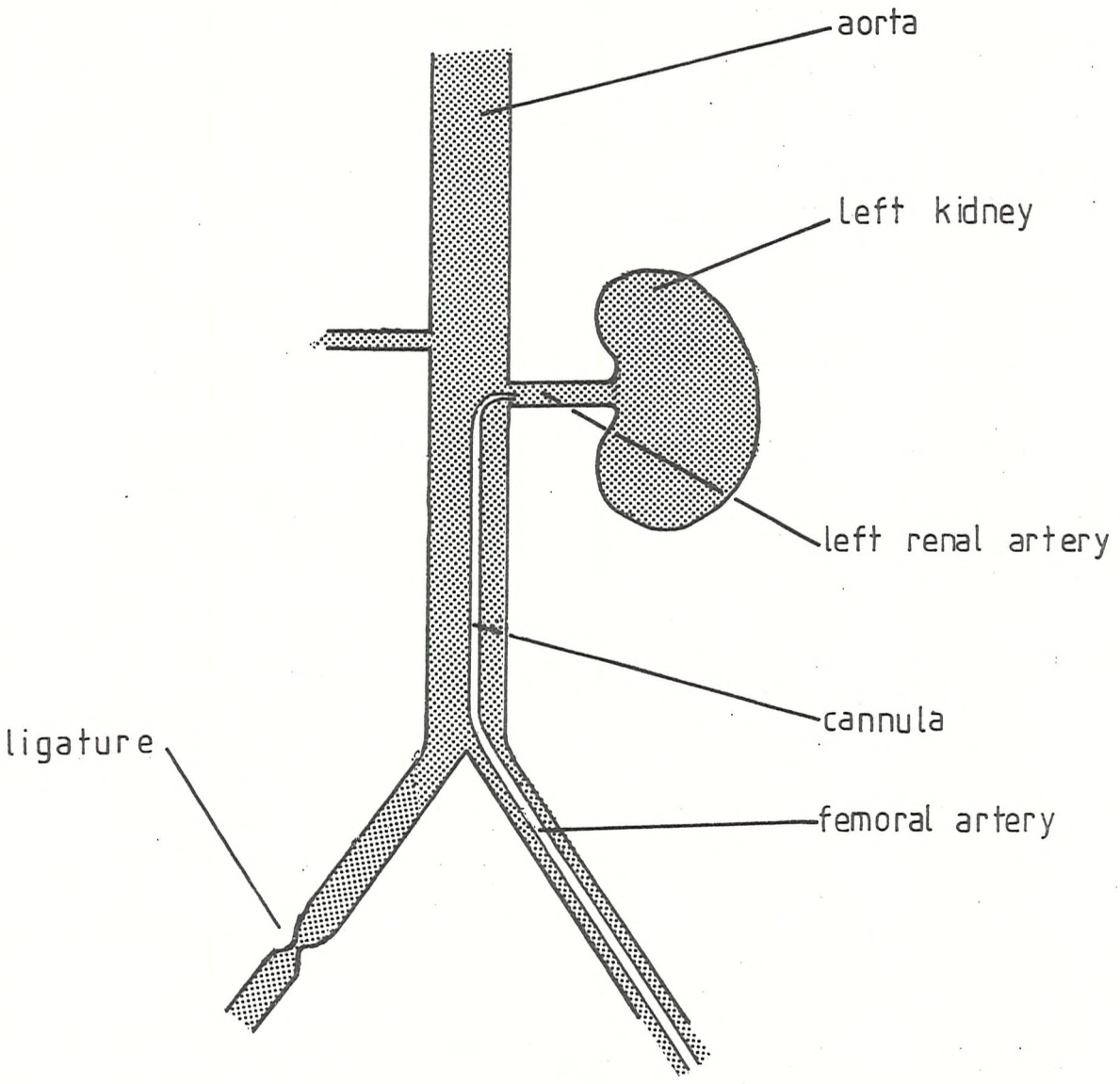


FIGURE 2.3 Schematic representation of positioning of cannula used for intra-renal artery drug administration.

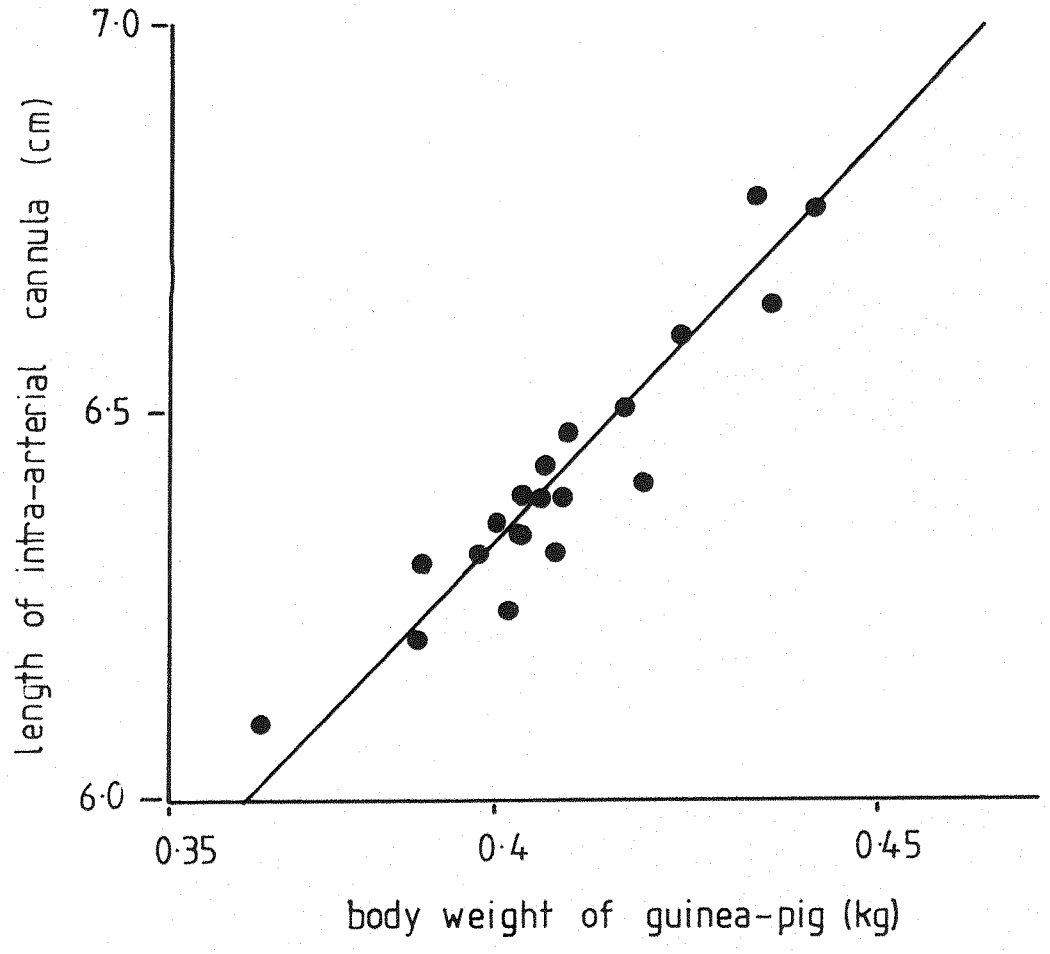


FIGURE 2.4 Correlation between guinea pig body weight and length of intra-renal artery cannulae. Drugs were administered at the bifurcation of the aorta and left renal artery.

PLATE I Typical platinum electrode used for the recording of renal hydrogen concentration in the guinea pig.

PLATE 2 Platinum polarography electrode tip. Following puncture of the renal capsule with a fine needle, electrodes were inserted to a depth of approximately 1 mm into the renal cortex.

Aukland (1965; 1980b) suggest that insertion of electrodes into the kidney only superficially affects the physiological characteristics of blood vessels in immediate surrounding tissue. A thin layer of damaged cells surrounding the electrode tip would slightly delay, but not alter, the gradient of the H_2 desaturation curve and autoregulation around the electrode tip would remain unaffected.

4) Validity of H_2 clearance for estimating local blood flow: The use of hydrogen desaturation rate as a measurement of local blood flow is based on two assumptions (Aukland et al, 1964), i) instantaneous equilibration between a tissue and its venous blood, and ii) no recirculation of hydrogen. Extensive studies of the H_2 clearance technique and a thorough discussion of its validity for estimating local blood flow have been made by Aukland et al (1964) and Aukland (1968), particularly for the measurement of cortical blood flow (Løyning, 1971; Aukland et al, 1973). These authors reported mono-exponential curves down to at least 90% H_2 desaturation of mixed venous blood indicating only a negligible amount of H_2 recirculation (well below 10%). Furthermore, there is good agreement between results obtained by the H_2 clearance method and those obtained by measuring total renal blood flow, using an electromagnetic flow probe (Løyning, 1974) or PAH clearance (Aukland et al, 1964) and local renal blood flow using radioactive probes such as the transit time of ^{32}P labelled erythrocytes (Aukland and Wolgast, 1968), clearance of ^{85}Kr and ^{133}Xe (Aukland and Wolgast, 1968; Passmore et al, 1977), or uptake of inert diffusible tracers such as ^{125}I-iodoantipyrine and $^{3}H_2O$ (Clausen et al, 1979; 1980; Hope et al, 1976; Aukland, 1978). Moreover the technique allows repeatable measurements of local capillary blood flow and is highly sensitive to intra renal flow redistribution induced by vaso-dilating agents (Aukland and Løyning, 1970; Tyssebotn and Kirkebø, 1979). Methods for measuring renal blood flow are extensively reviewed by Aukland (1980b).

To ensure that H_2 gas did not diffuse from the cortex into the interlobular arteries or from the surface of the kidney, and result in overestimation of blood flow, a hydrogen desaturation curve from three guinea pigs was interrupted by 1.0 ml i.v. saturated KCl solution. Figure 2.5 shows that KCl induced death and cessation of RBF was reflected by an instantaneous halt of the downward curve of H_2 washout. The logarithmic response representing H_2 activity in the

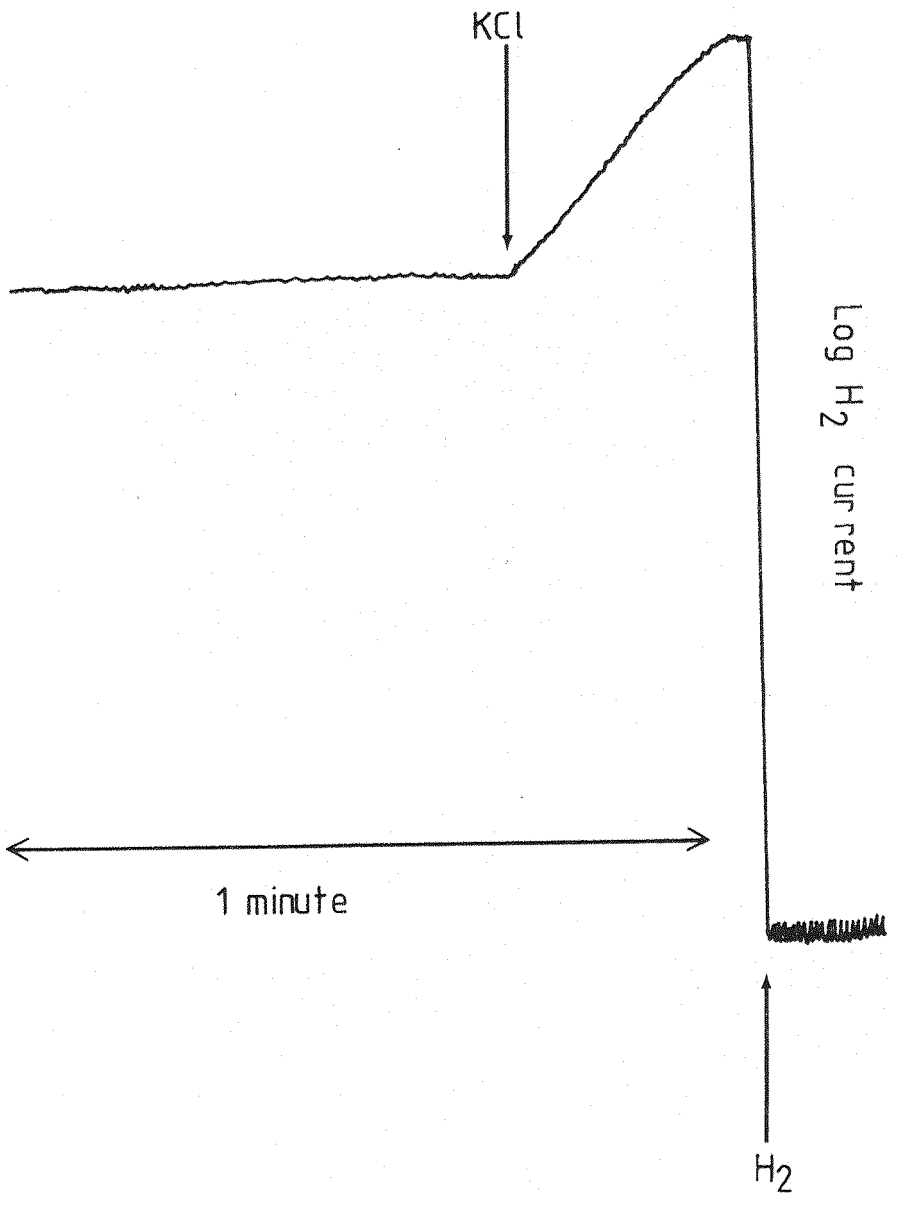


FIGURE 2.5 KCl induced cessation of renal blood flow. Hydrogen washout, recorded from a guinea pig renal cortex is immediately arrested following intra-venous administration of 1 ml of 1M HCl.

kidney remained parallel to the baseline after death, thus demonstrating that diffusion exchange of hydrogen gas out of the kidney is probably not significant. This finding confirms previous reports from our own laboratories (Robertson, 1981) and others (Løyning, 1971; Tyssebotn and Kirkebø, 1979) who reported a similar percentage reduction in total blood flow and in cortical H_2 clearance, when renal perfusion pressure was reduced below the level of blood flow autoregulation.

5) Drug solution:

i) Sulpiride was dissolved in 0.9% saline containing a trace of NaH_2PO_4.

ii) Prazosin (1 mg/ml) was dissolved by gentle warming in 1% ascorbic acid and stored for up to a week at 4°C before use. Aliquots were diluted as required in 0.9% saline to a final concentration of 0.1 mg/ml prazosin (thus containing 0.1% ascorbic acid) and kept at room temperature prior to experimentation.

iii) Haloperidol was dissolved in a minimum volume of lactic acid by heating to 80°C for one hour with occasional agitation. The mixture was then diluted with distilled water and the haloperidol solution could be stored in 1% lactic acid for up to a year at 4°C, with light excluded.

iv) All other drugs were dissolved in 0.9% saline.

2-2 Stimulation of dopamine sensitive adenylate cyclase in guinea pig renal particulate preparations

2-2-1 Preparation of kidney particulate

Renal particulate preparations, designed to contain an enriched composition of renal tubules, glomeruli and blood vessels, were prepared by differential filtration, essentially as described by Nakajima *et al* (1977). Male guinea pigs (300-500 g) were sacrificed by cervical dislocation and their kidneys immediately submerged in ice cold Krebs-Ringer bicarbonate buffer pH 7.4 (mM) (NaCl 118; KCl 4.7; $CaCl_2.6H_2O$ 2.54; KH_2PO_4 1.2; $MgSO_4.7H_2O$ 1.2; $NaHCO_3$ 24.8; glucose 11 and nialamide 0.075), which had previously been equilibrated with a mixture of O_2 (95%) and CO_2 (5%). Fat surrounding the kidneys was removed together with the renal capsules and pelvices. Renal cortical and medullary tissue was cut into pieces and manually homogenised in 5 vols. (V/W) ice cold buffer with a loosely fitting glass homogeniser. The homogenate

was filtered by suction, through a brass sieve with P/Bronze mesh, 150 μ pore size, and washed with 5 vols of ice cold buffer. Residue remaining on the sieve was rehomogenised and the tissue fraction containing renal tubules, glomeruli and blood vessels was further separated from cellular material by refiltration, followed by thorough washing with the same volume of buffer.

2-2-2 Incubation procedures

The particulate preparation retained on the sieve was immediately suspended in Krebs-Ringer bicarbonate buffer pH 7.4 (2.5 vols) and equilibrated with a mixture of O_2 (95%) and CO_2 (5%). Tissue cAMP levels were minimised by a 60 minute preincubation at 37°C with continuous agitation to allow enzymic degradation of the nucleotide to reduce basal cAMP concentrations. During incubation periods, the medium was aspirated and replaced once after 20 minutes and twice after 40 minutes. After 60 minutes the preparation was washed twice with Krebs-Ringer bicarbonate buffer containing theophylline (5 mM) to prevent further degradation of cAMP and resuspended in the same buffer (2.5 vol).

Incubation procedures took the same format as those described by Forn et al (1974). 0.5 ml aliquots (1-5 mg protein) were added to tubes containing 'Krebs' theophylline' buffer and the various drugs or vehicle to be tested resulting in a final volume of 0.6 ml. In experiments with antagonists, the kidney particulate preparation was added to tubes containing drugs or vehicle after only 50 minutes preincubation, other agents were added 10 minutes later. After aspiration with a mixture of O_2 (95%) and CO_2 (5%), renal tubules, glomeruli and blood vessels were incubated for a further 15 minutes in the presence or absence of test substances. The reaction was terminated by placing the tubes in boiling water for 5 minutes. Soluble cAMP was separated from tissue by centrifugation (MSE Minor, bench centrifuge) at approximately 3,000 g for 10 minutes; the resulting supernatant was removed for cAMP estimation whilst the pellet was dissolved in 1 ml 1N NaOH. Protein content of this was determined by the method of Lowry et al (1951), using bovine serum albumen as a protein standard. All drugs were dissolved in 10 μl 4 mM tartaric acid.

2-2-3 Estimation of tissue cAMP content

A 50 µl aliquot of each resulting supernatant was assayed in duplicate for cAMP content by a competitive protein binding assay as described by Brown et al (1971). Commercially available cAMP (0.5-10 pmol) and [^{3}H] cAMP (specific activity 25 Ci/mmol) were routinely used to construct standard curves. A typical standard curve for cAMP content is shown in Figure 2.6. Free ligand was separated from bound by millipore filtration using HAWP 02400 filters pre-soaked in 50 mM tris-Krebs' buffer containing 8 mM theophylline and 6 mM mercaptoethanol, pH 7.4. The filters were washed with 10 ml ice cold buffer and dissolved in 1 ml methyl cellusolve. Bound radioactivity was determined by liquid scintillation spectrometry after correction for background, quenching and machine efficiency. Results were expressed as pmol cAMP formed per mg protein or as the percentage of maximum response which was taken as that produced by 1 mM DA and represent the mean of 3-4 determinations at each drug concentration.

2-2-4 cAMP binding protein

cAMP binding protein was purified from bovine adrenals according to the method of Brown et al (1971) and stored in 0.5 or 1.0 ml aliquots at -20°C for up to six months. The sensitivity of individual preparations of cAMP binding protein was assessed by construction of protein dilution curves. The dilution of binding protein which specifically bound 25-30% [^{3}H] cAMP was routinely used for assays (Figure 2.7).

Since renal cAMP estimation was based upon competition for protein binding of the nucleotide ligand to a cAMP dependent protein kinase, the specificity of the assay was evaluated. The ability of various nucleotides and related compounds, which might have been present in the particulate preparation, to compete with cAMP as ligands for the binding protein was tested. Figure 2.8 demonstrates that of the compounds tested 3'5'cGMP was the most effective at competing for cAMP binding. In spite of this reaction a concentration of almost 0.5 µM 3'5'cGMP was required to displace 2 pmol [^{3}H] cAMP. ATP had no inhibitory effect on [^{3}H] cAMP binding at very high concentrations (890 µM) and virtually no effect at 100 µM. ADP, adenosine and theophylline displayed no significant affinity as ligands for cAMP dependent protein kinase. These nucleotides are not present in mammalian tissue at sufficiently

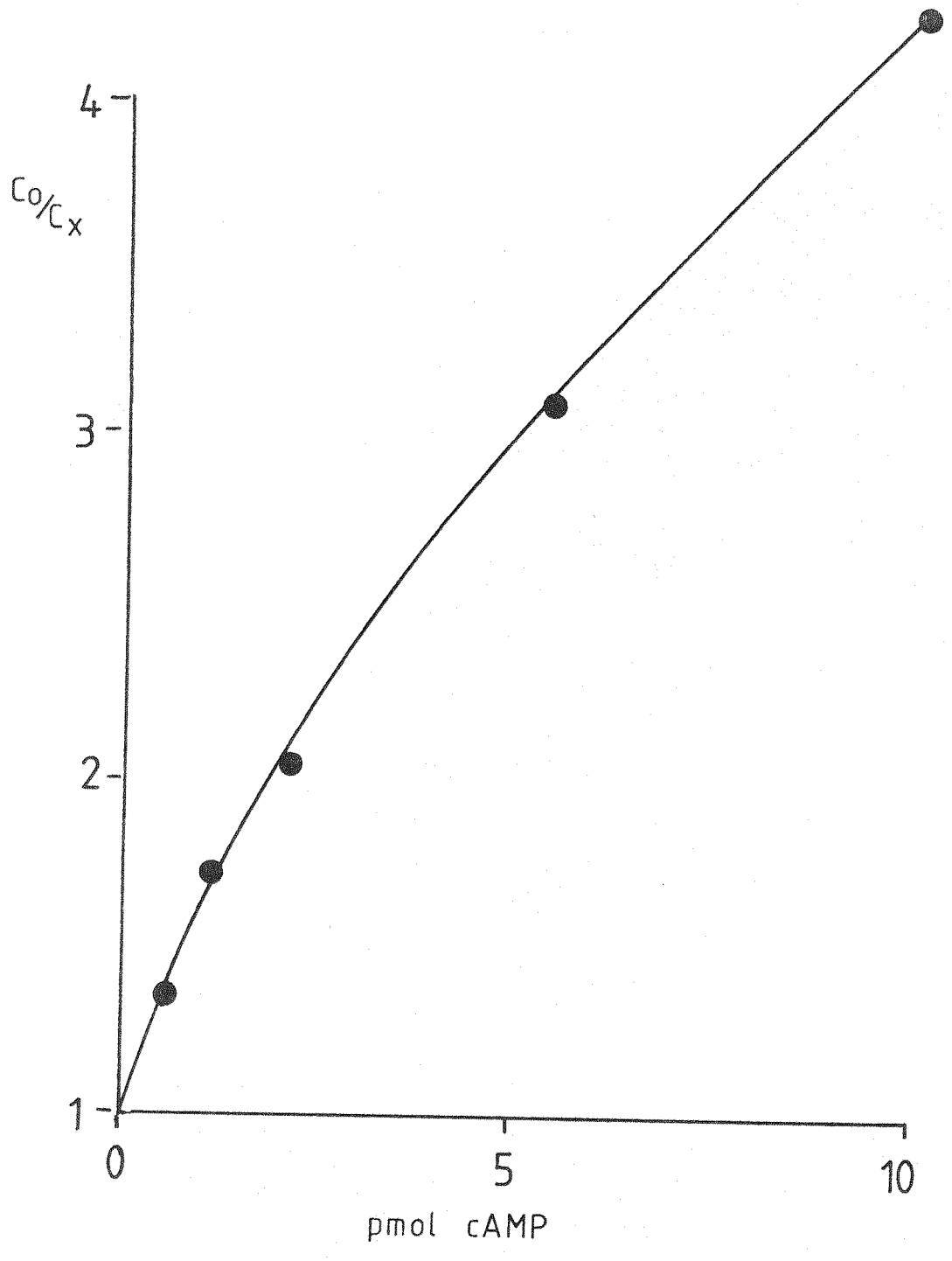


FIGURE 2.6 Typical cAMP standard curve. Co/Cx represents the proportion of a known quantity of [^{3}H] cAMP displaced by unlabelled cAMP.

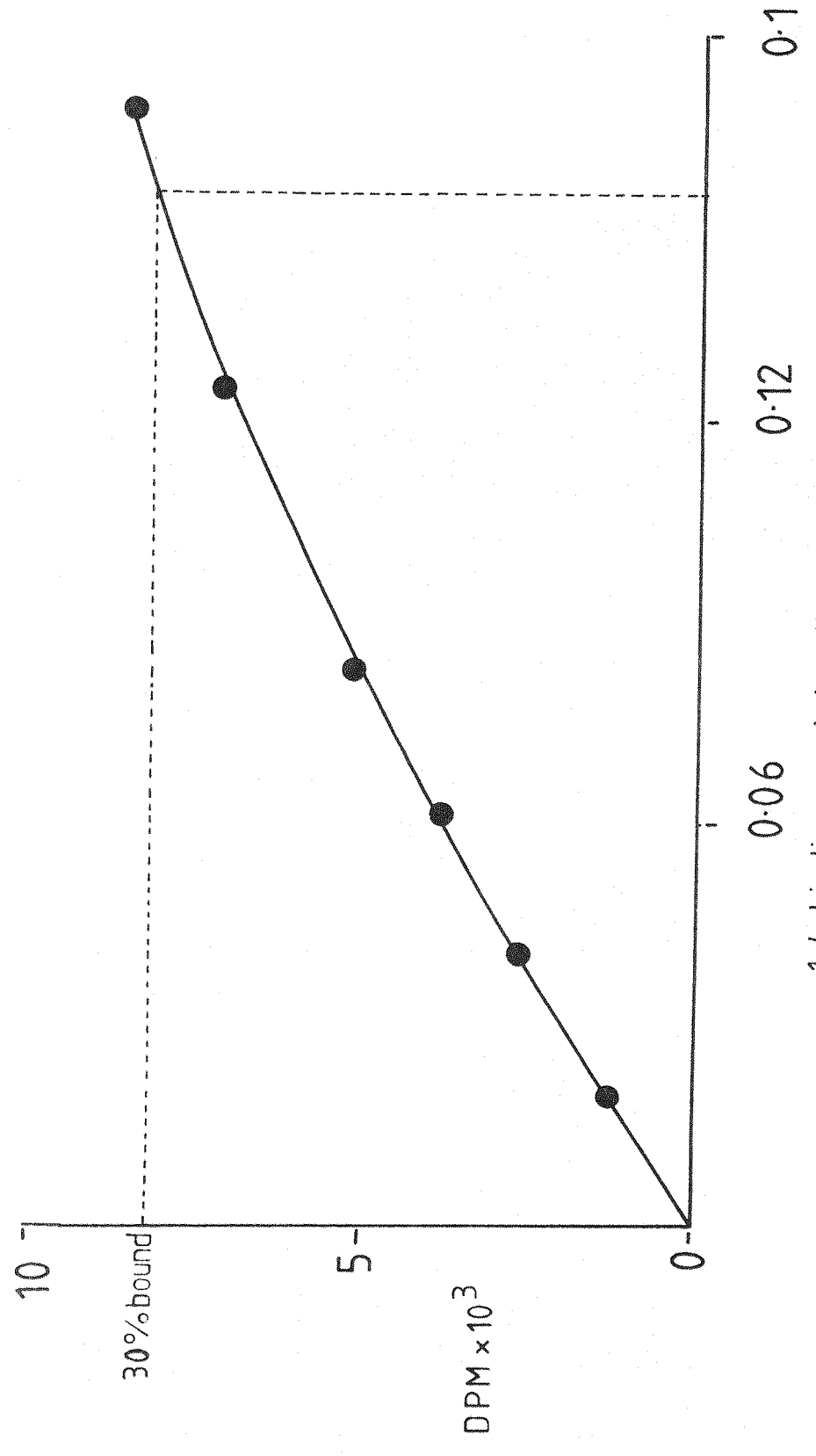


FIGURE 2.7 Typical cAMP binding protein dilution curve. Binding protein was diluted in Krebs'-bicarbonate buffer containing 5 mM theophylline, pH 7.4 at 0°C.

compound	[μM] required to displace 2 pmol [^{3}H] c AMP
cAMP	0·004
cGMP	0·046
ATP	890
ADP	no cross reactivity
adenosine	no cross reactivity
theophylline	no cross reactivity

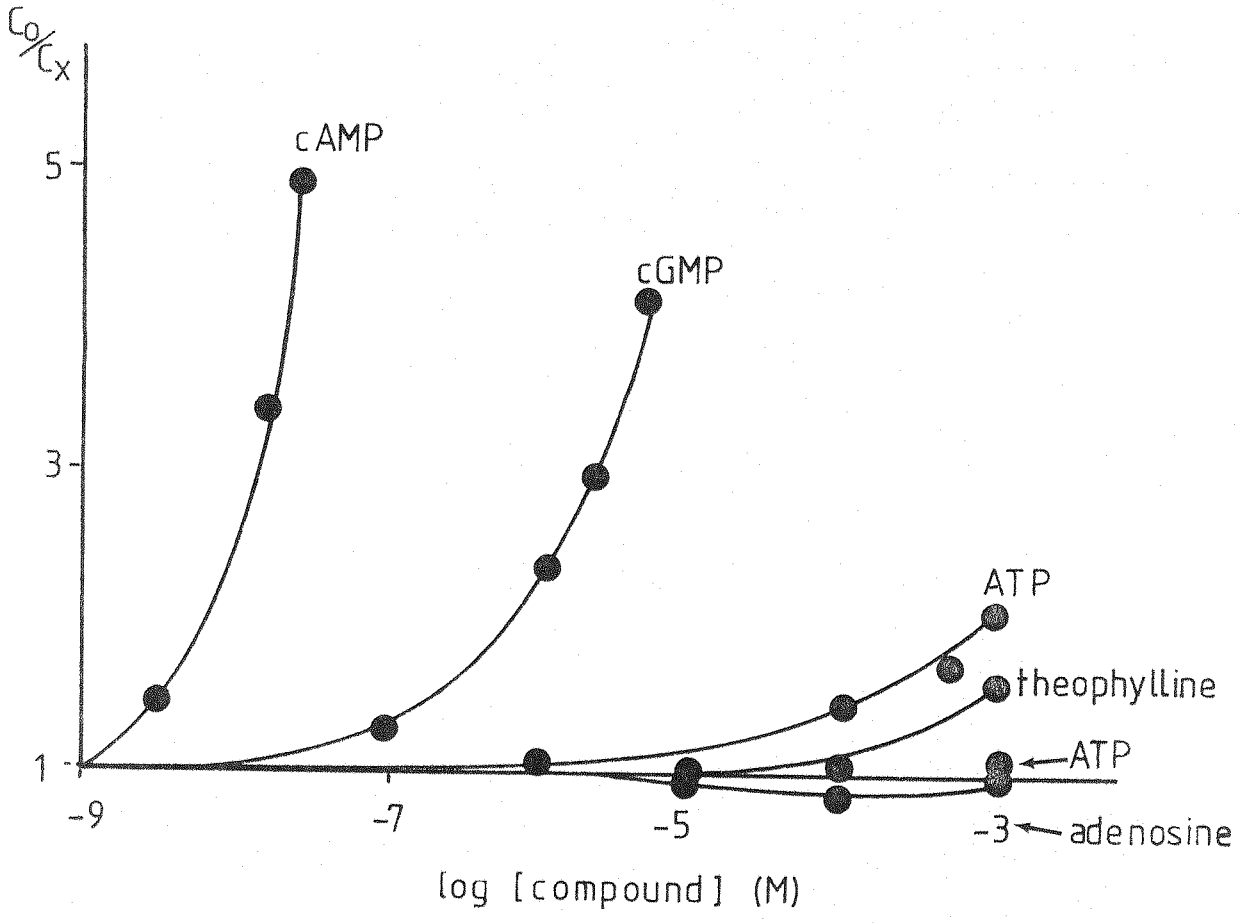


FIGURE 2.8 Specificity of cAMP binding protein.

high concentrations to interfere with cAMP binding. Moreover tissue nucleotide levels were diluted by a factor of 2.4 during cAMP estimation, therefore even ATP, which exists at concentrations approaching 500 µM, would be unable to cross-react with cAMP. For these reasons the protein binding assay was presumed to be specific for cAMP.

2-3 Receptor binding studies

2-3-1 Preparation of membranes

Renal cortical membranes were prepared from 400-500 g male guinea pigs by the method of Rosenblatt et al (1980). After decapitation the kidneys were rapidly removed and submerged in ice cold 50 mM tris HCl buffer, pH 7.3. Fat surrounding the kidneys was removed together with the renal capsule. After lateral bisection, renal cortical tissue (the most distal 1mm) was cut into 1 mm diameter strips and homogenised with 5 strokes (Tri-r instruments polytron, Model K41, setting 7 for 30 seconds) in 100 volumes (W/V) tris HCl buffer. The homogenates were centrifuged twice at 50,000 g for 10 minutes (Beckman J2-21 centrifuge, JA20 rotar head), with resuspension in fresh buffer. Final suspension was in 50 mM tris HCl buffer pH 7.3 at 25°C containing 0.1% ascorbic acid (mM) NaCl 120; KCl 5; CaCl 2; $MgCl_2$. When necessary renal medullary and pelvic membranes were prepared in the same way. Tissue preparations were immediately frozen in liquid nitrogen and stored at -20°C for up to a week before use.

2-3-2 Binding assay

Aliquots of renal tissue preparations (1.14 ± 0.035 mg) were pre-incubated for 3 min. at 25°C in the presence and absence of displacing drug, vehicle or 50 mM tris HCl to make a final volume of 1.2 ml. 10 µM s-(-)-sulpiride was used to define specific binding. Incubations were initiated by the addition of [^{3}H] sulpiride (specific activity 26.2 Ci/mmol) at a mean concentration of 20.8 ± 0.6 nM. The reaction was terminated 10 minutes later by placing the tubes on ice for 2 minutes. Free and bound ligand were separated by centrifugation in a previously cooled Beckman microfuge, for 1 min. Samples were placed on ice and the resulting supernatant quickly aspirated. After washing the pellet surface with 1 ml 50 mM tris HCl buffer to remove superfluous free ligand, the pellets were solubilised in 0.8 ml protosol with incubation

for one hour at 60-70°C. Bound radioactivity was determined by liquid scintillation counting after the addition of 0.40 ml glacial acetic acid to reduce phosphorescence. Radioactivity was corrected for quenching, background and machine efficiency. Protein was evaluated according to Lowry et al (1951).

In preliminary experiments free and bound radioactivity was separated by filtration using either millipore HAWP 02400 or Whatman GF/B filters. Millipore filters were presoaked in 50 mM tris-Krebs' buffer pH 7.3 and after filtration were washed with 10 ml ice cold buffer followed by solubilisation in 1 ml methyl cellusolve. Alternatively, following separation with Whatman GF/B filters, radioactivity was removed from the filters by standing overnight in 1 ml 2% sodium dodecyl sulphate. All drugs were dissolved in 4 mM tartaric acid or 10 µl glacial acetic acid followed by dilution to the correct volume with 4 mM tartaric acid. All subsequent dilutions were in tris-Krebs' buffer.

2-4-1 Dopamine level estimation

Regional DA content in the kidney was estimated by the spectrofluorometric method of Earley and Leonard (1978). Dissection procedures were the same as for receptor binding, after which each tissue sample was manually homogenised in 2.5 ml of 0.4 N perchloric acid and centrifuged (MSE Minor, bench centrifuge) at approximately 3,000 g for 5 minutes. One ml aliquots of the resulting supernatant were assayed in duplicate, following separation of the catecholamines by sephadex column chromatography. DA (0-100 ng/ml) standard curves were constructed simultaneously (Figure 2.9) and results were expressed as nmol DA/g wet weight of tissue and were the mean of three determinations.

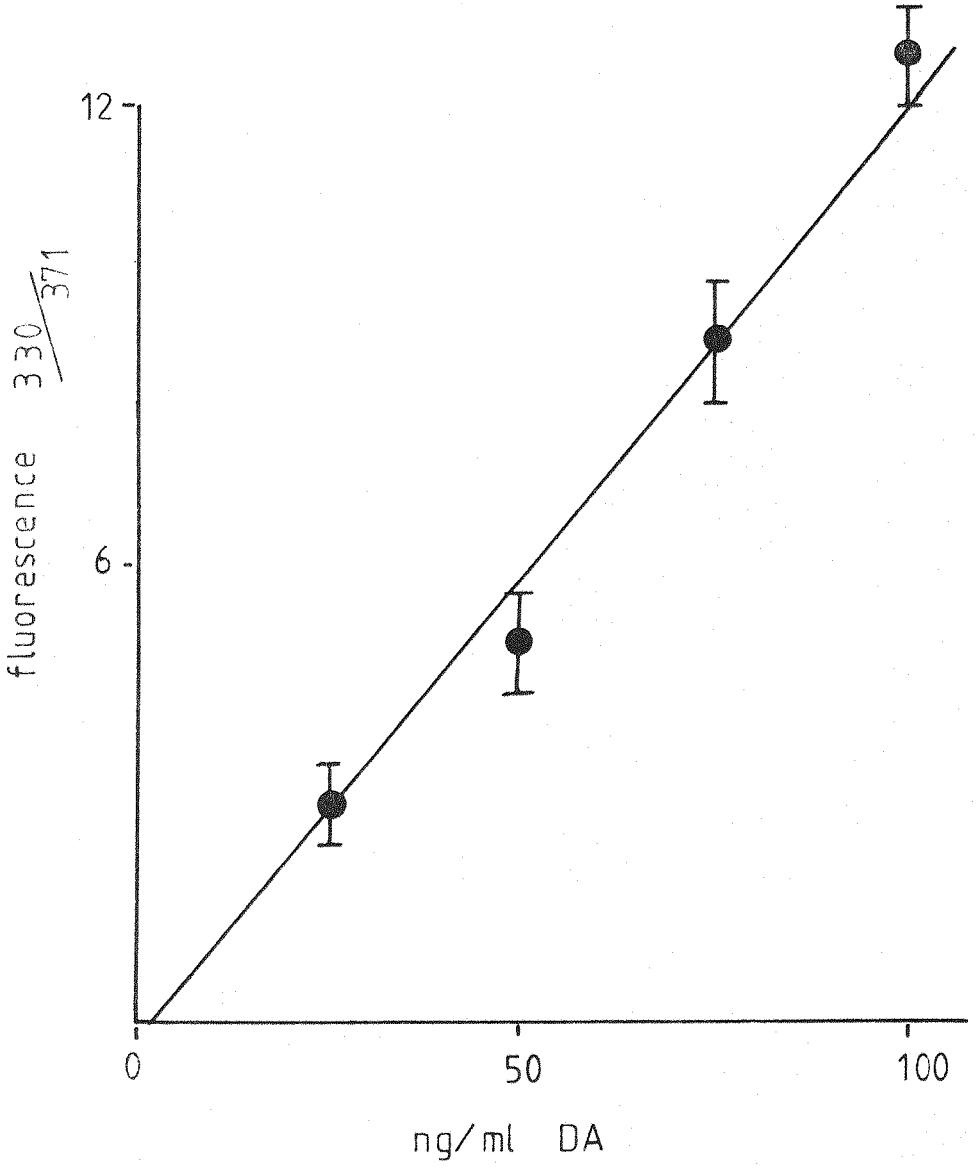


FIGURE 2.9 Typical standard curve for the estimation of dopamine content by the spectrofluorometric method of Earley and Leonard (1974).

Materials

Acetylcholine. Br	Koch-Light
Adenosine	Sigma
Adenosine-5'-diphosphoric acid, trisodium salt	BDH
Adenosine-5'-triphosphoric acid, disodium salt	BDH
Adenosine 3',5'-cyclic phosphate	Sigma
[8-^{3}H] Adenosine 3',5'-cyclic phosphate	Amersham Radiochemicals
4-Amino-n-butyric acid (GABA)	BDH
2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN). H.Br.	Calbiochem-Behring
Apomorphine. HCl	Sigma
Atropine sulphate	Koch-Light
Atenolol	I.C.I.
Bradykinin triacetrate	Sigma
Bromocryptine (2-bromo-α-ergocryptine)	Sandoz
(+)-Bulbocapnine	Research Biochemicals
(+)- and (-)-Butaclamol	Ayerst
Chlorpromazine	Sigma
Dopamine. HCl or HBr.	Aldrich
6-Hydroxydopamine. HBr.	Aldrich
Fluphenazine	Squibb
cis-(z)- and trans(Z)-Flupenthixol	H. Lundbeck and Co.
Guanine 3',5' cyclic phosphate	Sigma
Haloperidol	Janssen
Histamine diphosphate	Sigma
L-Isoproterenol HCl	Sigma
Metoclopramide	Berk
Nialamide (N-Isonicotinoyl-N'[β(N-benzylcarboxamido) ethyl] hydrazine	Sigma
Nicotine hydrogen tartrate	BDH
Nomifensine	Hoechst
L-Noradrenaline bitartrate	Koch-Light
Papaverine	BDH
Phenoxybenzamine	Smith, Klein & French
Phentolamine (Rogitine)	Ciba
L-Phenylephrine	Koch-Light
Propranolol HCl	I.C.I.
Prazosin	Pfizer

Serotonin creatine sulphate	BDH
SKF 38393	Smith, Kline & French
(+)-(-)- and (±)-sulpiride	Ravizza
Sultopride	de la Grange
[^{3}H] sulpiride	Amersham Radiochemicals
Theophylline hydrate anhydrous	Sigma

CHAPTER 3

RESULTS

3-1 Renal blood flow model

3-1-1 Measurement of renal function by the H_2 washout technique

Preliminary studies verified that H_2 washout was a reliable technique for the estimation of RBF. Flow values were reproducible and had stability, over many hours, comparable with those reported by other workers (Aukland et al, 1964; Aukland, 1980b; Løyning, 1974; Tyssebotn and Kirkebø, 1979; Robertson, 1981). A typical H_2 washout curve recorded with one electrode in the outer cortex of a guinea pig is shown in plate 3. Momentarily inspired H_2 was transported to the surface of the electrode where a current proportional to the peak tissue concentration of H_2 was obtained 1-3 seconds after stopping inhalation of H_2 gas (arrow). Monoexponential desaturation curves, similar to those observed by Løyning (1971) then followed. In control periods individual desaturation curves were practically identical.

As shown in table 3.1 H_2 clearance from cortical and medullary tissue was 455 ± 16 ml/100 g/min (n = 12) and 288 ± 29 ml/100 g/min (n = 6) respectively in the urethane anaesthetised rat; the corresponding values in the urethane anaesthetised guinea pig were much lower, cortical RBF was 212 ± 15 ml/100 tissue g/min (n = 31) and medullary RBF was 127.5 ± 3.68 ml/100 g tissue/min (n = 8). These values for RBF in rat compared favourably with those reported by other workers (Robertson, 1981; Haining and Turner, 1966) using the same technique. Other techniques produced similar values for rat RBF, for example Mercer and Zusko (1974) reported values of 678 ml/100 g/min for outer and 344 ml/100 g/min for inner cortical blood flow, using ^{86}Rb uptake. Källskog et al (1975) reported a total RBF value of 315 ml/100 g/min (after conversion from their units) using [^{141}Ce] and [^{85}Sr] labelled 15μ diameter microspheres. No comparable data exists with regard to the measurement of RBF in the guinea pig by the H_2 washout technique. Mean CRVR in rats and guinea-pigs was approximately 54% and 40% lower than MRVR respectively. However, there was no significant difference between species in CRVR and MRVR values, presumably a factor determined by MABP in both species.

RVR was carefully monitored throughout all experimental procedures to check for the absence of renal vascular autoregulation (see Section 2-1-1), but RVR values are omitted for clarity in Sections

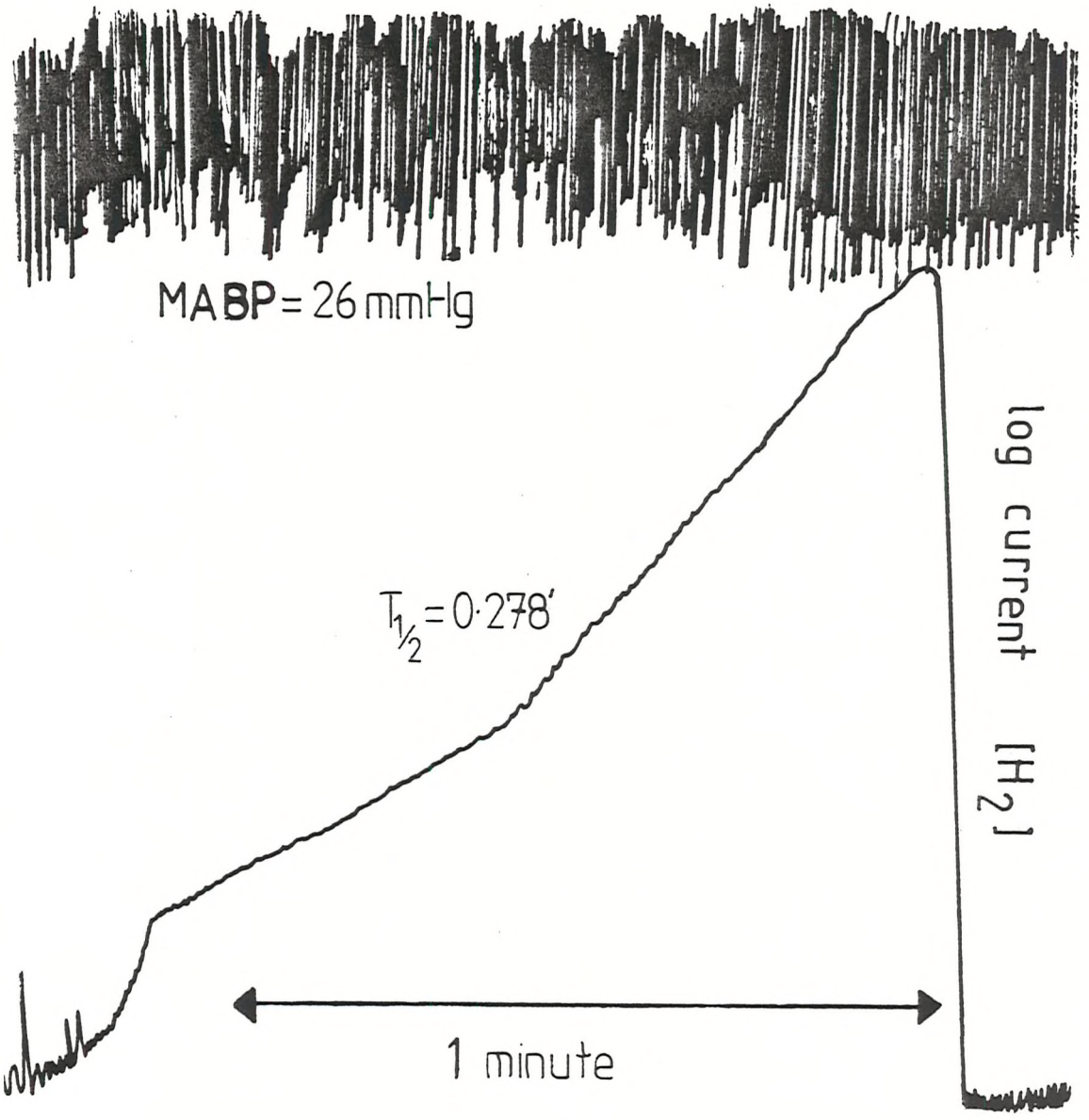


PLATE 3: A typical H_2 washout curve recorded with one electrode in the outer renal cortex of a guinea pig. Renal blood flow (RBF) and renal vascular resistance (RVR) are calculated from the following equations:-

$$RBF = \frac{69.3}{t_{\frac{1}{2}}} \text{ ml/100 g/min} \qquad RVR = \frac{MABP}{RBF}$$

TABLE 3.1 Renal parameters of urethane anaesthetised rats and guinea pigs as measured by hydrogen polarography.

	Rat			Guinea pig		
	mean	SE	(n)	mean	SE	(n)
CRBF	455	16	(12)	212	15.3	(9)
CRVR	0.242	0.03	(6)	0.285	0.002	(6)
MRBF	288	29	(12)	127.5	3.68	(8)
MRVR	0.515	0.07	(6)	0.475	0.005	(6)
MABP	113	4.3	(12)	60.6	2.5	(9)

Abbreviations: cortical renal blood flow (CRBF), medullary renal blood flow (MRBF), cortical renal vascular resistance (CRVR), medullary renal vascular resistance (MRVR), mean arterial blood pressure (MABP). RBF values are expressed as ml/100 g tissue/min, RVR values as mmHg/ml min^{-1} 100 g $tissue^{-1}$ and MABP as mmHg.

where pharmacological data is presented.

3-1-2 Effect of dopamine upon RBF and selection of α-adrenergic blocking agent

In dog the integrated cardiovascular response to large doses of DA is a dose-dependent increase in systemic blood pressure (Eble, 1964). In guinea pig the response to DA is a fall in blood pressure (Hornykiewicz, 1958). The blood pressure response is a result of a balance between DA stimulating its own receptors, in addition to modifying NA release and activating α- and β-adrenoceptors in the periphery (Goldberg et al, 1978b). Alterations in systemic blood pressure result in autoregulation of blood flow, accompanied by changes in the vascular resistance of blood vessels. The level to which this vasoconstriction or vasodilation proceeds in the vasculature depends upon the magnitude of pressure rise or fall. The arteriolar network is an arrangement of myogenic sensor-effector units reacting to keep intravascular pressure constant. The resulting effect is to maintain blood flow constant also. Therefore, when evaluating vascular responses to DA, it is essential to distinguish between blood flow increases resulting from the actions of DA upon a specific vascular DA receptor and increases arising from autoregulatory mechanisms serving to restore blood flow homeostasis after a change in blood pressure.

DA produces a pronounced renal vasoconstriction when injected directly into the renal artery of dog (McNay et al, 1965). This response is prevented by α-adrenergic blocking agents which unmask the renal vasodilatory actions of DA (see Section 1-5-5). In order to develop a model test system which exhibited clear-cut renal vasodilation responses to DA-like agents in the guinea pig, α-adrenoceptors were pharmacologically eliminated by specific α-antagonists. Reduction of sympathetic tone produced by the administration of α-blockers is usually followed by a reflex (autoregulatory) tachycardia serving to restore vascular blood flow.

In preliminary experiments prazosin, POB and phentolamine were compared for their duration and specificity as α-adrenergic blocking agents. Phentolamine was unsuitable due to its short duration of action. The effect of the two remaining antagonists, prazosin and

POB upon guinea pig RBF, MABP and RVR is shown in table 3.2. CRBF was reduced by approximately 45% following both prazosin (0.1 mg/kg) and POB (0.5 mg/kg) pretreatment. These blood flow values (ml/100 g/min) fell from 212 ± 15.3 (n = 31) to 118 ± 6.5 (n = 38) after prazosin administration and to 114.9 ± 4.8 (n = 10) after POB administration. However, RVR was reduced 22% by prazosin but raised 14.6% by POB. This differential effect upon systemic blood pressure is reflected by the lower prevailing systemic blood pressure in prazosin pretreated animals.

The effect of DA (200 nmol/kg/min) upon RBF was examined either in the presence of prazosin (0.1 mg/kg) or POB (0.5 mg/kg) (Table 3.3), in order to ascertain the capacity of each α-adrenergic blocking agent to inhibit renal vasoconstriction produced by DA-mediated activation of postsynaptic α-adrenoceptors Renal vasoconstriction in response to DA was completely abolished by both prazosin and POB pretreatment. The increased RBF responses to DA indicated that DA produced renal vasodilation but no autoregulation.

Prazosin and POB were equally effective at eliminating the renal vasoconstriction produced in response to α-receptor activation by DA. Prazosin was chosen in preference to POB for use in this model. Prazosin is a highly selective α_1 antagonist (Cambridge et al, 1977) whereas POB is a less selective, mixed adrenergic antagonist which can stimulate endogenous NA release. MABP fell rapidly after prazosin administration (plate 4) and quickly stabilised at a basal level. However, these cardiovascular changes were not accompanied by a reflex tachycardia (increase in heart rate). Adequacy of α-adrenergic blockade has been demonstrated in Section 2-1-4.

3-1-3 RBF responses to dopamine in the presence and absence of α-adrenergic blockade

Guinea pig RBF responses to intra-renal artery injections of DA (0.5-1,600 nmol/kg) in the absence and presence of α-adrenergic blockade by prazosin (0.1 mg/kg) are shown in figure 3.1. In the absence of α-adrenergic blockade renal vascular responses to DA were biphasic. Low doses of DA (0.5-16 nmol/kg) produced dose-related increments in RBF but high doses of DA (50-1,590 nmol/kg) predominantly produced vasoconstriction; presumably by activation of postsynaptic α-adrenoceptors present in the renal vasculature (McNay et al,

TABLE 3.2 The effect of prazosin and phenoxybenzamine (POB) upon guinea pig renal parameters and blood pressure. Values for prazosin represent base-line cardiovascular parameters used in subsequent studies with dopamine and other drugs.

	saline (1ml/kg)			prazosin (0.1mg/kg)			POB (0.5mg/kg)		
	mean	SE	(n)	mean	SE	(n)	mean	SE	(n)
CRBF	212	15.3	(31)	118	6.5	(38)	114.9	4.8	(10)
MABP	60	2.5	(30)	26.3	0.5	(26)	48	7.3	(11)
CRVR	0.285	0.03	(30)	0.222	0.04	(28)	0.327	0.17	(10)

Abbreviations: cortical renal blood flow (CRBF), mean arterial blood pressure (MABP), cortical renal vascular resistance (CRVR). RBF values are expressed as ml/100 g/min, MABP as mmHg and RVR values as mmHg/ml min^{-1} 100 g $tissue^{-1}$.

TABLE 3.3 Increases in renal blood flow (RBF) produced by intra-arterial infusions of dopamine (200 nmol/kg/hr) in anaesthetised guinea pigs following α-adrenergic blockade with prazosin (0.1 mg/kg) or phenoxybenzamine (POB) (0.5 mg/kg).

	RBF (ml/100 g/min)					
	vehicle (1.4 ml/kg/hr)			DA (200 nmol/kg/hr)		
TREATMENT	mean	SE	n	mean	SE	n
control	182	12	12	150	13	12
prazosin	114	8.2	9	147	12.5	6
POB	104	11	6	139	7.8	6

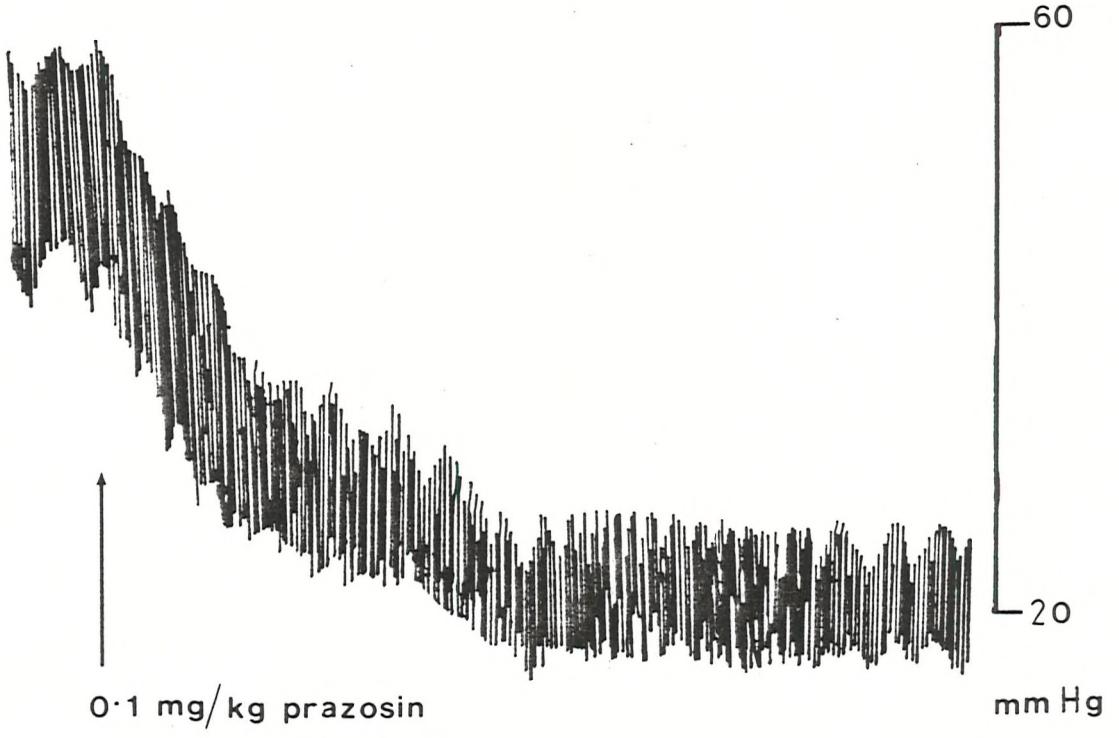


PLATE 4: A typical mean arterial blood pressure (MABP) response following prazosin (0.1 mg/kg) administration recorded from the carotid artery of a urethane anaesthetised guinea pig. The fall in MABP was rapid and stabilised without an accompanying reflex tacchycardia.

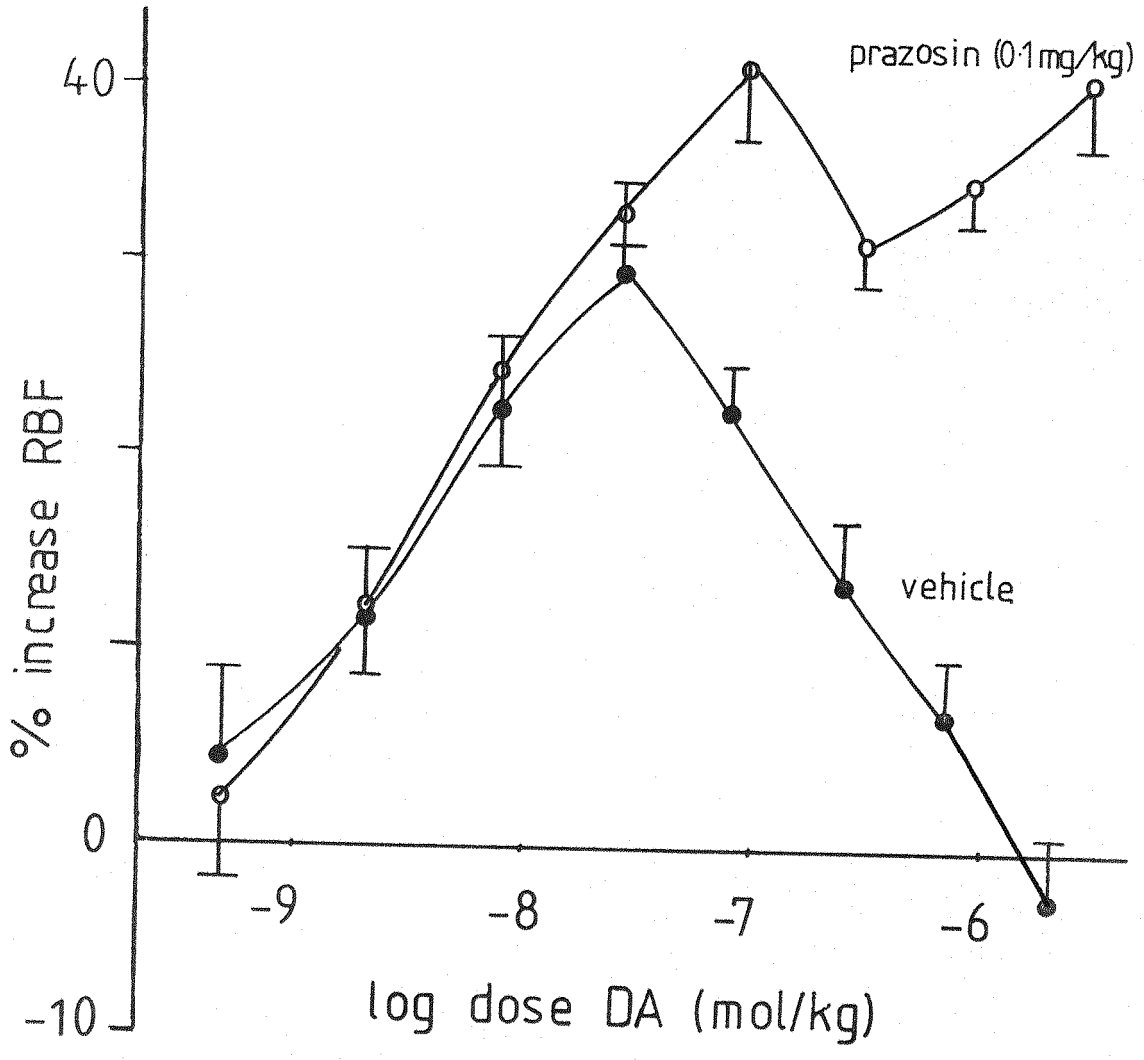


FIGURE 3.1 Guinea pig renal blood flow (RBF) responses to intra-renal artery injections of dopamine (DA) (0.5-1,600 nmol/kg) following ● vehicle or ○ prazosin (0.1 mg/kg) pretreatment. Ordinate responses are expressed as the % increase in renal blood flow. Each value is the mean response of 6-30 guinea pigs, and the vertical bars represent the standard errors of the means.

1965; Goldberg et al, 1968).

The injection of DA (0.5-1,590 nmol/kg) into the renal artery of prazosin (0.1 mg/kg) pretreated guinea pigs produced dose-related increases in RBF and falls in RVR (Figure 3.1). Basal RBF values were 118 ± 6.5 ml/100 g/min (n = 38) and rose to 156 ± 9.9 ml/100 g/min (n = 36) in the presence of DA (53 nmol/kg). This was the maximum response to DA and represented a 30-40% increase of RBF. Results in this and subsequent experiments are expressed as the percentage increase of RBF, in order to overcome animal variation in basal RBF and in responsiveness to vasoactive drugs.

3-1-4 RBF responses to dopamine and isoprenaline in the presence of β-adrenergic blockade

The presence of both β-adrenoceptors and DA receptors mediating vasodilation in the renal vascular bed is well documented (Goldberg, 1972; Fenyevsi and Kallay, 1970). Obviously the most direct characterisation of dopaminergic receptors would be the actual isolation of the receptor system to allow precise analysis of drug-receptor interaction. Since isolation cannot be accomplished in this preparation, and furthermore DA can stimulate β-adrenoceptors or its own receptors to produce vasodilation, indirect methods must be used. The simplest means of differentiating between β-adrenoceptor mediated and DA receptor mediated vascular responses is by the use of specific pharmacological blocking agents. Thus having obtained a RBF response to DA, the specificity of DA induced renal vasodilation was reassessed after pretreatment with the β-adrenergic blocking agent (±)-propranolol. Antagonism of the β-adrenergic vasodilating agent, isoprenaline, was used to determine adequacy of β-adrenergic blockade. The effect of (±)-propranolol (1.2 μmol/kg/hr) upon RBF responses to DA and isoprenaline is shown in figure 3.2. Isoprenaline (53 nmol/kg) and DA (53 nmol/kg) produced a 42 ± 5% (n = 6) and 39 ± 5% (n = 6) rise in RBF respectively. The response to DA was not affected by infusion of vehicle or (±)-propranolol (1.2 μmol/kg/hr). This same dose of (±)-propranolol produced a complete inhibition of the increased RBF responses to equiactive doses of isoprenaline.

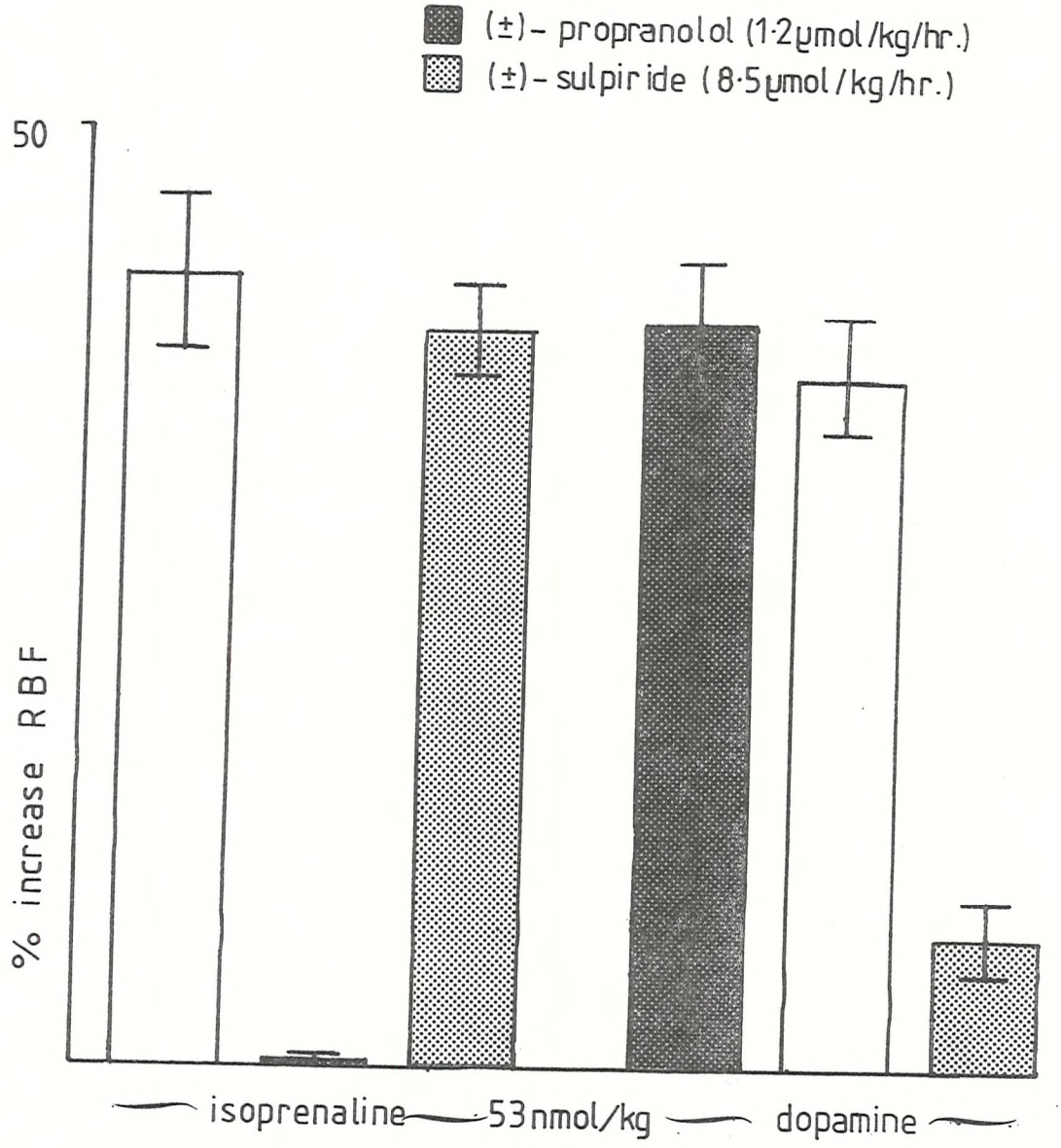


FIGURE 3.2 Selective inhibition of renal blood flow (RBF) responses to intra-renal artery injections of isoprenaline (53 nmol/kg) and dopamine (53 nmol/kg) following (±)-propranolol (1.2 µmol/kg/hr) and (±)-sulpiride (8.5 µmol/kg/hr) infusions. Prazosin (0.1 mg/kg) was administered before the vasodilating agents. Ordinate responses are expressed as the % increase in renal blood flow (RBF). Each value is the mean response of 6-10 guinea pigs, and the vertical bars represent the standard error of the means.

3-1-5 RBF responses to dopamine and isoprenaline in the presence of dopaminergic receptor blockade

The hypothesis that DA is acting upon specific receptors in the renal vascular bed relies heavily upon selective blockade of the DA induced response by a specific DA blocking agent. However, most classical neuroleptics such as haloperidol and fluphenazine used in CNS DA receptor model systems appear to lack the potency and specificity required for the quantitative investigation of the DA vascular receptor (Goldberg and Yeh, 1971). In the CNS the substituted benzamide sulpiride has received particular attention, because among its pharmacological spectrum of activities it has some properties which are characteristic of classical neuroleptics and others which are not. In particular, despite sulpiride's potency as an antipsychotic drug (Borenstein et al, 1969) and upon many animal models (see Sections 1-5-11 and 3-3-7) it was completely ineffective at antagonising the DA-sensitive adenylate cyclase model in brain homogenates (Trabucchi et al, 1975) and relatively weak in displacing [^{3}H] haloperidol binding (Spano et al, 1978). However, sulpiride was much more potent and specific at antagonising peripheral DA receptors in the canine RBF model (Goldberg et al, 1979) than classical neuroleptics. In order to establish whether guinea pig kidney vascular responses to DA were specifically antagonised by a neuroleptic and to examine whether these responses had a pharmacological profile for antagonists, similar to those of central, or of other peripheral DA receptor models, the effect of sulpiride upon RBF responses to dopaminergic agents was studied. The specific inhibition of increased RBF responses to DA (53 nmol/kg) but not isoprenaline (53 nmol/kg) by (±)-sulpiride (8.5 µmol/kg/hr) is also shown in figure 3.2. Increases in RBF produced by DA (53 nmol/kg) were unaffected by vehicle infusions but were strongly inhibited (70%) by the DA receptor antagonist (±)-sulpiride. Responses to equiactive doses of isoprenaline were unaffected by the same dose sulpiride.

3-1-6 RBF responses to ADTN and SKF 38393

The DA receptor agonists ADTN (Woodruff, 1978) and SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H3-benzazepine) (Pendleton et al, 1978) produced a similar propranolol resistant but sulpiride sensitive dose related increase in RBF and decrease in RVR. Dose response curves for ADTN (0.5-530 nmol/kg) and SKF 38393 (0.05-530

nmol/kg) are shown in figure 3.3. ADTN was approximately equipotent with DA at all concentrations and produced a maximum response of similar magnitude. SKF 38393 had a threshold vasoactive dose approximately 10 times lower than that of DA but became approximately equipotent with DA at higher doses. The effect of (±)-propranolol (1.2 µmol/kg/hr) upon RBF responses to ADTN and SKF 38393 is shown in figure 3.4. Responses to these drugs were not significantly affected by β-adrenergic blockade. In contrast, the increases in RBF produced by ADTN and SKF 38393 were antagonised by (±)-sulpiride (8.5 µmol/kg/hr) (Figure 3.5).

3-1-7 Inhibition of RBF responses to dopamine by haloperidol and fluphenazine

Preliminary experiments were performed to test the classical neuroleptic drugs haloperidol and fluphenazine for their DA-blocking actions in the guinea pig renal vasculature. Infusions of both haloperidol (7.5 nmol/kg/hr) and fluphenazine (15.6 nmol/kg/hr) reduced basal MABP (Figure 3.6a) and RBF (Figure 3.6b); RBF responses to DA (0.5-30 nmol/kg) were antagonised by haloperidol (7.5 µmol/kg/hr) (Figure 3.6b). However, the cardiovascular effects of haloperidol and fluphenazine were uncharacteristic of pure DA receptor antagonists because they produced a drastic reduction of MABP. This suggested that the drugs had significant α-antagonist activity. Haloperidol and chlorpromazine have previously been reported to be potent antagonists of NA induced contraction *in vitro* rabbit aorta (Kohli, 1969; Gokhale et al, 1964). The poor specificity of haloperidol as a peripheral DA receptor blocking agent was subsequently confirmed in a series of experiments (Figure 3.7), where the dose-related increase in MABP produced by phenylephrine (10-10,000 nmol/kg; an α-adrenergic agonist) was significantly inhibited by haloperidol (7.5 µmol/kg/hr), as was the dose-related fall in MABP produced by isoprenaline (0.1-100 nmol/kg; a β-adrenergic agonist) and by DA (0.5-500 nmol/kg).

3-1-8 Stereospecific antagonism of RBF responses to dopamine by S-(-)-sulpiride and R-(+)-sulpiride

(±)-sulpiride has recently been resolved into its enantiomers. The S-(-)-form has been reported to be between 6-20 times more active at antagonising central DA receptors than the R-(+)-form, depending upon which model systems were used to assess their potency. For example

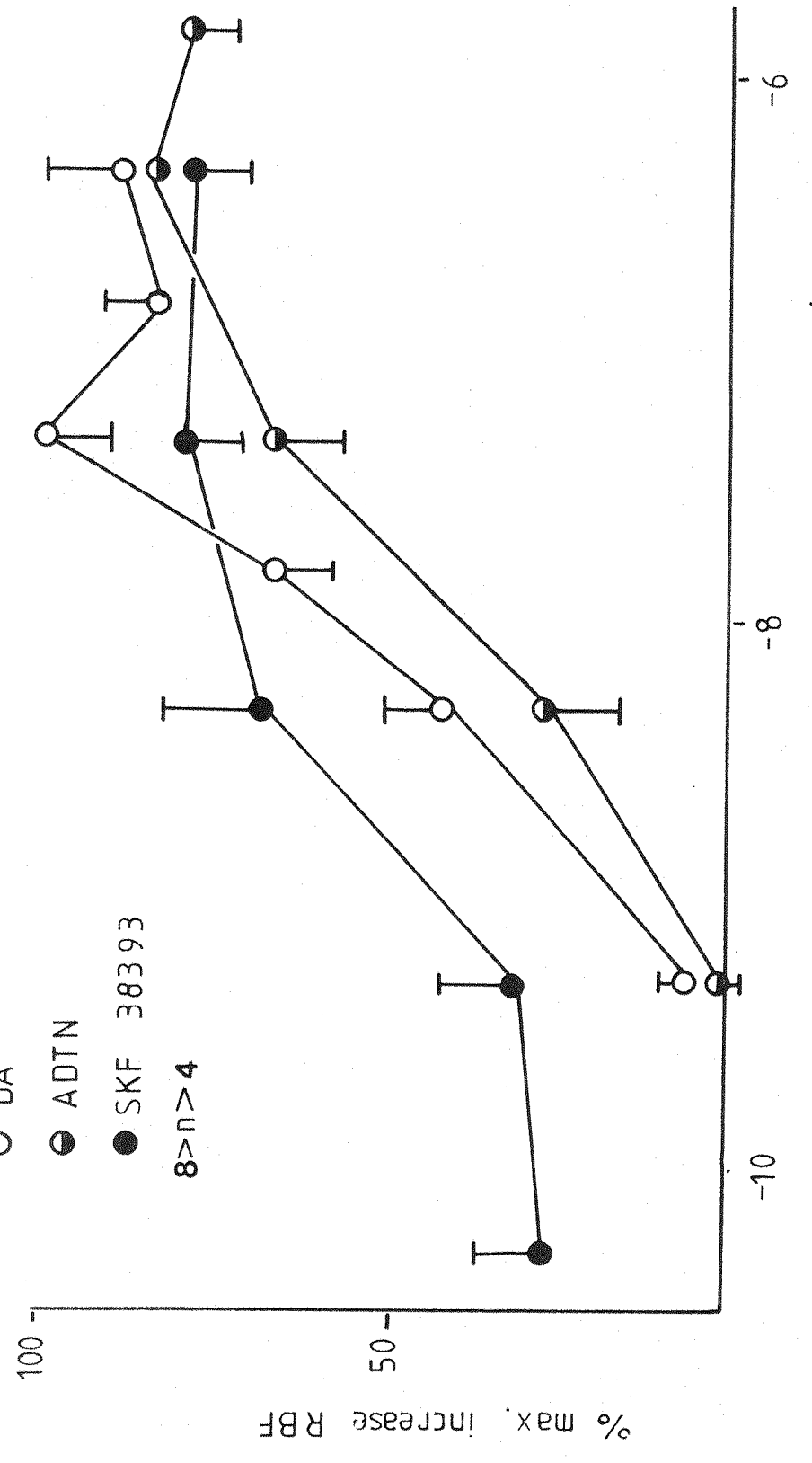


FIGURE 3.3 Dose-related increases in RBF (renal blood flow) produced by intra-arterial injections of ○ DA (dopamine), ◐ ADTN (2-NH_2-6,7-diOH -1,2,3,4-tetrahydronaphthalene) and ● SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine). Prazosin (0.1 mg/kg) was administered before the vasodilating agents. Ordinate responses are expressed as the % increase in RBF which was produced by 53 nmol/kg DA. DA was compared to ADTN in 4 experiments and to SKF 38393 in another 4 experiments. Responses were assessed in triplicate at each drug concentration and the vertical bars represent the standard error of the means.

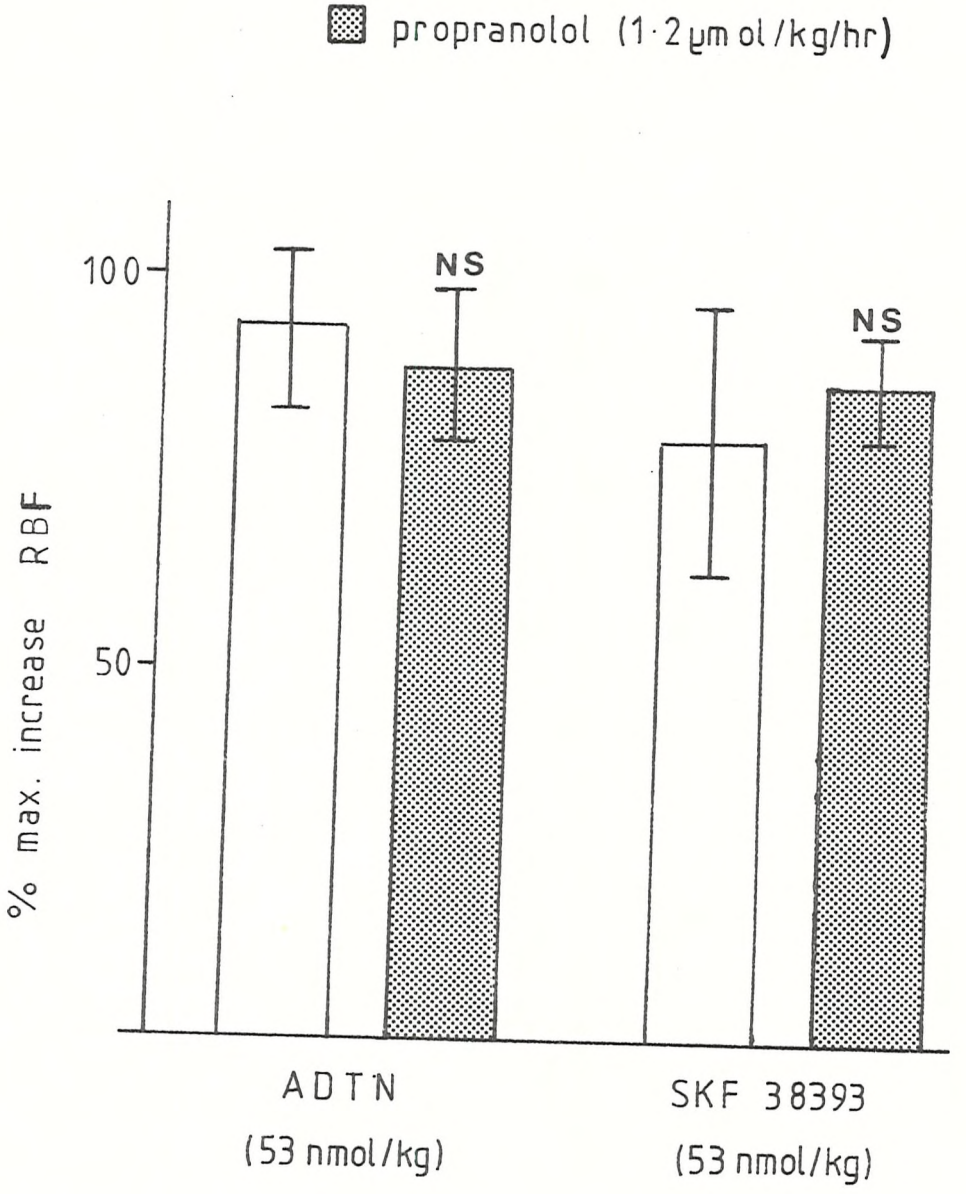


FIGURE 3.4 Lack of inhibition of RBF (renal blood flow) responses to ADTN and SKF 38393 following ▨ propranolol (1.2 µmol/kg/hr) infusions. Prazosin was administered before the vasodilating agents. Ordinate responses are expressed as the % increase of RBF. Each value is the mean response of 3-4 guinea pigs and the vertical bars represent the standard error of the means.

FIGURE 3.5 Inhibition of RBF (renal blood flow) responses to DA, ADTN and SKF 38393 following ▒ (±)-sulpiride (8.5 µmol/kg/hr) infusions. Prazosin (0.1 mg/kg) was administered before the vasodilating agents. Ordinate responses were expressed as the percentage increase of RBF which was produced by 53 nmol/kg DA. Each value is the mean response of 4-8 guinea pigs and the vertical bars represent the standard error of the means.

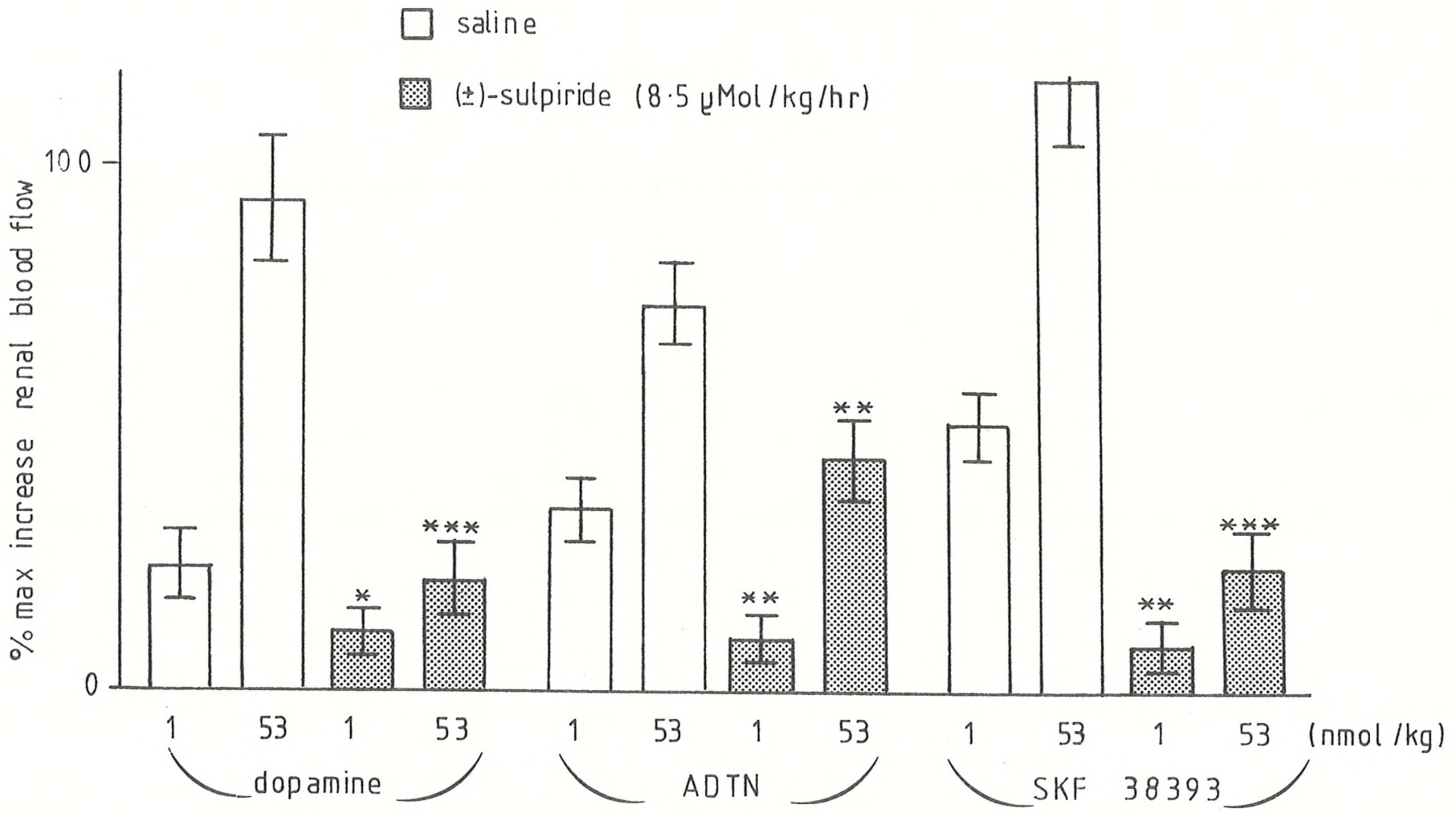
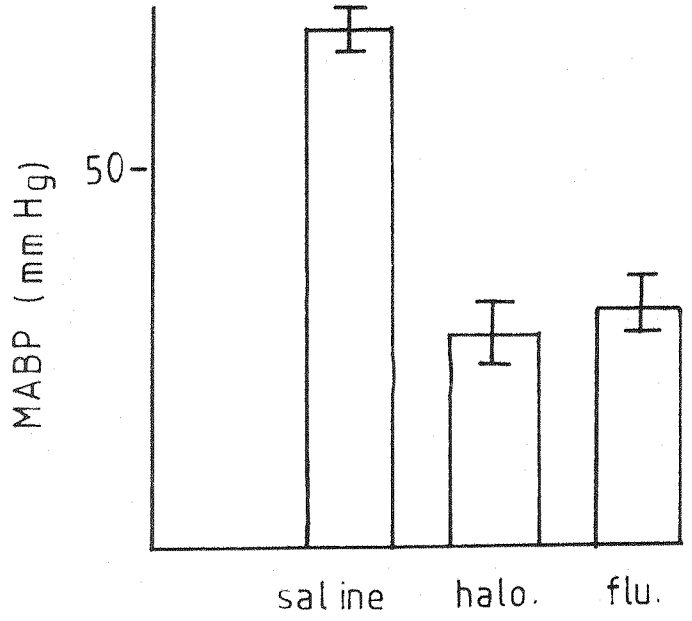
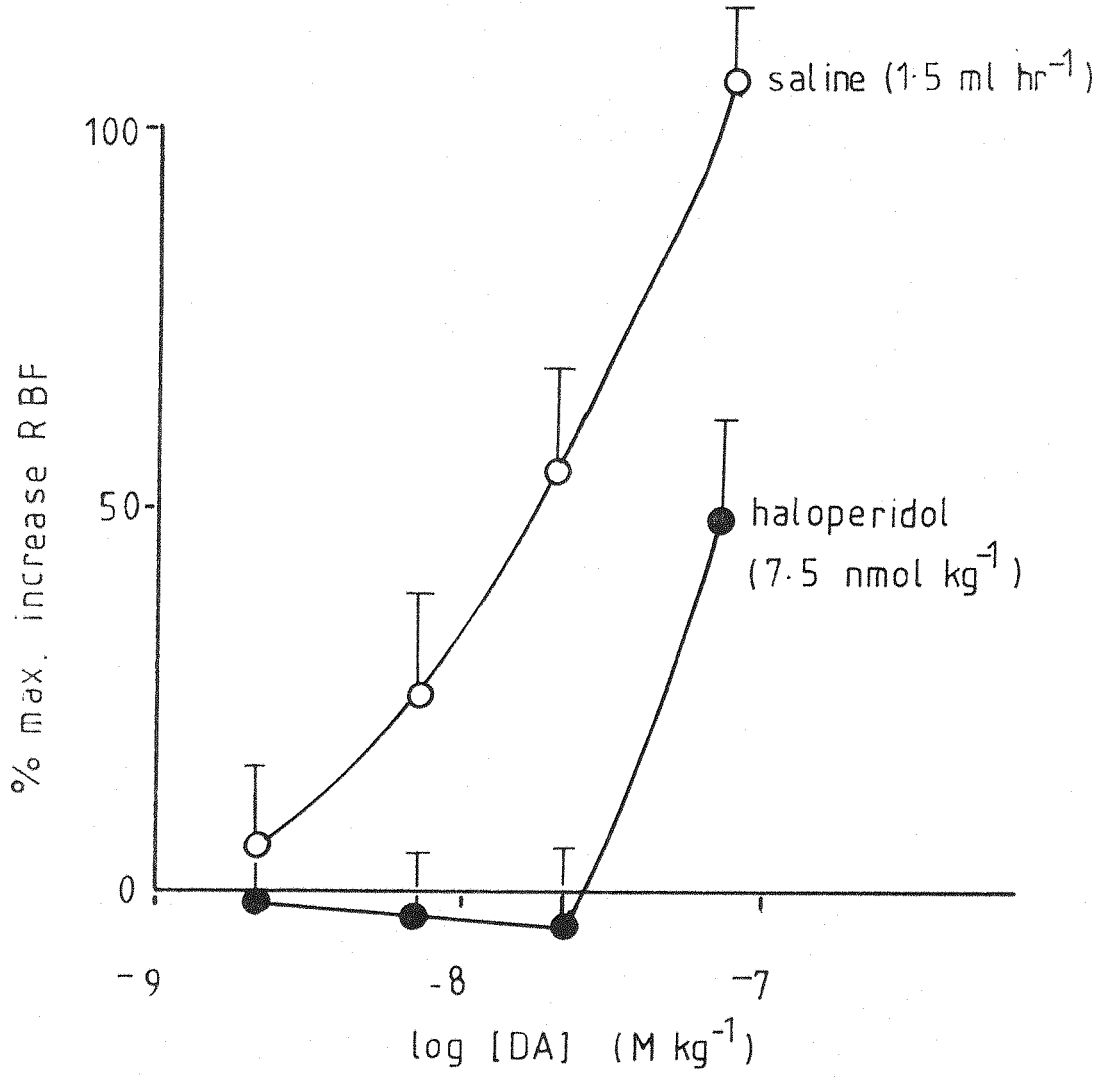

saline
(±)-sulpiride (8·5 µMol/kg/hr)
% max increase renal blood flow
100
0
*
***
**
**
**
***
1
53
1
53
1
53
1
53
1
53
1
53
(nmol/kg)
dopamine
ADTN
SKF 38393

FIGURE 3.6a Guinea pig MABP (mean arterial blood pressure) responses to intra-venous administration of haloperidol (7.5 nmol/kg/hr) and fluphenazine (15 nmol/kg/hr).

FIGURE 3.6b RBF (renal blood flow) responses to DA (2-53 nmol/kg) during ○ saline (1.4 ml/hr) and ● haloperidol (7.5 nmol/kg/hr) infusions. Prazosin (0.1 mg/kg) was administered before DA. Each value is the mean response of 3 guinea pigs and the vertical bars represent the standard error of the means.


MABP (mm Hg)
50
saline
halo.
flu.


saline (1·5 ml hr⁻¹)
haloperidol
(7·5 nmol kg⁻¹)
% max. increase RBF
100
50
0
-9
-8
-7
log [DA] (M kg⁻¹)

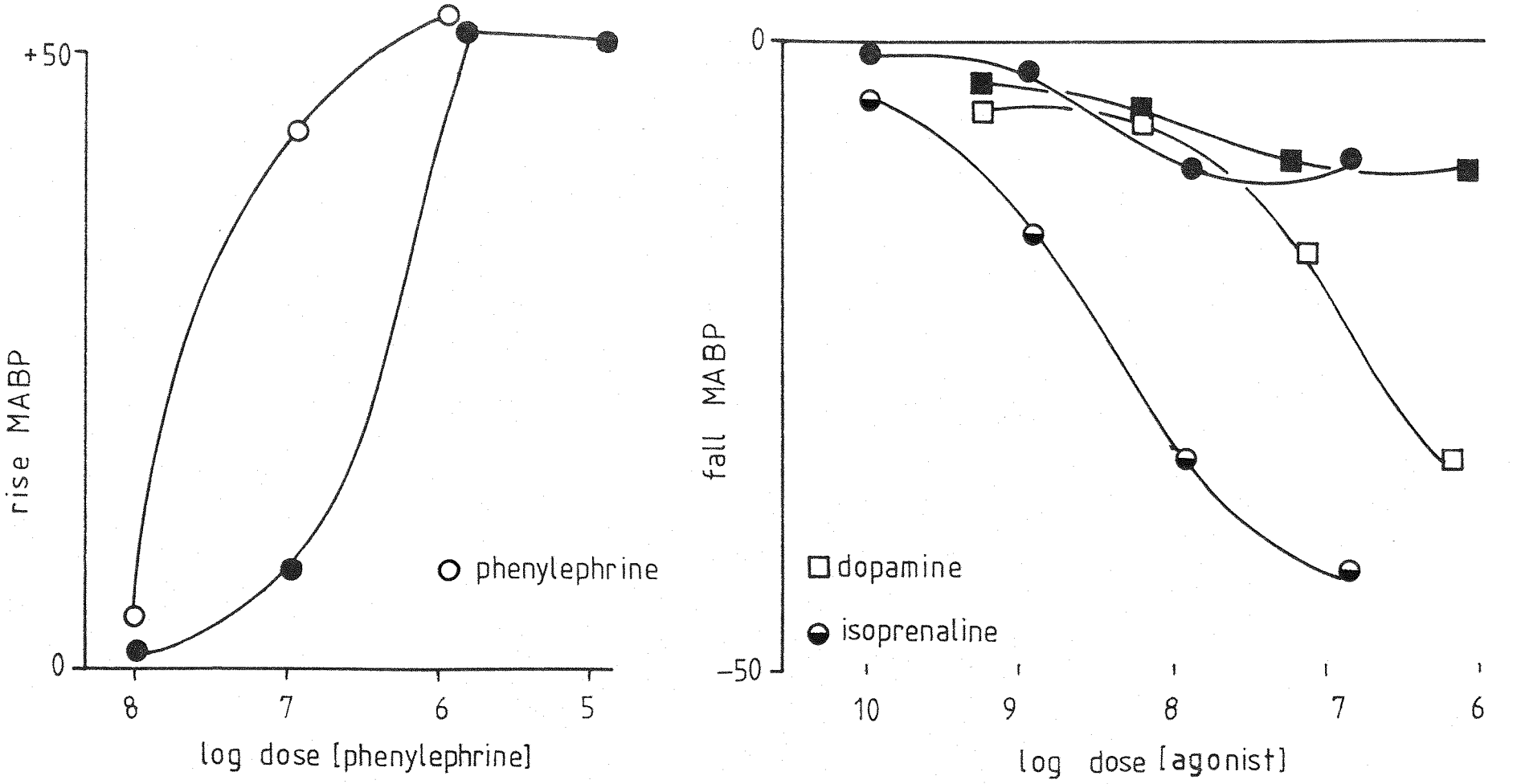


FIGURE 3.7 MABP (mean arterial blood pressure) responses to the α-adrenergic agent phenylephrine, the β-adrenergic agent isoprenaline and dopamine. Open symbols represent responses during saline infusions (1.4 ml/hr) and closed symbols responses during haloperidol (7.5 nmol/kg/hr).

S-(-)-sulpiride preferentially enhances brain DA metabolism; it elevates DOPAC levels and activates tyrosine hydroxylase activity (Tissari et al, 1979). In addition it preferentially inhibits [^{3}H] haloperidol binding in rat striatal preparations (Spano et al, 1979), displays antiemetic activity in dogs (Reina et al, 1979), antagonises DA inhibition of prolactin levels in rat, *in vitro* pituitary (Scapagnini et al, 1979), and man (Massara and Camanni, 1979), antagonises hyperpolarisation of dopaminergic cells in the substantia nigra of rat (Pinnock, 1981) and inhibits [^{3}H] sulpiride binding in the rat striatum (Woodruff and Freedman, 1981). Since the racemic mixture of sulpiride was a potent and specific antagonist of renal vascular responses to DA in the guinea pig (Section 3-1-6), it might be expected that the isomers of sulpiride would also exhibit a stereoisometric potency difference in this system. Furthermore, in order to compare the pharmacological characteristics of peripheral DA receptors with those in the CNS, it would be of interest to establish whether the R-(-)-enantiomer is similarly more active in the periphery or whether peripheral DA receptors might differ from CNS DA receptors and display stereoselectivity for S-(+)-sulpiride.

The effect of S-(-)-sulpiride and R-(+)-sulpiride upon RBF responses to DA are shown in figure 3.8. S-(-)-sulpiride (8.5 µmol/kg/hr) was between 6 and 10 times more potent than the same dose of R-(+)-sulpiride in its DA blocking actions in the guinea pig kidney. RBF responses to DA at 1 nmol/kg were not significantly altered by R-(+)-sulpiride (8.5 µmol/kg/hr), the responses to 53 nmol/kg were inhibited by 32%. In contrast, the same dose of S-(-)-sulpiride inhibited renal responses to 1 nmol/kg of DA completely and to 53 nmol/kg of DA by 72%.

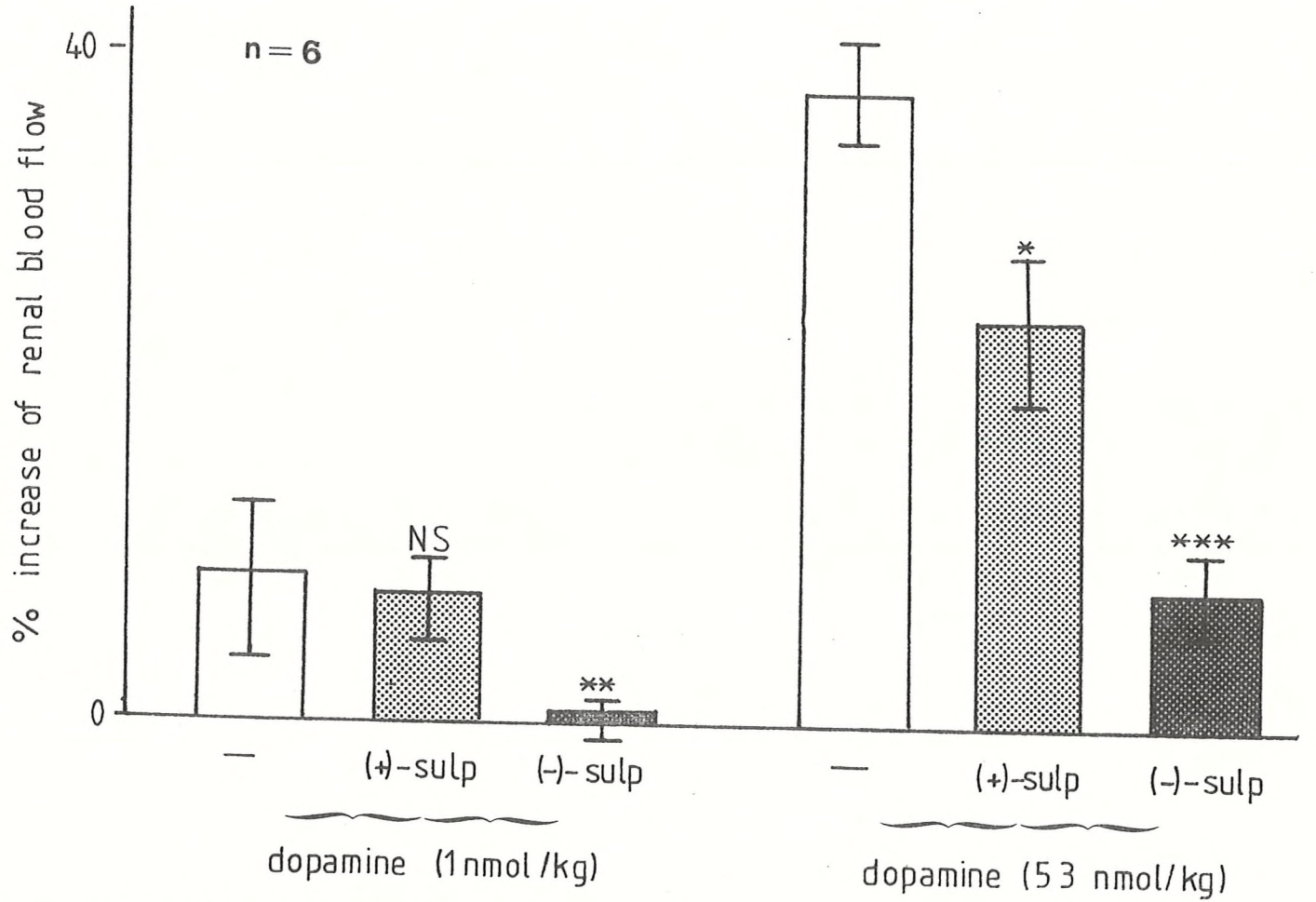


FIGURE 3.8 Differential inhibition of RBF (renal blood flow) responses produced by dopamine (1,53 nmol/kg) during ☐ saline (1.4 ml/hr), ▨ R-(+)-sulpiride (8.5 µmol/kg/hr) or ▩ S-(-)-sulpiride (8.5 µmol/kg/hr) infusions. Each value is the mean result of 6 guinea pigs. Ordinate responses are expressed as the % increase of RBF and the vertical bars represent the standard error of the means.

3-2 Dopamine sensitive adenylate cyclase

3-2-1 Effect of catecholamines upon cAMP content in guinea pig renal particulate preparations

The involvement of DA in renal excretion, glomerular filtration and RBF in man and dog (Goldberg, 1972; Dinerstein et al, 1979) appears to suggest the existence of DA receptors in the kidney. Previous studies of peripheral DA receptors have been limited to the physiological measurement of the vasodilation produced by a series of DA agonists.

In the CNS, DA receptors have been studied using a variety of techniques. Kebabian, Petzold and Greengard (1972) demonstrated the ability of DA to stimulate a specific DA-sensitive adenylate cyclase in rat striatal tissue. Subsequent searches for a DA-sensitive adenylate cyclase system have served as a valuable tool to evidentiate the existence of DA receptors in many regions of the CNS (see Section 1-5-9), where this enzyme has been implicated in the actions of DA at postsynaptic receptors (Kebabian and Calne, 1979). The DA-sensitive adenylate cyclase in the CNS shows a strong similarity to the DA receptors in the canine renal vasculature, in terms of the structure activity requirements for DA-like activity (Woodruff et al, 1977; Volkman et al, 1977). The most potent agonist on both systems is the semi-rigid DA analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) which contains the β-rotameric form of DA, the preferred conformation of DA at postsynaptic receptor sites (Woodruff et al, 1977). However, antagonist studies have revealed important discrepancies between the canine RBF model and the CNS DA-sensitive adenylate cyclase model. In particular, sulpiride is completely ineffective at antagonising the DA-sensitive adenylate cyclase (Trabucchi et al, 1975) whereas it is an extremely potent antagonist of DA-induced renal vasodilation (Kohli et al, 1978b). During the course of this study, Kebabian and Calne (1979) proposed a classification of central DA receptors into D_1 receptors which they claim to be adenylate cyclase linked and inhibited by cis-flupenthixol, and D_2 receptors, which in contrast are non-cyclase linked and inhibited by sulpiride. In order to investigate the possible role of cAMP in mediating the vascular responses to DA observed in Section 3-1, the drugs tested on the guinea pig RBF model were examined for their ability to produce cAMP in a guinea pig renal particulate preparation.

Basal cAMP content in untreated renal particulate preparations was 2.63 ± 0.24 pmol/mg protein (n = 13). The results obtained for DA and isoprenaline are shown in figure 3.9. The figure shows that isoprenaline was more potent than DA at enhancing cAMP formation and that both DA (10^{-5}-10^{-3} M) and isoprenaline (10^{-8}-10^{-6} M) stimulated the production of cAMP in a dose-related manner. The EC_{50} for DA stimulation was 63 μM and the EC_{50} for isoprenaline was 0.17 μM (Table 3.5). Maximal stimulation of adenylate cyclase activity was achieved by the highest concentration of catecholamine in both cases. The maximum cAMP accumulation produced by DA (1 mM) was 5.07 ± 0.56 pmol/mg protein (n = 11) which represented a 93% increase of adenylate cyclase activity. Maximum accumulation of cAMP by isoprenaline (1 μM) was 8.55 ± 1.45 pmol/mg protein (n = 10), which represented a 225% stimulation of adenylate cyclase activity. These results are of a similar order of magnitude as those reported by Nakajima et al (1977) using a similar rat renal particulate preparation.

3-2-2 Pharmacological distinction between β-adrenergic and dopaminergic stimulation of adenylate cyclase activity

The presence of dopaminergic and β-adrenoceptors mediating vasodilation in the renal vascular bed is well documented (Fenyvesi and Kállay, 1970; Goldberg, 1972). Additionally, it has been demonstrated that DA can activate both these receptors (see Section 1-5-2) and that β-adrenoceptors are associated with cAMP production in most tissues (Sutherland and Robinson, 1966; Bilezikian and Aurbach, 1973). We have demonstrated that the β-agonist isoprenaline produces a renal vasodilation of a similar order of magnitude to DA in guinea pig kidneys. Distinct differences between these responses were established with the use of specific antagonists in Sections 3-1-4 and 3-1-5 of this study. By analogy, it was necessary to first establish the lack of involvement of β-adrenergic mechanisms in DA stimulation of adenylate cyclase activity in renal glomeruli and blood vessels, before this system could be established as a specific DA receptor model.

Although as shown in figure 3.9 isoprenaline was very active in stimulating the production of cAMP in guinea pig glomeruli and blood vessels, the effect of antagonists on the response suggests that the stimulation is via a different mechanism from the effect of DA. The β-adrenergic blockers propranolol and atenolol were used to distinguish

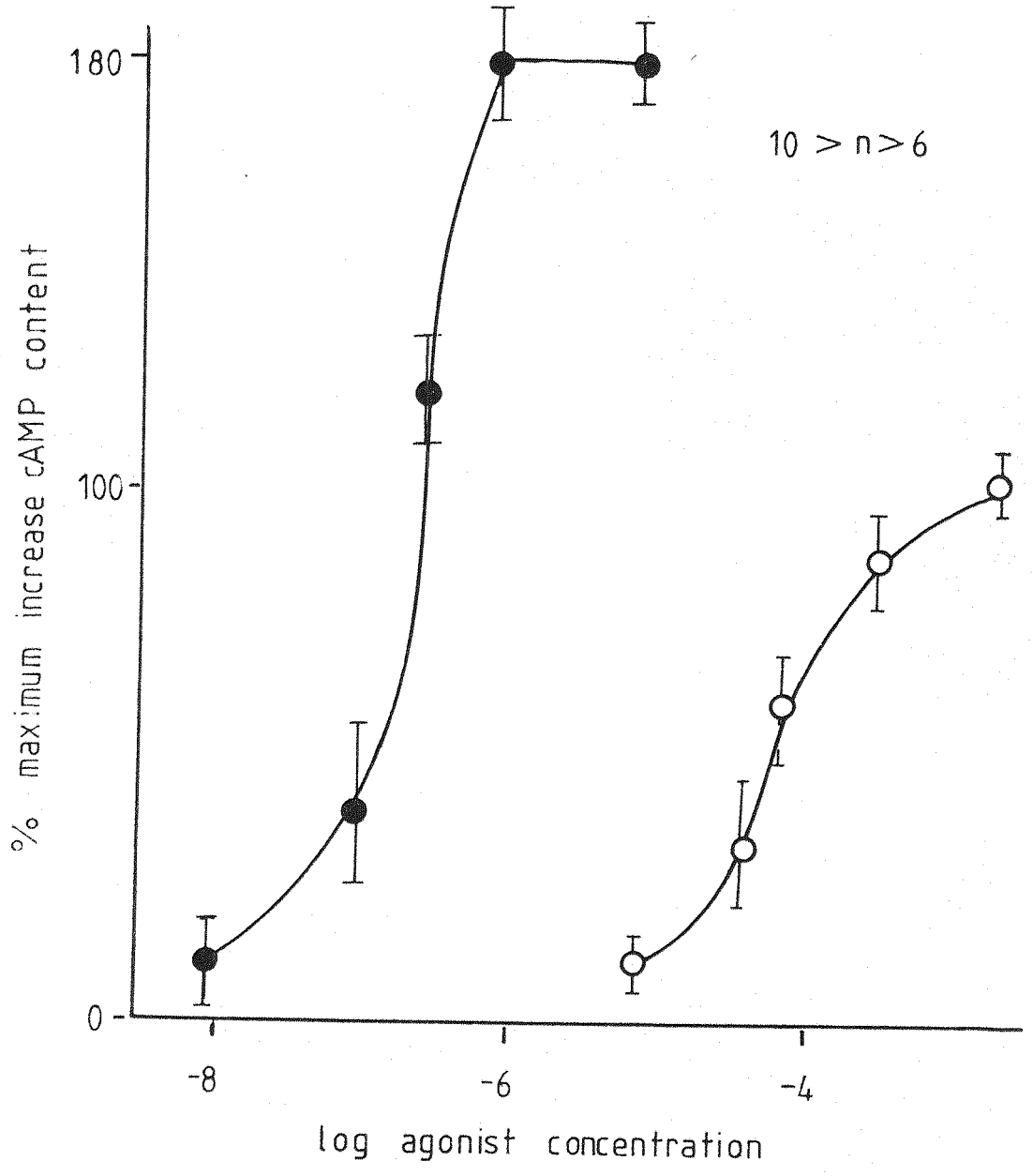


FIGURE 3.9 Dose-related increases in guinea pig renal particulate preparation cAMP content produced by ○ DA (10^{-5}-10^{-3}M) and ● isoprenaline (10^{-8}-10^{-5}M). Ordinate responses are expressed as the % increase of cAMP content which was produced by 1mM DA. Basal cAMP content was 3.5 ± 0.3 pmol cAMP/mg protein. Drugs were assessed in triplicate at each concentration and the vertical bars represent the standard error of the means.

between DA and isoprenaline stimulated adenylate cyclase activity. The effect of propranolol on the stimulation of cAMP produced by DA and isoprenaline is shown in figure 3.10. Here propranolol (50 μM) reduced the response to isoprenaline (1 μM) by 60% without affecting the response to DA (1 mM).

Despite adenylate cyclase responses to isoprenaline being selectively antagonised by propranolol, this result was not clear cut, because propranolol elevated cAMP content to the same extent as DA. Indeed, under conditions of propranolol blockade, responses to DA and isoprenaline were equal to those produced by propranolol alone. This partial agonist-like behaviour of propranolol might be attributed to an intrinsic sympathomimetic activity, characteristic of many β-antagonists (Scriabine, 1979) closely related in structure to β-agonists, which enables it to behave as an apparent agonist in some systems (Murthy, 1980). Therefore, in order to clarify this result further, these experiments were repeated under the conditions of atenolol (Barrett et al, 1973) blockade, a β-adrenergic antagonist lacking in membrane stabilising effects. Figure 3.11 shows that basal adenylate cyclase activity was unaffected by atenolol (5, 10 and 50 μM). This figure also shows that isoprenaline-sensitive adenylate cyclase activity was potently and selectively antagonised by atenolol (10 μM), whilst responses to DA were unaffected. cAMP accumulation produced by isoprenaline (1 μM) was 210 ± 40% (n = 3) of that produced by a standard concentration of DA (1 mM), and was 94% inhibited by atenolol (10 μM). In contrast DA-sensitive adenylate cyclase responses were insensitive (7% inhibited) to the same concentration of atenolol.

3-2-3 Effect of fluphenazine on renal adenylate cyclase activity

In order to effectively characterise the pharmacological properties of this enzyme system, the nature of the receptors mediating the stimulation of adenylate cyclase was further investigated using the classical neuroleptic, fluphenazine. Specific antagonism of the guinea pig renal DA-sensitive adenylate cyclase by fluphenazine is shown in figure 3.12. Here basal cAMP levels were unaffected by fluphenazine (10 μM) but the increase in cAMP content produced by DA (1 mM) was 90% inhibited by the DA receptor antagonist fluphenazine (10 μM). This figure also shows that the same dose of fluphenazine was ineffective at blocking cAMP formation produced by isoprenaline (1 μM).

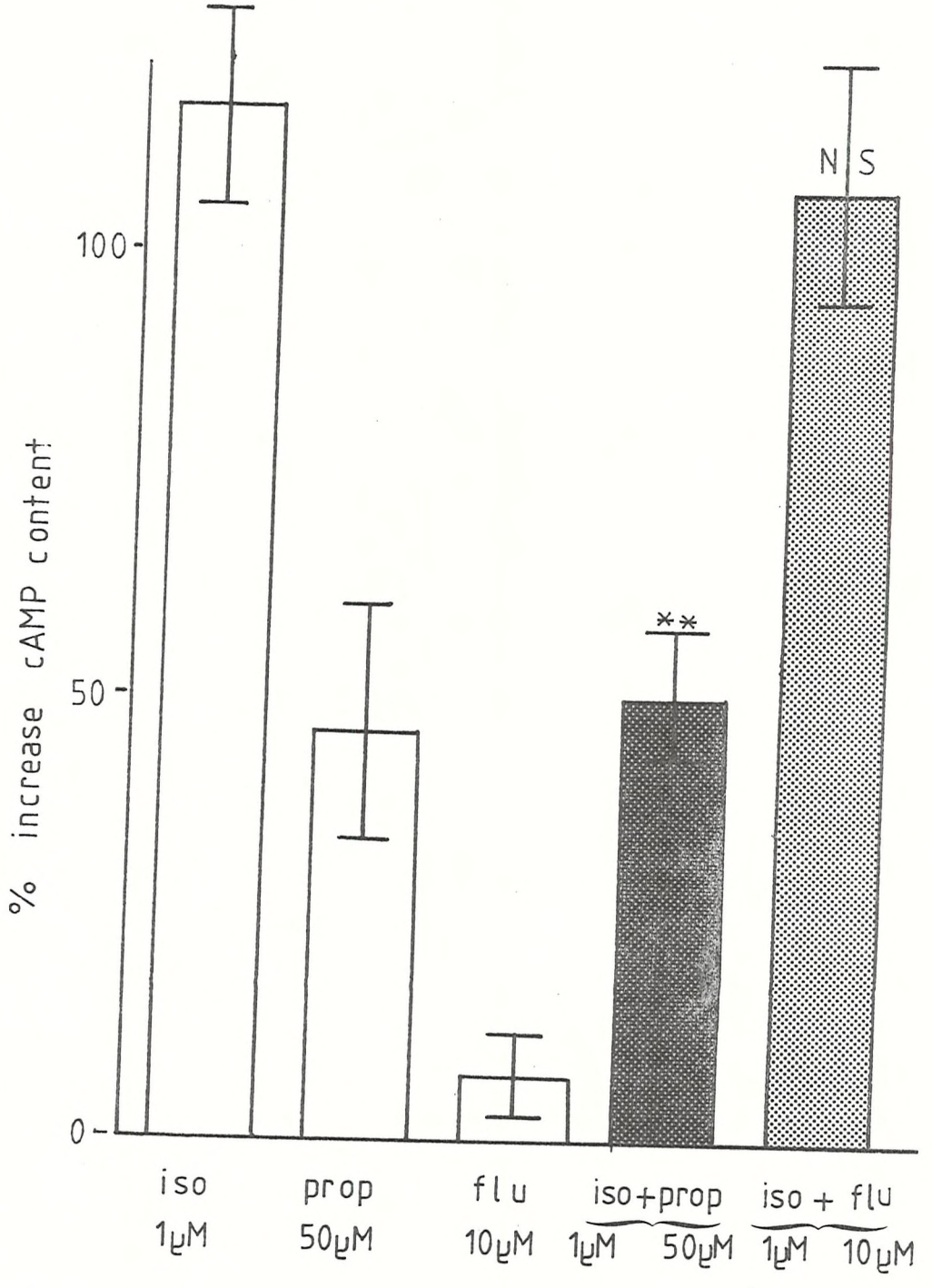


FIGURE 3.10 The effect of (±)-propranolol (50 μM) and fluphenazine (10 μM) on the increase of cAMP content produced by isoprenaline (1 μM) in guinea pig renal particulate preparations. Ordinate responses are expressed as the % increase in cAMP above basal levels, which were 4.2 ± 0.3 pmol/mg protein. Antagonist drugs were added to tubes containing membranes after 50 minutes preincubation and isoprenaline was added 10 minutes later. Each value is the mean response of 3 experiments, assessed in triplicate at each concentration.

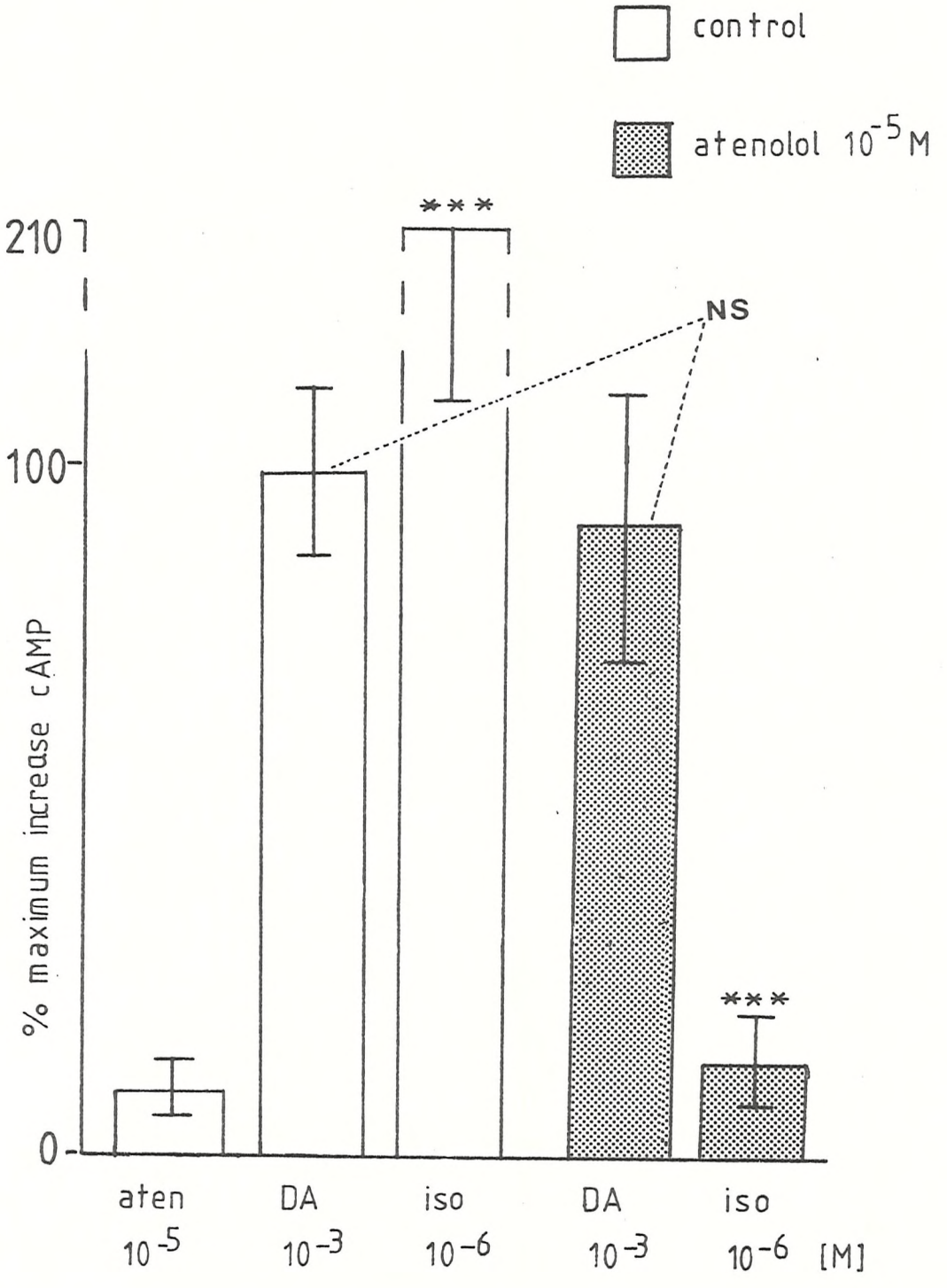


FIGURE 3.11 Effect of atenolol (10 μM) on the increase of cAMP content produced by DA (1 mM) and isoprenaline (1 μM) in guinea pig renal particulate preparations. Atenolol was added to tubes containing membranes after 50 minutes preincubation and agonist drugs were added 10 minutes later. Ordinate responses were expressed as the % increase of cAMP content which was produced by 1 mM DA. Basal cAMP content was 3.7 ± 0.3 pmol cAMP/mg protein. Each value is the mean response of 3 experiments assessed in triplicate at each concentration.

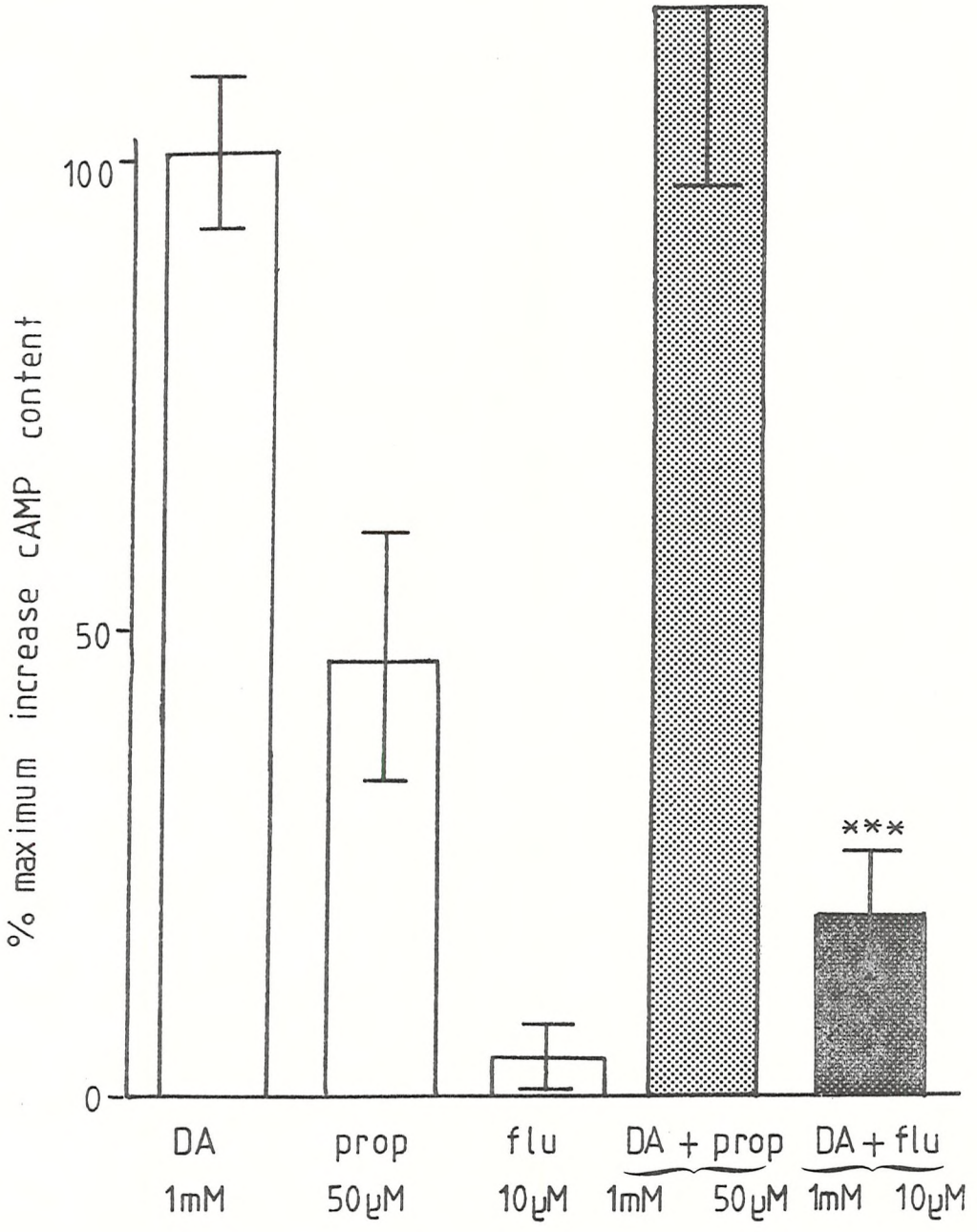


FIGURE 3.12 The effect of fluphenazine (10 μM) and (±)-propranolol (50 μM) on the increase of cAMP content produced by DA (1 mM) in guinea pig renal particulate preparations. Antagonist drugs were added to tubes containing membranes after 50 minutes preincubation and DA added 10 minutes later. Ordinate responses are expressed as the % increase of cAMP content which was produced by 1 mM DA. Basal cAMP content was 3.7 ± 0.3 pmol cAMP/mg protein. Each value is the mean response of 3 experiments assessed in triplicate at each concentration.

TABLE 3.4 Effect of dopamine (1 mM) on cAMP accumulation in preparations from various regions of the guinea pig kidney. Homogenisation and incubation procedures for medullary and pelvic preparations were the same as those described for the renal cortical particulate preparation. Results are expressed as the percentage increase of cAMP (pmol/mg protein) in individual regions.

	Basal cAMP levels pmol/mg protein			% increase of cAMP levels		
	mean	SE	n	mean	SE	n
CORTEX	2.375	1.1	(4)	116	12	(14)
MEDULLA	6.39	0.6	(4)	34	12.4	(4)
PELVIS	ND			ND		

ND = not detectable.

3-2-4 Regional distribution of dopamine-sensitive adenylate cyclase

It has been suggested that DA induced renal vasodilation is mediated through specific postsynaptic DA receptors located in the renal vascular bed (Goldberg, 1972). In the CNS a specific DA-sensitive adenylate cyclase has been implicated in postsynaptic dopaminergic mechanisms. If by analogy, peripheral DA receptors are associated with a DA-sensitive adenylate cyclase, it might be expected that the location of this enzyme is coincidental with regional distribution of endogenous DA content (see Section 3-4) and with renal zones displaying more sensitive vascular responses (increased RBF and decreased RVR) to exogenously administered DA. We therefore investigated the DA-sensitive adenylate cyclase in preparations from various regions of the guinea pig kidney. Table 3.4 shows the effect of DA (1 mM) on cAMP formation in renal cortical, medullary and pelvic particulate preparations. Here DA stimulated the production of cAMP most powerfully in cortical preparations where the absolute increase in cAMP content was 2.81 ± 0.9 pmol/mg protein, which represented a 116% increase. Although basal cAMP content was greater in medullary preparations, the increase in cAMP produced by DA was 2.175 ± 0.79 pmol/mg protein which represented only a 34% increase. DA had no significant effect on adenylate cyclase activity in renal pelvic preparations.

3-2-5 The effect of ADTN and SKF 38393 on dopamine-sensitive adenylate cyclase activity

In order to study the pharmacological similarities between renal vascular responses to DA and DA-sensitive adenylate cyclase, the DA receptor agonists ADTN and SKF 38393, which had produced a DA-like increase in RBF and decrease in RVR, were tested for their potency at elevating cAMP content in renal particulate preparations. ADTN (10^{-6} - 10^{-3} M) produced a dose-related increase of renal cAMP levels (Figure 3.13). The maximum response to ADTN (0.1 mM) was 88 ± 3.3% (n = 6) of that produced by a standard concentration of DA (1 mM) which was included in every experiment. ADTN was approximately six times more potent than DA at stimulating the production of cAMP, with an EC_{50} value of 10 µM (Table 3.5).

SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H3-benzazepine)

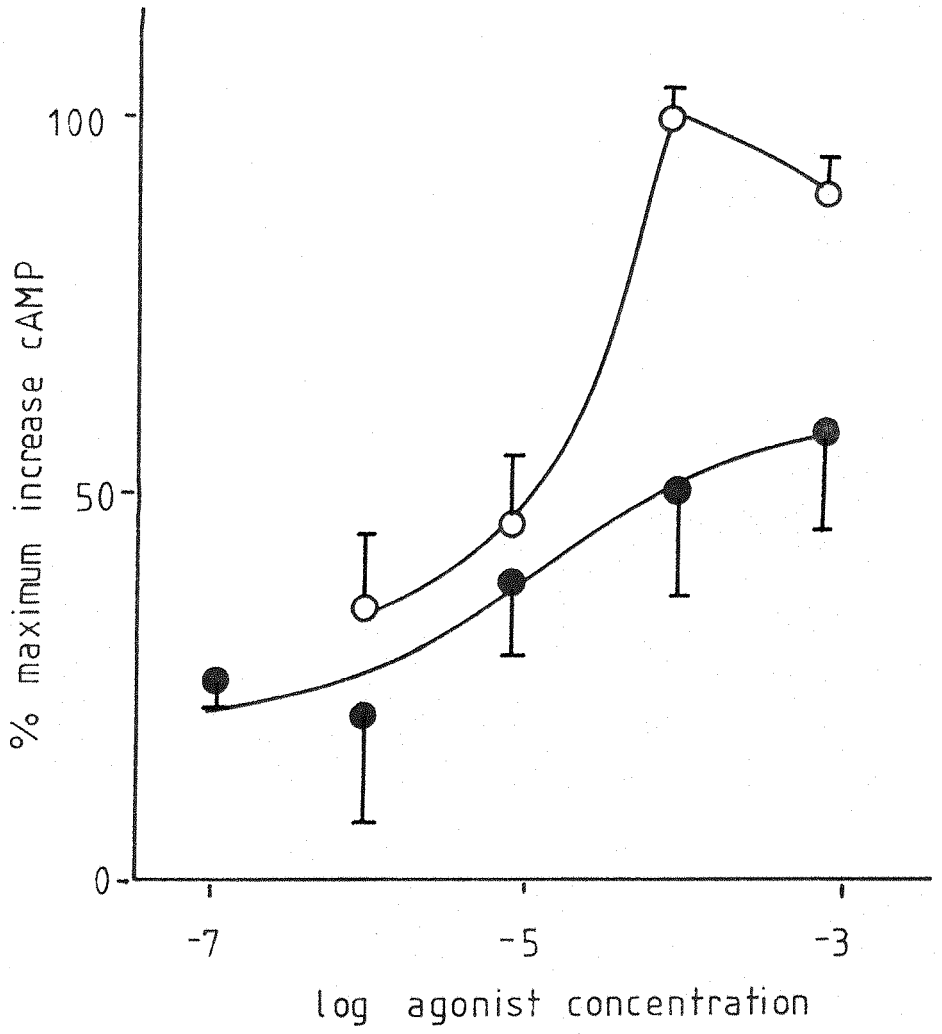


FIGURE 3.13 Dose-related increases in cAMP content produced by ○ ADTN (2-NH_2— 6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) and ● SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine) in guinea pig renal particulate preparations. Ordinate responses are expressed as the % increase of cAMP content which was produced by 1 mM DA. Each value is the mean response of 3 experiments assessed in triplicate at each concentration.

TABLE 3.5

Drug	EC_{50} (M)	µM	Relative Potency
DA	6.30×10^{-5}	63	1
ADTN	1.00×10^{-5}	10	6.3
SKF 38393	1.40×10^{-5}	14	4.5
bromocr ptine	1.58×10^{-7}	0.158	398
isoprenaline	1.70×10^{-7}	0.17	370

Agonists active on renal DA-sensitive adenylate cyclase and their relative potency. EC_{50} values were determined from dose-response curves of percentage maximum production of cAMP, which was taken as that produced by 1 mM DA.

Abbreviations: DA, dopamine; ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine.

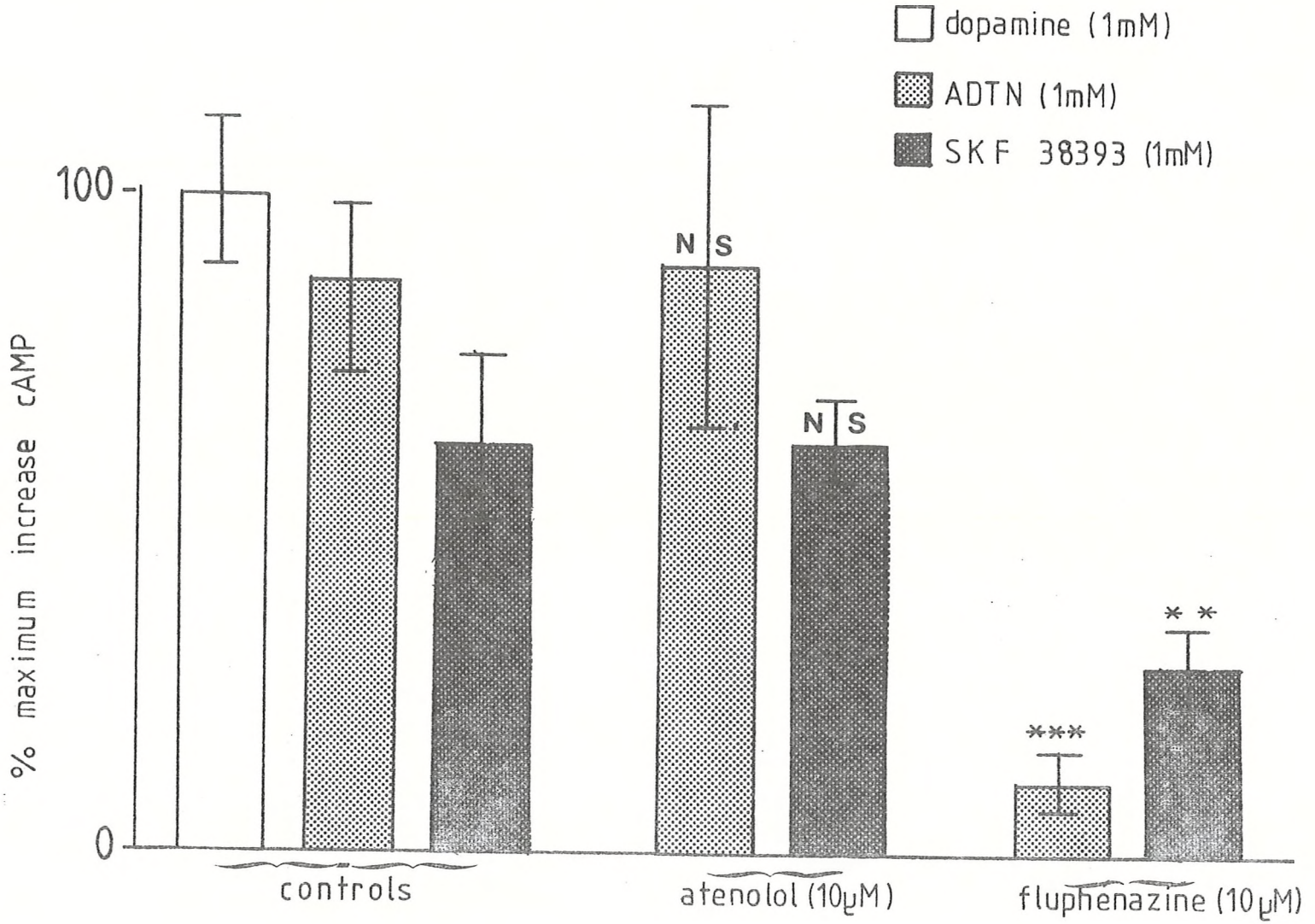
(10^{-7} - 10^{-3} M) enhanced cAMP formation in renal particulate preparations in a concentration dependent manner which was dissimilar to that of DA but characteristic of a partial DA receptor agonist drug (Figure 3.12). SKF 38393 had a threshold active concentration of 0.1 μM which was approximately 10 and 100 times lower than that of ADTN and DA respectively, but did not produce a maximum response equivalent to that produced by 1 mM DA. In the lower concentration range SKF 38393 (0.1-10 μM) was 4.5 times more potent than DA on the adenylate cyclase model and had an EC_{50} value of 1.4×10^{-5} M However, SKF 38393 lacked potency in the higher concentration ranges, for example 1 mM SKF 38393 only produced 69 ± 14% (n = 6) of the maximum in increase in cAMP levels, which was produced by 1 mM DA. This may suggest that, as in striatal tissue, SKF 38393 is behaving as a partial agonist (Freedman, Wait and Woodruff, 1979; Hahn and Wardell, 1980).

3-2-6 Specific antagonism of renal cAMP responses to ADTN and SKF 38393

In order to characterise the pharmacological specificity of DA receptor agonists upon renal DA-sensitive adenylate cyclase, it is important that responses produced by DA analogues, as well as DA itself, should be selectively inhibited by neuroleptics previously established as efficient antagonists of other DA receptor model systems. The effect of the typical neuroleptic fluphenazine, and the β-adrenergic blocking agent atenolol upon adenylate cyclase responses to ADTN and SKF 38393 was examined in renal particulate preparations. Responses are expressed as the percentage of maximum response which was taken as that produced by a standard concentration of DA (1 mM). Fluphenazine (10 μM) inhibited ADTN (0.1 mM) and SKF 38393 (0.1 mM) induced cAMP accumulation by 88 ± 6% (n = 3) and 35 ± 15% (n = 3) respectively (Figure 3.14). In contrast atenolol (10 μM), present at the same concentration which had potently inhibited the elevation of cAMP levels produced by the β-adrenergic agonist isoprenaline (1 μM) (Figure 3.11), was ineffective at inhibiting renal adenylate cyclase responses to ADTN (0.1 mM) and SKF 38393 (0.1 mM) (Figure 3.14). Fluphenazine was more potent at inhibiting renal adenylate cyclase responses to ADTN (Figure 3.14) and DA (Figure 3.12) than responses to SKF 38393 (Figure 3.14).

FIGURE 3.14 Effect of atenolol and fluphenazine on dopamine, ADTN and SKF 38393 stimulated elevation of cAMP content in guinea pig renal particulate preparations. Antagonists or vehicle were added to tubes after 50 minutes pre-incubation and agonist drugs added 10 minutes later. Results are expressed as the percentage of maximum cAMP formed (pmol per mg protein) which was taken as that produced by 1 mM DA. Each value represents the mean of 4 determinations. Basal cAMP content was 2.83 ± 0.21 pmol/mg protein.

Abbreviations: ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine.



3-2-7 Effect of (±)-sulpiride on renal adenylate cyclase

Having established that DA-sensitive adenylate cyclase in the guinea pig kidney has a pharmacological profile for agonists very similar to that of renal vascular responses to DA in both guinea pig and dog (Goldberg et al, 1978a) in that (1) DA elevated cAMP content, (2) this elevation was not inhibited by β-adrenergic blockade, (3) fluphenazine, a specific DA receptor blocker, potently and selectively inhibited the stimulatory effect of DA and (4) ADTN and SKF 38393, DA receptor agonists, also elevated cAMP content and were similarly selectively antagonised by fluphenazine. The logical progression was to investigate whether the pharmacological profile for antagonist drugs correlated equally as well in both systems.

The substituted benzamide sulpiride was the most potent and selective antagonist of DA induced renal vasodilation in guinea pig (see Section 3-1-6) and dog (Goldberg et al, 1979). However, it has been suggested that the functional relationship between sulpiride and DA receptors in the brain might differ fundamentally from that of classical antipsychotic drugs. This suggestion recently arose to explain why sulpiride conformed with typical neuroleptic characteristics in some animal models, but not in others. Of particular interest to this study was that despite sulpiride's proven potency in the clinic as an antiemetic (Laville and Margarit, 1968), antipsychotic drug (Benoit et al, 1969) which elevated DA turnover (Tagliamonte et al, 1975) and had strong prolactin releasing effects (Mancini et al, 1976) (see Section 1-5-11), it completely failed to inhibit cAMP accumulation induced by DA in striatal and limbic preparations (Trabucchi et al, 1975). One interpretation for these differences in potency was that they provided evidence for D_1 (cyclase-linked), sulpiride insensitive receptors and D_2 (non-cyclase-linked) receptors. Thus, sulpiride might interact with a population of DA receptors not associated with an adenylate cyclase system (Kebabian and Calne, 1979). Receptor linkage to an adenylate cyclase molecule (D_1) inferred an exclusively postsynaptic cellular location, whereas receptors lacking such a linkage (D_2) could be located pre- or postsynaptically or elsewhere on the nerve cell at a distance from the synaptic cleft. Analogous to postsynaptic DA receptors mediating responses in the CNS, renal vascular responses to DA are probably mediated by specific postsynaptic DA receptors (Goldberg, 1972) and according to classical

theory, it might be expected that the DA-sensitive adenylate cyclase associated with renal particulate preparations should also be located postsynaptically. Since sulpiride was an extremely potent antagonist of the RBF model, its effect upon cAMP accumulation induced by DA, ADTN and SKF 38393 was investigated in order to deduce whether the potency differences displayed by sulpiride in central DA receptor models would be similar in peripheral models. Basal adenylate cyclase activity was unaffected by vehicle or (±)-sulpiride (0.01, 0.1 and 1 mM) as shown in figure 3.15. cAMP accumulation responses to DA (1 mM) (Figure 3.15), isoprenaline (1 μM) (Figure 3.16), ADTN (0.1 mM) (Figure 3.17) and SKF 38393 (0.1 mM) (Figure 3.18) in renal particulate preparations were completely unaffected by pretreatment with these same high concentrations of (±)-sulpiride. In some experiments sulpiride slightly, but not significantly, enhanced DA stimulated cAMP formation, but never reduced it.

3-2-8 Effect of bromocriptine on renal dopamine-sensitive adenylate cyclase

DA-sensitive adenylate cyclase in renal particulate preparations was remarkably similar to that found in brain homogenates particularly regarding its agonist profile and insensitivity to sulpiride. Recently, another dopaminergic drug bromocriptine, has also been reported to yield surprising results in CNS adenylate cyclase models and has been implicated in the multiple DA receptors theory (Kebabian and Calne, 1979). Despite bromocriptine being an extremely potent agonist in many DA receptor models, this ergot derivative has been reported to have the reverse effect (ie inhibition) upon DA-sensitive adenylate cyclase activity (Trabucchi et al, 1976) at micromolar concentrations.

Among its spectrum of DA receptor agonist activities, bromocriptine depresses prolactin levels in man (Del Pozo et al, 1972) and in animals (Fuxe et al, 1973), inhibits DA synthesis (Corrodi et al, 1973; Di Chiara et al, 1977) and is active in many animal behavioural models (Johnson et al, 1976; for review see Fuxe et al, 1978). However, the actions of bromocriptine upon other DA receptor models are less clear. Bromocriptine lacks agonist activity on the adenylate cyclase model because it only weakly enhances cAMP accumulation in some tissue preparations, for example, rabbit retina (Schorderet, 1976), not at all in others, for example, rat striatal and pituitary gland homo-

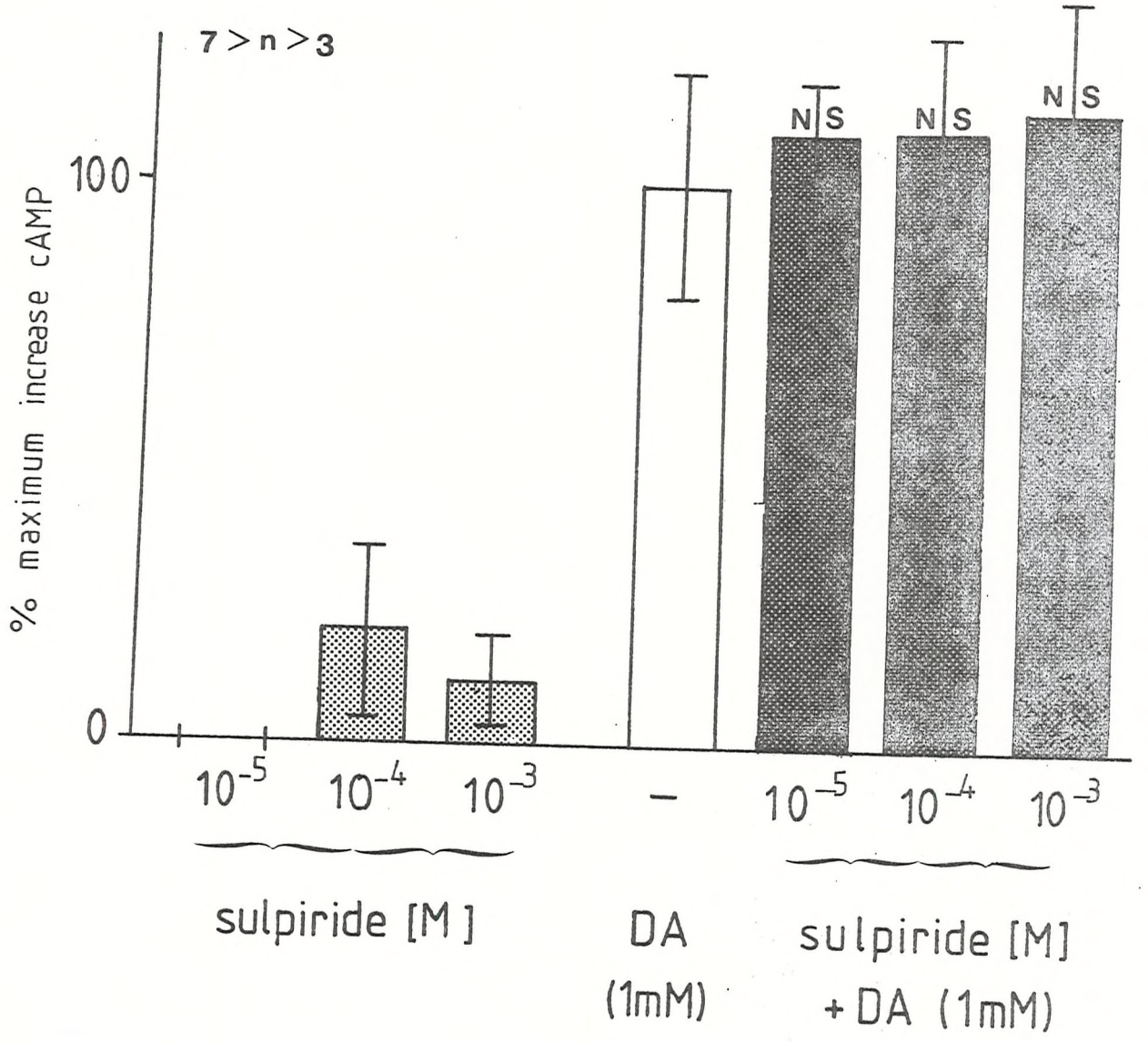


FIGURE 3.15 Effect of (±)-sulpiride (10,100 and 1000 μM) on basal cAMP content and on increases in cAMP content produced by DA in guinea pig renal particulate preparations. Ordinate responses are expressed as the % maximum increase in cAMP content which was produced by 1 mM DA. (±)-Sulpiride was added to tubes containing membranes after 50 minutes preincubation and DA was added 10 minutes later. Basal cAMP levels were 2.8 ± 0.21 pmol/mg protein (n = 6).

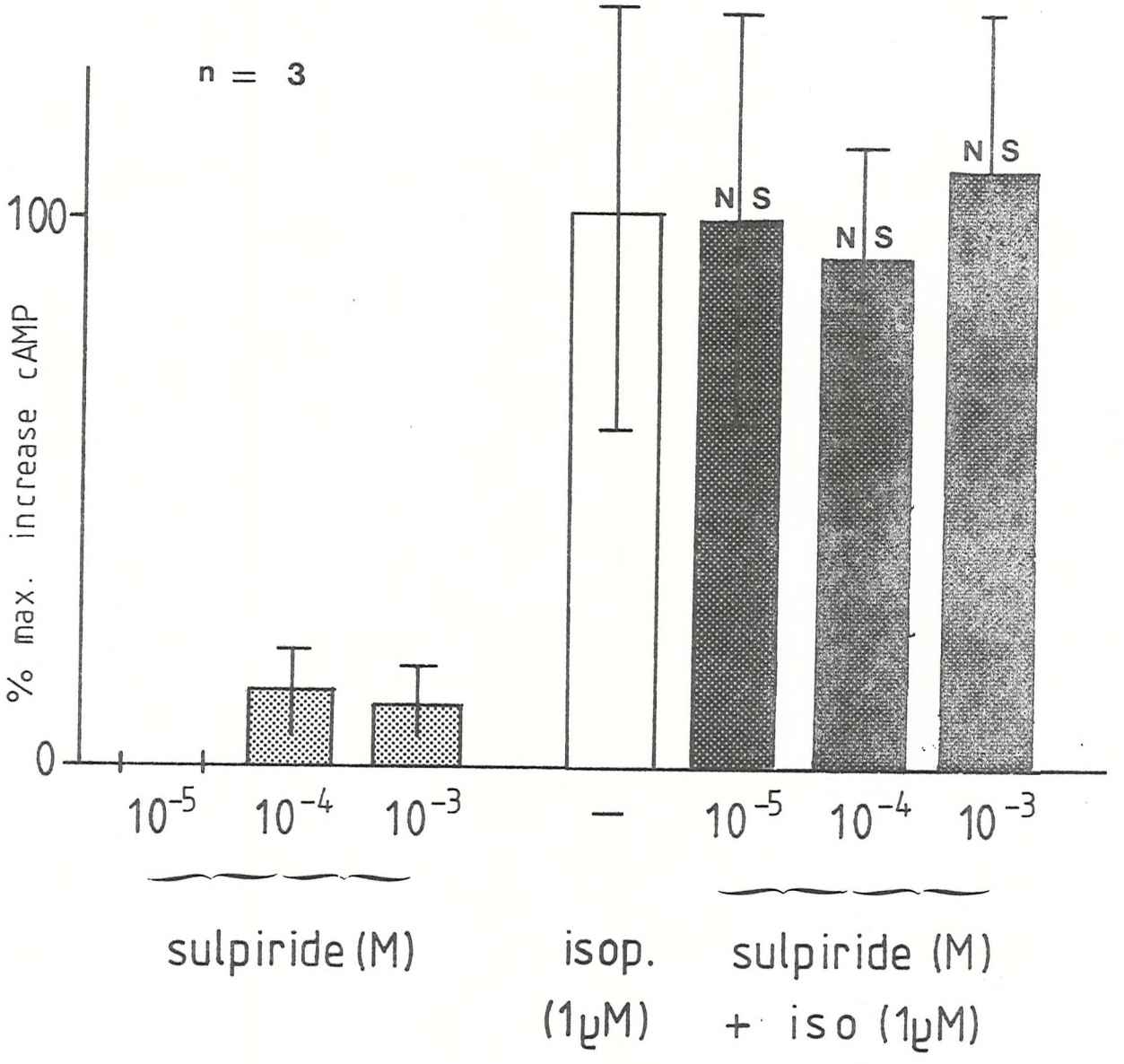


FIGURE 3.16 Effect of sulpiride on isoprenaline stimulated elevation of cAMP content in guinea pig renal particulate preparations. Sulpiride was added to tubes after 50 minutes preincubation and isoprenaline or vehicle added 10 minutes later. Results are expressed as the percentage of maximum cAMP formed (pmol per mg protein) which was taken as that produced by 1 μM isoprenaline. Basal cAMP content was 2.64 ± 0.28 pmol/mg protein.

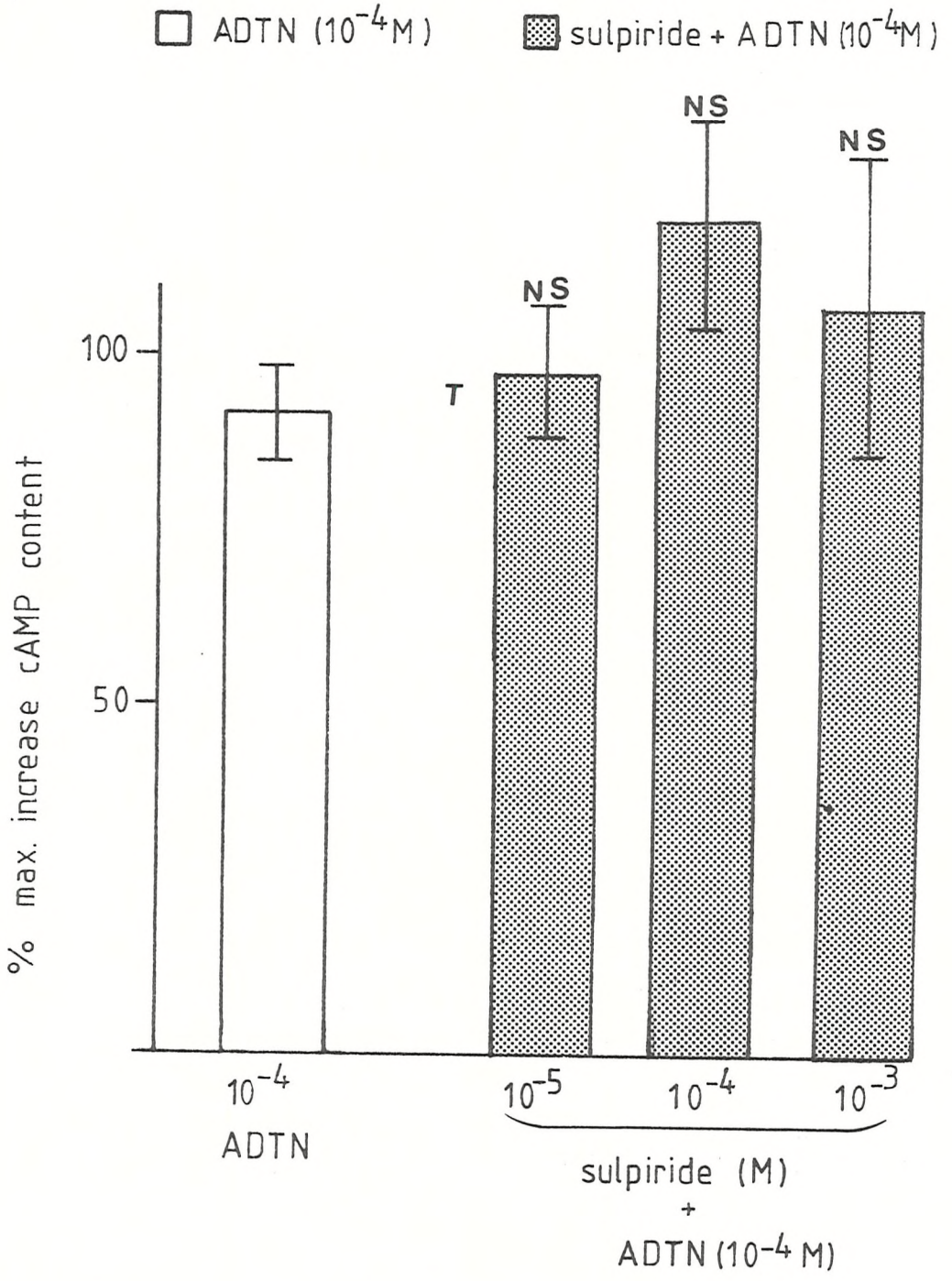


FIGURE 3.17 Effect of (±)-sulpiride (10, 100 and 1000 μM) on increases in cAMP content produced by ADTN (100 μM) in guinea pig renal particulate preparations. Ordinate responses are expressed as the % maximum increase in cAMP which was produced by 1 mM DA. Each value represents the mean of 3 observations, assayed in triplicate at each concentration; vertical bars represent the standard error of the means.

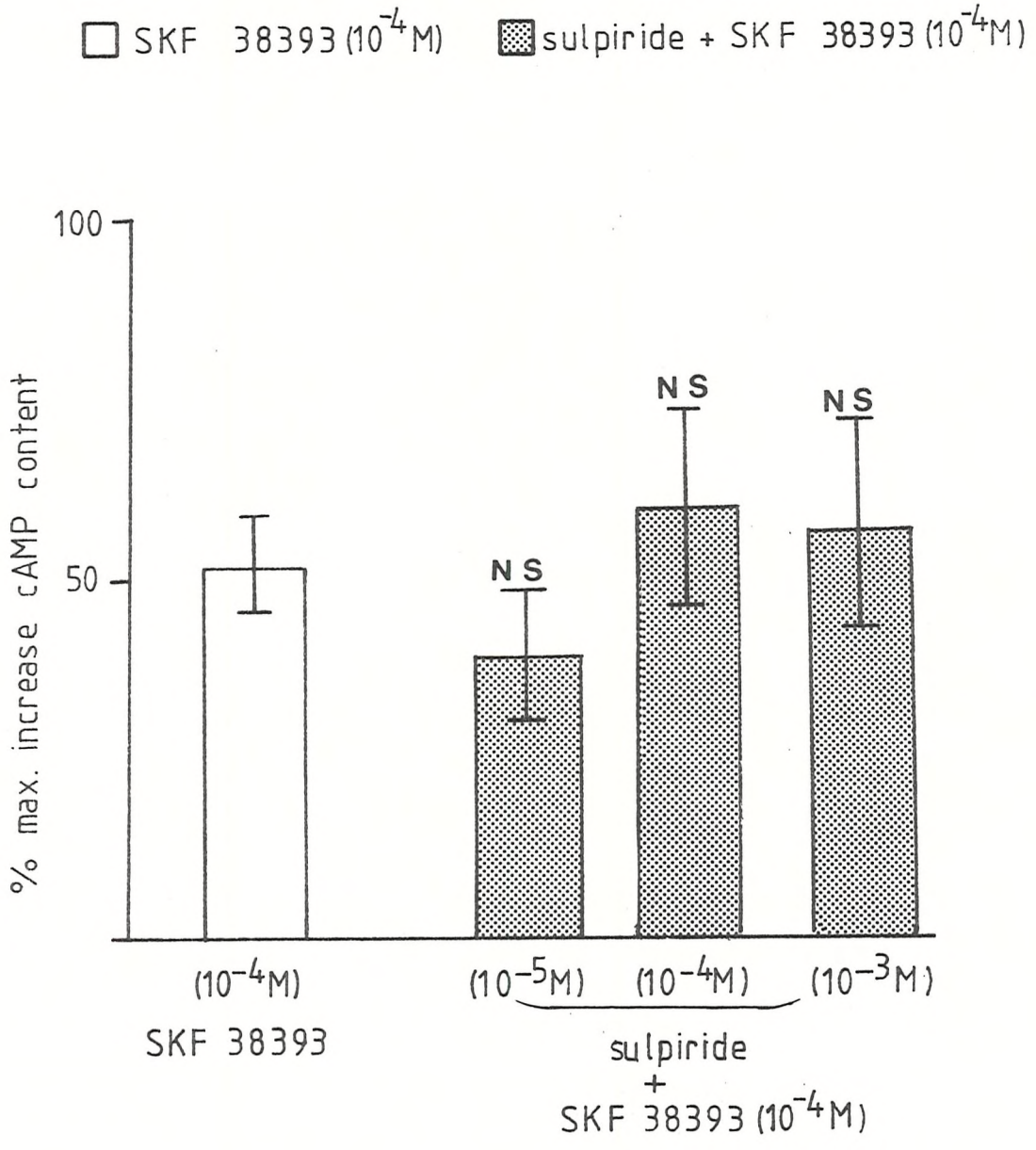


FIGURE 3.18 Effect of (±)-sulpiride (10, 100 and 1000 μM) on increases in cAMP content produced by SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine) (100 μM) in guinea pig renal particulate preparations. Ordinate responses are expressed as the % maximum increase in cAMP which was produced by 1 mM DA. Each value represents the mean of 3 observations, assayed in triplicate at each concentration; vertical bars represent the standard error of the means.

genates (Spano et al, 1978) and is inhibitory in others (Trabbucchi et al, 1976). Another indication that bromocriptine might have DA antagonist activity, is that it potently displaces [^{3}H] spiroperidol and [^{3}H] haloperidol binding to calf and rat striatal preparations (Seeman, 1980), but only weakly displaces [^{3}H] DA and [^{3}H] apomorphine binding to similar striatal preparations. Finally, it is extremely interesting to note that bromocriptine is totally inactive upon the canine renal vascular DA receptor model (Volkman and Goldberg, 1976).

As with sulpiride, these differences in bromocriptine's potency and mode of action upon various DA receptor models have been interpreted as evidence for distinct CNS DA subtypes; differentiated by their association with an adenylate cyclase molecule or not (see Section 3-2-7 and Chapter 4). Furthermore the lack of correlation between dopaminergic inhibition of prolactin release and stimulation of dopaminergic renal vasodilation by the ergots was interpreted as evidence that CNS DA receptors mediating prolactin release were different to peripheral DA receptors mediating renal vasodilation (Volkman and Goldberg, 1976). If there were truly such differences between peripheral and central DA receptors it might be expected that bromocriptine would be ineffective on the renal adenylate cyclase model. Such a possibility was investigated.

Bromocriptine (10^{-9} - 10^{-5} M) produced dose-related elevations in renal cAMP content (Figure 3.19) with partial agonist characteristics. Its threshold active concentration was in the nanomolar range which was approximately 500 times lower than that of DA but the maximum response to bromocriptine (1 μM) was only 63 ± 7.5% (n = 5) of that produced by a standard concentration of DA (1 mM). Bromocriptine was extremely potent in the adenylate cyclase system with an EC_{50} value of 1.58×10^{-7} (Table 3.5) which was approximately 400 times more powerful than DA at stimulating the production of cAMP. These agonist effects were unanticipated in view of bromocriptine's ineffectiveness upon the canine RBF model (Volkman and Goldberg, 1976) and upon striatal adenylate cyclase activity (Spano et al, 1978).

Having established that bromocriptine was a potent partial agonist in stimulating DA-sensitive adenylate cyclase activity in renal particulate preparations, bromocriptine was screened for any apparent DA antagonist activity. Adenylate cyclase responses to the β-adrenergic

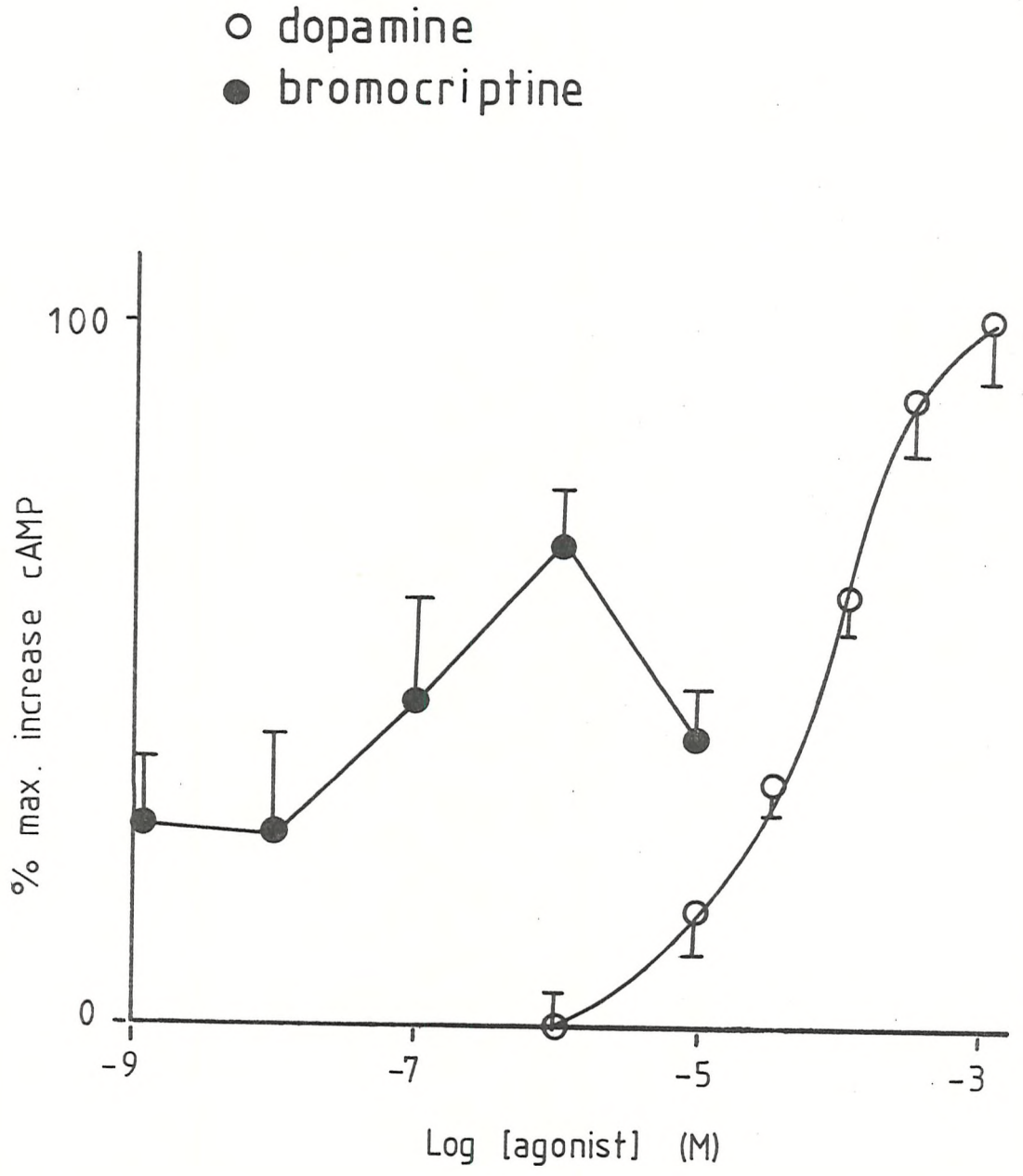


FIGURE 3.19 Dose response curves for the elevation of cAMP content produced by dopamine and bromocriptine in guinea pig renal particulate preparations. Results are expressed as the percentage of maximum cAMP formed (pmol per mg protein) which was taken as that produced by 1 mM DA and represent the mean of 2-4 determinations at each concentration. Basal cAMP content was 2.34 ± 0.18 pmol/mg protein.

agonist isoprenaline (1 μM) were 146 ± 35% (n = 5) and to bromocriptine (10 μM) were 47 ± 2.8% (n = 3) (Figure 3.20) of the increase in cAMP content which was produced by a standard concentration of DA (1 mM). This same concentration of bromocriptine inhibited adenylate cyclase responses to DA (1 mM) by 42 ± 17% (n = 3). Surprisingly responses to isoprenaline (1 μM) were enhanced 60 ± 28% (n = 3) by bromocriptine (10 μM). This additional ability of bromocriptine to exert an inhibitory effect upon renal DA-sensitive adenylate cyclase activity is in agreement with studies conducted in the brain (Trabucchi et al, 1976; Spano et al, 1978). However, the bromocriptine potentiation of cAMP accumulation produced by isoprenaline (the latter being presumably a β-adrenoceptor mediated event) was not anticipated in view of current concepts about receptor-mediated regulatory processes controlling the activity of single adenylate cyclase complexes in the cell membrane (Rodbell, 1980).

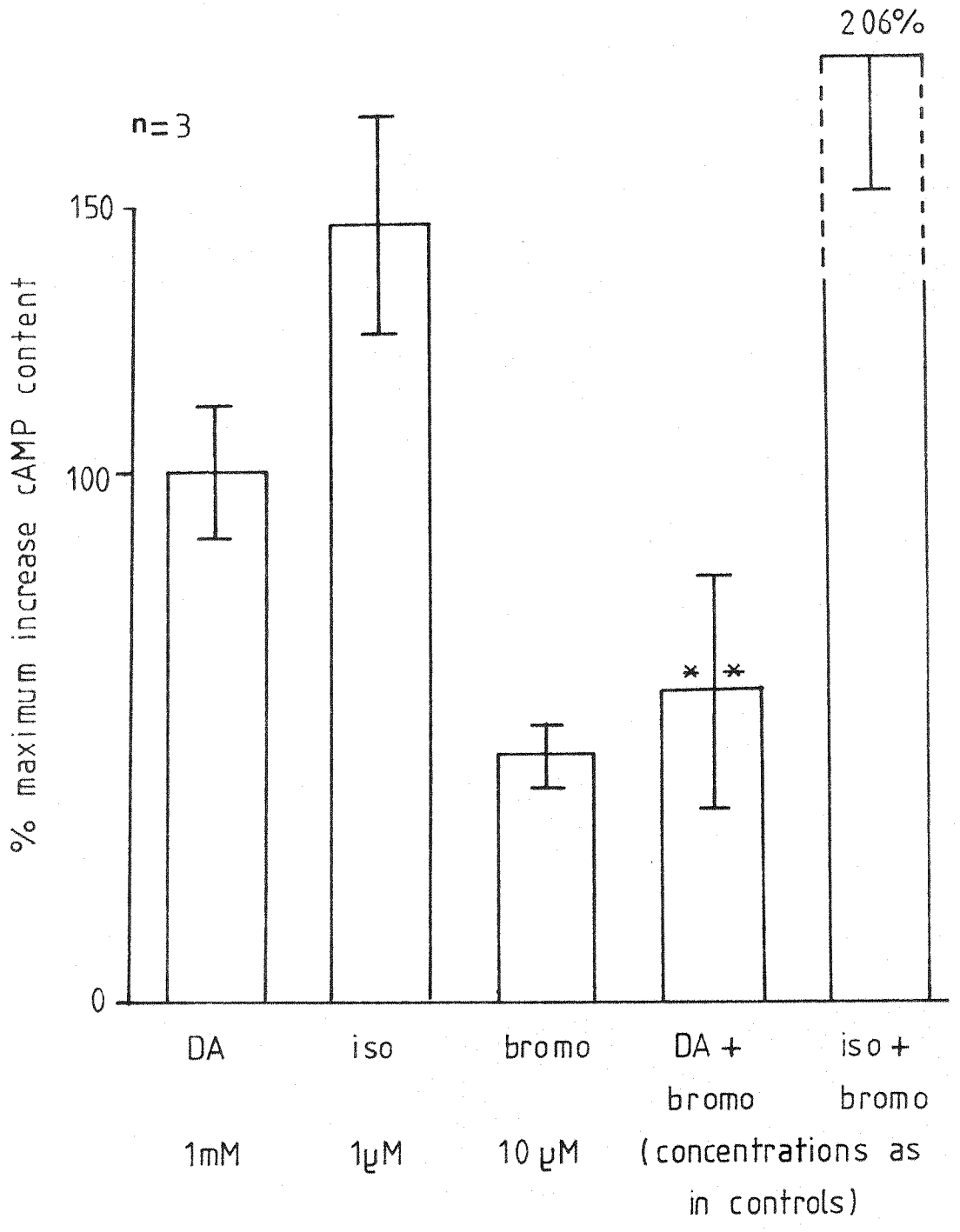


FIGURE 3.20 Effect of bromocriptine (10 μM) on the increase in cAMP content produced by DA (1 mM) and isoprenaline (1 μM). Ordinate responses are expressed as a % of the increase in cAMP responses produced by 1 mM DA. Bromocriptine was added to tubes containing membranes after a 50 minute preincubation period and agonist drugs were added 10 minutes later. The number of observations is shown and the vertical bars represent the standard error of the means.

3-3 Binding of [^{3}H] sulpiride to crude guinea pig renal cortical membranes

The development of receptor binding techniques has assisted the identification, localisation and pharmacological characterisation of DA receptors in the CNS. Previous reports from our laboratories have demonstrated that [^{3}H] sulpiride is a particularly suitable ligand for DA receptors (Woodruff and Freedman, 1981) which binds to rat striatal preparations in a highly specific, saturable manner. Moreover, neuroleptic drugs inhibit this high affinity binding in a rank order of potency which correlates well with their clinical efficiency as antipsychotic agents. Many lines of evidence suggest that DA has a physiological role in the kidney (see Sections 1-4-3 and 1-5-5), where it appears to be involved in the regulation of RBF. Elevations in RBF have been associated with a specific renal vascular DA receptor which is potently and specifically antagonised by sulpiride in the dog (Kohli et al, 1978b), rat (Chapman et al, 1980) and guinea pig (Section 3-1-5). Additionally the DA-sensitive adenylate cyclase in the kidney (Section 3-2) has a remarkable resemblance to the DA receptors mediating vasodilation in the renal vasculature (Section 3-1) in terms of agonist requirements for DA-like activity. 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) which contains the DA molecule held rigidly in its β-rotamer conformation was the most potent agonist on both models and 2,3,4,5-tetrahydro-6,7-dihydroxy-1-phenyl-1H-3-benzazepine (SKF 38393) appeared to behave as a partial agonist. However, the most striking discrepancy between these models was the complete inability of sulpiride to antagonise DA on the renal adenylate cyclase model (see Section 3-2-7), whereas it was an extremely potent antagonist of DA induced vasodilation (see Section 3-1-5). Similar discrepancies in the CNS (see Section 1-5-11) have given rise to the classification of DA receptors into D_1 receptors, linked to an adenylate cyclase and inhibited by cis-flupenthixol, and D_2 receptors, non-cyclase linked and inhibited by sulpiride (Kebabian and Calne, 1979). The kidney is a particularly suitable tissue for investigating the feasibility of such an explanation for the anomolous actions of sulpiride. One obvious reason for this suitability is that, in the kidney, sulpiride is simultaneously the most potent antagonist of the RBF model and the most ineffective antagonist of the renal adenylate cyclase model. In order to further investigate the mechanism by which sulpiride is involved in dopaminergic function in the kidney, we examined the

TABLE 3.6 Blank rate of [^{3}H] sulpiride binding to various separation materials in comparison with the specific binding of [^{3}H] sulpiride to renal cortical membranes. Specific binding was determined using 20 nM [^{3}H] sulpiride in the presence and absence of 10 µM s-(-)-sulpiride. Filters were washed with 10 ml ice cold tris-HCl buffer, pH 7.3 at 0°C, microfuge tubes were washed with 1 ml of the same buffer. Blanks were routinely incorporated into experiments.

	Millipore HAWP 02400	Whatman GF/B	Microfuge tubes
% specific binding to membranes	48%	41.7%	31.3%
binding to separation materials DPM/filter or tube	1021 ± 270	208 ± 162	4 ± 2
binding to membranes DPM/filter or tube	4120 ± 346	3980 ± 820	4837 ± 395
$\frac{\text{binding to filters}}{\text{binding to membranes}} \times 100$	24.8%	5.22%	0.08%

the characteristics of [^{3}H] sulpiride binding sites in guinea pig renal cortical homogenates.

3-3-1 Binding characteristics

a) Filter blanks

A major criticism of direct ligand binding assays for neurotransmitter and hormone receptors is that the radioligands used for binding to membrane preparations can interact stereospecifically with non-biological materials, such as filters and glassware in a manner which imitates some of the criteria used for the identification of specific binding (see Section 1-5-10). For example, [^{125}I] insulin binding to talc and glass test-tubes is specific, reversible and saturable in the same nanomolar concentration range in which insulin binds to membranes (Cuatrecasas and Hollenberg, 1975).

In order to establish the optimum conditions for the separation of free [^{3}H] sulpiride from bound, preliminary experiments were performed in which several ways of separating free and bound ligand were examined. These initial experiments were performed using 10 µM S-(-)-sulpiride to define specific binding. The blank rate, in the absence of guinea pig renal cortical tissue, for [^{3}H] sulpiride separation by filtration with i) millipore HAWP 02400 filters and ii) Whatman GF/B filters compared with rapid centrifugation in plastic microcentrifuge tubes is shown in table 3.6.

i) Millipore filters Table 3.6 shows that [^{3}H] sulpiride bound to millipore filters at a typical rate of 1020 ± 270 DPM/filter which represented 18.2 ± 4.8 fmol/filter. These extremely high blank rates were unanticipated in view of the low blank rates reported by Woodruff and Freedman (1981) using the same radioligand. They found only 1.4 ± 0.3 fmol/filter was attributable to filter blanks under assay conditions where 1 µM S-(-)-sulpiride was used to define specific binding.

ii) Microfuge tubes In the absence of tissue [^{3}H] sulpiride binding to plastic microfuge tubes was negligible as shown in table 3.6.

iii) Whatman GF/B filters Table 3.6 shows that [^{3}H] sulpiride bound to Whatman GF/B filters at a rate of 208 ± 162 DPM/filter which represented 3.7 ± 0.06 fmol/filter. Thus, apparent binding to

separation materials was highest for millipore HAWP 02400 filters, low for Whatman GF/B filters and negligible for plastic microfuge tubes.

b) Comparison of tissue specific binding using various separation techniques

The efficiency of individual separation techniques to produce a high percentage of specific binding depends upon how effectively non-specific binding and free radioligand can be eliminated. Thus the most successful separation methods produce high yield specific binding with minimal accompanying blank rates (less than 10% is acceptable). Table 3.6 also shows the amount of tissue specific binding obtained using these three separation techniques.

i) Millipore filters In the presence of tissue, a typical value for [^{3}H] sulpiride specific binding was 4120 ± 346 DPM/filter which represented 73.5 ± 6.1 fmol/filter. Table 3.6 also shows that specific binding represented 48% of total binding to guinea pig renal cortical membranes, but that a quarter of this specific binding was attributable to filter blanks. These high millipore filter blank rates are probably due to slow filtration procedures. Total time for filtration and washing was several minutes because the filtrate was retarded by the large amount of protein used in each assay. This problem could not be overcome by reducing tissue quantity as the amount of specific binding then fell below an unacceptable error margin. Speed is a crucial aspect of radioligand separation by filtration. Receptor-ligand complexes which dissociate rapidly require fast separation procedures to minimise the amount of radioligand dissociated, otherwise the result is a loss of binding, underestimation of the maximum binding capacity (Bmax) of the system and inaccurate determination of the binding affinity constant (K_D). Millipore filtration was unsuitable due to high blank binding rates.

ii) Microcentrifugation [^{3}H] sulpiride bound to membranes at a rate of 4837 ± 395 DPM/microfuge tube which represented 86.1 ± 7.05 fmol/microfuge tube. This amount of specific binding was lower than that obtained by filtration and only represented 31.3% of total binding to renal cortical membranes, in comparison with 42% for Whatman GF/B filters and 48% for millipore filters as shown in table 3.6. During the rapid centrifugation procedure, higher levels of non-eliminable

free [^{3}H] sulpiride were trapped within the pellet space and resulted in a proportionate reduction of specific binding. In contrast to filtration none of this binding was attributable to blanks, since blank rates were negligible.

iii) Whatman GF/B filtration [^{3}H] sulpiride bound to membranes at a rate of 3491 ± 720 DPM/filter which represented 71 ± 14.5 fmol/filter. As shown in table 3.6 only 5% of this binding was attributable to filter blanks; moreover specific binding represented approximately 42% of total binding to renal cortical membranes. Total time for filtration and washing procedureswas consistently less than one minute.

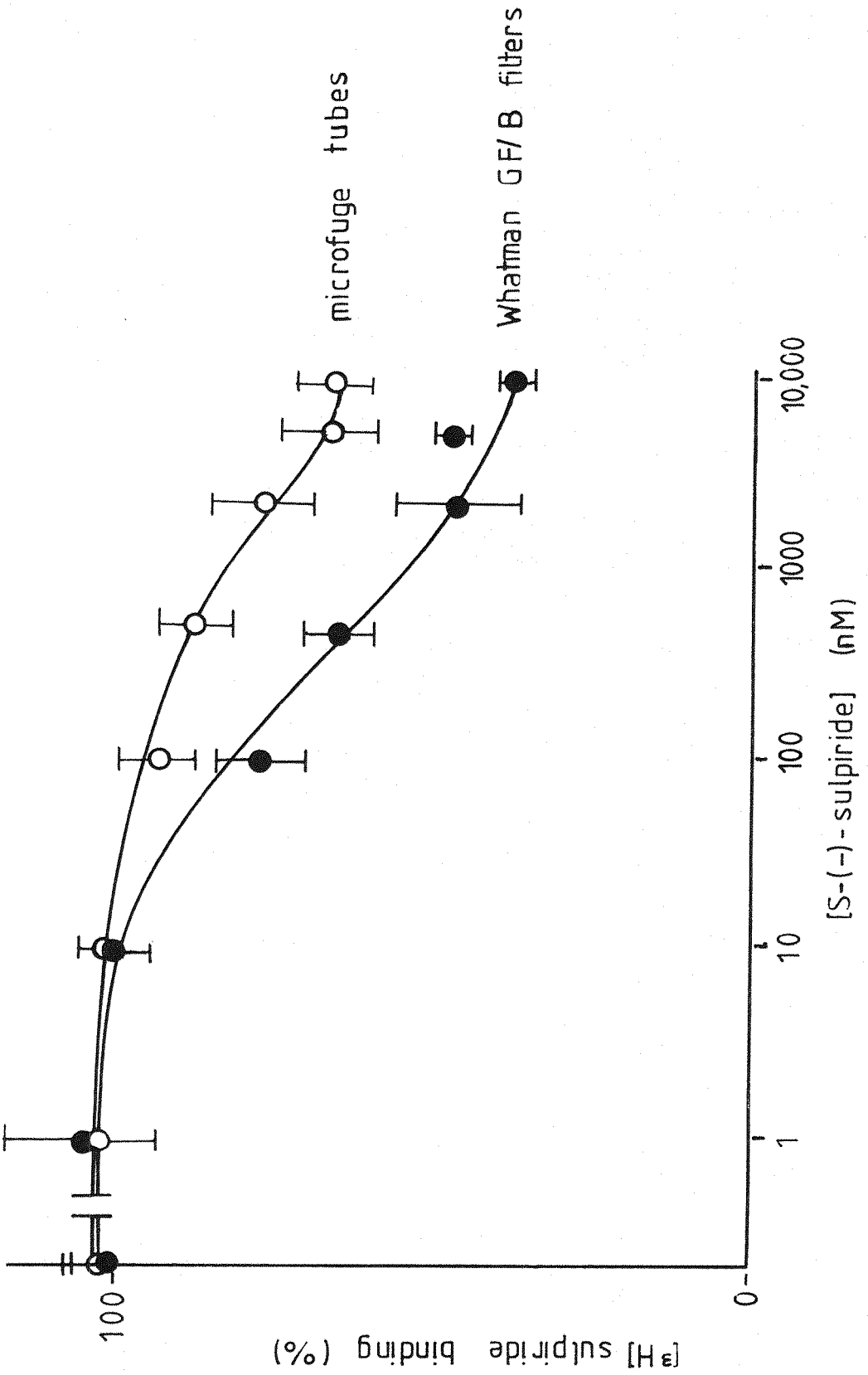
c) [^{3}H] sulpiride separation by Whatman GF/B filtration or by microcentrifugation?

Radiolabelled and non-radiolabelled ligand concentrations may effect blank rates. In further investigations specific binding in renal membrane preparations using Whatman GF/B filters was compared with that obtained using microcentrifuge tubes at varying concentrations of S-(-)-sulpiride and of [^{3}H] sulpiride.

i) Displacement curves The displacement of 20 nM [^{3}H] sulpiride by increasing concentrations of S-(-)-sulpiride in renal cortical preparations, using either Whatman GF/B filtration or microcentrifugation for radioligand separation, is shown in figure 3.22. These displacement curves show that maximum [^{3}H] sulpiride binding was obtained with 10 µM S-(-)-sulpiride and confirm that the separation of free and bound ligand by GF/B filtration yielded more binding (63%) than microcentrifugation (33%).

ii) Saturability [^{3}H] sulpiride bound specifically to guinea pig renal cortical membranes and to Whatman GF/B filters as shown in figures 3.23 and 3.24, but not to microfuge tubes as shown in figure 3.25. Figure 3.23 represents the specific and non-specific constituents of binding in preparations where Whatman GF/B filters were used to separate free and bound ligand. This figure also shows that [^{3}H] sulpiride binding even at very high concentrations was not completely saturable. Blank assays performed in the absence of tissue revealed a similar amount of binding to the filters themselves. Figure 3.24 reveals the residual specific binding component after Whatman GF/B blank binding values had been subtracted from membrane binding values.

FIGURE 3.22 Displacement of [^{3}H] sulpiride. A comparison between Whatman GF/B filtration and microcentrifugation for the separation of free and bound ligand. Assays were performed using 20 nM [^{3}H] sulpiride and incubated for 10 minutes at 25°C. Each value represents the mean of three experiments, each assayed in triplicate. Whatman GF/B filters were washed with 10 ml ice-cold tris-HCl buffer pH 7.3 at 0°C, microfuge tubes were washed with 1 ml of the same buffer.


microfuge tubes
Whatman GF/B filters
100
0
[³H] sulpiride binding (%)
1
10
100
1000
10,000
[S-(-)-sulpiride] (nM)

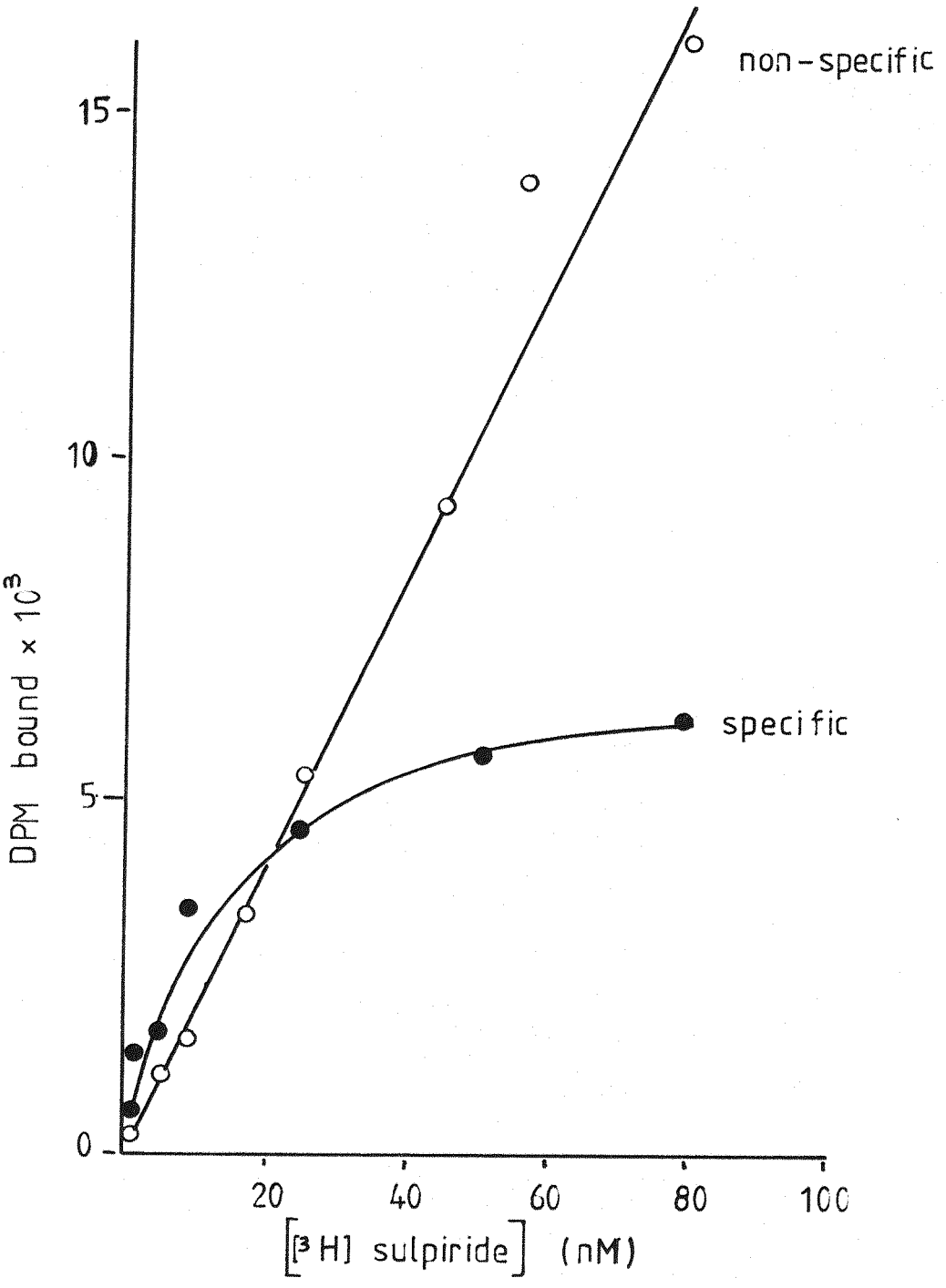


FIGURE 3.23 ○ Non-specific and ● specific binding of [^{3}H] sulpiride to guinea pig renal cortical membranes using Whatman GF/B filters for the separation of free and bound ligand. Membranes were incubated for 10 minutes at 25°C using 10 µM s-(-)-sulpiride to define specific binding. Each value represents the mean of 2 determinations assayed in triplicate.

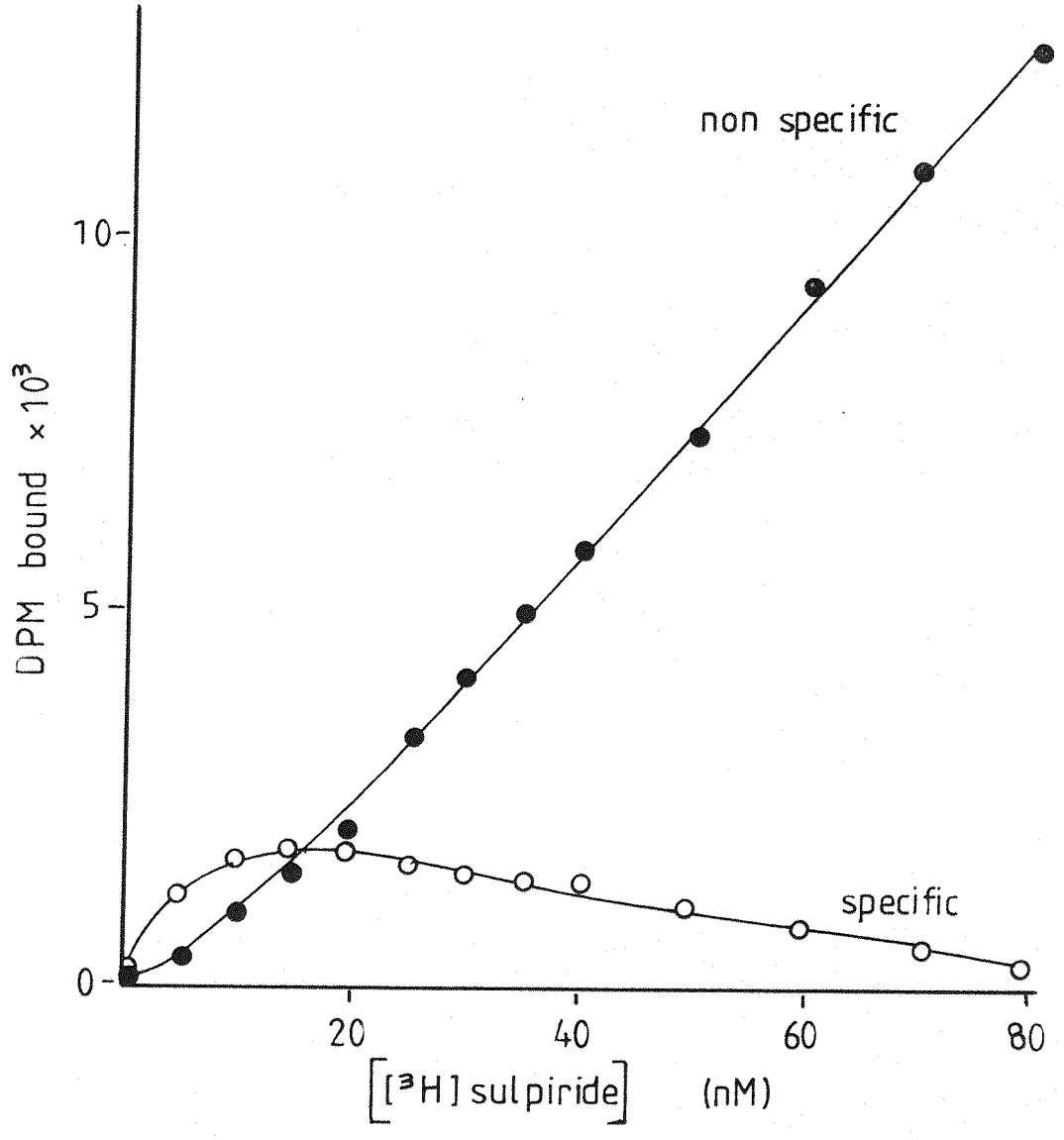


FIGURE 3.24 ○ Specific and ● non-specific binding of [^{3}H] sulpiride to guinea pig renal cortical preparations after the blank rate binding component for Whatman GF/B filters has been subtracted. Membranes were incubated for 10 minutes at 25°C using 10 µM s-(-)-sulpiride to define specific binding. In the case of blank tubes, membranes were substituted with an equal volume of tris-HCl buffer, pH 7.3 at 0°C.

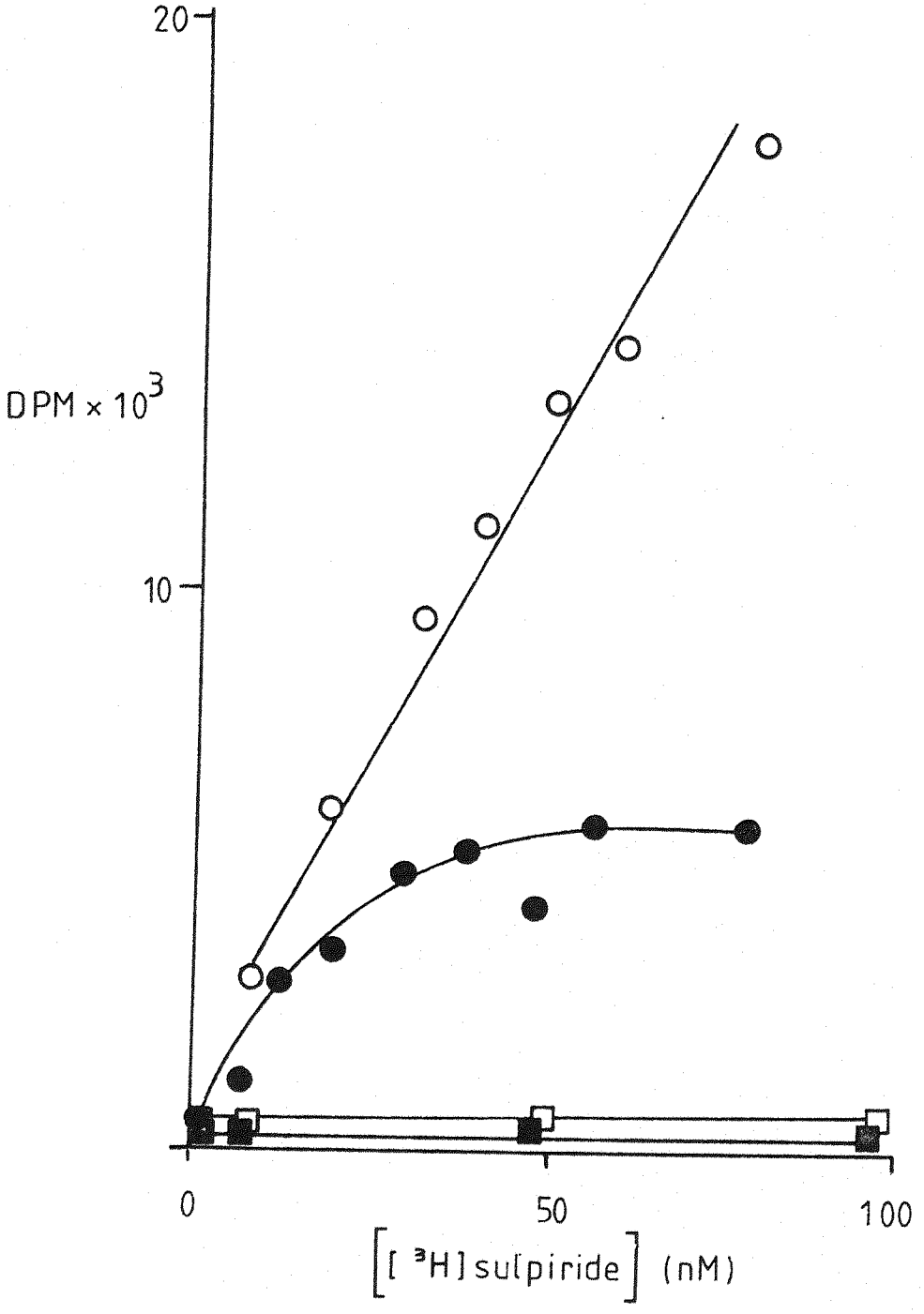


FIGURE 3.25 ○ Non-specific and ● specific binding of [^{3}H] sulpiride in guinea pig renal cortical membranes. □ Non-specific and ■ specific binding to microfuge tubes in the absence of tissue. Membranes were incubated for 10 minutes at 25°C using 10 µM s-(-)-sulpiride to define specific binding. Each value represents the mean of 2 determinations assayed in triplicate. In the case of blanks tubes, membranes were substituted with an equal volume of tris-HCl buffer, pH 7.3 at 0°C.

This component is equivalent to the proportion of binding to guinea pig renal cortical tissue and clearly demonstrates that specific binding to membranes was overwhelmingly masked by specific binding to filters.

In contrast figure 3.25 shows that binding performed in microfuge tubes was saturable in the ligand concentration range 1-50 nM. This figure also shows that [^{3}H] sulpiride binding to microfuge tubes was negligible in the ligand concentration range 1-100 nM. Scatchard analysis of the data derived from the microfuge studies (Figure 3.26) revealed a single binding component with an affinity constant (K_D) of 20.27 nM and a maximum binding capacity (Bmax) of 177 fmol/mg protein.

Lineweaver-Burk analysis of the same data (Figure 3.27) revealed a K_D of 27 nM and a Bmax of 113 fmol/mg. The differences in binding constant values derived for this system are probably due to inherent errors in the statistical methods used for evaluation. The Lineweaver-Burke plot is more liable to error than Scatchard analysis, since taking reciprocals leads to the smallest binding rate and radioligand concentration values becoming the largest values on the reciprocal plots and thus magnifies any errors in these points when fitting a straight line to the data.

Hill analysis of the data (Figure 3.28) revealed a linear plot with a Hill coefficient of 1.00. The Hill coefficient gives an indication of the presence of co-operativity in binding; in this study a Hill coefficient of unity suggests a lack of co-operativity in [^{3}H] sulpiride binding. Microcentrifugation was used to separate free ligand from bound in all subsequent experiments since binding to Whatman GF/B filters could not be eliminated.

d) Time course and protein concentration

Under the assay conditions described in Section 2-3, [^{3}H] sulpiride binding to guinea pig renal cortical preparations occurred too rapidly at 25°C to allow an accurate time course to be obtained. In order to reduce the rate of binding the experiment was repeated at 0°C and the results are shown in figures 3.29 and 3.30. Tissue specific [^{3}H] sulpiride binding under these conditions was rapid, reaching a maximal value at five minutes (Figure 3.29). The logarithmic plot in figure 3.30 shows that the half-life for the association of [^{3}H] sulpiride

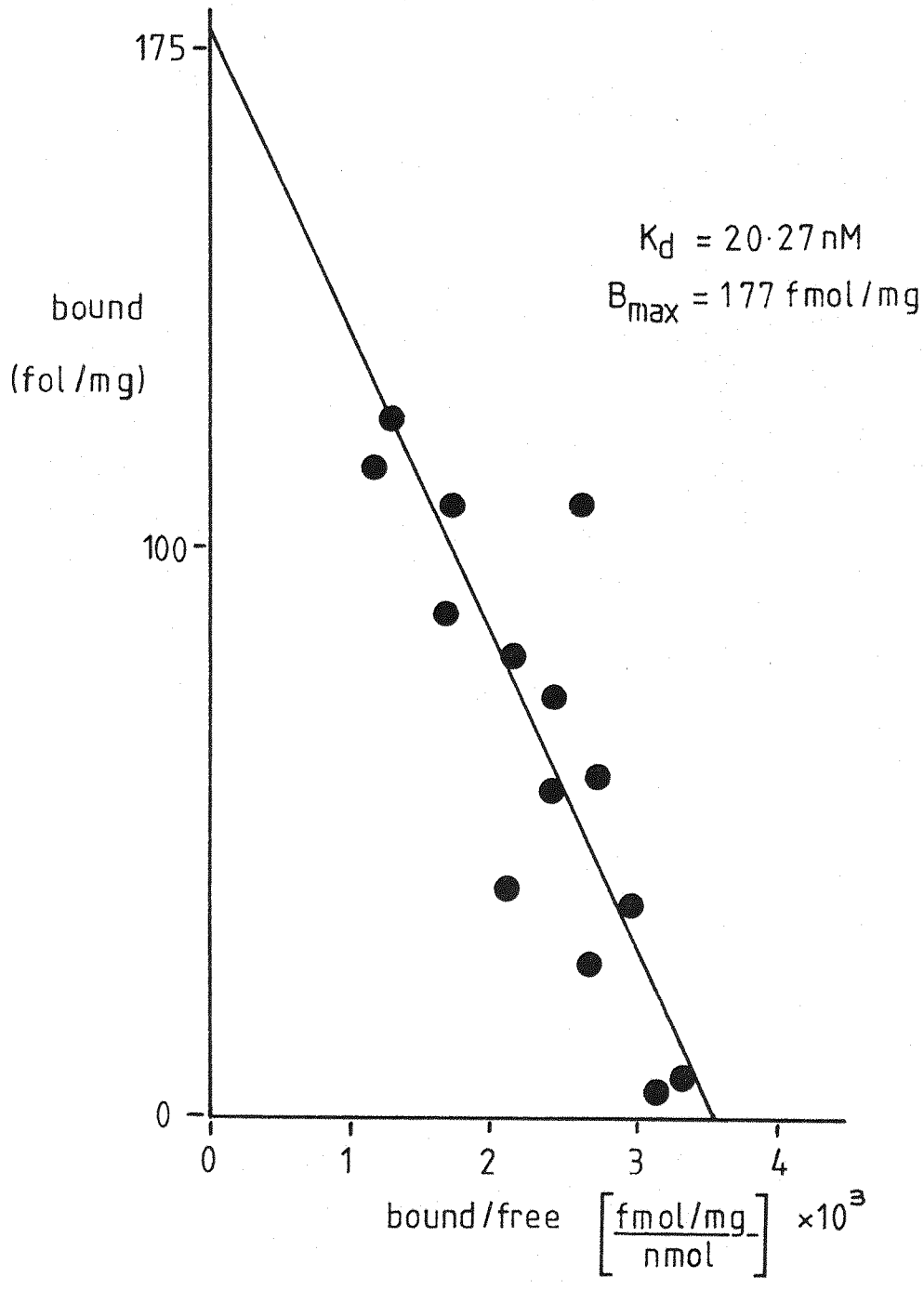


FIGURE 3.26 Scatchard analysis of specific binding of [^{3}H] sulpiride in guinea pig renal cortical membranes. The ordinate axis is expressed as bound radioligand (fmol/mg) and the abscissa as

$$\text{bound/free} \quad \left(\frac{\text{fmol/mg nM}^{-1}}{\text{nM}}\right).$$

The maximum binding capacity (Bmax) of the system is taken as the intercept of the ordinate axis and the affinity constant (K_d) evaluated from -1/gradient of the slope.

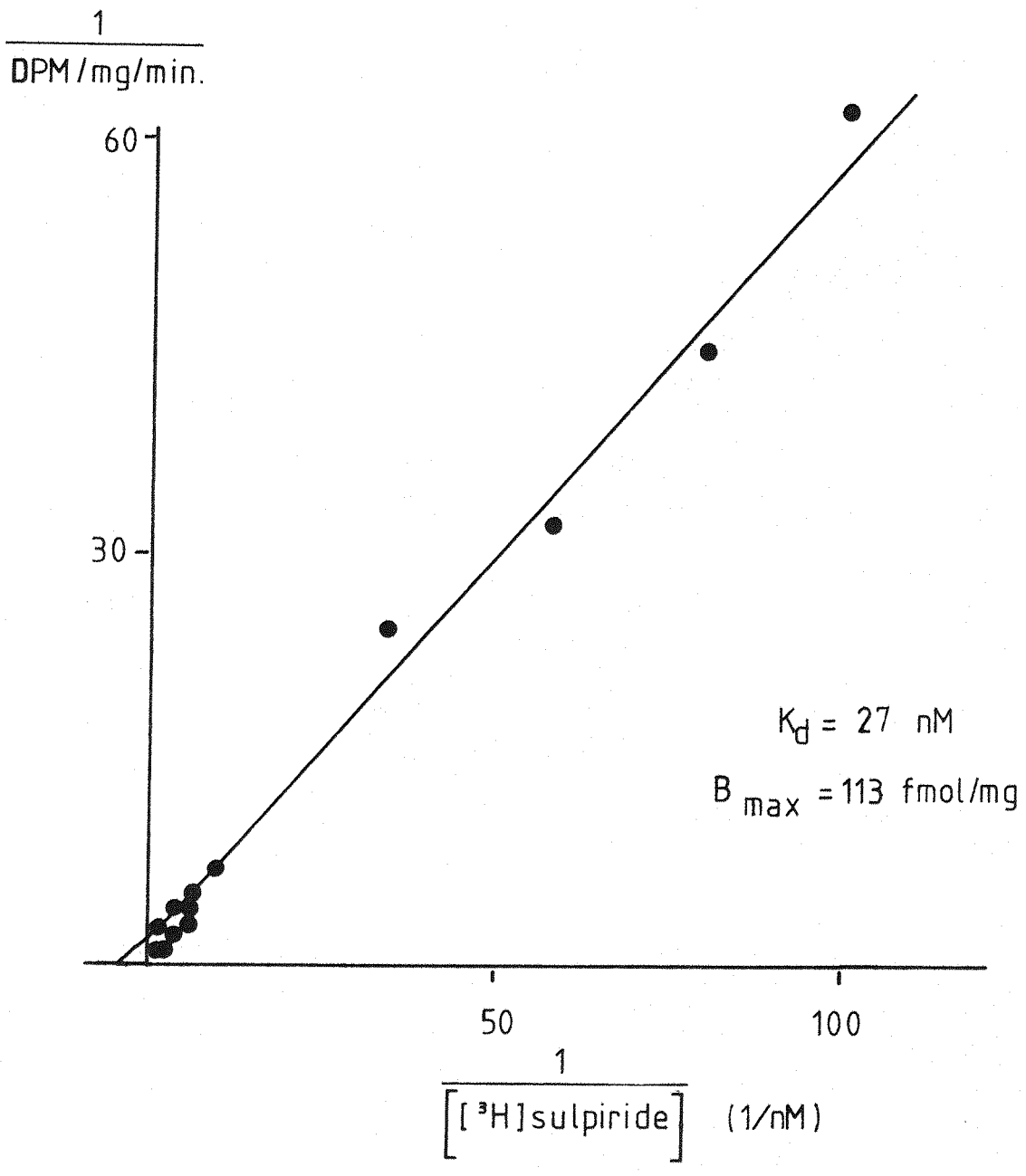


FIGURE 3.27 Lineweaver-Burk analysis of specific [^{3}H] sulpiride binding data. The ordinate axis is expressed as the reciprocal of the rate of radioligand bound/mg protein/minute and the ascissa as the reciprocal of radioligand concentration (1/nM). The maximum binding capacity of the system is derived from the reciprocal of the intercept on the ordinate axis and the affinity constant (K_d) evaluated from -1/intercept on the abscissa.

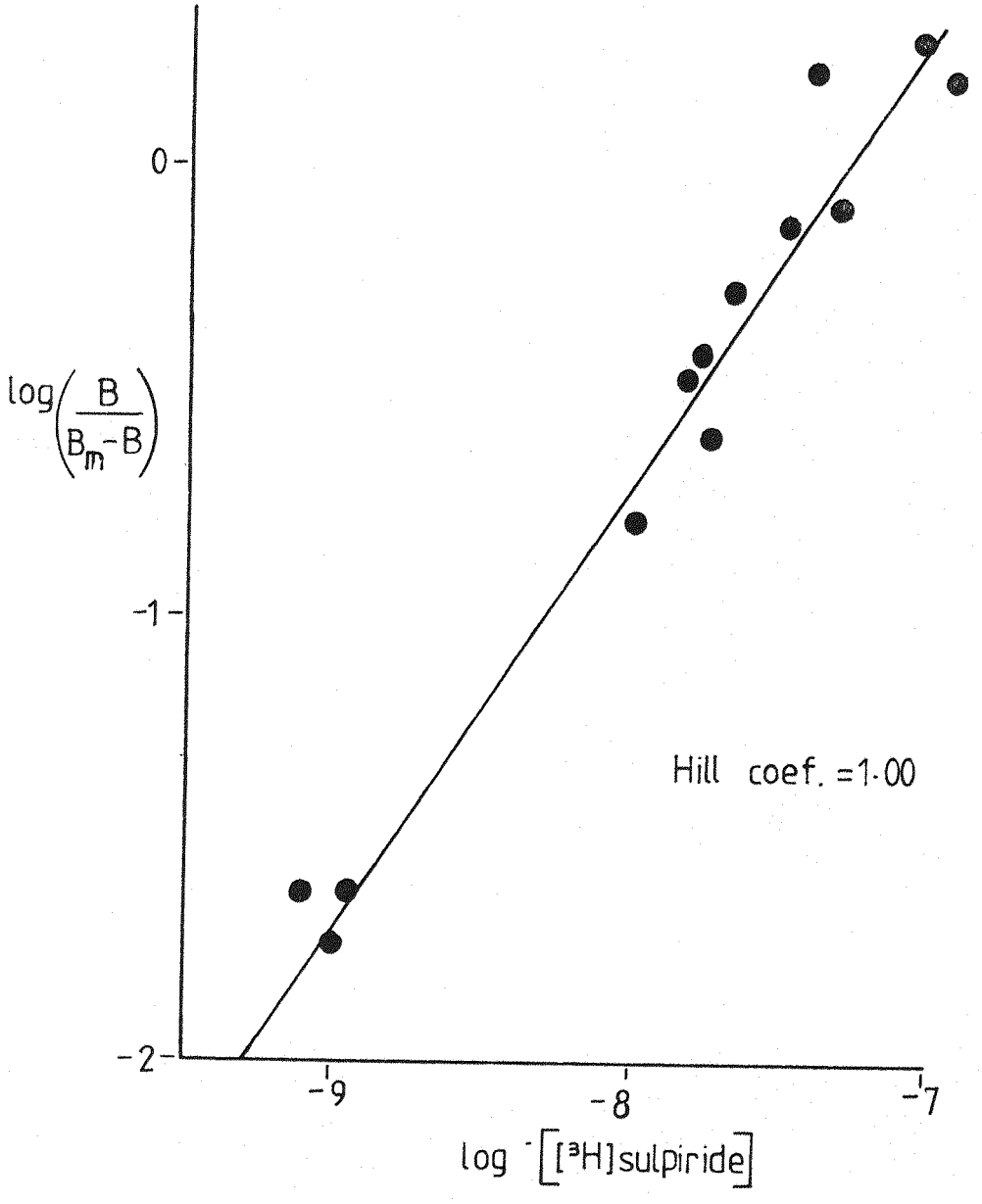
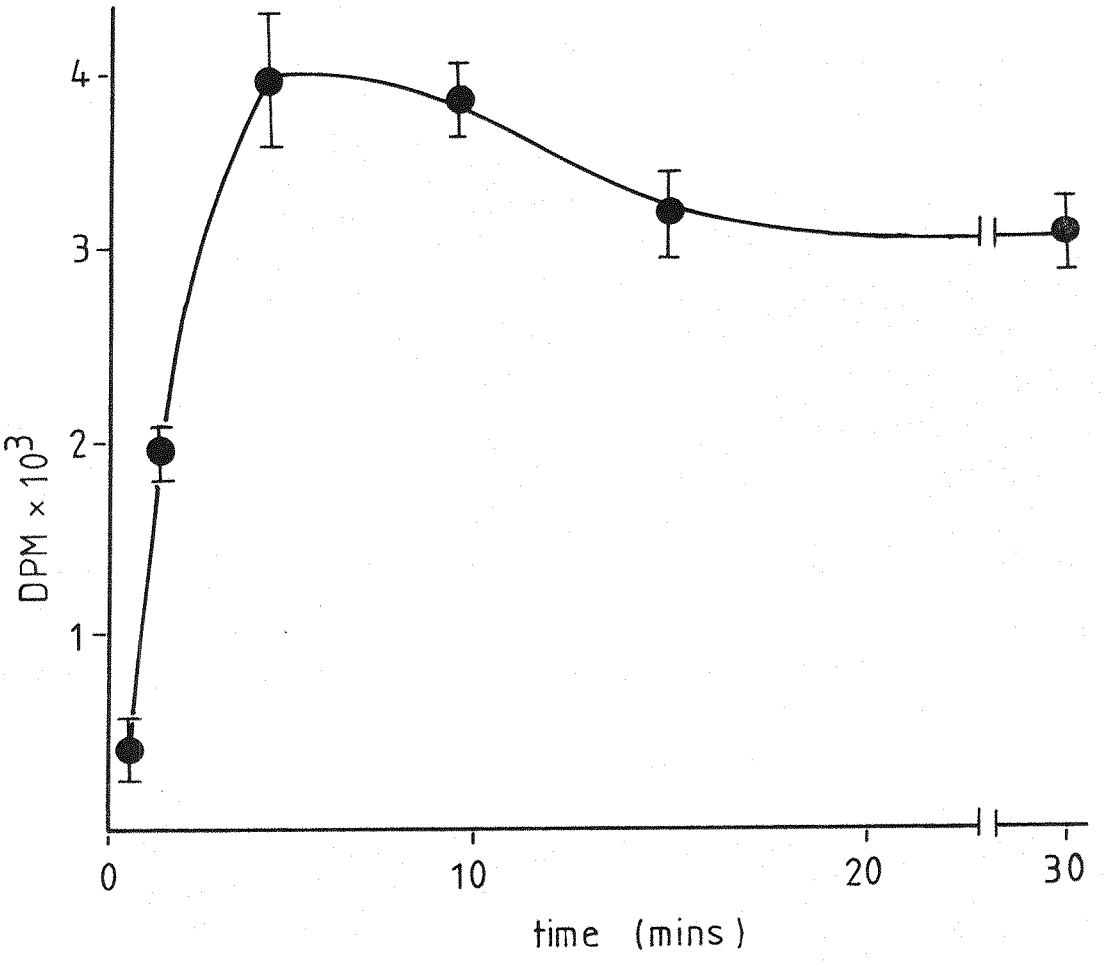


FIGURE 3.28 Hill analysis of [^{3}H] sulpiride binding to guinea pig renal cortical membranes. The graph was constructed by plotting log (B/Bmax - B) versus log [^{3}H] sulpiride concentration and the Hill coefficient was taken as the slope of the line. Bmax is the maximum binding capacity of the system (fmol/mg protein) and B is the radioligand bound (fmol/mg) at a given concentration of the radioligand.

FIGURE 3.29 Time course of [^{3}H] sulpiride binding to guinea pig renal cortical preparations. Assays were performed using 20 nM [^{3}H] sulpiride and 1 μM s-(-)-sulpiride to define maximum specific binding at 0°C. Each value represents the mean of two experiments, assayed in triplicate.

FIGURE 3.30 Logarithmic plot of time course data for [^{3}H] sulpiride binding.


4
3
2
1
DPM x 10^3
0
10
20
30
time (mins)

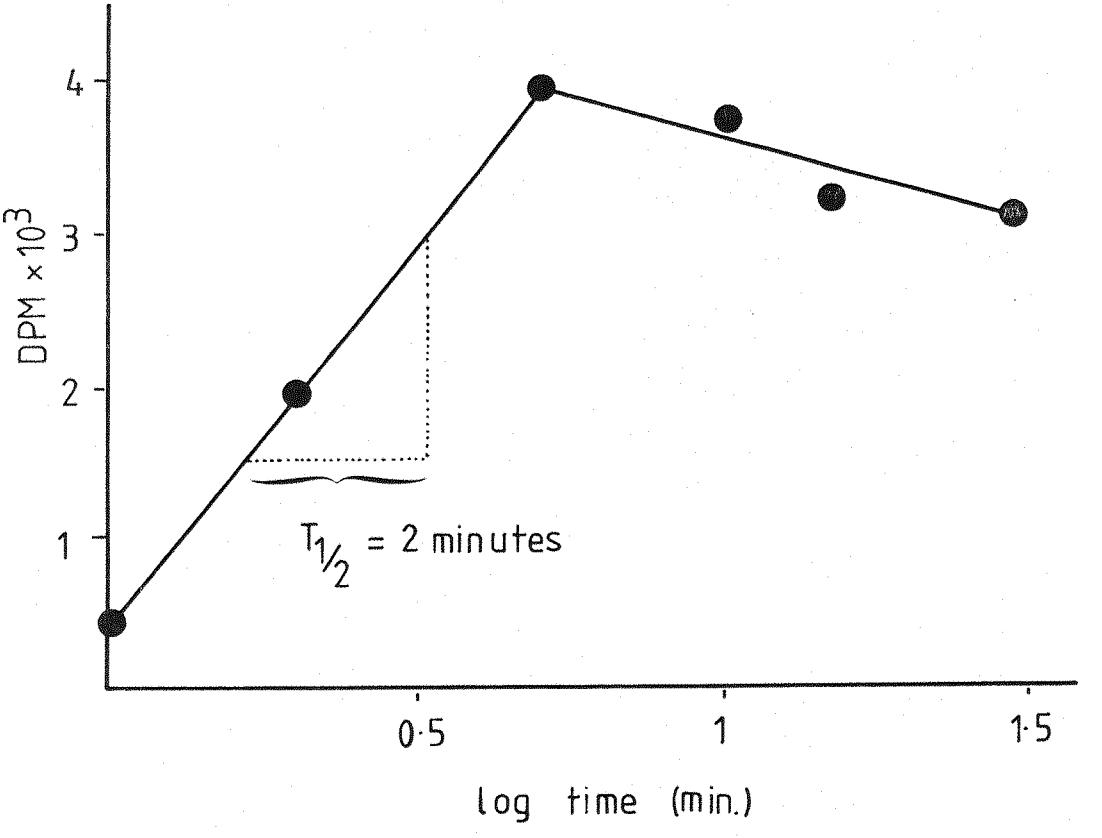

4
3
2
1
DPM x 10^3
T1/2 = 2 minutes
0·5
1
1·5
log time (min.)

TABLE 3.7

Tissue	Specific Binding fmol/mg protein
Kidney - cortex	129.8 ± 6.9
- medulla	47.6 ± 8.7
- pelvis	not detectable
Adrenal	31.9 ± 13.4
Liver	60.0 ± 5.2

$6 < n < 8$

Specific binding of [^{3}H] sulpiride to guinea pig membrane preparations from the above regions. Tissue preparations were incubated at 25°C with 20 nM [^{3}H] sulpiride for 10 minutes in a total volume of 1.2 ml containing 50 mM tris HCl buffer pH 7.3 at 0°C. Non-specific binding was determined in the presence of 10 µM s-(-)-sulpiride. Results are expressed as the mean ± SEM of two experiments assayed in quadruplet.

was approximately two minutes. Non-specific binding was independent of time. Furthermore, specific binding was proportional to protein concentration in the range used (0.75-1.0 mg/ml) whilst non-specific binding was independent of protein concentration.

3-3-2 Regional distribution of binding

The vasodilation produced by DA in the renal vascular bed appears to be mediated via interaction with a population of specific post-synaptic DA receptors (see Section 1-5-5). It has been suggested that in the dog, these receptors are associated principally with afferent and efferent juxtaglomerular arterioles in superficial cortical layers of the kidney (see Sections 3-4 and 3-2-4; Bell et al, 1978a; Dinerstein et al, 1979). Moreover, during DA infusion in dogs, some authors claim that intracortical blood flow is redistributed from inner to outer cortical areas of the cortex (Hardaker and Wechsler, 1973; see also Section 1-4-2). If [^{3}H] sulpiride binding sites are associated with specific DA receptors, then some correlation between specific [^{3}H] sulpiride binding, DA content and physiological function within the kidney might be anticipated. The distribution of [^{3}H] sulpiride binding in membranes prepared from guinea pig liver, adrenals and renal cortex, medulla and pelvis is shown in table 3.7. Binding was highest in renal cortical membranes and undetectable in those from pelvic tissue. [^{3}H] sulpiride binding to liver preparations was of the same order of magnitude as that for renal medullary preparations. Hepatic responses mediated by DA like receptors have been reported in canine hepatic portal and arterial vascular beds (Richardson and Withrington, 1978). Binding to adrenal preparations was low.

3-3-3 Effects of drugs on [^{3}H] sulpiride binding

An important criterion for assessing the specificity of [^{3}H] sulpiride as a ligand for DA receptors is the profile of drug displacement. If [^{3}H] sulpiride binding sites are really associated with specific DA receptors, then some correlation between the inhibition of binding by dopaminergic drugs and the potency of these drugs on other model systems might be anticipated. Furthermore, if sulpiride is truly labelling a D_2, non-adenylate-cyclase linked, receptor as suggested by Kebabian and Calne (1979) for the CNS, it might be expected that the neuroleptics of the thioxanthene group, cis- and

trans-flupenthixol, would only weakly displace [^{3}H] sulpiride binding in the kidney, whilst the substituted benzamides would potently displace binding.

Table 3.8 shows the rank order of potency of dopaminergic agonists and antagonists in displacing [^{3}H] sulpiride binding in guinea pig renal cortical preparations. IC_{50} values were determined from whole displacement curves; these values were in turn used to calculate the absolute concentration required to inhibit 50% of [^{3}H] sulpiride binding (K_i) according to the equation shown in table 3.8. Example displacement curves for the DA antagonist (+)-butaclamol and the DA agonist, apomorphine are shown in figure 3.31. The specific component of [^{3}H] sulpiride binding was reduced by 50% at 0.49 nM (+)-butaclamol and 33.9 nM apomorphine, which represent the IC_{50} values for these drugs.

The most potent inhibitors of [^{3}H] sulpiride binding were the neuroleptics, of which the classical neuroleptics, spiroperidol, fluphenazine, (+)-butaclamol and cis-(z)-flupenthixol were the most active. Marginally less potent displacers of binding were the ergot derivative bromocriptine, the butyrophenone haloperidol and the substituted benzamide (±)-sultopride. Next followed slightly weaker inhibitors of binding, the atypical neuroleptic S-(-)-sulpiride, the clinically inactive thioxanthene trans-(z)-flupenthixol, the phenothiazine chlorpromazine and the most active of the agonists, ADTN and apomorphine. This table also shows that agonist drugs were generally weaker than antagonists at displacing [^{3}H] sulpiride binding. Of the agonists 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) which is a rigid analogue of the β-rotamer of DA (see Sections 1-5-2 and 1-5-10) was the most potent. ADTN was approximately 50 times more potent than DA and marginally weaker than S-(-)-sulpiride. Apomorphine, a partial agonist in the canine vascular DA receptor model (Crumley et al, 1976), was 40 times more potent than DA and equipotent with the weak antipsychotic agent chlorpromazine. Bulbocapnine, a renal vascular DA receptor antagonist (Pendleton et al, 1975; Goldberg et al, 1979), was a weak displacer of binding. Bulbocapnine was less potent than ADTN and apomorphine but 1.6 times more potent than R-(+)-sulpiride and 7 times more potent than DA. The dopaminergic agonist 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine (SKF 38393) (Pendleton et al, 1978)

Drug	IC_{50} (nM)	Ki (nM)
fluphenazine	0.209	0.10
spiroperidol	0.37	0.18
(+)-butaclamol	0.49	0.24
cis-(z)-flupenthixol	0.905	0.48
bromocriptine	1.2	0.52
haloperidol	2.29	1.17
sultopride	3.4	1.77
trans-(z)-flupenthixol	14.8	8.71
S-(-)-sulpiride	21.38	9.96
ADTN	26.3	12.23
chlorpromazine	31.6	15.87
apomorphine	33.9	15.50
(+)-bulbocapnine	177.8	90.64
R-(+)-sulpiride	288.4	134.30
SKF 38393	1120	520.64
dopamine	1400	698.64
(±)-propranolol	1450	730.00
(-)-butaclamol prazosin isoprenaline phenylephrine nomifensine benztropine GABA 5HT (-)-noradrenaline atenolol histamine	> 10,000	

TABLE 3.8 Guinea pig renal cortical preparations were incubated with 20 nM [^{3}H] sulpiride for 10 minutes at 25°C using 10 µM S-(-)-supiride to define maximum specific binding. IC_{50} values were determined graphically as the concentration of drug producing 50% inhibition of specific [^{3}H] sulpiride binding. Ki values were calculated from the equation:-

$$Ki = \frac{IC_{50}}{(1+\frac{[ligand]}{Kd})}$$

All drugs were tested at between 5 and 8 concentrations, in triplicate in 2-4 experimental determinations. Abbreviations are GABA, γ-amino-butyric acid; 5HT, 5-hydroxytryptamine; ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine.

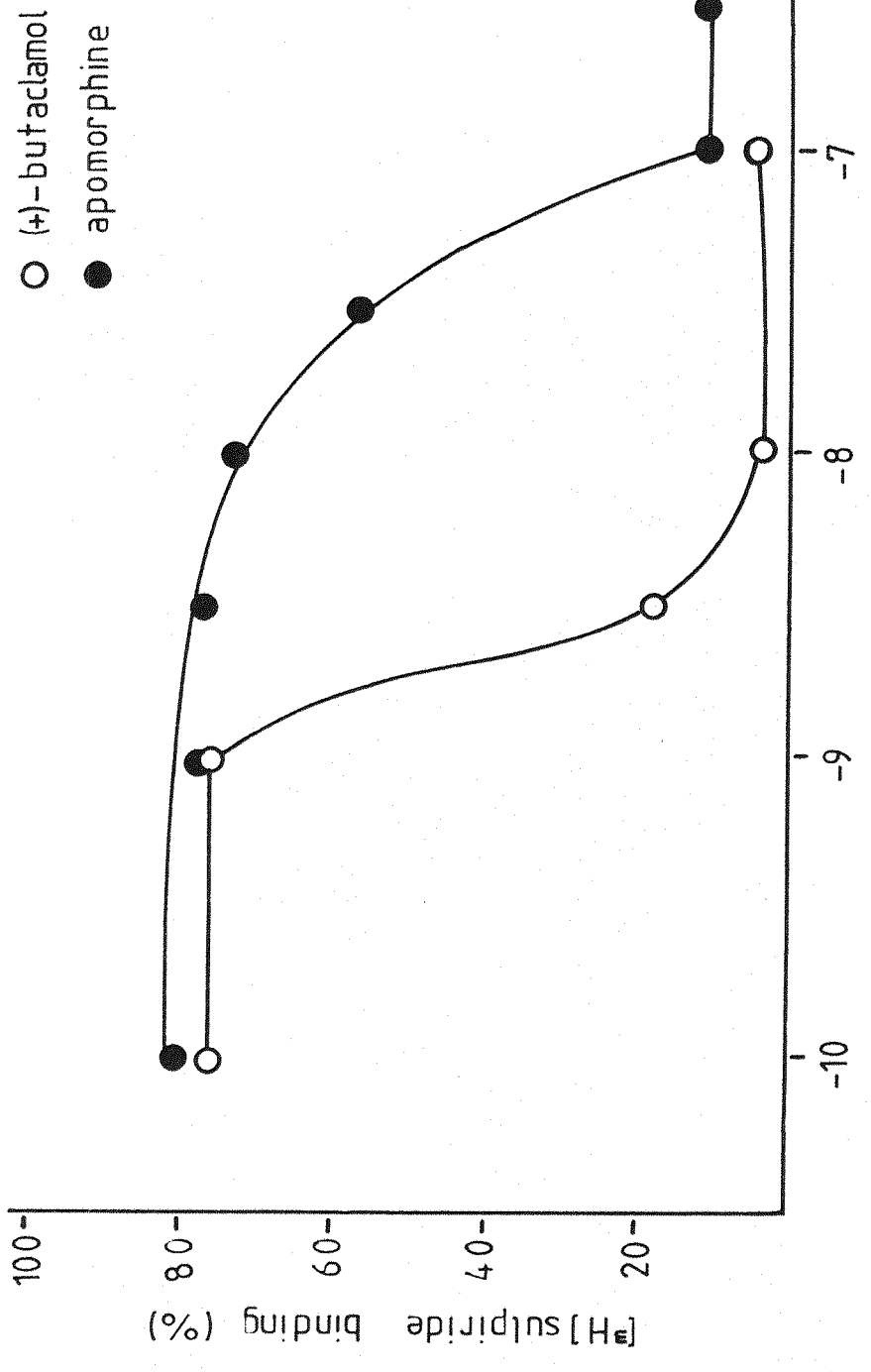


FIGURE 3.31 Displacement curves of [^{3}H] sulpiride binding by (+)-butaclamol and apomorphine. Assays were performed using 20 nM [^{3}H] sulpiride and 1 µM s-(-)-sulpiride to define maximum specific binding. Membranes were incubated for 10 minutes at 25°C. Each value represents the mean of 2 determinations assayed in quadruplet at each concentration.

was equipotent with DA, but only displaced 60% of [^{3}H] sulpiride binding.

[^{3}H] sulpiride binding exhibited distinct stereospecificity for neuroleptics; in particular, of the two enantiomers of sulpiride S-(-)-sulpiride was 13 times more powerful at displacing binding than R-(+)-sulpiride. (+)-Butaclamol, an effective antischizophrenic agent was 10,000 times more potent than its clinically inactive stereoisomer (-)-butaclamol. Similarly, the cis- and trans-geometric isomers of the potent neuroleptic flupenthixol, differ in their potencies for competing for [^{3}H] sulpiride binding by a factor of 16.

The Hill coefficients for agonist inhibition of [^{3}H] sulpiride binding were less than unity compared with values for antagonists which generally approximated to unity, as shown in table 3.9. The values for DA and ADTN were 0.7 and 0.44 respectively, whereas those for fluphenazine (1.09), cis-(z)-flupenthixol (0.92), (+)-butaclamol (0.91) and s-(-)-sulpiride (0.77) were closer to 1.0. The Hill coefficients for the butyrophenone haloperidol, the phenothiazine chlorpromazine and for (+)-bulbocapnine were low in comparison with other neuroleptics. Apart from these three discrepancies, similar Hill coefficients for dopaminergic agonists and antagonists have been reported for [^{3}H] spiroperidol binding (Zahniser and Molinoff, 1978; Creese et al, 1979a; Watling and Iversen, 1981) and for [^{3}H] sulpiride binding (Freedman, Poat and Woodruff, 1981b) in striatal preparations. Antagonist binding appears to be at a single class of sites, whereas agonist binding may be more complex. Zahniser and Molinoff suggested that these low Hill coefficient values for agonists might indicate interaction with multiple binding sites or negative co-operativity between individual receptor sites.

In order to substantiate the claim that [^{3}H] sulpiride was associated exclusively with dopaminergic sites, a variety of non-dopaminergic drugs and neurotransmitters were evaluated for their ability to displace binding. The transmitters noradrenaline (NA) 5-hydroxytryptamine (5HT), γ-amino-butyric acid (GABA) and histamine were inactive. Similarly the catecholamine uptake inhibitors, nomifensine and benztropine did not displace binding at concentrations as high as 10 μM. This lack of activity inferred that [^{3}H] sulpiride did not bind to other neurotransmitter receptor sites or to catecholamine

Agonists	Hill coefficient
Dopamine	0.70
ADTN	0.44
Apomorphine	0.63
SKF 38393	N.L.

Antagonists	Hill coefficient
Fluphenazine	1.09
Cis-(z)-flupenthixol	0.92
Trans-(z)-flupenthixol	1.04
Sultopride	0.88
S-(-)-sulpiride	0.77
R-(+)-sulpiride	0.74
Haloperidol	0.51
Chlorpromazine	0.50
Spiroperidol	0.82
(+)-Bulbocapnine	0.40
(+)-Butaclamol	0.91
Bromocriptine	1.21

TABLE 3.9 Hill coefficients for agonist and antagonist inhibition of [^{3}H] sulpiride binding. The displacement curves from experimental data in Table 3.8 were analysed graphically according to the Hill equation. The ordinate axis was log (B/Bmax - B) and the abscissa log [S].

Hill coefficients were determined from the slope of linear plots. Bmax (fmol/mg) is the maximum binding capacity of the system as determined from Scatchard analysis , B is radioligand bound (fmol/mg) at a given concentration of S, the drug concentration.

Abbreviations: ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene); SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine; NL (Not linear).

uptake sites. The α- and β-adrenergic agents phenylephrine, prazosin, isoprenaline and atenolol had no capacity to interfere with binding. Surprisingly the mixed β-adrenergic antagonist propranolol weakly displaced binding, but only when present at high concentrations. This effect is probably attributable to propranolol's membrane stabilising actions (Scriabine, 1979) rather than any affinity which sulpiride might have for β-receptors since another β-antagonist, atenolol, which lacks propranolol's local anaesthetic action (Saelens et al, 1977) was completely ineffective on binding.

In summary, the results demonstrated that [^{3}H] sulpiride binds to guinea pig renal cortical membranes in a specific saturable manner. The distribution of binding sites is consistent with the distribution of DA in the kidney (see also Section 3-4) as binding was greatest in the renal cortex. Furthermore, binding appears to be associated with dopaminergic sites since DA and its agonists ADTN and apomorphine inhibited [^{3}H] sulpiride binding in contrast to the inactivity of noradrenaline, histamine, 5-hydroxytryptamine and γ-amino-butyric acid. The DA antagonists were generally more potent in displacing [^{3}H] sulpiride binding than agonists, of which the classical neuroleptics were the most potent of all. The results presented also show that the inhibition of [^{3}H] sulpiride binding exhibited stereospecificity for (+)-butaclamol, cis-(z)-flupenthixol and S-(-)-sulpiride. Cis- and trans-flupenthixol powerfully displaced binding and although the substituted benzamides were active displacers, they did not appear to belong to any particular category. These results do not support the theory that sulpiride is labelling a non-adenylate cyclase-linked DA receptor, but are consistent with results obtained from other dopaminergic systems.

	ENDOGENOUS DA CONTENT			RENAL VASODILATATION		DA-SENSITIVE ADENYLATE CYCLASE		[^{3}H] SULPIRIDE BINDING	
				%increase RBF produced by 53 nmol/kg DA		% increase cAMP content			
	(ng/g wet wt)			(ml/100g/min)		(pmol cAMP/mg protein)		(fmol/mg protein)	
	mean	SE	(n)	mean	SE	mean	SE	mean	SE
CORTEX	137.7	± 38.5	(4)	39.7	± 3.9	116	± 12	130	± 6.9
MEDULLA	125.6	± 9	(4)	10.1	± 2.1	34	± 12.4	48	± 8.7
PELVIS	39.4	± 21	(4)	0		0		0	

TABLE 3.10 Comparison of endogenous dopamine content in various regions of the guinea pig kidney with regional vascular responses to dopamine, distribution of dopamine-sensitive adenylate cyclase and [^{3}H] sulpiride binding.

3-4 Regional distribution of dopamine in the guinea pig kidney

3-4-1

DA has been implicated as a peripheral neurotransmitter involved in the regulation of RBF. DA containing nerves have been detected by fluorescence histochemistry in the canine renal cortex (Bell et al, 1978a; Dinerstein et al, 1979). The level of DA found in cat, rabbit and dog renal cortices, relative to that of NA, was 5, 7.4 and 24% respectively (Bell et al, 1978a; Hattori et al, 1981; Bell and Gillespie, 1981). This is several times greater than the level which could be attributed to its role as a precursor in noradrenergic nerves alone. Halushka and Hoffmann (1966) investigated the distribution of [2-^{14}C] DA in guinea pigs and found that the kidney contained higher amounts of DA than all other tissues examined, despite its failure to take up NA.

In theguinea pig kidney, RBF responses to DA were greater in the cortex than in the medulla (see Table 3.10). Similarly, DA-sensitive cAMP accumulation and [^{3}H] sulpiride binding was greater in renal cortical preparations than in medullary preparations. These results support the suggestion that DA receptors are located predominantly in the renal cortex. Bell and Gillespie (1981) measured separately the DA/NA ratios in outer and inner halves of the renal cortex and in renal medulla in cats. In the medulla they found this ratio to be representative of typically noradrenergically innervated tissues. However, in the inner half of the cortex the DA/NA ratio was greater than the medulla and the ratio of DA in the outer cortex was almost twice that of the medulla. In order to see whether regional DA content in the guinea pig kidney paralleled [^{3}H] sulpiride binding, RBF and renal adenylate cyclase responses, the levels of DA in the renal cortex, medulla and pelvis were measured.

DA was detectable in all three regions examined, the absolute levels are listed in table 3.10. The relative levels of DA in the cortex: medulla ranged from 75% to 237% with a mean of 108%. This ratio was of a similar magnitude to those reported in the cat kidney which was 105% (Bell and Gillespie, 1981). It was not possible to measure DA content in the outer and inner cortical regions because the small size of the guinea pig kidney made it difficult to

accurately distinguish between the two. The small amount of DA detectable in the pelvis was probably representative of that excreted by the kidney.

Although the rate of DA excretion has not been measured in the guinea pig, Akpaffiong et al (1980) in their recent study of factors affecting release and excretion of DA in the rat, reported a value of 0.97 nmol/ml DA in urine collected over a 24 hour period.

CHAPTER 4

DISCUSSION

There is much evidence to suggest that endogenous DA may play a physiological role in the kidney as a neurotransmitter involved in the regulation of renal function and particularly of renal blood flow. The present study has examined the feasibility of using a small animal renal vasodilatation model (the guinea pig) for DA receptors.

The renal blood flow results obtained in the rat and guinea pig provide evidence that the hydrogen washout technique is a reliable method for the measurement of local blood flow. Flow values in the rat were 455 ± 16 ml/100 g/min; these are very similar to values reported by other workers using the same technique (Haining and Turner, 1966; Robertson, Ph.D. thesis, 1981) and are comparable with values obtained using ^{86}Rb uptake (Mercer and Zusko, 1974) and ^{141}Ce and ^{85}Sr labelled microspheres (Kallskog et al, 1975). Further evidence that hydrogen clearance accurately measures RBF was provided by the proportional increases and decreases of flow values recorded during renal vasodilatation and vasoconstriction respectively. These changes were similar to the findings of Aukland et al (1973), Aukland and Wolgast (1968) and Clausen et al (1977; 1978) in the dog. Flow values in the guinea pig under conditions of reduced systemic blood pressure produced by α-adrenoceptor blockade, fell from (ml/100g/min) 212 ± 15.3 to 118 ± 6.5 following prazosin treatment and to 114.9 ± 4.8 following phenoxybenzamine treatment. In contrast flow values rose from 114 ± 8.2 ml/100 g/min to 147 ± 12.5 ml/100 g/min during renal vasodilatation produced by DA. Furthermore, in their extensive studies of the credibility of this technique Løyning (1971; 1974), Aukland (1980a; b) and Clausen et al (1980) concluded that hydrogen washout is the most suitable method available for the measurement of local flows in the renal cortex.

The recording of hydrogen gas by implanted platinum electrodes as developed by Aukland et al (1964) has several advantages: 1) practically unlimited reproducibility of simultaneous measurements at several locations in the kidney, 2) the renal nerves are not disturbed during dissection procedures, 3) many serial measurements can be taken in a short period. The disadvantage of this system is that blood flow through the inner medulla cannot be measured because counter-current exchange mechanisms located in this region cause recirculation of the gas, resulting in an overestimation of hydrogen clearance time. This

problem, however, is not applicable to the present study since blood flow was measured in the outer renal cortex, a region of low gas recirculation. Furthermore, hydrogen desaturation curves were mono-exponential down to at least 10%. This observation suggests that most of the hydrogen was eliminated during the first circulation. The most obvious concern of this study was the possibility of flow disturbance due to the insertion of polarography electrodes into renal tissue. It has been suggested that such tissue trauma might explain why deep cortical flow measured with inert diffusible indicators such as hydrogen is lower than that obtained with microspheres during vaso-dilatation, in rats and dogs (Clausen et al, 1979). This explanation is unlikely, however, since local blood flow around the electrode tip is good (Løyning, 1974; Tyssebotn and Kirkebo, 1979) and blood flow distribution patterns obtained with [^{125}I] iodoantipyrene and tritiated water uptake recorded from excised dog kidneys are practically identical to those obtained with local hydrogen (Clausen et al, 1979; 1980; Aukland, 1976) and ^{85}Kr washout (Passmore et al, 1977). In a recent review Aukland (1980a) concluded that the failure of local hydrogen measurements to confirm the finding of an increased deep cortical flow fraction during vasodilatation was more likely due to maldistribution of microspheres.

In general the main problem with the evaluation of local blood flow is the absence of a well established reference method, hence the need to rely on indirect criteria for assessment of the suitability of a technique. Obviously non-invasive techniques would provide a better solution for this difficulty. Two recent techniques for measuring total RBF involve the detection of ultrasonic Doppler signals from the renal artery (Watanabe et al, 1976) and the external recording of the ^{81}Rb/^{81m}Kr ratio after intravenous injection of ^{81}Rb (van Herk and de Zeeuw, 1978). This latter method is based upon the principle that ^{81}Rb, taken up by the kidney, disintegrates rapidly ($T_{\frac{1}{2}}$ 4.6 hours) to form ^{81m}Kr gas, which is in turn removed at a rate dependent on blood flow.

The present study demonstrates that there are specific populations of α- and β-adrenoceptors and DA receptors located in the peripheral vasculature of the guinea pig. Studies with selective α- and β-adrenoceptor blocking agents and neuroleptics confirmed the finding made in other species that the DA molecule is highly specific for DA receptors

but may interact with α- and β-adrenoceptors at high concentrations (Goldberg et al, 1978b). The results, however, show that the guinea pig differs from the canine blood flow model pioneered by Goldberg and colleagues (1968; 1978a; b) and the rat (Aihara et al, 1972; Augustin et al, 1977; Chapman et al, 1980) for the study of peripheral DA receptors. In the guinea pig DA, at low concentrations, will cause renal vasodilatation in the absence of α-adrenoceptor blockade; although at higher concentrations of DA, α-activity predominates and vasoconstriction results. In the dog and rat kidney it is necessary to block α-adrenoceptors in order to see the vasodilator effect of DA. The reason for such a species difference is unclear but might be explained in terms of differences in regional DA receptor distribution within the cardiovascular system. For example, systemic administration of DA into the guinea pig causes a vasodepressor response (Holtz and Credner, 1942; Hornykiewicz, 1958; Woodruff and Sumners, 1979). In contrast DA causes a pressor response in the dog (Eble, 1964; McNay and Goldberg, 1966; Goldberg, 1972) and rat (Chapman et al, 1980).

This blood pressure change represents the integrated cardiovascular response to large doses of DA. It is the result of a complex balance between the vasodilatation resulting from the actions of DA on its own specific receptors and β-adrenoceptors, and the vasoconstriction resulting from its actions on postsynaptic α-adrenoceptors. These vascular effects are further complicated by DA's ability to stimulate presynaptic α-adrenoceptors and DA receptors on sympathetic nerve terminals and its mixed adrenergic cardiac effects. Since the vasodepressor effect produced by DA in guinea pigs is unaffected by α- and β-adrenoceptor blocking agents (Woodruff and Sumners, 1979) the actions of DA in this animal are possibly mediated by an action on specific DA receptors. This is similar to the effect in man but contrasts with the actions of DA in the dog (Goldberg, 1972; McNay et al, 1965; Bell and Mya, 1977; Bell et al, 1974) and rat (Aihara et al, 1972; Neuvonnen and Westermann, 1973; Augustin et al, 1977; Chapman et al, 1980) where DA has a predominantly pressor effect, mediated by activation of α-adrenoceptors.

The possibility that methodological differences might account for the contrasting renal vascular responses to DA detected in the dog and guinea pig cannot be discounted. In the present study guinea pigs were anaesthetised with urethane whereas in studies involving the rat

(Chapman et al, 1980) and dog (Goldberg et al, 1968) anaesthesia was induced with pentobarbitone. This explanation is unlikely, however, since in this study rats and guinea pigs were anaesthetised with the same agent (urethane), yet infusions of DA (200 nmol/kg/min) persistently produced renal vasodilatation in guinea pigs in contrast to renal vasoconstriction in rats (unreported data).

An alternative explanation for these differences may by due to the methods employed for the measurement of RBF. In the dog an electromagnetic flow probe placed around the renal artery is used to continuously monitor total blood flow through the kidney (McNay et al, 1965). Whereas in this preparation blood flow was monitored in a highly localised area in the renal cortex. As already mentioned, in studies using microsphere distribution to measure RBF, it has been suggested that vasodilator substances like DA may cause intrarenal redistribution of blood flow from inner to outer cortical areas (Mimram and Casellas, 1979), whilst total flow through the kidney remains unchanged. If DA caused such a localised vasodilatation, the electromagnetic method might not detect this regional increase of RBF whereas the hydrogen clearance technique is sensitive to regional variations of blood flow and would therefore be able to detect a cortical vasodilatation.

Such a possibility is unlikely, however, since extensive investigations have shown that H_2 clearance in the outer medullary and various cortical layers of the kidney varies directly in proportion with total RBF (Løyning, 1971; 1974; Aukland, 1976), also there is no evidence for zonal redistribution of blood flow in virtually all situations examined. Furthermore, in their recent study of the effects of DA in the rat, Chapman et al (1980) employed a practically identical methodology to that of the present study for the measurement of RBF, yet low doses of DA produced renal vasoconstriction similar to that reported in the dog. It is therefore concluded that the renal vasodilatation produced by low doses of DA in the guinea pig, in the absence of α-blockade is truly a species specific phenomenon. Hence, it appears that the guinea pig kidney is a particularly useful preparation on which to evaluate the activity of DA receptor agonists and antagonists, because it lacks the dominant α-adrenergic vasoconstrictor mechanism which masks specific vasodilator responses to DA in other species.

A further difference between the present study and those of Goldberg and colleagues (1978a; b) is the use of prazosin, a highly selective postsynaptic α_1-adrenoceptor antagonist (Cambridge et al, 1977; Davey, 1980). α-adrenoceptors have been classified as two pharmacological subtypes designated α_1 (postsynaptic) and α_2 (predominantly presynaptic). In the experimental protocol of Goldberg et al (1978a) phenoxybenzamine, an agent which preferentially blocks postsynaptic α_1-adrenoceptors at low doses, but can block both α_1- and α_2-adrenoceptors at high concentrations (Dubocovich and Langer, 1974), is used as the α-antagonist. Similarly, the increased RBF responses produced by high doses of DA in the present study are most likely mediated at postsynaptic DA receptors, since responses to DA were practically identical in the presence of either of the above α-blocking agents. Thus, animals pretreated with prazosin to block postsynaptic α_1-adrenoceptors, in doses which eliminate responses to a test dose of phenylephrine, still respond with an increase of RBF. The duel advantages of using prazosin are that it 1) lacks phenoxybenzamine's ability to interact non-specifically with biological membranes and 2) lacks a negative feedback inhibitory effect on presynaptic α_2-adrenoceptors and thus does not cause an indirect release of noradrenaline.

Lokhandwala and Jandhyala (1979) in their study of the cardiovascular effects of DA concluded that the vasodilator actions of DA may partly involve the inhibition of NA release from sympathetic nerve endings in the renal vasculature by activation of presynaptic DA receptors. Thus, DA can only produce a presynaptically initiated vasodilatation in the presence of sympathetic tone. In the urethane anaesthetised guinea pig sympathetic tone is extremely low (Woodruff and Sumners, discussion remark, 1979) hence the hypotensive effect of dopaminergic agents is unlikely to be mediated by activation of presynaptic receptors (either presynaptic α-adrenoceptors or DA receptors). In the present study sympathetic tone was reduced further by the administration of prazosin, a factor reflected by the low MABP in most animals (26-40 mmHg). It is unlikely therefore that the 30-40% increase of RBF (and decrease in vascular resistance) produced by DA in the α-blocked, urethane anaesthetised, guinea pig is due to passive withdrawal of the extremely diminished sympathetic tone by activation of presynaptic DA receptors. Furthermore, it is unlikely

that the renal vasodilatation is due to the effects of DA on the heart since vasodilator agents were administered directly into the kidney and RBF responses measured immediately.

The results show that increases of RBF produced by isoprenaline are approximately equal to those of DA. This observation suggests that in the guinea pig, renal vasodilatation is mediated equally by β-adrenergic and dopaminergic mechanisms. In this respect the guinea pig differs from the dog, where renal vasodilatation appears to be mediated predominantly by DA receptors (McNay and Goldberg, 1966; Bell, 1979) and from the rat, where renal vasodilatation seems to be mediated predominantly by β-adrenoceptors (Bell, 1979; Chapman et al, 1980). The renal vasodilatation produced by DA in this study is not due to the activation of β-adrenoceptors since (±)-propranolol (1.2 μmol/kg/hr) specifically abolished responses to isoprenaline but did not affect responses to DA. This dose provided a very effective blockade against the test dose of isoprenaline (53 nmol/kg) and it therefore seems reasonable to infer that the propranolol would also block the β-adrenergic activity of an equal dose of DA. Isoprenaline stimulates two types of β-adrenoceptors, β_1 is associated with heart rate and adipose tissue and β_2 with peripheral resistance. The variation in peripheral blood flow reflects vasodilatation and cardiac factors such as heart rate and contractility. Therefore systemic administration of isoprenaline would induce 1) tachycardia, as a direct result of stimulation of β_1-cardiac adrenoceptors and 2) a β_2-mediated peripheral vasodilatation which, in turn, causes an indirect reflex tachycardia. The vasodilatation produced by isoprenaline in this study is most likely mediated by β_2-postsynaptic vascular receptors since as already mentioned, RBF responses were measured directly as vasodilator agents were administered into the renal artery.

Interestingly, the major differences between vascular DA receptors such as those reported in this study and CNS DA receptors as identified by radioligand binding studies are in the structural-activity criteria. The vascular DA receptor has highly specific requirements; two hydroxyl groups located at positions 3 and 4 on the benzene nucleus of the DA molecule are compulsory for activation (Goldberg et al, 1968; 1978a), whereas monohydroxylated analogues of DA are able to displace

[^{3}H] neuroleptics from dopaminergic sites in the CNS (Tedesco et al, 1979; also see Section 1-5-10e). Both models, however, are in agreement that the active conformation of DA at postsynaptic receptor sites is the β-trans rotamer (Volkman et al, 1977; Woodruff et al, 1979) which is contained in the molecular structure of ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) whereas isoADTN (2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene) is an analogue of the α-trans rotamer of DA and exhibits β_2-adrenoceptor activity but has low activity as a DA agonist.

The results show that ADTN and SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine) are very active dopaminergic agonists. These agonists produced a propranolol-resistant increase of RBF similar to that obtained with DA. SKF 38393 (see Figure 4.1) is a potential DA receptor agonist which potently stimulates the striatal DA-sensitive adenylate cyclase (Setler et al, 1978) and produces renal vasodilatation in the dog (Pendleton et al, 1978). The slope of the dose response curves for RBF responses to DA and ADTN were practically identical and both agents produced the same maximum response. This finding is in agreement with observations in the CNS (Woodruff et al, 1979) and dog kidney (Crumley et al, 1976) where ADTN is an extremely potent DA receptor agonist. SKF 38393 had a threshold dose approximately 10 times lower than that of DA but became equipotent with the latter at higher doses. These observations are in agreement with those of Pendleton et al (1978) who found SKF 38393 to be a full agonist of postsynaptic DA receptors in the dog. In addition to its vasodilator activities, SKF 38393 powerfully enhanced urine and electrolyte (sodium, chloride and potassium) excretion and these effects were specifically inhibited by the dopaminergic antagonist bulbocapnine. Recently in their study of RBF in the non α-blocked dog Hahn and Wardell (1980) reported that SKF 38393 and DA have slightly different pharmacological profiles. They found the substituted benzazepine to possess sub-maximal renal vasodilator activity relative to DA itself and suggested that this partial agonist behaviour might be due to an exclusive postsynaptic action of SKF 38393. DA, however, might achieve greater vasodilatation by aiming at two targets, namely pre- and postsynaptic DA receptors. This latter observation provides further evidence that vasodilator responses to DA in the present study were mediated purely by postsynaptic receptors, since SKF 38393, which lacks pre-synaptic DA receptor activity, produced the same maximum

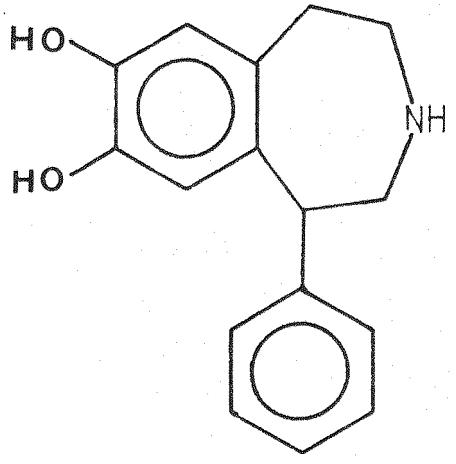


Figure 4.1 SKF 38393

(2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine)

response as DA.

Investigations of presynaptic DA receptors in the renal vasculature have mostly been confined to measurements of the reduction of vasoconstrictor responses elicited by renal nerve stimulation in the dog by agonists such as pirebidil (Laubie and Schmitt, 1978), dipropyldopamine (Massingham et al, 1980) and DA itself (Lokhandwala and Buckley, 1977). This inhibition of reduced RBF was antagonised by the DA receptor blocking agents, haloperidol (Laubie and Schmitt, 1978), (±)-sulpiride (Massingham et al, 1980) and pimozide (Lokhandwala and Buckley, 1977).

These observations have recently been incorporated into a theory which suggests that DA receptors mediating vasodilatation in the canine renal vascular bed can be differentiated into two populations, that of pre- and postsynaptic DA receptors which may differ from each other in terms of their responsiveness to agonists and antagonists. Thus, Goldberg and Kohli (1979) have suggested that vascular DA receptors located postsynaptically in the periphery, have strict structural requirements for agonists and are specifically blocked by R-(+)-sulpiride. These authors claim that in contrast S-(-)-sulpiride is a specific antagonist of presynaptic DA receptors located on sympathetic nerve terminals which have less specific requirements for agonist activity (Goldberg et al, 1978c; 1978d; for details of structure-activity relationships see Section 1-5-5d). Vasodilatation of the innervated canine femoral vascular bed due to the inhibition of NA release from sympathetic nerve terminals is used as an experimental model for presynaptic DA receptors. In studies with agonists this preparation is equally sensitive to dipropyldopamine and DA (Kohli et al, 1978a; Goldberg et al, 1978d), moreover both the α- and β-trans rotameric analogues of DA, isoADTN and ADTN respectively, are powerful agonists of the presynaptic DA receptor (Kohli et al, 1979). In contrast dipropyldopamine only displays approximately 1/30th of the activity of DA and ADTN in the renal vasculature model for postsynaptic DA receptors. Thus, it would appear that dipropyldopamine is a specific agonist of presynaptic DA receptors. This compound possesses unusual properties in that it does not exhibit β_1- or β_2-adrenoceptor activity and has weaker α-adrenoceptor activities than DA in the dog (Kohli et al, 1978a; 1980) and the rat (Cavero et al, 1981).

This study also shows that vasodilator responses to DA, ADTN and SKF 38393 in the guinea pig renal vasculature are potently inhibited by the atypical neuroleptic (±)-sulpiride whilst responses to isoprenaline are not affected. Thus responses to β-adrenergic and dopaminergic agonists are differentiated by pharmacological blockade with specific antagonists. It has previously been shown that of the neuroleptics, (±)-sulpiride is the most potent and specific antagonist in the dog renal vasculature (Goldberg et al, 1978a; Kohli et al, 1978b). In contrast to classical neuroleptics such as chlorpromazine and haloperidol (Gokhale et al, 1964; Kohli, 1969), (±)-sulpiride lacks α-adrenergic activity in up to 1 μM concentrations (Dubocovich and Langer, 1980) and is 300-1000 fold weaker than phentolamine at blocking serotonergic and α-adrenoceptors (Kohli and Cripe, 1979). The results of this study are in agreement with those of Kohli (1969) and Gokhale et al (1964) in that haloperidol had significant α- and β-adrenoceptor blocking activity in addition to its DA-blocking actions. Furthermore, sulpiride was a highly specific antagonist of renal vascular responses but lacked the non-specific blocking effects exhibited by classical neuroleptics.

As previously mentioned, Goldberg and Kohli (1979) have suggested that the enantiomers of sulpiride can differentiate between pre- and postsynaptic DA receptors. Central to this theory is the observation that S-(-)-sulpiride was more powerful than R-(+)-sulpiride at attenuating the vasodilatation produced by dipropyldopamine in the innervated femoral vascular bed (presynaptic model) (Goldberg et al, 1978c; Glock et al, 1981). Further evidence that S-(-)-sulpiride may be stereoselective for presynaptic DA receptors was recently reported by Dubocovich and Langer (1980) in their study of the reduction of [^{3}H] noradrenaline overflow by dopaminergic agents in the isolated cat spleen during nerve stimulation, and by Massingham et al (1980) in their *in vivo* study of the reduction of cat nictitating membrane contraction by dopaminergic agents during sympathetic nerve stimulation. In the former study S-(-)-sulpiride was more effective than R-(+)-sulpiride at antagonising the apomorphine-induced inhibition of [^{3}H] noradrenaline release. In the latter study, (±)-sulpiride completely blocked dipropyldopamine induced inhibition of responses, whereas R-(+)-sulpiride produced a partial, short-lived reversal of the effects of dipropyldopamine.

In this study, which has examined a predominantly postsynaptic DA receptor population, S-(-)-sulpiride was similarly found to be between 6 and 10 times more potent than R-(+)-sulpiride in antagonising the DA induced renal vasodilatation in the guinea pig renal vasculature. The reasons for this discrepancy are not clear. However, in many other dopaminergic systems it is the S-(-)-enantiomer of sulpiride which is the active form. Thus, S-(-)-sulpiride is more active than R-(+)-sulpiride in inhibiting ADTN induced locomotor activity (Woodruff and Andrews, 1979) and inhibiting the binding of [^{3}H] ADTN, [^{3}H] sulpiride and [^{3}H] haloperidol to striatal membrane preparations (Woodruff et al, 1979; Woodruff and Freedman, 1981; Spano et al, 1978). Recently, Horn et al (1980) reported that systemic administration of the R-(+)-enantiomer of sulpiride raised the blood pressure in guinea pigs possibly by reducing the inhibition of sympathetic ganglionic transmission, and potentiated the increase in heart rate by preganglionic stimulation of the heart possibly by facilitation of transmission through canine stellate ganglia (Horn et al, 1981). In contrast they reported that S-(-)-sulpiride lowers blood pressure in guinea pigs and does not effect the tachycardia induced by DA in the dog, whereas it inhibits that produced by noradrenaline by blockade of α-adrenoceptors. The results obtained from the present study do not, however, provide evidence of such changes in blood pressure, at least in the dose range tested, since neither enantiomer of sulpiride had a pressor nor depressor effect in prazosin pretreated guinea pigs. In the absence of α_1-adrenoceptor blockade (±)-sulpiride altered resting blood pressure by 1 or 2 mmHg, but this effect was never to consistently raise or lower blood pressure. A possible explanation for the effects reported by Horn et al (1980; 1981) might be due to an affinity of sulpiride for α_2-adrenoceptors. Lokhandwala and Barrett (1981) recently reported that (±)-sulpiride potentiated the positive chronotropic effect of cardioaccelerator nerve stimulation in a manner similar to that produced by yohimbine, a specific α_2-adrenoceptor antagonist. The effects of clonidine, an α_2-adrenoceptor agonist, on this response were similarly reversed by (±)-sulpiride and yohimbine. Since α_2-adrenoceptors are predominantly located presynaptically, these authors interpreted this observation as suggestive of an interaction between (±)-sulpiride and presynaptic α_2-adrenoceptors. This explanation is unlikely, however, since Kohli and Cripe (1979) and Dubocovich and Langer (1980) clearly demonstrated

that sulpiride was a highly specific DA receptor antagonist which lacks α-adrenoceptor activity.

The controversy concerning the stereospecificities of the enantiomers of sulpiride for pre- and postsynaptic DA receptors clearly merits further investigation. It would be of interest to see which of the enantiomers was the more potent displacer of [^{3}H] sulpiride binding in membranes isolated from 6-hydroxydopamine lesioned guinea pig kidneys. Such a preparation would be anticipated to contain a higher density of postsynaptic DA receptor binding sites than the one used in the present study and thus might provide evidence to indicate whether it is the R-(+)- or the S-(-)-enantiomer of sulpiride which is the more potent antagonist of postsynaptic DA receptors.

In summary, the renal vasodilatation produced by DA in the present study appears to be mediated by a postsynaptic DA receptor similar to that which has been extensively used for the study of peripheral DA receptors in the dog (Goldberg et al, 1978a; b).

Renal vascular DA receptor models are similar to *in vivo* CNS models such as ADTN induced hypermobility and inhibition of neuronal firing in the substantia nigra in that they are powerfully inhibited by sulpiride (Woodruff and Andrews, 1979; Pinnock et al, 1979). However, they are dissimilar to the striatal DA-sensitive adenylate cyclase in that sulpiride is completely ineffective at inhibiting the accumulation of cAMP produced by DA (Trabucchi et al, 1975). In the past the stimulation of a DA-sensitive adenylate cyclase has been extensively used as a model for postsynaptic DA receptors in the CNS. This was first described in rat striatal tissue (Kebabian et al, 1972) and subsequently in the olfactory tubercules (Clement-Cormier et al, 1974), nucleus accumbens (Watling et al, 1979) and retina (Brown and Makman, 1972). Furthermore, the DA-sensitive adenylate cyclase in the CNS shows a strong similarity to DA receptors mediating vasodilatation in the dog in terms of structure-activity requirements for DA-like activity (Goldberg et al, 1968; Woodruff et al, 1979).

Despite the fact that the DA-sensitive adenylate cyclase model was originally developed for the study of DA receptors almost 10 years ago and that this major discrepancy concerning the anomolous actions of

sulpiride exists in the CNS, the effects of sulpiride in the periphery, particularly in biochemical models have been completely overlooked. Since sulpiride is the most potent and specific antagonist of renal vascular responses to DA, there exists a strong possibility that its actions on a renal DA-sensitive adenylate cyclase might differ from those on the striatal cyclase. This possibility was investigated using a renal cortical particulate preparation and studying the effects of agonists and antagonists used in the blood flow model.

With respect to agonist activity the responses obtained in the *in vivo* blood flow model are similar to those obtained in the *in vitro* guinea pig adenylate cyclase preparation. This preparation contains both a β-adrenoceptor (stimulated by isoprenaline and specifically blocked by atenolol) and a DA receptor (stimulated by DA and specifically inhibited by fluphenazine). The identification of two receptors is in agreement with the observations of Nakajima et al (1977) using a similar preparation of rat renal glomeruli and blood vessels and those of Murthy et al (1976), using both homogenates and a particulate fraction of dog renal arteries. Such a duel stimulation of adenylate cyclase activity has similarly been demonstrated in the CNS (Kebabian et al, 1972). Interestingly the β-adrenoceptor sensitive adenylate cyclase in striatal slices is selectively destroyed during homogenisation procedures whereas the DA-sensitive adenylate cyclase remains unaffected (Iversen, 1975). In this respect the isoprenaline stimulated adenylate cyclase found in renal artery preparations differs from that in the brain, in its ability to survive homogenisation as intactly as the DA-sensitive adenylate cyclase (Murthy et al, 1976). The β-adrenoceptor agonist, isoprenaline has long been known to stimulate adenylate cyclase in broken cell preparations of vascular tissue (Triner et al, 1972) and renal cortex (Bell, 1974). Another similarity between the preparation used in the present study and intact striatal preparations is that the production of cAMP is more sensitive to β-adrenoceptor stimulation than to DA-receptor stimulation. Thus the EC_{50} for isoprenaline induced elevation of cAMP levels was 0.17 μM whilst that for DA was 63 μM. These values represent a 370 fold difference. The results, however, provide good evidence that the two receptor systems separately stimulate adenylate cyclase activity, since they are differentiated by the use of pharmacological blocking agents.

In the guinea pig preparation ADTN is a full agonist of the DA-

sensitive adenylate cyclase and SKF 38393 may act as a partial agonist. If this latter observation is correct, then this is similar to the action of this compound in the CNS where it behaves as a partial agonist in striatal adenylate cyclase preparations (Freedman et al, 1979; Setler et al, 1978). The responses to these agonists are blocked by fluphenazine but unaffected by β-adrenergic blockade with atenolol. Thus, ADTN, which is an analogue of the β-rotameric form of DA appears to behave as a pure DA agonist. This observation suggests that the β-trans conformation of DA is the active one and corroborates findings in the CNS, where conformational studies indicate that the active form of DA at postsynaptic DA receptors in the mammalian brain is the extended β-trans rotamer (Woodruff et al, 1979; Horn and Rodgers, 1980).

In their current study of DA-sensitive adenylate cyclase activity in isolated rat glomeruli, Kotake et al (1981) reported a slightly greater affinity of DA for β-adrenoceptors than that found in this study and that of Nakajma et al (1977). cAMP accumulation produced by ADTN was approximately 50% of that produced by DA (Kotake et al, 1981). Furthermore, responses to DA were reduced by propranolol to the level of responses obtained with ADTN. These findings differ in two major ways from the present results. Firstly responses to DA and ADTN were approximately equal at all concentrations and were unaffected by the specific β-blocking agent atenolol. Differences in methodology or species variations in the density of the β-adrenoceptor population mediating cAMP production in the kidney may explain this differing result. Also the β-adrenergic antagonist (±)-propranolol stimulated basal enzymic activity. During the course of this study Murphy (1980) reported that (±)-propranolol produced an increase of adenylate cyclase activity in femoral and renal artery preparations similar to that found in the present study. Further evidence that propranolol possesses intrinsic sympathomimetic activity was recently reported by Harada et al (1981) who found that propranolol exerted a negative chronotropic effect on the isolated guinea pig heart. These authors suggested that the degree of non-specific β-adrenergic activity of propranolol may be related to its high degree of lipid solubility. The results of the present study provide evidence to support this suggestion, since atenolol, a β-adrenergic blocking agent with a poor degree of lipid solubility selectively reduced the elevation in cAMP content produced by isoprenaline but did not affect basal adenylate cyclase activity.

The results show that the ergot derivative, bromocriptine, displays mixed agonist and antagonist activity on the renal adenylate cyclase model. Thus, it was a potent partial agonist, approximately 400 times more powerful than DA at stimulating the production of cAMP in renal blood vessels and glomeruli, but did not produce a maximal response similar to that of DA. In contrast bromocriptine in the micromolar concentration range inhibited the response produced by DA. Little is known of the pharmacological effects of bromocriptine in the periphery. However, it lowers systemic blood pressure and vascular resistance in the dog (Clark et al, 1978) and in man (Kaye et al, 1976). There is no good explanation for these effects, however, it has been suggested that *in vivo*, bromocriptine may interact with both pre- and postsynaptic DA receptors (Clark and Menninger, 1980; Lokhandwala, 1979), or ganglionic DA receptors (Montastruc and Montastruc, 1981). The results of this study support the suggestion that bromocriptine exerts some of its effects postsynaptically, since DA-sensitive adenylate cyclase is generally accepted as representative of postsynaptic DA receptors. Contrary to these suggestions is the finding that bromocriptine is inactive in the dog renal vasculature model for postsynaptic DA receptors (Volkman and Goldberg, 1976).

The actions of bromocriptine in the present study are similar to those in the CNS in that it displays mixed agonist and antagonist effects on central DA receptor models. For example, amongst its dopaminomimetic activities, bromocriptine exerts apomorphine-like effects in the rat 6-hydroxydopamine rotation model (Fuxe et al, 1973) and stereo type sniffing and licking in rats (Johnson et al, 1976), decreases DA turnover (Corrodi et al, 1973), strongly inhibits prolactin secretion in man (Del Pozo, 1972) and rats (Hökfelt and Fuxe, 1972), reduces Parkinsonian side effects in patients and animal models (Goldstein et al, 1978) and weakly stimulates DA-sensitive adenylate cyclase activity at high concentrations (Fuxe et al, 1978). In contrast bromocriptine behaves as an antagonist *in vitro* models for DA receptors in the CNS. Thus, it inhibits striatal DA-sensitive adenylate cyclase at low concentrations (Trabucchi et al, 1976) and potently displaces the binding of [^{3}H] haloperidol (Goldstein et al, 1978) and [^{3}H] spiroperidol in rat striatal tissue (Hruska and Silbergeld, 1978).

It has been suggested that these differences in the properties of

bromocriptine may be partly due to an action on central presynaptic DA receptors (Bannon et al, 1980). Thus bromocriptine prevents an increase in DA synthesis, following inhibition of impulse flow in striatal dopaminergic neurones with γ-butyrolactone. This prevention of a γ-butyrolactone-induced increase in dihydroxyphenylacetic acid was interpreted as suggestive of presynaptic DA receptor stimulation (Walter and Roth, 1976). An alternative explanation for the anomolous activity of bromocr ptine and the ergot derivatives in general has been proposed by Kebabian and Calne (1979) who interpreted these results as evidence for the existence of two subtypes of postsynaptic DA receptors in the CNS. Thus, it has been suggested that the DA receptors found in the pituitary gland, which function to inhibit prolactin release, are strongly stimulated by bromocriptine in the nanomolar concentration range and typify the D_2-type receptor. In contrast DA-receptors associated with a DA-sensitive adenylate cyclase in the striatum are inhibited by bromocriptine at high concentrations and typify the D_1-type receptor. The present results do not support such a theory since bromocriptine stimulated the renal DA-sensitive adenylate cyclase in the nanomolar concentration range yet inhibited the increase in cAMP produced by DA in the micromolar concentration range. These results are contrary to those which would be anticipated if bromocriptine was a selective agonist of the non-cyclase linked DA receptor. On the other hand bromocriptine behaved as an antagonist in the displacement of [^{3}H] sulpiride binding, in that it was as active as the neuroleptics, (+)-butaclamol, fluphenazine, cis-flupenthixol and haloperidol at inhibiting binding. According to the theory of Kebabian and Calne (1979) sulpiride is a suggested specific ligand of the D_2 receptor type, hence it could be argued that this would be the predicted result if bromocriptine were also a ligand of the same subclass of DA receptor. However, more recent evidence reported by Woodruff and Freedman (1981) has disputed the proposal that sulpiride is a specific ligand of the D_2 receptor.

Although the DA-sensitive adenylate cyclase is similar to the *in vivo* dog and guinea pig model, with respect to agonist specificity, there are some important discrepancies when antagonists are considered. The most striking of these is the complete inability of the substituted benzamide sulpiride to antagonise the stimulation of cAMP produced by DA although it was found to be extremely potent in inhibiting the DA

induced renal vasodilatation in the guinea pig. These results are similar to those first reported by Trabucchi et al (1975) in the CNS where sulpiride did not inhibit the striatal DA-sensitive adenylate cyclase. This observation has been confirmed by many investigations (Spano et al, 1978; Rupniak et al, 1981). Likewise the potency of sulpiride as a DA antagonist in renal blood flow models is well documented (Goldberg et al, 1978a; b; 1979). It therefore seems that the function of DA as measured by RBF changes may be inhibited by sulpiride whilst the adenylate cyclase may not be implicated directly in this response.

The results underline the anomolous behaviour of sulpiride in the periphery and are similar to those obtained in the CNS. Here sulpiride's potent actions as an antagonist on many *in vivo* models (see Section 1-5-11) are contrasted by its inactivity on striatal DA-sensitive adenylate cyclase (Trabucchi et al, 1975). In the CNS the controversial results obtained with sulpiride and the ergots have been incorporated into the classification of DA receptors into two receptor populations; that of D_1 receptors, which are said to be linked to a stimulatory adenylate cyclase and specifically inhibited by cis-flupenthixol, and D_2, non-cyclase-linked receptors, which are said to be specifically inhibited by sulpiride (Kebabian and Calne, 1979). Again more recent evidence reported by Freedman et al (1981a) in their study of [^{3}H] sulpiride binding in striatal preparations has suggested that such a classification is premature.

Although the DA-sensitive adenylate cyclase is specifically antagonised by certain neuroleptics, as confirmed by our results with fluphenazine, a major discrepancy of this model is that the rank order of potency of the neuroleptics based on IC_{50} values does not correlate with their clinical potency as antipsychotic agents (Seeman, 1980). Thus, there is a rough correlation for neuroleptics of the phenothiazine and thioxanthene groups and butaclamol for inhibiting the striatal DA-sensitive adenylate cyclase and alleviating schizophrenic symptoms in the clinic, but this does not hold for neuroleptics of the butyrophenone group. Such a correlation would not hold for the substituted benzamides, since they are unable to penetrate the blood brain barrier. The greatest weakness of the adenylate cyclase model is that the proposed physiological role of cAMP, as part of a regulatory system

which mediates the actions of DA, has never been conclusively demonstrated. According to classical theory the DA molecule binds to its receptor, presumably located on the external face of the membrane, which activates an adenylate cyclase molecule, located on the inside of the same membrane, to stimulate the production of cAMP. If the adenylate cyclase moiety and the DA receptor binding sites were subunits of one receptor complex it might be anticipated that neuroleptic antagonist potencies on both these systems would equally correlate with their antipsychotic potencies. In the CNS, however, the behavioural and antipsychotic potencies of neuroleptics correlate far better with their potencies at displacing the binding of dopaminergic ligands than with inhibiting the DA-sensitive adenylate cyclase (Seeman et al, 1976).

In view of the major discrepancies found in the actions of sulpiride in the guinea pig kidney, a search for renal [^{3}H] sulpiride binding sites was made. If, as has been suggested for the CNS, sulpiride is a specific antagonist of non-cyclase-linked DA receptors (Kebabian and Calne, 1979), then it might be expected that [^{3}H] sulpiride would label a specific category of DA receptors. If one considers that the canine renal vasculature model was originally developed for the study of DA receptors almost 20 years ago, it is surprising that so few radioreceptor binding studies utilising [^{3}H] neuroleptics have been made in the periphery. One of the major problems in peripheral ligand binding studies is the relatively low density of receptors available for labelling by radioligands, in comparison with CNS preparations. A further problem, particularly in the kidney, is the presence of mixed populations of α- and β-adrenoceptors in addition to DA receptors. This highlights the importance of selecting a highly specific [^{3}H] neuroleptic ligand for labelling dopaminergic sites. [^{3}H] sulpiride is such a ligand, and has been successfully used for binding studies in the CNS (Woodruff and Freedman, 1981). Two important properties of sulpiride make it a particularly suitable ligand. Firstly, it is highly specific for DA receptors but unlike the classical neuroleptics, spiroperidol and haloperidol, it lacks 5HT or α- and β-adrenoceptor activity (Kohli and Cripe, 1979; Dubocovich and Langer, 1980; Woodruff and Freedman, 1981; Leyson and Laduron, 1977a). Hence it might be anticipated that sulpiride has high affinity for dopaminergic binding sites but low affinity for other receptors. Secondly, sulpiride has a very low lipid solubility and thus low non-specific membrane binding

effects. This poor solubility is reflected by sulpiride's inability to cross the blood-brain barrier (Honda et al, 1977; Woodruff and Andrews, 1979) and extremely low water:octanol partition coefficient (Norman et al, 1979). In contrast most neuroleptics are highly lipid soluble and are less suitable than sulpiride for use in radiolabelling experiments because of their non-specific penetration into biological membranes.

The results demonstrate that [^{3}H] sulpiride bound to guinea pig renal cortical homogenates in a specific, saturable manner. There was a single binding component with an affinity constant (Kd) of 20.2 nM and a maximum binding capacity (Bmax) of 177 fmol/mg protein. These kinetic parameters are of a similar order of magnitude to those reported by Theodorou et al (1979) using crude striatal membranes, similar assay conditions and microcentrifugation for the separation of free and bound [^{3}H] sulpiride where the Bmax was 23.8 ± 5.3 pmol g^{-1} wet weight of tissue and the Kd was 26.9 ± 10.3 nM. The higher Bmax of 240 fmol/mg protein and lower Kd of 7 nM reported by Woodruff and Freedman (1981) in their study of [^{3}H] sulpiride binding to purified striatal membranes might be a reflection of differences in purity of the preparation. It is more likely, however, that these differences are due to the millipore filtration techniques used by Woodruff and Freedman (1981) since in a recent repeat of their experiments using partially purified striatal preparations and millipore filtration (Freedman et al, 1981b) there was no significant difference in the binding affinity constant (Kd was 8.8 ± 1.0 nM), however, the maximum binding capacity of the system increased by approximately 180% (Bmax was 433 ± 27 fmol/mg). This latter result is in contrast to that which would be anticipated if a reduction in the purity of striatal preparations led to a reduction in the Bmax. A further indication that differences in separation procedures may account for these variations is the percentage of specific [^{3}H] sulpiride binding obtained using the various separation techniques. In the present study using microcentrifugation, specific binding represented approximately 31% of total tissue binding, a value strikingly similar to that reported by Theodorou et al (1979) (29%), whereas Woodruff and Freedman (1981) reported that using millipore filtration, specific binding represented 55% of total binding in highly purified striatal membranes and approximately 60% in partially purified membranes (S. Freedman, personal communication).

Initial experiments revealed that [^{3}H] sulpiride bound to Whatman GF/B glass fibre filters and Millipore cellulose filters in a specific displaceable manner in the absence of membranes. Furthermore this 'false' specific binding was displaced stereospecifically by S-(-)-sulpiride in the same concentration range in which it was displaced from membranes. Such a phenomenon has previously been reported for [^{3}H] sulpiride binding to millipore filters (Woodruff and Freedman, 1981), but is by no means limited to the neuroleptics. As mentioned previously [^{125}I] insulin binds to talc (Cuatrecasas and Hollenberg, 1975) and [^{3}H] naloxone, a ligand for opiate receptor, binds stereospecifically to glass fibre filters (Snyder et al, 1975). In the present study this problem was overcome by the use of rapid microcentrifugation to separate free and bound [^{3}H] sulpiride, a solution similarly adopted by Theodorou et al (1979). In a third report of [^{3}H] sulpiride binding in the CNS (Spano et al, 1980) Whatman GF/B filters are used to separate bound radioactivity, after correction for binding to filters (E. Carboni, personal communication).

The distribution of [^{3}H] sulpiride binding sites suggests that the binding component is associated with dopaminergic sites. Regional binding parallels the distribution of endogenous DA, the DA-sensitive adenylate cyclase and regional increases of RBF produced by DA as shown in table 3.10. Binding was greatest in the renal cortex, lower in the medulla and not detectable in the pelvis, a region low in dopaminergic activity. This result is in agreement with that of Rosenblatt et al (1980) in their study of [^{3}H] spiroperidol binding in rat renal membranes. The low level of binding in the liver and adrenals might be attributable to the existence of a sparce population of DA receptors in these tissues. It was recently demonstrated that the adrenal glands are involved in the hypotensive response to the dopaminergic agent bromocryptine (Hamilton, 1981). This response was abolished by removal of the adrenal medulla in rats (Hamilton, 1981) or pretreatment with the DA antagonists, haloperidol, sulpiride and metaclopropamide in the dog (Montastruc and Montastruc, 1981). Alternatively this binding might be associated with adrenal DA uptake sites (Waldeck et al, 1975) located in sympathetic nerves of blood vessels, since it was recently shown that high levels of free and conjugated DA are released from the adrenal medulla following stress in dogs (Unger et al, 1979). In the dog, DA produces hepatic arterial vasodilatation which is antagonised by haloperidol but unaffected by

α- and β-adrenoceptor antagonists (Richardson and Withrington, 1978). It is likely therefore that the low level of binding in the liver might be associated with specific DA receptors located in the hepatic arterial and portal vascular beds.

The pharmacological profile of both agonists and antagonists provides further evidence that the specific binding component is associated with renal dopaminergic sites. The ability of DA and its agonists ADTN and apomorphine to displace binding, in contrast to the inactivity of noradrenaline, gamma-aminobutyric acid, 5-hydroxytryptamine, histamine and glutamic acid in this system, highlights the interaction of [^{3}H] sulpiride with DA receptor binding sites. The rigid DA analogue ADTN is the most potent agonist tested, this is consistent with its activity in many dopaminergic systems (Woodruff, 1978). During the course of this study Nakajima and Kuruma (1980) reported a [^{3}H] haloperidol binding assay using a sonicated rat kidney particulate preparation similar to the system from which they had previously isolated a DA-sensitive adenylate cyclase (Nakajima et al, 1977). They were unable to demonstrate displacement of binding by DA or its agonists but found an excellent correlation between the rank order of potency of neuroleptics at displacing [^{3}H] haloperidol binding in the kidney and that in the striatum. These authors interpreted this latter observation as suggestive of an adenylate cyclase-linked component of [^{3}H] haloperidol binding. However, the inability of agonists to displace binding suggests that [^{3}H] haloperidol is unable to specifically label dopaminergic sites in the assay of Nakajima and Kuruma (1980). Haloperidol is known to lack specificity in that it has approximately equal affinity for α-adrenoceptors and DA receptors in binding studies (Laduron, 1980) and *in vivo* as shown by its ability to antagonise MABP responses to phenylephrine and DA in the present study. In a mixed receptor preparation such as the kidney, which contains α- and β-adrenoceptors in addition to DA receptors, haloperidol is not a suitable ligand for the identification of DA receptors.

An alternative explanation for these conflicting results may lie in differences between the experimental protocol of Nakajima and Kuruma (1980) and that of the present study. Firstly, these workers allowed a two hour incubation period for the labelling of membranes

by [^{3}H] haloperidol, in comparison to 10 minutes employed in this study. It is clear from kinetic studies of [^{3}H] neuroleptic binding in the CNS (Bennett, 1978) that such an incubation period is too long, since virtually all of the specific binding occurs in the first few minutes. In the present study a rapid association between the ligand and the renal cortical membranes was demonstrated. Also in the methodology of Nakajima and Kuruma (1980) separation of free and bound radioactivity was achieved by glass fibre filters which, as already mentioned are able to bind [^{3}H] neuroleptic ligands. The authors do not make it clear whether or not the results are corrected for binding to filters.

The results provide further evidence for the specificity of sulpiride as a dopaminergic ligand since the α- and β-adrenoceptor agents, prazosin, phenylephrine, atenolol and isoprenaline were completely inactive at displacing [^{3}H] sulpiride binding. It is interesting to note, however, that the β-adrenergic antagonist (±)-propranolol was a weak displacer of binding. As previously mentioned the membrane stabilising effects of (±)-propranolol are well known (Vaughan Williams, 1966; Barrett and Cullum, 1968; Frishman, 1979; Kaiho et al, 1981). Of the β-blocking agents (±)-propranolol has the highest octanol:water partition coefficient (16.25; Harada et al, 1981) and is non-specifically membrane soluble in the 0.1-1 µM concentration range. Thus, the weak ability of (±)-propranolol to inhibit binding at 1 µM might be due to its high degree of non-specific penetration into membranes more than to the non-specific labelling of β-adrenoceptors by [^{3}H] sulpiride. This suggestion is supported by the observation that the poorly lipid soluble β-adrenoceptor blocking agent, atenolol (octanol:water partition coefficient of 0.01; Harada et al, 1981) is unable to inhibit binding even at concentrations as high as 10 µM.

The DA antagonists are generally more effective than agonists in displacing [^{3}H] sulpiride binding. This is consistent with results obtained using other neuroleptic radioligands such as [^{3}H] spiroperidol (Laduron and Leyson, 1977, Creese et al, 1977). Figure 4.2 shows that there is a strong correlation between the rank order of potency of agonists and neuroleptics in displacing [^{3}H] sulpiride binding in guinea pig renal cortical preparations and that in striatal preparations (Woodruff and Freedman, 1981). This agreement suggests that

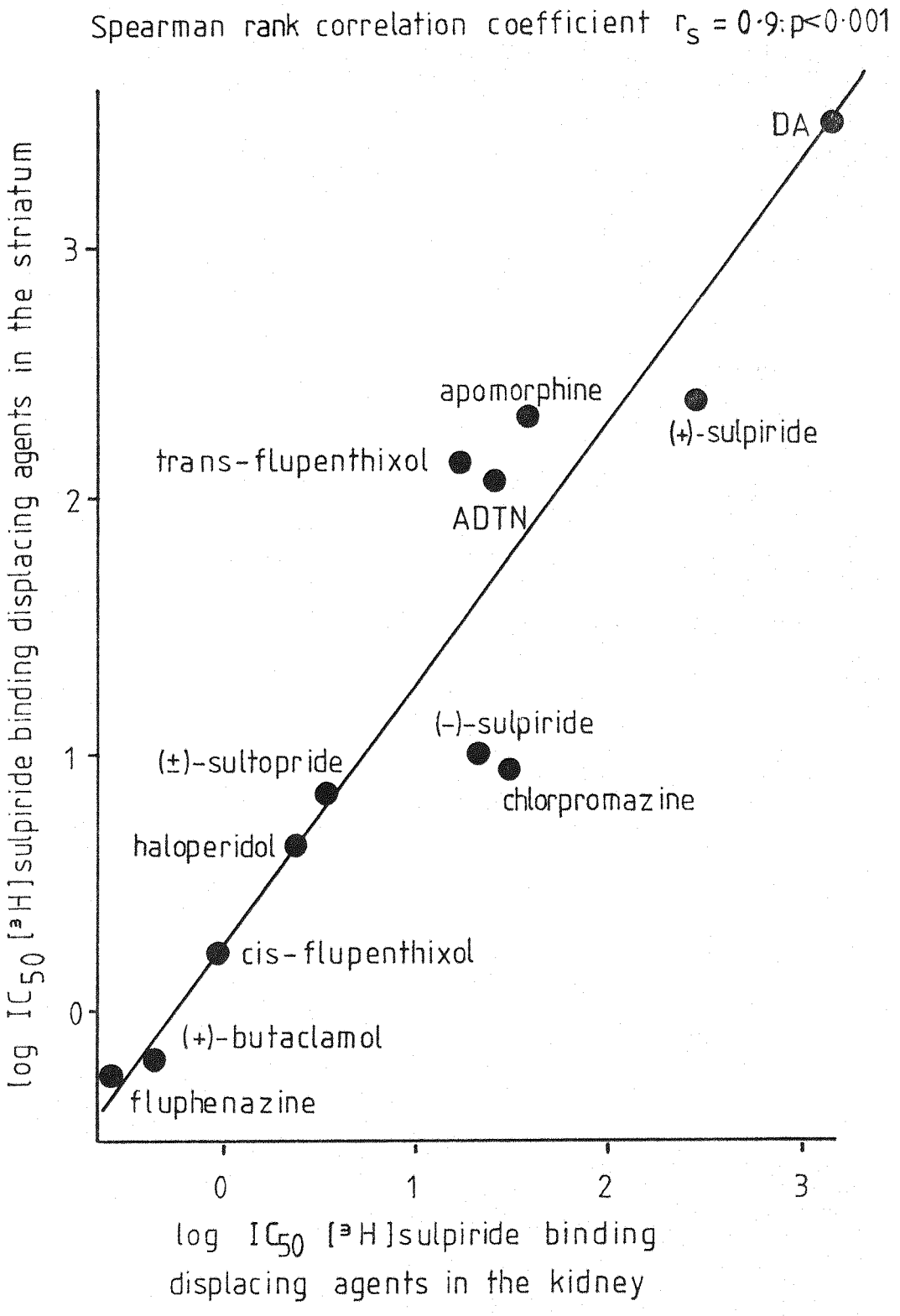


FIGURE 4.2 Correlation of drug affinity for [^{3}H] sulpiride binding sites in guinea pig kidney particulate preparations and that in rat striatal preparations. Striatal binding data is from Woodruff and Freedman (1981). IC_{50} is the concentration of the agent giving 50% inhibition of specific [^{3}H] sulpiride binding.

dopaminergic sites as labelled by [^{3}H] sulpiride in the kidney are similar to those in the CNS.

The results demonstrate that the displacement of binding by neuroleptics of the various classes is stereospecific. Thus, S-(-)-sulpiride is 13 times more potent than R-(+)-sulpiride, a finding which is consistent with S-(-)-sulpiride being more active in inhibiting renal vasodilator responses to DA. Furthermore, (+)-butaclamol is more than 10,000 times more active than its pharmacologically inactive (-)-enantiomer and cis-flupenthixol is 13 times more active than its clinically inactive trans-isomer.

The rank order of potency of the neuroleptics in displacing binding in the kidney is of particular interest. Classical neuroleptics of the phenothiazine, thioxanthene and butyrophenone groups such as fluphenazine, spiroperidol, cis-flupenthixol and (+)-butaclamol are extremely potent displacers of binding. The finding that cis-flupenthixol is powerful in inhibiting binding (20 times more potent than S-(-)-sulpiride) is especially significant since it was originally suggested as a selective antagonist of the D_1 type receptor (adenylate cyclase linked) (Hyttel, 1978; 1980; Cross and Owen, 1980) because it was weakly active in displacing [^{3}H] sulpiride binding in the assay of Theodorou et al (1979). These authors interpreted this latter observation as suggestive of sulpiride labelling a specific population of non-cyclase linked receptors. The theory of Kebabian and Calne (1979) suggests that binding sites associated with the putative D_2 receptor are non-cyclase-linked, specifically labelled by [^{3}H] sulpiride but unaffected by cis-flupenthixol, whereas binding sites associated with the D_1 receptor are cyclase-linked, preferentially labelled by [^{3}H] cis-flupenthixol but unaffected by sulpiride.

More recently Woodruff and Freedman (1981) using a highly purified rat striatal preparation found cis-flupenthixol to be a powerful displacer of [^{3}H] sulpiride binding, which is contrary to the anticipated result if cis-flupenthixol were a specific antagonist of the DA-sensitive adenylate cyclase. The results of the present study are in agreement with those of Woodruff and Freedman (1981), but do not support the theory of Kebabian and Calne (1979) which suggests that sulpiride and cis-flupenthixol selectively interact with two separate subclasses of DA receptors. Recently, Theodorou and colleagues have

repeated their experiments and now agree that cis-flupenthixol is a powerful displacer of binding. Furthermore, in a current report Hytell (1981) has refuted the claim that cis-flupenthixol is a specific ligand of the D_1 receptor subtype and suggests that the term 'preferential' labelling of the adenylate cyclase is a better description of the specificity of this antagonist.

If sulpiride was preferentially labelling a separate group of receptor sites, then one might expect that the substituted benzamides would fall into a well defined grouping in the potency profile of compounds in inhibiting [^{3}H] sulpiride binding. The results clearly demonstrate that this is not so in that (±)-sultopride, S-(-)-sulpiride and R-(+)-sulpiride are dispersed evenly throughout the potency range of displacing agents. This observation is also in agreement with the results of Woodruff and Freedman (1981) but does not support the suggestion of Theodorou et al (1979) and Spano et al (1979) that [^{3}H] sulpiride labels a population of non-cylcase linked receptors.

Creese, Usdin and Snyder (1979b) have extended the theory of Kebabian and Calne (1979) to incorporate the effects of guanine nucleotides as a further means of distinguishing between the proposed D_1 and D_2 receptors. Guanine nucleotides are known to be essential cofactors for the activity of many hormones and neurotransmitters linked to an adenylate cyclase system (Rodbell et al, 1971) such as the inhibitory opiate system (Blume et al, 1979) and the excitatory β-adrenergic system (Lefkowitz and Williams, 1978; Lefkowitz, 1980). This phenomenon has similarly been demonstrated for the DA-sensitive adenylate cyclase in the caudate nucleus (Clement-Cormier et al, 1975). It has also been reported that in the presence of GTP (guanosine 5'-triphosphate) or its non metabolisable analogue Gpp (NH)p (5'-guanylylimidodiphosphate) the affinity of dopaminergic binding sites for agonists is decreased. This effect is specific for agonists in that [^{3}H] apomorphine and [^{3}H] ADTN binding is inhibited by GTP whereas that of the antagonist [^{3}H] spiroperidol is unaffected (Creese et al, 1978). Furthermore, the ability of agonists to compete for binding is reduced, such that the displacement of [^{3}H] spiroperidol by agonists is decreased in the presence of GTP, whereas that of antagonists is unaffected (Zahniser and Molinoff,

1978). This effect of guanine nucleotides has been described as 'agonist specific' by Zahniser and Molinoff (1978) who suggested that agonist ligands affected by GTP label an adenylate cyclase-linked part of the DA receptor. Creese et al (1979b) have suggested that the receptors referred to as D_1 are cyclase-linked, regulated by GTP and unaffected by sulpiride, whereas those receptors referred to as D_2 are not cyclase-linked, unaffected by guanine nucleotides and specifically labelled by sulpiride.

However, on the basis of their recent study of the interaction between guanine nucleotides and [^{3}H] sulpiride binding, Freedman et al (1981a) have disputed such a proposal. According to the D_1 and D_2 classification, if sulpiride were a specific antagonist of non-cyclase linked DA receptors, then DA agonist affinity for [^{3}H] sulpiride binding would not be reduced by guanine nucleotides. However, these authors clearly demonstrated that GTP, GDP and Gpp(NH)p specifically decreased the affinity of ADTN, DA and apomorphine for [^{3}H] sulpiride binding sites. In contrast the nucleotides had little effect on competition for displacement of binding by the antagonists, cis-flupenthixol, fluphenazine and S-(-)-sulpiride. These observations underline the controversy associated with the classification of DA receptors and suggest that there is a basic flaw in the criteria used to distinguish between the putative D_1 and D_2 receptors. Either the use of guanine nucleotides to distinguish between cyclase-linked and non-cyclase linked DA receptors is wrong or alternatively the assumption that sulpiride is a specific antagonist of the non-cyclase linked receptor is wrong.

Collectively the results of the present study suggest that sulpiride is a highly specific antagonist of DA receptors. [^{3}H] sulpiride labelled dopaminergic binding sites *in vitro* guinea pig renal cortical preparations in a manner which was characteristic of other neuroleptics. Furthermore, sulpiride was an extremely powerful antagonist of renal vascular responses to DA, yet it was completely ineffective at inhibiting the elevations in renal cAMP content produced by DA and its agonists, ADTN and apomorphine. These contrasting results are analogous to the ones incorporated into Kebabian and Calne's (1979) D_1 and D_2 receptor classification for the CNS. Recently it has been proposed that D_2 receptors are the physiologically important post-synaptic ones (Seeman, 1980). If a tentative comparison is made between

central and peripheral studies, the renal DA-sensitive adenylate cyclase appears to resemble the D_1-receptor type while the renal vascular and [^{3}H] sulpiride binding models appear to resemble the functional D_2 receptor. It is clear, however, that the D_1 and D_2 theory does not comply with the anticipated effects of sulpiride on DA receptors associated with an adenylate cyclase and GTP linkage (Freedman et al, 1981a). If the D_1 and D_2 theory is rejected, what alternatives are there to explain the anomolous results obtained with sulpiride?

The most plausible hypothesis to explain the current controversies regarding the existence of adenylate cyclase- and non-cyclase-linked DA receptors is that of dual regulation of the adenylate cyclase complex as proposed by Rodbell (1980). Figure 4.3 shows that in addition to the classically acceptable two component systems, whereby a specific receptor is located on the outer membrane surface and an adenylate cyclase complex on the inner, he has suggested the possibility of (at least three) GTP regulatory proteins which mediate events between the receptor and the cyclase. These proteins are similar in that they are activated by binding to GTP and are located on the inner membrane surface, but different in that one mediates inhibition of the adenylate cyclase and the second excitation as shown in figure 4.4, whilst the third is not related to adenylate cyclase activity. This theory would then explain the necessity for GTP in the adenylate cyclase assay system, since in the absence of GTP, the protein mediating events between receptor and adenylate cyclase moiety is inactive. Hence in the presence of GTP, the stimulatory GTP binding protein may mediate β-adrenergic stimulation of cAMP formation, such as that observed in the present study by the β-agonist isoprenaline. In contrast the inhibitory GTP binding protein may mediate the inhibition of adenylate cyclase, as in the case of muscarinic receptors (Watanabe et al, 1978). Rodbell (1980) has additionally postulated that the presence of sodium ions is essential for the functioning of such an inhibitory cyclase mechanism. Similarly Freedman and Woodruff (1981), Theodorou et al (1980) and Stefanini et al (1980) have demonstrated that sodium ions are essential for [^{3}H] sulpiride binding. Thus, sodium is a pre-requisite for both [^{3}H] sulpiride binding and also for inhibition of the adenylate cyclase. Furthermore, agonist displacement of [^{3}H] sulpiride and [^{3}H] spiroperidol binding is reduced in the presence of GTP (Freedman et al, 1981a; Creese et al, 1979b). These results initially appear to be contradictory in that GTP is essential for

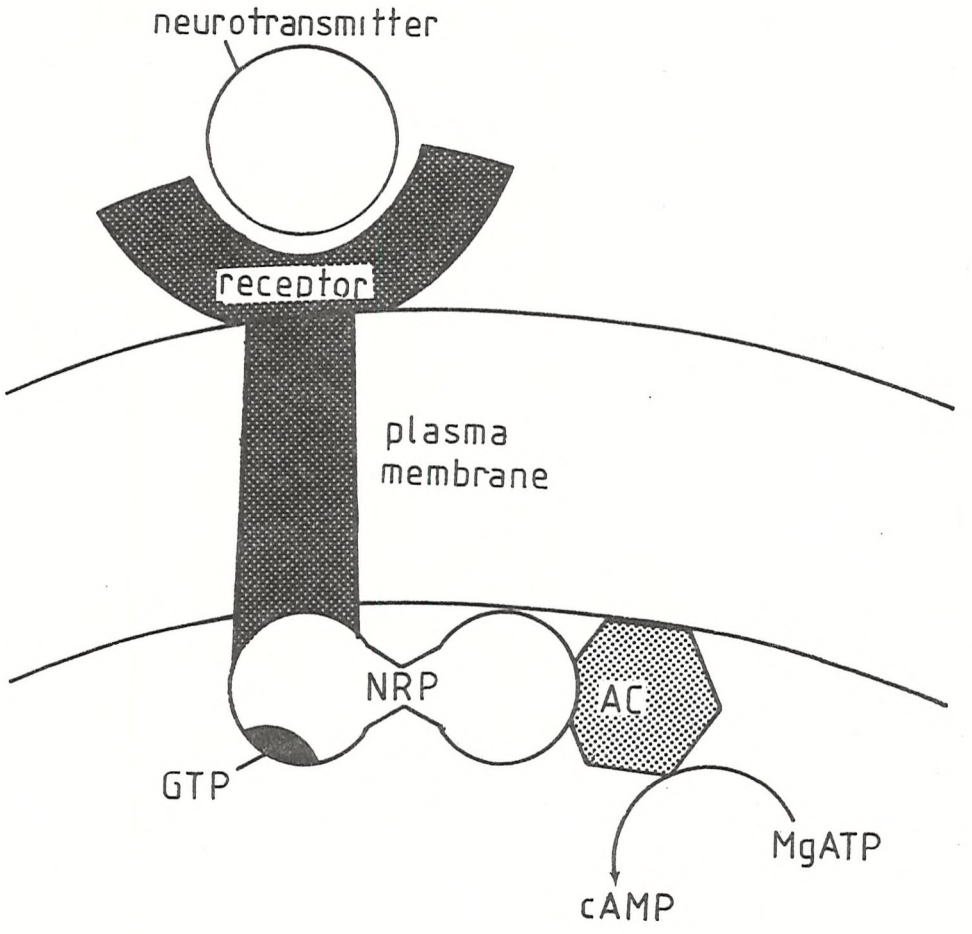


FIGURE 4.3 Schematic representation of the components and organisation of the adenylate cyclase moiety for regulation by hormones/neurotransmitters and GTP (guanine 5'-triphosphate) from Rodbell (1980). In this model the receptor bridges the membrane and possesses a neurotransmitter binding site located at the outer membrane surface and can attach to a nucleotide regulatory protein (NRP) at the inner face of the membrane. NRP binds GTP and forms a linkage between the receptor and the adenylate cyclase complex located at the inner face of the membrane.

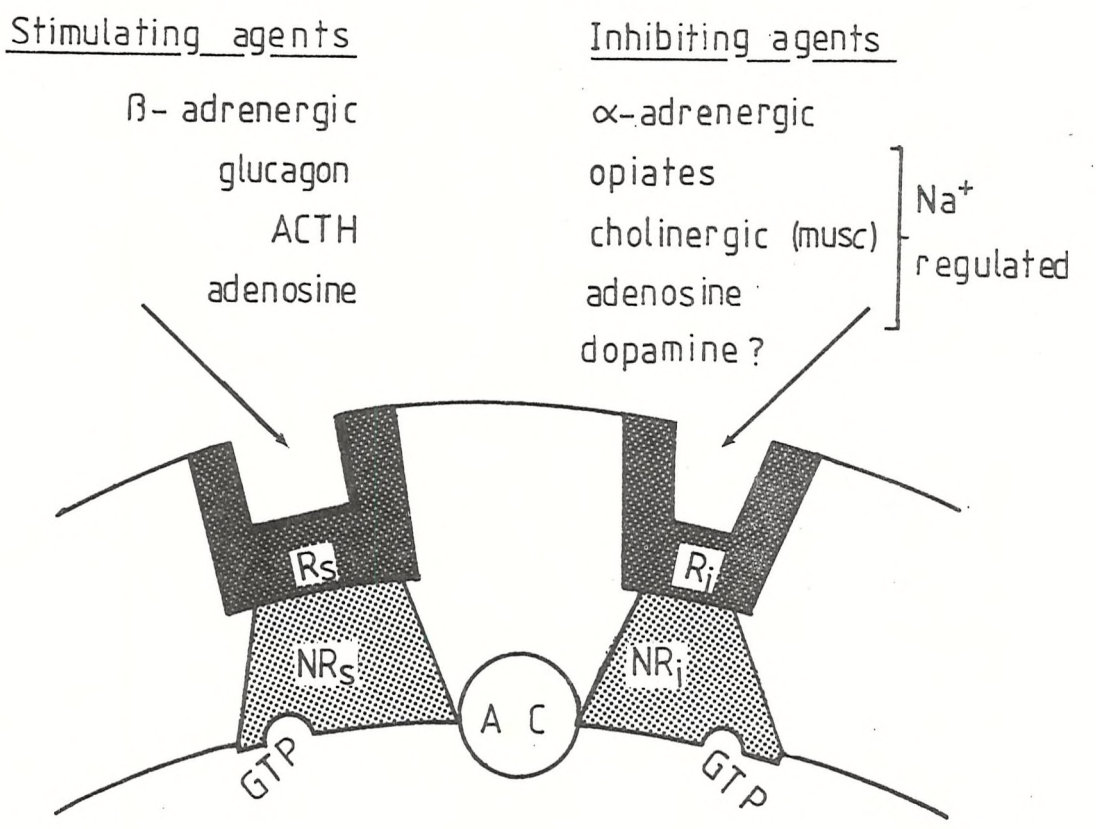


FIGURE 4.4 Diagramatic representation of the dual regulation of adenylate cyclase by stimulatory and inhibitory hormones and neurotransmitters from Rodbell (1980). Located at the outer membrane surface is the receptor (R) containing a specific neurotransmitter or hormone binding site. At the inner surface is the nucleotide regulatory unit (N) and the adenylate cyclase component (AC). One class of receptors are thought to mediate the stimulatory effects of agents (Rs) through stimulatory nucleotide regulatory proteins (Ns) on adenylate cyclase activity and a second class of receptors mediate the inhibitory effects of agents (Ri) through inhibitory nucleotide regulatory proteins (Ni) on adenylate cyclase activity. GTP binding to the neurotransmitter/hormone:receptor:nucleotide regulatory protein leads to a complex which preferentially links to the adenylate cyclase complex to form the activated holoenzyme. Thus the Ns and Ni units form a linkage between the receptor (Rs or Ri) and the adenylate cyclase (AC). The RiNi units require Na^+ for the effects of the various inhibitory agents on adenylate cyclase activity and dopamine may act via such an inhibitory mechanism.

adenylate cyclase activation and yet it decreases agonist binding to DA receptors. It is therefore suggested that sulpiride might function as an antagonist by affecting an inhibitory DA-sensitive adenylate cyclase.

Previous studies of the DA-sensitive adenylate cyclase have always searched for an elevation of cAMP content. For example in the present study cAMP levels were reduced to basal vaues by a 60 minute preincubation in order to record large stimulations of adenylate cyclase activity. Such a methodology may have masked the activation of an inhibitory DA-sensitive adenylate cyclase.

The first indication that DA receptors might be associated with an inhibitory adenylate cyclase originated from studies in the anterior pituitary gland. De Camilli et al (1979) demonstrated that the inhibition of adenylate cyclase activity in pituitary adenomas was antagonised by sulpiride. Furthermore, activation of DA receptors resulted in the inhibition of vaso-intestinal polypeptide (VIP) (Onali et al, 1981) and β-adrenoceptor (Cote et al, 1981) sensitive adenylate cyclase activity in rat pituitary, an effect which was blocked by DA-receptor antagonists.

Very recently a reduction of cAMP efflux by the DA agonist LY-141865 was reported in superfused rat neostriatum (Stoof and Kebabian, 1981). This is in contrast with the present study where SKF 38393 partially stimulated the production of cAMP in renal particulate preparations. Stoof and Kebabian (1981) interpreted these observations as a further means of differentiating between D_1 and D_2 receptors (Kebabian and Calne, 1979) and have modified the original proposal to incorporate a D_2-sensitive inhibitory cyclase. Thus, D_1 receptors are said to be associated with an excitatory adenylate cyclase whereas D_2 receptors are associated with an inhibitory cyclase. They suggested that SKF 38393 was a specific agonist of the D_1 receptor which was selectively antagonised by fluphenazine whereas LY-141865 was a specific agonist of the inhibitory cyclase (D_2) which reduced the SKF 38393 stimulated efflux of cAMP. Furthermore, S-(-)-sulpiride specifically antagonised activation of the inhibitory adenylate cyclase by LY-141865 and potentiated the efflux of cAMP produced by DA and apomorphine. Stoof and Kebabian

(1981) suggested that D_1 and D_2 receptors may be interconnected and affect each others' activity by 'processes' within the cell membrane. However, by analogy with Rodbell's model (1980) it is more likely that these 'processes' can be explained in terms of differing subunits of a single multicomponent receptor site, linked to a GTP-catalytic unit regulatory mechanism.

The theory of dual regulation of adenylate cyclase activity does not exclude the possibility that several other factors might contribute to the lack of interaction between sulpiride and a stimulatory adenylate cyclase moiety. Initially sulpiride's failure to inhibit the DA-sensitive adenylate cyclase was thought to be due to a lack of sodium ions in the assay medium. [^{3}H] sulpiride binding is extremely sensitive to sodium ions (Theodorou et al, 1980; Freedman and Woodruff, 1981; Stefanini et al, 1980) and it was suggested that sulpiride could not gain access to the adenylate cyclase-linked dopaminergic site since its binding to the receptor site was hampered by a lack of sodium ions. However, this suggestion does not explain why sulpiride cannot inhibit the formation of cAMP produced by DA in renal preparations, since these were particulate preparations and the assay was carried out in Krebs' buffer containing 120 mM sodium ions. It has been demonstrated that the addition of sodium to the sliced nucleus accumbens preparation, which is similar to the renal preparation, and to striatal homogenates failed to affect sulpiride's inactivity on the DA-sensitive adenylate cyclase (K. Watling, personal communication; Rupniak et al, 1981, Freedman, Ph.D. thesis, 1982). These results are consistent with those of this thesis in that the ineffectiveness of sulpiride is not due to a deficiency in sodium.

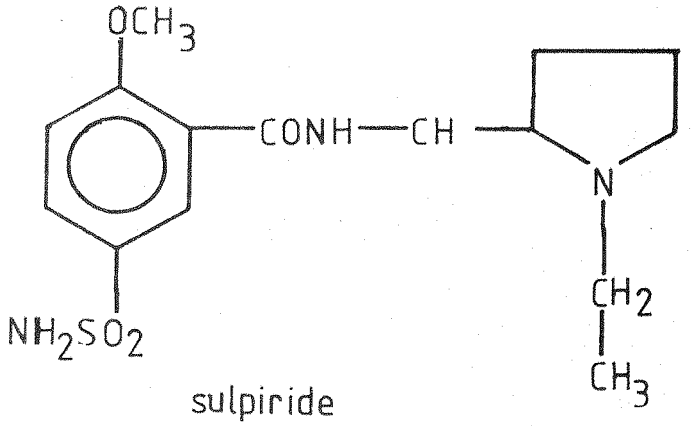
An alternative explanation for the inability of sulpiride to inhibit the accumulation of cAMP produced by DA may be related to its poor degree of penetration into membranes. Sulpiride is virtually insoluble in biological membranes as indicated by its low n-octanol: aqueous buffer partition coefficient (log P of -0.5; Norman et al, 1979). In contrast cis-flupenthixol is highly lipid soluble with a log P value of 4.25 (Norman et al, 1979) and is a potent antagonist of the striatal adenylate cyclase. Woodruff et al (1980) recently suggested that in order to be effective inhibitors of the adenylate cyclase, drugs should possess a certain degree of lipid solubility which would allow antagonist molecules to penetrate membranes, and come

into the vicinity of the receptor site associated with a membrane-bound adenylate cyclase complex. Their hypothesis is supported by the synthesis of two substituted benzamide compounds, N-(1-benzyl-3-pyrollidinyl)-5-chloro-2-methoxy-4-methylaminobenzamide (YM-08050) and cis-N-(1-benzyl-2-methyl-pyrolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide (YM-09151-2) which are structurally very similar to sulpiride as shown in figure 4.5. In contrast to sulpiride, these drugs can penetrate the blood brain barrier (Usada et al, 1979; 1980; Nishikori et al, 1980) and are antagonists of the DA-sensitive adenylate cyclase.

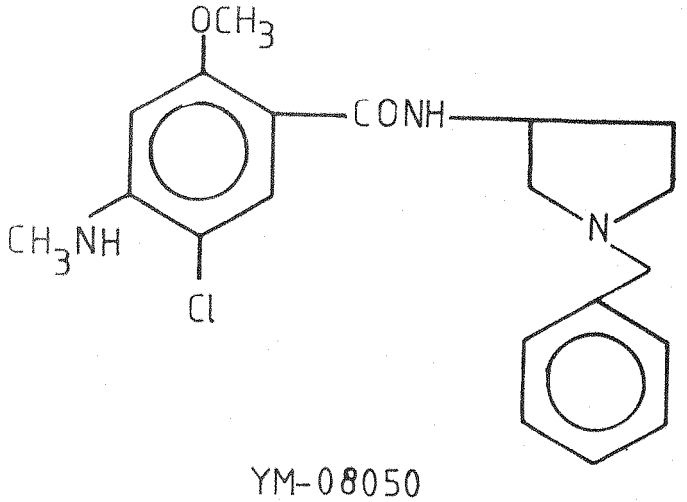
A fourth possibility for the ineffectiveness of sulpiride on the DA-sensitive adenylate cyclase is that sulpiride may interact with an ionophore or bind to protein complexes at, or near, the DA receptor site, resulting in an increased permeability to certain ions, possibly potassium. This seems unlikely, however, since in their study of the effect of cations on [^{3}H] sulpiride binding Freedman and Woodruff (1981) demonstrated that the removal of potassium ions from the assay medium had little effect on binding. Furthermore, tetraethylammonium, a potassium channel blocker was unable to displace [^{3}H] sulpiride binding. In contrast, sodium ions are essential for [^{3}H] sulpiride binding (Stefanini et al, 1980; Freedman and Woodruff, 1981; Hall et al, 1980), but again tetrodotoxin, a sodium channel blocker and ouabain, an inhibitor of the sodium potassium exchange pump did not displace binding (Freedman, Ph.D. thesis, 1982).

More recently it has been suggested that the DA receptor linked to a stimulatory adenylate cyclase (D_1) may not have a physiological function (Laduron, 1980). Studies with dopaminergic ligands imply that the non-cyclase linked binding site is the high affinity, functional receptor which is stimulated at low nanomolar concentrations. In contrast, D_1 may be a low affinity, non-functional receptor which requires high micromolar concentrations of DA for activation. The correlation between dopaminergic agonists at producing renal vasodilatation and displacing [^{3}H] sulpiride binding, in contrast to the lack of correlation between the physiological response and adenylate cyclase activity, provides further evidence that the DA receptor linked to a stimulatory cyclase is not physiologically significant. As shown in table 4.1, the EC_{50} for ADTN on the adenylate cyclase is 10 μM whereas the K_i for the displacement of [^{3}H] sulpiride binding

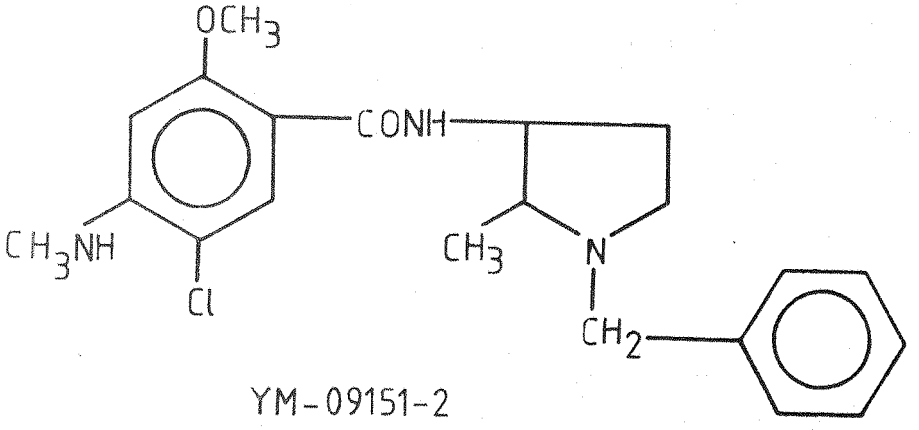
FIGURE 4.5 Structure of sulpiride and 2 structurally related lipid soluble benzamides YM08050, N-(1-benzyl-3-pyrollidinyl)-5-chloro-2-methoxy-4-methylamino-benzamide and YM-09151-2, cis-N-(1-benzyl-2-methyl-py rolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide.



sulpiride



YM-08050



YM-09151-2

Drug	renal blood flow ED_{50} (mol/kg)	dopamine-sensitive adenylate cyclase EC_{50} (M)	[^{3}H] sulpiride binding K_i (M)
Dopamine	2×10^{-9}	6.3×10^{-5}	6.98×10^{-7}
ADTN	2.3×10^{-9}	1.0×10^{-5}	0.122×10^{-7}
SKF	0.53×10^{-9}	$*1.4 \times 10^{-5}$	$*5.2 \times 10^{-7}$

* partial effects

TABLE 4.1 Comparison of the relative potencies of dopamine receptor agonists on renal vasodilator responses, adenylate cyclase responses and the displacement of [^{3}H] sulpiride binding in the guinea pig.
Abbreviations: ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene); SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine).

is 12.2 nM. These values represent an 800 fold difference in the sensitivities for agonists of the two DA model systems and in that respect support the suggestion that the dopaminergic binding site is more representative of dopaminergic function. The differences in the sensitivities of vascular responses and the DA-sensitive adenylate cyclase are even greater. For example ADTN produces an increase in RBF in the same nanomolar concentration range in which it inhibits binding (5 fold difference) whereas far higher concentrations (4350 fold difference) are required to stimulate cAMP formation.

However, these values are not directly comparable since the stimulation of DA receptors is studied using different parameters as a measure of their activation. Namely the D_1 receptor is assumed to be connected to the activation of an adenylate cyclase, whereas the process of ligand binding is assumed to be a function of the D_2 receptor. Recently solubilisation of the DA receptor has enabled binding of [^{3}H] DA to both the membrane fractions which are associated with adenylate cylase activity and those which are not (Nishikori et al, 1980). In these studies the fractions referred to as 'D_1' and 'D_2' had K_d values of 3.6 μM and 12 nM for DA respectively. This again implies that the affinity of the adenylate cyclase for DA is 300 times lower than that of the non-cyclase linked component. On the other hand the theory that the low-affinity adenylate-cyclase linked receptor may represent a functional entity should not be prematurely eliminated, without first considering the events which take place within the synaptic cleft itself. It is difficult to reconcile current concepts about whether 'high affinity' or 'low affinity' receptors represent the functional unit of the DA receptor without first knowing what concentration of DA is required to elicit a response by activation of receptors in the synapse. The K_d for [^{3}H] DA binding in calf and rat striata is between 2-3 nM (List et al, 1980) which is an indication of the low circulating levels of DA. However, a correlation between the circulating levels of a substance and its K_d on binding sites is only viable when dealing with hormone receptor sites, since tissues respond to the changes in continuous circulating hormone levels. In contrast the mechanism of action of neurotransmitter elicited responses function by the discontinuous discharge of quanta of transmitter into highly localised synaptic areas, followed by rapid removal via uptake processes. Thus, the concentration of DA at receptor sites within the synaptic cleft during

release may rise into the micromolar or even millimolar range and be sufficient to stimulate adenylate cyclase activity, whereas the circulating levels of DA remain low. In fact Titeler et al (1978) have supported such a proposal by speculating that the physiological concentration of DA released into the synapse may be as high as 1-10 μM. Thus, a low affinity DA-sensitive adenylate cyclase might be activated by very rapid elevations in local DA concentrations. For example, in the study of cholinergic receptors a similar situation arose where Birdsall et al (1978) first used the ligand [^{3}H] oxotremorine-M to label the muscarinic receptor. They concluded that brain membranes had three classes of receptors: 'super high', 'high' and 'low', which demonstrated different affinities for the agonist label, but were uniformly labelled by antagonist ligands. Recently from evidence provided by their study of [^{3}H] cis-methydioxolane binding to these varying affinity sites, Roeske and Yamamura (1980) suggested that modulators such as guanine nucleotides, sodium or isoprenaline (β-adrenoceptor occupancy) shift the super high and high affinity sites to a lower affinity which results in the reduction of cAMP production. Thus, the low affinity muscarinic receptor appears to be the more physiologically significant and operates via an inhibitory nucleotide-adenylate cyclase complex, similar to that in the models suggested by Rodbell (1980). Furthermore, Roeske and Yamamura (1980) suggested that the muscarinic receptor exists as a single complex which has multiple states of affinity for the agonist and that modulators can change this affinity. In the study of DA receptors, the problem of relating results obtained *in vitro* to the physiological mode of receptor regulation *in vivo* is analogous since we are dealing with a neurotransmitter agent rather than a hormone. In his review, Laduron (1980) similarly suggested that the DA receptor was most likely a single macromolecule which may contain different subunits within the receptor complex to mediate the effects of agonists and antagonists. Such a unitary concept is far more attractive than current speculations about the existence of up to four different types of dopaminergic binding sites (Seeman, 1980; Sokoloff et al, 1980).

The results of the present study suggest that there are specific DA receptors located in the guinea pig renal cortical vasculature which may mediate renal vasodilatation. These receptors are pharmacologically similar to those identified in the CNS by [^{3}H] sulpiride

binding studies and the stimulation of a DA-sensitive adenylate cyclase. The guinea pig renal vasculature is a valid model for the study of DA receptors which has certain advantages over the CNS where such studies are hampered by a lack of appropriate DA receptor model systems. For example, indirect behavioural responses which are attributed to DA receptors *in vivo* may not originate from the same brain area which is dissected out for *in vitro* binding and adenylate cyclase studies. In the periphery, it is possible to directly measure, the physiological responses produced by DA *in vivo* and to compare these with *in vitro* biochemical models for more accurate quantitative evaluation of the pharmacological characteristics of DA receptors in the same tissue.

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