SUBSTITUTED 2-AMINO-TETRAHYDRONAPHTHALENES AS AFFINITY AND PHOTOAFFINITY PROBES FOR DOPAMINE RECEPTORS

By

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SUBSTITUTED 2-AMINOTETRALINS AS DOPAMINE RECEPTOR PROBES

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ABSTRACT

In the current investigation several biochemical techniques, including solubilization, affinity chromatography and photoaffinity labelling, were used to purify the D-1 dopamine receptor. The design and synthesis of novel analogues of the dopamine agonist ADTN (2-amino-5,6-dihydroxy-tetrahydronaphthalene) to be used as affinity and photoaffinity probes was an integral part of this investigation.

Solubilization of the D-1 receptor was achieved with several detergents although cholic acid proved to be the most effective for receptor solubilization prior to affinity chromatography. Using this procedure, yields of solubilized receptors of greater than 30% were consistently obtained.

An affinity chromatography protocol utilizing an ADTN analogue covalently coupled to an affinity matrix was established for cholate-solubilized D-1 receptor. The affinity protocol developed during this investigation purified the D-1 receptor approximately 50-fold, while an average of 8% of the receptors were recovered. These results were superior to any previous literature reports of D-1 receptor purification.

Several photoactive compounds were synthesized and used to crosslink D-1 receptors. One compound in particular, a photoactive derivative of the dopamine agonist ADTN, proved to be a useful ligand for this purpose. A tritiated derivative of this compound was covalently and specifically incorporated into a protein of M.W. = 79 kDa. This was the first report that the D-1 receptor had a M.W. of greater than 70 kDa as determined by affinity crosslinking. Several other investigators have subsequently confirmed this observation.

Other photoactive compounds radiolabelled with ^{125}I were synthesized and examined for activity as D-1 and D-2 receptor probes. These compounds were not as useful as photoaffinity labels for dopamine receptors as had been originally proposed. The compounds did label a 50 kDa protein which was determined to be neither the D-1 nor D-2 receptor. This protein (originally designated Apo-50 and later CatNAP) has very interesting properties as it possesses binding activities with several catecholamines which are without precedent in the literature.

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LIST OF ABBREVIATIONS

Å	Angstrom
ADTN	2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydro-naphthalene
AP-ADTN	N-3-aminopropyl-ADTN
Apo	Apomorphine
Apo-50	50 kDa Apomorphine Binding Protein
ATP	Adenosine triphosphate
cAMP	cyclic-3',5'-Adenosine monophosphate
CatNAP	Catecholamine Absorbing Protein
Ci	Curie
CNS	Central Nervous System
CPM	Counts Per Minute
D-1	Adenylate Cyclase Linked Dopamine Receptor
D-2	Non-Adenylate Cyclase Linked or Negatively Linked Dopamine Receptor
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DPAT	Dipropyl Aminotetralin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g	Gram
x g	Times Gravitational Force
Gpp(NH)p	5'-Guanylyl Diphospho-imidophosphate

GTP	Guanosine Triphosphate
hr	Hour
IC_{50}	Inhibition Constant for 50% Response
K_d	Dissociation Constant
kDa	Kilo Daltons
L	Litre
m	Meter
M	Molar
MS	Mass Spectra
M.W.	Molecular Weight
N	Normal
NMR	Nuclear Magnetic Resonance
NPA	N-propyl-norapomorphine
PABA	para-Aminobenzoic Acid
PPHT	2-(N-phenethyl-N-propyl)amino-5-hydroxytetralin
PPP	3-(3-Hydroxyphenyl)-N-propylpiperidine
SDS-Page	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
TCA	Trichloroacetic acid
TEA	Triethylamine
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra Violet
Vol	Volume

CHAPTER 1

INTRODUCTION

1.1 CATECHOLAMINES IN THE CNS

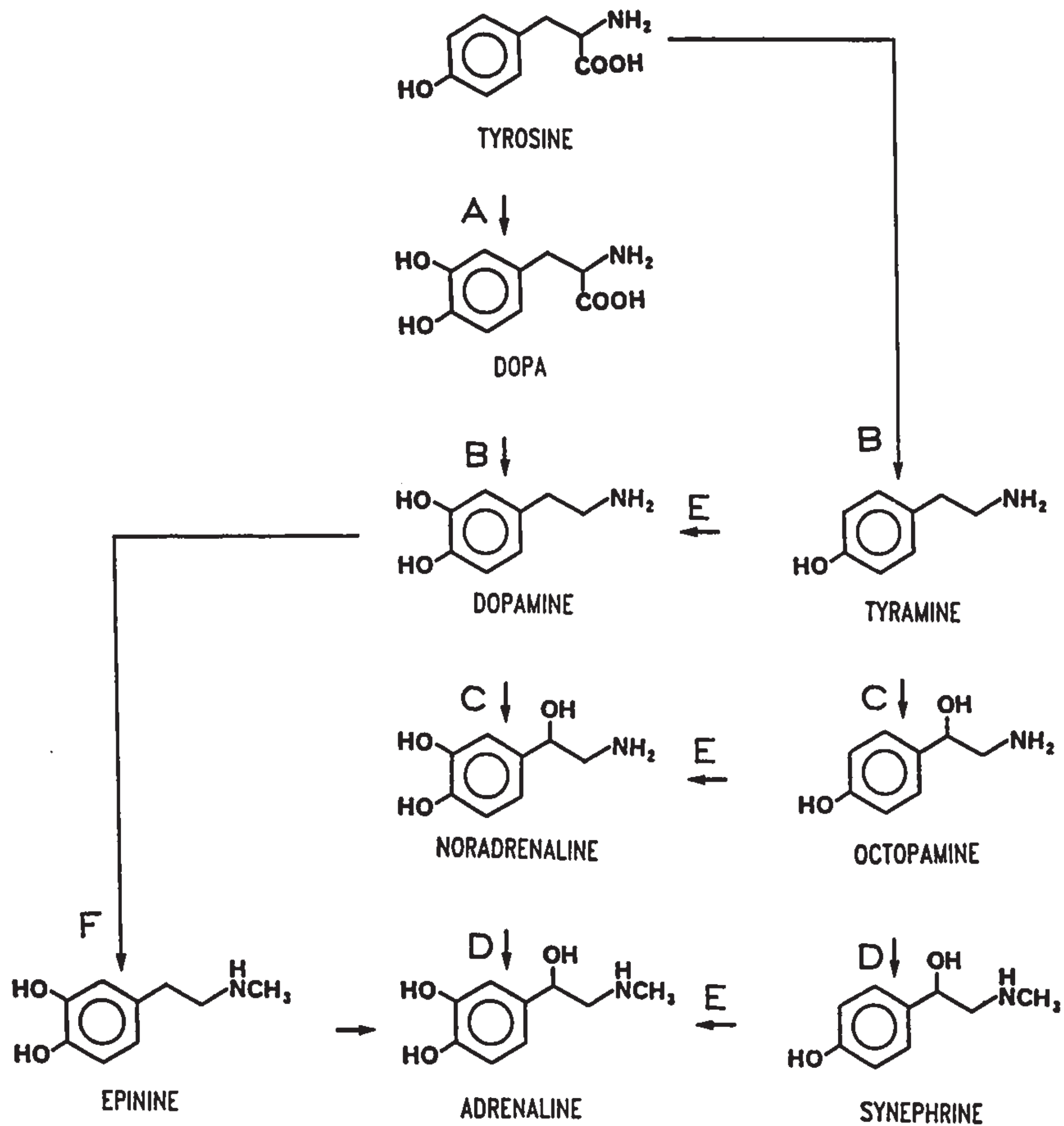
The term catecholamine can be used to describe any organic compound containing an amine and a catechol ring (a benzene with two adjacent hydroxyl groups). In general, the term catecholamine is used by investigators to imply dopamine, adrenaline or noradrenaline. In this thesis, however, the term will be used to describe catecholamines in the general chemical sense, rather than just to describe the three most common endogenous compounds mentioned above. On this basis, dopamine, adrenaline and noradrenaline will be designated endogenous catecholamines to distinguish them from the many other catecholamines which will be described in this thesis.

1.1.1 Metabolic Pathways of Endogenous Catecholamines in the CNS

Anabolic pathways of endogenous catecholamines have been studied for many years and have recently been summarized by Cooper et al (1986). The biosynthesis begins with the hydroxylation of tyrosine by the enzyme tyrosine

hydroxylase, which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Subsequently, DOPA is decarboxylated by the enzyme L-aromatic amino acid decarboxylase to form dopamine. Although this is the major synthetic pathway for dopamine, it can also be formed by decarboxylation of tyrosine to tyramine with subsequent hydroxylation to form dopamine. Dopamine can then serve as a substrate for dopamine-ß-hydroxylase, converting dopamine to noradrenaline. Again, alternate pathways for the biosynthesis of noradrenaline exist, as this compound can also be produced by hydroxylation of octopamine formed from tyramine. Noradrenaline in turn is the major precursor of adrenaline, which is produced by N-methylation of noradrenaline by the enzyme N-methyltransferase. Adrenaline, too, can be formed by alternate pathways, being produced by appropriate modifications of synephrine or epinine. The biosynthesis of endogenous catecholamines and the enzymes catalysing these reactions are illustrated in Figure 1.1.

Degradation of endogenous catecholamines in the brain is mediated by two major catabolic enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO converts catecholamines to their corresponding aldehydes, as indicated in Figure 1.2. There are at least two types of MAO, designated MAO-A and MAO-B, differentiated by substrate and inhibitor selectivity (Cooper et al, 1986). The other



TYROSINE
DOPA
DOPAMINE
TYRAMINE
NORADRENALINE
OCTOPAMINE
EPININE
ADRENALINE
SYNEPHRINE
A
B
B
C
C
D
D
E
E
E
F
HO
NH₂
COOH
OH
NCH₃

FIGURE 1.1

Biosynthesis of Endogenous Catecholamines

Several alternate pathways may be involved in the formation of the endogenous catecholamines dopamine, norepinephrine and epinephrine. The enzymes which catalyse the reactions are tyrosine hydroxylase (A), aromatic amino-acid decarboxylase (B), dopamine-ß-hydroxylase (C), N-methyl transferase (D) and catechol-forming enzyme (E). See text for details. Adapted from Cooper et al, 1986.

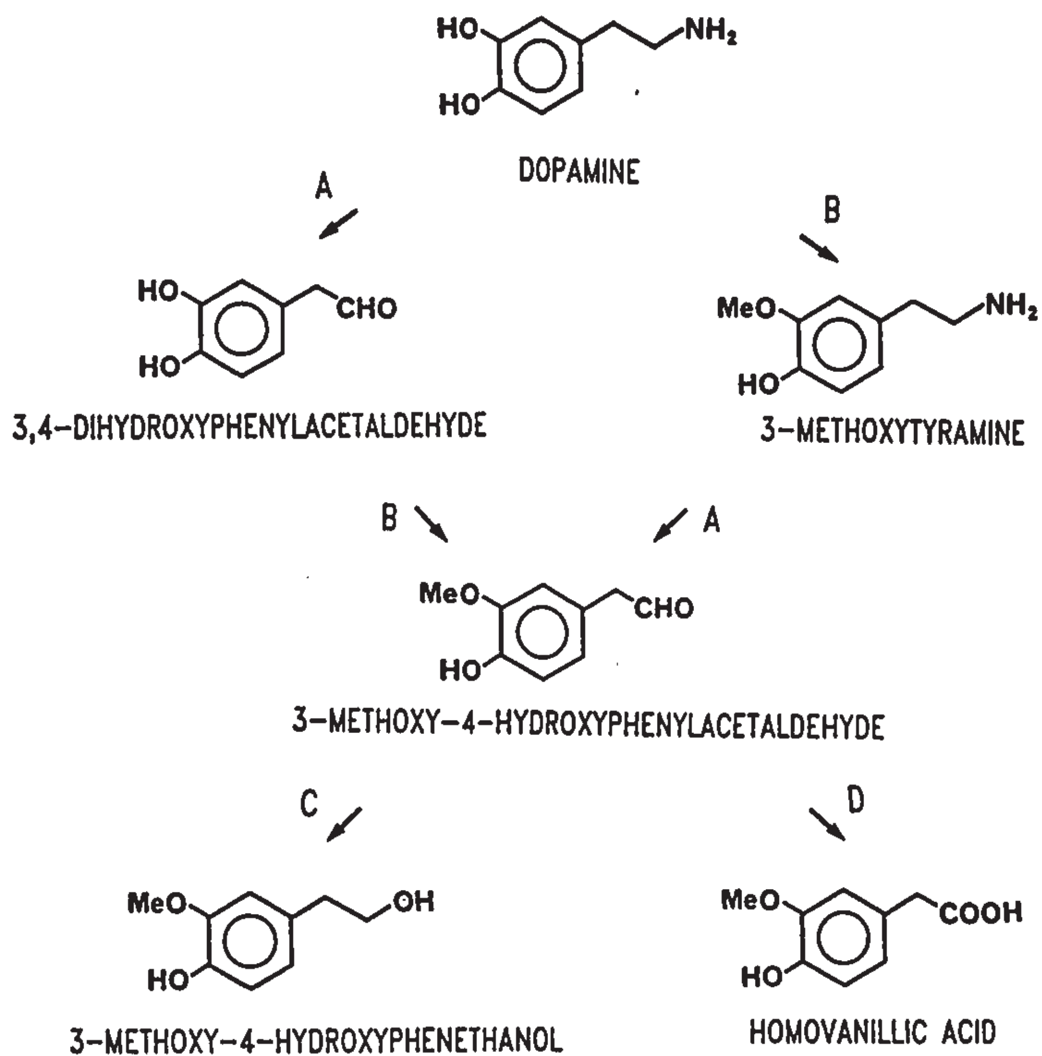

HO
HO
NH2
DOPAMINE
A
B
HO
HO
CHO
3,4-DIHYDROXYPHENYLACETALDEHYDE
MeO
HO
NH2
3-METHOXYTYRAMINE
B
A
MeO
HO
CHO
3-METHOXY-4-HYDROXYPHENYLACETALDEHYDE
C
D
MeO
HO
OH
3-METHOXY-4-HYDROXYPHENETHANOL
MeO
HO
COOH
HOMOVANILLIC ACID

FIGURE 1.2

Degradation of Endogenous Catecholamines

Endogenous catecholamines are degraded by several enzymes, including monoamine oxidase (MAO; A) and catechol-O-methyl transferase (COMT; B). These two enzymes can utilize each others products as substrates as shown. The products are then further metabolized by aldehyde reductase (C) or aldehyde dehydrogenase (D). The reactions are shown for dopamine only, norepinephrine and epinephrine also react in parallel pathways. Adapted from Cooper et al, 1986.

major enzyme of catechol inactivation is COMT. This enzyme was discovered by Axelrod *et al*, (1957) and catalyzes methylation of one of the aromatic hydroxyl groups as illustrated in Figure 1.2. The deaminated, methylated metabolites of catecholamines are subsequently reduced to the corresponding alcohol (by aldehyde reductase) or alternatively oxidized to the appropriate acid (by aldehyde dehydrogenase). These compounds generally leave the CNS *via* the circulatory system and are further degraded and excreted as free or conjugated metabolites.

1.1.2 The Role of Endogenous Catecholamines in the CNS

The presence of noradrenaline in peripheral adrenergic nerves was first demonstrated by von Euler (1946), who hypothesized that it may act as a neurotransmitter. Many subsequent studies confirmed this hypothesis and it was firmly established that noradrenaline was the neurotransmitter of peripheral adrenergic nerves. The presence of noradrenaline in the CNS was demonstrated by Vogt (1954) who suggested that noradrenaline may also act as a neurotransmitter in the CNS. This suggestion was based on the observation that noradrenaline was distributed in discrete regions of the CNS which did not correspond to the degree of vascularization in a specific region.

Subsequent investigation clearly demonstrated that noradrenaline plays a role as a neurotransmitter in the CNS. This conclusion is based on compelling evidence that noradrenaline fulfils all the criteria of a neurotransmitter; namely synthesis and storage in neurons, release in response to nerve stimulation, effector target response identical to nerve stimulation and appropriate pharmacological profile (Cooper et al, 1986 for review).

1.2 DOPAMINE IN THE CNS

1.2.1 Dopamine as a Neurotransmitter

The presence of dopamine in the mammalian brain was first demonstrated by Carlsson et al, (1958). These authors noted the presence of relatively high levels of dopamine in the brain, which led them to suggest that it may function as a "neurohumoral agent". Previous to this study dopamine was thought to be simply an intermediate in the biosynthesis of noradrenaline and adrenaline.

Many hallmark studies reported in the 1960's confirmed the hypothesis that dopamine did have an important role in brain function. The development of a fluorescent histochemical technique for dopamine allowed the mapping of dopamine neurons in the brain (Falk et al, 1962), and also

lead to the discovery in 1966 that levels of dopamine are depleted in certain regions of Parkinsonian brain tissue (Hornykiewicz, 1966). Other studies performed during that decade, including the discovery that antipsychotic drugs have a significant influence on levels of brain dopamine metabolites (Carlsson and Lindquist, 1963), established a relationship between dopaminergic function and psychosis. The advent of a histochemical technique also made it possible to localize dopamine neurons and led to an understanding of the extent and organization of dopaminergic pathways. Some of the major dopamine neuronal systems in the central nervous system (CNS) include the nigrostriatal, mesocortical, tubero-hypophysial and periventricular pathways (Cooper et al, 1986 for review). Different dopaminergic pathways are currently implicated as the locus for the pathogenesis of several disease states, to be discussed later.

1.2.2 Dopamine Receptors

Neurotransmitters are defined as "substances that are released from the axon terminal of a presynaptic neuron on excitation, and that travels across the synaptic cleft to either excite or inhibit the target cell" (Benjamin and Brackman Keane, 1987). Neurotransmitters exert their effect

on target cells by interacting with specific recognition sites, or receptors, which can be defined as "a molecule on the surface or within a cell that recognizes and binds with specific molecules" (Benjamin and Brackman Keane, 1987).

The concept of a "receptive substance" was originally proposed by Langley (1905) to account for the ability of nicotine to induce muscle contractions and the antagonistic effects of curare on these nicotine-induced contractions. In a subsequent classical report, Ehrlich (1913) introduced the term receptor, describing it as a molecular entity to which an introduced group could be anchored. These two landmark reports were the first in what has turned out to be many decades of exciting research into the biochemistry, physiology and pharmacology of receptor molecules.

Soon after the report of Ehrlich, Dale (also studying the actions of acetylcholine) described distinct responses of muscle to muscarine and nicotine (Dale, 1914). As both of these effects appeared to be mediated via the same mechanism as acetylcholine, Dale described a nicotinic and muscarinic subclass of the cholinergic receptor. Subclassification of a neurotransmitter receptor was later described by Alquist (1948) who grouped adrenergic receptors according to the order of potency of various catecholamines on different tissues. Receptors for many natural compounds have been described since that time and

today receptors and receptor subtypes for almost every biochemical class of compound (including biogenic amines, nucleotides, amino acids, sugars, steroids and oligomers of many of these) are known.

Most receptors currently being studied have been characterized using radioligand binding assays, a technique which became popular in the 1970's and has been used extensively to characterize a host of receptor proteins. It is important to realize, however, that radioligand binding does not, in itself, define a receptor. There are a number of criteria which have been developed and must be considered before a binding site can be considered a receptor. These have resulted from the observation that, at least to some extent, "everything binds everything". Clearly to define a true receptor site several criteria must be met to distinquish receptor binding from a simple adsorbtion process. These criteria include: 1) binding must be of high affinity and be saturable, 2) the affinity of competing agents must be relative to their pharmacological potency, 3) binding must have appropriate kinetics, time course and be reversible, 4) the distribution of binding should be appropriate and 5) binding should be stereoselective (Burt, 1978).

Soon after the advent of receptor binding studies in the early 1970's, Seeman (1974), Creese et al, (1975)

Trabucchi et al, (1975) and many others began to use this technique to study the dopamine receptor. These investigators demonstrated that [^{3}H]-dopamine and [^{3}H]-haloperidol appeared to label different populations of dopamine receptors and on this basis, suggested the existence of receptor subtypes. As additional dopaminergic ligands were discovered, many pharmacological classification schemes were developed and investigators have proposed as few as one to as many as four distinct subtypes (Stoof and Kebabian, 1984 for review). Classification schemes for dopamine receptors based solely on pharmacological criteria have not, however, produced a scheme acceptable to all investigators.

In the early 1970's dopamine was demonstrated to have the ability to stimulate the enzymatic activity of adenylate cyclase (Kebabian and Greengard, 1971). Spano et al, (1978) proposed that this feature distinguished two types of dopamine receptors either linked or not linked to adenylate cyclase. Kebabian and Calne (1979), however, were the first to propose a formal classification scheme based on association with cyclase activity and these investigators designated D-1 as the name for the receptor linked to adenylate cyclase and D-2 as the name for all dopamine receptors not linked to adenylate cyclase. The utility of this classification scheme quickly became apparent as

investigators found that many pharmacological and physiological effects were consistent with the D-1, D-2 nomenclature. Schachter et al, (1980) for example, demonstrated that dopaminergic compounds effective as anti-Parkinsonian agents appeared to interact with D-2 but not D-1 receptors. Although many other systems of dopamine receptor classification have been proposed, (e.g. Cooles and Rossum, 1976) none have proven as useful as the D-1, D-2 designation. Today essentially all investigators studying dopamine receptors use this method of classification.

Most researchers currently agree that there are only two dopamine receptor subtypes, however it is now clear that each can exist in more than one affinity state (Seeman et al, 1975, Creese et al, 1975). The affinity state of the receptor is modulated by guanyl nucleotide regulatory protein (G protein) as previously recognized for the ß-adrenergic (Lefkowitz et al, 1974), α-adrenergic (Williams and Lefkowitz, 1976) and opiate (Goldstein et al, 1971, Pert and Snyder, 1973) receptors. Literature reports which had previously suggested the existence of several dopamine receptor subtypes (including D-3 and D-4) are probably attributable to the different affinity states in which D-1 and D-2 receptors can exist.

1.2.3 Physiological Roles of Dopamine in the CNS

In the peripheral nervous system dopamine has several well defined roles in physiological processes, including stimulating the release of parathyroid hormone from bovine parathyroid gland (Dawson-Hughes et al, 1983 for review), and inhibition of prolactin release from the pituitary gland (Onali et al, 1981 for review). Other peripheral tissues (such as retina and carotid body) also utilize dopamine in well understood physiological processes. Conversely, relatively little is known about the physiology of dopamine in the CNS.

Although the relationship of the D-1 receptor and adenylate cyclase has been known for some time, only recently Walaas and Greengard (1984) identified a phosphoprotein (DARPP-32) which is found in cells possessing the D-1 receptor. Nestler and Greengard (1984) have postulated that phosphorylation of this protein may indirectly lead to a physiological response by regulating phosphorylation of other neuronal substrate proteins. Stoof and Kebabian (1984) have suggested that the discovery of this protein may advance our understanding of the role of the D-1 receptor. To date, however, the role of DARPP-32 in modulation of a physiological response is not understood.

Several physiological processes in the CNS have been identified which are mediated by the D-2 receptor. In the

striatum, stimulation of D-2 receptors on cholinergic interneurons blocks the release of acetylcholine (Euvrand _et al_, 1980). Release of dopamine from the terminal of nigrostriatal neurons is influenced by many substances, including dopamine and dopaminergic compounds (Langer, 1981). For example, the release of dopamine _in vitro_ can be blocked by dopamine and D-2 selective agonists (Creese _et al_, 1983). Dopamine and other agonists also inhibit the K^+ or electrically evoked release of dopamine, which can be restored by including D-2 antagonists.

In several brain regions, dopamine modulates the release of neuroactive brain peptides. In the hypothalamus, dopamine (mediated by D-2 receptors) inhibits the release of ß endorphine (Vermes _et al_, 1985). In the neostriatum, dopamine and D-2 specific agonists enhance the release of cholecystokinin (Conzelmann _et al_, 1984).

1.2.4 Pharmacology of Dopamine Receptors

Much of the evidence supporting the existence of two dopamine receptors has been obtained from studies on selective agonists and antagonists for D-1 and D-2 receptors (Spano _et al_, 1978). D-1 selective agonists and antagonists are usually identified by the effect they have on the dopamine-sensitive adenylate cyclase system, while D-2

selective compounds are usually characterized by behavioural or electrophysiological criteria. These differences in methodologies reflect the lack of a biochemical assay for the D-2 receptor or a behavioural model for the D-1 receptor. Although many compounds are known to interact with D-1 and D-2 receptors, only the most selective from each category will be described. The structures of several dopaminergic compounds discussed here can be found later in this chapter.

The most potent D-1 selective antagonist currently known is SCH 23390 (Iorio et al, 1983). This compound antagonizes the D-1 receptor at very low concentrations (sub-nanomolar), while high concentrations are required in order for it to have an effect on the D-2 receptor (Hyttel, 1983).

Several selective D-1 agonists are known including SKF 38393 and SKF 82526. These compounds mimic the ability of dopamine to stimulate adenylate cyclase activity (Watling and Dowling, 1981) and have essentially no D-2 activity. Both of these agonists are stereo-selective, as the S enantiomer of each compound is significantly less potent than the R enantiomer.

Many potent antagonists specific to the D-2 receptor have been described and include sulpride, domperidone and YM 09151-2. These compounds are highly specific for the D-2

receptor and have effectively no affinity for the D-1 receptor as measured by biochemical or radioligand binding data (Grewe et al, 1982).

Selective D-2 agonists have only been described in this decade, yet now include compounds from several distinct chemical families. RU 24926 and RU 24213 are currently the most potent D-2 agonists known. Although these compounds do not contain a chiral centre, other D-2 selective agonists (including [-]LY 141865) have been used to demonstrate stereo-selectivity of the D-2 receptor. A more detailed description of chemical features of these compounds is discussed later in this chapter.

1.2.5 Dopaminergic Dysfunction and Disease.

Alterations in CNS dopamine function is believed to underlie several disorders including schizophrenia, Parkinson's disease, Huntingtons' disease, tardive dyskinesia and Tourette's syndrome (Mishra, 1986 for review). Although each disorder arises from dopaminergic dysfunction, the etiology of each is very different. Schizophrenic symptoms, for example, are hypothesized to result from an overactivity of dopaminergic systems of the forebrain (Crow, 1980). Parkinson's disease, alternatively, is thought to be caused by a degeneration of dopaminergic

neurons of the nigrostriatal system (Barbeau, 1976, Price et al, 1978, for review). In several other disorders the etiology of the disease is not understood, yet dopaminergic compounds are effective in relieving the symptoms.

1.3 MOLECULAR CHARACTERISTICS OF DOPAMINERGIC LIGANDS

As discussed previously, the D-1 and D-2 dopamine receptors were originally distinguished on the ability of a number of agonists and antagonists to bind selectively to one or the other (Spano et al, 1978). Since that time many compounds have been identified which are selective for each subtype. Prior to the start of this study it was necessary to examine the structural features of dopamine agonists and antagonists selective for the D-1 or D-2 receptor. This information illustrated structural features observed in existing ligands, which was useful in the design of novel compounds used as dopamine receptor probes.

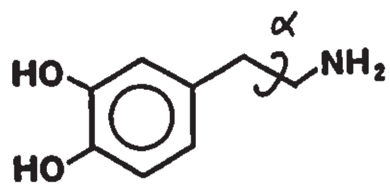
Dopaminergic compounds can loosely be grouped depending on whether they are D-1 or D-2 selective and whether they are agonists or antagonists. Although such a classification scheme is useful, radioligand binding experiments reveal most compounds are not exclusively D-1 or D-2, and many compounds have mixed agonist/antagonist properties. For the purposes of this discussion, compounds

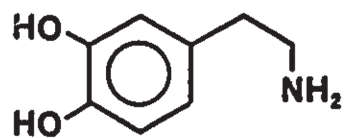
will be described as non-selective agonists, D-1 selective agonists, D-2 selective agonists, D-1 selective antagonists or D-2 selective antagonists depending on their most pronounced effect. This classification will provide a starting point from which to examine the stuctural features of these compounds.

1.3.1 Non-selective Agonists

Original investigations studying the structure-activity relationships of dopaminergic agonists focussed primarily on analogs of dopamine in a search for potent agonists. Studies reviewed by Woodruff, (1978) demonstrated that dopamine analogs that mimicked the extended conformation of dopamine (Figure 1.3) were of greater potency than those that mimicked more folded conformations. This conclusion was reached after the observation that ADTN was active in the CNS as a dopamine agonist while tetrahydroisoquinoline was inactive. Rotation of the α-bond in the dopamine molecule yields two conformations which can be compared to different isomers of ADTN (Figure 1.3) With respect to the hydroxyl positions in ADTN, the 6,7-dihydroxy isomer (A4) is more potent than the 5,6-dihydroxy compound (B4). This data would suggest that the extended (α)-rotomer, rather than the folded (β)-rotomer, is the

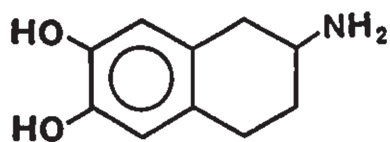
A

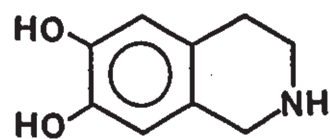




A 1

A 2

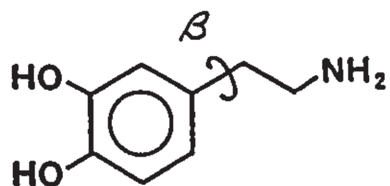


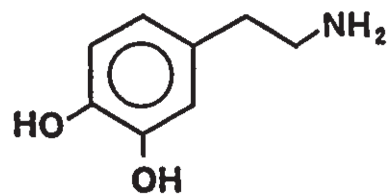


A3

A 4

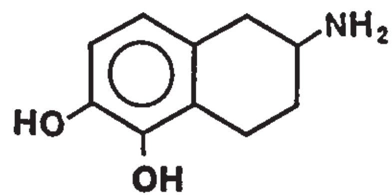
B





B 1

B 2



B 3

FIGURE 1.3

Rotamers of Dopamine and Their Rigid Analogues

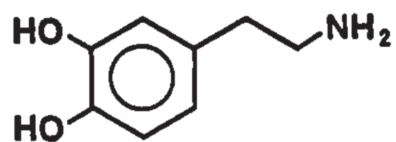
Rotation about the α bond of dopamine gives rise to an extended (A1) or folded (A2) conformation. ADTN (A3) is a rigid analogue of the extended conformation of dopamine, while tetrahydroisoquinoline (A4) is a rigid analogue of the folded rotomer of dopamine. Rotation about the ß bond of dopamine changes the position of the hydroxy group relative to the amine (B1 and B2). ADTN (A3) is a rigid analogue of the rotomer B1, while 2-amino-5,6-dihydroxy-tetrahydronaphthalene is a rigid analogue of dopamine in the conformation depicted by B2.

conformation which binds to the receptor.

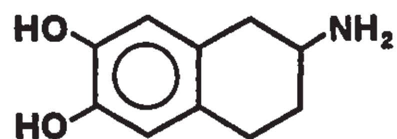
ADTN was identified as a potent dopamine agonist both *in vitro* (Woodruff *et al*, 1977) and *in vivo* (McDermed *et al*, 1975). Since these original studies, many analogs have been synthesized and today many potent compounds based on the 2-amino-1,2,3,4-tetrahydronaphthalene skeleton are known (Seeman, 1980; Cannon, 1985; Kaiser and Jain, 1985 for review). The structures of several non-selective agonists, including dopamine, ADTN, N,N-dipropyl-ADTN and apomorphine are shown in Figure 1.4.

Some of the ADTN analogs currently recognized as potent dopamine analogs include N,N-dipropyl-ADTN, N,N-diethyl-ADTN and N-phenethyl-ADTN. These structures illustrate some of the features of 2-aminotetralins consistent with dopaminergic activity, which in this case are derivatives obtained by substitution on the nitrogen atom. Other substitution patterns have been studied by several groups of investigators and can be summarized as derivatives obtained by substitution of 1) the aromatic ring, 2) the nitrogen or 3) the non-aromatic ring. Substitution patterns which either potentiate or reduce the activity of 2-aminotetralins (relative to ADTN) will be briefly examined.

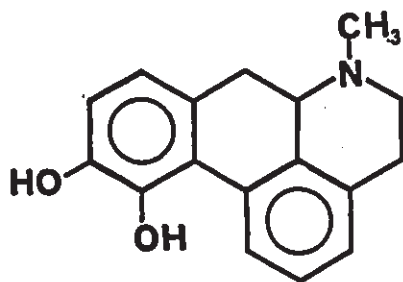
With respect to hydroxy substitution of the aromatic ring, detailed studies examining the phenolic (i.e.



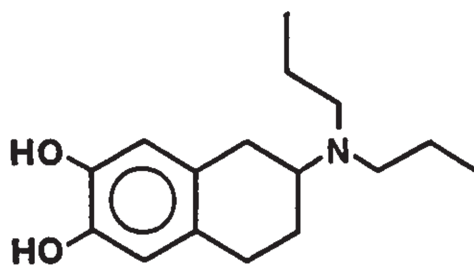
Dopamine



ADTN



Apomorphine



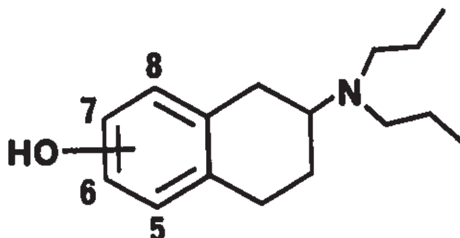
N,N–dipropyl–ADTN

FIGURE 1.4

Non-Selective Dopamine Agonists

Four examples of dopamine agonists which act at both the D-1 and D-2 dopamine receptors are dopamine, ADTN, Apomorphine and N,N-dipropyl-ADTN. All of these agonists have high affinity for the high affinity state of the receptor and relatively low affinity for the low affinity state of both receptor subtypes.

monohydroxy) compounds 5-, 6-, 7-, and 8-hydroxy-2-N-(di-N-propylamino)-tetralin (DPAT, see general structure below) have been performed.



hydroxy-DPAT

These studies concluded 5-OH-DPAT > 7-OH-DPAT > 6-OH-DPAT > 8-OH-DPAT in dopaminergic potency (Arvidsson <u>et al</u>, 1981 and references therein). Among catecholic 2-aminotetralins, hydroxy groups in the 6,7- positions are more potent than 5,6 (Woodruff, 1978), while the 7,8-, 5,7-, and 5,8-dihyroxylated isomers are either totally inactive or else have very little potency (Cannon <u>et al</u>, 1981).

Interesting results have been reported with several series of compounds substituted at the nitrogen position. McDermed <u>et al</u>, (1975) studied a series of 60 aminotetralins substituted at the N position with combinations of aliphatic chains of one to four carbons. Among this group of congeners, the N-(di-N-propyl) substitution pattern gave the

highest dopamine activity, which was about the same as ADTN itself. Either shortening (di-N-ethyl, di-N-methyl) or lengthening (di-N-butyl) the aliphatic chain reduced the potency of these compounds. In more recent studies several other N substituted aminotetralins, including N-phenethyl-ADTN, have also been demonstrated to possess potent dopaminergic activity (Beart et al, 1987)

Investigators have also examined the effect of increasing or decreasing the size of the saturated ring in the ADTN molecule (Cannon et al, 1984a and 1984b). These studies concluded that either increasing (to seven carbons) or decreasing (to five carbons) the size of the saturated ring decreased dopaminergic activity.

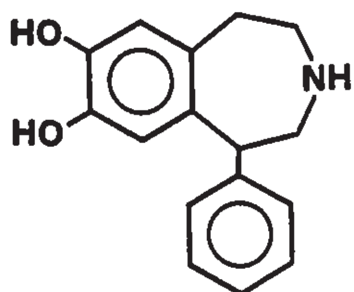
Other dopamine agonists, including apomorphine, are very potent compounds at both D-1 and D-2 receptors. Several of these compounds have many structural similarities with dopamine which Seeman (1980) has summarized as follows: 1) a hydrogen-bonding group (usually hydroxy) must be attached to the equivalent of the three position of dopamine on an aromatic ring, 2) there must be a nitrogen atom 0.6 Å out of the plane of the aromatic ring, which must also be 3) less than 7.3 Å from the hydroxyl group. Several other quantitative features, such as high fat solubility or an additional hydroxy group in the aromatic ring have been observed to enhance potency.

1.3.2 D-1 Selective Agonists

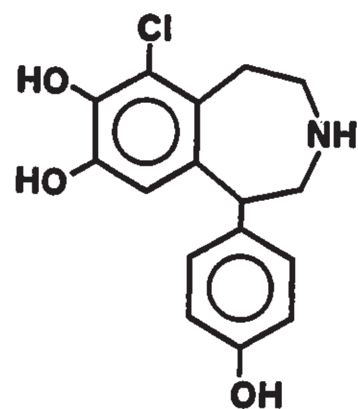
Several other catecholamine compounds, such as SKF 38393, SKF 82526 and dihydroxynomifensine (Figure 1.5) are potent dopamine agonists which act specifically at the D-1 receptor. Dihydroxynomifensine, in particular, shares many of the structural features previously described for the potent aminotetralin compounds. The two SKF compounds are also consistent with the structural limitations previously outlined. The benzazepine ring of these compounds permits the nitrogen to be an appropriate distance from the hydroxy groups as well as within the limitations with respect to the plane of the catechol ring.

1.3.3 D-2 Selective Agonists

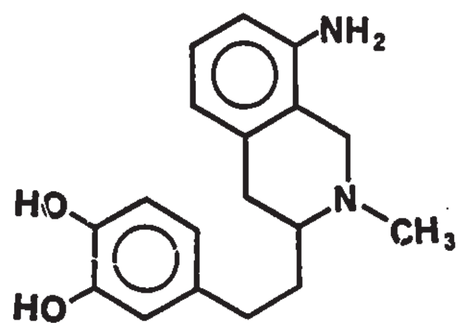
In the past few years several compounds have been shown to stimulate D-2 but not D-1 receptors. These compounds include several di-N-substituted phenethylamines (Horn et al, 1984) and di-N-substituted 5-hydroxy-2-aminotetralins (Beart et al, 1987). These compounds are usually not consistent with the structural features outlined earlier, and as they have no affinity for the D-1 receptor,



SKF 38393



SKF 82526



Dihydroxynomifensine

FIGURE 1.5

D-1 Selective Agonists

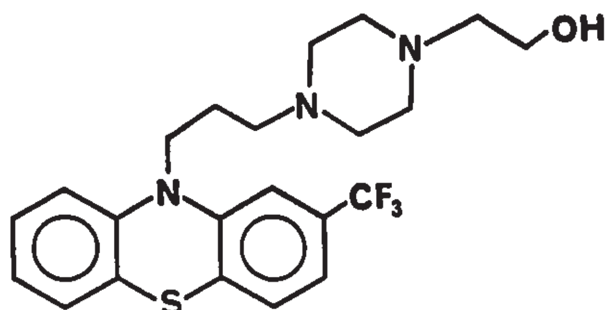
Three examples of selective D-1 agonists are SKF 38393, SKF 82526 and dihydroxynomifensine. All three of these molecules share many structural similarities with other dopaminergic agonists (see text).

were not thought to be appropriate model compounds for the current investigation.

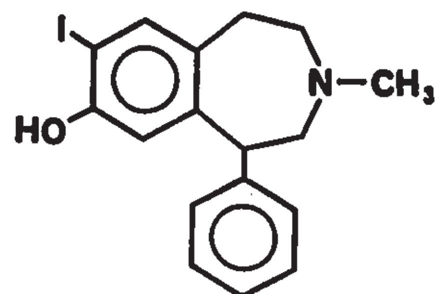
1.3.4 D-1 Selective Antagonists

The thioxanthines flupenthixol and fluphenazine (Figure 1.6) were the first compounds characterized as potent D-1 antagonists. While these two compounds bind to the D-1 receptor with very high affinity (Murrin, 1983), they also have reasonable affinity for the D-2 receptor (Montgomery, 1987). Like many other neurotransmitter antagonists, the thioanthines have very little in common structurally with the endogenous ligand and it is not possible to discuss similarities between them. As very little structure-activity data exists for these compounds, it is difficult to predict which functional groups on these molecules could be used for coupling in the synthesis of affinity and photoaffinity ligands. For this reason it was felt that they would not be good candidates for intermediates in the synthesis of D-1 receptor probes.

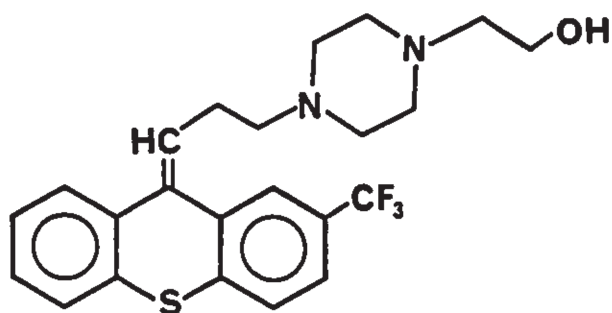
The most potent D-1 selective antagonist currently recognized is SCH 23390 (Figure 1.6). This compound has an extremely high affinity for the D-1 receptor (K_d = 0.1 nM, Hyttel, 1983). Like the aminotetralins already discussed, SCH 23390 has several structural features in common with



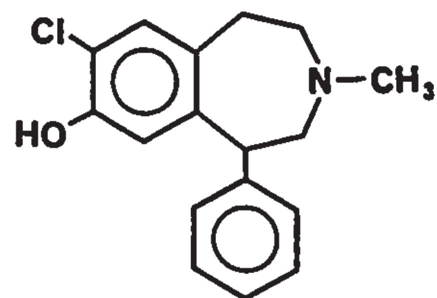
Fluphenazine



SCH 23982



Flupenthixol



SCH 23390

FIGURE 1.6

D-1 Selective Antagonists

Four examples of D-1 selective antagonists include Fluphenazine, SCH 23982, Flupenthixol and SCH 23390. The two benzazepines SCH 23982 and SCH 23390 share common features with other dopaminergic compounds, while the thioxanthines Fluphenazine and Flupenthixol have little in common with other dopaminergics.

dopamine. Firstly, both compounds have structural features consistent with the aminotetralins and other non-selective dopamine agonists described previously. Interestingly, antagonistic activity of these comounds is imparted by both the halogen and the N-methyl group. As can be seen from the stuctures of SCH 23390 and SCH 83566, either a Cl or an I can be substituted at the position of the halogen and potent antagonist activity is maintained (Itoh et al, 1984).

1.3.5 D-2 Selective Antagonists

The neuroleptic compounds (including chlorpromazine, haloperidol, spiperone, butaclamol etc.) were described in the early 1950's to be effective agents in ameliorating the symptoms of schizophrenia (Delay et al, 1952). These compounds were first proposed by Seeman et al. (1975) to bind to brain dopamine receptors. During the late 1970's many studies appeared (primarily using [^{3}H]-neuroleptic binding) which supported this hypothesis (Seeman, 1980 for review). Although the binding of radiolabelled neuroleptics is not used in the current definition of D-1 and D-2 receptors, Spano et al, (1978) used binding criteria in the original pharmacological definition. The currently accepted biochemical criteria of D-1 and D-2 as advanced by Kebabian and Calne (1979) was based on the effects of compounds on

dopamine sensitive adenylate cyclase, however it was evident at that time that [^{3}H]-neuroleptics bound to the non-adenylate linked, or D-2 receptor.

With respect to the structural characteristics of neuroleptics compared to those of dopamine, similarities are not immediately obvious. Several authors have presented structural studies where molecular features of dopamine are compared to those of neuroleptics. Seeman (1980) for example, has demonstrated a "structural fit" between phenethylamine and several neuroleptic compounds to show the similarities between the location of the amine and a phenyl ring of each compound. Although some similarities do exist, the relevance of these comparisons to the binding activity of these compounds has yet to be conclusively demonstrated.

The neuroleptic compounds have extremely high affinity for D-2 receptors, however have relatively poor affinity for D-1 receptors, which is the target protein for this study. Furthermore, the synthesis of affinity probes based on neuroleptic compounds would probably be a difficult task given the complexity of these molecules. Other detailed studies on the structural features of neuroleptics and dopaminergic activity have been presented but will not be described here, as these compounds were not felt to be suitable as intermediates for this study.

1.4 PURIFICATION OF NEUROTRANSMITTER RECEPTORS

Purification of any protein is prerequisite to detailed molecular characterization. Molecular characterization of a protein can include the primary amino acid sequence, secondary and tertiary structure, active site mapping and many other studies. An understanding of molecular features of a neurotransmitter receptor protein has been proposed by many investigators to be of great value in the design of more effective pharmaceutical agents.

Purification of neurotransmitter receptor proteins and subsequent protein sequencing also allows investigators to determine DNA sequence data. DNA probes can be used for a host of biochemical studies, which include gene regulation, post transcriptional processing and post-translational events such as covalent modification, transport, insertion into membranes, etc. Each of these steps employs processes which are potential targets for pharmaceuticals, raising the possibility that novel treatments for diseases caused by neurotransmitter receptor dysfunction may emerge.

1.5 PROBLEMS TO BE RESOLVED IN THE UNDERSTANDING OF DOPAMINE RECEPTORS

As previously described, several aspects of D-1 and D-

2 receptors are currently well understood. There are also, however, significant gaps in our understanding of many features of these two proteins. Among the more important aspects of these receptors which are not currently well understood are 1) function of the central D-1 receptor, 2) second messenger systems of the D-2 receptor, and 3) molecular aspects (including synthesis and regulation) of both the D-1 and D-2 receptors.

Each one of these aspects is, of course, a major area of research which may be pursued for many years in the future. The current study was aimed at purification of the D-1 receptor, as a necessary prerequisite to other investigations of this protein. These studies may yield insights into the biochemistry of the D-1 receptor which will provide a basis for the development of novel pharmaceuticals useful in the treatment of diseases which are a result of dopamine receptor dysfunction.

1.6 AIMS OF THIS STUDY

1.6.1 Purification of the D-1 receptor

In order to accomplish the purification of the D-1 receptor, it would be necessary to have a receptor probe which could be used as a precursor for the synthesis of an

affinity matrix or photoaffinity reagent. For either of these procedures, an intermediate is required which has two essential characteristics. The compound must 1) bind to the target protein with reasonable affinity and 2) must possess a functional group which can be used for coupling of the compound to an affinity matrix or a photoactive reagent. The functional group must also not participate in the recognition process with the target protein or the chances of the compound binding would be negligible.

As no compound existed at the outset of this study which fulfilled these criteria, it was necessary to first design and then synthesize a compound or compounds which would satisfy the requirements of an affinity probe for the D-1 receptor. It was therefore proposed that this study would include an examination of molecular features of existing receptor ligands, and the design of novel receptor ligands incorporating the required features. Subsequently, the designed compounds would be synthesized and examined for activity as D-1 receptor probes.

1.6.2 Solubilization of the D-1 Receptor

As previously discussed, solubilization is normally required before purification of an integral membrane protein can be accomplished. As existing literature procedures used

detergents not suitable for this study, an effective solubilization protocol needed to be developed.

1.6.3 Affinity Purification of the D-1 Receptor

In order to aid in the understanding of molecular aspects of the dopamine D-1 receptor, it was necessary to purify this protein. As purification by affinity chromatography has proven to be very useful in the purification of other neurotransmitter receptors, it was decided that the synthesis of an affinity matrix and development of a purification protocol would be a worthwhile objective for this study.

1.6.4 Photoaffinity Labelling of the D-1 Receptor

Photoaffinity labelling can also greatly aid in the understanding of molecular features of receptor proteins. As many important aspects of the D-1 receptor are not understood, and because photoaffinity labelling can greatly aid in the purification process, it was also proposed that photoaffinity crosslinking reagents would be synthesized. A flow chart of the proposed purification scheme is outlined in Figure 1.7. Two potential schemes (I and II) were identified which could be followed depending on the

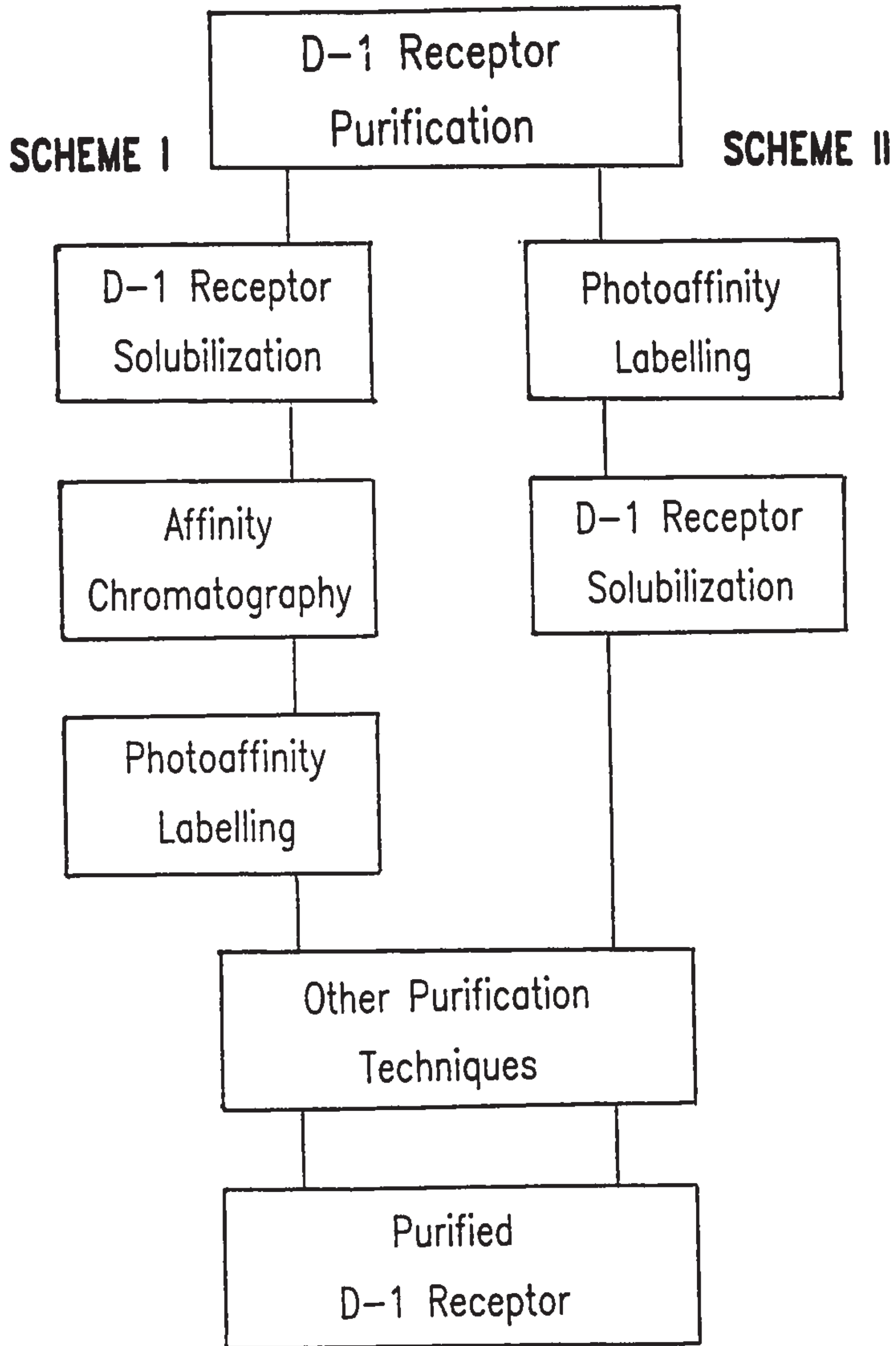

D-1 Receptor
Purification
SCHEME I
SCHEME II
D-1 Receptor
Solubilization
Photoaffinity
Labelling
Affinity
Chromatography
D-1 Receptor
Solubilization
Photoaffinity
Labelling
Other Purification
Techniques
Purified
D-1 Receptor

FIGURE 1.7

D-1 Receptor Purification Schemes

Two schemes were proposed for the purification of the D-1 receptor. In Scheme I the receptor would be first solubilized and subsequently purified using an affinity chromatography technique. The partially purified receptor could then be photoaffinity labelled and purified to homogeneity using such methods as ion exchange, hydroxy-apatite, hydrophobic interaction or lectin chromatography. Alternatively, the receptor could be photoaffinity labelled while still in the membrane bound state and then solubilized. Affinity chromatography could not subsequently be used for purification as the binding site of the receptor would be blocked; however, other chromatographic techniques could be used to further purify the protein. It was proposed that a radioactive photoaffinity label would be used, as this would greatly enhance detection of the receptor thoughout subsequent purification protocols.

successful development of an affinity and photoaffinity protocol.

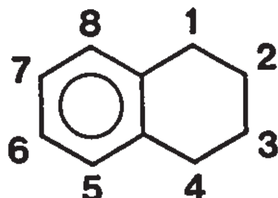
CHAPTER 2

DESIGN AND SYNTHESIS OF AFFINITY AND PHOTOAFFINITY LIGANDS

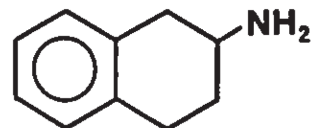
2.1 PROPOSED DOPAMINE RECEPTOR PROBES

For this study it was necessary to synthesize novel dopaminergic compounds requiring the following characteristics: 1) they must bind to the D-1 receptor, 2) they must have a functional group which can be utilized for coupling to an affinity matrix or heterobifunctional crosslinking reagent, 3) they must be amenable to the incorporation of radioactive isotopes to a high specific activity and 4) they must be relatively easy to synthesize. It was also hoped that the compounds would bind to the D-2 receptor, as they may also prove useful in D-2 receptor studies.

The stuctures envisioned at the start of this study which fulfilled all of these criteria were a series of substituted 2-aminotetralins, based on tetrahydronaphthalene:

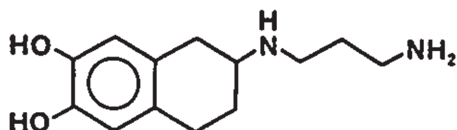


1,2,3,4-tetrahydronaphthalene



2-aminotetralin

Several observations were considered in the design of novel compounds, including the potency of 6,7-dihydroxy-2-aminotetralin (ADTN) and its N,N-dipropyl derivative, dipropyl-ADTN. From these compounds it was proposed that N-(aminopropyl)-2-amino-1,2,3,4-tetrahydronaphthalene (AP-ADTN, 7) would be a useful intermediate in the synthesis of D-1 receptor probes. AP-ADTN was the first compound synthesized for this study, after which additional 2-aminotetralins were proposed. This chapter details additional compounds proposed as affinity and photoaffinity ligands for D-1 receptor purification. Appendix 1 indicates the structures and numbering scheme used for all compounds proposed for this investigation.



7

2.1.1 Proposed Affinity Chromatography Matrix

The compound aminopropyl-ADTN (7) was proposed as a potential ligand for affinity chromatography. There are two features of this molecule which make it a likely candidate for such a role; (1) the compound possesses the structural features observed in other potent dopaminergic compounds and (2) it has a reactive primary amine which can be utilized in coupling the ligand to an activated affinity matrix. Most importantly, the terminal primary amino group used for coupling would not be intimately involved in the ligand-receptor interaction process.

Since the coupling reaction will occur *via* a primary amine, one of a host of affinity matrices could be chosen as the solid support. Most of the reactions that are effective in linking amine-containing ligands to solid supports are fairly lengthy (12 - 24 hr) and are carried out under basic conditions. Since catechols undergo facile air oxidation under basic conditions, it was decided that an alternate (milder) method would be required.

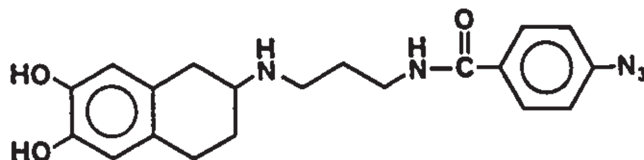
One such method available for the coupling of amine-containing ligands to solid supports involves reaction of the amine with active esters (such as N-hydroxysuccinimidyl esters) on the matrix. This reaction was first described by Cuatrecasas *et al*, (1970) who demonstrated the utility of

such a system in the purification of avidin by coupling biotin via such an ester. Although these reactions are also carried out under basic conditions, the reaction is very rapid (15-30 min), thereby minimizing the oxidation of the catechol system. An N-hydroxysuccinimidyl activated support is commercially available (BioRad Affi-Gel); AP-ADTN would be coupled to such a gel by the reaction shown in Figure 2.1.

2.1.2 Proposed Photoaffinity Crosslinking Reagents

As a result of structural studies such as those described in Chapter 1, several candidates were proposed as potentially useful photolabels for the D-1 receptor. The proposed stuctures could be derived from either AP-ADTN or one of the two monohydroxy analogs, the 6- or 7-hydroxy-(N-aminopropyl)-2-aminotetralins. Either of the two monohydroxy derivatives could easily be radioiodinated to produce iodophenolic photoaffinity ligands of high specific activity.

The photoaffinity ligand 10 was originally proposed



10

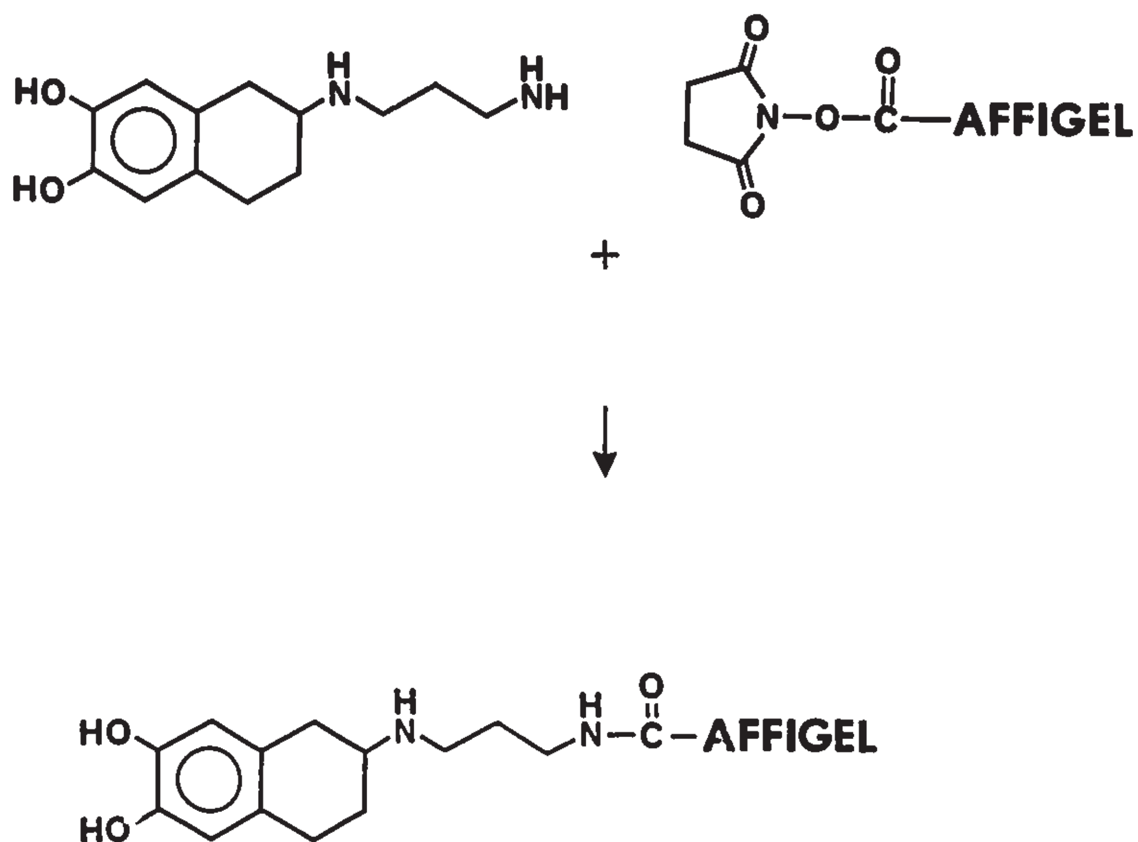

HO
HO
H
N
H
NH
O
N—O—C—AFFIGEL
O
O
+
HO
HO
H
N
H
N—C—AFFIGEL
O

FIGURE 2.1

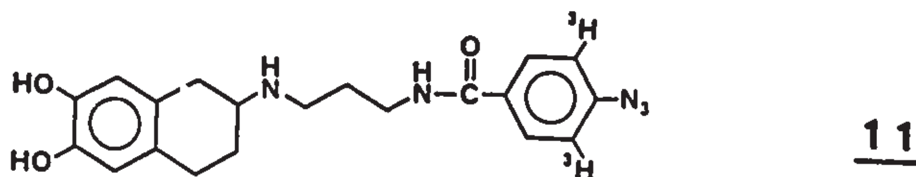
Synthesis of an AP-ADTN Afinity Matrix

AP-ADTN could be coupled to a N-hydroxysuccinimidyl ester activated affinity matrix directly as shown. The advantages to this chemistry are; (1) preservation of the basic nitrogen required for binding activity, and (2) mild conditions to prevent catechol oxidation.

as a relatively simple molecule which fulfils the desired molecular characteristics outlined in the previous chapter. This compound would also be relatively easy to synthesize from commercially available reagents.

The proposed synthesis of this compound is outlined in Figure 2.2. A radioactive derivative of 10 could be synthesized by utilizing tritiated N-hydroxy-succinimidyl azidobenzoate in Figure 2.2. The tritiated compound is also commercially available and was proposed to afford a simple route to a radiolabelled photoaffinity crosslinker.

Other catecholic photolabels (e.g. 11, 12 and 13) were also proposed which could be synthesized from the intermediate 7.



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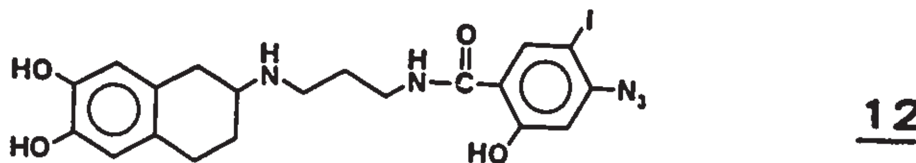

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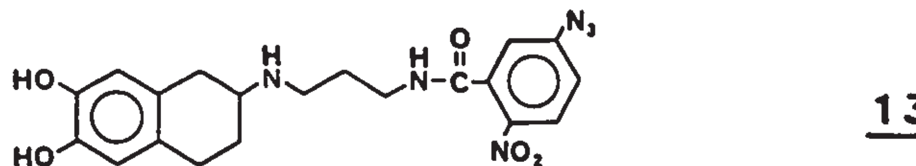

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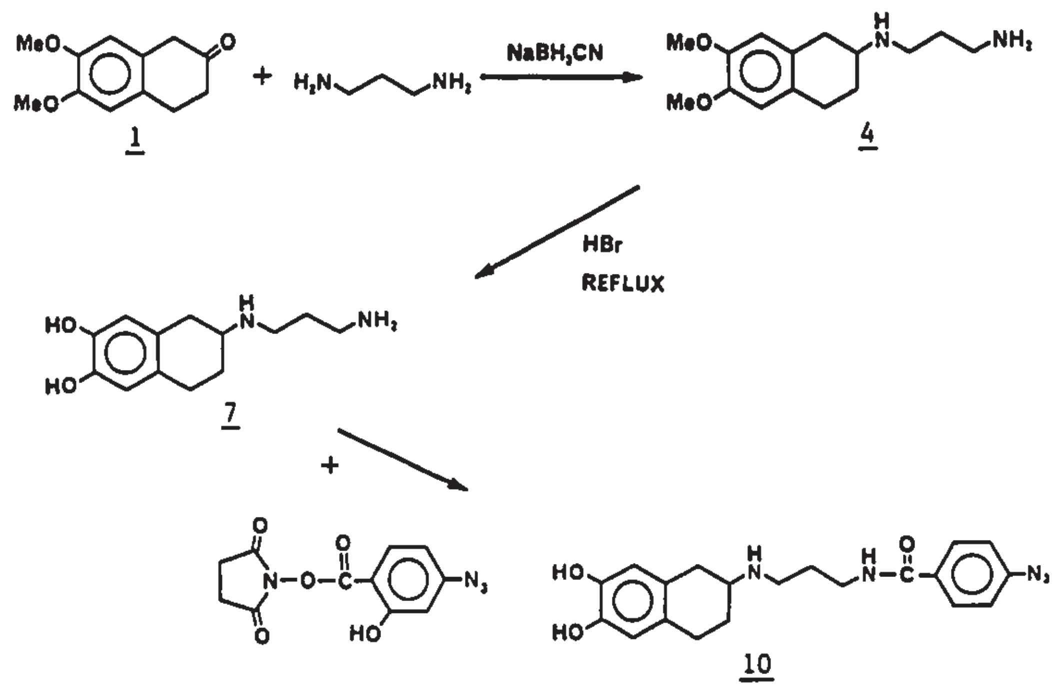
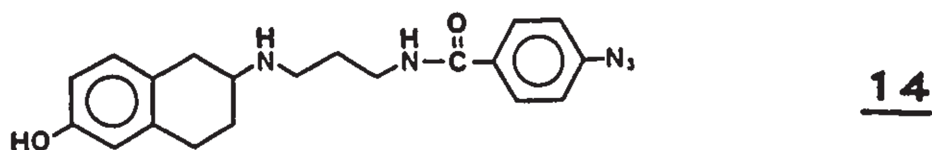

MeO
MeO
1
+
H_2N
NH_2
$NaBH_3CN$
MeO
MeO
H
N
NH_2
4
HBr
REFLUX
HO
HO
H
N
NH_2
7
+
O
N
O
O
C
O
HO
N_3
HO
HO
H
N
H
N
C
O
N_3
10

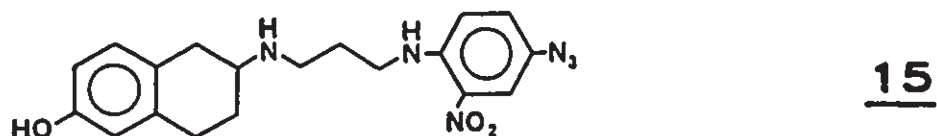
FIGURE 2.2

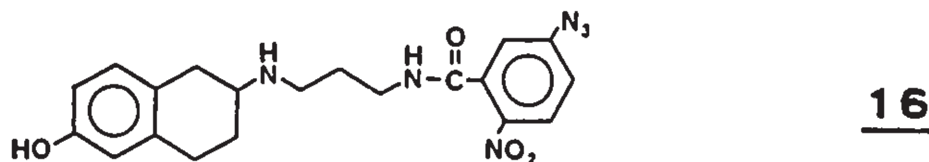
Synthesis of Photoactive ADTN Derivatives

Azidobenzamidyl-aminopropyl-ADTN could be synthesized by a three step reaction as shown. This compound was proposed as a photoaffinity ligand for the D-1 receptor, on the basis that it fulfills the molecular characteristics of compounds which bind to the D-1 receptor and posesses a photoactive functional group which could be used to alkylate the D-1 receptor.

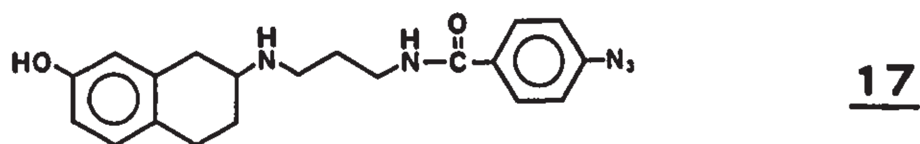
Substitution of appropriate starting compounds for 1 in Figure 2.2 would yield monohydroxy analogues of 7, providing the intermediates required for a distinct series of compounds. The intermediates could be used in a scheme similar to that outlined in Figure 2.2 to yield the 6-hydroxy compounds:



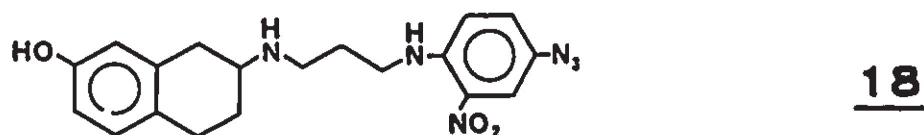




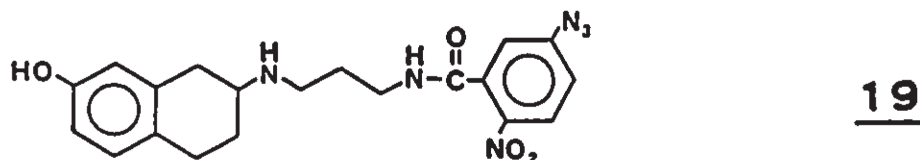
Alternatively, the 7-hydroxy tetralin be derivatized with the photoactive reagents to yield the analogues 17, 18 and 19.



17

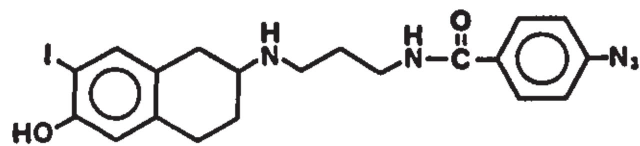


18

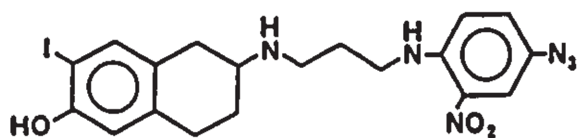


19

Although compounds 14 through 19 were expected to have low affinities for dopamine receptors, the introduction of an iodine at the 6- or 7- position of the 7- or 6- hydroxy compounds could produce compounds with substantially increased affinities toward the D-1 receptor, as these molecules would have considerable structural similarity with the benzazepines SCH 23390 and SCH 23982. Iodination of compounds 14 through 16 would presumably yield compounds 20-22; from iodination of 17, 18 and 19; compounds 23-25.



20



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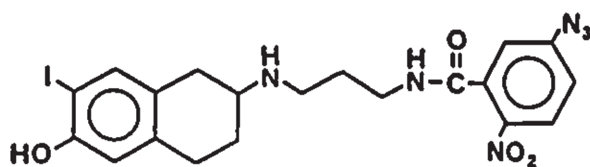
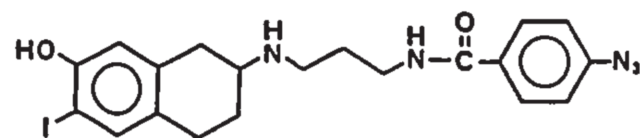
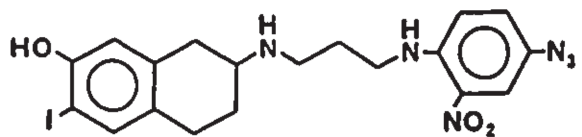


22



23



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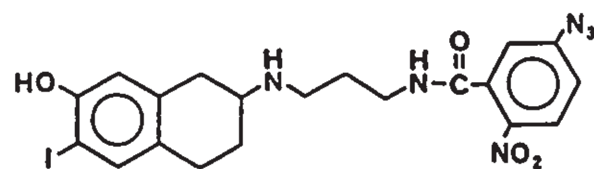


25

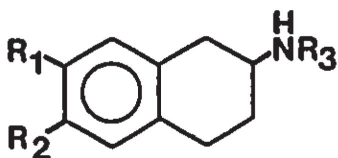
Two major advantages of compounds 20 through 25 were identified which would make them potentially superior to the corresponding catecholic photolabels for this study. Firstly, the compounds would have very high specific activities (greater than 2,000 Ci/mmol) which would greatly increase the sensitivity for detection of labelled proteins. Secondly, in all 6 compounds the radioiodine would be incorporated into the molecule as the last step in the synthesis. This avoids the technically difficult task of performing chemistry on trace quantities of radioiodinated compounds.

Table 2.1 lists the structures for photoaffinity labels proposed for this study and indicates the compound number used in this thesis.

2.2 SYNTHESIS OF DOPAMINE RECEPTOR PROBES

2.2.1 Materials and Methods

All reagents used in the synthesis of compounds described in this thesis were of the highest reagent grade available. The sources of all reagents are listed in Appendix 1.



R_3	R_1	R_2	COMPOUND
butyl–NH–C(=O)–(2-HO, 4-N_3, 5-I-phenyl)	OH	OH	12
butyl–NH–C(=O)–(4-N_3-phenyl)	OH	OH	10
	OH	H	17
	OH	I	23
	H	OH	14
	I	OH	20
butyl–NH–(2-NO_2, 4-N_3-phenyl)	OH	H	18
	OH	I	24
	H	OH	15
	I	OH	21
butyl–NH–C(=O)–(2-NO_2, 5-N_3-phenyl)	OH	H	19
	OH	I	25
	H	OH	16
	I	OH	22

TABLE 2.1

Proposed Photoactive 2-Aminotetralins

Several photoactive di- and mono-hydroxy-2-amino-tetrahydronaphthalenes were expected to have dopaminergic activity. Catecholic photoaffinity reagents could be synthesized as either non-radioactive or as tritiated compounds. Phenolic compounds could be synthesized as [^{125}I] derivatives which could potentially have greater utility due to their high specific activity.

2.2.2 Instrumentation

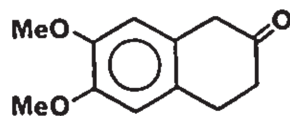
Instrumentation used for stuctural confirmation of all compounds synthesized is indicated in Appendix 2.

2.3 RESULTS

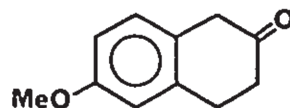
Spectroscopic data and other chemical characterization of the compounds synthesized for this investigation are included in Appendix 2 of this Thesis. Conditions for HPLC and TLC are also indicated in Appendix 2.

2.3.1 Synthesis of N-propyl Intermediates

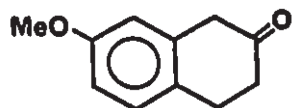
Compounds 10 through 25 were synthesized from one of the ketones 6,7-dimethoxy-2-tetralone (1), 6-methoxy-2-tetralone (2), or 7-methoxy-2-tetralone (3).



1



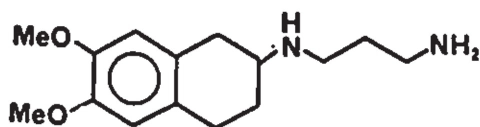
2



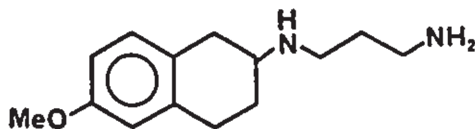
3

These compounds were either obtained from commercial suppliers or were prepared by Mr. K. Gatermann, a student in the laboratory of Dr. B.E. McCarry. A detailed description of the synthesis of these compounds is described by Gatermann (1987).

Reductive amination of 1, 2 and 3 with 1,3-diaminopropane to yield the intermediates 4, 5 and 6 was performed by a modification of the procedure described by Cannon et al, (1977).



4



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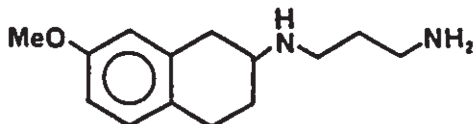


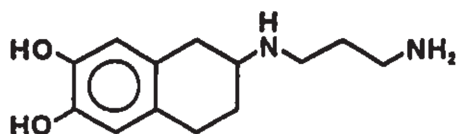
6

The procedure is illustrated for N-(3-aminopropyl)-2-amino-6,7-dimethoxy-(1,2,3,4)-tetrahydronaphthalene (4).

HCl (6M) was added to 1,3-diaminopropane (1 ml, 12 mmol) dropwise until pH 7.0 was reached. To this solution was added 0.25 g (1.2 mmol) of 6,7-dimethoxy-2-tetralone (1) dissolved in 5 mL methanol. The solution (total volume approximately 10 mL) was stirred for 10 min after which time 0.13 g (2 mmol) $NaBH_3CN$ was added. The mixture evolved gas briefly, after which the pH was checked and adjusted to pH 7.0 if required. The solution was stirred at room temperature for 24 hr and subsequently quenched with excess HCl. After H_2 evolution had ceased, the mixture was transfered to a 250 mL separatory funnel, basified with concentrated NaOH and the product extracted into CH_2Cl_2 (3 x 50 mL). The CH_2Cl_2 solution was extracted with 50 mM HCl (3 x 50 mL) to return the product to the aqueous phase. The combined acid extracts were pooled and washed with CH_2Cl_2 (3 x 50 mL) and the organic phase discarded. The acidic aqueous phase containing the product was again basified (conc NaOH) and the product re-extracted into CH_2Cl_2 (3 x 50 mL). The combined organic extracts were pooled, dried over anhydrous Na_2CO_3, filtered and the solvent removed under reduced pressure to give a green, oily product (average yield 0.22 g, 70%)

Cleavage of methyl ether groups from compounds 4, 5 and 6 to the catecholic or phenolic derivatives 7, 8 and 9 was performed by a modification of a reaction described by

Cannon et al, (1977). The product obtained from the previous reductive amination reaction was dissolved in 50 mL of 48% HBr and then refluxed for 4 hr under an N_2 atmosphere. The reaction mixture was then diluted with 200 mL of H_2O, heated to 70°C and the volatiles removed under reduced pressure. After the crude product was dry, the residue was dissolved in a minimal volume of methanol, to which ether was slowly added. The first product to precipitate was a dark brown tar from which the supernatant was removed by aspiration. The brown tar was dried and the procedure repeated. The supernatants obtained from both steps were pooled and the brown tar discarded. Additional ether was added to the combined supernatant and a fine white powder precipitated. The organic solvents were removed by aspiration and the product suspended in 2-3 drops of concentrated HCl for salt exchange. Addition of ether precipitated the HCl salt of the tetralins 7, 8 and 9 (average yield 83%).



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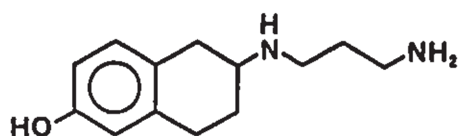
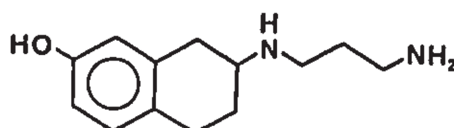


8



9

2.3.2 Synthesis of AP-ADTN Affinity Matrix

For affinity chromatography AP-ADTN (7) was coupled to Bio-Rad AffiGel 10 using a modification of the procedure described in Bio-Rad Bulletin 1099. For coupling, 25 mL of AffiGel-10 was filtered on a scintered glass filter funnel under vacuum and washed with 100 mL cold methanol. The Affi-gel cake was then transferred to a beaker containing 50 mL of methanol and 500 μL TEA. The mixture was placed on a stirrer in a glove box and purged with N_2. AP-ADTN (0.5 g) in 10 mL H_2O containing [^{125}I]-8 (as a coupling efficiency marker) was added and the reaction stirred for 30 min at room temperature. At the conclusion of coupling, the

affinity matrix was transfered back to the filtration funnel and washed with 500 mL of methanol, 500 mL H_2O and 200 mL of H_2O containing 0.1% ascorbate. An aliquot of the gel was counted for radioactivity and the amount of $[^{125}I]$-8 covalently coupled was determined. Using this procedure, 20 to 25 umol of AP-ADTN could be routinely coupled per mL bed volume of affinity matrix.

2.3.3 Synthesis of Photoactive Compounds

The catecholic photoactive compound N-(3-[N'-4-azidobenzamido]-aminopropyl)-ADTN (10) was synthesized from intermediate 7 and N-hydroxysuccinimidyl-4-azidobenzoate (NHS-AzB). For the reaction 50 mg (0.17 mmol) AP-ADTN was dissolved in 1 mL DMF and placed in a glove box purged with N_2. TEA (50 µL) and then 45 mg (0.18 mmol) NHS-AzB were added to the solution. The reaction was allowed to proceed for 15 min after which time the reaction was terminated with the addition of 100 µL of concentrated HCl. The product was recovered by precipitation with ether and was then recrystalized from methanol-ether to afford 53 mg (75% yield) of a white powder.

The phenolic photolabels were synthesized from the appropriate starting amines as described. For the affinity label 18 the 7-hydroxy-aminotetralin 9 was reacted with 4-

azido-1-fluoro-2-nitrobenzene (AzFNB). AzFNB was synthesized from 4-fluoro-3-nitroanaline as described by Levy (1973).

For the coupling reaction the diaminotetralin 9 (50 mg, 0.17 mmol) was dissolved in 0.5 mL of DMF containing 50 μL of TEA. To this solution AzFNB (28 mg, 0.17 mmol) was added and the mixture heated to 60°C for 24 hr in the dark. At the conclusion of the reaction 10 mL of H_2O containing 50 μL acetic acid was added to the mixture and was extracted with ethyl acetate (3 x 2 mL). Sodium bicarbonate was added to the aqueous phase (to pH > 7) and the product extracted into CH_2Cl_2 (3 x 10 mL). The solvent was dried over anhydrous sodium sulphate and then removed under reduced pressure to yield a red solid (average yield 63%).

Radioiodination of 18 was performed by a modification of the method of Bolton and Hunter (1973). Compound 18 (0.2 ug) in 10 μL of H_2O was added to 65 μL of 0.25 M phosphate buffer, pH 7.5. To this solution was added 50 μg chloramine-T in 10 μL H_2O and 1 mCi $Na^{125}I$. The reaction was allowed to proceed for 1 min and subsequently terminated by the addition of 120 ug sodium metabisulphate in 50 μL H_2O. The radioiodinated photolabel was purified by HPLC.

The 6-hydroxy-diaminotetralin 8 was used as a precursor to the proposed photolabel 22. It was originally proposed that 22 would be synthesized by iodination of 16,

however because of the limited solubility of 16 in water, compound 8 was first iodinated and than coupled to the photoactive moiety, rather then *vice-versa*.

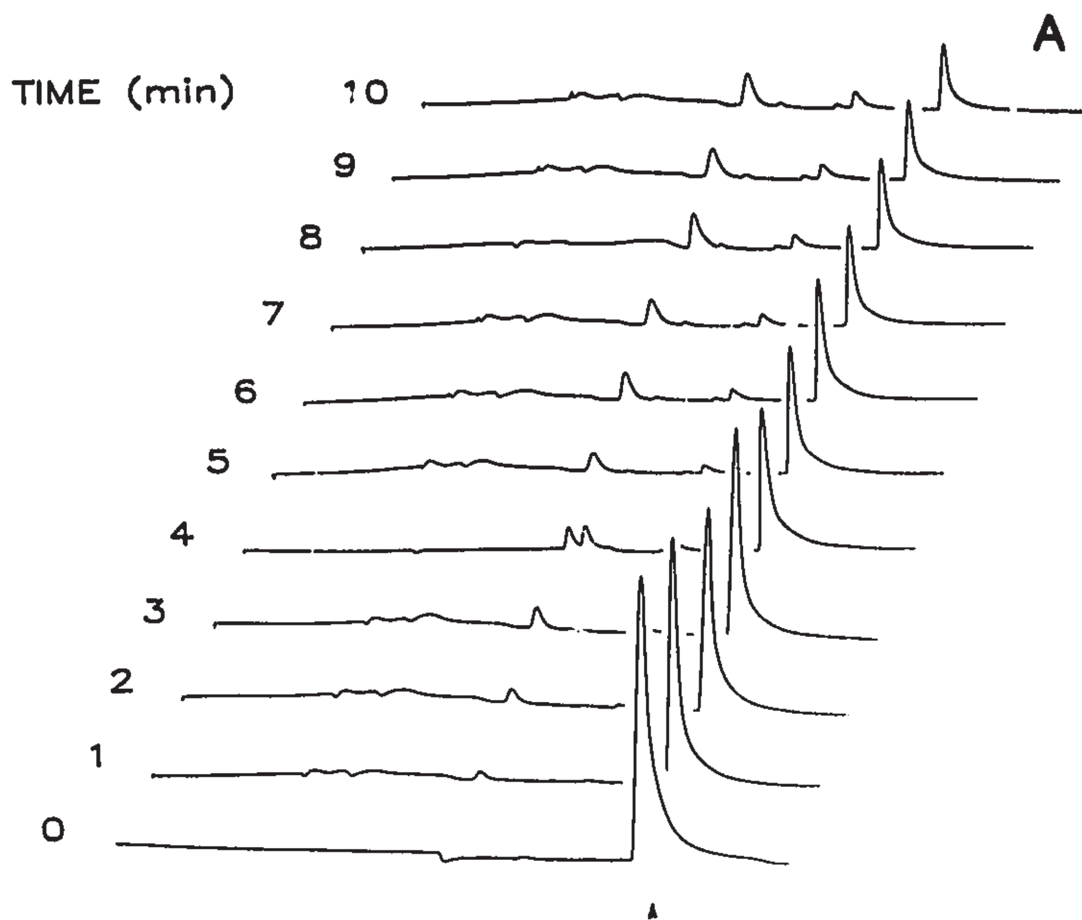
Iodination of 8 was performed as follows: compound 8 (0.5 g, 1.25 mmol) was dissolved in 10 mL H_2O and the pH adjusted to 9.0. In a separate tube I_2 (0.16 g, 1.25 mmol) and KI (0.31 g, 1.25 mmol) were dissolved in 1 mL water with gentle heating. After all of the I_2 had dissolved, it was added dropwise to the solution containing 8 with constant monitoring of the pH. Concentrated KOH was added during the iodination procedure to maintain a pH in the range of 9 - 9.5. After the addition of the I_2/KI solution was complete (approximately 30 min) the reaction was allowed to proceed for an additional hour. The solution was then acidified, and the solvent removed under reduced pressure. Methanol (20 mL) was added to the residue, which dissolved the diamines but not the potassium salts. The solution was then filtered, evaporated and recrystallized from methanol/ether as previously described for compounds 7 through 9.

Purification of the various products of the iodination reaction proved difficult, so the crude reaction product was used for subsequent derivatization to the photoactive product. For this reaction 50 mg of the iodination reaction crude product was dissolved in 1 mL DMF and 50 µL TEA was added to basify the solution. N-5-azido-2-

nitrobenzoyloxysuccinimide (38 mg, 0.125 mmol) was added and the reaction allowed to proceed for 15 min with occasional use of a vortex mixer. Concentrated HCl (50 ul) was added to terminate the reaction and ether (20 mL) was added to precipitate the products. The product (a yellow powder) was dissolved in 1 mL of methanol and purified by HPLC.

2.3.3 Photolysis of Photoactive Compounds

Both aryl azides and nitroaryl azides were photolysed with a mineralite UVSL-58. Short wave UV light was used for aryl azides, while long wave light was used for nitroaryl azides. At a distance of 2.5 cm from the source, photolysis of the compounds examined was essentially complete at 15 minutes (Figure 2.3).


A
TIME (min)
10
9
8
7
6
5
4
3
2
1
0

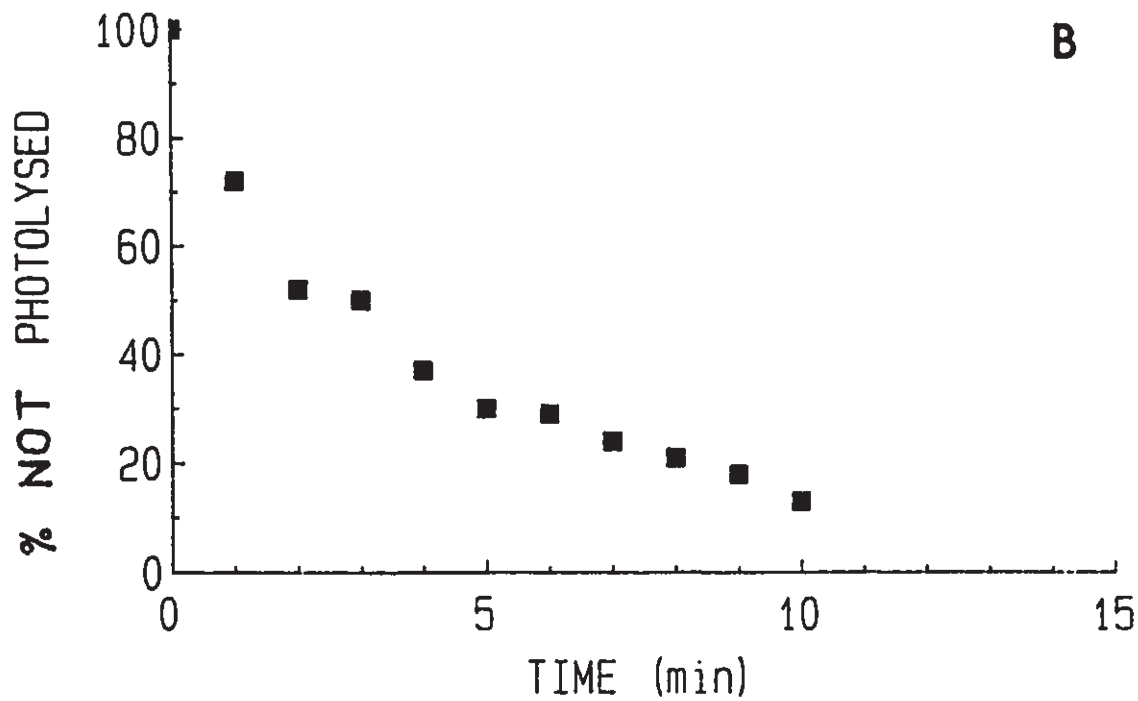

B
% NOT PHOTOLYSED
100
80
60
40
20
0
0
5
10
15
TIME (min)

FIGURE 2.3

Rate of Photolysis of Compound 16

Compound 16 (2 mg in 2 mL) was irradiated with long wave UV light from a Mineralight UVSL-58 at a distance of 2.5 cm. At the times indicated, an aliquot was removed and the products separated by HPLC as described in Appendix 4 (A). Compound 16 was photolysed as indicated by both the loss of 16 (arrow) and the appearance of more polar photolysis products (at earlier retention times). Over 90% of the 16 present was photolysed by 10 minutes, and only trace amounts could be detected after 15 minutes of irradiation. Compound 10 displayed a similar photolysis profile, however short wave UV light had to be used in order to consume all of the compound in 15 minutes. The decrease of compound 16 as a function of time (from A) is shown graphically in B.

CHAPTER 3

SOLUBILIZATION OF THE D-1 RECEPTOR

3.1 INTRODUCTION

One of the first steps in the purification of an intrinsic membrane protein is the removal of the protein from its lipid milieu. This process is normally performed with a solubilizing agent, or detergent, which facilitates the transition of the protein from its natural state within the membrane to an environment composed predominantly of detergent. For most purification schemes, it is essential that the solubilization process occur in a manner which minimizes loss of biological activity of the protein. It is normally essential that the protein maintain biological activity for two reasons: 1) the protein is normally identified by the presence of biological activity and 2) affinity chromatography is normally only possible if the integrity of the protein is maintained.

Many detergents are currently available to the biochemist for protein solubilization. The choice of detergent depends on many factors, including whether or not the chemical characteristics are compatible with the purification scheme. Some detergents can, for example, interfere with purification procedures, bind required ions,

or be difficult to remove. Any of these characteristics can result in a detergent not being suitable for a specific purification method. Of greater importance is the requirement that the detergent solubilize the protein in a biologically active state. This is a difficult feature to predict a priori and usually several detergents must be screened to obtain one which yields a significant amount of solubilized protein in a functional state.

Detergents are screened initially under relatively standard conditions, such as those described by Hjelmeland and Battersby (1984). Once a detergent is identified which proves useful for a solubilization, many other conditions must be optimized in order that the best yield of active protein can be obtained. Among the factors which contribute to the yield are the protein to detergent ratio, time and temperature of solubilization, salt concentration, the presence or absence of cofactors, ligands or substrates, and many other variables.

With respect to the D-1 receptor, studies have reported solubilization using the detergent cholic acid (Sidhu and Fishman, 1986). Cholic acid was not however felt to be an appropriate detergent for this study, as it tends to bind divalent cations. As divalent cations are required for D-1 agonist binding it was felt that this detergent would interfere with the affinity purification protocol.

Many studies have reported successful D-2 receptor solubilizations with a variety of detergents; most of the factors which influence solubilization yield have been optimized (Ramwani and Mishra, 1986; Elazar _et al_, 1988). Several groups of investigators were pursuing D-2 receptor purification at the onset of this study and since that time the receptor has been solubilized, purified to homogeneity (Senogles _et al_, 1986), sequenced and cloned (Bunzow _et al_, 1988).

3.2 METHODS

3.2.1 Caudate Membrane Preparation

For solubilization studies performed by Ross _et al_, (1985) using either octyl glucoside, digitonin, CHAPS or Triton X-100, membranes were prepared as follows: Bovine caudate tissue was homogenized with a polytron homogenizer (setting 6) for 20 seconds in 20 volumes of 50 mM Tris-HCl buffer (pH 7.35) containing 2 mM EDTA. The membrane suspension was subsequently centrifuged for 10 minutes at 50,000 x g, the pellet resuspended and centrifuged a second time in the same buffer. For radioligand binding assays, the pellet was resuspended in 40 volumes of 50 mM Tris-HCl (pH 7.35). For

solubilization, the pellet was suspended in 4 volumes of the same buffer containing 0.15 M KCl. Protein concentration was determined by the method of Lowrey *et al*, (1951).

3.2.2 Receptor Binding Assay

During solubilization studies, [^{3}H]-ADTN was used as a probe for the D-1 receptor. The procedure used was a modification of that used by Creese and Snyder (1978). For native membrane receptor, 8 nM [^{3}H]-ADTN (30 Ci/mmol) was incubated with tissue containing approximately 150 ug of protein. The standard binding assay contained 0.05 % ascorbic acid, 2mM $MnCl_2$ and 50 mM Tris-HCl (pH 7.4) in a final volume of 0.6 mL. The mixture was incubated for 10 minutes at 37°C. The reaction was terminated by filtering over Whatman GF/B filters. The filters were immediately rinsed with 15 mL of ice cold Tris-HCl buffer (pH 7.4) and transferred to appropriate vials for liquid scintillation counting. Non-specific binding was determined by the amount of bound radioactivity retained in the presence of 10 uM dopamine.

3.2.3 Receptor Solubilization

Receptor solubilization (as described by Ross et al, 1985) was performed in a 10 mL final volume with the indicated concentration of test detergent, 0.15 M KCl, 50 mM Tris-HCl (pH 7.40) and bovine caudate membranes with a final protein concentration of 5 mg/ml. Other compounds, including dopamine, $MnCl_2$, Gpp(NH)p and ascorbic acid were also included in some experiments. Each sample was gently stirred for 1 hr at 4°C and subsequently centrifuged for 1 hr at 100,000 x g to remove particulate material. The supernatant was removed and dialyzed against 3 changes of 50 volumes of Tris-HCl (pH 7.4) over a period of 16 hr. Each dialysis buffer also contained the same concentration of $MnCl_2$ as had been used in the solubilization as well as 0.01% detergent.

3.2.4 Solubilized Receptor Binding Assay

The binding assay employed for the solubilized receptor was identical to that used for the native membrane receptor with the exception that the incubation was carried out at 0°C for 8 hr (Ross et al, 1985). At the conclusion of the incubation period the protein was precipitated by the addition of 0.3 mL of 30% polyethylene glycol (final

concentration 10%) and 0.1% bovine γ-globulin. The mixture was allowed to stand for 10 minutes and was then filtered over GF/B filters pretreated overnight with 1% polyethylenimine. Filters were rinsed with 15 mL of 10% polyethylene glycol in Tris-HCl buffer, (pH 7.4). In the case of solubilized receptor, polyethylenimine pretreatment of the filters greatly reduced filter blank radioactivity.

3.2.5 Solubilization Protocol Used for Affinity Chromatography

Subsequent to the solubilization studies performed by Ross et al, (1985), a colleague (Dr L.K. Srivastava) continued to study D-1 receptor solubilization. The solubilization protocol developed by Srivastava et al, (1989) consistently yielded greater amounts of solubilized receptor with higher specific activities than did the solubilization method of Ross et al, (1985). For this reason the procedure developed by Srivastava et al, was used for solubilization prior to affinity chromatography.

For solubilization of the D-1 receptor with cholic acid, bovine caudate membranes were prepared by the method outlined by Srivastava et al, (1989). Briefly, caudate tissue was dissected from fresh bovine brain and immediately frozen on dry ice, after which the tissue was stored at

-70°C until required. The tissue was slowly warmed to 0°C, and all subsequent procedures were carried out at 0-4°C. Intact caudates were homogenized by hand with a Teflon and glass Potter-Elvehjem style homogenizer in 10 volumes of 0.25 M sucrose and subsequently centrifuged for 10 minutes at 1,000 x g. The supernatant was retained and the pellet was rehomogenized and centrifuged a second time as described. The supernatants from both steps were then combined and centrifuged at 105,000 x g for 1 hr. The resulting supernatant was discarded and the pellet suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA (Tris-EDTA buffer). The membrane suspension was centrifuged at 30,000 x g for 20 minutes and finally resuspended in Tris-EDTA buffer at a protein concentration af 12-14 mg/mL. The preparation was stored at -70°C and used within one week.

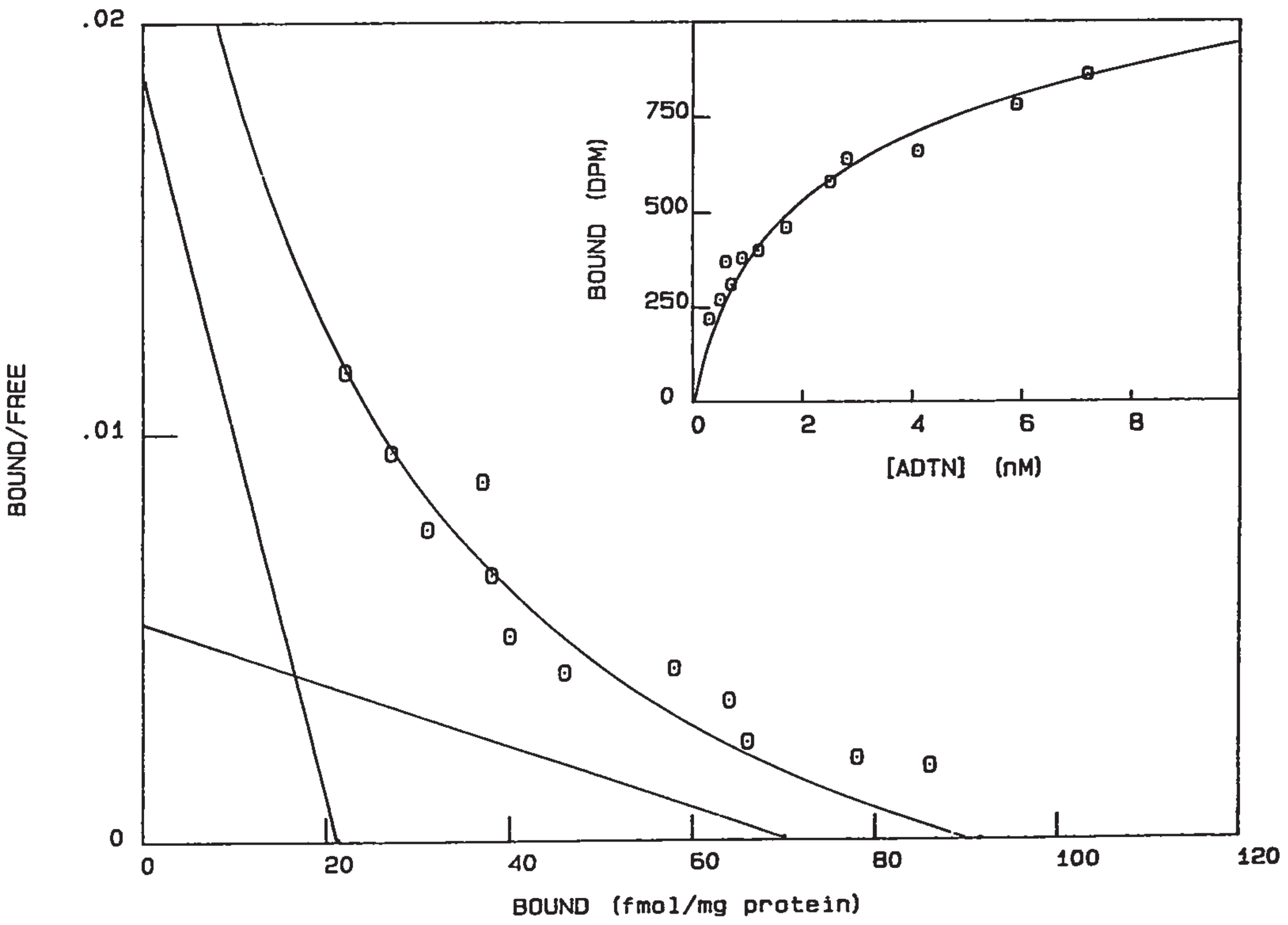
The solubilization procedure of Srivastava et al, (1989) was as follows: the caudate membrane preparation (described above) was diluted with two volumes of solubilization buffer (Tris-HCl [pH 7.4] containing 2 mM EDTA, 5mM $MgCl_2$, 0.1% ascorbic acid, and 10 uM SKF-82526-J). The solution was incubated for 30 minutes at 37°C with gentle mixing and subsequently centrifuged at 12,000 x g for 15 minutes. The pellet was washed twice in solubilization buffer using the same centrifugation steps and the resulting

pellet was finally suspended in the original volume of buffer. For solubilization, the preincubated membranes were mixed with an equal volume of the same buffer containing (final concentrations) 0.05% sodium cholate, 1 M NaCl, 0.1 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine and 1 mg/mL crude soya bean phosphatidylcholine. The solution was mixed at 4°C for 30 minutes and centrifuged at 130,000 x g for 60 min. The upper 80 % of the clear supernatant which contained the solubilized receptor was carefully aspirated for subsequent affinity chromatography studies. The solubilized receptor binding assay was performed essentially as described, with the exception that $[^3H]$-SCH 23390 was used as a radioligand.

3.3 RESULTS AND DISCUSSION

3.3.1 $[^3H]$-ADTN Binding to Caudate Membranes

Scatchard analysis of $[^3H]$-ADTN binding to caudate membranes revealed saturable, high affinity binding (Figure 3.1). A biphasic curve was obtained and computer analysis of the data (Program "Ligand") indicated the data best fit a two site model, one high affinity site (K_d = 0.24 nM, B_{max} = 20.4 fmol/mg protein) and one site of lower affinity (K_d = 3.5 nM, B_{max} = 71.6 fmol/mg protein).



.02
.01
0
BOUND/FREE
0
20
40
60
80
100
120
BOUND (fmol/mg protein)
750
500
250
0
BOUND (DPM)
0
2
4
6
8
[ADTN] (nM)

FIGURE 3.1

[^{3}H]-ADTN Binding to Caudate Membranes

Scatchard analysis of [^{3}H]-ADTN binding to bovine caudate membranes. Data best fit a two site model, with one high affinity site (B_{max} = 20.4 fmol/mg protein, K_d = 0.24 nM) and one lower affinity site (B_{max} = 71.6 fmol/mg protein, K_d = 3.5 nM). Inset: saturation isotherm of data transformed in Scatchard analysis.

The binding parameters for [^{3}H]-ADTN binding to bovine caudate membranes demonstrated properties similar to those described by authors using tissue from different species. Creese and Snyder (1978) for example, describe biphasic binding of [^{3}H]-ADTN to membranes prepared from rat caudate. The biphasic binding observed for [^{3}H]-ADTN is consistent with a receptor linked to the adenylate cyclase enzyme complex. The two affinity states probably represent two subpopulations, linked and non-linked to the regulatory G protein (Tsai and Leftkowitz, 1979; Pfueffer, 1979).

3.3.2 Receptor Solubilization using Octyl Glucoside

Early studies on solubilization of the D-1 receptor examined detergents which were thought to be suitable for agonist affinity chromatography. Although an effective solubilization protocol had been developed for the D-1 receptor (Sidhu and Fishman, 1986) the protocol required the use of cholic acid as a detergent. Cholic acid is well known to bind divalent cations (Hjelmeland and Charmbach, 1984) which are required for agonist binding, so alternate detergents were examined for their ability to solubilize the D-1 receptor.

For initial screening, four detergents (CHAPS, octyl glucoside, Triton X-100 and digitonin) were used for

solubilization. All four detergents were effective to a degree, however octyl glucoside consistently gave a higher yield of solubilized receptor and had the advantage of easy removal after solubilization. The ability of octyl glucoside to solubilize the D-1 receptor is shown in Figure 3.2. The optimal concentration of detergent was 1.0%.

To ensure ligand specificity, the ability of various dopaminergic drugs to decrease binding was examined. The compounds tested revealed an order of potency as follows: SCH 23390 > SKF 38393 > apomorphine > dopamine in both native and solubilized receptor preparations. Haloperidol and spiroperidol did not inhibit greater than 50% of [^{3}H]-ADTN binding (at concentrations up to 10 uM). The order of potency of these compounds in displacing [^{3}H]-ADTN binding supports the hypothesis that the ligand is indeed associated with the D-1 receptor. The observation that spiroperidol and haloperidol had little potency in inhibiting binding would exclude the possibility that there is a contribution to binding from D-2 type receptors. As the order of potency of the compounds tested did not change after solubilization, it was concluded that the binding activity in the solubilized preparation was attributed to the D-1 receptor and not an unrelated ADTN-binding protein.

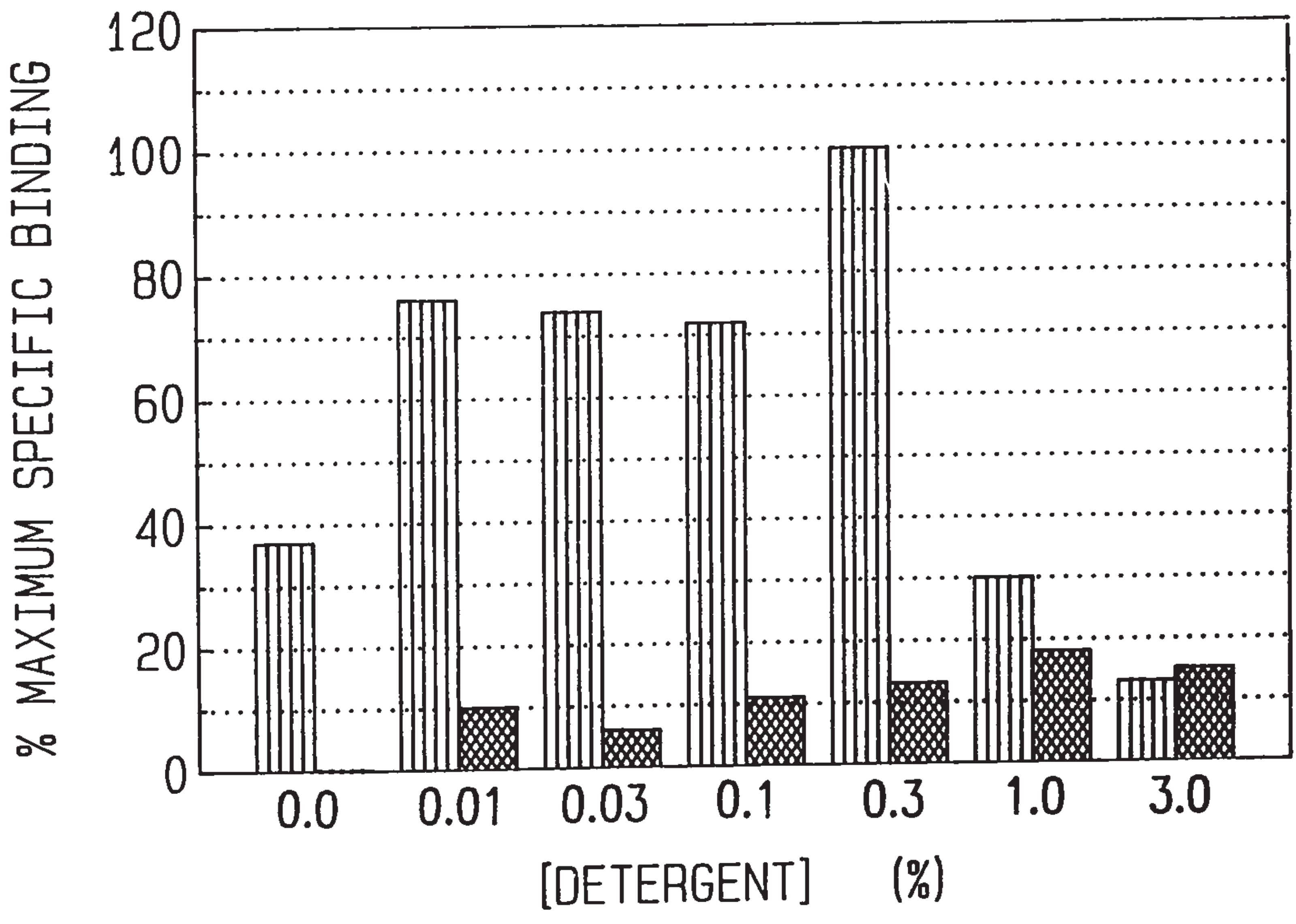

120
100
80
60
40
20
0
% MAXIMUM SPECIFIC BINDING
0.0
0.01
0.03
0.1
0.3
1.0
3.0
[DETERGENT] (%)

FIGURE 3.2

Solubilization of the D-1 Receptor with Octyl-Glucoside

Bovine caudate membranes were solubilized with octyl glucoside as described. This detergent was effective in increasing the density of receptors in membranes at low concentrations (to 0.3%). At higher concentrations, more receptors were solubilized, the optimal concentration for solubilization being 1.0%. Under these conditions, approximately 50% of the control binding (0% detergent) activity was recovered. Striped bars represent binding activity in the pellet and cross-hatched bars represent binding in the supernatant.

3.3.3 Effects of Agonist and Mg^{++} on D-1 Receptor Solubilization with Octyl Glucoside

Initial attempts at solubilization of the D-1 receptor using detergents alone were found to be totally ineffective unless the membranes were pretreated with dopamine and that dopamine was present during the solubilization procedure. Presumably, dopamine occupies the binding site of the receptor and allows greater recovery of receptors in a state capable of binding [^{3}H]-ADTN with high affinity. Many receptors have been solubilized in the presence of a ligand, and various groups have shown that the yields of α_2 (Smith and Limbird, 1981), ß (Limbird et al, 1980) and D-2 receptors (Kilpatrick and Caron, 1983) increase if solubilizations were carried out in the presence of an agonist.

As Creese and Snyder (1978) had previously described a divalent ion requirement (particularly Mn^{++}) for [^{3}H]-ADTN binding, solubilization was also attempted in the presence of Mn^{++}. The results (Table 3.1) clearly demonstrate that inclusion of Mn^{++} ions in the solubilization medium increased the yield of solubilized receptors. Also shown in Table 3.1 is the effect of dopamine on the yield of solubilized receptors. The data clearly indicated that the yield of solubilized receptors could be maximized by including both dopamine (1 uM) and Mn^{++} (1 mM) in the

Effects of Dopamine and Mn^{++} on Solubilization of the D-1 Receptor with Octyl-glucoside

[Dopamine] (μM)[a]	ADTN Bound[b]	[Mn^{++}] (mM)[c]	ADTN Bound[b]
0.0	0.208	0.0	0.111
0.1	0.293	0.1	0.205
1.0	0.371	1.0	0.371
10.0	0.154	10.0	0.062

[a] Solubilized in the presence of 1.0 mM Mn^{++}

[b] [^{3}H]-ADTN Bound in pmol/mg protein

[c] Solubilized in the presence of 1.0 μM Dopamine

Data is mean of triplicate determinations. SEM varied < 10%.

From Ross et al, 1985.

TABLE 3.1

Effect of Agonist and Mn^{++} on D-1 Solubilization

Bovine caudate membranes were solubilized with octyl-glucoside in the presence of varying concentrations of agonist (dopamine) and Mn^{++} as indicated. At the conclusion of solubilization, dopamine was removed by dialysis. The best yield of solubilized receptor was obtained when 1 μM dopamine and 10 mM Mn^{++} were included in the solubilization medium.

detergent solution.

3.3.4 Receptor Solubilization with Cholic Acid

Subsequent to the solubilization studies of Ross _et al_, (1985), Srivastava _et al_, (1989) re-evaluated the procedure and developed a new protocol for solubilization of the D-1 receptor using cholic acid. The cholic acid procedure was superior to solubilization with octyl glucoside in that the procedure of Srivastava _et al_, (1989) provided a greater yield of receptors (see Table 3.2). From the table it can be seen that the best yield of solubilized receptor (31.2%) was obtained when membranes were pretreated with SKF-82526-J (a D-1 agonist). Other significant changes to the previous procedure were the inclusion of exogenous lipid, a higher salt concentration and removal of the cholic acid after solubilization.

The solubilization protocol developed by Srivastava _et al_, (1989) was found to have several advantages over other published procedures. Sidhu and Fishman, (1986) reported a higher yield (48%) of solubilized D-1 receptors from rat striatum, however this procedure could not be reproduced in this laboratory; lower receptor yields were consistently obtained using this procedure.

More recently several investigators, including

Effect of Dopaminergic Ligands on Solubilization of the D-1 Receptor with Cholic Acid

Ligand	[Ligand] (M)	Solubilized[a]	SCH-23390 Binding[b]
SKF-82526-J	10^{-8}	7.9	220
	10^{-7}	18.1	489
	10^{-6}	22.3	621
	10^{-5}	31.2	860
	10^{-4}	31.1	824
SCH-23390	10^{-8}	14.9	402
	10^{-7}	17.8	526
	10^{-6}	21.3	643
	10^{-5}	29.7	884
	10^{-4}	30.0	889
Dopamine	10^{-5}	16.8	500
SKF-38393	10^{-5}	13.3	423
ADTN	10^{-5}	6.3	197

[a] % of Total

[b] [^{3}H]-SCH-23390 Binding in fmol/mg protein

Data is mean of triplicate determinations. SEM varied < 10%.

From Srivastava et al, 1990.

TABLE 3.2

Effect of Ligand Preincubation on D-1
Solubilization with Cholic Acid

Bovine caudate membranes were preincubated with the indicated concentration of ligand for 30 min at 37°C. The membranes were then solubilized as described and binding activity determined. The optimal receptor yield was obtained when membranes were preincubated with 10 μM SKF 82526-J.

Gingrich et al, (1988) and Niznik et al, (1986) have reported successful solubilizations of the D-1 receptor with digitonin. This laboratory had previously tested digitonin and failed to obtain significant solubilization (Ross et al, 1985). It now appears this lack of success was in part due to the quality of the detergent. It is now known that only the digitonin obtained from Waco Chemical Company leads to successful receptor solubilizaton, while digitonin from any other supplier (including Sigma, Cal-Biochem, Fisher or Boeringer-Manheim) is totally ineffective. The reason for this discrepancy is not currently understood.

CHAPTER 4

AFFINITY CHROMATOGRAPHY OF THE D-1 RECEPTOR

4.1 INTRODUCTION

Affinity chromotography was introduced over twenty years ago as a method of purification based on biological recognition (Cuatrecasas *et al*, 1968a, 1968b). This technique is, of course, one of the most popular methods of purification of many biological compounds. Currently a wealth of scientific papers are published every year which utilize this technique.

Of the several neurotransmitter receptors which have been purified, an affinity chromatography procedure has usually been an essential step in the purification scheme. There are many examples of receptors which have been successfully purified using this technique. Based on the success other investigators have had using affinity chromatography, it was proposed that an affinity matrix could be synthesized and used for purification of the D-1 receptor. Few studies have reported the sucessful purification of the D-1 receptor and at the start of this investigation no studies had reported a highly effective affinity chromatography protocol.

4.2 METHODS

4.2.1 Affinity Chromatography

Cholate solubilized D-1 receptor (as described in 3.3.3) was diluted in solubilization buffer to a final protein concentration of approximately 0.8 mg/mL. Solubilized receptor was batch adsorbed to the affinity matrix using the following procedure: 10 mL of solubilized caudate membrane preparation was incubated overnight with 2 mL bed volume of AP-ADTN-Affigel at 4°C. During the incubation the mixture was slowly rotated end-over-end. The mixture was then transferred to a 0.5 x 3.0 cm disposable polypropylene column and the solution slowly (0.1 mL/min) allowed to drain from the column. After the 10 mL of solubilization solution had passed from the column, the column was washed with 15 mL of wash buffer (containing 0.125% cholic acid, 0.25 M NaCl, 0.013% soya bean phospholipid, 50 mM Tris-HCl, 5mM $MgCl_2$, 5 mM EDTA and 0.1% ascorbate acid, pH 7.4) at the same flow rate. Bound proteins were eluted with 2.5 mL of wash buffer containing 100 nM SCH 23390. The SCH 23390 wash was at a discontinous flow, as the bed was allowed to equilibrate with the elution solution for 1 hr.

The collected eluates (containing receptor and SCH

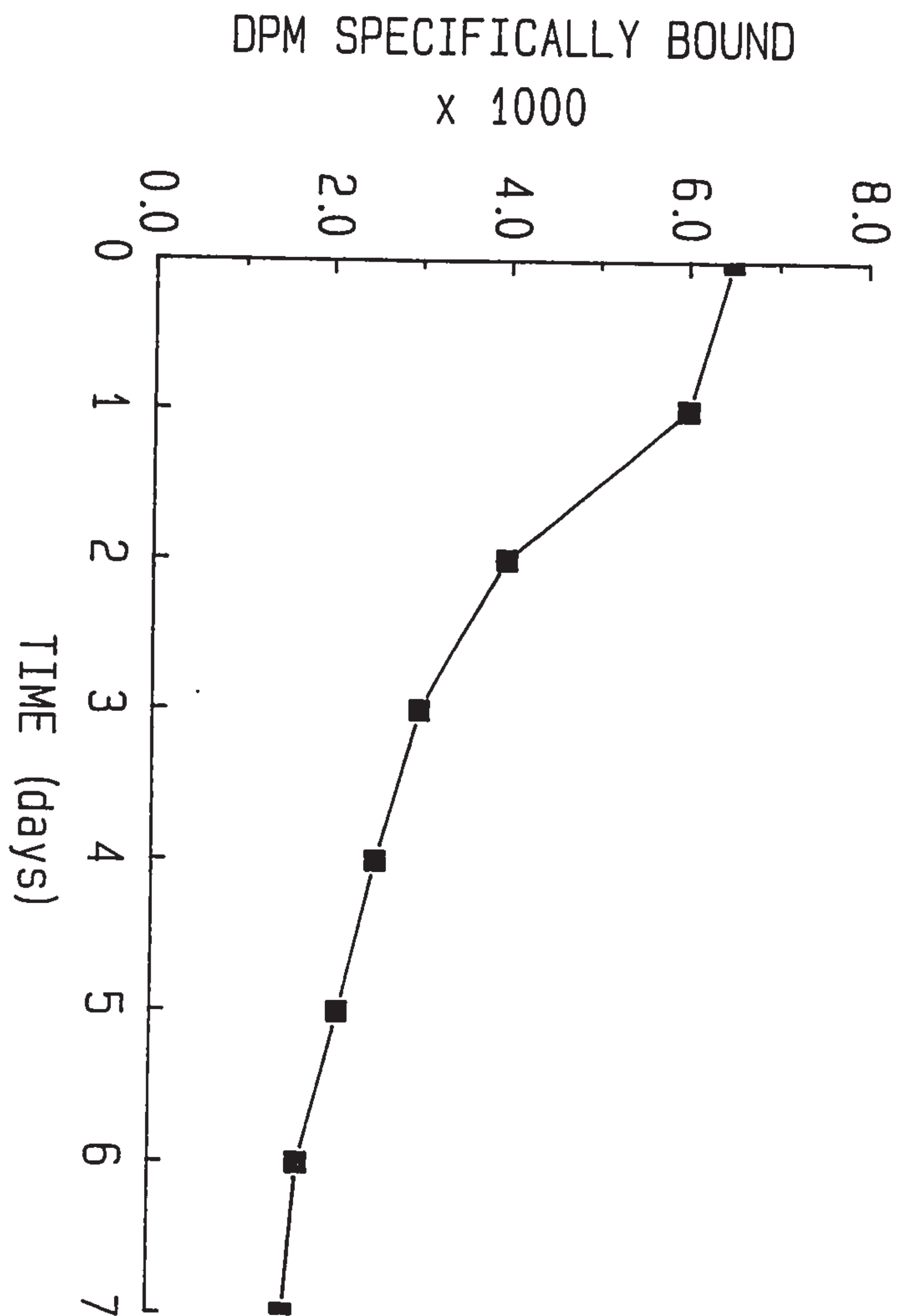
23390) were then chromatographed on Sephadex G-50 to remove the displacing agent. The G-50 size exclusion column (2.5 x 12 cm) was pre-equilibrated with wash buffer. The void volume from this column contained receptor binding activity, which eluted considerably before SCH 23390. Receptor binding assays on the affinity purified fractions were performed with an assay containing 1 nM [^{3}H]-SCH 23390. The assay was performed as described in 3.2.5, using 1 uM (+)-butaclamol to define non-specific binding.

Protein concentrations were determined by the method of Schaffner and Weissmann (1973).

4.3 RESULTS AND DISCUSSION

4.3.1 Stability of the Solubilized Receptor

Before starting affinity purification of the D-1 receptor, it was essential to establish the stability of the solubilized preparation over time. As affinity chromatography procedures typically take 24 hrs or more, it is essential that the solubilized receptor maintain binding activity for at least one and preferably several days. Representative results of one study examining the stability of cholate-solubilized D-1 receptor are shown in Figure 4.1. For the cholate solubilized preparation stored at 4°C, it



DPM SPECIFICALLY BOUND
x 1000
0.0
2.0
4.0
6.0
8.0
TIME (days)
0
1
2
3
4
5
6
7

FIGURE 4.1

Stability of Cholate Solubilized D-1 Receptor

The stability of cholate solubilized D-1 receptor over time was examined. Caudate membranes were solubilized as described and stored at 4°C. Each day for seven days a fraction was removed and the binding activity of [^{3}H]-SCH 23390 (0.5 nM) determined. DPM specifically bound was measured per assay tube.

was observed that very little [^{3}H]-SCH 23390 binding activity was lost in the first 24 hr after solubilization. However, at times greater than 24 hr considerable binding activity was lost and by 72 hrs after solubilization, less than 50% of the original activity remained in the preparation. It was interesting to note that after the first 36 hr binding decreased at a moderate rate and even five days after solubilization greater than 20% of the binding activity remained.

With reference to affinity chromatography, it was concluded that the stability of the preparation was sufficient for this study. In our hands many preparations were stable for one to two days and some were stable for only hours.

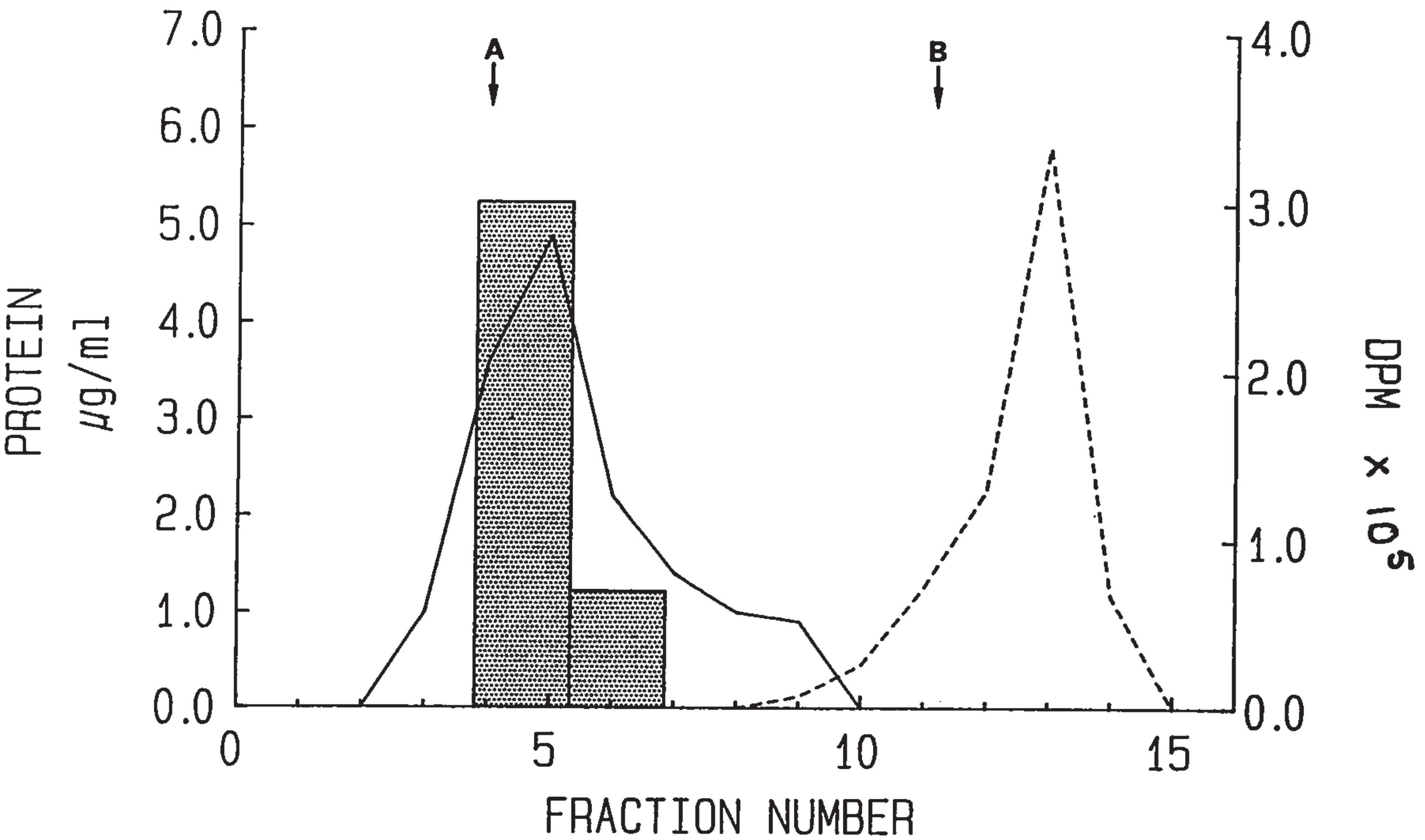
4.3.2 Removal of SCH 23390 from the Solubilized Preparation

One of the technical difficulties commonly encountered in the affinity chromatography of receptors is the removal of the displacing agent after the protein has been eluted from the affinity matrix. This removal is essential if receptor binding assays are to be carried out subsequently. It was proposed that for this study SCH 23390 would be used to displace the D-1 receptor bound to the column. As the affinity of SCH 23390 is extremely high (K_d less than 1 nM)

it was essential to develop an effective means by which the drug could be removed from the eluted proteins. Two methods (gel filtration and dialysis) were tested for their effectiveness in removing SCH 23390. While both techniques removed the drug from the column eluates, dialysis was found to be too time consuming. For this reason, gel filtration (Sephadex G-50) was the method of choice.

The efficiency of the G-50 separation was evaluated by spiking the eluated fractions from an affinity column with 10,000,000 dpm (10 nM) of [^{3}H]-SCH 23390, and chromatographing the mixture on the gel filtration column. The elution profile of protein, [^{3}H]-SCH 23390 and binding activity from the G-50 column are shown in Figure 4.2.

Using a Sephadex G-50 column as indicated, there was no significant overlap between the fractions which contained proteins and those which contained [^{3}H]-SCH 23390. Receptor binding activity eluted from the column in the same fractions as the protein, again considerably earlier than [^{3}H]-SCH 23390. In these experiments only background radioactivity was detected in the earlier fractions containing binding activity. Considering 10,000,000 DPM had been applied to the column with the affinity eluates, it was concluded that no significant quantity of SCH 23390 bound to and/or eluted with the protein fractions from the G-50 column.



PROTEIN
µg/ml
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
A
B
DPM x 10^5
4.0
3.0
2.0
1.0
0.0
0
5
10
15
FRACTION NUMBER

FIGURE 4.2

G-50 Chromatography of Solubilized D-1 Receptor
and SCH 23390

Cholate solubilized D-1 receptor was spiked with 10,000,000 DPM (10 nM) [^{3}H]-SCH 23390 and chromatographed on Sephadex G-50. D-1 receptor binding activity (bars) eluted with fractions containing protein (solid line). [^{3}H]-SCH 23390 (dotted line) eluted after receptor binding activity and most protein. Also shown are the elution positions of a high molecular weight standard (blue dextran, arrow A) and a low molecular weight standard (riboflavin, arrow B). Receptor binding activity (hatched bars) is in relative units.

4.3.3 Affinity Purification of the D-1 Receptor

The methodology for D-1 receptor purification by AP-ADTN affinity chromatography as outlined in 4.2.1 was the result of many subtle variations of the original protocol. The effects of salts, lipids, detergent removal, divalent cations and many other parameters were examined. Of the changes made in these variables, none consistently yielded better results than the method described.

The results of six separate purification experiments under the conditions described are summarized in Table 4.1. The binding activity in the AP-ADTN affinity purified preparation was approximately 50,000 fmol/mg protein, or about 50-fold purified with respect to the solubilized preparation. The average recovery after purification was 8%. This data (Ross et al, 1987) was the first report of affinity purification of the D-1 receptor with a specific ligand. Niznik et al, (1986) had previously reported affinity purification with Con-A Sepharose, obtaining 10- to 12-fold purification and 15% recovery. More recent studies by Gingrich et al, (1988) have reported a superior purification, observing a 200-250 fold purification using a SCH 23390 analog as an affinity ligand.

STEP	TOTAL ACTIVITY (fmol)	SPECIFIC ACTIVITY (fmol/mg protein)	% YIELD	PURIFICATION (fold)
SOLUBILIZED	772	1155	100	1
± SEM	±280	±842	-	-
PREWASH	435	1678	69	1.3
±SEM	±247	±1303	±23	±0.8
WASH	39	149	4.3	<1
± SEM	±28	±225	±2.6	-
ELUTION	66	46583	8.0	47.6
± SEM	±40	±22992	±3.1	±18.4

TABLE 4.1

Affinity Purification of the D-1 Receptor

Bovine caudate tissue was solubilized as described and adsorbed in a batch procedure to an AP-ADTN affinity column. After a prewash and wash steps, bound receptor was eluted with 2.5 ml of 100 nM SCH 23390. Eluates were chromatographed on Sephadex G-50 and receptor binding activity in the fractions containing protein was determined. Protein concentration was determined by the method of (Schaffner and Weissmann, 1973). Results are the mean +/- SD of six separate experiments.

CHAPTER 5

PHOTOAFFINITY CROSSLINKING THE D-1 RECEPTOR

5.1 INTRODUCTION

Like affinity chromatography, affinity labelling has proven to be a highly useful technique in the purification of many important proteins. Chemical affinity labelling was developed in the 1960's and was first used for investigation of ligand-receptor interactions (Singer, 1967). In chemical affinity labelling a reactive functional group (such as α-haloketone) is incubated with a "receptor" molecule during which time the two will form a covalent bond (Bayley, 1983 for review). Although there are many examples of very useful affinity ligands in the literature the technique is generally plagued with complications, which can arise as a result of the following: 1) chemically active alkylating agents will begin to react with groups outside of the binding site once incubation is initiated and 2) chemically reactive functional groups are generally specific for a certain functional group on the target molecule. If such a group is not present, then the affinity ligand has no chance of reacting with the binding site to which it is directed (Bayley, 1983).

Many of the difficulties encountered with chemical

affinity labelling can be overcome by using photoaffinity probes. The first photolabile molecules were reported in 1969 by Fleet _et al_, who used photoactive haptens in the study of hapten-immunoglobulin interactions. There are several advantages of photoaffinity labels over chemical affinity labels. These advantages (Bayley, 1983 for review) include the following: 1) until the ligand is irradiated with light, it is inert (this allows one to study kinetic and affinity parameters without complications caused by a reactive ligand; 2) the photolysis reaction can be initiated at any time, which eliminates many side reactions which can occur in the time taken to equilibrate the ligand with its target molecule; 3) the reactive intermediates formed as a result of photolysis are far more reactive than chemical alkylating agents. The benefit of this feature is that the ligand can covalently react with almost any functional group, including carbon-carbon, carbon-hydrogen and carbon-oxygen bonds. This eliminates the requirement of specific functional groups present in the binding site for alkylation to occur. There are numerous examples of photoaffinity ligands used to label a host of target molecules. Many of these uses have been reviewed elsewhere (_e.g._, Methods in Enzymology, Vol XLVI, 1977; Bayley, 1983) so will not be discussed here.

Many different photoactive functional groups are

available which can be used to make a specific ligand photoactive. Some of the more common ones include carbenes and carbene precursors (such as α-diazoketones and α-diazoacetals) as well as nitrenes and nitrene precursors (such as aryl azides and nitroarylazides, Bayley and Knowles, 1977 for review). Of the many photoactive derivatives available for the design of photoaffinity ligands, the most commonly used are aryl and nitroaryl azides.

Some of the recently reported uses of azides as neurotransmitter receptor photolabels include compounds specific for the ß-adrenergic (Lavin et al, 1981), D-2 (Amlaiky and Caron, 1985) and the α_2-adrenergic receptors (Kawahara et al, 1985). Aryl azides are popular functional groups for several reasons including: 1) they are stable in the absence of light, 2) they are simple to synthesize, 3) they can be photolysed at wavelengths above 300 nm, 4) the intermediates formed as a result of photolysis are highly reactive and 5) they form stable adducts with the target protein (Bayley 1983 for review). This technique is beneficial in protein purification for several reasons, particularly if the photoaffinity ligand is radiolabelled. By using a radiolabelled ligand to crosslink target proteins, it becomes possible to identify the protein even if biological activity is lost. This is a great advantage

in the puification of proteins (such as receptors) which exist in vanishingly small amounts in tissue and are not generally stable.

At the beginning of this study, there was only one report of D-1 photoaffinity labelling existed in the literature (Kuno and Tanaka, 1981). This study reported a molecular weight of 52 kDa for the D-1 receptor, which is not consistent with molecular weight determinations obtained from different techniques (Neilsen et al, 1984). Several significant problems were observed with the Kuno study, including the authors' failure to demonstrate specificity of labelling as well as the lack of important control experiments, such as demonstration of light dependence of labelling. Amlaiky et al, (1987) had reported photoaffinity crosslinking of the D-2 receptor with a derivative of spiroperidol, which was incorporated into a protein of M.W. 92 kDa.

As affinity chromatography and photoaffinity labelling are both very useful techniques, they are sometimes used together in a purification scheme. Both techniques have been used sucessfully together in the purification of a host of proteins. After a protein is covalently bound with a crosslinking reagent, however, it is not normally possible to further purify the protein by affinity chromatography as the binding site of the protein is blocked. For this reason

photoaffinity labelling is normally performed subsequent to affinity chromatography.

5.2 METHODS

5.2.1 Receptor Binding Assay

The [^{3}H]-SCH 23390 binding assay was essentially the same as outlined in 3.2.2, with the exception that the incubation time was increased to 90 min.

5.2.2 Adenylate Cyclase Assay

The assay chosen was a modification of that of Barbaccia et al, (1983). Briefly, the tissue of interest was homogenized in 20 volumes of 2 mM Tris-maleate buffer (pH 7.35) at 4°C with 10 strokes of a teflon-glass pestle. The buffer also contained 2 mM EDTA and 0.32 M sucrose. The homogenate was centrifuged at 1,000 x g to remove cellular debris. The supernatant was then centrifuged at 20,000 x g for 15 min to precipitate membrane material. The pellet was resuspended in 5 volumes of 20 mM Tris-maleate buffer (pH 7.35) containing 0.032 M sucrose and 2 mM EDTA.

The standard mixture (total volume 150 uL) contained the following: 85 mM Tris-maleate (pH 7.35), 1 mM cAMP, 0.5

mM IBMX, 1 mM ATP, 10 uCi [^{3}H]-ATP, 4 mM $MgSO_4$, 0.3 mM EDTA, 100 uM GTP, 0.5 mg/mL phosphoenolpyruvic acid and 0.12 mg/mL pyruvate kinase. The reaction was stopped by addition of 1.0 mL of 10% TCA and 1,000 CPM of [^{14}C]-cAMP was added as a recovery marker for product purification.

Purification of cAMP was done by the method of Haslam and McClenaghan (1981) with slight modification. Acidified samples were centrifuged at 9,000 x g for 5 min to remove precipitated proteins. The supernatant was applied onto columns (15 x 0.7 cm) containing 1.5 g alumina (neutral type WN-3) previously equilibrated with 10 % TCA. Columns were washed with 9 mL of 10% TCA, 10 mL H_2O and finally 2 mL of 0.2 M ammonium formate. cAMP was eluted into 13 x 75 cm test tubes with an additional 3 mL of 0.2 M ammonium formate. The eluates were decanted into columns (15 x 75 cm) containing 1.5 mL Dowex 50 ion exchange resin (Bio-Rad AG 50W-XB 100-200 mesh, H^+ form). Columns were washed with 6 mL of 1 mM potassium phosphate buffer (pH 7.0) and cAMP was eluted with 10 mL of the same buffer directly into 20 mL glass scintillation vials. Each sample was frozen in liquid N_2 and lyophilized. H_2O (0.5 mL) and 7.5 mL scintillation cocktail were added to each sample and ^{3}H and ^{14}C determined by liquid scintillation spectrophotometry. The tritium counts obtained were corrected for ^{14}C recovery. Adenylate cyclase activity was expressed as pmol/min/mg protein.

5.2.3 Photoaffinity Crosslinking

Caudate membranes were prepared as described in 5.1.1 or were homogenized from frozen caudate tissue in a Polytron tissue grinder (setting 6, 20 seconds) in 20 volumes of 50 mM Tris HCl (pH 7.4) containing 5 mM EDTA (Tris-EDTA buffer). The tissue suspension was centrifiged for 10 min at 50,000 x g and the supernatant discarded. The pellet was resuspended in the same buffer, centrifuged and resuspended in Tris-EDTA buffer as above. The protein concentration in this preparation varied from 500 ug to 750 ug per mL.

The standard photoaffinity incubation assay contained 150 to 200 ug protein, 50 mM Tris HCl (pH 7.4), varying concentrations of the photolabel and a variety of other additives as indicated in a total volume of 1.0 mL. Subsequent to preincubation and/or incubation, the solution was transferred to a 24 well microtitre plate (well dimension 1.6 cm diameter by 1.6 cm deep). Samples were then exposed for 15 min to long wave UV light (for nitroaryl azides) or short wave UV light (for aryl azides) from a Mineralight Model UVSL-58 at a distance of 2.5 cm. For some experiments photolyses were carried out at $0^{o}C$ and in some instances the reaction was carried out in an N_2 atmosphere.

For samples which were to be assayed for binding

activity after having been crosslinked with non-radioactive photolabel, dopamine was added to the mixture (10 uM final concentration) and incubated for 30 min at 0°C. This procedure was performed to displace any non-covalently bound photolabel from the D-1 receptor. The membranes were subsequently centrifuged for 10 min at 50,000 x g in Tris-EDTA buffer and resuspended in the same buffer. This procedure was repeated three times, after which [^{3}H]-SCH 23390 binding was performed on the membranes.

5.2.4 Electrophoresis and Autoradiography

For samples crosslinked with radioactive photolabels, the membranes were transferred to 1.5 mL microfuge tubes and TCA (0.5 mL of 30% solution) was added to each sample. The acid precipitated proteins were pelleted by centrifugation at 9,500 x g for 10 min. The supernatant containing non-covalently bound photolabel was aspirated and discarded while the protein pellet was processed for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed under denaturing conditions using the method of Laemeli (1977) as described in Bio-Rad Protein II product literature. Slab gels (20 x 16 cm) 1.5 mm thick containing a total monomer concentration of 10% were used for all gels. Proteins were prepared by

solubilization in 150 μl of sample buffer containing bromophenol blue, 10% glycerol, 2% SDS, and 0.5% β-mercaptoethanol in 125 mM Tris HCl (pH 6.8). Proteins were electrophoresed through the stacking gel at 25 mA per gel, after which the current was increased to 35 mA per gel until the dye front reached the bottom of the gel (about 5 hr). The electrophoresis apparatus was continually cooled with tap water, maintaining a temperature of approximately 10°C.

Once the dye front had reached the bottom of the gel, the gel was removed from the apparatus and transfered to Whatman filter paper, covered in Saran Wrap and dried under vacuum at 70°C. For samples which had been labelled with tritiated photolabel, the lanes were cut into bands 3 mm wide, transfered to scintillation vials and digested with 1 mL of 30% H_2O_2 containing 5% NH_4OH. Scintillation cocktail was added to each sample and the amount of radioactivity determined. Gels from ^{125}I-labelled proteins were loaded into X-ray cassettes with film (Kodak X-OMAT XAR 5) and an intensifying screen (Dupont Lightning Plus). Film was exposed for an appropriate length of time (usually 12 - 24 hr) at -70°C and processed manually.

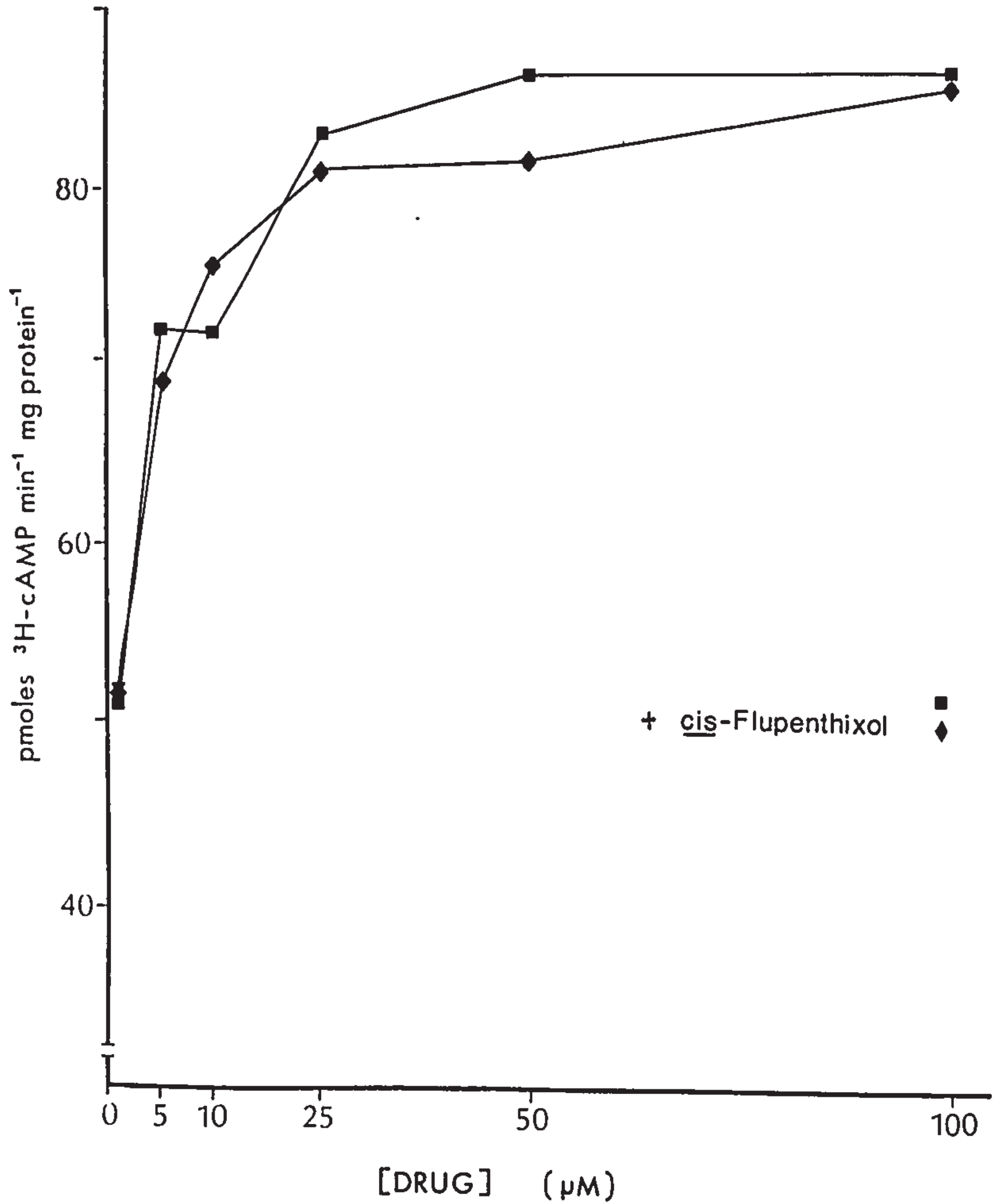
5.3 RESULTS AND DISCUSSION

Of the photolabels originally proposed for the study (10 and 20-25), three compounds were used in photoaffinity experiments. Compound 10 was used to study the light dependent inactivation of the D-1 receptor and tritiated 10 was used as a photoaffinity probe. The products of radioiodination of 15 and 16 (presumably 22 and 24) were used as probes to study the labelling patterns of these compound in bovine caudate membranes.

As indicated in Chapter 4, compound 24 was not the proposed structure, but iodination actually produced a different isomer, compound 26. The actual structure of compound 22 was not elucidated, so the position of the iodine in compound 22 is not known.

5.3.1 Stimulation of Dopamine-Sensitive Adenylate Cyclase With Compound 10

D-1 dopaminergic activity of compound 10 was established by examining the ability of this compound to (1) stimulate adenylate cyclase activity and (2) to irreversibly inhibit D-1 receptor binding in a light-dependant manner. The ability of compound 10 to stimulate adenylate cyclase activity is shown in Figure 5.1. This compound stimulated



80
60
40
pmoles ³H-cAMP min⁻¹ mg protein⁻¹
+ cis-Flupenthixol
0
5
10
25
50
100
[DRUG] (µM)

FIGURE 5.1

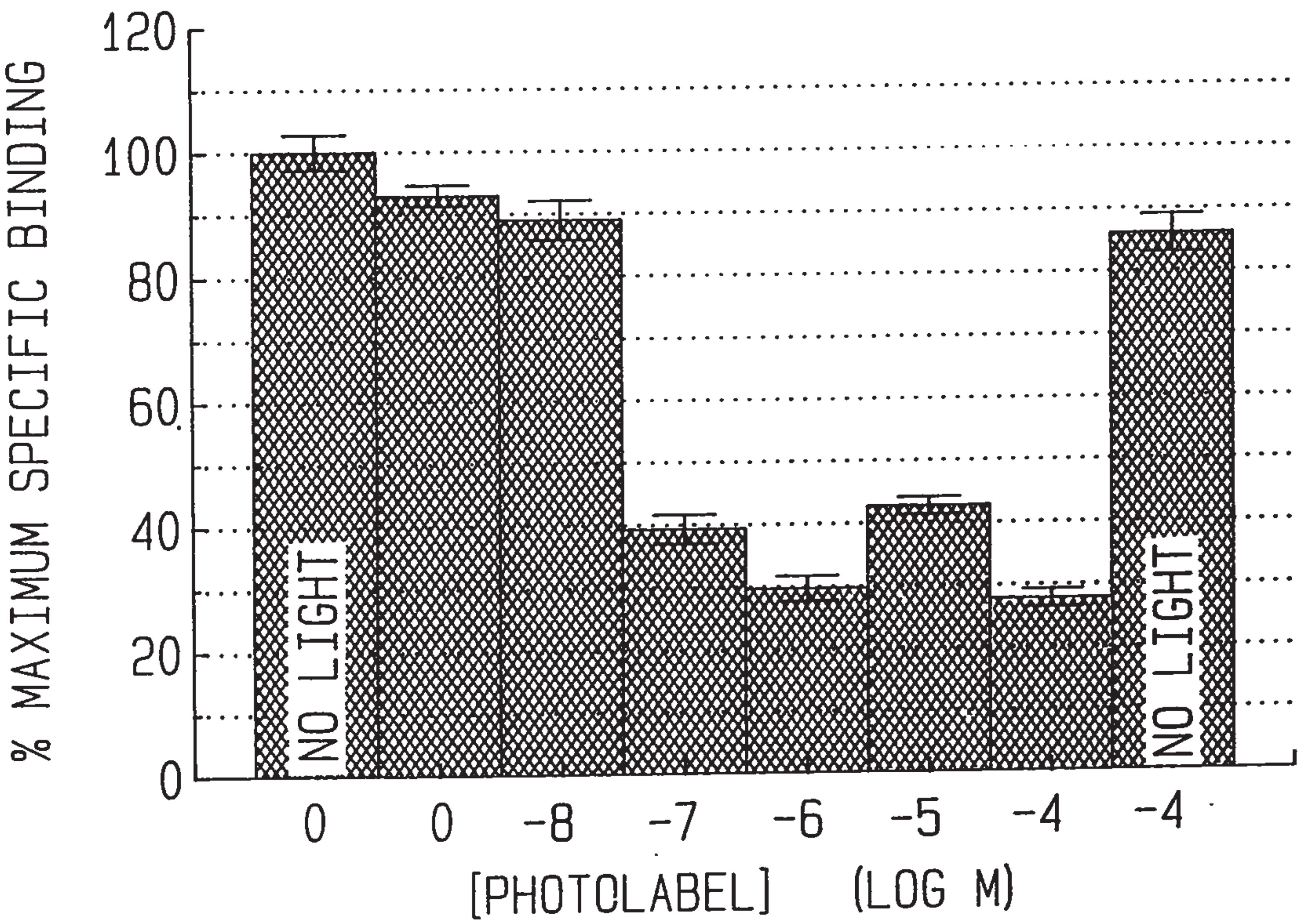
Stimulation of Adeylate Cyclase with
Dopamine and <u>10</u>

The effect of dopamine (■) and compound <u>10</u> (♦) on adenylate cyclase is shown. Compound <u>10</u> stimulated adenylate cyclase to the same extent as did dopamine (maximal stimulation 160% of basal) with a similar dose-response profile. cis-Flupenthixol (10 μM) effectively blocked the stimulation induced by both dopamine and <u>10</u> (agonist at 100 μM), demonstrating <u>10</u> is stimulating adenylate cyclase via the D-1 receptor. Data represents one representative experiment performed in triplicate. SEM of replicates varied less than 5%.

cyclase activity to the same degree as did dopamine, with a similar dose response profile. Also significant is that the increase in activity induced by 10 could be completely blocked by including cis-flupenthixol, a potent D-1 antagonist. This data clearly shows that compound 10 has D-1 receptor activity, specifically, that it is a full agonist. This compound also has a similar dose-response curve to that of dopamine, which is further evidence that 10 has a similar affinity for the D-1 receptor. The observation that cis-flupenthixol was able to block the stimulation of adenylate cyclase by 10 confirms that the compound is acting at the D-1 receptor and not at one of the many other receptors coupled to adenylate cyclase.

5.3.2 Photoinactivation of the D-1 Receptor with 10

The ability of compound 10 to irreversibly block SCH-23390 binding activity was used to evaluate the utility of this compound as a D-1 photoaffinity probe. As illustrated in Figure 5.3, 10 was effective in irreversibly inhibiting [^{3}H]-SCH 23390 binding. The concentration range at which 10 was effective in blocking labelling (0.1 uM and greater) did not indicate that the compound had an extremely high affinity for the D-1 receptor. Considering that the photoactive moiety of 10 is an aryl azide and further that



% MAXIMUM SPECIFIC BINDING
120
100
80
60
40
20
0
NO LIGHT
NO LIGHT
0
0
-8
-7
-6
-5
-4
-4
[PHOTOLABEL] (LOG M)

FIGURE 5.3

Ability of 10 to Irreversibly Inhibit
[^{3}H]-SCH 23390 Binding

The ability of compound 10 to irreversibly inhibit [^{3}H]-SCH 23390 binding to bovine caudate membranes is shown. Membranes were incubated with the indicated concentration of 10, photolysed and assayed for D-1 binding activity. No light controls were performed at 0 and 100 μM 10. No significant decrease in binding activity was observed when membranes were incubated with 10 in the absence of light. A significant decrease in binding activity was observed in samples photolysed with 100 nM (or more) photolabel ($p<0.01$). Data is the average of three experiments, each done in triplicate +/- SD.

the proposed radioactive derivative of 10 is a tritiated compound, it was felt that this compound would probably have low efficiency as a D-1 photolabel. As experiments did show that 10 had modest affinity for the D-1 receptor, however, the tritiated analog was synthesized and used in photoaffinity labelling experiments.

5.3.3 Photoaffinity Labelling the D-1 Receptor with [^{3}H] 10

The tritiated derivative of compound 10 was used to label bovine caudate membranes. The labelling pattern of covalent [^{3}H]-10 into caudate proteins is shown in Fig 5.4. Upon photolysis of caudate membranes in the presence of [^{3}H]-10, only a single protein band specifically incorporated radioactivity. SDS-PAGE of the photolabelled proteins showed minor labelling of many proteins, with one protein (M.W. = 79 kDa) which was protected from [^{3}H] incorporation by 10 uM dopamine. The amount of protein labelled by 10 represents a small amount (<1%) of the total receptor present (as determined by Scatchard analysis using [^{3}H]-SCH 23390). In control experiments (with no irradiation of the mixture) no specifically bound radioactivity could be detected in any location of the gel.

At the time when these studies were reported (Ross *et*

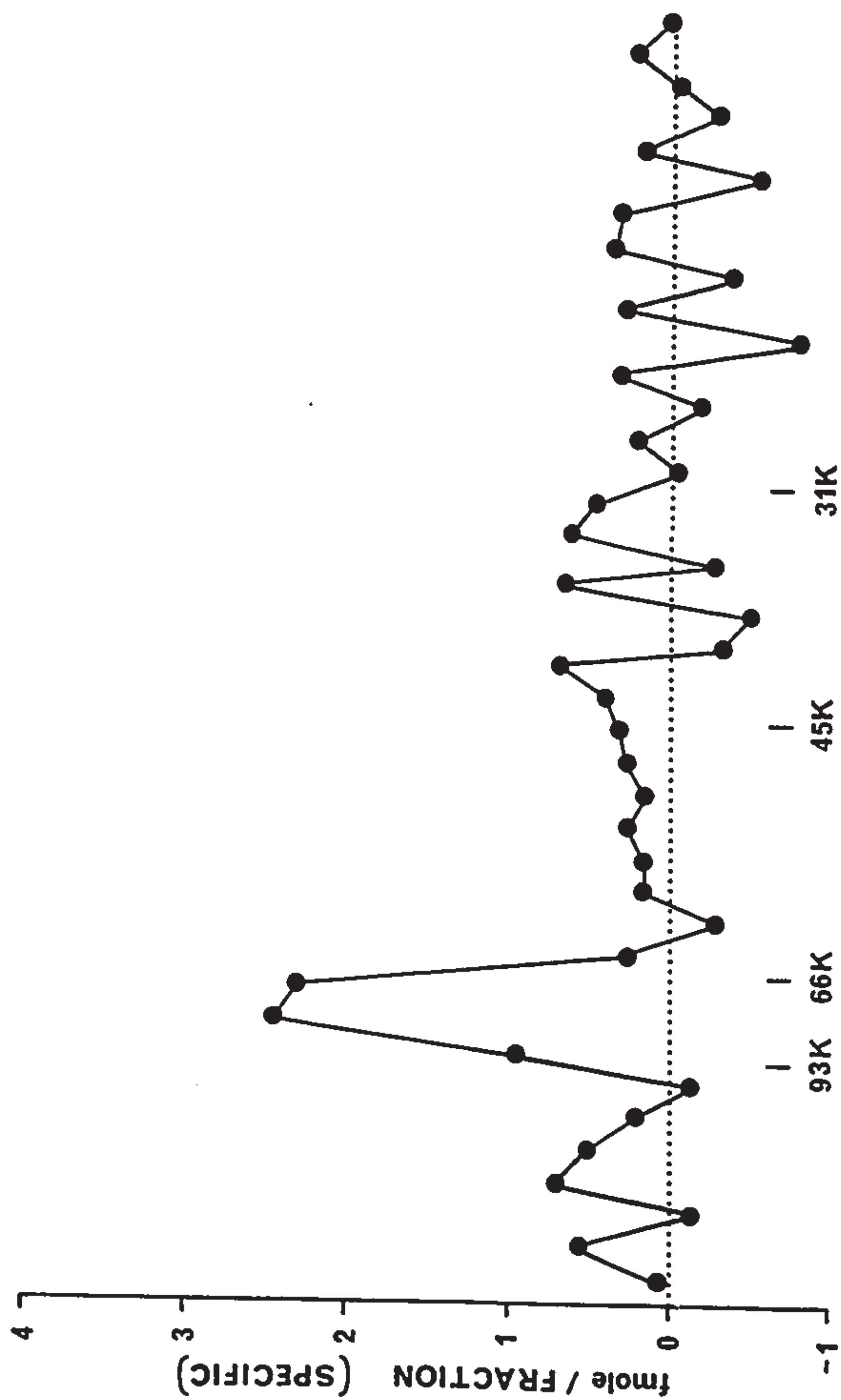

fmole / FRACTION (SPECIFIC)
4
3
2
1
0
-1
93K
66K
45K
31K

FIGURE 5.4

Covalent Incorporation of $[^3H]$-10
into Caudate Membranes.

$[^3H]$-10 was incubated with caudate membranes, photolysed and products seperated by SDS-PAGE as described. Gels were sliced into 3 mm bands, digested and $[^3H]$-10 covalently bound was determined by liquid scintillation spectroscopy. A parallel lane was run which contained 10 µM dopamine (non-specific labelling). Data shown is specific incorporation (total lane - non-specific lane) from one representative experiment. The specific activity of $[^3H]$-10 was 68 DPM/fmol.

al, 1986) there was only one literature report of photoaffinity labelling the D-1 receptor. That particular study used [^{3}H]-dopamine as a photolabel and reported specific incorporation into a protein of molecular weight of 57 kDa (Kuno and Tanaka, 1981). Several major problems were evident in their study including the authors' failure to demonstrate specificity of labelling. These authors based their identification of the D-1 receptor solely on the observation that haloperidol (a D-2 antagonist) did not inhibit labelling. One additional estimate of the D-1 receptor molecular weight was proposed by (Nielsen et al, 1984) who calculated a theoretical Mr of 77 kDa based on radiation inactivation studies.

Since the report by Ross et al, (1986) that compound 10 photolabeled the D-1 receptor, Amlaiky et al, (1987) have published a report describing the use of an iodinated SCH 23390 derivative which could be covalently coupled to the D-1 receptor using a heterobifunctional crosslinking reagent. These authors reported a molecular weight of 72 kDa (well within the error of the molecular weight obtained in this study). In another recent study, Niznik et al, (1988) used an azido SCH 23390 derivative to photoaffinity label the D-1 receptor, a protein of M.W.= 74 kDa; minor labelling of proteins of 62 and 51 kDa was also observed. Both groups studied the specificity of labelling with their respective

cross-linking reagents and concluded that the protein was the D-1 receptor based on the ability of specific pharmacological agents to prevent radioincorporation of crosslinker into the D-1 receptor. In all three reports of D-1 crosslinking a broad band of labelled protein was observed, so the three reported molecular weights (79 kDa, Ross et al, 1986; 72 kDa, Amlaiky et al, 1987; 74 kDa, Niznik et al, 1988) could easily represent the same protein. Discrepancies in reported molecular weight could result from different calibration standards or from where within the radiolabelled band the measurement is taken.

Several conclusions were obvious from labelling studies using [^{3}H]-10. Although the compound did specifically and covalently label a small amount of protein with a molecular weight consistent with literature reports for the D-1 recptor, the amount of labelling was so low that any autoradiographic studies with this compound would take a long time to complete. For this reason, it was decided not to pursue 10 as a D-1 photolabel but rather examine some of the radioiodinated compounds 20 through 25 as possible D-1 receptor probes.

5.3.4 Photoaffinity Labelling Caudate Membranes with [^{125}I]-18

The first compound synthesized as a precursor to a radioiodinated photolabel was compound 18, which was prepared by Mr. K. Gatterman. Before the non-radioactive product was synthesized, compound 18 was radioiodinated, presumably to yield 24. The radioiodinated product of compound 18 was screened for D-1 receptor binding activity by utilizing the compound in a binding assay. For these experiments, SCH-23390 was used to define non-specific binding. From these experiments it was not possible to conclude whether the compound had any affinity for the D-1 receptor, because non-specific binding of the ligand to the membrane preparation was excessive. As specific binding of any ligand is usually a small percentage of the total counts present (less than 10%) it is impossible to observe specific binding with a ligand which has such high non-specific binding. The compound [^{125}I]-18 had over 80% non-specific binding to the membrane preparation.

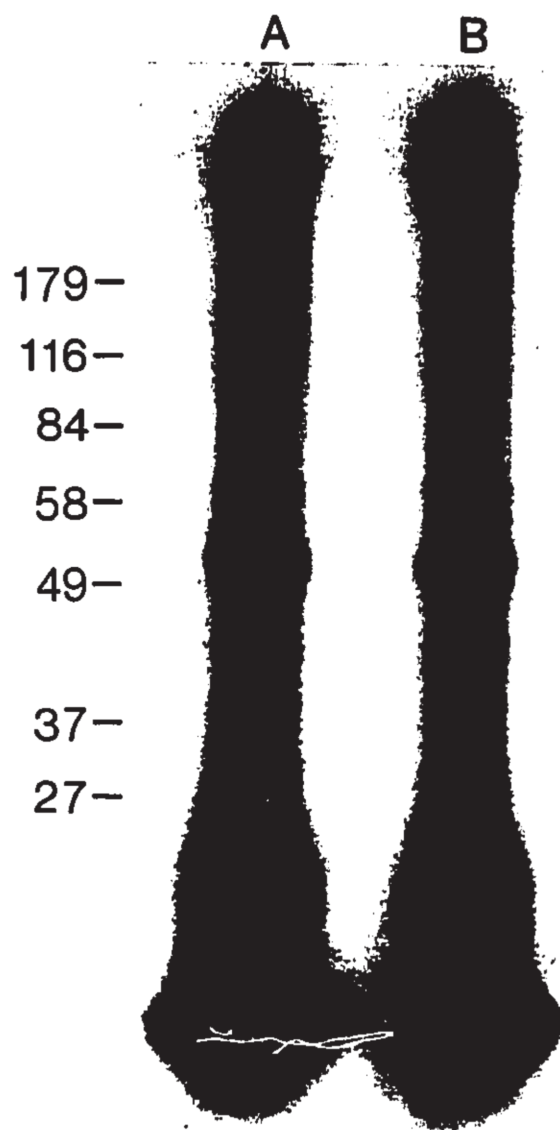
It is possible, however, that a ligand with high non-specific binding could be useful as a photoaffinity probe. This situation could occur if the non-specific binding were to components that do not comigrate with the protein of interest during SDS-PAGE. In the event that this situation

was the case, the radioiodinated product was used to label membranes in the presence and absence of (+)-butaclamol. A typical autoradiograph from SDS-PAGE of the labelled products is shown in Fig 5.5. In the autoradiograph shown, there are no protein bands labelled in the absence of (+)-butaclamol that are not labelled in the presence of (+)-butaclamol. For this reason, it was concluded that radiolabeled 18 is not an effective photoaffinity label for the D-1 receptor.

5.3.5 Affinity of 26 for D-1 and D-2 Receptors

As one of the problems observed with iodinated 18 was the excess non-specific labelling, a less lipophilic analog was synthesized. The compound 19 and its radioiodinated derivative were tested for dopamine receptor binding as well as labelling patterns in caudate tissue.

In competition binding experiments both compound 19 and 26 had effectively no affinity for the D-1 receptor as measured by their ability to block [^{3}H]-SCH 23390 binding (Figure 5.6). One interesting observation from these studies was that compound 26 had surprising affinity for the D-2 receptor. The ability of these two compounds to inhibit [^{3}H]-SCH 23390 (D-1) and [^{3}H]-spiroperidol (D-2) binding is indicated in Figure 5.6.

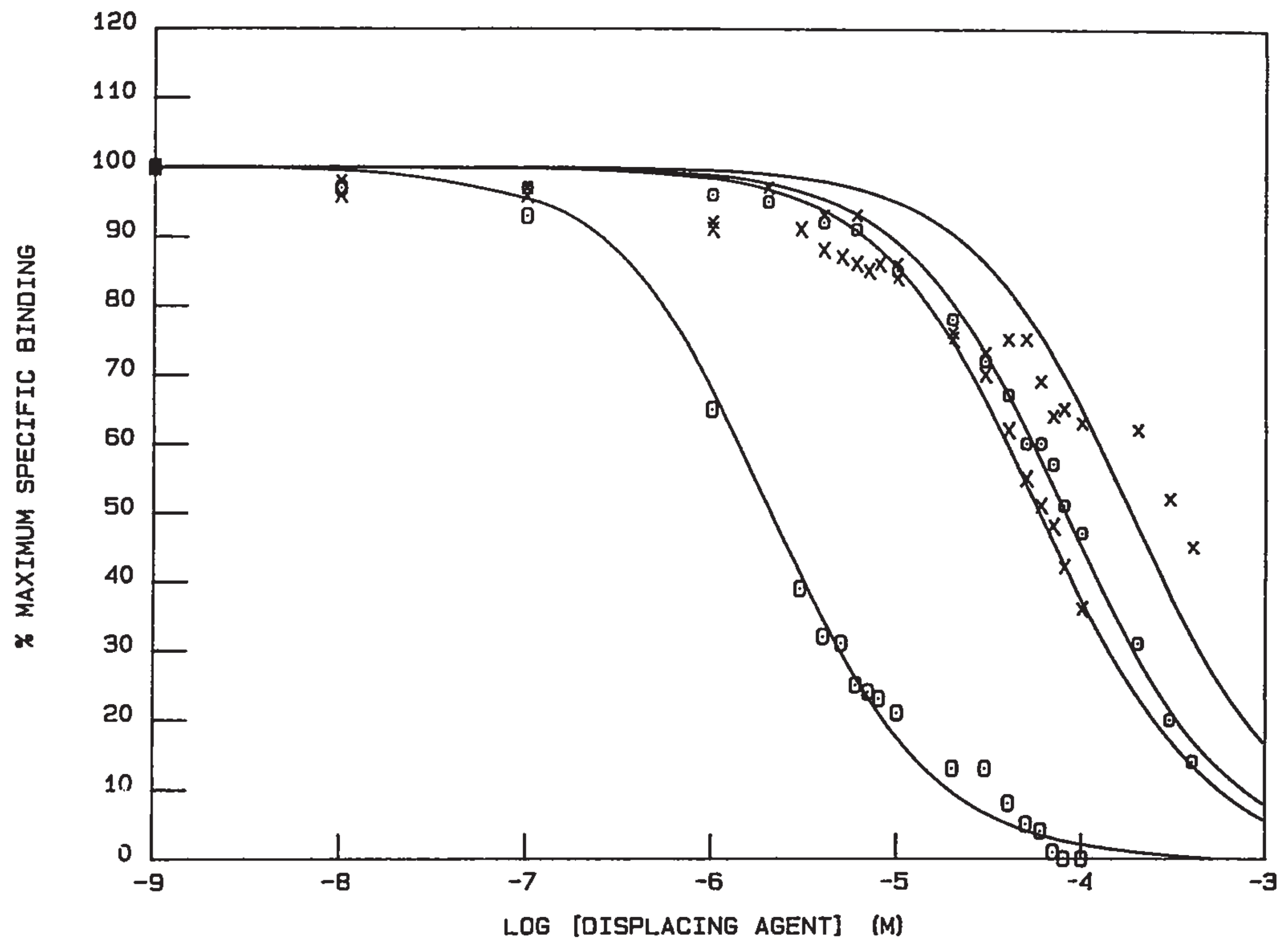


A: Control
B: (+) Butaclamol

FIGURE 5.5

Covalent Incorporation of [^{125}I]-18
into Caudate Membranes

Caudate membranes were incubated with [^{125}I]-18, photolysed, processed for SDS-PAGE and autoradiographed as described. Lane A represents total labelling, while lane B is labelling in the presence of 10 μM (+)-butaclamol. Molecular weight standards (in kDa) are indicated.



% MAXIMUM SPECIFIC BINDING
120
110
100
90
80
70
60
50
40
30
20
10
0
-9
-8
-7
-6
-5
-4
-3
LOG [DISPLACING AGENT] (M)

FIGURE 5.6

Competition Curve of 19 and 26 Against
[^{3}H]-spiroperidol and [^{3}H]-SCH 23390

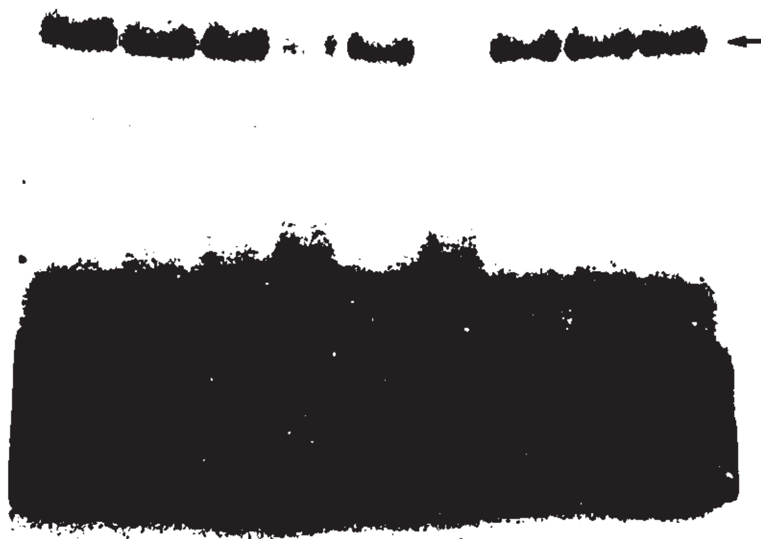
Compounds 19 and 26 were studied for their ability to inhibit [^{3}H]-spiroperidol (D2) and [^{3}H]-SCH 23390 (D-1) binding. Both compounds bound to a single affinity class of both receptor types. Compound 19 had an IC_{50} of 180 μM and 81 μM at the D-1 (x) and D-2 (o) receptor respectively. 26 had an IC_{50} of 59 μM at the D-1 receptor (X) and 2.1 μM at the D-2 receptor (O). Data is from one representative experiment performed in triplicate.

5.3.6 Inhibition of [^{125}I]-26 Incorporation and the Discovery of Apo-50

Compound 26 exibited a very similar labelling pattern to radiolabelled 18 in that a considerable amount of photolabel was incorporated into the 50 kDa protein band, while no specific binding was observed. Due to the high efficiency of labelling of the 50 kDa band, studies were undertaken to find an agent which would block incorporation into that protein. While screening a number of compounds for one with the ability to block labelling at 50 kDa, one compound proved to be effective. Apomorphine, a classical dopaminergic agonist, had the ability to inhibit labelling of the 50 kDa protein. A typical experiment illustrating this observation is shown (Figure 5.7). Densitometry of control labelling and labelling in the presence of apomorphine is shown in Figure 5.8.

This was a most interesting observation in that apomorphine, one of the most studied of all neuroactive compounds, is not well understood in terms of its mechanisms of action. Due to the potential importance of this finding, as well as the limited success obtained at D-1 receptor photolabelling, further experiments were aimed at elucidating the molecular characteristics of the 50 kDa

A B C D E F G H I



A: Control
B: + Pyrogallol
C: + Dopamine
D: + Dipropyl ADTN
E: + SKF 38393
F: + Apo
G: + ADTN
H: + Propranolol
I: Control

FIGURE 5.7

Covalent Incorporation of [^{125}I]-26 into
Bovine Caudate Membranes

Caudate membranes were incubated with [^{125}I]-26 in the presence of the indicated compounds (all at 100 μM), photolysed and proteins separated by SDS-PAGE. [^{125}I]-26 had a similar labelling pattern to [^{125}I]-18, in that one protein of 50 kDa (arrow) was highly crosslinked. Apomorphine (lane F) blocked covalent incorporation of [^{125}I]-26 into the 50 kDa protein.

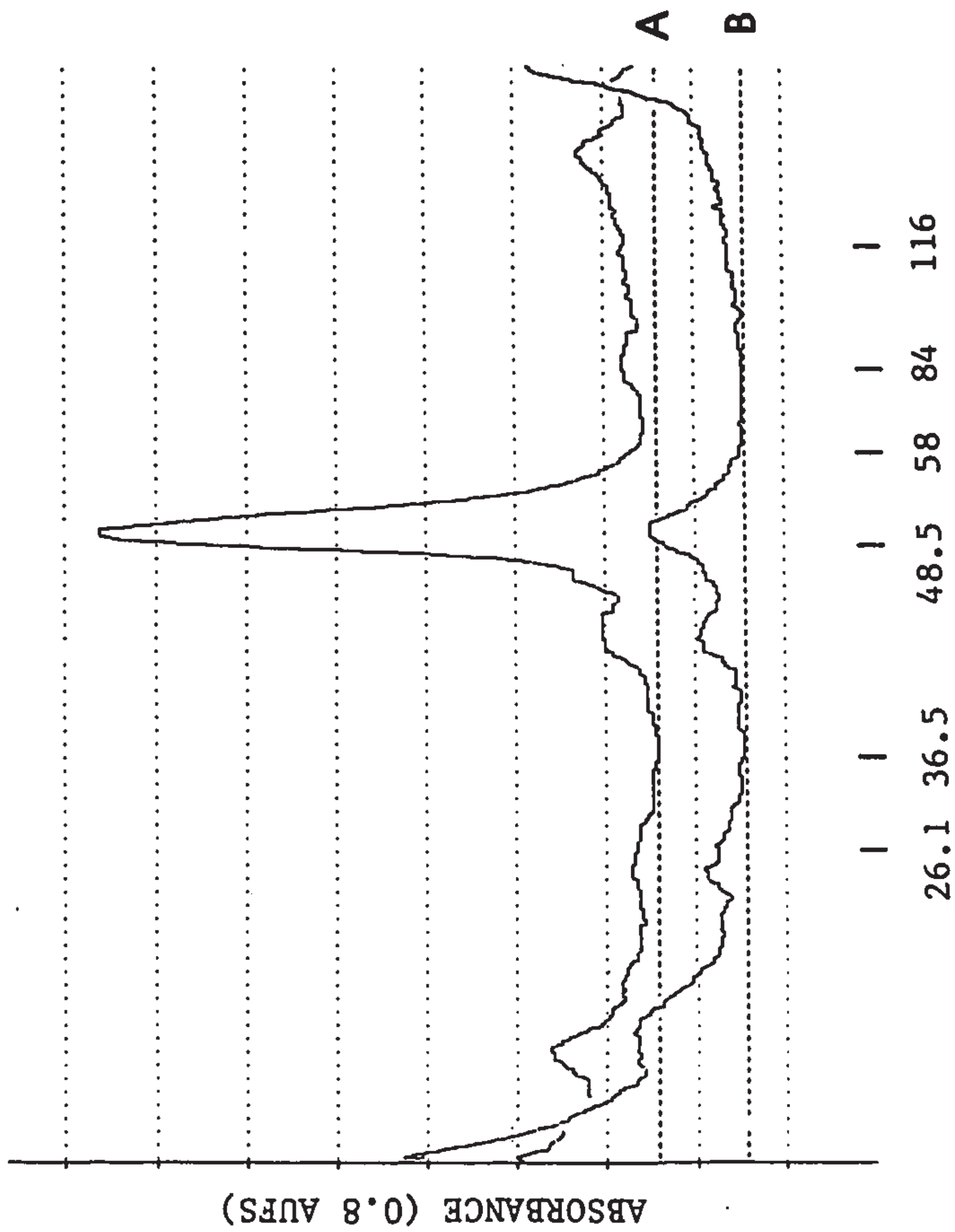

A
B
26.1
36.5
48.5
58
84
116
M.W. STANDARDS
ABSORBANCE (0.8 AUFS)

FIGURE 5.8

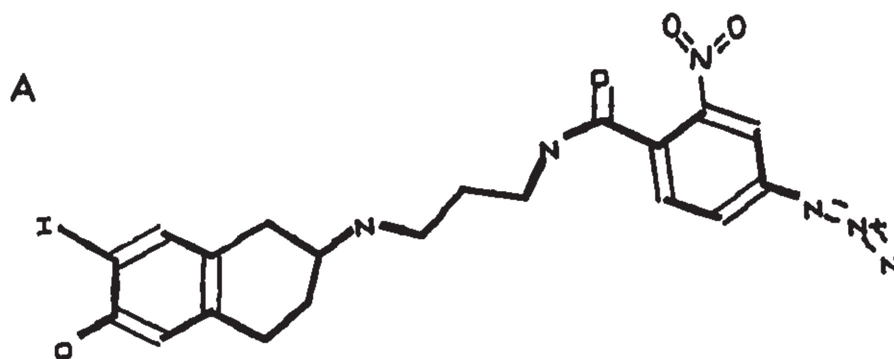
Densitometric Scan of [^{125}I]-26
Crosslinked Proteins.

Densitometric scan of control labelling (A) and labelling in the presence of 100 μM apomorphine (B) from lane F of the autoradiograph in Figure 5.7. Densitometric scans were performed with an LKB Ultrascan densitometer.

apomorphine binding protein (named Apo-50, Ross _et al_, 1988). The results to those studies are presented in Chapter 6.

In order to propose explanations as to why covalent incorporation of compound _26_ into Apo-50 could be blocked by apomorphine but not by SCH 23390, two drugs which bind to the D-1 receptor with high affinity, a more detailed structural study was made. The molecular modelling software package PC Model V1.0 was used to examine 3-dimensional features of these compounds. PC Model can be used to calculate minimum energy conformations, inter- and intra-atomic distances, van der Waal's radii and other parameters of interest. The compound _26_ is shown in its minimum energy conformation (Figure 5.9A) and with a dot density representation of the van der Waal's radius of each atom (Figure 5.9B). Minimum energy conformations can be of use in predicting stucture-activity relationships of biologically active molecules (Marshall, 1987).

In Figure 5.10, _26_ is superimposed with apomorphine, a compound which potently protects Apo-50 from photoaffinity labelling. The superimposed structures are shown both in the plane depicted by panel A and rotated 90° about the x axis (B). From both views A and B the aromatic substitutions and the superimposed nitrogens of both molecules are in very close proximity, illustrating the


A
I
O
N
N
O
O
N
O
N
N
N

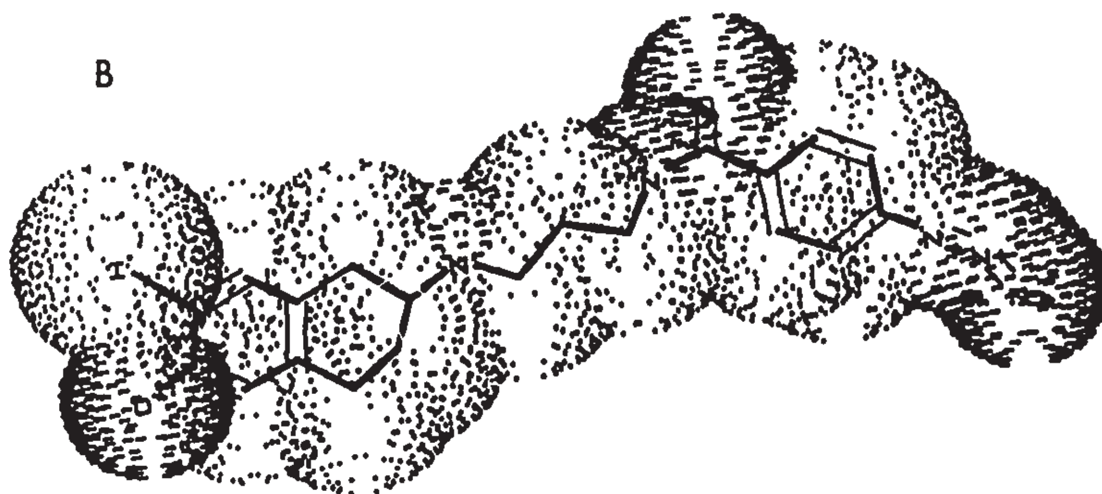
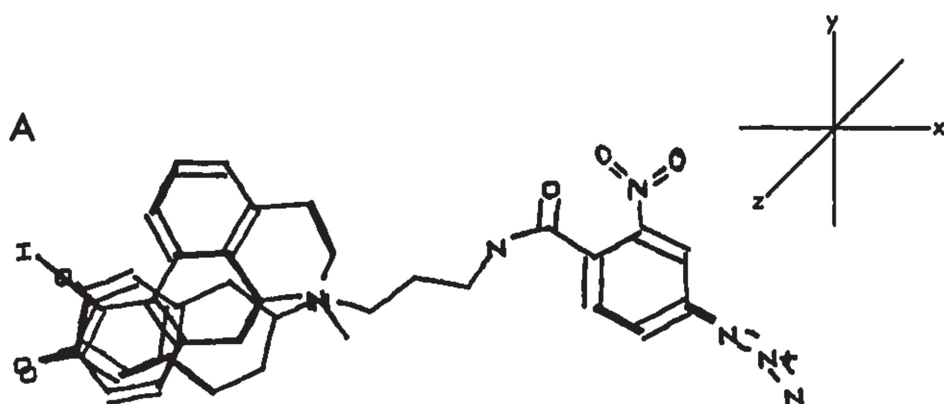

B
I
O

FIGURE 5.9

Stuctural Features of 26

Compound 26 shown in a minmum energy conformation (A) and a dot-density illustration of the van der Waal's radius of each atom (B). Both were calculated and drawn using the computer software PC Model. For simplicity, the structures are shown without the H atoms included.


A
y
x
z
I
O
N
O
N
O
N

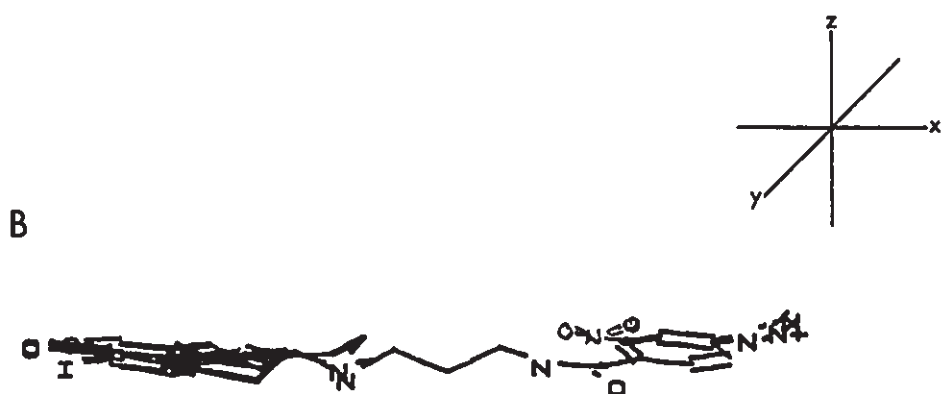

B
z
x
y
O
I
N
N
O
N
O
O

FIGURE 5.10

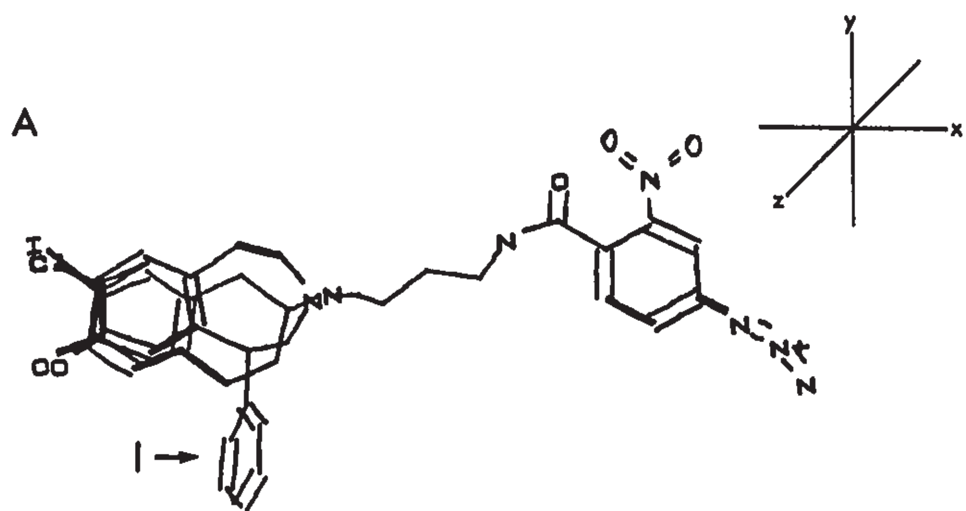
Stuctural Comparison of Apomorphine and 26

Apomorphine and 26 superimposed (A) illustrates that both the benzylic substitutions and amines are in close proximity. Rotation about the x axis (B) shows that these functional groups are in still in very close proximity when veiwed from an angle 90° from that depicted in A. Both compounds are in minimum energy conformations, with all hydrogen atoms removed.

similarity of these molecules. Although each compound has regions which do not correspond to the other, these groups apparently do not sterically hinder the binding of either apomorphine or 26 to Apo-50.

Similarly the structures of SCH 23390 and 26 have also been compared (Figure 5.11). Examing the superimposed structures as depicted in A, one can see that aromatic substitutions as well as the two nitrogens appear to have very similar spatial characteristics. SCH 23390 does have an additional aromatic ring (1) however, which does not share spatial features with either 26 or apomorphine. Furthermore, when these structures are rotated about the x axis as depicted in Figure 5.11B, it becomes apparent that the nitrogen of SCH 23390 (2) lies considerably outside of the plane of the molecule.

Although firm conclusions can not be drawn vis a vis structural features of these molecules and how binding to Apo-50 is enchanced or inhibited, these studies illustrate some of the structural characteristics of these compounds which may play a role in Apo-50 recognition.


A
y
x
z
O
O
O
N
N
N
N
N
N
1

B
z
x
y
N
N
N
2

FIGURE 5.11

Stuctural Comparison of 26 and SCH 23390

SCH 23390 superimposed with (A) shows that both benzylic substitutions as well as the amines are in close proximity. Rotation of the complex 90° about the x axis (B) shows that the amine of SCH 23390 (2) is now considerably outside of the plane of the aromatic ring (for this illustration the aromatic rings were forced to stay in one plane). Both compounds are in minimum energy conformations, with all hydrogen atoms removed.

CHAPTER 6

PHOTOAFFINITY LABELLING "APO-50"

6.1 INTRODUCTION

As described in the previous chapter, compound [^{125}I]-26 exhibited a unique labelling pattern when used to photoaffinity label caudate membrane proteins. Specifically, this compound is covalently incorporated into a 50 kDa protein, which can be blocked by apomorphine. As this finding may help to elucidate mechanisms of apomorphine actions, experiments were performed to characterize this protein. This chapter presents the results to experiments conducted which were aimed at characterization of pharmacological and molecular aspects of Apo-50

6.1.1 Methods

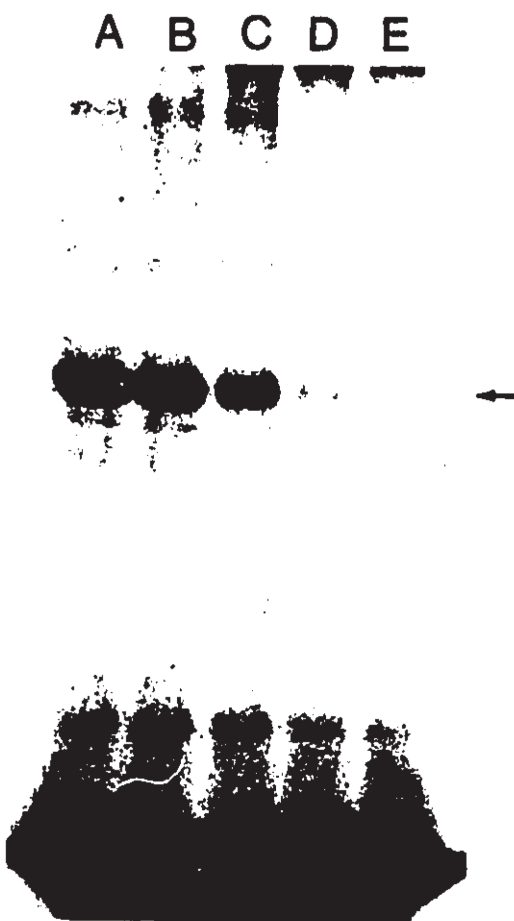
Tissue preparations and the binding assays were performed as described in 3.2.1. The standard photolysis mixture for Apo-50 labelling contained 0.5 nM of [^{125}I]-26 and additional compounds as indicated in the figure legends. The apparatus used for photoaffinity labelling as well as electrophoretic and autoradiographic techniques were as described in the previous chapter.

6.2 RESULTS AND DISCUSSION

6.2.1 Affinity of Apo-50 for Apomorphine

A significant feature of many biological interactions is that the components involved have high affinity for one another. This is particularly true in the case of receptor-hormone and receptor-neurotransmitter interactions, where components have binding constants (K_d) in the sub-micromolar to nanomolar ranges. In order to evaluate the affinity of apomorphine for Apo-50, experiments were conducted to determine the ability of apomorphine to block covalent incorporation of 26 into Apo-50 with respect to the dose of apomorphine.

The results of one such experiment are shown in Figure 6.1. Several such experiments were analysed quantitatively and the results were computer fitted using the competition curve analysis software CDATA. This analysis yielded an average IC_{50} for apomorphine against 26 of 1.3 +/- 2.1 uM. Although this affinity is not extremely high in terms of many biological interactions, it is nonetheless within the range reported for many important processes. While data of this nature does not imply that biologically significant interactions are occurring, this type of data does suggest that the affinity of apomorphine for Apo-50 is such that



A: Control
B: + 0.1 uM Apo
C: + 1.0 uM Apo
D: + 10 uM Apo
E: + 100 uM Apo

FIGURE 6.1

Affinity of Apo-50 for Apomorphine

Representative autoradiograph showing the ability of apomorphine to block covalent incorporation of $[^{125}I]$-26 into Apo-50. Apomorphine (in varying concentrations as indicated) was coincubated with bovine caudate tissue under standard conditions as described. The mixture was subsequently photolysed and the proteins separated by SDS-PAGE. Covalent incorporation of 26 into the 50 kDa protein band (arrow) was quantitated and the IC_{50} of apomorphine calculated. Data from three experiments revealed the IC_{50} of apomorphine for the protein to be 1.3 +/- 2.1 uM.

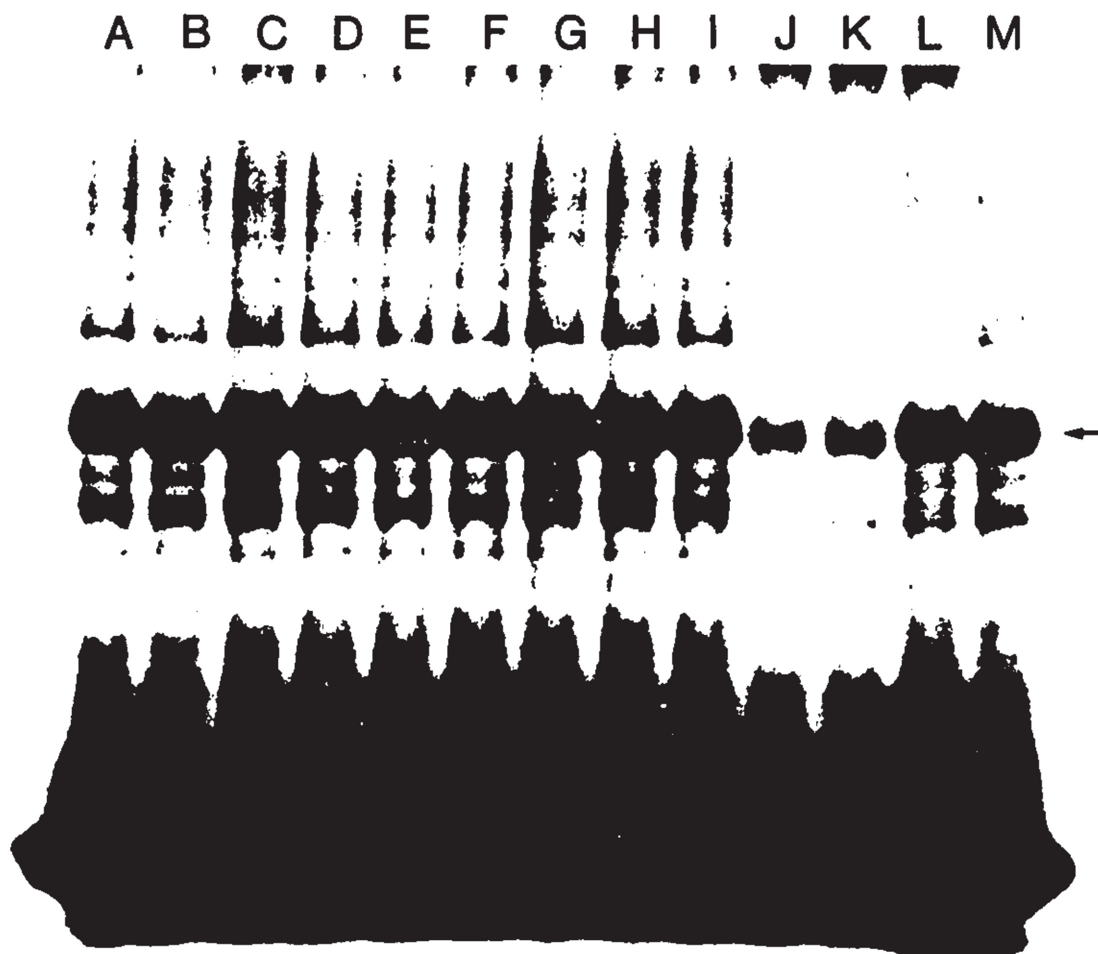
important processes may occur.

6.2.2 Ability of Dopaminergic Compounds to Block Apo-50 Labelling

As described, apomorphine is well recognized as having a wide range of effects on the central nervous system (CNS) as well as other tissues in the body. Apomorphine is considered a classical dopaminergic compound due to its well documented effects on dopaminergic neurotransmission. In addition to D-1 and D-2 receptors, several other proteins are known to play a role in dopamine function at the synapse. These include a presynaptic dopamine autoreceptor (Oene et al, 1984) and a specific dopamine uptake protein (Heikkila and Manzino, 1984). These are in addition to proteins involved in the metabolism of dopamine such as monoamine oxidases (MAO's) and catechol-O-methyl transferase (COMT). To examine the possibility that the identity of Apo-50 may in fact be any of these proteins, compounds known to interact with these proteins were tested for their ability to block photoincorporation of 26 into Apo-50. The compounds tested included SKF-38393 (a D-1 agonist), SCH-23390 (a D-1 antagonist), LY 171555 (a D-2 agonist), Spiperone (a D-2 antagonist), GBR 12909 (a dopamine uptake blocker), as well as (+)3-PPP, (-)3-PPP, and PPHT (all

putative dopamine autoreceptor ligands). The results of this experiment are presented in Figure 6.2. As none of the above compounds had any effect on the labelling of Apo-50, it was concluded that this protein was neither the D-1 receptor, the D-2 receptor, the uptake site or the autoreceptor.

Also examined and presented in Figure 6.2 was the effect of (-)NPA (a D-2 specific mixed agonist), (+)NPA (a less active isomer), dipropyl ADTN and ADTN (two non-specific dopamine agonists). Of these four compounds, only the two isomers of NPA were effective in reducing the labelling of Apo-50. NPA (N-propyl-norapomorphine) differs from apomorphine only in the substitution of a propyl group for a methyl group at the N position of apomorphine, so it is reasonable that this compound would also be effective in blocking photoincorporation into Apo-50. The only documented effects of NPA in the CNS are its interaction with the D-2 receptor, yet Apo-50 is clearly not the D-2 receptor as evidenced by the inability of either spiperone or LY 171555 to inhibit labelling. Therefore this information did not contribute significantly to the identity of Apo-50.



A: Control
B: + SKF 38393
C: + SCH 23390
D: + Spiperone
E: + LY 171555
F: + GBR 12909
G: + (+)3–PPP
H: + (–)3–PPP
I: + PPHT
J: + (–) NPA
K: + (+) NPA
L: + Dipropyl ADTN
M: + ADTN

FIGURE 6.2

Ability of Dopaminergic Compounds
to Block Apo-50 Labelling

Autoradiographic data illustrating the inability of other dopaminergic compounds to block covalent incorporation of 26 into Apo-50. Several dopaminergic compounds (all at 10 μM) were coincubated with caudate membranes and [^{125}I]-26 under standard conditions and subsequently photolysed. All compounds (including SKF 38393, a D-1 agonist; SCH 23390, a D-1 antagonist; spiperone, a D-2 antagonist; LY 171555, a D-2 agonist; GBR 12909, a dopamine uptake blocker; (+)3-PPP, (-)-3PPP and PPHT, all dopamine autoreceptor ligands; dipropyl-ADTN and ADTN, both non-selective agonists) except (-)NPA and (+)NPA had effectively no effect on incorporation of the photolabel into Apo-50 (arrow).

6.2.3 Ability of Serotonergic, Adrenergic and Opiate Compounds to Block Apo-50 Labelling

In addition to the well-studied effects of apomorphine on dopaminergic function, this compound has also been demonstrated to interact with several other neurotransmitter systems. These include adrenergic (Cools, 1980), serotonergic (Lee and Geyer, 1986) and opiate (Szechtman, 1986) systems. As each of these neurotransmitter systems potentially represent several classes and subclasses of receptor types, representative non-selective ligands from each class were chosen and tested for their ability to prevent 26 incorporation into Apo-50.

The compounds selected were Metergoline and serotonin (serotonergic), phentolamine and isoproterinol (adrenergic), and Dipenorphine and Etorphine (opiate). As shown in Figure 6.3, none of the above compounds were effective in reducing photoincorporation in concentrations of 100 μM, suggesting that Apo-50 is not a receptor of the serotonergic, adrenergic or opiate systems.

A: Control
B: + Apo
C: + Metergoline
D: + Serotonin
E: + Phentolamine
F: + Isoproterinol
G: + Dipenorphine
H: + Etorphone

FIGURE 6.3

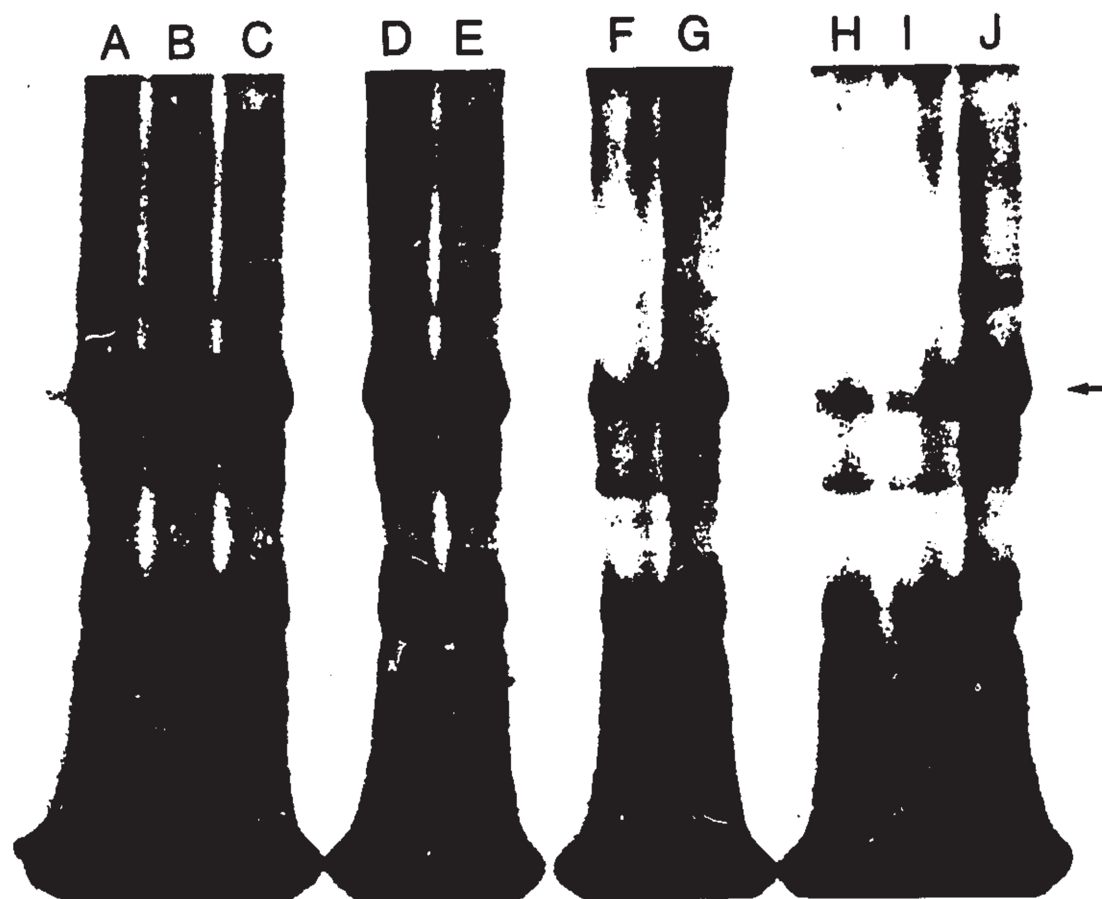
Ability of Serotonergic, Adrenergic and Opiate Compounds to Block Apo-50 Labelling.

The ability of drugs which act at several classes of neurotransmitters were examined for their ability to block Apo-50 labelling as described in the previous figures. The compounds tested (all at 10 μM) were Metergoline (a non-selective serotonergic antagonist), serotonin (a non-selective serotonergic agonist), Phentolamine (an adrenergic antagonist), Isoproterinol (an adrenergic agonist), Dipenorphine (an opiate antagonist), and Etorphine (an opiate agonist). None of these compounds had any effect on the incorporation of [^{125}I]26 into Apo-50 (arrow). Apomorphine (lane B) is included as a positive control.

6.2.4 Stereo-selectivity of Apo-50

One of the features often noted in the interactions of biological molecules is that of stereo-selectivity. Although stereo-selectivity is not necessarily observed in all biological processes, it is an important feature which distinguishes most biochemical reactions from reactions not mediated by biological molecules. Stereo-selectivity has been described in almost all ligand-receptor interactions and is often a useful criterion in distinguishing receptor sites from non-specific binding sites.

To determine whether or not the binding of Apo-50 to apomorphine was stereo-selective, two commercially available isomers of NPA were used to block incorporation. The two isomers were used in a range of concentrations and the IC_{50} for each isomer calculated. Representative data (Figure 6.4) shows that Apo-50 has a higher affinity for (-)NPA than it does for (+)NPA. Several such experiments yielded the following data: (-)NPA IC_{50} = 3.5 +/- 1.2 uM; (+)NPA IC_{50} = 24 +/- 4.7 uM. From these studies it is clear that the interaction of NPA with Apo-50 is stereo-selective, suggesting that the protein is not interacting with NPA in a non-specific manner.



A: Control
B: + 0.1 uM (−)NPA
C: + 0.1 uM (+)NPA
D: + 1.0 uM (−)NPA
E: + 1.0 uM (+)NPA
F: + 10 uM (−)NPA
G: + 10 uM (+)NPA
H: + 100 uM (−)NPA
I: + 100 uM (+)NPA
J: Control

FIGURE 6.4

Stereoselectivity of Apo-50 Labelling

Two pure isomers of N-propylnorapomorphine (NPA) were studied for their ability to block labelling of Apo-50 (arrow) in a dose related manner. Both isomers were used in an identical dose range and the IC_{50} for each isomer was calculated. The more active of the two isomers was (-)NPA, having an IC_{50} of 3.5 +/- 1.2 μM (average +/- SD of three experiments). The other isomer, (+)NPA, was less effective, having an IC_{50} of 44 +/- 4.7 (average +/- SD of three experiments). Experimental conditions were as described for previous figures.

6.2.5 Effect of ß-mercaptoethanol and Glutathione on Apo-50 Labelling

An important control experiment which must be considered in all photolabelling studies is labelling in the presence of radical scavengers (Bayley, 1983). The reactive species which results from photolysis of an azido compound (a nitrene) can easily be quenched with appropriate scavengers. Photolabel bound tightly to a target molecule can not be readily quenched by scavengers. Several radical scavengers, such as PABA, DTT or glutathione can be used for this purpose. In the case of true photoaffinity labelling, the amount of label incorporation should not be decreased by the presence of a scavenger molecule. Not all scavengers can be used in every situation, however, as some very effective scavenger molecules (such as DTT) can modify the ability of a protein to bind the photolabel by reduction of disulfides required for binding activity. As some proteins can lose binding activity in the presence of disulfide reducing compounds, PABA was also chosen as a scavenger molecule for control experiments. PABA, in concentrations up to 10 mM (the solubility limit) had no effect on incorporation of 26 into Apo-50 (data not shown).

In other experiments sulfhydryl compounds were tested as scavenger molecules and a representative autoradiograph

is shown in Figure 6.5. From the data it is obvious that either ß-mercaptothanol or glutathione totally abolished the ability of [^{125}I]-26 to photolabel Apo-50. In addition to being effective radical scavengers, both compounds are potent reducing agents of disulfides. As PABA was ineffective in blocking labelling yet the sulfhydryl compounds were effective, it was concluded that the sulfhydryl compounds probably inhibit labelling by reduction of disulfides required by Apo-50 for [^{125}I]-26 binding, rather than by scavenging radicals.

The sensitivity of Apo-50 to reduction by sulfhydryls is not uncommon among proteins. Both the D-1 and D-2 receptors are sensitive to reducing agents such as DTT or ß-mercaptoethanol and agonist binding in particular is abolished in even low concentrations of disulfide reducing compounds.

A B C D E F G H

A: Control
B: A + Apo
C: + β–Mercaptoethanol
D: C + Apo
E: + Glutathione
F: E + Apo
G: + Both
H: G + Apo

FIGURE 6.5

Effect of Sulphydryl Containing Compounds
on Apo-50 Labelling

The effect of ß-mercaptoethanol and glutathione on Apo-50 labelling was examined. Both compounds (at 1 mM) were incubated with caudate membranes and [^{125}I]-26 as described, photolysed and separated on SDS-PAGE. ß-mercaptoethanol and glutathione both inhibited ^{125}I incorporation into Apo-50 (arrow).

6.2.6 Effects of Divalent Cations and GTP on Apo-50

Many neurotransmitter receptor proteins are intimately coupled to guanyl nucleotide regulatory protein (or G-protein, see introduction). G-proteins are considered to be a link in the signal transduction pathway, mediating the events from receptor occupancy to physiological effects. Receptors which are coupled to G-proteins are often modulated by the presence of GTP, GppNHp.
Including any of these compounds in a receptor binding assay will decrease the affinity of the receptor and could therefore change the labelling pattern of the ligand if Apo-50 is coupled to a G-protein. The activity of guanyl nucleotides is dependent on the presence of divalent cations, so it was essential to examine their effects as well.

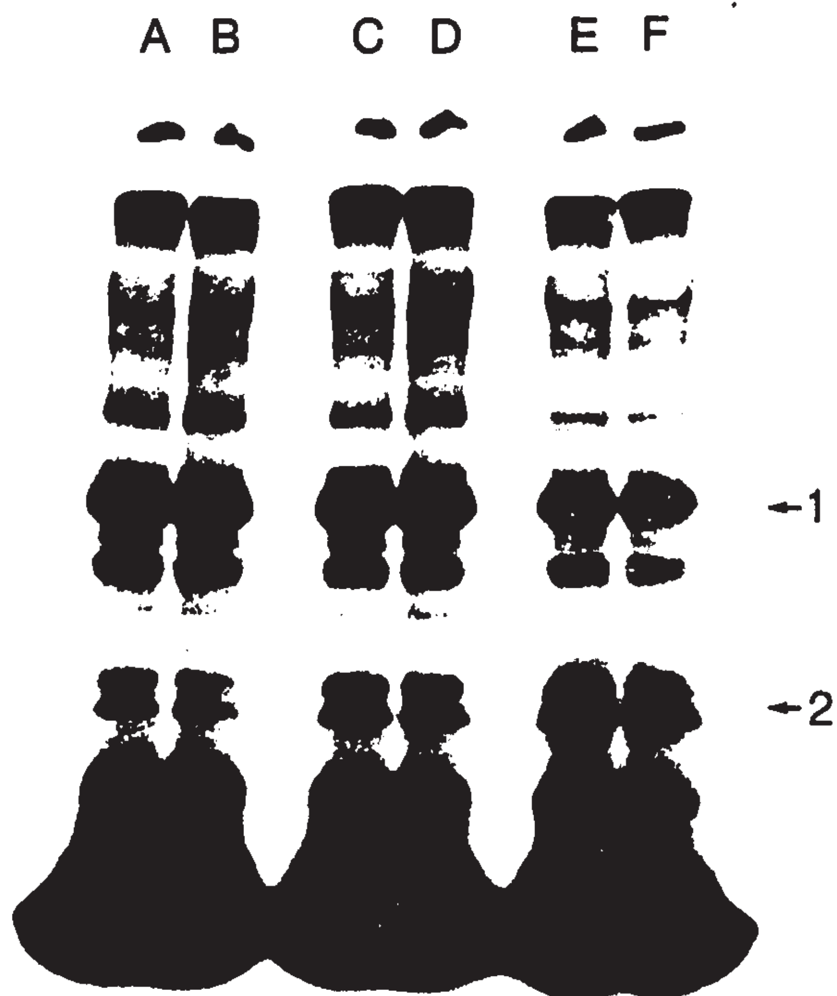
From previous experiments it was obvious that divalent cations had a significant effect on the labelling pattern observed with photoincorporation of [^{125}I]-26. In general, the presence of EDTA-enhanced incorporation into Apo-50, while the absence of EDTA showed diminished radioincorporation. From this observation it was suspected that some type of degradation (probably proteolytic) was occurring in the presence of divalent cations.

To explore the possibility of proteolytic degradation,

the effect of divalent cations (Mg^{++} and Mn^{++}) on the labelling pattern of Apo-50 was examined. Representative data is illustrated in Figure 6.6. From the autoradiograph two features of the labelling pattern are apparent. Firstly, the amount of radioactivity incorporated into the 50 kDa band (arrow 1) decreases in the presence of either Mg^{++} or Mn^{++}. Secondly, two lower molecular weight proteins (arrow 2) increase concomitantly with the loss of labelling at 50 kDa. The lower molecular weight proteins probably represent cleavage products of Apo-50 by a divalent cation-dependent protease. Many proteolytic enzymes, including carboxypeptidases, require divalent cations for full enzymatic activity (Metzler, 1977).

The effects of GTP on Apo-50 are shown in Figure 6.7. From the data, there was no significant change in the amount of photolabel incorporated into Apo-50 either in the absence or in the presence of GTP. Although Mg^{++} again significantly reduced incorporation, labelling in the presence of Mg^{++} and GTP was not different than labelling in the presence of Mg^{++} alone. In experiments where GppNHp or GTPγS was substituted for GTP, no effect on the labelling of Apo-50 was observed.

Also shown in Figure 6.7 is the effect of NaCl on Apo-50 labelling. From labelling experiments performed either in the absence or the presence of NaCl it was concluded that



A: Control

B: A + (+) But.

C: + $MgCl_2$

D: C + (+) But.

E: + $MnCl_2$

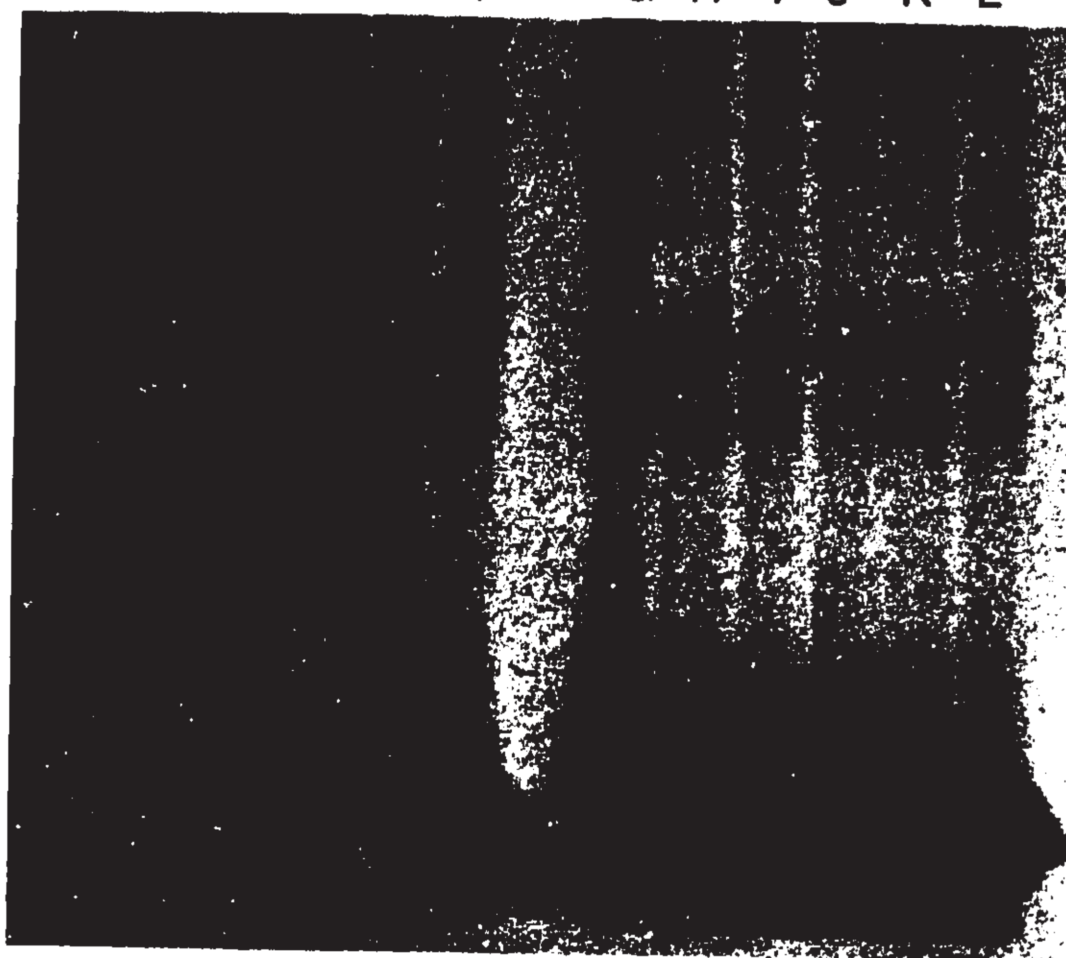
F: E + (+) But.

FIGURE 6.6

Effect of Divalent Cations on
Apo-50 Labelling

$MgCl_2$ and $MnCl_2$ (both at 10 mM) were added to the standard photoaffinity labelling mixture and photolysed as described. SDS-PAGE of the labelled proteins followed by autoradiography revealed that both Mg^{++} and Mn^{++} reduced ^{125}I incorporation into Apo-50 (arrow 1), while additional labelling appeared in a lower molecular weight region (arrow 2). (+)But indicates the presence of 10 μM (+) butaclamol.

A B C D E F G H I J K L



A: Control
B: + GTP and MgCl2
C: B + Apo
D: A + Apo
E: D + Apo
F: A + MgCl2

G: A + NaCl
H: B + NaCl
I: C + NaCl
J: D + NaCl
K: E + NaCl
L: F + NaCl

FIGURE 6.7

Effect of GTP on the
Labelling of Apo-50.

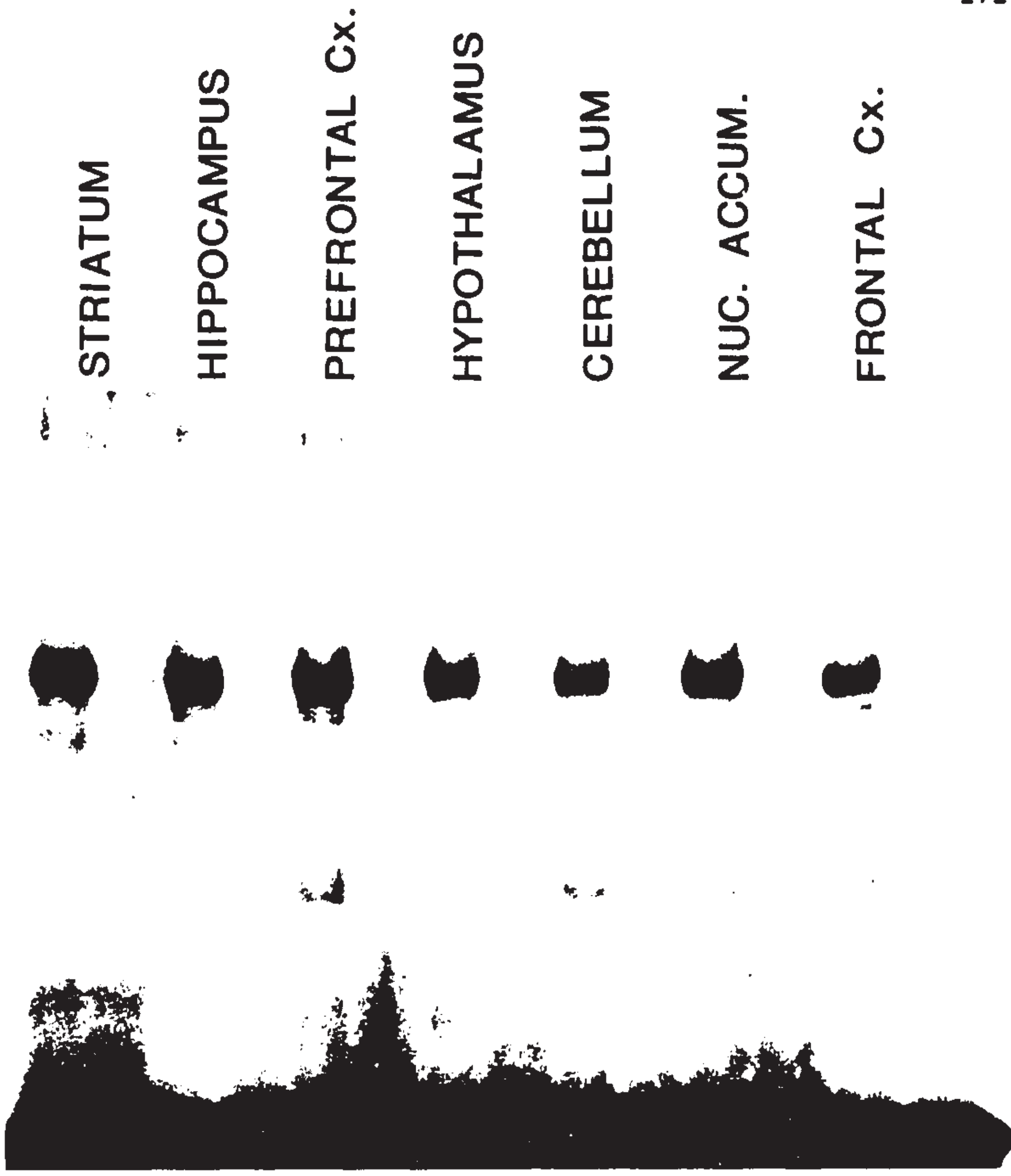
The effects of GTP (100 μM), $MgCl_2$ (10 mM) and NaCl (120 mM) on the labelling of Apo-50 (arrow) was examined. GTP had no significant effect on reducing labelling in the presence of Mg^{++} (lane B vs F). Mg^{++} did reduce labelling as already observed, while Na^+ had no effect on the labelling pattern.

this salt had no effect on the labelling of Apo-50.

6.2.7 Apo-50 in the Brain

One of the hallmark features of receptor proteins is their discrete localization within the brain. Receptors for a given neurotransmitter are localized within specific pathways of certain brain nuclei which utilize that neurotransmitter. Other proteins, such as those which play a more general metabolic role or function as structural elements, tend not to be discretely localized but rather are more generally distributed.

To study the distribution of Apo-50 a bovine brain was disected and several regions assayed for the presence of this protein. The regions examined included the striatum (caudate), hippocampus, prefrontal cortex, hypothalamus, cerebellum, nucleus accumbens and prefrontal cortex. As can be seen in Figure 6.8, Apo-50 is present in each of the areas examined. Although from this data it cannot be concluded that Apo-50 is not a receptor protein, it suggests that Apo-50 is more likely to be involved in a function which is less discrete with respect to localization than a neurotransmitter pathway.



— Control Labelling

\+ Labelling in the presence of 10 uM Apo

FIGURE 6.8

Distribution of Apo-50 in the Brain

Several regions of bovine brain, including striatum (caudate), hippocampus, prefrontal cortex, hypothalamus, cerebellum, nucleus accumbens and prefrontal cortex were assayed for the presence of Apo-50 (arrow). The protein was present in all regions examined.

6.2.8 Apo-50 in Other Species

Further information on the general nature of a protein may also be obtained from examining the inter-species distribution of the protein. Although a very small number of species were studied, Figure 6.9 illustrates that Apo-50 is present in three species, namely cow (bovine) rat and dog.

6.2.9 Apo-50 in Other Organs

Several pieces of evidence suggested that Apo-50 may in fact be the catabolic enzyme catechol-O-methyl-transferase (COMT). The evidence upon which this suggestion was based was: 1) COMT is widely localized in high concentrations in the brain, (Axelrod, 1971) 2) apomorphine is a substrate for COMT (Sourks and Lal, 1975) and 3) the molecular weight of purified COMT is approximately 50 kDa (Moo-On Huh and Friedhoff, 1979). COMT, as well as being found in the brain, is present in high amounts in both the liver and kidney. This enzyme is currently thought to exist in both a soluble (cytoplasmic) and membrane bound form (Martin and Veser, 1986). In Figure 6.10, both the soluble and membrane fractions of liver, kidney and brain were assayed for the presence of Apo-50. From this experiment,

A: Cow Caudate Membranes
B: A + Apo
C: Rat Caudate Membranes
D: C + Apo
E: Dog Caudate Membranes
F: E + Apo

FIGURE 6.9

Apo-50 in Different Species

Several species of mammals, including cow (bovine), rat and dog were assayed for the presence of Apo-50 (arrow). The protein was observed all three species, and apomorphine (10 μM) decreased incorporation in each.

A: Liver Cytosolic
B: A + Apo
C: Liver Membranes
D: C + Apo
E: Kidney Cytosolic
F: E + Apo
G: Kidney Membranes
H: G + Apo
I: Brain Cytosolic
J: I + Apo
K: Brain Membranes
L: K + Apo

FIGURE 6.10

Apo-50 in Other Organs

Cytosolic and particulate (membrane) fractions of homogenized liver, kidney and brain tissue from rat was assayed for the presence of Apo-50 (arrow). The protein was present in both fractions of brain tissue, while none could be observed in either kidney or liver.

no evidence of Apo-50 could be found in either the cytoplasmic or membrane fractions of the liver or kidney, while considerable amounts were present in both fractions of the brain homogenate. This data (along with that in section 6.2.2) suggests that Apo-50 is not the enzyme COMT. The observation that Apo-50 is brain specific (at least with respect to liver and kidney) again offers no evidence as to the identity or function of this protein, however it does apear that Apo-50 is involved in a brain function, one not shared by either the liver or kidney (such as COMT activity).

6.2.10 Light Dependence of Apo-50 Labelling

Early control experiments examining the covalent incorporation of 26 into Apo-50 showed no evidence of labelling in the absence of light. This later proved to be an incorrect conclusion, however, in that some incorporation could be observed with sufficiently long film exposure times (Figure 6.11). This was a very interesting result as it demonstrated that a component of the incorporation of $[^{125}I]$-26 into Apo-50 was not light dependent. From Figure 6.11, it can also be seen that apomorphine blocks both the light-dependent and the light-independent components of Apo-50 labelling.

A B C

A: Control (with U.V. Light)
B: A + Apo
C: No U.V. Light

FIGURE 6.11

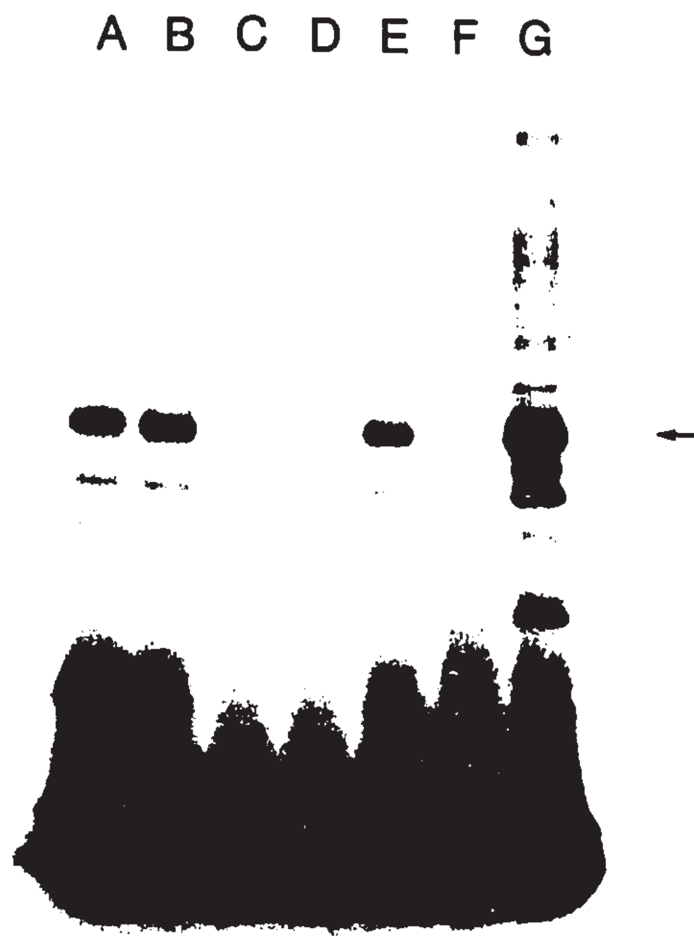
Light-independant Crosslinking of Apo-50

Bovine caudate membranes were incubated in the dark and photolysed (lane A), photolysed with apomorphine (lane B), or not photolysed (lane C). Some covalent incorporation of [^{125}I]-26 into the 50 kDa protein band (arrow) could be observed in the absence of irradiation with UV light. Quantitative autoradiography demonstrated that less than 10% of the radioactivity in the 50 kDa band in lane A was present in the same band in lane C.

The light-independent component of Apo-50 labelling was a very surprising finding and suggests one of the possible functions of Apo-50. As the biological stability of nitroaryl azides has been established by many investigators, it is likely that compound 26 is covalently bound to Apo-50 by one of the functional groups remote from the photoactive moiety. In addition to the photoactive end of 26, the functional groups likely to participate in a covalent reaction are the 2-amino secondary amine, or the phenolic hydroxy group at position 6. Interestingly, both of these functional groups are also present in apomorphine and share many spatial molecular characteristics.

Based on the observation that Apo-50 is capable of covalently coupling [^{125}I]-26 even in the absence of light, it is possible that one of the functions of this protein is a role as a scavenger molecule. The protein may recognize apomorphine-like molecules and covalently bind them, which would prevent them from interfering with neuronal transmission.

Several sulfhydryl compounds also inhibit the light independent component of labelling (Figure 6.12). This would support the suggestion proposed earlier that sulfhydryls block labelling by reducing required disulfides in the protein rather than by a radical scavenging mechanism. If sulfhydryls blocked labelling by quenching



A: Control
B: A + NaNO2
C: A + DTT
D: A + B—Mercaptoethanol
E: Control
F: E + Pyrogallol
G: + U.V. Light

FIGURE 6.12

Effect of Reducing Agents on Light-independant Labelling of Apo-50

The effect of DTT, ß-mercaptoethanol and pyrogallol on light-independant crosslinking of Apo-50 (arrow) was examined. All three reducing agents decreased the light-independant component of covalent labelling.

radicals, then they should not be effective in inhibiting the light independent incorporation of 26.

6.2.11 The CatNAP Hypothesis

During experiments aimed at elucidating the nature of parameters affecting the labelling of Apo-50 by 26, several anomolies became apparent. The first was the observation that a significant amount of 26 could be covalently incorporated into Apo-50 even in the absence of light. As discussed in the previous chapter, this result was certainly not expected as one of the crucial control experiments in any photoaffinity studies includes the demonstration that the reaction is light dependent.

An interesting concept was raised by the observation that a compound such as the photolabel used could spontaneously and covalently react with a protein. Indeed if 26 could, perhaps other catecholamines such as ADTN or NPA would also react under the appropriate conditions. To test this hypothesis caudate membranes were incubated with [^{3}H]-NPA and [^{3}H]-ADTN in the absence and presence of reducing agent (DTT) and proteins separated by SDS-Page. Gels were then processed for fluorography and exposed as previously described. The results of this experiment (Figure 6.13) clearly demonstrate covalent labelling of a

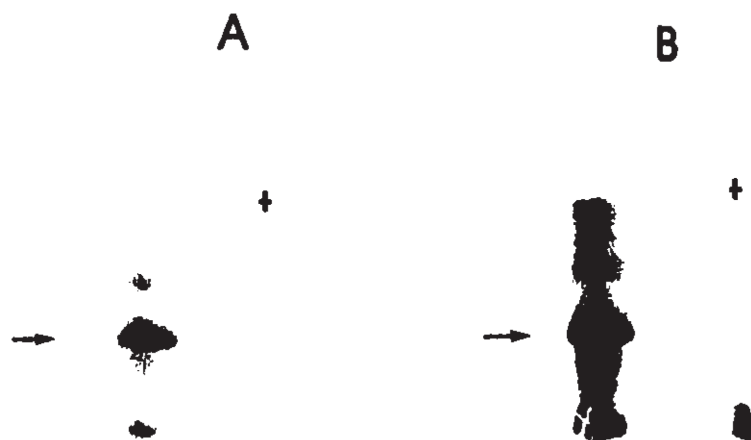

A
B
+
+

FIGURE 6.13

Covalent Incorporation of [^{3}H]-ADTN and [^{3}H]-NPA into Caudate Membranes

Both [^{3}H]-ADTN (A) and [^{3}H]-NPA (B) were incubated with caudate membranes under standard conditions in the absence of light. Proteins were then separated by SDS-PAGE and processed for flourography. Both [^{3}H]-ADTN and [^{3}H]-NPA (each at 50 nM) covalently incorporated into a protein with a M.W. of 50 kDa (arrow). In both cases, covalent incorporation could be eliminated by including ß-mercaptoethanol (lane indicated by +).

protein with a M.W. of 50 kDa. It is postulated that this protein is, in fact, Apo-50.

From the results of this experiment it appears likely that Apo-50 is actually a catecholamine binding protein, rather than a specific apomorphine binding protein. Furthermore, the binding of catecholamines to the protein can be covalent. In light of these experiments, Apo-50 has been renamed CatNAP-50 (for Catechol-[Cat] amine [N] absorbing [A] protein [P]-50 [50 kDa]) to more accurately reflect the observation that it reacts with catecholamines other than apomorphine.

Certainly after considering these observations many questions come to mind. Among them is the nature of the reaction which may be occuring. There appears to be no precedent in the literature for a covalent reaction of catecholamines with proteins, yet there is one relatively well known reaction, phenolic coupling, which could be involved in this process. Phenolic coupling occurs when two phenols dimerize oxidatively and form biphenyls as shown:

This reaction has been implicated to occur in Parkinsonian patients receiving large doses of l-DOPA (Sourkes and Lal, 1975), and has been demonstrated to occur with tyrosine units in peptides and histone (Prutz *et al*, 1983).

Data obtained during this investigation is consistant with a radical-mediated reaction, such as phenolic coupling, occuring between *26* and the 50 kDa protein. The evidence can be summarized as follows: 1) thiols (potent radical scavengers) inhibit the covalent incorporation of *26* into CatNAP-50; 2) ascorbate (also a radical scavenger) inhibits incorporation; 3) catecholamines (including ADTN and NPA) covalently label CatNAP in the absence of radical scavengers.

Although there is no evidence for it at this time, it is interesting to consider that CatNAP may be involved in CNS processes. Clearly under appropriate conditions this protein can absorb catecholamines from its surrounding milieu, which may play a key role in brain function. As this protein could potentially modulate the concentration of neurotransmitters such as dopamine, it may be involed in signal transduction mechanisms. Given also that dopamine dysfunction is involved in several disease processes, CatNAP may play a role in the pathogenesis of schizophrenia,

Parkinson's disease and others.

It is well known that radicals are toxic to many biological systems. Organisms are normally equiped with mechanisms (such as Vitamine E) which can scavenge radicals as they form, thereby preventing damage to more sensitive components. It would be logical to imagine that the brain could have efficient mechanisms for radical scavenging, given the high concentrations of radical forming compounds (such as catecholamines) and the sensitivity of brain tissues. CatNAP may therefore act as a specific catecholamine scavenging molecule, having the ability to both bind these molecules and react with any that may have formed potentially harmful radicals.

CHAPTER 7

CONCLUSIONS

CONCLUSIONS

The current investigation was aimed at developing novel probes to be used in the purification of the dopamine D-1 receptor. Several new compounds were synthesized for this investigation, to be used as affinity and photoaffinity ligands for the D-1 receptor. The first compound designed for this investigation was AP-ADTN. This compound was successfully synthesized and covalently coupled to an affinity matrix.

Prior to beginning affinity purification, it was necessary to develop an effective solubilization protocol. Several detergents were tested for their ability to solubilize the D-1 receptor, but few proved to be effective. A protocol using cholic acid resulted in the best solubilization, with greater than 30% of the total receptors recovered in a soluble form.

Using the affinity matrix synthesized from AP-ADTN, it was possible to purify the D-1 receptor approximately 50-fold, however recovery was less than 10%. Although this level of recovery was better than any other reported at that

time, it precluded further purification of the protein using other chromatographic techniques.

A photoactive analogue of AP-ADTN was synthesized and used to photoaffinity crosslink the D-1 receptor. This compound effectively crosslinked greater than 70% of the D-1 receptor as measured by the decrease in binding activity. A tritiated form of the same compound was used to photoaffinity label striatal proteins. A protein of M.W. = 79 kDa was specifically labelled in the absence of dopamine, and was presumed to be the D-1 receptor. At the time this data was reported, only one literature report of photoaffinity labelling of the D-1 receptor existed, and these investigators reported a M.W. = 59 kDa. Since reporting the findings of this investigation, several other investigators have reported a D-1 M.W. of greater than 70 kDa as determined by photoaffinity crosslinking. As the amount of radioactivity incorporated into the 79 kDa protein was very low, it was felt that the photoaffinity procedure developed would not be suitable as a step in purification. For this reason, other photolabels were synthesized with ^{125}I, to produce compounds with higher specific activities.

Two radioiodinated photolabels were synthesized and tested as probes for the D-1 receptor. Neither compound proved effective as a photoaffinity label, however both compounds were non-specifically incorporated into a 50 kDa

protein. The 50 kDa protein was later discovered to have binding activity with several catecholamines. This protein (named CatNAP-50) has now been shown to have covalent binding activity with several pharmacologically active compounds. The role, if any, of this protein in CNS function has yet to be elucidated and is beyond the scope of this thesis.

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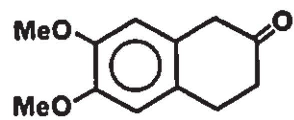
APPENDIX 1



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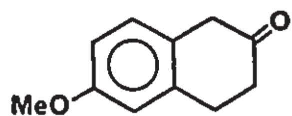

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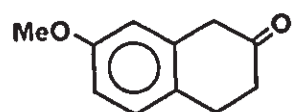

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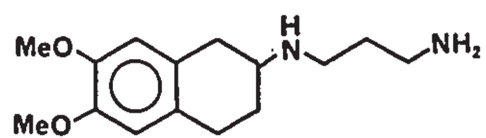

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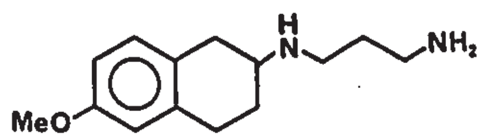

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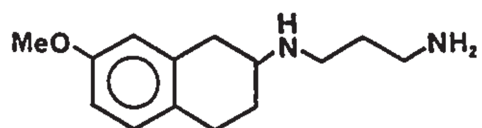

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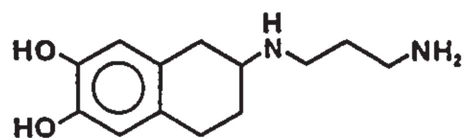

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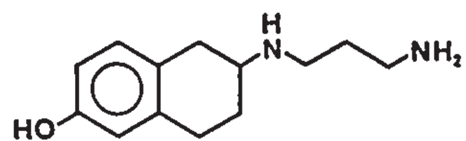

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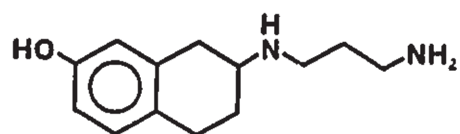

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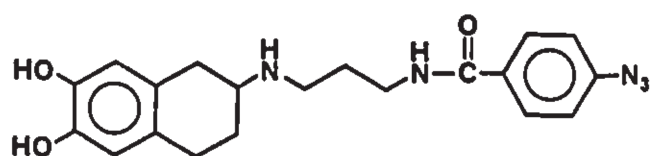

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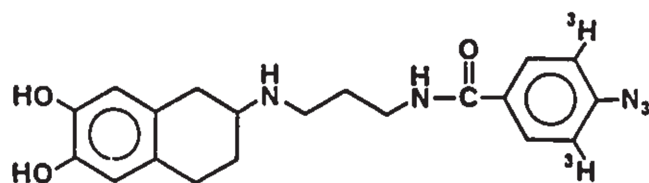

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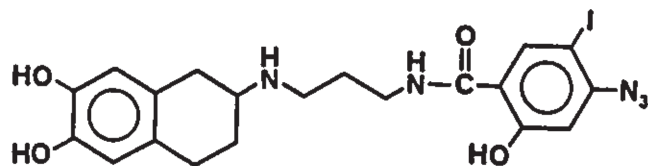

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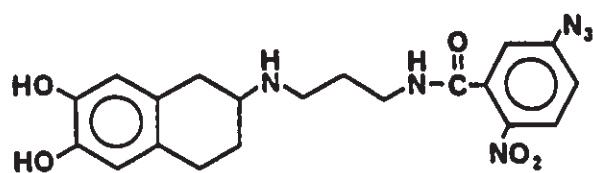

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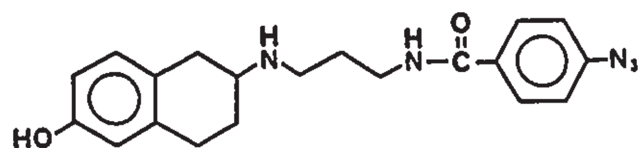

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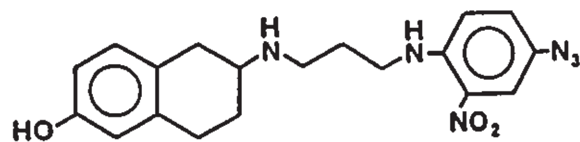

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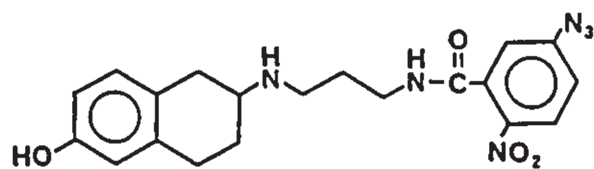

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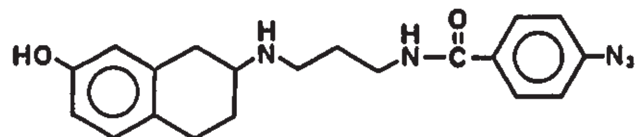

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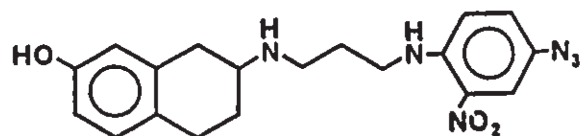

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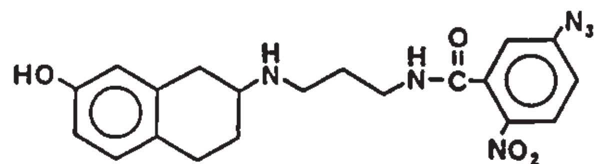

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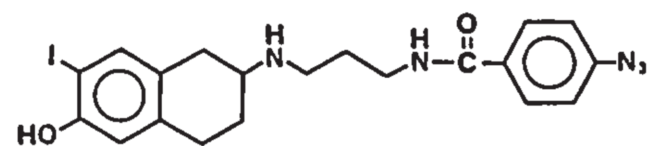

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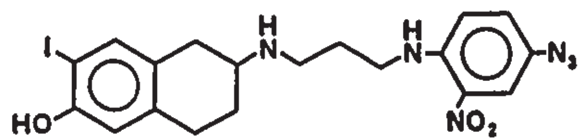

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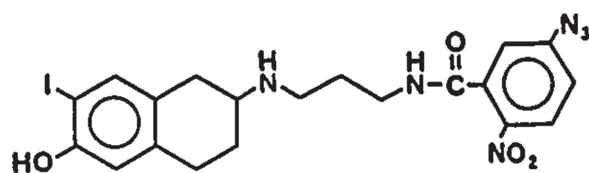

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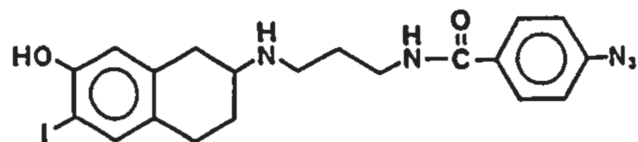

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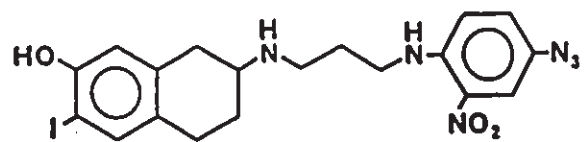

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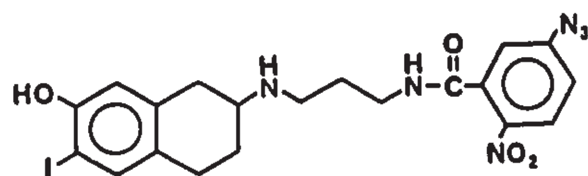

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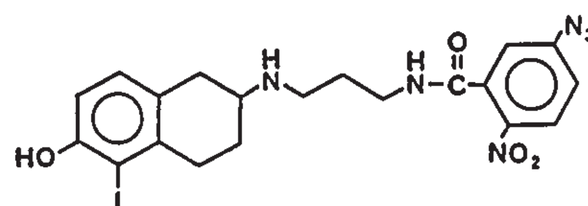

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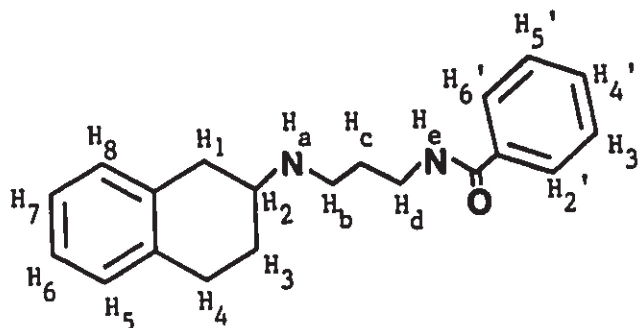
APPENDIX 2

Chemical Analysis of Novel Compounds Synthesized for this Thesis

Thin layer chromatography (TLC) was performed on silica plates (Merck) using butanol/acetic acid/water (25:5:9) as a mobile phase. High performance liquid chromatography (HPLC) separations were performed using 4.6 x 250 mm C_{18} reverse phase columns (Beckman) with a mobile phase of 50% acetonitrile, 49.9% water and 0.1% trifluoroacetic acid. Mass Spectra (MS) were obtained on a VG ZAB-E mass spectrometer using chemical ionization (ammonia gas). Infrared spectra (IR) were obtaind from KBr pellets using a Varian IR spectophotometer. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AC-200 200 MHz or AM-500 500 MHz spectrometer as indicated. All NMR spectra were obtained in DMSO-d_6 at 2.49 ppm. Chemical shift values (δ) are in parts per million, using the abreviations s (singlet), d (doublet), dd (doublet of doublets), p (pentet) and m (multiplet) for the coupling pattern. Coupling constants (J) are expressed in Hz, where J_o is ortho coupling and J_m is meta coupling. Proton assignments are based on the following numbering scheme:



Compound 4

TLC: R_f=0.13

MS:	m/z:	265	$(M+H)^+$	(molecular ion $+H^+$)
		208	$(M+H)^+-57$	$-(C_3H_7N)$
		191	$(M+H)^+-57-17$	$-(H_4N)$
		164		

NMR:	δ:	1.80	m	1H	(H_3ax)
		2.04	p	2H	(H_c)
		2.27	br d	1H	(H_3eq)
		2.28	m	3H	
		2.91	m	3H	(H_1, H_2eq, H_4
		3.09	m	2H	H_b, H_d)
		3.40	br s	1H	(H_2ax)
		3.69	s	6H	(methoxy)
		6.65	s	2H	(H_5, H_8)
		8.16	br s	3H	(H_e)
		9.44	br s	2H	(H_a)

Compound 5

TLC: R_f=0.15

MS: m/z:

m/z		
235	$(M+H)^+$	(molecular ion $+H^+$)
198	$(M+H)^+$-57	$-(C_3H_7N)$
161	$(M+H)^+$-57-17	$-(H_4N)$
160		
134		

NMR: δ:

δ			
1.80	m	1H	(H_3ax)
2.04	p	2H	(H_c)
2.27	br d	1H	(H_3eq)
2.28	m	3H	
2.91	m	3H	(H_1, H_2eq, H_4
3.09	m	2H	H_b, H_d)
3.40	br s	1H	(H_2ax)
3.69	s	3H	(methoxy)
6.68	s	1H	(H_5)
6.71	dd J_o=11.0 J_m=2.6	1H	(H_7)
7.01	d J_o=8.3	1H	(H_8)
8.16	br s	3H	(H_e)
9.44	br s	2H	(H_a)

Compound _7_

TLC: R_f=0.10

MS: m/z:

m/z		
237	$(M+H)^+$	(molecular ion $+H^+$)
180	$(M+H)^+$-57	$-(C_3H_7N)$
163	$(M+H)^+$-57-17	$-(H_4N)$

IR:

3300(b)	(phenyl, amine)
2980(b)	(amine)
2820(b)	(amine)
1620	(primary amine)
1590	(phenyl)
1460	(CH)
1150	(amine)
1350, 1280, 1240, 1000	

NMR: δ:

δ			
1.80	m	1H	(H_3ax)
2.04	p	2H	(H_c)
2.27	br d	1H	(H_3eq)
2.28	m	3H	
2.91	m	3H	(H_1, H_2eq, H_4
3.09	m	2H	H_b, H_d)
3.40	br s	1H	(H_2ax)
6.45	s	2H	(H_5, H_8)
7.93	br s	5H	(hydroxy, H_e)

8.84	br s		2H	(H_a)

Compound 8

TLC: R_f=0.12

MS: m/z:

221	$(M+H)^+$	(molecular ion $+H^+$)
164	$(M+H)^+-57$	$-(C_3H_7N)$
147	$(M+H)^+-57-17$	$-(H_4N)$
103		

IR:

3280(b)	(phenol, amine)
2960(b)	(amine)
1600	(primary amine)
1580	(phenyl)
1450	(CH)
1180	(amine)

1990, 1520, 1320, 1280, 1150, 1100

NMR: δ:

δ	mult.	J		H	assignment
1.80	m			1H	(H_3ax)
2.04	p			2H	(H_c)
2.27	br d			1H	(H_3eq)
2.28	m			3H	
2.91	m			3H	(H_1, H_2eq, H_4
3.09	m			2H	H_b, H_d)
3.40	br s			1H	(H_2ax)
6.49	d	J_m=2.3		1H	(H_5)
6.56	dd	J_o=8.2	J_m=2.5	1H	(H_7)
7.89	d	J_o=8.3		1H	(H_8)
7.90	br s			4H	(H_e, hydroxy)
9.44	br s			2H	(H_a)

Compound 10

HPLC: k'=2.4

MS: m/z:

382	$(M+H)^+$	(molecular ion $+H^+$)
356	$(M+H)^+-26$	(N_3 reduction)
237		
220		

IR:

3260(b)	(phenol, secondary amine)
2940	(CH)
2110	(azide)
1640	(secondary amine)
1530	(secondary amide)
1500	(phenyl)

2760, 2680, 2500, 1610, 1480, 1290, 1190, 1030

NMR:	δ:				
	1.80	m		1H	(H_3ax)
	2.04	p		2H	(H_c)
	2.27	br d		1H	(H_3eq)
	2.28	m		3H	
	2.91	m		3H	(H_1, H_2eq, H_4
	3.09	m		2H	H_b, H_d)
	3.40	br s		1H	(H_2ax)
	6.45	s		2H	(H_5, H_8)
	7.19	d	J_o=8.4	2H	(H_2', H_6')
	7.94	d	J_o=8.5	2H	(H_3', H_5')
	9.44	br s		2H	(H_a)

Compound 15

HPLC: k'=3.2

MS:	m/z:		
	411	$(M+H)^+$	(molecular ion $+H^+$)
	385	$(M+H)^+-26$	(N_3 reduction)
	355	$(M+H)^+-26-30$	(NO_2 reduction)
	326		
	263		
	247		
	222		

IR:		
	3240(b)	(phenol, amine)
	2990	(CH)
	2110	(azide)
	1650	(secondary amide)
	1520	(phenyl)
	1440	(CH)
	1350	(nitro)
	1170	(amine)

2740, 2680, 2500, 1620, 1520, 1480, 1280, 1080

NMR:	δ:				
	1.80	m		1H	(H_3ax)
	2.04	p		2H	(H_c)
	2.27	br d		1H	(H_3eq)
	2.28	m		3H	
	2.91	m		3H	(H_1, H_2eq, H_4
	3.09	m		2H	H_b, H_d)
	3.40	br s		1H	(H_2ax)
	6.56	s		1H	(H_5)
	6.62	d	J_o=8.0	1H	(H_7)
	6.97	d	J_o=8.4	1H	(H_8)
	7.34	d	J_m=2.4	1H	(H_3')
	7.47	dd	J_o=8.8 Jo=2.4	1H	(H_5')
	8.18	d	J_o=8.8	1H	(H_6')

9.44	br s	2H	(H_a)

Compound 26

HPLC: k'=6.5

MS: m/z:

m/z	Ion	Assignment
537	$(M+H)^+$	(molecular ion $+H^+$)
511	$(M+H)^+-26$	(N_3 reduction)
481	$(M+H)^+-26-30$	(NO_2 reduction)
443		
411		
385		
355		

NMR: δ:

δ	Multiplicity	Integration	Assignment
1.80	m	1H	(H_3ax)
2.04	p	2H	(H_c)
2.27	br d	1H	(H_3eq)
2.28	m	3H	
2.91	m	3H	(H_1, H_2eq, H_4
3.09	m	2H	H_b, H_d)
3.40	br s	1H	(H_2ax)
6.62	d J_o=8.0	1H	(H_7)
6.97	d J_o=8.4	1H	(H_8)
7.34	d J_m=2.4	1H	(H_3')
7.47	dd J_o=8.8 Jo=2.4	1H	(H_5')
8.18	d J_o=8.8	1H	(H_6')
9.44	br s	2H	(H_a)