Solubilization, Purification and Pharmacological Characterization of Bovine Striatal Dopamine D-2 Receptor

By

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ABSTRACT

With the advent of radioligand binding assays, central nervous system dopamine receptors have been well characterized in their membrane bound state. These receptors have been grouped into D-1 and D-2 subclasses on the basis of their relationship to the enzyme adenylate cyclase and affinities for dopamine agonists and antagonists. The dopamine D-2 receptor is considered relevant to the behavioral and pharmacological effects of neuroleptic drugs.

The studies presented in this dissertation describe a successful method of solubilization of bovine striatal membrane bound dopamine D-2 receptor. The solubilized receptor exhibited typical pharmacological characteristics to that of membrane bound dopamine D-2 receptor. The rank order potency of agonists and antagonists to displace [^{3}H]spiroperidol binding was the same as those observed with the membrane bound receptor. Analysis of the [^{3}H]spiroperidol/agonist competition curves and the [^{3}H]NPA binding revealed the retention of high and low affinity states of dopamine D-2 receptor in the solubilized preparation.

This study demonstrated for the first time, a successful affinity chromatography method for the purification of dopamine D-2 receptor. One cycle affinity purification resulted in a 2000-fold enrichment of dopamine D-2 receptor activity with a recovery of 12% from the membrane-bound state and a specific activity of 169,600 fmol/mg protein (assayed with [^{3}H]spiroperidol). The order of potency of D-2 agonists (N-propylnorapomorphine > N0434 >apomorphine >dopamine) and antagonists (spiroperidol > (+)-butaclamol >domperidone) with a purified preparation

was found to be similar to that of the membrane bound or solubilized dopamine D-2 receptor. The adsorption of receptor to the affinity matrix was biospecific as pre-incubation of the solubilized preparation with D-2 receptor agonists or antagonists blocked retention of receptor activity. Elution of receptor was also biospecific as dopaminergic drugs were effective in eluting the bound receptor.

Affinity purified preprations should be useful in producing monoclonal antibody to dopamine D-2 receptor and also prove to be important in understanding the molecular events from receptor drug binding to final response.

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LIST OF ABBREVIATIONS

AC	Adenylate Cyclase
ADTN	2-Amino 6,7 dihydroxy (1,2,3,4) tetrahydronaphthalene
C	Centigrade
CAMP	3':5' Cyclic adenosine monophosphate
CHAPS	3-(3-cholamidopropyl)-dimethyl ammonio-2 hydroxy-1-propane sulfonate
Ci	Curie
CNS	Central nervous system
CPM	Counts per minute
DA	Dopamine
DPM	Disintegration per minute
DOPA	3,4 dihydroxy phenylalanine
DMF	Dimethyl formamide
DTT	Dithiothreitol
EDTA	Ethylene diaminotetra acetic acid
fm	Femtomoles
g	Centrifugal force
gm	Gram
Gpp(NH)p	5'-guanylyl imidodiphosphate
GTP	Guanosine triphosphate
hr	Hour

LIST OF ABBREVIATIONS (cont'd)

K_D	Equilibrium dissociation constant
M	Molar
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
N	Normality
Ni or Gi	Guanine nucleotide inhibitory regulatory protein
Ns or Gs	Guanine nucleotide stimulatory regulatory protein
NPA	N-propylnorapomorphine
PC	Phosphatidyl choline
PM	Picomolar
R_H	High affinity receptor binding
R_L	Low affinity receptor binding
Tris	Tris (hydroxy methyl) amino methane
Vol	Volume

1. INTRODUCTION

1.1 General Orientation of Thesis

The past two decades have percieved the role of dopamine in the brain, from being a precursor for other catecholamines, principally norepinephrine, to a neurotransmitter in its own right. Alteration in the dopaminergic neurotransmission has been directly linked to various neurological and psychiatric disorders. The use of dopaminergic agonists in the treatment of Parkinson's disease and dopaminergic antagonists for the treatment of schizophrenia, Huntington's disease and Gilles de la Tourette's syndrome have now been established. In recent years, the focus of research has been directed towards the dopamine receptors, by which dopaminergic drugs act directly or indirectly (e.g. L-DOPA --> dopamine). Alterations in these receptors have also been correlated with various behavioural and psychiatric disorders.

In the last decade, with the development of radioligand binding assay, progress has been made in the understanding and classification of dopamine receptors. These studies have divided dopamine receptors into distinct subtypes, as was done for the adrenergic and cholinergic receptors. Such studies have now characterized two dopamine receptor subtypes known as D-1 and D-2 in the central nervous system (CNS). This classification is, as yet, preliminary and sometimes controversial in the literature, however, it has provided a basis for implicating dopamine D-2 receptor with various neurological and psychiatric disorders.

The present investigation was carried out in order to i) establish a method for the solubilization of dopamine-D-2 receptor from CNS membranes, particularly from striatum; ii) design a method of

affinity chromotagraphy for the purification of dopamine-D-2 receptor; iii) pharmacologically characterize the receptor in the solubilized as well as in the purified form, vis-a-vis the membrane bound form.

2.1 Historical Perspective of Neurotransmitter Receptor

Historically, a receptor was considered as a physiological entity accounting for the ability of a tissue to respond to minute quantities of drugs or endogenous compounds. Three striking characteristics have led to the hypothesis that cells possess specific receptors for drugs or endogenous compounds: a) High potency: many drugs act at 10^{-9} M or lower concentration, b) Chemical selectivity: exemplified by marked differences in potencies observed between optical isomers, c) Biological specificity; eg. cholinergic receptors respond to acetylcholine, adrenoceptors respond to epinephrine or norepinephrine.

Paul Ehrlich (1913) introduced the term receptor viewing it as a "combining group of protoplasmic molecule to which the introduced group is anchored". However, it was Langley (1905) who introduced the term receptive substance in the context that now appears to be the most appropriate. He proposed this term to account for the action of nicotine in causing contraction of voluntary muscle when applied to a particular region of the muscle, and antagonism of this effect by curare. Later, Dale (1914) distinguished two distinct types of receptor responses; one sensitive to muscarine, and the other sensitive to nicotine. Both of these receptors utilized acetylcholine, henceforth, cholinergic receptors were divided into two classes; the nicotinic receptor and muscarinic receptor. In a similar fashion, Ahlquist (1948) distinguished the

adrenergic receptors based on the order of potency for catecholamine on different tissues. When arranged according to their potencies, the group of catecholamines divided the adrenergic receptors into two series; the α-adrenergic receptor, and the β-adrenergic receptor. Although the receptor concept was mainly of theoretical significance in the past, it has evolved into a concrete reality and receptor can now be studied as a tangible molecular entity.

Receptors for neurotransmitters are membrane bound proteins composed of at least two sites; a recognition site that determines the ligand specificity with exquisite sensitivity and chemical selectivity and an associated processing site that converts the process of recognition into a signal that results in a final response. This dual functionality of a neurotransmitter and other hormone receptors, distinguishes itself from other highly functional cell surface molecules which serve important cellular functions by specific recognition such as transport of nutrients or the selective pinocytotic uptake of carrier bound cell regulators (e.g. transferrin, or low density lipoprotein). Recognition molecules that mediate the uptake of ligands have been termed receptors of the "Class II" or acceptors (Kaplan, 1981; O'Connor and Hollenberg, 1983).

To describe the dual recognition-activation function of a receptor, a number of models have been developed that relate receptor occupation to the generation of a cellular signal. In early studies, Clarke (1926, 1937) and Gaddum (1926) proposed an occupancy model; according to this model the magnitude of the pharmacological effect of a drug was directly proportional to the number of receptors occupied by the

drug, and the maximum response was obtained only when all the receptors were occupied.

This simple occupation theory was modified by Ariens (1954) to incorporate the concept of intrinsic activity (ability of drugs to elicit a biological response after binding to the receptor) which enabled the quantitation of the response after binding of a drug to the receptor. Stephenson (1956), Furchgott (1955) and Nickerson (1956) further extended this theory and introduced the concept of the "spare receptor". They demonstrated that in certain tissues an excess number of receptors was present, and that the maximum response in these tissues could be obtained with a strong agonist at a concentration well below the concentration required for the saturation of the total receptor population.

Paton (1961) suggested a new theory, the rate theory, to account for the desensitization phenomenon. According to this theory, the effect a drug produces depends on the kinetics of receptor occupation by the drug rather than the number of receptors occupied, and the response in certain tissues may be observed as an "on-off" phenomenon when the tissue is continually exposed to the stimulant. In other words, this desensitization phenomenon expresses a decrease in response on continuous exposure to a agonist or as a diminished response of a tissue to a repetitive exposure of the same concentration of the agonist.

Although the rate theory and the occupancy theory differ with regard to the immediate function of the ligand-receptor complex, both theories consider the complex as a distinct micro-chemical entity with properties different from those of the uncomplexed component. These and other theories (reviewed by Hollenberg, 1985) for the activation of

receptor by a drug, gave a new insight into the subject.

Recently, the characteristics of a receptor have been experimentally established by selective binding studies with radiolabelled ligands. These studies have resulted in localization, identification and characterization of receptors and their functional properties.

1.3 Characterization of Receptors by Radiologand Binding Assay

Over the past two decades, with the introduction of radioactively labelled ligands of high specific activity, the direct measurement of hormone or neurotransmitter receptor binding became possible. This direct binding technique has enabled investigators to label biologically active compounds and quantitatively assay the receptor sites which are thought to mediate the agonist or antagonist effects of putative neurotransmitters and many psychoactive drugs in the brain. Such studies have led to the identification and establishment of receptor sites for various hormones, neurotransmitters, peptides and drugs in their respective target tissues. A few examples are: insulin receptor, growth hormone receptor, α-adrenergic receptors, β-adrenergic receptors, dopamine receptors, opiate receptors and benzodiazepine receptors (review articles, Braastrap and Neilsan, 1980; Ross and Gilman, 1980; Mishra and Cleghorn, 1980; Seeman, 1980; Terenius, 1980; Brown and Aurbach, 1982; Lefkowitz, 1982).

The fundamental principles and methodology of neurotransmitter receptor binding have been reviewed in many excellent monographs (Titler, 1983; Bennett and Yamamura, 1985). Binding experiments consist of

exposing a suitable tissue (receptor) preparation to radiolabelled ligand (agonist or antagonist) under appropriate buffer, optimal time and temperature conditions, followed by separation of the free ligand from the bound ligand and determination of the bound radioactivity. The assumption made in most receptor binding studies is that ligand-receptor binding is a reversible bimolecular reaction which at equilibrium obeys the law of mass action and can be described as follows:

$$[L] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [LR] \qquad (1)$$

where [L] is the concentration of the free ligand, [R] the concentration of the free receptor, [LR] the concentration of ligand receptor complex, k_1 and k_2 are the association and dissociation rate constants respectively. In order to be considered as a ligand interacting with a receptor, the binding of a radioligand must satisfy the following criteria: i) Binding must be specific, saturable, reversible and displaceable by drugs known to act at the receptor; ii) Binding should show high affinity i.e. compatible with concentrations of the ligand observed in the physiological situation; iii) Temporal parameters of the binding must match the onset-offset of the biological response; iv) Binding should be found only in tissues where the biological receptor exists; v) Binding should display the proper pharmacological profile such that drugs with greater biological potency at the receptor should have higher affinity. Also, if the biological response to the drug is stereospecific, the binding site must show this property. Once the specific parameters of a binding can be determined (with confidence) with

appropriate radioligand, tissue, and cold drug concentration, mathematical models can be applied to the data to yield biochemical information about the receptor. It is beyond the scope of this dissertation to describe and derive various equations for the estimation of kinetic parameters, as they are dealt with in several recent reviews (Williams and Lefkowitz, 1978; Furchgott, 1978; Bennett and Yamamura, 1985). This section will largely focus on the application of those principles which are directly relevant to the estimation of binding parameters commonly used in the radioligand binding studies such as Scatchard plot for the estimation of dissociation constant (k_D) and maximum binding (B_{max}), competition curves for determining the IC_{50} values for different agonists and antagonists, and Hill plot for resolving the one or more binding sites and association and dissociation binding kinetics.

1.3.1 Scatchard Plot

In most binding studies, the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) is estimated from the Scatchard plot of the saturation isotherm obtained by using the regression equation as proposed by Scatchard (1949) for the analysis of binding data. This mathematical model is based on the assumption that ligand-receptor binding is a bimolecular interaction based on the law of mass action with kinetics similar to that of an enzyme-substrate interaction.

The equation is:

$$\frac{B}{F} = \frac{B_{max} - B}{K_D} \qquad (2)$$

By measuring the specifically bound ligand (B) and knowing the concentration of the free ligand (F) in the incubation medium at

equilibrium, one can plot the ratio of bound and free ligand (B/F) against the amount of bound ligand (B) and fit a best line by linear regression. The equilibrium dissociation constant (K_D) is then estimated from the plot as the negative reciprocal of the slope ($-1/B$) and the maximum number of binding sites (B_{max}) is given by the intercept of the line on the abscissa (x-intercept). In the case of the simple bimolecular interaction with the ligand binding to only one type of site with a constant affinity, a straight line will be generated from which K_D and B_{max} can be determined. However, in many instances plotting the saturation data results in a non-linear or curved Scatchard plot. This would indicate either heterogeneity of the receptor site i.e. binding of a ligand to two or more sites with different affinities, or a cooperative interaction between the receptor sites. If this coopertivity is positive (binding to one site facilitated by binding to the other site), the Scatchard plot is curvilinear upwards. In contrast, a Scatchard plot curvilinear downwards is indicative of possible negative cooperativity. A non-linear Scatchard plot in the case of binding to two sets of independent non-interacting sites can be resolved into two components by specific computer programs e.g. "Ligand" by Munson and Rodbard, (1980).

1.3.2 Hill Plot

In order to check the deviation of receptor-ligand binding from the classic mass action law, simple data are usually converted into the Hill equation (Hill, 1913)

$$\log \left[\frac{B}{B_{max}-B} \right] = n \log(L) - n \log K0.5 \qquad (3)$$

This equation can be employed to determine the presence or

absence of cooperativity in the saturation binding data. The Hill plot can be obtained from saturation binding data by plotting the log of radioligand concentration (Log [L]) against the ratio log [B/(B_{max}-B)]. Where [L] is the concentration of the free ligand, B is the amount of the bound ligand and B_{max} is the maximum number of binding sites estimated by Scatchard analysis. The slope of the resulting line is the Hill coefficient (n). A Hill coefficient of less than unity indicates negative cooperativity or multiple sites. A Hill number of unity indicates a single class of binding sites, while a Hill number of greater than unity indicates multiple subsites.

1.3.3 Kinetics of Binding

The equilbrium binding constant (K_D) can also be determined from experiments where the constants for association (k_1) and dissociation (k_2) of ligand receptor binding are estimated since K_D is equal to the ratio k_2/k_1 (see equation 1). The K_D derived from such experiments should, within the experimental error, be similar to the K_D obtained from the saturation experiment (Scatchard plot).

In practice, it is extremely difficult to measure association rate directly as high affinity drugs have very rapid rates of association and usually only less than 10% of the ligand is bound at equilibrium. However, dissociation rates for high affinity drugs can be measured directly. The procedure involves incubating the tissue preparation with a particular concentration of the radioligand until steady state is reached, and then either by diluting the ligand with an excess of buffer (100-fold) or adding an excess (1000-fold) of non-radioactive ligand. Specific binding is measured at various time intervals after dilution (or

addition of excess displacer) and the half life of specifically bound radioligand is estimated from a plot of log bound vs. time. Once the dissociation rate has been determined, the association rate can be determined indirectly by equation 2: $K_D = k_2/k_1$, so $k_1 = k_2/K_D$. The K_D is determined independently from equilibrium experiments. The second method used for determining association kinetics takes into consideration the contribution of ligand receptor dissociation to the eventual reaching of a steady state. Bound ligand is assayed at various time intervals up to the steady state level (Beq). Ln (Beq/(Beq-Bt) is plotted versus time where Bt is the amount of specifically bound ligand at time t. The slope of this line (Kobs) is related to the association (k_1) and dissociation (k_2) rate constant and free ligand concentration (L).

$$k_1 = \frac{Kobs - k_2}{[L]} \qquad (4)$$

This equation is valid only when the binding reaction is performed with no more than 10% initial free ligand bound at equilibrium.

1.3.4 Competition Curves

One of the important criteria for recognizing binding site as a receptor is its pharmacological specificity. This is determined by incubating a fixed concentration of radioligand with an increasing concentration of unlabelled ligand. If the unlabelled drug binds to the same site as the radioactive drug, it will interfere with the binding of the radioactive drug. This is the molecular basis of direct pharmacological competition. A set of competition curves are generated, and the concentration of unlabelled ligand displacing 50% of specifically bound radioligand (IC_{50}) is determined. The binding data are usually

plotted as percent of specific binding remaining against log of displacing drug concentration. As the concentration of radioligand [L] utilized in inhibition studies increases, the difference between the IC_{50} values of a ligand and the binding affinity constant of unlabelled ligand (Ki) progressively widens at fixed tissue concentration according to the equation described by Cheng and Prusoff, 1973.

$$Ki = \frac{IC_{50}}{1 + [L]/K_D} \qquad (5)$$

where [L] = free concentration of radioactive drug in incubation solution;

K_D = Equilibrium dissociation constant of the radioactive drug for binding site;

IC_{50} = concentration of the unlabelled drug that inhibits 50% of the radioactive ligand binding;

Ki = apparent equilibrium association constant.

This equation applies only to the binding phenomenon where labelled and unlabelled ligands interact competitively at the same receptor site.

Hill coefficient can be calculated for the competition curves just as they can be calculated for the saturation curves. The equation is as follows:

$$\text{Log } \frac{\text{\% inhibition}}{\text{100\% inhibition}} = \text{Hill coefficient x log (L)} \qquad (6)$$

In recent years, shallow or biphasic competition curves describing the interaction of an agonist with the radioligand antagonist have been the subject of considerable interest in receptor binding, posing complexities to the receptor classification and functional heterogeneity. These shallow biphasic competition curves of

radiolabelled antagonist against agonist or vice versa have been interpreted differently by different investigators e.g. Seeman and his co-workers (Seeman 1980;1982) investigating the dopamine binding sites in the central nervous system of various species have interpreted these findings as showing existence of multiple receptor binding sites for the dopamine neurotransmitter, e.g. D-1, D-2, D-3 and D-4. However, similar findings by Sibley et al., (1982) in the anterior pituitary have been interpreted as suggestive of two states of dopamine receptors (i.e. high or low affinity state), inducible by agonist, but not by antagonist, and modulated by guanine nucleotide.

1.4 Receptors and Signal Transduction

Receptors are large protein molecules composed of complex structures for both binding the transmitter and transducing the message into the biologically relevant effect. Investigations into the molecular mechanisms that translate the drug, neurotransmitter or hormone receptor interactions into biochemical responses have greatly enhanced our understanding of the mechanism of receptor-coupling in physiological systems. The actual transmitter or hormone binding sites comprise a very small part of the molecule. The transducer involved in the signal processing is an integral part of the receptor structure and may differ in different systems. At least four different models of transduction have been described in the literature and diagrammatic representation of these models have been presented in Figure 1a-d (review: Hollenberg, 1982,1985).

1. In the case of the nicotinic receptor, acetylcholine

stimulation of a cell is mediated by the activation of an ion channel (Conti-Toronconi and Raftery, 1982). Receptors of this type are involved in signaling of short latencies and duration of milliseconds (Neumann and Bernhardt, 1977).

2. There are other types of receptors that may interact with an independent enzyme system acting as transducers i.e. adenylate cyclase system. In this system, the mechanism is different from the manner whereby the nicotinic receptors regulate the ion flux. The adenylate cyclase system is composed of at least two subunits; the GTP binding subunit or G protein and the catalytic subunit or C protein (Ross and Gilman, 1980). Receptors for neurotransmitters such as β-adrenergic, dopamine D-1, and hormones such as glucagon that stimulate the adenylate cyclase interact via an oligomeric stimulatory GTP binding sub-unit (the G_s or N_s sub-unit) to modulate the activity of the catalytic sub-unit C (Fig. 1a). Whereas, receptors for α_2 adrenergic, dopamine D-2 and hormones like angiotensin (reviewed by Helmreich and Pfuffer, 1985) that cause inhibition of cyclase activity do so via interaction with an inhibitory GTP binding sub-unit designated N_i or G_i. Thus, two kinds of GTP binding proteins, G_s (N_s) and G_i (N_i), exist whose composition has recently been described as heterotrimer consisting of of α_s, β, γ and α_i, β, γ subunits respectively (Northup et al., 1980, 1982). GTP binding to α_s (mol. wt. 42,000 dalton) or α_i (mol. wt. 39,000 dalton) mediates activation or inhibition of adenylate cyclase systems respectively. Thus, the cyclase activity is subjected to a bidirectional control, from the interaction of either stimulatory or inhibitory sub-units. Since GTP promotes the interactions of a receptor with N_s or N_i sub-unit, it has an

Figure 1: Molecular models of receptor mediated cell regulation, modified from Hollenberg (1985). Panel A represents the neurotransmitter or hormone mediated action through stimulatory or inhibitory adenylate cyclase. Panel B represents the insulin hormone action. Panel C describes the possible mechanism of acetylcholine action, and Panel D for epidermal growth factor action. R_s - stimulatory receptor protein, G - stimulating hormone or neurotransmitter, N_s - cyclase stimulatory regulatory protein, N_i - inhibitory regulatory protein, C - catalytic subunit, I - insulin, α, β, subunit of insulin receptor, GPR - glycoprotein regulatory site of insulin receptor affinity, TYR-(R) - phosphotyrosine on putative kinase site of the insulin receptor, A - acetylcholine receptor, α, β, γ, δ subunit of nicotine receptor forming the ion channel, E - epidermal growth factor, P-TYR - phosphotyrosine S-S disulfide bond that link receptor regulator.

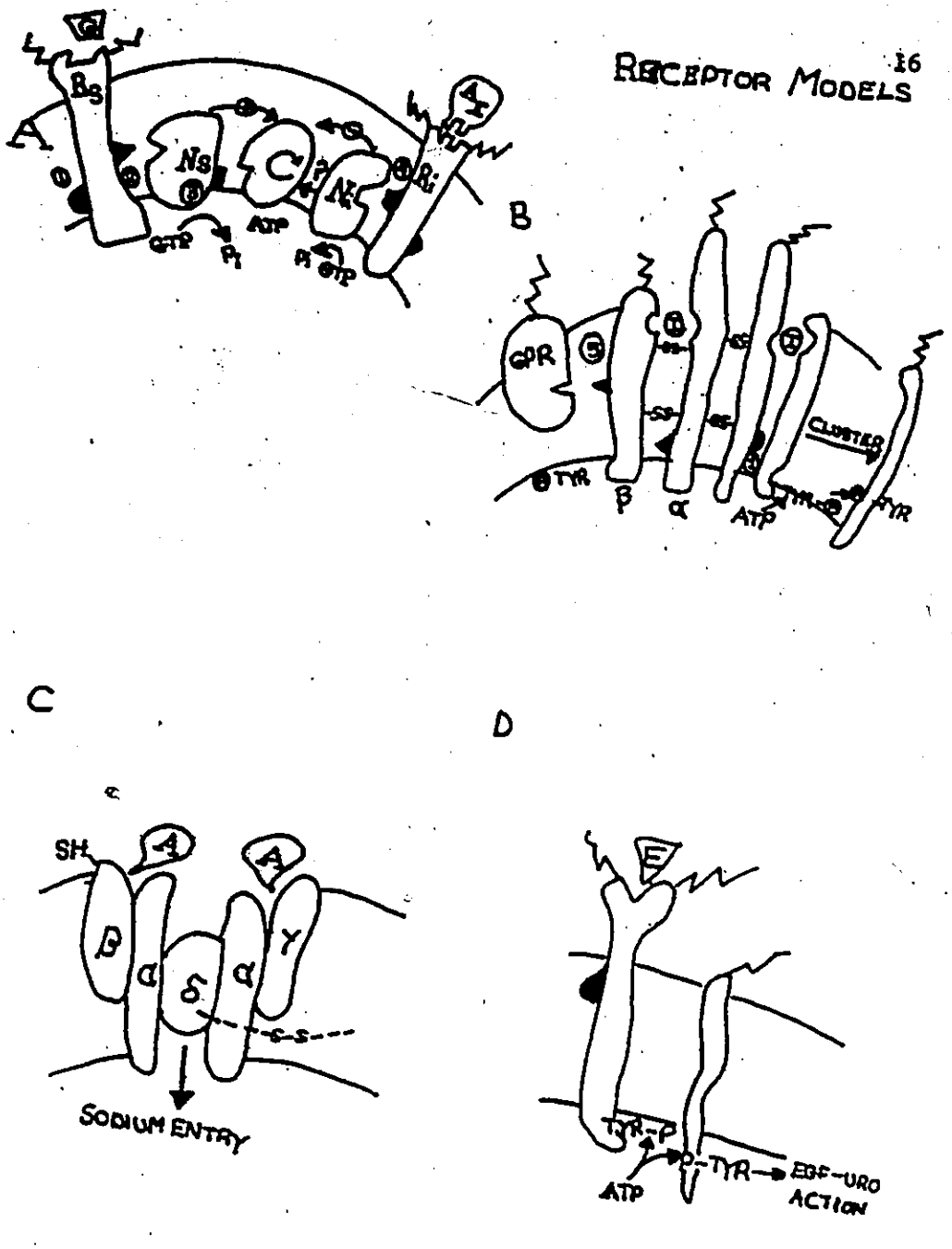

A
B
C
D
SH
SODIUM ENTRY
CLUSTER
TYR
ATP
TYR-P
P-TYR
EGF-URO
ACTION

FIGURE 1

observable effect on the ligand-receptor affinities measured in a binding experiment. As will be described later in this section, GTP converts the high affinity agonist binding site to the low affinity binding site.

3. Insulin (Fig. 1b) may act, in part, via the liberation of an intracellular peptide mediator generated by proteolysis of a membrane protein (Czech, 1981). These mediators, in turn via modulation of phosphatase and/or cAMP phosphodiestrase activity can regulate the activities of insulin responsive enzymes.

4. In contrast to the above models, the epidermal growth factor-urogastrone (EGF-URO) receptor is a single chain glycoprotein whose activity resides on the inner aspect of the plasma membrane. It is suggested that the receptor possesses an intracellular catalytic tyrosine kinase site which is responsible for the phosphorylation of the membrane protein tyrosine residues (Cohen et al., 1980) and this phosphorylation represents the first step in the action of EGF-URO effects. Thus, four distinct membrane localized reaction pathways (activation of ion transport, cyclase activation, activation of membranes to liberate low molecular weight chemical mediators and intrinsic receptor kinase activity leading to membrane protein phosphorylation) appear to mediate the cell activation. The mechanism by which these four pathways of signal transduction operate are explained in Figure (1 a-d).

1.5 Dopamine as a Neurotransmitter in the Central Nervous System

Dopamine has long been recognized as an intermediary compound in the norepinephrine biosynthetic pathway. However, in the late 1950's, dopamine was shown to occur in high concentrations in the brain and

peripheral nervous system (Montagu, 1957; Carlsson et al., 1958; Bertler and Rosengren, 1959), which suggested that dopamine might have a function other than serving just as an intermediate in the synthesis of norepinephrine or epinephrine. In other studies, Carlsson (1959) observed that L-DOPA injection reversed reserpine-induced pharmacological effects without affecting the depleted levels of norepinephrine. This effect was directly attributed to dopamine and was suggested that dopamine may be a neurotransmitter involved in motor coordination in the central nervous system.

In the early 1960's, three pivotal studies led to the conclusion that dopamine has a separate major role in normal and abnormal brain functions. Hornykiewicz (1966) first reported depleted levels of dopamine in the nigrostriatal regions of post mortum brains from patients with Parkinson's disease. The subsequent clinical success of therapy using L-DOPA (dopamine's immediate precursor), underlined the importance of dopamine deficiency in this disorder (Hornykiewicz, 1973). Secondly, Falck et al., (1962) developed the fluorescence histochemical technique which allowed visualization of dopamine in the brain, along with other neurotransmitters like norepinephrine and serotonin. These histochemical studies later clearly demonstrated that dopamine neurons were an intimate part of the extrapyramidal motor system and the limbic forebrain (Fuxe, 1965). Thirdly, it was shown by Carlsson and Lingvist (1963) that antipsychotic agents have the ability to cause an increase in the levels of brain dopamine metabolites. These findings have been further supported by the fact that antipsychotic drugs (neuroleptics) block the behavioural effects of dopamine agonists in animals (for review see Seeman, 1980).

The anatomical localization of the dopamine neuronal system has been documented with the mapping of neurotransmitter pathways by fluorescence techniques of Dalhstrom and Fuxe (1964). Dopamine neuronal pathways are outlined in Table I. Details of these pathways are reviewed elsewhere in the literature (Lindvall and Bjarklund, 1977; Moore and Bloom, 1978). The nigrostriatal pathway accounts for about 70% of the total brain content of dopamine. This pathway is the major focus of dopamine research. The degeneration of DA (dopamine) containing nigrostriatal neurons have been directly linked with the neuropathology of Parkinson's disease (Denny-Brown, 1962; Bernheimer et al., 1973). The abnormality of this pathway has also been implicated in other psychiatric and behavioural disorders such as schizophrenia (Creese et al., 1976; Angvist et al., 1980) and Huntington's chorea (Klawan, 1973).

In animal studies, akinesia and rigidity which are characteristics of Parkinson's disease, can be produced by lesions of the nigrostrial pathway (Ungerstedt, 1971a; Silbergold and Calne, 1981). Lesion studies with intracerebral micro-injection of dopamine, dopamine agonists or antagonists into various dopamine terminal areas have suggested that stereotyped gnawing and chewing in rats evoked by amphetamine and apomorphine are initiated in the striatum (Creese and Iversen, 1974) by increased dopaminergic activity.

Unilateral lesion of the nigrostriatal pathway in rats with 6-hydroxy dopamine (a selective catecholamine destroying agent) caused an ipsilateral rotational behaviour. However when these rats were challenged with apomorphine (DA agonist) they rotated in a direction contralateral to the lesion (Ungerstadt, 1971). This directional change

TABLE 1

Dopamine Neuron Systems in the Mammalian Brain

System	Nucleus of origin	Site(s) of termination
Meso-telencephalic		
Nigrostriatal	Substantia nigra, pars compacta; ventral tegmental area	Neostriatum (caudate-putamen); globus pallidus
Mesocortical	Ventral tegmental area; substantia nigra, pars compacta	Isocortex (mesial frontal, anterior cingulate, entorhinal, perirhinal)
		Allocortex (olfactory bulb, anterior olfactory nucleus, olfactory tubercle, piriform cortex, septal area, nucleus accumbens, amygdaloid complex)
Tubero-hypophysial	Arcuate and periventricular hypothalamic nuclei	Neuro-intermediate lobe of pituitary, median eminence
Retinal	Interplexiform cells of retina	Inner and outer plexiform layers of retina
Incerto-hypothalamic	Zona incerta, posterior hypothalamus	Dorsal hypothalamic area, septum
Periventricular	Medulla in area of dorsal motor vagus, nucleus tractus solitarius, periaqueductal and periventricular gray	Periventricular and periaqueductal gray, tegmentum, tectum, thalamus, hypothalamus
Olfactory bulb	Periglomerular cells	Glomeruli (mitral cells)

From: Moore and Bloom (1978).

was interpreted as an indication of greater post-synaptic dopaminergic activity of lesioned striatum and was suggested to be due to the supersensitization of the lesioned striatum to dopamine or its agonist. This post-synaptic supersensitivity in rats has been reconfirmed in many other studies by injection with various dopaminergic antagonists (Mishra et al., 1978; Ungerstadt, 1971a).

These findings in animal models, and evidence that antipsychotic and anti-Parkinsonian drugs minimize the symptoms of these disorders by blocking or activating the dopaminergic system respectively gave a major impetus in the growth of dopamine neurotransmitter research leading to the identification and characterization of dopamine receptors.

1.6 Multiple Dopamine Receptors (Pharmacological characterization).

The mammalian striatum has the highest concentration of dopamine and been extensively used for studying the characteristics of dopamine receptors in the central nervous system (Seeman, 1980). During the 1970's, two major developments made it possible to study the pharmacology, the kinetic properties and the distribution of recognition sites for dopamine; 1) The measurement of DA sensitive adenylate cyclase (cAMP) levels; 2) Radioligand binding to dopamine receptors.

1.6. A) Dopamine sensitive adenylate cyclase.

The first biochemical evidence for the existence of dopamine receptors in mammalian brain was derived from the identification of dopamine sensitive adenylate cyclase (DA sensitive AC) activity in different brain tissue, eg. DA sensitive AC in the bovine superior cervical ganglion (reviewed in Greengard, 1976), a similar enzyme in the

rat striatum (Miller et al., 1974), retina (Brown and Makmen, 1972), and also in the cortical region of the primate (Mishra et al., 1974). This enzyme was found to be maximally stimulated by dopamine at 100 μM concentration with half maximal effect at about 2 μM (Greengard, 1976). The regional distribution of this enzyme in brain tissue also suggested an association with the dopamine synapse. Higher enzymatic activity was observed in the corpus striatum, olfactory tubercle and nucleus accumbens (Leysen and Laduron, 1977), the three brain regions richest in dopamine innervation. These findings indicated that this enzyme may be directly linked with the dopaminergic behavioural actions (Nathanson, 1977). Furthermore, neuroleptic phenothiazines (e.g. chlorpramazine) used for the treatment of psychiatric disorders were found to be potent competitive inhibitors of dopamine sensitive adenylate cyclase activity (Clement-Cormier et al., 1974; Miller et al., 1974; Iversen, 1976). In a series of competitive inhibition experiments with phenothiazines, there was a correlation between their pharmacological potencies as dopamine antagonists and their influences on adenylate cyclase activity (Clement-Cormier et al., 1975). Based on this cumulative evidence, it was hypothesized that antipsychotic drugs were acting via blocking the dopamine sensitive adenylate cyclase activity, and this enzyme was a receptor linked enzyme involved in the antipsychotic effects of neuroleptics (Greengard, 1976).

In studies with butyrophenone neuroleptics (which as a group are more potent than phenothiazine neuroleptics in blocking dopamine receptors mediated behavioural actions), it was found that they were less effective in inhibiting the adenylate cyclase activity (Iversen, 1975;

Snyder et al., 1975). For example, the butyrophenone spiroperidol (spiperone), one of the most potent neuroleptics (100 times more potent than chlorpramazine) in blocking brain dopamine receptors in animal models of dopaminergic activity was found to be 10-fold less potent than chlorpramazine in blocking the dopamine sensitive adenylate cyclase. Furthermore, ergot derivatives that have been found to stimulate brain dopamine receptors in vivo have also been found to antagonize the dopamine sensitive adenylate cyclase in vitro, e.g. bromocryptine, a clinically useful anti-Parkinsonian agent and a useful drug used in the brain dopamine receptor stimulation model can inhibit the cyclase activity (Kebabian, 1978; Berde and Sturman, 1978). The evidence became inconsistent with the earlier hypothesis that the dopamine sensitive adenylate cyclase was a receptor linked enzyme system in the brain through which anti-psychotic and anti-Pakinsoniah drugs may exert their effects. These discrepancies raised the possibility that there might exist more than one type of dopamine receptor. Thus, butyrophenones would exhibit weak affinity for the receptors responsible for eliciting an increase in cAMP whereas they would exhibit higher potencies at those receptors responsible for behavioural and clinical effects. This hypothesis of more than one type of dopamine receptor was further supported by the binding data for DA agonists and antagonists to striatal membranes.

1.6.2 Radioligand Binding Studies

In the mid-seventies, two independent laboratories, Seemen et al., (1975) and Burt et al., (1976) successfully used [^{3}H]haloperidol and [^{3}H]dopamine to label dopamine-related specific binding sites in calf

caudate membrane. These binding sites have been well characterized, and densities of these binding sites have been found to be rich in those areas where more dopaminergic neurotransmission was localized, e.g. striatum and tubercle olfactorium (Fields et al., 1979; Seeman, 1980). Brain regions where little or no dopamine has been detected, such as the frontal cortex or the cerebellum displayed no significant amount of specific receptor binding. According to the pharmacological potency ratio with [^{3}H]haloperidol it was found that dopamine and apomorphine (dopamine agonist) were more potent as displaying agent than norepinephrine or isoproterenol (β adrenergic agonist); however neuroleptic antagonists were found to be even more potent in competing for [^{3}H]haloperidol specific binding sites than agonists (Burt et al., 1975). Stereoisomers of neuroleptics such as (+) and (-)-butaclamol and a cis-trans-thiozanthine inhibited [^{3}H]haloperidol binding with different potencies indicating that the [^{3}H]haloperidol binding is stereospecific. Furthermore, the potencies of neuroleptic drugs in competing for [^{3}H]haloperidol specific binding in the calf caudate membrane homogenate correlated well with the clinical potencies of the neuroleptics as antipsychotic drugs (Seeman et al., 1976; Creese et al., 1976). Thus, it was found that butyrophenones, such as spiroperidol and haloperidol, the most potent neuroleptics, were also extremely potent in competing with the binding of [^{3}H]spiroperidol or [^{3}H]haloperidol to dopamine receptors, but were weak as inhibitors of the adenylate cyclase (Creese et el., 1975). In other drug specificity studies of receptors labelled by agonists like [^{3}H]dopamine, [^{3}H]apomorphine (Seeman et al., 1976) or [^{3}H]ADTN (Creese and Snyder, 1978) it was found that these binding sites

have potency ratios similar to that of the dopamine sensitive adenylate cyclase. Also, careful examination of binding data revealed that the competition of an [^{3}H]agonist with antagonist assumed a sigmoid Hill plot with a coefficient of less than unity, an indication of multiple receptor binding sites (Beld et al., 1978; Creese, et al., 1978). Thus, binding studies have provided further support for the existence of more than one type of dopamine receptor.

In lesion experiments, chemical lesions by kainic acid (which selectively destroys intrinsic neurons of the striatum) resulted in almost complete loss of dopamine stimulated adenylate cyclase and produced a major loss of [^{3}H]apomorphine binding, but elicited only 50% decline in [^{3}H]spiroperidol or [^{3}H]haloperidol binding (Schwarcz et al., 1978; Fields et al., 1979). On the other hand, surgical ablation of the cerebral cortex in the rat resulted in decreased striatal [^{3}H]spiroperidol binding without any change of the DA stimulated AC (Garau et al., 1978). The cerebral cortical lesion and a kainate lesion effects were additive and together almost totally depleted striatal [^{3}H]spiroperidol binding (Schwarcz et al., 1978). Since the dopamine sensitive AC is not reduced by cerebral cortical ablation, this may further support the notion that the dopamine receptors on corticostriatal nerve endings are not linked to the adenylate cyclase. Based on these pharmacological and behavioural evidence, Kebabian and Calne (1979) have clearly distinguished dopamine receptors into two subtypes: a) dopamine receptor that activates adenylate cyclase termed D-1 receptor (in the periphery as DA_1 receptor). The prototype D-1 receptor is found in the parathyroid gland where dopamine causes a release of parathyroid hormone

through an increase in cAMP level (Brown et al., 1977); b) a dopamine receptor that does not activate, but may inhibit adenylate cyclase termed D-2 receptor (in periphery DA_2 receptors). Prototype D-2 receptor is found in the anterior pituitary, where dopamine mediated inhibition of prolactin hormone secretion is caused by a decrease, rather than an increase, in adenylate cyclase activity (Clement-Cormier, 1974). Both D-1 and D-2 receptors are present in the caudate nucleus (Zahinser and Molinoff, 1978; Seeman, 1980; Mishra and Cleghorn, 1980). However, this classification has created many controversies in the recent literature. On the basis of binding data, as many as 4 distinct subtypes of dopamine receptors have been identified (Seeman 1980,1982) depending on their affinities for different agonists and antagonists. Similar findings have been argued by others (Creese and Sibly, 1982) who have classified the dopamine receptors into three subtypes. Nonetheless, now the classification scheme for DA-receptors based on binding studies is beginning to be brought into an agreement with the original classification of D-1 and D-2 subclasses based on more conventional pharmacological techniques.

Thus, in more recent studies it has been proposed that the D-1 receptor may exist in two interchangeable states differing in their affinities for agonists (i.e. low affinity and high affinity states); Leff and Creese (1983) have suggested that the "D-3" binding site orginally proposed (Creese and Sibley, 1982) represents high affinity agonist state of the D-1 receptor. Likewise, the D-2 receptor in the pituitary gland may have high and low forms (Sibley et al., 1982) which were originally thought to be D-2 and D-4 sites (Seeman, 1980). It

should be pointed out that Laduron (1983) still argues for the existence of a single dopamine receptor, whereas Martaress et al. (1984) and Joyce (1983) continue to consider the existence of three types of dopamine receptors. Without going into any details of controversy, this chapter will be limited to D-1 and D-2 classification.

The D-1 and D-2 dopamine receptor hypothesis is further strengthened by recent identification of the drugs discriminating between the two receptors. A number of compounds which are found to be potent agonists of the D-2 receptor have negligible affinity for the D-1 receptor. For example, RU24213 and RU24926 are potent D-2 agonists, but display no agonistic activity on the D-1 receptor (Euvrad, et al., 1980). Similarly, D-1 receptor agonists SK&F 389393 or SK&F 82526 (Hahn et al., 1982; Brown and Dawson-Hughes, 1983), have failed to inhibit the release of prolactin, from this evidence it was concluded that D-1 agonists do not interact with the D-2 receptor. In a similar fashion, there are some antagonists which show higher potency in inhibiting D-1 receptor than D-2 receptor, eg. SCH 23390 has a ki of 0.66 nM for the D-1 receptor, but is inactive at this concentration on the D-2 receptor (Iorio, et al., 1983). This classification of dopamine receptor is summarized in Table 2.

1.7 Dopamine D-1 Receptor

As discussed in the preceding section, dopamine D-1 receptor is directly linked with the stimulation of adenylate cyclase (Kebabian and Calne, 1979). This receptor site has been characterized on the basis of the ability of drugs to mimic or antagonize the stimulatory effects of dopamine on adenylate cyclase activity. Neuroleptics of the

TABLE 2

Two Dopamine Receptors: Classification, Biochemical and Pharmacological Characteristics*

	D-1		D-2	
	R_H	R_L	R_H	R_L
Radio ligands				
[^{3}H]Thioxanthene	+	+	+	+
[^{3}H]Butyrophenone	-	-	+	+
[^{3}H]Agonist	+	-	+	-
Agonist affinity	nM	uM	nM	uM
Selective agonist	SKF38393; SKF82526 Dihydroxy nomifensine		Ru24926; Ru24213; NO434; No437	
Selective Antagonist	SCH23390		(-)sulpiride; YM09151-2 Domperidone	
Function	Parathyroid hormone Release Striatum: unknown		Inhibition of pituitary hormone release DA mediated behavioural responses and their antagonism by neuroleptics	
Striatal location Pituitary location	Intrinsic neurons		Intrinsic neurons present both in anterior and Intermediate lobe of pituitary	
	GTP GDP R_H ⇄ R_L Ca^{++} or Mg^{++}		GTP GDP R_H ⇄ R_L Ca^{++} or Mg^{++}	

* Modified from Creese et al., 1983.

phenothiazine family (e.g. chloropramazine, fluphenozine) are known to block the activity of adenylate cyclase and exhibit good correlation with blocking the D-1 binding sites (Hyttal, 1979). D-1 binding sites can be labelled directly with the thioxanthine antagonists [^{3}H]flupenthixol or [^{3}H]pifluthixol (Hyttal, 1978). These radioactive ligands exhibit nanomolar affinities for D-1 receptor in the striatum and the potencies of the dopaminergic antagonists in competing for[^{3}H]flupenthixol correlate well with their potencies in inhibiting DA sensitive AC (Hyttal, 1978). However, it should be cautioned that these ligands could also label D-2 receptor (Sibley and Creese, 1983). A low concentration of an unlabelled butyrophenone should be included in the assay system to block D-2 receptor activity.

1.7.1 Tissue Localization

Dopamine receptor of the parathyroid gland is considered a prototype of D-1 receptor. Dopamine elicits the biochemical response in parathyroid gland characteristic of D-1 activation (i.e. enhanced adenylate cyclase activity, increased cAMP production and activation of cAMP dependent protein kinases), followed by an increase in the release of parathyroid hormone, a physiologic response (Brown et al., 1977; Attie et al., 1980). Response is stimulated by dopamine, SK&F 38393 (dopamine agonist), and blocked by neuroleptics and the ergot lergotril and lisuride (Brown et al., 1980). Dopamine D-2 antagonist sulpiride had a very weak effect in blocking the receptor activity (Stoof and Kebabian, 1984).

The D-1 receptor also occurs in the retina of several species (Schorderet and McDermot, 1978). The cellular localization and

physiologic significance of stimulating the retinal D-1 receptor is unknown. However, in Teleost fish retina, D-1 binding sites located on the external horizontal cells; upon stimulation with dopamine or D-1 agonist SK&F 38393, caused 5 to 7-fold increase in the adenylate cyclase activity (Van Buskirk and Dowling, 1981). This effect was blocked by the D-1 specific antagonist SCH 23390, conversely sulpiride a specific D-2 antagonist was without any effect (Walting and Dowling, 1981).

In the central nervous system, D-1 receptor is found in those regions which are rich in dopaminergic innervation. The striatum and olfactory turbercle have the highest dopamine sensitive adenylate cyclase activity and an increased D-1 radiolabelled ligand binding (Seeman,1980). In the striatum, dopamine binding sites appear to be localized on cell bodies within the striatal neuron and these sites can be completely eliminated with kainic acid lesion (Minnerman et al., 1978). In the olfactory tubercle, adenylate cyclase occurs upon the pyramidal cells and is found to be associated with a phosphoprotein of apparent molecular weight of 32000 daltons known as 3'5 adenosine monophosphate regulated phosphoprotein DARPP-32 (Wallas and Greengard, 1984). Although D-1 binding sites have been localized in different regions of the central nervous system, however, their functional importance is not clearly understood. D-1 receptor in brain is described as receptor in search of a function (Joyce, 1983).

1.7.2 Effects of Guanine Nucleotides on D-1 Receptor Binding

D-1 dopamine receptor appears to be linked to a guanine nucleotide binding protein, since GTP (or its analogue Gpp(NH)p) alters the affinity (converts high affinity to low affinity) of the receptor for

agonists (Creese, 1982). Furthermore, stimulation of striatal dopamine sensitive adenylate cyclase requires GTP (or its analogue) for its stimulatory effect on dopamine. The relationship between dopamine D-1 receptor, the guanine nucleotide binding regulatory sub-unit and the required co-factors in the stimulation of the dopamine sensitive adenylate cyclase has been investigated by several laboratories (Chen et al., 1980; McSaigan et al., 1980; Joyce, 1983). Addition of GTP, and Mg^{2+} to the striatal homogenates results in activation of dopamine stimulated adenylate cyclase immediately followed by the formation of the GTP bound low affinity receptor complex. These GTP modulatory effects are illustrated in Figure 2.

1.8 Dopamine D-2 Receptor

Dopamine D-2 receptor is functionally classified as the receptor which does not stimulate but inhibits adenylate cyclase activity upon agonist occupation. The consequence of D-2 receptor stimulation is shown to cause either decrease or to have no effect on the formation of cAMP (Creese et al., 1983, 1984). The activation of CNS dopamine D-2 receptor with agonists like apomorphine leads to an increase in the motor activity and stereotyped motor behaviour (reviewed by Seeman, 1980). Butyrophenone neuroleptics (e.g. spiroperidol, haloperidol) are the most potent antagonists for the D-2 receptor, substituted benzamide derivatives (e.g. sulpiride) exhibit moderate affinity at D-2 site (reviewed by Creese et al., 1983). The rank order potency of various agonists for D-2 receptor is exhibited as NPA > ADTN = apomorphine > DA and for antagonists is spiroperidol > (+)-butaclanol > domperidone > haloperidol (Reviewed by

Seeman, 1980).

Radioligand binding studies have allowed direct correlation of binding data with the behavioural action of dopamine D-2 receptor. The affinities of a number of structurally diverse dopamine antagonists for [^{3}H]butyrophenone binding sites agree with their potency in antagonism of apomorphine and amphetamine induced stereotypical behaviour in rat (Creese et al., 1978; Mishra and Cleghorn, 1980) and antipsychotic activity in man (Seeman et al., 1976). The plasma concentration of drugs measured by neuroleptic radioreceptor assay and by other methods at therapeutic dose level correlated well with the nanomolar affinity of the antipsychotic drugs for dopamine receptor binding sites (Creese et al., 1976). Similar correlation between the plasma concentration and the therapeutic efficacy for anti-Parkinsonian effects of dopamine agonists have been reported, and the effects are to be mediated through the butyrophenone labelled D-2 receptor (Titler and Seeman, 1978).

1.8.1 Tissue Localization

Dopamine D-2 receptor occurs both in the anterior and intermediate lobes of pituitary and upon stimulation, with dopamine agonist, inhibits prolactin and MSH release, respectively (Onali et al., 1981; Cote et al., 1982). Radioligand binding data with dopamine antagonists and agonists in the pituitary gland revealed a single dopamine receptor subtype with affinity and stereoselectivity as observed for the dopamine D-2 type (Creese et al., 1977a; Caron et al., 1978; Cronin and Weiner, 1979). Characterization of receptors with [^{3}H]spiroperidol/agonist competition experiments revealed that dopamine D-2 receptor exists in two states; the high affinity and the low affinity

states for agonists, (Sibley et al., 1982) that are modulated by GTP or its analogue. GTP at saturating concentration shifts the agonist curves to the right, implying the conversion of dopamine D-2 receptor from high to low affinity state. The high affinity binding sites have been characterized by a specific radiolabelled agonist [^{3}H]N-propylnorapomorphine [^{3}H]NPA binding in bovine anterior pituitary membranes (Sibley et al., 1982). The binding was shown to be saturable and homogenous with single affinity corresponding to the antagonist [^{3}H]spiroperidol binding. The saturating concentration of guanine nucleotide (100μM) completely abolished the specific [^{3}H]NPA binding to the pituitary membrane preparation (Creese et al., 1983).

Moreover, dopamine agonists have been shown to decrease cAMP levels in both the anterior (Labrie et al., 1981; Giannattasio et al., 1980) and the intermediate lobes of the pituitary (Cote et al., 1982). The sodium ions appear to modulate the dopamine D-2 inhibition, by amplifying the inhibition of adenylate cyclase enzyme activity (George et al., 1985). This negative coupling between dopamine and adenylate cyclase is GTP regulated (Cote et al., 1982), and stimulation of D-2 receptor results in the inhibition of cAMP formation, this may serve as a mechanism by which dopamine decreases the synthesis and release of pituitary hormones, like prolactin from the anterior pituitary and α-melanophore stimulatory hormone (α-MSH) from the intermediate lobe of pituitary (Engalbert and Bockaert, 1982).

1.8.2 Striatal Dopamine D-2 Receptor

The very first dopamine binding studies utilizing [^{3}H]dopamine and [^{3}H]haloperidol were performed in the mammalian striatum (Creese et

al., 1975; Seeman et al., 1975). Since then, the number of radioligands used in binding studies have multiplied, and in fact far exceed those for many other neurotransmitters. At present, the following [^{3}H]labelled ligands have been shown to bind to the dopamine D-2 receptor: Spiroperidol, domperidone, pimozide, sulpiride, flupenthixol, dihydroergocryptine, apomorphine and N-propylnorapomorphine (NPA) (for reviews see Seeman, 1980; Sibley and Creese, 1983; Memo et al, 1983). There are quantitative and qualitative differences in the binding properties of these structurally diverse ligands. However, these ligands satisfy all or most of the criteria necessary to support the contention that they label dopamine D-2 receptor. 1. The regional distribution of binding sites labelled with these ligands parallel dopamine innervation; 2. The dopamine D-2 agonists and antagonists are more potent than other non-dopaminergic neurotransmitters; 3. They exhibit stereospecificity with respect to the dopamine selective antagonist (+) and (-)-butaclamol. The dopamine D-2 receptor is situated in post-synaptic processes in the striatum (Seeman, 1980). Kainic acid lesions invariably reduced the density of the D-2 receptor (Creese et al., 1977; Mishra et al., 1978). However, lesions with the 6-hydroxy dopamine (destroys pre-synaptic terminals) do not cause any decrease in the receptor density (Creese et al., 1977).

As in the pituitary, [^{3}H]agonist ligands in striatum also label dopamine D-2 sites with high affinity under appropriate conditions. The affinity of the agonist binding is reduced by guanine nucleotide with a specificity similar to that of the pituitary (Zahnisav and Molinoff, 1978; Creese et al., 1979). Striatal [^{3}H]agonist D-2 binding is enhanced

by either preincubation of the tissue homogenates with Mg^{2+} (or other divalent metal cations) or inclusion of these ions in the assay system (Hamblin and Creese, 1982).

There is now evidence that the D-2 receptor in the striatum may also be negatively coupled to the adenylate cyclase. Stoof and Kebabian (1981) have demonstrated that the D-2 receptor specific agonist LY 141865 could reduce the SKF 38393 (D-1 specific agonist) stimulated cAMP efflux from rat striatal slices. This inhibitory effect was reversed by (-) sulpiride. In other studies, inclusion of D-1 antagonist and an elaborate preparation of rat striatal homogenate resulted in a significant D-2 receptor mediated inhibition of the basal adenylate cyclase activity (Onali et al., 1984). This inhibition was dependent on the presence of GTP.

In recent studies, it was further shown that D-2 receptors may be directly linked to the Ni (or Gi) protein of the adenylate cyclase. Fugita et al. (1985) have demonstrated that injection of Ni specific inhibitor IAP (islet activating protein) from pertussis toxin in rat striatum blocked the apomorphine induced stereotyped behaviour. There was also a concomitant shift to the right in the affinity of apomorphine for the [^{3}H]spiroperidol binding site in an IAP treated rats. In other studies Tanaka et al., (1984) have shown that inhibition of adenylate cyclase by dopamine required several-folds more GTP than that required for stimulation of adenylate cyclase which is consistent with the bimodel N_i and N_s regulation in many other systems (Cooper et al., 1982). Furthermore, Battagdla et al. (1985) have demonstrated that forskolin which stimulates directly the catalytic site of AC, caused enhancement of

Figure 2: A model of D-1, D-2 dopamine receptor regulation of adenylate cyclase (reproduced in modified from Helmreich and Pfeuffer, 1985) system. R_s and R_i represent activating and inhibiting receptors respectively; G guanylyl nucleotide binding proteins; G_s = α_s 42000 mol. wt. activating subunit, G_i and α_i, 39000 mol. wt. inhibitory subunit β, γ, 35000 and 8000 mol. wt. subunits respectively, common to both G_s and G_i subunits. C_i catalytic moity of adenylate cyclase. GN, non-hydrolysable guanylyl neuclotide analogues. Release of activated G_s or activated G_i results in formation of stimulated or inhibited adenylate cyclase, C_s or C_i respectively. In the presence of dopamine or other agonists, α, β, subunits will be released from G_s and G_i unit will activate or inhibit the cyclase system depends on the type of receptor stimulation.

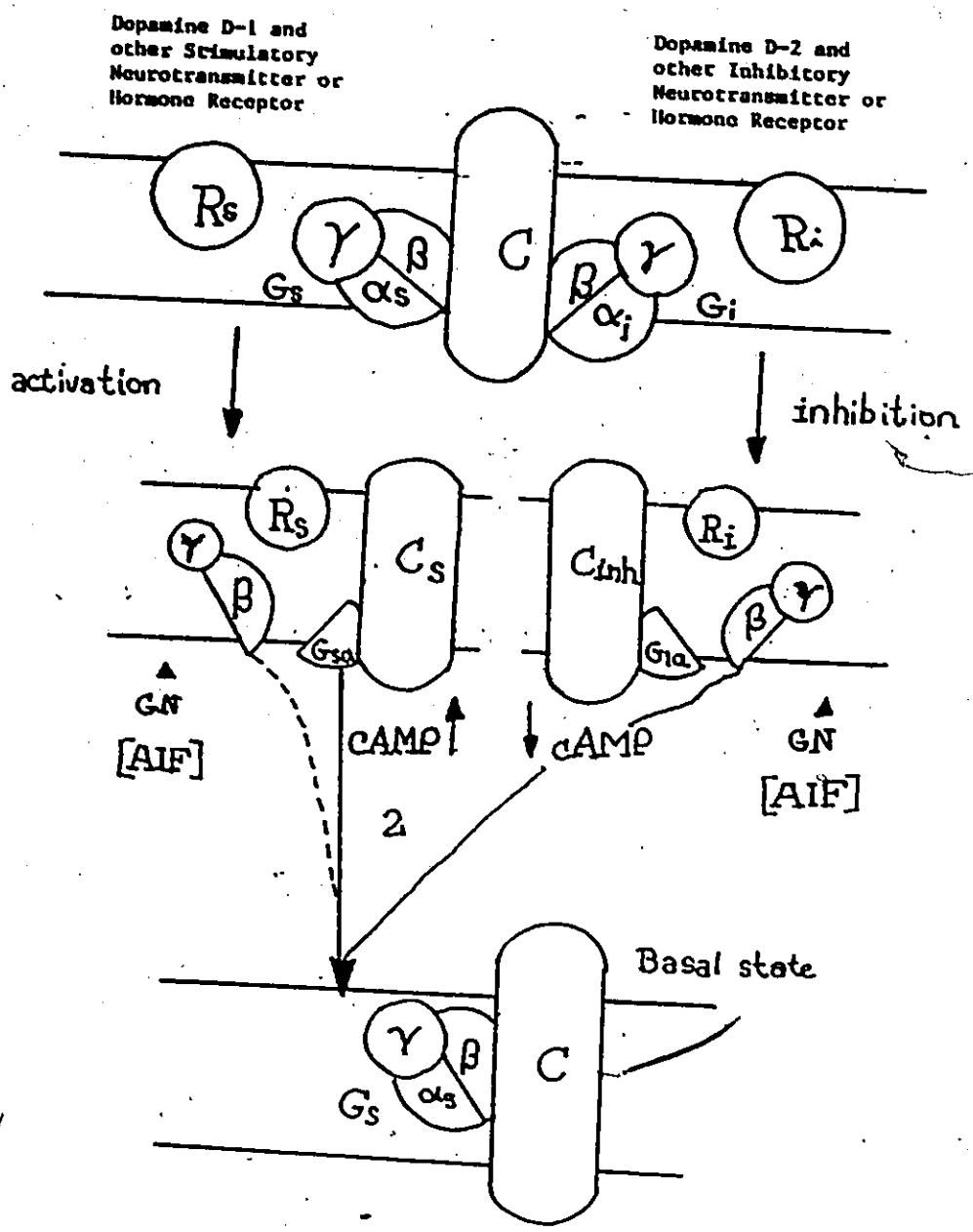

Dopamine D-1 and other Stimulatory Neurotransmitter or Hormone Receptor
Dopamine D-2 and other Inhibitory Neurotransmitter or Hormone Receptor
Rs
γ
β
αs
Gs
C
β
γ
αi
Gi
Ri
activation
inhibition
Rs
γ
β
Gsα
Cs
Cinh
Ri
β
γ
Giα
GN
[AIF]
cAMP
cAMP
GN
[AIF]
2
Basal state
γ
β
αs
Gs
C

FIGURE 2

absolute inhibition of the striatal adenylate cyclase enzyme activity that was induced by the dopamine D-2 receptor agonists. This inhibition was concentration dependent with respect to forskolin and the agonist, and the inhibitory effect was antagonized by spiroperidol.

Based on all the above observations one may conclude that: i) the dopamine D-2 receptor is negatively linked to adenylate cyclase activity; ii) the receptor is directly linked with a Gi (or Ni) unit of adenylate cyclase. The mechanism by which this receptor-adenylate cyclase coupling influences the response is not clearly understood. How GTP is involved in the conversion of the high affinity to the low affinity state is also not yet understood. However, a model illustrated by Helmreich and Pfuffer (1985) has been modified to explain the possible modulatory effects of GTP on dopamine D-2 receptor, (Figure 2). A final mechanism of receptor-effector system will only be understood when each component of the system is isolated, purified and then reconstituted in a simulated membrane system.

1.9 Isolation and Purification of Dopamine D-2 Receptor

As discussed in the preceding sections, with the advent of radioligand binding studies, considerable progress has been made in the understanding and pharmacological characterization of dopamine receptors. However, at the same time, this binding technique has created controversies about their classification and the affinities of different forms towards their agonist and antagonist (Seeman, 1982; Creese and Sibley, 1982). Furthermore, this technique has provided very little information about the cascade of molecular event which begins with the

formation of the ligand receptor complex terminating to a physiological response. In order to enhance our understanding of molecular mechanisms of drug receptor interaction and the final response, two prerequisite steps are essential: first, the isolation/solubilization of the receptor from its membrane bound form into a solubilized form, and second, to have it purified.

1.9.1 Detergent Solubilization of Receptors

Membrane proteins exist in nature at the interface between a phospholipid bilayer and the adjacent aqueous medium. It has been thought that the protein associated with the membrane can be roughly classified into two categories the peripheral or extrinsic and the integral or intrinsic. The peripheral protein can easily be dislodged from membranes either by chelating agents or by a change in ionic strength or pH without dissociating the lipid matrix of the membrane. These proteins are presumably bound by polar interactions to the other protein or lipids in the membrane (for review Helenius and Simon, 1975). On the other hand, the intrinsic proteins are tightly bound to the membrane and can only be solubilized by disrupting the membrane with an organic solvent or a detergent. These proteins have been suggested to have an amphiphilic structure (Tanford and Reynold, 1976).

The neurotransmitter receptor proteins are intrinsic membrane bound proteins. Solubilization of these proteins can be defined as a "conversion of the complex membrane system into a relatively simpler state without disruption of its functional characteristic" (Tanford andReynolds, 1976). In order to achieve this, a solvent medium must be able to simulate the natural environment of the protein at the

lipid/aqueous interface. In most instances, this goal can be met by the use of a suitable detergent or a combination of detergents as a solubilizing agent.

Detergents: By definition detergents are a special class of lipid containing polar and apolar groups, i.e. they are soluble amphiphiles (possessing both polar and non-polar moieties) which above a critical concentration and temperature form micelles (compact aggregates with apolar groups sequestered in the centre and polar group facing outwards). The solubilizing effect of detergents stem from their amphiphilic character, which enables them to interact with both hydrophilic and lipophilic regions in an essentially disruptive fashion, but not a denaturing one.

The Critical Micelle Concentration (CMC): CMC is very important in detergent solubilization, and is defined as the narrow concentration range of the surfactant below which no micelles are detected and above which all additional surfactant form micelles. Ionic surfactants often have higher CMC values than those of their non-ionic analogues. The CMC of ionic detergents can be reduced by increasing the counter ion concentration with sodium salt (Shackland, 1970). This is mainly due to the reduction of the electrostatic repulsion between the head groups allowing association at a lower monomer concentration. The effect of temperature between 0°C and 37°C on the CMC of the ionic surfactant is fairly small, but there is significant lowering of the CMC of the non-ionic surfactant with increasing temperature (Helenius and Simon, 1975). Appropriate detergents can fully substitute for membrane phospholipids (phospholipid simulation) and can offer the same local

environment to the protein as a phospholipid bilayer.

The choice of a detergent for the solubilization of a membrane receptor protein at present is rather empirical. Among the detergents most widely used in membrane neurotransmitter receptor solubilization and reconstitution are sodium cholate, deoxycholate, zwitterionic detergent CHAPS, digitonin and octyl-glucoside. These detergents have thus far proven to be the most effective in solubilizing many functional neurotransmitter receptor sites, (For review, see Hjelmeland and Chrambech, 1984). Ionic detergents (e.g. cholic acid), form small micelles in aqueous media and are capable of solubilizing large quantities of phospholipids by forming mixed micelles with them (Lichtenberg et al., 1983).

1.9.2 Solubilization Criteria of Specific Receptor Site

To assess that a specific receptor site has been solubilized, certain biochemical and morphological criteria must be satisfied. These criteria have been put forth by Laduron and Ilien (1982) and a modified form is presented in Table 3a,b. These criteria if satisfied will help in differentiating the membrane bound state from the soluble state of a receptor. Non-sedimentation at 100,000 g for 60 min represents the most important criterion for solubilization. However, when a high salt concentration is used as a solubilizing agent, the density of the medium becomes so high as to prevent certain membrane elements from being spun down. In this situation, membrane-like structures may still remain in the solution or above the solution and may lead to the false impression of solubilization (Laduron and Ilien, 1982). Along with this criterion and others as described in Table 3a for solubilization, it must also be

TABLE 3

Criteria of Solubilization of Specific Brain Receptors*

a. Operational criteria to assess receptor solubilization

1. Lack of sedimentation in low density media (100,000 g for 60 min)
2. No retention on small pore size filters (millipore 0.22 μ)
3. Higher retention on gel filtration than membranes
4. Lower sedimentation coefficient than membranes
5. Disappearance of lamellar membrane structure under electron microscopy
6. Decrease of thermostability

b. Criteria of receptor specificity

1. High affinity
2. Saturability
3. Reversibility
4. Stereospecificity
5. Regional distribution
6. Drug displacement (agonists and antagonists belonging to different chemical and pharmacological classes)
7. Correlation between drug affinity in solubilized and membrane preparations
8. Correlation between drug affinity in vitro and pharmacological potency in in vivo

* Modified from Laduron and Ilien, 1982.

ascertained that the solubilized receptor protein satisfies all the receptor binding characteristics as measured with in vitro radiolabelled binding techniques. These characteristics are listed in Table 3b. In the solubilized form a particular receptor must exhibit the same pharmacological characteristices such as affinity, specificity, saturability, stereospecificity and reversibility as the membrane bound receptor. Furthermore, these receptors must satisfy the regional distribution in the brain and the drug displacement curves must correlate with their pharmacological and clinical doses.

1.9.3 Solubilization of Dopamine (D-2) Receptor (A review of literature)

In the past few years, several investigators have reported the solubilization of [^{3}H]butyrophenone binding sites. Initially, Gorrisen and Laduron (1979) employed 1% digitonin treatment to dog striatal membrane followed by ultracentrifugation. With this technique, they were able to solubilize the D-2 receptor which retained similar affinity and specificity to that of the membrane bound receptor. Similar findings have been reported in other species, as in rat (Gorrisen et al., 1980), and human striatum (Madras et al., 1980). However, by this solubilization procedure the yield was very low ($\leq$ 20%, Lerner et al., 1981) and also has given a very high nonspecific binding. Furthermore, it has also been reported that solubilization with digitonin has changed some of the pharmacological characteristics in some species e.g. loss of stereoselectivity and a change in the affinity for butyrophenone antagonists in bovine striatum (Madras et al., 1982).

In recent years, various laboratories have attempted to use different detergents to solubilize dopamine D-2 receptor, e.g.

lysolecithin (Lerner et al., 1981), lubrol (Withey et al., 1981) and a zwitterionic detergent CHAPS (Lew and Goldstein, 1981, 1984). In these detergent solubilization procedures, the yield and the reproducibility of a solubilized material still seemed to be of concern, (Lerner et al., 1981). Clement-Cormier and Kendrick (1980) have reported solubilization of dopamine receptors with 50% (w/v) KCl. However, at this point it is not clear whether they actually have solubilized the receptors or have simply suspended the membrane fragments into a thick solution (commentary by Laduron and Ilien, 1982).

In this thesis, an efficient procedure has been developed for the solubilization of a dopamine D-2 receptor. The new method of cholic acid: NaCl solubilization was 2-3 times more effective in solubilizing dopamine-D-2 receptor (Ramwani and Mishra, 1983) than the other methods. This method was equally effective in solubilizing the striatal dopamine-D-2 receptor from different species e.g. human, rat, dog and bovine (Ramwani and Mishra, 1983a). Similar findings have also been reported from other laboratories (Hall et al., 1983, Wouter et al., 1984). Furthermore, by this method, it has also been demonstrated that solubilized receptor from the bovine striatum have retained high affinity dopamine receptor binding sites, the receptor-Gi protein complex (Kazmi et al., 1986).

1.10 Purification of Neurotransmitter Receptors

In general, purification of membrane bound proteins involves a cascade of steps, each with a number of inherent problems. As mentioned earlier in this section, the first and foremost step is to solubilize

these proteins from the membrane with the help of detergents or other agents. Once isolated, these proteins, in many instances, lose their biological activity which may be due to a loss of co-factors or associated phospholipids, without which the solubilized proteins may not express their biological activity. Furthermore, the membrane proteins which are not involved in the frame-work structure are generally present in minute quantities. This is especially true with the membrane bound neurotransmitter receptor protein.

All the above reasons have generated the design of the specific chromotogaphic methods necessary for purification of the essential components of the cell membranes. Affinity chromatography has been the preferred method amongst all other chromatographic techniques for the purification of enzymes and many membrane bound proteins (Lowe, 1979).

Affinity chromatography is an extension of adsorption chromatography, which involves a complex set of Van der Waals, hydrophobic, steric and electrostatic forces for binding of affinity ligands to the proteins to be purified (Turkova, 1978). It exploits the unique biological specificity inherent in a ligand-protein interaction. This ligand could refer to a substrate, product, inhibitor, coenzyme, allosteric effector or any other molecule that interacts specifically and reversibly with the protein to be purified. Affinity chromatography requires the covalent attachment of a ligand to an insoluble and stable matrix, packed into a chromatographic bed. When the mixture comprising several proteins is applied to the column, only that protein which displays appreciable affinity for the ligand will be retained and the remaining protein which do not show the recognition will pass through

unattached. The specifically adsorbed proteins can subsequently be eluted by an appropriate method which may include another ligand of higher affinity, a change in pH, or increased salt concentration. Clearly, this technique can be applied wherever a specific interaction occurs between any two biological molecules.

This technique has a number of inherent advantages over the classical methods of protein purification (reviewed by Lowe, 1979). First, it involves a design and construction of an adsorbent specifically suitable for the protein to be purified. Secondly, it permits a rapid separation of the desired protein from inhibitors and contaminants. Thirdly, in many instances it can lead to a "single step" purification of a protein by several thousand-folds. Finally, this technique is especially suitable for the isolation of a protein present in minute quantities, e.g. neurotransmitter receptor protein. Since this chromatographic procedure involves a specific selection of matrix, ligand and elution procedure, it is beyond the scope of present dissertation, to review every step in detail designed for different affinity ligand matrix attachments. Excellent reviews on affinity adsorption techniques are available (Turkova, 1978; Trayer, 1978; Lowe, 1979). The essential requirement of a successful affinity adsorbent are the following: a) The matrix - The solid support to which the affinity ligand couples is generally a cross-linked polymer routinely referred as a gel. Almost any macromolecule, synthetic or natural may form a gel in a suitable liquid when cross-linked with a bifunctional reagent. This gel or insoluble support must possess certain characteristics; (1) it should form a loose porous network which may permit the uniform entry and exit of a large

macromolecule. The ease of penetration of the macromolecule through the gel determines the concentration of the ligand freely available to the macromolecule. 2) The gel particle should be uniform, spherical and rigid. 3) The gel matrix must be chemically inert, so as to minimize the non-specific adsorption. 4) Finally, the gel must be physically and chemically stable to the condition employed for covalent coupling of the selected ligand and for the adsorption and subsequent elution of complementary molecules.

b) The ligand - The ligand should exhibit specific and reversible binding affinity for the protein to be purified. It should also possess chemically modifiable groups which allow it to be attached to the gel without destroying its binding specificity to the protein. It is important to consider the region of the ligand that is attached to the matrix. The ligand should be coupled via the group least likely to be involved in its specific biological interaction with the molecule to be isolated. Finally, the linkage should be stable to the conditions likely to be used during the chromatographic procedure including the recycling for repeated use of the gel.

c) Spacer arms - The spacer arm between the matrix and ligand facilitates the effective binding of the ligand to the protein. It sets the ligand away from the matrix so as to make it more accessible to the protein to be purified. The compounds most commonly employed as spacer molecules are linear aliphatic hydrocarbons with ω-terminal functional groups providing points of attachment to the ligand (Lowe, 1979). The length of the spacer arm is critical. If it is too short, the arm is ineffective and binding capacity becomes negligible. If it is too long,

non-specific effects become pronounced reducing the selectivity of separation. Many affinity adsorbant matrices either "activated" or with spacer arms are commercially available. It should be pointed out that the affinity system is a specifically designed system for a specific protein purification. It is a "trial and error" that optimizes the final yield of purification folds.

1.10.1 Literature Review on Purification of Neurotransmitter Receptors:

In order to elucidate the molecular mechnisms whereby a neurotransmitter alters the signal transduction, it is essential to isolate and purify the receptor protein. Although, high affinity, reversibility and specificity of a neurotransmitter-receptor interaction make affinity chromatography an attractive method for purification, yet, except for a few, not much progress has been reported towards the purification of neurotransmitter receptors. The major hindrance seems to be the minute quantities of these receptors present in a given tissue source (Caron et al., 1979). The greatest success in this area has been with the purification of a nicotinic cholinergic receptor from the electric organ (source of Ach receptor) of various invertebrates (reviewed by Heidmann and Changeux, 1978). Several laboratories have isolated the protein subunits of the Ach receptor and reconstituted into an artificial lipid membrane capable of mediating sodium flux in a fashion similar to the membrane bound Ach receptors (Briley and Changeux, 1978). The other neurotransmitter which has been purified extensively is β-adrenergic receptor. This receptor has been purified to homogeneity and its neurotransmitter binding site has been identified and characterized (Caron et al., 1979; Shorr et al., 1981, 1982). This

purified receptor has also been reconstituted in lipid vesicles and subsequently been fused with the adenylate cyclase of Xenopus laevis erythrocyte (Cerione et al., 1983). Recently, some progress has been reported in purification of other neurotransmitters , e.g. α_1 (Graham et al., 1982), β_2 (Regen et al., 1982), adrenergic receptors, and muscarinic cholinergic receptor (Haga and Haga, 1985). However, so far, there is no single report in the literature on the substantial purification of the dopamine receptor. Preliminary findings on purification of the dopamine binding protein have recently been reported (Moroi and Hsu, 1984). However, it was only a four-fold purification and detailed characterization of this protein as a D-2 receptor was not established in the report.

1.11 Aims of The Thesis:

In the past two decades major impetus was given to the field of dopamine D-2 receptor research. This receptor has been characterized and implicated in various neurological and psychiatric disorders. At the start of this project in 1981, the membrane bound dopamine-D-2 receptor has been characterized from the different regions of brain and also from the different species including post-mortem human brain. The pharmacological specificity, affinity and behavioural correlations of D-2 binding sites have also been established. However, molecular characterization of the receptor and events leading to the final response have not yet been understood. The logical approach to study these events was to isolate the receptor from membranes and purify it from the rest of the proteins and study it in the isolated form. Henceforth the specific

aims for the thesis were as follows:

a) To establish an efficient method of solubilization for the bovine striatal dopamine D-2 receptor.

b) To determine the pharmacological characteristics (affinity specificity and stereoselectivity) of the solubilized receptor and establish the D-2 receptor binding characteristics of the solubilized preparation.

c) To examine different detergent solubilization procedures in various species and compare their effectiveness in solubilizing striatal dopamine D-2 receptor.

d) To establish an affinity chromatographic procedure for the purification of bovine striatal dopamine D-2 receptor.

e) To pharmacologically characterize the purified receptor and demonstrate its similarity to the membrane bound or solubilized dopamine D-2 receptor.

2. MATERIALS AND METHODS

2.1 Materials

General Chemicals

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO.): Trizma base, L-ascorbic acid, DL-dithiothrietol, bovine serum albumin, bovine brain total lipid extract, soybean crude lipid extract, 1,4, butanedioldiglycidyl either, sodium borohydride, polyethylene glycol, (6000), bovine gamma globulin, and Sephadex G-50. Coomassie blue reagent kit for protein assay was purchased from BioRad Laboratories (Mississauga, Canada). Sepharose CL-6B was obtained from Pharmacia Chemicals (Montreal, Canada). Other chemicals used in the present study were of standard or analytical grade and purchased from commercial sources. Buffers and solutions were made in deionized distilled water.

Detergents: CHAPS and cholic acid were purchased from Calbiochem (LaJolla, California). Lysolecithin and digitonin were obtained from Fisher (Toronto, Canada) and Sigma Chemicals respectively.

Radioligands: The radioligands used in the present study, [^{3}H]spiroperidol, [^{3}H]haloperidol and [^{3}H]NPA were purchased from New England Nuclear (Boston, Mass.).

Drugs: The following unlabelled drugs were generous gifts: haloperidol (McNeil, Canada); spiroperidol, domperidone, ketanserin and R5260 (Janssen, Belgium); NO343 (Nelson, U.S.A.); SCH23390 (Schering, U.S.A.); SK&F 38393 (Smith, Kline and French, U.S.A.). N-propylnorapomorphine (NPA), (+) and (-)-butaclamol were obtained from Research Biochemical Inc. (Wayland, MA) and dopamine, apomorphine, phentolamine and

propranolol were from Sigma Chemicals.

All dopamine agonists were dissolved in 50 mM Tris - 1 mM EDTA buffer containing 0.1% ascorbic acid (pH 7.4). The antagonists were first dissolved in 20 µl 50% acetic acid and then working concentrations were made with assay buffer (50 mM Tris, 1mM EDTA, pH 7.4). All drugs were prepared just before use.

Tissues: Fresh bovine brains (within two hrs. of sacrifice of the animals) were obtained from Fearman and Company, a local slaughter house. Human brains were obtained post-mortem, within 12 hrs, from individuals with no previous neurological or psychiatric history. Normal Wistar rat brains and canine brains were obtained from the animal quarters of our institute. The striatal tissue was removed by standard dissection techniques and stored at -70°C until further use.

2.2 Methods

2.2.1 Membrane Preparation: Frozen striatal tissue from different species was thawed slowly at 4°C and weighed. Tissues were cut into small pieces and homogenized in 10 vol. of 0.25M sucrose at 4°C using teflon glass homogenizer with 20 strokes of tight fitting pestle. The homogenate was centrifuged at 1000 g for 10 min. in a Sorvall RC-5 centrifuge. The supernatant was saved and the pellet was resuspended in 10 vol. of sucrose solution and recentrifuged at 1000 g for 10 min. The supernatants from both centrifugations were collected and centrifuged at 100,000 g for 1 hr. in a Beckman ultracentrifuge. The resulting P_2-P_3 (mitochondrial-microsomal) pellet was washed in 50 mM Tris - 1 mM EDTA buffer, pH 7.4 (30 vol.) and centrifuged at 30,000g for 20 min. The

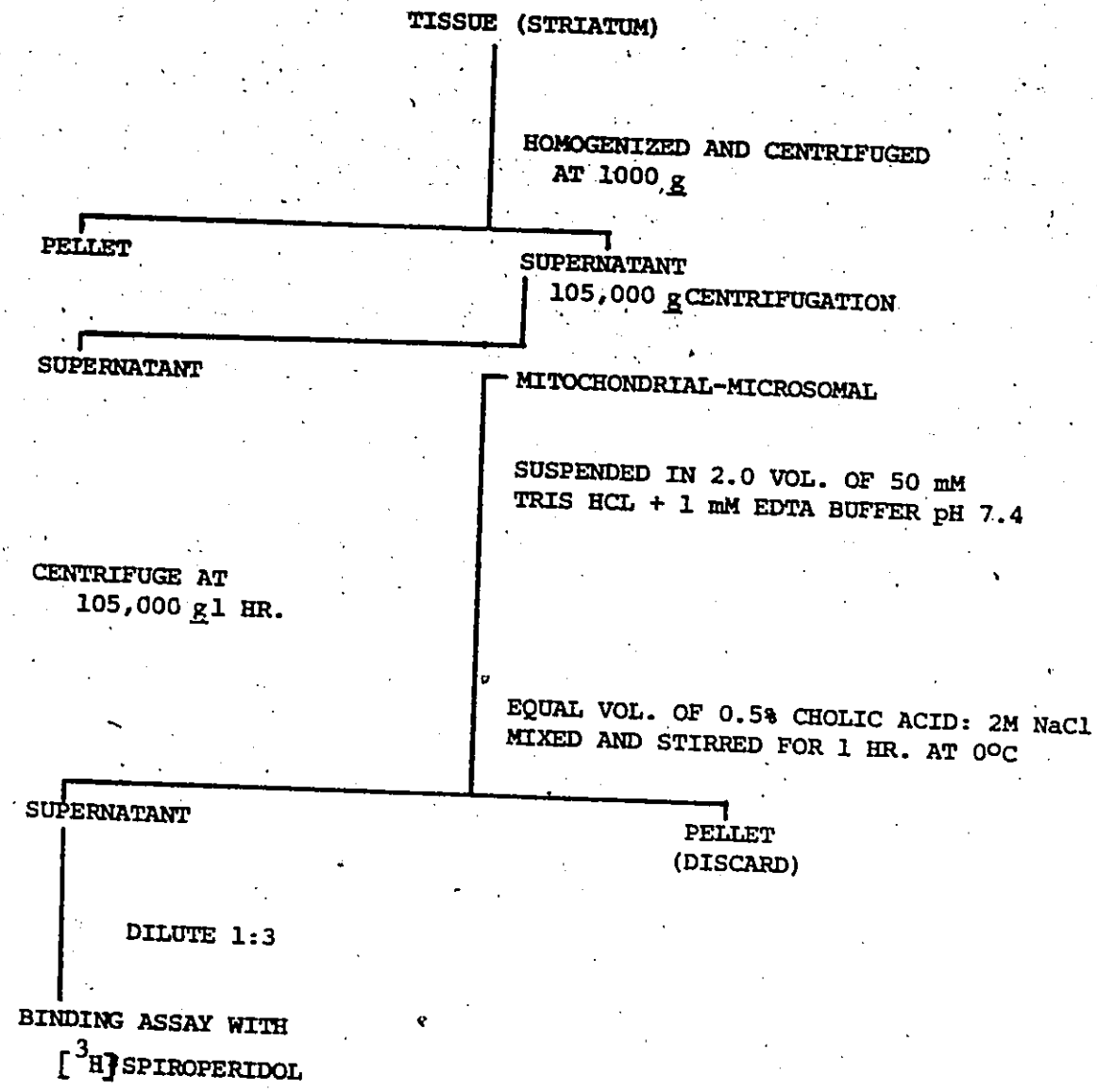


FIGURE 3: Flow Diagram for the Solubilization of Striatal Dopamine D-2 Receptors.

final washed pellet was suspended in two vol. of the same buffer and frozen at -70°C until further use. Membrane preparation steps are described diagrammatically in Figure 3. The preparation was stable over a one month period at -70°C.

2.2.1.1 **Binding Assay in the Membrane:** The binding assay for the P_2-P_3 membrane fraction was performed in 50 mM Tris-1mM EDTA buffer, pH 7.4 (assay buffer). The membrane preparation was diluted 1:10 with assay buffer to give a final protein concentration of 0.8 - 1 mg/ml. [^{3}H]spiroperidol was used as a radioligand in most of the studies. In some experiments, [^{3}H]NPA was used as a radioligand to identify the high affinity binding sites. [^{3}H]spiroperidol dilution was made in assay buffer. [^{3}H]NPA solution was prepared in the same assay buffer, except that it also contained 0.1% ascorbic acid and 5 mM $MgCl_2$. Ascorbic acid prevents the oxidation of NPA, and $MgCl_2$ helps to retain the high affinity binding sites. The binding assay was performed in 12 x 75 mm glass tubes in triplicates in 1.0 ml vol. Non-specific binding was determined a in parallel assay in the presence of 1 μM (+)-butaclamol. The incubation was carried out either at room temp (22°C) for one hr. or at 4°C for 16 hrs. The bound ligand was separated from the free ligand by filtration using GF/B filters (Whatman). The filters were washed with 4 x 2.5 ml, 50 mM Tris buffer, pH 7.4 at 4°C and then counted with aquasol in a Beckman scintillation counter. The specific binding is defined as the difference between counts bound in the presence and absence of 1 μM (+)-butaclamol. The buffer blanks under the assay conditions gave no specific binding. The total binding in the blank was less than 10% of that obtained with the membranes.

2.2.2 Solubilization of Dopamine D-2 Receptor: The P_2-P_3 membrane fraction was solubilized by various detergents.

Digitonin Solubilization: Solubilization with digitonin was carried out essentially by the method of Madras et al. (1980). The membrane suspension was diluted in 0.1 M phosphate buffer, pH 7.4 and concentration adjusted to 3-4 mg protein per ml and solubilized with 1.0% digitonin final concentration (digitonin was dissolved by warming digitonin suspension in hot water). The extraction time with digitonin was 30 min at 4°C. A clear supernatant was obtained by centrifugation at 100,000 g for 60 min. The solubilized material was used immediately for [^{3}H]spiroperidol binding.

Lysolecithin Solubilization: It was carried out according to the method of Withey et al. (1982). Membrane P_2-P_3 suspension was diluted with 50 mM Tris, 1 mM EDTA buffer, pH 7.4 to the concentration of 3-4 mg/ml protein and mixed with 0.2% lysolecithin. The mixture was agitated for 30 min and centrifuged at 100,000 g for 1 hr. The clear supernatant was collected and used immediately for the binding assay.

Cholic Acid Ammonium Sulfate Solubilization: It was carried out with some modifications to the previously published procedure from this laboratory (Varmuza and Mishra, 1981). Briefly, striata were homogenized in Tris-Maleate-EDTA-Mg buffer, pH 7.4, solubilized with cholic acid and simultaneously precipitated fractionally with varying concentrations (31-49%) of ammonium sulfate. The 49% ammonium sulfate precipitate was collected and suspended in 0.05% egg lysolecithin-Tris-EDTA buffer pH 7.4. This solubilized preparation was either used immediately for [^{3}H]spiroperidol binding or stored at -70°C for future use.

Cholic Acid - NaCl Solubilization: It was carried out by mixing equal volumes of the P_2-P_3 (mitochondrial-microsomal) preparation and cholic acid-sodium chloride mixture to give a final concentration of 0.25% cholic acid - 1 M sodium chloride and 5-7 mg of protein in the suspension. Phenylmethylsulphonyl flouride (0.1 mM, dissolved by warming in a boiling water bath) as a protease inhibitor, and 0.5 mM dithiothreitol were included during solubilization. The suspension was agitated for 1 hr. at 0°C and centrifuged at 105,000 g for 60 min. The clear supernatant was collected and diluted 1:3 with 50 mM Tris - 1 mM EDTA buffer for binding studies. This method is diagrammatically outlined in Figure 3.

2.2.2.1 **Binding Assay for Solubilized or Affinity Purified Preparation:** The solutions of [^{3}H]ligands and drugs were prepared in the assay buffer (50 mM Tris 1 mM EDTA buffer pH 7.4) and added to 12 mm x 75 mm glass tubes in triplicate. The total binding of [^{3}H]spiroperidol to solubilized or affinity purified receptor was determined in 1.0 ml assay mixture containing 1 nM [^{3}H]spiroperidol. Nonspecific binding was determined in parallel assays in the presence of 1 µM (+)-butaclamol. The incubation was carried out at 4°C for 16 hrs. At the end of incubation, the bound ligand was separated from the free by a modified polyethylene glycol (PEG) precipitation method (Cuatrecasas, 1972). Bovine gamma globulin was added to each tube (0.01% final concentration) followed by immediate precipitation with PEG 6000 (10% final concentration). The contents of the tube were thoroughly mixed and placed on ice for 10-15 min. The suspension was then filtered through GF/C filters (Whatman) under a low vacuum. The filters were washed with 4 x 2.5 ml of 10% PEG

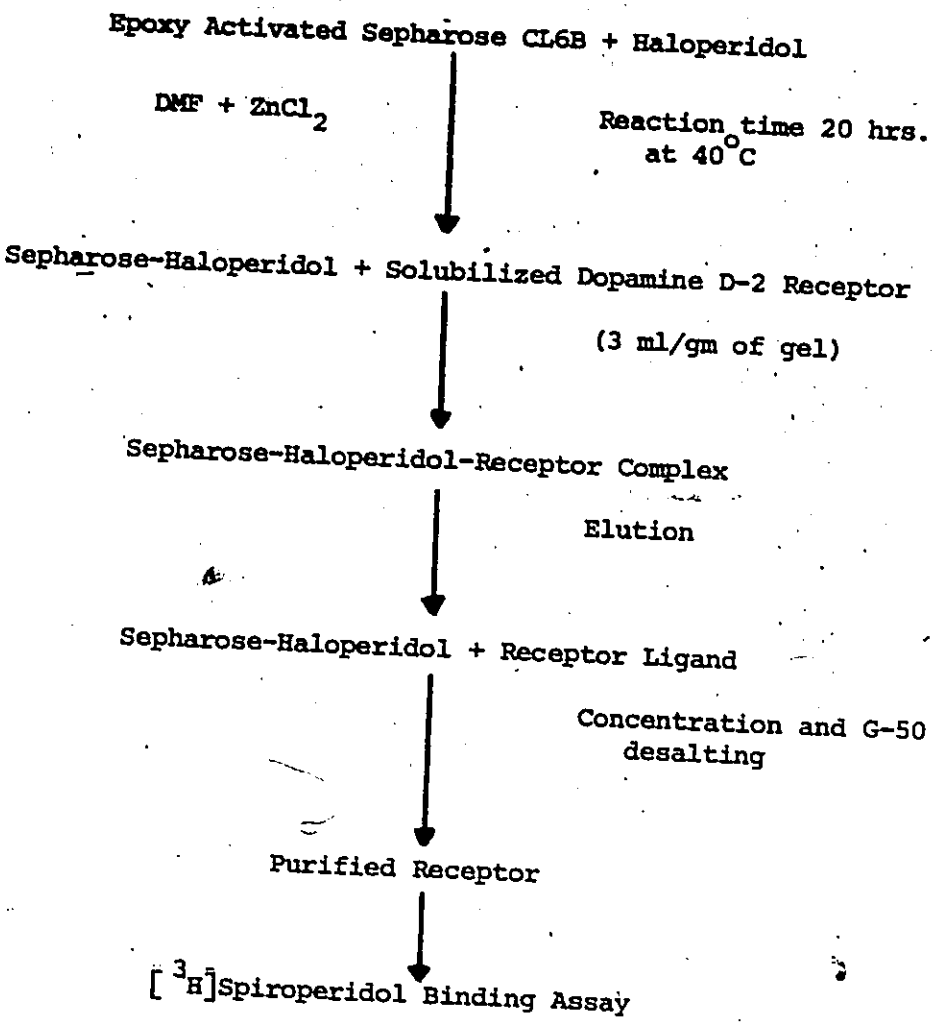


FIGURE 4: Flow Diagram of the Affinity Purification of Bovine Striatal Dopamine D-2 Receptor

in 0.05 M Tris buffer, pH 7.4 at 4°C and then counted in Aquasol using a Beckman scintillation counter.

In some experiments, [^{3}H]NPA was used as a radioligand to determine the high affinity binding sites. [^{3}H]NPA solution was prepared in assay buffer containing 0.1% ascorbic acid and 5 mM $MgCl_2$.

2.2.3 Affinity Matrix Preparation

2.2.3.1 Preparation of Epoxy-Activated Sepharose-CL6B: Epoxy-activated Sepharose CL-6B was prepared according to the method of Sandberg and Porath (1974). Routinely, 30 gm of Sepharose CL-6B was washed with H_2O and mixed with 30 ml of 0.6 N NaOH containing 2mg/ml sodium borohydride and 30 ml of bisoxirane, 1,4 butane diol diglycidylether and the reaction was carried out at 25°C for 8 hrs. using a rotary flask. Under these conditions, one end of the bisoxirane is attached to the Sepharose CL-6B and the other epoxide end is available for coupling to the ligand. Normally, 10-15 micro equivalents of the epoxide groups were attached to 1 ml of wet Sepharose CL-6B gel as determined by acid-base titration (Sandberg and Parth, 1974). This epoxy-activated Sepharose was then coupled to haloperidol under the conditions described below.

2.2.3.2 Preparation of the Affinity Adsorbent for D-2 Receptor: In a typical affinity adsorbent preparation, epoxy-activated Sepharose (20 gm) was soaked in deionized water and then washed on a sintered glass funnel. The washed gel was acetone dried and equilibrated with dimethylformamide (DMF). The gel was then mixed with 600 mg of haloperidol (dissolved in 40 ml of DMF) and the coupling reaction was carried out for 20 hrs. at 40°C in a rotary flask with continuous gentle rotation. $ZnCl_2$ (2.0 gm) was added to the reaction as a catalyst. At the end of incubation period

the haloperidol-coupled gel was washed on a sintered glass funnel with 3 x 50 ml DMF, excess water with acetate buffer, pH 4.5, and then with 50 mM Tris buffer, pH 7.4 containing 1.0 M NaCl. This gel was finally washed with water and stored in 0.01% sodium azide. In some experiments [^{3}H]haloperidol was added to the reaction mixture for the estimation of haloperidol coupling to the gel. The preparation was stable without leaching over a one-month period.

2.2.4 Affinity Chromatography: Routinely, 10 ml of gel was packed into a 0.5 x 30 cm column and equilibrated with 50 mM Tris - 1 mM ETDA buffer containing 0.15 M NaCl, 0.02% cholic acid at pH 7.4 (equilibrating buffer). Approximately 40.0 ml (3-5 pmol) of solubilized receptor was loaded at 4°C at a flow rate of 1.5-2 ml/hr, and the column was washed with 5 vol. of equilibrating buffer at 20 ml/hr. The column was eluted with 50 ml of equilibrating buffer containing 500 nM spiroperidol and 0.1 mg/ml bovine brain total lipid extract (Sigma) at a flow rate of 5 ml/hr. The lipids were found to stabilize the receptor activity. The eluting ligand was removed either by extensive dialysis or by concentrating the eluate with Centriflo CF25 cones and then desalting it on a Sephadex G-50 column. The receptor activity of the desalted fraction was assayed by the [^{3}H]spiroperidol binding. The affinity chromatography steps are summarized in the flow diagram (Figure 4).

2.2.5 Protein Determination: In the membrane preparation, protein was determined by the method of Lowry et al. (1951). In the solubilized preparation, protein was determined by the method of Bradford (1976). During affinity chromatography protein absorbance of the flow-through fraction was monitored continuously at 280 nm with LKB-Uvicord SII

monitor system. In addition, protein concentration was determined by the micro assay based on the Bradford method. The sensitivity of this method was found to be at $\leq$ 1.0 µg of bovine serum albumin. In some affinity purified fractions, protein concentration was estimated either by the amido-black staining procedure of Schaffner and Weissman (1973) or by amino acid analysis. In amino acid analysis procedure the affinity purified fraction was hydrolyzed in 6N HCl for 20 hrs. at 110°C in vacuo and amino acid analysis was performed with a Beckman 6300 amino acid analyzer. S-2 amino ethyl-L-cysteine was used an an internal standard. The recovery of the amino acid was determined by running in parallel known concentration of albumin.

2.2.6 Preparation of Phospholipid Suspension: Bovine brain total lipid extract or soyabean crude phosphatidyl choline was used in the present study. Routinely, 100 mg of the lipids were suspended in 10 ml of the equilibrating buffer of the affinity column and then vortexed until large particles disappeared. The tubes were flushed with nitrogen for 30 seconds and sonicated to clarity (approximately 30 min.) in a bath type sonicator. The sonicated lipids (10 mg/ml) were then suspended in equilibrating buffer to adjust the final lipid concentration to 0.1 mg/ml.

2.2.7 Target Size Determination by Radiation Inactivation: The molecular size of dopamine D-2 receptor was estimated by target size analysis using radiation inactivation with some modification to the procedure by Lo et al. (1982). Under appropriate conditions, the molecular size determined by this method is the size required for the functional activity of a protein. It could represent the oligomeric size

of the multi-unit substructure (Jung, 1984). With this method, functional size of a protein can be determined in a membrane bound form and can also be compared with the solubilized protein. The principles of this method are described elsewhere (Pollard et al., 1951; Lo et al., 1982; Jung, 1984) and are based upon the observation that a relationship exists between a dose dependent inactivation and a functional size of macromolecule or protein. The biological activity of a protein can be destroyed by a single "hit" of high energy radiation occurring at its molecular size, and this concept referred to as the "one target - one hit theory of radiation inactivation" (Pollard et al., 1951). In the present studies, the freshly prepared (mitochondrial-microsomal) membrane suspension or the cholic acid: NaCl solubilized preparation were frozen in an aluminum tray under liquid nitrogen. The depth of the sample was 1 to 2 mm. The frozen samples were stored at -70°C, transported to Buffalo N.Y. in closed, plastic bags on dry ice and then irradiated at -45 to -65°C with a 0.5 mA beam of 1.5 MeV electrons produced by a Van de Graff generator (Jung, 1984). The control samples were not irradiated, but otherwise treated identically. The radiation dose was measured at the radiation temperature with the bleaching of blue cellophane. The following enzyme molecular weight standards were also irradiated: pyruvate kinase, molecular weight, 224,000 daltons (Lo et al., 1982), yeast alcohol dehydrogenase, 160,000 daltons (Lo et al., 1982), and galactose oxidase, 68,000 daltons (Kosman et al., 1974). The assays for standard enzymes were performed according to the established procedures (Lo et al., 1982; Kosman et al., 1974). The samples after irradiation were stored at -70°C until assayed.

[^{3}H]Spiperone binding assay was performed as described earlier in this section. For determination of [^{3}H]spiperone binding, the trays were first warmed to room temperature and then diluted to a protein concentration of 0.8 mg/ml. The inactivation data of the dopamine D-2 receptor as well as the standard enzymes in each experiment were computed as percent control (no radiation) and then the data were pooled from 4-5 such experiments and plotted as percent residual activity vs. radiation dose in Mrad. Linear least square regression analysis was used in all the cases. The values of D37 (dose in Mrad required for 37% inactivation) were computed from the slopes of such plots.

3. RESULTS

3.1 Comparative Solubilization of Dopamine D-2 Receptor from Different Species Using Various Detergents

In order to obtain an effective solubilization procedure, various detergents at different concentrations and combinations were examined to solubilize the striatal dopamine D-2 receptor from four different species (Table 4). Among all the detergents tested, cholic acid: NaCl combination yielded maximum solubilization in all the different species examined. The recovery of the binding sites were 45% in bovine, 25% in canine, 26% in human and 30% in rat striatal membrane preparations. This recovery was significantly higher than any other detergent or their combinations. One percent digitonin yielded less than 20% recovery of binding sites among all the species. Cholic acid alone yielded the recovery of only 8-10% of the [^{3}H]spiroperidol binding when compared with the membrane. Also, the percentage specific binding of [^{3}H]spiroperidol with cholic acid: NaCl procedure was much higher than other solubilization methods. It was 85% in bovine, 62% in canine, 65% in human, and 62% in rat striata.

3.2 The Solubilization Criteria of Dopamine Receptors

Striatal dopamine D-2 receptor solubilized with cholic acid: NaCl satisfied the criteria of solubility as described by Ilien and Laduron (1982). There was no sedimentation on centrifugation of the solubilized preparation at 100,000 g for 4 hrs, and filtration through GF/C filters demonstrated no loss in the binding activity. Sucrose density gradient (15-30%) produced one major peak with maximum [^{3}H]spiroperidol binding (Figure 5). There was no sedimentation at the bottom of the tube on

TABLE 4

Comparative Solubility of Dopamine D-2 Receptors of Striata from Different Species by Various Detergents

Species	Detergent	% of Specific Binding	% of Binding Sites Recovered
Bovine	0.25% Cholic Acid + 1.0M NaCl	85 ± 6.5	45 ± 4.0
	0.5% Cholic Acid + 49% Ammonium Sulphate	65 ± 5.0	15 ± 2.5
	1% Digitonin	30 ± 2.8	8 ± 12.0
	1:5 Digitonin: Cholic Acid	29 ± 2.1	12 ± 1.8
	0.25% Cholic Acid	25 ± 2.0	8 ± 0.7
	0.25% Lysolecithin	30 ± 2.0	15 ± 1.0
Canine	0.25% Cholic Acid + 1.0 M NaCl	62 ± 4.5	25 ± 2.0
	0.5% Cholic Acid + 49% Ammonium Sulphate	45 ± 3.5	15 ± 1.8
	1% Digitonin	35 ± 3.0	11 ± 1.0
Human	0.25% Cholic Acid + 1.0 m NaCl	65 ± 4.2	26 ± 2.2
	0.5% Cholic Acid + 49% Ammonium	57 ± 3.8	--
Rat	0.25% Cholic Acid + 1.0 M NaCl Sulphate	62 ± 5.5	30 ± 4.0

Values shown are the mean of triplicate determinations for 3-5 separate experiments.

Figure 5: Sucrose density gradient profile of bovine striatal cholic acid-NaCl [^{3}H]spiroperidol binding sites. The soluble extract was layered on 13 ml of sucrose gradient (15-30%) buffered with 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 100 mM NaCl, 0.05% sodium cholate. Centrifugation was carried out at 4°C in a Beckman L50 centrifuge using SW 40 rotor at 40,000 rpm (201,800 g) for 16 hrs. A 250 ul aliquot of each 1.0 ml fraction was assayed for [^{3}H]sprioperidol binding as described in methods. The specifically bound DPM are plotted on the graph.

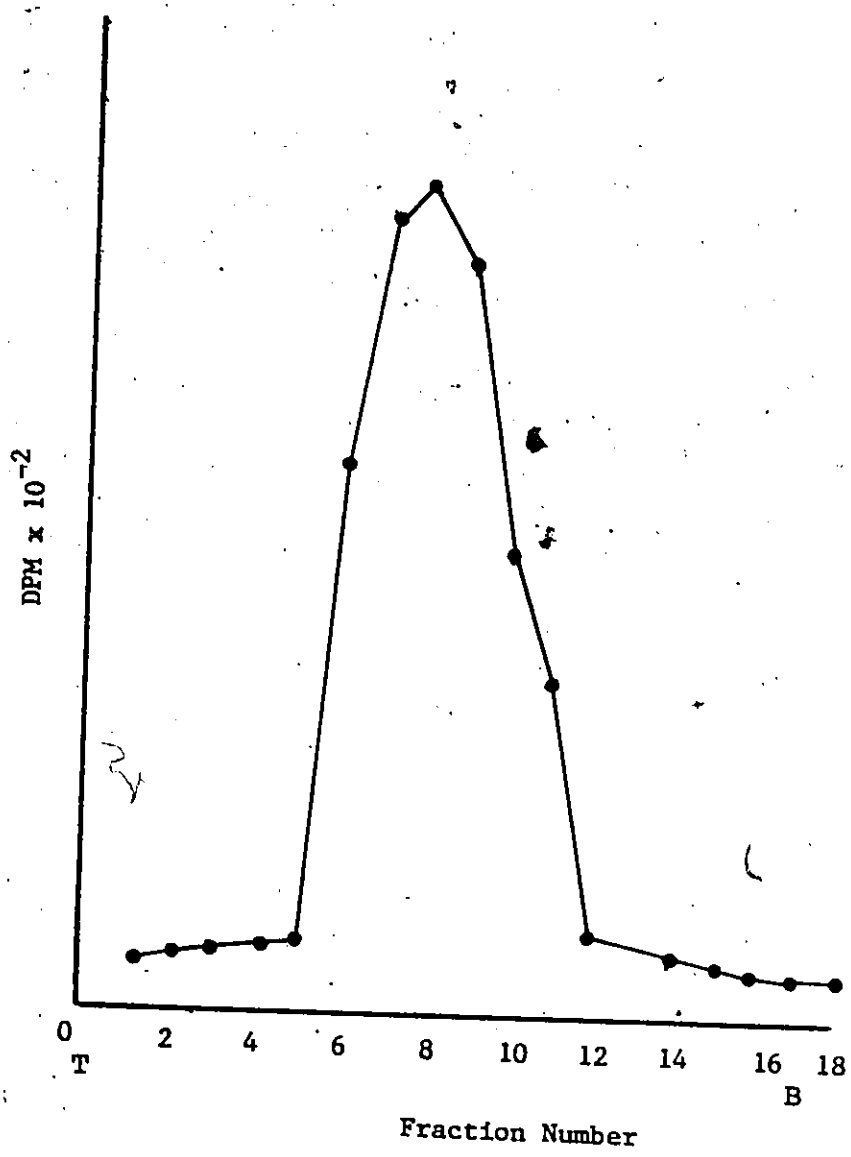


FIGURE 3

density centrifugation.

[^{3}H]Spiroperidol has been suggested by investigators (List and Seeman, 1981) to label a small proportion of serotonin binding sites in the striatum. To rule out the possibility of serotonin binding in the solubilized preparation 100 nM of ketanserin or mianserin (potent serotonergic antagonists) were included in the binding assay in the first few experiments and there was no significant effect on the [^{3}H]spiroperidol binding. Subsequent experiments were carried out in the absence of any serotonin antagonist.

3.3 [3H] Spiroperidol Binding Characteristics of Solubilized D-2 Receptor

The binding of [^{3}H]spiroperidol to the cholic acid: NaCl solubilized dopamine D-2 receptor was saturable and was of high affinity. The representative results from the bovine striata are illustrated in Figure 6. The saturation curve was obtained by incubating solubilized material with increasing concentrations (.05 nM - 1 nM) of [^{3}H]spiroperidol. Saturation occurred at approximately 0.6 nM. The Scatchard analysis of the data indicated a straight line with a K_D of 0.30 nM and a B_{max} of 300 f mol/mg protein. The values are in close agreement with the membrane preparation of bovine striata (K_D = 0.25 nM). The Hill coefficient of these data indicated an apparent absence of cooperative interaction or of binding to other receptors (Figure 7).

Table 5 compares the affinities (K_D values) and maximum binding sites (B_{max}) of the D-2 receptor in both the membrane and the solubilized preparations from different species. In all these species, the cholic acid: NaCl solubilized receptor exhibited good correlation in their

Figure 6: Scatchard analysis and saturation isotherm (insset) of [^{3}H]spiroperidol binding in bovine solubilized preparation. The [^{3}H]spiroperidol binding was saturable at 0.8 nM, showing a single class of binding site with a K_D of 0.30 nM and a receptor density of 300 fmol/mg protein. Values represent the mean of triplicate determination of an experiment for three such experiments.

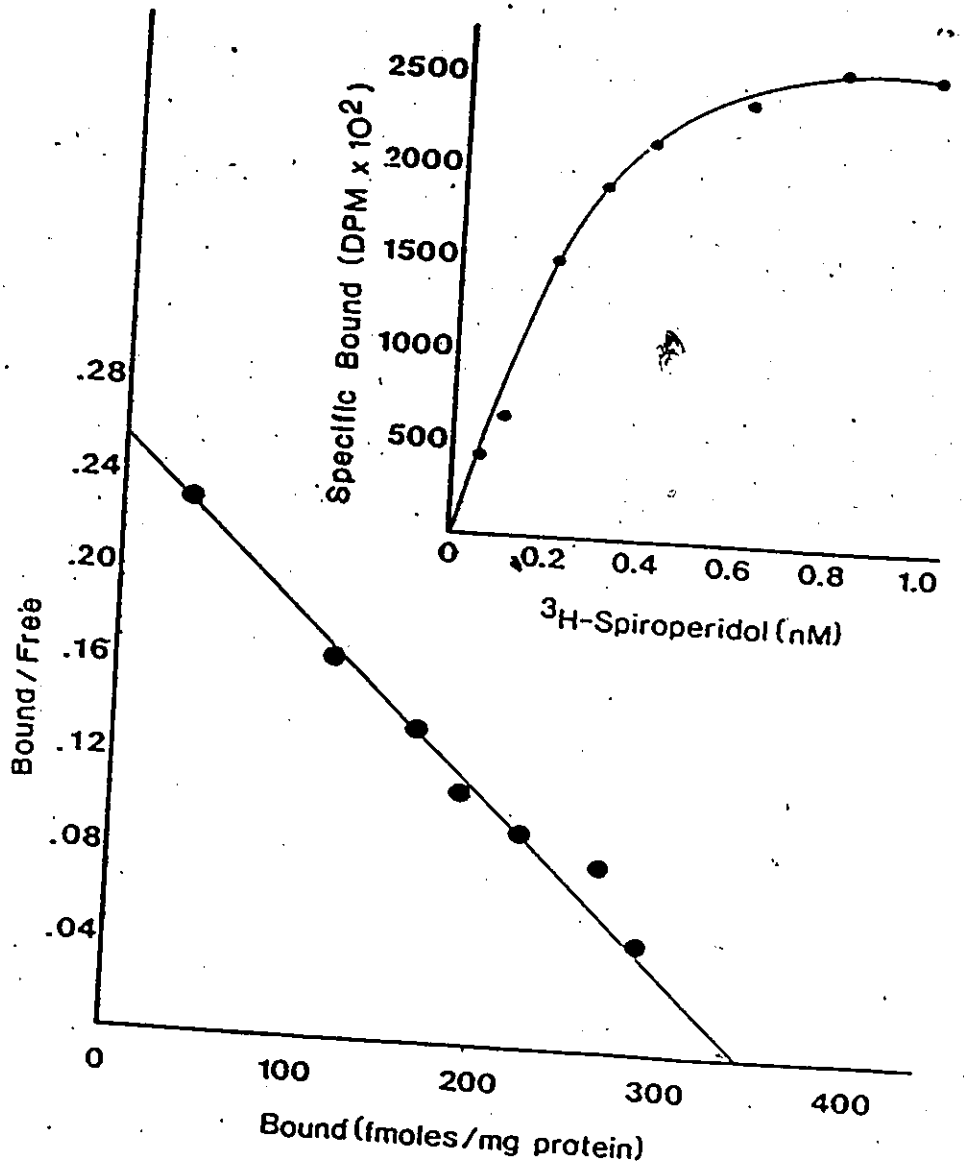

Specific Bound (DPM x 10²)
2500
2000
1500
1000
500
0
0.2
0.4
0.6
0.8
1.0
³H-Spiroperidol (nM)
Bound / Free
.28
.24
.20
.16
.12
.08
.04
0
100
200
300
400
Bound (fmoles/mg protein)

FIGURE 6

Figure 7: The Hill plot of [^{3}H]spiroperidol binding to the solubilized bovine striatal dopamine D-2 receptor. The plot represents the data from the saturation binding experiment shown in Figure 6. The value of Hill coefficient is 0.976 indicating independent binding of the drug to the site with single affinity.

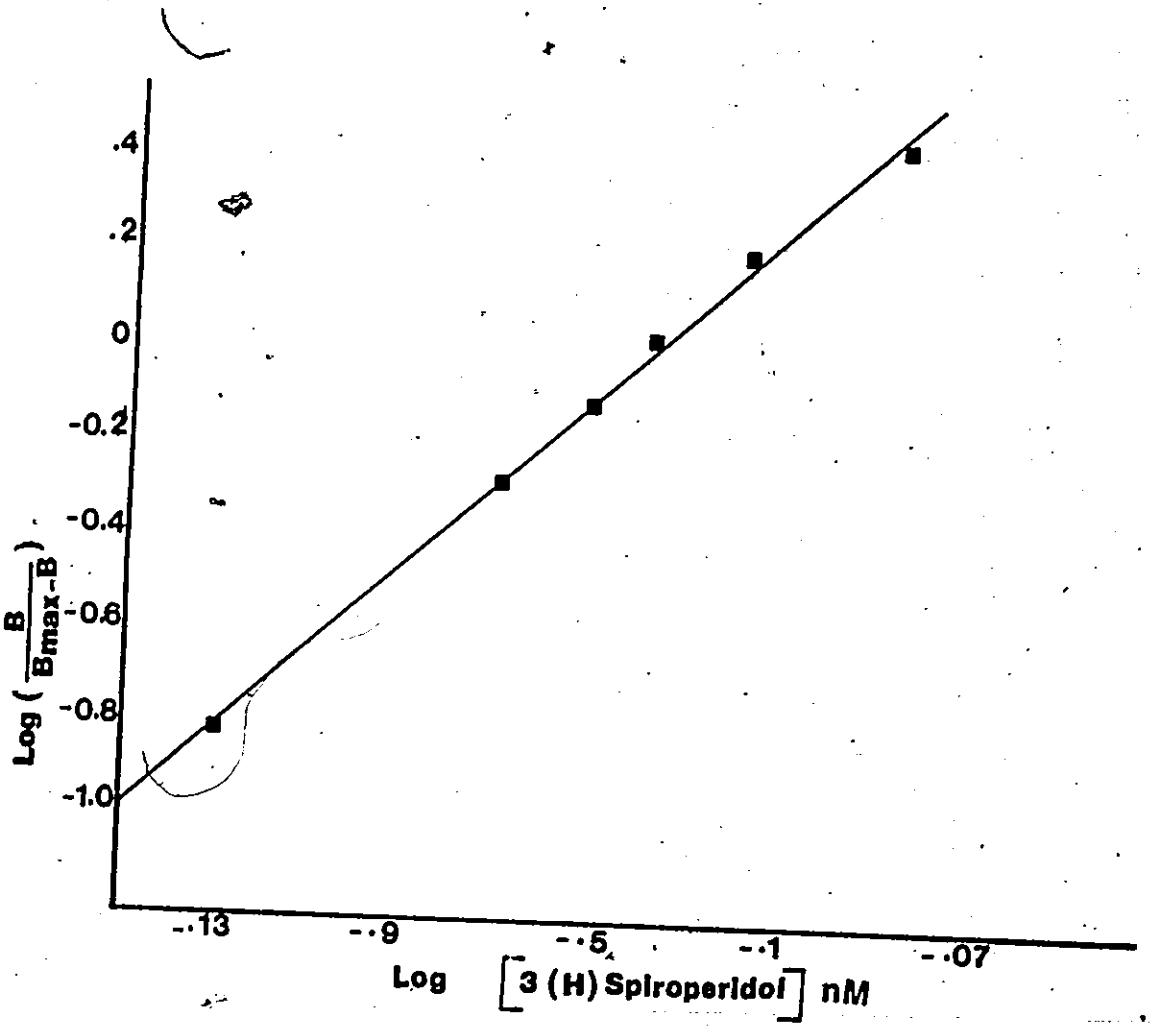


FIGURE 7

TABLE 5

Comparison of Binding Characteristics of ^{3}[H]Spiroperidol to Membrane Bound and Solubilized Receptor Preparation Among Different Species

	Bovine		Canine		Rat		Human	
	Membrane	Solubilized	Membrane	Solubilized	Membrane	Solubilized	Membrane	Solubilized
KD nM	.25 ±.05	0.30 ±.18	.30 ±.035	.28 ±.028	0.4 ±.099	.37 ±.17	.51 ±.1	.54 ±.04
B_{max} fmol/mg protein	279 ± 80	300 ± 80	209 ± 40	158 ± 40	200 ± 75	94 ± 15	112 ± 33	96 ± 19

Values shown are mean of triplicate determinations for 2-4 separate experiments.

TABLE 6

Dopaminergic Radioligand Binding in the Bovine Striatal Cholic Acid: NaCl Solubilized Preparation

Ligand	Total Binding DPM	Specific Binding DPM	Percent Binding
[^{3}H]spiroperidol (1.0 nM)	3500 ± 210	3000 ± 160	85 ± 3.0
[^{3}H]NPA binding (0.25 nM)	2965 ± 180	2275 ± 200	77 ± 5.0
D_1 specific [^{3}H] Flupenthixol binding (1 nM)	455 ± 50	95 ± 10	20 ± 2

Mean values of 2-5 experiments.

affinities with the membrane-bound striatal dopamine D-2 receptor. The specific binding in these experiments was defined as a binding remaining in the presence of 1 μM (+)-butaclamol.

Table 6 demonstrates the different dopaminergic radioligand binding in the bovine striatal cholic acid: NaCl solubilized preparation. Only dopamine D-2 specific radioligands, spiroperidol and NPA, exhibited maximum percentage specific binding (>75%). The dopamine D-1 specific ligands showed only 20% specific binding.

3.4 Specificity of Dopamine D-2 Receptor

The displacing potencies of various dopaminergic antagonists and agonist drugs against [^{3}H]spiroperidol (1.0 nM) binding in the solubilized and the membrane bound receptor among different species are given in Table 7. The IC_{50} values calculated by log probit analysis for antagonists, spiroperidol, haloperidol and (+)-butaclamol, and agonists dopamine and apomorphine in the solubilized preparation are in close agreement with the membrane bound D-2 receptor among all species.

The detailed binding specificity of the bovine striatal cholic acid: NaCl solubilized preparation is provided in Table 15. The order of potency for the dopamine antagonists (spiroperidol > (+)-butaclamol > domperidone > haloperidol) and for the agonists (NPA>NO434>apomorphine>dopamine) in the solubilized preparation was similar to that of the membrane bound receptor (Seeman, 1980). The solubilized receptor retained its stereoselectivity for (+)- and (-)-butaclamol in that IC_{50} value for the former is 1000 fold less than the latter. The dopamine D-1 agonist SKF 38393 and antagonist SCH 23390

TABLE 7

Comparative Inhibition (IC_{50} in nM) of $^{3}[H]$ Spiroperidol Binding By Dopamine Agonists and Antagonists in Membrane and Soluble Preparations from Different Species

Displacing Agent	Bovine		Canine		Rat		Human	
	Membrane	Solubilized	Membrane	Solubilized	Membrane	Solubilized	Membrane	Solubilized
Spiroperidol	0.93 ±.2	1.11 ±.29	0.94 ±.15	.79 ±.08	0.73 ±.06	1.49 ±.15	0.73±.069	1.25 ±0.44
Haloperidol	4.6 ±.62	7.0 ±1.1	19.0 ±2.2	33.0 ±3.5	4.3 ±.42	5.0 ±.52	-	12.0 ±1.3
(+)-butaclamol	4.0 ±.51	3.96 ±1.15	6.33 ±.56	4.84 ±.08	5.0 ±.45	4.3 ±.38	6.6 ±.66	10.0 ±2.9
Sulpiride	3000 ± 30	2426 ±15.8	105.3±10.51	288 ±12.8	87.0 ±.78	79.3 ±8.0	500 ± 45	445 ±165
Fluphenazine	5.0 ±.50	7.0 ± 1.5	-	4.47 ±.44	4.6 ±.41	25.0 ±2.5	3.5 ±.35	2.5 ±.18
Dopamine	7000 ±710	5300 ±619	16887	8300 ±800			9485	38144
Apomorphine	86.0 ± 15	147 ±11.6						

Values are mean of triplicate determinations of 1-4 experiments.
(-) represents values not determined

exhibited potencies in micromolar and high nanomolar concentrations respectively (IC_{50} value of SCH 23780 for D-1 receptor is <1.0 nM). Non-dopaminergic agents, ketanserin (serotonin antagonist) phentolamine (α antagonist), and propranolol (β antagonist), showed IC_{50} value greater than 1 μM.

3.5 Characterisation of D-2 High Affinity Binding Sites in the Bovine Striatal Solubilized Preparation

Bovine striatal dopamine D-2 receptor solubilized with cholic acid: NaCl exhibited the retention of high affinity binding sites as previously reported in the membrane bound dopamine D-2 receptor from the bovine anterior pituitary and striatum (Sibley et al., 1982; Kilpatrick and Caron, 1983; Grigoriadis and Seeman, 1985). These solubilized high affinity binding sites revealed similar characteristics as membrane bound receptors. The high affinity receptor sites are shown to be coupled to the guanine nucleotide regulatory protein.

To ascertain that the sites in the solubilized preparation represent the high affinity binding sites, the following experiments were carried out.

3.5.1 [^{3}H]NPA Binding

Figure 8 represents the saturation isotherm of [^{3}H]NPA binding which is a highly specific ligand for the dopamine D-2 high affinity binding sites (Titler and Seeman, 1979). The solubilized preparation displayed very high [^{3}H]NPA binding when (+)-butaclamol (1 μM) was used as the displacing agent. The saturation isotherm with different concentrations of the radioligand (25 pM - 1000 pM) demonstrated a saturation at 0.7 nM. The Scatchard plot of the binding data gave a

Figure 8: Scatchard analysis of [^{3}H]NPA binding in the solubilized preparations. The specific binding of [^{3}H]NPA was saturable (inset) and represented a single homogenous population of binding sites with a K_D of 0.28 ± 0.06 nM and receptor density (B_{max}) of 195 ± 25 fmol/mg protein. Gpp(NH)p at 100 µM concentration almost completely abolished the specific binding of [^{3}H]NPA as shown in saturation curve (▲——▲). The points are a Mean ± S.E.M. of triplicate determinations of a typical experiment, representing three similar experiments.

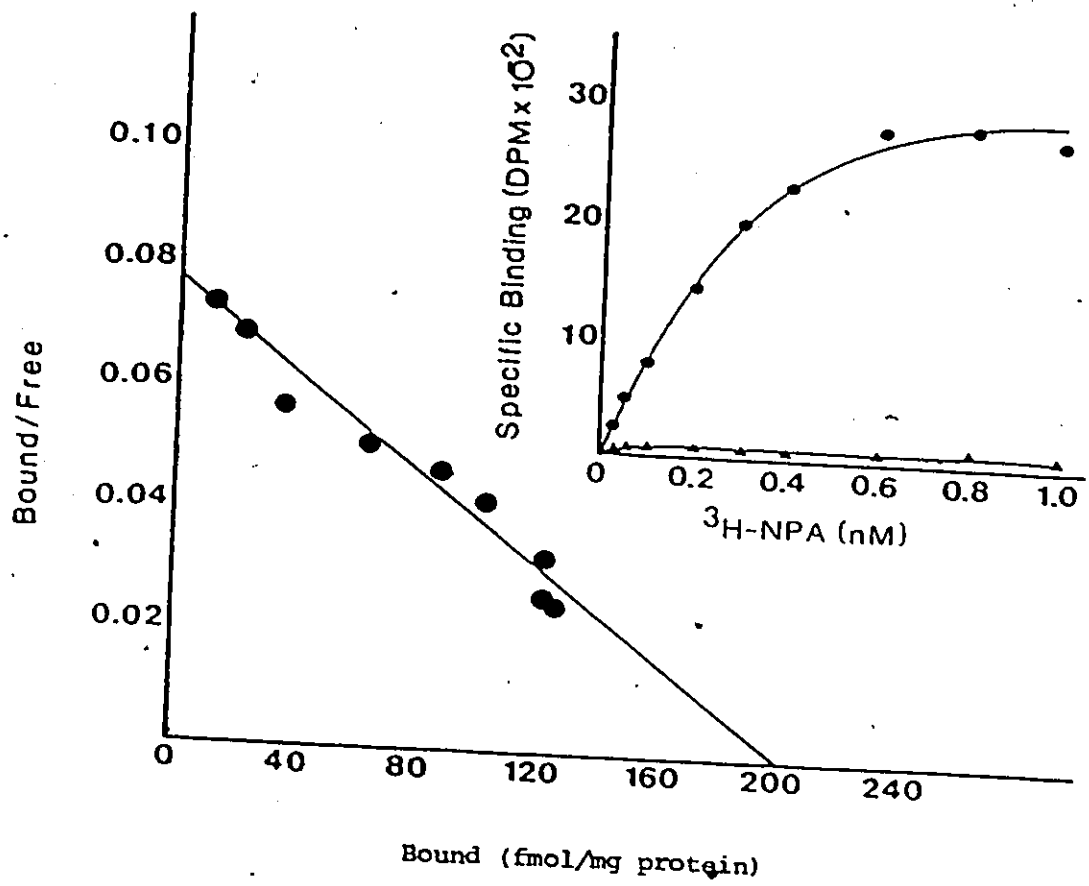

Bound/Free
0.10
0.08
0.06
0.04
0.02
0
40
80
120
160
200
240
Bound (fmol/mg protein)
Specific Binding (DPM x 10²)
30
20
10
0
0.2
0.4
0.6
0.8
1.0
³H-NPA (nM)

FIGURE 8

Figure 9: Effect of 100 μM Gpp(NH)p on Scatchard analysis of [^{3}H]spiroperidol binding in the cholic acid-NaCl solubilized preparation. The points are mean of triplicate determinations of a typical experiment, representing three similar experiments. (●——●) represents the control experiment without Gpp(NH)p and (●——▲) represents the binding data with Gpp(NH)p.

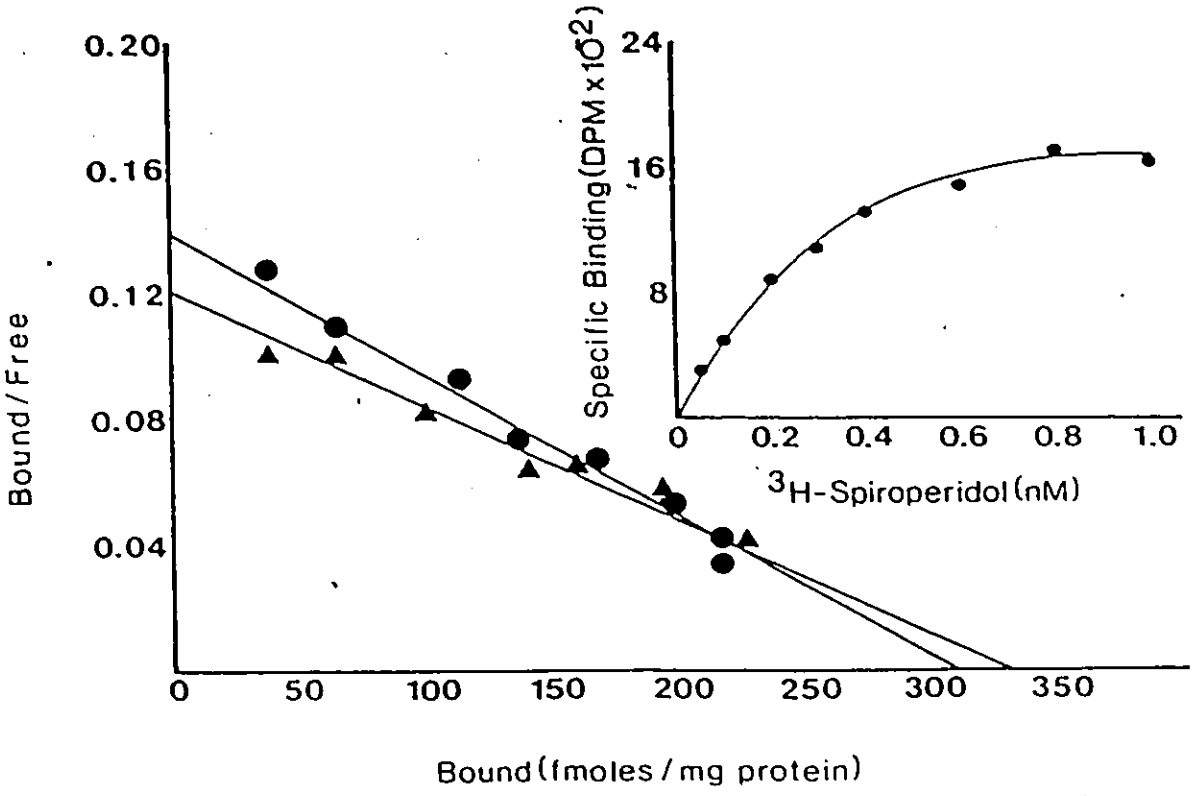

0.20
0.16
0.12
0.08
0.04
Bound / Free
0
50
100
150
200
250
300
350
Bound (fmoles / mg protein)
Specific Binding (DPM x 10^2)
24
16
8
0
0.2
0.4
0.6
0.8
1.0
3H-Spiroperidol (nM)

FIGURE 9

straight line with a K_D of 0.3 ± .02 nM and a B_{max} of 270 ± 90fmol/mg protein . Addition of 100 µM of the GTP analogue Gpp(NH)p almost completely abolished the specific [^{3}H]NPA binding at all the concentrations of the radioligand used (i.e., from .05 nM - 1nM, Figure 8).

Gpp(NH)p at 100 µM showed no effect on the [^{3}H]spiroperidol binding and the values of K_D (.35 ± .05 nM) and B_{max} remained unchanged with Gpp(NH)p (Figure 9).

3.5.2 [^{3}H] Spiroperidol/Agonist Competition and Effects of Guanine Nucleotide

The high affinity binding sites were also characterized with the agonists competition curves against [^{3}H]spiroperidol (0.5 nM) in the presence and absence of 100 µM Gpp(NH)p. The competition curves of [^{3}H]spiroperidol versus agonists in the bovine striatal solubilized preparation in presence and absence of guanine nucleotide Gpp(NH)p in an assay system containing 5 mM $MgCl_2$ are represented in Figures 10-12.

Figure 10 illustrates the displacement of [^{3}H]spiroperidol binding by dopamine. Gpp(NH)p caused a significant shift of competition curve to the right with greater than 10-fold increase in the IC_{50} value. This shift in the IC_{50} value with the GTP analogue may indicate a conversion of the high affinity state to the low affinity state as has been observed in the membrane preparations (Kilpartick and Caron, 1983; Grigoriadis and Seeman, 1985). The Hill coefficient has also been increased significantly in this case. Similarly, apomorphine displaced [^{3}H]spiroperidol binding in the nanomolar concentrations (IC_{50}, 78 nM) and the guanine nucleotides lowered the agonist affinity causing the IC_{50} values to rise to micromolar range (Figure 11). (-)NPA was the most

Figure 10: Competition curves of [^{3}H]spiroperidol/dopamine in the solubilized preparation in the absence (●——●) and presence (■——■) of 100 µM guanine nucleotide Gpp(NH)p. The values represent the mean of 3-9 determinations.

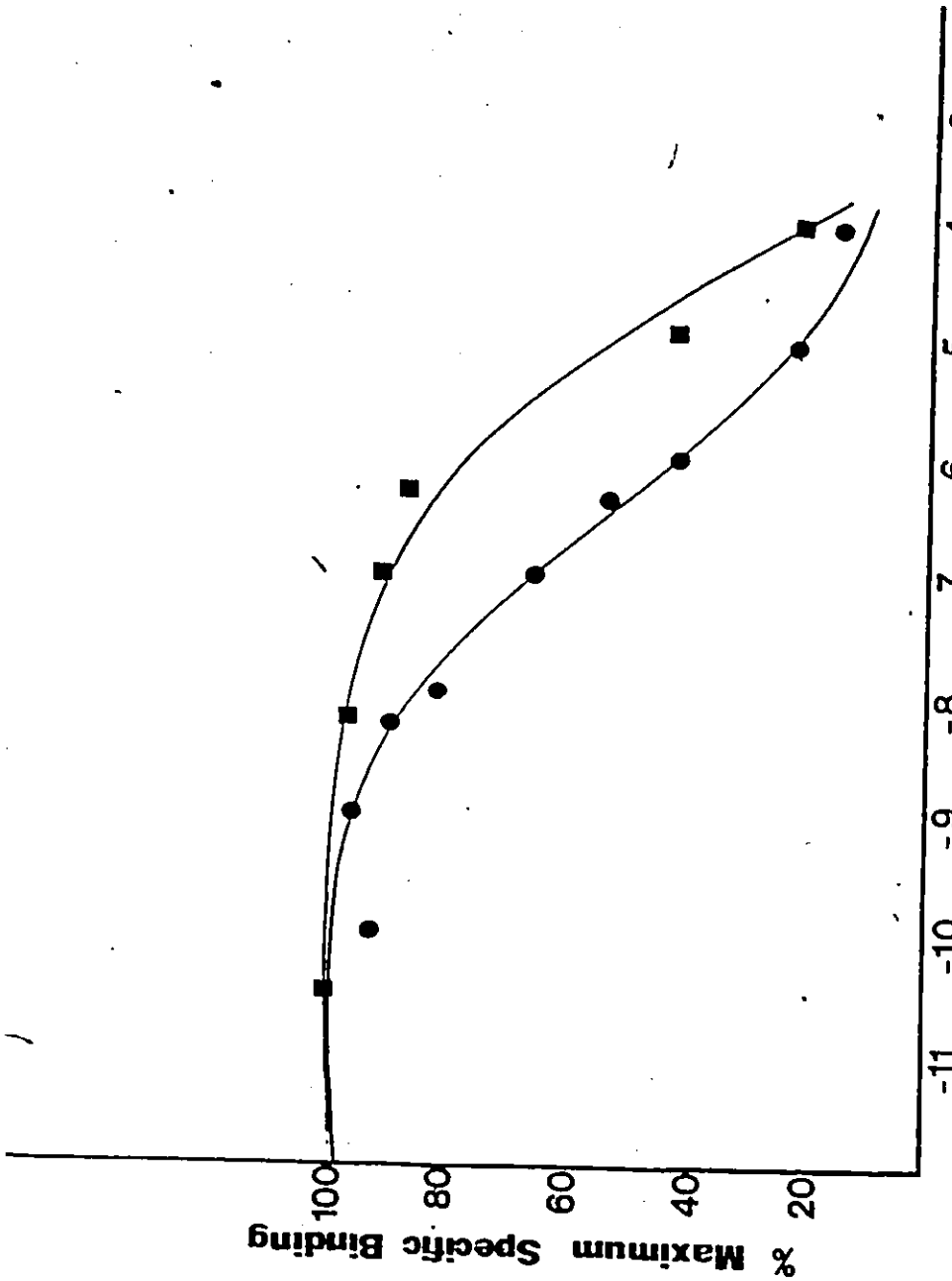

% Maximum Specific Binding
100
80
60
40
20
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Dopamine] M

FIGURE 10

Figure 11: Competition curves of [^{3}H]spiroperidol/apomorphine in the absence (●——●) and presence (■——■) of Gpp(NH)p. The IC_{50} values analyzed by log probit analysis showed a shift of greater than 10 fold from (78 nM to 1.1 μM) in the presence of Gpp(NH)p. The values are mean of triplicate with 2-4 such experiments.

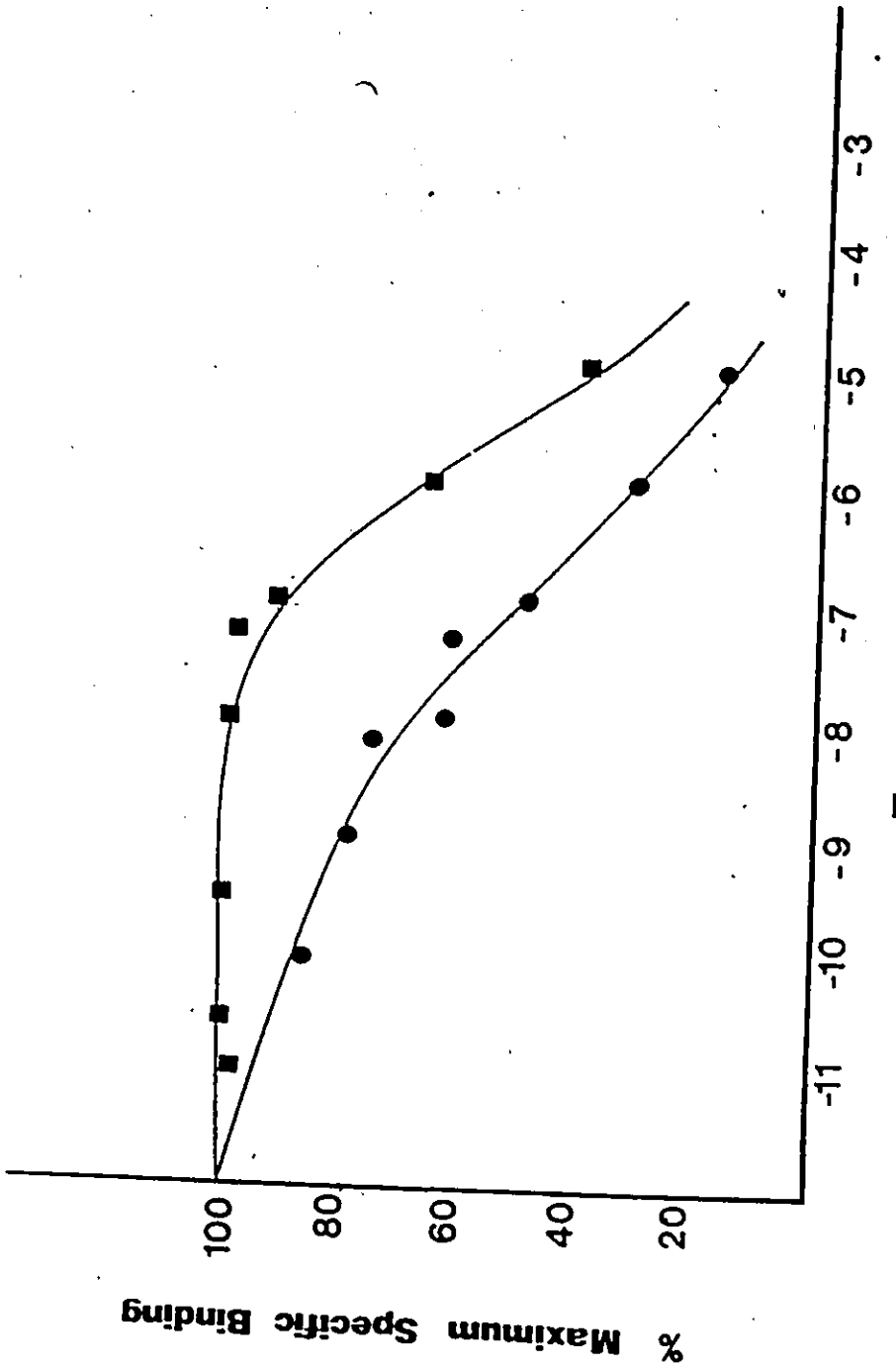

% Maximum Specific Binding
100
80
60
40
20
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Apomorphine] M

FIGURE 11

Figure 12: Competition of specific [^{3}H]spiroperidol binding by NPA in the absence (●——●) and presence (■——■) of 100 μM Gpp(NH)p. The IC_{50} value of NPA increased greater than 100-fold with Gpp(NH)p (from 1.2 nM to 121 nM). Values are mean of triplicate determination with 3-5 such experiments.

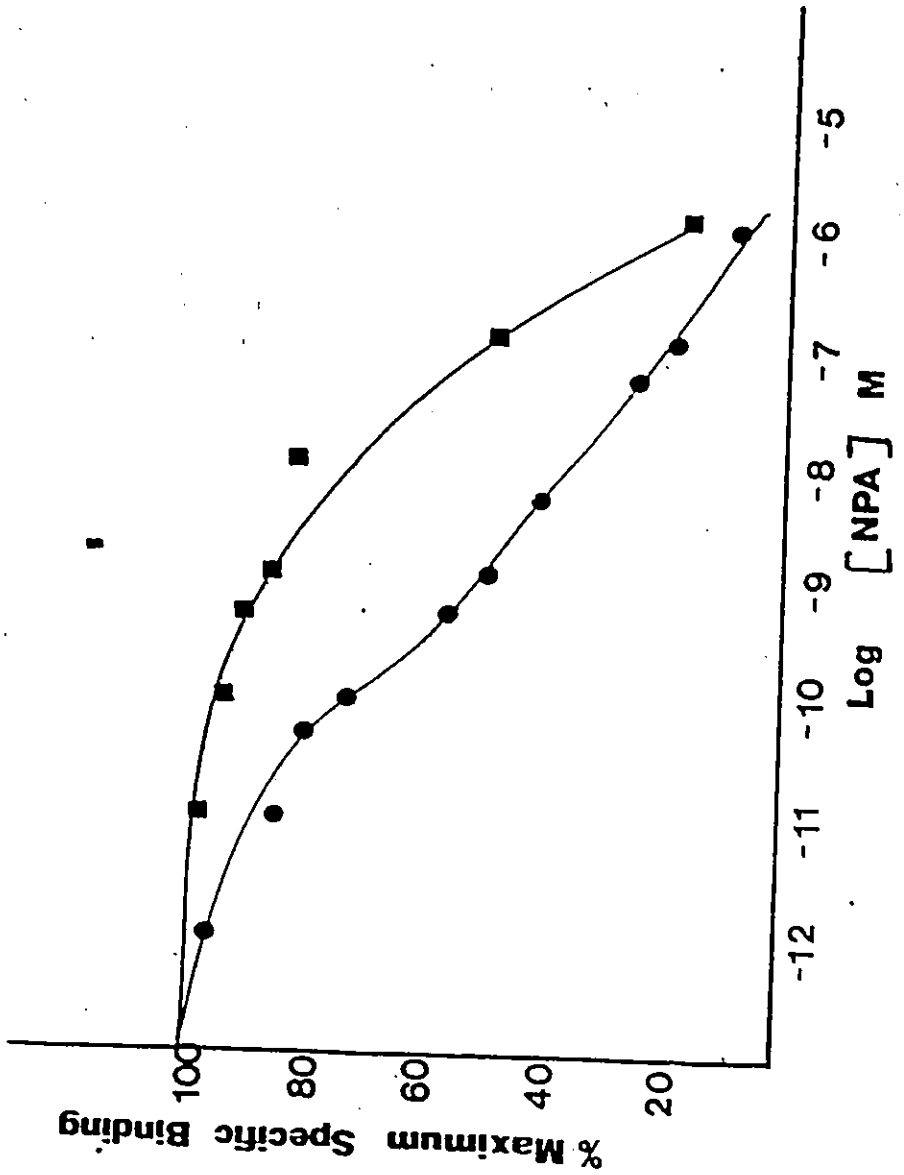

% Maximum Specific Binding
100
80
60
40
20
-12
-11
-10
-9
-8
-7
-6
-5
Log [NPA] M

FIGURE 12

TABLE 8

Inhibition of [^{3}H]spiroperidol Binding by Dopamine D-2 Receptor Antagonists in the Absence and Presence of Gpp(NH)p

DRUGS	K_D (nM) -Gpp(NH)p	K_D (nM) +Gpp(NH)p
Spiroperidol	0.45 ± 0.04	0.68 ± 0.08
Haloperidol	5.3 ± 0.6	6.4 ± 1.1
Domperidone	6.8 ± 0.8	8.3 ± 1.5

The displacement of [^{3}H]spiroperidol (0.5 nM) binding by different concentrations of antagonists (10^{-12}-10^{-6}M) was not affected significantly by the addition of 100 μM Gpp(NH)p. The IC_{50} values (converted to K_D, using the Cheng and Prusoff equation) are mean ± S.E.M. of four separate experiments, each performed in triplicate.

potent among the agonists tested (IC_{50}, 1.2 nM) and again, Gpp(NH)p caused a significant fall in the inhibitory potency (IC_{50}, 170 nM) of the agonist (Figure 12).

3.5.3 [^{3}H]Spiroperidol/Antagonist Competition and Effects of Guanine Nucleotide

Three antagonists, namely, spiroperidol, haloperidol and domperidone were tested for their ability to displace [^{3}H]spiroperidol binding from the solubilized dopamine D-2 receptor in the presence and absence of Gpp(NH)p (Table 8). Unlike agonist competition curves, the binding parameters of the antagonists displacement were best represented by a single affinity state of binding sites. Addition of the saturating concentration (100 µM) of Gpp(NH)p had little or no effect on the K_D values of the antagonists with respect to the [^{3}H]spiroperidol binding.

3.5.4 [^{35}S] - GTPγs Binding

To test the possibility of the coupling of receptor with G proteins at the high affinity state, specific radioligand binding studies with the GTP analogue [^{35}S]GTP γS were performed. The solubilized preparation exhibited high specific binding of [^{35}S]GTPγs in the presence of 100µM Gpp(NH)p used as the displacing agent. The Scatchard analysis of the binding data revealed a K_D value of 90 ± 12 nM and a B_{max} of 45 ± 8 pmol/mg protein (Figure 13).

3.6 Molecular Weight Determination by Target Size Analysis

The target size of the membrane bound and the solubilized bovine striatal dopamine D-2 receptor was determined by radiation inactivation and the results are shown in Figures 14-15. From the inactivation pattern of the receptor binding and the molecular weight standards, the

Figure 13: Scatchard analysis of [^{35}S]GTPγS binding in the solubilized preparation. Binding assyas were performed with [^{35}S]GTP S mixed with the corresponding unlabelled GTPγS (10 nM - 250 nM). The Scatchard plot represents a single class of binding sites where the K_D and B_{max} were 90 ± 12 nM and 45 ± 8 pmol/mg protein. The points are the mean ± S.E.M. of two experiments, each performed in triplicate.

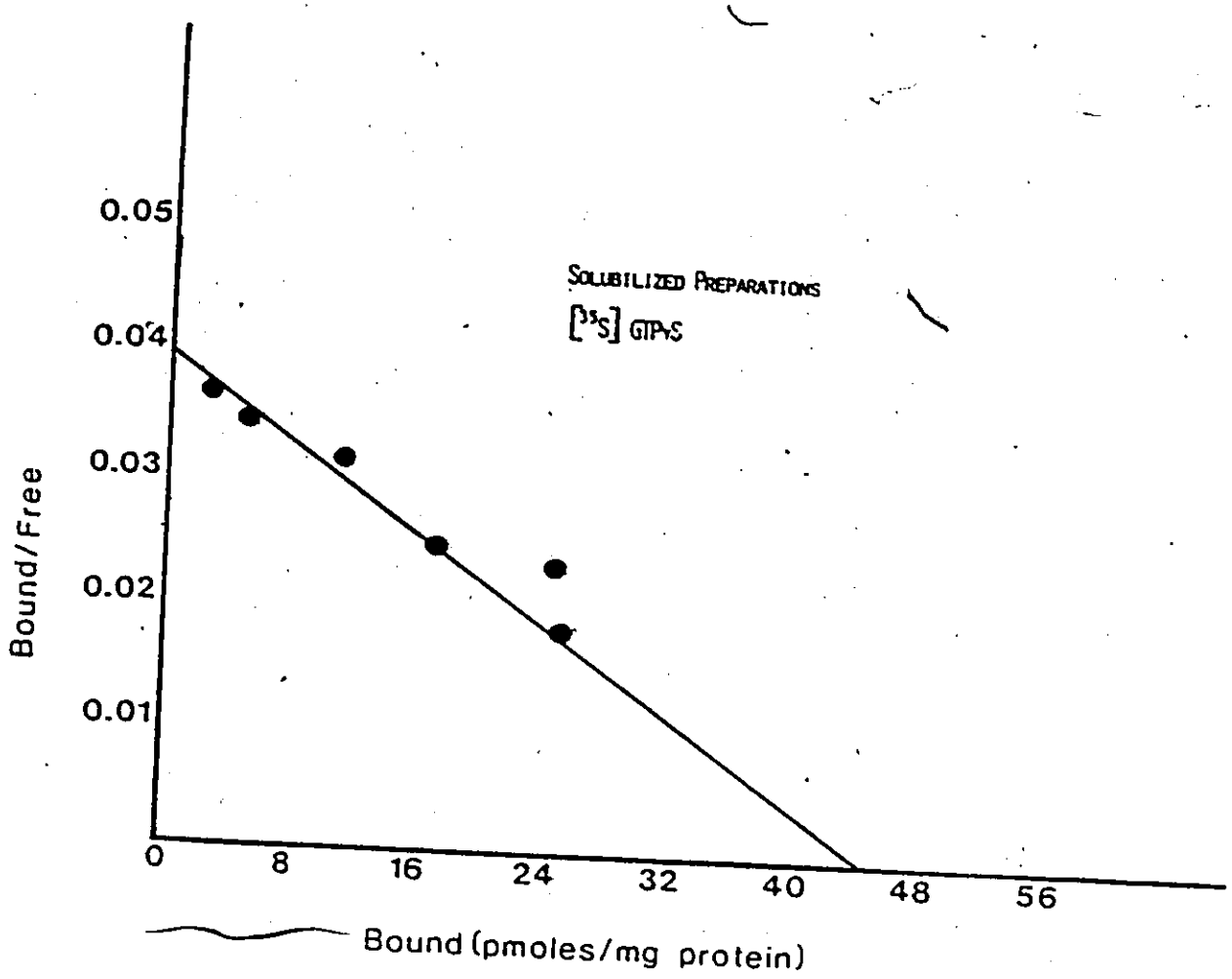

0.05
0.04
0.03
0.02
0.01
Bound/Free
SOLUBILIZED PREPARATIONS
[35S] GTPγS
0
8
16
24
32
40
48
56
Bound (pmoles/mg protein)

FIGURE 13

target size of the membrane bound dopamine receptor was determined to be 146 x 10_3 ± 17.1 x 10_3 daltons and for the solubilized receptor 139 x 10_3 ± 16.2 x 10_3 daltons (Table 9). The slopes for the inactivation of the standard enzymes were: pyruvate kinase, 0.116 ± .0066; yeast alcohol dehydrogenase, 0.0946 ± 0.0038; and galactose oxidase, 0.0357 ± 0.0014 (mean ± S.E.M. of 4 to 5 experiments). The slopes for the membrane bound and the solubilized receptor were .07304 and .0769 respectively. There was an excellent linear correlation (= 0.99) between the molecular weights of the standards and their slopes for the radiation inactivation. The slopes of these plots corresponded to the following D-37 (dose in Mrad required for 37% inactivation) values: pyruvate kinase, 3.78 ±. 22; yeast alcohol dehydrogenase, 4.62± 0.18; and galactose oxidase 12.2 ± 0.5 Mrad. The D-37 values for the membrane bound and solubilized receptor were 5.643 and 5.965 Mrad respectively.

[^{3}H]Spiroperidol binding in the membrane preparation decayed linearly with the dose of radiation (Figure 14) indicating a single target size. However, in the solubilized preparation the radiation data for [^{3}H]spiroperidol specific binding exhibited 25% decay at ≤ 1 Mrad and showed linear decay with further radiation dose indicating more than one target size. The loss of receptor activity at or below 1 Mrad may represent a major complex (a high-affinity complex) contributing approximately 20-30% to the total binding activity at zero dose. Because of the decay of high affinity binding sites, there was only a slightly detectable [^{3}H]NPA binding in the irradiated solubilized preparation even at a dose of <1 Mrad (Figure 15). The specific binding of [^{3}H]NPA at 0.25 Mrad was 20%. However, in the membrane preparation [^{3}H]NPA linearly

Figure 14: Radiation inactivation: target size analysis of bovine striatal membrane bound dopamine D-2 receptor. The activity is expressed as % of control activity present in samples not irradiated but otherwise treated exactly as the irradiated samples. The survival of specific [^{3}H]spiroperidol is represented by (⊟——⊟) and of [^{3}H]NPA by (✕——✕). Each point is a mean of triplicate determination. The data have been pooled from 3-5 preparations.

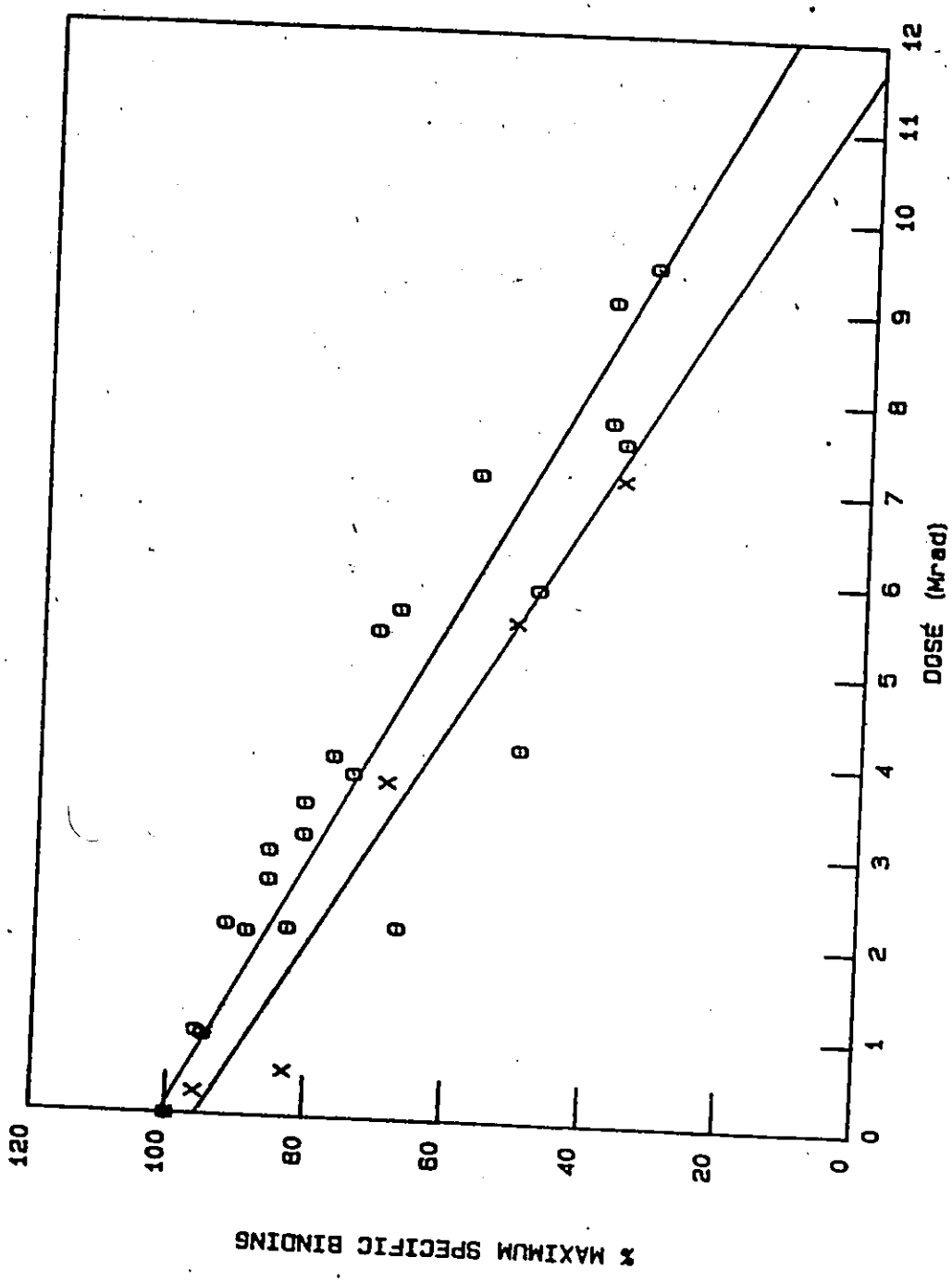

% MAXIMUM SPECIFIC BINDING
120
100
80
60
40
20
0
DOSE (Mrad)
0
1
2
3
4
5
6
7
8
9
10
11
12

FIGURE 14

Figure 15: Radiation inactivation data of bovine striatal cholic acid: NaCl solubilized dopamine D-2 receptor. (X——X) represents the [^{3}H]spiroperidol binding and (□——□) represents the [^{3}H]NPA binding. The activity is expressed as % of control activity present in samples not irradiated but otherwise treated exactly as the irradiated samples. Each point is a mean of triplicate determination. The data have been pooled from 2-4 experiments.

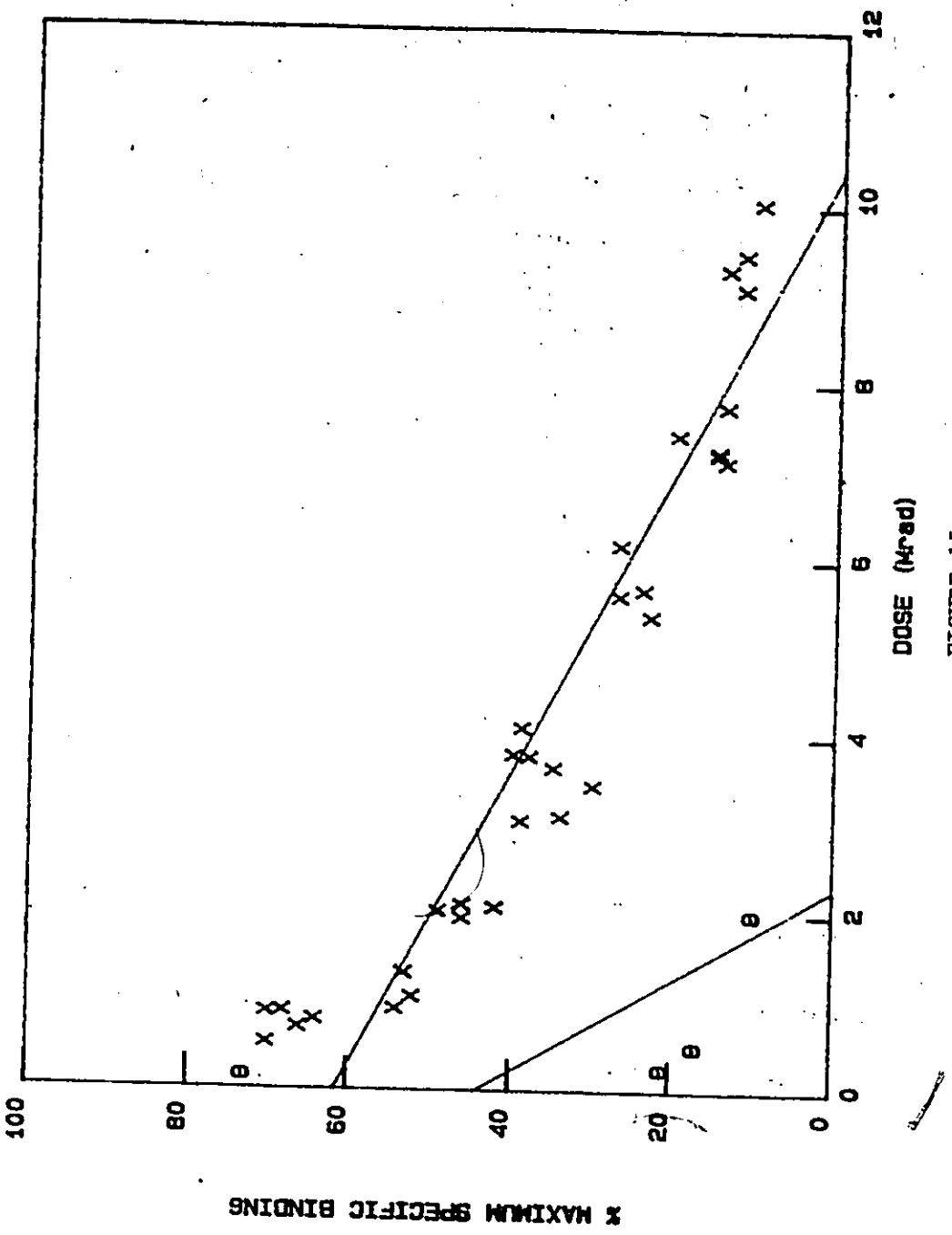
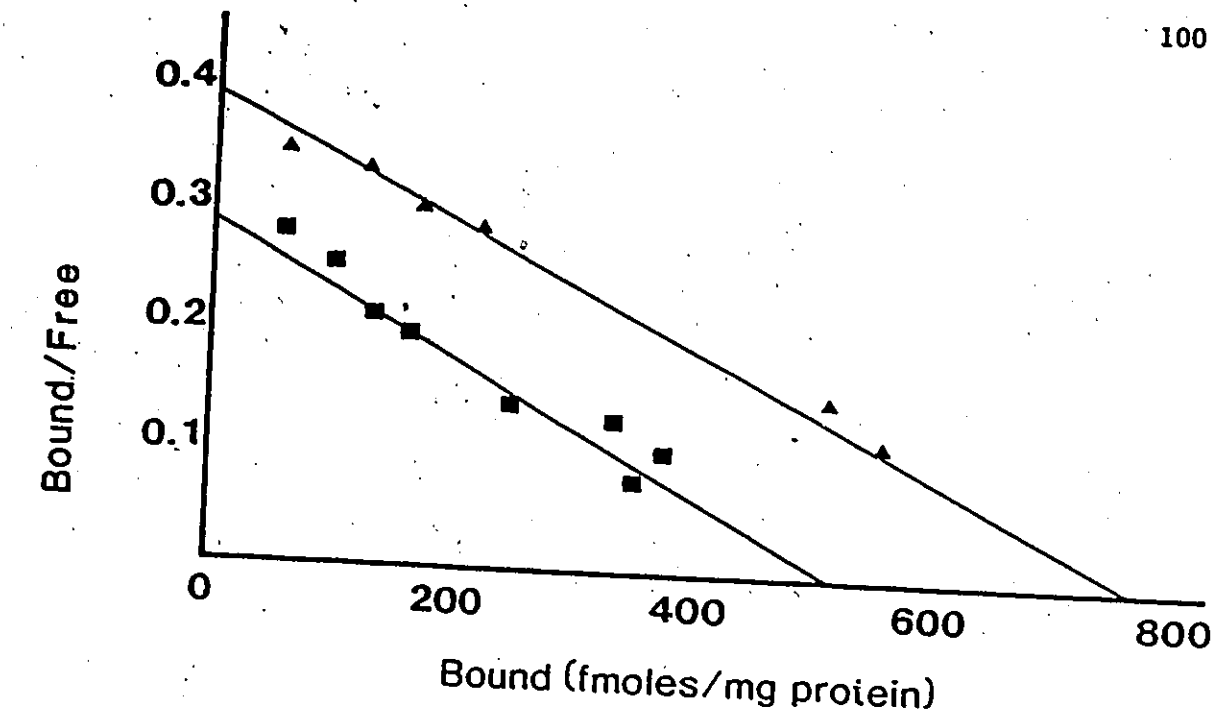

% MAXIMUM SPECIFIC BINDING
100
80
60
40
20
0
0
2
4
6
8
10
12
DOSE (Mrad)

FIGURE 15

Figure 16: Representative Scatchard analysis of [^{3}H]spiroperidol binding in non-irradiated (●——●) and irradiated (■——■) bovine striatal membrane (Figure 16a), and cholic acid-NaCl solubilized preparation (Figure 16b). Each point is a mean of triplicate determination. Two experiments from different preparations were carried out. The parallel shift in the curve represents the reduction in the bound value to about 60% of control.


0.4
0.3
0.2
0.1
Bound./Free
0
200
400
600
800
Bound (fmoles/mg protein)

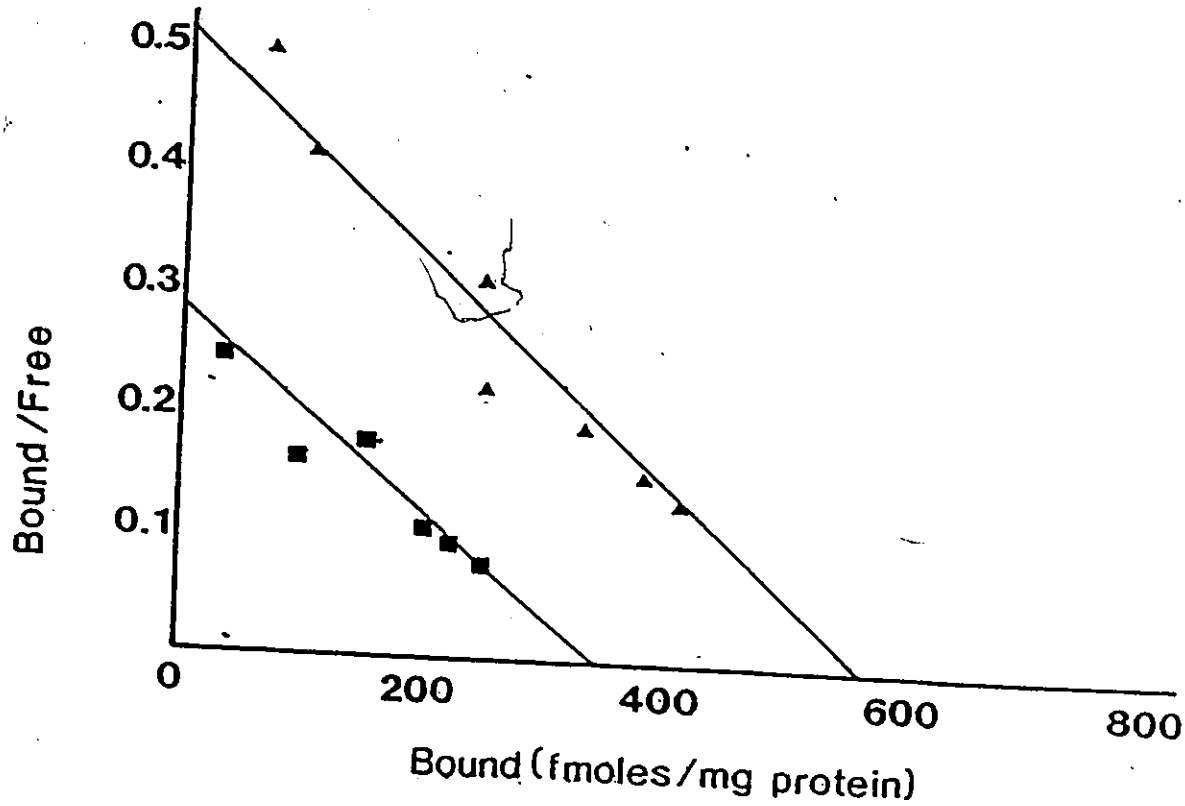

0.5
0.4
0.3
0.2
0.1
Bound /Free
0
200
400
600
800
Bound (fmoles/mg protein)

FIGURE 16

TABLE 9

Comparison of Target Size Analysis of Membrane Bound and Solubilized Dopamine (D-2) Receptor

Parameter	Membrane Bound D-2 Receptor	Solubilized (D-2) Receptor
Molecular Size in Daltons	$146 \times 10^3 \pm 17.1 \times 10^3$	$139 + 10^3 \pm 16.2 \times 10^3$
Slope	.07304 + 0.0089	.0769 + 0.0085
D_{37} Value in Mrad	5.643 + 0.657	5.965 + 0.694

Mean value of 3-4 experiments.

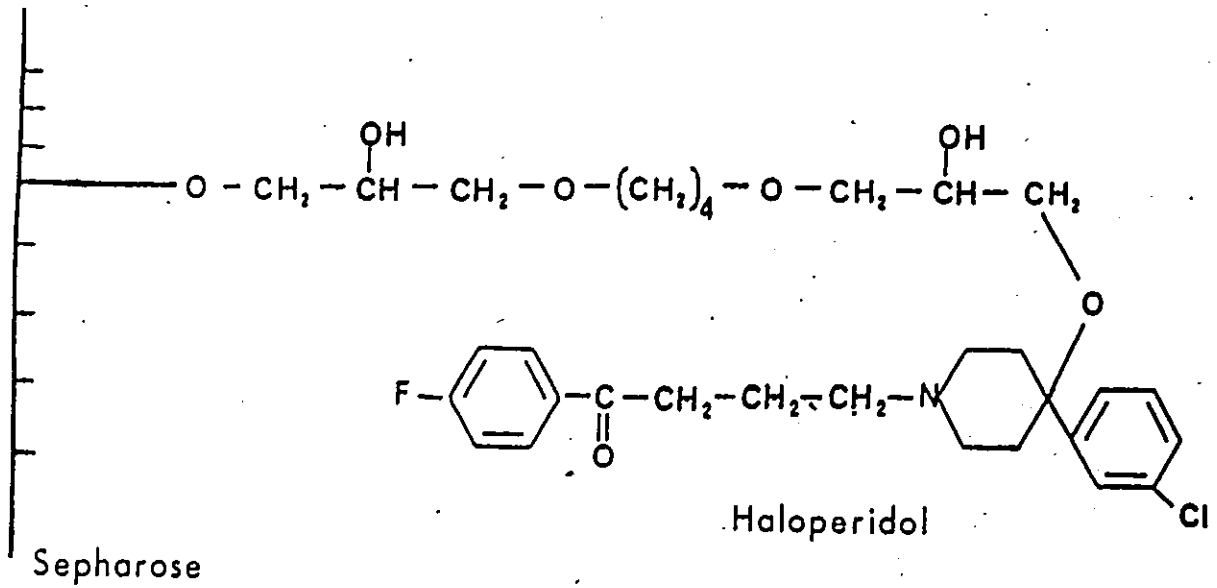


FIGURE 17: Presumed structure of the sepharose 6B-haloperidol gel. Specific conditions for the reaction are described under Methods.

decayed in parallel with the [^{3}H]spiroperidol binding indicating a lack of multiple high affinity complexes. Similar finding with the target size of 150 x 10_4 daltons has been reported in the bovine striatal lyophilized membrane preparation (Kuno and Tanaka, 1983).

The Scatchard analysis of the control (non-irradiated) and radiation inactivated preparations at 5 Mrad revealed that both membrane bound and solubilized dopamine D-2 receptor exhibited a parallel shift in the line with no change in the K_D value but a significant change in the B_{max} value with irradiation (Figure 16). The B_{max} value for the membrane bound irradiated receptor changed from 750 to 450 fmol/mg protein, and for the solubilized receptor the B_{max} changed from 580 to 360 fmol/mg protein.

3.7 Haloperidol Linkage to Epoxy Activated Sepharose CL-6B

The bovine striatal cholic acid: NaCl solubilized dopamine D-2 receptor has been purified by haloperidol-linked Sepharose CL-6B gel. The linkage of the tertiary alcoholic group of haloperidol with the epoxide arm of Sepharose was completed in 20 hrs. at 40°C. The linkage of haloperidol to Sepharose is shown in Figure 17. In a typical experiment approximately 1 µmol of haloperidol was linked to a gram of wet Sepharose. A possible covalent nature of the linkage was established by counting the [^{3}H]haloperidol remained bound to the gel after thorough washing with the reaction solvent dimethyl formamide, 1 M sodium chloride, acetete buffer (pH 4.5), Tris (pH 8.0) buffer and finally with excess water. The non-specific sites on the gel were blocked by treating the gel with 1 M ethanolamine (pH 8.0) for 8 hrs. The prepared gel was

stable over a period of 2 months without any significant loss of radioactivity.

The reaction time for maximum linkage was found to be 20 hrs. (Table 10). Prolonged incubation for 40 hr. did not show an increase in the linkage of haloperidol to the gel, whereas a 6 hr. reaction time exhibited 30% of the maximum linkage.

3.8 Affinity Chromatography of the Solubilized Receptor

The cholic acid: NaCl solubilized receptor preparation was purified on the haloperidol-linked Sepharose column. Figure 18 represents the affinity chromatography profile of the solubilized receptor through the haloperidol-linked column. Based on the difference between the [^{3}H]spiroperidol specific binding activity of the starting material and that of the flow through fraction, 60-70% of the dopamine D-2 receptor activity was found to be adsorbed on the affinity gel. In contrast, no significant adsorption of the [^{3}H]spiroperidol binding activity occurred when the solubilized receptor preparation was passed over epoxy-activated Sepharose CL-6B containing only spacer moiety (Table 12).

Washing of the column with 5 volumes of 50 mM Tris-1 mM EDTA 0.02% cholic acid, 150 mM NaCl pH 7.4 (equilibrating buffer) at 4°C eluted most of the applied protein as indicated by absorbance at 280 nm. [^{3}H]spiroperidol binding activity was present in the first few fractions after washing, which may be due to the elution of the unadsorbed receptor present in the void volume of the column rather than desorption of the bound receptor from the gel. The elution with 50 ml of the same

TABLE 10

Time Requirement for the Coupling of Haloperidol to the Epoxy Activated Sepharose CL-6B

Reaction Time in Hours	Haloperidol Linkage (DPM/g gel)	(μM haloperidol/g gel)
6	1960	0.26
20	3000	0.8
40	5600	0.75

One gm of epoxya-ctivated Sehparose CL-6B was mixed with 300 mg of haloperidol and 100 mg of $ZnCl_2$ in DMF and the reaction was stopped at varying times by washing with DMF.

Figure 18: Affinity chromatography of the cholic acid: NaCl solubilized preparation of bovine striatum on haloperidol-linked Sepharose CL6B affinity gel. Soluble preparation (40 ml) was passed for 16 hours at 4°C with a flow rate of 1.5-2 ml/hr. on a 0.5 x 30 cms column containing 10.0 ml of affinity gel. The column was washed at a flow rate of 20 ml/hr. (indicated by buffer) with 5 volumes of 50 mM Tris-1 mM EDTA 0.2% cholic acid, 150 mM NaCl pH 7.4 at 4°C until no protein was observed by absorbance at 280 nM using LKB 2138 Unicord S. The column was then eluted at 4°C with 50 ml of the same wash buffer containing 500 nM spiroperidol and 0.01% bovine brain lipid. 3.0 ml fractions were collected at the flow rate of 5.0 ml/hr. The fractions were concentrated through amicon CF25 centriflo cones and desalted on Sephadex G-50 column equilibrated with equilibration buffer and assayed for [^{3}H]spiroperidol binding. The protein concentration was determined by the Bio-Rad micro assay as per manufacturer's instructions.

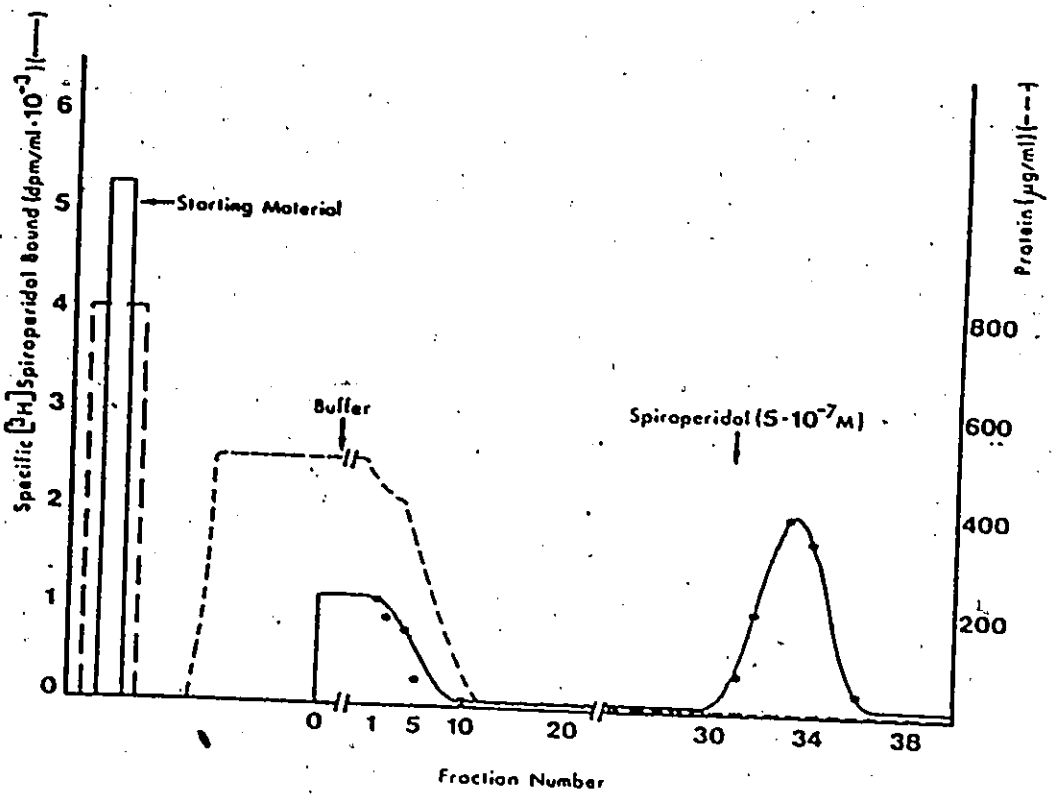

Specific [3H]Spiroperidol Bound (dpm/ml·10-3)(—)
Protein (μg/ml)(- - -)
Starting Material
Buffer
Spiroperidol (5·10-7M)
0
1
2
3
4
5
6
200
400
600
800
0
1
5
10
20
30
34
38
Fraction Number

FIGURE 18

TABLE 11

Purification of D-2 Receptor

[^{3}H]spiroperdol Binding

Preparation	Volume	Total Protein	Total Activity	Specific Activity	Purification	Yield
	ml	mg	fmol	fmol/mg	Fold	Percent
Crude Homogenate	10.0	140	11,200	80	1	100%
Solubilized	40.0	30	3,960	165	2	36
One cycel of affinity gel	10.0*	0.008	1,356	169,600	2,120	12.1(35)

Typically, 10.0 g of bovine brain striatal tissue was homogenized in 0.25 M sucrose and the mitochondrial-microsomal (P_2-P_3) preparation was solubilized with cholic acid - NaCl as described in Methods. Affinity chromatography was performed as described. The [^{3}H]sprioperidol binding assay was performed and the specific binding was defined as the difference between the counts bound in the presence and absence of 1.0 µM (+)-butaclamol. Protein was estimated by the method of Lowry et al. (23) in membranes, and by Bio-Rad micro assay (18) in solubilized and purified preparations. The numbers in parentheses indicate the percent recovery of receptor as compared to the solubilized preparation.

*This volume represents the pooled desalted fractions from the Sephadex G-50 column. Receptor activity from the gel was eluted with 50 ml of 500 nM spiroperidol and concentrated to 1.0 ml through Amicon CF25 Centriflo cones; 100 l of this concentrate was kept for protein estimation and the remainder was desalted and used for [^{3}H]spiroperidol binding assay.

equilibrating buffer containing 500 nM spiroperidol and 0.01% bovine brain lipid extract at 4°C at a flow rate of 5.0 ml/hr. caused an increase in the [^{3}H]spiroperidol binding activity with no significant increase in protein.

The data from a typical purification experiment are shown in Table 11. Solubilization of the membrane preparation resulted in an apparent two fold purification. The affinity chromatography on haloperidol-linked Sepharose yielded greater than 2000 fold purification. [^{3}H]spiroperidol binding assay of the purified receptor showed a recovery of 12% and 35% binding sites as compared to the membrane bound and the solubilized receptors respectively. Specific activity of the purified preparation was 169,600 fmol/mg protein.

3.8.1 Optimization of the Condition for the Adsorption of Receptor Activity to the Gel

Adsorption of the receptor activity to the gel was optimized by incubating varying amounts of the gel with a fixed amount of the receptor preparation. The gel and the receptor preparations were incubated either at 4°C or at room temperature as indicated in Table 12. The optimum adsorption was obtained when 3ml (300 fmol) of the solubilized preparation was incubated with 1 gram of wet haloperidol-linked Sepharose CL-6B gel (Table 12). At room temperature, adsorption was less than at 4°C which could be due to the denaturation of receptor activity on prolonged incubation. When preparations were dialyzed to remove excess salt or detergent, there was a significant decrease in the adsorption of receptor activity to the column which could have been due to an aggregation of the receptor during dialysis. Table 13 compares the adsorption of solubilized dopamine D-2 receptors to different gels. The

TABLE 12

Optimization of the Condition for the Adsorption of the Receptor Activity to the Affinity Gel

Gel	Receptor Activity in the Flow through Fractions fmol/mg protein	Bound to Gel %
Control 1 gm epoxy gel (no haloperidol coupling)	305	0
0.1 gm hal-gel*	250	18
0.2 gm hal-gel	228	25
0.5 gm hal-gel	193	37
1.0 gm hal-gel	119	60
1.0 gm hal-gel + undiluted 1 ml prep.	173	43
1.0 gm hal-gel + dialyzed prep.	207	30
1.0 gm hal-gel + prep. at room temp. for 4 hrs.	153	50

Mean of 2-3 experiments, S.E.M. varied less than 10%.

Various amounts of the haloperidol-linked Sepharose were incubated with 3.0 ml of 1:3 diluted solubilized preparation (or otherwise as marked) for 6 hr. in a batch-wise procedure with gentle mixing at 4°C. The gel-preparation mixture was than passed through a pasteur pipette column. The pass-through fractions were used for [^{3}H]spiroperidol binding.

*hal-gel: Haloperidol-linked Sepharose CL6B

haloperidol-linked Sepharose CL-6B showed better adsorption of receptor activity than the other gels.

3.8.2 Adsorption Specificity

The biospecificity of the haloperidol-Sepharose CL-6B affinity gel was determined by studying the effect of different dopaminergic and non-dopaminergic drugs on the adsorption of [^{3}H]spiroperidol binding activity. In these studies, a number of drugs that had varying degrees of selectivity for the dopamine D-2 receptor were used. To test the stereo selectivity, (-)- and (+)-butaclamol were used. The abilities of these drugs to inhibit the adsorption of [^{3}H]spiroperidol activity to the affinity gel are presented in Figure 19. The dopamine D-2 receptor antagonists spiroperidol and domperidone (tested at 10 μM) were effective in inhibiting >90% of the adsorption of [^{3}H]spiroperidol binding activity. Stereoselective (+)-butaclamol, at 10 μM concentration, inhibited 90% of the activity, whereas (-)-butaclamol was ineffective at the same concentration in blocking the adsorption of receptor activity. Other non-dopaminergic drugs, mianserin (serotonin S_2 antagonist) and propronalol (β-adrenergic blocker) were ineffective at the same concentration. Agonists N-Propylnorapomorphine (NPA) and apomorphine (tested at 100 μM) blocked 70% of the [^{3}H]spiroperidol binding activity.

3.8.3 Elution of Dopamine D-2 Receptor from Affinity Column

Different eluting agents were tried to elute the affinity adsorbed dopamine D-2 receptor from the column. Table 14 and Figure 20 show the comparative elution with dopaminergic and non-dopaminergic eluting agents. Spiroperidol (500 nM) was approximately 4 times more effective than dopamine (2 mM) in eluting the bound receptor. Haloperidol was also

TABLE 13

Adsorption of Receptor Activity to Different Gels

Gel Type	Receptor Activity Loaded Total fmol	Activity in Pre-wash Total fmol	%Bound to the Gel
Haloperidol Linked Epoxy Gel	460	165	64
Fluphenazine Linked Epoxy Gel	400	250	37
Clebopride Linked Affinity Gel	450	267	42

Mean value of 2-3 experiments. One gram of each gel was incubated with 3.0 ml of 1:3 diluted solubilized preparation at 4°C for 6 hr. with gentle mixing in a batchwise procedure. The mixture was passed through pasteur pipette columns. The pass-through fractions were used for [^{3}H]spiroperidol binding.

Figure 19: Ability of doperminergic agonists, antagonists and other agents to inhibit the adsorption of [^{3}H]spiroperidol binding activity. The height of the bar represents the total activity present in 2.0 ml of the starting material (left) or 2.0 ml of the soluble preparation containing the indicated drugs which have been passed through the haloperidol-gel. Two ml of the soluble receptor preparation was incubated with 10 μM of antagonist drugs (spiroperidol, domperidone, (+)-butaclamol, (-)-butaclamol, propranolol and mianserin) or 100 μM of agonist drugs (apomorphine and N-propylnorapomorphine for 3 hours at 4°C and was then applied to a column (5 x 0.2 cm) containing 0.8 ml of gel, and equilibrated for 3 hr. The column was washed with 1.5 ml of wash buffer (50 mM Tris-HCl, 1 mM EDTA and 0.02% cholic acid, pH 7.4 at 4°C). The total eluate was collected; 1.5 ml of this eluate was then desalted by Sephadex G-50 column and the binding of [^{3}H]spiroperidol was performed as given Material and Methods. Results shown are of a single experiment representative of 2-3 experiments.

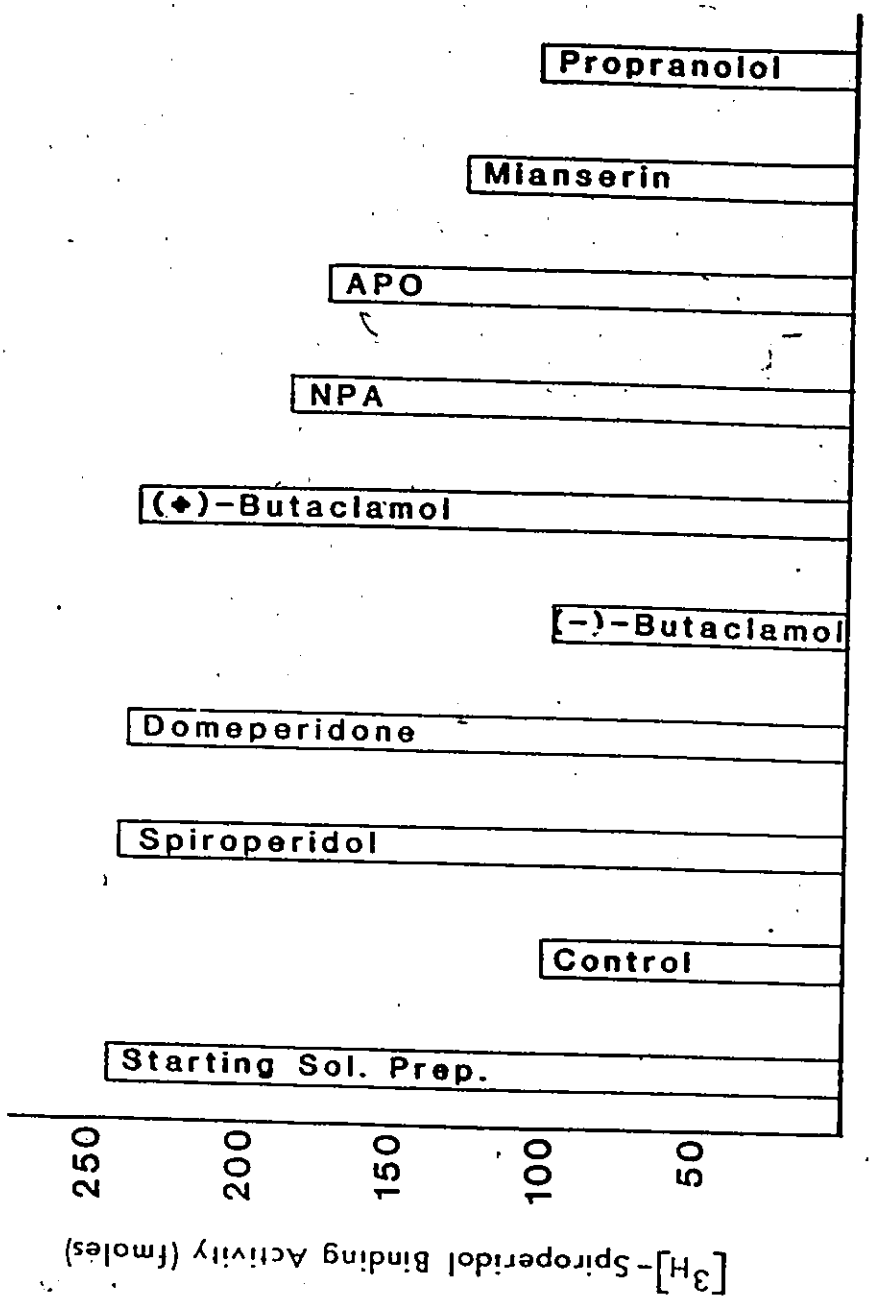

Propranolol
Mianserin
APO
NPA
(+)-Butaclamol
(-)-Butaclamol
Domeperidone
Spiroperidol
Control
Starting Sol. Prep.
250
200
150
100
50
[3H]-Spiroperidol Binding Activity (fmoles)

FIGURE 19

less effective than spiroperidol as an eluting agent. Non-dopaminergic drugs such as mianserin and propranolol were only slightly effective (<10%) in eluting the bound receptor; this was also the case with 1M $MgCl_2$ and 0.1M acetic acid (Figure 20). Providing lipid environment improved the specific binding of the receptor eluted with both agonists and antagonists (Table 14). However, when lipid concentration was increased to 1 mg/ml, higher non-specific, and blank values were obtained. Bovine brain total lipids (sigma) and soybean crude phosphatidylcholine (PC) were also compared in elution buffer, and both were found to be effective in maintaining the specific binding. However, soy bean lipids eluted 25% more dopamine receptor binding activity than bovine brain lipids. Blank values were higher (20-30% of total binding) with the soybean lipids than with the bovine brain lipids (10-15% of total binding). There was less than 10% specific binding in the blanks. On balance, the soybean lipid gave better results than the bovine brain lipids (Table 14).

Dopamine was removed from the eluate by either extensive dialysis against the assay buffer or by concentrating with CF25 Amicon cones and then desalting through G-50 column. By using [^{3}H]dopamine as marker in the elution buffer, it was calculated that 99.98% dopamine could be removed by this technique. With spiroperidol elution, after extensive dialysis for 24 hrs. and 4-5 changes of the buffer, there was still significant amount of spiroperidol remaining in the eluate which interfered with the binding. However, concentration with Amicon CF25 cones and then desalting removed almost all the spiroperidol and did not seem to interfere with the binding. The non-dopaminergic drugs were

TABLE 14

Elution of Dopamine Receptors from the Affinity Column by Different Eluting Agents

Eluting Agent	Total DPM	Nonspecific DPM	Specific DMP	% Binding
2 mM DA + .1 M $MgCl_2$	1142	784	358	31
2 mM DA + .1 M $MgCl_2$ + .1 mg.ml bovine brain lipids	1172	644	528	45
500 nM Spiroperidol + 0.1 mg/ml bovine brain lipds	1928	811	1117	58
100 nM Spiroperidol	906	941	42	50
100 nM Spiroperidol + .1 mg/ml lipids (soy PC)	1890	980	910	48
500 nM Spiroperidol + .1 mg/ml lipids (soy PC)	2564	895	1669	65
500 nM Spiroperidol + 1.0 mg/ml lipids (soy PC)	3026	2908	118	5

Results are mean of 2-3 experiments.

S.E.M. varied less than 10%.

Figure 20: The ability of dopaminergic and other agents to elute the dopamine D-2 receptor activity adsorbed to the Sepharose-haloperidol gel. Routinely, in these experiments, 8 ml. of a soluble preparation containing 0.7-1.0 pmol of receptor activity was loaded on each 1.5-2 ml of haloperidol-gel columns at 4°C with a flow rate of 2 ml/hr. About 60-70% of the receptor activity was bound to the gel. After washing the columns with wash buffer, (50 mM Tris-1 mM EDTA - .02% cholic acid - 1 mM DTT - 150 mM NaCl) they were eluted with 10 ml of the same buffer containing different eluting agents.

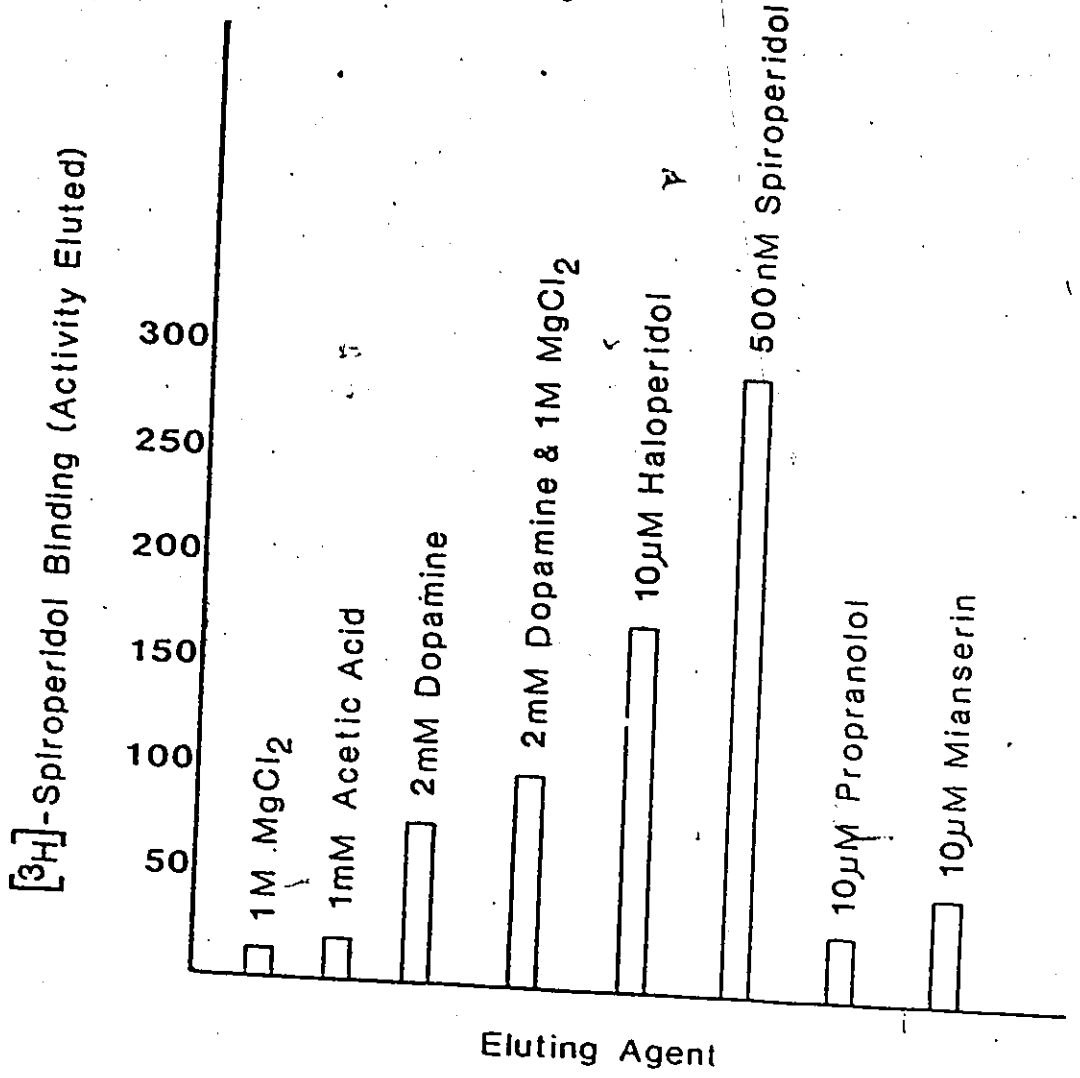

[3H]-Spiroperidol Binding (Activity Eluted)
300
250
200
150
100
50
1M MgCl2
1mM Acetic Acid
2mM Dopamine
2mM Dopamine & 1M MgCl2
10μM Haloperidol
500nM Spiroperidol
10μM Propranolol
10μM Mianserin
Eluting Agent

FIGURE 20

separated from the eluate by similar techniques of concentration and desalting on G-50.

3.8.4 Binding Specificity

The purified receptor exhibited a K_D of 0.15 nM and a B_{max} of 62 fmol/ml by Scatchard analysis as shown in Figure 21. The K_D value corresponded well to the value obtained for the solubilized receptor under similar conditions.

Table 15 shows the binding specificities of the purified and the solubilized receptor. The IC_{50} values for antagonists obtained with the purified preparation were in close agreement to that of the solubilized preparation. The purified receptor retained its stereo-specificity for (+)- and (-)-butaclamol, in that the former was 100- times more potent than the later. The order of potency for the agonists in the purified preparation was NPA>NO434>APO>dopamine, which was similar to the solubilized receptor. However, it should be pointed out that agonist IC_{50} values for the affinity-purified receptor were higher than those for the solubilized receptor which may be due to an increased number of low affinity sites in the purified preparation. The presence of an increased number of low affinity sites can be supported from the observation that Gpp(NH)p had no effect on the [^{3}H]spiroperidol/NPA competition curve (Figure 22).

The dopamine D-1 specific agonist SK&F 38393 (Stoof and Kebabian, 1984), serotonin receptor antagonist, ketanserin, β-adrenergic receptor antagonist, propranolol, and α-adrenergic receptor antagonist, phentolamine, tested with the solubilized as well as the purified preparations exhibited displacement of the [^{3}H]spiroperidol in

Figure 21: Saturation isotherm and Scatchard plot for the specific binding of [^{3}H]sprioperidol to the pooled, concentrated and desalted fractions from the affinity column. Receptor binding assay was performed using the PEG method as described in Materials and Methods. Values are mean of triplicates. Saturation was achieved at 0.8 nM with a K_D of 0.15 nM and a B_{max} of 62 fmol/ml.

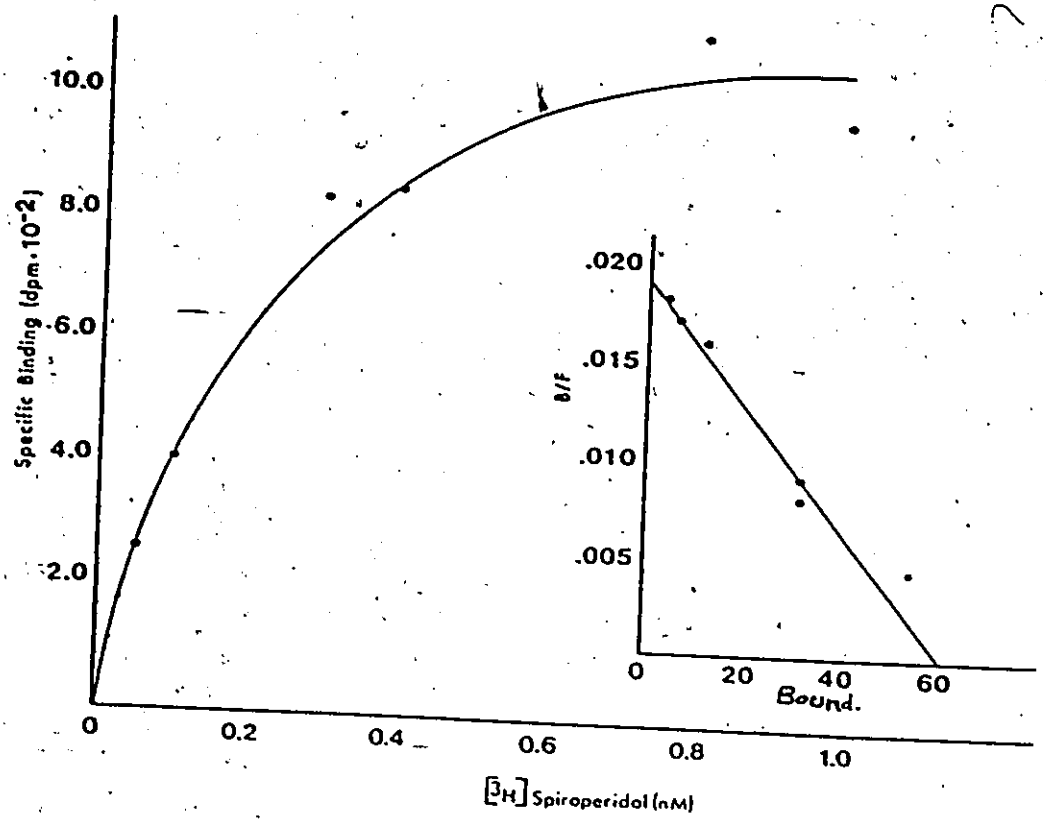

10.0
8.0
6.0
4.0
2.0
Specific Binding (dpm.10⁻²)
0
0.2
0.4
0.6
0.8
1.0
[³H] Spiroperidol (nM)
.020
.015
.010
.005
B/F
0
20
40
60
Bound.

FIGURE 21

TABLE 15

Inhibition of [^{3}H]Spiroperidol Binding to Solubilized and Purified Bovine Dopamine Receptor

AGENTS	Solubilized Receptor IC_{50} nM	Affinity Purified Receptor IC_{50} nM
Dopamine Receptor Antagonists and Agonists		
Spiroperidol	0.84	0.7
Haloperidol	7.0	24.0
(+)-Butaclamol	3.99	9.66
(-)-Butaclamol	5,000	2,400
Domperidone	18.0	15.0
SCH 23390 (D-1 antagonist)	421	440
N-Propylnorapomorphine (NPA)	1.3	33
NO434	50	167
Apomorphine	147	500
Dopamine	5,300	30,000
SKF 38393 (D-1 agonist)	6,000	26,440
Others		
Ketanserin	1,000	1,700
Phentolamine	15,800	10,000
Propranolol	20,000	10,000
R5260 (spirodecanone specific)	1,480	50,000

The IC_{50} values (concentration of drugs, that inhibit 50% of the [^{3}H]spiroperidol binding) were obtained by using 7 different concentrations of drugs (10^{-10} to 10^{-4}M) and were calculated by log probit analysis. The IC_{50} values are the mean of 3-9 determinations. S.E.M. was less than 10%.

Figure 22: Competition curve of [^{3}H]spiroperidol/NPA in the purified preparation, (●——●) in the presence and (■——■) absence of Gpp(NH)p. The values represent the mean of triplicates of 3-9 determinations. Addition of 100 μM Gpp(NH)p to the purified preparation showed no shift of the curve.

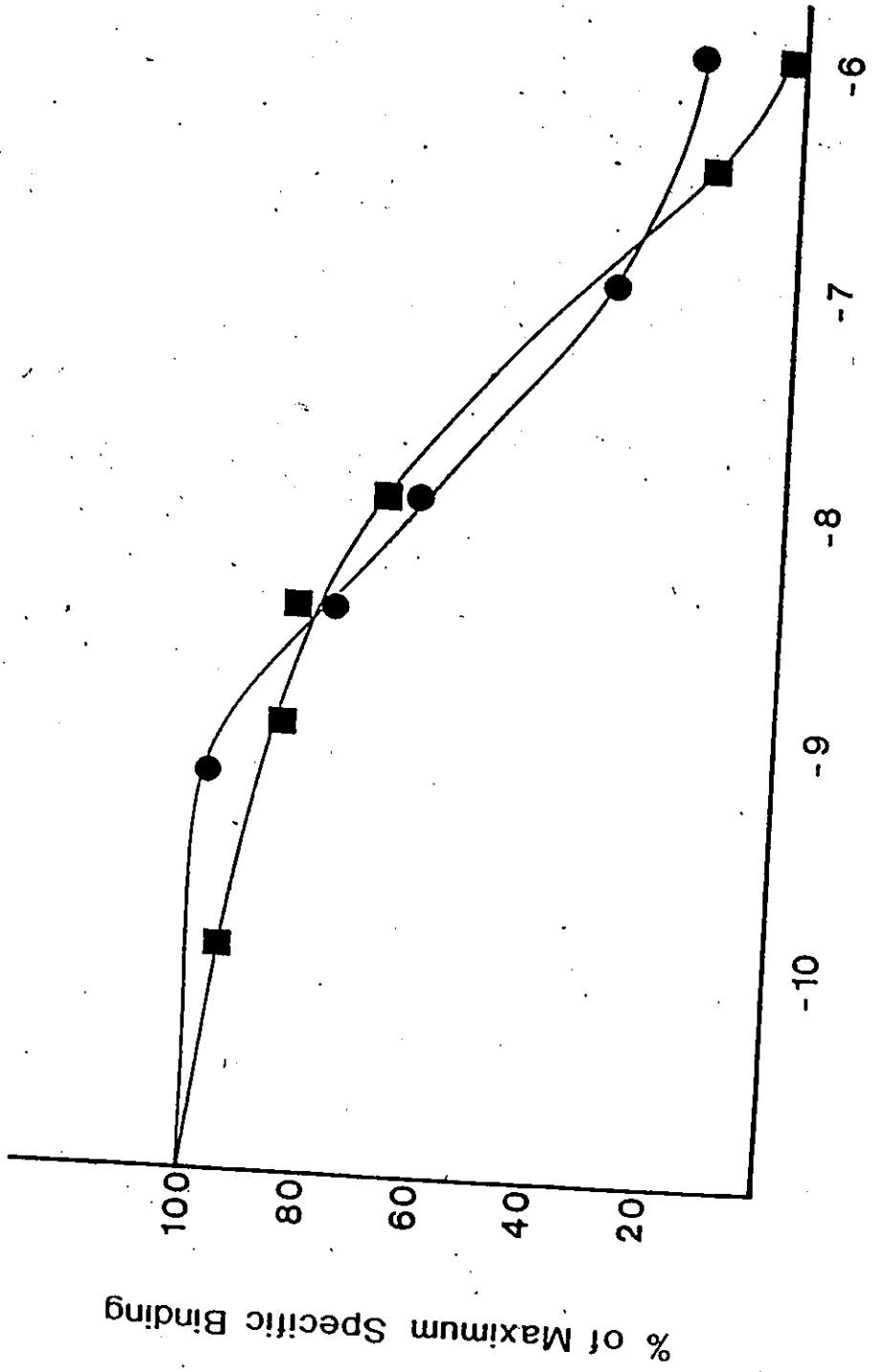


FIGURE 22

micromolar concentrations. R5260, a specific ligand for spiroperidol sites did not displace [^{3}H]spiroperidol binding up to a concentration of 10μM showing a lack of spirodecanone sites in the purified preparation.

4. DISCUSSION

The objectives of the present study were (i) to develop an effective procedure for solubilization of dopamine D-2 receptor from its membrane bound form and (ii) to establish a suitable method for subsequent purification. At the start of the project, the dopamine receptor had been extensively characterized by the radioligand binding assays in the membrane bound form from various species. However, solubilization of dopamine D-2 receptor was still at a developing stage. The reported procedures (Gorrison et al., 1979; Madras et al., 1980; Lerner et al., 1981) on solubilization were not only inconsistent but the recovery was considerably low(<15%). Furthermore, in most cases, the solubilized preparation could not satisfy the criteria of solubility (Ilein and Laduron, 1982). The widely reported detergent, digitonin, could solubilize the receptor from canine and human striata, but not from bovine striatum (Madras et al., 1982). In the later species, solubilized receptor lost the stereospecificity and there was an increase in the K_D value for spiroperidol binding.

It was therefore, important to establish a procedure which would effectively solubilize the D-2 receptor from bovine striatum, since it is a good source of dopamine D-2 receptor, and is easily available with minimal cost. In Table 4 solubilization from four different species, namely bovine, canine, rat and human brain striata is compared. When various detergents were tested, 0.25% cholic acid in combination with 1 M NaCl yielded better solubilization (45%) of active binding sites in bovine striatum. Low yield (<15%) obtained with digitonin solubilization (Table 4) is in agreement with other reports in literature (Gorrisen et

al, 1979; Meuinier and Labrie, 1982). The yield of 45% with cholic acid: NaCl solubilization is higher than any other reported procedure, e.g. 10% for lysophosphatidylcholine (Wheatley and Strange , 1983) and 23% for CHAPs (Kilpatrick et al., 1984; Lew and Goldstein, 1984).

The high yield of 25-45% of dopamine receptor (depending on species) solubilization by 0.25% cholic acid and 1 M NaCl could be due to its solubility below the critical micelle concentration (CMC). The normal CMC of cholic acid is around 2% (Tanford and Reynold, 1976). Usually solubilization of intrinsic membrane protein (eg., receptors) requires a detergent concentration at or above the CMC of the detergent. At that concentration, protein can be solubilized but most of the receptor activity is lost due to denaturation. As mentioned in the introduction section, CMC of cholic acid is salt dependent; Shackland (1970) reported that a high concentration of NaCl lowers the monomer concentration in solution, by allowing more cholate to be incorporated into mixed micelles which decreases their average size. Also low concentration of cholate may be essential to avoid further stripping of phospholipid than minimally required for removing the protein from the membrane. The phospholipids are essential for maintaining the receptor activity (Hall et al., 1983). Effective solubilization with cholic acid: NaCl combination of dopamine D-2 receptor (Ramwani and Mishra, 1983) has been further confirmed by other laboratories (Hall et al, 1983; Wouters et al., 1984).

The dopamine receptor recovered from striatal membranes by detergent-salt treatment, satisfied the generally accepted criteria for solubilization as suggested by Laduron and Ilein, (1982), namely absence

of a sedimentation after centrifugation at 105,000 g for 6 hrs., passage through glass fiber filter (0.22 μm) and one major peak with no sedimentation at the bottom of the tube following sucrose density gradient (15-30%) profile (Figure 5). Futhermore, as recommended by Laduron and Ilien (1982) in their commentary that solubilization procedures be able to solubilize the receptor activity from different species has also been satisifed in the present study.

The soluble D-2 receptor was stable at 4°C. There was no significant (<10%) loss of the [^{3}H]spiroperidol binding activity even after storage for over 72 hrs. This stability of solubilized receptor is an important advantage for further purification, since normal purification procedure by affinity chromatography takes more than 48 hrs. There was minimal loss of (<10%) binding activity in the solubilized preparation on freezing and thawing; such treatment did not affect the binding parameters of the [^{3}H]spiroperidol assay (The K_D of [^{3}H]spiroperidol remained 0.3 nM.)

Pharmacological characteristics of the soluble preparation indicated that [^{3}H]spiroperidol exhibited binding to a single population of receptor sites with high affinity. The K_D value for the soluble receptor corresponded to the membrane bound D-2 receptor. The cholic acid: NaCl solubilized binding sites in the bovine striatum, in contrast to the digitonin solubilization (Madras et al., 1982), retained their stereoselectivity for (+)- and (-)-butaclamol and the specificity for the dopamine agonists and antagonists also exhibited similarity to the membrane bound D-2 receptor (Table 7 results). The IC_{50} values for non-dopaminergic agents, e.g. ketanserin (serotonin antagonist), GABA,

muscimol (GABA agonist), diazapam and phentolamine (β adrenegric antagonist) were in the micromolar concentration range.

The possibility of D-1 binding sites in the solubilized preparation was ruled out by performing binding assay with D-1 specific antagonist, [^{3}H]flupenthixol which gave a specific binding of less than 20% (Table 6): the displacement of [^{3}H]spiroperidol binding by the D-1 specific agonist SK&F 38393 yielded an IC_{50} in micromolar range (Table 13). Furthermore, the D-1 specific antagonist, SCH 23390, gave IC_{50} value in high nanomolar range (IC_{50} of SCH 23390 for D-1 is <1 nM).

Several lines of evidence for the existence of high affinity dopamine D-2 receptor binding sites in the solubilized preparation have been provided in this thesis (Figures 8-13). These sites are also shown to be associated with guanine nucleotide binding (G protein) protein (Figure 13).

High affinity dopamine D-2 sites were demonstrated with [^{3}H]NPA (a specific high affinity ligand) binding in the soluble preparation. This is the first direct evidence for the presence of high affinity binding sites in the soluble preparation. [^{3}H]NPA binding was satuarable with a K_D of 0.3 nM and was with an appreciable specific binding (75%) at a very low concentration (0.25 nM) of [^{3}H]NPA (Figure 8). Unlike [^{3}H]spiroperidol binding, [^{3}H]NPA binding was highly sensitive to guanine nucleotides. Gpp(NH)p was capable of almost completely abolishing the [^{3}H]NPA binding, probably by converting the high affinity state to low affinity state (Figure 8). These findings in the soluble preparation were in agreement with the reports on the membrane bound dopamine D-2 receptor (Hamblin et al., 1984; Girgoridis and Seeman, 1985), where it

was demonstrated that guanine nucleotides convert high affinity state to low affinity state. The possibility of [^{3}H]NPA binding to dopamine D-1 receptor was ruled out by the fact that in the solubilized preparation there was less than 20% of D-1 sites determined by the [^{3}H]flupentihixol binding (Table 6). Furthermore, when [^{3}H]NPA binding assay was performed in the presence of 1 nM D-1 specific antagonist SCH 23390, there was no noticeable difference in the binding.

The presence of G protein in the solubilized preparation was evident with GTP analogue [^{35}S]GTPγs binding assay. The binding was saturable with a K_D of 100 nM and a B_{max} of 56 pmol/mg protein. These parameters of [^{35}S]GTP γs binding in the solubilized preparation corresponded well with the earlier report on [^{3}H]Gpp(NH)p binding in the brain membrane preparation (Hamon et al., 1982). Since the binding sites labelled by [^{35}S]GTPγs were approximately 200 times more than the D-2 receptor density, only a small proportion of guanine nucleotide binding sites appear to interact with dopamine receptor. However, from the K_D values of [^{35}S]GTPγs (Figure 13) and of [^{3}H]Gpp(NH)p (Hamon et al., 1982) one can assume that the inhibitory concentration of Gpp(NH)p would fall in the range required to saturate the guanine nucleotide binding sites, suggesting the coupling of G protein with the solubilized dopamine D-2 receptor. Direct association of the Gi protein with the dopamine D-2 receptor has recently been provided by selective inhibition of Gi protein with the pertussis toxin (catalyses ADP ribosylation of Gi subunit and inhibits agonist stimulated GTPase activity) which decreased the potency of NPA in the membrane binding assay and abolished the ability of GTP to convert the high affinity state to the low affinity state (Cote et al.,

1984; Tanaka et al., 1984).

The observed retention of receptor-G protein complex in the solubilized preparation is in contrast to the earlier reports on solubilization of striatal dopamine D-2 receptor using digitonon as the detergent (Leff and Creese, 1982; Kilpatrick and Caron, 1983). In these studies, investigators could find the high affinity binding site only when solubilized in the presence of dopaminergic agonist. The discrepent finding in the present study suggests that the presence of NaCl may be helping to retain the receptor-G protein complex. Furthermore, in this investigation, the concentration of cholic acid used is 5-10 times less than CMC of cholic acid, hence the detergent may be having a minimal overall effect on the structural components of the receptor. Kuno et al. (1983) have reported a right shift in the [^{3}H]spiroperidol/agonist competition curves with GTP in a CHAPS-NaCl solubilized pituitary dopamine D-2 receptor preparation. However, in their studies, the IC_{50} values for agonists were higher than those reported in the literature (Seeman, 1980). Furthermore, in their studies, high affinity binding with [^{3}H]NPA was not performed and the GTP effect on the shift of IC_{50} values was much less pronounced than in the present investigation. This could be due to the absence of Mg^{++} from their buffer system. Mg^{++} is reported to induce nanomolar potency to dopaminergic agonist for inhibiting [^{3}H]spiroperidol binding (DeVries and Beart, 1985). Further support for high affinity binding sites in the solubilized preparation can be derived from a statement by Kilpatrick et al. (1984) that "NaCl allows reassociation of the receptor with G protein in the solubilized preparation". The presence of high affinity binding sites in the

solubilized preparation (with receptor-G protein complex intact) is an interesting finding and should enhance the understanding of events from receptor drug interaction to final response.

Molecular Size by Radiation Inactivation

This dissertation provides the first information on the molecular size of the solubilized dopamine D-2 receptor as determined by the radiation inactivation technique. Target size analysis is the only known method whereby molecular size of the receptor or any other functional protein can be determined directly in the membrane bound form and can also be compared to the solubilized form. In this technique, high energy electrons destroy the functional activity of a protein and radiation sensitivity of a protein is proportional to the molecular size of the functional protein (Jung, 1984). The molecular size of the solubilized receptor determined by radiation inactivation was in close agreement with the membrane bound receptor. The reported values of $146 \times 10^3 \pm 17 \times 10^3$ and $139 \times 10^3 \pm 16 \times 10^3$ daltons in the membrane and solubilized preparation respectively corresponded closely to those reported earlier, e.g. $136.9 \times 10^3 \pm 5.2 \times 10^3$ daltons for rat striatal membrane (Nielsen et al., 1984) and 123×10^3 daltons for human and dog striatal membranes respectively (Lilly et al., 1983). However, it should be pointed out that in the solubilized preparation, there was approximately 20-30% of the portion which was a high molecular weight complex and was inactivated below 1 Mrad dose. This portion (high molecular weight complex) could represent the multiples of receptor - Gi protein units and the presence of NaCl in the solubilized preparation may enhance the formation of these multiple R-Ni complexes. Similar R-Ni multiple complexes have been

reported for the adenosine-receptor (Rodbell, 1980). These high molecular weight complexes have also been reported in the lyophilized irradiated bovine membrane preparation (Kuno and Tanaka, 1983). The formation of these complexes in the CHAPS solublized preparation has been reported by Kilpatrick et al. (1984).

The molecular size of D-2 receptor in the solubilized preparation determined by target size analysis is much lower than the reported molecular size of 200 K daltons by the gel filtration method (Hall et al., 1983). This discrepency could be due to the interference of detergent when the molecular size is determined by gel filtration. In another study, using photo-affinity labelling of dopamine D-2 receptor with $^{125}IN_3$-NAPS followed by SDS polyacrylamide gel electrophoresis, the molecular size of the dopamine receptor was reported to be 94,000 daltons (Amlaiky and Caron, 1985) which may represent the molecular size of the dopamine D-2 receptor without G protein. In the present solublized preparation, receptor-Gi protein complex was evident. The molecular size for α sub-unit of G protein is reported to be 42,000 dalton (Narthup et al., 1982). Taking this into consideration the values reported here are likely to represent the molecular size of receptor-αi subunit of G protein and are in agreement with the previous reports (Lilly et al., 1983; Neilsen et al., 1984).

Scatchard analysis of [^{3}H]spiroperidol binding (Figure 16) in the non-irradiated and irradiated membrane as well as solubilized preparation showed a parallel shift in the curve with no change in the K_D value. These data indicate that the high energy electrons are directly destroying the functional activity of the receptor as proposed by the

target theory (Pollard, 1951).

Affinity Purification

Affinity chromatography is a highly specific technique. Each system must have its own custom made affinity adsorbent which reflects and utilizes the chemical, physical and biochemical parameters of the system under study. This allows for a great deal of versatility in application, but also requires special effort in order to obtain the best possible adsorbent.

In the past two decades, affinity chromatography has proven to be a successful technique for the purification of enzymes and hormones. However, except for β adrenergic (Caron et al., 1979; Shorr et al., 1981) and acetylcholine nicotinic receptor (Heidman and Changeux, 1978) purification it has still a limited success in the purification of other neurotransmitter receptors. In this dissertation, for the first time, a successful affinity method for the purification of the dopamine D-2 receptor has been provided. Each step in this scheme was specifically designed for the dopamine D-2 receptor purification.

Selection of the affinity adsorbent is a prerequisite for the purification of any hormone or drug receptor. In these studies, the ligand haloperidol has a functional tertiary hydroxyl group for reaction, hence bis-oxirane activated Sepharose was selected as the matrix. The synthesis of bis-oxirane or epoxy-activated Sepharose was carried out routinely in the laboratory. Typically 10-15 μM of epoxide groups were reacted with 1 gm of Sepharose. This reaction generates a hydrophilic spacer arm of 12 carbons linked to the matrix via a stable ether linkage with an active terminal oxirane group available for spontaneously

coupling to the hydroxyl group of ligand (Lowe, 1979). The spacer arm is essential for setting the ligand away from the matrix. Since the ligand, haloperidol itself is a small molecule, the spacer arm made it more accessible to the receptor protein and also avoided any non-specific interaction.

Selection of the suitable ligand is central to the preparation of immobilized ligand matrix. It must possess a suitable functional group capable of coupling to a support, must be chemically stable to the coupling process and must retain the biological activity during the process of coupling. Ligand selection was the most difficult step in the whole affinity procedure of dopamine D-2 receptor purification. An effort was made to understand the molecular structure of dopamine receptor ligands and the availability of functional groups for there in coupling with the gel. Almost all of the dopaminergic agonists are unstable and easily oxidizable and any modification of the structure could have caused the loss of binding to the receptor sites. Attempts for the linkage of dopaminergic agonists ADTN or dopamine itself have been reported by Morof and Hsu (1984). In their report it was suggested that the change in the color of the gel beads indicated the success of the coupling reaction. At this point, it is not clear whether the change in the color was an indication of oxidation of the ligand or the true coupling. Moreover, the extent of receptor activity adsorbed on such gel has not been mentioned in their study. Dopaminergic antagonist, clebopride which has a free amino group, was coupled with the commercially available Affigel 10 (having succinimide ester as the reactive group), but the success with such linkage was very limited and

it was difficult to quantitate the linkage. In addition, the adsorption of receptor activity on the clebopride linked gel was much less than that on the haloperidol linked gel (Table 13). Another dopaminergic ligand, fluphenazine (having a primary alcohol group for coupling) was attempted to link with the epoxy-activated Sepharose. In this reaction also it was difficult to quantitate the coupling of fluphenazine to the gel. Furthermore, adsorption of the receptor activity to the gel was less than 30% of the total receptor activity applied. Though haloperidol has a tertiary alcohol as the functionally reactive group, nonetheless it proved to be better than other ligands tested. It can be coupled to the gel with sufficient stability and affinity to act as an adsorbent. As mentioned in the results (Table 10), coupling in this case was roughly 0.8 mol per gram gel, which is less than optimum requirement for the affinity system (Lowe, 1979). However, the adsorption of the receptor activity was significantly higher (>60%) than other affinity adsorbent prepared in the laboratory (Table 12). Furthermore, the linkage could easily be quantitatively estimated by using [^{3}H]haloperidol as the marker. Haloperidol was easily soluble in DMF. Sepharose CL-6B was also stable in this medium. $ZnCl_2$ as catalyst was an important step in this reaction. Without $ZnCl_2$, no haloperidol linkage was detected in the gel. The stability and the covalent nature of the reaction was established by washing the gel repeatedly with DMF and excess water and finally with 500 mM NaCl in Tris-Hcl, pH 8.0 [^{3}H]haloperidol was included as the marker to quantitate the haloperidol linkage to the gel. Non-specific sites on the adsorbent were blocked by reacting the gel with 1 M ethanolamine, pH 8.0 for over 10 hrs. Such linkage was stable and

there was no significant loss (<10%) on storage over 2 months at 4°C.

It should also be mentioned that the haloperidol coupling to epoxy-activated Sepharose CL-6B is reproducible and can be carried out easily.

In a typical assay, 60-70% of the receptor activity applied could be adsorbed to the affinity column (Table 12). The adsorption of greater than 70% was not possible, which could be due to the presence of 150 mM NaCl in the solubilized preparation (NaCl is known to retard the adsorption of protein to the column). However, presence of NaCl in the preparation was essential to maintain the receptor activity, since on dialysis for removal of NaCl, (Table 11), percentage specific binding was decreased to half that of undialyzed diluted prepartion. It should be pointed out that this adsorption efficiency is in agreement with that of other receptors, e.g. β adrenergic receptor 70% adsorption (Caron et al., 1979) and α_2 adrenergic receptors, 73% adsorption (Regan et al., 1982). In contrast to the adsorption of receptor activity on haloperidol linked gel, there was no significant adsorption of binding activity to the control gel, i.e. epoxy activated Sepharose without haloperidol linkage.

The bioselective nature of the affinity gel was examined by blocking the receptor sites with different dopaminergic and non-dopaminergic drugs. In these investigations, when the solubilized receptors were blocked with highly potent dopamine D-2 receptor antagonists, there was almost no adsorption to the affinity column (Figure 19) whereas the receptor sites blocked with non-dopaminergic drugs, e.g. ketansenin (serotonin receptor antagonist) and β adrenergic

blocker propranolol could be adsorbed to the extent similar to that with the control gel. Further, in these studies, stereoselectivity of the D-2 receptor was also obvious. (-)-Butaclamol was ineffective, whereas (+)-butaclamol blocked the adsorption of receptor activity to the gel (Figure 19). Dopaminergic agonists also inhibited receptor adsorption with similar dopamine D-2 potency ratio to the gel; NPA was more potent than apomorphine in blocking the adsorption of receptor activity to the gel.

Elution of the adsorbed receptor activity was optimized by trial and error with different eluting agents (Table 14 and Figure 20). As it is clear from the results, spiroperidol in the presence of lipids was more effective as the eluting agent than others tested. Spiroperidol was able to elute almost 70% of the bound receptor activity. Also, the elution was concentration dependent, as 500 nM spiroperidol (Table 14) with lipids eluted more receptor activity than 100 nM spiroperidol with lipids. There was no significant difference between 500 nM and 1 M spiroperidol elution. Higher concentration of eluting agent interfered with the binding assay, since it became difficult to remove the ligand completely from the eluted fractions. To ensure the separation of eluting agent, 100,000 DPM of [^{3}H]spiroperidol was added as a the marker to the eluting buffer. Routinely, 99.98% eluting agent (free and bound) got removed by concentration with Amicon cones and desalting (G-50). The elution profile also seemed to be quite biospecific. Only dopaminergic agents were able to elute the receptor activity from the column. Among dopaminergic agents spiroperidol was 3-4 times more effective than dopamine in eluting the bound receptor (Figure 20 and Table 14).

Haloperidol was also proportionately less effective than spiroperidol in eluting the bound receptor. Other agents like mianserin, propranolol, 1 M MgCl and 1 mM acetic acid had little effect on the elution of the bound receptor activity.

Maximum activity of the eluted receptor could only be restored when elution was performed with lipids. Both soybean crude lipids (containing 16% PC) and bovine total brain lipids (chloroform-methanol extract) were effective in restoring the receptor activity. However, soybean lipids repeatedly restored 15-20% more activity than bovine brain lipids. It should be pointed out that soybean lipids gave higher blank values (20-30% of the total counts of ligand binding with no significant specific binding) than bovine brain lipids (15-20% of the total counts). The concentration of lipid (0.01%) and the formation of lipid vesicles seemed to be the most critical. In routine procedure, as mentioned in materials and methods, 10 mg/ml lipids (either soybean or bovine brain) were suspended in equilibrating buffer under nitrogen gas and were sonicated in a bath type sonicator for 30 minutes with periodic stirring. If the concentration of lipid was increased beyond 0.01% it interferred with the binding assay, gave high blanks and increased non-specific binding.

Addition of lipids to the eluted fractions and, concentration and passing through the G-50 column appeared to be necessary for providing proper configuration to the regions for binding. Similar requirement of lipid was found for immunoglobulin IgE purified receptor binding (Rivnay et al., 1984). It is evident from these studies that some molecular changes occur upon purification which may cause the stripping of certain

phospholipids from the receptor protein, the absence of necessary lipid domain would make reconstitution a prerequisite for the functional D-2 receptor binding. The requirement of phospholipids has previously been alluded to in improving D-2 receptor binding activity in the solubilized preparation (Wheatley and Strange, 1983; Wheatley et al., 1984).

As shown in Figure 18, elution of the receptor activity was not accompanied by a significant increase in the amount of protein, yielding a purification of approximately 2000 fold compared to the membrane bound receptor activity. This single cycle affinity chromatography yielded specific activity of greater than 150 pmol/mg protein. The overall recovery was 12-15%, calculated from bovine striatal membrane preparation and 35-40% compared to solubilized preparation (Ramwani and Mishra, 1985, 1986). The purification folds and the recovery were based on the assumption that there was no interference of residual spiroperidol from elution in the binding assay. Also, it was assumed that 100% of the receptor activity was reconstituted with phospholipids. The true efficacy of reconstitution could not be assessed in this study. Keeping these above mentioned variables in consideration, it could be stated that the present result may be an underestimation of the true specific activity, the folds of purification and the recovery of receptor activity from the affinity column. The actual purification fold and recovery could be more than mentioned in the results.

In a routine assay, protein contents in the solubilized as well the as in purified preparation were determined by Bradford's (1976) Coomassie blue dye method (commerically available from Bio- Rad). The microassay, as suggested by manufacturer, was carried out for protein

determination. The purified preparation was first concentrated to 1 ml and then required amount (100 µl) was taken for protein assay. Portion of elution buffer was treated the same way as purified preparation for blank, i.e. it was concentrated and then 100 µl was used for the blank. Treatment of the blank in similar manner should avoid any interference of the detergent in the estimation of protein. Furthermore, protein concentration was checked periodically by amido black method (Schaffner and Weissman, 1973). An attempt was also made to determine the protein concentration by total amino acid analysis. Because of the degradation of certain amino acids during analysis mainly of tryptophan and cysteine and also of threonine and serine to a smaller extent, it became difficult to exactly quantitate the protein concentration. However, arbitrary calculation showed the concentration of protein to be 300-500 nanograms per ml of concentrated fractions by amino acid analysis.

The purified receptor exhibited similar pharmacological characteristics as solubilized dopamine D-2 receptor. The dissociation constant (K_D) obtained from Scatchard analysis for [^{3}H]spiroperidol was similar to that of the solubilized receptor (Figure 21). Purified receptor also showed similar specificity as that of the solubilized receptor. The antagonists, namely, spiroperidol, haloperidol, butaclamol and domperidone exhibited IC_{50} values in low nanomolar range with close agreement to the solublized preparation. The stereoselectivity of the receptor was retained throughout the solubilization and purification procedures. (-)-Butaclamol had IC_{50} values in micromolar range. Agonists showed similar rank order of potency in the purified preparation as compared to the solubilized

preparation (NPA>N0434>apomorphine>dopamine.) However, it should be pointed out that the IC_{50} values are higher in the purified than in the solubilized preparation. The reason for higher IC_{50} values for agonist in the purified preparation could be following:

i) The cholic acid NaCl solubilized preparation exhibited biphasic antagonist/agonist displacement curves indicating the presence of high and low affinity binding sites. Furthermore, the high affinity sites were associated with GTP binding protein. It is possible that during purification there was a detachment of G-protein from the receptor and henceforth high affinity sites either were lost or converted to low affinity sites, which is reflected by a shift in the IC_{50} values. This hypothesis can be supported by the evidence that in the purified preparation there was no significant binding (<15%) of [^{3}H]NPA at concentrations ranging from 0.1 nM to 1 nM. Furthermore, there was no effect of 100 μM Gpp(NH)p on the displacement of [^{3}H]spiroperidol by NPA (Figure 22).

ii) In the purified D-2 receptor, [^{3}H]spiroperidol binding could only be demonstrated when the elution was performed in the presence of lipids. This may suggest a conformational change in the receptor configuration upon purification and addition of lipids restored the ligand binding conformation. This change may contribute to the shift in the IC_{50} values, also it could have led to the dissociation of G-protein from the receptor. The lack of GTP binding protein in the purified β adrenergic receptor preparation has been reported by Caron et al. (1979).

The dopamine D-1 specific agonist SK&F 38393 (Stoof and Kebabian, 1984) showed a IC_{50} value in high micromolar concentration (IC_{50} value

for D-1 is in nanomolar) confirming the non-existence of the D-1 binding sites in the purified preparation. Furthermore, as reported in the results section, our solubilized preparation had minimal D-1 binding sites, since there was less then 20% specific binding of D-1 ligand [^{3}H]flupenthixol. Non-dopaminergic drugs, e.g. the serotonin receptor antagonist (ketanserin), β adrenergic antagonist (propranolol) and adrenergic receptor antagonist (phentoloamine) tested in solubilized as well as in the purified preparation showed displacement of [^{3}H]spiroperidol in the micromolar concentration range. In the purified as well as the solubilized preparation, R5200, a specific ligand for spirodecanone site (Gorissen et al., 1979) displaced [^{3}H]spiroperidol only above 10 μM concentration, showing the lack of these nonspecific bindingr sites in the preparation.

Recently, Moroi and Hsu (1984) have reported the isolation and partial purification of a dopamine binding protein (using a dopamine-linked affinity column) and subsequent characterization with photo-affinity labelled dopamine. However, it remains to be ascertained whether the binding protein is linked to dopamine or serotonin, since spiroperidol has high affinity for serotonin (S-2) receptors as well. Furthermore, the tissue source (cortex) is rich in serotonin and relatively deficient in dopamine receptors. The reported K_D values (4-12 nM) for spiroperidol appeared to be higher than values in the literature (Seeman, 1980) for the dopamine D-2 receptor. In the present study, the K_D value of purified receptor (0.15 nM) is in close agreement with the binding kinetics of dopamine D-2 receptor. In addition the authors' (Moroi and Hsu, 1984) claim to have purified the binding protein to

homogeneity is difficult to appreciate, particularly when there was just a 4-fold purification after affinity chromatography. In other studies, Lilly et al. (1985) have partially purified the receptor by a combination of gel filtration and isoelectric focussing, showing a 20-fold purification from the starting material. In another study using lectin affinity chromatography (specific for glycoprotein), Lew and Goldstein (1984) have purified CHAPS solubilized dopamine D-2 receptor up to 12-fold. The adsorbtion of the receptor activity to lectin suggests that receptor is a glycoprotein. Similar findings (i.e. D-2 receptor is a glycoprotein) have been reported in cholic acid: NaCl solubilized preparation (Hall et al., 1983).

The affinity chromatography procedure reported in this thesis has many advantages over other methods used for purification: (1) the haloperidol derivatized support described here appears to be much simpler to synthesize, since it involves only a two-step reaction which could be completed in two days; and (2) the purification obtained with this procedure is significantly higher than any other procedure reported earlier (Moroi and Hsu, 1984; Lilly et al., 1985). Simple concentration with Amicon cones and desalting on Sephadex G-50 have facilitated the measurement of binding activity in the receptor enriched preparation, particularly when spiroperidol was used as an eluting agent from the affinity column. Although receptors purified by one step affinity procedure have not reached the homogeneity state of purified protein, it will serve as a key tool in achieving the eventual purification to homogeneity of the dopamine D-2 receptor. It will require another 10-20 fold purification to reach the homogeneity state of purification, which

can be achieved by combining affinity chromatogaphy with other techniques, e.g. molecular sieving on high pressure liquid chromatography (HPLC) or lectin chromatography. Reconstitution of purified D-2 receptor with the subunit of adenylate cyclase system should enhance the understanding of the molecular events of drug-receptor interaction and the final response. Reconstitution may also help in understanding the basis of dopamine receptor classification and may resolve controversies surrounding the subclasses of receptor.

Summary and Conclusions

This thesis provides key procedures for solubilization and purification of dopamine D-2 receptor from bovine striatum. An efficient solubilization procedure and a highly biospecific affinity chromatography procedure for the purification of dopamine D-2 receptor have been established in this thesis. I submit that experiments reported here test and support the following conclusions.

i) Dopamine D-2 receptor has been solubilized from striatal membranes by a cholic acid - NaCl combination.

ii) Recovery of the solubilized receptor by this procedure from the four species tested was greater than any other presently available procedure in the literature.

iii) Solubilized receptor satisifed the criteria of solubility as suggested in the literature.

iv) Solubilized receptor demonstrated similar pharmacological characteristics, i.e. affinity, specificity and stereoslectivity to that of membrane bound receptor.

v) Solubilized D-2 receptor exhibited high affinity binding sites associated with GTP binding protein of the adenylate cyclase system.

vi) The presence of high affinity sites in the solubilized preparation has been established by a shift of [^{3}H]antagonist/agonist curve to the right with Gpp(NH)p, a GTP analogue. Solubilized receptor binding to the dopamine D-2 high affinity ligand, [^{3}H]NPA was saturable and occurred with high affinity (K_D 0.30 nM).

vii) Target size analysis by radiation inactiviation exhibited similarity of molecular size in both the membrane bound and the solubilized dopamine D-2 receptor from bovine striatum.

viii) Affinity chromatographic technique has been established for the purification of solubilized dopamine D-2 receptor. Affinity gel (haloperidol-linked epoxy-sepharose) satisfied the criteria of adsorption and elution specificities.

ix) Using this technique, more than 2,000 folds enrichment of the dopamine receptor with good recovery and specific activity (169,900 fmol/mg protein) has been achieved.

x) Purified receptor exhibited similar pharmacological characteristics as that of solubilized and membrane bound dopamine D-2 receptor.

xi) Requirement of lipid seemed essential for restoring the [^{3}H]spiroperidol binding activity of the purified receptor. Finally, the receptor purified by affinity chromatography should be useful in producing monoclonal antibody to the dopamine receptor, also it should be pivotal in understanding the molecular events from receptor drug binding to the final response. The results described in this thesis should open

additional avenues of research in the area of dopamine receptor effector interactions.

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