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by

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Abstract

NEUROLEPTIC DRUGS: A COMPARISON OF THEIR EFFECTS IN VIVO AND INTRO ON BRAIN DOPAMINERGIC SYSTEMS

by

Chun Wei Lin

Advisor: Professor Sherwin Wilk

The objective of this research was to examine the relationship between the antidopamine effects in vivo of antipsychotic drugs with their antidopamine effects in vitro. Antipsychotic drugs are believed to exert their clinical effect by the blockade of dopamine (DA) receptors. Biochemically, this effect can be monitored by measuring the increase in the turnover of DA neurons produced by these drugs, by the competition for the binding sites labelled with (3H)-neuroleptic drugs, or by the reversal of stimulation of adenylate cyclase by DA. The relative clinical potencies of neuroleptic drugs in general parallels their potencies in increasing DA turnover and in competing for binding sites labelled by (3H)-neuroleptics. Antagonism of adenylate cyclase stimulation correlates poorly with clinical potency. There are a number of antipsycho-

tic drugs whose properties differ from typical neuroleptics. The benzamide neuroleptics metoclopramide and sulpiride, for example, increase DA turnover in vivo, but are weak in competing for (3H)spiroperidol(Spiro) sites and are ineffective in reversing the stimulation of adenylate cyclase. The anomalous properties of these benzamide drugs prompted us to (1) re-evaluate the validity of using the radioreceptor binding technique to predict antidopamine properties in vivo and (2) to develop a radioreceptor binding system which would help to elucidate the sites of action of the benzamide neuroleptics. In addition to binding studies in membrane fractions, we also studied binding to cryostat-cut slide mounted brain slices. In the first study, the effect of an extensive series of typical and atypical neuroleptic drugs on the binding of (3H)Spiro in calf striatal membrane fractions was assessed. Using 1 uM d-butaclamol to define the non-specific binding of (3H)Spiro and a rapid filtration technique, saturation of (3H)Spiro, kinetics of association and dissociation, and competition experiments were run. Results were analysed with the aid of the PROPHET computer, and methods of Hill, Scatchard, and Dixon were used to analyse the saturation and competition experiments. The potencies of antipsychotic drugs in this system were compared to their clinical potencies and their potencies in increasing the turnover of DA neurons in rat striatum and tuberculum olfactorium. Levels of DA, 3,4-dihydroxyphenylacetic acid and homovanillic acid were determined by

gas chromatography. In the second study, (3H)dihydroergocryptine (DHE) was used as the radioligand. The specificity of binding was enhanced using a low concentration of the displacer, d-butaclamol. DHE was proposed to label a subpopulation of DA receptors which are independent of adenylate cyclase activity. Under these conditions, apparent DAergic sites have been defined which have high affinity for the benzamide neuroleptics as well as for a majority of typical neuroleptics. This study confirmed the antiDA properties of benzamide neuroleptics in vitro. In another study, binding of (3H)Spiro was assessed using a nM concentration of d-butaclamol as the displacer. The properties of this system was compared to the system using 1 uM d-butaclamol as the displacer. In the brain slice experiments, sites were labeled by (3H)Spiro and (3H)DHE and patterns of displacement of these sites by various typical and atypical neuroleptics were also examined.

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Like many other drugs used today, the discovery of antipsychotic drugs occurred by chance. In an effort to synthesize a strong barbiturate with prolonged sleeping time, Charpentier(1950) synthesized chlorpromazine based on the structure of a phenothiazine derivative, promethazine, a known antihistamine agent. Two years later Delay et al(1952)., noted this compound's efficacy in the treatment of schizophrenia. In 1959 Janssen et al., reported the synthesis and discovery of butyrophenones which differ structurally from chlorpromazine, but are pharmacologically and clinically similar. Today there are several classes of clinically used antipsychotics. Among these are the phenothiazines(such as chlorpromazine), the thioxanthenes(cis-flupenthixol), the dibenzoheteroepines(clozapine and loxapine), the butyrophenones(haloperidol and spiroperidol), the diarylbutylamines(pimozide), the benzamides(sulpiride and metoclopramide), the pentacyclics(d-butaclamol), and the indoles(molindone). The development of these structurally distinct compounds having similar clinical effects has allowed pharmacologists to decipher and select, from the various actions of these drugs, a common neurochemical effect elicited by these compounds. This effect may be directly related to the mechanism(s) of action of antipsychotic drugs, and may be of significance in elucidating the neuropathological basis of schizophrenia.

In the 1960's many studies on the biochemical properties of neuroleptic drugs were carried out. Among the effects described were their electron donating ability(Foster and Fyfe, 1966), membrane

stablizing effect(Seeman and Bialy, 1963), inhibition of oxidative phosphorylation(Medina et al; 1964); prevention of mitochondrial swelling(Spirtes and Girth, 1963), and inhibition of Na^+-K^+ activated ATPase(Davis and Brody, 1966). More recently, the inhibition of calmodulin activated cyclic nucleotide phosphodiesterase by neuroleptics has also been described(Levin and Weiss, 1977). However, these properties do not seem to be relevant to the therapeutic action of neuroleptics because (1) the concentrations required to produce an effect in these systems were generally high(> uM); (2) these properties were also found in clinically inactive compounds.

It was in 1963 when Carlsson and Lindqvist described a specific action of chlorpromazine(CPZ) and haloperidol(HAL) on catecholamine metabolism that a plausible mechanism for antipsychotic drug action was enunciated. They observed that low doses of CPZ and HAL but not the clinically inactive phenothiazine promethazine stimulated the accumulation of the O-methylated metabolites of dopamine(DA) and norepinephrine(NE) following monoamine oxidase inhibition. Since the levels of DA and NE were unchanged, it was inferred that not only the metabolism but also the synthesis of the catecholamines was accelerated by the antipsychotic drugs. It was known at that time that the awakening action of DA and NE(formed from administration of their precursor 3,4-dihydroxyphenylalanine) was

weakened by antipsychotics and CPZ was known to have alpha-blocking actions. They therefore hypothesized that CPZ and HAL acted by blocking DA and NE receptors and the acceleration of the synthesis and metabolism of these catecholamines was due to feedback activation of the respective neurons. This hypothesis was later confirmed by a variety of turnover studies in vivo, and the role of antipsychotic drugs as DA antagonists was firmly established. For example, Anden et al (1964) showed that levels of homovanillic acid(HVA) and 3,4-dihydroxyphenylacetic acid(DOPAC), two major DA metabolites, were markedly elevated in brains of rabbits treated with neuroleptics. With the use of ^{14}C-labeled tyrosine and phenylalanine, Nyback and Sedvall(1968) demonstrated that the synthesis and turnover of ^{14}C-DA in the striatum of mice were stimulated severalfold following neuroleptic treatment. Apomorphine, the DA agonist, produced the opposite changes. Although many neuroleptics are able to increase NE turnover, this action does not seem to be essential for antipsychotic efficacy. Pimozide and spiroperidol (Spiro) are two potent antipsychotics, but they produced less effects on NE turnover than clozapine, a weaker neuroleptic drug. Morover, phenoxybenzamine, a centrally active alpha-adrenergic blocking agent, appears to lack antipsychotic activity.

A number of behavioral tests have also been described for neuroleptic drugs which may be related to their anti-DA properties. For example, DA agonists apomorphine and amphetamine elicit beha-

vioral stereotypy(Randrup et al; 1963), a state of continuous repetition of sniffing, head bobbing, gnawing, and scratching. Neuroleptic drugs specifically antagonize stereotypy. Neuroleptic drugs induce catalepsy in animals, an abnormal motor state in which an animal can be maintained in bizarre postures(Munkvard et al., 1968), and this behavior has been ascribed to hypoactivity in the corpus striaum, a DA-rich brain area . Antipsychotic drugs also impair conditioned avoidance behavior in animals without affecting escape behavior(Cook and Catania, 1964). Conditioned avoidance response refers to a learned behavior in which an animal is trained to escape, when a signal, usually followed by a noxious stimulus is presented. In addition, neuroleptic drugs induce ptosis and inhibit apomorphine-induced emesis in dogs. These behavioral tests are commonly employed for screening potential antipsychotic drugs.

Electrophysiological studies also support the anti-DA properties of neuroleptic agents. Bunney et al(1973) studied the effects of d-amphetamine(AMP), an indirect DA agonist and neuroleptic drugs on the activity of DAergic neurons in the substantia nigra(SN) and ventral tegmental area(areas A9 and A10). d-AMP administered i.v. markedly decreased the spontaneous activity of DA neurons in these areas. Antipsychotic drugs increased the firing rate of these cells and reversed the AMP-induced depression. Groves et al(1975) studied the effects of iontophoretically administered HAL and AMP on the firing rates of a population of neurons

in the striatum and pars compacta of SN simultaneously. They found that caudal injection of AMP resulted in a decrease in the firing rate of striatal neurons but an increase in the firing rate of nigral neurons. Caudal injection of HAL caused an increase in the firing rate of striatal neurons and a decrease in the activity of nigral cells. Although their results on the effect of AMP on nigral neurons differed from that of Bunney et al , the effects of neuroleptic drugs on DA cells were always opposite to that of DA agonists.

In the past decade, research on neuroleptic drugs has centered on an attempt to directly characterize DA receptors by enzymatic and radioreceptor binding techniques. In addition to complementing previous turnover and behavioral studies in vivo, these assays in vitro have helped to reaffirm the relationship between the anti-DA properties and clinical efficacy of antipsychotic drugs. They have also contributed significantly to the realization of the existence of multiple DA receptors in brain. In the future, these assays may contribute significantly to the development of drugs with specific clinical efficacy and little untoward side-effects. The purpose of the following sections is to review studies on : (1) the regional selectivity of neuroleptic drugs. (2) The various DA receptor models in the periphery and brain. (3) The properties of DA-sensitive adenylate cyclase in brain. (4) the characterization of DA/neuroleptic receptors in the CNS by radioligand

binding assay and the controversies about the identity of these DA/ neuroleptic binding sites. (5) the classification of multiple DA receptors by their association with or independence from DA-sensitive adenylate cyclase and (6) the properties of DA receptors in the CNS and periphery. A brief outline of DAergic pathways in the CNS and DA synthesis and metabolism is included. Special emphasis will be placed on the properties of atypical neuroleptics such as the benzamide derivatives metoclopramide and sulpiride. Unlike the typical neuroleptics CPZ and HAL, these drugs possess contrasting properties on DA receptor models that have been taken as evidence for the existence of multiple DA receptors.

The following discussion deals only with antipsychotic drugs acting primarily as DA receptor blockers. The properties of reserpine, a drug with antipsychotic efficacy but acting presynaptically to deplete catecholamines, will not be discussed. The term 'neuroleptic' is used interchangeably with 'antipsychotic'. Proposed by Delay and Deniker, the word 'neuroletic' came from the Greek 'going to the nerve'. It is used to define an agent which induces behavioral patterns similar to CPZ-like drugs. Typically, these patterns include a true antipsychotic action in patients with acute and chronic psychosis, a state of affective indifference, and motor disturbance with Parkinsonian and other extrapyramidal symptoms. In addition, catalepsy is observed in animals(Carlsson, 1978).

Central DA Pathways

The discovery and morphological description of DA neurons was made by Falck et al(1962) and Hokfelt et al(1976) using histochemical fluorescence and immunohistochemical techniques. The greatest number of DA-containing cells are found in the brain stem at mesencephalic levels, with two main groups of DAergic cells. The first is known as the nigrostriatal system. Its cells bodies are in the pars comparta of the substantia nigra, which projects to the striatum. This pathway degenerates in patients with idiopathic and post-encephalitic Parkinson's disease(Hornykiewiz, 1974). The second major group of DAergic fibers is known as the mesolimbic system, with cell bodies found in midbrain but caudal and medial to the substantia nigra. The cells project to the nucleus accumbens, olfactory tubercle and cerebral cortex. These mesolimbic and mesocortical pathways have been implicated in the etiology of schizophrenia(Matthysse, 1973). Another DA pathway important to endocrinology has cell bodies in the arcuate nucleus of the the hypothalamus and nerve terminals synapsing on the portal vessels in the median eminence. It is involved in the control of pituitary hormone release, particularly prolactin(MacLeod, 1976).

DA Synthesis and Metabolism

DA is synthesized from tyrosine via two enzymatic steps. The first enzyme, tyrosine hydroxylase(TH) converts L-tyrosine to dihy-

pterindine cofactor, molecular oxygen, and Fe^{++} for catalytic activity. The stoichiometry of this reaction is formulated as follows(Nagatsu et al; 1964):

$$\text{L-tyrosine} + BH_4 + O_2 \text{ ----> } \text{Dopa} + QH_2B + H_2O$$

where BH_4 is tetrahydrobiopterin and QH_2B is the quinoid form of dihydrobiopterin. It is the rate-limiting step in the synthesis of catecholamines. A soluble enzyme, TH is found in all tissues that synthesize catecholamines. In brain the enzyme is concentrated in regions known to contain high levels of catecholamines (Coyle, 1972).

There are at least four classes of compounds that can inhibit TH: (1) amino acids and amino acid analogs such as alpha-methyl-tyrosine are competitive inhibitors of TH(Udenfriend et al; 1965). The inhibition is competitive with tyrosine(Nagatsu et al; 1964). (2) Catecholamines and other catechol derivatives inhibit TH competitively(Musacchio et al; 1973) with the reduced pteridine cofactor, and this end-product inhibition provids the mechanism for feedback regulation of catecholamine biosynthesis. (3) Iron chelating agents such as alphs,alpha'-dipyridyl(Nagatsu et al; 1964). (4) Naphthoquinones such as deoxyfrenolicin, an antibiotic produced by streptomyces fradiae, inhibit TH competively with tyrosine(Taylor et al; 1970).

The second enzyme, aromatic L-amino acid decarboxylase, con-

verts L-Dopa to DA. It is found in the cytoplasm and requires pyridoxal phosphate as a cofactor. Alpha-methyldopa and its hydrazino derivative, MK-486, are inhibitors of this enzyme.

In brain, DA is metabolized to DOPAC and HVA. Monoamine oxidase(MAO) oxidizes DA to an intermediate aldehyde which is further degraded by aldehyde dehydrogenase to DOPAC. Catechol-O-methyltransferase (COMT) methylates DOPAC to HVA. Alternately, DA may be metabolized first by COMT to 3-methoxytyramine and then oxidized by MAO to form HVA. Studies in rats have demonstrated that the turnover of DOPAC is greater than HVA, and it is believed that DOPAC is the major brain metabolite of DA in that species (Wilk et al; 1975). In humans, however, HVA is the major brain metabolite of DA(Wilk and Stanley, 1978).

MAO is a flavin-containing enzyme. It is found primarily in the outer membrane of mitochondria. Studies with rat brain MAO have shown that there are at least two types of MAO that can be differentiated by substrate and inbititor specificity(Squires,1976) NE and serotonin are preferentially deaminated by MAO type A, and DA is deaminated by both MAO A and B(Neff et al; 1973). The type A enzyme is selectively blocked by low concentrations of clorgyline and harmine. Type B is selectively blocked by deprenyl.

COMT is a soluble enzyme. It requires S-adenosylmethionine and Mg^{++} ion for activity. Methylation of the substrate is predominantly on the meta position of the catechol. Pyrogallol and

tropolone are examples of inhibitors(Billeau and Burba, 1961; Axelrod and Laroche, 1959).

Regional Selectivity

Many antipsychotic drugs including haloperidol(HAL) produce an extrapyramidal syndrome(EPS) indistinguishable from Parkinson's disease. On the other hand, clozapine(CLOZ), a dibenzodiazepine derivative, is an effective antipsychotic drug but it seems to lack the EPS-inducing property(Matz et al , 1974). Such a dissociation between EPS-inducing and antipsychotic properties of neuroleptic drugs has led to the hypothesis that these effects are due to blockade of different DA receptors in the brain. The antipsychotic effect has been proposed to be due to the blockade of mesolimbic DA receptors (Matthysse, 1973; Stevens, 1973), while the EPS effect has been attributed to the blockade of nigrostriatal DA receptors (Crow et al; 1976).

Anden and Stock(1973) compared the effects of CLOZ and HAL on DA metabolism in rabbit mesolimbic and striatal brain regions. They reported that CLOZ at 2.5 to 10 mg/kg i.v., produced a greater increase of HVA in the limbic area than in the striatum. In contrast, HAL at 0.005 to 0.05 mg/kg i.v., produced a similar HVA elevation in both brain regions. They concluded that DA receptors in the limbic areas may be blocked to a greater extent than those

in the striatum by CLOZ but not by HAL.

However, subsequent studies in rats have failed to substantiate the greater sensitivity of mesolimbic DA receptors toward CLOZ. For example, Wiesel and Sedvall(1975) studied the effects of HAL, chlorpromazine, CLOZ and thioridazine on HVA levels in the striatum and tuberculum olfactorium, a discrete mesolimbic region. They observed that the relative elevation of the content of HVA was significantly greater in the striatum than in the olfactory tubercle for all doses of drugs except thioridazine, which produced similar increases in both regions. Furthermore, a lower dose was required to double the content of HVA in striatum than TO, indicating that striatal DA receptors are more sensitive to these drugs. They concluded that although the antipsychotic effect may be localized to the limbic DA areas, their data can not disqualify the striatum as the site for the antipsychotic effect. In fact, their finding that CLOZ, which does not produce EPS but shows a marked effect in the striatum, may implicate the striatum as the area of antipsychotic action.

In our laboratory, Wilk et al(1975) also examined the effects of HAL and CLOZ on HVA and DOPAC levels in rat striatum and TO. From dose-response curves generated at the time of peak response for each drug, they observed, similar to Wiesel and Sedvall, that a greater dose of either drug was needed to achieve a half-maximal metabolite elevation in the TO as compared to the striatum. CLOZ

was shown to be more sensitive toward striatal DA receptors than those in the TO. In a subsequent study, Wilk and Glick(1976) found that the sensitivity of DA receptors to CLOZ was similar in the striatum and nucleus accumbens . These studies clearly demonstrated that the lack of EPS after admistration of CLOZ can not be ascribed to its selective interaction with DA receptors in the mesolimbic regions. Furthermore, in the DA-sensitive adenylate cyclase assay and in radioligand binding studies(Clement-Cormier et al ; 1974; Burt et al; 1976), no regional differences in drug potency have been observed for CLOZ. The lack of EPS following treatment with CLOZ has been ascribed to its anti-cholingergic properties(Snyder et al, 1974).

Tolerance

Clinically, neuroleptic drug treatment requires more than one week for a therapeutic response and treatment is continued on a chronic basis(NIMH, 1964). By contrast, neuroleptic-induced extrapyramidal syndromes disappear after repeated administration. If the mesolimbic area represents the site of antipsychotic action and the nigrostriatal area represents the site responsible for the extrapyramidal syndrome of neuroleptic drugs, it can be inferred that after chronic treatment with neuroleptic agents, the nigrostriatal area may develop a diminished response or tolerance to the effect of neuroleptic drugs, while the mesolimbic region may not. In our labo-

ratory, Stanley and Wilk(1980) have examined the chronic effects of CLOZ and HAL on DA metabolism in the striatum and TO. They found that in both regions chronic treatment with a high dose of HAL produced a diminished elevation of DOPAC levels as compared to acute treatment in both regions. By contrast, at a ED50 dose, no tolerance developed in either region. Chronic CLOZ treatment at 20 or 40 mg/kg i.p., did not affect the DOPAC elevating property of this drug administered acutely. These results do not support the hypothesis that mesolimbic and nigrostriatal DA neurons mediate the antipsychotic and EPS effects of neuroleptic drugs, respectively. Similar findings have been observed by Racagni et al in their studies on CLOZ and HAL(1980).

However, in contrast to results obtained by Stanley and Wilk (1980), Scatton(1977) reported that after 11 days, tolerance to the increase in DA turnover occurred at the ED50 dose of HAL and sulpiride. This phenomenon occurred in the striatum, nucleus accumbens, TO and frontal cortex of the rat. The threshold dose inducing this effect was found to be lower in the striatum than in the limbic and cortical regions. With a single dose of trifluorperazine(2.5-3.5 mg/kg/day), Clow et al(1979) also observed that the increase in HVA and DOPAC level after acute administration(1-2 weeks) disappeared after 6 months of treatment. This effect was accompanied by the development of DA receptor supersensitivity(as seen in increased

DA-adenylate cyclase sensitivity, in increased affinity for (3H)Spiro binding sites, and in apomorphine induced rotation).

Recently Bacopoulous et al reported that treatment with HAL(0.5 mg/kg) for 3 or 5 weeks induced no change in the level of HVA(the major DA metabolite in monkeys) in the olfactory cortex, basal ganglia, cisternal cerebrospinal fluid or plasma of monkeys. By contrast, significant increases of HVA were seen in dorsal and orbital frontal cortex and cingulate cortex. The continued elevated HVA content in frontal cortical regions demonstrated that tolerance to the effects of HAL does not occur uniformly throughout the brain but is characterized by some regional specificity. Since the therapeutic effects of antipsychotic agents are not susceptible to tolerance , these authors contended that the persistent alteration in cortical DA metabolism may be related to the therapeutic efficacy of neuroleptic drugs. Therefore, it would seen that the development of tolerance is dependent upon the dose of drug administered, on the brain regions studied, and seems to be corelated with the development of DA receptor supersensitivity.

DA-Stimulated Adenylate Cyclase

Many studies have shown that various hormones affect the intracellular content of cyclic AMP(adenosine 3'5'-monophosphate) through actions on tissue-specific receptors and thereby initiate a tissue-

specific response. Because of the difficulty in quantitating the physiological response elicited by neurotransmitters in the CNS, studies on the effects of antipsychotic drugs on DA neurotransmission were made only indirectly. Therefore, the discovery of a DA-sensitive adenylate cyclase enzyme evoked the promise of a possible clue as to the biochemical mechanism of DA action in the CNS. However, as more studies were performed, it became clear that the many physiological roles implicated for DA could not all be explained on the basis of its interaction with DA-sensitive adenylate cyclase alone. To date, the physiological role of this enzyme remains unclear.

DA-sensitive adenylate cyclase was first demonstrated in postganglionic sympathetic neurons(Kebabian and Greengard, 1971) and later characterized in DA-rich brain areas such as caudate, nucleus accumbens, tuberculum olfactorium, medium eminence, and in areas containing DAergic dentrites, the zona reticulata of substantia nigra, and retina(Clement-Cormier et al; 1974; Kebabian et al; 1972; Clement-Cormier and Robinson, 1977; Kebabian and Saavedra, 1976; Brown et al; 1972). In their original report Kebabian et al(1972) showed that in the striatum the sensitivity of DA for this enzyme was about 10 times greater than l-norepinephrine and that l-isoproterenol was inactive in stimulating this enzyme. The activity of this enzyme, measured by cAMP content, is doubled in the presence of 10 uM DA, and a half-maximal increase in enzyme activity was achieved with about 4 uM DA. Of interest, the DA agonist apomor-

phine behaved as a partial agonist in this system(30 % increase in basal activity). At low concentrations(<10 uM) apomorphine increased the cAMP level, but at higher concentrations, it decreased the cAMP content. This antagonistic action in vitro by apomorphine on this enzyme was unanticipated, as this compound behaved as a pure agonist in vivo(Ungerstedt et al; 1969; Anden et al; 1967).

Lergotrile and bromocryptine are two ergot derivatives which mimic the DAergic inhibition of prolactin release from anterior pituitary(Clemens et al; 1972). Their efficacy in the treatment of Parkinsonism and in animal behavioral models indicates that these compounds function as agonists on the postsynaptic DA receptor in the corpus striatum(Lieberman et al; 1975; Calne et al; 1978; Silbergeld and Pferffer, 1977). However, in the adenylate cyclase assay, lergotrile was inactive. Furthermore, lergotrile inhibited uncompetitively the activation of this enzyme by DA(Kebabian et al; 1977). This observation indicates that DA receptors linked to adenylate cyclase may not be involved in relieving the symptoms of Parkinsonism.

Various antipsychotic phenothiazine derivatives caused a dose-dependent competitive inhibition of DA-stimulated adenylate cyclase activity, and their affinity paralleled their clinical potencies(Kebabian et al; 1972; Clement-Cormier and Robinson, 1977). Promethazine, a non-antipsychotic phenothiazine is an inactive antagonist. In addition, the receptor site of this enzyme is stereospecific. Thus, the clinically active d-butaclamol and cis-flupenthixol are

more potent than their clinically inactive enantiomers. However, it should be noted that the clinically potent butyrophenone antipsychotic drug, haloperidol, which is 20 times more potent than chlorpromazine in increasing DA turnover (Anden et al; 1970), is relatively weak in the DA-adenylate cyclase assay. It is three times weaker than chlorpromazine(Clement-Cormier et al., 1974). In addition, benzamide antipsychotic drugs sulpiride and metoclopramide are virturally inactive as DA antagonists in this assay(Roufogalis et al; 1976; Spano et al; 1978), despite their activity in enhancing DA turnover and prolactin release(Peringer et al; 1976; Stanley et al; 1979; Meltzer et al; 1979), suggesting that these compounds elicit their anti-DA effects independent of a cyclase-linked receptor. These discrepancies between the in vivo and in vitro activities of the benzamide and butyrophenone antipsychotics and ergot derivatives suggested that in addition to the DA cyclase-linked receptors, there must exist DA receptors which are independent of cyclase activity.

DA Vascular Receptors

In the kidney, infusion of DA into the renal artery results in vasodilation(Yeh et al; 1969). This DA-induced effect can be quantitated in the laboratory by measuring the decrease in renal blood pressure and increase in renal blood flow. As this effect is not blocked by propranolol, atropine, phenoxybenzamine, pretreatment with reserpine, monoamine oxidase inhibitors, histamine deple-

ting agents, or interruption of sympathetic nerve control, but is antagonized by haloperidol and phenothiazines, this phenomenon has been explained as a post-synaptic DA receptor mediated event. For some 15 years Goldberg et al(1978) have studied the structure-activity relationships of several hundred analogs of DA on this post-synaptic DA receptor model. It was found that the structural requirements for activation of DA vascular receptors is extremely limited, and seems to be similar to the DA-sensitive adenylate cyclase receptors in brain. Thus active DA vascular agents studied thus far require two hydroxyl groups on positions 3 and 4 of the catechol nucleus and unsubstituted alpha and beta carbons. Substitutions of the amino group is limited to one methyl in mono-substituted derivatives(as in epinine) and to a propyl or butyl group as one of the substituents in di-N-substituted compounds. Studies of the rigid analogs of DA suggest that the preferred conformation of activation of DA vascular receptors is the transoid beta rotamer of DA. Thus 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene(A-6,7-DTN) is active while the alpha rotamer(A-5,6-DTN) is not. Apomorphine is only a partial agonist on DA vascular receptors. However, the similarity between the post-synaptic DA vascular receptor and the DA-sensitive adenylate cyclase receptor is not found for the antagonists(Goldberg and Kohli, 1979). The substituted benzamide sulpiride and bulbocapnine are the most potent antagonists on the DA vascular receptor, followed by the phenothi-

azines chlorpromazine, fluphenazine and thioridazine which are in the same potency range. The butyrophenone derivative haloperidol is equipotent to chlorpromazine. It should be noted that in the DA-sensitive adenylate cyclase assay, sulpiride is virturally inactive(Roufogalis et al; 1976; Spano et al; 1978), and that the phenothiazine fluphenazine is much more potent than chlorpromazine and thioridazine(Clement-Cormier et al; 1974). Differences in potencies of antagonists acting on the DA vascular receptor and DA-sensitive adenylate cyclase in the striatum and the similarity in agonist potencies for these two systems exemplifies the differences between the peripheral and central DA receptors.

The differences between the DA vascular receptors, DA receptors influencing prolactin release and receptors mediating DA turnover can also be seen with enantiomers of sulpiride. The (-) enantiomer of sulpiride is much more potent than the (+) enantiomer in influencing prolactin release and DA turnover in the CNS(Hofmann et al; 1979). In contrast, the (+) enantiomer is much more potent than (-) as an antagonist of DA vascular receptors.

DA/Neuroleptic Radioreceptor Binding Studies

Since 1975 when Seeman(1975) and Burt et al(1975) first described independently the characterization of DA receptors by radioreceptor binding techniques, there has been much controversy regarding the molecular identity of DA binding sites. In their

original studies Burt et al (1975) showed that binding sites labeled by the DA agonist (3H)DA and the antagonist (3H)haloperidol(Hal) were saturable and stereospecific, existed only in membranes prepared from DA rich areas such as the striatum, nucleus accumbens, and olfactory tubercle, and that a good correlation was found between the affinities of DA antagonists, i.e., neuroleptic drugs, for (3H)Hal sites and their average clinical dose(Snyder et al; 1975; Creese et al; 1976; Burt et al; 1976). As sites labeled by the (3H)DA were not diminished after 6-hydroxydopamine(6-OHDA) infusion which destroys DA neurons, they postulated that these sites labeled by the DA agonist and antagonist were postsynaptic receptors(Burt et al; 1975). However, it was revealed that while the binding of (3H)DA, the agonist, was strongly antagonized by the agonists in a competitive manner(Hill coefficient n=1), it was weakly antagonized by the antipsychotic drugs in a noncompetitive fashion(n= 0.5). The converse happened for the antagonism of (3H)Hal binding(Burt et al; 1975; Burt et al; 1976). As DA antagonists were supposed to act via DA receptors, Burt et al proposed that (3H)DA and (3H)Hal were labeling the same DA receptors, and that (3H)DA and (3H)Hal labeled the agonist and antagonist state of the interconvertible DA receptors, respectively(Creese et al; 1975). This hypothesis, although plausible because of the similar distribution in the binding sites for these ligands, does not preclude the po-

ssibility that different populations of binding sites may have been labeled by these ligands.

Such a two-state hypothesis for DA receptors has been challenged recently. For example, Nagy et al(1978) studied binding of the labeled DA agonist, apomorphine, and the antagonist, Spiro in the striatum and substantia nigra(SN) following unilateral 6-OHDA infusion. They observed that compared to the unlesioned side, there was a decrease in the number of binding sites for (3H)apomorphine in the striatum(56%) and SN(76%) in the lesioned side, while binding of (3H)Hal or (3H)Spiro were increased in the striatum(20%) and no change was detected in SN of the lesioned side. This differential change in the binding sites for DA agonists and antagonists following 6-OHDA lesion suggests that there is a difference in the location of binding sites for these radioligands. Other studies also have shown that properties of binding sites for (3H)agonist and antagonist are different. For example, Titeler et al(1978a) showed that (3H)apomorphine and (3H)Spiro binding sites are located in different subcellular fractions. Lew and Goldstein (1979) demonstrated that binding proteins for (3H)DA were more heat labile than (3H)Spiro binding proteins. Based on these observations, Seeman has proposed that high affinity receptor binding proteins for agonists and antagonists are located in different areas; DA agonists preferentially bind to presynaptic DA receptors and DA antagonists bind primarily to postsynaptic receptors in the striatum (Nagy et al; 1978; Titeler et al; 1978b). However, this hypothesis

was challenged by Creese and Snyder(1979) who showed that, in contrast to results obtained by Nagy et al, binding of (3H)apomorphine and (3H)Spiro was increased following 6-OHDA lesion, and they have maintained that both types of ligand bind to post-synaptic DA receptors.

In several studies the anatomical location, pharmacological specificity and regulation by ions in receptor binding were investigated. It was shown, for example, that the binding of (3H)Hal and (3H)Spiro were heterogeneous in the striatum(Briley and Langer, 1978; Pedigo et al; 1978; Clement-Cormier and George, 1979). These ligands, in addition to labeling sites intrinsic to receptors in the striatum, also labeled sites on neurons projecting from cortical regions(Schwartz et al; 1978; Garau et al; 1978). Kainic acid injection, which destroyed neurons in the caudate but spared fibers passing through the region, decreased binding of (3H)Hal and (3H) Spiro by 50% and the majority of the remaining sites were abolished after cortical ablation. In addition, binding of the agonists (3H) apomorphine and (3H)2-amino, 6,7-dihydroxytetrahydrotetralin(ADTN) to striatal tissues were decreased in the presence of guanine nucleotides, guaninosine triphosphate(GTP) and guanosine diphosphate, while binding of the antagonist (3H)Spiro was not. This guanine-induced decrease in binding of (3H)apomorphine was shown to be due to a decrease in the affinity of this ligand while the maximal binding capacity was unaffected(Creese et al; 1979a; Creese et al;

1979b). In competition experiments guanine nucleotides selectively decreased the potencies of DA and apomorphine, for (3H)Spiro sites, while the affinities of the DA antagonists and ergot derivatives were not changed in the presence of guanine nucleotides. Furthermore, after kainic acid lesion the remaining sites labeled by (3H)Spiro and (3H)apomorphine were no longer sensitive to the effects of guanine nucleotides. As kainic acid lesion completely abolished DA-sensitive adenylate cyclase activity in the striatum, and as guanine nucleotides played an important role in the coupling of receptor and enzyme activation, it was speculated that kainic acid sensitive binding of (3H)apomorphine and (3H)Spiro may be linked to DA-sensitive adenylate cyclase(Creese et al; 1979a), whereas sites originating from cortical areas were independent of cyclase activity and therefore insensitive to guanine nuceleotide regulation.

One other significant aspect of (3H)Spiro binding was discovered by Leysen et al(1978a). They showed that although affinities of antipsychotic drugs for (3H)Spiro binding sites paralleled their anti-DA activities in the striatum, competition against (3H)Spiro binding by these drugs in the frontal cortex was highly correlated with their anti-5-HT activities. This observation has been extended to 5-HT rich areas such as the hippocampus(Creese and Snyder, 1978). Therefore, the heterogeneous nature of (3H)Spiro binding may be in part due to its interaction with serotonergic sites.

Although a portion of the (3H)Spiro binding sites in the

striatum have been proposed to be linked to DA-stimulated adenylate cyclase(Creese et al; 1979a), it should be noted that these two systems are not identical. First, DA-stimulated adenylate cyclase activity is concentrated in the mitochondrial fraction, whereas most of the (3H)Hal binding activity is found in the microsomal synaptosomal fractions(Clement-Cormier and George,1979; Leysen and Laduron; 1977). Secondly, there is a good correlation between antagonism of (3H)Spiro binding and clinical and pharmacological activities in vivo for antipsychotic drugs, but no such correlation was found to exist between antagonism of DA-stimulated cyclase and properties in vivo of neuroleptics(Creese et al; 1976; Leysen et al; 1978a).

More recently Hyttel characterized binding of (3H)cis-flupenthixol(Flu) to DA rich areas in striatum and found that there was a good agreement between the ability of antipsychotic drugs to displace (3H)Flu and their ability to reverse DA-stimulated adenylate cyclase(Hyttel, 1978). He proposed that cis-Flu may label predominantly cyclase-linked DA receptors. This hypothesis was supported by a kainic acid study(Creese and Sibley, 1979). Kainic acid lesion which reduced most of DA-sensitive adenylate cyclase activity in the striatum also decreased most of cis-Flu binding. Cross and Owen (1980) studied (3H)Flu binding and found that antagonism by butyrophenone drugs against (3H)Flu exhibited biphasic inhibition curves. They estimated that 20% of the (3H)Flu binding may be to sites not linked to the cyclase. Despite this evidence, it is difficult to explain why cis-Flu when used as a radioligand should selectively

label cyclase-dependent sites. This drug has high affintiy for sites labelled by (3H)Spiro(Kd=3.2 nM), and (3H)Hal(Kd=0.8 nM), two ligands believed to label mostly cyclase independent sites (Cross and Owen; 1980; Burt et al; 1976; Kebabian and Calne; 1979). Therefore, one would not expect that, when assay conditions were similar for (3H)Spiro and (3H)Flu, binding of (3H)-Flu could selectively interact with cyclase linked DA receptors.

Postganglionic Sympathetic DA Receptors

Recently Steinsland and Hieble (1978) reported on an appropriate model for studies of DA receptor mechanisms in the isolated rabbit ear artery. In this system electrical stimulation induces a vasoconstrictor response by releasing NE from postganglionic sympathetic nerve terminals which activate postsynaptic alpha-adrenoreceptors. DA receptors localized in the postganglionic neurons can inhibit the electrically evoked release of NE and thereby reduce its vasoconstrictor effect. This action of DA is not blocked by propranolol, the beta-adrenergic antagonist. Also DA does not inhibit the vasoconstrictor response to exogeneously administered NE, in agreement with the concept that inhibition of neurotransmission by DA appears to result from an interference with the release of NE. DA and apomorphine(K= 40 nM) are equipotent as agonists in this system, and in the concentration range of 3-1000 nM they reduced the perfusion pressure in a dose-dependent manner.

Higher doses of either drug produced vasoconstriction due to activation of alpha-adrenoreceptors. Antipsychotic drugs haloperidol, perphenazine, d- and not l-butaclamol and other neuroleptics caused parallel shifts to the right in the dose-response curve for DA, suggesting that they act as competitive antagonists in this system. In addition, their Ki values were in good agreement with their reported values in antagonizing (3H)Hal binding in the CNS. It should be noted that this response to DA can not be due to activation of presynaptic alpha-receptors(alpha2), as the response to clonidine, a selective alpha2 agonist, which also inhibits NE release, is not antagonized by high concentrations of haloperidol. Similarly, tolazoline, shown to be a selective alpha2 antagonist in this system, failed to reverse DAinduced vasodilation. These observations suggest that DAergic inhibition of adrenergic neurotransmission is a good model for studies on DAergic receptor mechanisms. However, this model has not been used extensively probably due to (1) the slow onset for antagonist effect(1 hr) before antagonism of DA action by haloperidol, (2) long washing out periods(7 hrs for haloperidol), (3) limited number of drugs and tissues that can be monitored at the same time.

It should be noted, however, that the affinities of neuroleptic drugs for the postganglionic sympathetic DA receptor are similar to the affinities of these drugs for postsnaptic (3H)Hal binding sites in the striatum. This is in marked contrast to the DA-sensitive adenylate cyclase receptor in the striatum where the butyrophenones

are relatively weak antagonists and to the postsynaptic DA vascular receptors where similar potencies for various neuroleptic drugs were found.

The Classification Of Multiple DA Receptors

Recently Kebabian and Calne(1979) classified DA receptors in the brain and periphery into two subclasses depending on their relationship to adenylate cyclase activity. DA receptors that are coupled with adenylate cyclase activation are designated as D1, and those that are not coupled with cyclase activation are termed D2. This biochemical classification for multiple DA receptor subtypes was based on the realization that : (1) mechanisms controlling secretions of parathyroid hormone(PTH) and prolactin by DA receptors are different, (2) DA receptors in the striatum can be subdivided and selectively examined by different chemical lesions, and (3) the apparent selective nature of benzamide antipsychotic drugs and ergot derivatives point to more than one class of DA receptors.

DA receptors in the anterior pituitary mediate the inhibition of prolactin release(Bishop et al; 1972; Takahara et al; 1974). Radioreceptor binding studies using (3H)dihydroergocryptine(DHE) (Caron et al; 1978) have demonstrated the existence of DAergic binding sites with properties expected of DA receptors: There is a good correlation between the affinities of DA agonists and antagonists for (3H)DHE sites and their potencies in decreasing and elevating prolactin levels, respectively. However, these DA receptors do not seem to mediate their physiological effects via

activation of adenylate cyclase, as high concentrations of DA, apomorphine and DA ergots all failed to elevate cAMP levels(Schmidt and Hill, 1977; Spano et al; 1978). Cholera toxin, the nonspecific activator of adenylate cyclase, has no effect on prolactin release (Gill, 1977). Bovine parathyroid cells, on the other hand, seem to possess DA receptors that are coupled to adenylate cyclase and activation of this enzyme has been correlated with increases in the release of parathyroid hormone(Brown et al; 1978). These studies on the release of PTH and prolactin indicate that DA receptor mediated events in a given tissue may or may not be linked to cAMP activity.

Chemical lesion and radioreceptor binding studies have also suggested the presence of separate D1 and D2 receptors in the striatum. As mentioned, DA and DA analogs can stimulate the activity of adenylate cyclase, leading to an increase in cAMP levels in the striatum(Kebabian et al; 1972; Clement-Cormier et al; 1974). Intrastriatal injection of kainic acid, a glutamate analog which seletively destroys neuronal cell bodies but spares the DAergic nerve terminals, completely abolishes the DA-stimulation of adenylate cyclase(Schwartz et al; 1978; Garau et al; 1978; Creese et al; 1979a). This indicates that DA receptors coupled to the cyclase are located postsynaptically and are intrinsic to neuronal elements in the striatum. In contrast, nigral injection of 6-hydroxydopamine(6-OHDA) which selectively eliminates nigrostriatal DA neurons, does not reduce DA stimulation(Krueger et al; 1976). This indicates that DA receptors located on DA nerve terminals are not coupled to cyclase activity(D2 receptors).

Radioreceptor binding studies with (3H)Spiro, Hal, DA, apomorphine, and DHE have shown that properties of these binding sites are different from the properties of DA-stimulated adenylate cyclase in the striatum(Burt et al; 1976; Creese et al; 1976; Leysen et al; 1978a; Tittler et al; 1977). On the other hand, (3H)Flupenthixol (Flu) seems to label sites with properties similar to DA-stimulated adenylate cyclase(Hyttel, 1978; Cross and Owen, 1980). These observations have led to the suggestion that (3H)Spiro and (3H)Flu label predominantly the D2 and D1 receptors, respectively(Creese et al; 1979a; Cross and Owen ; 1980).

Several ergot derivatives seem to be able to distinguish different DA receptors. Bromocryptine, lisuride and lergotrile are extremely potent DA agonists in the anterior pituitary (Caron et al; 1978; Clemens et al; 1972). These compounds also decrease the turnover of DA (Keller and Da Prada ; 1979) and are used clinically as DA agonists in the treatment of Parkinsonism(Calne, 1978; Fuxe et al; 1978). However, lisuride and lergotrile do not stimulate DA-sensitive adenylate cyclase(Kebabian et al; 1977). Furthermore, at high concentrations, they are antagonists of DA-stimulated adenylate cyclase. Although bromocryptine has been shown to activate adenylate cyclase, this activation occurs at a very high concentration(Schorderet, 1976). Because at low concentrations ergot alkaloids seem to possess DA-agonist properties in the anterior pituitary without affecting adenylate cyclase activity, it may be assumed that these drugs may interact specifically with D2 receptor sites at

low concentrations.

Among the various classes of antipsychotic drugs, only butyrophenones, molindone and benzamides seem to discriminate between various DA receptors. Antipsychotic drugs of the phenothiazine and thioxanthene classes strongly antagonize DA receptors linked to adenylate cyclase activity(Kebabian et al; 1972; Clement-Cormier; 1974). In contrast, butyrophenones are relatively weak as DA antagonists of the cyclase system compared with their strong potency in other DA receptor systems(Caron et al; 1978; Burt et al ; 1976; Wilk et al; 1975; Kebabian et al; 1972). The benzamides such as metoclopramide and sulpiride, and the indole-derivative molindone are antipsychotic agents(Stanley et al; 1979; Benoit et al; 1969; Gallant and Bishop; 1968). They increase DA turnover in vivo(Peringer et al; 1976; Stanley and Wilk; 1979; Bunney et al; 1975) . Moreover, metoclopramide and sulpiride have been shown to be potent DA antagonists in the anterior pituitary(Meltzer et al; 1979). However, these drugs are virtually inactive in the DA-stimulated adenylate cyclase assay(Roufogalis et al; 1976; Spano et al; 1978; Cross and Owen; 1980) and are weak in (3H)neuroleptic binding assays.

In summary, the significance of D1 receptors in the control of parathyroid hormone release has been established, but its role in the CNS is unclear. The importance of D2 receptors in the control of prolactin is well characterized, and it may be involved with symptoms of Parkinsonism and schizoprenia. The ergot derivative bromocryptine, which seems to be a specific D2 agonist, has been used with some success in the treatment of Parkinsonism. It has also

been reported to induce a florid paranoid psychosis(Kebabian and Calne, 1979). The antipsychotics molindone and the benzamide derivatives behave as specific D2 antagonists; their efficacy in the treatment of schizophrenia implicates D2 receptor dysfunction in schizophrenic patients.

Although this biochemical classification of DA receptors provides a simple means of designating various DA receptors, there are several important issues needed to be considered: First, the observed pharmacological properties of a drug may be dependent on the tissue in which they occur. For example, apomorphine behaves as a partial D1 receptor agonist in the striatum(Kebabian et al; 1974), but it lacks any agonist activity on the parathyroid D1 receptor. The IC50 of fluphenazine in antagonizing the DA-stimulated adenylate cyclase in the parathyroid gland is about 60 nM (Brown et al; 1977). This is ten times weaker than its potency in antagonizing the DA stimulated adenylate cyclase in the striatum. Similarly, the butyrophenone spiroperidol is 20 times weaker in the parathyroid system than in the striatal system. Secondly, although there are selective D2 receptor agonists and antagonists, there are no known selective D1 receptor agonists and antagonists at present. Binding studies using (3H)Flu have indicated that this ligand may predominantly label D1 sites, but studies have reported that this compound is a potent antagonist on the D2 receptor in the anterior pituitary(Caron et al; 1978), and in (3H)Spiro binding (Cross and Owen, 1980). Thirdly, it is difficult to classify DA receptors in the renal artery using this biochemical approach. DA vascular receptors in the renal artery have an agonist profile similar to

that found in DA receptors in the striatum(Goldberg and Kohli, 1979). Thus, DA, epinine and the DA rigid analog 2-amino,6,7-dihydroxytetrahydrotetralin are full agonists in both systems (Kebabian, 1978; Goldberg and Kholi; 1979). However, the benzamides sulpiride and metoclopramide, two reported specific D2 antagonists, have been shown to be most specific antagonists on the DA vascular receptors(Goldberg et al; 1978) . Furthermore, whereas the potency of fluphenazine was greater than chlropromazine on the D1 system in the striatum, the potency of fluphenazine in the vascular system is comparable to chlorpromazine. Although the measurement of potencies for drugs in the DA vascular receptor is based on a physiological response rather than a biochemical measurement of cAMP levels, it is expected that when two receptor systems possess similar agonist profiles, their antagonist profiles can be expected to be similar. This has not been observed for the DA vascular and striatal D1 sites.

Objectives Of The Experiments

The objectives of this research were to (1) compare the antiDA properties in vivo of antipsychotic drugs with their ability to compete for (3H)Spiro binding sites. This study could test the reliability of binding assays in predicting the anti-DA properties in vivo of antipsychotics. (2) To examine the properties of binding sites labeled by (3H)DHE in the striatum. Proposed by Kebabian and Calne(1979) as a specific D2 receptor ligand, (3H)DHE sites may show high affinity for the benzamide neuroleptics, meto-

(see pg. 163)

Some neuroleptics can be classified as atypical on the basis of apparently anomalous properties. For example, drugs such as clozapine and sulpiride are antipsychotic agents but they produce few if any extrapyramidal side effects, a prominent sign of DA receptor blockade(Gross and Langer 1966, 1970; Benoit et al., 1969; Haase et al., 1974). The benzamide derivative, metoclopramide, produces catalepsy and extrapyramidal effects(Jenner et al., 1978 a, b), elevates homovanillic acid and DOPAC levels in striatum and tuberculum olfactorium(Peringer et al., 1976; Stanley and Wilk, 1979), possesses antipsychotic activity(Stanley et al., 1979), yet it does not antagonize DA stimulation of adenylate cyclase (Roufogalis et al., 1976; Jenner et al., 1978a, b) and competes poorly with (3H)Spiro for binding sites in rat striatum(Howard et al., 1978). The dibenzoheteroepine derivative, perlapine, increases DA turnover (Wilk and Stanley, 1977), induces catalepsy(Burki et al., 1975), increases prolactin secretion(Meltzer et al., 1977), but only weakly antagonizes DA stimulation of adenylate cyclase(Miller and Hiley, 1976) and is considered to lack antipsychotic efficacy(Wander Ltd., Berne, Personal communication). Because of their anomalous effects on DA systems, these atypical neuroleptics have become valuable probes in elucidating the relationship between DA and antipsychotic effects(see Table 1A for a summary of the properties exhibited by the atypical neuroleptics). (page 164)

As an extension of studies on DA turnover in this laboratory, we were interested in examining the effects of these atypical as well as typical neuroleptics on binding of (3H)Spiro and to

compare their effects with their properties in vivo. This study could show the value of binding assays in predicting properties in vivo of neuroleptic drugs. Accordingly, we characterized (3H)Spiro binding to calf striatal membranes and examined the effects of an extensive series of atypical and typical neuroleptic drugs and two clinically inactive butyrophenones on (3H)Spiro binding. This work constituted the first series of experiments.

In the second study, we characterized (3H)DHE binding to calf striatal membranes using a low concentration of d-butaclamol as the masking drug. Proposed by Kebabian and Calne as the specific radioligand for D2 receptor sites, (3H)DHE binding sites are of particular interest because they may label sites where the benzamide neuroleptics act in vivo. These drugs are inactive in the adenylate cyclase assay and are exceptionally weak in the (3H)Spiro binding assay, despite their activity in vivo. Since (3H)DHE and d-butaclamol have high affinities for other receptor sites, we decided to lower the concentration of d-butaclamol, the displacing drug, to define a specific binding DA binding site. This approach seemed to have enhanced the specificity of the (3H)DHE binding system.

In the third study, we characterized (3H)Spiro binding to calf striatal membranes using a low concentration of d-butaclamol as the masking drug. (3H)Spiro binding to striatal tissues was heterogeneous when a high d-butaclamol concentration was used as the masking drug. In an effort to enhance the specificity of binding , we lowered the concentration of the displacing drug and examined the properties of (3H)Spiro binding under these conditions. This

study demonstrates the superiority of using low masking ligand concentrations in radioreceptor binding assays.

In the fourth study, we examined binding of radioligands to cryostat cut slide-mounted brain slices. Patterns of displacement of radiolabeled sites were studied by atypical and typical neuroleptics. A comparison was made between the results of this system with that of the membrane preparations.

In the final experiments, we studied the effects of metoclopramide and sulpiride on DA turnover in vivo in hypophysectomised rats . Portaleone et al(1978) reported that the ability of these drugs to increase DA turnover in the brain is lost in hypophysectomized rats, suggesting that hormones secreted by the pituitary were responsible for the central antiDA properties of these drugs. To confirm their results, we examined the effects of these agents on DA metabolism in hypophysectomised rats and compared them to the results obtained in the intact animal. In addition, we studied the effects of sultopride and tiapride, two benzamide derivatives, on DA turnover.

Materials for Radioreceptor Binding Studies

Fresh calf brains were obtained on ice from a slughterhouse. Striata and other regions were dissected out and frozen in a -80°C freezer for periods of up to 2 weeks. (3H)Spiro(specific activity 23 Ci/mmol) , (3H)DHE(specific activity 31 Ci/mmol), and Formula-963 scintillation cocktail were purchased from New England Nuclear, Boston, MA. The purity of (3H)Spiro (>93 %) was checked by a thin layer chromatography system(chloroform:methanol(9:1)). The purity of (3H)DHE was checked by two thin layer chromatography systems (chloroform:toluene:ethanol: ammonium hydroxide(4:2:1: 0.1) ; chloroform:ethanol: glacial acetic acid(9:5:1)). Whatman glass fiber GF/C filters and ascorbic acid were purchased from Fisher Scientific Co. Springfield, NH. The drugs were generously supplied by the following companies: DHE, clozapine, perlapine, thiethylperazine(Sandoz, East Hanover, NJ); lergotrile(Eli Lilly, Indianapolis, IN); loxapine(Lederle, Pearl River, NY); sulpiride(Warren-Teed, Horsham, PA); metoclopramide (A.H. Robbins, Richmond, VA); spiroperidol, domperidone, and penfluridol (Janssen, Beerse, Belgium); tiapride and sultopride(Delagrange, Paris, France); chlorpromazine (Smith, Kline, and French, Philadelphia, PA); d- and l-butaclamol (Ayerst, Montreal, Canada); fluphenazine(E.R. Squibb, Princeton, NJ); U-25, 927(Upjohn, Kalamazoo, MI); haloperidol(McNeil, Fort Washington; PA); cis- and trans-flupenthixol(H.Lundbeck, Copenhagen, Denmark); pargyline(Abbott, North Chicago, IL). Phentolamine (Ciba Geigy, Summit, NJ). Serotonin(5-HT), l-norepinephrine((-)NE), procainamide, methysergide, and bovine serum albumin were purchased from Sigma, St.Louis, MO.

Membrane Preparations

Bovine striata and other regions were homogenized in 50 volumes of ice-cold 50 mM Tris HCl buffer, pH 7.7 at 25°C, with a Brinkman polytron PT-10(setting 3, 15 sec). The homogenate was centrifuged at 1000 x g for 10 min(Sorvall RC 2B, rotor-34). The pellet (P1) was discarded and the supernatant (S1) was centrifuged at 39,000 x g for 10 min. The 39,000 x g pellet(P2) was washed and rehomogenized with a Teflon glass homogenizer(setting 7, 15 up and down strokes) in fresh Tris-HCl buffer and recentrifuged at the same speed. The final pellet was then rewashed and rehomogenized at a final concentration of 20 mg original wet weight per ml in a cold, fresh 50 mM Tris HCl buffer containing 0.1% ascorbic acid, 10 uM pargyline, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.1 at 37°C. This final suspension was placed in a 37°C bath for 5 min and returned to ice.

Procedures of Radioreceptor Binding Studies

In saturation experiments, 100 ul of increasing concentrations of radioligand, diluted with 0.1% ascorbic acid, 100 ul of 0.1% ascorbic acid or d-butaclamol and 800 ul of tissue suspension were added to each tube. The range of radiolabel used was 10^{-11} to 10^{-9} M. All determinations were in triplicate.

In competition experiments, 100 ul of radioligand, 50 ul of 0.1% ascorbic acid or d-butaclamol solution and 800 ul of tissue suspension were added to each tube.

All tubes were incubated at 37°C when (3H)Spiro was used. Tubes were incubated for 25 min when 1 uM d-butaclamol was used to define nonspecific binding and 30 min when 4 nM d-butaclamol was used to define nonspecific binding. When (3H)DHE was used, incubation was done at 25°C for 100 min under dim light. Incubation was terminated by rapidly filtering the contents through Whatman glass fiber GF/C filters under vacuum . After three 5 ml rinses with ice-cold 50 mM Tris HCl buffer, pH 7.7 at 23°C, the filters were placed in glass scintillation vials with 9 ml of Formula963 scintillation cocktail, shaken in the dark for 15 min and analysed by scintillation spectrometry(Beckman LS-250) at 45-50 % counting efficiency.

Analysis of Binding Data

Data from binding experiments were analysed by the PROPHET computer of the National Institute of Health. Based on the Michaelis-Menten equation, B = Bmax (S) / (Kd + S), a computerized procedure calculates the dissociation constant (Kd) and the total receptor density(Bmax) from the data of saturation experiments. It also provides the user with the Scatchard(1949) and the Hill analysis(1913).

The Hill equation is : Log(B/(Bmax-B)) = n log(S) - log Kd, where B is the amount bound by the radioligand, Bmax is the total receptor bound, n is the Hill coefficient , Kd is the dissociation constant, and S is the concentration of radioligand. When the Hill coefficient is close to unity, binding is assumed to be competitive and that sites are non-interacting. When n is non-unity, binding is interpreted as exhibiting cooperativity (negative or positive)

or that the sites are heterogeneous.

The Scatchard analysis is based on the equation: B/S = Bmax/Kd - B/Kd. Plotting B vs. B/S would yield Bmax on the x-intercept. Kd is the negative of the inverse of the slope. A linear Scatchard plot indicates that the binding sites are homogeneous. On the other hand, a curvilinear Scatchard is indicative of multiple binding sites or cooperativity is involved in the binding process.

The inhibition constants(Ki) for drugs with unity Hill coefficients were analysed by the methods of Dixon(1953), where

$$Ki = \frac{Kd}{(Bmax)\ (S)} \cdot \frac{1}{slope} ,$$

Ki is the inhibition constant of the unlabeled competitor, Kd and Bmax are the dissociation constant and the total receptor density of the system, S is the concentration of the radioligand used in the competition experiment, and the (slope) is the slope of the Dixon plot, depicting competitor concentration vs the inverse of the amount bound by the radioligand in the presence of the competitor. In addition, the competition experiments were analysed by the methods of Scatchard and Hill. These analyses assume that the amount bound by the competitor is equal to the difference between binding in the absence and in the presence of the competitor. The IC50's for all drugs were computed from the Hill analysis.

Protein Determinations

Protein concentrations were measured by the method of Lowry et al (1951) with crystalline bovine serum albumin as the standard.

Procedures For Binding to Brain Slices

Calf striatum or rat brain were mounted onto a cryostat chuck with Lipshaw M-1 embedding matrix, and frozen on the quick-freeze stage of a Damon/IEC cryostat. 32 uM thick sections were cut in the coronal plane at -20°C, and thaw-mounted onto glass microscope slides. Approximately 150 sections were prepared from a calf striatum and 80 sections from rat brains containing striatal tissues. Sections were stored at -20°C for up to two weeks. In binding experiments, striatal slices were incubated in a Coplin staining jar with (3H)Spiro or (3H)DHE in 25 ml of the same Tris HCl salt buffer(50 mM, pH 7.1 at 4°C) used in the membrane experiments. Incubations were carried out at room temperature for 60 min with (3H)Spiro and 100 min with (3H)DHE. (3H)DHE incubations were conducted under dim light. After incubation, the slices were washed in three changes of ice-cold buffer for 10 min each. The sections were removed from the glass slides while still wet, with Whatman GF/C glass fiber papers attached to a hemostat. The filter paper was then placed into a scintillation vial with 9 ml of Formula-963 cocktail. After 20 min shaking in the dark, the radioactivity was determined by liquid scintillation spectrometry(LS-250) with 45-50% counting efficiency. Background values, determined by wiping the back of each slide with filter paper, were typically 20$\pm$ 3 cpm.

Materials and Methods for Determination of DA metabolites

Pentafluoropropionic anhydride was obtained from Pierce Chemical, Rockford, IL. 1-chloro-1,1,3,3,3-pentafluoro-2-propanol

was obtained from the Penisula Chemical Research Co., Gainesville, FL. Both were purified by fractional distillation. Homovanillic acid(HVA) and 3,4-dihydroxyphenylacetic acid(DOPAC) were obtained from the Sigma Chemical Company, St. Louis, MO. 3,4-dihydroxyphenylpropionic acid and 4-hydroxy3-methoxycinnamic acid were obtained from the Aldrich Chemical Co., Milwaukee, WI. 4-hydroxy-3-methoxyphenylpropionic acid was prepared by catalytic hydrogenation of the cinnamic acid, using methanol as solvent and 10% palladium on carbon as catalyst. JXR(3%) coated on gas chrom Q 100/120 mesh was obtained from the Applied Science Laboratories, Inc., State College, PA.

Male Sprague-Dawley rats weighing 175-225 g were used in these studies. Animals were kept at room temperature with free access to food and water. Stock solutions of drugs were prepared by dissolving drugs in a minimal amount of glacial acetic acid, and serial dilutions were made with normal saline. Drugs were administered i.p. on a mg/kg basis. Rats were killed by decapitation and brains rapidly removed. A transverse scalpel cut was made anterior to the hypothalamus at the level of the anterior commissure through the optic chiasm. Left and right striata on either side of the cut were dissected out and combined. Left and right tuberculi olfactorium(TO) were dissected out from the anterior brain sections and combined. The TO was defined laterally by the lateral olfactory tract, medially by the most medial part of the anterior commissure and dorsally by a plane tangential to the lateral olfactory tract. Typically, combined striata weighed 45 mg and combined TO weighed 10 mg per rat brain.

Quantitation of DOPAC and HVA

Striata and TO from individual rat brains were homogenized separately in 1 ml of cold 1 N HCl and centrifuged at 4°C for 15 min at 15,000 rpm in a Sorvall RC-2B refrigerated centrifuge. DOPAC and HVA were simultaneously analysed in 0.1 ml of the supernatant from the striatum and 0.25 ml of the supernatant from the TO. After additions of 25 ul H_2O, 10 ul diethyldithiocarbamate and internal standards, 4-hydroxy-3-methoxyphenylpropionic acid for HVA and 3,4-dihydroxyphenylpropionic acid for DOPAC, the acids were extracted into 1 ml of cold ether. The ether layer was transfered to a 3-ml ground-glass-stoppered centrifuge tube and evaporated under a stream of nitrogen. The acids were derivatized with 10 ul of 1-chloro-1,1,3,3,3-pentafluoro-2-propanol and 50 ul of pentafluoropropionic anhydride by heating in the stoppered tube for 15 min at 75°C. After evaporation of the reagents by a stream of nitrogen, the reaction was completed by adding 40 ul pentafluoropropionic anhydride and heating in the stoppered tube for an additional 5 min at 75°C. The excess anhydride was removed under a stream of nitrogen and the derivatized samples were then dissolved in toluene. The derivatives were chromatographed on a 3% JXR column at 135°C in a Packard 1400 series gas chromatograph equipped with an electron capture detector which has a 150 mCi tritium foil as the electron source. The flow rate of nitrogen carrier gas was 60 ml/min. The temperatures were: inlet 175°C, column 175°C, and the detector 180°C.

Quantitation of DA

For quantitation of DA, to 0.2 ml of striatal sample, were added 4 ml of 0.5 M Tris HCl buffer(pH 8.5), 100 mg alumina, 0.1 ml 10% EDTA and 50 ng of the internal standard, alpha-methyldopamine. The samples were agitated by hand for 3 min to adsorb the catecholamines and the solutions aspirated and discarded. The alumina was washed 3 times with 5 ml of distilled water, each with 1 min shaking, centrifuging, and the water removed by aspiration. 1 ml of 0.25 M acetic acid dissolved in methanol was then added to the alumina and shaken for 3 min to elute DA. The solution was then carefully transfered to 3 ml silanized centrifuge tubes and dried under nitrogen. To the residues was then added 10 ul of 1.25 mg/ml diethyldithiocarbamate, 50 ul of pentafluoropropionic anhydride and 100 ul ether . The samples were allowed to react at room temperature for 5 min and dried under nitrogen. Afterward, derivatives were dissolved by adding 500 ul of toluene to the residue . DA was detected by chromatography on a 3% OV-17 column at 132°C.

The (3H)Spiro / 1 uM d-Butaclamol Study

Results

Specific binding of (3H)Spiro to calf striatal membranes, defined as the difference between binding in the absence and presence of 1 uM d-butaclamol, was saturable, nonhomogeneous, reversible, stereospecific and heat sensitive. After a 25 min incubation at 37°C, specific binding plateaued at approximately 3 nM (3H)Spiro (Fig 1). The total receptor density(Bmax) and dissociation constant(Kd), determined by the PROPHET computer, were 230 $\pm$ 10 fmole/ mg protein and 0.69 $\pm$ 0.09 nM, respectively for an average of 11 determinations. The Scatchard plot(Fig 2) revealed heterogeneity in these binding sites. By assuming the system to be the sum of two Michaelis-Menten equations, it was calculated that the higher affinity component represented about 20 to 30% of the saturable sites, with a Kd of approximately 0.12 nM, whereas the remaining lower affinity component had a Kd of about 1.3 nM. Despite the nonhomogeneous behavior, as revealed by Scatchard analysis, the Hill analysis of the data gave a Hill slope of close to unity(1.03) and a straight line was obtained with the Lineweaver-Burke plot.

The kinetics of association of (3H)Spiro at 37°C were carried out with three concentrations of labeled ligand. At a concentration of about 1 Kd(0.58 nM), total binding reached equilibrium by 18 min, although at 0.1 Kd(0.07) and 10 Kd , equilibrium was reached by 20 and 15 min respectively. The nonspecific binding at all three concentrations reached equilibrium within 3 min. The kinetics of

association were analyzed at 0.58 nM. A graph of Ln(Beq/(Beq - B(t))) vs. time yielded a k_{obs} of 0.155 min^{-1}; k_1, the association rate constant, calculated from k_{obs} and k_{-1}, the dissociation rate constant (see below), was 1.67 x 10^8 M^{-1} min^{-1}. It was assumed that in the saturation experiments all substrate concentrations used (0.02 to 10 nM) were at equilibria after a 25 min incubation.

The dissociation kinetics were carried out after a 25-min incubation with (3H)Spiro by the addition of 1.5 uM d-butaclamol. Half of the bound (3H)Spiro was displaced after 9 min, and by 20 min, no further displacement was seen. The nonspecific binding at these conditions stabilized within 3 min. A plot of Ln(B(t)) vs. time yielded k_{-1} of 0.0589 min^{-1}. The rate of dissociation was monophasic. The dissociation constant Kd, calculated from k_{-1}/k_1 was 0.36 nM, a value similar to the Kd from saturation experiments.

Specific binding of (3H)Spiro is heat sensitive and linearly dependent on protein concentration. Preheating of the final homogenate in boiling water for 2 min eliminated specific binding. There was a steady linear relationship between protein concentration and specific binding up to 1.8 mg of protein per test tube. In a routine experiment, the final concentration of protein used was approximately 0.8 mg/test tube.

Stereospecificity of binding was observed in the competition experiments, for the d-isomer of butaclamol was much more potent than the l-isomer in competing for (3H)Spiro binding sites(see Table 2). Since (3H)Spiro binds to more than one class of binding sites, it was critical to learn if the specific binding of (3H)Spiro was completely inhibited at 1 uM d-butaclamol, the concentration

selected to define specific binding. Accordingly, the inhibition of specific binding with 1 nM (3H)Spiro was titrated with increasing concentrations of d-butaclamol. It was observed that 1 uM d-butaclamol completely suppressed specific binding and no further inhibition was seen with higher d-butaclamol concentrations. (Fig 3).

The effect of vaious typical and atypical neuroleptics on binding of (3H)Spiro was studied with a fixed concentration of the unlabeled agents(Fig . 3). At 1 to 1.5 nM of (3H)Spiro, specific binding accounted for approximately 70 to 85% of the total binding. In a preliminary study, with haloperidol as the competing drug, it was found that by 25 min of incubation, the system had equilibrated with regard to the reduction of the specific binding of (3H)Spiro. Data from competition experiments were analysed by the Dixon, Scatchard and Hill plots. As shown in Figures 4, 5a and 5b, competition by unlabeled spiroperidol for (3H)Spiro yielded similar inhibition constants(Ki) in these different analyses, and the Scatchard analysis of the competition experiment again revealed a deviation from linearity, despite a Hill slope near unity(1.06).

The Ki and IC50 values determined from Dixon's method and Hill plot for various typical and atypical neuroleptics are listed in Tables 1 and 2. Since the Dixon method described was suitable only to calculate the Ki values for simple competitive inhibitors, the IC50's for drugs with low Hill coefficients are listed separately in Table 2 instead of Ki's. For neuroleptic agents with Hill coefficients close to unity, the Ki values ranged from 1.2 nM for spiroperidol to 29,000 nM for sulpiride; AHR-1900 had a

value of 820 nM. Among the butyrophenones, spiroperidol was 20 times more potent than haloperidol and 700 times more potent than the clinically inactive congener AHR-1900. Among the phenothiazines, fluphenazine was 3 times more potent than thiethylperazine and 4 times more potent than chlorpromazine. The benzamide derivatives, metoclopramide and sulpiride, were virtually inactive in competing for the (3H)Spiro sites, showing Ki's of about 5800 and 29000 nM, respectively.

Of the neuroleptic agents with low Hill coefficients, the clinically active d-butaclamol was 500 times more potent than the clinically inactive butyrophenone U-25,927. Among the dibenzoheteroepines, the antipsychotic loxapine was 500 times more potent than perlapine and clozapine was four times weaker than its 'cis' isomer, HF-2046 and 8 times weaker than loxapine. It is important to note that the binding potencies of clozapine,an antipsychotic agent, and the clinically inactive butyrophenone, U-25,927, were similar. The two clinically active benzamides were much weaker than the clinically inactive butyrophenone, AHR-1900. Perlapine, an agent with various DA antagonistic properties in vivo, was also weaker than the clinically inactive butyrophenone U-25,927 in the binding assay.

In Table 3, the effects of various typical and atypical neuroleptics on binding and on DOPAC elevation are compared and correlated to the average clinical dose of these compounds. For the neuroleptics listed, there is a significant correlation between the ED50's for DOPAC elevation in rat striatum and clinical dose ($R= 0.86$; $P< 0.005$).

In comparison, a somewhat lower correlation was found between IC50's for (3H)Spiro sites and clinical dose($R= 0.72$; $P< 0.015$). It is important to point out that for typical neuroleptics there is a good agreement between IC50 values and clincial dose. It is for the atypical neuroleptics that a considerable disagreement exists between binding data and antipsychotic efficacy.

Discussion

This study demonstrates that (3H)Spiro labels more than one class of binding sites in calf striatum. As shown by Scatchard analysis(Fig.2), there seems to be a high affinity component comprising 20 to 30% of the binding sites and a higher capacity, lower affinity component. This is in contrast to previous results obtained by Creese et al(1977) showing a single population of binding sites by Scatchard analysis. The reason for the discrepancy may be related to the relatively small proportion of high affinity sites. If insufficient points and a wide spacing of substrate concentrations are used to define the saturation curve, the smaller component may be masked by the higher capacity and lower affinity sites. Other investigators have shown more than one class of binding sites labeled by (3H)Spiro in rat striatum(Briley and Langer, 1978; Pedigo et al., 1978). At present, the meaning of these multiple binding sites is not known, but it may represent (3H)Spiro binding to other neurotransmitter binding sites, in addition to DAergic sites. Since (3H)Spiro labels more than one class of binding sites in calf caudate, it is more prudent to regard the Kd of (3H)Spiro and Ki's of various drugs in this study as the average affinity constants for these sites.

Despite the nonhomogeneous behavior revealed by Scatchard analysis, the Hill analysis of the saturation data gave a Hill slope of close to unity and a straight line was observed in the Lineweaver-Burke plot. These descrepancies may be due to: (1) the logarithmic treatment in the Hill plot which tends to linearize points in the region of 50% saturation(10 to 90%) such that the lower capacity, higher affinity sites are being concealed; (2) the double-receprocal treatment of the Lineweaver-Burke plot which compresses points at high substrate concentrations so that the line is heavily weighted in favor of the lower concentration points; (3) the Scatchard plot is more accurate in that it tends to exaggerate deviation from the theoretical relationship(Dowd and Riggs, 1965).

Since there is at present no evidence favoring either the higher or lower afinity sites as relevant to the pharmacological activity of neuroleptics, we felt that it would be more meaningful to examine the effects of neuroleptic drugs under conditions in which both sites were labeled(1- 1.5 nM (3H)Spiro).

As shown in Tables 1 and 2, the Hill coefficients determined from competion experiments for various classes of neuroleptics are close to unity, but for d-butaclamol and the dibenzoheteroepines, perlapine, clozapine, HF-2046 and loxapine, the Hill coefficients are significantly less than one. A low Hill coefficient (<1) could indicate negative cooperativity between individual receptor sites or the existence of multiple binding sites. Since the binding potencies of these dibezoheteroepines are weaker than would be expected from their effects in vivo(see below), it is conceivable

that this class of agents binds less avidly to both or one class of sites labeled by (3H)Spiro, whereas other groups of neuroleptics bind in a manner similar to spiroperidol.

Previous binding studies with (3H)DA antagonists have revealed several pharmacological and physiological properties of these receptor sites. (1) Binding of (3H)Hal and (3H)Spiro to rat striatum is not confined to receptors intrinsic to nigro-striatal neurons but also includes receptors for neurons originating from cortical regions(Schwartz et al., 1978; Garau et al., 1978; Creese et al., 1979a). (2) Antagonism of (3H)Hal and (3H)Spiro binding in frontal cortex and hippocampus correlates with their anti-serotonergic properties(Leysen et al., 1978b; Creese and Snyder, 1978). (3) Inhibition of (3H)Spiro binding by DA agonists in rat striatum is modified by guanine nucleotides(Zahniser and Molinoff, 1978; Creese et al., 1978, 1979a,b). (4) Antagonism of butyrophenone binding does not correlate with the effect of neuroleptics on DA-stimulated adenylate cyclase. However, when (3H)Flu is used as the ligand, a significant correlation is obtained(Hyttel, 1978; Cross and Owen, 1980). Therefore, it appears that receptor sites defined by various classes of labeled ligands are not necessarily identical in a given brain region and that sites labeled by a particular ligand are probably heterogeneous. Further, since neuroleptic agents are comprised of many classes of compounds with varying pharmacolgical and clinical properties, it would seem rather unlikely for a study in vitro based on competition with a labeled ligand to account for the in vivo anti-DAergic effects of all neuroleptics. In this regard, the present study with atypical

neuroleptics has revealed that the uncritical use of neuroleptic binding assays to predict anti-DAergic properties in vivo may yield misleading results.

As shown in Tables 1 to 3, the clincially inactive butyrophenone, AHR-1900, is much weaker than the active butyrophenones, spiroperidol and haloperidol, in agreement with the data in vivo. However, the clinically active dibenzodiapzepine clozapine, which elevates DOPAC levels in rat(Wilk et al., 1975), has an IC50 value similar to the clinically inactive butyrophenone U,25-927, which does not elevate DOPAC(Stanley and Wilk, 1977). Perlapine, an agent exhibiting many anti-DAergic properties in vivo(Burki et al., 1975; Wilk and Stanley, 1977; Meltzer et al., 1977) is found to be weaker than the clinically inactive butyrophenone U-25,927 in binding. The most striking discrepancy between binding and effects in vivo is seen with the two benzamides, sulpiride and metoclopramide. Sulpiride is a drug with established antipsychotic efficacy(Benoit et al., 1969; Mielke et al., 1977) and metoclopramide in recent studies has also been shown to be clinically active(Stanley et al., 1979). Both agents characteristically increase DOPAC levels in vivo(Stanley and Wilk, 1979). However, in binding assays sulpiride is 40 times and metoclopramide 7 times weaker than the clincally inactive butyrophenone, AHR-1900, and less active than l-butaclamol. These discrepancies between binding and properties in vivo of atypical neuroleptic drugs underscore the inadequacy of neuroleptic binding assays in predicting anti-DA properties of neuroleptic drugs.

In contrast to the (3H)Spiro assay, a significant correlation was found between the ED50's for DOPAC elevation and the clinical

dose for the typical as well as atypical neuroleptics($r>0.86$). An exeception is perlapine, a drug which elevates DOPAC, but on the basis of unpublished clinical trials was reported to lack antipsychotic activity. On the basis of its ability to elevate DOPAC and prolactin levels, Wilk and Stanley(1977) and Meltzer et al (1977) have independently predicted that this drug would be found to be clinically active if reevaluated at a dose range similar to chlorpromazine.

In order to establish a relationship between binding and clinical properties of antipsychotic drugs, we have made correlations between the ability of drugs to compete for (3H)Spiro sites with the average clinical dose of these compounds. However, the 'average clinical dose' may not represent the true pharmacological potency of these drugs. Factors such as differences in the individual sensitivity to drug, in drug inactivation and in the formation of active metabolites will affect their potency. In addition, the reported dosage of a compound may vary widely from one study to another. Despite these limitations, the high correlation between the ability of antipsychotic drugs to compete for (3H)Spiro binding sites with their clinical doses suggests that competition for DAergic binding sites is associated with the therapeutic efficacy of these drugs. It should be mentioned that the ability of neuroleptic drugs to compete against other putative neurotransmitter binding sites and the antagonism of DA-stimulated adenylate cyclase by these neuroleptics does not correlate with their clinical efficacy.

In summary, it appears that assays of (3H)Spiro binding to calf striatal membranes can accurately predict the pharmacological

(see page 165)

Fig. 1. Saturation of specific spiroperidol binding sites in calf striatal membranes. Triplicate tubes containing homogenate(0.8 mg of protein per tube) and the indicated amounts of (3H)Spiro were incubated in 1 ml of incubation medium at 37°C for 25 min. Total binding(■) and non-specific binding(▼) were determined in the absence and in the presence of 1 uM d-butaclamol, respectively; The difference between the two is defined as specific binding(△). Total receptor density(Bmax) and dissociation constant(Kd) were 0.17 pmole/mg protein and 0.47 nM, respectively. Vertical bars represent S.D. of triplicate tubes in one experiment.

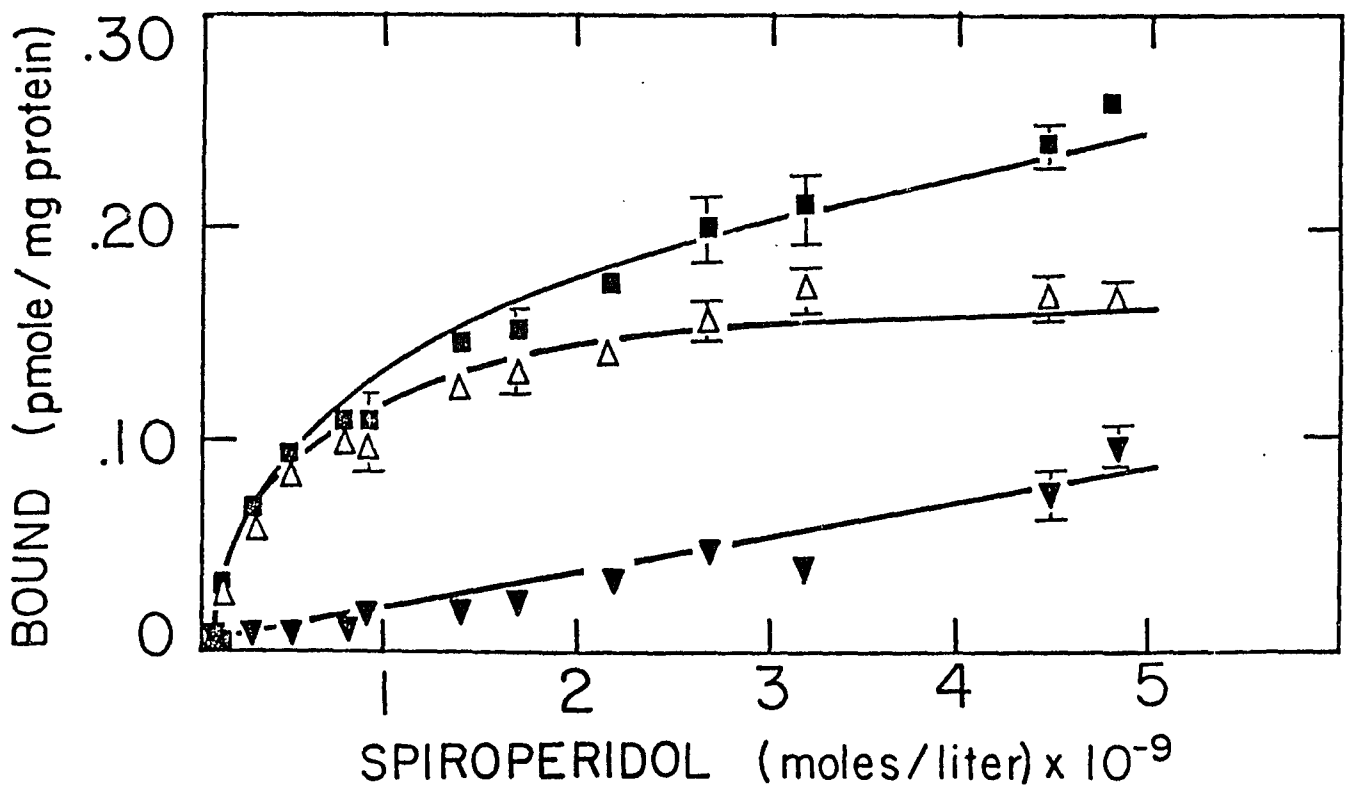
BOUND (pmole/mg protein)
.30
.20
.10
0
1
2
3
4
5
SPIROPERIDOL (moles/liter) x 10^{-9}

Fig. 2. A Scatchard plot of the specific binding in Fig. 1. Ordinate is $\frac{\text{Bound(pmole/mg protein)}}{\text{Free(Spiro(M))}}$. Kd and Bmax for high (H) and low(L) affinity componets were obtained by fitting the binding data into the equation:

$$B = \frac{B^{H}max\ (D^*)}{D^* + K_dH} + \frac{B^{L}max\ (D^*)}{D^* + K_dL}$$

where $B^{H}max = 0.05$, $B^{L}max = 0.155$ pmol/mg protein, $K_dH = 0.12$ nM, $K_dL = 1.3$ nM and D^* is the concentration of (3H)Spiro used.

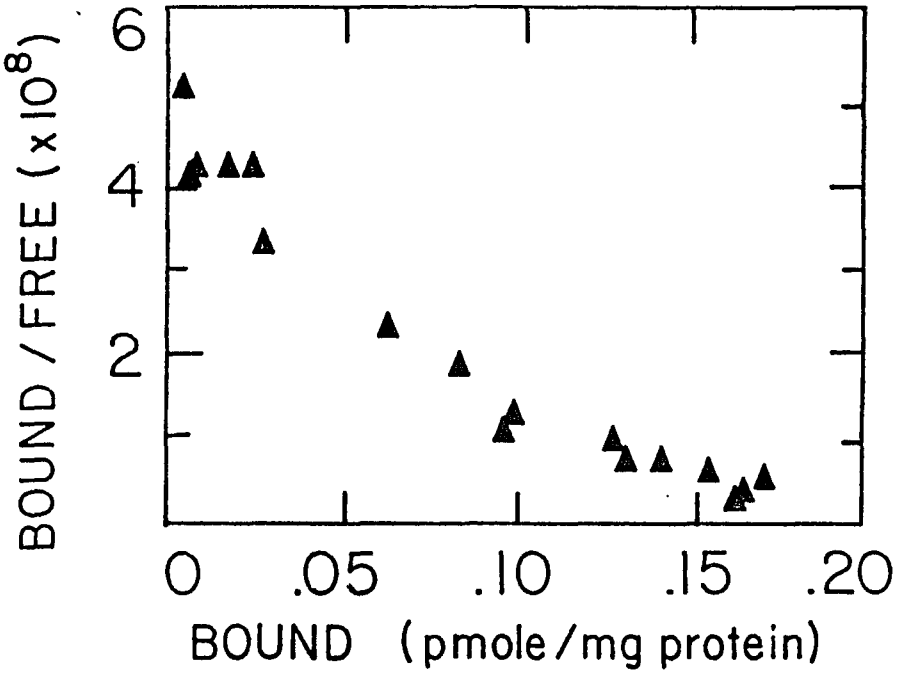
BOUND / FREE (x10^8)
6
4
2
0
.05
.10
.15
.20
BOUND (pmole/mg protein)

Fig. 3. Inhibition of (3H)Spiro binding in calf striatal membranes by various neuroleptics. d-Butaclamol and other drugs were dissolved in a minimal amount of glacial acetic acid and diluted with 0.1% ascorbic acid. Incubation conditions are as indicated in Fig. 1. Percentage of inhibition is the precentage reduction of specific binding by the indicated amount of unlabeled ligand. d-Butaclamol (○); thiethylperazine(▲); chlorpromazine(△); clozapine(■); AHR-1900(□); metoclopramide(●); sulpiride(×).

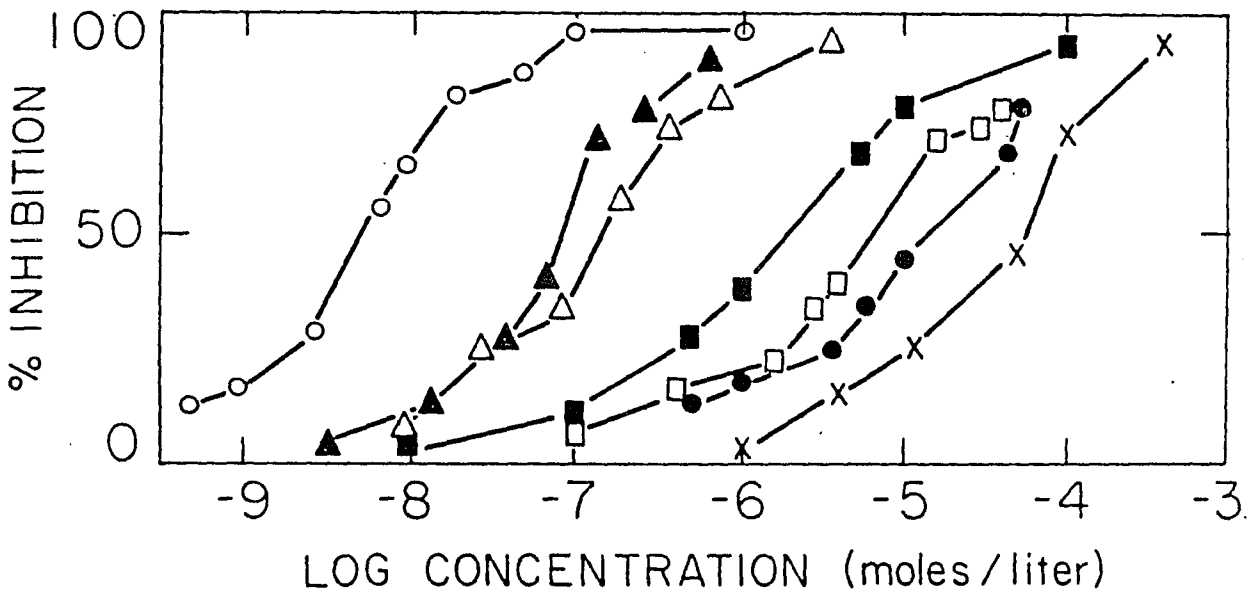
100
50
0
% INHIBITION
-9
-8
-7
-6
-5
-4
-3
LOG CONCENTRATION (moles / liter)

Fig. 4. A Dixon plot of unlabeled Spiro competing for (3H)Spiro sites in calf striatal membranes. Triplicate tubes containing tissue homogenate(0.8 mg protein/ tube) and a fixed amount of (3H)Spiro(1 to 1.5 nM) were incubated with indicated amount of unlabeled ligand at 37°C for 25 min. At 1 to 1.5 nM of (3H)Spiro, specific binding represented about 70 to 85% of the total binding. The Ki values for the unlabeled ligand were calculated from

$$Ki = \frac{Kd}{(Bmax)\ (D^*)} \times \frac{1}{(slope)},$$

where Ki is the inhibition constant of the unlabeled ligand, Ki and (Bmax) are the dissociation constant and the total receptor density determined from saturation experiments(Fig. 1), (D*) is the concentration of (3H)Spiro used. Vertical bars represent S.D. for triplicates in one experiment.

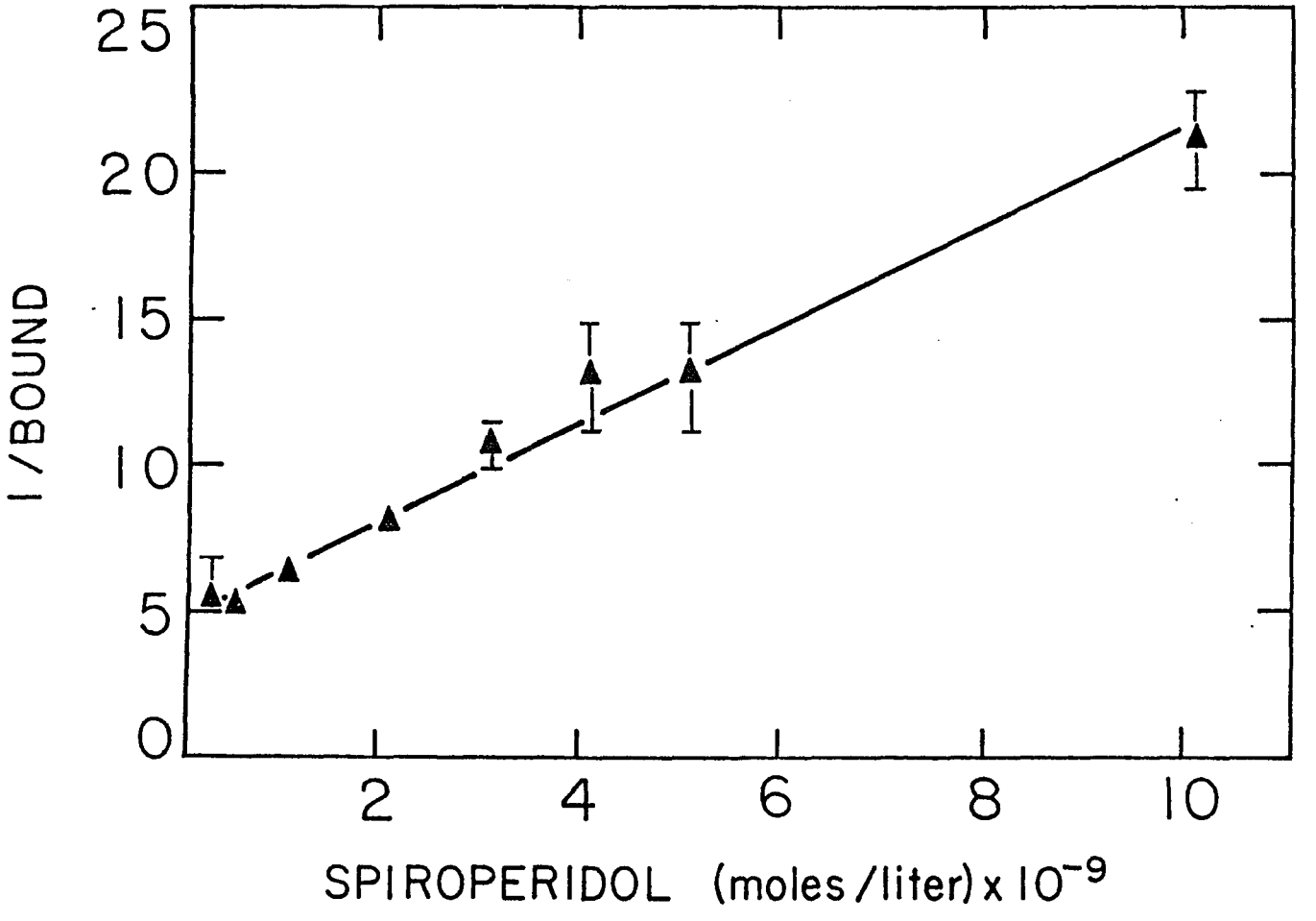
25
20
15
10
5
0
1/BOUND
2
4
6
8
10
SPIROPERIDOL (moles/liter) x 10^{-9}

Fig. 5.a, A Scatchard plot of the competition experiment in Fig. 4. The amount bound(B) represents the reduction in the specific binding in the presence of a specific concentration of unlabeled Spiro. b, a Hill plot of the competition experiment in Fig. 4. B is defined as above. B_M is the amount of specific binding in the absence of unlabeled Spiro. IC50(2 nM) and Hill coefficient(1.06) were calculated from the equation of the fitted line. The Ki value(0.7 nM) is obtained from

$$Ki = \frac{IC50}{1 + \frac{D^*}{Kd}}$$

where D* is the amount of (3H)Spiro used(1.3 nM) and Kd(0.69 nM) the dissociation constant from the saturation experiment.

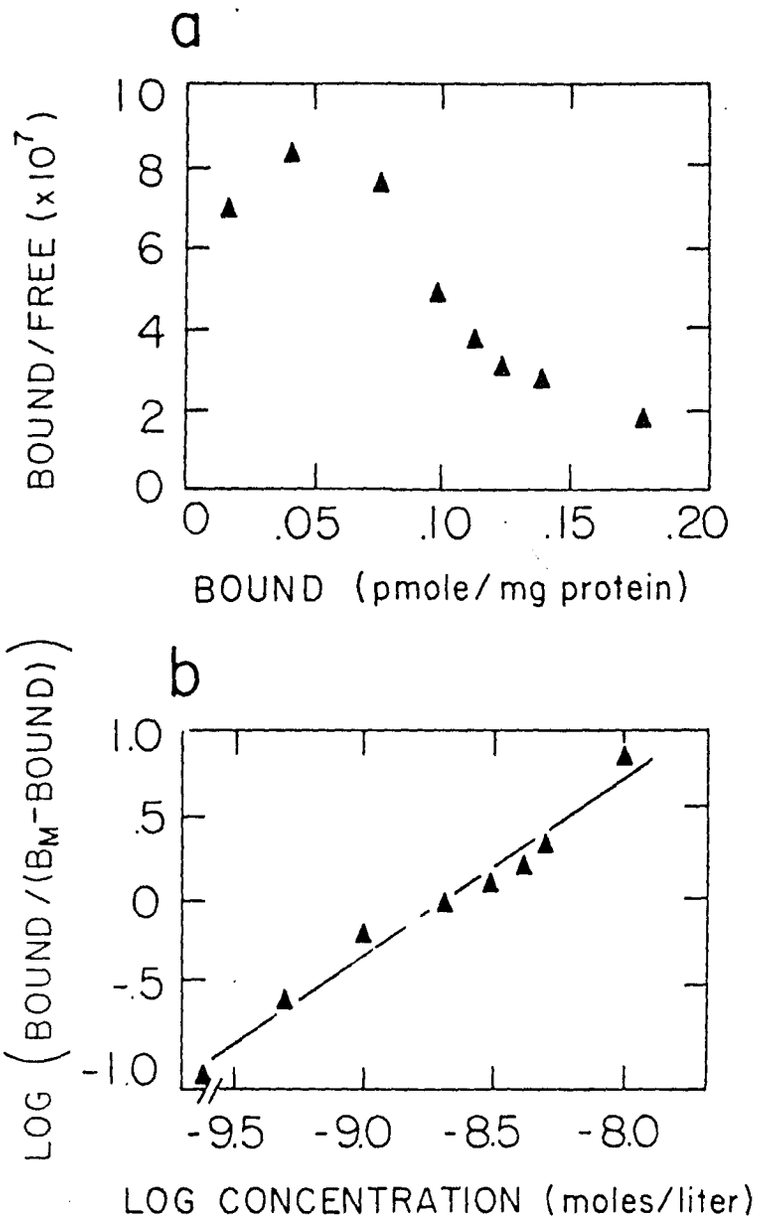
a
BOUND/FREE (x10^7)
10
8
6
4
2
0
0
.05
.10
.15
.20
BOUND (pmole/mg protein)
b
LOG (BOUND/(B_M-BOUND))
1.0
.5
0
-.5
-1.0
-9.5
-9.0
-8.5
-8.0
LOG CONCENTRATION (moles/liter)

Table 1: Apparent inhibition constants(Ki) and Hill coefficients of some neuroleptic agents for (3H)Spiro sites

Drug	Ki ± SE(n)[a]		Hill Coefficient ± SE(n)[b]	
	nM			
Spiroperidol	1.2 ± 0.13	(4)	1.01	(2)
Haloperidol	23 ± 4.7	(3)	0.83 ± 0.06	(3)
AHR-1900	829 ± 95	(3)	0.87 ± 0.12	(3)
Fluphenazine	10.8 ± 3.4	(3)	1.0 ± 0.14	(3)
Thiethylperazine	30 ± 8.5	(3)	0.98 ± 0.08	(3)
Chlorpromazine	40 ± 2.3	(3)	0.92 ± 0.05	(3)
Penfluridol	30 ± 10	(3)	0.82 ± 0.06	(3)
Sulpiride	29000 ± 14000	(3)	0.87 ± 0.24	(3)
Metoclopramide	5800 ± 2000	(3)	0.83 ± 0.12	(3)

[a] Calculated by the method of Dixon; numbers in parentheses, number of experiments.

[b] Calculated from Hill plot, see Fig. 5b; number in parentheses, number of analyses.

Table 2: IC50's and Hill coefficients of neuroleptic agents not exhibiting simple competitive kinetics on (3H)Spiro binding

Drug	IC50 ± SE(n)[a]		Hill Coefficient ± SE(n)[a]	
d-Butaclamol	4.16 ± 0.5	(3)	0.74 ± 0.05	(4)
l-Butaclamol	5290 ± 1273	(3)	1.56 ± 0.10	(3)
Loxapine	78 ± 9.5	(3)	0.72 ± 0.03	(3)
HF-2046	265 ± 95	(3)	0.62 ± 0.01	(3)
Clozapine	1420 ± 170	(3)	0.64 ± 0.07	(3)
Perlapine	3990 ± 810	(4)	0.51 ± 0.14	(4)
U-25,927	1430 ± 270	(4)	0.72 ± 0.08	(4)

[a] Calculated from Hill plot, see Fig 5b.

Table 3: Comparison of in vitro and in vivo effects of neuroleptics[a]

Drug	IC50[b]	ED50[c]	Clinical dose[d]
	nM	um/kg	um/kg
Spiroperidol	3.81	0.4	0.06(6)
d-Butaclamol	4.16	0.34	0.4 (7)
Fluphenazine	26	0.17	0.09(8)
Haloperidol	104	0.43 (1)	0.14(8)
Thiethylperazine	73	1.58 (2)	6.4 (2)
Loxapine	83		3 (9)
Chlorpromazine	151	8.4	9.87(8)
Clozapine	1420	61 (3)	6.12(10)
Perlapine	3990	11 (4)	?
Metoclopramine	16900	8.9 (5)	12 (11)
Sulpiride	29000	176 (5)	40 (12)
AHR-1900	2130	inactive(3)	inactive

[a] Correlation between IC50 and clinical dose($r=0.79$; $P<0.01$); correlation between ED50 for DOPAC elevation and clinical dose($r=0.86$, $P<0.005$).

[b] IC50 was calculated from the Hill plot.

[c] ED50 values represent the dose of drug producing a half-maximal increase in DOPAC levels in rat striatum measured at the time of peak response

[d] (1)Wilk et al., 1975; (2) Rotrosen et al., 1978; (3) Stanley and Wilk, 1977; (4) Wilk and Stanley, 1977; (5) Stanley and Wilk, 1979; (6) Usdin and Efron, 1972; (7) Mielke et al; 1975. (8) Davis, J.M., 1974. (9) Heel et al; 1978. (10) Simpson and Varga 1974. (11) Stanley et al; 1979. (12) Mielke et al; 1977.

Specific Methods For (3H)DHE Study

Saturable or specific binding was defined as the difference between binding in the absence and presence of 4 nM d-butaclamol. This low concentration of d-butaclamol was selected to define apparent DA sites for the following reasons: (1) DHE has been shown to possess high affinity for alpha-noradrenergic, 5-HT and DA sites (Table 4). Therefore, at the nM range, (3H)DHE would label more than one receptor site. (2) d-butaclamol interacts with DA sites in the nM range, and interacts with alpha-noradrenergic and 5-HT sites in the 10^{-8}M range(Table 4). At a concentration greater than 10^{-8} M, d-butaclamol would certainly bind to more than one receptor population. Therefore, in order to define a subclass of DA sites within the multiple (3H)DHE binding sites, it is necessary to lower the concentration of d-butaclamol. Based on its affinity for various receptor sites, 4 nM d-butaclamol would preferentially bind to the DA sites with minimal interaction with the alpha-noradrenergic or 5-HT sites. As shown below, although this concentration of d-butaclamol seems to underestimate the number of binding sites in the saturation experiment, experimentally this error does not greatly affect the calculated values of Kd and Bmax for (3H)DHE.

The displacement of (3H)DHE binding by d-butaclamol was studied. As shown in Fig. 6, the suppression of (3H)DHE binding required more than five log concentration units of d-butaclamol, and a 50% inhibition occurred at 4 nM. There was no apparent plateau over the entire range studied(10^{-10} to 10^{-5} M), and it was

not possible to select a concentration of d-butaclamol which would appear to suppress only one component of binding sites labelled by (3H)DHE. However, the slope of the displacement curve of (3H)DHE binding by d-butaclamol in the nM range was steeper than at higher concentrations. Since d-butaclamol in the nM range was so effective in competing against (3H)DHE binding , and since d-butaclamol in the nM range shows selectivity for DAergic sites(Table 4), we decided to investigate the properties of (3H)DHE binding sites using 4 nM d-butaclamol as the masking drug.

Based on the affinity of d-butaclamol for the 4 nM d-butaclamol sensitive (3H)DHE sites given in Table 5, the % receptor occupancy by 4 nM d-butaclamol in the presence of 0.25 nM (3H)DHE, the concentration used in the competition experiments, is:

$$B/Bm = L/(L + Km(1 + I/Ki)) \qquad (1)$$

$$= 4/(4 + 0.78(1+0.25/0.25)) = 72\%.$$

In the saturation experiments, the highest concentration of (3H)DHE used was 2.5 nM, and 4 nM d-butaclamol could theoretically occupy only 32% of these sites(eq. 1). It would seem therefore that the Bmax of (3H)DHE sites, defined as the difference in binding in the absence of presence of 4 nM d-butaclamol is underestimated and that Kd values are also affected by this error. However, the validity of using 4 nM d-butaclamol as the masking drug is supported by the following experiments. First, a saturation experiment was performed using 20 nM d-butaclamol to define non-

specific binding. At this concentration d-butaclamol occupies 70% of the receptor sites when (3H)DHE is at 2.5 nM, and 93% when (3H)DHE is 0.25 nM (eq.1). Under these conditions the Kd was 0.2 nM, Bm was 60 fmol/mg protein, the Scatchard plot was linear, and the Hill coefficient was 0.93. These values do not differ significantly from the Kd(0.25 nM), Bm(75 fmol/mg protein), linear Scatchard , and unity Hill coefficient obtained with 4 nM d-butaclamol(see results). Second, a saturation experiment was performed using various d-butaclamol concentrations as masking ligand to define specific binding. The concentrations were chosen to provide a fixed percentage of receptor sites occupied(72 %) at various (3H)DHE concentrations. A linear Scatchard plot and unity Hill coefficient were observed. The Kd(0.4 nM) and Bm(80 fmol/mg protein) values again did not vary significantly from results obtained with 4 nM d-butaclamol. Thirdly,we have reanalysed the saturation experiments done with 4 nM d-butaclamol by eliminating points with high (3H)DHE concentrations, as these points were supposed to contain greater errors. We found that the Kd and Bm were virtually unchanged. Thus, although 4 nM d-butaclamol may have underestimated the number of binding sites, experimentally these errors do not seem to affect greatly the calculated values of Kd and the Bmax of (3H)DHE, and the apparent homogeneity of the binding sites.

In competition experiments it can be shown that in the

presence of the competitor,the specific binding, defined as the difference between binding in absence and presence of 4 nM d-butaclamol, is :

total binding - non-specific binding =

$$\frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{A}{Ka}\right)} - \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{A}{Ka} + \frac{B}{Kb}\right)}, \qquad (2)$$

Where D and Kd are the concentration and dissociation constant of (3H)DHE, A and Ka are the concentration and dissociation constant of the competitor, and B and Kb are the concentration and dissociation constant of d-butaclamol. The amount of specific binding for (3H) DHE in the absence of competitor is

Total binding - Non-specific binding

$$= \frac{1}{1 + \frac{Kd}{D}} - \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{B}{Kb}\right)}. \qquad (3)$$

The amount bound by the competitor is therefore (3)-(2)

$$\frac{1}{1 + \frac{Kd}{D}} - \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{B}{Kb}\right)} - \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{A}{Ka}\right)} + \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{A}{Ka} + \frac{B}{Kb}\right)} \qquad (4)$$

When D=Kd, B>>>Kb, the amount bound by the competitor when A=Ka is :

$$\frac{1}{1 + 1} - \frac{1}{1 + 1(1 + \infty)} - \frac{1}{1 + 1\ (1 + 1)} \qquad \frac{1}{1 + 1\ (1 + 1 + \infty)}$$

(equa.4)

$= 1/2 - 1/3 = 1/6 = 0.167$. When D=Kd, B>>>Kb, the amount bound by the competitor when A=100 Ka is

$$\frac{1}{1+\frac{1}{1}} - \frac{1}{1+1(1+\infty)} - \frac{1}{1+1(1+100)} + \frac{1}{1+1(1+100+\infty)} \qquad \text{(eq.4)}$$

$= 1/2 - 1/102 = 0.49$.

In our experiments when D=Kd, B=4, Kb=0.78, the amount bound by the competitor when A=Ka is

$$\frac{1}{1+1} - \frac{1}{1+(1+\frac{4}{0.78})} - \frac{1}{1+1(1+1)} + \frac{1}{1+1(1+1+\frac{4}{0.78})}$$

$= 1/2 - 1/7.13 - 1/3 + 1/8.13 = 0.147$. When D=Kd, B=4, Kb=0.78, the amount bound by the competitor when A=100Ka is

$$\frac{1}{1+1} - \frac{1}{1+(1+\frac{4}{0.78})} - \frac{1}{1+1(1+100)} + \frac{1}{1+1(1+100+\frac{4}{0.78})}$$

$= 1/2 - 1/7.13 - 1/102 + 1/107.13 = 0.3592$. The magnitude of error when A=Ka is therefore $(0.147-0.167)/ 0.167 = -0.12 =$ 12%. The magnitude of error when A=100Ka is $(0.3592-0.49)/ 0.49 = -0.27 = 27\%$. Thus the magnitude of error introduced is 12 to 27 % when the range of the concentration of the competitor increases from 1 Ka to 100 Ka. This amount of error is not sufficient to invalidate the measurement of Ki for various competitors when specific (3H)DHE binding is defined with 4 nM d-butaclamol as masking drug.

Results Of (3H)DHE Study

Our results showed that the specific binding of (3H) DHE to membrane preparations from bovine striatum, defined as the difference between binding in the absence and presence of 4 nM d-butaclamol, is apparently homogeneous, reversible, stereospecific and heat sensitive. In saturation experiments, specific binding reached a plateau at approximately 0.5 nM (3H)DHE (Fig 7) after a 100 min incubation period at 23°C. The total receptor density (Bmax) and dissociation constant (Kd), determined by the hyperbolic fit, using the PROPHET computer based on the Michaelis-Menten equation, were 0.075 $\pm$ 0.006 pmol/mg protein and 0.244 $\pm$ 0.06 nM, respectively, for an average of 5 determinations. In each determination the Scatchard plot was linear($R>0.91$)(Fig.8) and the Hill analysis did not show any indication of multiple binding sites or cooperativity (Hill coefficient= 0.98 $\pm$ 0.04). Thus, despite its high affinity for various receptor types, (3H)DHE binding sites defined with a low d-butaclamol concentration appeared to be homogeneous. It should be noted that the non-specific binding, or binding in the presence of 4 nM d-butaclamol, was non-linear in 4 out of 5 cases. At 0.24 nM, the concentration used in the competition experiments, specific binding was 45-55% of the total binding.

The kinetics of (3H)DHE binding was carried out at a radioligand concentration of 0.16 nM at 23°C. In the association experiment, there was no specific binding in the first 15 min, but after 100 min specific binding had reached a plateau. The level of specific binding at 80 min was 85% of the binding at

100 min. As most of the concentrations employed in the saturation experiments were close to or above 0.16 nM, it was assumed that the system was at equilibrium after a 100 min incubation period. The displacement of (3H)DHE binding was carried out after the association experiment by the addition of 4 nM d-butaclamol. Bound (3H)DHE was slowly displaced by 4 nM d-butaclamol. A 15% reduction of specific binding was observed after 5 hours. However, (3H)DHE binding is clearly reversible, as 1 uM d-butaclamol displaced half of the bound (3H)DHE in one hour at 30°C. Similar slow reversal of binding has been observed for other ligands such as LSD and QNB (Maayani, in preparation; Galper et al; 1977; Ben-Bark et al;1979) and problems with (3H)DHE kinetics have also been reported (Greenberg and Snyder, 1978) .

Specific binding of (3H)DHE is heat sensitive and linearly dependent on protein concentration. Heating the final homogenate in boiling water for 5 min eliminated specific binding. There was a linear relationship between protein concentration and specific binding up to 1.2 mg of protein per tube. In routine experiments, the amount of protein used was 0.7 mg/test tube. There was no specific binding to filters in the absence of tissue. The amount of binding to the filter was 5% of (3H)DHE added and it was not displaceable by d-butaclamol. Specific binding was stable during the experiment.

Stereospecificity of (3H)DHE binding was observed in the competition experiments (Fig 9): d-Butaclamol, the clinically active isomer, was 500 times more potent in competing for

(3H)DHE sites than the clinically inactive l-isomer. cis-Flupenthixol, the clinically active thioxanthene, was also more potent than the trans-isomer, but the difference in potency was only 8-fold(Table 5).

The regional distribution of high affinity (3H)DHE sites was studied using various ligand concentrations. Specific binding in the striatum, including part of nucleus accumbens, was 75 fmole/mg protein. This was 3 times that of tuberculum olfactorium (23 fmole/mg protein) and 5 times that of substantia nigra and frontal cortex (14 fmole/mg protein). Compared to striatum, only negligible specific binding was detected in medulla (3%), occipital cortex (3%), hippocampus (3%), hypothalamus (6%) and cerebellum (<1%).

The competition for (3H)DHE binding sites at 0.24 nM (3H)DHE was studied with the agonists DA, apomorphine, 5-HT,and (-)NE(Fig 10), and various classes of DA antagonists (Fig 11 and Table 5). In general, DA antagonists competed effectively for (3H)DHE sites and agonists competed poorly. As shown in Table 5, the IC_{50}'s of agonists were in the uM range, while the IC_{50}'s for the majority of DA antagonists were in the nM range. Among the agonists, the order of potency was compatible with DAergic sites as apomorphine (IC_{50}=100 nM) was 50 times more active than DA, which in turn was 8 times more potent than (-)NE and 5-HT.

The antipsychotic agents were active in the DHE binding assay, and for the most part, the potency of drugs within a given class agreed well with their properties in vivo (Table 5). For example,

the butyrophenone,spiroperidol(IC50= 0.22 nM),was 20 times more active than haloperidol(4.5 nM); the phenothiazine,fluphenazine(2.6 nM),was two fold more potent than thiethylperazine(5.9 nM) and 4 times as potent than chlorpromazine(10 nM) ; the dibenzoheteroepine, loxapine(15 nM),was 20 times more potent than perlapine(320 nM) or clozapine(320 nM). The most interesting finding was that the benzamides were active in competing for sites labelled by (3H)DHE. The IC50's of metoclopramide, sulpiride, and sultopride were close to 100 nM. The ergot derivatives DHE, lergotrile and methysergide competed well with (3H)DHE for binding sites. The inhibition constant of DHE (Ki=0.44 nM), calculated from the Dixon plot (Fig 12), agreed well with the Kd of (3H)DHE (0.24 nM) obtained in saturation experiments.

Phentolamine, a strong alpha -noradrenergic blocker, was weak in the (3H)DHE binding assay, with an IC_{50} in the uM range. This indicates that (3H)DHE sites defined with 4 nM d-butaclamol are not significantly contaminated with alpha -noradrenergic sites. The benzamide antiarrhythmic agent, procainamide, was inactive against (3H) DHE sites. At uM concentrations, it displaced only 20% of the specifically bound ligand . It is interesting to note that U-25,927, a clinically inactive butyrophenone which does not increase DA turnover in vivo (Stanley and Wilk, 1977), was fairly active in competing with (3H)DHE for binding sites. Although it is much weaker than spiroperidol or haloperidol, it is still stronger than clozapine, a drug with proven antipsychotic

efficacy. Domperidone, a butyrophenone derivative with anti-emetic properties but which does not cross the blood-brain barrier was also very active in the present assay.

In Table 6, the IC_{50}'s and Hill coefficients of various neuroleptic agents in the (3H)DHE assay were compared with results which we previously obtained in the (3H)Spiro binding assay, using 1 uM d-butaclamol as the masking ligand (Lin et al., 1980). These two systems seem to differ in the following respects: (1) Except for sulpiride and metoclopramide, the IC_{50}'s for drugs in the (3H)DHE assay were about one order of magnitude lower than in the (3H)Spiro assay. (2) The affinity of sulpiride and metoclopramide for (3H)DHE sites was about two orders of magnitude higher than for (3H)Spiro sites. (3) Judged by the Hill coefficients, an apparent competitive inhibition was generally observed for antipsychotic drugs competing for DHE sites, in contrast to several drugs with low Hill coefficients competing for (3H)Spiro sites.

In Table 7, the effects of various neuroleptics in competing for (3H)DHE sites, (3H)Spiro sites and on DOPAC elevation in rat are compared and correlated to the average clinical dose of these compounds. There is a significant correlation between the IC_{50}'s for (3H)DHE sites and clinical dose ($R=0.86$, $P<0.002$). A similar correlation exists between the ED_{50}'s for DOPAC elevation in rat striatum and clinical dose ($R=0.86$, $P<0.005$). In comparison, a somewhat lower correlation was found between IC_{50}'s for (3H)Spiro sites and clinical dose ($R=0.76$, $P<0.015$).

Discussion

The characteristics of a population of binding sites are determined by the selectivity and concentration range of both the radioligand and masking drug utilized. (3H)DHE, the radioligand used in this study,was reported to interact with DA, alpha-noradrenergic, and 5-HT sites(Table 4). Similarly, d-butaclamol, the masking drug selected for this study, also interacts with these sites(Table 4). To enhance the specificity of binding to DAergic sites from the multiple binding sites labelled by (3H)DHE, it was necessary to select a concentration of d-butaclamol which would preferentially mask these sites. A concentration of 4 nM d-butaclamol was selected for the present study because it selectively interacts with DA sites(Table 4) and it provides a measurable "net" binding, i.e., a sufficient difference between binding in its presence and its absence. As shown in the Specific Methods section, although this use of a low concentration of masking drug seems to underestimate the number of binding sites, the results obtained do not differ when the d-butaclamol concentration is varied from 4-20 nM.

We have demonstrated that specific (3H)DHE binding sites, defined as the difference between binding in the absence and presence of 4 nM d-butaclamol, are saturable and stereospecific, and that ligand binding is slowly reversible and heat sensitive. The affinity of apomorphine exceeds that of DA and both have a greater affinity than (-)NE and 5-HT(Table 5) . The regional distribution of 4 nM d-butaclamol sensitive (3H)DHE binding

sites parallels the distribution of DA in brain, and the affinity of a variety of DA antagonists, i.e., antipsychotic agents, for these sites agrees well with their pharmacological and clinical potencies(Table 7). These sites appear to be related to the DA D2 (cyclase independent) receptors, since the benzamide derivatives which are DA antagonists in vivo, but do not antagonize the stimulation of adenylate cyclase by DA, are effective in competing for (3H) DHE binding.

The non-specific binding of (3H)DHE, or binding in the presence of 4 nM d-butaclamol, was consistently nonlinear in our studies(Fig 7). This could be due to (3H) DHE binding to other saturable sites. Since (3H) DHE binding reverses slowly and since a lag occurs in the association experiment , we were unable to determine the Kd of (3H)DHE from kinetic experiments. Further studies are required to resolve these complexities.

The antagonism of (3H)DHE binding by the benzamide derivatives is of particular interest. Previously, the benzamide derivatives metoclopramide and sulpiride were shown to be inactive in antagonizing DA stimulated adenylate cyclase (Roufogalis et al; 1976; Peringer et al, 1976) and very weak in antagonizing (3H)Spiro binding (Howard et al., 1978; Lin et al; 1980; Creese et al., 1979c). As these systems are believed to represent DA receptors, the inactivity of these drugs was in contrast to their known anti- DA properties in vivo. For example, metoclopramide, recently shown to be an effective antipsychotic (Stanley et al., 1979), increases DA turnover (Peringer et al., 1976; Elliott et al., 1977; Stanley and

Wilk, 1979) stimulates prolactin release(Yamauchi et al., 1977; Meltzer et al., 1979), reverses apomorphine induced stereotypy Niemegeers and Janssen, 1979; Jenner et al., 1978a,b) and also antagonizes the inhibitory effects of DA on vasculature (Day and Blower, 1975; Kohli et al., 1978). The antagonism of (3H)DHE sites by these benzamides has therefore reinforced their anti-DA properties. It should be noted that with respect to its clinical dose or its ability to elevate striatal DOPAC,metoclopramide is 20 times less potent than haloperidol, but in the (3H)Spiro assay, metoclopramide is 150 times weaker than haloperidol. By comparison, metoclopramide is only 15 times weaker than haloperidol in the (3H)DHE binding assay, in agreement with the studies in vivo (Table 7). Therefore, (3H) DHE sites appear to be a good marker in vitro for the antiDAergic properties of the benzamide antipsychotics. Recently, Creese et al.,(1979c) reported that substituted benzamides are more potent in striatal membranes from rat and human than from calf in competing for (3H)Spiro sites. It is possible that these agents may be even more potent in competing for (3H) DHE sites in rat.

In contrast to classical neuroleptics such as haloperidol and chlorpromazine, the benzamide derivatives are relatively selective DA antagonists. For example, sulpiride is a weak alpha-NE and 5-HT antagonist on rabbit aortic strips (Kohli and Cripe, 1979), competes poorly against cholinergic muscarinic sites labelled by (3H)propylbenzylcholine and (3H)QNB (Fjalland et al., 1977; Spano et al., 1978), and is a weak inhibitor of (3H)GABA sites (Spano et al., 1978). Metoclopramide and sulpiride are weak

in competing for 5-HT sites labelled by 3H-Spiro in rat frontal cortex (Leysen et al., 1978a,b). Further, metoclopramide is inactive in antagonizing the stimulation of adenylate cyclase by NE (Robinson et al., 1979). In studies on the antagonism of the effect of DA on renal vasculature, both sulpiride and metoclopramide were shown to be the most specific of the various DA antagonists evaluated(Kohli et al., 1978).

The selective effect on DA receptors by these benzamides may explain their weak interaction with (3H)Spiro binding sites. Previously, we have studied binding sites labelled with (3H)Spiro as defined by 1 uM d-butaclamol. As spiroperidol possesses a high affinity for DA, 5-HT and alpha-NE sites (Table 4), the use of 1 uM d-butaclamol, an agent also with high affinity for those sites (Table 4), as a masking drug, would be expected to result in a mixed population of binding sites defined as "specific". We and others have found that (3H)Spiro sites are heterogeneous when 1 uM d-butaclamol is used to define specific binding (Lin et al; 1980; Briley and Langer, 1978; Pedigo et al., 1978) . In such a system, a relatively non-selective drug may be expected to compete well, while a more selective drug would appear weaker. Our results are consistent with this proposition. In the present study we have found differences in the IC50's of antipsychotic drugs in the (3H)Spiro and (3H) DHE systems. Compared with the (3H)Spiro system, the potency of all neuroleptics has increased. This increase is approximately 10 fold for butyrophenones and phenothiazines, 5 fold for dibenzoheteroepines, whereas the increase for the more

selective substituted benzamides is 200 fold(Table 6). In addition, judged by the Hill analysis, an apparent competitive inhibition was observed in general for antipsychotic drugs competing for DHE sites, whereas in the Spiro system, many drugs have Hill coefficients less than one, an indication of multiple binding sites. In part, the increase of potency in the DHE assay can be attributed to the use of a lower concentration of DHE(0.25 nM) compared to a concentration of 1-1.5 nM in the Spiro assay.

The success in demonstrating an apparently homogeneous receptor population using a low d-butaclamol concentration and (3H)DHE, a non-selective radioligand, offers an alternative method to define a subpopulation of binding sites. Previously, DA receptor binding using (3H)DHE has been studied by Tittler et al(1977) by using phentolamine to mask alpha -NE sites. However, biphasic inhibition curves were found for DA and (-)NE and the potency of DA was only two times that of (-)NE for the high affinity component of (3H)DHE sites. In contrast, we found monophasic inhibition curves for DA and (-)NE with low Hill coefficients. Moreover, DA was 8 times more potent than (-)NE.

In spite of the high correlation between binding and pharmacological activities for various neuroleptics (Table 7), the (3H)DHE binding assay does not reflect the anti-DA properties in vivo of several agents. For example, trans-flupenthixol has no clinical efficacy (Crow et al., 1978), but is quite active in the (3H)DHE assay (IC50=45 nM). U-25,927, a clinically inactive butyrophenone (O'Meallie et al., 1969), has an IC50 similar to the clinically

active drug, clozapine. These discrepancies between in vivo and in vitro properties of antipsychotic drugs have been observed in the (3H)SPIRO assay. Therefore, although the DHE binding assay can better account for the anti-DA properties of substituted benzamides than can the (3H)Spiro system, it is not possible to predict the antiDA properties of a drug based on the DHE assay alone. In this respect, it is best to combine a system using an effect in vivo such as elevation of striatal DOPAC levels(Stanley and Wilk, 1977) with a binding assay to reach a more valid conclusion concerning the anti-DA properties of a proposed neuroleptic agent. A similar conclusion was reached by Koe(1979) in his studies on molindone.

In summary, this study demonstrated an apparent DA D2 receptor system using (3H)DHE and a low concentration of d-butaclamol. These sites are antagonized effectively by the benzamide antipsychotics in accordance with their anti-DA potencies in vivo, and represent an improvement over the system labelled by (3H)Spiro as defined with a high d-butaclamol concentration.

Fig 6. Inhibition of (3H)DHE binding by d-butaclamol. Triplicate test tubes containing tissue homogenate, (3H)DHE(0.23 nM) and increasing concentrations of d-butaclamol were incubated under dim light at 23C for 100 min. The radioactivity in the absence of d-butaclamol(100%) was 3200 dpm. Each point on the figure was the average of three samples in one experiment. The standard deviation for each point was within the range of the symbol.

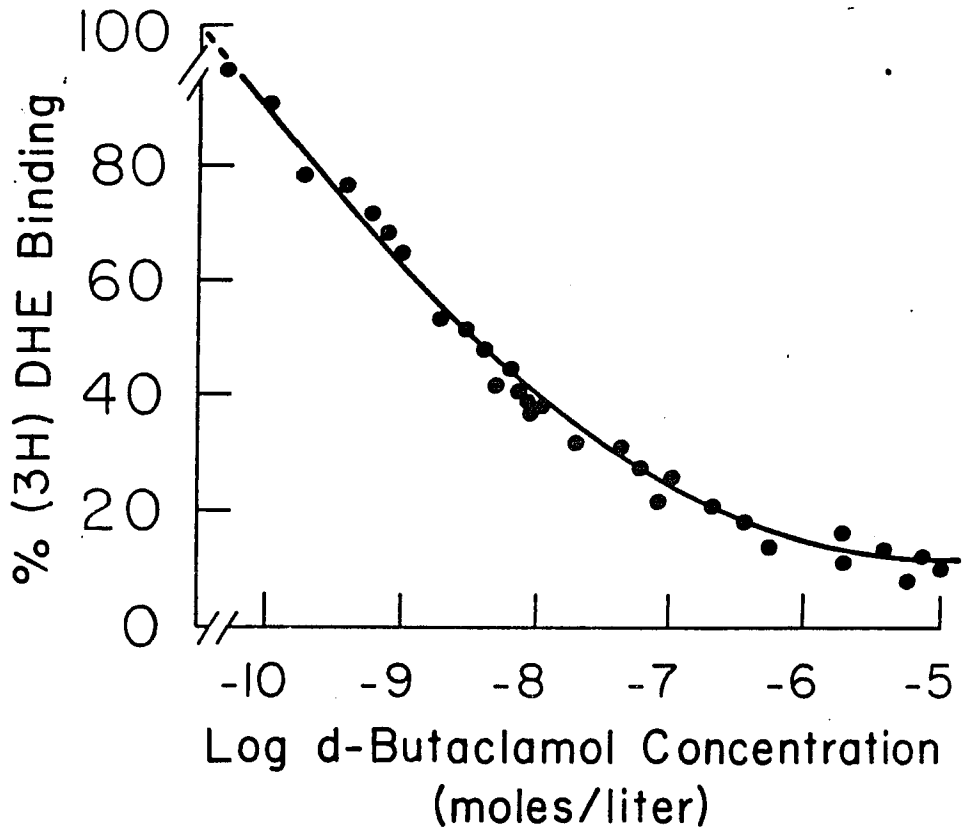
100
80
60
40
20
0
% (3H) DHE Binding
-10
-9
-8
-7
-6
-5
Log d-Butaclamol Concentration
(moles/liter)

Fig. 7

Saturation of specific [3H]DHE binding sites in bovine caudate membranes. Triplicate tubes containing tissue homogenate (0.7 mg protein/ assay) and the indicated amount of [3H]DHE were incubated in 1 ml of incubation medium at 23°C for 100 min. Total binding (●) and non-specific binding (△) were defined as binding in the absence and the presence of 4 nM d-butaclamol, respectively; the difference between the two is defined as specific binding (▲). Total receptor density (Bmax) and dissociation constant (Kd) were 0.075 pmole/mg and 0.25 nM, respectively. Vertical bars represent standard deviation.

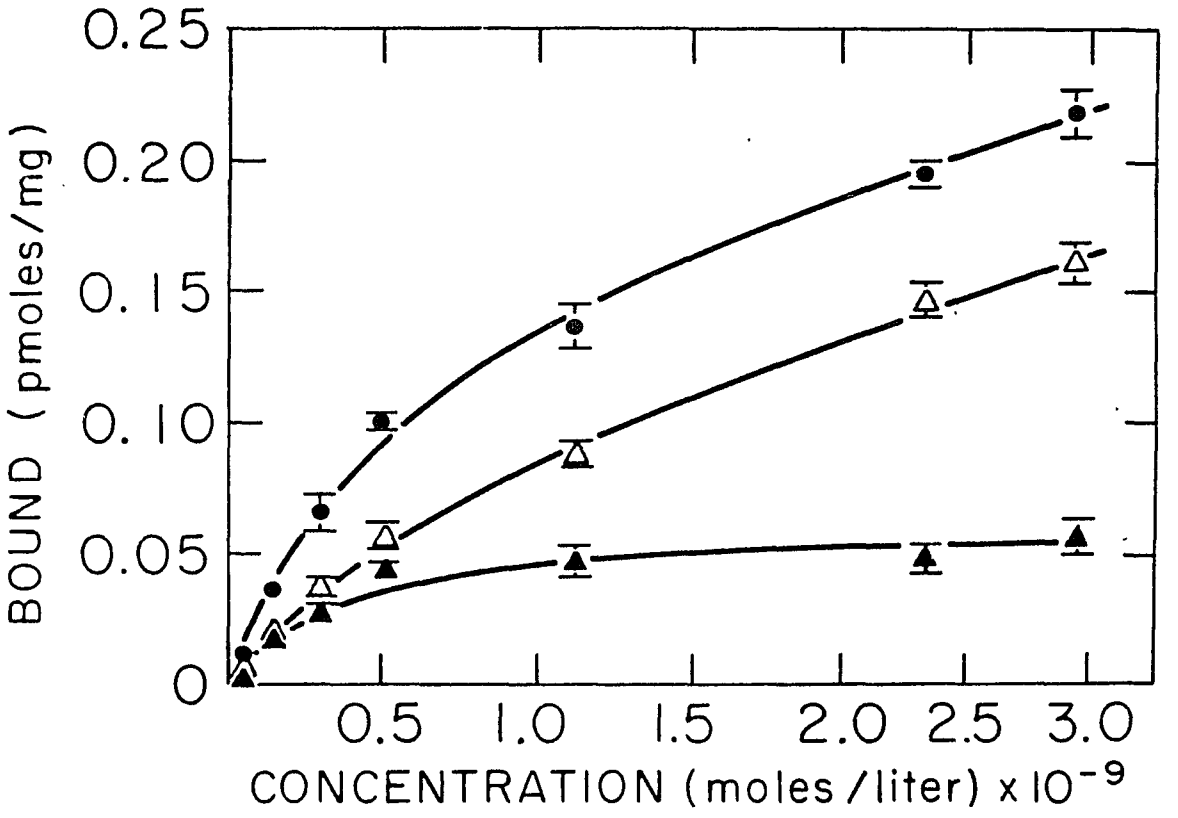
0.25
0.20
0.15
0.10
0.05
0
BOUND (pmoles/mg)
0.5
1.0
1.5
2.0
2.5
3.0
CONCENTRATION (moles/liter) x 10⁻⁹

Fig. 8

A Scatchard plot of the specific binding in Fig. 7.

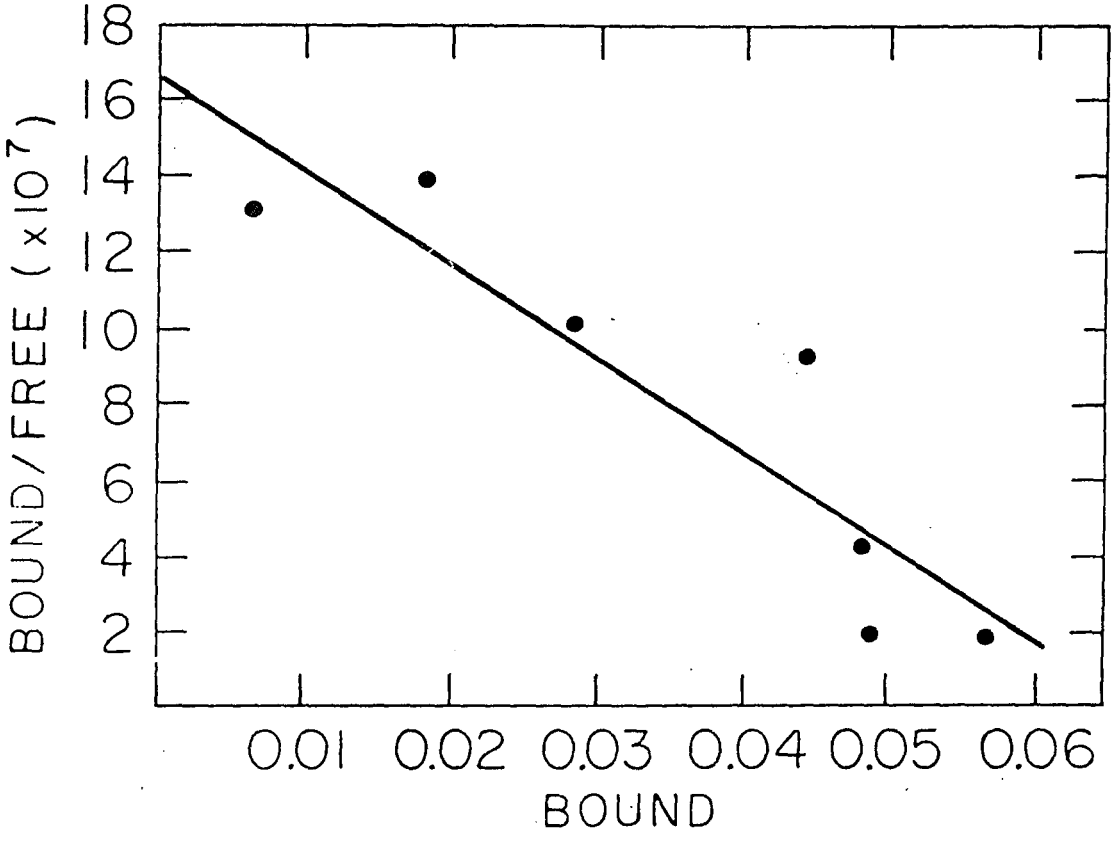
BOUND/FREE (x10^7)
18
16
14
12
10
8
6
4
2
0.01
0.02
0.03
0.04
0.05
0.06
BOUND

Fig. 9

Effect of some isomers of neuroleptic agents on [3H]DHE sites. d-Butaclamol and other drugs were dissolved in a minimal amount of glacial acetic acid and diluted with 0.1% ascorbic acid. Specific binding of [3H] DHE at 0.25 nM (Kd) represents 45-55% of the total binding. Incubation conditions are the same as indicated in Fig. 7. d-Butaclamol (O); cis-flupenthixol (△); trans-flupenthixol (▲); l-butaclamol (●).

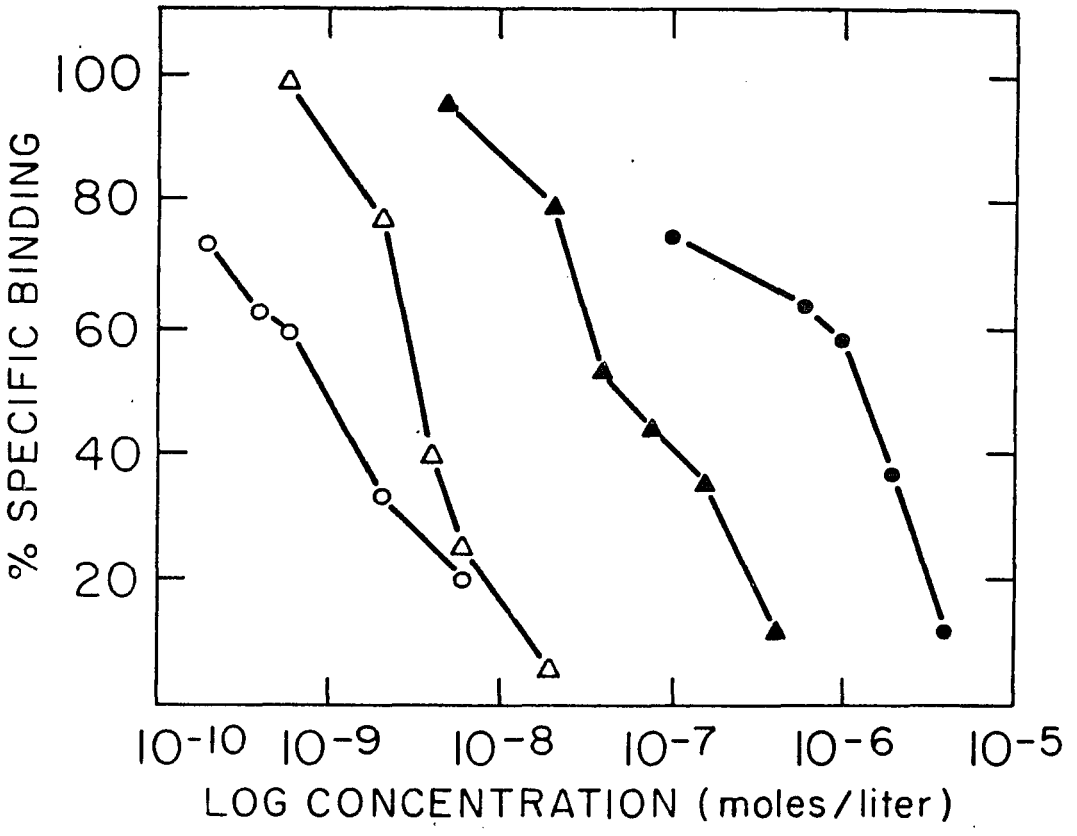
100
80
60
40
20
% SPECIFIC BINDING
10-10
10-9
10-8
10-7
10-6
10-5
LOG CONCENTRATION (moles/liter)

Fig. 10.

Effect of various agonists on [3H]DHE sites. Conditions as indicated in Fig. 7. Apomorphine (●); dopamine (○); (-)norepinephrine (▲); 5-HT(□).

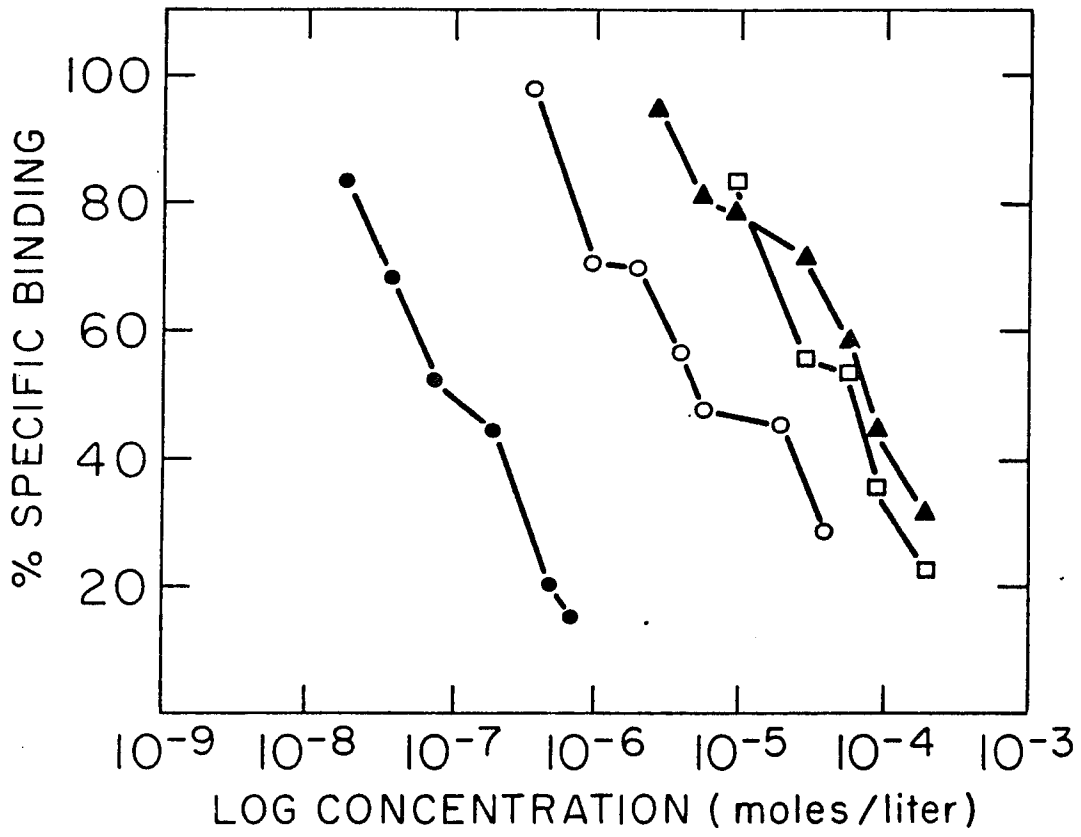
% SPECIFIC BINDING
100
80
60
40
20
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
LOG CONCENTRATION (moles / liter)

Fig. 11

Effects of DHE, neuroleptics and phentolamine on [3]DHE sites. Conditions as indicated in Fig. 7. DHE(○); haloperidol(●); chlorpromazine(□); metoclopramide(■); sulpiride(△); phentolamine (▲).

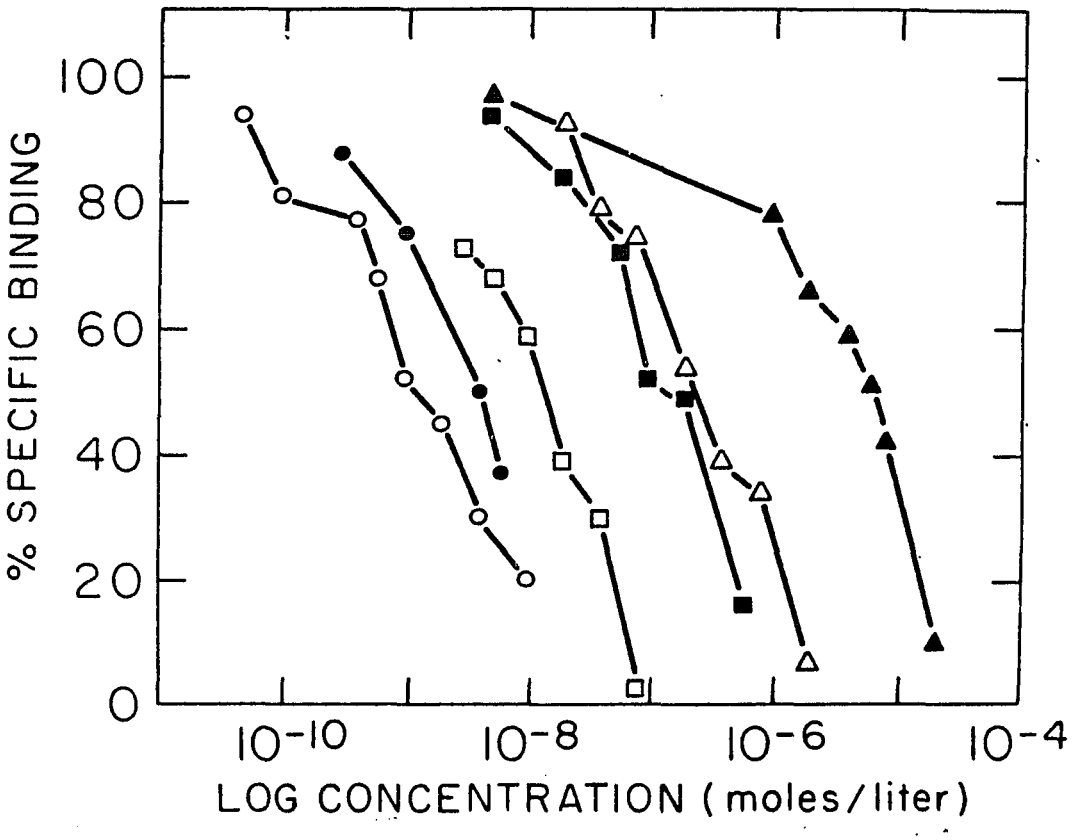
% SPECIFIC BINDING
100
80
60
40
20
0
10-10
10-8
10-6
10-4
LOG CONCENTRATION (moles/liter)

Fig. 12

A Dixon plot of unlabelled DHE competing for [3H]DHE sites in bovine caudate membranes. Conditions as indicated in Fig. 7. The Ki for the unlabelled ligand was calculated from Ki = Kd/((Bmax)(D*)) x 1/(slope), where Ki is the inhibition constant of the unlabelled ligand, Kd and (Bmax) are the dissociation constant and the total receptor density determined from the saturation experiment (Fig. 6). (D*) is the concentration of [3H]DHE used. Vertical bars represent standard deviation.

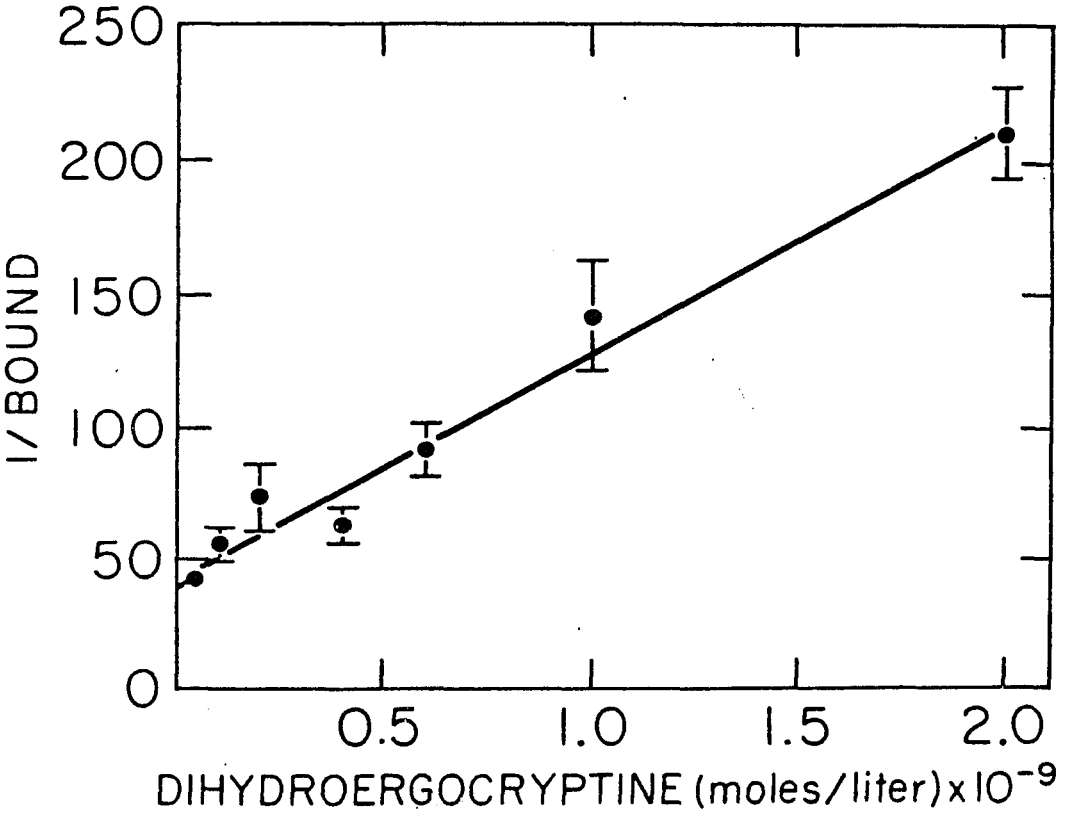
250
200
150
100
50
0
1/BOUND
0.5
1.0
1.5
2.0
DIHYDROERGOCRYPTINE (moles/liter) x 10⁻⁹

Table 4: Affinity of Spiroperidol, d-Butaclamol, and DHE for various Binding sites

Compound	Radioligand	Putative Receptor	Ki(nM)
Spiroperidol	Haloperidol	DA	0.2 [a]
	WB-4101	alpha-NE	18 [b]
	LSD	5-HT	6.3 [c]
d-Butaclamol	Haloperidol	DA	0.54[a]
	WB-4101	alpha-NE	24. [b]
	LSD	5-HT	42. [c]
DHE	DHE	DA	0.54[d]
	DHE	alpha-NE	1.55[e]
	LSD	5-HT	7.5 [f]

a Burt et al., 1976.

b Peroutka et al., 1977.

c Leysen et al ., 1978a.

d Tittler et al., 1977.

e Holck et al., 1979.

f Greenberg and Snyder, 1977.

Table 5: Competition for [3H]DHE sites by drugs

	Ki*(nM)+ SE(n)		IC50†(nM) +SE(n)		Hill Coefficient†+ SE(n)	
A. Agonists						
Apomorphine	62	(2)	98	(2)	0.87	(1)
DA			6200 ± 1520	(3)	0.58 ± 0.07	(3)
(-)NE			47700	(2)	0.64	(2)
5-HT			43220	(1)	0.63	(1)
B. Antipsychotic Agents						
Spiroperidol	0.1	(2)	0.22	(2)	0.94	(2)
Domperidone	0.5 ± 0.12	(3)	1.68 ± 0.75	(3)	0.9	(2)
Haloperidol			4.5 ± 1.02	(3)	0.76	(2)
Fluphenazine	0.49 ± 0.18	(3)	2.59 ± 1.77	(3)	0.94	(2)
Thiethylperane	2.97 ± 0.67	(3)	5.93 ± 2.15	(3)	1.07	(2)
Chlorpromazine	3.3 ± 0.47	(3)	9.96 ± 1.76	(3)	1.08 ± 0.09	(3)
Cis-Flupenthixol	2.43 ± 1.4	(3)	5.87 ± 2.47	(3)	0.85	(2)
Trans-Flupenthixol	30 ± 6.8	(3)	43	(2)	0.87	(2)
d-Butaclamol	0.78	(2)	1.26	(2)	0.93	(2)
l-Butaclamol	463	(2)	547	(2)	0.82	(2)
Loxapine	5.91 ± 0.67	(3)	15 ± 1.75	(3)	0.97 ± 0.13	(3)
Clozapine	125	(2)	317	(2)	1.	(1)
Perlapine	306 + 36	(3)	321	(2)	0.92	(2)
Metoclopramide	44 ± 13	(5)	70 ± 28	(5)	0.90 ± 0.08	(5)
Sultopride	50 ± 12	(5)	91 ± 28	(5)	0.95 ± 0.08	(4)
Sulpiride	99 ± 31	(3)	143 ± 48	(3)	0.81 ± 0.05	(3)
Tiapride			2908	(2)	0.68	(2)
C. Ergots						
DHE	0.44 ± 0.22	(3)	0.66 ± 0.13	(3)	1.03 ± 0.01	(3)
Lergotrile			81	(2)	0.62	(2)
Methysergide			231	(1)	1.72	(1)
D. Others						
Phentolamine			4000 ± 1300	(3)	0.75	(2)
U-25,927			324	(2)	1.22	(2)
Procainamide			20,000	(1)		

* Ki's for drugs with unit Hill coefficients were calculated from the Dixon plot. The amount of (3H)DHE used was 0.25 nM.

† IC50's and Hill coefficients were obtained from the Hill plot.

Table 6: IC_{50}'s and Hill coefficients of neuroleptic agents in the (3H) DHE and (3H) Spiroperidol binding assays

	(^{3}H) DHE		(^{3}H) Spiro*		IC_{50}(Spiro)
Drug	IC_{50} (nM)	Hill coefficient	IC_{50} (nM)	Hill coefficient	IC_{50} (DHE)
Spiroperidol	0.22	0.94	3.81	1.01	17
Haloperidol	4.5	0.76	104	0.83	23
Fluphenazine	2.59	0.94	26	1.0	10
Thiethylperazine	5.93	1.07	73	0.98	12
Chlorpromazine	10.	1.08	151	0.92	15
d-Butaclamol	1.26	0.93	4.16	0.74	3
l-Butaclamol	547	0.82	5290	1.56	10
Loxapine	15	0.97	83	0.72	5.5
Perlapine	321	0.92	3990	0.51	12
Clozapine	317	1	1420	0.64	4.5
Metoclopramide	70	0.94	16900	0.83	241
Sulpiride	143	0.95	29000	0.87	201
U-25,927	324	1.22	1429	0.72	44

* Values taken from Table 3. The amount of (3H)Spiro was 1-1.5 nM; the Kd of (3H)SPIRO was 0.69 nM.

Table 7: Comparison of effects of some neuroleptics on [^{3}H] DHE and [^{3}H] Spiro sites,and on DOPAC elevation in the rat, and to average clinical dose*

Drug	[^{3}H] DHE IC_{50}*	[^{3}H] Spiro IC_{50}	Striatal DOPAC Elevation ED_{50}	Average Clinical Dose
	nM	nM	um/Kg	um/Kg
Spiroperidol	0.22	3.81	0.4	0.06
d-Butaclamol	1.26	4.16	0.34	2.14
Fluphenazine	2.59	26	0.17	0.17
Haloperidol	4.5	104	0.43	0.51
Thiethylperazine	5.93	73	1.58	6.4
Loxapine	15	83	n.d.	3
Chlorpromazine	10	151	8.4	12
Clozapine	317	1420	61	24.5
Perlapine	321	3990	11	?
Metoclopramide	70	16900	8.9	12
Sulpiride	143	290000	176	40
U-25,927	324	1429	inactive	inactive

* Correlation between IC_{50} for [^{3}H] DHE sites and clinical dose ($R = 0.86$, $P < 0.002$); correlation between IC_{50}'s for [^{3}H] SPIRO sites and clinical dose ($R = 0.76$, $P < 0.015$). Correlation between IC_{50} for (3H) DHE sites and ED_{50} for DOPAC elevation ($R = 0.86$, $P < 0.0015$); correlation between IC_{50}'s for (3H) Spiro sites and ED_{50}'s for DOPAC elevation ($R = 0.85$, $P < 0.002$).

† IC_{50} was calculated from the Hill plot; (n) is the number of analyses. IC_{50}'s for (3H)Spiro sites were calculated from the Hill plot. ED_{50} values represent the dose of drug producing a half-maximal increase in DOPAC levels in rat striatum measured at the time of peak response. n.d. = not determined .

(^{3}H) Spiro was 1-1.5 nM; The K_d of (^{3}H) Spiro was 0.69 nM.

Results of the (3H)Spiro/ 4 nM d-Butaclamol Study

The specific binding of (3H)Spiro to membrane preparations from calf striatum, defined as the difference between binding in the absence and presence of 4 nM d-butaclamol, is apparently homogeneous, reversible, stereospecific, and heat sensitive. In saturation experiments, specific binding reached a plateau at approximately 0.4 nM (3H)Spiro (Fig 13) after a 30 min incubation period at 37°C. The total receptor density (Bmax) and dissociation constant (Kd), determined by the hyperbolic fit, using the PROPHET computer based on the Michaelis-Menten equation , were 78 $\pm$ 3 fmole/mg protein and 0.08 $\pm$ 0.008 nM, respectively, for an average of 4 determinations. In each determination the Scatchard plot was linear (Fig 14a) and the Hill analysis did not show any indication of multiple binding sites or cooperativity(Hill coefficient= 0.99 $\pm$ 0.05(3)). Thus, in contrast to the heterogeneous (3H)Spiro binding sites displaceable by 1 uM d-butaclamol(Fig 14b), sites labeled by (3H)Spiro using 4 nM d-butaclamol as the masking drug are apparently homogeneous. At 0.1 nM, the concentration used in the competition experiments, specific binding was 70-80% of the total binding.

The kinetics of (3H)Spiro was studied with 0.076 nM (3H)Spiro at 37°C. As shown in Fig 15, specific binding of (3H)Spiro to bovine striatum reached equilibrium within 20 min at 37°C. A plot of Ln(B(t)/(B(eq)-B(t)) vs. time yielded a Kobs of 0.1324 min^{-1}(Fig 16a) . In the same preparation, 4 nM d-butaclamol was introduced 30 min after (3H)Spiro incubation. 4 nM d-butaclamol rapidly reversed bound (3H)Spiro. 28 min after d-butaclamol addition, specific binding of (3H)Spiro was virtually totally reversed(Fig

15). A plot of Ln(B(t)) vs. time (Fig 16b) yielded k_{-1}= 0.102 min^{-1}. The association rate, k_1, calculated from $k_1 = K_{obs} - k_{-1} / (L^*) = 4 \times 10^8\ M^{-1}\ min^{-1}$. The dissociation constant Kd, calculated from k_{-1} / k_1 , was 0.26 nM, a value greater than the Kd(0.08 nM) from the saturation experiments.

In Table 8, a comparison between (3H)Spiro binding in the presence of 4 nM and 1000 nM d-butaclamol as the masking drug is shown. It can be seen that there are differences in the Scatchard plot and the rate constants. In the presence of 4 nM d-butaclamol, (3H)Spiro binding was homogeneous, whereas in the 1 uM system, (3H)Spiro binding was heterogeneous. In the kinetic experiments, the association rate (k_1) and dissociation rate(k_{-1}) constants of the 4 nM system are slightly greater than that of 1 uM system.

Specific binding of (3H)Spiro/4 nM d-butaclamol is heat sensitive and linearly dependent on protein concentration. Preheating of the membrane preparation in boiling water for 2 min eliminated specific binding. There was a linear relationship between protein concentration and specific binding up to 1.2 mg protein/test tube. A protein concentration of 0.7 mg/tube was routinely used.

The competition for (3H)Spiro binding at 0.1 nM (3H)Spiro was studied with the agonists DA, NE, and 5-HT, the ergot derivative DHE, and various DA antagonists(Fig 17,18 and Table 9). Among the agonists(Fig 17), the order of potency was compatible with DAergic sites as DA (IC50= 19 uM) was 10 times more active than NE and 25 times more active than 5-HT. DHE had an IC50 close to 1 nM(Table 9).

The antipsychotic agents were active in the (3H)Spiro assay, and for the most part, the potency of drugs within a given class agreed well with their anti-DA potencies in vivo. For example, the butyrophenone spiroperidol(IC50=0.27 nM) was 25 times more active than haloperidol(6.7 nM) which in turn was 60 times more active than the clinically inactive butyrophenone U-25,927. The phenothiazine fluphenazine (1.4 nM) was 20 times more potent than chlorpromazine; the dibenzoheteroepine loxapine (33 nM) was 10 times more active than clozapine(295 nM). The benzamide derivatives were exceptionally weak in the (3H)Spiro assay. The IC50 of metoclopramide was 1500 nM, and the IC50's of sultopride and sulpiride were 1240 and 4480 nM, respectively(Table 9). Stereospecificity of (3H)Spiro binding was observed. d-Butaclamol (IC50=0.54 nM) was 1500 times more potent than the l-isomer.

In Table 10, the IC50's and Hill coefficients of various neuroleptic drugs in the (3H)Spiro binding assay sensitive to 4 nM d-butaclamol and (3H)Spiro binding assay sensitive to 1 uM d-butaclamol are compared. There is a high correlation between the IC50's for 4 nM d-butaclamol sensitive (3H)Spiro sites and 1 uM d-butaclamol sensitive (3H)Spiro sites(R=0.98, slope=0.92, Fig 19). Compared to potencies in the 1 uM d-butaclamol sensitive system, the potencies of various drugs in the 4 nM d-butaclamol displaceable (3H)Spiro system have increased 10 fold.

Discussion

The % receptor occupancy by 4 nM d-butaclamol in the presence of 0.1 nM (3H)Spiro, the concentration used in the competition experiments, is given by :

$$B/Bmax = L / (L+ Kd(1+ D^*/K^*)) \qquad (1)$$

$$= 4/(4 + 0.12 (1 + 0.1/0.08)) = 94\ \%.$$

In the saturation experiments, the highest concentration of (3H)Spiro used was 2 nM, and under these conditions,4 nM d-butaclamol could theoretically occupy only 60% of the receptor sites (equa. 1). The use of 4 nM d-butaclamol as the masking drug however does not underestimate the Bmax of the system nor does it yield an erroneous value for Kd. we have previously shown for the (3H)DHE system that when a saturation experiment was done by using 20 nM d-butaclamol as the masking drug, the Bmax and Kd were the same as when 4 nM d-butaclamol was used. In addition, the Hill coefficient and the Scatchard plot showed similar properties as the 4 nM system. Reanalysis of the saturation data by eliminating points for high (3H)Spiro concentration, which contain most errors, have also yielded the same Bmax and Kd . It appears that the Kd and Bmax are insensitive to changes in the d-butaclamol concentration when it varies from 4 to 20 nM.

Similarly, in the competition experiments, it can be shown that the amount bound by the competitor is :

$$\frac{1}{1 + \frac{Kd}{D}} - \frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{B}{Kb}\right)} - \frac{1}{1 + \frac{Kd}{D}\left(1+ \frac{A}{Ka} \right)} +$$

$$\frac{1}{1 + \frac{Kd}{D}\left(1 + \frac{A}{Ka} + \frac{B}{Kb}\right)} \qquad (2)$$

where A is the concentration of the competitor, B is the concentration of d-butaclamol, and D is the concentration of (3H)Spiro. Ka, Kb and Kd are their respective dissociation constants. It can be calculated that the errors in the amount bound by the competitor from the use of 4 nM d-butaclamol instead of a high masking ligand concentration is 1% when A=Ka, and 3% when A=100 Ka. These errors are too small to have a significant effect on the measurement of the Ka in the competition experiments.

In radioreceptor binding studies, the characteristics of binding sites are heavily dependent on the specificity of the radioligand and the displacing drug. In an ideal situation when a relatively specific radioligand and a specific masking drug are used, the resultant binding can be expected to be homogeneous. On the other hand, binding studies using antipsychotic drugs pose many problems since these drugs are always non-specific, and when a non-selective radioligand and a non-selective displacing drug are utilized, the resulting binding sites can be expected to be heterogeneous. This seems to be the case with (3H)Spiro and d-butaclamol. These drugs have high affinity toward DAergic, 5-HT, and NE sites (Table 4). When (3H)Spiro is used as the radioligand, it can label several types of receptor sites. If a high concentration of d-butaclamol is used to displace (3H)Spiro binding, it would displace more than just the DAergic component of (3H)Spiro sites.

The specific binding, taken as the difference of these two, can then be expected to be heterogeneous, as shown by us and others (Lin et al.,1980; Briley and Langer, 1978; Howlett and Nahorski, 1980; Andorn and Maguire, 1980). In an effort to enhance the specificity of the binding system, we have now used a relatively low concentration of d-butaclamol(4 nM) as the masking drug. This is based on the assumption that this low concentration of d-butaclamol would displace predominnantly the DAergic component of (3H)Spiro binding.

As shown in Fig 14, the specific binding of (3H)Spiro to calf striatal membranes, defined as the difference between binding in the presence and the absence of 4 nM d-butaclamol, is apparently homogeneous over the range of (3H)Spiro concentration used(10^{-11} to 10^{-9} M). In contrast , specific binding of (3H)Spiro, when 1 uM d-butaclamol is used as the displacing drug, is heterogeneous over the same range of concentrations. The Bmax(80 fmole/mg protein) of the 4 nM d-butaclamol sensitive (3H)Spiro binding sites is about 35% of the Bmax of the 1 uM d-butaclamol sensitive sites(Table 2). The Kd of the 4 nM d-butaclamol sensitive (3H)Spiro sites is about the same as the high affinity component of the 1 uM d-butaclamol sensitive (3H)Spiro sites($B^{H}max$=50 , B^{L}=155 fmole/ mg protein; Kd^{H} = 0.12, Kd^{L}= 1.3 nM). It may be speculated that the 4 nM d-butaclamol sensitive (3H)Spiro sites represent predominantly the high affinity component of the 1 uM sensitive sites.

(3H)Spiro binding sites sensitive to 4 nM d-butaclamol have properties similar to those sensitive to 1 uM d-butaclamol and satisfy several criteria expected of DA receptors(Table 9). For

example, the potency of DA is greater than (-)NE and 5-HT; the clincially active d-butaclamol is more potent than the clinically inactive (-)isomer; and these sites are potently antagonized by the antipsychotic drugs.

As shown in Table 10, there is a high correlation between the IC50's of drugs for the 4 nM d-butaclamol sensitive (3H)Spiro sites and 1 uM d-butaclamol sites(R=0.98, slope=0.92, Fig.19). The unity slope suggests that these two binding sites are similar. Compared to the potencies in the 1 uM d-butaclamol sensitive (3H)-Spiro system, the potencies of various drugs have increased 10 fold in the 4 nM d-butaclamol displaceable (3H)Spiro system. This may be related to the use of a higher (3H)Spiro concentration(1-1.5 nM) in the competition experiments for the 1 uM d-butaclamol sensitive (3H)Spiro assay.

An examination of the Hill coefficients of neuroleptic drugs (Table 10) reveals that for many neuroleptic drugs the non-unity Hill coefficients observed in the 1 uM system have changed to unity Hill coefficients in the 4 nM d-butaclamol system. These are U-25,927, loxapine, clozapine, and d-butaclamol. The non-competitive nature of these drugs in the 1 uM d-butaclamol sensitive (3H)Spiro binding may be related to the heterogeneous nature of that system.

It should however be noted that the potencies of neuroleptic drugs in the present system do not necessarily reflect their potencies in vivo. For example, clozapine, metoclopramide, sulpiride and sultopride all increase DA turnover, elevate prolactin levels and are clinically effective antipsychotics. These drugs are all

weaker than the clinically inactive l-butaclamol in the binding assay. Clozapine also has similar affinity for (3H)Spiro sites as the clinically inactive butyrophenone U-25,927, which does not increase DA turnover. These discrepancies between properties in vivo and binding of neuroleptic drugs have been observed in the 1-uM d-butaclamol sensitive (3H)Spiro system(Lin et al., 1980).

In summary, the homogeneity of 4 nM d-butaclamol displaceable (3H)Spiro binding sites and the generally competitive nature of neuroleptic drugs in competing against these sites indicates that the use of a low concentration of displacing drug has enhanced the specificity of (3H)Spiro binding. Further studies on the neuroanatomical locations and pharmacological significance of these high affinity (3H)Spiro binding sites may give clearer meaning as to the nature of (3H)Spiro binding sites than previous studies done with a heterogeneous population of binding sites.

Fig. 13 : Saturation of specific (3H)Spiro binding to calf striatal membranes. Specific binding is defined as the difference between binding in the absence and the presence of 4 nM d-butaclamol. Samples were incubated for 30 min at 37°C. All determinations were made in triplicate. Total receptor density(Bmax) and dissociation constant(Kd) , calculated from the hyperbolic fit, were 78 fmole/mg protein and 0.1 nM, respectively. Concentrations of the labeled drug had been corrected for the amount bound. Vertical bars represent S.D. of triplicate tubes in one experiment.

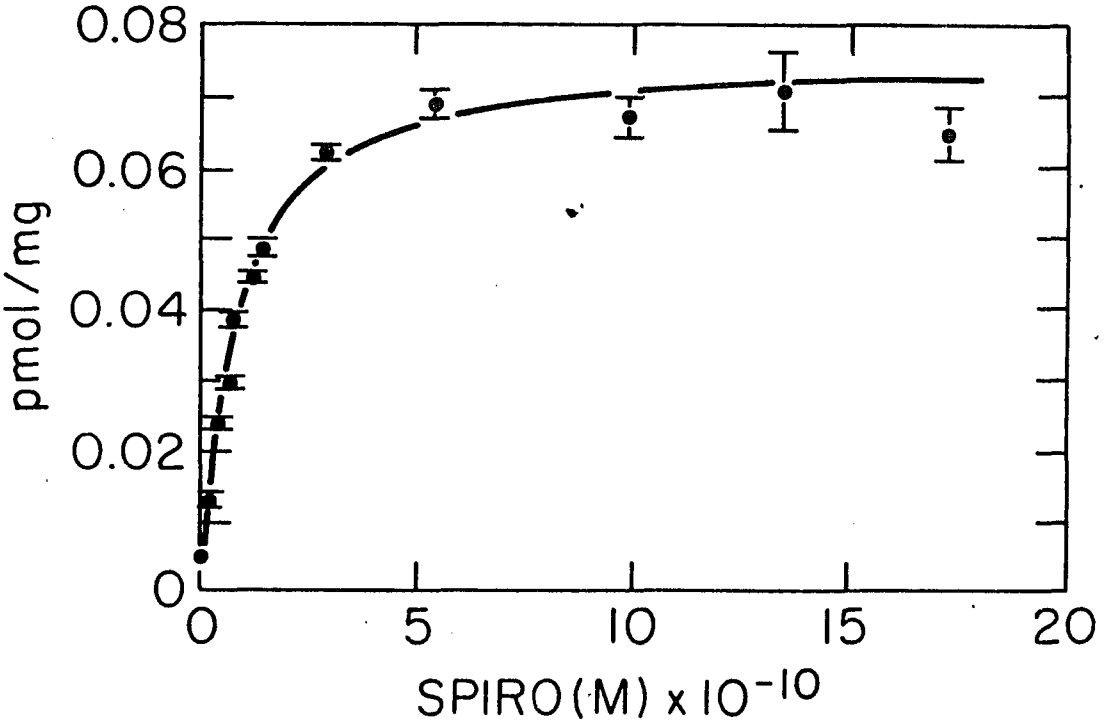
0.08
0.06
0.04
0.02
0
pmol/mg
0
5
10
15
20
SPIRO(M) x 10⁻¹⁰

Fig 14 a : The Scatchard plot of the specific binding in Fig. 13.

Fig 14 b : The Scatchard plot of a saturation experiment using (3H)Spiro and 1 uM d-butaclamol as the masking drug. Kd and Bmax for high(H) and low(L) affinity components were obtained by fitting the binding data into the equation:

$$B = \frac{B^{H}max\ (D^*)}{D^* + Kd^{H}} + \frac{B^{L}max\ (D^*)}{D^* + Kd^{L}}$$

where $B^{H}max$ = 50, $B^{L}max$ = 155 fmole/mg protein, Kd^{H} = 0.12, Kd^{L} = 1.3 nM. D* is the concentration of unbound (3H)Spiro.

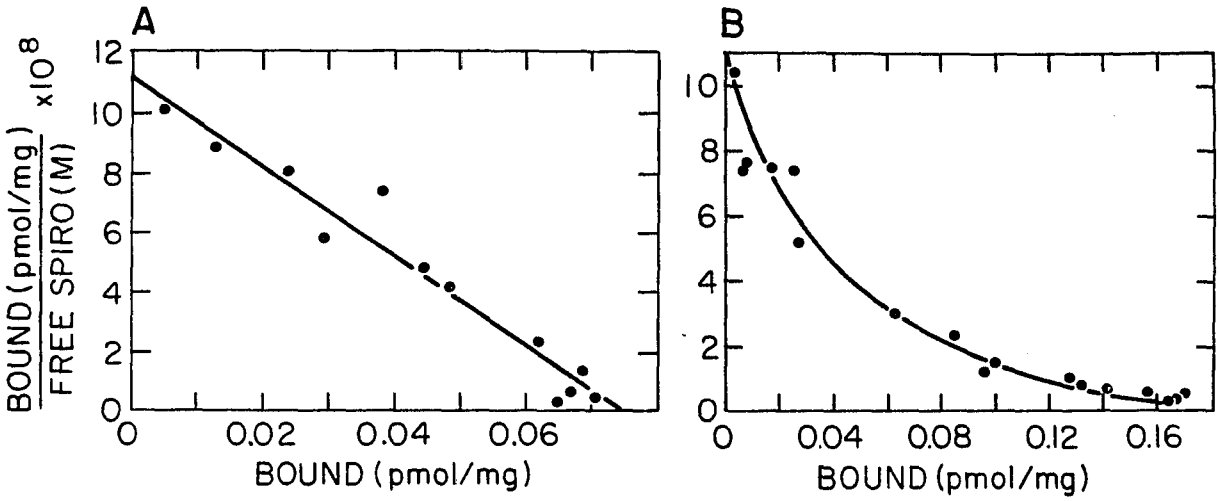
A
B
BOUND (pmol/mg) / FREE SPIRO (M) x10^8
BOUND (pmol/mg)
BOUND (pmol/mg)
0
2
4
6
8
10
12
0.02
0.04
0.06
0.08
0.12
0.16

Fig 15: The kinetics of (3H)Spiro binding to calf striatal membranes. Specific binding is defined as in Fig 13. Dissociation experiment was initiated by adding 4 nM d-butaclamol to the flask containing only (3H)Spiro. All determinations were in duplicate.

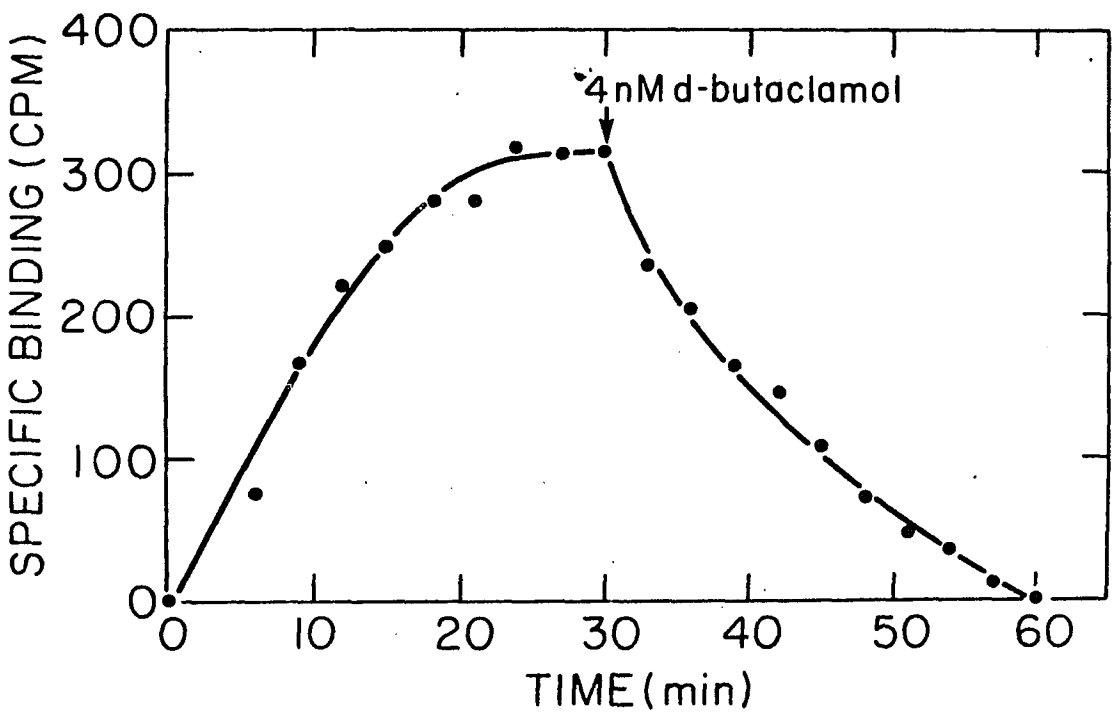
400
300
200
100
0
SPECIFIC BINDING (CPM)
4 nM d-butaclamol
0
10
20
30
40
50
60
TIME (min)

Fig 16 a : The plot of Ln(Beq/Beq -B(t)) vs. time(min) for the association of (3H)Spiro in Fig 15. The observed rate constant, K_{obs} = 0.1324 min^{-1}, was the slope of the plot.

Fig 16 b : The plot of Ln(B(t)) vs time(min) for the dissociation of (3H)Spiro in Fig 15. The dissociation rate, K_{-1} = 0.102 min^{-1}, was the slope of the plot.

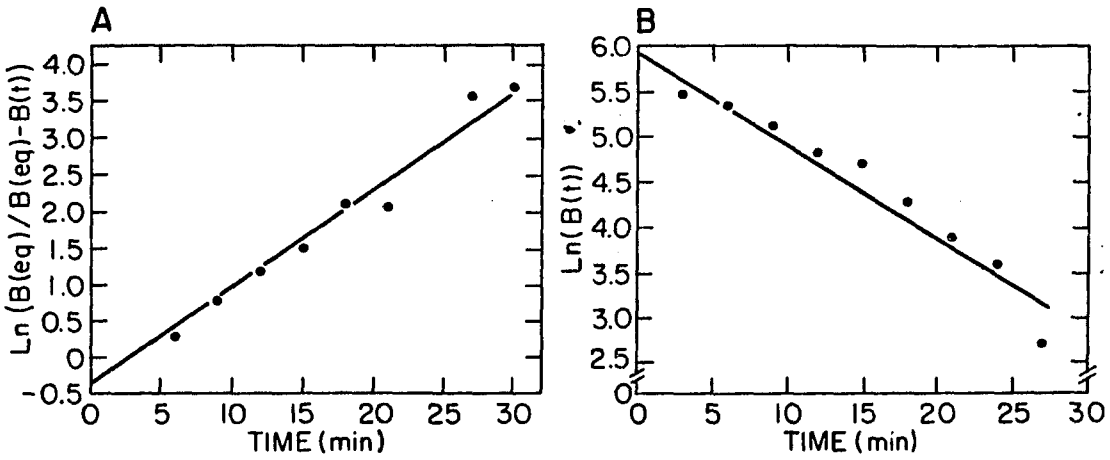
A
Ln (B(eq)/B(eq)-B(t))
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
0
5
10
15
20
25
30
TIME (min)
B
Ln(B(t))
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
0
5
10
15
20
25
30
TIME (min)

Fig. 17: Inhibition of specific (3H)Spiro binding in calf striatal membranes by the agonists, DA, 5-HT, and (-)NE. The antagonist, d-BUT = d-butaclamol, was included for comparison. Specific binding of (3H)Spiro at 0.1 nM represented 70-80% of the total binding. Incubation conditions were identical to that of Fig. 13. The S.E.M. for triplicates were within 15 % of the average.

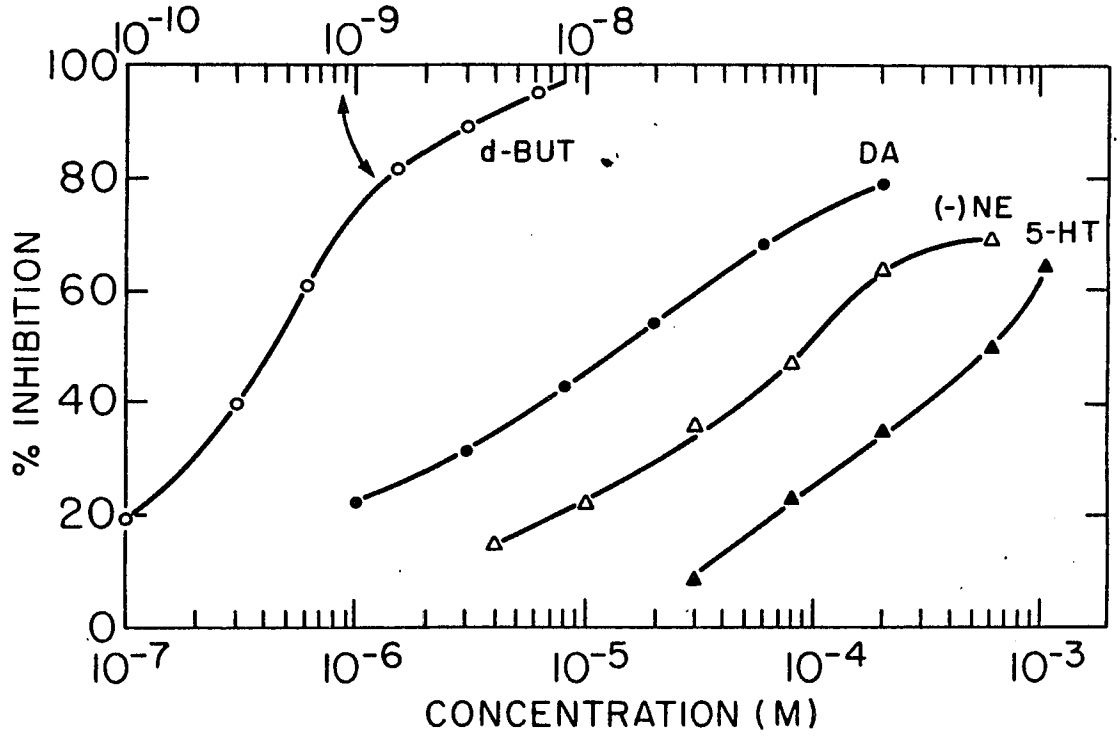
10^-10
10^-9
10^-8
100
80
60
40
20
0
% INHIBITION
d-BUT
DA
(-)NE
5-HT
10^-7
10^-6
10^-5
10^-4
10^-3
CONCENTRATION (M)

Fig.18: Effects of neuroleptic drugs on specific (3H)Spiro sites. Conditions were indicated in Fig 13. d-BUT = d-butaclamol; HAL = haloperidol; LOX = loxapine; CLOZ = clozapine ; l-BUT = l-butaclamol; MET = metoclopramide.

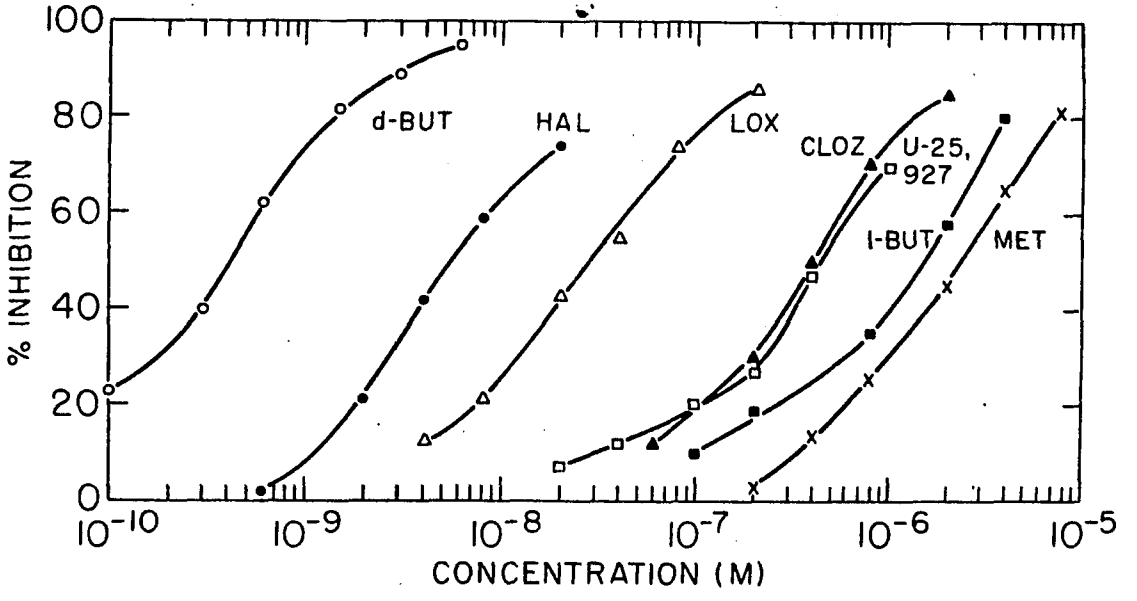
100
80
60
40
20
0
% INHIBITION
d-BUT
HAL
LOX
CLOZ
U-25,
927
I-BUT
MET
10-10
10-9
10-8
10-7
10-6
10-5
CONCENTRATION (M)

Fig.19: The correlation between the affinities of drugs for the (3H)Spiro binding sites sensitive to 4 nM d-butaclamol and (3H)Spiro binding sites sensitive to 1 uM d-butaclamol. (R= 0.98, slope = 0.92, P< 0.0001).

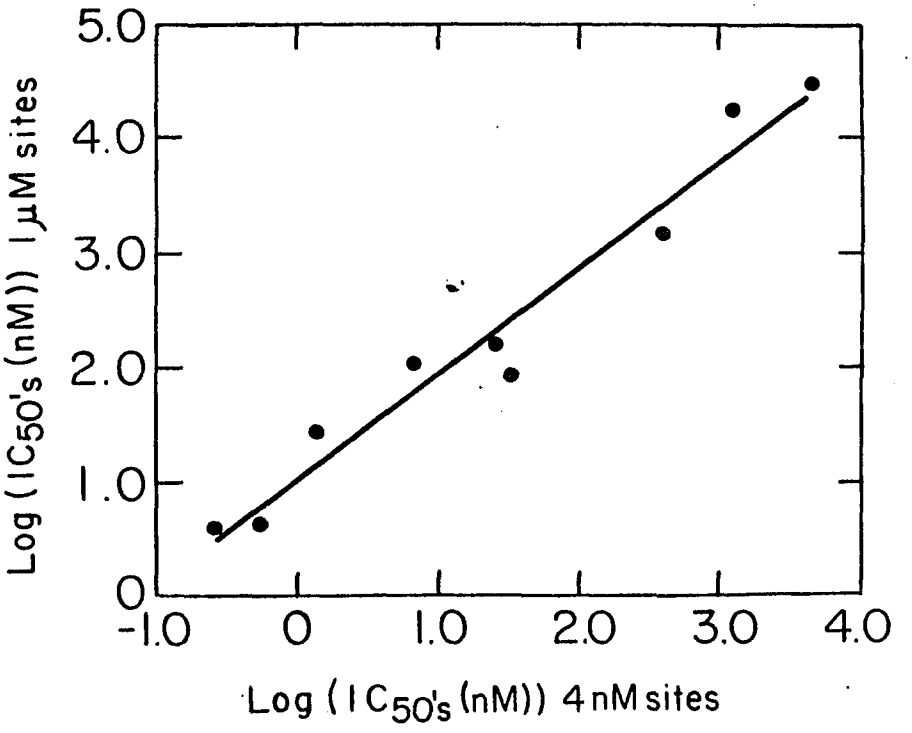
5.0
4.0
3.0
2.0
1.0
0
-1.0
0
1.0
2.0
3.0
4.0
Log (IC50's (nM)) 1 μM sites
Log (IC50's (nM)) 4 nM sites

Table 8: Comparison of the properties of (3H)Spiro sites displaceable by 4 nM d-butaclamol and by 1 uM d-butaclamol

Parameter	(3H)Spiro/ 4 nM d-butaclamol sensitive sites	(3H)Spiro/1 uM d-butaclamol sensitive sites
Kd	0.08 nM $\pm$ 0.008 (4)†	0.69 nM + 0.09 (11)
k_1	4×10^8 M^{-1} min^{-1}	1.67×10^8 M^{-1} min^{-1}
k_{-1}	0.102 min^{-1}	0.059 min^{-1}
Bmax	78 + 4 (4) fmole/mg	*230 + 10 (11) fmol/mg
Scatchard	Linear	Curvilinear
Hill coefficient	0.99 + 0.05 (3)	1.03 (11)

* Assuming a two component system, the Bmax and Kd of the high affinity component were estimated to be 50 fmole/mg protein and 0.12 nM, respectively. The Bmax and Kd of the low affnity component were 155 fmole/ mg and 1.3 nM, respectively. These values were calculated by a computerized iterative procedure based on the date from a representative experiment.

† Numbers in parentheses represent number of experiments.

Table 9: Competition for 4 nM d-butaclamol sensitive (3H)Spiro sites by drugs

Drugs	Ki(nM) ± SE(n)	IC50(nM) ± SE(n)	Hill coefficients ± SE(n)
A. Agonists			
DHE	0.25 ± 0.1(3)	0.73 (2)	0.85 (2)
DA		19,000 ± 7800 (3)	0.48 ± 0.01 (3)
(-)NE		196,000 ± 80000 (3)	0.5 ± 0.04 (3)
5-HT		502,000 ±129000 (3)	
B. Antagonists			
Spiroperidol	0.08 ± 0.01 (3)	0.27 ± 0.04 (4)	1.11 ± 0.08 (3)
d-Butaclamol	0.12 ± 0.026(3)	0.54 ± 0.1 (3)	1.11 ± 0.04 (3)
Fluphenazine	0.57 ± 0.12 (3)	1.4 (2)	0.91 (2)
Haloperidol	1.44 ± 0.14 (3)	6.7 ± 1.22 (3)	1.07 ± 0.03 (3)
Chlorpromazine	8.1 ± 2.5 (3)	26 ± 2.3 (3)	1.08 ± 0.06 (3)
Loxapine	10.2 ± 1.8 (3)	33 ± 3 (3)	1.14 ± 0.15 (3)
Clozapine	89 ± 23 (3)	295 ± 20 (3)	1.03 ± 0.11 (4)
Metoclopramide	440 ± 49 (5)	1500 ± 450 (5)	1.03 ± 0.11 (4)
Sultopride	500 ± 100 (3)	1240 ± 285 (3)	0.81 ± 0.04 (3)
Sulpiride	2038 ± 800 (3)	4480 ± 1500 (3)	0.84 ± 0.04 (3)
C. Clinically Inactive Durgs			
l-Butaclamol	185 ± 61 (4)	960 ± 210 (4)	1.15 ± 0.1 (4)
U-25,927	130 ± 24 (3)	410 ± 60 (3)	0.95 ± 0.05 (3)

Table 10: IC50's and Hill coefficients of neuroleptic agents in the (3H)Spiro/ 4 nM d-butaclamol and (3H)Spiro/1 uM d-butaclamol systems

	(3H)Spiro/4 nM d-Butaclamol		*(3H)Spiro/1 uM d-butaclamol	
Drugs	IC50 (nM)	Hill Coefficients	IC50 (nM)	Hill Coefficients
Spiroperidol	0.27	1.11	3.80	1.01
d-Butaclamol	0.54	1.11	4.16	0.74
Fluphenazine	1.4	0.91	26	1.0
Haloperidol	6.7	1.07	104	0.83
Chlorpromazine	26.	1.08	151	0.92
Loxapine	33	1.14	83	0.72
Clozapine	295	1.03	1420	0.64
Metoclopramide	1500	1.03	16900	0.83
Sulpiride	4500	1.03	29000	0.87
l-Butaclamol	960	1.15	5300	1.56
U-25,927	410	0.95	1430	0.72

* Values taken from Tables 1 and 2.

Results and Discussion of (3H)DHE Binding to Brain Slices

Binding of (3H)DHE to striatal slices of rat and calf showed stereoselectivity. In competing against total (3H)DHE binding, the clinically active d-butaclamol was 1000 times more potent than the l-isomer(Table 11). Competition by the agonists, DA, (-)NE and serotonin did not show that (3H)DHE binding was DAergic. The potency of DA(IC50= 20 uM) was about 3 times weaker than (-)NE and 5-HT(Table 17). The benzamide derivatives metoclopramide(IC50= 3 uM) and sulpiride(5 uM) were 100 times weaker than chlorpromazine(7 x 10^{-8} M) in competing against total (3H)DHE binding(Fig 20).

These data support the conclusions reached in studies done in membrane preparations. It was shown, for example, that (3H)DHE has high affinity toward DA, (-)NE and 5-HT sites and binding of this ligand can be expected to be non-selective. The present study in calf striatal slices indicates that (3H)DHE binding does not exhibit preferential affinity toward DA and may involve other neurotransmitter sites. Furthermore, this study also exemplifies that stereoselectivity alone is insufficient to indicate the nature of a particular binding sites(Enna et al; 1976).

Results and Discussion of (3H)Spiro Binding to Brain Slices

Kinetics of binding of (3H)Spiro to rat striatal slices was studied at 23°C. After a 40 min incubation, the specific binding, defined as the difference between binding in the absence and the presence of 400 nM d-butaclamol, reached a plateau and continued to stablize after 60 min. The displacement of the bound (3H)Spiro by 400 nM d-butaclamol showed that the specific binding declined steadily,

and a 50% reduction was observed after 50 min. In a saturation experiment using 400 nM d-butaclamol as the masking drug, the Kd and the Bmax were determined to be 0.25 nM and 35 fmole/2 slices.

In competing against total (3H)Spiro binding to slices, metoclopramide (IC50= 7 uM) and DA (20 uM) (table 12, Fig 21) were both ineffective. The d-isomer of butaclamol was 100 times more potent than the l-isomer. No specific binding was observed after the slices were boiled for 2 min.

The stereospecificity, reversibility, heat sensitivity and saturability of (3H)Spiro binding to slice preparations indicate that (3H)Spiro binding may be suitable for further characterization by autoradiographic means to visualize (3H)Spiro binding sites.

Fig. 20: Inhibition of total (3H)DHE binding to calf striatal slices by drugs. Incubation was done under dim light at room temperature for 90 min. The amount of (3H)DHE was 0.35 nM. d-But = d-butaclamol; CPZ = chlorpromazine; MET = metoclopramide; DA = dopamine.

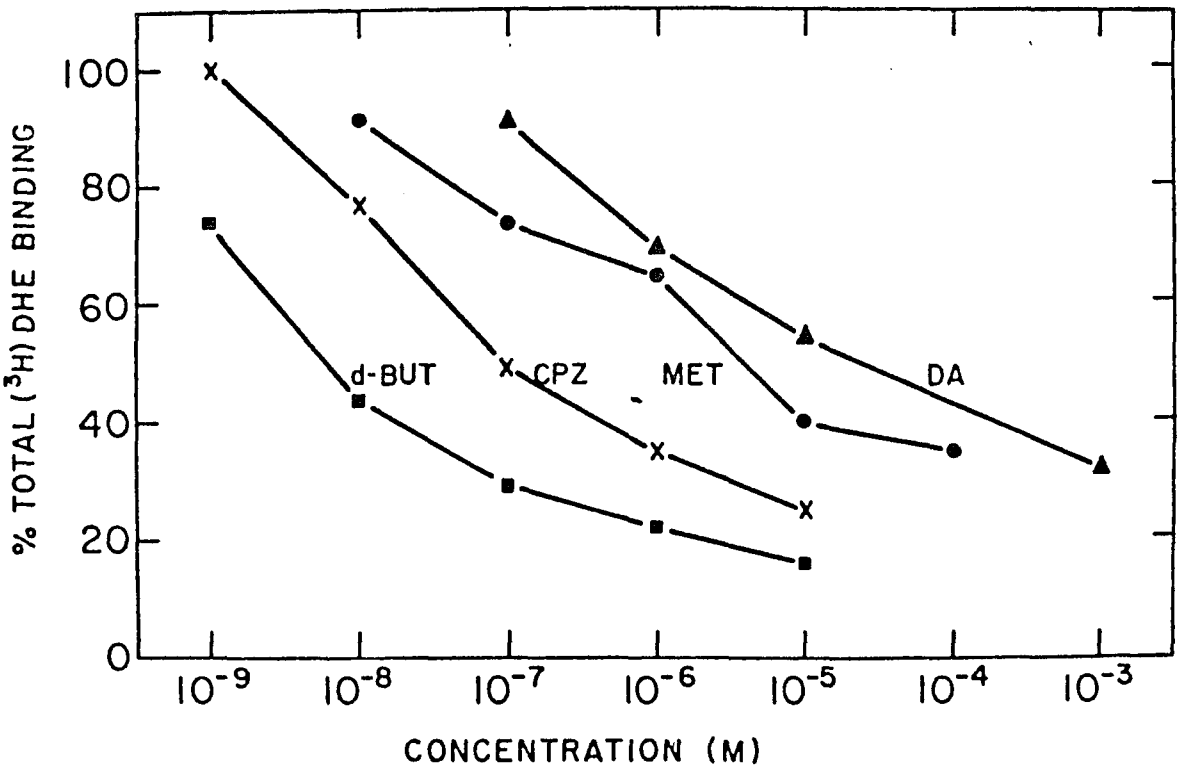
% TOTAL (^{3}H) DHE BINDING
100
80
60
40
20
0
d-BUT
CPZ
MET
DA
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION (M)

Fig. 21: Inhibition of (3H)Spiro binding to calf striatal slices by drugs. Incubation was done at room temperature for 60 min. The amount of (3H)Spiro used was 0.25 nM. d-But = d-butaclamol; CPZ = chlorpromazine; MET = metoclopramide; DA = dopamine.

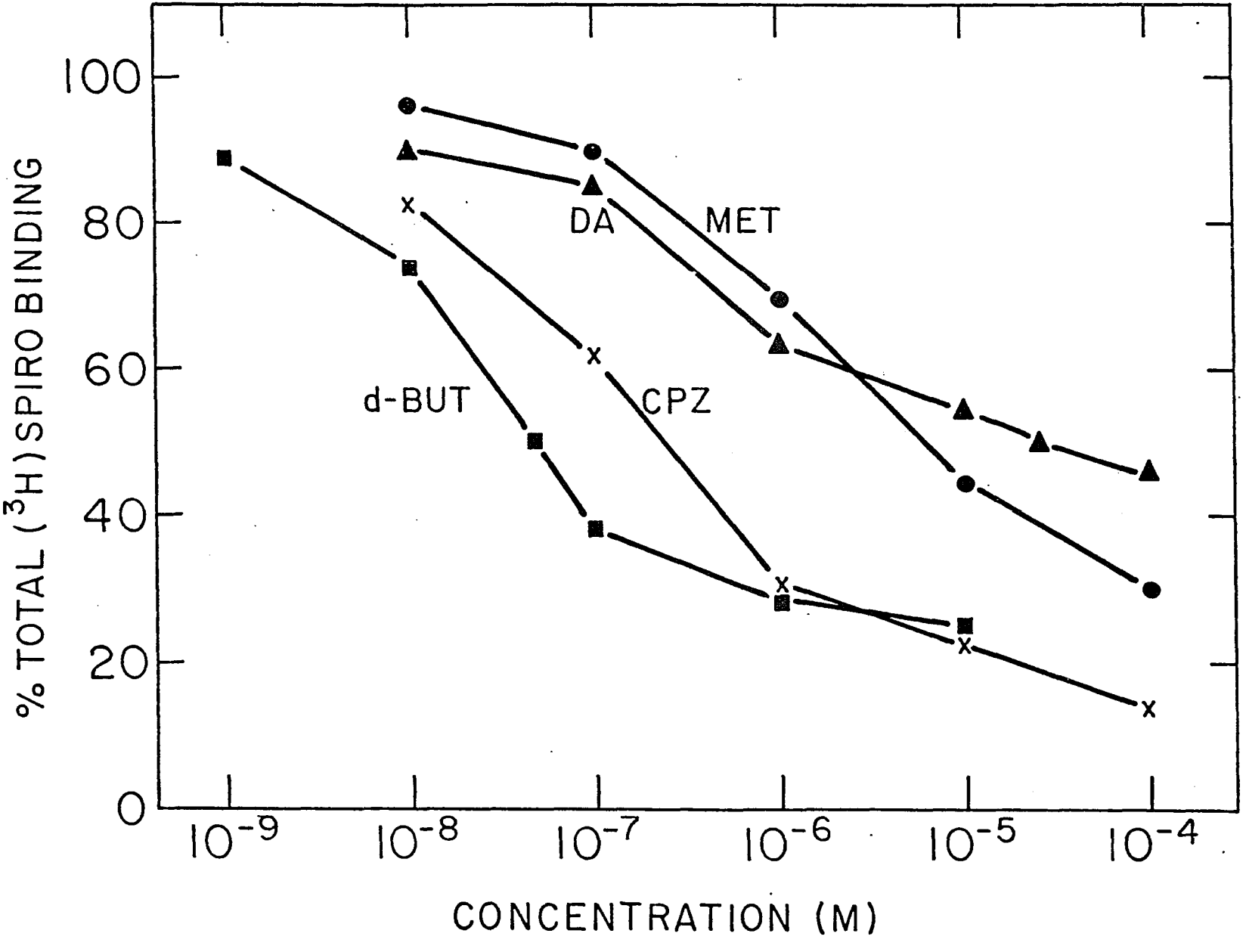
100
80
60
40
20
0
% TOTAL (^{3}H) SPIRO BINDING
DA
MET
d-BUT
CPZ
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
CONCENTRATION (M)

Table 11: IC50's* of drugs for Binding of (3H)DHE in brain slices

Drug	Striatal Calf Slices IC50(nM)
d-Butaclamol	60
Chlorpromazine	73 ± 2.5 (3)
Metoclopramie	3100 ± 1000 (3)
Sulpiride	4800
DA	21000 ± 6000 (3)
5-HT	6900
(-) NE	5300
l-butaclamol	44000

* The amount of (3H)DHE used was 0.2 to 0.25 nM.
IC50 values represent the concentration of drugs needed to displace 50% of the total (3H)DHE binding

Table 12: IC50's* of drugs for (3H)Spirot binding to slices

Drugs	Calf striatal slices IC50(nM)		Rat slices IC50(nM)	
d-Butaclamol	33	(2)	58	(2)
Chlorpromazine	230	(2)		
Metoclopramide	6700 ± 500	(3)	3500	
DA	19000	(2)		
l-Butaclamol			8000	

* IC50 represents the concentration of drugs needed to displace 50% of total (3H)Spiro binding.

† The amount of (3H)Spiro used was 0.3 nM.

Effects of Sultopride and Tiapride on DA Turnover

As shown in Tables 13 and 14 , sultopride and tiapride caused dose-dependent increases in DOPAC levels in rat striatum. The ED50 values for DOPAC increases were approximately 8 mg/kg for sultopride and 25 mg/kg for tiapride. However, it should be noted that the dose-response curves for these drugs were conducted 4 hr after injection. In subsequent studies it was determined that 1 hr after injection, both compounds showed greater effects on DA turnover(Table 13,14). Therefore, the ED50's for these compounds cited above could be lower.

In hypophysectomized rates, sulpiride(50 mg/kg) and metoclopramide(10 mg/kg) increased DOPAC levels in the striatum and TO. The levels of increases were comparable to unlesioned rats receiving the same doses (Stanley and Wilk, 1979). Hypophysectomy, therefore, does not influence the reponse of DA neurons to the benzamide injections. This is in contrast to results obtained by Portaleone et al(1978) who observed that the ability of sulpiride to increase DA turnover in rat brain was abolished after hypophysectomy. They hypothesized that the ability of sulpiride to affect DA turnover in the striatum was dependent on the drugs's ability to modify pituitary prolactin levels. However, their findings have not been substantiated here or by others(Jenner et al; 1978b).

Table 13: Effect of Tiapride on DA metabolism

Tiapride		Striatum		
Dose mg/kg	Time	DOPAC ug/g	HVA ug/g	DA ug/g
10	4 hr	1.59 ± 0.1 (4)	1.22 ± 0.09 (4)	
20		1.78 ± 0.17 (3)	1.73 ± 0.23 (3)	12.5 (2)
40		2.80 ± 0.22 (4)	2.48 ± 0.20 (4)	11.1 (2)
60		3.22 ± 0.31 (4)	3.28 ± 0.31 (4)	10.9 (2)
20	1 hr	2.24 ± 0.13 (4)	1.66 ± 0.26 (4)	
Saline		1.10 ± 0.07 (9)	0.80 ± 0.06 (9)	8.72 ± 0.26(3)

Tiapride		TO	
Dose mg/kg	Time	DOPAC ug/g	HVA ug/g
10	4 hr	1.18 ± 0.07 (4)	0.71 ± 0.03 (3)
20		0.97 ± 0.02 (3)	0.91 ± 0.08 (3)
40		1.43 ± 0.14 (3)	0.97 ± 0.09 (4)
60		1.5 ± 0.16 (4)	1.05 ± 0.14 (4)
20	1 hr	1.28 ± 0.16 (4)	0.87 ± 0.13 (4)
	2 hr	1.01 ± 0.23 (3)	0.76 ± 0.15 (3)
Saline		0.62 ± 0.05 (4)	0.35 ± 0.09 (4)

Table 14: Effect of sultopride on DA metabolism

Sultopride		Striatum	
Dose mg/kg	Time	Dopac ug/kg	HVA ug/kg
2.5	4 hr	1.82 ± 0.38 (4)	1.47 ± 0.22 (4)
5		2.32 ± 0.26 (4)	1.75 ± 0.18 (4)
10		2.9 ± 0.12 (4)	2.31 ± 0.02 (3)
20		2.95 ± 0.3 (3)	3.33 ± 0.16 (3)
30		3.85 ± 0.45 (4)	3.91 ± 0.21 (4)
Saline	1 hr	1.10 ± 0.09 (9)	0.8 ± 0.06 (9)

Sultopride		TO	
Dose mg/kg	Time	Dopac ug/kg	HVA ug/kg
2.5	4 hr	1.0 ± 0.06 (4)	0.51 ± 0.07 (4)
5		1.21 ± 0.17 (4)	0.56 ± 0.06 (4)
10		1.67 ± 0.24 (4)	0.82 ± 0.15 (4)
20		0.84 ± 0.06 (3)	1.26 ± 0.08 (3)
30		2.01 ± 0.10 (4)	1.26 ± 0.03 (4)
20	1 hr	1.81 ± 0.36 (4)	1.13 ± 0.06 (4)
	2 hr	0.82 ± 0.12 (3)	1.15 ± 0.4 (3)
	4 hr	0.84 ± 0.06 (3)	1.26 ± 0.08 (3)
Saline		0.62 ± 0.05 (4)	0.35 ± 0.09 (4)

Table 15: Effects of sulpiride and metoclopramide on DA metabolism in hypophysectomised rats

		Striatum		
Drug	Dose mg/kg	DOPAC ug/mg	HVA ug/mg	DA ug/mg
Sulpiride*	50	3.09 ± 0.24(6)	2.86 ± 0.2(6)	10.01 ± 0.68(5)
Metoclopramide†	10	2.73 (2)	3.25 (2)	
Saline		1.6 ± 0.1 (4)	1.06 ± 0.06(4)	10.2 ± 0.22(3)

		TO	
Drug	Dose mg/kg	DOPAC ug/kg	HVA ug/kg
Saline		0.88 ± 0.09(4)	0.61 ± 0.18(4)
Sulpiride*	50	1.41 ± 0.12(4)	1.13 ± 0.1 (5)
Metoclopramide†	10	1.65 (2)	1.67 (2)

* Rats were killed 4 hr after i.p. injection.

† Rats were killed 1 hr after i.p. injection.

¶ Values taken from Stanley and Wilk, 1979.

Radioreceptor Binding Assay as a Model to Predict Anti-DA Properties of Neuroleptic Drugs

Studies on (3H)Spiro binding sites sensitive to 1 uM or 4 nM d-butaclamol in calf striatal membranes demonstrate that (1) the affinities of typical neuroleptic drugs for (3H)Spiro binding sites parallels their clinical potenices and their ability to increase DA turnover. (2) the affinities of atypical neuroleptic drugs such as the dibenzoheteroepine, clozapine, and the benzamide derivatives, metoclopramide and sulpiride, for (3H)Spiro binding sites are not consistent with their clinical potencies and their ability to increase DA turnover. For example, l-butaclamol is a clinically inactive neuroleptic drug and it does not increase DA turnover. However, this compound is more potent than clozapine, sulpiride and metoclopramide in competing against (3H)Spiro binding. Similarly, U-25,927 is a clinically inactive butyrophenone, but it is more potent than the clinically active drugs sulpiride and metoclopramide. (3) Among neuroleptics such as the butyrophenones, phenothiazines, pentacyclics, and dibenzoheteroepines, there is a good agreement between binding and anti-DA properties in vivo of these drugs within a given class of compounds. Thus, the butyrophenone, spiroperidol, is more potent than haloperidol which in turn is much more potent than U-25-927. The phenothiazine, fluphenazine, is a stronger competitor for (3H)Spiro sites than thiethylperazine and chlorpromazine. Similarly, cis-flupenthixol has much higher

affinity for (3H)Spiro sites than trans-flupenthixol, d-butaclamol is more potent than the l-isomer, and loxapine is stronger than clozapine. These results indicate that when using (3H)Spiro assays to predict the anti-DA properties in vivo of a potential neuroleptic drug, it is best to compare its potency within a given class of compounds. However, if the drug belongs to the benzamide class, (3H)Spiro assays in calf striatal membranes are not suitable for predicting its anti-DA properties in vivo.

Although the benzamides metoclopramide, sultopride, and sulpiride are active in competing against (3H)DHE sites sensitive to 4 nM d-butaclamol, in agreement with their ability to increase DA turnover and their clinical activity, (3H)DHE assays can also yield misleading results as to the anti-DA properties of an unknown compound. Thus, tiapride, a benzamide derivative which increases DA turnover, is weaker in (3H)DHE assays than the clinically inactive U-25,927. Perlapine, a dibenzoheteroepine derivative which increases DA turnover in vivo, is equipotent with U-25,927, a drug which does not increase DA turnover. Trans-flupenthixol, a clinically inactive neuroleptic, has a much higher affinity for (3H)DHE sites than the clinically active clozapine. Therefore, despite its ability to reflect the anti-DA properties in vivo of several benzamide drugs, the use of the (3H)DHE binding assay to predict anti-DA properties of drugs has many drawbacks similar to the (3H)Spiro assays. To assess the anti-DA properties of a potential neuroleptic drug, it is best to combine binding assays with some measurements in vivo

(such as turnover studies). Such a combined approach would avoid misleading results from binding studies.

The Relationship Between (3H)DHE and (3H)Spiro Binding Sites

Studies using (3H)DHE and (3H)Spiro show that unlabeled DHE competes effectively against (3H)DHE and (3H)Spiro sites. Similarly, unlabeled Spiro is potent in competing against (3H)Spiro and (3H)DHE binding. These data indicate that (3H)DHE and (3H)Spiro interact with similar populations of binding sites. However, the benzamides such as metoclopramide and sulpiride are weak in competing against (3H)Spiro binding, but in the (3H)DHE assay, they were found to be active. The explanation for these apparent paradoxical properties of benzamides in the binding systems is unclear at present. Studies have shown that (3H)Spiro binding consists of two major components: (1) the guanine nucleotide sensitive component which disappears after kainic acid injection, (2) the guanine nucleotide insensitive component which disappears after cortical ablation. These data suggest that the guanine nucleotide sensitive population of (3H)Spiro binding may be linked to adenylate cyclase (D1 sites). The benzamides, being selective D2 antagonists, are inactive in reversing ligand binding to D1 sites, and therefore, are weak in (3H)Spiro assays. On the other hand, DHE binding may be exclusively to the D2 sites, as guanine nucleotides do not influence the competition of DHE for (3H)Spiro sites. The benzamides appear stronger because of

the selective D2 nature of DHE binding. However, it should be noted that guanine nucleotides may exert their regulatory role on (3H)Spiro binding independent of cyclase function. Studies have shown that in the anterior pituitary, where no DA-sensitive adenylate cyclase activity has been characterized, guanine nucleotides still modify binding of (3H)Spiro(Creese and Sibley, 1979).

Sodium Dependency of Benzamide Binding

Recently, Stefanini et al(1980) showed that the ability of sulpiride and other benzamides to compete for (3H)Spiro sites is selectively dependent on the presence of NaCl(> 0.1 M). In contrast, the typical neuroleptics butaclamol, haloperidol, and fluphenazine can displace (3H)Spiro binding either in the presence or absence of NaCl. Other ions such as $CaCl_2$, $MgCl_2$, and KCl do not change or only enhance slightly the ability of benzamides to compete against (3H)Spiro binding. They concluded that there are at least two populations of DA receptors present in the striatum, one of which is Na-dependent through which benzamides interact and the other is Na-independent, through which classical neuroleptic interact. Theodorou et al(1980) have also shown that NaCl can selectively enhance binding of (3H)Sulpiride to binding sites in rat striatum. However, it should be noted that the specific enhancement of benzamide binding by Na may be due to a non-specific effect. For example, Usdin et al(1980) showed that monovalent and

divalent cations as well as several chelating agents can increase the number of (3H)Spiro binding sites. This effect has been shown to be due to the prevention of the time-dependent decline of (3H)Spiro binding. In the absence of these ions, (3H)Spiro binding in rat membrane preparations starts to decline after 5 min incubation at 37°C. In Stefanini's study, (3H)Spiro was allowed to incubate for 12 min. If the benzamides can interact with a high affinity with sites lost in the incubation procedure, binding of (3H)Spiro in the absence of Na or other ions could lead to an apparent decrease in affinity for benzamide drugs. To determine a specific effect for Na activation of benzamide binding, it is crucial to establish stable binding conditions .

The Use Of A Low Masking Ligand Concentration In Radioreceptor Binding Studies

Studies with (3H)Spiro and (3H)DHE have demonstrated the value of using a low concentration of masking drug to enhance the specificity of radioreceptor binding sites. Previously, (3H)-Spiro binding has been assessed using 1 uM d-butaclamol as the masking drug. In that system, the Scatchard analysis reveals heterogeneity of binding sites. In addition, several neuroleptic drugs show non-competitive behavior in competing against (3H)Spiro binding. In contrast, when 4 nM d-butaclamol is used as the masking drug, (3H)Spiro binding sites become homogeneous. Moreover, drugs that show non-competitive behavior in the 1 uM system are exhibiting competitive inhibition of (3H)Spiro binding. Furthermore, the affi-

nity constant and the total receptor density of the 4 nM system are very close to the high affinity component of the 1 uM sensitive sites. These results indicate that the 4 nM d-butaclamol sensitive (3H)Spiro binding sites may represent the high affinity component of (3H)Spiro binding defined by 1 uM d-butaclamol. The use of a low concentration of masking drug can therefore, selectively define the high affinity component of radioligand binding sites. It should be appreciated that most of the radioligands used in characterizing binding sites are non-specific, i.e. they can interact with several neurotransmitter sites. The same holds true for many of the masking drugs. Thus, in order to define a homogeneous population of binding sites, it is crucial to establish an optimal condition where homogeneous binding can be favored. This can be achieved either by the inclusion of drugs that prevent binding to other transmitter sites or as in our studies by selecting a low concentration of the masking drug that preferentially interacts with sites of interest.

Conclusions

In the past few years, the DA-sensitive adenylate cyclase assay and the neuroleptic/DA radioreceptor binding assay have sustantially increased our understanding of multiple DA receptor mechanisms in the CNS. It is now appreciated that neuroleptic drugs such as the benzamides and the indole derivative, molindone,

may relieve schizophrenic symptoms by selectively blocking a population of DA receptors which are independent of DA-stimulated adenylate cyclase (D2 receptors). Similarly, D2 receptors are now implicated in the anti-Parkinsonian action of bromocryptine and other ergot derivatives. In the striatum, radioreceptor binding assays have sucessfully identified different DAergic binding sites. These different elements have been selectively characterized by 6-OHDA lesions, kainic acid lesions, and by cortical ablation. The discovery of a population of DAergic binding sites originating from cortical areas has opened another possibility for the site(s) of action of neuroleptic drugs. The finding that guanine nucleotides can seletively impede agonist binding and that sodium ions may seletively enhance the affinity of the benzamide neuroleptics for DAergic sites have also introduced us to the complexities of cellular regulation in vivo.

However, it should be noted that the physiological role of the DA-stimulated adenylate cyclase in the CNS is unclear at present. Further, because of the discrepancies between anti-DA properties in vivo of neuroleptics and their potencies in antagonizing DA-stimulated adenylate cyclase, and the difference in the subcellular localizations of DA-stimulated cyclase and neuroleptic binding activity, Laduron(1980) has maintained that the DA-stimulated adenylate cyclase is only an enzyme which may not be coupled to a specific receptor mechanism.

Although there is a good agreement between the anti-DA properties in vivo and potencies of neuroleptic drugs in competing for (3H)neuroleptic binding sites , several limitations of binding assays have also been uncovered. For example, we have found a discrepancy between the anti-DA properties in vivo and binding properties of atypical neuroleptic drugs. This discrepancy is not abolished despite the use of a low masking ligand concentration which can enhance the specificity of binding. In addition, sites labeled by the (3H)DA agonists and the (3H)DA antagonists have different pharmacological properties and the relationship between (3H)neuroleptic binding sites and DA receptors is still unclear at present. It is therefore often difficult to unequivocally relate the meaning of binding studies to DA receptor mechanisms. Further studies are needed to delineate the nature of binding sites.

Many lines of evidence suggest that sites labeled by (3H)DA-agonists and (3H)neuroleptics are distinct entities. For example, (3H)agonist binding is poorly reversed by unlabeled antagonist whereas the converse is true for (3H)-antagonist binding. Studies have also shown that binding of (3H)apomorphine and (3H)Spiro is differentially affected by kaninic acid lesions, which selectively destroys neuronal cell bodies. After kainic acid lesions, binding of (3H)apomorphine is decreased by 70-80%, whereas binding of (3H)Spiro is decreased only 40-50%. The remaining (3H)Spiro sites

have been shown to disappear after cortical ablation. Thus, it appears that the majority of (3H)apomorphine sites are located on cells intrinsic to the striatum. In contrast, half of (3H)Spiro sites are located on cells intrinsic to the striatum and the rest seems to be on nerve terminals originating from the cortex. Whether apomorphine labels pre-synaptic sites on nigro-striatal DA nerve terminals is unclear at present.

There are three distinct types of DAergic binding sites known at present: Sites having high affinity for antagonist and low affinity for agonist, sites with high affinity for agonist and low affinity for antagonist, and sites related to D1 receptors. At present, it is unclear whether these binding sites represent distinct DA receptro sites or that they represent different conformations of a DA receptor population. The high affinity antagonist and low affinity agonist sites have been examined by ligands such as (3H)Spiro, (3H)Hal and (3H)DHE. Of the three ligands, DHE is the only drug with agonist activity in vivo and the other two are potent butyrophenone neuroleptics. In our studies the benzamide neuroleptics are weak in the (3H)Spiro system and active in the (3H)DHE system. However, there has been one report which demonstrates a high potency of sulpiride for (3H)Spiro sites when (3H)Spiro concentration used is extremely low(Howlett and Nahorski; 1980).

The high affinity agonist and low affinity antagonist sites have been characterized by (3H)DA and (3H)apomorphine, and the D1 binding sites have been characterized with (3H)Cis-Flupenthixol.

It is apparent that the (3H)apomorphine and parts of (3H)Spiro binding sites are associated with guanine nucleotide binding protein. It has been shown that binding of (3H)apomorphine is impeded by GTP and GDP, and the affinities of DA agonists for (3H)Spiro sites are decreased by ions. It is unclear whether the guanine nucleotide sensitive protion of (3H)Spiro binding is related to D1 receptors. However, if they represent D1 receptors, they would be expected to have properties similar to the (3H)Flupenthixol binding sites. It has also been shown that most of the guanine nucleotide insensitive (3H)Spiro binding sites are from the cortical region. To study the relationship between guanine sensitive (3H)-Spiro binding and D1 receptors, it may be valuable to examine properties of (3H)Spiro sites in decorticated rats.

In conclusion, although the DA/neuroleptic binding assay can better account for the in vivo properties of neuroleptic drugs than can the DA-stimulated cyclase assay, the uncertainties of the nature of binding sites have limited their value for interpreting DA receptor mechanisms.

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clopramide and sulpiride, which are selective D2 antagonists. (3) To examine properties of (3H)Spiro binding sites using a low concentration of d-butaclamol as the masking drug and to compare them with properties of (3H)Spiro binding sites that used a high concentration of d-butaclamol as the masking ligand. As mentioned, antipsychotic drugs are believed to exert their clinical effect by the blockade of DA receptors. Biochemically, this effect can be monitored by measuring the increase in the turnover of DA neurons produced by these drugs, by the competition for the binding sites labeled by (3H)neuroleptic drugs, or by the reversal of stimulation of adenylate cyclase by DA. The relative clinical potencies of neuroleptic drugs in general parallels their potencies in increasing DA turnover and in competing for binding sites labeled by (3H)neuroleptics(Creese et al., 1976; Seeman et al., 1976) . Antagonism of adenylate cyclase stimulation correlates poorly with clinical potency. In our laboratory Wilk and Stanley have studied the metabolism of DAergic neurons in rat striatum and tuberculum olfactorium after i.p. administration of typical and atypical agents(Wilk et al., 1975; Wilk and Stanley , 1977; Stanley and Wilk, 1977, 1979). These studies have shown that antipsychotic drugs produce a characteristic dose-dependent increase in the level of 3,4-dihydroxyphenylacetic acid (DOPAC) in the brain regions described, whereas non-antipsychotic congeners or other classes of centrally acting drugs fail to elicit a similar increase in DOPAC levels. This dose-dependent elevation in DOPAC forms the basis of a predictive system for antipsychotic efficacy.

Table 1A: Properties of Some Typical and Atypical Neuroleptics on Various Dopaminergic Model Systems

Drug	(3H)Spiro pIC50[1]	DA-Stimulated Adenylate Cyclase Ki	Striatal DOPAC Elevation ED50	Catalepsy	Extrapyramidal Side Effects
		nM	um/kg		
Haloperidol	8.5	220[2]	0.43	+	+
Chlorpromazine	6.9	66 [2]	8.4	+	+
Clozapine	6.4	170[3]	61	-	-
Perlapine		480[3]	11	+	?
Sulpiride	7.1	inactive[4]	175	-	+
Metoclopramide	7.0	inactive[4]	8.5	+	+

(1) Leysen et al; 1978b.

(2) Clement-Cormier et al; 1974.

(3) Miller and Hiley, 1976.

(4) Roufagalis et al; 1976.

and clinical potencies of typical antipsychotics, but these assays are less reliable for neuroleptics belonging to the dibenzoheteroepines and inaccurate for the benzamides. Moreover, the ability of a drug to produce an elevation of striatal DOPAC in vivo more accurately reflects its antipsychotic properties than its ability to compete with (3H)Spiro binding to calf striatal membranes.