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DOPAMINE RECEPTORS IN RAT BRAIN REGIONS

OPTIMAL CONDITIONS FOR ^{3}H-AGONIST BINDING, pH DEPENDENCY AND LACK OF INHIBITION BY ASCORBIC ACID*

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Abstract—The binding of dopaminergic agonist and antagonist radioligands was investigated in rat brain regions. A 30-min preincubation of homogenates of caudate nucleus or mesolimbic brain regions at 37° induced a several-fold increase in the stereospecific binding of [^{3}H]-dopamine or [^{3}H]apomorphine to the subsequently washed particulate fraction, whereas it induced a slight decrease in [^{3}H]spiroperidol binding. Stereospecific ^{3}H-agonist binding was not observed in brain regions devoid of dopaminergic innervation. Guanosyl nucleotides, EDTA or ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetate (EGTA), included in the preincubation buffer, antagonized the stimulation of ^{3}H-agonist binding. The stereospecific binding sites of ^{3}H-agonists were saturable with equilibrium dissociation constants (K_d) of 1–2 nM. High-affinity binding was pH dependent, with different pH optima for each radioligand. Several dopamine receptor agonists and antagonists were potent inhibitors of stereospecific [^{3}H]dopamine binding, whereas *l*-ascorbic acid was inactive at concentrations as high as 1.0 mM. The stereospecific binding of [^{3}H]apomorphine or [^{3}H]spiroperidol was also unaffected by ascorbic acid. The nonspecific (*d*-butaclamol-insensitive) portion of ^{3}H-agonist binding was weakly inhibited by concentrations of ascorbic acid higher than 0.01 mM. This effect was also observed in the cerebellum and spinal cord, where none of the ^{3}H-agonist binding was stereospecific. It is concluded that the portion of ^{3}H-agonist or ^{3}H-antagonist radioligand binding which is related to dopamine receptors is unaffected by ascorbic acid.

The possible involvement of ascorbic acid in the regulation of dopamine receptor function has been suggested by several kinds of evidence. Ascorbic acid was found to inhibit the dopamine-stimulated adenylate cyclase from rat caudate nucleus [1] and to antagonize the behavioural effects of dopaminergic stimulants [2, 3]. Recently, it was reported that ascorbic acid inhibits the association of dopamine agonists with binding sites in rat caudate nucleus, spinal cord [4, 5] and other tissues. In contrast, Zahniser *et al.* [6] reported that the stereospecific binding of [^{3}H]amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]ADTN), a dopamine receptor agonist, to rat striatal membranes is not affected by ascorbic acid. Furthermore, Leff *et al.* [7] have reported that ascorbic acid is necessary for the detection of ^{3}H-agonist binding to dopamine receptors in calf caudate membranes.

The resolution of this discrepancy is important because ascorbic acid has been included in several assays of dopamine receptor binding [7–10], and a physiologic role of ascorbic acid in dopaminergic transmission would be of great interest. Several authors have cautioned that dopaminergic agonist radioligands may bind to nonspecific sites in brain membrane preparations [7, 8]. It is therefore necessary to establish whether ascorbic acid inhibits ^{3}H-agonist binding to dopamine receptors or to nonspecific sites.

In this paper, we describe optimal assay conditions for the measurement of ^{3}H-agonist radioligand binding to high-affinity stereospecific sites in rat brain regions. These sites are saturable and have a regional distribution and pharmacologic properties consistent with dopamine receptors. A wide range of ascorbic acid concentrations had negligible effects on the stereospecific binding of agonist or antagonist radioligands to dopamine receptors.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River, Boston, MA) weighing 250–300 g were used in all experiments. Caudate nuclei, mesolimbic regions (nucleus accumbens and olfactory tubercles) and other brain areas were dissected rapidly on an ice-cold surface as previously described [11] and frozen on dry ice until homogenization. Brain regions were homogenized with a Teflon pestle and glass homogenizer in 100 vol. of ice-cold 50 mM Tris–HCl buffer (pH 7.0) containing 3.0 mM $CaCl_2$. Homogenates were preincubated at 37° for 30 min and centrifuged at 20,000 *g* for 15 min. Pellets were washed twice with 100 vol. of fresh ice-cold buffer, and the final pellet was suspended in $CaCl_2$-free buffer (2.0 mg protein/ml) and used in the binding assay.

Standard binding assay conditions. Aliquots of the tissue suspension containing 0.2 mg protein were diluted to a final assay volume of 1.0 ml containing (final concentration) 50 mM Tris–HCl (pH 7.1),

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15 μM pargyline, 5.0 mM EDTA and the indicated concentrations of [^{3}H]dopamine (20–33.5 Ci/mmole, New England Nuclear Corp., Boston, MA). Sample tubes were placed in a 23° water bath for 30 min in the dark and rapidly passed through GF/B filters under suction. Previous experiments had demonstrated that stereospecific binding reached equilibrium by 20 min. Filters were washed with three 5-ml portions of assay buffer, allowed to dry for 30 min, and suspended in 5% Protosol-Econofluor (New England Nuclear Corp.) by vigorous mixing on a Vortex mixer. Radioactivity was measured with a Beckman LS7500 counter at 50% efficiency for tritium. Nonspecific binding was measured in the presence of 10 μM *d*-butaclamol. The same concentration of *l*-butaclamol had no effect on binding.

The binding of [^{3}H]apomorphine (30–40 Ci/mmole, New England Nuclear Corp.) was measured under the same conditions, with the exception of the pH of the binding assay, which was 6.1. [^{3}H]Spiroperidol (24–33 Ci/mmole, New England Nuclear Corp.) binding was measured with 0.1 mg tissue protein in 0.5 ml final assay volume, containing 5.0 mM EDTA, 15 μM pargyline and 50 mM Tris–HCl (pH 7.7) and the indicated concentration of radioligand. Samples were incubated at 37° for 15 min. Filtration and counting were done as described above. Nonspecific binding was measured in the presence of 1.0 μM *d*-butaclamol. In experiments designed to determine the effects of different preincubation conditions on binding, the above standard assay conditions were used. In experiments where the conditions of the binding assay itself were changed, preincubation conditions were kept constant [30 min at 37°, 1:100 homogenate in 50 mM Tris–HCl, 3.0 mM $CaCl_2$ (pH 7.0)]. The pH of all buffers indicated above was adjusted at room temperature.

Drugs and chemicals. GTP, GDP, ATP, dopamine, apomorphine and serotonin were purchased from the Sigma Chemical Co., St. Louis, MO, Butaclamol isomers were a gift of Ayerst Laboratories, Montreal, Canada. All other antipsychotic drugs were donated by the manufacturers. The GTP analog, guanylyl-imidodiphosphate (GPP(NH)P) was purchased from Boehringer-Mannheim, Indianapolis, IN. *l*-Ascorbic acid was obtained from the Fisher Scientific Co., Fair Lawn, NJ. Protein concentration was measured by the method of Lowry *et al.* [12] using bovine serum albumin as a standard.

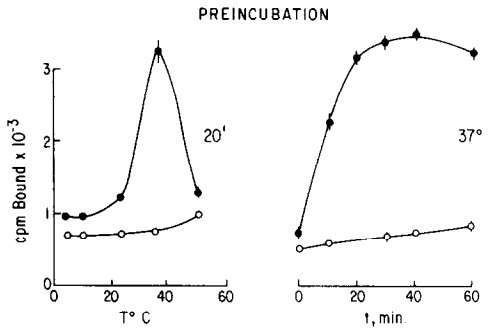

Fig. 1. Effect of preincubation temperature and duration on the binding of [^{3}H]dopamine. Caudate nuclei were homogenized in 100 vol. of Tris–HCl buffer (pH 7.0) containing 3.0 mM $CaCl_2$. Aliquots of the homogenate were preincubated at the indicated temperatures for 20 min. Following centrifugation and washing, binding was measured with 3.0 nM [^{3}H]dopamine under standard assay conditions. The right panel shows the effect of preincubation time on [^{3}H]dopamine binding. Open circles denote binding in the presence of 10 μM *d*-butaclamol. Closed circles denote total binding (mean ± S.D. of quadruplicate determinations). The difference between total and nonspecific binding was significant in each case [multiple analyses of variance (ANOVA), $P < 0.05$].

RESULTS

Effects of tissue preparation on radioligand binding. We have reported previously that preincubation of homogenates of rat caudate nucleus at 37° produced a drastic increase in the sterospecific binding of [^{3}H]dopamine to subsequently washed membranes [13, 14]. Further experiments were performed to determine the effects of this procedure on the stereospecific binding of [^{3}H]-apomorphine, the direct dopamine receptor agonist, and of [^{3}H]spiroperidol, the antagonist. It was found that preincubation at 37° increased the stereospecific binding of the ^{3}H-agonist by 5- to 6-fold, whereas it reduced [^{3}H]spiroperidol binding slightly (15%).

The increase in [^{3}H]dopamine binding was both time and temperature dependent; preincubation for 30 min at 37° resulted in the highest amount of stereospecific binding to the subsequently washed pellet (Fig. 1). Temperatures below or above the physiologic range were not effective in increasing [^{3}H]dopamine binding. The same pattern of results

Table 1. Parameters of saturable binding of dopamine receptor radioligands*

	Caudate nucleus		Mesolimbic regions	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
[^{3}H]DA	550 ± 23	1.7 ± 0.17	220 ± 32	2.2 ± 0.20
[^{3}H]APO	860 ± 60	1.6 ± 0.21	340 ± 43	1.5 ± 0.11

* Stereospecific binding was measured as described in the text under standard preincubation and assay conditions, using 0.5 to 10.0 nM [^{3}H]dopamine ([^{3}H]DA) or [^{3}H]apomorphine ([^{3}H]APO). The K_d was the slope and the B_{max} was the y-axis intercept of Scatchard plots obtained from saturation isotherms such as those shown in Fig. 4. The mean ± S.D. of three to seven separate experiments is shown in each case.

Table 2. Effects of ascorbic acid and dopamine receptor blockers on [^{3}H]dopamine binding in rat spinal cord or cerebellum*

Drug	Concentration in assay	% Total binding Spinal cord	Cerebellum
d-Butaclamol	100 nM	110 ± 11	105 ± 9
	1000 nM	94 ± 8	93 ± 5
Haloperidol	100 nM	105 ± 7	93 ± 10
	1000 nM	96 ± 5	95 ± 8
Ascorbic acid	0.01 mM	90 ± 9	110 ± 14
	0.1 mM	81 ± 4†	80 ± 5†
	1.0 mM	70 ± 5†	71 ± 4†

* Tissues were homogenized, preincubated, and washed as described in the text. Binding was measured with 3.0 nM [^{3}H]dopamine. Total binding was comparable in both regions (160 ± 20 fmoles/mg protein). Data are shown as the mean ± S.D. of quadruplicate determinations.

† Significant difference ($P < 0.05$, multiple ANOVA).

was obtained with [^{3}H]apomorphine binding, which was increased from 150 fmoles/mg protein in non-preincubated caudate nucleus to 600 fmoles/mg protein after a 30-min preincubation in experiments similar to those shown in Fig. 1, conducted with 3.0 nM [^{3}H]apomorphine.

Preincubation increased ^{3}H-agonist binding in the caudate nucleus and in mesolimbic regions (Table 1). In all cases, preincubation increased the stereospecific portion of the binding selectively, without altering nonspecific binding. No stereospecific binding of either ^{3}H-agonist was observed in the thalamus, hypothalamus, pons-medulla, hippocampus, occipital cortex, cerebellum or spinal cord (data from the last two regions are shown in Table 2). When tissue homogenates were washed by repeated centrifugations and resuspensions prior to the preincubation step, the increase in ^{3}H-agonist binding still occurred, but was reduced and had a shorter time course [14].

Several compounds exerted different effects on ^{3}H-agonist binding, depending on whether they were added to the preincubation medium or directly to the binding assay. GTP, GDP and GPP(NH)P antagonized the effect of preincubation on [^{3}H]dopamine binding, whereas ATP did not (Table 3). In contrast, these compounds had only a slight inhibitory effect on the stereospecific binding of [^{3}H]dopamine when added directly to the binding assay.

Table 3. Effects of nucleotides on the preincubation-induced increase in stereospecific [^{3}H]dopamine binding in rat caudate nucleus*

Nucleotide	concn	% Inhibition of SSB
GTP	0.1 mM	41 ± 3†
	1.0 mM	52 ± 4†
GPP(NH)P	0.1 mM	34 ± 3†
	1.0 mM	40 ± 3†
GDP	0.1 mM	45 ± 4†
	1.0 mM	51 ± 3†
ATP	1.0 mM	0

* Nucleotides were added to the preincubation of the homogenate at 37°. Binding assays of the washed pellets were performed under standard conditions. Control levels of stereospecific binding (SSB) of [^{3}H]dopamine (3.0 nM) were 355 ± 40 fmoles/mg protein. The means ± S.E.M. of quadruplicate determinations are shown.

† Significant inhibition ($P < 0.05$, multiple ANOVA).

The effect of preincubation on ^{3}H-agonist binding was also reduced in the presence of the divalent ion chelators EDTA or ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetate (EGTA; Fig. 2). A markedly different effect of EDTA and EGTA was observed when these compounds were added directly to the binding assay of ^{3}H-agonists. A drastic decrease in total binding was observed, which appeared to be due to a selective reduction of nonspecific binding (Fig. 2). The chelators reduced the variation between duplicate assay samples, which was very high in their absence.

Effect of assay pH on stereospecific binding. The pH of the binding assay was controlled with Tris–HCl buffers, 50 mM, containing 5.0 mM EDTA. Tris concentrations of up to 80 mM did not inhibit the binding of any radioligand. The stereospecific binding to membranes from mesolimbic brain regions or caudate nucleus was very sensitive to changes in pH (Fig. 3). At acidic pH, very low amounts of binding were detectable. As the pH approached neutrality, total binding increased much faster than nonspecific binding, yielding a large stereospecific difference. The decreases in stereospecific ^{3}H-agonist binding at alkaline pH were due to a sharp increase in nonspecific binding without a concomitant increase in total binding. In contrast, nonspecific [^{3}H]spiroperidol binding remained low at alkaline pH, resulting in a prolonged plateau of specific [^{3}H]spiroperidol binding. All subsequent binding assays were carried out at the optimal pH of each radioligand binding ([^{3}H]dopamine, pH 7.1; [^{3}H]apomorphine, pH 6.1; [^{3}H]spiroperiodol, pH 7.7).

Parameters of saturable binding. The stereospecific binding sites of the three radioligands were saturable in the caudate nucleus and mesolimbic regions. We have described the characteristics of [^{3}H]spiroperidol binding in detail elsewhere [15]. Scatchard analysis of saturation isotherms suggested a single class of binding sites of [^{3}H]dopamine or [^{3}H]apomorphine

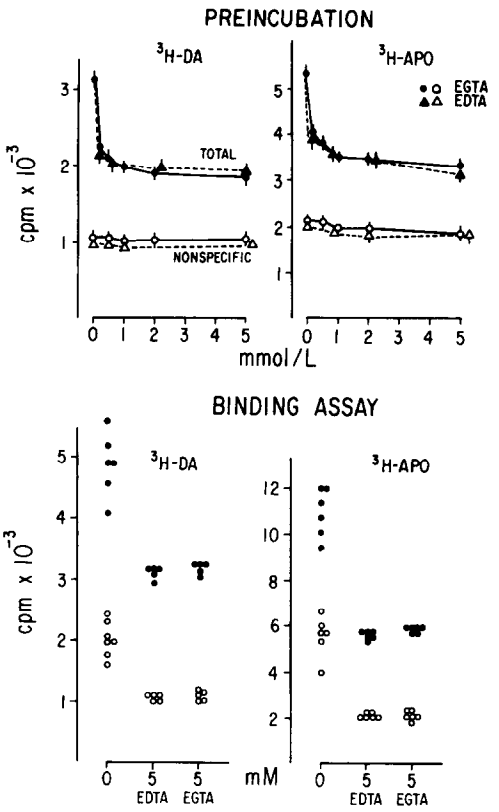

Fig. 2. Top panel: Effects of EDTA or EGTA during preincubation. Tissues were homogenized in 100 vol. of Tris–HCl buffer (pH 7.0) containing the indicated concentrations of chelators at constant pH. Homogenates were preincubated for 30 min at 37°, and binding to the subsequently washed pellets was measured under standard assay conditions, with 3.0 nM [^{3}H]dopamine or [^{3}H]apomorphine. Nonspecific binding was measured in the presence of 10 μM *d*-butaclamol. Results are the mean ± S.D. of quadruplicate determinations. The decrease in binding induced by EDTA or EGTA was statistically significant (multiple ANOVA, $P < 0.05$) at each concentration tested. Bottom panel: Effects of EDTA or EGTA on the binding assay of ^{3}H-agonists. Caudate nuclei were homogenized in 100 vol. of 50 mM Tris–HCl (pH 7.0), 3.0 mM $CaCl_2$ and preincubated at 37° for 30 min. The homogenate was centrifuged and washed as described in Materials and Methods. Binding was measured in aliquots of the final suspension with or without EDTA and EGTA. The concentration of [^{3}H]dopamine was 3.0 nM and that of [^{3}H]apomorphine was 2.8 nM.

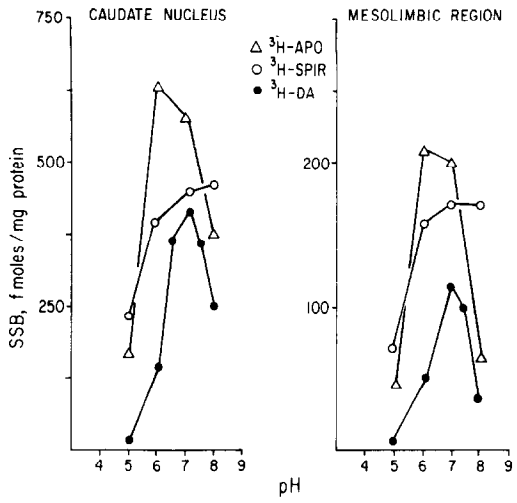

Fig. 3. Effect of the pH of the binding assay on the stereospecific binding (SSB) of dopaminergic radioligands. Standard preincubation and binding assay conditions were used, except for the indicated pH of the assay buffer, which was controlled with Tris–HCl buffers, 50 mM. Stereospecific binding was measured as described in Materials and Methods, with 1.0 nM [^{3}H]spiroperidol and 3.0 nM [^{3}H]dopamine or [^{3}H]apomorphine.

in the caudate nucleus or in mesolimbic regions (Table 1). The drastic effect of preincubation at 37° on the parameters of [^{3}H]dopamine binding is illustrated in Fig. 4. In non-preincubated caudate nucleus the number of binding sites (B_{max}) was equal to one-fifth of the B_{max} observed in preincubated tissues, which was 490 fmoles/mg protein. The K_d was reduced, by a 30-min preincubation, from 4.4 ± 0.5 to 1.53 ± 0.2 nM.

Pharmacologic properties of the ^{3}H-agonist binding site. The stereospecificity of a large portion of ^{3}H-agonist binding was easily demonstrable with *d*- and *l*-butaclamol (Fig. 5). Using 3.0 nM [^{3}H]dopamine or [^{3}H]apomorphine and 0.2 mg preincubated caudate nucleus protein, about 2000 and 5000 stereospecific cpm could be obtained with each radioligand, respectively, enabling the accurate and reproducible performance of competition studies. The stereospecific sites of both ^{3}H-agonists had much higher affinities for dopamine than they did for serotonin (Fig. 5). The abilities of several additional dopamine receptor agonists and antagonists to inhibit the stereospecific binding of [^{3}H]dopamine (3.0 nM) to particulate fractions of caudate nucleus or mesolimbic region were determined. Relative drug potencies

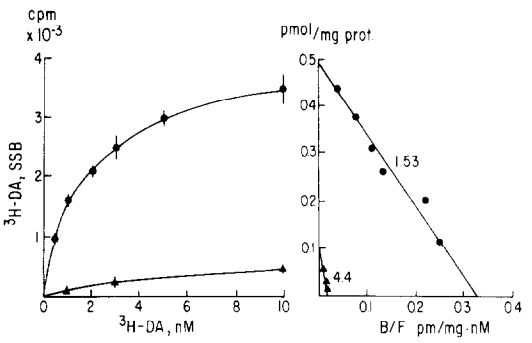

Fig. 4. Saturation and Scatchard analysis of stereospecific [^{3}H]dopamine binding: effect of preincubation on the binding parameters. The binding of the indicated concentrations of [^{3}H]dopamine was measured with 0.28 mg caudate nucleus protein, under standard assay conditions. The y-axis of the left panel shows stereospecific binding (SSB) in cpm. The same data are shown as pmoles/mg protein on the y-axis of the right panel. Triangles: 30-min preincubation at 4°; circles: 30-min preincubation at 37°. The B_{max} values (x-axis intercept ± S.D. by linear regression analysis) were 95 ± 14 and 490 ± 34 fmoles/mg protein. K_d values (slope of the Scatchard plot ± S.D.) were 4.4 ± 0.5 and 1.53 ± 0.2 nM respectively.

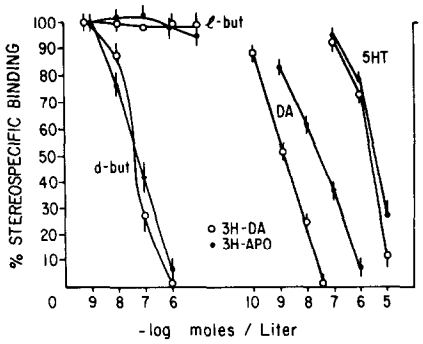

Fig. 5. Stereospecific displacement of ^{3}H-agonist binding in rat caudate nucleus. Standard preincubation and assay conditions were used, with a 3.0 nM radioligand concentration. Stereospecific binding was 2150 ± 60 cpm for [^{3}H]dopamine ([^{3}H]DA) and 4950 ± 110 for [^{3}H]apomorphine ([^{3}H]APO). Results shown are the mean ± S.D. of quadruplicate determinations. The average IC_{50} of several experiments such as these shown here is given in the text. Abbreviations: *l*-but: *l*-butaclamol; *d*-but: *d*-butaclamol; DA: dopamine; and 5-HT: serotonin.

were as follows (IC_{50}, nM, mean ± S.E.M. of two to four experiments, caudate nucleus, mesolimbic region): dopamine, 2.3 ± 0.4, 2.1 ± 0.3; apomorphine, 5.5 ± 0.5, 6.1 ± 0.4; serotonin, 2500 ± 150, 3100 ± 200; norepinephrine, 65 ± 12, 70 ± 10; epinephrine, 70 ± 13, 76 ± 10; *d*-butaclamol, 75 ± 11, 90 ± 20; fluphenazine, 100 ± 17, 120 ± 30, haloperidol, 120 ± 22, 400 ± 75; and chlorpromazine, 250 ± 60, 460 ± 60.

^{3}H-Agonist binding was also measured in particulate fractions of cerebellum or spinal cord. Total binding in these regions was equal to the amount of nonspecific binding measured in the caudate nucleus with comparable radioligand concentrations. This binding was not inhibited by concentrations of *d*-butaclamol or haloperidol which abolished stereospecific ^{3}H-agonist binding in the caudate nucleus or in mesolimbic regions (Table 2).

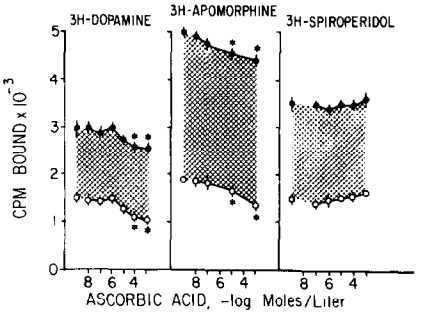

Fig. 6. Effects of ascorbic acid on dopaminergic radioligand binding. Binding was measured under standard assay conditions, with 3.0 nM ^{3}H-agonists or 1.0 nM [^{3}H]spiroperidol. Assay pH was constant at all concentrations of ascorbic acid. Results shown are the mean ± S.D. of quadruplicate determinations. Closed circles denote total binding and open circles denote binding measured in the presence of 10 μM *d*-butaclamol. Asterisks denote a statistically significant difference from control (measured in the absence of ascorbic acid and shown as the first point on the left-hand side of each panel) ($P < 0.05$, multiple ANOVA). The shaded area in each graph represents the stereospecific portion of binding.

Effects of ascorbic acid on radioligand binding. Ascorbic acid included in the standard binding assay at constant pH had no effect on the stereospecific binding of any of the three radioligands tested. Neither the total nor the butaclamol-insensitive portion of [^{3}H]spiroperidol binding to particulate fractions of caudate nucleus was affected by the above concentrations of ascorbic acid (Fig. 6). Similar results were obtained with particulate fractions of mesolimbic brain regions (data not shown).

The stereospecific portion of [^{3}H]dopamine or [^{3}H]apomorphine binding was similarly unaffected by ascorbic acid. However, total and nonspecific (butaclamol-insensitive) binding of these two radioligands declined in parallel when ascorbic acid concentrations above 10 μM were present in the binding assay. The magnitude of this change was small (15–20%) but statistically significant (Fig. 6). Ascorbic acid also had a weak inhibitory effect on ^{3}H-agonist binding in the spinal cord and in the cerebellum (Table 2).

DISCUSSION

Although the conditions which affect radioligand binding to dopamine receptors *in vitro* have been investigated in several laboratories [7, 8, 10], the method of tissue preparation prior to binding assay has not been as thoroughly evaluated. However, it has become apparent, from studies of γ-aminobutyric acid (GABA) [16], serotonin [17, 18] and dopamine [13, 14] receptors, that the prior treatment of the tissue can markedly alter the outcome of binding assays. In this paper, we present further evidence that preincubation of rat brain homogenates at 37° is critical for the measurement of dopaminergic ^{3}H-agonist receptors. Preincubation increased the binding of ^{3}H-agonists by several-fold while it reduced the binding of [^{3}H]spiroperidol by 15%. This phenomenon appears to be unrelated to the heat-inactivation of ^{3}H-agonist binding reported by Hamblin and Creese [19]. The effects observed here had a strict requirement for physiologic temperature, whereas those described by the above authors were obtained at 53°. Moreover, GTP and its analogues antagonized the preincubation-induced increase in stereospecific ^{3}H-agonist binding (Table 3), whereas they mimicked the effects induced by heat treatment [19]. Similarly, there is no apparent relationship between the present findings and the heat-denaturation phenomena reported by Lew and Goldstein [20].

Several kinds of evidence suggest that the preincubation-induced increase in ^{3}H-agonist binding is associated with dopamine receptors. Preincubation increased binding only in those brain regions containing a dense dopaminergic innervation, such as the caudate nucleus and mesolimbic regions. The increased binding was stereospecifically displaced by *d*-butaclamol, a dopamine receptor blocker. The stereospecific ^{3}H-agonist binding sites were saturable with K_d values in the low nanomolar range. *d*-Butaclamol is also a stereospecific blocker

of serotonergic and α-adrenergic receptors [21–23] but [^{3}H]dopamine did not appear to bind these receptors under the assay conditions used in the present study. For example, stereospecific binding of [^{3}H]dopamine was not observed in brain regions containining noradrenergic or serotonergic innervation, such as the hippocampus or the cerebellum. Moreover, dopamine and apomorphine were several hundred times more potent than serotonin as inhibitors of stereospecific [^{3}H]dopamine binding and thirty times more potent than norepinephrine or epinephrine. Several dopamine receptor antagonists inhibited the entire stereospecific portion of [^{3}H]dopamine binding both in the caudate nucleus and in the mesolimbic region. The higher relative affinity of ^{3}H-agonist binding sites for agonists as compared to antagonists suggest that they may be "D-3" receptors, in accordance with a currently used classification scheme [19, 24]. It appears that preincubation at 37° increases binding to D-3 receptors but not to the D-2 receptors labeled by [^{3}H]spiroperidol, in agreement with the view that these are separate entities [19, 24].

Under our experimental conditions, preincubation at 37° was essential for the precise measurement of stereospecific ^{3}H-agonist binding in rat brain regions. Other techniques have been reported for the measurement of [^{3}H]apomorphine binding in rat brain [8, 10, 25–28]. Advantages of the present method include consistently high amounts of stereospecific ^{3}H-agonist binding and the elimination of the anomalous kinetics obtained with previous methods [8]. Saturable binding of both ^{3}H-agonists, measured as described here, was characterized by linear Scatchard plots and Hill coefficients near unity. The [^{3}H]apomorphine binding sites we reported prior to the development of the method described here were nonspecific [29]. In that study, tissues were not preincubated prior to binding assay, and "blanks" were determined with micromolar concentrations of nonradioactive agonists.

Because the stereospecific binding of ^{3}H-agonists was sensitive to changes in pH, it was ensured that ascorbic acid or other drug solutions did not change the pH of the binding assay. Under these conditions, ascorbic acid did not alter the stereospecific binding of either antagonist or agonist radioligands to particulate fractions of caudate nucleus or mesolimbic brain regions, whereas several dopamine receptor agonists and antagonists did. A small decrease in total binding of ^{3}H-agonists was observed with higher concentrations of ascorbic acid, but this could be attributed to a decrease in nonspecific binding (Fig. 6). Accordingly, ascorbic acid induced the same weak inhibition of nonspecific binding in cerebellum and spinal cord as it did in the caudate nucleus (Table 2).

The discrepancy between the present results and previous reports that ascorbic acid inhibits ^{3}H-agonist binding to dopamine receptors may be due to methodological factors. Previous investigators [4, 5] used micromolar concentrations of nonradioactive dopamine or apomorphine to define the specific binding of [^{3}H]dopamine or [^{3}H]apomorphine. However, high concentrations of nonradioactive agonists may displace ^{3}H-agonists from nonspecific sites not related to dopamine receptors [7, 8]. Moreover, the dopaminergic properties of the sites inhibited by ascorbic acid were not demonstrated [4, 5]. The results of these investigators may, therefore, be explained by the ability of ascorbic acid to inhibit nonspecific ^{3}H-agonist binding. This view is supported by their observation that ascorbic acid inhibited [^{3}H]dopamine binding in the spinal cord, a tissue devoid of stereospecific [^{3}H]dopamine sites, as potently as it did in the caudate nucleus [5].

Leff *et al.* [7] reported that ascorbic acid was necessary for the measurement of specific ^{3}H-agonist binding in caudate nucleus membranes. In the present study, the EDTA included in the binding assay may have served as an antioxidant, thus obviating the use of ascorbic acid. Leysen [25] has suggested that EDTA facilitates the assay of stereospecific ^{3}H-agonist binding by lowering nonspecific binding, a view which is supported by our results (Fig. 2). The finding that antioxidants and ion chelators facilitate the binding of ^{3}H-agonist radioligands [7, 8] may be due to the chemical properties of radioligands rather than physiological properties of the receptors. For example ascorbic acid does not alter the stereospecific binding of [^{3}H]ADTN to rat caudate nucleus [6].

There is agreement between previous studies [4, 7] and the present results that ascorbic acid does not alter specific [^{3}H]spiroperidol binding. This may be due to the fact that all three laboratories defined the specific binding of [^{3}H]spiroperidol with *d*-butaclamol. This further emphasizes the necessity of defining "specific" binding rigorously, so that results from different laboratories can be compared. It appears that when strict criteria of dopamine receptor identification are applied to radioligand binding sites, the effects of ascorbic acid on either ^{3}H-agonist or ^{3}H-antagonist binding are negligible. The hypothesis that ascorbic acid regulates dopamine receptor function needs to be cautiously reevaluated.

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