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EFFECTS OF HEAVY METAL CATIONS, SULFHYDRYL REAGENTS AND OTHER CHEMICAL AGENTS ON STRIATAL D_2 DOPAMINE RECEPTORS*

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Abstract—To investigate aspects of the biochemical nature of the membrane-bound D_2 dopamine receptor, rat striatal homogenates were pretreated with heavy metal cations and a variety of other chemical agents, and their effects on D_2 receptor density were subsequently determined using a standard [^{3}H]spiperone binding assay. Preincubation of striatal membranes in the presence of 3 mM Mn^{2+}, Fe^{2+}, Co^{2+}, EDTA, or ascorbate enhanced subsequently measured stereospecific binding of [^{3}H]spiperone compared to control (e.g. control: B_{max} = 140 fmoles/mg protein, K_D = 0.21 nM; Mn^{2+}-treated: B_{max} = 253 fmoles/mg protein, K_D = 0.20 nM). Another group of metal cations, that is Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and CH_3Hg^+, all of which have significant −SH reactivity, as well as the −SH alkylating agent *N*-ethylmaleimide (NEM), caused a decrease in specific binding sites. Pretreatment with 3 mM Cd^{2+} or Cu^{2+} resulted in a 40–60% reduction in the subsequently measured stereospecific binding of [^{3}H]-spiperone, whereas 1 mM Hg^{2+} or 3 mM NEM completely abolished specific [^{3}H]spiperone binding. The effect of Hg^{2+} could not be reversed by washing the membranes, nor by further incubation of the membranes in the presence of excess EDTA or 2,3-dimercapto-1-propanesulfonic acid (DMPS). Further incubation in the presence of 3 mM dithioerythritol (DTE) resulted in the regeneration of about 40% of lost sites. Agents which enhanced receptor density, such as Mn^{2+} or EDTA, could not antagonize the effect of Hg^{2+}, nor could the mercury-chelating agent DMPS, when added to crude homogenates prior to Hg^{2+}. Ascorbate protected 25–35% of specific binding sites by virtue of its ability to reduce Hg^{2+} to insoluble Hg^+. Only 3 mM DTE afforded complete protection against 1 mM Hg^{2+}. Prior formation of the spiperone/receptor complex also demonstrated considerable ability to protect receptors from destruction by Hg^{2+}. Preincubation of striatal membranes in the presence of 0.5 mM spiperone protected about 80% of sites from the subsequent addition of 1 mM Hg^{2+}. A major conclusion of these studies is that one or more free −SH groups on or adjacent to the active site may be a requirement for specific antagonist binding to the membrane-bound D_2 receptor. Occlusion of these −SH groups by sulfhydryl reagents results in partial to complete abolition of subsequently measured specific ^{3}H-antagonist binding. Only agents which can regenerate free −SH groups, such as DTE, are able to induce any recovery in specific binding sites. Other substances, such as Mn^{2+}, EDTA and ascorbate which, on their own, cause an enhanced density of specific antagonist binding sites are generally ineffective in preventing the action of sulfhydryl binding agents. Their enhancing effect is probably due to the protection of receptors from normal degradation during incubation and to an improved recovery of receptor sites after centrifugation procedures.

The D_2 dopamine receptor [1] is characterized pharmacologically by its nanomolar affinity for neuroleptic antagonists such as the phenothiazines, thioxanthenes and butyrophenones. Specific agonist binding to the D_2 receptor can be resolved into a high (nanomolar) affinity (D_2^{high}) and a low (micromolar) affinity (D_2^{low}) component. Guanine nucleotides are able to convert most if not all of the D_2^{high} sites into D_2^{low} sites [2, 3]. Antagonists are unable to resolve these two sites. Recent advances in D_2 receptor biochemistry and pharmacology include its solubilization and partial purification [4–6].

An aspect of the D_2 receptor which has not yet been well characterized is the biochemical nature of the specific binding site. In the present study, heavy metal cations, sulfhydryl reagents, and other chemical agents were used as probes to investigate the biochemical properties of the membrane bound D_2 receptor molecule with regards to specific antagonist binding. Initially, these experiments were designed to supplement *in vivo* studies assessing the effects of Mn^{2+} and other heavy metal cations on central D_2 receptor density. However, as interesting results were accumulated, the scope of the studies was broadened to include investigations aimed at elucidating some of the structural requirements of dopamine receptors for the specific binding of tritiated dopamine antagonists such as [^{3}H]spiperone ([^{3}H]-SPIRO). In addition, it is hoped that these investigations may bear relevance to the neurotoxicology of certain agents, such as manganese, in which the involvement of central dopaminergic parameters has been implicated [7, 8].

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EXPERIMENTAL PROCEDURE

Rat striatal membrane preparation and treatment. Sprague–Dawley rats (150–250 g) were killed by exsanguination through the abdominal aorta while under light pentobarbital anesthesia. The brain was removed immediately and placed on dry ice, and then stored at −80° until use. Immediately before commencement of individual experiments, striatal tissue (as defined previously) [9] from a sufficient number of brains was dissected over dry ice, pooled, weighed and homogenized for 20 sec with a Tekmar tissumizer set at a speed of 90 in 30 vol. of cold minimum essential medium (ascorbate-free)*, pH 7.4. Metal cations and other reagents were added to aliquots of homogenate from stock solutions made up fresh in 20 mM acetate buffer and adjusted to pH 7.4. Cu^{2+} and Fe^{2+}, however, have extremely low solubilities in the physiological pH range; therefore, stock solutions of these metal cations were prepared fresh using 50–100 mM Tris buffers. This system allows for the loose chelation of the metals by Tris with sufficient excess buffer to maintain the pH at 7.4. Homogenates with added reagents were incubated for various time periods at 37°. Incubations were terminated by centrifugation at 20,000 *g* for 20 min in a refrigerated centrifuge. The supernatant fraction was discarded and the pellet was resuspended in assay buffer, which was 15 mM Tris/5 mM EDTA, pH 7.4, unless otherwise stated.

Radioligand binding assays. D_2 receptor densities were determined by quadruplicate incubations of 2–4 mg tissue for 20 min at 20° in 0.6 ml of 15 mM Tris/5 mM EDTA, pH 7.4, in the presence of 0.5 nM [^{3}H]SPIRO and 1 μM (+)- or (−)-butaclamol. This combination of assay conditions had been determined previously to be optimal for the complete saturation of available specific [^{3}H]SPIRO binding sites. Incubation was terminated by rapid filtration over Whatman GF/B glass fiber filters under vacuum. Filters were then rinsed three times with 5.0 ml of cold Tris buffer, and radioactivity trapped on the filters was measured on a Packard liquid scintillation counter. Protein was measured by the modified Lowry method of Markwell *et al.* [16].

Preparation of homogenates for electron microscopy. To study the morphological appearance of control striatal homogenates and those incubated in the presence of heavy metal cations, homogenates were incubated for 1 hr at 37° in the presence of 3 mM Mn^{2+} or Hg^{2+}, or in the absence of added metal. Incubation was terminated by centrifugation at 700 *g* for 10 min. The pellet was discarded and the supernatant fraction was further centrifuged at 20,000 *g* for 5 min. The supernatant fraction was discarded and the pellets were fixed overnight at 4° in 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4. The pellets were washed in several changes of 0.05 M cacodylate, pH 7.4, with 4% sucrose, and postfixed in buffered 1% osmium tetroxide at 4° overnight. After several washes in distilled water, the pellets were dehydrated in graded ethanols, cleared in acetone, and embedded in Polybed resin. Thin silver-grey sections were stained in uranyl acetate and viewed on a Philips EM 300, at 80 kV.

RESULTS

Effects of heavy metals on D_2 receptor density. Pretreatment of striatal homogenates with heavy metal cations markedly affected D_2 receptor density as measured by the stereospecific binding of [^{3}H]-SPIRO (Table 1). Mn^{2+}, Fe^{2+} and Co^{2+} at 3 mM all enhanced subsequently measured specific binding of [^{3}H]SPIRO, with Mn^{2+} exhibiting the most pronounced effect. Estimation of the Mn^{2+} content of striatal membranes from control and Mn^{2+}-treated homogenates by atomic absorption spectrophotometry revealed that much of the exogenous Mn^{2+} had become membrane-bound during the incubation period, increasing in concentration from 6.0 μg Mn^{2+}/g tissue in control membranes to 4.4 × 10^3 μg/g in Mn^{2+}-treated membranes. Scatchard analysis

Table 1. Effects of heavy metal cations on striatal D_2 receptor density*

Addition	[^{3}H]SPIRO bound (% of control)	
	Stereospecific binding	Non-specific binding
None (control)	100	100
Mn^{2+}	170 ± 32†	187 ± 14
Fe^{2+}	127 ± 18	170 ± 17
Co^{2+}	125 ± 6	136 ± 18
Zn^{2+}	86 ± 10	122 ± 1
Cd^{2+}	53 ± 5	145 ± 25
Cu^{2+}	43 ± 19	172 ± 18
Hg^{2+}	7 ± 6	139 ± 12
CH_3Hg^+	15 ± 5	115 ± 15

* Equal volume aliquots of rat striatal homogenate were incubated for 1 hr at 37° in the presence or absence of 3 mM metals. Dopamine receptor density was determined subsequently in the 20,000 *g* pellet using the standard [^{3}H]SPIRO binding assay described in Experimental Procedures.

† Mean ± S.D., N = 3.

* Lipid peroxidation can produce deleterious effects on membrane function *in vitro* [10]. The non-enzymatic formation of lipid peroxides in tissue homogenates is catalyzed by Fe^{2+} which is oxidized to Fe^{3+} in the process. When Fe^{2+} has been depleted by conversion to Fe^{3+}, the production of lipid peroxides falls off [11]. However, ascorbate, over a specific range of low concentrations, selectively reduces Fe^{3+} to Fe^{2+}, thus effectively regenerating the catalytic agent and causing a sustained enhancement of lipid peroxide production [11]. Ascorbate-induced lipid peroxidation has been implicated as an important factor leading to the unwanted reduction of specific ^{3}H-antagonist binding to D_2 dopamine receptors [12, 13]. The contention that this reduced binding may be due to lipid peroxidation is supported by the finding that the dose–response curves for the ascorbate-related enhancement of lipid peroxidation as well as the ascorbate-induced destruction of ^{3}H-ligand binding to receptors both display a biphasic character, and both processes are inhibited by EDTA [13–15]. The biphasic character is due to the fact that, at higher concentrations, ascorbate ceases to selectively reduce Fe^{3+}, but rather acts as a general antioxidant thus inhibiting lipid peroxide formation. Because of these complicating factors, ascorbate was not included as a standard component in the incubation medium, nor in the standard assay buffer used in the present series of investigations.

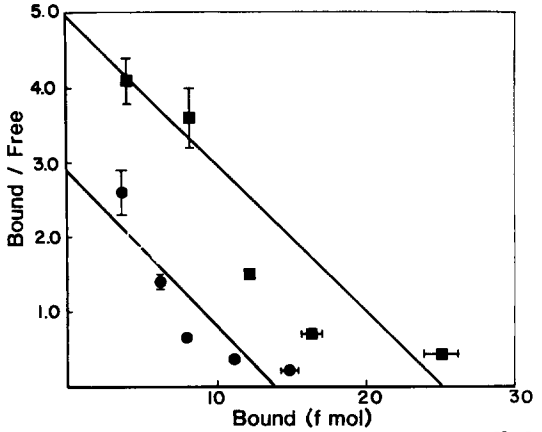

Fig. 1. Scatchard plot of stereospecific binding of [^{3}H]-SPIRO. Striatal membranes were preincubated for 1 hr at 37° in the presence or absence of 3 mM Mn^{2+}. The [^{3}H]-SPIRO binding assay was performed under standard conditions using 1.7 mg tissue per incubation tube and five concentrations of [^{3}H]SPIRO (0.03 to 0.50 nM). Linearity is assumed. Control (—●—): B_{max} = 140 fmoles/mg protein, K_D = 0.21 nM; Mn^{2+} (—■—): B_{max} = 253 fmoles/mg protein, K_D = 0.20 nM. It was not possible to perform meaningful Scatchard analysis on Hg^{2+}-treated membranes. Data points are means ± S.D., N = 3.

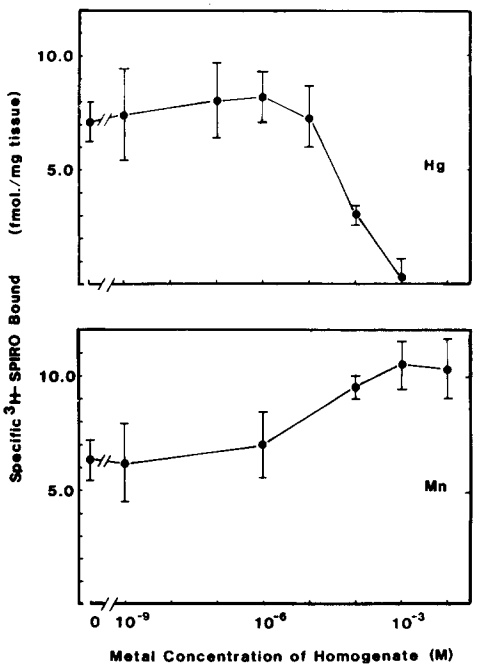

Fig. 2. Effects of increasing concentrations of Hg^{2+} and Mn^{2+}. Incubations were for 1 hr at 37°. The 20,000 *g* pellet was washed once before a final resuspension in standard assay buffer (15 mM Tris/5 mM EDTA, pH 7.4). Effects on the stereospecific binding of [^{3}H]SPIRO due to the preincubation treatments were then determined. Data points are means ± S.D., N = 3.

revealed that the influence of Mn^{2+} was to increase the maximum number of binding sites compared to control levels, while the affinity of [^{3}H]SPIRO for the receptor was unchanged (Fig. 1). Some other metals, particularly those with a high sulfhydryl affinity such as Cd^{2+}, Cu^{2+} and Hg^{2+}, caused a decrease in receptor sites. Pretreatment with 3 mM Cd^{2+} or Cu^{2+} resulted in a 40–60% reduction in the subsequently measured stereospecific binding of [^{3}H]-SPIRO, while 3 mM Hg^{2+} completely abolished specific [^{3}H]SPIRO binding. The effect of monovalent mercury in the form of CH_3Hg^+ was similar to that of the inorganic divalent form. In addition, all metals had the common effect of increasing the nonspecific binding of [^{3}H]SPIRO compared to control.

Concentration effects of Mn^{2+} *and* Hg^{2+}. Since Mn^{2+} and Hg^{2+} showed the most pronounced effects of the metals tested in the preliminary investigation, they were chosen for further study. As shown in Fig. 2, the destructive effect of Hg^{2+} was maximal between 10^{-4} and 10^{-3} M, as was the enhancing effect of Mn^{2+}. Increasing the concentration of Mn^{2+} above 10^{-3} M caused no further enhancement of specific [^{3}H]SPIRO binding.

Time course of the effects of Mn^{2+} *and* Hg^{2+}. In the absence of added metals, the incubation of striatal homogenates at 37° caused a rapid disappearance of 10–20% of D_2 receptor sites, after which loss of the remaining sites was more gradual with an extrapolated half-time of about 14 hr (Fig. 3). The addition of 3 mM Mn^{2+} not only protected receptors from the rapid short-term loss, but it also resulted in significantly higher initial receptor densities as measured by [^{3}H]SPIRO. However, to observe the enhancing effect of Mn^{2+}, it was essential to add the metal before the incubation was begun. The addition of Mn^{2+} to striatal homogenates which had already undergone 0.5 hr of incubation at 37° was not effective in increasing the subsequently measured specific binding of [^{3}H]SPIRO compared to control (Table 2). The addition of 3 mM Hg^{2+} caused a complete and virtually instantaneous elimination of specific [^{3}H]SPIRO binding sites.

Effects of EDTA, ascorbate, $Na_2S_3O_5$ *and NEM.* Preincubation of striatal homogenates in the presence of EDTA or ascorbate enhanced D_2 receptor density compared to control (Table 3). The effect was of a magnitude similar to that seen with Mn^{2+}, and, as illustrated in Fig. 4, incubation in the presence of two "enhancers" (Mn^{2+} and ascorbate) resulted in an increase which was no greater than the maximum effect produced by one agent alone. Although ascorbate produced significant increases in receptor density, incubation of homogenates with another reducing agent, $Na_2S_3O_5$, had no effect on subsequently measured D_2 receptor density (Table 3). The −SH alkylating agent, *N*-ethylmaleimide (NEM) produced results similar to those of Hg^{2+}. Treatment of homogenates with 3 mM NEM completely abolished the subsequently measured specific binding of [^{3}H]SPIRO. This effect was not antagonized by the presence of Mn^{2+}.

Recovery of specific [^{3}H]*SPIRO binding after* Hg^{2+}. Various manipulations and additions were attempted in order to recover specific [^{3}H]SPIRO binding sites subsequent to their elimination by Hg^{2+}. It was reasoned that, if Hg^{2+} could be removed from the membrane, then some or all of the lost binding sites might be regenerated. However, washing the membranes with Tris/EDTA or Tris/2,3-dimercapto-1-propanesulfonic acid (DMPS) buffers

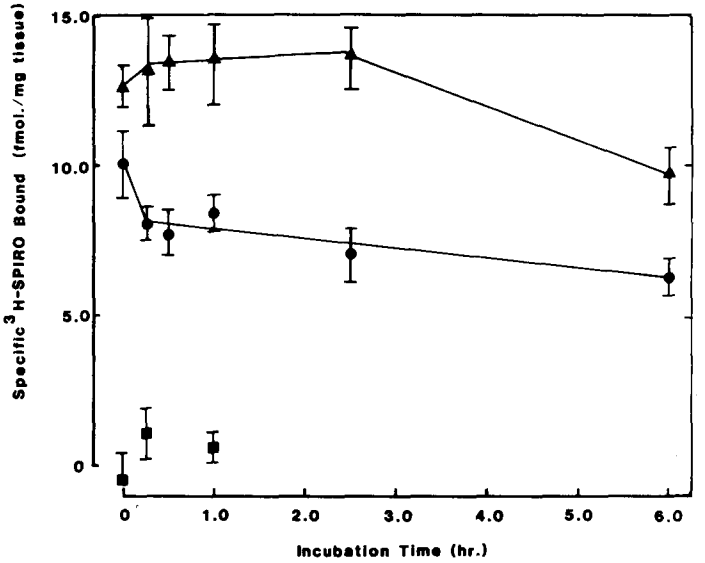

Fig. 3. Time course of the loss of specific [^{3}H]SPIRO binding sites. Striatal homogenates were preincubated for 0 to 6.0 hr in the presence or absence of 3 mM metals. Control (—●—); Mn^{2+} (—▲—); and Hg^{2+} (—■—). Data points are means ± S.D., N = 3.

Table 2. Effect of Mn^{2+} on striatal D_2 receptor density*

Additions	[^{3}H]SPIRO bound (% of control)	
	Stereospecific binding	Non-specific binding
None (control)	100	100
Mn^{2+} (at 0 min)	142 ± 13†	119 ± 5
Mn^{2+} (at 30 min)	106 ± 7	101 ± 5

* Homogenates were incubated for a total of 1 hr at 37°. A 3 mM concentration of Mn^{2+}, when added before commencement of incubation, enhanced subsequently measured [^{3}H]SPIRO binding, but had no effect if added after 30 min of incubation in the absence of metal.

† Mean ± S.D., N = 3.

did not result in the recovery of any lost binding sites, nor did the incubation of Hg^{2+}-treated membranes with excess EDTA or DMPS for an additional 0.5 hr (Fig. 5). A similar incubation in the presence of 3 mM DTE, however, was effective in regenerating approximately 40% of lost binding sites.

Protection of D_2 receptors from destruction by Hg^{2+}. Generally, agents which tended to enhance specific [^{3}H]SPIRO binding to striatal membranes, such as Mn^{2+} and EDTA, were unable to protect receptors from the destructive effect of Hg^{2+} when added to striatal homogenates prior to Hg^{2+} (Fig. 6). Ascorbate protected slightly, but this was due to its ability to remove Hg^{2+} from the system by reducing Hg^{2+} to Hg^{+} which was promptly precipitated as dark grey Hg_2O or HgOH. The chelating agent

Table 3. Effects of EDTA, ascorbate, $Na_2S_3O_5$ and NEM on specific [^{3}H]SPIRO binding sites*

Additions	[^{3}H]SPIRO bound (% of control)	
	Stereospecific binding	Non-specific binding
None (control)	100	100
EDTA (1 mM)	152 ± 25†	111 ± 5
Ascorbate (5 mM)	150 ± 16	105 ± 4
$Na_2S_3O_5$ (3 mM)	94 ± 9	95 ± 4
NEM (3 mM)	9 ± 13	106 ± 15
NEM‡ + Mn^{2+} (3 mM)	10 ± 10	123 ± 10

* Crude striatal homogenates were incubated for 1 hr at 37° in the presence or absence of the indicated agents. Incubation was terminated by centrifugation, and [^{3}H]SPIRO binding was determined in the resuspended 20,000 *g* pellet.

† Mean ± S.D., N = 3.

‡ Mn^{2+} was added 1 min prior to NEM.

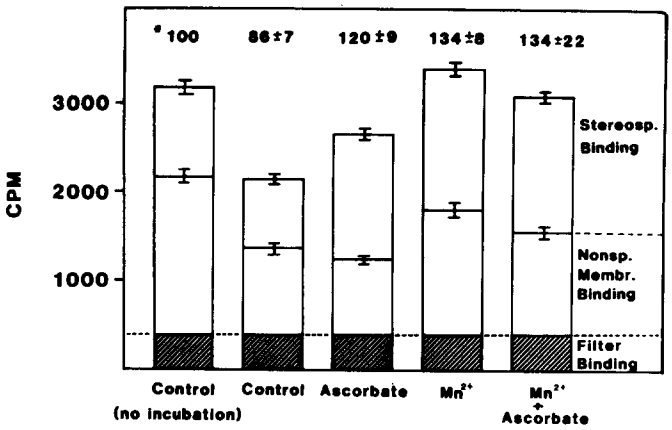

Fig. 4. Combined effect of Mn^{2+} and ascorbate on [^{3}H]SPIRO binding. Equal volume aliquots of a striatal homogenate were either centrifuged immediately (control, no incubation), or incubated at 37° for 1.5 hr in the presence of no additions (control), Mn^{2+} (3 mM), ascorbate (1 mM), or Mn^{2+} + ascorbate. [^{3}H]SPIRO binding was determined subsequent to centrifugation and resuspension of pellets in 15 mM Tris/5 mM EDTA, pH 7.4. Enhancement of D_2 receptor density was maximal with Mn^{2+} alone. Key: (*) stereospecific binding of [^{3}H]SPIRO as a percentage of non-incubated control; mean ± S.D., N = 3.

DMPS (5 mM), a water-soluble derivative of British anti-lewisite (BAL) with high affinity for Hg^{2+}, was unable to protect receptors against 1 mM Hg^{2+}, but the disulfide-bridge reducing agent dithioerythritol (DTE) (3 mM), although it caused a 30% reduction in specific [^{3}H]SPIRO binding sites when added alone, afforded complete protection to receptors when added to homogenates immediately prior to Hg^{2+}. Formation of the SPIRO/receptor complex prior to the addition of Hg^{2+} also tended to protect specific binding sites from destruction by Hg^{2+}. As depicted in Fig. 7, the normal dissociation rate of [^{3}H]SPIRO in the absence of added metal was not rapid under the conditions used in the present study.

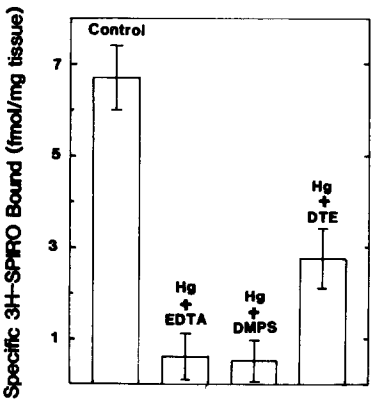

Fig. 5. Recovery of specific [^{3}H]SPIRO binding sites after pretreatment with Hg^{2+}. Equal volume aliquots of a striatal homogenate were incubated for 0.5 hr in the presence of 1 mM Hg^{2+} and then for an additional 0.5 hr in the presence of 5 mM EDTA, 5 mM DMPS, or 3 mM DTE. Specific [^{3}H]SPIRO binding was subsequently determined under standard assay conditions. Control = incubation for 1 hr in the absence of any additions. DTE was the only agent tested which caused a significant regeneration of specific binding sites lost due to treatment with Hg^{2+}. Data are means ± S.D., N = 3.

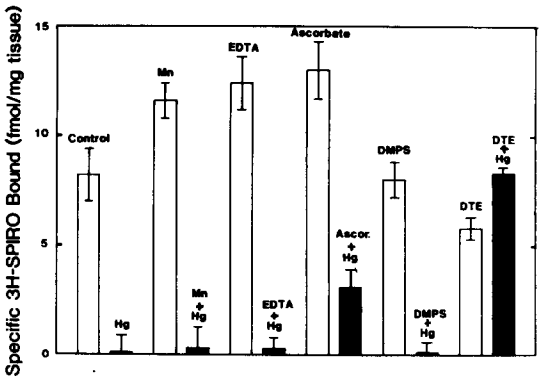

Fig. 6. Protection of specific [^{3}H]SPIRO binding sites from destruction by Hg^{2+}. Substances were added to crude striatal homogenates 1 min prior to Hg^{2+}. Concentrations used were: Hg^{2+}, EDTA, 1 mM; Mn^{2+}, DTE, 3 mM; DMPS, ascorbate; 5 mM. Incubation was for 1 hr at 37°. Only 3 mM DTE protected 100% of specific binding sites from 1 mM Hg^{2+}. Data are means ± S.D., N = 3.

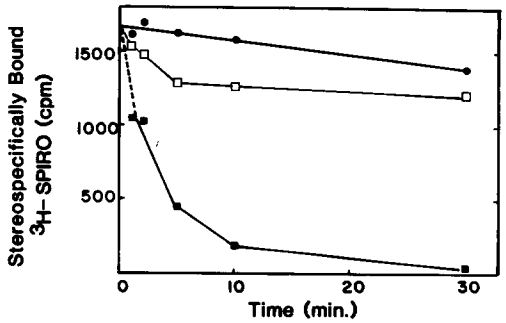

Fig. 7. Dissociation of specific [^{3}H]SPIRO bound to striatal membranes. The standard 20-min binding assay was carried out before the addition of 0 (—●—), 1 (—□—), or 3 (—■—) mM Hg^{2+} and incubation was then allowed to continue for 1, 2, 5, 10 or 30 min. By extrapolation, the $T_{1/2}$ of the SPIRO/receptor complex under control conditions was estimated to be 90 min.

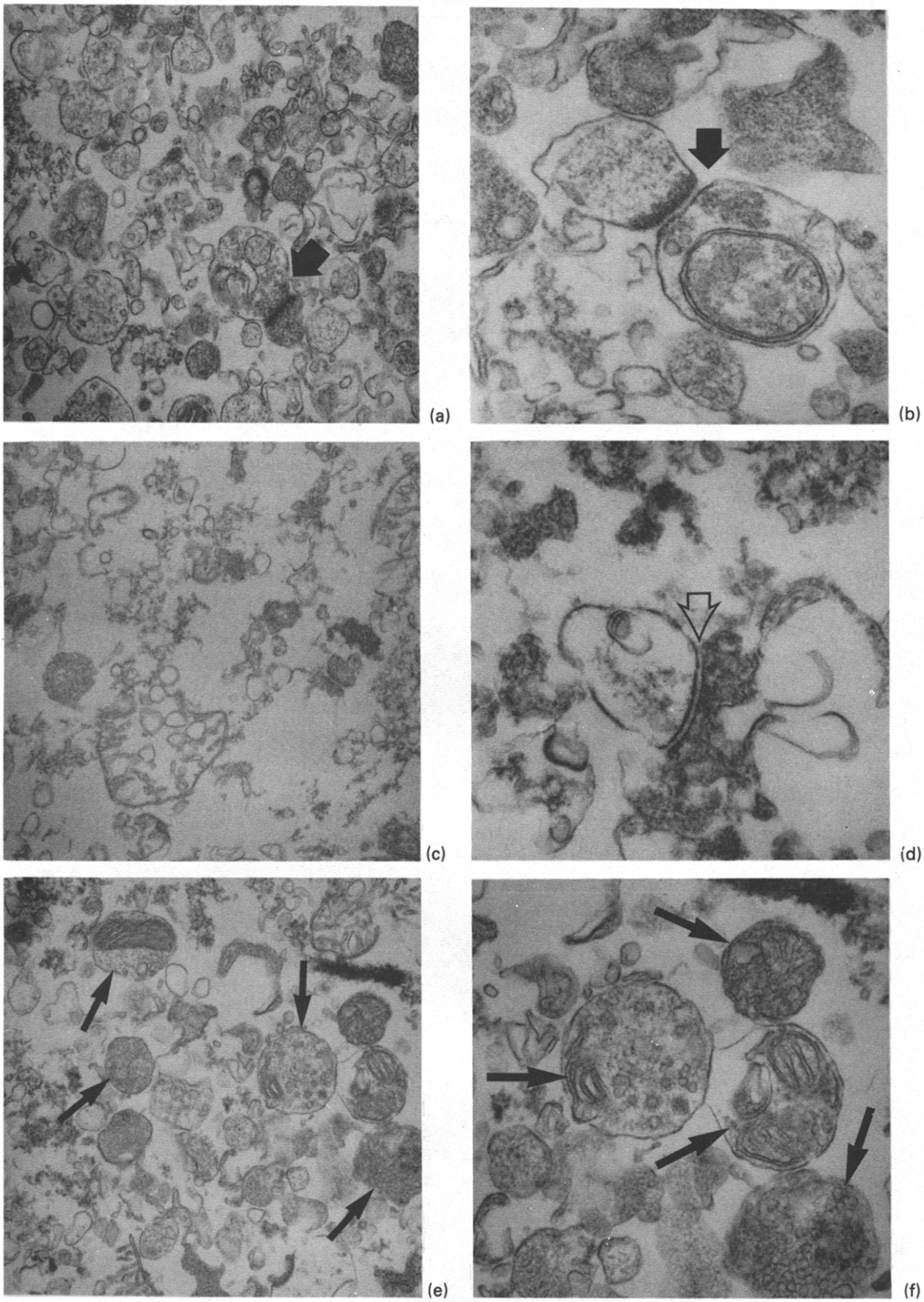

Fig. 8. Morphological appearance of striatal membranes. Striatal homogenates were preincubated for 1 hr, 37°, in the presence of no metal (control; panels 8a and b), 3 mM Hg^{2+} (panels c and d), or 3 mM Mn^{2+} (panels e and f) and later processed for electron microscopy. The control homogenates were characterized by the presence of abundant vesicular bodies many of which were judged to be synaptosomes (closed arrows) containing synaptic vesicles. Membranes from Hg^{2+}-treated homogenates were broken and fragmented. Vesicular structures, when present, were usually devoid of contents. Panel d depicts what may be the remnants of a synaptic cleft (open arrow). Membranes from Mn^{2+}-treated homogenates retained the general characteristics of the control membranes with the presence of numerous synaptosomal-like structures (arrows).

The extrapolated half time of the [^{3}H]SPIRO/receptor complex was 1.5 hr after the initial 20-min incubation. Hg^{2+} at 1 mM, a concentration which completely abolished specific binding when added to crude striatal homogenates, when added to membrane suspensions after the standard 20-min [^{3}H]SPIRO binding incubation caused a rapid dissociation of 20% of specifically bound [^{3}H]SPIRO, within 5 min of the addition of Hg^{2+}, but thereafter the dissociation rate was similar to the control rate. The effect of 3 mM Hg^{2+} was more dramatic, causing a loss of 90% of specific [^{3}H]SPIRO binding within 10 min of the addition of the metal.

Morphological appearance of striatal membranes. Transmission electron microscopy of the 20,000 *g* pellet from control, Hg^{2+}-treated and Mn^{2+}-treated striatal homogenates revealed significant differences among the groups (Fig. 8). Controls were characterized by large numbers of vesicular structures per field. Synaptosomes were frequently observed. In stark contrast, Hg^{2+}-treated membranes were characterized by a much lower density of vesicles, most of which were devoid of contents. Rather than being organized into vesicular structures, membranes from Hg^{2+}-treated homogenates were fragmented, and the fields were strewn with debris. Intact synaptosomes were never observed. Mn^{2+}-treated homogenates, on the other hand, retained a morphological appearance similar to that of the control. Morphometric analysis of striatal membranes confirmed statistically that 50–70% fewer vesicular structures were present in Hg^{2+}-treated membrane samples, and a higher proportion were empty as compared to control or Mn^{2+}-treated membranes ($P < 0.01$). These results indicate that even relatively high concentrations of Mn^{2+} do not disrupt the normal vesicular organization of striatal membrane suspensions.

DISCUSSION

Previous reports [17–19] have demonstrated the apparent enhancing effects of various cations (Na^+, K^+, Li^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}) and EDTA on the specific binding of [^{3}H]SPIRO in membrane suspensions when these reagents are present as constituents of the assay buffer. Under such experimental conditions, it is unclear whether the reported effects of these substances were mediated through their action on the receptor itself, or through some chemical interaction with the tritiated ligand, or both. Since in all the above-mentioned studies, ascorbate was also present in the assay buffer, yet another possibility is that these reagents acted simply by modulating the destructive effect of ascorbate [12]. In the present study, we demonstrate that preincubation of striatal homogenates in the presence of millimolar Mn^{2+}, EDTA, or ascorbate resulted in an enhancement of the subsequently measured stereospecific binding of [^{3}H]SPIRO after removal of the reagents from the system. The simplest explanation for this effect is to hypothesize that these agents protect receptor sites from degradation during the preincubation period. Indeed, Fig. 3 demonstrates the ability of 3 mM Mn^{2+} to inhibit the rapid short-term loss of specific [^{3}H]SPIRO binding sites which was observed in control homogenates incubated in the absence of added Mn^{2+}. This initial loss of sites may be due to the release of proteolytic enzymes and/or to lipid peroxidation in the membranes [13]. Mn^{2+}, if added to homogenates subsequent to a period of preincubation in the absence of Mn^{2+}, was unable to induce recovery of these lost sites. However, as both Figs. 3 and 4 indicate, the addition of 3 mM Mn^{2+} to striatal homogenates resulted in a significantly increased density of specific [^{3}H]SPIRO binding sites even when compared to control levels measured before any incubation had taken place. Thus, it may be that, in addition to protecting receptors from degradation, Mn^{2+} (or EDTA or ascorbate) may somehow enhance the recovery of specific binding sites, sites which are lost in the absence of these agents. Corroborating evidence for this possibility is presented in Table 4. It was found that the 20,000 *g* pellet from aliquots of homogenate incubated in the presence of Mn^{2+}, when resuspended in buffer and analyzed for protein content contained approximately 130% of the protein measured in controls. Hg^{2+} treatment resulted in a similar elevation in recovered protein (data not shown), in spite of its detrimental effect on specific [^{3}H]SPIRO binding sites, as did $(NH_4)_2SO_4$ precipitation. The concurrent enhancement of [^{3}H]-SPIRO binding sites observed in Mn^{2+}-treated homogenates was of a similar magnitude (~130%) in the absence of any preincubation, and even greater when homogenates were incubated at 37° prior to the measurement of specific [^{3}H]SPIRO binding. Treatment of homogenates with $(NH_4)_2SO_4$, while enhancing protein recovery, caused a 40–50% reduction in specific [^{3}H]SPIRO binding sites (data not shown). Thus, agents which increase the apparent D_2 receptor density in striatal membranes probably do so by a process of protection against receptor degradation combined with an enhancement in the recovery of specific binding sites during centrifugation. The exact mechanisms by which these effects occur are not known. It was thought that the production of these effects might be a general property of antioxidants, but incubation of striatal homogenates in the presence of the reducing agent $Na_2S_3O_5$ did not result in any enhancement of the

Table 4. Protein content of striatal membranes*

	Protein (mg/g original wet tissue wt)
Control	69 ± 2†
$(NH_4)_2SO_4$	87 ± 1
Mn^{2+} (3 mM)	91 ± 6

* Protein was measured in the resuspended 20,000 *g* pellet from equal volume aliquots of rat striatal homogenate incubated for 1 hr at 37° in the presence or absence of 3 mM Mn^{2+}. A drop of saturated $(NH_4)_2SO_4$ was added to one control homogenate immediately prior to centrifugation. Incubation with Mn^{2+} had the same effect as $(NH_4)_2SO_4$ precipitation in raising the apparent protein content of striatal membranes by enhancing recovery of protein.

† Mean ± S.D. of three replications.

subsequently measured D_2 receptor density (Table 3). However, regarding the protection of receptors from degradation, it may be significant to note that both enzymatic and non-enzymatic lipid peroxidation occur in brain synaptosomal and microsomal preparations incubated at 37° [20], and that, at appropriate concentrations, Mn^{2+}, EDTA and ascorbate all inhibit lipid peroxidation in membrane suspensions [14, 20]. Lipid peroxidation has been implicated as an important factor causing the unwanted reduction of specific ^{3}H-ligand binding to D_2 receptors [12, 13]. Regarding the ability of Mn^{2+} to enhance the recovery of receptors in the absence of any preincubation of homogenates, it should be noted that the microsomal fraction from striatal homogenates, which would not normally be expected to sediment at 20,000 *g*, is enriched with specific [^{3}H]SPIRO binding sites [21, 22]. Thus, the addition of Mn^{2+} (or EDTA or ascorbate) at appropriate concentrations may cause sedimentation of subcellular components not normally recovered at 20,000 *g*, resulting in enhanced recovery of both total protein (Table 4) and specific [^{3}H]SPIRO binding sites. This effect may also explain the enhancement of receptor density caused by Fe^{2+} and Co^{2+}, and the fact that heavy metals in general cause increases in the nonspecific binding of [^{3}H]SPIRO (Table 1).

A second major finding of the present series of investigations was that the incubation of striatal homogenates in the presence of sulfhydryl reagents including the sulfhydryl-reactive metal cations Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and CH_3Hg^+, as well as the sulfhydryl-alkylating agent NEM, caused variable losses of D_2 receptors as measured by subsequent [^{3}H]SPIRO binding assays. The degree of receptor loss caused by heavy metal cations ($Hg^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$) was correlated with their affinity for sulfhydryl groups. The results obtained in the present study for D_2 receptors are in accord with those of Aronstam *et al.* [23] who found a similar order of potencies for heavy metal inhibition of specific [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to cerebral muscarinic receptors, whereas metals with lower sulfhydryl reactivity such as Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Sn^{2+} were unable to inhibit specific [^{3}H]QNB binding.

The destructive effect of Hg^{2+} on the membrane-bound D_2 receptor was immediate upon the addition of the metal, and the effect could not be antagonized by the prior addition of substrates which, by themselves, caused an enhancement of D_2 receptor density such as Mn^{2+} or EDTA. Similarly, Mn^{2+} was unable to protect specific [^{3}H]SPIRO sites from destruction by NEM. These results indicate that the detrimental effects of Hg^{2+} and NEM are not simply due to a stimulation of the normal degradation rate of the receptors. Rather, it is likely that Hg^{2+} and NEM bind to one or more vital −SH groups located on or in close proximity to the receptor site, resulting in a conformational change which prevents subsequently measured specific [^{3}H]SPIRO binding. Prior formation of the SPIRO/receptor complex rendered these −SH groups less accessible to Hg^{2+}. Likewise, prior addition of DTE to striatal homogenates protected these essential −SH groups from occlusion by Hg^{2+}. DMPS, however, was ineffective in protecting specific [^{3}H]SPIRO sites from destruction by Hg^{2+}. This is surprising since DMPS, unlike EDTA, has a high affinity for Hg^{2+}, and has been found to be an effective *in vivo* chelating agent for both inorganic and organic forms of mercury [24, 25]. It was therefore predicted that DMPS would be able to protect all or most of the specific [^{3}H]SPIRO binding sites from destruction by Hg^{2+}. The fact that it was unable to do so indicates that the simple ability to complex Hg^{2+} is not a sufficient condition to enable a substance to protect receptors from occlusion by Hg^{2+}. Further studies are required to determine more precisely the biochemical properties which are important in protecting specific D_2-antagonist binding sites from damage by sulfhydryl reagents.

The high affinity agonist conformation of the D_2 receptor (D_2^{high}) may be more sensitive to −SH binding agents than the D_2^{low} site. Suen *et al.* [26] found that specific high-affinity [^{3}H]dopamine binding to calf striatal membranes is reduced to 40% of the control level in the presence of 0.1 mM NEM, a concentration which had no effect on specific [^{3}H]-SPIRO binding. The fact that specific ^{3}H-antagonist binding remained unaffected indicates that low concentrations of NEM may cause conversion of receptors from a D_2^{high} to a D_2^{low} configuration. Guanine nucleotides have been shown to convert D_2^{high} to D_2^{low} sites [2]. A complicating factor in the Suen *et al.* study is that under the agonist assay conditions used in that report [40 nM [^{3}H]dopamine in the presence or absence of 10 μM (−)-butaclamol], [^{3}H]-dopamine will label D_3 sites [27] as well as D_2^{high} sites. Labeling of D_3 sites may comprise as much as 50% of the specific ^{3}H-agonist binding to striatal membranes [28]; thus, it becomes difficult to clearly interpret the effect of NEM on the D_2 agonist site. However, Sibley and Creese [29] have shown that, after treatment of pituitary membranes with low concentrations of NEM (0.2 mM), agonist/[^{3}H]SPIRO competition curves are right-shifted and the curve assumes a one-site fit rather than the two-site (high and low affinity) fit observed in the absence of NEM treatment. An almost identical effect is seen in the presence of guanine nucleotides [2] which cause conversion of D_2^{high} sites to D_2^{low} sites. Reportedly, NEM exerts an opposite effect on ^{3}H-agonist binding to muscarinic receptors, converting receptors from a state of low agonist affinity to high agonist affinity [23]. In the present study, pretreatment of striatal homogenates with a higher concentration (3 mM) of NEM resulted in the complete abolition of ^{3}H-antagonist ([^{3}H]SPIRO) binding. This supports and extends the findings of Freedman *et al.* [30] who reported that preincubation of striatal membranes in 1 mM NEM caused a reduction of subsequently measured specific [^{3}H]sulpiride binding to 36% of the control level. Receptors could be protected from the effect of NEM by the prior addition of dithiothreitol. Similarly, we have demonstrated here that DTE was the only agent of several which were examined that could completely inhibit the destructive effect of Hg^{2+} on D_2 receptor density, and the only agent which could partially reverse the loss of specific [^{3}H]SPIRO sites after treatment of homogenates with Hg^{2+}. The ability of a substance to regenerate free −SH groups may thus be a requirement to

protect receptors from sulfhydryl reagents or to reverse their detrimental effects.

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