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High-Affinity Dextromethorphan Binding Sites in Guinea Pig Brain

I. Initial Characterization

GALE L. CRAVISO[1] AND JOSÉ M. MUSACCHIO[2]

Department of Pharmacology, New York University Medical Center, New York, New York 10016

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SUMMARY

Tritiated dextromethorphan ([^{3}H]DM) binds to two distinct sites in guinea pig brain, a high-affinity site (K_d = 13–20 nM) and a low-affinity site (K_d > 200 nM). Binding of [^{3}H]DM to the high-affinity site is rapid, reversible, saturable, proportional to tissue concentration, and pH-dependent. The sites have a protein-like component, since preincubating brain homogenate in the presence of proteolytic enzymes and protein-modifying reagents significantly reduces binding. There is also a progressive loss of binding when brain homogenate is heated to temperatures in excess of 37°. Millimolar concentrations of lithium, calcium, magnesium, and manganese decrease DM binding while sodium, in concentrations as high as 100 mM, has little effect; calcium in micromolar concentrations slightly enhances binding. The pons-medulla and cerebellum contain the highest density of sites. Subcellular localization studies have shown that high-affinity sites are confined almost exclusively to the microsomal fraction. Binding of DM to brain microsomes does not appear to be related to drug-metabolizing enzymes. The characteristics of DM binding suggest that DM sites are not a subclass of opiate receptors. Studies using tritiated dextrorphan as radioligand failed to reveal a high-affinity binding site for this compound in brain.

INTRODUCTION

DM[3] [(+)-3-methoxy-*N*-methylmorphinan] is a synthetic dextrorotatory analogue of codeine. While it lacks the analgesic, the respiratory-depressant, and the addictive properties of opiate analgesic drugs (1, 2), numerous animal and clinical tests have shown that DM is an effective, centrally acting antitussive, in some cases equal in potency to codeine (3). Although the mechanism by which DM exerts its antitussive effects is unknown, the fact that DM is structurally related to codeine (Fig. 1), the most widely used opiate cough suppressant, may suggest that DM acts at the same central sites as codeine to elevate the cough threshold. Since DM and codeine possess opposite stereoconfigurations, it could be implied that receptors responsible for cough suppression lack the strict stereospecificity associated with other pharmacological actions of opiates and therefore represent a distinct subclass of opiate binding sites. This suggestion has been made by Mansky (4) and more recently by Chau and Harris (5), who reported that (+)-codeine is antitussive.

Direct evidence demonstrating that DM does interact with a specific subpopulation of opiate binding sites has never been reported. However, Cavanagh *et al.* (6) have indicated that the sites at which DM acts to suppress cough are probably different from those at which codeine and other analgesics act. This is based on their observation that the antitussive activity of codeine and other opiate analgesics are naloxone-reversible whereas the antitussive actions of DM are not affected by even large doses (40 mg/kg) of this drug. While these results do not rule out a nonspecific interaction of DM at opiate sites, an interaction which is not naloxone-reversible, a more likely possibility is that DM acts at other sites to elicit its antitussive effects.

In an attempt to elucidate the site of action for the antitussive activity of DM, we have looked for specific binding sites for DM in brain. Using tritium-labeled DM as radioligand, we have developed a binding assay and have detected high-affinity DM sites which may be related to the cough-suppressant activity of this drug (7). This first paper describes some of the general characteristics of these sites in guinea-pig brain. The second paper (8) describes the interaction of antitussives and several neurotransmitter receptor ligands with DM sites.

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[2] Recipient of Research Scientist Award MH-17785.

[3] The abbreviations used are: DM, dextromethorphan; TLC, thin-layer chromatography.

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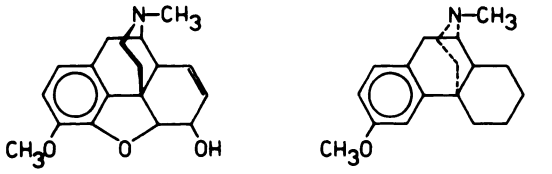

FIG. 1. *Structure of codeine and DM*

MATERIALS AND METHODS

Preparation of radioactive ligands. An iodo derivative of dextrorphan was prepared (9) and subjected to catalytic reduction with tritium gas at New England Nuclear Corporation (Boston, Mass.). This procedure yielded high specific activity [^{3}H]dextrorphan (60.4 mCi/mg) which was purified by TLC on silica gel in the solvent butanol/acetic acid/water (60:15:25). [^{3}H]DM was prepared from [^{3}H]dextrorphan as described (9). The specific activity of the radiolabeled DM was 26 Ci/mmole.

[^{3}H]DM binding assay. Male English short-hair guinea pigs were killed by decapitation and the brains were rapidly removed and placed in ice-cold saline. For routine binding studies the pons and medulla were homogenized with 24 volumes of ice-cold 50 mM Tris-Cl buffer (pH 7.7 at 25°) using a Brinkmann Polytron tissue disruptor (speed 5, 30 sec). Binding assays were carried out by incubating 3–4 mg of brain tissue (wet weight) with 2–4 nM [^{3}H]DM at 0° in a final incubation volume of 1 ml. Even though equilibrium was reached in 30 min, an incubation time of 2.5 hr was used throughout our studies since we found that, at 0°, a prolonged incubation was necessary for measuring the maximal effects of various drugs on [^{3}H]DM binding (8). At the end of the incubation period, Whatman GF/B glass-fiber filters were washed with 5 ml of ice-cold 50 mM Tris-Cl buffer (pH 7.7) containing 100 mM choline chloride and 0.01% Triton X-100, and the samples were rapidly filtered under vacuum. After three 5-ml rinses of the same wash buffer, filters were placed in scintillation vials and shaken with 1 ml of NCS tissue solubilizer (Amersham) for 40 min. Ten milliliters of a toluene-based scintillation fluor (0.4% 2,5-diphenyloxazole and 0.02% *p*-bis[2-(5-phenyloxazolyl)]benzene) were added to each vial, and radioactivity was determined by liquid scintillation spectrometry at a tritium-counting efficiency of 40%. Specific [^{3}H]DM binding was defined as the difference in binding in the presence and absence of 10 μM unlabeled DM and constituted approximately 80–90% of the total tissue binding at 2–4 nM [^{3}H]DM. Experimental conditions were chosen whereby total [^{3}H]DM bound to tissue did not exceed 10% of the total [^{3}H]DM added. Results were obtained from duplicate or triplicate determinations which varied less than 10%, and each experiment was performed at least twice. The specific activity of [^{3}H]DM was diluted 5- or 10-fold with unlabeled DM for saturation experiments using [^{3}H]DM in the concentration range of 2–100 nM. Binding site maxima (B_{max}) and equilibrium dissociation constants (K_d) were determined by linear regression analysis of Scatchard plots.

Preparation of subcellular fractions. Subcellular fractions were prepared essentially as described by De Robertis *et al.* (10). Briefly stated, whole guinea pig brains without cerebellum were homogenized in 10 volumes of 0.32 M sucrose using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 900 × *g* and the pellet (P_1) was washed twice with 0.32 M sucrose. The crude mitochondrial pellet (P_2) was obtained by centrifuging the combined 900 × *g* supernatant fractions for 20 min at 11,500 × *g*. P_2 was washed once with 0.32 M sucrose, and the combined 11,500 × *g* supernatant fractions were centrifuged at 100,000 × *g* for 1 hr to obtain the microsomal pellet (P_3). The crude P_2 fraction was osmotically shocked with water and the mitochondria were sedimented at 11,500 × *g* for 20 min. The resulting supernatant and fluffy layer were centrifuged at 100,000 × *g* for 1 hr to obtain a "synaptic plasma membrane" pellet. All fractions were resuspended in 10 volumes (based on original tissue wet weight) of 50 mM Tris-Cl buffer (pH 7.7) and assayed for specific [^{3}H]DM binding. In order to ensure a greater retention of small membranes isolated during the subcellular fractionation, the filtration step of the binding assay was carried out using GF/F filters (0.7-μm pore size) in place of the GF/B filters (1.0-μm pore size) routinely used in our studies. An aliquot of the original 1:10 homogenate was diluted with 10 volumes of 50 mM Tris-Cl buffer, centrifuged at 100,000 × *g* for 60 min, and resuspended in Tris-Cl buffer to its original volume. All recoveries of binding activity and protein were referred to this washed homogenate. Specific [^{3}H]DM binding was not significantly altered when subcellular fractions were stored at −80° for 1 month.

Microsomal subfractions were prepared by slight modification of the method described by Kiang *et al.* (11). Cerebral cortices from guinea pig brains were scraped free of white matter and homogenized, using a glass-Teflon homogenizer, in 10 volumes of 0.3 M sucrose containing 1 mM NaH_2PO_4/0.1 mM EDTA, buffered to pH 7.5. The homogenate was centrifuged at 12,000 × *g* for 30 min and the pellet was washed once with the buffered 0.3 M sucrose. The combined 12,000 × *g* supernatant fractions were centrifuged at 100,000 × *g* for 1 hr. The microsomal pellet obtained was resuspended in the 0.3 M buffered sucrose to a concentration of 2 ml/g of starting tissue using the glass-Teflon homogenizer. Three milliliters of the resuspended microsomes were layered onto discontinuous sucrose gradients consisting of 9 ml each of 0.5, 0.8, 1.0, and 1.3 M sucrose, respectively. All sucrose solutions contained 1 mM NaH_2PO_4/0.1 mM EDTA and were buffered to pH 7.5. After centrifugation for 16 hr at 97,000 × *g* (Beckman SW-27 swinging bucket rotor), the areas at each sucrose interface were collected and combined with the sucrose solution halfway above and halfway below the particular interface. Each fraction was then diluted to 10% sucrose with 50 mM Tris-Cl buffer (pH 7.7) and pelleted at 100,000 × *g* for 1 hr. After resuspension in Tris-Cl buffer, each fraction was immediately assayed for [^{3}H]DM binding as described, using Whatman GF/F glass-fiber filters for the filtration step.

Opiate receptor binding assay. The method used to determine opiate receptor binding is similar to that previously described (12). Aliquots of subcellular fractions and microsomal subfractions were incubated in 50 mM Tris-Cl buffer (pH 7.7) with 2 nM [^{3}H]naltrexone for 40 min at 25°. The final incubation volume was 1 ml. Following incubation, samples were cooled in ice and filtered through Whatman GF/F glass-fiber filters, using three 5-ml rinses of ice-cold 50 mM Tris-Cl buffer (pH 7.7). Radioactivity retained on the filters was determined by liquid scintillation spectrometry as described for the [^{3}H]DM binding studies. Specific [^{3}H]naltrexone binding was defined as the difference in binding in the presence and absence of 0.1 μM levorphanol. Specific binding was linear with respect to tissue concentration, and all results were obtained from duplicate determinations.

Metabolism of DM by brain and liver microsomes. Microsomes were prepared from guinea pig brain cortex and liver using the procedure previously described for the microsomal subfractionation studies, except that 0.32 M sucrose was used as the homogenizing medium. The microsomal pellets obtained after the final 100,000 × *g* centrifugation step were resuspended in 50 mM Na_2HPO_4/H_3PO_4 buffer (pH 7.4) to a final concentration of 1.5 ml/g of brain and 4 ml/g of liver. Aliquots of each (50 μl) were incubated with 80 nM [^{3}H]DM and 100 μM NADPH for 30–60 min at 37°. The final incubation volume was 125 μl. At the end of the incubation, each sample received 25 μl of 2 N HCl containing 8 μg of nonradioactive DM. Following centrifugation for 10 min, 50 μl were spotted onto silica gel plates, and TLC was carried out using chloroform/ethanol/ammonium hydroxide (70:30:0.4) as the developing solvent. Radioactivity at the area of the plate corresponding to DM was determined by liquid scintillation spectrometry.

Other methods. For regional distribution studies, guinea pig brains were dissected according to the method of Glowinski and Iversen (13). NADPH-cytochrome *c* reductase was assayed using the method of Ernster *et al.* (14), and RNA was measured by the procedure of Fleck and Begg (15). All enzyme and RNA determinations were linear with respect to tissue concentration. Protein was determined by the method of Lowry *et al.* (16), using bovine serum albumin as standard.

Materials. Dextromethorphan and dextrorphan were generous gifts from Hoffmann-La Roche (Nutley, N. J.). [^{3}H]Naltrexone (9.72 Ci/mmole) was obtained from the National Institute of Drug Abuse

(Bethesda, Md.). All other chemicals and reagents were obtained from commercial sources.

RESULTS

Dextrorphan binding studies. Since we assumed that DM could be *O*-demethylated as codeine (17), our initial binding studies were performed with [^{3}H]dextrorphan as radioligand instead of [^{3}H]DM. The results of these studies indicated that dextrorphan does not have a high-affinity binding site in guinea pig or rat brain; less than 25% of the total [^{3}H]dextrorphan bound to brain homogenates could be displaced by concentrations of unlabeled dextrorphan up to 10^{-6} M.

In the course of these experiments, we observed that there was a high level of adsorption of [^{3}H]dextrorphan to the glass-fiber filters used in the filtration step of the binding assay. A method to avoid this problem was devised and is the same one described below for the [^{3}H]DM binding studies.

Adsorption of [^{3}H]DM to glass-fiber filters. In contrast to the results obtained with [^{3}H]dextrorphan, specific high-affinity [^{3}H]DM binding sites could be detected in guinea pig brain stem homogenates. However, our control samples indicated that a significant amount of the radioactivity measured was due to the binding of [^{3}H]DM to the glass-fiber filters used in the binding assay. As the experiment in Table 1 illustrates, in the absence of tissue considerable [^{3}H]DM adhered to the filters when plain 50 mM Tris-Cl buffer (pH 7.7) was used to rinse the filters. Moreover, part of this filter-bound radioactivity was displaced by 10 μM unlabeled DM. Similar results were obtained when the filters were prewet with buffer containing brain tissue (6 mg wet weight), indicating that the presence of tissue does not prevent the adsorption of the ^{3}H-ligand to the filters. Law *et al.* (18) reported that the addition of 100 mM choline chloride, 0.01% Triton X-100, and 0.1% bovine serum albumin to their wash buffer reduced the binding of [^{3}H]β-endorphin to the glass-fiber filters used in their binding assays. When these same three reagents were included in our wash buffer (Buffer A), filter binding of [^{3}H]DM was significantly reduced while specific binding of [^{3}H]DM to brain tissue was apparently not altered. In comparing each of these components of the wash buffer alone or in various combinations it was found that the addition of albumin was not required. Therefore, all subsequent experiments were carried out using 50 mM Tris-Cl buffer (pH 7.7) containing only choline chloride and Triton X-100. Specific [^{3}H]DM binding to brain tissue was stable over the course of one to four filter washings, indicating that specifically bound [^{3}H]DM is not lost during the washing procedure.

TABLE 1

Binding of [^{3}H]DM to Whatman GF/B glass-fiber filters

The amount of labeled DM bound to the glass-fiber filters in the presence and absence of 10 μM unlabeled DM was determined after filtering samples containing 30,000 cpm of [^{3}H]DM and washing the filters three times with 5 ml of 50 mM Tris-Cl buffer (pH 7.7) or 50 mM Tris-Cl buffer (pH 7.7) containing 100 mM choline chloride, 0.01% Triton X-100, and 0.1% bovine serum albumin (Buffer A). In Experiment 1, samples contained 1 ml of 50 mM Tris-Cl buffer (pH 7.7) and [^{3}H]DM with or without unlabeled DM. The composition of samples in Experiment 2 was identical with that of samples in Experiment 1 except that, immediately before filtration, 1 ml of buffer containing brain homogenate (6 mg of tissue, wet weight) was poured onto the filter. In Experiment 3, tissue binding of [^{3}H]DM was determined by incubating brain homogenate (6 mg of tissue, wet weight) in 1 ml of buffer for 1 hr at 0°C with [^{3}H]DM in the presence and absence of unlabeled DM. All values are the means of duplicate determinations.

Experiment	Binding					
	50 mM Tris-Cl buffer			Buffer A		
	Total	+10 μM DM	Displaceable	Total	+10 μM DM	Displaceable
	cpm			*cpm*		
1. Filters (no homogenate)	1563	465	1098	149	105	44
2. Filters coated with homogenate	984	451	533	175	159	16
3. Filters + incubated homogenate	2565	940	1624	1636	433	1203

Tissue linearity of [^{3}H]DM binding and identity of bound radioactivity. Specific binding of [^{3}H]DM to guinea pig brain stem homogenates was linear with respect to tissue concentration and extrapolated to zero over the range of 0.4–4 mg of tissue (wet weight). High-performance liquid chromatographic analysis of the tritiated material bound to brain tissue incubated with 4 nM [^{3}H]DM for 1–2.5 hr at 0° indicated that the radioactive ligand was not metabolized during the binding process. Metabolism of DM was also not demonstrated in the supernatant fluid after 4 nM [^{3}H]DM was incubated with brain tissue for 30 min at 37°.

Saturation of [^{3}H]DM binding. Displacement of total [^{3}H]DM bound to brain tissue by unlabeled DM is illustrated in Fig. 2. When binding in the presence of 10 μM unlabeled DM was subtracted from all values to obtain specific [^{3}H]DM binding and the data were analyzed by log-probit plots, an IC_{50} value of 25 nM was obtained. Scatchard analysis of these binding data (Fig. 2) revealed the presence of two binding components, a high-affinity component (K_d = 17.7 nM) and a component of much lower affinity (K_d > 200 nM). The B_{max} value calculated for the high-affinity component was 2.9 pmoles/mg of protein. The slope obtained from a Hill plot of the same data (Fig. 2) was significantly less than 1 (n_H = 0.78), again indicating binding of [^{3}H]DM to more than one site.

Incubation of brain tissue with increasing concentrations of [^{3}H]DM up to 100 nM demonstrated that specific [^{3}H]DM binding was saturable, whereas nonspecific binding increased linearly with increasing concentrations of radioactivity (Fig. 3). Scatchard analysis of the specific binding data yielded a linear plot (Fig. 3), indicating that when low concentrations of ligand (5–100 nM [^{3}H]DM) are used only the high-affinity DM sites are labeled. A Hill coefficient on n_H = 1 was obtained (Fig. 3), further suggesting a single population of high-affinity, noninteracting binding sites. From these data an apparent dissociation constant (K_d) of 13.39 ± 0.51 nM (n = 8) and a maximal number of binding sites (B_{max}) of 2.48 ± 0.11 pmoles (n = 8) [^{3}H]DM bound per milligram of protein were obtained. These values agree with those obtained from the displacement studies described previously for

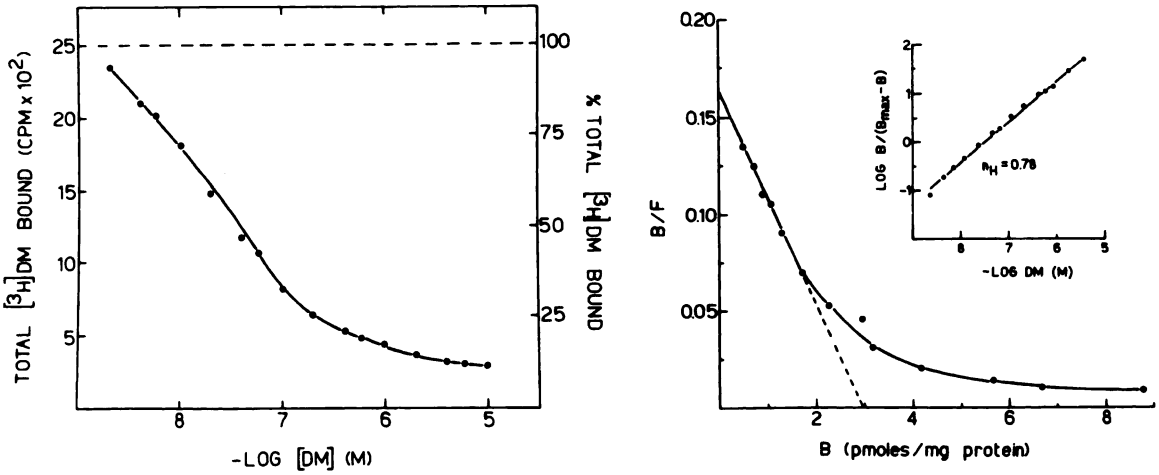

FIG. 2. *Displacement of [³H]DM from guinea pig brain stem homogenate by unlabeled DM*

Brain homogenate (3 mg of tissue, wet weight) was incubated for 2.5 hr at 0° with 4 nM [³H]DM and increasing concentrations of unlabeled DM ranging from 2 nM to 10 μM. Binding was determined as described under Materials and Methods. The results are representative of two experiments performed in duplicate. Left, Data are expressed as displacement of total [³H]DM bound. Right, Scatchard analysis of the same data. Nonspecific binding occurring in the presence of 10 μM unlabeled DM was subtracted from all values to obtain specific [³H]DM binding as described under Materials and Methods. The K_d calculated for the high-affinity component was 17.7 nM and the B_{max} value was 2.9 pmoles/mg of protein. *Inset*, Hill plot of the same data.

the high-affinity [³H]DM binding component (see Fig. 2).

Kinetics of [³H]DM binding. Figure 4 shows the time course of specific and nonspecific binding of [³H]DM to brain homogenate at 0°. Specific [³H]DM binding occurred rapidly, being 50% complete after approximately 3 min and reaching equilibrium at between 12 and 30 min; nonspecific binding reached a maximum in 2 min. Since the total binding of [³H]DM to the tissue at equilibrium never exceeded 10% of the total [³H]DM added, the association process could be considered a pseudo-first order reaction and a plot of the pseudo-first order kinetic data obtained by graphing ln $[B_{eq}/(B_{eq}-B)]$ versus time, where B is the amount of specific [³H]DM bound at time t and B_{eq} is the amount bound at equilibrium (Fig. 4). The slope of the line (k_{obs}) obtained from this plot is the observed forward rate constant for the pseudo-first order reaction, which was used to calculate the second-order association rate constant, k_1, by the equation $k_{obs} = k_1 \times [([^3H]DM)[R_t]/[B_{eq}]]$, where R_t equals the total concentration of receptor. Using [³H]DM concentrations of 1.2–4.0 nM, a mean k_1 of 0.0149 ± 0.007 nM^{-1} min^{-1} ($n = 7$) was obtained for specific [³H]DM binding at 0°. Once equilibrium has been reached, specific binding is stable for up to 3 hr at 0°.

The dissociation of specifically bound [³H]DM, determined by adding excess unlabeled DM to a sample of brain homogenate incubated with 4 nM [³H]DM to equilibrium, was very rapid at 25°, with a $t_{1/2}$ of approximately 1.6 min (data not shown). At 0°, dissociation was slower and biphasic (Fig. 4), with half-lives for the fast and slow components of dissociation of approximately 2.5 min and

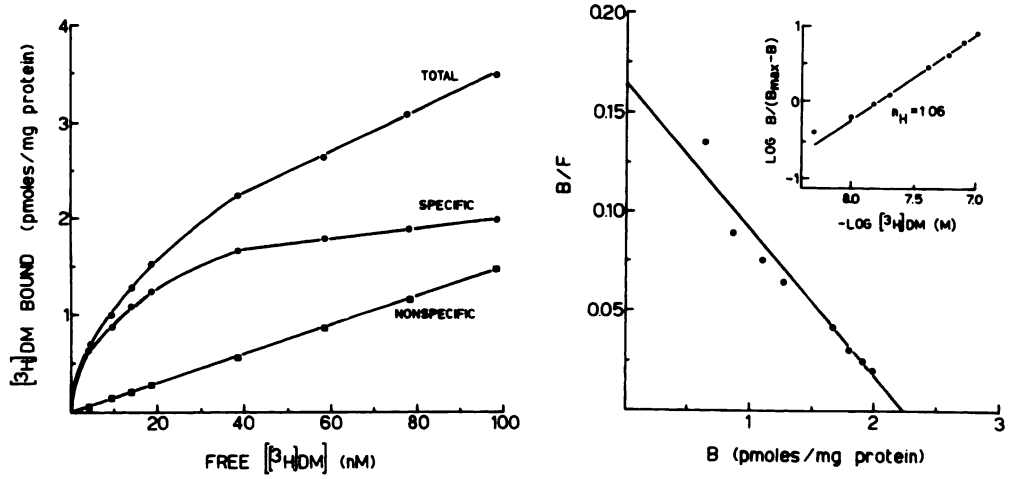

FIG. 3. *Saturation of specific [³H]DM binding to guinea pig brain stem homogenate*

Brain stem homogenate (3 mg of tissue, wet weight) was incubated with increasing concentrations of [³H]DM (5–100 nM) and binding was determined as described under Materials and Methods. Each value is the mean of triplicate determinations, and the data shown are representative of eight such experiments. Left, A direct plot of the data. Specific [³H]DM binding (●) represents the difference between total (○) and nonspecific (■) binding ([³H]DM bound in the presence of 10 μM unlabeled DM). Right, Scatchard analysis of the same specific [³H]DM binding data. The K_d was 13.51 nM and the B_{max} value was 2.22 pmoles/mg of protein. Inset, Hill plot of the same data.

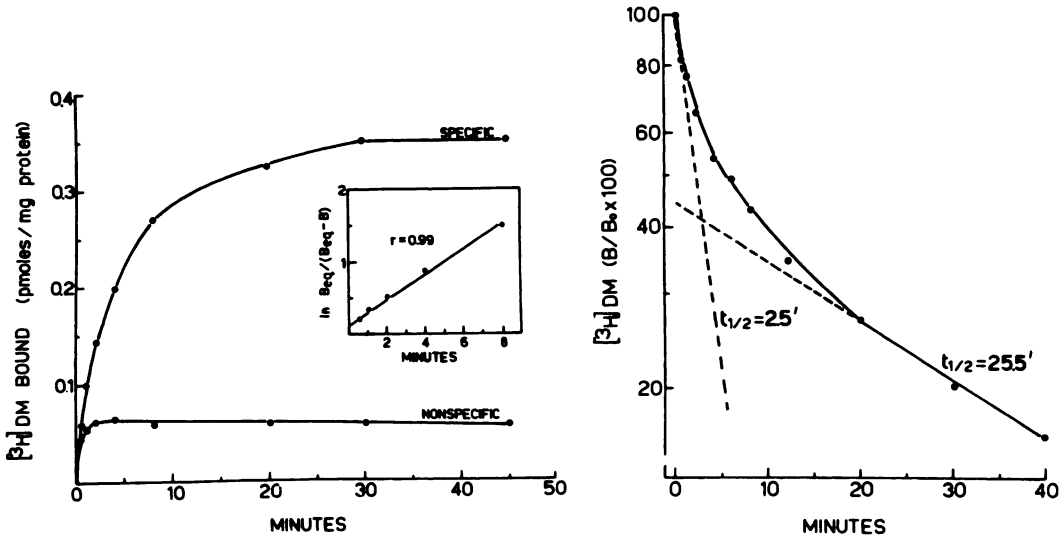

FIG. 4. *Kinetic analysis of specific [³H]DM binding to guinea pig brain stem homogenate*

Left. Time course of specific and nonspecific binding of [³H]DM. Brain homogenate [3 mg of tissue (wet weight) per milliliter of incubation volume] was incubated with 4 nM [³H]DM at 0° in the presence and absence of 10 μM unlabeled DM. At the times indicated, 800-μl aliquots were removed from the incubation mixture and specific (●) and nonspecific (○) [³H]DM binding was determined as described under Materials and Methods. The data are representative of seven such experiments. Inset, a plot of the pseudo-first order kinetics of association for specific [³H]DM binding, where B_{eq} equals the amount of specific [³H]DM bound at equilibrium and B equals the amount bound at each time.

Right. Dissociation of specific [³H]DM binding. 4 nM [³H]DM was incubated with brain homogenate [3 mg tissue (wet weight) per milliliter of incubation volume] to equilibrium at 0°. At time zero, a large excess of unlabeled DM (10 μM) was rapidly added to the incubation mixture to initiate dissociation. Aliquots of 1.0 ml were filtered at the times indicated and specific [³H]DM binding was determined as described under Materials and Methods. The data shown are representative of seven such experiments.

TABLE 2

Effect of enzymes and protein-modifying reagents on specific [³H] DM binding

The pons-medulla from guinea pig brain was homogenized with 100 volumes of 50 mM Tris-Cl buffer (pH 7.7), and aliquots were incubated for 30 min at 25° in the absence (control sample) or presence of the appropriate enzyme or protein-modifying reagent. At the end of the incubation each sample was cooled in ice and centrifuged at 39,000 × *g* for 40 min. The resulting pellets were resuspended in Tris buffer, and a tissue content of 4 mg (wet weight) was assayed for [³H]DM binding as described under Materials and Methods, using 4 nM [³H]DM. Values are the means of triplicate determinations and are typical of at least two experiments.

Enzyme or reagent	Concentration	[³H]DM binding
		% of control
Experiment 1		
Trypsin	3 μg/ml	78
	30 μg/ml	62
	300 μg/ml	66
α-Chymotrypsin	3 μg/ml	95
	30 μg/ml	77
	300 μg/ml	57
Experiment 2		
Trypsin	200 μg/ml	30
Trypsin + soybean trypsin inhibitor (600 μg/ml)	200 μg/ml	70
Experiment 3		
N-Bromosuccinimide	1.0 mM	16
5,5′-Dithiobis[2-nitrobenzoic acid]	1.0 mM	66
N-Ethylmaleimide	2.0 mM	73
Iodoacetamide	10.0 mM	91
p-Chloromercuribenzoic acid	0.1 mM	74

25.5 min, respectively. From seven separate experiments the rate constants for dissociation (k_{-1}) of the fast and slow components were 0.3043 ± 0.038 min^{-1} and 0.031 ± 0.004 min^{-1}, respectively. Using these k_{-1} values, the apparent dissociation constants (K_d) calculated from the ratio k_{-1}/k_1 for the rapidly and slowly dissociating components of [³H]DM specific binding were 20.42 nM and 2.02 nM, respectively. The K_d calculated from the rapidly dissociating component (20 nM) agreed quite well with that determined by Scatchard analysis (13–18 nM).

Characteristics of [³H]DM binding. [³H]DM binding sites in brain are temperature-sensitive. When aliquots of brain homogenate were maintained for 5 min at various temperatures and then assayed for specific [³H]DM binding at 0°, there was a noticeable decrease (10–20%) in binding in tissue samples preincubated at 25° and 37°. These findings agree with the observation that conducting DM binding assays at 25° and 37° and cooling samples in ice prior to filtration results in specific [³H]DM binding which is only 80% and 75%, respectively, of that obtained from identical samples incubated at 0°. Thus, binding was maximal at 0–4°, and binding assays were routinely carried out at this temperature. Preincubating brain homogenate at temperatures in excess of 37° resulted in a progressive decrease in binding, so that tissue heated to 60° exhibited only 20% of the binding of control samples maintained at 0°. At temperatures greater than 60°, all high-affinity DM binding was abolished.

The pH profile for specific [³H]DM binding at 0° indicated that binding is maximal at pH 8.5. There was a sharp decrease in binding below pH 8.0, and a gradual

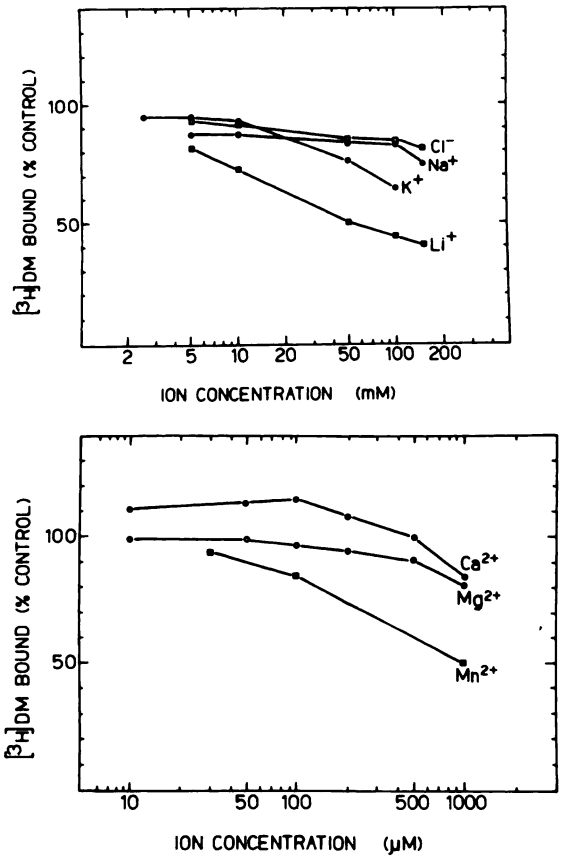

FIG. 5. *Effect of monovalent and divalent ions on specific [³H]DM binding to guinea pig brain stem homogenate*

The pons-medulla from guinea pig brain was homogenized with 100 volumes of 50 mM Tris-Cl buffer (pH 7.7) and centrifuged at 39,000 × *g* for 40 min. The resulting pellets were resuspended in Tris-Cl buffer, and a tissue content of 4 mg (wet weight) was assayed for specific [³H]DM binding as described under Materials and Methods using 4 nM [³H]DM in the absence (control sample) or presence of the various monovalent (top) or divalent (bottom) ions indicated. The results shown are representative of two such experiments performed in duplicate. All cations were used in the form of their chloride salts. When chloride was the ion being tested, brain tissue was prepared in 50 mM Tris-acetate buffer (pH 7.7) and the binding assay was conducted in the same buffer. The binding of control samples in Tris-acetate buffer was identical with those in Tris-Cl buffer.

decline in binding was observed at pH values greater than 8.5. Since the binding assay was carried out at 0° using 50 mM Tris-Cl buffer (pH 7.7 at 25°), the actual pH of the incubation mixture was close to 8.0 and binding was roughly 80% of the maximal binding observed at pH 8.5.

Preincubation of brain homogenate with the proteolytic enzymes trypsin and α-chymotrypsin significantly reduced specific [³H]DM binding (Table 2). Some loss of binding activity was also observed after pretreatment of brain tissue with several protein-modifying reagents (Table 2).

Specific [³H]DM binding was altered when binding assays were conducted in the presence of various ions (Fig. 5). Sodium inhibited specific [³H]DM binding about 25% at 150 mM while at lower concentrations it had no marked effect. Potassium did not significantly affect DM binding at physiological concentrations but did produce a gradual decline in binding at concentrations in excess of 10 mM. Lithium was the most inhibitory of the monovalent ions tested, producing a 50% decrease in binding at 50 mM. Calcium had a biphasic effect: concentrations ranging from 5 μM to 200 μM produced a reproducible and statistically significant increase in specific [³H]DM binding, while concentrations of 1 mM and greater were inhibitory. When brain homogenate was pretreated with ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid in order to remove endogenous free calcium, the magnitude of the enhancement of DM binding was the same as in untreated tissue, indicating that the effects of added calcium could not be further augmented by reducing endogenous levels of the divalent cation. In contrast to calcium, magnesium did not produce a biphasic effect, and only inhibition of binding occurred. Manganese in high concentration (100–1000 μM) was inhibitory. The effects of calcium, magnesium, and manganese in concentrations greater than 1 mM could not be accurately determined since nonspecific binding was also decreased.

Regional distribution of [³H]DM binding. Regional distribution studies in guinea pig brain (Table 3) revealed that specific [³H]DM binding is ubiquitous in the central nervous system. However, there was a 2.5-fold difference between the region of highest density of [³H]DM binding sites, the pons-medulla, and the region of lowest density, the hippocampus. For three of the regions examined, Scatchard analysis showed that the differences in binding were due to B_{max} values and not differences in affinity (Table 3).

Subcellular localization of [³H]DM binding. When specific [³H]DM binding was measured in subcellular fractions obtained by differential centrifugation of guinea pig whole-brain homogenate, binding displaceable by 10^{-5} M unlabeled DM was found in all of the subcellular fractions analyzed, with an apparent enrichment of binding occurring in the membrane fraction obtained from lysed synaptosomes (Table 4). In order to determine

TABLE 3

Regional distribution of specific [³H]DM binding in guinea pig brain

Fresh guinea pig brains were dissected into individual regions and each area was homogenized with 24 volumes of 50 mM Tris-Cl buffer (pH 7.7). Specific [³H]DM binding was measured as described under Materials and Methods, using 4 nM [³H]DM and 3 mg of tissue (wet weight) of each brain region. Values represent the mean of two separate experiments performed in duplicate. K_d and B_{max} values were determined from Scatchard plots of the binding data obtained when 3 mg of tissue (wet weight) were incubated with concentrations of [³H]DM ranging from 2 to 30 nM.

Region	[³H]DM bound	K_d	B_{max}
	fmoles/mg protein	*nM*	*pmoles/mg protein*
Corpus striatum	237	15.79	1.24
Cerebral cortex	279	15.61	1.24
Midbrain	402	ND[a]	ND
Thalamus-hypothalamus	385	ND	ND
Cerebellum	483	12.24	1.88
Medulla-pons	515	13.39	2.48
Hippocampus	200	ND	ND

[a] ND, Not determined.

TABLE 4

Subcellular distribution of specific [^{3}H]DM and [^{3}H]naltrexone binding in guinea pig brain

Subcellular fractions of guinea pig brain were prepared as described under Materials and Methods, and specific binding of [^{3}H]DM (4 nM) and [^{3}H]naltrexone (2 nM) was determined in each fraction. Values for [^{3}H]DM binding are the means of triplicate determinations and are representative of two experiments. The type of DM binding in each fraction was determined from Scathard plots of the binding data obtained after incubation of fractions with concentrations of [^{3}H]DM ranging from 2 to 30 nM. Values for [^{3}H]naltrexone binding were obtained from duplicate determinations and represent the mean of [^{3}H]naltrexone binding at two tissue concentrations. The experiment has been replicated once.

Fraction	Specific [^{3}H]DM Binding			Specific [^{3}H]Naltrexone Binding	
	pmoles/mg of protein	pmoles/g (wet wt)	Type of binding	pmoles/mg of protein	pmoles/g (wet wt)
Nuclear (900 × *g* pellet)	1.65	17.92	Low-affinity, high-capacity	0.079	0.62
Lysed P_2					
Mitochondria (11,500 × *g* pellet)	1.40	14.22	Low-affinity, high-capacity	0.068	1.00
Synaptic plasma membranes (100,000 × *g* pellet)	1.73	27.59	Low-affinity, high-capacity	0.223	2.93
Microsomes (100,000 × *g* pellet)	0.59	14.63	K_d = 30 nM, B_{max} = 6 pmoles/mg protein	0.380	7.31

whether binding in each of the subcellular fractions was due to high- or low-affinity DM sites, saturation binding studies were carried out using [^{3}H]DM in the concentration range of 2–30 nM. Scatchard analysis of the data derived from these studies revealed that high-affinity DM sites were present in the microsomal fraction and that, based on the B_{max} value calculated for DM binding in this fraction (B_{max} = 6 pmoles/mg of protein), there was a 2.4-fold enrichment relative to the total homogenate (B_{max} = 2.5 pmoles/mg of protein). On the other hand, no high-affinity sites were detected in the nuclear, mitochondrial, and synaptic plasma membrane fractions; additional binding studies measuring the displacement of [^{3}H]DM by unlabeled DM (10^{-9}–10^{-5} M) indicated that binding in these subcellular fractions was of low affinity (IC_{50} values in the micromolar range) and saturable only at micromolar concentrations of DM. However, even though high-affinity DM sites appeared to be confined almost exclusively to the microsomal fraction, it is possible that they were also present in other subcellular fractions but were not detected because of the large capacity of low-affinity sites.

The subcellular distribution of opiate receptor binding determined in the very same subcellular fractions (see Table 4) is consistent with reports that opiate binding is present on both synaptic plasma membranes and microsomal membranes, with the greatest enrichment of binding activity occurring in the microsomal fraction (19). To determine whether DM binding sites are located on membranes similar to those containing microsomal opiate receptors, microsomes were subfractionated on discontinuous sucrose density gradients and the membranes collected at each interface were assayed for [^{3}H]DM binding and [^{3}H]naltrexone binding. As Table 5 illustrates, the distribution pattern for [^{3}H]DM binding activity was very similar to that of NADPH-cytochrome *c* reductase, a smooth endoplasmic reticulum marker, and followed less closely the pattern for [^{3}H]naltrexone binding.

Metabolism of DM. Studies were carried out to determine whether the microsomal binding of [^{3}H]DM was in any way related to a drug-metabolizing enzyme. First, binding assays were conducted at 0° and 25° in the presence and absence of 0.1 mM NADPH, the cofactor used in demonstrating drug-metabolizing activity in brain (e.g., see refs. 20 and 21). The results showed that NADPH had a negligible effect on specific [^{3}H]DM binding at either temperature. Second, [^{3}H]DM was incubated with brain and liver microsomes in the presence of NADPH as described under Materials and Methods, and aliquots of the incubated samples were analyzed by TLC for degradation of DM. Similar to what has been reported (22), we found that DM can be substantially degraded by

TABLE 5

Distribution of [^{3}H]DM and [^{3}H]naltrexone binding, NADPH-cytochrome c reductase, and RNA in microsomal subfractions of guinea pig brain cortex

Specific binding of [^{3}H]DM at 4 nM and [^{3}H]naltrexone at 2 nM was determined in microsomal subfractions of brain cortex prepared as described under Materials and Methods. Binding assays were conducted immediately after preparation of the subfractions; NADPH-cytochrome c reductase and RNA were measured in microsomal subfractions frozen at −80° for 1–2 days. All determinations were performed in duplicate and at two sample concentrations. Relative specific activity (RSA) equals percentage activity recovered/percentage protein recovered.

	Sucrose molarities			
	0.3–0.5	0.5–0.8	0.8–1.0	1.0–1.3
[^{3}H]DM binding				
pmoles/mg protein	1.05	1.36	0.39	0.36
% total recovered	3.5	10.1	45.6	40.8
RSA	2.5	3.26	0.94	0.86
[^{3}H]Naltrexone binding				
fmoles/mg protein	0	140	404	225
% total recovered	0	1.5	63.7	34.8
RSA	—	0.48	1.31	0.74
NADPH-cytochrome *c* reductase				
nmoles/mg protein/hr	145	417	130	133
% total recovered	1.5	8.9	45.6	44.0
RSA	1.07	2.87	0.94	0.93
RNA				
μg/mg protein	0	0	1.15	1.18
% total recovered	0	0	50	50
RSA	—	—	1.06	1.06

enzymes in liver microsomes, with less than 5% of the radioactivity applied to the TLC plate recovered in the DM spot. In contrast, [^{3}H]DM incubated with brain microsomes was not metabolized, and recovery of radioactivity at the DM spot was generally 95–100%.

DISCUSSION

The data presented in this paper clearly support the existence of high-affinity binding sites for the antitussive DM in guinea pig brain. These sites are saturable and bind DM in a reversible manner. The K_d calculated from radioligand saturation experiments was 13 nM, and a similar value (18 nM) had been determined from studies using nonradioactive DM to displace [^{3}H]DM binding. Although low-affinity DM binding sites (K_d > 200 nM) have also been detected in brain homogenates, they have not been studied since the levels of DM measured in human serum after single therapeutic oral doses are in the nanomolar range (23) and one would expect the high-affinity sites to be more relevant to the pharmacological actions of DM.

The maximal number of high-affinity [^{3}H]DM binding sites in guinea pig brain has been calculated at 2.5 pmoles/mg of protein, which is equivalent to 240 pmoles/g of brain (wet weight). This binding capacity is significantly greater than that found for opiate receptors (24) or central neurotransmitter receptors, where the total number of binding sites is generally in the range of 10–100 pmoles of ligand bound per gram of tissue. Based on this observation alone, it would appear that DM is not labeling opiate receptors or receptor sites for central neurotransmitters, a point which receives more attention in the following paper (8). However, the capacity of DM sites is similar to values reported for other drugs which bind to brain tissue. For example, Vincent *et al.* (25) have found that phencyclidine binding sites display a maximal binding capacity of 250 pmoles/g of brain.

The rapid filtration technique used in our studies was a potential source of artifacts, since the glass-fiber filters themselves were able to bind significant amounts of tritiated DM which were displaceable by unlabeled drug. As we have shown, we were able to overcome this problem and obtain reliable binding data by using a wash buffer similar to that described by Law *et al.* (18) for reducing filter binding of [^{3}H]β-endorphin.

During the kinetic studies we observed a biphasic pattern for the dissociation of DM bound to guinea pig brain homogenate. When the rate constant for the rapidly dissociating component was used to calculate the apparent dissociation constant, a K_d of 20 nM was obtained, a value which agrees with that determined from equilibrium studies. However, similar calculations using the rate constant for the slowly dissociating component indicated the presence of a binding site with much higher affinity (2 nM). Scatchard analysis of the data obtained from saturation experiments utilizing [^{3}H]DM concentrations of 0.18–30 nM has not demonstrated another high-affinity DM binding site. Therefore, our results could mean that DM binds to two sites having similar affinities but different rate constants. Alternately, it could be postulated that the site which binds DM exists in two conformations which display different dissociation rates. Obviously, more detailed kinetic analyses are necessary in order to resolve this question.

Several of our findings suggest that protein is a component of DM binding sites. First, DM sites are temperature-sensitive and binding progressively declines as tissue is heated to temperatures greater than 37°. Second, significant decreases in DM binding are seen after brain homogenate is preincubated with the proteolytic enzymes trypsin and α-chymotrypsin. Third, pretreatment of brain homogenate with several protein-modifying reagents also results in a loss of DM binding. The fact that the effects of the sulfhydryl reagents *N*-ethylmaleimide, iodoacetamide, and 5,5′-dithiobis[2-nitrobenzoic acid] are relatively weak and are seen only at high concentration may indicate that sulfhydryl groups do not play an important role in the DM binding process.

In addition to finding that DM binding is pH-dependent, we have also observed that various monovalent and divalent cations can alter specific [^{3}H]DM binding. For example, lithium and manganese are very inhibitory at millimolar concentrations whereas calcium has a biphasic effect, enhancing binding at concentrations of 5–200 μM and decreasing binding at concentrations greater than 1 mM. However, sodium, in concentrations up to 100 mM, does not appreciably alter DM binding, a finding which clearly distinguishes DM sites from opiate receptor binding sites (26). Chloride also produces minimal changes in DM binding, suggesting that DM sites are not coupled to chloride channels.

Regional distribution studies indicate that high-affinity DM binding sites are present in all major brain areas, with the greatest number of sites in the pons-medulla, the region in which the cough center is localized (27). Although there is only a 2.5-fold difference between the regions containing the highest and the lowest density of DM sites, autoradiographic studies may reveal more pronounced regional variations. It is conceivable that DM binding in areas other than the lower brain stem reflects the sites at which DM exerts pharmacological effects other than cough suppression. For example, in man, single large doses of DM produce psychotomimetic effects and sedative-hypnotic effects, as well as feelings of drunkedness, nervousness, and dysphoria (28); in monkeys, chronic administration produces a physical dependence which is unlike that produced by morphine (29). In behavioral studies, DM acts as a discriminative stimulus in rats (30) and antagonizes the limb flick behavior in cats following lysergic acid diethylamide administration (31). Additionally, microiontophoretic experiments in rat brain have shown that DM inhibits the firing of midbrain raphe serotonergic neurons as well as postsynaptic neurons receiving a serotonergic input from these cells (32).

After subcellular fractionation of guinea pig whole brain, high-affinity DM binding sites are found almost exclusively in the microsomal fraction. However, in preliminary studies we also detected high-affinity sites in unwashed crude P_2 pellets and synaptic plasma membranes obtained from this fraction (7). Gurd *et al.* (33) have shown that considerable amounts of microsomal particles are trapped in crude P_2 pellets during differential centrifugation and that washing the P_2 fraction by

resuspension and recentrifugation significantly reduces the presence of these contaminating microsomal elements. In the present report, the crude P_2 pellet was washed, and no high-affinity binding was identified in this subcellular fraction. Thus, it can be concluded that the binding we previously observed in this fraction (7) was due to microsomal contamination.

Opiate receptors are present in microsomal fractions prepared from brain (19), and Roth *et al.* (34) have reported that after subfractionation of microsomes, opiate binding sites are concentrated in fractions that are enriched in smooth endoplasmic reticulum. In similar microsomal subfractionation experiments, we have found that DM binding sites are also localized in smooth endoplasmic reticulum-enriched fractions since the distribution pattern for binding coincides with the distribution pattern for NADPH-cytochrome *c* reductase activity. However, the distribution profile for opiate receptors that we observed is slightly different from that of DM binding, and therefore it is likely that the microsomal membranes containing opiate receptors are different from those containing DM binding sites.

The demonstration of drug-metabolizing activity in brain microsomal preparations (20, 21) prompted us to investigate whether DM sites may represent binding to drug-degrading enzymes. The results of these studies have demonstrated that DM is extensively metabolized by liver microsomal enzymes but not by brain microsomes. We also observed that NADPH, the cofactor required for brain and liver drug-metabolizing enzymes, has no effect on DM binding to brain microsomes. These results suggest (but do not prove) that DM does not bind to a microsomal drug-metabolizing enzyme in brain.

Dextrorphan is the major metabolite of DM found in urine and serum of several species, including man (ref. 35 and references therein), and Benson *et al.* (1) have reported that it has antitussive activity in the dog. These facts suggested the possibility that DM, like codeine, may be *O*-demethylated before it exerts its pharmacological effects and that dextrorphan, rather than the parent compound, might be the actual antitussive agent (35). As we have indicated, our attempts to demonstrate specific binding sites for tritiated dextrorphan in brains of rats and guinea pigs were unsuccessful. This finding is of some interest since, like DM, dextrorphan in high doses produces a number of behavioral effects in animals (29, 31, 36, 37). The existence of specific binding sites in central nervous system tissue where (+)-isomers of opioids such as dextrorphan and (+)-morphine exert their effects has been postulated (37, 38), but our results using [^{3}H]dextrorphan as radioligand were negative.

In contrast to brain, we have found specific binding sites for [^{3}H]dextrorphan in homogenates of guinea pig kidney.[4] These sites are not stereoselective, since levorphanol is a potent displacer of dextrorphan binding. Simantov *et al.* (39) have described binding of [^{3}H]opiates to guinea pig kidney membranes that is potently inhibited by the dextrorotatory compounds dextrorphan and dextrallorphan. The dextrorphan sites we have labeled in the kidney may be similar to the nonstereospecific binding sites described by these investigators.

[4] G. L. Craviso and J. M. Musacchio, unpublished observation.

In conclusion, we have described some of the general characteristics of DM binding sites in brain. The following paper (8) presents the results obtained from competition studies in which we have investigated the nature of DM binding sites as well as their possible physiological and pharmacological relevance.

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Send reprint requests to: Dr. José M. Musacchio, Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, N. Y. 10016.