Biochemical Pharmacology, Vol. 28, pp. 3361–3366.

BINDING OF A LABELED ANTIDEPRESSANT TO RAT BRAIN TISSUE

ANAT BIEGON and DAVID SAMUEL
Department of Isotope Research, The Weizmann Institute of Science, Rehovot, Israel

(*Received* 31 *March* 1979; *accepted* 11 *June* 1979)

Abstract—[^{3}H]desipramine, a labeled tricyclic antidepressant, binds specifically to rat brain crude synaptosomal and membranal preparations. The binding is rapid, displaceable and saturable with a IC_{50} value of 4×10^{-6}M. The maximal number of sites is 18–26 nmoles/g original tissue in the synaptosomal preparation and only 6–8 nmoles/g in the membranal preparation. Scatchard plots of the binding data are not linear, suggesting a heterogenous set of binding sites. Pargyline and a number of neurotransmitters and agonists have only a small effect on desipramine binding; whereas various neurotransmitter antagonists inhibit the binding at the μM concentration range.

Tricyclic antidepressants are known as inhibitors of norepinephrine (NE) and serotonin (5HT) reuptake into nerve terminals. This action was also believed to be responsible for their therapeutic effect [1], and probably involves presynaptic sites. Recently, some doubts have been raised as to the relevance of uptake inhibition to the mechanism of therapeutic action of these drugs [2, 3]. In addition, recent reports suggest a direct interaction of tricyclic antidepressants with a variety of seemingly postsynaptic sites. Thus, evidence has been obtained for the interaction of tricyclics with central muscarinic [4, 5], histaminergic [6], serotonergic [7] and α-adrenergic [8] receptors.

However, these studies were performed separately, using, each time, a ligand specific for the receptor system investigated. We felt it was important to investigate the direct interaction of a labelled antidepressant with brain tissue. Previous studies have demonstrated binding of labelled antidepressants to synaptosomal preparations [5, 9, 10]. We wanted to confirm and extend these studies. We used tritiated desipramine [^{3}H]DMI to elucidate the characteristics, pharmacology, subcellular localization and regional distribution of antidepressant binding.

MATERIALS AND METHODS

[^{3}H]DMI, 44Ci/mmole, was obtained from I.N.C. Beer-Sheva, Israel. DMI.HCl (Ciba-Geigy), amitriptyline (Teva), mianserine (Organon) and fluxethine (Eli Lilly) were gifts from the respective companies. The other drugs and chemicals used were obtained from commercial sources. Wistar rats from the breeding colony of the Hormone Research department of the Weizmann Institute of Science were used throughout this study.

Binding studies

Animals were killed by decapitation. Forebrains were quickly removed and homogenised in 10 vol of ice cold, isotonic (0.32M) sucrose, using a glass-teflon homogeniser. The homogenate was spun at 1000*g* for 10 min and 50 μl of the supernatant (S_1) were added to test tubes containing modified Krebs–Henseleit buffer (pH 7.4) and the desired concentrations of label and cold ligand to a final volume of 2 ml. Following an incubation for 20 min at room temperature, the mixture was filtered through GF/C paper and the filters were washed with 4 × 3 ml ice cold buffer. The filters were then transfered to vials, shaken with scintillation fluid and counted in a Packard Tri-Carb scintillation counter, using a toluene–triton scintillation mixture, at 36% efficiency.

Crude membranes were prepared by homogenising rat forebrains in 10 vol of 50 mM Tris–HCl buffer (pH 7.4), using a polytron homogeniser. The homogenate was spun at 30,000 *g* for 10 min, the supernatant decanted and the pellet resuspended and spun again under the same conditions. The washed pellet was resuspended and homogenised in the original volume of buffer. Therefore, the S_1 and membrane preparations represent the same amount of original tissue. Fifty μl of the membrane preparation were taken for the binding assay. When DA, NE or 5HT were used as inhibitors, pargyline (10^{-4}M) and ascorbic acid (0.2%) were added to the buffer.

Subcellular fractionation

Synaptic plasma membranes (SPM) were isolated and purified essentially by the method of Morgan *et al.* [11]. Microsomes were sedimented from the S_2 fraction by spinning at 100,000 *g* for 60 min. Samples of each fraction were taken for binding assays and protein determination [12]. For triton solubilization, a sample of P_2 was left overnight at 4° in 1% triton, then spun at 100,000 *g* for 60 min. The pellet was washed with Tris buffer, 50 mM, (pH 7.4), spun again at 100,000 *g* for 60 min and resuspended in buffer for the binding assay. A parallel sample of P_2 (lysed P_2) was left overnight at 4° in Tris buffer, and then treated in the same manner.

RESULTS

Binding studies

Temperature, time and concentration dependence. The binding of [^{3}H]DMI is not dependent on temperature: when different concentrations of

Table 1. The accumulation of [^{3}H]DMI by brain tissue as a function of temperature

[^{3}H]DMI in medium (M)	[^{3}H]DMI in tissue (mole/mg protein)		Tissue/medium ratio
	0°	37°	
5×10^{-8}	7.35×10^{-11}	7.8×10^{-11}	0.60
1.5×10^{-7}	2.23×10^{-10}	2.3×10^{-10}	0.62
1.0×10^{-6}	1.45×10^{-9}	1.45×10^{-9}	0.59
1.0×10^{-5}	1.20×10^{-8}	1.21×10^{-8}	0.48

[^{3}H]DMI, between 5×10^{-8}M and 10^{-5}M, were incubated for 5 min in 0° or 37°, the amount of tissue bound [^{3}H]DMI was the same (Table 1). This is in agreement with the results of Hunt *et al.* [9], who have shown that imipramine binding does not depend on temperature and is not inhibited by ouabain.

The binding process is rapid, reaching equilibrium within 1 min. When 50nM[^{3}H]DMI were incubated for 1, 2, 5, 10, 20, or 30 min at 25°, the amount of specific binding did not increase. Specific binding is defined as the difference between total binding and binding in the presence of 5×10^{-4}M cold DMI or amitriptyline. The incubation time for all the binding studies was set at 20 min.

Saturation is reached at about 10^{-4}M, at which concentration only 30 per cent of the binding is specific. However, specific binding can be detected at concentrations as low as 0.1nM, at which point nonspecific binding is so low as to be indistinguishable from the binding to the filters alone, which amounts to 1 per cent of the applied radioactivity (Fig. 1). The Scatchard plot for the binding of labeled DMI is not linear, suggesting a heterogenous set of binding sites (Fig. 2). This is true for both low (nM) and high (μM) concentrations. Therefore, no single *Kd* value can be obtained from this plot. The maximal number of binding sites, however, may be calculated, and is rather high: 18–26 nmoles/g original wet weight.

The binding to a crude membranal preparation (i.e. no intact synaptosomes) obeys the same kinetics and saturation parameters as the binding to the S_1 fraction, but the number of available sites is markedly reduced, being 6–8 nmoles/g (Fig. 3).

Pharmacology. Table 2 summarises the IC_{50} values of various drugs and transmitters towards displacement of [^{3}H]DMI binding. The displacement curves of the monoamines do not parallel those of the antidepressants; and they were not capable of displacing more than 30 per cent of specific [^{3}H]DMI binding. Two novel antidepressants were included in the study: mianserine, a tetracyclic drug, and dibenzepine (Fig. 4). Mianserine was as active as the tricyclics, while dibenzepine exhibited higher IC_{50} values. The other drugs included in Table 2 do not conform to any predictable pattern, based on their known affinity for a single receptor. As a rule, antagonists were more active than agonists (e.g. phentolamine vs clonidine). None of the agonists was capable of inhibiting 100% of the specific binding, but quite a few antagonists were reasonably efficient. However, the IC_{50} values of the antagonists are much higher than their affinities towards the receptor they are known to interact with 'specifically'.

Some of the antagonists were tested in combination with others belonging to different classes. In no case were the effects totally additive (Table 3). This fact strongly suggests a large degree of overlap among the sites for which the different antagonists compete with [^{3}H]DMI.

Subcellular fractionation

The specific binding of [^{3}H]DMI to different subcellular fractions is summarized in Table 4. Two concentrations of the label were used: 2×10^{-9}M and 10^{-4}M. The low concentration should indicate the relative affinity of the different fractions, whereas the high concentration should give an approximate value for the maximal number of binding sites.

The amount of specifically bound [^{3}H]DMI

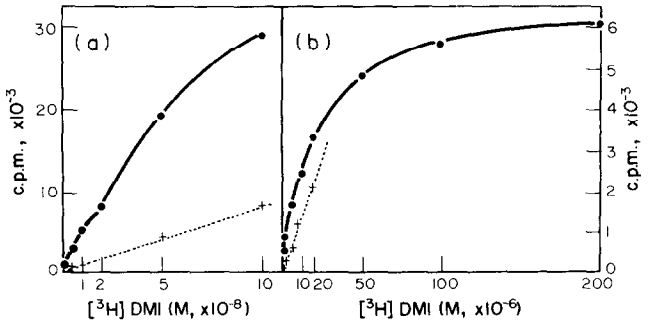

Fig. 1. The specific (●—●) and non specific (×—×) binding of [^{3}H]DMI to the S_1 fraction from rat forebrain. (a) binding at low concentrations; (b) binding at high concentrations.

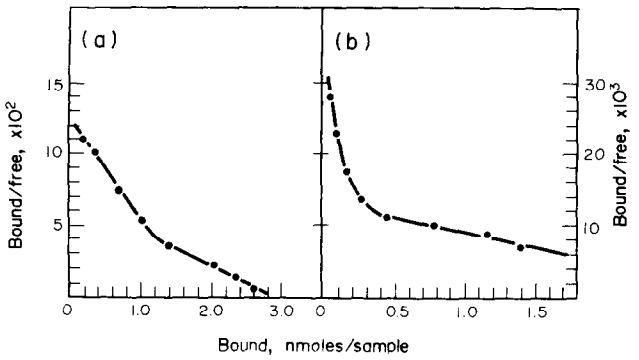

Fig. 2. Scatchard plots of [³H]DMI binding to (a) the S1 fraction; (b) a crude membranal preparation, from rat forebrain.

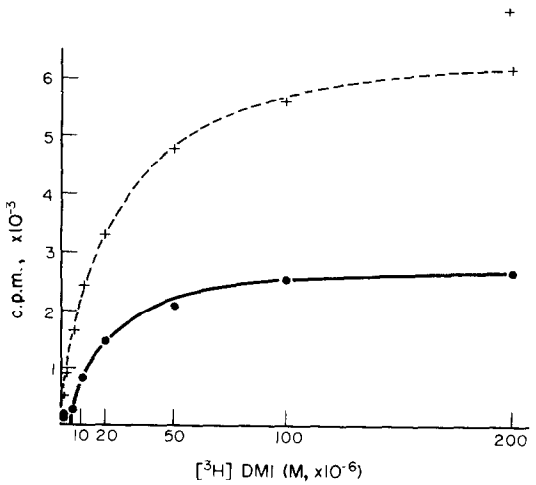

Fig. 3. Comparison of the specific binding of [³H]DMI to the S1 fraction (+—+) and a crude membranal preparation (●—●) prepared from one rat forebrain.

increases with the degree of purification of the synaptosomes. Lysis of the synaptosomes causes a considerable loss of binding. Thus, SPM bind only 30% of the radioactivity bound by whole synaptosomes when incubated with 2nM. The binding to SPM at 10^{-4}M is only 3 per cent of the binding to whole synaptosomes, which suggests that the lower binding at 2nM is not due to reduced affinity but rather to a large decrease in the number of available sites.

Membranal binding is reduced by another 60 per cent on treatment with triton, which suggests that this binding is related to membranal receptors.

Regional distribution. The lowest binding of [³H]DMI (5nM) occurs in the cerebellum, which has only half of the specific binding in the cortex. The distribution in the other brain regions studied is fairly uniform, with the cortex and caudate tending towards higher values than septum, hypothalamus, hippocampus and medulla-pons (Table 5).

DISCUSSION

The salient points of the present study may be summarized as follows:

1. The binding of [³H]DMI to brain tissue is independent of temperature, very rapid and occurs over a wide concentration range. Scatchard analysis of the binding data suggests interaction with more than one binding, or receptor, site. The maximal number of sites is large; namely 18–26 nmoles/g tissue in the S1 fraction and 6–8 nmoles/g in the crude membrane preparation.

Table 2. Inhibition of [³H]DMI (25 nM) specific binding by various drugs

Ligand	$IC_{50}(\mu M)$	Ligand	$IC_{50}(\mu M)$
Nortriptyline	2.7 ± 0.8	Norepinephrine	1.0 (IC_{30})
Amitriptyline	5.5 ± 1.5	Clonidine	>100
DMI	4.0 ± 2.0	Phentolamine	7.0 ± 3
Imipramine	8.5 ± 2.0	Propranolol	18.0 ± 6
Fluxethine	2.0 ± 0.5	Serotonin	1.0 (IC_{25})
Mianserine	12.0 ± 4.0	Methysergide	>100
Doxepine	13.0 ± 4.0	Methiothepin	1.8 ± 0.8
Dibenzepine	116 ± 20	Histamine	10.0 (IC_{25})
Dopamine	1.0 (IC_{30})	Cyproheptidine	8.5 ± 3.0
Haloperidol	22.5 ± 8.0	Gaba	>100
Chlorpromazine	2.3 ± 1.0	Picrotoxin	>100
Atropine	>100	Morphine	1.0 (IC_{33})
Diazepam	>100	Naloxone	1.0 (IC_{33})
		Pargyline	>100

Results are the Mean ± S.E.M. of 3–8 determinations, using the S1 fraction.

Table 3. Inhibition of [^{3}H]DMI (25 nM) binding by combinations of drugs

Inhibitor (M)	Per cent inhibition
Methiothepin 10^{-6}	36
Propranolol 10^{-4}	80
Methiothepin 10^{-6} + Propranolol 10^{-4}	88
Propranolol 10^{-5}	50
Methiothepin 10^{-5}	78
Propranolol 10^{-5} + Methiothepin 10^{-5}	86
Cyproheptidine 5×10^{-6}	40
Cyproheptidine 5×10^{-6} + 10^{-6}Methiothepin 10^{-6}	54
Cyproheptidine 5×10^{-5}	75
Cyproheptidine 5×10^{-5} + Methiothepin 10^{-5}	90
Cyproheptidine 5×10^{-6} + Propranolol 10^{-5}	53
Phentolamine 3×10^{-6}	25
Haloperidol 3×10^{-6}	23
Phentolamine 3×10^{-6} + Haloperidol 3×10^{-6}	25
Phentolamine 10^{-5}	60
Haloperidol 10^{-5}	35
Phentolamine 10^{-5} + Haloperidol 10^{-5}	65

2. A large proportion (approx. 60 per cent) of the binding of [^{3}H]DMI apparently occurs inside the nerve terminal, since lysis of synaptosomes results in a reduction in binding capacity.

3. A number of pharmacological and neurotransmitter agents exhibit some affinity towards [^{3}H]DMI binding sites. However, no single receptor agonist is capable of inhibiting 100% of the specific binding of DMI. Some antagonists are capable of complete inhibition of [^{3}H]DMI binding. The later belong to diverse receptor systems, and their IC_{50} values for [^{3}H]DMI are, generally, a few orders of magnitude larger than their respective IC_{50} values for their own receptors. Furthermore, the effects of antagonists related to different receptor systems are not additive.

The only agents that can inhibit [^{3}H]DMI at concentrations close to the ones used to elicit their known clinical and pharmacological actions, are the other antidepressants.

4. The binding of [^{3}H]DMI to subcellular fractions increases with the degree of purification of synaptosomes. Upon lysis, or purification of synaptic plasma membranes, a large proportion of the binding is lost. The membranal binding sites are sensitive to mild triton treatment.

5.There is no significant regional distribution of specific binding throughout the forebrain, while the cerebellum has only half of the binding activity of the other regions.

It has been suggested that tricyclics can enter nerve terminals, not using an active uptake mechanism [9]. Support for the intra-neuronal site of tricyclic drug binding comes from several additional sources: When rats were injected with [^{3}H]DMI *in vivo*, and their brains subjected to sub-cellular fractionation, the pattern of radioactivity found per mg protein was essentially similar to the *in vitro* results reported here. High values were found in whole synaptosomes, which were considerably reduced upon lysis and purification of synaptic plasma membranes. Using E.M autoradiography, we have found that a large proportion of the grains were inside nerve

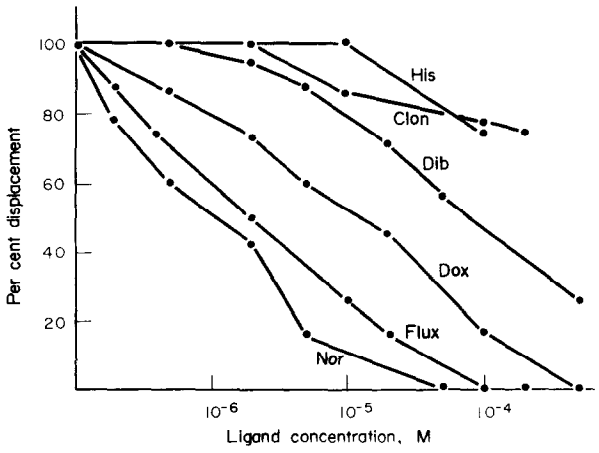

Fig. 4. Displacement of [^{3}H]DMI specific binding by several drugs. HIS = histamine, Clon = Clonidine, Dib. = dibenzepine, Dox = doxepine, Flux = fluxethine, Nor = nortriptyline.

Table 4. Subcellular distribution of specific [^{3}H]DMI binding at a low (2nM) and high (100μM) concentration

Fraction	pmole specifically bound/mg protein 2 nM	100 nM
Homogenate	1.25	103
S_1 (1000g × 10 min)	1.49	64.4
P_2 (20,000g × 25 min)	2.25	140
Synaptosomes	2.32	153
Microsomes	0.19	12.4
Mitochondria	1.1	88.7
SPM	0.69	4.4
Lysed P_2	0.32	5.8
Triton treated P_2	0.14	n.d

n.d = not detectable.

Table 5. Regional distribution of [^{3}H]DMI (5 nM) specific binding. The results are the means ± S.D. of 4–5 determinations

Brain region	Relative binding per cent
Cortex	87 ± 6
Hippocampus	88 ± 23
Caudate	100
Septum	80 ± 10
Hypothalamus	80 ± 12
Pons-medulla	96 ± 17
Cerebellum	44 ± 4*

* $P < 0.01$, student's t-test.

terminals [13]. Similar autoradiographic localization has been reported using another labelled antidepressant, dimetacrine [14]. Microsomes could be possible candidates for intracellular binding of [^{3}H]DMI, because of the well known binding of tricyclics to liver microsomes [15]. This notion is not supported by the results (Table 4). The nature of the intracellular component which binds DMI is still unknown to us. The large capacity and non membranal character suggests that it may be an intracellular enzyme, perhaps one involved in the regulation of uptake.

The evidence for the heterogenous nature of tricyclic binding is supported from various sources. Recent reports from different laboratories have shown that antidepressants interact with muscarinic, histaminergic, serotonergic and adrenergic receptors [4–8] and all within a range of fairly similar concentrations. These data, from several sources, are summarized in Table 6. This suggestion is supported by the wide range of [^{3}H]DMI binding activity and the non linear Scatchard plot we have observed. Multireceptor interaction is also supported indirectly by the fact that the maximal number of binding sites observed in a crude membrane preparation is larger than the figures reported for any single receptor, and may represent a combination of a number of them. Indirect proof of this is the lack of regional distribution of [^{3}H]DMI binding: since most specific receptors appear to have a unique regional distribution, such a pattern will not be apparent when a drug is bound to several different receptors in a given brain region.

It is still an open question which of the several receptor interactions, if any, is essential for antidepressive action. Tricyclic antidepressants are not 'high affinity' drugs. In depressed patients, large doses over prolonged periods (2–3 weeks) are necessary for clinical improvement [16]. We cannot therefore assume that the highest affinity interaction, i.e. in the nM region, is more important than the lower affinity interactions occuring at higher concentrations. Perhaps the unique feature of tricyclics is their ability to act upon several systems simultaneously, at both presynaptic (uptake) and postsynaptic (receptor) sites. Only additional detailed structure activity studies of antidepressants on different receptors related to their relative clinical efficacies, may provide a more conclusive answer to this question.

Note: After this work was completed, a similar study of the binding of [^{3}H]imipramine was reported (O'Brien *et al.*, *Soc. Neurosci. Abstr.* **4**, 430, 1978), which is in very good agreement with our results.

Acknowledgement— We wish to thank Mr. and Mrs. Erwin Gudelsky for the generous support of this project.

REFERENCES

1. J. M. Davis, *Int. Rev. Neurobiol.* **12**, 145 (1970).
2. A. Frazer and J. Mendels, *Am. J. Psychiat.* **134**, 1040 (1977).

Table 6. Reported affinities of DMI for different systems, and relevant brain concentrations

Target of action	IC_{50}	Ref.
NE uptake	5×10^{-8}	[17]
5HT uptake	2×10^{-6}	[17]
Muscarinic binding	4×10^{-6}	[18]
Histaminergic binding	3.2×10^{-7}	[6]
α-adrenergic binding	1.4×10^{-7}	[8]
5HT binding	1×10^{-6}	[7]
β-adrenergic binding	5×10^{-6}	[19]
Dopaminergic binding	4×10^{-6}	*
Opiate binding	2×10^{-5}	*
DMI binding	4×10^{-6}	
Concentration of DMI in rat brain, 30 min after 15 mg/kg i.p.	4.1×10^{-5}M	[20,24]
Plasma levels found in patients (unrelated to therapeutic outcome)	$0.02–2.1 \times 10^{-6}$M	[21]
Estimated brain levels	$0.02–2.1 \times 10^{-5}$M	†
Therapeutic range of Nortriptyline plasma levels	$1.6–6.6 \times 10^{-7}$M	[22]
Estimated brain levels	$1.6–6.6 \times 10^{-6}$M	†

* Unpublished results.

† Estimation of brain level was done employing a plasma: brain ratio of 1:10, found in the rat by ourselves and Nagy [23].

3. K. Ghose and A. Coppen, *Psychopharmacology* **54,** 57 (1977).
4. M. Rehavi, S. Maayani and M. Sokolovsky, *Biochem Pharmac.* **26,** 1559 (1977).
5. M. Rehavi and M. Sokolovsky, *Brain Res.* **149,** 525 (1978).
6. P. D. Kanof and P. Greengard, *Nature, New Biol.* **272,** 329 (1978).
7. K. Fuxe, S. O. Ogren, L. Agnati, J. A. Gustafsson and G. Jonsson, *Neurosci. Lett.* **6,** 339 (1977).
8. D. C. U'prichard, D. A. Greenberg, P. P. Sheehan and S. H. Snyder, *Science* **199,** 197 (1978).
9. P. F. Hunt, M. H. Kannengiesser and J. P. Raynaud, *Biochem. Pharmac.* **24,** 681 (1975).
10. R. Ishitani and T. Iwamoto, *Japan J. Pharmac.* **28,** 85 (1978).
11. I. G. Morgan, L. S. Wolfe, P. Mendel and G. Gombos, *BBA* **241,** 737 (1971).
12. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
13. Z. Yavin, A. Biegon, R. Hofstein and D. Samuel, *Experientia*, in press (1979).
14. R. Ishitani and T. Iwamoto, *Experientia* **32,** 1036 (1976).
15. L. F. Gram, *Dan. Med. Bull.* **24,** 81 (1977).
16. J. Koch-Weser and L. E. Hollister, *New Engl. J. Med.* **299,** 1106 (1978).
17. T. Segawa, Y. Nomura, A. Tanaka and H. Murakami, *J. Pharm. Pharmac.* **29,** 139 (1977).
18. K. Shein and S. E. Smith, *Br. J. Pharmac.* **62,** 567 (1978).
19. B. B. Wolfe, T. K. Harden, J. R. Sporn and P. B. Molinoff, *J. Pharmac. exp. Ther.* **207,** 446 (1978).
20. J. Vetulani, R. J. Stawarsz, J. V. Dingell and F. Sulser, *Naunyn-Schmiedeberg's Archs. exp. Path. Pharmak.* **293,** 109 (1976).
21. A. Nagy and L. Treiber, *J. Pharm. Pharmac.* **25,** 599 (1973).
22. P. Kragh-Sorensen, M. Asberg and C. Eggert-Hansen, *Lancet* **1,** 113 (1973).
23. A. J. Nagy, *Pharm. Pharmac.* **29,** 104 (1977).
24. A. Biegon and D. Samuel, *Psychopharmacology*, in press (1979).