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# THE NATURE OF [$^3$H]IMIPRAMINE BINDING TO SYNAPTOSOMES

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**Abstract**—In contrast to serotonin "uptake", the saturable binding of imipramine to rat brain synaptosomes does not involve an active transport process, since it is independent of time, temperature and $Na^+$, $K^+$-ATPase. It is moreover unaffected by a high concentration of serotonin, confirming that the "uptake" site for this amine is not implicated. Analysis of the saturation curve for binding of imipramine to intact synaptosomes gives a binding constant of $3{\cdot}5 \pm 1{\cdot}0 \times 10^{-9}$ M. Identical binding characteristics are found with a synaptosomal membrane "ghost" fraction. When the membrane fraction is solubilized in Triton X-100, saturable binding is still observed but the affinity for imipramine decreases while the number of binding sites increases. Diverse psychoactive compounds effectively compete with imipramine for binding to synaptosomal membranes and the order of their activity is correlated with their lipophilicity. It is suggested that these compounds interact with a lipophilic pocket of low affinity and low specificity. If a more specific interaction exists and is associated with inhibition of transmitter amine "uptake" by tricyclic antidepressants, it could be masked by this process.

The clinical activity of imipramine-like tricyclic antidepressants has been correlated with their inhibitory action on the re-uptake of noradrenalin and serotonin released by neuronal activity into the synaptic cleft [1–4]. The process of re-uptake and its inhibition by tricyclic antidepressants can be conveniently studied *in vitro* using a preparation of synaptosomes. Inhibition of amine uptake into synaptosomes occurs at relatively low drug concentrations ($\sim 10^{-7}$ M) and shows structural specificity. At much higher drug concentrations ($\sim 10^{-4}$ M) a number of membrane-associated enzymes are inhibited by tricyclics [5], as well as by related compounds such as phenothiazines [6, 7] suggesting a much more general phenomenon.

In order to investigate the mechanism of action of imipramine, we have compared the nature of its binding to a non-purified synaptosome preparation with that of serotonin. Secondly, we have examined the characteristics of its binding to purified synaptosomes and to a partially purified synaptosomal membrane preparation.

## MATERIALS AND METHODS

All reagents used were of analytical grade. [10,11-$^3$H]imipramine (specific activity 7·7 Ci/m-mole) and the corresponding hydrochloride (sp. act. 7·5 mCi/m-mole) were prepared at Roussel-Uclaf. Radioactivity measurements were made by liquid scintillation as previously described [4].

*Preparation of synaptosomes.* Rat brain synaptosomes were prepared according to the method of Whittaker [8] as previously described [4] and the preparation was checked by electron microscopy.

*Incubation with crude synaptosomes.* The crude mitochondrial pellet ($P_2$) from one brain was resuspended in 40 mM Na phosphate buffer pH 7·0 (40 ml) containing 100 mM NaCl, 4 mM KCl and 11 mM glucose. Aliquots (2 ml) were incubated with [$^3$H]imipramine as described, layered on a two-phase sucrose gradient (equal vol 1·2 and 0·8 M sucrose) and centrifuged (Beckman, model L2 75B ultracentrifuge) at 50,000 *g* for 2 hr at 4° (SW 50.1 rotor). The gradient was fractionated and radioactivity in each fraction was measured.

*Incubation of [$^3$H]imipramine with purified synaptosomes.* Purified synaptosomes from two brains were resuspended in 40 mM Na phosphate buffer (as above) (40 ml). Aliquots (2 ml) were incubated as described with [$^3$H]imipramine. After incubation the samples were immediately cooled in ice, an aliquot removed for radioactivity measurement and the remainder centrifuged (ECCO, Model E2/12 centrifuge) at 7000 *g* for 20 min. The synaptosome pellet was rinsed twice with cold phosphate buffer and digested in 1% Triton X-100 (1 ml) for radioactivity measurement.*

*Preparation of plasma membranes by lysis of synaptosomes.* The crude mitochondrial pellet ($P_2$) from two brains, after resuspension in water (2 ml/g brain tissue) to lyse the synaptosomes, was layered on a two-phase sucrose gradient and centrifuged as above. The opaque layer of membranes and synaptosome "ghosts" at the 1·2–0·8 M interface was recovered, diluted with an equal volume of distilled water and centrifuged at

* It should be noted that too extensive rinsing could result in some desorption of bound radio-activity from the surface of the pellet.

100,000 *g* for 1 hr at 4°. The membrane pellet was rinsed twice with 10 mM Na phosphate buffer pH 7·0 (containing 100 mM NaCl and 4 mM KCl) and resuspended in the same buffer for binding experiments as described. Incubation, centrifugation and radioactivity measurements were carried out as described for purified synaptosomes. An electron microscopic examination confirmed the absence of complete synaptosome structures.

*Solubilization with Triton X-100.* The rinsed membrane pellets from four brains (~ 20 mg protein) were taken up in 0·2% Triton X-100 in the same phosphate buffer (40 ml). A small insoluble residue was removed by centrifugation at 100,000 *g* for 45 min. The clear supernatant was used for equilibrium dialysis with a dialysis bag volume of 1 ml and a total volume of 15 ml. Dialysis was carried out for 16 hr at 4° and with continuous stirring.

## RESULTS

### *Binding of [$^3$H]imipramine to synaptosomes*

[$^3$H]imipramine was incubated with the crude mitochondrial pellet, under conditions where an active uptake of serotonin is observed. The suspension was layered on a two-phase sucrose gradient and centrifuged. As shown in Fig. 1 binding is clearly associated with the synaptosome fraction sedimenting at the interface. Increasing concentrations of unlabelled imipramine displace [$^3$H]imipramine from the synaptosome peak, thus showing that the binding is saturable. No displacement occurs however with increasing concentrations of serotonin. This result would suggest that the serotonin uptake site is not responsible for the observed binding. Furthermore, [$^3$H]imipramine binding is not affected by a high concentration of ouabain ($10^{-5}$ M), inhibitor of $Na^+$, $K^+$-ATPase, which blocks serotonin uptake into synaptosomes [4]. Further experiments to determine the characteristics of imipramine binding, were carried out with a purified preparation of synaptosomes, using a direct centrifugation technique.

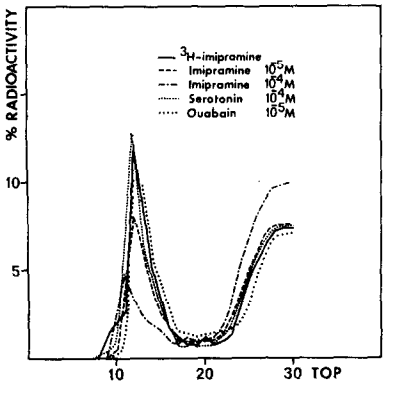


Fig. 1. Distribution of [$^3$H]imipramine activity after sucrose gradient separation of synaptosomes. Effect of various compounds. [$^3$H]imipramine ($10^{-8}$ M) was incubated for 15 min at 37° with the crude synaptosome suspension with or without unlabelled imipramine, serotonin or ouabain (pre-incubation 15 min at 37°). The suspension was layered directly on a two-phase gradient (1·2, 0·8 M sucrose).

Table 1. Percentage binding of [$^3$H]imipramine as a function of time and temperature

| Temp. (°C) | | Incubation time (min) 2 | 15 | 60 |
|---|---|---|---|---|
| 4 | (a) | 54 | 54 | — |
| | (b) | 32 | 28 | 28 |
| 37 | (a) | 52 | 50 | 47 |
| | (b) | 30 | 25 | 24 |

Binding of [$^3$H]imipramine to (a) purified synaptosomes and (b) synaptosomal membranes, was measured as described. [$^3$H]imipramine concentration was $10^{-8}$ M.

*Binding of [$^3$H]imipramine as a function of time and temperature.* [$^3$H]imipramine was incubated with purified synaptosomes for various periods of time at 4° or 37°. As shown in Table 1 (a), binding to the particulate fraction was the same at both temperatures and very rapid, reaching a plateau in less than 2 min. At 37° no significant modification was observed up to 60 min incubation.

The contrast with serotonin uptake into synaptosomes, which is both time and temperature dependent [4], and moreover is inhibited by ouabain, serves to demonstrate that the binding of imipramine to synaptosomes does not involve an active transport process. This does not exclude however a possible intraneuronal site of action of imipramine [9], which could still be accumulated within the nerve-ending by diffusion.

*Binding of [$^3$H]imipramine as a function of concentration.* The incorporation of [$^3$H]imipramine into synaptosomes at 4° has been measured as a function of imipramine concentration. Figure 2a shows the saturation curve which is similar to the one obtained by Weinstein *et al.* [10] using a crude synaptosome preparation from mouse brain.

The nature of the curve suggests that the binding may be resolved into a saturable (S) component and a non-saturable (NS) component. As previously described for serotonin uptake [4] the experimental data can be represented as an (S + NS) system on a proportion graph [11] which is shown in Fig. 2b and which on analysis gives for the S system a binding constant of $3{\cdot}5 \pm 1{\cdot}0 \times 10^{-5}$ M. The maximum specific binding capacity of the preparation is $3{\cdot}7 \pm 0{\cdot}9 \times 10^{-5}$ M.

The results presented so far show that imipramine binds specifically, but with low affinity, to purified synaptosomes. The process is rapid and takes place at 4° as well as at 37°. In order to see whether the nature of the binding is modified after removal of the soluble cytoplasmic content of the synaptosomes, similar experiments were carried out with a partially purified membrane fraction. The membrane preparation was

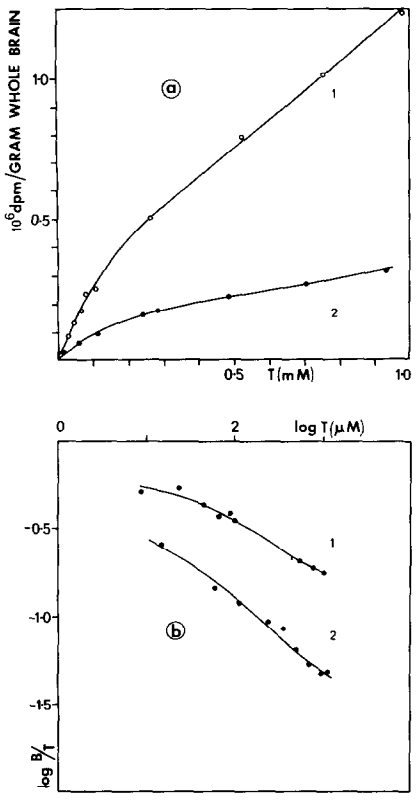


Fig. 2. (a) Binding of [$^3$H]imipramine as a function of concentration. Varying concentrations of [$^3$H]imipramine HCl were incubated for 15 min at 4° with purified synaptosomes (curve 1) or the synaptosomal membrane preparation (curve 2). Incorporation of radioactivity into the particulate fraction was measured by centrifugation at 7000 *g* for 20 min and digestion of the pellet in 1% Triton X-100. (b) Proportion graph. Log *B*/*T* (the proportion of radioactivity bound) is plotted against log *T* (the total imipramine concentration). Both curves can be fitted by an S + NS system from which an initial estimate of the binding parameters has been graphically deduced and subsequently evaluated more precisely using the computer method described in Ref. 11.

obtained by osmotic lysis of the crude mitochondrial pellet and centrifugation of the lysate on a two-phase sucrose gradient. The fraction ("ghosts" etc.) sedimenting at the interface was recovered and used throughout the following experiments. We have previously shown [4], by assay of the soluble cytoplasmic marker, lactate dehydrogenase, that this method effectively eliminates the soluble contents of the synaptosomes.

* The concentrations of the initial homogenates and the volumes of the final suspensions were the same in each case, allowing an approximate comparison.

† "Intrinsic dissociation constant" is used here because imipramine binding was measured by equilibrium dialysis. "Binding constant" is used when bound imipramine has been separated from unbound imipramine by centrifugation.

*Binding of [$^3$H]imipramine to a synaptosomal membrane preparation*

(*a*) *As a function of time and temperature.* The membrane preparation was incubated with [$^3$H]imipramine at 4° or 37° for various periods of time. A result identical to that for intact synaptosomes was obtained, that is, at both temperatures binding was very rapid and reached a maximum in less than 2 min (Table 1 b).

(*b*) *As a function of imipramine concentration.* The membrane preparation was incubated with various concentrations of [$^3$H]imipramine as for purified synaptosomes. The saturation curve was very similar to that obtained previously (Fig. 2a) and was analysed in the same way by the proportion graph method (Fig. 2b). The binding constant ($2{\cdot}5 \pm 0{\cdot}5 \times 10^{-5}$ M) for the saturable (S) system was not significantly different from that obtained using whole synaptosomes. The maximum specific binding capacity ($1{\cdot}0 \pm 0{\cdot}2 \times 10^{-5}$ M) is of the same order as that for whole synaptosomes* which means that soluble cytoplasmic contamination of the membrane preparation is unlikely to account for the observed binding.

It is to be noted that non-saturable binding, as represented by the slope of the linear portion of the curve, is appreciably less (by a factor of ~ 5) than that associated with whole synaptosomes. Possibly in the latter case there is a diffusion into the interior of the intact structure where the imipramine remains trapped.

(*c*) *Solubilization by Triton X.* Membranes were solubilized in 0·2% Triton X-100 as described and the resulting opaque suspension was ultra-centrifuged to remove undissolved material. The binding of [$^3$H]imipramine to the supernatant was measured by equilibrium dialysis. Analysis of the results by the proportion graph method (Fig. 3) showed that the experimental points are fitted by an S system only. In other words, non-saturable NS binding is virtually eliminated in the presence of Triton. The intrinsic dissociation constant† ($3{\cdot}3 \pm 0{\cdot}3 \times 10^{-4}$ M) obtained is however an order of magnitude higher than the binding constant obtained with untreated membranes. The maximum specific binding capacity ($0{\cdot}8 \pm 0{\cdot}1 \times 10^{-4}$ M) for the same membrane concentration as before, is similarly higher. Thus if the binding measured in 0·2% Triton corresponds to the binding previously observed, one must assume that the presence of Triton diminishes the affinity for imipramine while at the same time exposing binding sites hitherto inaccessible.

(*d*) *Effects of various pharmacological agents.* A number of pharmacological agents were tested as competitors of the synaptosomal membrane binding of [$^3$H]imipramine. The compounds chosen represented the series of imipramine-like antidepressants and also included several neuroleptics, two of which, chlorpromazine and fluphenazine, bear a structural resemblance to the tricyclics and a third, haloperidol, having a distinct structure.

The concentration $IC_{50}$ of a given compound required for 50 per cent inhibition has been calculated

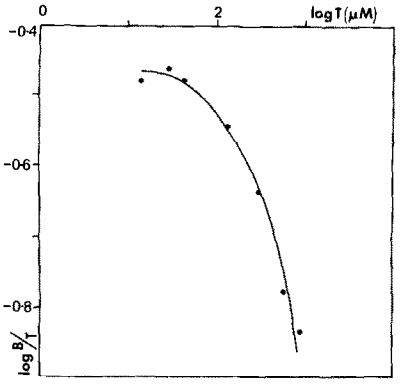


*Fig. 3.* Equilibrium dialysis of Triton X solubilized synaptosomal membrane fraction with [$^3$H]imipramine. The concentration of [$^3$H]imipramine was $10^{-9}$ M and dialysis was carried out as described. In the proportion graph, log $B/T$ ($B$ = bound imipramine, $T$ = total concentration of imipramine, within the dialysis bag) is plotted against log $T$. The curve is fitted by an S system only (see legend Fig. 2b). For comparison of the maximum specific binding capacity for Triton X solubilized membranes (from four brains) with that obtained for non-treated membranes (from two brains), it is assumed that the number of binding sites is directly proportional to the quantity of starting tissue.

in each case by measuring the binding of [$^3$H]imipramine at various concentrations of inhibitor (Table 2) and is compared with the $IC_{50}$ for unlabelled imipramine. Apart from serotonin which has no effect, all the compounds tested, effectively compete; the order of activity being CPZ > FLU > CLI > TRI > AMI $\geqslant$ DMI > IMI = HAL. It is evident that activity is totally unrelated to inhibitory activity on the uptake process for serotonin or nor-adrenalin, where the tricyclic antidepressants are generally more active than the phenothiazines. The most active compounds (CPZ, FLU, CLI) are tricyclic structures having an electronegative substituent. In general, electronegative substitution of an aromatic compound increases its lipophilicity. A direct comparison of the lipophilicity of the majority of the compounds tested can be made from the partition coefficient data of Frisk-Holmberg [12]. The order of activity in the membrane system indeed shows a certain correspondance with the apparent partition coefficients measured in octanol (FLU > CPZ > CLI > AMI = IMI > DMI) or in chloroform (FLU > CLI > CPZ > AMI > IMI > DMI). Empirical calculations by the method of Leo *et al.* [13] indicate that both trimipramine and haloperidol have approximately the same partition coefficient (octanol) as amitriptyline.

These observations would suggest that the observed imipramine binding is associated with a "lipophilic pocket" of low specificity.

* For inhibition of serotonin uptake into synaptosomes, the $IC_{50}$ ($\simeq K_i$) for imipramine is $\sim 2 \times 10^{-7}$ M [4].

Table 2. Effect of various compounds on [$^3$H]imipramine binding

| Compound | $IC_{50}$ ($10^{-5}$ M) |
|---|---|
| Imipramine (IMI) | 12·0 |
| Chlorimipramine (CLI) | 5·8 |
| Desmethylimipramine (DMI) | 8·5 |
| Trimipramine (TRI) | 7·8 |
| Amitriptyline (AMI) | 8·4 |
| Chlorpromazine (CPZ) | 2·6 |
| Fluphenazine (FLU) | 3·6 |
| Haloperidol (HAL) | 11·5 |
| Serotonin | |

Binding of [$^3$H]imipramine to the membrane preparation was measured as described. Incubation was at 4° for 30 min with [$^3$H]imipramine concentration $10^{-8}$ M. Compounds were incubated simultaneously with [$^3$H]imipramine, at concentrations of $10^{-4}$, $3 \times 10^{-5}$, $10^{-5}$ and $3 \times 10^{-6}$ M. $IC_{50}$ values are an average from two separate experiments and were calculated from logarithmic probability plots of percentage inhibition.

## DISCUSSION

We have shown that imipramine binds to synaptosomes by a saturable process and that binding to a synaptosomal "ghost" fraction, depleted of cytoplasmic contents, has essentially the same characteristics. The binding does not appear to be associated with the uptake site for serotonin and imipramine is not accumulated by an active transport process. Affinity as well as specificity for imipramine is low as indicated by the binding constant and the number of different compounds that can effectively compete. The concentrations involved fall in the range where numerous lipophilic, biologically active molecules have an inhibitory effect on membrane-bound enzymes such as acetylcholine-esterase and ATP-ase [5, 14], and also affect membrane stability [15]. The molecular nature of these interactions is not known. That imipramine binds by a saturable process at these concentrations suggests that the overall effect exerted on the membrane by this type of lipophilic molecule is not due to a random solubility phenomenon, but involves a low affinity interaction with a discrete and probably structural, as distinct from functional, entity.

The low affinity interaction of various steroids with serum albumin (for review see Westphal [16]) would seem to be a closely analogous situation.

Several recent publications concerning specific opiate receptors [17, 18] have highlighted the difficulties of demonstrating high-affinity binding of psychoactive compounds whose lipophilicity leads to extensive low-affinity interaction. If imipramine inhibition of the uptake process for serotonin or noradrenalin involves binding of moderate affinity ($K_i \sim 10^{-7}$ M)* to the synaptosomal membrane, then this binding has been masked by the low-affinity interaction observed.

Subcellular distribution studies with [$^3$H]imipramine have presented similar problems. At each stage of separation the low-affinity complex dissociates and the compound is redistributed throughout membrane rich fractions, thereby masking any specific localization which may have occurred.

It is interesting to note that after solubilization of the membrane fraction in Triton X-100 a saturable binding still exists, albeit of lower affinity, while non-saturable binding is virtually eliminated. The number of binding sites observed is also much greater than in the untreated membrane. Presumably, buried hydrophobic sites are exposed by detergent treatment which also modifies the structure of the lipophilic site of interaction of imipramine, thereby reducing its affinity.

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