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Molecular aspects of the imipramine 'receptor'

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1. *Introduction*

The imipramine binding site has been characterized for only 4–5 years[99], and yet in that short time, it has emerged as a potentially powerful tool for investigation of depression. Because its possible usefulness was recognized from the start imipramine was used in clinical studies almost immediately[7,14]. As traditional paths of investigation were by-passed in the case of imipramine, a real gap in basic knowledge about the imipramine binding site exists. During the last year, studies in this and other laboratories have attempted to fill part of this gap with the intention of providing a better rationale for the use of this drug in the clinical situation.

The aim of this review, therefore, is to draw together the existing pharmacological and biochemical data in order to summarize the current status of the imipramine binding site. While the clinical results have drawn considerable attention in the past, they will be discussed here only in so far as they contribute to the picture as a whole. In addition, hypotheses of depression are many and since they have been adequately reviewed (see Lingjaerde[75] for references), will not be further explored in this text. Rather, the emphasis here is to analyze one potential mechanism of action of a single antidepressant drug. Accordingly, the interaction of the imipramine binding site with the 5-hydroxytryptamine (5-HT) transport mechanism will be discussed rather than how imipramine may modify CNS activity by its action at this site. I believe that this approach, in the long-term, will be more fruitful than the 'blanket' approach since the total mode of action of the antidepressant drugs will most probably involve an integration of many actions – all of which have not yet been adequately defined.

2. *Can the imipramine binding site be called a receptor?*

2.1 *Criteria for a receptor*

There are at least 4 fundamental criteria by which receptors are defined:
1. High affinity and saturability
2. Stereospecificity
3. Regional localization
4. Pharmacological selectivity.

These have all essentially been satisfied for the imipramine binding site. In most cases, [^{3}H] imipramine has been shown to bind to a single, saturable population of sites in the 300–1000 fmol/mg density range with an affinity of 0.5–9 nM (see section 4.1). In our hands, slight curvilinearity is present when using data from rat cortex suggesting the existence of a 2nd, lower-affinity binding site (see also Grabowsky et al.[52]). Such a site has not been investigated in detail and is beyond the scope of this review.

The 2nd criterion, that of stereospecificity, is interesting from a theoretical standpoint. The imipramine binding site may label the recognition site for the 5-HT carrier, and uptake mechanisms are generally less stereospecific than receptors. This question was addressed by Langer et al.[65]. Using 4 pairs of stereoenantiomers of antidepressant drugs, varying degrees of selectivity were obtained. A 70-fold difference in potency was observed for zimelidine enantiomers (Z and E), sufficient to satisfy this criterion for a receptor. It would be beneficial to assess the potencies of these enantiomers on 5-HT uptake.

In keeping with a specific 'receptor', [^{3}H] imipramine binding should be regionally localised. This has been demonstrated in both rat[88] and human brain[68], binding being highest in the hypothalamus, cortex and hippocampus. This distribution was highly correlated with the endogenous levels of 5-HT. More specifically, the sites are located on the terminals of 5-HT neurons as has been shown by lesion studies[17,20,42,90,115]. Binding was decreased in direct relationship to the loss of 5-HT uptake and 5-HT levels.

The imipramine binding site shows pharmacological selectivity towards agents that inhibit 5-HT uptake. This includes many antidepressant drugs but not monoamine oxidase inhibitors or the 'atypical' antidepressants (for example, mianserin, iprindole)[50,66,67,100]. It remains to be determined if all potent 5-HT uptake inhibitors exhibit antidepressant action. It is therefore premature to call this binding site an 'antidepressant receptor'. The potencies of those antidepressants that are active on this site do appear to correlate with the mean daily clinical doses[14], but caution is needed when so many factors (for example, plasma protein binding, lipophilicity and metabolism) intervene between oral administration and the binding to these sites.

These data suggest a relationship between high-affinity [^{3}H] imipramine binding and the 5-HT uptake system. Since imipramine itself inhibits 5-HT uptake[23,73,131], it presumably binds and prevents transport. Such a model was supported by the findings of Talvenheimo et al.[129]. This group reported [^{3}H] imipramine binding but found that imipramine was not itself carried across the membrane. The requirement that Na+ was required for maximal binding to occur[16] would tend to support this hypothesis. Bogdanski et al.[12] found that 5-HT uptake was dependent on Na+ and Sneddon[118,119] found that this dependency was reflected in the carrier affinity for serotonin (K_m) rather than in the maximal transport capacity (V_{max}). He therefore postulated that Na+ was required for the binding of 5-HT to the recognition site. A parallel situation can be seen in the sodium-dependent component of [^{3}H] GABA binding which is related to GABA uptake sites[44]. However, the affinity and capacity of this GABA binding are more akin to that of a transport system whereas that for [^{3}H] imipramine is not. In addition, if [^{3}H] imipramine is labelling the 5-HT uptake recognition site, one would expect 5-HT to competitively displace this binding. It is now becoming obvious that despite the earlier findings of Talvenheimo et al.[129], this is not the situation. Wennogle and Meyerson[144] found that while 5-HT was displacing [^{3}H] imipramine binding, it was at the same time decreasing the dissociation rate of the ligand. Briley et al.[18,116] and Abbot et al.[3] reported an allosteric, non-competitive interaction between 5-HT and imipramine. These studies revealed that there was a relationship between the Na+ dependency and the nature of the competition for [^{3}H] imipramine binding. Removal of Na+ led to a 4-fold drop in affinity but no change in the number of binding sites. The ability of tricyclic antidepressant drugs to inhibit the binding was effectively unaltered by this Na+ removal. In contrast, the 5-HT uptake blockers fluoxetine, citalopram and paroxetine suffered a 40–100-fold loss in potency. These blockers and 5-HT itself, both in the presence and absence of Na+ ions, inhibited binding non-competitively with Hill coefficients less than 1.0. Some confusion exists with regards to the effects of tricyclic antidepressant drugs on the uptake of 5-HT. Most reports favor a competitive inhibition of uptake[73,74,76,111,120,129,135] while a few report non-competitive kinetics[69,146,147]. Lingjaerde[74] concluded that the methodology employed[69,146] may have led to false conclusions. He did, however, find that cloimipramine had a non-competitive component to its inhibitory ac-

tion on 5-HT uptake. The true relationship between the imipramine binding site and 5-HT uptake remains unclear.

In summary, the imipramine binding site satisfies the criteria of saturability, regional localization (as well as cellular and subcellular localization), stereospecificity and pharmacological selectivity. These analyses have previously been pursued by Langer and Briley[64]; however, these authors refrained from calling this site a receptor since, at that time, all evidence pointed to a mutual identity with the 5-HT transporter recognition site. The increasing accumulation of data suggesting discrepencies between the recognition site and the binding site does, however, change the situation. It is now conceivable that the binding site can be considered as a presynaptic receptor that modulates 5-HT transport, a concept which is favored by the group of Costa[8]. While this concept may be considered premature, I think the receptor terminology will aid the design of future experiments. The uptake recognition site may best be thought of as a 'secondary messenger' to the imipramine receptor. The most important question that the idea of an imipramine receptor raises is one of natural ligands. If the receptor is distinct from 5-HT uptake, is 5-HT still the endogenous ligand?

2.2 *Imipramine: agonist or antagonist?*

This question must be definitively answered to gain acceptance of the imipramine binding site as a receptor. As yet, only circumstantial evidence indicates that imipramine is an agonist at this site. The main evidence is that chronic administration (and withdrawal) of imipramine to either rats[8,61,95] or cats[19] decreased cortical [^{3}H] imipramine binding. Chronic desipramine produced similar results[8,100]. Receptor down-regulation is always associated with chronic agonist administration; antagonists produce supersensitivity.

Support for the receptor concept would be increased if the imipramine binding site could be demonstrated to be physiologically relevant. Using the model of a presynaptic receptor modulating 5-HT uptake one would predict that down-regulation of these receptors would lead to enhancement (dis-inhibition) of uptake.

A recent report by Barbaccia et al.[8] supports this concept. 5-HT uptake was measured in hippocampal slices of rats which had previously been chronically treated with imipramine. As discussed above, after chronic treatment, the numbers of imipramine binding sites are decreased. If physiologically relevant in controlling the uptake of 5-HT, one would expect an increased 5-HT uptake under these circumstances, and this is exactly what was observed. The change in uptake was due to an increased V_{max}. Of course, the conclusion is that there is an endogenous ligand for this receptor exerting control over 5-HT uptake.

Conformational changes can occur in imipramine receptors. These were revealed by the temperature-sensitivity of the binding (which will be discussed later in more detail). In other systems, agonists and antagonists displayed differential effects on the temperature sensitivity of receptors – an example being the β-adrenoceptor system[139-141]. The rationale is that agonists bind to their receptors and induce conformational changes within the receptor leading to activation of a secondary messenger (for example, activation of an adenylate cyclase, opening of an ion channel, control of phospholipid methylation). In contrast, antagonists supposedly bind without inducing any such changes. The degree of enthalpy and entropy change associated with ligand binding may therefore be different with agonists and antagonists as in the case of the β-adrenoceptor. Segregation into enthalpy and entropy changes for [^{3}H] imipramine binding has not yet been rigorously performed. It is possible that events not associated with signal transduction may account for the temperature sensitivity of the binding. Entropy-driven reactions may be associated with a ligand's ability to displace ordered water molecules from around the ligand itself and the binding site. Alternatively, changes in viscosities of lipids as a result of increasing temperature may alter receptor affinity directly, or indirectly, by altering the partition coefficient of the ligand. However, since imipramine and its derivatives are the only ligands available for labelling the site, it is impossible to say whether or not the ligand per se is agonistic and therefore inducing the conformational change. Only if a ligand is found which labels this site yet does not show this conformational change can we conclude that imipramine is an agonist.

In summary, there are 3 lines of evidence suggesting that imipramine is acting as an agonist: 1. down-regulation of its own receptors, 2. converse relationships between binding and 5-HT uptake and 3. receptor conformation changes. However, all this evidence is indirect. The isolation of a natural ligand, and the synthesis of antagonists would be conclusive evidence. At this point it is worth mentioning the possibility of multiple binding sites related to the 5-HT transporter/imipramine receptor supramolecular complex. Recently it has been postulated that so-called calcium channel blockers can be classified according to their ability to bind up to at least three different binding sites located in and around the calcium ion channels[45,49,87]. The nicotinic cholinergic receptor sodium ion channel also contains different binding sites for agonists, antagonists, local anesthetics and neurotoxins (see, for example, Kistler et al.[63]). Experiments on conformational changes indicate that there may be heterogeneity of the imipramine binding site population in the rat cortex. In addition [^{3}H] Ro 11-2465[24], a ligand exhibiting the same pharmacological binding profile as [^{3}H] imipramine[21], binds to only a subpopulation of these sites[43]. At least 2 populations have been defined on the basis of differential temperature and sulphur bond-modifying reagent sensitivity[29,36,38,43]. Furthermore, as mentioned in the last section, Lingjaerde[74] reported that imipramine inhibited 5-HT uptake by purely competitive means while clomipramine had an additional, noncompetitive, inhibitory component. It is tempting to suggest 2 sites of action for clomipramine on this basis and direct binding studies using the tritiated ligand may be useful. Lastly, evidence of multiple sites also have arisen from direct binding studies performed with [^{3}H] cocaine[107,108]. The pharmacological profile was similar to that for 5-HT uptake and the sites were found to be localised on sero-

tonergic nerve terminals and on human platelets. Despite these similarities, the [^{3}H] cocaine binding sites are probably not identical to those for [^{3}H] imipramine because a) [^{3}H] cocaine binding is not Na+-dependent and b) imipramine displaced [^{3}H] cocaine binding at concentrations higher than those required to displace [^{3}H] imipramine binding itself.

Yet another group[54] has investigated the binding of [^{3}H] norzimelidine, a potent 5-HT uptake inhibitor. These sites are probably not related to those labelled by [^{3}H] imipramine since 5-HT is a much more potent inhibitor of [^{3}H] norzimelidine binding (IC_{50} 43 nM) than it is of [^{3}H] imipramine binding (IC_{50} 1 μM).

In conclusion, the possibility of multiple sites of action must be borne in mind when investigating the interactions between the imipramine binding site and the 5-HT transporter.

3. *Clinical relevance of the imipramine 'receptor' and the relationship to 5-HT uptake*

3.1 *[^{3}H] Imipramine binding and depression*

Since the initial description on the assay for human platelet [^{3}H] imipramine binding sites[14], there have been a number of reports studying this 'biological marker' in various psychiatric illnesses. The hypothesis is that the imipramine binding site may be a possible site of action for imipramine-related antidepressants, and that abnormalities in its density may be a reflection of a genetic predisposition to depression. Platelet binding is postulated to mimic CNS binding, and being a non-invasive technique, it can readily be applied to the clinical situation.

To date, only one paper has presented evidence that platelet and neuronal imipramine binding sites are regulated in the same manner. Briley et al.[19] reported that after chronic treatment of cats with imipramine, platelet binding was decreased 54% while that of the hypothalamus was decreased 68%. More data is required to see if these values are qualitatively identical and whether other brain regions (cortex, striatum) show similar decreases. In a rat strain which shows a genetic defect in platelet 5-hydroxytryptamine transport[28], [^{3}H] imipramine binding was decreased in both the platelets and cortex[39]. These results, however, have been disputed[6,86].

Before further discussing platelet imipramine binding as used clinically, it would be useful to review the evidence for the concept of the platelet as a model for the serotonergic neuron. The qualitative similarities between platelets and 5-HT neurons have been amply documented[119,122]. Less frequently discussed is the *mechanism* by which this similarity may have come about. There is some evidence that platelets and 5-HT neurons share a common embryological origin.

Platelets have been shown to contain an enolase enzyme[79] which is found only in neurons and in cells of the APUD (amine precursor, uptake, and decarboxylation) system as defined by Pearse[92-94]. This classification includes cells of the adrenal medulla and peptide-containing cells in the pancreas and gut. The diffuse neuroendocrine system (DNE) includes the APUD and makes up one of the 3 sections of the nervous system (along with the somatic and autonomic systems)[22]. Here then is a possible explanation for any correlation between platelet and CNS imipramine binding.

Numerous reports now indicate that platelet imipramine binding sites are decreased in depressives[7,15,91,101,127,128] (but note Berrettini et al.[10] and Mellerup et al.[83]). Parallel decreases in 5-HT uptake have also been observed[25,136,137] but it is becoming increasingly clear that the two systems are controlled differently. This is seen in the lack of correlation found between either the affinity constant or B_{max}/V_{max} values for the binding and transport mechanisms of individual subjects[101,148]. In cirrhotic patients, uptake of 5-HT was reduced but [^{3}H] imipramine binding was not[4]. Twin studies (Paul et al., unpublished observations) implied a simple inheritance pattern for [^{3}H] imipramine binding with a marked concordance between monozygotic twins, but the picture was more complicated for 5-HT uptake. Finally, development studies have identified different control mechanisms over these 2 systems[84].

3.2 *State or trait marker of depression?*

The discordance between [^{3}H] imipramine binding and 5-HT uptake is easy to understand in view of the many steps which are involved in transport, each one probably under separate genetic control. Thus binding is a simpler system to study to answer the question of whether a biochemical abnormality exists in depressives which may reflect a genetic predisposition to depression (i.e., a trait marker) or is present only when people are depressed (i.e., a state marker). Longitudinal studies, possibly involving relatives of depressives, will ultimately be needed. Here it should be stated that there is considerable, but still controversial evidence for a genetic component to depression (see, for example, Propert et al.[98] and Weitkamp et al.[142]). At the present time, the 'trait or state dispute' has been studied mainly by following the pattern of [^{3}H] imipramine binding upon remission of depressives. In the discussion of Paul et al.[91], it was mentioned that the number of binding sites remained decreased even after clinical recovery (favoring a trait marker) but no further details were given. Suranyi-Cadotte et al.[127] gave preliminary findings of a return to normal binding site numbers in both unmedicated and medicated patients during remission for 3 weeks. Gay et al.[48] gave details of a more complete study. In order to overcome the problem of circulating tricyclic antidepressants interfering with binding, this group used unmedicated patients who showed recovery subsequent to either electroconvulsive therapy (ECT) or treatment with maprotiline, a selective noradrenaline uptake inhibitor. Despite the clinical improvement observed in both treatment groups, [^{3}H] imipramine binding remained low, this favoring a trait-marker model.

3.3 *[^{3}H] Imipramine binding in other psychiatric disorders*

It is necessary to mention that depression is not the only psychiatric illness in which altered 5-HT transport has been detected. It may be worthwile to explore [^{3}H]

imipramine binding in these other mental conditions. Uptake of 5-HT has been extensively studied in blood platelets of schizophrenics[5,85,109,110,123,124,149] but the results are not conclusive. 5-HT transport has been shown to be decreased in Huntington's chorea[82] and Down's syndrome[9,13,77,81,134]. In migraine, a decreased V_{max} for 5-HT has been detected (postulated to be due to a circulating blood factor)[26]. Assessment of [^{3}H] imipramine binding may also be interesting in the blood platelets of hypertensives, in which decreased 5-HT transport has been observed both clinically[11] and in an animal model[97].

4. *Biochemical approaches to the investigation of the [^{3}H] imipramine binding site*

4.1 *Ligands available as probes*

At this moment, only 3 ligands are available to assay the imipramine binding site. These are [^{3}H] imipramine itself (20–75 Ci/mmol), [^{3}H] 2-nitroimipramine (65 Ci/mmol) and [^{3}H] Ro 11-2465 (17 Ci/mmol). The structures of these compounds are shown in figure 1.

a) *[^{3}H] Imipramine.* The affinity of this ligand for the imipramine binding site has been reported to vary from 0.5 to 9 nM. In general, higher values have been reported in rat cortical preparations than in human platelets, the 2 most widely used tissue preparations. At least one variable is the amount of protein/assay – the K_D increasing as the protein is increased. The minimal value obtained in our laboratories was approximately 0.5 nM. Such a relationship between K_D and protein has been elegantly demonstrated for [^{3}H] spiperone binding to dopamine receptors[114]. However, it became apparent in our studies that varying protein could not explain all the discrepancies.

In the cortex, high K_D values are always reported and, in addition, Scatchard analyses frequently reveal curvilinearity in the plot[52].

Kinetically, [^{3}H] imipramine acts as a competitive, reversible ligand. Most compounds inhibit platelet imipramine binding with Hill coefficients of unity and the affinity constant derived from measurements of rates of association and dissociation (2.1 nM)[30] correspond to those obtained by saturation analyses (2.15 nM)[37].

b) *[^{3}H] 2-Nitroimipramine.* 2-Nitroimipramine was initially reported to be an irreversible blocker of 5-HT uptake and [^{3}H] imipramine binding[103]. However, the use of the tritiated compound revealed that this was not so[104,106] and that this ligand was essentially identical to [^{3}H] imipramine in terms of its binding affinity and capacity, pharmacological profile and sodium ion dependency. The apparent irreversibility was due to its slow dissociation rate.

More detailed studies in these laboratories have revealed that the situation is not quite that simple[30]. I confirmed the slow dissociation rate but was puzzled by the similar affinities displayed by [^{3}H] imipramine and [^{3}H] 2-nitroimipramine. Surely the affinity for the latter ligand should be higher? Saturation analyses revealed a K_D of 3.6 nM for [^{3}H] 2-nitroimipramine[30] compared to a value of 2–4 nM for [^{3}H] imipramine (at comparable protein concentrations[36,37]). These values were similar to those of Rehavi et al.[106]. The association kinetics were effectively identical for both ligands; however, the dissociation rate was found to be dependent on the length of incubation. After 20-min incubation, addition of excess desipramine displaced [^{3}H] 2-nitroimipramine with a half-life ($t_{1/2}$) of 8.3 h. After a 2-h association, the $t_{1/2}$ was increased to 18.3 h and after overnight incubation, the value was 21.9 h (see fig. 2). Parallel experiments with [^{3}H] imipramine revealed that the $t_{1/2}$ remained effectively constant with association times ranging from 0.5–1.5 h. The K_D as derived from the kinetic data was 2.1 nM for [^{3}H] imipramine but for [^{3}H] 2-nitroimipramine, the kinetically-derived affinity constant *decreased* as the incubation time *increased.* These data are summarized in table 1. This ligand is clearly not just a 'slowly-dissociating' ligand. It is apparent that it is not behaving in a competitive, reversible fashion except, perhaps, at very short incubation times. These peculiar binding characteristics can be interpreted in the light of other studies in these laboratories concerning another imipramine binding site ligand, [^{3}H] Ro 11-2465, and may involve the existence of possible conformational changes in this site.

c) *[^{3}H] Ro 11-2465 (5-[3-dimethylamino-propyl]-10,11-dihydro-5H-dibenz[b,f]azepine-3-carbonitrile).* This compound is a structural analogue of chlorimipramine, the chlorine being substituted by a cyano group (see fig. 1). The first reports of its use[21,53,70] revealed it to be the most potent compound yet tested for inhibiting 5-HT uptake. The sites labelled by [^{3}H] Ro 11-2465 are located on pre-synaptic serotonergic nerve terminals

Table 1. Kinetics of [^{3}H]imipramine and [^{3}H]2-nitroimipramine binding to platelets

Ligand	Association time (h)	Dissociation $t_{1/2}$ (h)	K_d (nM) by Kinetic analysis	Saturation analysis
[^{3}H]Imipramine	2*	0.5–1.5	2.1	2.15
	16–20	0.5–1.5	2.1	
[^{3}H]2-Nitroimipramine	0.3	8.3	0.14	3.6
	2	18.3	0.065	
	16–20	21.9	0.054	

* Data taken from Davis[30] and Davis et al.[37].

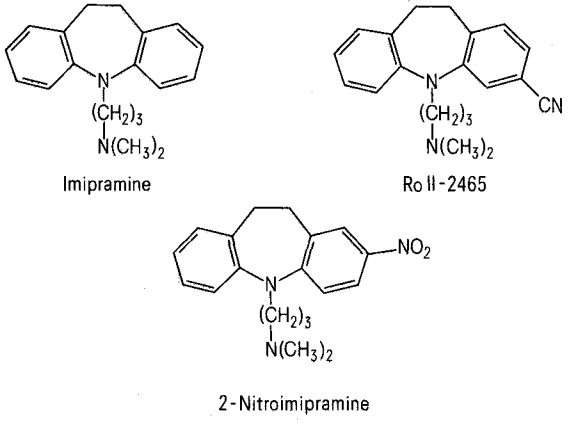

Figure 1. Ligands used to label the imipramine binding site.

and the binding is Na+ dependent[40,41], thus favoring a mutual identity with the [^{3}H] imipramine binding site. This radioligand is now sold by New England Nuclear as [^{3}H] 3-cyanoimipramine (NET-818). Since all the data discussed in this review utilised the original radioactive batch, it was considered wise to maintain the Ro 11-2465 terminology.

In outdated human platelets, the maximal binding capacity was 323 fmol/mg protein as compared to a value of 360 fmol/mg for [^{3}H] imipramine[41]. In rat cerebral cortex homogenates however, [^{3}H] Ro 11-2465 bound to only approximately 50% of the number of [^{3}H] imipramine sites. This observation led these authors to suggest that [^{3}H] Ro 11-2465 binds to only a subpopulation of cerebral imipramine binding sites. Data on the temperature dependency of both [^{3}H] Ro 11-2465 and [^{3}H] imipramine binding supported this hypothesis (see section 4.6).

4.2 *Solubilization studies*

Up to the present day solubilization of the imipramine binding site has been successful with three different detergents. The first report by Talvenheimo and Rudnick[130] screened 21 detergents and found digitonin to be the most effective. Solubilized platelet [^{3}H] imipramine binding sites were assessed by flow dialysis and were found to be saturable, sodium ion-dependent and displaceable by 5-HT. Unfortunately, high concentrations of ligand were used (100 nM) and the K_D obtained (20 nM) is not consistent with the values others have reported in platelet membranes. In addition, the B_{max} was very high (9.5 pmol/mg protein).

Under more appropriate conditions, however, digitonin was still found to be of use[36]. In this report digitonin solubilization resulted in an affinity constant change from 4.6 nM in the platelet membranes to 7.65 nM in the solubilized preparations. The recovery was high and the pharmacological profile remained unchanged with a change in Ki value of four-fold (for desipramine). Kinnier et al.[62] failed in their use of digitonin, probably due to the use of too low a concentration of detergent (0.1%).

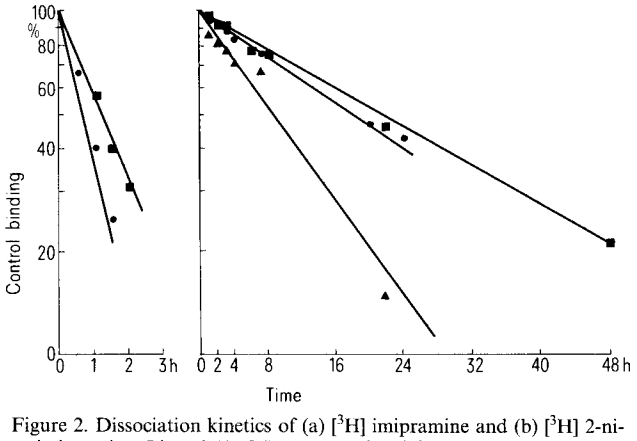

Figure 2. Dissociation kinetics of (a) [^{3}H] imipramine and (b) [^{3}H] 2-nitroimipramine. Ligand (4 nM) was associated for 20 min (Δ), 2 h (●) or overnight (16–20 h) before addition of 100 μM desipramine in a small volume (25 μl to assay volume of 300 μl). Binding was assessed at increasing times thereafter. Data taken from Davis[30].

In 1980 Hjelmeland[57] described the synthesis and properties of a detergent designed to be superior to existing ones. This detergent was specifically designed to be capable of solubilising proteins functionally intact with the added benefit of easy removability for subsequent reconstitution studies. CHAPS (3-[3-cholamidopropyl]dimethylammonium-1-propanesulphonate) has been used successfully for solubilization of, for example, opiate[117] and dopamine[71] receptors. Rehavi et al.[105] described its use for solubilization of imipramine binding sites (using [^{3}H] 2-nitroimipramine) and concluded that it was better than digitonin. Curiously, we have found the reverse situation to be true[36]; in terms of solubilized binding site density, digitonin yielded 537 fmol/mg as compared to the 175 fmol/mg reported by Rehavi et al.[105].

It is probable that the difference between the two groups can be explained by the differing assay methods employed, since in both cases the same tissue source (human platelets) was used. Davis et al.[36] used a modification of the polyethylene glycol precipitation assay[27] while Rehavi et al.[105] used a charcoal adsorption method.

The ultimate aim of solubilization studies is to eventually purify and reconstitute the molecule under investigation; in the present discussion with the primary intention of studying the interaction of the [^{3}H] imipramine binding site with the 5-HT transporter molecule. Unfortunately, the detergents that tend to be best for obtaining solubilized proteins that still show intact pharmacological profiles are not always the best for subsequent removal for reconstitution studies. For example, digitonin exists in a large micelle (aggregation number 60, micellar molecular weight 70,000 daltons)[56] and has a low critical micellar concentration. Like other non-ionic detergents, however, it is difficult to remove by such methods as gel permeation chromatography or dialysis (see review by Furth[47]). It is nevertheless, the detergent of choice for solubilization of numerous receptors (for example, β-adrenoceptors[24,125]; dopamine receptors[34,35,51,78]; and opiate receptors[113]).

The ionic bile salt detergents (cholate, deoxycholate) are much more frequently used for reconstitution studies (of, for example, enzymes involved in oxidative phosphorylation[98a], and nicotinic cholinergic receptors[96] due to their ease of removal[47,58]. However, they tend to be more denaturing than, say, digitonin.

Sodium cholate has now been used to successfully solubilise and reconstitute the imipramine binding site. This will be dealt with in depth in section 4.4.

4.3 *Characteristics of solubilized imipramine binding sites*

Only very preliminary results are available concerning the molecular properties of the solubilized imipramine binding sites. The three groups which have reported successful solubilization all carried out gel permeation chromatographic studies, but these are difficult to compare.

Eluting in 0.06% digitonin, Talvenheimo and Rudnick[130] found a single peak of binding which eluted be-

tween thyroglobulin (Stokes' radius 8.5 nm, 669,000 daltons) and ferritin (Stokes' radius 6.1 nm, 440,000 daltons). While they draw the only possible conclusion, that this value is compatible with those found for other soluble proteins, molecular mass cannot be deduced with any degree of certainty due to the probable large contribution of digitonin. Davis[32] and Davis, Morris and Tang (submitted), using [^{3}H] Ro 11-2465 to label the imipramine binding sites, and 0.1% digitonin to solubilize, also found a similar value (6.3 nm). Interestingly, Rehavi et al.[105] found that imipramine binding sites, as labelled by [^{3}H] 2-nitroimipramine, eluted (in 0.05% CHAPS) prior to thyroglobulin. This occurred despite the fact that CHAPS probably has a lower micellar molecular weight than digitonin[31]. When solubilized in sodium cholate a much smaller complex was obtained. Eluted in 0.1% of this detergent, the Stokes' radius was 4.1 nm. Clearly, no definitive statement can yet be made about the size of the imipramine binding site and, as is common with such studies, no appreciable degree of purification was attained in any of these reports.

The only other purification approach used up to now has been isoelectric focusing using [^{3}H] Ro 11-2465 labelled sites (Davis, Morris and Tang, submitted). However, due to the presence of Ampholines (which form the pH gradient) protein is difficult to accurately determine and therefore purification was not assessed. An isoelectric point was found at pH 5.3 indicating that the binding site is negatively charged at physiological pH values; this is in keeping with similar results found for other integral membrane proteins[72].

A more direct investigation of the imipramine 'receptor' has been carried out by Wennogle et al.[145]. This group have photoaffinity labelled the sites using [^{3}H] 2-nitroimipramine. On SDS-PAGE, an apparent molecular weight of 30,000 daltons was obtained. An approach such as this will yield very interesting results as would the application of irradiation inactivation studies to compare the platelet and cortical binding sites.

4.4 *Composition of the imipramine binding site*

Very little is known about the protein, lipid and carbohydrate composition of the imipramine binding site. Some work has been reported on the 5-HT uptake system; however, with such a complex system involving transmembrane transport linked to ion gradients, substances such as proteases and phosphilipases would be expected to have very complex consequences.

The [^{3}H] imipramine binding sites are heat labile (see, for example, Kinnier et al.[62]); however, it appears that there is both a reversible and an irreversible component of heat-induced binding loss. Wennogle et al.[143] showed that with as little as a 1-min exposure of platelet membranes to 65 °C, a 50% loss of subsequent [^{3}H] imipramine binding was observed (as compared to incubations at 4 °C). This loss was time dependent and, at 37 °C, stimulated by calcium ions. This was shown by the fact that the calcium ionophore A23187 enhanced this binding loss while EDTA inhibited the Ca^{++} effect. After a 1-h incubation at 37 °C, the maximal number of hippocampal binding sites was decreased from 660 to 73 fmol/mg protein[62]. Leupeptin, an inhibitor of a calcium ion-dependent protease[132] prevented this (supposed) degradation of binding sites as did EGTA. A 3rd group[43], has confirmed some of these results, but disagrees in other respects. It was found that there are 2 components to this binding site loss. The Ca^{++}-dependent, EDTA-prevented loss of binding was seen only after prolonged 20-h incubations at either 23 °C or 37 °C. The loss in binding observed up to 3 h of preincubation was completely reversed by simply cooling to 4 °C. The reversible and irreversible binding losses were observed in rat hippocampal and cortical tissue as well as platelets, eliminating the possibility of regional differences in proteolytic activity. In addition, Dumbrille-Ross et al.[43] controlled for the possibility of changes in binding caused by alteration in pH which occurs in the buffer between 4 °C and 37 °C. pH-induced changes in binding are especially important when the effects of Ca^{++} are investigated, since Ca^{++} appeared to radically alter the pH of the Tris-ions buffer used. The reversible loss in binding seen between 4 °C and 37 °C has been extensively studied (see next section).

Apart from the proteinaceous nature of the binding site for [^{3}H] imipramine, Kinnier et al.[62] demonstrated that certain lipids are crucial since phospholipase A_2 completely eliminated subsequent [^{3}H] imipramine binding. In the hands of Wennogle et al.[143] dithiothreitol (which specifically reduces disulphide bonds) had no effect on platelet membrane binding when preincubated for 30 min at 25 °C. However, Davis[29] found that dithioerythreitol, the stereoisomer of dithiothreitol, caused a significant 50% increase in binding when platelet membranes were preincubated and subsequently assayed at 23 °C. The EC_{50} value was 1.6 mM. As stated above, there is a reversible change that occurs when binding is assayed at different temperatures. Indeed, when the experiment was repeated at 4 °C throughout, only a maximal 13% increase was observed with an EC_{50} of 3 mM. Clearly, the disulphide bonds are more accessible at the higher temperature. β-Mercaptoethanol showed the same trend but only caused a significant increase in specific binding at 10 mM.

An equilibrium between free sulphydryl groups and disulphide bonds was suggested by the finding that N-ethylmaleimide (NEM), which alkyates sulphydyrl groups, caused a *decrease* in [^{3}H] imipramine binding[29]. NEM degraded the binding by a pseudo first-order reaction rate and did not do so by interacting with the ligand itself. Saturation experiments carried out in the presence of 0.5 mM NEM caused a shift in the K_D value (1.6 to 2.23 nM) implying that the sulphur-containing bonds are located directly in, or very close to, the [^{3}H] imipramine binding site. In platelets, NEM could cause loss of all the binding sites if left up to 20 h. Separate studies involving DTE also suggested the importance of disulphide bonds at the binding site. Simultaneous presence of 500 nM fluoxetine (which is sufficient to occupy all the imipramine binding sites) prevented the DTE activation of the binding[29]. In addition, the accessibility of the disulphide bonds are affected by the temperature sensitive conformational change (see section 4.6).

4.5 *Reconstitution of the imipramine binding site*

Reconstitution studies provide a means of investigating molecular interactions between different mechanisms; thus, reconstitution will probably be the only definitive method by which the molecular relationship between the imipramine binding site and the 5-HT carrier protein can be defined. The contribution of the membrane environment to the functioning of the binding and uptake mechanisms could also be assessed. For example, in studies concerning reconstitution of adenylate cyclase, specific phospholipids were required for optimal recovery of enzyme activity[55]. The methods commonly used for reconstitution studies differ from each other mainly in respect to source of lipids into which the reconstitution is to occur and in the varying ways by which the solubilizing detergent is removed (see reviews by Razin[102], Furth[47] and Hokin[58]).

In many cases, a 'soup' of lipids is used to provide the membrane 'framework' for reconstitution, Azolectin (Associated Concentrates Inc., Woodside, New York), a lipid extract of soyabean, being of popular use. Azolectin has been the lipid source for the two reports to date of reconstitution of the imipramine binding site. Removal of unbound detergent may leave the protein contained within a detergent micelle, as a phosphilipid vesicle or as a completely detergent free protein. The form which results will depend on the hydrophobicity of the protein and the nature of the detergent. As discussed above, additional phospholipid is generally added to the initial detergent protein complex so that when detergent is removed, it will be replaced by phospholipid. As detergent is removed a concentration is reached below which micelles can no longer be maintained and they change conformation to form monomers (the critical micellar concentration (CMC)). For non-ionic detergents this can be very low making their removal difficult. This is why sodium cholate, which has a CMC of 0.57% (for a comparison with other detergents see Davis[31]), is chosen rather than digitonin (CMC of 0.01–0.04%). Razin[102] summarized a number of other useful variables. Most membrane-bound proteins are acidic and therefore negatively charged at physiological pH values[72]. Therefore it is not surprising that a divalent cation is usually necessary for reattachment of the protein into a membrane form (to minimize electrostatic repulsion during reassembly). 20 mM Mg^{++} was used for such a purpose in both reports on reconstitution of the imipramine binding site; in addition, the presence of Mg^{++} may also favor vesicle formation.

Finally, it appears that certain methods of detergent removal favor formation of stable vesicles. Since the aim is to reconstitute 5-HT transport, these methods are obviously preferred. Detergents may be removed by dialysis or gel permeation chromatography. Dilution can also be used but obviously requires high initial concentrations of protein.

Gel permeation chromatography exploits the size differences between detergent micelles and monomers. The small aggregates formed by cholate, as used by Rudnick and Nelson[112] and Davis[32,33], may be removed by Sephadex G-50 (for example, as used by the former group). Aggregated protein elutes in the void volume. With dialysis (as used by Davis[32,33]), the detergent is dialyzed so as to fall below its CMC. This is by far the most gentle method but may lead to denaturating of the protein due to prolonged exposure to the detergent. However, it is believed that this approach is more likely to yield vesicles than is the gel chromatography method.

Using sodium cholate as the detergent and gel chromatography to subsequently remove the detergent, Rudnick and Nelson[112] failed to reconstitute human blood platelet membranes. Indeed, the failure occurred in the initial solubilization step where small membrane fragments were observed. Razin[102] commented (see references contained therein) that rather than the absolute concentration of detergent being important, it is the weight ratio of detergent to membrane protein that ensures successful solubilization. In addition, Im et al.[59] showed that a concentration of 0.7% cholate solubilized mainly peripheral proteins of renal brush border membranes. Such events may explain the failure of Rudnick and Nelson[112] who used 1% cholate. To overcome both these potential difficulties Davis[30,32,33] used 2% cholate. By the criteria of non-sedimentation at 100,000 × g for 1 h and inclusion within Sepharose 4B gel, solubilization was effective. It was interesting to note that cholate precipitated under the negative staining fixation conditions used for electron microscopy[33]. Clearly, freeze-etching is a more applicable method.

[^{3}H] Ro 11-2465, used under conditions where it exhibited persistent binding (see above), was used to label the binding site in these reconstitution studies. Overall 16% of the original receptor sites and protein were reconstituted into the Azolectin acceptor lipids. Electron microscopy revealed apparent 1-μm diameter vesicles. However, this needs to be verified by more appropriate techniques (by, for example, measuring the inclusion of ^{14}C inulin).

Recent results (unpublished) have demonstrated [^{3}H] imipramine binding in reconstituted membranes formed in the absence of [^{3}H] Ro 11-2465. In addition, the binding was found to be temperature-dependent.

4.6 *Temperature effects on the binding: Differentiation between cortex and platelets*

The binding parameters of all imipramine binding site ligands are markedly sensitive to temperature ([^{3}H]-imipramine[37,38,43,90]; [^{3}H]-nitroimipramine[105,106]; [^{3}H] Ro 11-2465, Dumbrille-Ross and Tang[41]). Over short incubation periods (up to 4 h) the changes in [^{3}H] imipramine binding induced by temperature were fully reversible. It is necessary to distinguish this reversible change from the irreversible one described by Kinnier et al.[62] which was postulated to be due to the action of a calcium-dependent protease. In their hands this loss occurred within 1 h whereas Dumbrille-Ross et al.[43] reported that it required a much longer incubation period.

[^{3}H] Imipramine. In platelets, the reversible loss in binding which resulted from increasing incubation temperature from 4 °C to 23 °C was due to a loss in affinity for [^{3}H] imipramine with no significant loss in the total number of sites. This has been demonstrated in fresh[38], outdated, and solubilized[37] human platelets. Table 2

shows the changes that occur. The change in affinity is probably due to an increased dissociation rate as suggested by data obtained on solubilized platelets[37]. It was difficult to obtain consistent binding results at temperatures above 23 °C, due probably to a further loss in affinity.

In cortex, however, the results were found to be different. Here there was an initial loss in the total number of binding sites (as the incubation temperature was raised from 4 °C to 23 °C) followed by an affinity loss between 30 °C and 37 °C incubations (see table 2). The reversible nature of this loss in binding was demonstrated by preincubation experiments followed by a 4 °C incubation period. Changes in binding due to altered pH of the buffer at these temperatures could not explain the reported degree of binding loss[43].

[^{3}H] 2-Nitroimipramine. Originally reported to be an irreversible ligand at the imipramine binding site[103], [^{3}H] 2-nitroimipramine is now better described as a 'slowly-dissociating' ligand[30,104-106]. It is clearly not a competitive ligand since the affinity constant derived from saturation studies was not the same as that obtained from kinetic studies (as described above). Like Ro 11-2465 when incubated at temperatures greater than 4 °C, [^{3}H] 2-nitroimipramine becomes more readily reversible[106].

[^{3}H] Ro 11-2465. The binding of [^{3}H] Ro 11-2465 to rat cortical membranes shows an even more extreme change upon raising the incubation temperature. At 4 °C the binding ligand displays irreversibly kinetics but at 23 °C and above it exhibits reversible kinetics. In addition, the binding affinity of the ligand decreases between 23 °C and 37 °C[41]. In platelets the temperature sensitivity has not been investigated, but at 4 °C the ligand exhibits the same pseudo-irreversible binding as in earlier studies[32,33].

Involvement of sulphydryl bonds

There are several possible explanations for these temperature-sensitive changes in the binding parameters. The 2 most obvious ones are intra-molecular and inter-molecular conformational changes within the binding site molecule. The first option would involve a change in the tertiary structure whereas the second could be an alteration between the binding molecule and regulatory (sub)units (for example, aggregation or disssociation). A third option covers events extraneous to the binding molecule, such as decreased access of the ligand due to lipid viscosity changes or changing lipid solubilities.

The existence of the temperature-sensitive affinity change in solubilized human platelets tends to favor the first model, but this is very circumstantial evidence. Direct evidence for a conformational change *within* the receptor came from data on the effects of sulphur bond modifying reagents.

The significance of sulphydryl bonds within the binding site was first demonstrated by Wennogle et al.[143]. N-Ethylmaleimide (NEM) was found to inhibit binding to a maximal 74% with an IC_{50} of 41 µM (at 25 °C). This reagent selectively alkylates free sulphydryl bonds (−SH). The lack of importance of disulphide bonds (−S−S−) to the [^{3}H] imipramine binding was indicated by the insensitivity of binding to dithiothreitol (DTT). Sulphydryl and disulphide bonds have been found to participate in the binding of many different receptors; for example, in β-adrenoceptors[121,138], dopamine receptors[46,126], opiate receptors[89] and benzodiazepine receptors[80].

5. *Future studies*

5.1 *Clinical aspects*

There are 4 main areas into which research efforts are presently or perhaps should be directed. Firstly, is the question of whether the imipramine binding site is a state or a trait marker for depression. This could be investigated by longitudinal studies involving patients who exhibit relapses and remissions into, and from depression. Of further interest would be the study of relatives at risk.

Second, is the question of whether these changes in platelet imipramine binding sites accurately reflect any changes in the CNS. If they do, are these generalized changes or are they localised to any one specific region? Evidence has been presented which indicates that cortical imipramine binding sites may be heterogeneous. What are the functions of these proposed multiple binding sites?

Third, is the problem of definition. Throughout this review, I have carefully avoided the topic of what constitutes depression and how many subtypes can be distinguished. Such definition problems represent probably the greatest difficulty in attempting to apply the use of the imipramine binding site data to the clinical situation.

Fourth, is the question of alterations in imipramine binding in other clinical situations. Some possibilities

Table 2. Influence of temperature on the binding parameters of [^{3}H] imipramine

Tissue	4 °C		23 °C		37 °C	
	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)
Rat cortex	8 ± 1	323 ± 12 (4)	9 ± 1	173 ± 16 (4)	24 ± 4	211 ± 9 (3)
Human platelets (fresh)	1.3 ± 0.2	974 ± 39 (3)	7.9 ± 2.6	1113 ± 216 (3)		
Human platelets (outdated)	2.4 ± 0.7	704 ± 84 (7)	6.1 ± 1.0	611 ± 42 (7)		
Human platelets (digitonin solubilized)	100% (3)		10%* (3)			

Values in brackets = number of experiments.

* Percent binding of 3 nM [3H] imipramine after 10 min at 23 °C, n = 3.

Kinetic parameters were obtained by Scatchard analyses of saturation data (0.1–10 nM, 4–8 concentrations) using displacement by 10–100 µM desipramine to define specific binding. All incubations were for 2 h. See text for references to data source.

were outlined in section 3.3, but these are by no means exhaustive.

5.2 *Molecular aspects*

Of more immediate concern is the physiological relevence of the imipramine binding site. Does it function as a presynaptic receptor controlling uptake of 5-HT? If so, what is the natural agonist at this site? Or does the binding site reside on the 5-HT carrier molecule and alter uptake simply by allosteric conformational changes which are not physiologically relevant?
These questions, and those involving heterogeneity of cortical imipramine binding sites, will best be answered by more pharmacological and biochemical studies. In particular, the reconstitution of the binding site and the carrier protein promises to yield information crucial to clarifying their interactions. These studies are as yet in the early stages, but will probably be the source of much effort in the near future.
Further aid will probably also come from the field of immunology. Monoclonal antibodies to the imipramine binding site could be used to further probe the binding site and the carrier protein and, in addition, antibodies will aid in the purification of these molecules. For an example of how far these approaches can go, one need only look at progress in the field of nicotinic cholinergic receptors (see, for example, Popot et al.[96]). Indeed, at least one group is actively searching for antibodies to the imipramine 'receptor'[133].

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Nicotianamine, the 'normalizing factor' for the auxotroph tomato mutant *Chloronerva*; a representative of a new class of plant effectors

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Summary. The article surveys our knowledge of the 'normalizing factor', gained from its discovery a quarter of a century ago up to the present time, under the following headings: Discovery; Physiological properties; Isolation and characterization; Structure determination (Identity of the 'normalizing factor' with nicotianamine); Chemical properties; Analysis; Synthesis; Occurrence and physiological role; Related compounds; Prospects.

Discovery

More than 25 years ago genetic and plant-physiological experiments were conducted at the Central Institute for Genetics and Crop Plant Research at Gatersleben (GDR) in the course of which tomato plants (*Lycopersicon esculentum* Mill., var. 'Bonner Beste') were grafted on to tobacco (*Nicotiana tabacum* L.) rootstocks[1]. Among the fruits obtained, 1 contained seeds which gave 67 normal and 22 mutated plants. The mutant was spontaneous, recessive and monogenic, characterized by severely retarded growth and distorted leaves of abnormal shape, and exhibited a pale yellowish chlorosis of intercostal areas of the leaves, which was most distinctly expressed in young leaves and more or less subdued in older ones. Flower buds very rarely developed, did not unfold and eventually died off. The mutant was given the name *chloronerva*[2].

Physiological properties

Normal growth and development could, however, be completely restored by grafts, in which it was irrelevant whether the mutant was used as scion or as rootstock[2] (fig. 1). This normalization of the phenotype also occurred in grafts between the mutant and other species[3]. Scholz and Böhme showed that grafting could be re-