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2-Iodoimipramine, a Novel Ligand for the Serotonin Transporter

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SUMMARY

Iodoimipramine was synthesized by iodinating imipramine with ICl. Iodoimipramine competitively inhibits [^{3}H]imipramine binding with a K_I of 0.52 nM and also inhibits [^{3}H]serotonin transport competitively, suggesting that serotonin, imipramine, and iodoimipramine all bind to the same site on the serotonin transporter. Association of [^{125}I]iodoimipramine to platelet membranes in Na^+ requires 20 min to reach equilibrium at 25° and 1.5 hr at 0°. [^{125}I] Iodoimipramine binding at equilibrium is saturable and Na^+ dependent, with a K_D of 0.58 nM and a B_{max} of 1.3 pmol/mg at 25°. Serotonin competitively inhibits [^{125}I]iodoimipramine binding, with a K_I of 1.3 μM. [^{125}I]Iodoimipramine bound at 0° in the presence of Na^+ does not dissociate unless the temperature is raised or Na^+ is removed from the medium. At 25°, dissociation of [^{125}I] iodoimipramine from platelet membranes in the presence of Na^+ is only partial, with 40% of the ligand remaining persistently bound over 5 hr after a 50-fold dilution.

Serotonin transport into platelets and neurons is catalyzed by a saturable, Na^+-dependent, plasma membrane transporter (1, 2). Imipramine and many other drugs used clinically to treat depression selectively inhibit serotonin transport. Moreover, platelets and post-mortem brain tissue from depressed patients transport serotonin with reduced V_{max} and bind less imipramine than controls (3–5).

Imipramine competitively inhibits serotonin transport with a K_I identical to its K_D for binding to platelet plasma membranes, and serotonin competes with imipramine for binding to the same membranes (6). The potencies of a variety of antidepressant drugs for inhibition of serotonin transport correlate with their ability to displace [^{3}H]imipramine binding (7, 8), and the regional localization of [^{3}H]imipramine binding sites coincides with areas of serotonergic innervation in the brain (9). These findings led to the proposal that imipramine binds to the substrate site of the serotonin transporter. Consequently, [^{3}H]imipramine, and subsequently other tritium-labeled serotonin uptake inhibitors, have been used as specific ligands for the serotonin transport system in further characterization and purification studies. An alternative explanation by Sette *et al.* (10) and Barbaccia *et al.* (11) proposed that imipramine binds to a site distinct from the serotonin transport site, possibly on a separate modulatory protein. This proposal is supported by the observation (12) that serotonin inhibits imipramine dissociation. More recent results, however, suggest that this effect is mediated by a second serotonin site unrelated to the transport site (13).

Several ligands, including 3-cyanoimipramine, 2-nitroimipramine, and paroxetine, bind extremely tightly to the transporter, with very slow dissociation rates (14–16), but the sensitivity of assays using these radioligands is limited by the specific radioactivity of the ^{3}H with which they are labeled. In the present study, we describe the synthesis, purification, and binding characteristics of 2-[^{125}I]iodoimipramine. This novel ligand inhibits both imipramine binding and serotonin transport by platelet plasma membrane vesicles. Due to its high specific radioactivity and high affinity for the transporter, [^{125}I] iodoimipramine may be a valuable tool in future purification and characterization of the serotonin transporter.

Experimental Procedures

Preparation of membrane vesicles. Outdated human platelet concentrates were purchased from the Connecticut Red Cross. Platelets from 50 to 100 individuals were pooled for each membrane preparation. Platelet plasma membrane vesicles were isolated by the method of Barber and Jamieson (17), with the modifications described previously (18).

Synthesis and purification of 2-iodoimipramine. Iodoimipramine was synthesized by iodinating imipramine with ICl following previously described procedures (19, 20). The ICl reagent was prepared as an aqueous solution containing 0.75 M KCl, 0.5 mM KI, 1.5 mM KIO_3, and 50 mM HCl and was incubated 10 min at room temperature. Over a period of 15 sec, 40 ml of this reagent were added to an equal volume of vigorously stirring 2.5 mM imipramine hydrochloride in 0.25 M potassium acetate, pH 5. The iodination reaction was terminated after 5 min by adding $NaHSO_3$ to a concentration of 10 mM. The reaction mixture was extracted three times with 10 ml of ethyl acetate (spectroscopic grade), the combined extracts were evaporated to dryness, and the residue was dissolved in 1 ml of 75% acetonitrile, 25%

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ABBREVIATION: HPLC, high pressure liquid chromatography.

0.1 M ammonium formate, 0.1% triethylamine, adjusted to pH 5 with formic acid (HPLC mobile phase). Samples containing 0.1 ml of the solubilized extract was applied to a 250 × 4 mm C-18 HPLC column and eluted with the same mobile phase, at a rate of 2 ml/min. The last major peak, which starts eluting at approximately 5 min, was collected and the same fractions from 10 repetitive runs were combined and evaporated. The residue was dissolved in 1 ml of water and extracted twice with 1 ml of ethyl acetate, and the organic phases were combined and evaporated. Finally, the product was dissolved in 1 ml of 25 mM HCl and lyophilized. The yield of iodoimipramine hydrochloride was about 3 mg. Its molar extinction coefficient in water was 1970 cm^{-1} at 261 nm.

Structural analysis. An excess of imipramine over ICl was present during the iodination reaction, leading to the expectation that only one iodine atom would be incorporated. This was tested directly by including a tracer amount of ^{125}I in the reaction to give ICl reagent of known specific radioactivity. The amount of radioactivity incorporated into the final product was determined and found to correspond to the presence of one iodine atom/iodoimipramine molecule. Electrophilic substitution of the aromatic ring of imipramine by ICl is expected to occur either *ortho* or *para* to the bridgehead nitrogen (at the C-4 or C-2 positions, respectively). Comparison of the NMR spectrum of iodoimipramine with that of imipramine reveals a decrease in the intensity corresponding to the C-2 and C-8 hydrogens and an upfield shift of the C-4 hydrogen (which in imipramine is chemically equivalent to the C-6 hydrogen), due to the well known effect of a *meta*-halogen. Iodination also induces a split in the chemical shift of the previously identical hydrogen atoms on C-10 and C-11, due to asymmetry introduced by the iodine atom, and the appearance of a downfield-shifted singlet, which corresponds to the C-1 hydrogen. All of these changes suggest that the position of the iodine atom is on the C-2 carbon, *para* to the bridgehead nitrogen, as expected for an electrophilic substitution.

Synthesis of [^{125}I]iodoimipramine. High specific activity [^{125}I] iodoimipramine was synthesized by iodinating imipramine with [^{125}I] iodine monochloride. Na^{125}I (1 mCi) in 1 M KCl, 1 mM KIO_3 (30 μl), was mixed with 0.2 N HCl (10 μl) and incubated at room temperature for 10 min. An equal volume of 1 mM imipramine hydrochloride in 0.25 M sodium acetate, pH 6.0, was added and the iodination reaction was allowed to proceed for 5 min before it was stopped by the addition of 0.1 M sodium sulfite (10 μl). [^{125}I]Iodoimipramine was extracted from the reaction mixture with ethyl acetate (3 × 100 μl), the organic phase was removed and evaporated by bulb to bulb distillation, and the residue was dissolved in HPLC mobile phase. The crude [^{125}I]iodoimipramine solution was purified by HPLC, as described above for unlabeled iodoimipramine. Fractions containing the peak of radioactivity were pooled, concentrated under vacuum to a volume less than 200 μl, diluted with an equal volume of ethanol, and stored at −20°. [^{125}I] Iodoimipramine comigrated with unlabeled iodoimipramine in this HPLC system. The specific radioactivity of the product synthesized using carrier-free Na^{125}I was estimated as 1450 Ci/mmol and the yield was 10–20%. [^{125}I]Iodoimipramine of defined specific activity was prepared by diluting Na^{125}I in the reaction mixture with unlabeled potassium iodide to a measured specific radioactivity. Iodoimipramine synthesized under these conditions should contain ^{125}I at a specific activity 2/3 that of the I^- in the reaction mixture, due to the fact that one iodine atom in three molecules of ICl is derived from KIO_3 (19).

Initial transport rate measurements. Serotonin transport was assayed at 25°, as described previously (18). Briefly, vesicular contents were equilibrated with K^+ by diluting vesicles 20-fold into a solution of 133 mM K_2SO_4 containing 10 mM KH_2PO_4 and 1 mM $MgSO_4$, pH 6.7, followed by incubation at 25° for 10 min, centrifugation at 43,000 × *g* for 20 min at 4°, and resuspension to a protein concentration of approximately 5 mg/ml with the same buffer. Reactions were initiated by 40-fold dilution of this preequilibrated vesicle suspension into 200 μl of 200 mM NaCl containing 10 mM LiH_2PO_4, pH 6.7, 1 mM $MgSO_4$, the indicated concentration of [^{3}H]serotonin and, in some samples, 39 nM iodoimipramine. Reactions were terminated after 15 sec by rapid dilution with 2 ml ice-cold 0.212 M NaCl, followed by immediate filtration through Gelman GN-6 filters (25-mm diameter) and three 2-ml washes of tube and filter with cold NaCl solution. In parallel control experiments, all of the Na^+ was replaced with Li^+, which does not substitute for Na^+ at the serotonin transporter (18). These Li^+ samples were used to correct for nonspecific serotonin influx or binding. Filters were placed in Optifluor (Packard, Downers Grove, IL) and counted after 5 hr.

Iodoimipramine and imipramine binding. Iodoimipramine binding and its displacement by both serotonin and imipramine, as well as imipramine binding and its inhibition by iodoimipramine, were measured at 25° or 0° using the filtration assay described previously (13) for measurement of [^{3}H]imipramine binding. Membrane vesicles were suspended at a protein concentration of 0.33 to 1 mg/ml in 200 mM NaCl containing 10 mM LiH_2PO_4, pH 6.7, 1 mM $MgSO_4$, and either [^{125}I]iodoimipramine or [^{3}H]imipramine at the indicated concentrations. In some samples, unlabeled serotonin, imipramine, or iodoimipramine were added at the indicated concentrations. After incubation for the specified time, the reactions (300 μl/assay) were terminated by dilution with 4 ml of ice-cold 0.212 M NaCl and filtered through Whatman GF/B filters that were pretreated with 0.3% polyethyleneimine. The tube and filters were washed three times with 4 ml of ice-cold NaCl solution. Filters were placed in Optifluor and counted after 5 hr. Binding in the absence of Na^+ (replacement by Li^+) or in the presence of 100 μM serotonin was taken as a control for nonspecific binding. Binding is expressed as mol of ligand bound/mg of membrane protein.

Iodoimipramine dissociation. Plasma membrane vesicles were suspended at a concentration of 1 mg/ml in 200 mM NaCl containing 10 mM LiH_2PO_4, pH 6.7, and 1 mM $MgSO_4$. [^{125}I]Iodoimipramine was added to a final concentration of 0.1 to 0.4 nM and the suspension was incubated at 0° or 25° for 25 min. At this time, the suspension was diluted 50-fold into the indicated solution at 0° or 25° and, at later specified times, 2.5-ml samples were removed and filtered, as described above, to assess the amount of [^{125}I]iodoimipramine that remained associated with the membranes.

Protein measurements. Protein concentration was determined by the method of Lowry *et al.* (21), using bovine serum albumin as a standard.

Data analysis. The data presented in the figures were fit using Enzfitter (Elsevier Biosoft). Uncertainties are presented as standard errors (calculated by the matrix inversion method). The lines in the figures are drawn to the parameters determined by these fits.

Materials. Na^{125}I (carrier-free) was obtained from New England Nuclear and [^{3}H]serotonin (12–14 Ci/mmol) and [^{3}H]imipramine (20–22 Ci/mmol) were purchased from Amersham. All other reagents were reagent grade, purchased from commercial sources.

Results and Discussion

Inhibition of imipramine binding. To determine whether iodination significantly alters the affinity of imipramine for the serotonin transporter, we examined the ability of iodoimipramine to compete with [^{3}H]imipramine for binding to platelet plasma membranes at 25° during a 15-min incubation. As shown in Fig. 1, where the data are plotted according to the method of Scatchard (22), iodoimipramine inhibits imipramine binding competitively. Although the intercepts of the two lines on the abscissa (B_{max}) are, to within experimental error, the same, the absolute value of the slope ($1/K_D$) is decreased in the presence of 3.7 nM iodoimipramine. The inhibitory dissociation constant, K_I, for iodoimipramine calculated from the data is 0.52 ± 0.14 nM. This K_I is lower than the K_D of 1.86 nM measured for imipramine binding in the same experiment, indicating that iodination of imipramine does not decrease its affinity for the transporter.

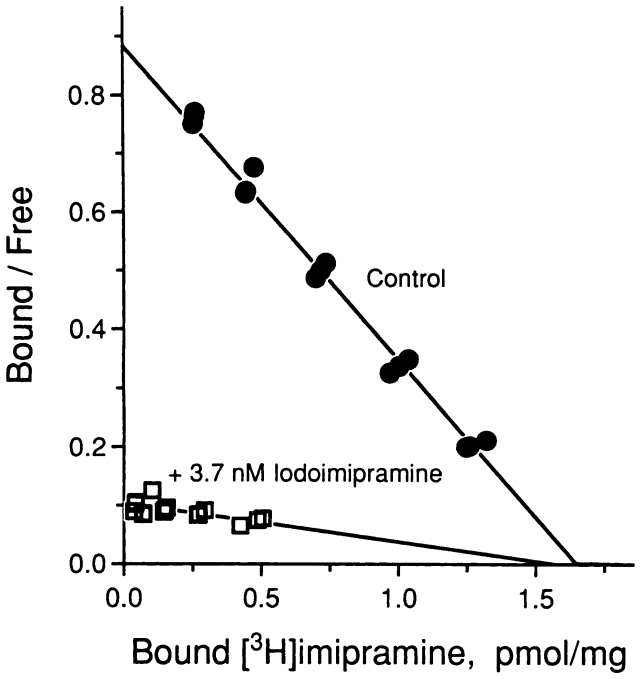

Fig. 1. Competitive inhibition of imipramine binding by iodoimipramine. Equilibrium imipramine binding (15 min) to platelet plasma membrane vesicles at 25° was determined over a range from 0.25 to 6 nM free [^{3}H] imipramine, as described under Experimental Procedures, in the presence (□) or absence (●) of 3.7 nM iodoimipramine. The data are plotted according to the method of Scatchard (22). The calculated value of K_I for iodoimipramine is 0.52 ± 0.14 nM. The calculated values of K_D and B_{max} for imipramine binding in this experiment were 1.86 ± 0.08 nM and 1.65 ± 0.03 pmol/mg, respectively, in the absence of iodoimipramine and 15.15 ± 4.0 nM and 1.57 ± 0.31 pmol/mg, respectively, in the presence of 3.7 nM iodoimipramine.

Kinetics of [^{125}I]iodoimipramine binding. In the presence of Na^+, [^{125}I]iodoimipramine binds specifically to platelet plasma membranes. The data shown in Fig. 2A demonstrate that association of iodoimipramine is slow (k_{obs} = 3.6 ± 0.2 × 10^{-3} sec^{-1}), taking 20 min to reach equilibrium at 25°. The nonspecific binding, defined as binding in the absence of Na^+ or in the presence of 100 μM serotonin, comprises about 10 to 15% of total [^{125}I]iodoimipramine binding, which is comparable to the level observed with [^{3}H]imipramine (data not shown). Association of iodoimipramine was much slower at 0°, taking almost 2 hr to reach equilibrium (Fig. 2B).

Concentration dependence of iodoimipramine binding. Equilibrium binding of [^{125}I]iodoimipramine by platelet plasma membrane vesicles at 25° is a saturable function of iodoimipramine concentration. As shown in Fig. 3A, iodoimipramine binding in the absence of Na^+ increases linearly with concentration. When Na^+ is present, bound iodoimipramine increases more steeply at low iodoimipramine concentrations and, at higher concentrations, approaches a maximum of 1.3 pmol of iodoimipramine bound/mg of membrane protein in excess over the amount bound in the absence of Na^+. This compares favorably with the abundance of imipramine binding sites on platelet plasma membranes under the same conditions (Fig. 1). The calculated K_D for iodoimipramine at 25° is 0.58 ± 0.03 nM. The presence of 2 μM serotonin increases the apparent K_D to 1.45 ± 0.21 nM without significantly affecting the B_{max}, indicating that serotonin competitively inhibits iodoimipramine binding to platelet plasma membranes. Fig. 3B presents the same data, corrected for nonspecific binding, plotted according to the method of Scatchard (22). The apparent K_I for serotonin, 1.3 ± 0.2 μM, is slightly higher than its K_M for transport, 0.35 μM, in agreement with previous results using imipramine, where the difference between K_D and K_M for serotonin was attributed to a kinetic step that is rate limiting for transport but not binding (6, 23).

Inhibition of serotonin transport. Iodoimipramine competitively inhibits the initial rate of Na^+-dependent serotonin transport. The Hofstee plot (24) of serotonin transport into platelet plasma membrane vesicles in the presence and absence of iodoimipramine (Fig. 4) demonstrates that the inhibitory effect is strongest at low serotonin concentrations and is overcome at higher concentrations. Thus, iodoimipramine at 39 nM does not change V_{max}. The intercept on the ordinate is not significantly changed but the slope ($-K_M$) is 3-fold steeper. The K_I for iodoimipramine's inhibition of the initial rate of serotonin transport is 19.5 ± 5.8 nM, 1 order of magnitude greater than both the K_D measured for iodoimipramine binding to the same plasma membrane vesicles and the K_I for iodoimi-

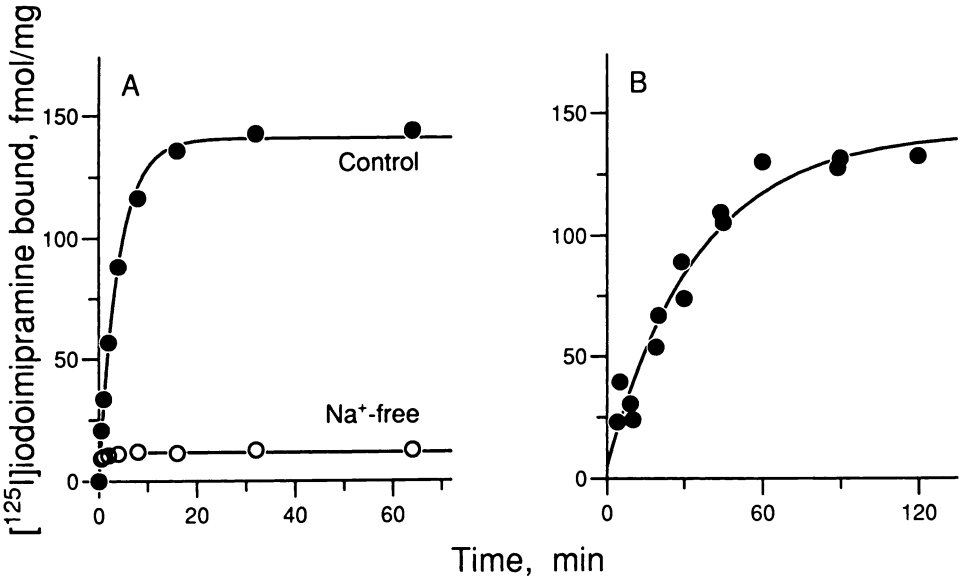

Fig. 2. Time courses of iodoimipramine binding at 25° (A) and 0° (B). Iodoimipramine binding was measured as described under Experimental Procedures, using 0.16 nM [^{125}I]iodoimipramine. A, Association of iodoimipramine at 25°. Iodoimipramine binding was measured in the presence (●) and absence (○) of sodium. The calculated pseudo-first-order rate constant, k_{obs}, for association of iodoimipramine in Na^+ at 25° is 3.6 ± 0.2 × 10^{-3} sec^{-1}. B, Association of iodoimipramine at 0°. Specific [^{125}I]iodoimipramine binding was calculated as the difference between binding measured in the absence and presence of 100 μM serotonin. The calculated value of k_{obs} at 0° is 5.2 ± 0.6 × 10^{-4} sec^{-1}.

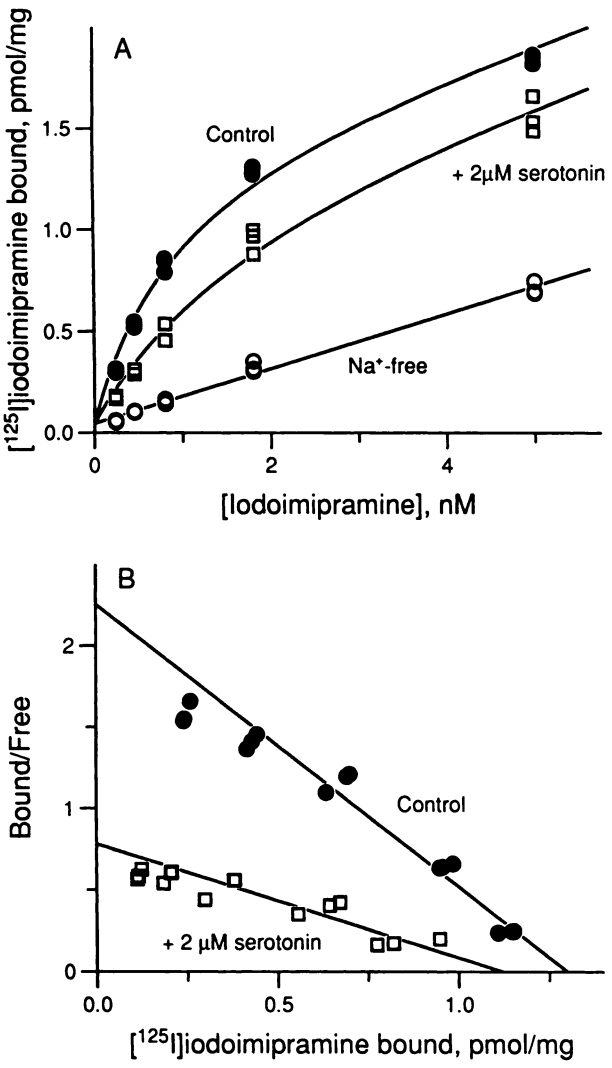

Fig. 3. Concentration dependence of iodoimipramine binding and its competitive inhibition by serotonin at 25°. A, Equilibrium [^{125}I]iodoimipramine binding (25 min) to plasma membrane vesicles was measured, as described under Experimental Procedures, over the indicated range of iodoimipramine concentrations. The assay solution contained either 200 mM NaCl (*filled circles, squares*) or LiCl (*open circles*) and either 0 (*circles*) or 2 μM serotonin (*squares*). B, The amounts of iodoimipramine binding measured in A were corrected for nonspecific association by subtracting the *lower curve* from each of the *upper curves*. The resulting data, showing specific binding in the absence (*circles*) and presence (*squares*) of serotonin, are plotted according to the method of Scatchard (22). The K_D and B_{max} values calculated for iodoimipramine binding are 0.58 ± 0.03 nM and 1.33 ± 0.03 pmol/mg, respectively, in the absence of serotonin and 1.45 ± 0.21 nM and 1.12 ± 0.07 pmol/mg, respectively, in the presence of 2 μM serotonin. The calculated value of K_i for serotonin is 1.3 ± 0.2 μM.

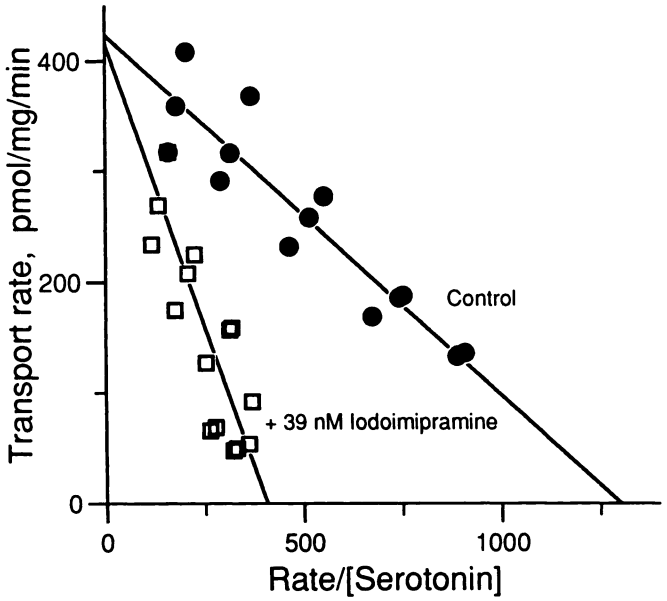

Fig. 4. Competitive inhibition of serotonin transport by iodoimipramine. Plasma membrane vesicles were assayed for initial rates of serotonin transport in the absence (●) and presence (□) of iodoimipramine, as described under Experimental Procedures, over a range from 0.15 to 2 μM [^{3}H]serotonin. The experimental data, having been corrected for nonspecific association, are plotted here according to the method of Hofstee (24). The K_i calculated for iodoimipramine is 19.5 ± 5.8 nM. The calculated values of K_M and V_{max} for serotonin are 0.33 ± 0.06 μM and 424 ± 28 pmol/mg/min, respectively, in the absence of iodoimipramine and 0.98 ± 0.27 μM and 408 ± 54 pmol/min/mg, respectively, in the presence of 39 nM iodoimipramine.

pramine inhibition of imipramine binding. This discrepancy is a direct consequence of the slow association kinetics for iodoimipramine (Fig. 2A). During the 15-sec transport assay, iodoimipramine binding does not reach equilibrium and the observed inhibition is consequently much less than that expected if iodoimipramine binding were rapid. Previous reports described discrepancies between potencies for inhibition of serotonin transport and imipramine binding for a number of drugs (10, 13, 25). Although for some drugs the difference could be explained by different temperatures used for transport and binding assays (26), in other cases (13) the discrepancy was not explained. It is likely that slow drug binding to the transporter was responsible also in some of those cases.

Iodoimipramine dissociation at 0° and 25°. We examined the dissociation of [^{125}I]iodoimipramine from platelet plasma membranes at 0° by diluting membranes that were equilibrated with [^{125}I]iodoimipramine into medium free of ligand and then measuring the loss of membrane-bound radioactivity with time. The semilogarithmic plot of these data is shown in Fig. 5A. The results demonstrate the [^{125}I]iodoimipramine does not dissociate following 50-fold dilution into medium containing Na^+. When Na^+ in the dilution buffer is replaced with Li^+, however, iodoimipramine dissociates in a first-order process with an approximate $t_{1/2}$ of 1.5 hr. This is in marked contrast to the behavior of imipramine, which dissociates completely from human platelet plasma membrane following dilution into buffer containing or lacking Na^+. Imipramine dissociation in the absence of Na^+, however, is 5 times faster than in its presence (13). An extremely slow dissociation from the serotonin transporter, at 0° and in the presence of Na^+, has also been observed with 3-cyanoimipramine (14) and paroxetine (16). Like iodoimipramine, both 3-cyanoimipramine (14) and paroxetine[1] readily dissociate when Na^+ is removed from the dilution medium.

To characterize more completely iodoimipramine binding to the serotonin transporter, we examined iodoimipramine dissociation kinetics at the same temperature used for association and equilibrium measurements. Membranes were incubated with [^{125}I]iodoimipramine at 25° for 25 min and diluted 50-fold into the same medium free of [^{125}I]iodoimipramine or into

[1] C. J. Humphreys and G. Rudnick, unpublished observation.

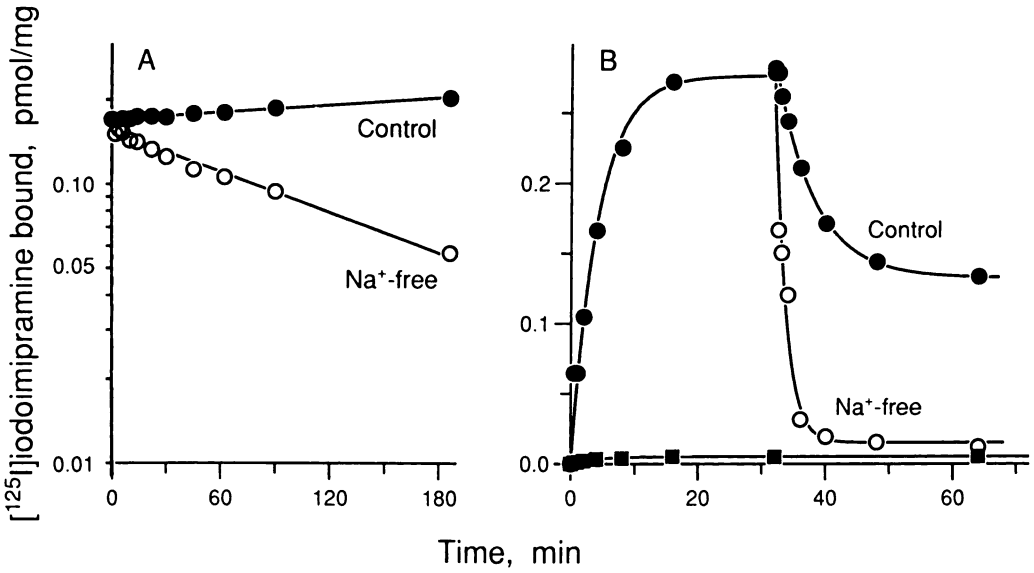

Fig. 5. Dissociation of iodoimipramine. A, Semilogarithmic plot of iodoimipramine dissociation at 0°. Membrane vesicles were equilibrated in 0.16 nM [^{125}I]iodoimipramine for 25 min and subsequent dissociation was measured following 50-fold dilution with either 200 mM NaCl (*filled circles*) or 200 mM LiCl (*open circles*) containing 10 mM lithium phosphate buffer, pH 6.7, and 1 mM $MgSO_4$. The calculated value of the rate for iodoimipramine dissociation at 0° in the absence of Na^+ is $7.4 \pm 0.5 \times 10^{-3}$ min^{-1}. B, Iodoimipramine dissociation at 25°. The time courses of iodoimipramine binding and dissociation were measured as described under Experimental Procedures, using 0.29 nM (*circles*) or 5.2 pM (*squares*) [^{125}I]iodoimipramine in the association assays. After 32 min, the membranes incubated with 0.29 nM [^{125}I]iodoimipramine were diluted 50-fold (final concentration of [^{125}I] iodoimipramine, 5.8 pM) into 200 mM NaCl (*filled circles*) or LiCl (*open circles*) containing 10 mM lithium phosphate buffer, pH 6.7, and 1 mM $MgSO_4$. The calculated rates of iodoimipramine dissociation in Na^+-containing and Na^+-free media are 0.16 ± 0.01 min^{-1} and 0.60 ± 0.01 min^{-1}, respectively.

medium in which Na^+ was replaced by Li^+. As at 0°, specifically bound iodoimipramine dissociates completely from the membranes following dilution into Na^+-free medium (Fig. 5B), although at 25° the rate is 80 times faster ($t_{1/2}$ is 1.2 min). Dilution of membranes with bound iodoimipramine into Na^+-containing medium results in dissociation of approximately 60% of the bound iodoimipramine at a rate of 0.16 ± 0.01 min^{-1}. This dissociation process is complete within 40 min and no further dissociation is observed at times up to 5 hr after dilution. The K_D for iodoimipramine binding, based on this measured dissociation rate and the calculated K_{on} of $3.15 \pm 0.26 \times 10^6$ $sec^{-1}M^{-1}$ is 0.89 nM, close to the value obtained from the equilibrium binding measurements shown in Fig. 3.

Among the potential reasons for persistent binding of a fraction of bound iodoimipramine is the possibility that the iodoimipramine remaining associated with the membranes after dilution is actually inside the vesicles, having been transported across the membrane during the predilution incubation. We have ruled out this possibility by measuring dissociation in the presence of digitonin. In separate experiments, we determined that a detergent to protein ratio of 1:1 (w/w) was sufficient to collapse a serotonin gradient across these vesicles within 30 sec. The time course of [^{125}I]iodoimipramine dissociation is the same in the presence and absence of this digitonin concentration (data not shown), indicating that iodoimipramine, which remains persistently associated with the membranes under these conditions, is not trapped inside the membrane vesicles.

An alternative explanation for the persistent binding observed in Fig. 5B is that the transporter-iodoimipramine complex undergoes a time-dependent increase in affinity. The suggestion that persistent binding reflects a change in affinity and not occlusion of bound iodoimipramine is supported by the observation that the extent of iodoimipramine dissociation depends on the extent of dilution. If vesicles with persistently bound iodoimipramine are further diluted, a new lower steady state is reached with the same time course. At every dilution, the amount of binding apparently represents an equilibrium. In the experiment shown in Fig. 6, membranes with bound [^{125}I]iodoimipramine were diluted to different extents, and the amount of label remaining bound 40 min after dilution was measured. We found that, to dissociate a significant fraction of bound iodoimipramine, it was necessary to dilute the suspension to well below the previously measured K_D of 0.58 nM. The results shown in Fig. 6 describe a binding isotherm with a K_D 50 to 100 times lower than measured in Fig. 3. Another indication that persistent binding represents an affinity change comes from the observation that competitive ligands such as serotonin and imipramine cause rapid dissociation of persistently bound iodoimipramine (see Fig. 7 below).

The affinity change leading to persistent iodoimipramine binding requires previous incubation with iodoimipramine. In the experiment shown in Fig. 5B, platelet membrane vesicles were either preincubated with 0.29 nM [^{125}I]iodoimipramine and then diluted 50-fold or incubated for the same period of time with iodoimipramine at a 60-fold lower concentration. The difference between the final levels of bound iodoimipramine is dramatic, despite the fact that the final iodoimipramine concentration was essentially the same. In both cases, binding had reached equilibrium and was not changing with time at the end of the experiment. The history of iodoimipramine binding by the transporter clearly affects the final level bound. Data (not shown) from other experiments indicate that the increase in the amount of persistently bound iodoimipramine develops in a time-dependent manner.

It is difficult to evaluate the ability of persistently bound

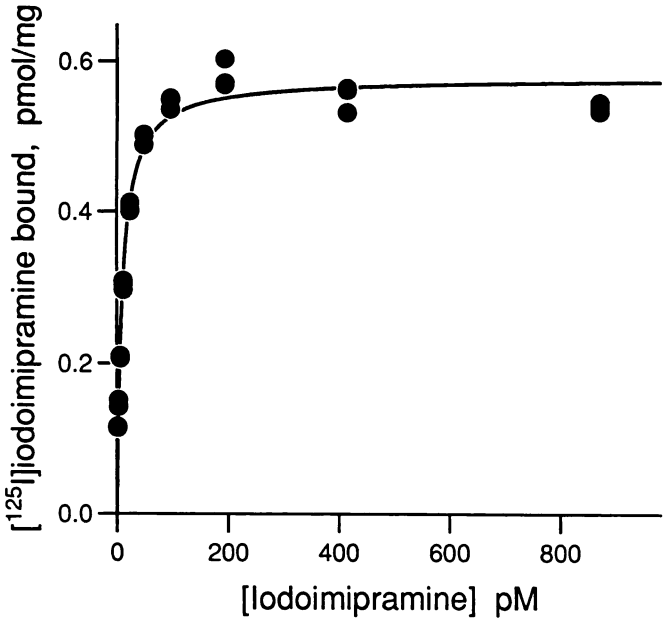

Fig. 6. Dilution of [^{125}I]iodoimipramine-bound membrane vesicles at 25°. Membranes were incubated with iodoimipramine for 25 min at 25°, as described under Experimental Procedures, using 0.9 nM [^{125}I]iodoimipramine. Portions of the membrane suspension were diluted with 200 mM NaCl containing 10 mM lithium phosphate, pH 6.7, and 1 mM $MgSO_4$, to the indicated final free iodoimipramine concentrations. After dilution, the membranes were incubated for 40 min at 25° and then assayed for bound iodoimipramine. The K_D for iodoimipramine calculated from these data is 9.5 ± 0.6 pM.

iodoimipramine to inhibit serotonin transport, because the incubation time required to demonstrate persistent binding is long enough to allow dissipation of the ion gradients required for transport. The data shown in Fig. 7, however, demonstrate that persistently bound iodoimipramine is competitively displaced by concentrations of serotonin close to the K_M for transport. In this experiment, vesicles were allowed to bind [^{125}I]iodoimipramine and were then diluted 50-fold with buffer containing 200 meq/liter Na^+. After the new binding equilibrium was established, the indicated concentrations of serotonin were added and portions of the suspension were filtered at the indicated times. The results, shown in Fig. 7A, demonstrate that serotonin induces rapid dissociation of iodoimipramine in a time- and concentration-dependent manner. Fig. 7B shows that the extent of dissociation saturates with increasing serotonin concentration, consistent with competitive displacement. The K_M for serotonin in this process, 0.88 ± 0.11 μM, is close to the K_I of 1.3 μM determined for competitive inhibition (Fig. 3). These results also demonstrate that the lack of complete iodoimipramine dissociation is not due to a kinetic barrier for dissociation but rather an increased affinity.

The high affinity and persistent binding of iodoimipramine, particularly at low temperature, together with a high specific activity of the radioiodinated derivative provide a uniquely potent probe for the serotonin transporter. These properties should prove useful in the identification and purification of the serotonin reuptake system.

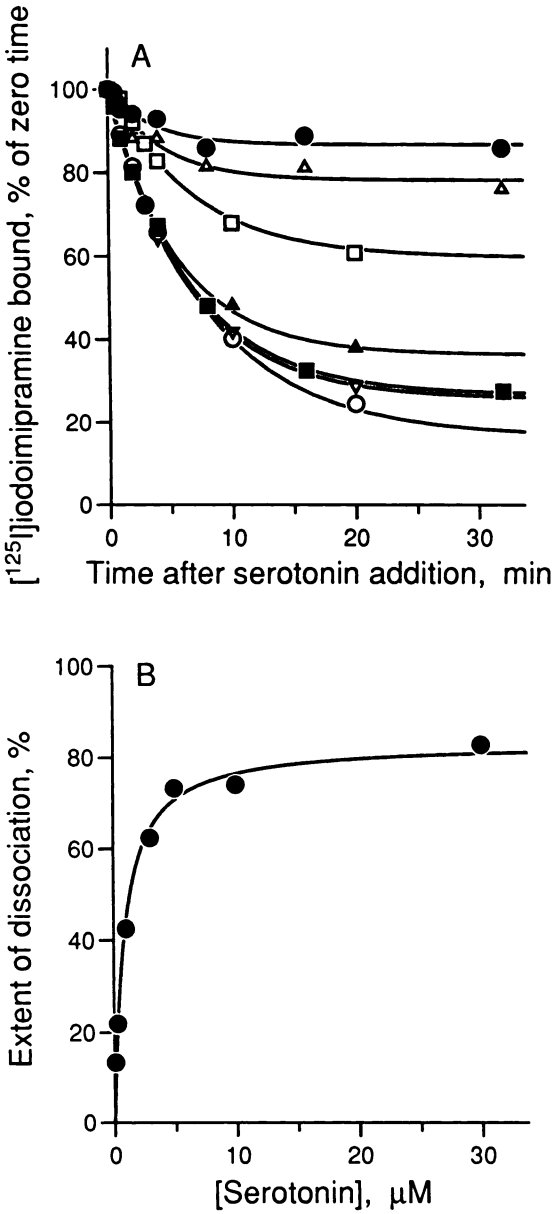

Fig. 7. Displacement of tightly bound iodoimipramine by serotonin. A, Time course, Vesicles were incubated with either 0.17 or 0.35 nM [^{125}I] iodoimipramine for 25 min and then diluted 50-fold, as described in the legend to Fig. 5B. After a further incubation of 35 min, serotonin was added to the following concentrations: ●, 0.1 μM; △, 0.3 μM, □, 1 μM; ▲, 3 μM; ■, 5 μM; ▽, 10 μM; ○, 30 μM. Samples were filtered and counted at the indicated times after serotonin addition. The 100% control value (after dilution) was 72.6 fmol/mg when 0.17 nM [^{125}I]iodoimipramine was used and 160 fmol/mg with 0.35 nM [^{125}I]iodoimipramine. B, The extent of serotonin-induced dissociation plotted as a function of serotonin concentration. Half-maximal dissociation occurred at 0.88 ± 0.11 μM serotonin. The calculated maximal displacement was 83.3 ± 2.3% of the tightly bound iodoimipramine.

References

1. Pletscher, A. Platelets as models: use and limitations. *Experentia (Basel)* **44**:152–155 (1988).
2. Sneddon, J. M. Blood platelets as a model for monoamine-containing neurones. *Prog. Neurobiol.* **1**:153–198 (1973).
3. Tuomisto, J., and E. Tukiainen. Decreased uptake of 5-hydroxytryptamine in blood platelets from depressed patients. *Nature (Lond.)* **262**:596–598 (1976).
4. Briley, M. S., S. Z. Langer, R. Raisman, D. Sechter, and E. Zarifian. Tritiated imipramine binding sites are decreased in platelets of untreated depressed patients. *Science (Wash. D. C.)* **209**:303–305 (1980).
5. Perry, E. K., E. F. Marshall, G. Blessed, B. E. Tomlinson, and R. H. Perry. Decreased imipramine binding in the brains of patients with depressive illness. *Br. J. Psychiatry* **142**:188–192 (1983).
6. Talvenheimo, J., P. J. Nelson, and G. Rudnick. Mechanism of imipramine inhibition of platelet 5-hydroxytryptamine transport. *J. Biol. Chem.* **254**:4631–4635 (1979).

7. Langer, S. Z., C. Moret, R. Raisman, M. C. Dubocovich, and M. Briley. High-affinity [^{3}H]imipramine binding in rat hypothalamus: association with uptake of serotonin but not of norepinephrine. *Science (Wash. D. C.)* **210:**1133–1135 (1980).
8. Paul, S. M., M. Rehavi, P. Skolnick, and F. K. Goodwin. Demonstration of specific 'high-affinity' binding sites for [^{3}H]imipramine on human platelets. *Life Sci.* **26:**953–959 (1980).
9. Palkovits, M., R. Raisman, M. S. Briley, and S. Z. Langer. Regional distribution of [^{3}H]imipramine binding in rat brain. *Brain Res.* **210:**493–498 (1981).
10. Sette, M., M. S. Briley, and S. Z. Langer. Complex inhibition of [^{3}H] imipramine binding by serotonin and nontricyclic serotonin uptake blockers. *J. Neurochem.* **40:**622–628 (1983).
11. Barbaccia, M. L., D. M. Brunello, D. M. Chuang, and E. Costa. On the mode of action of imipramine: relationship between serotonergic axon terminal function and down-regulation of β-adrenergic receptors. *Neuropharmacology* **22:**373–383 (1983).
12. Wennogle, L. P., and L. R. Meyerson. Serotonin modulates the dissociation of [^{3}H]imipramine from human platelet recognition sites. *Eur. J. Pharmacol.* **86:**303–307 (1983).
13. Humphreys, C. J., J. Levin, and G. Rudnick. Antidepressant binding to the porcine and human platelet serotonin transporters. *Mol. Pharmacol.* **33:**657–663 (1988).
14. Dumbrille-Ross, A., and S. W. Tang. Binding of [^{3}H]Ro 11-2465: possible identification of a subclass of [^{3}H]imipramine binding sites. *Mol. Pharmacol.* **23:**607–613 (1983).
15. Davis, A. Kinetic properties of [^{3}H]2-nitroimipramine binding to human platelets. *Eur. J. Pharmacol.* **96:**105–110 (1983).
16. Plenge, E. T., P. Plenge, and M. Engelstoft. High affinity binding of [^{3}H] paroxetine and [^{3}H]imipramine to human platelet membranes. *Eur. J. Pharmacol.* **96:**303–309 (1983).
17. Barber, A. J., and G. A. Jamieson. Isolation and characterization of plasma membranes from human blood platelets. *J. Biol. Chem.* **245:**6357–6365 (1970).
18. Rudnick, G. Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. *J. Biol. Chem.* **252:**2170–2174 (1977).
19. Helmkamp, R. W., M. A. Contreras, and M. J. Izzo. ^{131}I-labeling of proteins at high activity level with ^{131}ICl produced by oxidation of total iodine in Na^{131}I preparations. *Int. J. Appl. Radiat. Isotopes* **18:**747–754 (1967).
20. Cassel, D., M. Rotman, E. J. Cragoe, Jr., and P. Igarashi. Preparation of 6-^{125}I-labeled amiloride derivatives. *Anal. Biochem.* **170:**63–67 (1988).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
22. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:**660–672 (1949).
23. Nelson, P. J., and G. Rudnick. Coupling between platelet 5-hydroxytryptamine and potassium transport. *J. Biol. Chem.* **254:**10084–10089 (1979).
24. Hofstee, B. H. J. On the evaluation of the constants V_m and K_m in enzyme reactions. *Science (Wash. D. C.)* **116:**329–331 (1952).
25. Meyerson, L. R., J. R. Ieni, and L. P. Wennogle. Allosteric interaction between the site labeled by [^{3}H]imipramine and the serotonin transporter in human platelets. *J. Neurochem.* **48:**560–565 (1987).
26. Segonzac, A., H. Schoemaker, and S. Z. Langer. Temperature dependence of drug interaction with the platelet 5-hydroxytryptamine transporter: a clue to the imipramine selectivity paradox. *J. Neurochem.* **48:**331–339 (1987).

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