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# AN ARTIFACTUAL COMPONENT OF DRUG-PROTEIN BINDING GENERATED IN VITRO

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## Summary

In the course of investigating the binding of imipramine to soluble cellular fractions from brain and leukocytes using equilibrium dialysis, an artifactual binding component was produced. On Scatchard and double-reciprocal plots of the data, the component appeared as a homogenous population of sites displaying a binding affinity of 170 nM. The obtained pattern of biphasic interaction bore a marked resemblance to reported Scatchard plots representing the interaction of drugs with bovine serum albumin, and depicting two components of widely differing binding affinity and capacity. The artifact occurred when solutions were transferred after dialysis and before quantitation to intermediate containers, and resulted from binding of $^{3}$H-imipramine to the walls of these containers. The latter interaction decreased the concentration of radiolabeled drug in the dialysate but not in the dialyzed solution, and thus mimicked increased imipramine binding to the biological material under study. The effect was particularly pronounced at low drug concentrations, and was prevented by the presence of either proteinaceous material, or of an excess of another basic compound such as methadone. The concentration dependence of the phenomenon led to its appearance as a discrete binding component. The artifact was eliminated either by applying an appropriate correction factor, or by transferring the dialyzed solutions directly into scintillation vials for counting.

Earlier work in this laboratory has provided evidence for the active transport of drugs, with the structural feature of basic amines, in leukocytes (1,2) and synaptosomes (3), and for the mediated transport of similar drugs in the isolated retina (4). The evidence included saturable transport components, competitive inhibition by structural analogues, specific dependence on metabolic energy and temperature, and a demonstration of trans-acceleration. In order to assess the possible contribution of intracellular binding to the observed cellular accumulation of basic drugs, e.g., benzomorphans, tricyclic antidepressants and

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methadone, we have investigated their binding in soluble cellular fractions from brain and leukocytes using equilibrium dialysis. The binding data were evaluated on double reciprocal (5) and Scatchard (6) plots. In this report we describe experimental conditions for equilibrium dialysis under which a well defined but artifactual binding component for imipramine was generated, mimicking drug-protein interaction of apparent high affinity. In addition to imipramine other basic compounds, such as methadone, have the potential to display this misleading phenomenon.

## Materials and Methods

[2,4,6,8-$^{3}$H]-Imipramine. HCl and [Methylene-$^{14}$C]-Imipramine.HCl were purchased from New England Nuclear, Boston, MA, and Amersham/Searle, Arlington Heights, IL, respectively. The radiochemical purity of these compounds was greater than 98%, as confirmed in our laboratory by thin-layer chromatography. Unlabeled imipramine and methadone were obtained through the Drug Abuse Basic Research Program at the University of Michigan. Bovine serum albumin, fraction V and crystalline, were obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals were of reagent grade. Karush-type dialysis chambers (7) were purchased from Bellco, Vinland, NJ. Dialysis tubing with flat diameters of 9 mm and 24 mm was purchased from the Films and Packing Division of Union Carbide Corporation, Chicago, IL. The buffer medium used throughout this work had the following composition (mM): NaCl, 123; KCl, 5; $MgSO_4$, 1.5; $Na_2HPO_4$, 16. The pH was adjusted to 7.4 with HCl.

Membranes for dialysis were prepared by cutting the dialysis tubing open to yield single-layer rectangles of 4 cm x 2.4 cm. The cut pieces were repeatedly washed and soaked overnight in distilled water at 4°. The membranes were then rinsed again and were left standing in distilled water until assembly into the Karush-type glass dialysis chamber. In some experiments dialysis was carried out in bags, after rinsing the dialysis tubing as described above.

A soluble, predominantly cytoplasmic, fraction from rat brain was prepared as follows: male Sprague-Dawley rats weighing 200-300 g were lightly anesthetized with diethyl ether, decapitated, and the heads placed on ice. All subsequent steps were carried out at 4°. The brain was excised, and the cerebrum was dissected and cleaned of meninges. The cerebra from 2-3 rats were disrupted in a Potter-Elvehjem type homogenizer with 10 volumes of ice-cold buffer using eight full strokes of a Teflon pestle at 500 rpm. The homogenate was centrifuged at 25,000 x g for 20 min, and the supernatant was collected. The latter was then centrifuged at 105,000 x g for 60 min, and the supernatant diluted with buffer to the protein concentration desired. Protein was determined according to Lowry et al. (8) with crystalline bovine serum albumin as standard.

Formation of rat polymorphonuclear leukocytes was induced by the intraperitoneal injection of sodium caseinate[1]. A

[1]E.I. Cullen, C.J. Spears, and F. Medzihradsky, unpublished observations.

soluble cellular fraction was prepared by thrice freezing in dry ice-acetone (-70°) and thawing to room temperature. Following centrifugations as described above at 25,000 x g and 105,000 x g for 20 min and 60 min, respectively, equilibrium dialysis was carried out with the resulting supernatant.

The general protocol for equilibrium dialysis was as follows: 1.0 ml of the freshly prepared soluble fraction from brain or leukocytes was pipetted into one side of the Karush chamber or into a dialysis bag of 90 mm flat diameter. To the other side of the Karush assembly or the outside of the dialysis bag were added 1 ml or 2 ml, respectively, of the buffer containing 0.2 µCi of $^{3}H$ or $^{14}C$-imipramine and different concentrations of the unlabeled drug. In experiments investigating competitive drug interactions that solution also contained 1 mM unlabeled d,l-methadone. The chambers were placed on a Rotating Mixer (9) designed to accept the Karush-type dialysis chambers. Dialysis was carried out at 3 rpm for 18 hours at 4°, a time period shown to be sufficient to reach equilibrium. The solutions from each side of the apparatus were then transferred into separate polypropylene tubes from which aliquots were pipetted into scintillation vials for counting. The radioactivity in the dialysate represented free drug, and the concentration of bound drug was calculated from the difference between the radioactivity measured in the two dialysis compartments. Apparent $K_D$'s were calculated from the binding curves by the method of least squares. The contributions from the separate binding components were calculated according to Neal (10), and were also analyzed using a nonlinear least squares regression computer program (11).

## Results and Discussion

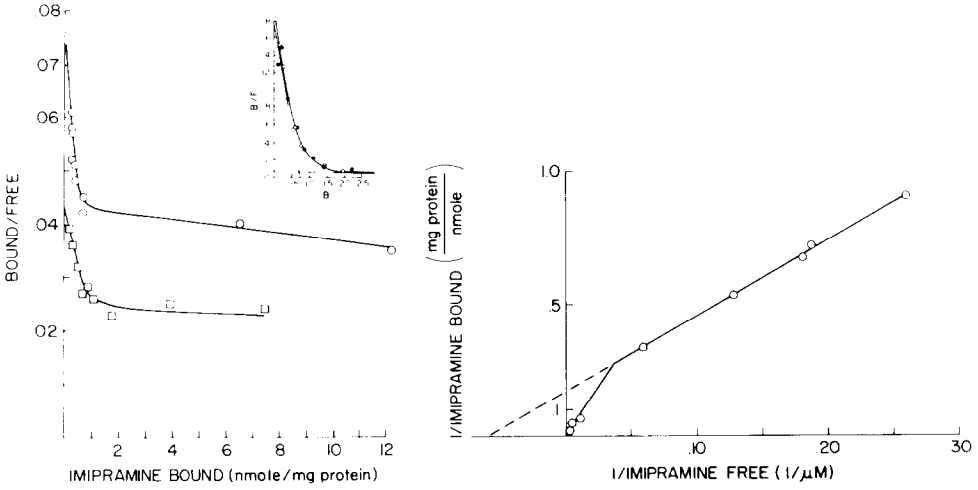
Data of initial experiments depicted two binding components for imipramine in soluble fractions from brain and leukocytes. Scatchard plots were biphasic, showing in both fractions two distinct, homogeneous populations of binding sites with apparent $K_D$'s of 24 µM (brain) and 44 µM (leukocytes), in addition to a binding component of much lower affinity and considerably higher binding capacity (Fig. 1, left graph). The resolution of the data, considering the mutual interference of the components (10), markedly altered the values of the apparent binding constants for the high affinity component, while not affecting low affinity binding (Table 1).

The patterns obtained were markedly similar to published Scatchard plots of drug binding to bovine serum albumin. In the example shown here (Fig. 1. inset) salicylate was bound to 2 classes of sites with $K_D$'s of 40 µM and 6.6 mM (12). Furthermore, it was of interest to note the similarly high affinity of apparent intracellular imipramine binding and of desipramine and methadone transport described in leukocytes (1,2) and synaptosomes (3).

Our suspicion about the validity of these results was raised by the observation that heat treatment had little effect on the high affinity binding component described above. The soluble fraction from brain was exposed to 70° for 20 min and then centrifuged to remove the precipitate. Equilibrium dialysis with the heated material yielded a double-reciprocal plot

depicting a high affinity $K_D$ still in the submicromolar range (Fig. 1, right graph). Further investigation identified a procedural step as the source for the obtained biphasic drug binding. As described above, following equilibrium dialysis the solutions from each compartment were transferred into test tubes prior to the quantitative removal of aliquots for liquid scintillation counting. In samples without protein, a concentration dependent interaction between imipramine and the tube material occurred (Fig. 2).

FIG. 1




Equilibrium dialysis with soluble tissue fractions. In all the experiments shown in this figure, equilibrium dialysis was terminated by transferring the solutions to polypropylene tubes before aliquots were pipetted into scintillation vials for quantitation of radioactivity. The results shown were replicated at least 3 times. The standard deviation around the calculated mean of the affinity constants was 11%. The Scatchard plots (left graph) depict data obtained from equilibrium dialysis performed as described in the text using either Karush dialysis chambers, [$^3$H]-imipramine, and soluble fraction from rat brain (O), or using dialysis bags, [$^{14}$C]-imipramine, and soluble extract prepared from leukocytes (□). The inset depicts a Scatchard plot of salicylate binding to bovine serum albumin in phosphate buffer at pH 7.4 (reproduced by permission from reference 12). The double-reciprocal plot (right graph) represents results of equilibrium dialysis performed with $^3$H-imipramine in Karush dialysis chambers using the soluble brain fraction which had been treated for 20 min at 70° and centrifuged to remove the resulting precipitate.

As a consequence, the difference between the radioactivity from the samples with and without protein was large at lower drug concentrations (Fig. 2, inset). The effect decreased the free imipramine concentration and, thus, mimicked drug binding to the biological material under study. This artifactual binding component was reflected on both Scatchard and double reciprocal plots (Fig. 1).

TABLE 1

Apparent and corrected values of the binding constants

| Preparation | Apparent values | | Corrected values | |
|---|---|---|---|---|
| | $K_D$ (μM) | Bmax (nmole/mg pr) | $K_D$ (μM) | Bmax (nmole/mg pr) |
| Soluble fraction from brain | | | | |
| A. High affinity component | 24 | 1.6 | 0.14 | 0.60 |
| B. Low affinity component | 2400 | 100 | 2415 | 99.4 |
| Soluble fraction from leukocytes | | | | |
| A. High affinity component | 51 | 2.2 | 0.15 | 0.89 |
| B. Low affinity component | 5650 | 140 | 5686 | 139 |

The apparent values were obtained from the respective binding curves. The corrections were implemented according to Neal (10), considering the mutual interference of the two binding components. (Bmax = maximal binding; pr = protein)

The interaction between drug and tube material was prevented by proteinaceous material. Both soluble cellular fractions and bovine serum albumin were effective, and resulted in different amounts of recoverable radioactivity in the two dialysis compartments, i.e., samples with and without protein (Table 2 and Fig. 2). Binding of $^3$H-imipramine to the tube wall occurred rapidly, and long-range incubation added little to the initial effect (Table 2). The phenomenon was observed with three different types of tube surfaces: polypropylene, glass that had been acid washed, and glass initially siliconized according to a procedure described previously (13).

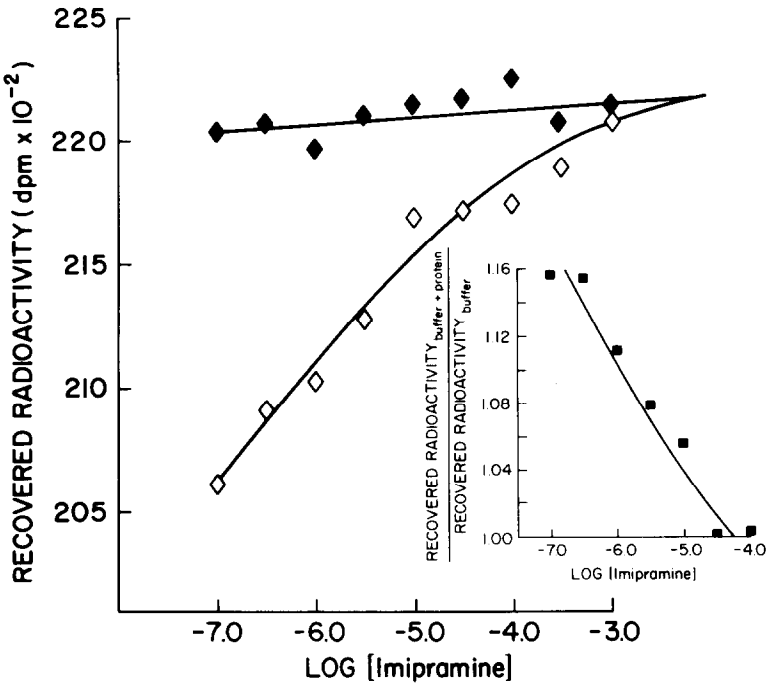
TABLE 2

Recovery of $^{3}$H-imipramine from solutions in the presence and absence of protein

| Experimental conditions | $^{3}$H-Imipramine recovered per aliquot (cpm) | Recovered radioactivity (+ protein) / Recovered radioactivity (- protein) |
|---|---|---|
| Polypropylene tubes, overnight incubation | | |
| Buffer alone | 2334 | --- |
| 0.2 mg/ml SBF | 2511 | 1.076 |
| 0.6 mg/ml SBF | 2526 | 1.082 |
| 1.0 mg/ml SBF | 2528 | 1.083 |
| Acid washed tubes, overnight incubation | | |
| Buffer alone | 2369 | --- |
| 0.2 mg/ml SBF | 2560 | 1.081 |
| 0.6 mg/ml SBF | 2531 | 1.068 |
| 1.0 mg/ml SBF | 2522 | 1.064 |
| Siliconized glass tubes, overnight incubation | | |
| Buffer alone | 2356 | --- |
| 0.2 mg/ml SBF | 2489 | 1.051 |
| 0.6 mg/ml SBF | 2487 | 1.047 |
| 1.0 mg/ml SBF | 2533 | 1.069 |
| Polypropylene tubes, 15 min incubation | | |
| Buffer alone | 3731 | --- |
| 4.0 mg/ml BSA | 3963 | 1.062 |
| Polypropylene tubes, overnight incubation | | |
| Buffer alone | 3654 | --- |
| 4.0 mg/ml BSA | 3941 | 1.079 |
| Mix directly in counting vial, 15 min incubation | | |
| Buffer alone | 4024 | --- |
| 4.0 mg/ml BSA | 4034 | 1.002 |
| Mix directly in counting vial, overnight incubation | | |
| Buffer alone | 3994 | --- |
| 4.0 mg/ml BSA | 4029 | 1.009 |

Solutions were mixed in the containers indicated in order to give a final concentration of 5 µM imipramine and the protein concentrations shown (BSA = bovine serum albumin; SBF = soluble brain fraction). The final volume in the mixing tubes was 1 ml. Three 100 µl aliquots were taken from each tube for liquid scintillation counting. The incubation in counting vials was carried out with 100 µl volumes. The incubations were performed at 4°. Each value shown represents the mean obtained from 3 tubes or vials.

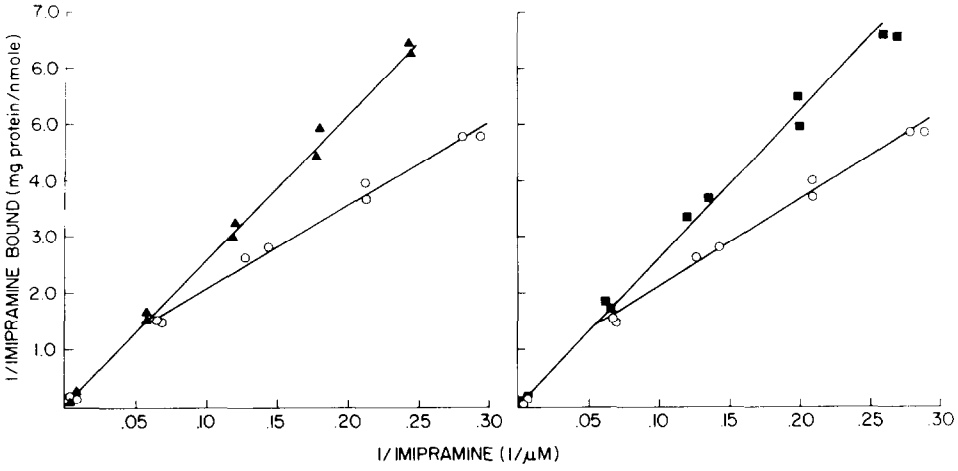
FIG. 2




$^{3}$H-imipramine binding to test tube walls. To a series of polypropylene tubes were added 200 µl buffer (◇) or 200 µl of 8 mg/ml BSA (◆), followed by 160 µl of an appropriate solution of unlabeled imipramine to yield the indicated final concentrations, and 40 µl of 1 µCi/ml $^{3}$H-imipramine. The tubes were then incubated at room temperature for 2 hours. At that time, three 120 µl aliquots were removed from each tube for the determination of radioactivity. Plotted is the measured radioactivity per aliquot against the logarithm of the imipramine concentration. Each point is the mean of triplicate determinations. The average standard deviation around the mean was 1.6%. The inset shows results of an experiment performed exactly as described above except that soluble brain fraction, at 2 mg protein/ml, was used instead of BSA. Plotted are the ratio of radioactivities recovered from the tubes with and without the biological material, against the logarithm of the initial imipramine concentration.

Considering that imipramine and methadone were competitive structural analogues in the active transport system described in leukocytes (1,2), it was our original aim to investigate whether competitive inhibition could be demonstrated during the course of drug binding to soluble cellular fractions. Indeed, in initial experiments the double-reciprocal plots of imipramine binding in the presence of excess methadone were monophasic, suggesting the inhibition of the apparent high affinity binding component (Fig. 3, left graph).

FIG. 3




Double-reciprocal plot of $^{3}$H-imipramine binding: effect of methadone, and correction of artifact. Equilibrium dialysis was performed with the soluble brain fraction and $^{3}$H-imipramine in the Karush dialysis chambers in the presence (▲) or absence (○) of 500 μM methadone (left graph). After dialysis, the solutions were transferred to polypropylene tubes before aliquots were taken for the determination of radioactivity. Shown in the right graph are the data for $^{3}$H-imipramine binding obtained in the absence of methadone before (○) and after correction (■) for the extent of drug interaction with container material, considering the findings depicted in Fig. 2.

However, appropriate control experiments revealed, as in the experiments without competitive drug interaction, the error of that conclusion. Aliquots of either soluble cellular material, bovine serum albumin, or buffer alone were added to polypropylene tubes with or without a solution of methadone that yielded a final concentration of 750 μM. After the addition of equal amounts of $^{3}$H-imipramine, the tubes were incubated overnight at 4°. Aliquots from these tubes were then pipetted into scintillation vials for counting. At 5 μM imipramine, the data indicated a 7.4% difference in counts between the samples containing proteinaceous material and buffer alone. That difference was reduced to 1.8% in the presence of 750 μM methadone. When 400 μM unlabeled imipramine was initially present, the maximal difference in counts was reduced to 1.2% and 750 μM methadone had little additional effect. If the components of the above incubation medium were mixed directly in the scintillation vials in which they were subsequently counted, there was no difference in counts between samples with and without protein.

The artificial binding component was also eliminated by computation. The results of equilibrium dialysis of $^{3}$H-imipramine with soluble cellular fractions were corrected by a factor representing the difference in recoverable radioactivity between samples without and with protein (Fig. 2). Double-reciprocal plots of such revised binding data depicted a single binding component (Fig. 3, right graph) of low affinity and very high capacity, indistinguishable from that obtained for $^{3}$H-imipramine binding in the presence of excess methadone (Fig. 3, left graph). Corresponding Scatchard plots (not shown) indicated the same result. On both of these plots the previously seen high affinity apparent binding component for $^{3}$H-imipramine was absent. A one component non-saturable model was used to calculate empirical binding constants relating bound (nmol/mg protein) to free (nmole/ml) imipramine. The values obtained for the methadone inhibited group (Fig. 3, left graph) and the binding data corrected by computation (Fig. 3, right graph) were indistinguishable at 0.0355 ± 0.0003 ml/mg protein.

Although this work was limited to the use of imipramine and methadone, the nature of the artifact implies that it can occur with any compound capable of binding to plastic material or to glass under similar circumstances. Significant binding to container walls has been reported for methotrexate, promazine, thiopental and other compounds (14-16). The interaction of basic drugs with binding sites on glass-fiber filters has been reported (17,18) and was in the focus of a recent controversy on the biological specificity of the observed phenomenon (19). The appearance of the artifactual binding component, described here, as a distinct population of sites with significant binding affinity carries an impressive potential for misleading interpretations in assessing drug-protein interactions by the widespread use of equilibrium dialysis.

## Acknowledgements

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