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Binding of Drugs with a Quaternary Ammonium Group to *Alpha*-1 Acid Glycoprotein and Asialo *Alpha*-1 Acid Glycoprotein[1]

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ABSTRACT

The interaction of eight mono- and four bisquaternary ammonium compounds with *alpha*-1 acid glycoprotein and its desialylated derivative was investigated. Protein binding was performed *in vitro* by equilibrium dialysis at 37°C. The simple monoquaternary ammonium compounds tributylmethylammonium, tripropylmethylammonium, triethylmethylammonium and procainethobromide were not appreciably bound (unbound fraction > 0.99). Excellent negative correlations of unbound fraction and the log of the octanol/Krebs' partition coefficient were obtained for thiazinamium, N-methylimipramine, N-methyldeptropine and d-tubocurarine, both for *alpha*-1 acid glycoprotein ($r = -0.99$) and asialo *alpha*-1 acid glycoprotein ($r = -0.94$). Bisquaternary ammonium compounds, with the exception of hexafluorenium, were only poorly bound. Our binding data on N-methyl-deptropine, N-methylimipramine and thiazinamium reveal that these compounds are more avidly bound to *alpha*-1 acid glycoprotein than to albumin, in spite of their polar character. The interaction of N-methyldeptropine with *alpha*-1 acid glycoprotein was studied in more detail. Scatchard plots revealed the presence of two classes of binding sites. N-Methyldeptropine could effectively be displaced by imipramine from its high-affinity binding site. This points to the presence of a common high-affinity binding site for tertiary and quaternary ammonium compounds on *alpha*-1 acid glycoprotein.

During the last decade much interest has been focused on the drug-binding properties of plasma proteins not included in the albumin fraction (Brinkschulte and Breyer-Pfaff, 1980; Paxton, 1983). With respect to these biological macromolecules α_1-AGP has been shown to bind lipophilic basic drugs far more avidly than albumin (De Leve and Piafsky, 1981; Glasson *et al.*, 1980) although its binding capacity for these drugs is lower. Moreover, binding of acidic and uncharged drugs to α_1-AGP has also been reported (Milsap and Jusko, 1983; Urien *et al.*, 1982). The liver is thought to be the major site of α_1-AGP synthesis, especially during the so-called acute-phase reaction. However a variety of human tumors also secrete α_1-AGP as well as antigenically cross-reacting glycoproteins (Baumann *et al.*, 1983; Ganz *et al.*, 1983), "indicating" that sources of plasma α_1-AGP may vary in healthy and diseased subjects. Plasma levels of α_1-AGP and other acute-phase reactants are increased severalfold in a variety of nonrelated diseases (Piafsky, 1980); this has been shown to have consequences for the free fraction of certain drugs in plasma (Piafsky, 1980; Abramson, 1982). In addition to binding to α_1-AGP, being a sialic acid-containing glycoprotein, there is also interest in binding of drugs to desialylated or asialoglycoproteins (Robert *et al.*, 1983; El Gamal *et al.*, 1981). In asialoglycoproteins, the terminal sugar of the oligosaccharide domain is galactose instead of sialic acid. Circulating asialoglycoproteins (Marshall and Williams, 1978; Bordas *et al.*, 1981; Sawamura *et al.*, 1981) interact with a highly specific galactose-recognizing receptor (Ashwell and Harford, 1982) situated on liver parenchymal cells. This interaction initiates the very efficient removal of asialoglycoproteins from the circulation. After internalization these ligands are generally trafficked to lysosomes (Bridges *et al.*, 1982) whereupon they are degraded by proteases and glycosidases. Therefore drugs bound to such proteins may in principle be taken up by the liver along with the asialoglycoprotein. This may significantly alter the disposition of such drugs in general and hepatic distribution in particular.

Previously we (Weitering *et al.*, 1975, 1977) and others (Echigoya *et al.*, 1972) observed that, after injection into rats, certain quaternary ammonium compounds accumulated in the hepatic lysosomal fraction. This was demonstrated in subcellular fractionation studies and supported by electron microscopical data (Weitering *et al.*, 1975). One hypothesis to explain this phenomenon involves the endocytosis of such cationic drugs bound to (asialo) glycoproteins by a receptor-mediated mechanism. In this paper we investigate the binding of a series of mono- and bisquaternary ammonium compounds to both α_1-AGP and its asialo derivative. This may shed light on the factors involved in the interaction between quaternary ammonium compounds

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ABBREVIATION: α_1-AGP, *alpha*-1 acid glycoprotein.

and these two types of glycoproteins and also may provide a basis for further studies with respect to the above-mentioned hypothesis. Structures of the quaternary ammonium compounds we used in our investigation are shown in figure 1.

Methods

Chemicals. α_1-AGP was obtained from Sigma Chemical Co. (St. Louis, MO), and pancuronium (Pavulon) and ORG 6368 were from Organon (Oss, The Netherlands). Hexafluorenium was purchased through Mallinckrodt Inc. (St. Louis, MO) and alcuronium (Alloferine) through Hoffmann-La Roche (Mijdrecht, The Netherlands). Imipramine HCl was supplied by the Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands). [^{3}H]-*d*-Tubocurarine (2.35 μCi/μmol) and [^{14}C]procainethobromide (4.15 μCi/μmol) were from New England Nuclear (Dreieichenhain, West Germany). The following quaternary ammonium compounds were synthesized (as iodide salts) according to procedures described by Neef and Meijer (1984b): [^{14}C] triethylmethylammonium (0.19 μCi/μmol), [^{14}C]tripropylmethylammonium (0.24 μCi/μmol), [^{14}C]tributylmethylammonium (0.17 μCi/μmol), N-[^{14}C]methylimipramine (0.15 μCi/μmol) and [^{14}C]thiazinamium (0.06 μCi/μmol). N-[^{14}C]Methyldeptropine (1.21 μCi/μmol) was prepared according to a procedure described by Ruifrok *et al.* (1979). Radiochemical purity was assessed by thin-layer chromatography on Merck silica plates with the solvent system methanol-chloroform (80:20) in which NaBr to 0.5 M was dissolved. All the compounds had >98% radiochemical purity, as determined by this method. All other chemicals were of analytical grade or the best grade available.

Experimental design. α_1-AGP was nonenzymatically desialylated as outlined by Spiro (1960). Sialic acid release was monitored by the thiobarbituric acid assay (Aminoff, 1961) and was virtually complete after 1.5 hr. The asialo compound was extensively dialyzed (3 days) against distilled water and lyophilized (water content about 10%).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (10% running gel) of α_1-AGP and asialo α_1-AGP was performed according to Laemmli (1970), proteins were stained with Coomassie Brilliant Blue R250. These preparations were free of impurities as assessed by this method.

Protein binding experiments were performed at 37°C by equilibrium dialysis in homemade Teflon cells for 17 hr. Control experiments in which no protein was added ascertained that this period was sufficient for all ligands to equilibrate between the two compartments. Spectrapor 1 membranes (Spectrum Medical Industries, Inc., Los Angles, CA) (exclusion limit 6–8 kilodaltons) were sandwiched between the two halves of each cell (compartment volume was 0.9 ml) after they had been thoroughly soaked for 15 min in the following solvents: running tap water, distilled water, 30% ethanol, distilled water and 70 mM sodium phosphate, 150 mM NaCl buffer, pH = 7.35. The same buffer was used to dissolve ligands and proteins. Each ligand concentration (2.27, 22.67 and 226.76 μM) was run in triplicate, asialo α_1-AGP and α_1-AGP were dissolved to 22.67 μM, being the mean physiological plasma concentration of the latter in humans (Romach *et al.*, 1981). In a different series of experiments, equilibrium binding of N-[^{14}C]methyldeptropine to α_1-AGP was studied in the presence and absence of imipramine.

Post dialysis protein concentrations were determined by the method of Bradford (1976) with α_1-AGP and asialo α_1-AGP as standards. These experiments ascertained the absence of protein leakage and osmotic fluid shifts. Nonspecific binding of ligands to the dialysis cell and membrane amounted up to 5%.

Partition experiments were performed in an octanol/Krebs pH 7.4 system as described by Neef *et al.* (1984b).

Pancuronium, ORG 6368, hexafluorenium and alcuronium were assayed by fluorometric procedures described by Kersten *et al.* (1973).

Radioligand samples (400 μl) were mixed with 6 ml of Plasmasol and counted for 10 min in a liquid scintillation counter (Isocap 300; Nuclear-Chicago Corporation, Chicago, IL). Quenching was corrected for by external standardization.

Counting efficiencies of the ^{3}H and ^{14}C samples were 39 and 91%.

Data presentation. Binding parameters were computed by means of the CFT3 multiparameter curve-fitting program (Meites, 1974) modified to our needs. The following equation for noncooperative binding was used:

$$r = \sum_{i=1}^{p} \frac{N_i K_i [D]}{1 + K_i [D]}$$

where r denotes moles of bound ligand per mole of protein, p the number of binding site classes, N_i the number of binding sites with an intrinsic binding constant K_i and $[D]$ the molar concentration of free ligand. Data were fitted for two classes of binding sites.

Results

Procainethobromide and the model compounds we used previously to study hepatic transport of monoquaternary ammonium compounds (Neef *et al.*, 1984b), triethylmethylammon-

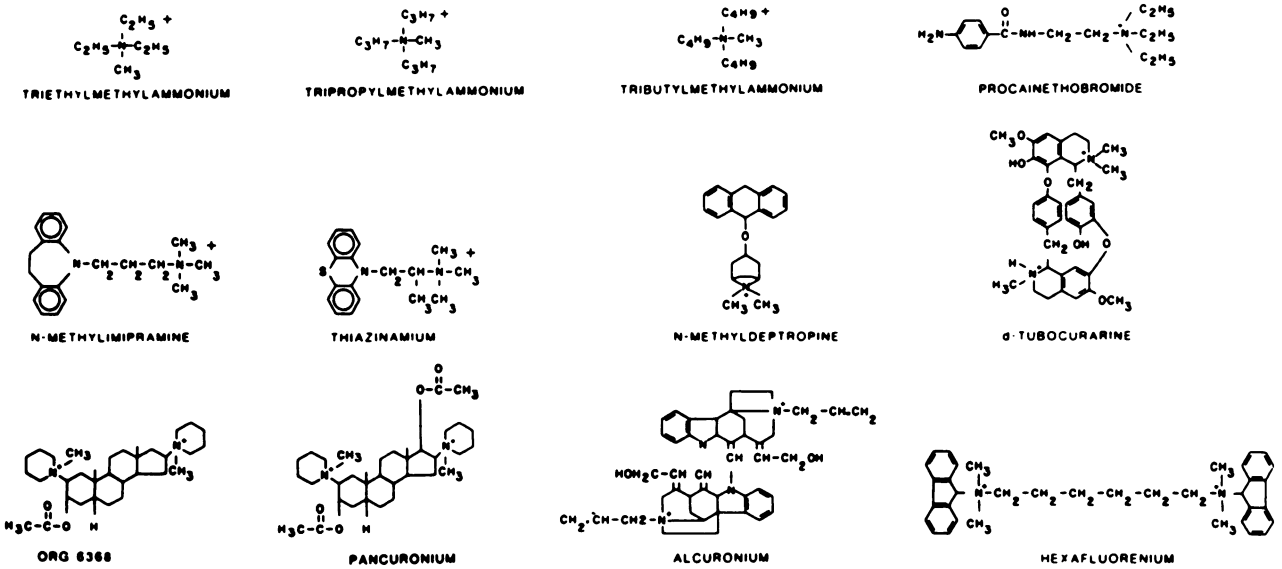

Fig. 1. Structural formulas of the quaternary ammonium ligands.

ium, tripropylmethylammonium and tributylmethylammonium, actually did not bind to asialo α_1-AGP and α_1-AGP at the concentrations used by us. The unbound fraction of this class of highly polar organic cations was always higher than 99%. The results obtained with the other more lipophilic monoquaternary ammonium compounds are summarized in table 1 and figure 2. These data demonstrate dose-dependent binding phenomena for N-methyldeptropine, thiazinamium, N-methylimipramine and *d*-tubocurarine; moreover it is evident from these data that binding of these ligands to α_1-AGP is not sensitive to manipulations carried out at the termini of the oligosaccharide moiety of this glycoprotein. To investigate whether lipophilicity is a determinant in monoquaternary ammonium compound binding, we correlated partition coefficients with the free fraction of N-methyldeptropine, thiazinamium, N-methylimipramine and *d*-tubocurarine. These binding experiments, in which total ligand concentration was equimolar to α_1-AGP or asialo α_1-AGP concentrations, showed a very high negative correlation ($r = 0.99$ and 0.94) between partition coefficient and free fraction within this group (fig. 2). To gain more insight into the binding aspects of monoquaternary ammonium compounds to α_1-AGP, we selected N-methyldeptropine as a representative substance of this class of compounds and investigated binding in a concentration range between 2 and 1000 μM with a fixed α_1-AGP concentration of 22.6 μM. Binding data were transformed according to Scatchard (1949) and plotted in figure 3; in the same figure data are presented from a parallel experiment in which the α_1-AGP solution also contained 25 μM of nonquaternized imipramine. Clearly in both cases interaction between N-methyldeptropine and α_1-AGP involves more than one class of binding sites.

Computer fitting of these data yielded a model with two sets of binding sites: $K_1 = 5.93 \cdot 10^5\ M^{-1}$, $n_1 = 0.40$, and a low-affinity site with $K_2 = 0.18 \cdot 10^5\ M^{-1}$, $n_2 = 1.72$. Experiments in which imipramine was added gave the following binding parameters: $K_1 = 2.20 \cdot 10^5\ M^{-1}$, $n_1 = 0.55$; and $K_2 = 0.14 \cdot 10^5\ M^{-1}$, $n_2 = 1.56$. To substantiate our finding of more than one class of binding sites, inhibition experiments were performed with a fixed N-methyldeptropine concentration (10 μM) and variable concentrations of the inhibitor imipramine. The influence of imipramine on the bound fraction of N-methyldeptropine is shown in figure 4. For a model with a single class of binding sites a smooth log concentration-effect relationship would be expected. However, in these experiments a flattening of the curve around 70 μM could clearly be demonstrated.

Results obtained in binding experiments with the bis-quaternary ammonium compounds hexafluorenium, pancuronium, alcuronium and ORG 6368 to α_1-AGP and asialo α_1-AGP are depicted in table 1. Unbound fractions of these bisquaternary ammonium compounds at the 2.26 μM level could not be reliably detected. However, it is evident from these data that, also for the bisquaternary ammonium compounds, binding behavior toward α_1-AGP is not changed upon desialylation. With the exception of hexafluorenium, which shows remarkably high binding, the polar bisquaternary ammonium compounds are poorly bound at the investigated concentrations.

Discussion

In this paper evidence is presented for the binding of mono- and bisquaternary ammonium compounds to α_1-AGP. It has been shown by others that lipophilicity may play a role in the interaction between α_1-AGP and many tertiary amines such as phenothiazines and tricyclic antidepressants (Paxton, 1983), whereas other investigators emphasized the importance of the structural features of the ligands (El-Gamal *et al.*, 1983). The relevance of both factors is evident from our data on the interaction between quaternary ammonium compounds and α_1-AGP. The excellent correlations ($r = -0.99$ and -0.94) between log P and free fraction (fig. 2) of N-methyldeptropine, d-tubocurarine, N-methylimipramine and thiazinamium emphasizes that also for quaternary ammonium compounds binding strongly depends on lipophilicity. The simple quaternary ammonium compounds we synthesized, triethylmethylammonium, tripropylmethylammonium and tributylmethylammonium, did not bind to α_1-AGP, unbound fraction >0.99 at all concentrations tested. This can be explained in terms of their high ionic character, which is reflected by very low partition coefficients (Neef and Meijer, 1984b). However, the situation is more complicated because hexafluorenium, pancuronium, alcuronium and ORG 6368 had lower partition coefficients than tributylmethylammonium, while they showed considerable binding, up to 64% for hexafluorenium at equimolar concentrations as α_1-AGP. This anomalous behavior can be explained by the presence of condensed ring systems participating in hydrophobic interactions with α_1-AGP.

Removal of the strong negatively charged sialic acid residues does not affect binding of either mono- or bisquaternary ammonium compounds to α_1-AGP, as shown throughout table 1. This phenomenon indicates the absence of electrostatic interactions between the positively charged quaternary ammonium compounds and sialic acid, although interactions with acidic

TABLE 1

Binding of quaternary ammonium compounds to α_1-AGP and asialo α_1-AGP

Values represent mean ± S.E.M., $n = 3$. Protein was present at 22.67 μM, ligands at indicated concentrations.

	Percentage Unbound						Partition Coefficient Octanol/Krebs' NaOH
	2.27 μM		22.67 μM		226.76 μM		
	α_1-AGP	Asialo α_1-AGP	α_1-AGP	Asialo α_1-AGP	α_1-AGP	Asialo α_1-AGP	
N-Methylimipramine	39.2 ± 2.4	40.1 ± 3.4	58.2 ± 3.5	61.8 ± 2.5	84.1 ± 0.7	86.3 ± 0.8	2.28[a]
Thiazinamium	28.8 ± 5.6	29.4 ± 4.3	55.3 ± 3.0	54.7 ± 2.9	76.9 ± 1.5	84.2 ± 0.9	3.48[a]
N-Methyldeptropine	15.4 ± 1.5	15.3 ± 2.4	46.1 ± 3.1	48.0 ± 1.3	87.8 ± 2.6	88.4 ± 3.2	9.86[a]
d-Tubocurarine	82.3 ± 4.8	77.8 ± 4.1	87.6 ± 3.4	88.0 ± 2.4	91.1 ± 2.4	94.9 ± 1.8	0.176
Hexafluorenium			35.8 ± 4.7	43.0 ± 5.4	72.9 ± 1.5	72.9 ± 1.6	0.06
Pancuronium			89.9 ± 4.1	87.8 ± 3.8	90.6 ± 3.4	91.5 ± 2.5	0.013
Alcuronium			64.6 ± 5.8	77.0 ± 8.2	81.7 ± 5.8	90.4 ± 6.1	<0.01
ORG 6368			85.5 ± 3.0	82.2 ± 5.5	93.2 ± 2.5	92.9 ± 1.6	<0.01

[a] Taken from Neef and Meijer (1985).

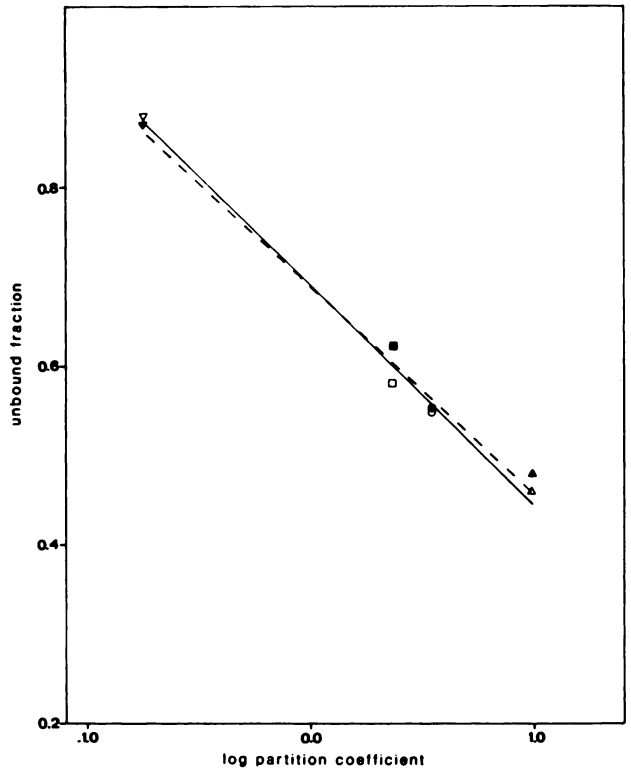

Fig. 2. Correlation plot of log partition coefficient and the unbound fraction of N-methyldeptropine (▲), thiazinamium (●), N-methylimipramine (■) and d-tubocurarine (▼). Closed symbols and solid line refer to α_1-AGP binding, and open symbols and dashed line to asialo α_1-AGP binding. Protein and ligand concentrations were fixed at 22.67 μM.

groups in the protein domain cannot be excluded. Recently, the group of Müller presented evidence for a single high-affinity binding site (El-Gamal *et al.*, 1981) located on a remote hydrophobic part of the protein moiety of the glycoprotein.

The secondary phase in both Scatchard plots very likely does not represent binding to other proteins, because sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) did not reveal contaminating proteins in our α_1-AGP and asialo α_1-AGP preparations. Our data on N-methyldeptropine binding to α_1-AGP rather demonstrate the existence of a high-affinity site and low-affinity site for this monoquaternary ammonium compound. We calculated a high-affinity association constant $5.93 \cdot 10^5$ M^{-1}, which is in the same order of magnitude as values reported for binding of phenothiazines (tertiary amines) to this glycoprotein (Müller and Stillbauer, 1983). Nonintegral values for the number of binding sites in drug binding to α_1-AGP have also been described by others (Abramson, 1982; Denson *et al.*, 1984). This might arise from negative cooperativity in binding of drugs to α_1-AGP. Neef and Meijer (1984b) reported dissociation constants for the binding of N-methylimipramine and thiazinamium to albumin in the millimolar range. If we compare their data with our binding data to α_1-AGP, it illustrates that these monoquaternary ammonium compounds also are more avidly bound to α_1-AGP than to albumin. However, Neef and Meijer (1984b) derived their binding parameters on the presumption that albumin is the sole determinant in binding to plasma; the present study contradicts this assumption. N-Methyldetropine could effectively be displaced from its binding sites on α_1-AGP by unlabeled imipramine as is shown in figure 4; displacement probably occurs through a competitive mechanism. Evidence is provided by our data in figure 3, in which the high-affinity association constant is lowered to one third of its control value, whereas the apparent number of high-affinity binding sites is essentially unchanged. These findings indicate that monoquaternary ammonium compounds and their tertiary amine counterparts might occupy the same binding site, although more experiments have to be performed to substantiate this hypothesis. The high-affinity constant for the interaction between N-methyldeptropine and α_1-AGP indicates that in principle this monoquaternary ammonium compound could be translocated from the circulation as a noncovalent complex along an endocytic pathway into the liver. Further studies are in progress to investigate this interesting phenomenon in more detail.

Caution must be exercised in extrapolating our binding data to the situation in the complex mixture of plasma proteins *in vivo*. Nevertheless, the observed extent of binding of the monoquaternary ammonium compounds to α_1-AGP correlates remarkably well with percentages bound in whole plasma (Neef and Meijer, 1984a). Because, for this class of compounds, affinity for α_1-AGP is much higher than for albumin, it can be predicted that at relatively low concentrations the overall bind-

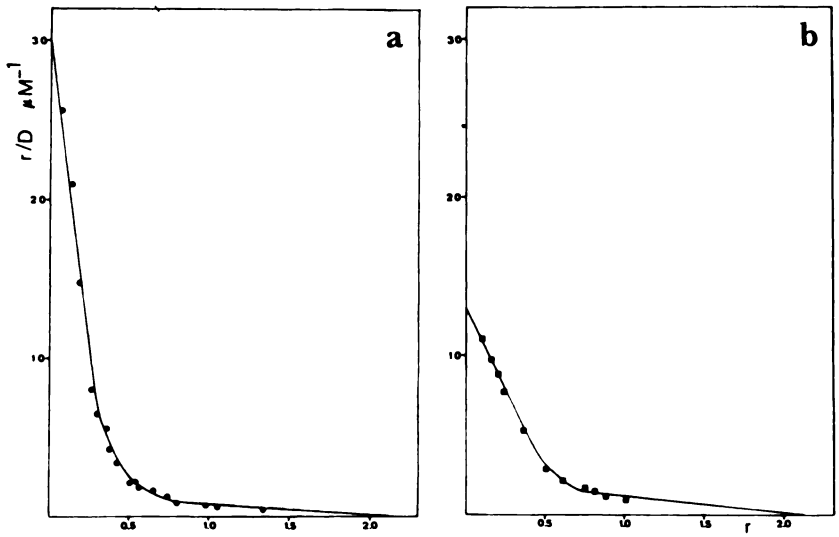

Fig. 3. Scatchard plot of N-methyldeptropine binding to α_1-AGP in the absence (a) and presence of 25 μM imipramine (b).

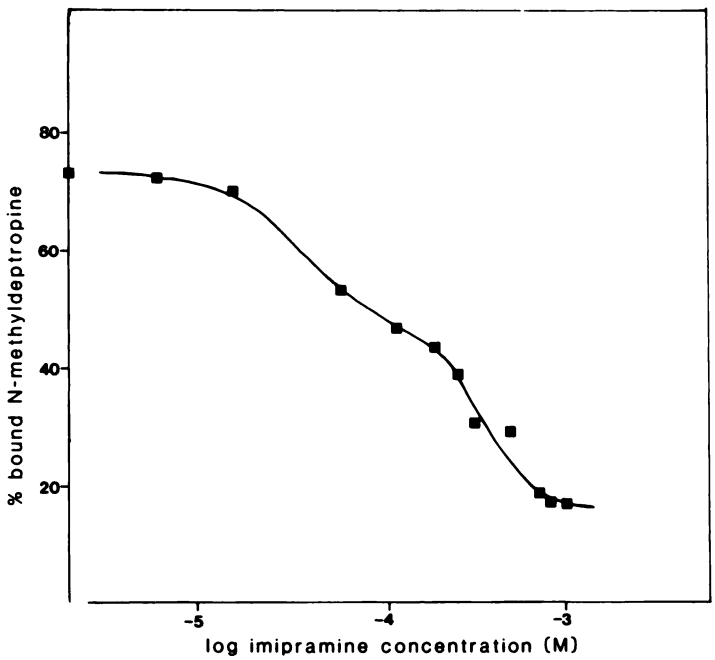

Fig. 4. Relationship between the bound fraction of N-methyldeptropine and the concentration of the displacer imipramine.

ing in plasma will be primarily governed by α_1-AGP. For *d*-tubocurarine the situation seems to be different. At plasma concentrations below 13 μM the bound fraction of *d*-tubocurarine to plasma proteins is twice as high (Meijer *et al.*, 1976) as we obtained for α_1-AGP in the present study. In addition to binding to α_1-AGP, very likely binding to albumin and globulin (Aladjemoff *et al.*, 1958) occurs, indicating a considerable reserve capacity for binding to other proteins than α_1-AGP over a wide range of concentrations.

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