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AROMATIC AMINO ACID METABOLITES AS POTENTIAL PROTEIN BINDING INHIBITORS IN HUMAN UREMIC PLASMA

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Abstract—Decreased binding of aromatic acidic drugs and endogenous metabolites to plasma proteins of patients with severe renal failure appears to be due to accumulation of unknown solutes. Both the warfarin and indole binding sites of albumin, the principal binding protein for these ligands, are affected. We used a large number of endogenous aromatic acids and synthetic congeners as displacers (a) better to characterize the chemical requirements for binding to each site and (b) to derive clues to the chemical structure of the undefined binding inhibitors in uremic plasma. ^{14}C-tryptophan, ^{14}C-warfarin and ^{14}C-salicylate were used as bound ligands.

Numerous indoles, quinolines and phenyl derivatives were moderate to strong displacers with several structural correlates. Increasing apolar side chain length enhanced displacing potency. A hydroxyl group at the 5 position of indoles and at the para position of phenyl derivatives severely reduced activity. The two ends of amphophilic molecules showed opposite requirements for displacement of tryptophan: the greater the polarity at the hydrophilic end, the greater the tryptophan displacing potency. Conversely, the greater the total hydrophobic mass of the remainder of the molecule, the more potent the inhibition of binding. The dipeptides *l*-tryptophyl-*l*-tryptophan and *l*-tryptophyl-*l*-phenylalanine were potent displacers. Computer-assisted analysis of warfarin binding in the presence of xanthurenic acid revealed inhibition by a mechanism other than simple competition, probably via a third albumin binding locus. We conclude that decreased binding in uremic plasma is most likely the summation effect of a number of retained aromatic acids, peptides, or both types of ligands.

Plasma binding of many drugs (phenytoin, salicylate, warfarin) and some endogenous ligands (tryptophan, bilirubin) is reduced in patients with advanced renal failure [1–7]. The chemical basis for reduced ligand binding by uremic plasma is not well understood. Unknown metabolites accumulate in renal failure and impair binding of test ligands [8–10]. We have prepared a potent extract from uremic fluids, which caused impaired binding of phenytoin and tryptophan to normal plasma [11]. This extract contains three or more inhibitory ligands, as demonstrated by anion-exchange chromatography [12]. The extract inhibits the uptake of para-aminohippurate (PAH) by kidney slices and excretion of PAH by the isolated perfused rat kidney [13, 14]. Aromatic acids, including hippurate, appear to be the active components in the extract [12].

Because tryptophan and some of its metabolites are known to bind to one major locus on albumin [15, 16], we tested a large series of tryptophan metabolites and related synthetic compounds as possible binding inhibitors. We also tested a large series of phenyl and phenolic acid metabolites of phenylalanine and tyrosine (and related synthetic compounds) as displacers, because several of these compounds as well as a number of drugs of similar structure are known to bind strongly to serum albumin and to accumulate in uremia [17–21]. ^{14}C-tryptophan, ^{14}C-warfarin and ^{14}C-salicylate were used as bound ligands. These studies provide clues to the possible chemical structure of the uremic binding inhibitors. In addition, by testing series of compounds with systematic structural variations (length and polarity of side chains, location of hydroxyl group on ring structures), new insights have been obtained into the structural requirements for binding of indole, quinoline and phenyl acids to human plasma. Finally, because of its unusual effects, xanthurenic acid has been studied in detail as an inhibitor of warfarin binding, with computer-assisted calculation of binding parameters.

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MATERIALS AND METHODS

Heparinized normal human plasma samples from five to nine subjects were pooled and kept frozen in small aliquots at −70° until used. In the final mixture used for ultrafiltration the albumin content was 40–80 μM. Albumin was measured with the bromcresol green method as previously described [22] except that readings were made 1 min after mixing.

(a) *Binding assay composition.* For studies with multiple indole, phenyl, quinoline and pyridine displacers (Tables 1–3) 0.30–0.34 ml of pooled plasma was mixed with 0.1 ml of test radioactive

ligand, 0.1 ml of unlabelled test ligand, 1.5 ml of displacer in the buffer and sufficient buffer to yield a final volume of 2.5 ml. The concentrations in these studies were 80 μM for albumin, 400 μM for displacers and 41 μM for the sum of labelled plus unlabelled test ligands (salicylate, warfarin and tryptophan). The contribution of endogenous tryptophan was taken to be 1.1 mg/dl [6].

The diluent buffer was a 50 mM mixture of Na_2HPO_4 and NaH_2PO_4 which yielded a final pH of 7.4. Unlabelled sodium salicylate and tryptophan were dissolved in the phosphate buffer. Because of its poor solubility warfarin was first dissolved in 0.1 M NaOH to which was added NaH_2PO_4 to yield a 50 mM phosphate solution with a pH of 7.4. For studies of the displacing effect of 100 to 1200 μM xanthurenic acid on the binding of ^{14}C-warfarin, the total final warfarin concentration for 16 data points ranged from 1.7 to 801 μM. Because xanthurenic acid was much more soluble than warfarin, it was added in 0.15 ml of the standard 50 mM phosphate buffer. Unlabelled warfarin (dissolved as described above) was added in 2.0 ml, mixed with 0.1 ml of ^{14}C-warfarin, 0.15–0.17 ml of pooled plasma and standard phosphate buffer to a final volume of 2.5 ml. The final albumin concentration in these studies was 40 μM.

The 7-^{14}C-salicylic acid (New England Nuclear, Boston, MA) of specific activity 51.7 mCi/mmole, *l*-tryptophan-[side chain-3-^{14}C] (New England Nuclear) of specific activity 51.9 mCi/mmole and α-^{14}C-warfarin (Amersham Corp., Arlington Heights, IL) of specific activity 55 mCi/mmole, were the test ligands (diluted with distilled water), which together with unlabelled test ligand yielded a final concentration of 41 μM. Purity of all ^{14}C ligands, checked by thin layer chromatography, was >98%.

All test ligands and displacers were of the highest purity available and were obtained from Sigma Chemical Co. (St Louis, MO) except for the following: *m*-hydroxybenzoic acid, *o*-hydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, 4-phenylbutyric acid, 5-phenylvaleric acid, 2,6-dihyroxy benzoic acid, and 2-chlorobenzoic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI) and *l*-phenylalanyl-*l*-phenylalanine was obtained from Vega Biochemicals (Tucson, AZ).

(b) *Ultrafiltration and HPLC procedures.* The technique for ultrafiltration has been described in detail [4, 23]. In brief, after three 20 μl samples of the final mixture had been removed for counting, the remaining solution (2.4 ml) was pipetted into a dry cellophane sac, suspended in a 15 ml tapered centrifuge tube, sealed, equilibrated for 35 min and centrifuged at 800 *g* for 60 min at 37°. About 0.4 ml of ultrafiltrate was obtained, of which three 0.1 ml samples were assayed for ^{14}C. The fraction of ^{14}C ligand bound was calculated as the [(total CPM/ml)-(ultrafiltrate CPM/ml)]/(total CPM/ml). Because ^{14}C efficiency was the same for both types of samples, CPM/ml were used in all calculations. Samples were mixed with a xylene based/detergent scintillation mixture and counted in a Beckman Model LS-330 liquid scintillation counter (Irvine, CA).

For octadecylsilane reversed-phase high pressure liquid chromatography (HPLC) we used a 25 × 0.46 cm Beckman RP C-18 Ultrasphere® column with 5 μm particles, a microprocessor controlled Altex–Beckman HPLC pump system and a Beckman UV monitor. Samples were dissolved in the initial mobile phase—10% acetonitrile/0.8% acetic acid. After injection of the sample, the initial mobile phase was pumped for 20 min at 1.0 ml/min. Then a linear gradient was pumped at the same rate over 120 min from the initial mobile phase to 50% acetonitrile/0.8% acetic acid. The eluate was monitored at 254 nm and absorbance was continuously recorded.

(c) *Data analysis.* For purposes of illustration only, the detailed studies of displacement by xanthurenic acid are presented as Scatchard plots (Fig. 1). Binding parameters were estimated using a weighted, non-linear least squares fit of the true independent variable, total warfarin concentration (W_t), to the true dependent variable, bound warfarin concentration (W_b), using a two-site model [24]. Residuals were weighted by dividing them by the total concentration prior to fitting. Fitting was done with the program LIGAND developed by Munson and Rodbard [25] which we modified and recompiled for use with a TRS 80/Model II desk-top computer (Tandy Corp., Fort Worth, TX). The model is derived from the law of mass action for two independent sites and includes a term for non-specific binding which is also fitted. These relationships are summarized as follows:

$$W_t = W_f + W_{b1} + W_{b2} + NS$$

where W_t = total warfarin concentration
W_f = concentration of free warfarin
W_{b1}, W_{b2} = concentration of warfarin at sites 1 and 2
NS = non-specific binding

$$\bar{r} = r_1 + r_2 + NS = \frac{n_1 k_1 W_f}{1 + k_1 W_f} + \frac{n_2 k_2 W_f}{1 + k_2 W_f} + NS$$

where k_1 and k_2 are the apparent association constants for the first and second sites respectively; n_1 and n_2 are the number of sites/mole albumin having the corresponding association constant and r is the ratio of bound warfarin to albumin (mole/mole). Statistical comparisons were made by Student's *t*-test applied to natural log transforms of the fitting parameters and by the Z test [25].

RESULTS

In preliminary studies we tested for inhibition of ^{14}C-salicylate binding by multiple indole, quinoline, phenyl and pyridine compounds under conditions listed in Table 1. At a concentration of 20 μM there was little or no inhibition and at 100 μM half of the compounds showed modest to moderate inhibition. All subsequent studies were done at a displacer concentration of 400 μM.

Tables 1 and 2 give the results of inhibition by tryptophan metabolites of ^{14}C-tryptophan, ^{14}C-salicylate, and ^{14}C-warfarin binding to human plasma. The concentration of labelled probes was made half that of the albumin to test the effect of the various metabolites mainly on the specific strong binding

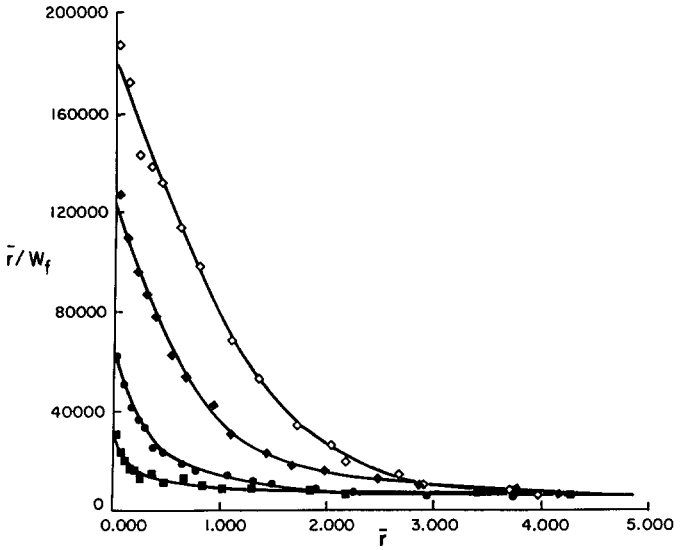

Fig. 1. Scatchard plots for effect of xanthurenic acid on binding of ^{14}C-warfarin to diluted human plasma. Lines were derived with the computer program "LIGAND" [25]. Concentrations of xanthurenic acid were: —◇— (zero), —◆— (100 μM), —●— (400 μM), —■— (1200 μM). Albumin concentration was 40 μM and total warfarin concentration ranged from 1.7 to 801 μM. $\bar{r}$ = moles warfarin bound/mole albumin; W_f = unbound warfarin concentration.

sites for the probes. The terminal group of the side chain attached to the 3 position of the indole ring had a major influence on binding. When the terminal group of the side chain was an uncharged methyl group (melatonin) or was positively charged (tryptamine, gramine) there was little or no inhibitory activity. The number of methylene groups in the side chain was critical, the more groups present the greater the inhibition as shown in the series 3-indole acetate, 3-indole propionate and 3-indole butyrate. Hydroxyl substitution on the 5 position of the indole ring completely abolished activity at the concentrations tested as seen by a comparison of effects on ^{14}C-salicylate binding by 5-OH-3-indole acetate (−1.9%) with 3-indole acetate (−11.2%) and 5-OH-tryptophan (−1.1%) with tryptophan (−6.6%). Effects were similar for tryptophan and warfarin binding. Substitution of a non-polar methoxy group

Table 1. Displacement by indoles of ^{14}C-tryptophan, ^{14}C-salicylate and ^{14}C-warfarin bound to diluted human plasma*

Displacer	Tryptophan	Salicylate	Warfarin
3-Indoxyl sulfate†	−34.4	−17.0	−5.8
3-Methyl indole (skatole)	−21.6	−6.7	−2.0
5-Methoxy-3-indole acetate	−21.9	−11.4	−4.0
3-Indole acetate	−25.8	−11.2	−3.2
3-Indole proprionate	−34.6	−19.7	−4.0
3-Indole butyrate	−36.9	−27.8	−6.2
3-Indole pyruvate	−21.9	−13.6	−4.6
Tryptophan	−20.6	−6.6	(−1.1)
Tryptophol	−22.1	−8.0	−2.1
N-acetyl tryptophan	−26.6	−9.4	−2.0
l-Tryptophyl-*l*-tryptophan	−29.4	−24.8	(+0.2)
l-Tryptophyl-*l*-phenylalanine	−22.2	−26.4	−9.1

* The final total concentration of the labeled probes was 41 μM, of the displacers was 400 μM and of albumin was 80 μM. Mean control binding of probes without displacer was 47.1 ± S.D. 1.7% for tryptophan (N = 42), 80.0 ± S.D. 1.3% for salicylate (N = 41), and 89.8 ± S.D. 0.7% for warfarin (N = 24). Values given (means of duplicates) are the % decrease in binding compared to the control without displacer. All values except those in parenthesis were significantly different from the control ($P < 0.05$) by the Z test.

† At higher concentrations inhibition of salicylate binding increased to −43.0% (2000 μM) and −56.4% (4000 μM) with 3-indoxylsulfate. The following compounds produced minimal or no inhibition: 5-OH-3-indole acetate, 5-OH tryptophan, tryptamine, melatonin and gramine.

Table 2. Displacement by quinoline compounds of ^{14}C-tryptophan, ^{14}C-salicylate, and ^{14}C-warfarin bound to human plasma*

Displacer	Tryptophan	Salicylate	Warfarin
Quinaldate	−26.1	−24.3	−12.5
Kynurenate	−3.7	−16.9	−7.8
Xanthurenate	−3.8	−27.3	−18.8

* Conventions and conditions are as in Table 1.

in the 5 position on the indole ring, however, did not effect activity.

Substitutions in the side chain at the 3 position of the indole ring had variable effects. A positively charged amino group in the alpha position, as in tryptophan, reduced inhibitory potency as compared to the unsubstituted compound. Likewise, a keto group in the side chain, as in 3-indole pyruvate, reduced activity as compared to 3-indole propionate. Finally a bulky substitution, as seen in *N*-acetyl tryptophan, diminished activity.

Although the inhibition of warfarin binding was in general weak, some of the effects of structural changes could also be seen with this probe. All the compounds which showed more than 3% inhibition of warfarin binding showed very strong tryptophan binding inhibition. It is noteworthy that the dipeptide *l*-tryptophyl-*l*-tryptophan produced strong inhibition of tryptophan binding but had no effect on warfarin binding at the same concentration.

Table 2 shows that the quinolines exhibited moderate to marked inhibitory activity. The structure/function relationships for this group were puzzling. Quinaldate, which has no substituents other than the carboxyl group, was strongly inhibitory (−24.3%). Kynurenate, which has a hydroxyl group in the four position, was considerably less active. Finally, xanthurenate which has hydroxyl groups at the four and eight positions was at least as potent as quinaldate in displacing salicylate and warfarin binding. The three pyridine derivatives tested, quinolinate, picolinate and nicotinate, all had only minimal inhibitory activity.

Our studies to date on uremic fluids indicate that acidic phenyl derivatives are present in the binding inhibitory extract of uremic fluids [11, 12]. Also, acidic phenyl derivatives are known to bind to albumin [17, 26]. We, therefore, expanded our studies to include additional phenyl derivatives, the results of which are given in Tables 3a and 3b. When the phenyl ring is without other substitutions, the side chain length correlates positively with inhibition of tryptophan binding. This inhibition increased from −11.8% for benzoate to −37.1%, in the series to 4-phenylbutyrate. Four carbons seem to be near the optimum length of the side chain as no greater inhibition occurred with 5-phenylvalerate. In regard to inhibition of salicylate and warfarin binding, benzoate showed anomalous behavior as its activity was greater than that of phenylacetate. The effect of hydroxyl substitution in the phenyl ring was very much dependent upon its position. The *ortho* hydroxyl substitution of benzoate (salicylate) enhanced inhibition for all three ligands as compared to the unsubstituted parent compound; with −OH in both *ortho* positions very potent inhibition resulted. A similar effect was seen when −OH was in the *ortho* position of phenylacetate and of hippurate.

When the hydroxyl substitution was in the meta position for benzoate, phenylacetate and cinnamate, it reduced inhibition of tryptophan and salicylate binding compared with the parent compound. *Para* hydroxyl substitution for both benzoate and phenyl acetate virtually abolished effects on binding of all three test ligands. When the *para* substituent was a hydrophobic methoxy or phenyl group, inhibition was enhanced compared to benzoate and phenyl acetate. The major urinary catechol metabolite, 4-OH-3-CH_3O-mandelate, was totally inactive.

The carboxylate anion in the side chain was important for activity. Saligenin (*o*-OH-benzyl alcohol) was totally inactive and 2-phenylethanol was inactive against salicylate and warfarin, while weakly active against tryptophan (−6.0%). The dipeptide *l*-phenylalanyl-*l*-phenylalanine showed only moderate inhibition. None of these compounds approached the self inhibition potency of warfarin which was −41.6%.

Table 3a. Displacement by phenyl derivatives of ^{14}C-tryptophan, ^{14}C-salicylate and ^{14}C-warfarin bound to human plasma*

Displacer	Tryptophan	Salicylate	Warfarin
Benzoate	−11.8	−17.6	−7.1
o-OH-Benzoate (salicylate)	−23.4	−31.3	−15.2
o-Cl-Benzoate	−12.8	−15.3	−8.8
m-OH-Benzoate	(−2.4)	−13.0	−10.8
p-CH_3O Benzoate	−26.8	−29.7	−14.1
2,6-Dihydroxy benzoate	−29.2	−38.9	−23.2
Phenylacetate	−22.1	−11.1	−2.9
o-OH-Phenylacetate	−21.2	−15.8	−4.5
m-OH-Phenylacetate	−8.0	−6.0	−1.3
p-CH_3O-Phenylacetate	−31.6	−15.2	−2.8
p-Phenyl-phenylacetate	−25.2	−35.6	−19.2

* Conventions and conditions are as in Table 1. The following compounds produced little or no inhibition: *p*-OH-benzoate, *o*-NH_2-*m*-OH benzoate, 2-phenylethanol, *p*-OH-phenylacetate, 4-OH-3-CH_3O-phenylacetate, *o*-OH-benzyl alcohol.

Table 3b. Displacement by phenyl derivatives of ^{14}C-tryptophan, ^{14}C-salicylate and ^{14}C-warfarin bound to human plasma*

Displacer	Tryptophan	Salicylate	Warfarin
3-Phenylpropionate (hydrocinnamate)	−33.4	−20.0	−4.7
3-Phenylpyruvate	−30.7	−18.0	−4.6
l-3-Phenylacetate	−20.0	−12.0	−2.2
4-OH-3-CH_3O-Cinnamate (ferulate)†	−12.0	−7.4	−3.2
Trans-cinnamate†	−33.8	−30.6	−6.8
m-OH-Cinnamate (*m*-coumarate)†	−21.3	−25.4	−13.0
l-Kynurenine	−24.0	−3.2	(+0.1)
Hippurate	−7.8	−7.0	−2.0
o-OH Hippurate	−21.8	−19.0	−11.5
4-Phenylbutyrate	−37.1	−24.8	−5.8
5-Phenylvalerate	−36.2	−27.2	−5.5
l-Phenylalanyl-*l*-phenylalanine	−9.7	−13.2	−5.3
Warfarin	−15.4	−37.4	−41.6

* Conventions and conditions are as in Table 1. *p*-OH-3-Phenylpyruvate reduced only tryptophan binding (−8.0%).

† Ferulate was in *trans* form; *m*-coumarate was a mixture, predominately in *trans* form.

To evaluate further the relationship of hydrophobic/hydrophilic properties to ligand binding inhibition, we determined the chromatographic retention time of selected displacers during reversed phase chromatography with hydrophobic octadecylsilane particles. For compounds with only a carboxyl group at the ionic end and no *ortho* substitutions*, the order of inhibition of tryptophan binding was the same as the order of elution (in minutes) from the reversed phase column (slope = −0.429, $r = 0.95$). For three compounds with *ortho* substitution†, there was an inverse correlation between binding inhibition and elution time (slope = 0.26, $r = 0.80$).

Figure 1 shows the effects of increasing concentration of the displacer xanthurenic acid on the binding of warfarin by dilute human plasma. The highest line represents the average of two control studies of warfarin alone without displacer. It appears that the control curve consists of two segments and a nonspecific binding component in order from left to right. Increasing quantities of xanthurenic acid produced a shift to the left of the initial slope and a steepening of the slope. Table 4 gives the computer-fitted parameters for studies illustrated in Fig. 1. The computed binding parameters for warfarin alone are in good general agreement with published results of others for serum albumin under similar conditions [2, 27]. The most striking effect of increasing concentrations of xanthurenic acid was that the parameter N1 (the number of the first class of binding sites) progressively declined to near zero. In contrast, the apparent association constant for the first binding site, K_1, increased from 1.36 to 3.92×10^5 L/mole. This increase, however, was shown not to be significant as none of the four curves showed a significant worsening of fit (i.e. a significant increase in the residual sum of squares) when K_1 was fixed at the average value of 2.21. An attempt to hold N1 constant led to significant degeneration of curve fitting using the least squares technique. Xanthurenic acid had only minor and irregular effects on the computed parameters for the second binding loci.

* *p*-OH benzoate, *p*-OH phenylacetate, benzoate, 3-phenylpropionate, 4-phenylbutyrate, 5-phenylvalerate.

† 2,6-dihydroxybenzoate, salicylate, *o*-Cl benzoate.

DISCUSSION

Acidic, aromatic drugs bind to one or two specific binding sites on serum albumin. One of these, designated Site I by Sudlow *et al.* [28, 29], has also been called the warfarin/azapropazone binding site. The other locus, Site II, has also been called the indole/benzodiazepine binding site. Many drugs including salicylate bind to both sites.

Considerable evidence suggests that retention of one or more small to intermediate size ligands accounts for abnormal albumin binding in uremia [8, 9, 11]. The concentration in plasma of innumerable organic compounds is increased in patients with advanced kidney failure [32, 33]. Because of the paucity of information in the published literature concerning the potential for interaction of serum albumin with these numerous organic solutes we undertook the present study. The aromatic acids seemed to be particularly likely candidates as binding displacers in uremia.

We studied effects on whole human plasma rather than on albumin preparations for several reasons. The aromatic acids we planned to use as probes bind almost exclusively to albumin among plasma proteins [34–36]. Several constituents of normal plasma which are bound to albumin and determine its binding properties (fatty acids, bilirubin, calcium) are removed during purification procedures. Even the mildest methods for purifying albumin from plasma lead to subtle alterations in some of its properties and results, therefore, may not apply to albumin *in vivo* [37]. We have found marked variations in the displacement by our uremic extract of ^{14}C-tryptophan from four different albumin preparations [11]. By using human plasma and a low ratio of the probe ligands to albumin we believe that the most

Table 4. Effect of xanthurenic acid on parameters for binding of ^{14}C-warfarin to diluted human plasma[a]

	$k_1 \times 10^5$		$k_2 \times 10^4$		n_1		n_2		NS
[b]Warfarin alone	1.36 ± 0.29	*	1.60 ± 1.16	*	1.17 ± 0.33	*	1.28 ± 0.21	*	0.11 ± 0.02
[b]Warfarin + 100 μM Xanthurenic acid	1.34 ± 0.19		0.56 ± 0.41		0.81 ± 0.12		2.04 ± 1.04		0.12 ± 0.05
		c		*		f		*	
[b]Warfarin + 400 μM Xanthurenic acid	2.20 ± 0.34		0.81 ± 0.27		0.23 ± 0.04		1.54 ± 0.35		0.13 ± 0.02
		e		*		c		*	d
Warfarin + 1200 μM Xanthurenic acid	3.92 ± 0.22		1.10 ± 1.03		0.06 ± 0.03		0.61 ± 0.26		0.23 ± 0.04

[a] k_1 and k_2 are the apparent association constants (L/mole) for the first and second sets of sites; n_1 and n_2 are the number of each type of site per albumin molecule; NS is the non-specific binding. Albumin concentration was 40 μM in all studies. Warfarin concentration ranged from 1.7 to 801 μM. Numbers are means ± approximate standard errors.

[b] Data were computed from averages of two studies.

c = $P < 0.05$; d = $P < 0.02$; e = $P < 0.005$; f = $P < 0.001$; * = not significant.

valid insight into binding displacement from native albumin was obtained. The effects of non-specific binding to other proteins should be minimal. Neither in this study using warfarin nor in a parallel study with salicylate [38] was there any evidence that saturation of the non-specific binding of these probes occurred even at extremely high concentrations.

For our initial set of screening studies we chose salicylate because it binds to both albumin sites I and II. In addition to providing clues to the nature of the inhibitory solutes in uremic plasma a major advantage of the study of these natural constituents and related derivatives was that homologous series could be studied which would also provide insights into the configurations required for binding to serum albumin. A number of insights into chemical: functional relations have been obtained. The length and the nature of the side chain which generates activity for both indole and phenyl derivatives has been shown. The optimum configuration is a combination of a carboxyl group at the end of a side chain of up to four methylene groups attached to a purely hydrophobic ring. The ligands which best fit this ideal configuration were 3-indole butyrate and 4-phenyl butyrate. Both of these compounds were potent displacers of both tryptophan and salicylate.

A configuration which is optimal for the indole binding site (Site II) has been suggested by studies of drugs and synthetic compounds by Sudlow *et al.* [28, 29]. Most drugs which have been shown to be specific for Site II are more complex than those we studied and consist in many cases of two or more ring structures. It is, therefore, of interest that *p*-phenyl-phenyl acetate and the dipeptides *l*-tryptophyl-*l*-tryptophan and *l*-tryptophyl-*l*-phenylalanine, whose molecular weights fall in the range of the postulated uremic "middle molecule" toxins [39], were potent displacers of both tryptophan and salicylate. These results also show that the hydrophobic crevice is flexible, accommodating to the size of the bound ligand.

An absolute requirement for a hydrophobic structure in the *para* position of phenyl compounds and in the five position of indoles was demonstrated. This finding supports the hypothesis that one end of these amphophiles [40] fits into a hydrophobic region either within one of the loops or in a crevice between adjacent loops [41, 42]. A positive charge on the protein at the entrance to such a crevice precludes the binding of any ligand which bears only a positive charge at the other end, such as tryptamine and gramine. The inverse chemical: functional requirements at the two ends of these amphophiles for inhibition at the indole site was confirmed in studies correlating displacement of ^{14}C-tryptophan and elution sequence during reversed phase HPLC.

The parallel pattern for displacement of tryptophan and salicylate confirm that the indole site is one of the major loci for binding of salicylate [43]. When a displacer showed a stronger effect on salicylate than on tryptophan binding, the compound also showed significant displacement of warfarin. Examples of this pattern were seen with kynurenate, xanthurenate, benzoate, salicylate, *m*-coumarate and, as to be expected, warfarin. Thus the other binding locus for salicylate is at or near the warfarin site. In a separate line of studies in our laboratory we have found that under similar conditions computer analysis of the binding of salicylate fits a model with one dominant class of binding sites of which there are two per mole of albumin with K_1 of 3×10^4 L/mole [38].

Particularly interesting were the effects of the quinolines, which to our knowledge have not been previously investigated as binding inhibitors. All three of the quinolines tested had moderate to substantial ability to displace warfarin. Quinaldate also was a potent displacer of tryptophan, which is not surprising as it fulfills the criteria given above for the indole site. The addition of a hydroxyl group in the four position in kynurenate almost totally destroyed affinity for the tryptophan site; in fact, the residual displacing ability probably reflected displacement of tryptophan bound to the warfarin site. The addition

of a second hydroxyl group in the eight position for xanthurenate resulted in a potent displacer of the warfarin site. This result was most unexpected for a compound with polar groups at both ends (cf. 5-OH-3-indole acetate). The detailed studies of the xanthurenate:warfarin interaction clarified this paradox.

With increasing concentrations of xanthurenic acid there is an effect both on the slope and the projected intercept on the horizontal axis for the binding isotherms (Fig. 1). This pattern indicates that the displacement of warfarin is not one of simple competition for the first binding site. The locus to which xanthurenic acid binds seems clearly different from either the indole or the warfarin sites. Such a third site has been postulated by Sudlow *et al.* [28] based on her extensive studies of dansylamino acids.

We have shown that hippurate is one component of our binding inhibitory extract of uremic sera [12]. Although the intrinsic inhibitory activity of hippurate is relatively weak (Table 3b), hippurate is the most abundant aromatic organic acid in normal human urine [44] and accumulates to high concentrations in uremic serum [45]. Hippurate's effect in uremia could be appreciable if the product of its concentration and intrinsic activity is sufficient. Another likely uremic inhibitor is indoxyl sulfate. These studies and those of Bowmer and Lindup [46] indicate that it is a potent inhibitor of the indole binding locus. We have recently developed and applied an ion pairing HPLC method to plasma of patients with advanced uremia. The mean indoxyl sulfate level we found of 167 ± 69 (S.D) μmole/L [45] should yield at least modest binding inhibition. The metabolites yielding most of the binding inhibition in uremia remain, however, to be identified.

The concentrations of a number of other aromatic acids, although shown to be markedly elevated in uremic compared to normal plasma, are very low compared to the concentration of albumin of 0.6 mM [47]. Other uremic binding inhibitors, therefore, would have to be much more potent than hippurate and indoxyl sulfate. Alternatively, the total concentration and potency of a large number of ligands may be sufficient to have a significant effect. One of these compounds may be 2-hydroxybenzoylglycine (salicyluric acid), which was recently isolated from uremic serum [48]. The importance of this finding, however, cannot be determined until the absolute concentrations of the compound have been determined and aspirin (acetylsalicylic acid) has been rigorously ruled out as its source.

Finally, some of these small aromatic acids, especially the phenolic acids, may react covalently with albumin thereby altering its binding properties [49]. Covalent binding of one phenolic acid, acetylsalicyclic acid, has been clearly shown [50].

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REFERENCES

1. M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.* **57**, 895 (1968).
2. U. Kragh-Hansen, *Pharmac. Rev.* **33**, 17 (1981).
3. M. M. Reidenberg, I. Odar-Cederlof, C. Bahr, O. Borga and F. Sjoquist, *New Engl. J. Med.* **285**, 264 (1971).
4. A. De Torrente, G. B. Glazer and P. F. Gulyassy, *Kidney Internat.* **6**, 222 (1974).
5. P. F. Gulyassy and T. A. Depner, *Dial. Transpl.* **8**, 19 (1979).
6. P. F. Gulyassy, J. H. Peters and P. Schoenfeld, in *Uremia: An International conference on Pathogenesis, Diagnosis and Therapy* (Eds. R. Kluthe, G. Berlyne and B. Burton), p. 163. Thieme, Stuttgart (1972).
7. M. M. Reidenberg and M. Affrime, *N. Y. Acad. Sci.* **226**, 115 (1973).
8. I. Sjoholm, A. Kober, I. Odar-Cederlof and O. Borga, *Biochem. Pharmac.* **25**, 1205 (1976).
9. W. A. Craig, M. A. Evenson, K. P. Sarver and J. P. Wagnild, *J. Lab. clin. Med.* **87**, 637 (1976).
10. T. A. Depner, L. A. Stanfel, E. A. Jarrard and P. F. Gulyassy, *Nephron* **25**, 231 (1980).
11. T. A. Depner and P. F. Gulyassy, *Kidney Internat.* **18**, 86 (1980).
12. P. F. Gulyassy, A. T. Bottini, E. A. Jarrard and L. A. Stanfel, *Kidney Internat.* **26**, (*Suppl* 16), S238 (1983).
13. T. A. Depner, *Kidney Internat.* **20**, 511 (1981).
14. T. A. Depner, T. Sanaka and L. A. Stanfel, *Am. J. Kidney Dis.* **III**, 280 (1984).
15. R. H. McMenamy, *J. biol. Chem.* **239**, 2835 (1964).
16. R. A. McMenamy, in *Albumin Structure, Function and Uses* (Eds. V. M. Rosenoer, M. Orsatz and M. A. Rothschild), p. 143. Pergamon Press, Oxford (1977).
17. C. Davison and P. K. Smith, *J. Pharmac. Exp. Ther.* **133**, 161 (1961).
18. J. Steinhardt and J. A. Reynolds, in *Multiple Equilibria in Proteins*, p. 310. Academic Press, New York (1969).
19. A. Kober and I. Sjoholm, *Molec. Pharmac.* **18**, 421 (1980).
20. A. Saito, T. Niwa, K. Maeda, K. Kobayashi, Y. Yamamoto and K. Ohta, *Am. J. clin. Nutr.* **33**, 1402 (1980).
21. T. Niwa, T. Ohki, K. Maeda, A. Saito, K. Ohta and K. Kobayashi, *Clin. Chem. Acta.* **96**, 247 (1979).
22. B. T. Doumas, W. A. Watson and H. C. Biggs, *Clinica chim. Acta* **31**, 87 (1971).
23. K. M. Lunde, A. Rane, S. J. Yaffe, L. Lund and F. Sjoquist, *Clin. Pharmac. Ther.* **11**, 846 (1970).
24. J. T. Edsall and J. Wyman, in *Biophysical Chemistry*, p. 610. Academic Press, New York (1958).
25. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
26. J. M. Luck and A. S. Schmit, *Stanford med. Bull.* **6**, 133 (1948).
27. R. A. O'Reilly, *J. clin. Invest.* **48**, 193 (1969).
28. G. Sudlow, D. J. Birkett and D. N. Wade, *Molec. Pharmac.* **11**, 824 (1975).
29. G. Sudlow, D. J. Birkett and D. N. Wade, *Molec. Pharmac.* **12**, 1052 (1976).
30. I. Sjoholm, B. Ekman, A. Kober, I. Ljungstedt-Pahlman, B. Seiving and T. Sjodin, *Molec. Pharmac.* **16**, 767 (1979).
31. K. J. Fehske, W. E. Muller and U. Wollert, *Biochem. Pharmac.* **30**, 687 (1981).
32. F. C. Senftleber, A. G. Halline, H. Veening and D. A. Dayton, *Clin. Chem.* **22**, 1522 (1976).
33. J. Menyhart and J. Grof, *Clin. Chem.* **27**, 1712 (1981).
34. R. C. Reynolds and L. E. Cluff, *Bull. Johns Hopkins Hosp.* **107**, 278 (1960).
35. F. Fadda, G, Biggio and G. Liguori, *Experientia* **30**, 635 (1974).

36. O. Odar-Cederlof and O. Borga, *Clin. Pharmac. Ther.* **20**, 36 (1976).
37. J. Janatova, *J. Med.* **5**, 149 (1974).
38. S. Roman, P. F. Gulyassy and T. A. Depner, *Am. J. Kidney Dis.* **IV**, 153 (1984).
39. J. Bergstrom and P. Furst, *Clin. Nephrol.* **5**, 143 (1976).
40. C. Tanford, in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, p. 12. John Wiley and Sons, New York (1973).
41. J. R. Brown, in *Albumin Structure, Function and Uses* (Eds. V. M. Rosenoer, M. Oratz and M. A. Rothschild), p. 27. Pergamon Press, Oxford (1977).
42. J. Peters and R. G. Reed, *FEBS* **50**, 11 (1977).
43. J. N. McArthur, P. D. Dawkins and J. N. Smith, *J. Pharm. Pharmac.* **23**, 393 (1971).
44. R. A. Chalmers and A. M. Lawson, in *Organic Acids in Man* (Eds. Chapman and Hall, Ltd.), p. 181. University Press, Cambridge, London (1982).
45. P. Gulyassy, P. Igarashi, L. Stanfel, E. Jarrard and T. Depner, *Abstr. Am. Soc. Nephrol 17th Ann. Meeting* (1984).
46. C. J. Bowmer and W. E. Lindup, *Biochem. Pharmac.* **31**, 319 (1982).
47. V. R. Bajaj, B. B. Garg, A. S. Saini, P. S. Bhatt, P. C. Singh and I. D. Singh, *Indian J. med. Sci.* **27**, 235 (1973).
48. D. M. Lichtenwalner and B. Suh, *J. clin. Invest.* **71**, 1289 (1983).
49. S. W. Boobis, *Clin. Pharmac.* **22**, 147 (1977).
50. D. Hawkins, R. N. Pinckard, I. P. Crawford and R. S. Farr, *J. clin. Invest.* **48**, 536 (1969).
51. P. F. Gulyassy and T. A. Depner, *Am. J. Kidney Dis.* **II**, 578 (1983).