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CONTRIBUTIONS OF HIPPURATE, INDOXYL SULFATE, AND *o*-HYDROXYHIPPURATE TO IMPAIRED LIGAND BINDING BY PLASMA IN AZOTEMIC HUMANS

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Abstract—We have evaluated pH, chloride, calcium and several endogenous aromatic acids as possible causes of the impaired binding of drugs by plasma albumin in renal failure. Changes in pH, chloride and calcium over the range found in renal failure had minimal or no effects on the binding of [^{14}C]salicylate, a model probe which binds to both of the major drug-binding loci of human albumin. Hippurate and indoxyl sulfate were weak inhibitors of binding by normal plasma. Ortho-hydroxyhippurate was undetectable or minimally elevated, except among patients with elevated plasma salicylate concentration. Although plasma hippurate and indoxyl sulfate concentrations were elevated markedly in patients with renal failure, inhibition of salicylate binding was significantly correlated only with the concentration of indoxyl sulfate. Addition of hippurate and indoxyl sulfate separately and together to normal plasma showed that these ligands could account for only 15% of the impaired binding of salicylate by azotemic plasma. The retained solutes which account for most of this binding defect remain to be identified. This uremic disorder (and perhaps others) is due not to a single chemical but to the additive effect of a family of chemicals.

Impaired plasma protein binding of acidic, aromatic drugs and endogenous metabolites is a consistent finding among patients and experimental animals with severe renal failure [1–3]. Substantial evidence for the hypothesis that retained azotemic solutes, which also bind to the plasma proteins, account for this disorder has been reported by us and several other investigators [4–6]. The nature of these solutes had remained poorly defined until recently. We have prepared an extract from azotemic body fluids which appears to contain aromatic acids as the active component and generates the binding abnormality when added to normal plasma or plasma albumin [7]. In addition, we have shown that these aromatic acids are secreted by the isolated perfused rat kidney and are present in normal urine [8–10]. To date we have isolated and rigorously determined the structures of three of these ligands: hippuric, β-(*m*-hydroxyphenyl)-hydracrylic and *p*-hydroxyphenylacetic acids. Of these, hippuric acid is the most abundant [9, 11]. Another likely candidate as an azotemic binding inhibitor is indoxyl sulfate, which binds strongly to plasma albumin and accumulates to high levels in uremia [12–14]. Finally, *o*-hydroxyhippurate has been identified in azotemic plasma and could be an additional contributor to the binding defect [15].

Clear proof of the role of any of these aromatic acids in the azotemic binding defect would require several pieces of evidence. The concentrations of the purported inhibitors must be measured in azotemic plasma and correlated with the binding of model probes. The effect should be reproducible by addition of the ligands individually and together to normal plasma. Finally, the extent to which the binding defect of azotemic plasma can be attributed to the aggregate effect of all the inhibitors known to date should be determined. These studies were designed to provide such evidence.

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MATERIALS AND METHODS

Salicylate binding assay. Heparinized plasma was obtained in the fasting state from normal volunteers and from patients with renal failure, with informed consent according to standards of the Human Subjects Committee of the University of California, Davis. The plasma samples were diluted with buffer solutions as detailed below to achieve identical concentrations of albumin in control and test samples. To 2.25 ml of normal control plasma, modified normal plasma, and plasma from azotemic patients, we added unlabeled salicylate and [^{14}C]salicylate as the test probe. The final total salicylate concentrations was 6.3 μM, with 1.2 μmol/l due to the [^{14}C]-salicylate. A very low salicylate : albumin ratio was used to demonstrate effects at the primary, high affinity binding sites. In addition, concentrated solutions of modifiers were added to achieve wide ranges of concentrations of pH, calcium, chloride, hippurate and indoxyl sulfate for specific studies of normal and azotemic plasma. The total volume of all the solutions added to the diluted plasma was 0.125 ml. The concentration of unbound [^{14}C]salicylate was determined in an ultrafiltrate of the mixture, obtained by centrifugation at 800 *g* for 60 min in cellophane sacs at 37° as previously described [13, 16], and percent bound was calculated.

Effects of pH, chloride and calcium. For study

of the possible effects of abnormalities of these variables, wide ranges of pH, chloride and calcium were generated in both normal and azotemic plasma. Plasma pools were made from three to five normal and azotemic plasma samples for the separate studies. The plasma pool from the normal subjects, which was used in all studies, had initial concentrations of 108 mEq/l of chloride, 17 mg/dl of urea nitrogen, 1.0 mg/dl of creatinine and 4.51 g/dl of albumin. To 1.625 ml of this pool various amounts of water and 0.5 M NaCl were added to achieve final chloride concentrations of 74–112 mEq/l and an albumin concentration identical to that of the azotemic pool. After addition of salicylate, binding was determined.

To determine the effect of abnormal chloride concentrations in azotemia, a plasma pool was made from patients with hypochloremia. The concentrations in the pool were 81 mEq/l of chloride, 22.5 mg/dl of creatinine, 246 mg/dl of urea nitrogen and 2.82 g/dl of albumin. Water or concentrated stock solutions of NaCl (0.6 to 1.8 M) were added to the plasma pool to achieve final chloride concentrations of 77–106 mEq/l.

In the study of the effects of calcium on binding, concentrations in the azotemic plasma pool were: calcium, 6.1 mg/dl; creatinine, 17.6 mg/dl; urea nitrogen, 138 mg/dl; and albumin, 3.20 g/dl. To achieve identical albumin concentrations the normal plasma pool was diluted with a buffer solution containing 25 mmol $NaHCO_3$ and 110 mmol NaCl per liter. Concentrated solutions of $CaCl_2$ were then added to both plasma pools to achieve ranges of 5.8 to 14.9 and 6.0 to 15.1 mg calcium/dl. After standing at room temperature for 1 hr, salicylate was added and binding was determined.

The effect of pH was studied in ambient air with azotemic and normal plasma pools, initial pH values of which were 7.93 and 7.92. The initial concentrations for the azotemic pool were 88 mg/dl for urea nitrogen, 11.7 mg/dl for creatinine and 3.41 g/dl for albumin. The lowest pH of 6.0 was achieved by addition of 1.0 N HCl followed by gentle aeration and use of vacuum. This step required 0.55 ml of acid/ml for normal plasma and 0.38 ml/ml for azotemic plasma. To achieve higher values of pH ranging from 6.5 to 8.5 at a constant concentration of sodium, appropriate volumes of 1.0 M NaOH and 1.0 M NaCl were added. The total volume of base and salt added was 30 μl for the patient pool and 40 μl for the normal pool. After addition of salicylate, binding was determined.

Effects of aromatic acids on salicylate binding by normal plasma. We tested the effects on salicylate binding of hippurate, indoxyl sulfate and *o*-hydroxyhippurate added to normal plasma by augmenting the low concentrations of these aromatic acids in normal plasma to the range found in patients with a wide range of azotemia. To simulate the hypoalbuminemia found in the azotemic patients, normal plasma pools were diluted to 3.0 g/dl of albumin by adding buffer consisting of 1.25 mmol $CaCl_2$, 110 mmol NaCl, and 25 mmol $NaHCO_3$ per liter. Concentrated solutions of the aromatic acids and salicylate were added, the mixture was incubated for 1 hr at room temperature, and binding was determined. Studies at each concentration were done in duplicate. The albumin concentration of the final mixture was 2.84 g/dl, comparable to the albumin concentration of the azotemic plasma samples whose endogenous content of these aromatic acids had been measured (see below).

Hippurate, indoxyl sulfate, p-hydroxyphenylacetate, and o-hydroxyhippurate concentrations in azotemic plasma. We used previously developed, specific reversed-phase HPLC methods to measure hippurate and indoxyl sulfate in plasma samples [14, 17]. We initially attempted to determine *o*-hydroxyhippurate using an octadecylsilane column and aqueous acetonitrile buffered to pH 3.0 as the eluant. Tryptophan seriously interfered with this method as it overlapped with *o*-hydroxyhippurate and the elution position of its broad, tailing peak varied widely depending on its concentration. We succeeded in eliminating the tryptophan interference by adding 5 mM sodium 1-octanesulfonate, which markedly delayed the elution of tryptophan by an ion-pairing effect. The aqueous eluant in addition contained 9% acetonitrile and 0.8% acetic acid. Plasma was deproteinized by adding 0.75 ml of acetonitrile to 0.5 ml of plasma followed by centrifugation for 20 min at 8000 *g*. A 0.75-ml aliquot of the supernatant fraction was dried with a stream of nitrogen. The residue was dissolved with 0.3 ml of the eluant, and 0.050 ml was injected into the chromatographic system. Recovery of *o*-hydroxyhippurate was evaluated for this method by determining its concentration in plasma before and after addition of known quantities of pure standard.

The HPLC method for measuring hippurate allowed us to make an estimation of the concentration of *p*-hydroxyphenylacetate, which eluted about 1 min later than hippurate. The minimal concentration of *p*-hydroxyphenylacetate which could be detected in normal plasma was 15 μmol/l, which gave a deflection of about 20,000 area units.

Combined effect of hippurate and indoxyl sulfate: Normal vs uremic plasma. To test for possible additive effects of these aromatic acids, nine pairs of normal : severely azotemic plasma samples were prepared by diluting the normal plasma with water and concentrated solutions of hippurate and indoxyl sulfate. As a result, the concentrations of albumin, hippurate and indoxyl sulfate were identical for each pair. Salicylate binding of each pair as well as that of the unmodified, diluted normal plasma control were determined. The deviation in binding from the unmodified normal control was calculated.

Miscellaneous. Albumin was determined by the method of Doumas [18] and salicylate by a modification [19] of the method of Trinder. The [7-^{14}C]salicylate, from New England Nuclear, Boston, MA, had a specific activity of 53.8 mCi/mmol. The unlabeled aromatic acids were purchased from the Sigma Chemical Co., St. Louis, MO. The cellophane tubing used for ultrafiltration was Spectrapor No. 4, from Spectrum Medical Industries Inc., Los Angeles, CA. The chromatographic system consisted of a 4.6 × 250 mm Beckman Ultrasphere octadecylsilane column (Beckman Instruments Inc., Berkeley, CA), a 4.6 × 30 mm guard column (type OD-GU, Brownlee Laboratories, Santa Clara, CA), a

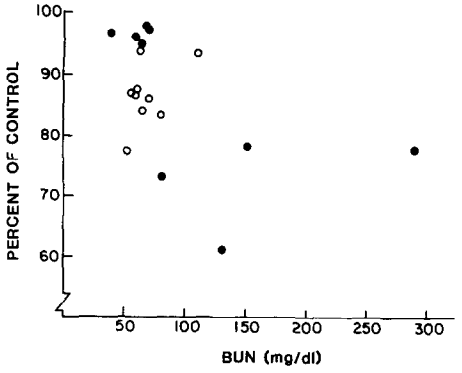

Fig. 1. Binding of a model probe, [^{14}C]salicylate, by plasma of chronically azotemic patients, who had either never been dialyzed (●) or were receiving regular dialysis treatment (○). Binding is expressed as a percent of the binding by a pool of normal control plasma, that had been diluted to identical albumin concentration, and is related to the blood urea nitrogen concentration (BUN).

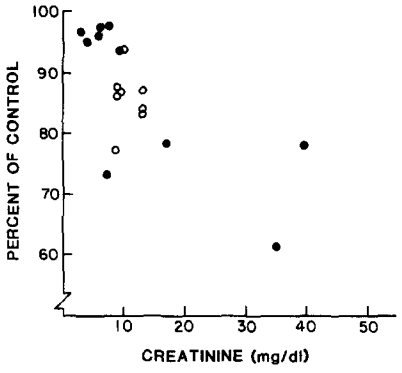

Fig. 2. Correlation of salicylate binding with plasma creatinine concentration. Symbols are defined in the legend of Fig. 1.

Beckman 332 liquid chromatograph, a model HM variable-wavelength detector (Gilson Medical Electronics Inc., Middleton, WI) set at 250 or 260 nm and 0.1 absorbance full scale, a model 4270 computing integrator (Spectra-Physics, San Jose, CA 95134), and a model 7125 injector (Rheodyne Inc., Cotati, CA).

RESULTS

Salicylate binding by azotemic plasma. The urea nitrogen (BUN) and creatinine concentrations in the plasma of the patients ranged from 40 to 305 and 3.3 to 42 mg/dl respectively. Salicylate binding by plasma from patients with a wide range of azotemia is related to BUN and serum creatinine in Figs. 1 and 2. Diluted normal control plasma bound 89.0 ± 0.54% (mean ± SD) of added salicylate. Binding was subnormal in both patient groups, ranging from 61.1 to 97.4% of the binding by the control normal plasma of equal albumin concentration. There was a weak negative correlation between salicylate bound (percent of control, y) and BUN (mg/dl), which was of borderline significance ($y = 92.9 - 0.076x$, $r = -0.44$, $0.1 > P > 0.05$) but a significant correlation with creatinine: $y = 94.9 - 0.70x$, $r = -0.69$, $P < 0.01$.

Effects of chloride, calcium and pH on salicylate binding. Over the chloride concentration range of 77 to 106 meQ/l, salicylate binding by azotemic plasma was constant (66.3 to 66.8%). Binding by normal plasma declined slightly from 90.8% at 74 mEq/l to 89.4% at 112 mEq/l (Table 1). These changes were trivial compared to the difference between binding by normal and azotemic plasma (Fig. 1). Over the wide range of 5.8 to 15.1 mg/dl, calcium variations had little effect on binding by either normal or azotemic plasma (Table 2). Alkalinization over a very wide range produced moderate inhibition of binding, which fell from 85.9% at pH 6.5 to 82.0% at pH 8.5 for azotemic plasma and from 93.4% at pH 6.5 to 91.4% at pH 8.5 for normal subjects (Table 3).

Effects on salicylate binding of hippurate, indoxyl sulfate and o-hydroxyhippurate added to normal plasma. As shown in Figs. 3 and 4, there was a progressive but slight decline in salicylate binding (y) as percent of control binding over the range of 25 to 1000 μM added hippurate (x) ($y = 100 - 0.0047x$, $r = -0.998$, $P < 0.01$) and from 50 to 300 μM added indoxyl sulfate (x) ($y = 99.9 - 0.010x$, $r = -0.966$, $P < 0.01$). Addition of *o*-hydroxyhippurate (x) to normal plasma also only slightly inhibited salicylate binding, which fell from 99.6% of the control at 50 μM to 98.0% at 300 μM. The regression relation of $y = 100.0 - 0.007x$ showed a correlation coefficient of −0.991 ($P < 0.001$).

Correlation of salicylate binding with plasma hippurate and indoxyl concentrations in azotemic plasma. The concentrations of both hippurate and indoxyl sulfate were elevated markedly in both patient groups compared to normal levels of <10 μM (Figs. 3 and 4), ranging from 18 to 883 μM and 45 to

Table 1. Effect of variations in plasma chloride concentration on binding of salicylate to plasma from normal subjects and patients with renal failure

	Normal					Renal failure			
Chloride (mEq/l)	74	83	93	102	112	77	86	96	106
Salicylate bound (percent)	90.8	90.6	90.2	89.9	89.4	66.7	66.4	66.3	66.8

Each result in Tables 1–3 represents the mean of two separate studies.

Table 2. Effect of variations in plasma calcium concentration on binding of salicylate to plasma from normal subjects and patients with renal failure

	Normal					Renal failure			
Calcium (mg/dl)	6.0	8.2	10.5	12.8	15.1	5.8	8.8	11.8	14.9
Salicylate bound percent	88.6	88.9	88.7	88.8	89.0	76.3	76.4	76.8	76.6

Table 3. Effect of variations in plasma pH on the binding of salicylate to plasma from normal subjects and patients with renal failure

	Normal					Renal failure				
pH	6.5	7.0	7.5	8.0	8.5	6.5	7.0	7.5	8.0	8.5
Salicylate bound (percent)	93.4	93.1	92.8	91.9	91.4	85.9	85.4	84.4	83.3	82.0

202 μM. The correlation between salicylate binding and level of hippurate was not significant ($y = 89.6 - 0.015x$, $r = -0.34$, $P > 0.1$), but there was a significant negative correlation with plasma indoxyl sulfate ($y = 98.7 - 0.11x$, $r = -0.73$, $P < 0.001$). Binding by plasma from azotemic patients was far less than that by normal plasma containing added hippurate or indoxyl sulfate. In both Figs. 3 and 4, the salicylate binding for all the patient samples fell below the 95% confidence limit of the regression line for the normal plasma enriched with hippurate or indoxyl sulfate.

Concentrations of p-*hydroxyphenylacetate and* o-*hydroxyhippurate in azotemic plasma.* Recovery of *o*-hydroxyhippurate was assessed by addition of 24 μmol/l of this acid to five normal and four azotemic plasma samples. Recovery was comparable in the two groups, with a mean and standard deviation of 94.5 ± 5.0%. The concentrations of both of these compounds were below detectable limits in normal plasma. The concentration of *o*-hydroxyhippurate in most of the azotemic plasmas was <2 μmol/l, the lower level of reliable detection. In those samples with measurable amounts there was a very wide range, from 8 to 467 μmol/l. Sufficient plasma was left in six cases to also measure levels of salicylate, the precursor of *o*-hydroxyhippurate. There was a correlation between the concentrations of salicylate and *o*-hydroxyhippurate as shown in Table 4 for those samples in which both compounds were measured ($r = 0.886$, $P < 0.05$).

Among samples from ten patients with advanced renal failure, who had never received dialysis treatment, all had undetectable levels of *p*-hydroxyphenylacetate. Among samples from ten patients receiving regular dialysis therapy for chronic renal failure, the level of this acid was undetectable in six and minimally elevated in two at 32 and 72 μmol/l.

Combined effect of hippurate and indoxyl sulfate: Normal vs uremic plasma. The results in studies of modified normal plasma compared to paired azotemic plasma are shown in Fig. 5. The salicylate bound as percent of binding by normal control plasma was 78.8% for the patients and 96.8% for the normal plasma samples modified to contain the same levels of hippurate and indoxyl sulfate as the patient samples. The mean (± SD) deviation from the normal control plasma binding was 21.2 ± 8.2% for the patient samples and 3.2 ± 1.1% for the modified normal plasma. The difference between the two

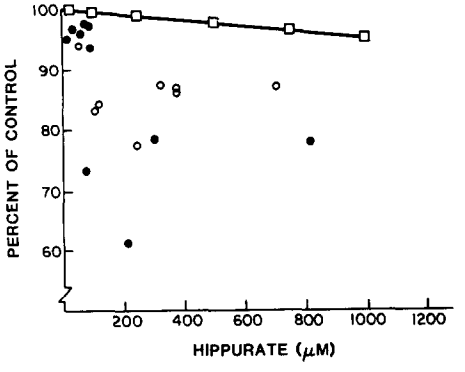

Fig. 3. Correlation of salicylate binding with plasma hippurate concentration. Symbols are defined in the legend of Fig. 1. Binding by normal plasma enriched with hippurate is shown at the top of the graph (−□−); each symbol for normal plasma represents the mean of two separate determinations.

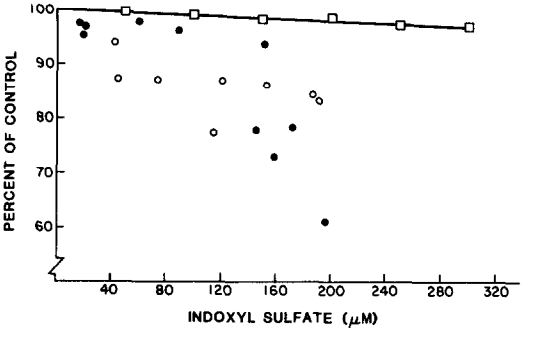

Fig. 4. Correlation of salicylate binding with plasma indoxyl sulfate concentration. Symbols are defined in the legend of Fig. 1. Binding by normal plasma enriched with indoxyl sulfate is shown at the top of the graph (−□−); each symbol for normal plasma represents the mean of two separate determinations.

Table 4. Plasma concentrations of *o*-hydroxyhippurate (oHH) and salicylate (SAL) in patients who had advanced renal failure (F) or were receiving regular hemodialysis therapy (H)

Patient	Creatinine (mg/dl)	Urea nitrogen (mg/dl)	oHH (μmol/l)	SAL (μmol/l)
F1	9.9	55	<2	ND*
F2	18.9	168	<2	ND
F3	8.6	83	<2	ND
F4	10.5	93	<2	ND
F5	8.0	86	<2	ND
F6	3.3	40	<2	ND
F7	43.0	312	57	ND
F8	8.7	119	66	ND
F9	35.8	134	467	1166
H1	12.9	100	<2	Undetectable
H2	11.4	76	<2	Undetectable
H3	15.5	77	<2	ND
H4	14.5	90	<2	Undetectable
H5	16.9	102	<2	ND
H6	15.0	94	<2	ND
H7	17.1	73	8	ND
H8	19.6	100	9	ND†
H9	20.1	125	12	ND†
H10	10.1	62	14	ND
H11	13.9	142	99	420
H12	8.2	90	116	876

Determinations were made on undiluted plasma.
* Not determined.
† Patients prescribed low dose aspirin.

groups, even treating them as independent samples with unequal variances, was highly significant ($t = 6.53$, df = 8, $P < 0.001$). Thus, hippurate and indoxyl sulfate accounted for only 15.1% of the total salicylate binding defect in uremic plasma.

DISCUSSION

In previous reports, binding of salicylate by plasma of patients with severe renal failure was reduced markedly [20, 21]. In the present study, we determined plasma salicylate binding over a wide range of renal failure, from minimal impairment to essentially total loss of function requiring regular dialysis therapy. The impairment in salicylate binding correlated with the degree of renal failure as reflected by the BUN and serum creatinine, but there was wider scatter in the data. Earlier *in vitro* studies have failed to show inhibition of binding of several drugs to plasma albumin by high concentrations of urea, creatinine and other small ligands, which accumulate to high levels in renal failure [5, 12, 22]. We, therefore, searched for other chemical causes of this defect.

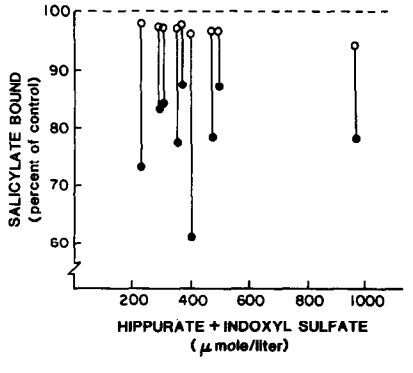

Fig. 5. Correlation of salicylate binding (as percent of binding by normal, unmodified plasma) with the sum of plasma hippurate and indoxyl sulfate concentrations for matched pairs of normal (○) and azotemic (●) plasma. Normal plasma samples were diluted with buffer and enriched with both organic acids so that concentrations of albumin, hippurate and indoxyl sulfate were equal for each pair of plasma samples.

The binding capacity of albumin is known to be modified by a number of small ions, especially the proton, calcium and chloride [23, 24]. Previous studies of the effects of these ions have utilized extreme changes in concentration of these ions and highly purified albumin preparations. In the present study albumin was in, or close to, its native state in plasma, and changes in pH, chloride and calcium were made over the range found in the most severe disturbances of these ions in human disease. These changes either failed to affect salicylate binding significantly or caused improvement in binding, a change opposite to that expected in renal disease. Acidemia and hypochloremia, common disorders in advanced renal failure, had no effect or slightly enhanced rather than depressed salicylate binding. Hypocalcemia produced a slight reduction in binding.

We next investigated the possible role of aromatic acids as the cause of impaired ligand binding in azotemic plasma. Tavares-Almeida *et al.* [13] have shown that many aromatic acids with specific structural features inhibit plasma binding of salicylate, warfarin and tryptophan. We first determined the plasma concentrations of potential aromatic acid inhibitors. Lichtenwalner and Suh [15] have implicated *o*-hydroxyhippurate as the cause of impaired plasma binding in uremia. The concentration of this acid was insignificant except among patients with substantial plasma levels of one of its precursors, salicylate (Table 4). Likewise, we found the amounts of *p*-hydroxyphenylacetate, a weak binding inhibitor present in normal human urine [11, 13], to be insignificant.

The concentrations of hippurate and indoxyl sulfate, especially in advanced azotemia, were quite substantial (Figs. 3 and 4), and there was a strong negative correlation between the binding of salicylate and the concentration of indoxyl sulfate. Addition of these aromatic acids to normal plasma produced a dose-dependent but very modest degree of inhibition of salicylate binding. Finally, we tested for possible interactions between hippurate and indoxyl sulfate by adding both ligands to normal plasma samples at the same concentrations found in paired azotemic plasma samples. No synergistic effect was found, and the two aromatic acids together produced only 15% of the depression found in azotemic plasma containing the same total concentrations of these two inhibitors.

The large residual inhibitory activity not explained by hippurate and indoxyl sulfate remains to be determined. Covalent modification of albumin or a change

in its amino acid composition in uremia has been proposed based on limited studies [22, 25]. Confirmation of these studies has not appeared. Carbamoylation of albumin has also been postulated as a cause of this drug-binding defect, but this theory was not confirmed by rigorous studies [26]. Another possible binding inhibitor found in normal urine is β-(*m*-hydroxyphenyl)hydracrylic acid [11]. We were unable to measure the concentration of this compound with the HPLC methods used in this study, as it eluted in the early complex portion of the chromatogram along with many polar compounds.

The present studies indicate that none of the aromatic acids implicated to date, alone or in combination, account for the impaired binding of acidic drugs and endogenous ligands by azotemic plasma. They imply that a number of other ligands remain to be identified as the major determinants of this disorder. Recent preparative scale chromatographic studies in our laboratory have shown that there are at least two binding inhibitors more hydrophobic than hippurate in azotemic plasma extracts. The recently identified furanoid acids, which accumulate in renal failure [27], may be among these inhibitors as Collier *et al.* have shown that one of these, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, is a very potent inhibitor of binding to albumin [28].

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