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Plasma protein binding in uremia: Extraction and characterization of an inhibitor

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Plasma protein binding in uremia: Extraction and characterization of an inhibitor. The impairment of binding of drugs and other substances to serum albumin in patients with uremia can be restored to normal or near normal levels by adsorption with charcoal or synthetic polymers at pH 3. We used a nonionic polystyrene-divinylbenzene copolymer to treat uremic plasma at pH 3. We observed a marked improvement of binding. Subsequent elution of this resin with ethanol produced a substance that, when dried and recombined with normal plasma, caused dose-dependent impairment of phenytoin and tryptophan binding. Restoration of normal binding affinity occurred after retreatment of this abnormalized plasma with resin at pH 3. Plasma and pleural fluid exudate from patients with uremia yielded, after extraction by the above technique, an inhibitor(s) of phenytoin binding in amounts averaging five times that extracted from equal volumes of normal plasma. This inhibitor (I_x) is water soluble, heat stable, and dialyzable across cellophane membranes. Unlike fatty acids, which can also interfere with binding, I_x partitions primarily in the water phase in solvent partition studies but undergoes a sharp transition in the pH 4 to 5 range, suggesting the presence of a carboxyl group. These findings lend further support to the hypothesis that a retained ligand(s) is responsible for impaired plasma binding associated with uremia and suggests a role for organic acids known to accumulate in renal failure.

Liaison aux protéines plasmatiques dans l'urémie: Extraction et caractérisation d'un inhibiteur. L'altération de la liaison de drogues et d'autres substances à l'albumine sérique au cours de l'urémie peut être complètement ou presque complètement supprimée par l'adsorption sur du charbon ou des polymères synthétiques à pH 3. Nous avons utilisé un co-polymère non ionique polystyrènedivinylbenzène pour traiter le plasma urémique à pH 3 et observé une amélioration importante de la liaison. L'élution ultérieure de cette résine par l'éthanol produit une substance qui, lorsqu'elle est séchée et recombinée avec du plasma normal, détermine une altération dose dépendante de la liaison de la diphényl-hydantoïne et du tryptophane. La récupération d'une affinité de liaison normale a été obtenue après un nouveau traitement du plasma par la résine à pH 3. Le plasma et le liquide pleural de malades urémiques a donné, après extraction par la technique ci-dessus, un inhibiteur(s) de la liaison de la phényl-hydantoïne en quantité cinq fois plus grande que celle extraite de volumes identiques de plasma normal. Cet inhibiteur (I_x) est soluble dans l'eau, thermostable et dialysable à travers des membranes de cellophane. A la différence des acides gras, qui peuvent aussi interférer avec la liaison, I_x passe dans la phase aqueuse au cours des études de partition dans des solvants, mais subit une transition brusque dans la gamme de pH 4 à 5, ce qui suggère la présence d'un groupe carboxyle. Ces constatations apportent des arguments supplémentaires à l'hypothèse selon laquelle un ligand (ou des ligands) retenus au cours de l'urémie est responsable de l'altération de la liaison plasmatique et suggère un rôle des acides organiques dont l'accumulation est connue dans l'insuffisance rénale.

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Extensive in vitro studies of plasma from patients with renal failure have documented defective binding of many drugs and several endogenous substances [1, 2]. In patients with advanced uremia, the degree of binding inhibition for some drugs is of sufficient magnitude as to require modification of drug administration. Much effort has been directed toward identification of individual drugs whose binding in uremia is critically impaired, but relatively few investigations have been devoted to uncovering the nature and causes of this binding inhibition [3–5].

Although several plasma proteins are known to bind a variety of drugs and other substances, the plasma protein that is most prominent in this role is albumin. It is also clear that the reduced albumin concentration, which is found especially in symptomatic uremic patients or poorly dialyzed patients, contributes to, but is not the major cause of, impaired plasma binding in uremia [6, 7]. Reduced binding is found in patients with renal failure when compared with normal persons with equal albumin concentrations. This binding defect has been postulated to result either from alteration of the primary structure of albumin or from accumulation of an unknown solute that interferes with binding [3–5].

Strong support for the latter hypothesis has come from studies demonstrating normalization of binding of several ligands by modified uremic plasma. Such normalization was achieved by Craig et al [5] using charcoal adsorption and by us using anion exchange resin treatment [7], both techniques being applied to plasma at pH 3.0. These agents may remove from uremic albumin a modifying ligand, which is hydrolyzed, is more loosely bound, or is more readily accessible at low pH. The last possibility is suggested by the fact that albumin undergoes a series of reversible configurational transformations between pH 3 to 4.5 [8]. The mechanism by which charcoal or resin restores binding by uremic plasma to normal has not been determined.

In this study we have attempted to recover inhibitory material from the resin used to normalize binding by uremic plasma. Because it has been well characterized for uremic plasma, we used binding of phenytoin by normal plasma as the test system to assay for inhibitory activity. By using a nonionic, hydrophobic resin for adsorbing uremic plasma and eluting it with ethanol, we obtained an extract containing an inhibitor(s) of phenytoin binding. We have determined a number of in vitro properties of the resin extract, which is free of proteins, serum electrolytes, and major small solutes.

Methods

Plasma was obtained from normal and hemodialyzed subjects in the fasting state after full consent was obtained according to the standards set forth by the Human Rights Committee at the University of California, Davis. Patients selected were stable outpatients who had been dialyzed for 1 to 3 years, ranged in age from 22 to 74 years, required a minimum number of drugs, and most had not taken drugs or caffeine for 48 hours prior to the venipuncture. A large amount of pleural fluid was collected from one hemodialyzed patient, who required therapeutic thoracentesis on two separate occasions. This fluid was drawn in both instances without anticoagulants while the patient was in the fasting state and after drug abstinence as above. Heparinized plasma and pleural fluid were stored at −70° C for less than 3 months prior to use.

Albumin concentration. Plasma albumin concentration of most samples was measured, as described by Doumas, with bromcresol green (BCG) except that readings were taken at 1 min after mixing [9]. This method was evaluated previously and found to give equivalent results in normal subjects and patients with renal failure [7].

Plasma binding of ^{14}C-phenytoin and ^{14}C-tryptophan. Binding of ^{14}C-phenytoin by whole plasma or purified albumin was measured by centrifugal ultrafiltration with techniques previously described [3, 7]. When inhibitors of phenytoin binding were added to normal test plasma, the sensitivity of the assay (to inhibition) was found to increase when the normal plasma was diluted 1:4 with isotonic 0.030 M Tris buffer [7] prior to the assay. (The use of this diluted assay is indicated in the text and figures.) ^{14}C-tryptophan binding was measured in a similar manner by using normal plasma diluted 1:4 with the isotonic 0.030 M Tris buffer. 3-^{14}C-L-tryptophan (specific activity, 49.4 mCi/mM, from New England Nuclear, Boston, Massachusetts) was added to the diluted plasma to achieve a concentration of 0.05 μCi/ml. No unlabeled tryptophan was added. Plasma ^{14}C-tryptophan was measured *after* centrifugation to compensate for slight binding of tryptophan to the cellophane bag.

Adsorption of uremic plasma with nonionized resin. Plasma was acidified to pH 3, then passed through resin columns as previously described [7], except that in most studies, for reasons given in the Results section, the uncharged XAD-2 polystyrene-divinyl benzene copolymer, Biobeads® (from Bio-Rad Laboratories, Richmond, California), was used in place of the anion exchange resin. This hydrophobic resin was prepared prior to use by sequential washing with 95% ethanol, distilled water, and 0.1 N hydrochloric acid. Initially, the effect of the uncharged resin on binding by plasma was evaluated in five pairs of normal and uremic subjects. With gentle stirring and aeration, these plasma samples were slowly acidified with 3 N hydrochloric acid to pH 3. Aliquots were then set aside as acidified controls. Approximately 12 to 14 ml of the remaining plasma was passed at 3 ml/hr through 1 × 5-cm columns (approximately 4.0 ml volume) of resin at 4° C, and 3-ml fractions were collected. The first fraction, diluted with column water, and the second fraction were discarded. The third and fourth column fractions and the acidified control were titrated to pH 8.0 with small volumes of 3 N sodium hydroxide, and, when necessary, the more concentrated plasma of the pair was diluted with Tris buffer so that each pair had the same final albumin concentration. Phenytoin was added to all samples, and binding was determined as described above for whole plasma.

Elution of ligand-binding inhibitor from the resin. The above columns were washed with water until no precipitate formed in the wash after addition of

trichloroacetic acid (TCA). Each column was then washed with 10 to 30 ml of 100% ethanol. This material subsequently will be referred to as the ethanol eluate. The ethanol eluate was evaporated to dryness under vacuum at room temperature by a rotary evaporator. The dried residue was dissolved and incubated at room temperature with 1.5 or 3.0 ml of normal plasma. No pH changes were observed on dissolving the residue with plasma. At least 50% of the total binding inhibitory activity was found in the first 10 ml of ethanol eluted from the column.

Isolation of inhibitor from large volumes of plasma and pleural fluid. To further characterize the inhibitor, we accumulated a pool of uremic plasma from six donors, and large quantities of pleural fluid were obtained from a single hemodialyzed patient. The high protein content and reduced phenytoin binding of the pleural fluid suggested to us that it might be a rich source of the protein-bound inhibitor previously demonstrated in uremic plasma. Solute content of the pleural fluid was as follows: creatinine, 11.7 mg/dl; bilirubin, 0.5 mg/dl; total protein, 5.6 g/dl; albumin, 3.0 g/dl. We applied 100 to 300 ml of pooled plasma or pleural fluid to larger columns (1.5 × 15 cm, total volume 26 ml) containing XAD-2 resin as described above. The columns were washed with distilled water until protein was no longer detectable by TCA and were then eluted with a volume of ethanol approximately one-half to one-fourth the volume of plasma or pleural fluid applied to the column. This ethanolic solution was stored at 4° C for subsequent studies. For dose-response assays, 0.3 to 12.0 ml of the ethanolic resin eluate was evaporated at room temperature under vacuum. We added 3 ml of a pool of normal plasma to each flask, mixed it at room temperature with gentle agitation, and then incubated it overnight at 4° C. After addition of phenytoin, binding was measured as above.

Characteristics of the inhibitor. Gravimetric analysis was done by the method of Kupke and Dorrier [10] on the dried ethanol eluate, which was solubilized by extraction with water. Solute content was evaluated by Technicon SMAC 20-channel analysis of a 0.5-ml sample of ethanol eluate that had been dried and reconstituted with 1.0 ml of water. Dialysis of the inhibitor was performed in 5-ml acrylic plastic cells with a cellophane membrane (Spectraphor 2 membrane, Spectrum Medical Industries, Inc., New York, New York; mol wt cutoff, 12,000 to 14,000 daltons). Two-milliliter samples of ethanolic eluate isolated from uremic plasma and pleural fluid were evaporated and reconstituted in 5 ml of water. These solutions were dialyzed against 5 ml of distilled water on a rocker at 4° C overnight. Samples from both sides of the membrane were lyophilized, and the residues were dissolved with normal plasma for analysis of binding inhibition as described above. Heat stability of an aqueous solution of the inhibitor was studied at pH 3.3 and 10. Two 4-ml samples of ethanol column wash were evaporated and reconstituted with 4 ml of water. The pH of the solution was 3.3. One 4-ml sample was titrated to pH 10.0 with 0.1 N sodium hydroxide. Both samples were incubated at 100° C for 30 min. These samples and unheated controls were then lyophilized and dissolved with normal plasma. Binding of phenytoin was measured as above. Fluorescence excitation and emission spectra were obtained with a spectroflurometer (Farrand Mark I).

Solvent partition studies were performed to determine the lipid solubility of inhibitory activity as a function of pH. Aliquots of the ethanol extract were dried, dissolved in distilled water, and titrated with hydrochloric acid or sodium hydroxide to pH values from 1.0 to 10. These aqueous solutions of inhibitory activity were added to equal volumes of ethylene dichloride or diisopropyl ether in separatory funnels and mixed vigorously. The phases were allowed to separate overnight at 4° C. The aqueous and organic phases were separated, dried, combined with normal test plasma, and binding of phenytoin was assayed in the samples and controls without inhibitor. The simultaneous partitioning of 1-^{14}C-oleic acid (54 mCi/mmole; Amersham, Arlington Heights, Illinois) was determined in a similar fashion, except that the ^{14}C-oleic acid was dried and dissolved in the organic phase before being mixed with aqueous solutions of the inhibitor, titrated as described above.

Results

We previously used anion exchange resin AG1-X8 from Bio-Rad Laboratories to normalize binding. After this resin was exposed to uremic plasma, several attempts were made to remove an inhibitor from it by eluting with hydrochloric acid at concentrations up to 200 mM, 200 mM ammonium hydroxide, and 100% ethanol. Failing in these attempts, we turned to a nonionic copolymer of styrene and divinyl benzene, XAD-2, which like AG-1 also produced effective improvement of phenytoin binding in uremic plasma. Table 1 shows phenytoin binding of five pairs of uremic and normal plasma studied after acidification alone and after resin treatment. The ratio of percent phenytoin unbound for each

Table 1. Effect of acid-resin treatment on phenytoin binding by normal and uremic plasma[a]

		Acidified control			Acid-resin treated		
		Albumin	Unbound phenytoin		Albumin	Unbound phenytoin	
Study	Status	*g/dl*	*%*	Ratio U/N	*g/dl*	*%*	Ratio U/N
1	U	4.54	23.0	1.41	4.37	18.5	1.10
	N	4.48	16.3		4.40	16.8	
2	U	2.80	27.3	1.35	3.03	19.6	1.09
	N	2.90	20.2		2.84	18.0	
3	U	3.79	27.2	1.43	3.80	20.5	1.11
	N	3.80	19.0		3.79	18.5	
4	U	3.78	29.7	1.49	3.82	24.5	1.19
	N	3.87	19.9		3.79	20.6	
5	U	4.44	23.7	1.47	4.31	18.2	1.14
	N	4.22	16.1		4.23	16.0	
	Mean			*1.43*			*1.13*
	± SEM			*± 0.02*			*± 0.02*
	Significance (paired difference)				*P < 0.001*		
	Significance (vs. 1.00)			*P < 0.001*			*P < 0.001*

[a] U denotes uremic plasma; N, normal.

uremic/normal pair improved significantly, from 1.43 after acidification alone to 1.13 after the acidified plasma was passed through the resin. The improvement of binding was similar to our previous findings with the AG-1 anion exchange resin [7].

Elution of inhibitor. When the dried ethanol eluate from the resin was combined with normal plasma, significant impairment of phenytoin binding by normal plasma resulted. Ethanol eluates from resin columns exposed to normal plasma produced a small but significant decrease in binding of 3.3 ± 0.8% (Table 2), possibly due to inhibitors present in and subsequently removed from normal plasma by the resin. The resin that had been exposed to uremic plasma, however, yielded much larger quantities, averaging 5.4 ± 2.5 times the amount in the normal plasma. Ethanol blank eluates from resin that had not been exposed to plasma yielded no appreciable inhibitory activity.

Dose-response relationship. We examined the relationship between the amount of plasma or pleural fluid and the degree of binding inhibition achieved by incubating a constant volume of normal plasma with varying quantities of the ethanol extract. A curvilinear relationship between response and the volume of pleural fluid extracted indicates a plateau in the effect of the inhibitor at higher concentrations (Fig. 1). Absolute binding by undiluted normal plasma combined with the inhibitor dropped from 87% to approximately 60% and appeared to approach a plateau at that level or slightly below.

Phenytoin binding by the pleural fluid used in these studies (albumin concentration, 3.0 g/dl) was

Table 2. Inhibition of phenytoin binding by dried ethanol eluate from resins that adsorbed normal or uremic plasma[a]

		Phenytoin bound	Decrement[b]	
Study	Samples	%	%	Ratio RU/RN
1	PN	87.6		
	RN	84.9	2.7	4.2
	RU	76.3	11.3	
2	PN	86.3		
	RN	81.2	5.1	4.5
	RU	63.2	23.1	
3	PN	88.4		
	RN	83.1	5.3	2.0
	RU	77.5	10.9	
4	PN	88.5		
	RN	86.7	1.8	5.7
	RU	78.3	10.2	
5	PN	87.9		
	RN	86.1	1.8	10.2
	RU	69.6	18.3	
Mean				*5.3*
± SEM				*± 1.4*

[a] PN denotes normal test plasma; RN, normal test plasma combined with elute from XAD-2 resin exposed to normal plasma; RU, normal plasma combined with eluate from XAD-2 resin exposed to uremic plasma.

[b] Decrements are the differences between PN and RN, and between PN and RU. The mean ± SEM decrements were: RN, 3.3 ± 0.8%; RU, 14.8 ± 2.5%

68 to 70% at 37° C and improved by 5 to 6% after resin treatment. Binding by plasma from this same patient (albumin concentration, 3.32 g/dl) was 72.1% at 37° C.

Reversibility. The "synthetic uremic plasma" produced by combining normal plasma with eluate from resin previously exposed to uremic plasma

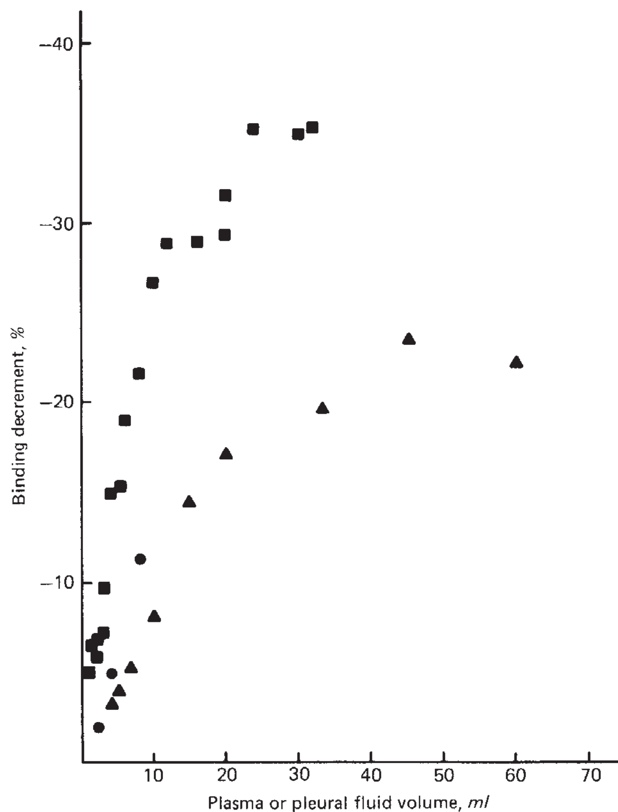

Fig. 1. *Dose-response relationships for inhibitor of plasma phenytoin binding extracted from uremic plasma (●) or uremic pleural fluid (■, ▲).* The normal *test* plasma for each dose-response series was used either undiluted (●, ▲) or after 1 : 4 dilution (■).

was treated with anion-exchange resin at pH 3 to test the important question of whether the induced inhibition was reversible, as in uremic plasma, or was a nonspecific, irreversible effect. Table 3 shows that complete normalization was achieved as occurs for natural, uremic plasma [7], a finding which supports the concept that a retained substance(s) is responsible for inhibition of ligand binding in uremia.

Specificity of binding inhibition. In Table 4 the inhibitory effect is shown not to be unique to phenytoin binding and in this respect resembles uremic plasma itself, which exhibits decreased binding to a number of drugs and other substances including tryptophan [2, 3].

In addition, plasma from other animal sources and purified human albumin preparations, which vary in their affinity for phenytoin, are affected by the resin eluate (Table 5). Of note is the poor binding by isolated human albumin of 15% to 35% compared with around 60% for equally diluted normal plasma at 37° C. The pure albumin preparations used here were isolated by different techniques, including alcohol and salt fractionation and affinity chromatography.

Solvent partitioning and other characteristics of the inhibitor. Relative solubility in organic compared with aqueous solvents was examined by partitioning of an aqueous buffered solution of the inhibitor with an equal volume of ethylene dichloride or diisopropyl ether. Partitioning into the organic phase increased as pH was lowered (Fig. 2). There was a change of partitioning in the range of pH 4 to 5, the inhibitor being totally in the aqueous phase above pH 5.0, but 15 to 33% being in the organic phase below pH 4.0.

For reasons discussed below a similar partitioning was carried out with the most abundant plasma fatty acid, oleic acid [13]. In contrast with the uremic plasma extract, oleic acid is much more soluble in the organic phase over the pH range 3 to 10. The percentage of total ^{14}C-oleic acid in ethylene dichloride ranged from 99.1% at pH 3 to 96.0% at pH 6.5. Partitioning of ^{14}C-oleic acid between diisopropyl ether and an aqueous solution (data not shown) showed 99.1% and 93.2% of the carbon 14 in the organic phase at pH 3 and 10, respectively.

Titration of the crude extract from pH 2 to 10 with 0.2 N hydrochloric acid revealed significant buffering in the 3 to 5 pH range, adding further evi-

Table 3. Reversibility of phenytoin binding inhibition[a]

	% Phenytoin bound
A. Untreated normal plasma[a]	56.0
B. Plasma and inhibitor	30.7
C. Acidified only	38.8
D. Acidified and resin treated	54.0

[a] Sample A was an untreated diluted normal plasma. Sample B (the same plasma as sample A) was used to dissolve one half volume of dried ethanol eluate containing uremic inhibitor. Sample C was equivalent to sample B but had been acidified to pH 3.0 and then titrated back to pH 8.0 before phenytoin binding was tested. Sample D was treated as was sample C except that it was passed over an AG-1 column at pH 3.0 and then titrated back to pH 8.0. All studies were done with plasma diluted 1:4 with isotonic Tris buffer (see Methods).

Table 4. Effect of inhibitor on tryptophan binding[a]

Ethanol eluate added[b] *ml*	Tryptophan bound %	Inhibition %
0	49.5	—
0.5	32.9	−16.6
1.0	24.3	−25.2
2.5	18.4	−31.1

[a] All binding measurements were done with 1.5 ml of a normal plasma after 1 : 4 dilution with Tris buffer (see Methods).
[b] Eluate was evaporated to dryness before being dissolved with the diluted plasma (see Methods).

Table 5. Effect of inhibitor on phenytoin binding by plasma from various species and on purified human albumin[a]

Type of specimen[b]	Albumin *g/dl*	^{14}C-phenytoin bound %	Inhibition %
Bovine plasma			
Undiluted	4.07	83.5	
Diluted	1.07	45.3	−10.3
Diluted + inhibitor	1.07	35.0	
Ovine plasma			
Undiluted	3.09	83.6	
Diluted	1.00	49.3	−13.6
Diluted + inhibitor	1.00	35.7	
Equine plasma			
Undiluted	3.42	59.5	
Diluted	0.92	23.4	−8.0
Diluted + inhibitor	0.92	15.4	
Human albumin A			
Alone	0.89	18.6	−11.0
+ Inhibitor	0.89	7.6	
Human Albumin B			
Alone	0.90	34.5	−31.5
+ Inhibitor	0.90	3.0	
Human Albumin C			
Alone	0.88	15.1	−15.1
+ Inhibitor	0.88	0	
Human Albumin D			
Alone	0.86	28.9	−22.8
+ Inhibitor	0.86	6.1	

[a] Source of inhibitor was 1.0 ml of ethanol eluate, derived from 4.0 ml of uremic pleural fluid, dried and combined with 1.5 ml of plasma or albumin solution. Plasma samples were diluted with Tris buffer (see methods). Albumins were dissolved in water.
[b] The human albumins were: A, Sigma fraction V; B, Sigma fraction V, fatty acid free; C, Sigma fraction V, crystalized and lypophilized; D, Albumin from Calbiochem prepared by affinity chromatography.

dence that an acidic group with a pK of 4 to 5 is a component of the inhibitor. Other characteristics of the crude ethanol eluate that contains the inhibitor are listed in Table 6. When the ethanolic elute is evaporated and the residue is extracted with water, the water extract contains all of the binding inhibitory capacity. This water extract had no detectable sodium, potassium, chloride, urea, creatinine, bilirubin, uric acid, phosphorus, calcium, glucose, or cholesterol. When this protein-free water extract of the ethanol elute was dialyzed against an equal volume of water across a cellophane membrane with a mol wt cutoff of 12,000 to 14,000 daltons, the inhibitory activity was equal in the two compartments after 16 hours of dialysis. Gravimetric analysis of the lyophilized aqueous extract of the ethanol elute yielded a weight of approximately 0.35 mg/ml of pleural fluid extracted. There is no loss of activity when an aqueous solution is heated at 100° C for 30 min at pH 3 and 10. Fluorescence of the aqueous extract is observed with an excitation peak at 340 mμ and an emission peak at 420 mμ.

Discussion

Our previous studies on treating uremic plasma with an anion exchange resin and studies by others using charcoal, both at pH 3.0, proved that impaired binding of small organic ligands by uremic plasma proteins was a reversible defect and, therefore, not the result of irreversible damage to the binding protein by some aspect of the uremic state [4, 5, 7]. The failure of both in vivo and in vitro dialysis to correct this binding abnormality [3, 5] suggested several possible mechanisms that could lead to this abnormality.

Having failed to elute from the anion exchange resin, AG-1, material that would reduce binding of small ligands[1] by normal plasma, we tested the ability of the nonionic core of the AG-1 resin, that is, the polystyrene/divinyl-benzene resin XAD-2, to correct the defective binding of uremic plasma. This type of resin is known to strongly adsorb organic compounds from aqueous solution and fatty acids from acidified plasma [11, 14]. Table 1 shows that the XAD-2 resin produced almost as much improvement in binding by uremic plasma as did AG-1 following acidification alone and acidification plus res-

[1]Unless otherwise stated, the ligands to which we refer is the group of acidic, aromatic ligands, which are bound less avidly by uremic than normal plasma (for example; tryptophan, phenytoin, salicylate, warfarin, phenylbutazone).

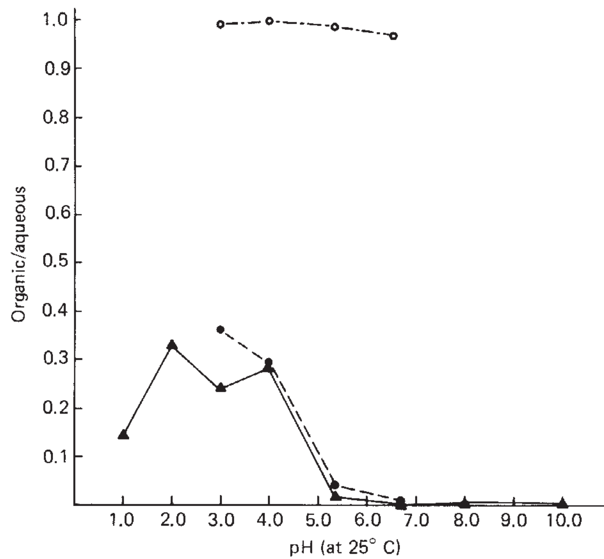

Fig. 2. *Partitioning between distilled water solution and ethylene dichloride of inhibitory activity* (▲, ●) *and of added* ^{14}*C-oleic acid* (○). Inhibitory activity was extracted either from uremic pleural fluid (○, ▲) or from uremic plasma (●).

in treatment. In previous studies using AG-1, the ratios of percent unbound phenytoin for uremic to normal plasma were 1.43 for the acidified control and 1.08 for the acid-resin treated group [7].

An ethanol wash of the XAD-2 resin column yielded a crude extract, which was a potent inhibitor of phenytoin binding by normal plasma. The early, nearly linear portion of the dose response curves (Fig. 1) can be used to quantitate the content of inhibitor in biologic fluids. It should be noted also that the sensitivity of the assay was nearly doubled when test plasma that had been diluted 1:4 was used. That the inhibitory material is not unique to uremic fluids is shown in Table 2; extracts from normal plasma produce about 20% as much inhibition as extracts of uremic plasma.

The specificity of the inhibitory extract was tested in several ways. The extract produced a dose-dependent inhibition of tryptophan binding, an abnormality characteristic of uremia [3]. Limited data suggests that albumin is the major binding protein for these acidic/aromatic ligands [15]. The inhibitor was active against four different pure preparations of commercial albumin (Table 5). It is noteworthy that none of these albumin preparations bound phenytoin as effectively as did normal plasma containing an equivalent concentration of albumin. In 35 control studies, whole plasma diluted 1:4 has shown 52% to 67% binding of ^{14}C-phenytoin. It is possible that other plasma proteins do, in fact, also bind phenytoin or that the purified albumins have been subtly altered during the purification process. Finally, there may be cofactors in plasma that are necessary for optimum binding but are removed during purification [16].

That albumins from different animal species have different primary structures is well established [8]. They also show different binding properties. Because it would be very useful to be able to use animal plasma in future studies because of the large volume of plasma required in many studies planned, we tested the effect of the inhibitory extract on plasma from three large animals. As seen in Table 5, a good effect was produced on bovine, ovine, and equine plasma.

A number of properties of the inhibitory component in the crude ethanol extract have been determined. At the time these studies were begun, we regarded plasma free fatty acids to be highly likely candidates as the inhibitor. They are extremely tightly bound to plasma albumin at pH 7.4, they displace many albumin-bound ligands, and they can be totally removed at pH 3.0 by adsorption with charcoal or AG-1 resin [11, 12]. The complete water solubility of the inhibitory component of the crude ethanol extract and its behavior on solvent partitioning (Fig. 2) show that it is not a long-chain fatty acid. This conclusion agrees with the finding of both normal total concentrations of free fatty acids and a normal pattern of individual long-chain fatty acids in uremic plasma [13, 17].

That the inhibitor is not a very large molecule (that is, > 10,000 daltons) is shown by its ready passage across cellophane from an aqueous, protein-free solution. Its stability in strong acid and alkali and at high temperature will greatly simplify future

Table 6. Properties of a crude preparation of binding inhibitor

Property	Value
Concentration of water-solubilized extract/ml pleural fluid, *mg/ml*	0.35
Solubility in water, *mg/ml*	≥6
Odor	Foul
Color	Yellow-brown
Heat stability	Aqueous solution stable at 100° C × 30 min at pH 3 and 10
Dialyzability	Completely equilibrates across cellophane in 16 hr
Fluorescence excitation maximum, *mμ*	340
Fluorescence emission maximum, *mμ*	420

attempts at isolation and purification. Because the material at this stage is quite crude, we cannot determine whether the strong fluorescence is due to the inhibitor or a contaminant. Recently, it has been shown that uremic plasma is abnormally fluorescent, and it is quite interesting that this fluorescence moves with albumin on electrophoresis [18]. The yellow-brown color and foul odor both are suggestive of retained substances normally present in urine. Finally, its adsorption to the XAD-2 resin suggests that it has a hydrophobic component and perhaps binds in part to albumin by hydrophobic interaction. It does appear also to have, however, a polar component including an ionizing group, perhaps a carboxyl group, with a pK of 4 to 5 (Fig. 2). Organic acids are retained in uremia [19].

Although the ethanol extract is clearly quite crude, the total mass of material requiring further purification is quite small. Of roughly 66.8 mg of dry weight per milliliter of the pleural fluid extracted, the water extract of the ethanol eluate contained only 0.35 mg/ml, that is, 0.5% of the starting solid. In future studies we will optimize efficiency of recovery of inhibitory activity up to this step, which we roughly estimate to be 25% with the present method.

The nature of the interaction of the inhibitor and plasma binding protein cannot be stated. In the present study and the studies by Sjoholm and by Craig [4, 5] a very low pH was required to normalize binding by uremic plasma. Our previous study of tryptophan binding showed normal tryptophan binding by albumin isolated from uremic plasma by salting out globulins with concentrated ammonium sulfate and DEAE-Sephadex chromatography. Thus, unfolding of albumin induced by both of these treatments seems to be a requirement to remove the inhibitor, perhaps by exposing bound inhibitor to the adsorbent resin. Alternatively, these maneuvers may displace an extremely tightly bound ligand from plasma proteins. Finally, it is possible that the inhibitor is covalently linked to protein at pH 7.4 with an acid labile bond. Covalently modified proteins are known to occur after prolonged exposure to glucose and aspirin [20, 21]. Boobis has recently reported finding abnormal amino acid composition and isoelectric focusing patterns for albumin isolated from uremic patients [22]. We cannot state at present whether our results and hers are interrelated. Purification of the inhibitor to homogeneity appears quite feasible and should allow us to determine the precise nature of the interaction between single plasma proteins and the inhibitor.

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