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DECREASED DRUG BINDING IN URAEMIA: EFFECT OF INDOXYL SULPHATE AND OTHER ENDOGENOUS SUBSTANCES ON THE BINDING OF DRUGS AND DYES TO HUMAN ALBUMIN

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Abstract—Ten uraemic metabolites, alone or in combination, have been investigated by equilibrium dialysis for their effect on the binding of methyl red, methyl orange, 2-(4′-hydroxybenzeneazo)benzoic acid (HABA), phenytoin and L-tryptophan to human albumin (HSA). Indoxyl sulphate emerges as a substance likely to inhibit binding *in vivo* while the other metabolites were unlikely to be implicated in the binding defect of uraemic plasma. The effects of indoxyl sulphate, on the binding of HABA and methyl red, studied by equilibrium dialysis and spectroscopy respectively, indicated competitive inhibition. The results suggest that indoxyl sulphate and indole carboxylic acids may contribute to the binding defect of uraemic plasma.

The reason for the defective binding of anionic compounds to uraemic plasma-proteins is not clear, but has been attributed to endogenous inhibitors of binding and/or a defective albumin molecule [1]. The ability of plasma from patients with renal insufficiency to bind anionic ligands can be improved by both haemodialysis [2] and renal transplantation [3, 4]. Attempts to remove supposed binding inhibitors from uraemic human [5] and rat [6] plasma by exhaustive pre-dialysis, prior to measurement of binding, were not particularly effective for the restoration of binding. However, isolation of albumin from uraemic plasma removed the binding defect for L-tryptophan [7] and exposure of uraemic plasma or sera to activated charcoal at acid pH largely removed the binding defect of both human [8–10] and rat plasma [6]. Furthermore, extraction of uraemic human sera with either a hydrophobic resin [11] or 1-chlorobutane [12] rectified the binding defect. It seems, therefore, that accumulated endogenous metabolites are either responsible for, or contribute to, the binding defect, but the identity of these inhibitors is unknown.

o-Methyl red, methyl orange, 2-(4′-hydroxybenzeneazo)benzoic acid (HABA), phenytoin and L-tryptophan all undergo a typical decrease in binding to uraemic plasma-proteins [1, 13], and so the effect of various uraemic metabolites on the interaction of these ligands with human serum albumin (HSA) has been studied. Indoxyl sulphate was found to inhibit the binding of methyl red [14] and so this substance has been studied in more detail.

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Abbreviations: HABA, 2-(4′-hydroxybenzeneazo)benzoic acid; HSA, human serum albumin.

MATERIALS AND METHODS

Chemicals. Crystalline human serum albumin (fraction V, Sigma Chemical Co., Poole, U.K.), several lot numbers) and defatted human albumin (<0.1 mole fatty acid per mole of albumin, Miles Laboratories, batch No. 13) were used. The source and purity of the dyes and 4-[^{14}C]phenytoin has been described previously [13]. L-[5-^{3}H]Tryptophan (sp. act. 27 Ci/mmole) with a stated radiochemical purity of 97% and containing 5% D-[5-^{3}H]tryptophan was purchased from the Radiochemical Centre, Amersham, U.K.

Unlabelled sodium phenytoin was kindly donated by Parke Davis & Co., and L-tryptophan and other metabolites were supplied by either B.D.H. Ltd (Poole, U.K.), or Sigma Chemical Co.

Solutions for dialysis. Experiments with *o*-methyl red, HABA and L-tryptophan were done with 0.05 M sodium phosphate buffer pH 7.4. In the phenytoin experiments the 0.05 M phosphate buffer was diluted with 5×10^{-4} M NaOH (containing sodium phenytoin) to give 0.025 M sodium phosphate buffer pH 7.4, containing 2.5×10^{-4} M NaOH. Experiments with methyl orange were done with 0.05 M sodium phosphate buffer containing 2.075 g NaCl/l.

Equal volumes (5 ml) of ligand solution, buffer with or without metabolites and HSA (3% w/v; 435 μM) in buffer were mixed and incubated for 20 min before an aliquot (3 ml) was dialysed against an equal volume of buffer at 37° [15]. The final concentration of HSA was 1% w/v (145 μM). Defatted HSA, which gave higher binding than ordinary crystalline HSA, was used in the experiments with L-tryptophan.

Measurement of radioactivity. Radioactivity in samples (0.25 ml) from the albumin and non-albumin compartments was measured by liquid scintillation

counting in an Intertechnique SL33 or Beckman LS-3133T liquid scintillation counter. Internal standards of [^{3}H] water and [^{14}C]*n*-hexadecane (Radiochemical Centre) were used for assessment of quenching and an appropriate correction was made for tritium decay. The mean recoveries (± S.D.) of L-[5-^{3}H]tryptophan and 4-[^{14}C] phenytoin from the dialysis cells were 93.8 ± 3.8% ($N = 22$) and 92.8 ± 6.9% ($N = 231$), respectively.

Determination of dyes. Unbound dyes were measured spectrophotometrically and the mean percentage recovery of each dye in absence of albumin was >97% [13].

Difference spectrophotometry. The methyl red and HSA solutions prepared for dialysis were both diluted with buffer to 26.7 μM and 29 μM (0.2% w/v), respectively. Difference spectra were recorded with a Cecil CE 505 Double Beam Ultraviolet Spectrophotometer using the tandem cell technique [16]. Four quartz cuvettes each with a light path of 1 cm were used. Spectra were recorded from 310 to 610 nm at a scan speed of 2.5 nm/sec with a scan length of 30 cm and a full scale deflection of 0.2 absorbance units.

Analysis of results. Results are expressed as the mean (±S.D.) and statistical comparisons were made with the non-paired Student's *t*-test. The effect of indoxyl sulphate on the binding of methyl red, methyl orange and HABA was analysed by the method of Scatchard [17]: $r/D_u = nk - rk$ where r is the molar ratio of bound ligand to albumin, D_u the unbound ligand concentration, n the number of binding sites and k the apparent association constant. The apparent association constant (k_i) for indoxyl sulphate was calculated by the method of Klotz, Triwush and Walker [18].

RESULTS

Effect of nitrogenous metabolites. An extensive investigation of five such metabolites showed that they produced no significant inhibition of either methyl red, methyl orange or phenytoin binding to HSA (Table 1). In addition, a mixture of these compounds (creatine, 0.07 mM; creatinine, 2.1 mM; methylguanidine, 0.09 mM; and urea, 33.3 mM) did not displace methyl red from HSA.

Methylguanidine had a small but significant effect upon the binding of methyl red. At low concentrations (0.036 and 0.073 mM) binding was unchanged, but at higher concentrations (0.091 and 1.83 mM) binding increased from 91.1 to 92.6 and 94%, respectively. However, methylguanidine did not affect the binding of methyl orange to HSA (Table 1).

Exposure of HSA to urea for less than 5 hr did not alter either dye or phenytoin binding (Table 1). But prior exposure of albumin to urea (16.6 mM) for 48 hr at 2°; followed by equilibrium dialysis for 24 hr at 37°, increased the binding of phenytoin from 37.4 ± 0.6 ($N = 5$) to 55.7 ± 0.7% ($N = 10$). A similar urea-induced increase in the binding of sulphaethylthiazole, doxycycline, metoclopramide and progesterone to bovine albumin has been observed [19].

Effect of phenols and other metabolites. Several compounds were tested for their ability to displace methyl red but of these only *p*-cresol produced a significant decrease in bound dye (Table 2). A large number of phenolic acids accumulate in uraemia [20] but the concentration required to inhibit is probably much greater than that known to occur. For example, the concentration of *p*-cresol that significantly displaced methyl red, was probably more than twenty-fold greater than that found in uraemic plasma [21].

A mixture of acidic compounds (guanidinosuccinic acid, 0.23 mM; indoxyl sulphate, 0.16 mM; and uric acid, 0.71 mM) decreased the binding of methyl red from 91.8 ± 0.2% (control; $N = 5$) to 90.3 ± 0.3% ($N = 5$; $P < 0.001$), a decrease which can be accounted for by the effect of indoxyl sulphate alone (see Table 3).

Table 1. Binding of methyl red (133 μM), methyl orange (145 μM) and phenytoin (21.7 μM) to human albumin (1% w/v, 145 μM) in the absence and presence of various nitrogenous uraemic metabolites

Metabolite	Concentration range (mM)*	Ligand	% of ligand bound with metabolite : ABSENT	PRESENT**	Effect
Creatine	0.67-3.35	methyl red	91.8±0.6	91.8±0.5	N.S.
Creatinine	0.35-1.06	methyl red	91.9±0.3	92.2±0.2	N.S.
	0.35-1.06	methyl orange	96.6±0.1	96.7±0.1	N.S.
	0.14-1.11	phenytoin	41.4±0.8	42.0±0.5	N.S.
Methylguanidine	0.036-1.83	methyl red	91.1±1.1	94.0±0.4	P < 0.001
	0.091-1.83	methyl orange	96.7±0.1	96.7±0.1	N.S.
Urea	8.3-33.3	methyl red	91.7±0.2	91.6±0.3	N.S.
	8.3-33.3	methyl orange	96.5±0.0	96.5±0.0	N.S.
	3.3-99.8	phenytoin	41.1±0.7	40.6±0.4	N.S.
Uric acid	0.36-1.07	methyl red	91.4±0.2	91.4±0.1	N.S.
	0.36-1.07	methyl orange	96.4±0.1	96.6±0.0	N.S.

* 3–8 concentrations in each range.
** Mean ± S.D. of 3–5 experiments at the highest concentration of metabolites.

Table 2. Binding of methyl red (133 μM) and methyl orange (145 μM) to human albumin (1% w/v, 145 μM) in the absence and presence of various uraemic metabolites

Metabolite	Concentration range (mM)	Ligand	% of Ligand bound with metabolite		Effect
			ABSENT	PRESENT**	
Benzyl alcohol	1.85*	methyl red	91.3±1.3	91.6±0.6	N.S.
p-Cresol	1.85*	methyl red	91.8±0.2	90.3±0.2	$p < 0.001$
Guanidinosuccinic acid	1.14*	methyl red	92.3±0.6	91.8±0.4	N.S.
Phenol	0.011-0.053	methyl red	91.6±0.2	91.3±0.3	N.S.
	0.011-0.106	methyl orange	96.6±0.0	96.8±0.1	N.S.

* Single concentration.
** Mean ± S.D. of 3–5 experiments at the highest concentration of metabolite.

Table 3. Effect of indoxyl sulphate on the binding of methyl red, methyl orange, HABA and L-tryptophan to 1% w/v human albumin *in vitro*

Indoxyl sulphate concentration (mM)	Percentage of ligand bound†			
	Methyl red (133μM)	Methyl orange (145μM)	HABA (145μM)	L-Tryptophan ‡ (4.9μM)
0	91.8±0.6	96.5±0.1	74.3±0.5	76.4±1.3
0.2	90.3±0.5**	95.8±0.1**	69.0±1.1*	25.9±5.3**
0.4	89.4±0.3	95.5±0.1	66.1±1.2**	19.8±1.7
0.8	87.3±0.4	94.9±0.0	62.5±0.2	
1.2	85.9±0.4	-	-	
1.4	84.3±1.2	-	-	
2.0	81.3±1.0	93.4±0.1	54.3±0.4	

* $P < 0.01$.
** $P < 0.001$.
† Mean ± S.D. of 3–5 experiments.
‡ Defatted HSA (145 μM) used.

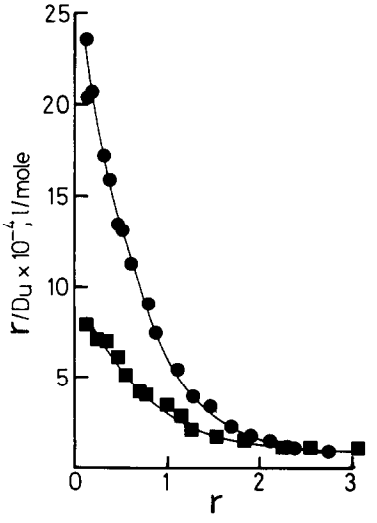

Fig. 1. Scatchard plot for the binding of methyl red (0.2–1 mM) to HSA (0.145 mM) at 37° in the absence (●) and presence (■) of indoxyl sulphate (1.2 mM). Each point is the mean of three or more determinations.

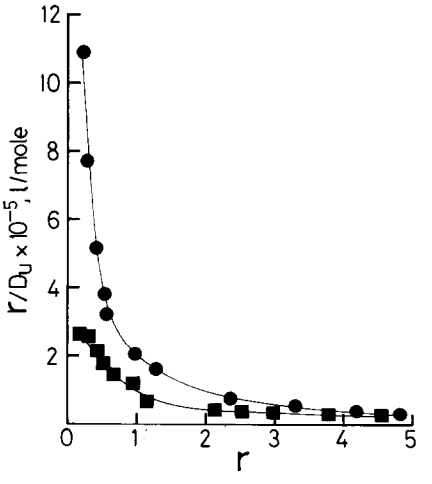

Fig. 2. Scatchard plot for the binding of methyl orange (0.03–1 mM) to HSA (0.145 mM) at 37° in the absence (●) and presence (■) of indoxyl sulphate (1.2 mM). Each point is the mean of three or more determinations.

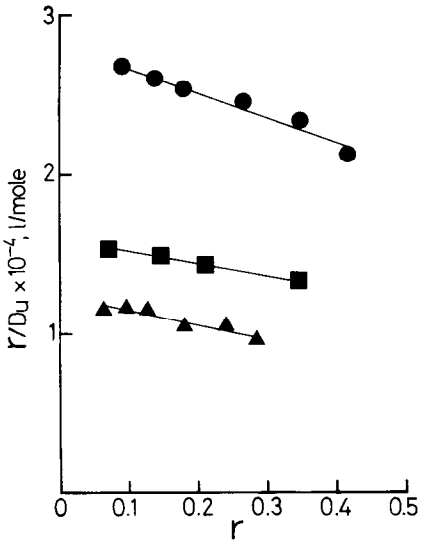

Fig. 3. Scatchard plot for the binding of HABA (0.02–0.1 mM) to HSA (0.145 mM) at 37° in the absence (●) and presence of 0.6 mM (■) and 1.2 mM (▲) indoxyl sulphate, respectively. Each point is the mean of three or more determinations.

Effect of indoxyl sulphate. Indoxyl sulphate at concentrations of 0.2 mM and higher significantly inhibited the binding of methyl red, methyl orange, HABA and L-tryptophan to 1% w/v HSA (Table 3). The effect of indoxyl sulphate on methyl red binding was greater at 37° than at 22°. For example, indoxyl sulphate (2.0 mM) at 37° increased the unbound fraction of methyl red (from the control value) by 128% whereas at 22° the increase was only 91%.

Indoxyl sulphate markedly depressed the initial steep portion of the Scatchard plot of methyl red and methyl orange binding to 1% w/v HSA (Figs. 1 and 2). This suggests that both dyes were displaced from their primary binding sites. Indoxyl sulphate had a more pronounced effect upon the binding of HABA to 1% w/v HSA (Fig. 3). Linear regression analysis of these Scatchard plots showed that indoxyl sulphate principally caused a decrease in the apparent affinity of HABA for albumin (Table 4). The binding constant, k_i, for the inhibitor indoxyl sulphate calculated from the HABA data in Table 3, on the assumption of competitive inhibition and one class of binding site, was found to be $8.4 \pm 0.8 \times 10^2$ l/mole.

Table 4. Effect of indoxyl sulphate on the binding constants for the interaction of HABA (0.02–0.1 mM) with HSA (0.145 mM) at 37°

Concentration of indoxyl sulphate (mM)	Binding constants for HABA	
	n	k (l/mol x 10^{-4})
0	1.8	1.54
0.6	2.0	0.78
1.2	1.4	0.91

The difference spectrum for the interaction between methyl red and HSA was characterized by a trough at 378 nm followed by a peak at 443 nm with an isosbestic point at 413 nm. These were unaffected by indoxyl sulphate which produced only quantitative changes in the difference spectrum, so that plots of the absorbance changes at either 378 nm (λ_{min}) or 443 nm (λ_{max}) versus indoxyl sulphate concentration were linear.

DISCUSSION

A large number and variety of plasma solutes are retained in uraemia and although the identity of a considerable number of these is known, it is our experience that there is a lack of adequate quantitative data. As far as possible the concentrations of metabolites which were investigated were those likely to occur in uraemia. A correlation between the unbound fraction of drug and blood urea concentration has been reported in some studies [22–25] but not others [26, 27]. The results reported here show that urea at pathological concentrations does not decrease binding and supports previous findings for ligands including phenolsulphophthalein [28], thiopentone [29], digitoxin [30] and sulphamethoxazole [8]. Concentrations of urea between 0.2 and 1.0 M were found to decrease the affinity of methyl orange for bovine serum albumin [31], but those concentrations are much higher than those known to occur in uraemic patients and experimental animals.

Although these data suggest that urea is not responsible for the binding defect in uraemia, it is possible that urea, at concentrations found in uraemia, can cause subtle alterations of the conformation of albumin. Prolonged exposure of albumin to low concentrations of urea increased the binding of phenytoin and similar observations have been made for other ligands [19]. In uraemia, albumin would suffer prolonged exposure to urea, and carbamylation has been suggested as a cause of the binding defect [32, 33]. However, binding can be fully restored by extraction of uraemic plasma with charcoal [6, 8–10], hydrophobic resin [11] or 1-chlorobutane [12].

Several of the metabolites included in the present study have been investigated previously for effects on drug binding [23, 28, 34]. The accumulated evidence now strongly suggests that along with urea, the following compounds are not directly responsible for the binding defect of uraemia: creatine–creatinine, methylguanidine, guanidinosuccinic acid, uric acid and a number of phenols.

Indoxyl sulphate decreased the apparent association constant (k) for HABA but had no consistent effect on the number of binding sites (n), which implies that inhibition was of a competitive nature. Similarly, the slopes of the initial steep portion of the Scatchard plots for methyl red and methyl orange were decreased, which suggests that the apparent affinity of these dyes for their primary binding site was decreased. Further evidence for competitive displacement of bound dye is suggested by the quantitative changes in the difference spectrum of methyl red. The limited information on the effect of uraemia

on the values of n and k for drug binding to plasma, indicate that it is mainly k which is decreased. The decreased binding of salicylate and warfarin to uraemic human serum was due largely to a fall in k [9] and although not conclusive, the data of Campion [23] point to a similar change for methyl red. We have found that the diminished plasma-protein binding of salicylate, methyl red and methyl orange to plasma from rats with renal failure is mainly due to a fall in k [6].

The binding of L-tryptophan is decreased in uraemia [7] and its structural similarity to indoxyl sulphate suggests that competition for binding sites may occur. However, the k value for the L-tryptophan–human albumin interaction at 37° is 12×10^3 l/mole [35] which is much higher than the derived value of k_i for indoxyl sulphate. Nevertheless, indoxyl sulphate did decrease the binding of L-tryptophan to HSA and at concentrations known to occur in renal failure. Indoxyl sulphate (0.1–0.8 mM) can displace phenytoin, sulphamethoxine, sulphamethoxazole, fluorescein, salicylate and thiopentone from human albumin (Ali and Lindup, unpublished results). Indoxyl sulphate may, therefore, contribute to the drug binding defect since concentrations of up to about 0.2 mM can occur in uraemia [36–38].

Renal failure increases the plasma concentration of several other indoles [38] and the properties of a crude preparation of the unidentified binding inhibitor described by Depner and Gulyassy [11] are not inconsistent with it being an indole derivative. We have found in preliminary experiments that the following indoles inhibit the binding of methyl red to HSA: indole-5-carboxylic acid; 5-hydroxy-3-indole acetic acid; indole-2-carboxylic acid; indole-3-lactic, -propionic, -butyric, -pyruvic and -acrylic acids, the last three being among the most potent. The contribution of these indoles to the binding defect, particularly with regard to L-tryptophan, needs further evaluation.

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