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PROTEIN BINDING OF DRUGS IN UREMIC AND NORMAL SERUM: THE ROLE OF ENDOGENOUS BINDING INHIBITORS

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Abstract The protein binding of diazepam, indomethacin, salicylic acid, sulfadimetoxine and warfarin in serum of uremic patients has been studied by equilibrium dialysis and circular dichroism measurements and compared with that in normal serum. Comparisons have also been made with isolated human serum albumin (HSA) from uremic patients and healthy individuals. The binding of diazepam, salicylic acid, sulfadimetoxine and warfarin is impaired in the uremic sera, while the binding of indomethacin is apparently unchanged. The apparent binding constants of salicylic acid and warfarin in both uremic and normal sera are affected by dilution of the sera in buffer. The binding constants obtained with isolated albumins, however, are unaffected by dilution. The albumin isolated from uremic serum shows lower binding affinity for salicylic acid and warfarin than normal HSA, but the affinity was normalized by charcoal treatment at pH 3.0. It is shown that the binding both in normal and uremic sera is impaired compared with isolated defatted serum albumin due to the presence of competitive inhibitors. The inhibition is more pronounced in uremic serum. In addition, the binding to albumin in uremic sera is impaired by strongly bound allosteric inhibitors. It is also emphasized that determinations of association constants have to be related to the dilution of the serum, plasma or blood, respectively.

Plasma from uremic patients has a decreased ability to bind certain drugs, which was first observed for some sulfa drugs [1, 2]. Reidenberg and associates [3] also observed a decreased binding of diphenylhydantoin in uremic plasma, the unbound fraction of the drug being strongly correlated to the levels of creatinine and blood urea nitrogen. They concluded that the lowered binding was not explained by low plasma albumin levels, since it occurred also in uremic patients with normal albumin levels. Subsequently, a decreased binding in uremic plasma or serum has been demonstrated for several other drugs, most of which have the common feature that they are weak acids (cf Table 1 where some earlier findings as well as the results from the present study are included). It should be observed that the bases diazepam and dapsone, which are uncharged at pH 7.4, contain electronegative centres, which may promote the binding.

Two hypothetical mechanisms are advanced to explain the observed phenomenon: (1) The serum from the uremic patients contains abnormal albumin

Table 1. The binding of various drugs in uremic plasma or serum

Drug	pK_a*	Binding compared with normal serum	Reference
Dapsone	1.0†	decreased or unchanged	[4]
Desmethylimipramine	10.1†	unchanged	[3]
Digitoxin	neutral	decreased	[6, 7]
Diphenylhydantoin	8.3	,,	[4, 6, 8–12]
Furosemide‡	3.7	,,	[13]
Pentobarbital	7.9	,,	[5]
Quinidine	4.3†, 8.4†	increased or unchanged	[4, 12]
Sulfaisodimidine	7.5	decreased	[2]
Sulfamethazine	6.9	,,	[1]
Sulfamoxole	7.2	,,	[2]
Triamterene	6.0†	,,	[4]
Trimethoprime	7.3†	unchanged	[14]
Diazepam	3.3†	decreased	This work
Indomethacin	4.5	unchanged	,, ,,
Salicylic acid	3.0	decreased	,, ,, [8]
Sulfadimethoxine	5.9	,,	,, ,,
Warfarin	5.1	,,	,, ,,

* Approximative pK_a-values.

† Basic compound. The pK_a is given for the corresponding acid, HB^+.

‡ The study was made on plasma from blood drawn immediately after hemodialysis.

species. (2) The albumin in the uremic serum is normal, but the binding of certain drugs to the albumin is impaired due to the presence of one or more inhibiting agents. The inhibition may be either competitive or noncompetitive.

The purpose of the present investigation was to study the mechanisms responsible for the impaired drug binding of uremic serum. Binding properties of three drugs with different affinities for albumin were studied in parallel by two different techniques, namely equilibrium dialysis and circular dichroism measurements. Binding experiments were undertaken with different dilutions of serum in buffer, to study if the impaired binding is due to inhibitors or to changed properties of the albumin.

MATERIALS AND METHODS

Serum. Samples were collected on three occasions, pooled and stored frozen until the experiments were undertaken. Pools 1 A and 1 B were used for experiments on intact serum, pools 2 A and 2 B for dilution experiments whilst pools 3 A and 3 B were used for the preparation of albumin. (A denotes normal serum pools and B uremic serum pools).

Altogether 13 healthy persons and 15 uremic patients participated in the study. Nine of the uremic patients were treated at the Renal Unit, Department of Internal Medicine, Karolinska Hospital, Stockholm. Sera from 4 of the uremic patients were kindly supplied by Drs. J. Bergström and B. Wehle, St. Eriks Hospital, Stockholm. The diagnosis, serum creatinine values and pharmacotherapy of the patients are given in Table 2 as well as the serum creatinine values of the pools. Most uremic patients included in this study were selected so as to avoid patients treated with drugs which fulfill the criteria of having high therapeutic serum levels and high binding to serum albumin. The presence of serum from one patient treated with a sulfonamide preparation in pool 2 B was thought to be of little significance for the overall

Table 2. Clinical data of the uremic patients

Serum pool number	Patient	Diagnosis	Serum creatinine Individual values (mg/100 ml)	Serum creatinine Pool values (mg/100 ml)	Serum albumin Pool values (mg/ml)	Drugs (g/day, orally)
1 B	K.H.	Chronic pyelonephritis	20.0	13.7	30.0	Hydralazine 0.075, Alprenolol 0.15, Allopurinol 0.1, Furosemide 0.08
	H.V.	Polycystic kidneys	12.8			Hydralazine 0.05, Furosemide 0.08
	A.S.	Chronic pyelonephritis	13.2			Propranolol 0.08
2 B	B.R.	Chronic glomerulonephritis	13.9	10.9	31.8	Digoxin 0.00013 every third day, Furosemide 0.5
	B.O.	Hypoplastic kidney Nephrosclerosis	13.6			Alphamethyldopa 1.5, Allopurinol 0.1, Sulfamethizole 0.4 and Sulfamethoxypyridazine 0.1 every second day, Digoxin 0.000006, Furosemide, 0.08, Ferrous succinate 0.075
	S.R.	Aplastic kidney Hydronephrosis	9.2			Ferrous succinate 0.075
	S.K.	Dysplastic kidneys	12.6			Furosemide 1.0, Cephalosporin 2.0
3 B	K.H.	Chronic pyelonephritis	9.0	9.4	29.5	Digoxin 0.00013, Cortisonacetate 0.05, Clemastin 0.003, Dicumarol 0.05, Allopurinol 0.1, Noscapine 0.075, Promethazine 0.025
	E.F.	Chronic glomerulonephritis	12.8			Bendroflumethiazide 0.005, Hydralazine 0.075, Alprenolol 0.6, Clofibrate 1.0
	R.S.*	Chronic pyelonephritis	8.9			Digoxin 0.00013, Ampicillin 0.5, Furosemide 0.008 Dihydrotachysterol 0.0012
	K.L.	Chronic pyelonephritis	6.6			Digoxin 0.00013, Allopurinol 0.1, Amitriptylin 0.03, Trimethoprim 0.08, Sulfamethoxazole 0.4
	E.E.	Nephrosclerosis Hypertension	6.8			Digoxin 0.00013, Clemastin 0.003, Promethazine 0.025, Prednisolone 0.02, Levomepromazine 0.015, Furosemide 0.25, Ascorbic acid 1.5
	B.P.*	Polycystic kidneys	12.0			Alphamethyldopa 1.5
	A.L.	Chronic nephritis	20.0			Ferrous succinate 0.037
	M.B.*	Chronic glomerulonephritis	9.5			Cephalexin 1.0, Clonidine 0.0003

* These patients were also treated by chronic intermittent hemodialysis.

results. Serum creatinine was determined according to Heinegard and Tiderström [15]. Some circular dichroism studies were also made on individual sera as specified in the figure legends.

Human serum albumin. HSA was prepared from normal blood-bank plasma by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography mainly according to McMenamy *et al.* [16]. The albumin was treated with activated charcoal at pH 3.0 according to Chen [17]. The albumin monomer was then isolated by gel-filtration on Sephadex G-100 in 0.02 M sodium phosphate buffer, pH 7.4. The concentration of HSA was determined from the extinction at 280 nm ($E^{1\%}_{1\,cm} = 5.80$). Albumin from pooled uremic serum was prepared by the same method.

Drugs. [^{14}C]Salicylic acid (31.4 mCi/m-mole) and [^{14}C]warfarin (23.5 mCi/m-mole) were purchased from The Radiochemical Centre, Amersham, England. [^{14}C]Indomethacin (26.3 mCi/m-mole) was a gift from Merck, Sharpe and Dohme. The radiochemical purity (>98%) was checked by thin layer chromatography. After purification of [^{14}C]warfarin less than 0.5% radiochemical impurities were detected. The corresponding unlabelled compounds were added to the isotope solutions to achieve suitable drug concentrations (0.1–2 mM). Unlabelled drugs were gifts from the different manufacturers.

Equilibrium dialysis. The serum protein binding was determined by equilibrium dialysis at 37° against isotonic phosphate buffer, pH 7.4 [18], using Technicon Type A standard membranes. For each drug concentration, duplicate determinations using 500 µl of serum and buffer were performed. The time used for equilibration was 5 hr for salicylic acid, 17 hr for warfarin and 8 hr for indomethacin. After equilibration, radioactivity (expressed in dis/min) was determined in duplicate with 100 µl of sample aliquots from both sides of the dialysis cells in 10 ml of Instagel® (Packard Instrument Company) using a Packard Tricarb Scintillation Spectrometer 3375 or a Beckman Scintillation Counter, LS 100-C. The binding was related to the serum albumin concentration, which was determined by immunochemical quantitation according to Mancini *et al.* [19].

Circular dichroism (CD) measurements. These were made on a Jasco J-20 spectropolarimeter, Japan Spectroscopic Co., Tokyo. The instrument was calibrated with D-camphorsulphonic acid and tested daily with a built-in test-signal system. Rectangular cells with path-lengths of 0.5–10 mm were used, in order to optimize the measuring conditions. The temperature was kept between 25° and 27°.

The sera were diluted with 0.005 M phosphate in 0.1 M KCl, pH 7.4, to a suitable albumin concentration. The drugs, dissolved in the same buffer or in a mixture of buffer and ethanol (final ethanol concentration never exceeded 1%) were then added to the diluted sera in a 1–3-fold molar excess at pH 7.4.

The results are expressed as molar ellipticity $[\theta]$ in degrees·cm^2·dmole^{-1}, calculated with reference to the albumin concentration, assuming a molecular weight of 65,100 [16]. The molar ellipticity $[\theta]$ is defined according to the equation:

$$[\theta] = \frac{\theta \cdot M}{c \cdot l \cdot 10}$$

where θ is the observed ellipticity in degrees, M is the molecular weight, c the concentration in g/ml and l the path-length in cm.

MATHEMATICAL ANALYSIS

The serum protein binding data for salicylic acid and warfarin were analyzed according to Scatchard, assuming binding mainly on albumin and assuming one class of binding sites [20]. The equation:

$$\frac{r}{(D)} = n \cdot K_{app} - rK_{app} \qquad (1)$$

was used where r = moles of bound drug/moles of albumin, D = moles of unbound drug, n = number of binding sites, K_{app} the apparent association constant. In the Scatchard plots, the points were fitted to a straight line by linear regression using r as the independent and $r/(D)$ as the dependent variable. Linear regression analysis was also used to characterize other linear relationships.

Characterization of inhibition of drug binding. When the binding of a ligand to a macromolecule is influenced by a competitive inhibitor, the following relationship exists [21] between the apparent association constant K_{app}, determined in the presence of the inhibitor, I, and the 'true' association constant, K_a^x.

$$K_{app} = \frac{K_a^x}{1 + (I) \cdot K_i} \qquad (2)$$

where K_i is the association constant for the binding of the inhibitor to the same site on the macromolecule and (I) is the unbound inhibitor concentration. The equation can be rearranged to:

$$K_{app} = K_a^x - K_{app} \cdot (I) \cdot K_i \qquad (3)$$

The true association constant, K_a^x, for a particular drug can be obtained from a series of diluted sera by estimating the apparent association constants in these sera. When the concentration of the inhibitor is unknown, it may be practical to introduce the approximate substitution:

$$(I) = I_o \cdot C_{se} \qquad (4)$$

where I_o is the initial concentration of the inhibitor in the undiluted serum and C_{se} is the fractional concentration of the serum in the diluted samples. One then obtains:

$$K_{app} = K_a^x - K_{app} \cdot C_{se} \cdot I_o \cdot K_i \qquad (5)$$

When K_{app} is plotted against $K_{app} \cdot C_{se}$, a straight line is obtained if the binding is competitively inhibited. The ordinate intercept corresponds to the K_a^x of the particular drug–protein system. The slope of the line $(-I_o \cdot K_i)$ is characteristic for a particular inhibitor in a particular serum. The numerical value of the slope is directly proportional to the initial inhibitor concentration and the association constant, K_i, of the inhibitor for the binding to the particular site, that binds the drug.

RESULTS

Equilibrium dialysis studies. Serum protein binding data for the lowest drug concentrations of indomethacin, salicylic acid and warfarin are given in Table 3.

Table 3. Serum protein binding in per cent of total drug concentration

Serum	Salicylic acid 0.10 mM	Warfarin 0.13 mM	Indomethacin 0.10 mM
Normal 1A	93.5	99.2	96.1
Uremic 1B	74.7	97.8	96.0
Normal 2A	94.3	99.1	96.7
Uremic 2B	88.9	97.5	94.3*

* At the time of the investigation the albumin concentration had decreased from 31.8 to 25.3 mg/ml.

Examples of the Scatchard plots are given in Figs. 1–3. As seen from the table and Figs. 1 and 2, the protein binding of salicylic acid and warfarin was lower in the uremic serum than in the normal serum.

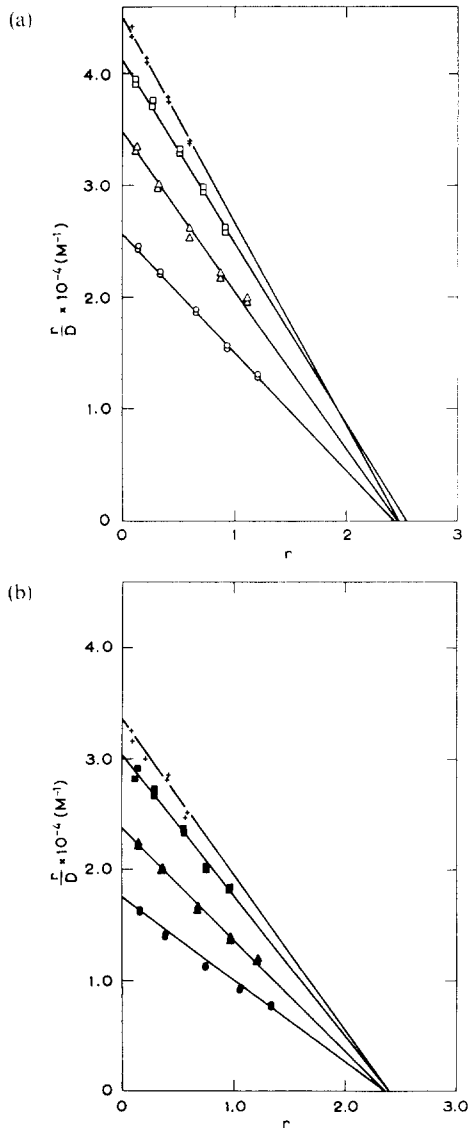

Fig. 1. Scatchard plots of the binding of salicylic acid to (a) normal serum 2 A (○○○, undiluted; △△△, diluted 1 + 1; □□□, diluted 1 + 4; + + +, diluted 1 + 9) and (b) uremic serum 2 B (●●●, undiluted; ▲▲▲, diluted 1 + 1; ■■■, diluted 1 + 4; + + +, diluted 1 + 9) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

The binding in the sera was also lower than to corresponding amounts of isolated and charcoal-treated HSA taken from normal serum or uremic serum (see Table 4 and Figs. 4 and 5). The Scatchard plots for warfarin (Fig. 2) showed a slightly curved form and only the points with $r < 0.6$ were used to estimate the product $n \cdot K_{...}$, n and $K_{...}$. For salicylic acid the intercept on the x-axis gave n around 2.5. In this case the estimated $K_{...}$ (eqn. 1) is a composed constant

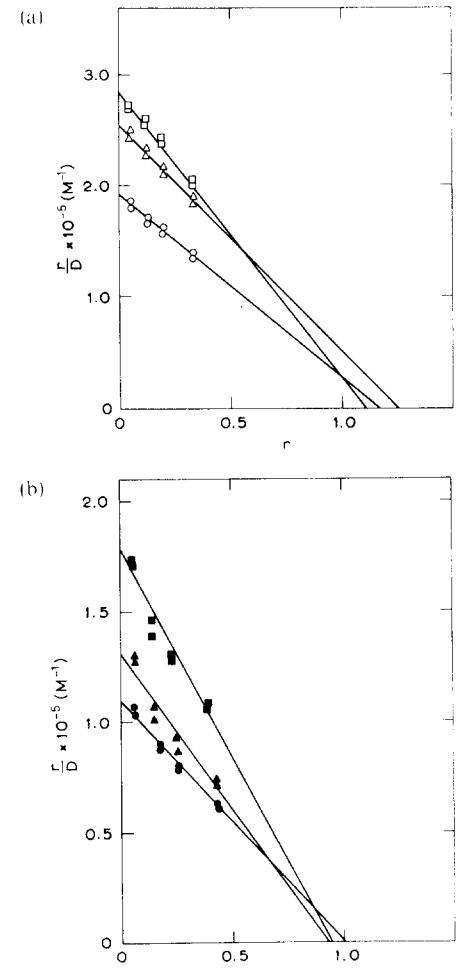

Fig. 2. Scatchard plots of the binding of warfarin to (a) normal serum 2 A (○○○, undiluted; △△△, diluted 1 + 1; □□□, diluted 1 + 4) and (b) uremic serum 2 B (●●●, undiluted; ▲▲▲, diluted 1 + 1; ■■■, diluted 1 + 4) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

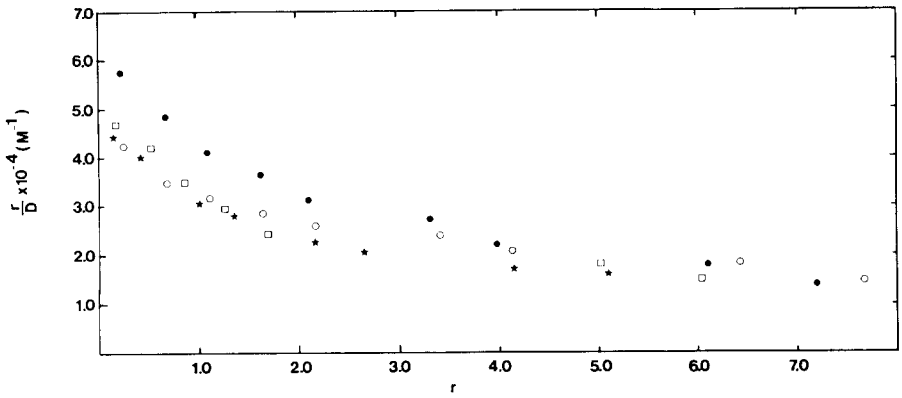

Fig. 3. Scatchard plots of the binding of indomethacin to normal serum pools 1 A (□□□) and 2 A (★★★) and uremic serum pools 1 B (●●●) and 2 B (○○○).

Table 4. Data obtained from Scatchard plots

Serum pool	Salicylic acid $n \cdot K_{ass}$ $M^{-1} \cdot 10^{-4}$	n	K_{ass} $M^{-1} \cdot 10^{-4}$	Warfarin $n \cdot K_{ass}$ $M^{-1} \cdot 10^{-5}$	n	K_{ass} $M^{-1} \cdot 10^{-5}$
Normal 1A	2.3	2.9	0.8	2.2	1.3	1.8
Uremic 1B	0.7	2.8	0.2	1.5	0.9	1.6
Normal 2A	2.6	2.5	1.1	1.9	1.2	1.6
Uremic 2B	1.7	2.4	0.8	1.1	1.0	1.1
Charcoal treated HSA from normal serum	4.8	2.7	1.8	3.4	1.1	3.0
Untreated HSA from uremic serum	3.0	2.3	1.3	2.3	0.9	2.4
Charcoal treated HSA from uremic serum	4.7	2.7	1.8	3.5	1.0	3.4

with contributions from at least two binding sites. This means that the mathematical discussion above giving equations (2)–(5) can only be used for a comparative study of the binding of salicylic acid in different sera and HSA-solutions. The results summarized in Table 4 demonstrate that for both warfarin and salicylic acid there is a significant decrease in K_{ass} in uremic serum. The decrease in the product $n \cdot K_{ass}$ is even more pronounced, due to an effect on the n values.

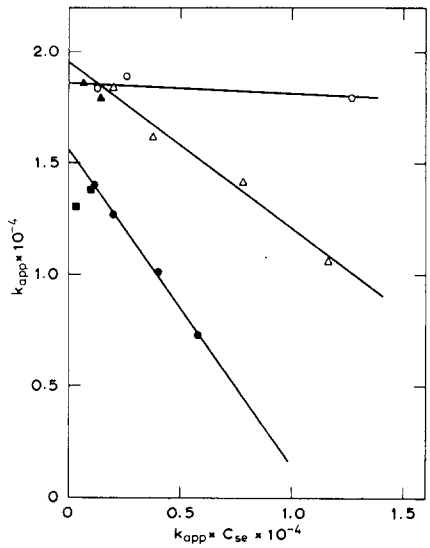

Fig. 4. Graphs of K_{app} versus K_{app} x C_{se} for salicylic acid in normal serum 2 A (△△△), uremic serum 2 B (●●●), human serum albumin (○○○), isolated uremic albumin (■■■) and charcoal-treated uremic albumin (▲▲▲). The product K_{app} x C_{se} in the different sera is corrected for the different albumin concentrations, defining $C_{se} = 1$ as 40 mg albumin per ml.

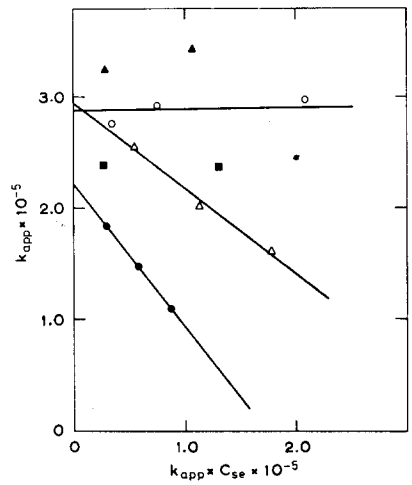

Fig. 5. Graphs of K_{app} versus K_{app} x C_{se} for warfarin in normal serum 2A (△△△), uremic serum 2B (●●●), human serum albumin (○○○), isolated uremic albumin (■■■) and charcoal treated uremic albumin (▲▲▲). The product K_{app} x C_{se} in the different sera is corrected for the different albumin concentrations, defining $C_{se} = 1$ as 40 mg albumin per ml.

In contrast to the findings with salicylic acid and warfarin, no clear differences in protein binding of indomethacin could be demonstrated between uremic and normal serum (Table 3, Fig. 3). The Scatchard plots indicated a considerable contribution of secondary binding sites. The data were therefore not analyzed according to eqn. 1.

The binding of salicylic acid and warfarin was also studied in different dilutions in buffer of a uremic serum pool, a normal serum pool and HSA. The Scatchard plots for the binding are shown in Figs. 1 and 2. The lines drawn are the linear regression lines. The intercepts on the r-D-axis from which the apparent binding constants, K_{app}, are obtained, changed with the dilution of sera, while they were constant within experimental errors with different concentrations of isolated HSA. With increasing dilution of the serum K_{app} increased, whereas the intercepts on the r-axis remained constant.

The effect on K_{app} from the Donnan equilibrium will be small and will not change the results. This is evident from the results with isolated HSA giving the same K_{app}-values with widely different HSA-concentrations (Figs. 4 and 5) and from the fact that the main portion of the negatively charged proteins in serum is HSA, as the isoelectric points of the immunoglobulins vary around 7. With undiluted serum samples the maximal error in the calculation of the concentration of the negatively charged, free drugs, and thus the K_{app} value, will then be about 5%, under our experimental conditions, which apparently will not significantly influence the results. Figs. 4 and 5 show the plots of K_{app} versus $K_{app} \cdot C_w$ for the binding of salicylic acid and warfarin, respectively, to uremic serum, normal serum and isolated normal HSA. All the experimental data fall on straight lines. The slope of the lines obtained with the uremic serum is steeper than with the normal serum for both drugs. According to eqn. 5 this means that the product $K_i \cdot I_n$ is larger.

The apparent binding constants with isolated HSA were the same with different concentrations. For both salicylic acid and warfarin, the intercepts, K_a^λ, on the Y-axes coincided with those from the normal serum, which evidently means that the binding in the sera can be explained entirely by the albumin contents. However, the intercepts, K_a^λ, obtained with the two drugs from the uremic serum pool were considerably lower.

In Figs. 4 and 5 the results obtained with albumin isolated from the uremic serum pool 3 B are also included. As is evident, the affinity of this albumin for salicylic acid and warfarin was significantly lower

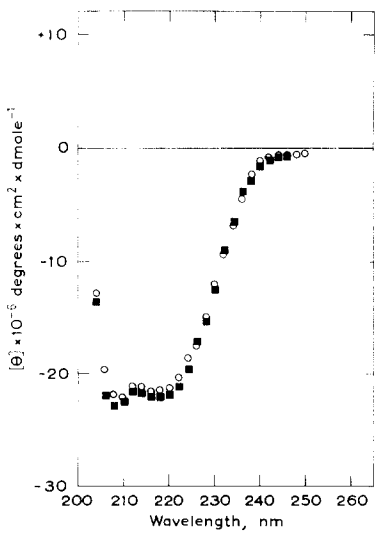

Fig. 6. Circular dichroism spectra of diluted serum from a uremic patient M.B. (■■■) and a healthy person (○○○) recorded between 200 and 250 nm in a 0.5 mm cell, pH 7.4. The albumin concentration was 0.5 mg/ml.

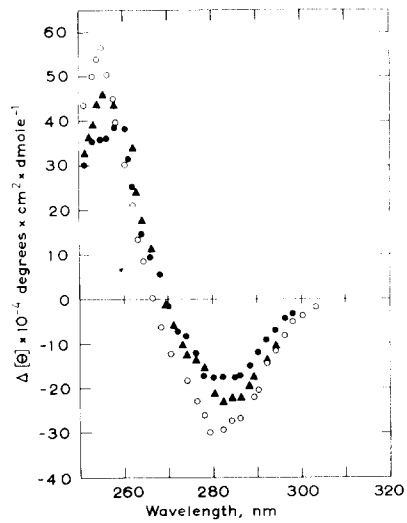

Fig. 7a. Circular dichroism spectra, recorded with 10 mm cells at pH 7.4, of the drug protein complexes between sulfadimetoxin and two uremic sera from patients A.L. (▲▲▲) and K.H. (●●●) and one normal serum (○○○) obtained as the difference spectra after subtraction of the CD-spectra for the sera alone. The albumin concentration was 1.5 mg/ml and the drug albumin ratio was 0.5.

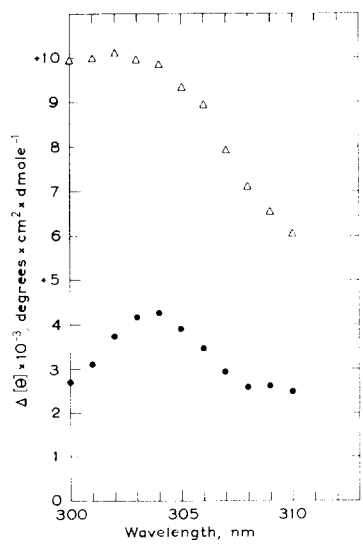

Fig. 7b. Circular dichroism spectra, recorded with 2 mm cells at pH 7.4, of the drug protein complexes between salicylic acid and one uremic serum pool 1 B (●●●) and one normal serum (△△△) obtained as the difference spectra after subtraction of the CD spectra for the sera alone. The albumin concentration was 20 mg/ml and the drug was present in a 2-fold molar excess.

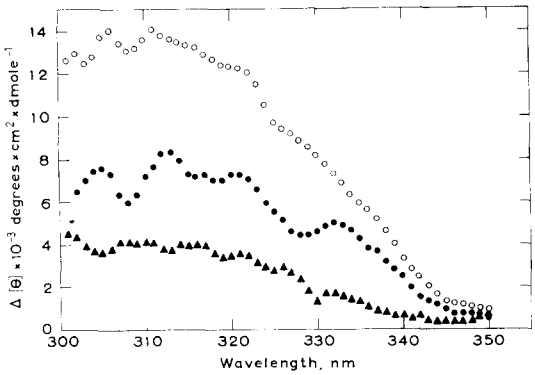

Fig. 7c. Circular dichroism spectra, recorded with 2 mm cells at pH 7.4, of the drug protein complexes between warfarin and two uremic sera, pool 1 B (●●●), patient A.L. (▲▲▲) and one normal serum (○○○) obtained as the difference spectra after subtraction of the CD-spectra for the sera alone. The albumin concentration was 20 mg/ml and the drug was present in a 2-fold molar excess.

than that of normal HSA. However, the binding properties of the albumin were the same as those of the normal HSA after treatment with activated charcoal according to Chen [17].

Circular dichroism studies. To study secondary and tertiary structures of the proteins in uremic and normal sera, the CD spectra of the sera were recorded in the far ultraviolet region at 200–250 nm. The ellipticity in this wavelength region is dominated by the contribution from the albumin present. As is shown in Fig. 6, there are no significant differences between the CD spectra of normal and uremic sera.

The binding of salicylic acid, warfarin, sulfadimethoxine and diazepam to normal and uremic serum was studied in the wavelength region 250–350 nm. When the drugs are bound to the proteins in the sera, new

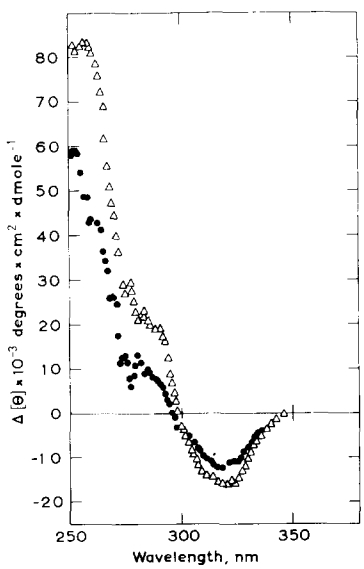

Fig. 7d. Circular dichroism spectra, recorded with 10 mm cells at pH 7.4, of the drug-protein complexes between diazepam and one uremic serum pool 1 B (●●●) and one normal serum (△△△) obtained as the difference spectra after subtraction of the CD-spectra for the sera alone. The albumin concentration was 0.9 mg/ml and the drug was present in a 2-fold molar excess.

extrinsic Cotton effects are created at the wavelengths where the substances have absorption maxima. The magnitude of the extrinsic Cotton effects are directly proportional to the concentration of the drug protein complexes. In Fig. 7, the difference CD spectra for the different drug protein complexes are shown. The difference spectra were obtained by subtracting the CD spectra given by the respective sera alone from those obtained when the respective drug was present. As is evident from the figures, the uremic sera produced significantly smaller extrinsic Cotton effects than the normal sera. This means that they have an impaired binding ability. It should be pointed out that the HSA and drug concentrations were the same in the two sera.

DISCUSSION

Studies on the binding of a drug to plasma proteins are of fundamental importance in pharmacokinetic investigations [22] since it affects the apparent volume of distribution (V_d) of the drug [23] a parameter which usually is based on total plasma concentration data. For drugs with very high protein binding and small V_d the binding will also have profound influence on the pharmacological effects and the elimination. One representative of this group of drugs is warfarin [24]. Salicylic acid, indomethacin, and sulfadimetoxin, also studied in this work, are interesting as potential displacing agents in protein-binding interactions. Moreover, diazepam, which is a weak base, uncharged at physiologic pH-values, is of great theoretical interest in this connection, since it is strongly bound to HSA at only one primary site [25, 26].

Many studies on protein binding of drugs have been performed on purified plasma fractions, especially the albumin fraction. While necessary for the understanding of the mechanism of binding, such data do not allow a direct extrapolation to the *in vivo* situation. Therefore parallel studies have to be performed with serum or plasma from patients in which the drugs in question are used, in order to obtain a basis for conclusions about the practical significance of drug protein binding. In the present study, it is unequivocally shown by both equilibrium dialysis and circular dichroism that the binding of salicylic acid, warfarin, diazepam and sulfadimetoxin is impaired in uremic patients suffering from chronic renal failure of different genesis.

In contrast to the drugs mentioned, the binding of indomethacin apparently was unchanged. An abnormal binding behaviour of indomethacin has been described by Mason and McQueen [27] who found that phenylbutazone did not displace indomethacin, despite the fact that indomethacin displaces phenylbutazone [28]. Studies by Dollery *et al.* [29] and Mason and McQueen [27] as well as the present study indicate, however, that indomethacin binds to several sites on HSA, which might explain why the binding degree of indomethacin is not significantly changed when the binding is disturbed at one site.

It is thus well established that the binding of certain drugs is significantly decreased in uremic patients. However, the mechanisms behind this effect are at present not well understood. Two possible explanations seem feasible, either the presence of inhibitors

in the serum or a modified albumin pool. The Scatchard plots (Figs. 1 and 2) clearly show that K_{app} increases when the sera are diluted. This indicates the presence of competitive inhibitors, the effect of which is decreased with dilution resulting in higher K_{app} values. Even normal serum contains inhibitors of the drug protein binding. This fact is hardly surprising considering the transport function of albumin and must be taken into account in all drug binding studies with serum, plasma or blood. With the accumulation of metabolic products in uremia the 'load' on the albumin increases and predisposes to an increased inhibition of drug protein binding. This concept is in consistence with the findings by Andreasen, who obtained increased protein binding capacity of both normal and uremic serum after *in vitro* dialysis [8].

For both salicylic acid and warfarin the affinity constant for the binding to isolated HSA in buffer was almost twice as high as the constant for the binding in normal serum. By successive dilution of the serum with buffer, the latter constant gradually increased so that at infinite dilution the two constants became identical. These results prove that albumin is the only binding protein of importance in serum for these two drugs.

The dilution experiments performed with uremic serum gave K_a^∞-values at infinite serum dilution which were considerably lower than those for normal sera. The decreased protein binding in uremic serum can thus be explained only to a certain degree by competitive inhibition. Additional factors must therefore be considered. The interest is then focused on the idea, advanced by several authors [1, 3], that there is a qualitative change in the uremic serum albumin. The microheterogeneity among albumins isolated from serum from normal volunteers and uremic patients observed by Shoeman and Azarnoff is especially interesting in this context [6]. By isoelectric focusing they observed the presence of two separate bands of albumin, bands A and B, in plasma from normal volunteers and uremic patients and in commercially available HSA. The relative amount of the B-band was highest in normal plasma and appeared to correlate to the binding capacity.

Also in other diseased [30] or abnormal states [31], as well as in normal HSA [32–34] heterogeneities have been detected in the albumin pool. It is therefore not unreasonable that there should be a changed drug binding capacity of the albumin in uremic patients. The CD studies in the far ultraviolet region (200–250 nm), where the contributions from secondary and tertiary structures of the polypeptide backbone of the albumin determine the ellipticity, were performed to detect any conformational differences between the albumin in the uremic and the normal serum (Fig. 6). However, no such differences were established. Unfortunately, the sensitivity of the method is limited, and minor changes of the conformation around the binding sites, e.g. due to an allosteric inhibition or minor alterations in the secondary or tertiary structures might not be detected. This means that the absence of any detectable CD differences is not conclusive evidence that no conformational differences exist.

By analogy with non-competitive Michaelis Menten kinetics, it is easy to show that irreversibly inhibited or allosterically changed albumin will give constant but lower K_{app}-values than normal albumin at different dilutions. At infinite dilution the uremic serum pool also gave lower binding constants, K_a^∞, with salicylic acid and warfarin than those obtained with normal serum. Moreover, the binding to isolated uremic albumin was impaired, but charcoal treatment of the albumin at pH 3.0 normalized the binding. A similar effect of the charcoal treatment on the binding properties of neonatal plasma albumin has been observed by Chignell *et al.* [35]. Most probably, the charcoal treatment removed strongly bound inhibitors.

The present study thus shows that the binding of salicylic acid and warfarin is normally considerably inhibited in serum by competitive mechanisms involving endogenous compounds as displacers. In uremic sera the displacement is even greater, but the decreased binding cannot be fully accounted for by the presence of reversibly bound displacing agents. The possibility of distinctly different albumin species is contradicted by the fact that charcoal treatment of the uremic albumin at acidic pH restored the binding properties and by the unchanged CD spectra obtained with uremic serum or isolated uremic albumin. The findings are best explained by the presence of a binding inhibitor irreversibly bound at normal pH but reversibly bound at pH 3.0. Studies are under way to isolate and identify the inhibitor(s).

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