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CORRECTION OF DRUG BINDING DEFECTS IN UREMIA *IN VITRO* BY ANION EXCHANGE RESIN TREATMENT*

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Abstract—Serum protein binding of weakly acidic drugs is impaired in uremia, but that of basic drugs tends to be normal. Treatment of uremic serum with anion exchange resin (Amberlite CG-400, acetate form) corrected binding defects for three acidic drugs (nafcillin, salicylate and sulfamethoxazole) but did not affect the binding of two basic drugs (trimethoprim and quinidine). Resin treatment of normal human serum did not alter the binding of these five drugs. Extraction of the acetate buffer eluate from resin exposed to uremic serum with *n*-butyl chloride at acidic pH (3.0) resulted in a fraction that could induce similar binding defects in normal human serum. The factor(s) responsible for binding defects in uremia appears to be lipid soluble, weakly acidic, and dialyzable. It is believed to be tightly bound to albumin at physiologic pH, but dissociates from it at acidic pH. These findings further support the previously proposed hypothesis that drug-binding defects in uremia are due to accumulation of certain endogenous metabolic product(s).

The decrease in serum protein binding of many drugs, primarily organic acids, in uremic patients is now widely recognized [1–4]. Many investigations have been carried out to study the chemical basis of the binding defects, but the precise mechanism which accounts for the phenomenon is still unknown. The degree of reduction in binding in this patient population is greater than can be accounted for by hypoalbuminemia [4–6], and the defect in binding is not corrected by hemodialysis, or prolonged *in vitro* dialysis [2, 7].

Two major hypotheses have been proposed to explain the binding defect phenomena. One hypothesis is that there may be intrinsic abnormalities in the structure of the binding protein, namely albumin, in the uremic state [8–10]. Another hypothesis is that certain endogenous metabolic products, which are present in low concentrations and are normally excreted by the kidneys in healthy individuals, accumulate in renal failure. These endogenous metabolic products would tightly bind to albumin molecules and, by so doing, modify the configuration of the protein molecule or bind to the primary binding sites and compete with weakly acidic drugs [11–14]. The latter hypothesis is supported by the fact that the binding defect is rapidly corrected by successful renal transplantation [15] and by activated charcoal treatment of uremic sera at acidic pH (3.0) [11]. However, the factor responsible for binding defects has not been recoverable from activated charcoal.

Recently, an organic solvent, *n*-butyl chloride, has been employed successfully to correct the binding defects by extraction of uremic serum at acidic pH (3.0). The binding inhibitor contained in the organic solvent layer was partially purified to homogeneity by thin-layer chromatography. Further characterization of the compound(s) reveal that binding defects similar to those seen in uremia can be induced by the compound when added to either normal serum or purified human serum albumin. Furthermore, the compound was shown to be dialyzable, lipid soluble and weakly acidic, with a molecular weight of approximately 500 or less [14].

Since the binding inhibitor was demonstrated to be an acidic compound, it appeared quite logical to apply an anion exchange resin treatment to correct the binding defects. The anion exchange resin, Amberlite CG-400 (acetate form), has been used successfully to correct the binding defects present in uremic serum. The compound(s) capable of inducing binding effects similar to those seen in uremia was easily eluted from the resin and partially characterized. This confirms previous findings that the binding defects in uremia are due to accumulation of endogenous metabolic products rather than any intrinsic defects in albumin structure.

METHODS

Serum specimen collections. All blood samples were collected in red top Vacutainer tubes (Becton, Dickinson & Co., Rutherford, NJ) with informed consent. The uremic specimens were obtained from

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thirty-five patients (ages ranging from 25 to 80), with chronic renal failure, maintained on hemodialysis; the specimens were taken immediately before receiving heparin, prior to a routine hemodialysis. Normal healthy volunteers (ages ranging from 20 to 40 of either sex) were employed to obtained either pooled or individual serum specimens to be used as controls. Serum specimens after separation were stored at −20° until use.

Assay procedures. Total serum protein [16], albumin [17], free fatty acid levels [18] and bilirubin [19] concentration were determined. α_1-Acid-glycoprotein levels were determined using radial diffusion plates purchased from Behring Diagnostics, American Hoechst Corp., Somerville, NJ. Fluorometric assays for nafcillin [20], sulfamethoxazole [21], trimethoprim [21], and quinidine [22] were employed. Salicylate was measured by the method of Trinder [23]. For penicillin G, benzyl[^{14}C]penicillin potassium (51 mCi/mole) was utilized in trace amounts to correlate the radioactivity to corresponding drug concentrations. Thin-layer chromatography was performed as described previously [14], and compounds were visualized using long and short wave ultraviolet light by the aid of an ultraviolet lamp (model UVSL-25, Ultraviolet Products, Inc., San Gabriel, CA). The R_f values of the compounds in the different extracts were compared.

Preparation of anion exchange resin. Resin was prepared according to the method of Scheider and Fuller [24]. Dry Amberlite CG-400 resin 100–200 mesh (Mallinckrodt Chemical Works, St. Louis, MO) was immersed overnight in 0.1 N NaOH, washed with deionized water, and then washed with isopropyl alcohol–heptane–2.5 M H_2SO_4 (40:10:1, by vol.) until the visible front reached the bottom of the column. Another three additional bed volumes of the above solvent mixture were passed through the resin. Isopropyl alcohol and then water were used to wash the resin. One molar acetic acid was passed through the column to convert it to the acetate form.

Protein binding measurements. Equilibrium dialysis as originally described by Klotz [25] and modified by Kunin [26] was utilized to determine the degree of protein binding of serum specimens. Two milliliters of serum was introduced into a nonnitrated cellulose dialysis tubing that had been boiled in deionized water for 15 min and rinsed twice with deionized water and was dialyzed against 3 ml of Krebs–Ringer phosphate buffer, pH 7.4, which contained the drug to be studied. Dialysis was performed on a roller drum (model TC-2, New Brunswick Scientific Co., New Brunswick, NJ) at 4° until equilibrium was reached (usually 36–48 hr). A buffer control (i.e. buffer dialyzed against buffer) was included to ensure that the drug had reached equilibrium across the membrane. An additional control was included in which the same amount of drug being studied was added to the serum and dialyzed against drug-free buffer. If equilibrium was reached, the assayed concentration of drug in serum should be identical whether drug is added inside or outside the bag. This latter control is necessary, as the rate of dialysis would be slower with protein binding [27]. Binding studies were run at 4°, since at this temperature bacterial contamination is not likely to occur and interfere with binding values. Furthermore, the effect of temperature (i.e. 4°, 25°, and 37°) on binding values of a number of drugs has been shown to be rather insignificant [27].

Drugs utilized in the binding studies and their concentrations are given below. Acidic drugs included penicillin G (10 μg/ml), nafcillin (20 μg/ml), sulfamethoxazole (20 μg/ml) and salicylate (100 μg/ml). Basic drugs employed in the study were trimethoprim (20 μg/ml) and quinidine (5 μg/ml).

The effect of serum albumin concentration on each drug studied was evaluated by diluting pooled normal human serum with Krebs–Ringer phosphate buffer, pH 7.4, to achieve 75, 50 and 25% serum. Five separate binding measurements for each dilution were determined by equilibrium dialysis to calculate mean values as well as standard deviations for each dilution.

Anion exchange resin treatment. Resin treatment of normal and uremic serum specimens was performed as follows. Approximately 2 ml of the resin suspension (CG-400, acetate form) was added to 15 ml graduated, ground glass stoppered Pyrex conical centrifuge tubes. Following centrifugation at 1000 *g* for 5 min, the liquid above the resin was aspirated and adjustments were made, if necessary, so that there was approximately 1 ml of resin per tube. Three milliliters of either normal human or uremic serum was added to the tubes containing the resin, and the tubes were mechanically shaken for 15 min with approximately 100 to-and-fro horizontal motions per min. After centrifugation for 10 min at 1000 *g*, the serum was removed from the resin and dialyzed overnight against Krebs–Ringer phosphate buffer, pH 7.4, to restore physiological pH. The resin was saved for isolation of the binding inhibitor. The pH of the treated serum samples was checked to ensure that it was 7.4. Samples of the pre-treatment and post-treatment normal and uremic serum were analyzed for total serum protein [16], albumin [17], and free fatty acids [18] to determine the effect of the resin treatment procedure on the levels of these components. Treated normal and uremic specimens were then tested for their binding capacity for acidic and basic drugs by equilibrium dialysis, and these values were compared to their pre-treatment values. Standard statistical analyses such as F-test and Student's *t*-test were employed to evaluate the significance of changes in drug binding values.

Inducement of binding defects. To determine whether the substance from uremic serum that adheres to the anion exchange resin can be eluted and used to induce binding defects for acidic drugs in normal human serum, the resin which had been exposed to normal or uremic serum was transferred to a 5-ml capacity disposable glass pipette. The column was rinsed with deionized water until no traces of albumin could be detected [19]. Five milliliters of 1 M sodium acetate buffer, pH 8.0, was applied to the column, and the eluate was collected. After acidification of the eluate to pH 3.0, the eluate was then extracted by mechanical shaking with 3 ml of *n*-butyl chloride for 10 min (100 to-and-fro motions/min). The *n*-butyl chloride layer was removed after centrifugation of the extraction mix-

Table 1. Effect of anion exchange resin treatment on serum protein binding of six drugs*

Drug	Per cent protein bound			
	Normal serum		Uremic serum	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Sulfamethoxazole	73.9 ± 0.1	74.2 ± 1.0	50.3 ± 7.6	73.1 ± 0.5†
Nafcillin	87.7 ± 1.9	85.4 ± 0.6	80.5 ± 4.6	86.1 ± 1.05†
Salicylate	93.8 ± 0.04	93.7 ± 0.1	85.3 ± 4.0	94.0 ± 1.3†
Trimethoprim	70.2 ± 1.5	69.5 ± 0.7	69.8 ± 0.5	69.5 ± 1.2
Quinidine	83.3 ± 1.8	83.9 ± 3.2	83.5 ± 7.8	82.1 ± 9.0
Penicillin G	71.3 ± 2.1	70.5 ± 2.4	36.0 ± 1.8	36.8 ± 3.3

* Serum specimens (3-ml aliquots) were mechanically shaken for 15 min with anion exchange resin (1 ml packed volume). After centrifugation, the serum was removed from the resin. Serum specimens were dialyzed against Krebs-Ringer phosphate buffer, pH 7.4, overnight and then were used for binding studies. Values are means ± S.D. and are derived from six different sera per value.

† Values are statistically greater than pre-treatment values with $P < 0.005$.

ture for 5 min at 1000 *g*. The extraction procedure was repeated once more and the organic solvent fractions were pooled. The combined organic extract was added to 3 ml of pooled normal human serum and the samples were shaken mechanically for 15 min at room temperature. The organic layer was removed after centrifugation of the mixture at 2000 *g* for 10 min. Serum samples were dialyzed overnight against Krebs–Ringer phosphate buffer (pH 7.4) at 4°. The pH after dialysis was checked to ensure that it was 7.4. Specimens were tested for their protein binding capacity by equilibrium dialysis.

RESULTS

Effect of anion exchange resin treatment on drug–protein binding. Serum specimens obtained from both normal volunteers and uremic patients were treated with anion exchange resin as described in Methods. After centrifugation, the serum layer was subjected to dialysis against Krebs–Ringer phosphate buffer, pH 7.4. Protein binding values for six pharmacological agents (nafcillin, salicylate, sulfamethoxazole, penicillin G, quinidine, and trimethoprim) were then determined as described in Methods.

The protein binding values for the weakly acidic drugs nafcillin, sodium salicylate and sulfamethoxazole in relation to serum albumin concentrations before and after the resin treatment are illustrated in Figs. 1–3. The reductions in albumin concentration to variable degrees, as reflected in the figures, were due to the dilutional effect of the resin treatment, as the resin was stored in water. The resin treatment resulted in an average reduction of free fatty acid levels by 14–21%. Bilirubin levels in all specimens were within the normal limits.

It can be seen that the binding defects for nafcillin, salicylate, and sulfamethoxazole, which were observed in the uremic serum specimens, were fully corrected by the resin treatment (Table 1). Results of the studies with penicillin G were inconclusive (Table 1). On the other hand, similar resin treatment did not alter the binding values for the two basic

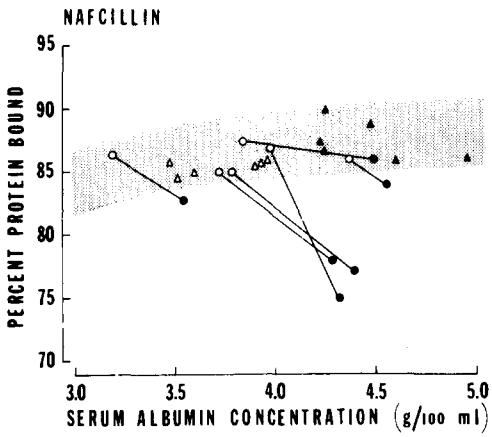

Fig. 1. Effect of anion exchange resin treatment on the relationship between serum albumin concentration and protein binding of nafcillin. The dotted area represents the 95% confidence limits for protein binding of sera (from five normal subjects) diluted with Krebs–Ringer phosphate buffer, pH 7.4, to lower the albumin concentration. Closed symbols (● and ▲), represent the protein binding values of untreated sera of uremic and normal subjects respectively. Open symbols (○ and △) and the binding values of uremic and normal sera, respectively, following the resin treatment.

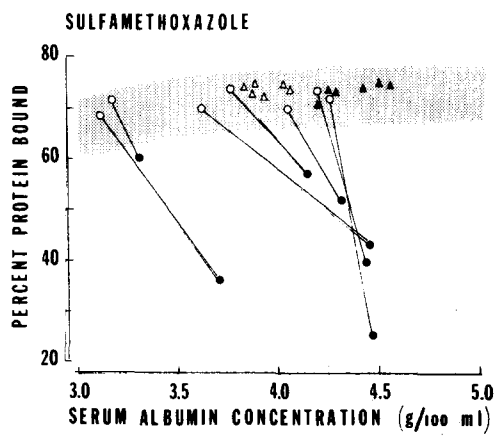

Fig. 2. Effect of anion exchange resin treatment on the relationship between serum albumin concentration and protein binding of sulfamethoxazole. Symbols are identical to those in Fig. 1.

Table 2. Inducement of drug binding defects by extracts of column eluates from uremic sera*

Drug	Per cent protein bound	
	Normal human serum	Normal serum plus extract
Sulfamethoxazole	74.4 ± 0.5	59.4 ± 4.3†
Nafcillin	86.0 ± 0.4	81.7 ± 4.0†
Salicylate	94.0 ± 0.5	87.3 ± 2.5†
Trimethoprim	70.0 ± 1.5	69.8 ± 1.5
Quinidine	82.5 ± 2.6	81.8 ± 2.9
Penicillin G	72.0 ± 1.5	67.3 ± 1.7

* *n*-Butyl chloride extracts of acidified column eluates were added to normal human serum specimens (3 ml) and gently mixed at pH 7.4 for 15 min at room temperature. After aspiration of the organic solvent, the specimens were dialyzed against Krebs–Ringer phosphate buffer, pH 7.4, overnight at 4° and used for binding studies. Values are means ± S.D.

† Values are statistically smaller than normal human serum binding values with $P < 0.01$.

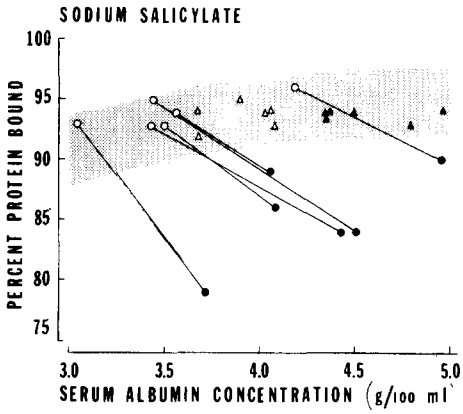

Fig. 3. Effect of anion exchange resin treatment on the relationship between serum albumin concentration and protein binding of salicylate. Symbols are identical to those in Fig. 1.

drugs, quinidine and trimethoprim, as shown in Table 1. The three uremic serum samples which exhibited elevated quinidine binding values also had elevated α_1-acid-glycoprotein values (220, 184 and 180 mg/dl) compared to normal values (55–140 mg/dl). When the binding values before and after resin treatment were statistically analyzed, the results which are summarized in Table 1 were obtained.

Inducement of binding defects. To determine if there was a substance that was removed from uremic serum by the resin, which could induce binding defects in normal human serum, *n*-butyl chloride extracts of acidified column eluates from normal and uremic serum were gently mixed with normal human serum as detailed in Methods. Following removal of the *n*-butyl chloride layer, samples were dialyzed against Krebs–Ringer phosphate buffer, and drug binding values for the six drugs studied were determined as described in Methods. The results are summarized in Table 2.

As can be observed, the *n*-butyl chloride extracts of column eluates from resin exposed to uremic serum contained a component which could induce binding defects for the acidic drugs nafcillin, salicylate, and sulfamethoxazole. On the other hand, the binding values for the basic drugs quinidine and trimethoprim were not altered.

Table 3. Effect of dialysis of uremic serum against normal human serum on protein binding of sulfamethoxazole*

Time (hr)	Per cent sulfamethoxazole bound	
	Normal serum side	Uremic serum side
0	72.0 ± 0.6	50.5 ± 3.9
24	64.5 ± 0.9	59.7 ± 2.6
48	63.0 ± 1.0	60.5 ± 2.5
69	61.2 ± 0.3	60.0 ± 1.2

* Binding of sulfamethoxazole in normal human serum remained the same throughout the experiment.

Effect of dialysis of uremic serum against normal serum. Dialysis of uremic serum against normal human serum resulted in a progressive improvement in the protein-binding of sulfamethoxazole in uremic serum, and equilibrium between the two sera was reached with a prolonged dialysis (i.e. 96 hr), as illustrated in Table 3. These results clearly demonstrate that the binding defect inducer is dialyzable under proper conditions.

DISCUSSION

Defective drug binding for acidic drugs in uremia is a well-established phenomenon and has been widely studied by many investigators [1–4]. Two major hypotheses have been given to explain the drug binding defect associated with severe renal failure: one theory deals with possible intrinsic structural abnormalities in the binding proteins (i.e. albumin) [8–10], and the other hypothesis speculates that there may be accumulation of certain endogenous metabolic products in uremia [11–14].

Several studies support the hypothesis that binding defects are due to the accumulation of endogenous metabolic products [2, 11, 14, 15]. One study used organic solvent extraction of acidified uremic serum to correct the binding defects [14]. Analysis of the organic extract showed the compounds to be weakly acidic, lipid soluble, and dialyzable with a molecular weight of 500 or less [14]. Similar treatment of uremic

serum did not affect the binding of the basic drugs quinidine and trimethoprim. Resin treatment of normal human serum did not alter the binding of these five drugs. The findings described above for both the three acidic and the two basic drugs were quite compatible with what was expected from uremic serum.

The effect of resin treatment on penicillin G binding was quite unpredictable, and the results were essentially identical to those obtained from either activated charcoal treatment [11] or *n*-butyl chloride extraction at acidic pH [14]. Recently, the effect of free fatty acids on protein binding of antimicrobials was investigated, and a strong positive correlation between the levels of free fatty acids and the degree of penicillin G binding was demonstrated [28]. Anion exchange resin treatment of serum specimens was shown to remove free fatty acids from serum and therefore to reduce penicillin G binding.

To investigate the possibility of recovering the binding defect inducer from the resin exposed to uremic serum, the resin eluate was analyzed for its ability to induce similar binding defects in normal human serum. *n*-Butyl chloride extract of the acidified eluate was able to induce the expected binding defects for three acidic drugs (nafcillin, sulfamethoxazole and salicylate) when added to either normal pooled human serum or purified human serum albumin solution at pH 7.4. On the other hand, the binding values for two basic drugs (quinidine and trimethoprim) were not altered by similar treatment.

These results document a second method which enabled recovery of the component responsible for drug binding defects in uremia. Thus, further investigation was carried out to compare this property of the compound with the fraction obtained by the organic solvent, *n*-butyl chloride, extraction procedure. The component obtained from the column was shown to have a similar mobility on a thin-layer chromatogram with fluorescence similar to that of the binding inhibitor recovered from *n*-butyl chloride extraction.

In summary, the anion exchange resin, Amberlite CG-400, has been used successfully to correct the drug binding defects seen in uremia. Recovery of the defect inducer by two separate methods, *n*-butyl chloride extraction procedure and anion exchange resin treatment of uremic serum, was shown to be possible. These findings support the hypothesis that the binding defect in uremia is due to accumulation of certain toxic metabolic products rather than to an intrinsic defect in albumin structure. Furthermore, the component most likely represents a compound or class of compounds that are weakly acidic, lipid soluble, dialyzable, and tightly bound to albumin at physiological pH but free at acidic pH. It is speculated that they most likely represent some metabolic products of aromatic amino acids, and further studies are in progress.

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