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CONTRIBUTION OF CYANATE TO THE ALBUMIN BINDING DEFECT OF UREMIA

KENNETH BACHMANN,* MONICA VALENTOVIC and RONALD SHAPIRO
The University of Toledo College of Pharmacy and The Medical College of Ohio, Toledo, OH 43606, U.S.A.

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Abstract—The binding of [^{14}C]warfarin to normal human serum albumin (HSA), carbamoylated albumin, and plasma of patients with renal failure was investigated. Warfarin binding to extensively carbamoylated albumin (exhibiting a molar ratio of homocitrulline : albumin of *ca.* 7) was markedly diminished. The decrease in binding was apparently due to a decrease in the primary binding affinity constant. The K_a for warfarin binding to HSA was 1.6×10^5 M^{-1} compared to 0.88×10^5 M^{-1} for carbamoylated HSA. Binding to normal plasma was significantly greater than to plasma from uremic patients. Mean fractions bound (±S.E.M.) were 98.0 ± 0.2 and 94.1 ± 1.6 respectively ($P < 0.02$). The extent of carbamoylation of albumin from patients with renal failure, however, was low, exhibiting a homocitrulline : albumin molar ratio of 0.27. The binding of [^{14}C]warfarin to carbamoylated albumin with a homocitrulline : albumin molar ratio of 0.5 was marginally diminished, suggesting that the carbamoylation of albumin that occurs during renal failure contributes only slightly to the defective plasma binding of warfarin in uremia.

Decreased binding of acidic drugs to plasma or albumin of patients with poor renal function is a well documented phenomenon [1, 2]. It has been attributed to changes in the nature of albumin itself [3, 4], to the presence of reversibly bound endogenous inhibitors of drug binding [5], and to the presence of irreversibly bound endogenous inhibitors of drug binding [6]. The addition to normal serum or plasma of guanidinosuccinic acid, *p*-aminohippuric acid, creatinine, or phenolic substances failed to give rise to the binding defect characteristic of uremia [7, 8]. The role of cyanate in the binding defect seen during uremia was initially investigated because this putative uremic toxin evolves spontaneously from urea [9] and is capable of reacting irreversibly with free amino groups on protein [10] as has been demonstrated, for example, with the carbamoylation of hemoglobin [11]. Our preliminary data suggested that cyanate may be responsible for the warfarin-binding defect [12]. Others have recently reported a possible role for cyanate in the defective binding of sulfadiazine and salicylate during uremia [13]. The present investigation was undertaken to extend our preliminary observations and to delineate more clearly the role of cyanate in the decreased binding of warfarin during uremia.

MATERIALS AND METHODS

Albumin. Normal serum albumin (HSA) fraction V was obtained from the Sigma Chemical Co. (St. Louis, MO). Albumin from six individuals with renal failure [serum creatinine 9.0 to 25.6 mg/100 ml (mean, 16.3), and BUN 53 to 106 mg/100 ml (mean, 84.3)] was isolated from plasma and purified by repeated ammonium sulfate precipitation and dialysis. Ammonium sulfate precipitation was performed as described by Kendall [14]; however, filtrates were dialyzed against running water for 48 hr and then lyophilized. Freeze-dried material was tested for homogeneity by electrophoresis on cellulose acetate strips [12]. A single spot was usually detected after five successive precipitation-dialysis cycles.

Derivatization of normal HSA. Carbamoylation of HSA was performed by incubating HSA with several concentrations of potassium cyanate under sterile conditions for 24 hr as described previously [12]. The difference between the distance that derivatized-albumin migrated upon electrophoresis and the migration distance of HSA was taken as a measure of the extent of carbamoylation [12].

Binding of [^{14}C]*warfarin to albumin and plasma.* Racemic [^{14}C]warfarin (51 mCi/mmole, 99 per cent radiochemically pure, Amersham/Searle, Arlington Heights, IL) binding to HSA, to derivatized HSA, and to uremic and normal plasma was assessed by ultrafiltration (23°, pH 7.4) and liquid scintillation spectrometry as described previously [12]. Binding of [^{14}C]warfarin (0.6 mg/l) to 4% solutions of albumin in Normosol-R† (Abbott Laboratories, North Chicago, IL) and to plasma was expressed as fraction bound, or as normalized fraction bound. Normalized fraction bound (normalized to 4% albumin) was computed as described previously [8]. Binding of

* Send correspondence to: Kenneth Bachmann, Ph.D., Associate Professor of Pharmacology, The University of Toledo College of Pharmacy, 2801 W. Bancroft St., Toledo, OH 43606, U.S.A.

† Normosol-R contains the following per liter: 5.26 g sodium chloride, 2.22 g sodium acetate, 5.02 g sodium gluconate, 370 mg potassium chloride, and 140 mg magnesium chloride.

[^{14}C]warfarin to 0.04% albumin solutions was analyzed according to the method of Scatchard [15].

Protein hydrolysis and detection of homocitrulline residues. The hydrolysis of normal HSA, derivatized HSA (dHSA), and HSA isolated from the plasma of uremic patients (uHSA), and the subsequent collection of amino acid residues were performed as described by Stark and Smyth [16]. Hydrolysates of 0.5 μmole albumin were placed on columns containing a cation exchange resin (Dowex-50W, 200–400 mesh, Sigma Chemical Co.) and were eluted first with 70 ml of water and then with 40 ml of 1 N ammonium hydroxide. The ammonium hydroxide eluate was evaporated to dryness at 40° using a Flash Evaporator (Buchler Instruments, Ft. Lee, NJ). Residues were dissolved in 2 ml of distilled water and then lyophilized (Virtis Uni-Trap, Gardiner, NY). Lyophilized material was reconstituted, and the amount of homocitrulline was measured spectrophotometrically with a Bausch and Lomb Spectronic 700 using a procedure modified from that described for citrulline [17], with thiosemicarbazide added to increase the sensitivity [18]. Ten milligrams of the lyophilized amino acid fraction of normal or derivatized HSA hydrolysates was reconstituted in 1 ml of distilled water, and 200 μl of this solution was added to a 4 ml solution containing 0.125% diacetylmonoxime, 0.005% thiosemicarbazide, 0.0093% ferric chloride hexahydrate, and 4.4% sulfuric acid (Pierce Chemical Co., Rockford, IL). The solution was mixed on a vortex mixer and incubated at 100° for 30 min. The absorption of the resulting pink chromophore was read at 525 nm after cooling for 5 min in an ice bath. Homocitrulline content of HSA from uremic patients was determined by dissolving 10 mg of the lyophilized amino acid fraction in 4 ml of chromogenic reagent and measuring the resulting absorbance as described above. Homocitrulline content was expressed as the molar ratio of homocitrulline : albumin.

Charcoal treatment of normal and derivatized HSA. Normal HSA and HSA derivatized by incubation with 125 mg/100 ml or 200 mg/100 ml potassium cyanate were charcoal treated by the method of Chen [19]. Charcoal-treated albumin was dialyzed against running water for 24 hr at 23° and then lyophilized. Homocitrulline content and warfarin binding were measured before and after charcoal treatment.

Fluorescence of normal HSA and uHSA. Normal HSA or HSA from each of five uremic patients was dissolved in distilled water (1 mg/ml). Fluorescence was read at an excitation wavelength of 345 nm and an emission wavelength of 410 nm using a Turner model 430 spectrofluorometer (Turner Instruments, Palo Alto, CA), with distilled water as the blank.

Cyanate detection in plasma ultrafiltrates. Ultrafiltrates of plasma from six patients with renal dysfunction and of normal plasma were assayed for cyanate by the spectrophotometric method of Marier and Rose [20] with the following modifications: (1) buffering to pH 6.3 was accomplished with 0.004 M phosphate buffer and 1 N HCl; (2) the copper–pyridine–cyanate complex was extracted into 1,2-dichloroethane, spectroquality (Mallinckrodt, St. Louis, MO), rather than chloroform.

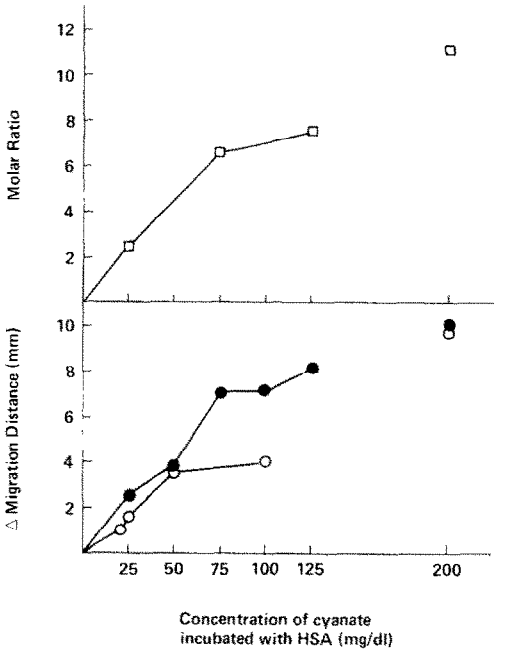

Fig. 1. Impact of cyanate incubation on the extent of carbamoylation of HSA. Lower panel depicts extent of carbamoylation represented by differences in electrophoretic migration distances between normal and derivatized albumin; HSA (●); BSA (○). Upper panel shows extent of carbamoylation estimated as molar ratio of homocitrulline : albumin for derivatized HSA.

Statistical analysis. Comparisons were made using Student's *t*-test. Differences were considered significant at $P < 0.05$ (two-tailed).

RESULTS

Derivatization of HSA. The extent of carbamoylation of HSA, indicated by migration distance upon electrophoresis, increased as the concentration of cyanate incubated with HSA was increased. Figure 1 depicts the migration distances of carbamoylated HSA and bovine serum albumin (BSA) relative to normal HSA and BSA as a function of the concentration of potassium cyanate with which they were derivatized. A sharp increase in derivatization occurred when HSA was incubated with 25, 50, or 75 mg/100 ml cyanate. The extent of derivatization, however, appeared to plateau at higher cyanate concentrations, until concentrations of 200 mg/100 ml cyanate were incubated with albumin. Figure 1 also shows the extent of carbamoylation expressed more directly as the molar ratio of homocitrulline : albumin plotted as a function of the concentration of cyanate with which HSA was incubated. The increase in the molar ratio was lowest in the range of 75–125 mg/100 ml cyanate. The correlation between the molar ratios of homocitrulline : albumin and the electrophoretic migration of derivatized HSA was good (correlation coefficient, 0.994).

Homocitrulline : albumin ratios of HSA and albumin from renal patients. The molar ratio of homo-

Table 1. Extent of [^{14}C]warfarin binding to HSA, carbamoylated HSA, normal plasma, and renal failure plasma*

Sample	Albumin concn (g/l)	Fraction bound (β)	Normalized fraction bound† (β')
T.T.‡	38	94.3	94.6
C.N.	31	91.7	93.4
J.W.	30	96.2	97.1
J.P.	40	93.0	93.0
J.H.	35	95.3	95.9
Mean		94.1 ± 1.60§	94.8 ± 1.53
Normal plasma	40	98.0 ± 0.20	98.0
HSA	40	97.8 ± 0.12	
Carbamoylated HSA‖	40	94.3 ± 0.12§	
Carbamoylated HSA¶	40	93.7 ± 1.68§	

* Values are means ± S.E.M.
† Normalized to an albumin concentration of 40 g/l.
‡ Initials are of patients with uremia.
§ $P < 0.02$ (compared with normal plasma or HSA).
‖ Molar homocitrulline:albumin ratio = 6.6
¶ Molar homocitrulline:albumin ratio = 7.3

citrulline:albumin was three times greater for albumin isolated from uremic patients than for normal HSA. The ratio for normal HSA was 0.08 (0.06 to 0.12) compared to 0.27 (0.20 to 0.33) for albumin from uremic patients ($P < 0.001$).

Binding of [^{14}C]warfarin to albumin and plasma. The extent of [^{14}C]warfarin binding to 4% solutions of HSA, dHSA (molar ratio of homocitrulline:albumin = 6.6), and dHSA (molar ratio = 7.3) was 97.8, 94.3, and 93.7 per cent respectively (Table 1). Also shown in Table 1 are the measured and normalized bound fractions of warfarin for normal plasma and plasma from five uremic patients. Binding was less extensive to carbamoylated albumin than to normal HSA, and it was less extensive to plasma from uremic patients (94.8 per cent) than to normal plasma (98.03 per cent). In Fig. 2, a comparison of warfarin binding parameters for normal HSA (0.04 per cent) and dHSA (0.04 per cent, molar ratio = 7.6) shows a 45 per cent reduction in K_a for the carbamoylated HSA, from 1.60×10^5 M^{-1} to 0.88×10^5 M^{-1} with the number of primary binding sites increasing slightly from 1.6 to 2.1.

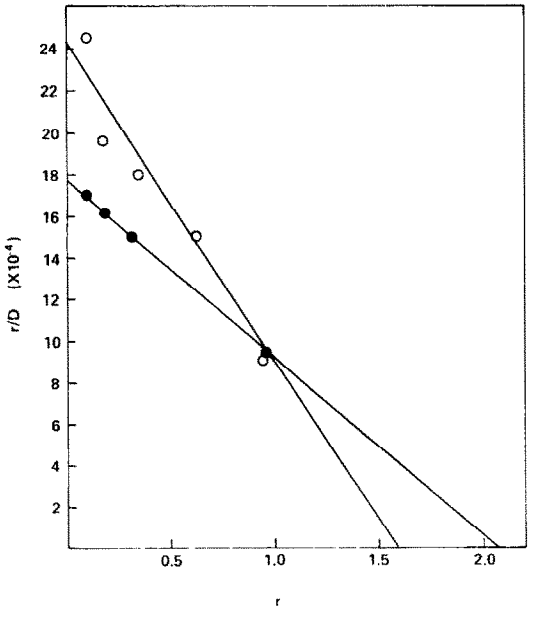

Fig. 2. Scatchard plots for [^{14}C]warfarin binding to HSA (○) and to carbamoylated HSA (●). The molar ratio of homocitrulline:albumin of carbamoylated HSA was 7.6.

Only a slight decrease in warfarin binding, however, was discernable at the very low molar ratio of homocitrulline:albumin of 0.5. This derivative exhibited a bound fraction of 98.7 per cent compared to 98.9 per cent for normal HSA.

Charcoal treatment. The molar ratio of homocitrulline:albumin decreased slightly following charcoal treatment of dHSA. Albumin, derivatized with 125 mg/100 ml potassium cyanate for 24 hr, exhibited an initial homocitrulline:albumin ratio of 7.8. The ratio for albumin derivatized with 200 mg/100 ml cyanate was initially 11.6. The ratios decreased to 6.33 and 7.70, respectively, following charcoal treatment, an average decline of 26 per cent. Charcoal treatment of dHSA (molar ratio of 7.8) increased the bound fraction of warfarin from 92.7 to 96.3 per cent.

Fluorescence of normal HSA and renal failure HSA. The fluorescence of albumin isolated from plasma of uremic patients was three to five times greater than the fluorescence of normal HSA (Fig. 3).

Cyanate concentrations in plasma ultrafiltrates. Concentrations of cyanate in plasma ultrafiltrates from six uremic patients averaged 8.4 mg/100 ml ± 2.71 (S.E.M.). However, no cyanate could be detected in two of the ultrafiltrates.

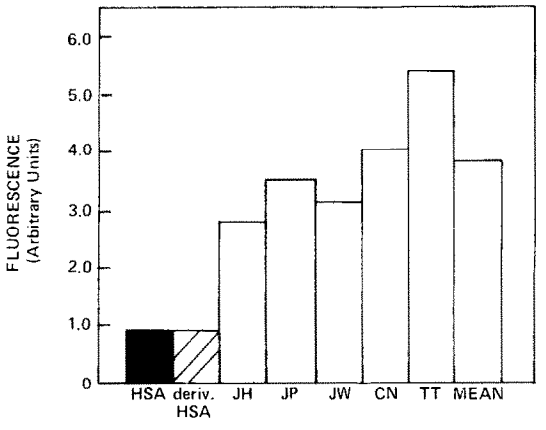

Fig. 3. Relative fluorescence of HSA, carbamoylated HSA, and albumin isolated from five patients with renal failure. The molar ratio of homocitrulline : albumin for derivatized HSA = 11.6. Emission and excitation wavelengths were 345 and 410 nm respectively.

DISCUSSION

This investigation was conducted with a view toward defining more clearly the role of cyanate in the uremic albumin-binding defect. The selection of warfarin as the binding ligand was based upon its high degree of binding which is diminished in uremia [6]. Having determined that cyanate-induced carbamoylation of albumin is capable of reducing warfarin binding [12], it seemed prudent to determine whether uHSA was indeed more carbamoylated than HSA and, if so, whether carbamoylation of uHSA would be sufficient to elicit a decrease in warfarin binding.

The extent of carbamoylation was estimated by determining the molar ratio of homocitrulline : albumin. Carbamoylation of lysine ε-amino groups by cyanate to form homocitrulline would be anticipated since the $T_{\frac{1}{2}}$ of cyanate-mediated lysine carbamoylation is 2–3 hr [21]. Furthermore, Tse and Pesce [22] observed, using amino acid analysis, a marked decrease only of lysine residues when HSA was treated with hexamethylene diisocyanate; isocyanate treatment had no impact on other amino acid residues. The molar ratio of homocitrulline : albumin correlated well with the electrophoretic properties of dHSA. Increasing the concentration of cyanate with which albumin was incubated up to 75 mg/100 ml produced a sharp increase in the extent of carbamoylation. Carbamoylation, however, was increased only slightly when cyanate was incubated in concentrations of 75–125 mg/100 ml.

Clearly, dHSA exhibiting molar homocitrulline : albumin ratios of 6.6 to 7.6 bound warfarin less extensively than did normal HSA. The decreased binding of [^{14}C]warfarin evolved principally from a decrease in binding affinity. Although the molar ratio of homocitrulline : albumin was significantly higher for uHSA than for normal HSA, it was approximately twenty times lower than for samples of dHSA that exhibited diminished warfarin binding comparable to that which occurs in uremia. A low molar ratio of homocitrulline : albumin for uHSA (0.27) seems to be in accord with the low concentrations of cyanate detected in plasma ultrafiltrates. The concentrations of cyanate in plasma ultrafiltrates from uremic patients were about at the limit of the spectrophotometric assay, with a mean value of approximately 2.5 mM. No cyanate could be detected in two uremic plasma ultrafiltrates or in ultrafiltrates of normal plasma. The molar homocitrulline : albumin ratio for uHSA was approximately 60 per cent of the 0.45 ratio computed from the 6.6 μmoles carbomyl groups per g protein reported by Erill *et al.* [13]. Erill *et al.* [13], however, evaluated carbamoylation of uremic plasma, and did not specify whether or not carbamoylation occurred exclusively on albumin.

It was important to determine whether or not a molar ratio of 0.27 could be functionally significant in terms of perturbing warfarin binding. The effectiveness of the small extent of carbamoylation of hemoglobin (0.16 to 0.39 carbamoyl groups per hemoglobin tetramer) in the suppression of hemoglobin sickling in low oxygen-tension blood [23] suggested that fractional carbamoylation might be functionally significant. Similarly, a molar ratio of carbamoyl groups : brain protein as low as 0.1 has been reputed to interfere with maze learning behavior in rats [24]. The impact of slight carbamoylation, as occurs on uHSA, on warfarin binding was evaluated by preparing dHSA with a molar homocitrulline : albumin ratio of 0.5. This derivative bound warfarin only slightly less than it bound normal HSA, suggesting that carbamoylation contributes to, but does not in itself account for, the binding defect.

Since charcoal treatment of uremic plasma at pH 3 tends to normalize drug binding [7, 8], its impact on extensively carbamoylated dHSA exhibiting a pronounced decrease in [^{14}C]warfarin binding was investigated. The line of reasoning was that acid treatment of dHSA might hydrolyze carbamoyl groups, thereby normalizing binding. In contrast to others [25], we observed a 26 per cent decrease in the molar ratio of homocitrulline : albumin, following charcoal treatment of dHSA, attended by an improvement in warfarin binding.

Schwertner and Hawthorne [26] have recently reported albumin-bound fluorescence, in uremic sera, that was 3.5 times as high as for normal sera. Charcoal treatment of uremic sera removed 51 per cent of the fluorescence, suggesting a connection between fluorescence and the uremia binding defect [26]. Our analysis of albumin from patients with renal failure also revealed fluorescence approximately three to five times greater than that of normal HSA. dHSA, however, did not exhibit increased fluorescence relative to HSA. Thus, any connection between fluorescence of uremic albumin and the binding defect must be independent of carbamoylation.

Erill *et al.* [13] reported decreases in salicylate and sulfadiazine binding to carbamoylated plasma derivatized by incubation with 40 mM potassium cyanate. Interpolating from their data, changes in salicylate binding appeared to be effected by molar

ratios of carbamoyl groups:albumin averaging approximately 0.9 (assuming that only albumin was carbamoylated). Unfortunately, a ratio cannot be estimated for the derivatized plasma that exhibited a diminished K_a for sulfadiazine, since the μmoles of carbamoyl groups per g protein were not reported. Nor was the duration of incubation of those plasma samples with 40 mM potassium cyanate reported.

In conclusion, our data suggest that, although extensive carbamoylation of albumin can significantly decrease warfarin binding, the carbamoylation of lysine residues of albumin by cyanate occurring in uremia contributes only slightly to the uremia albumin binding defect which has been reported for warfarin. These observations afford a better perspective on the magnitude of the contribution of cyanate to the uremia binding defect than do earlier observations for warfarin [12], as well as those for salicylate, sulfadiazine, and sulfisoxazole [13, 25]. Carbamoylated albumin, exhibiting a molar homocitrulline:albumin ratio of 0.5, binds warfarin only marginally less extensively than normal HSA. This apparently small extent of carbamoylation approaches that which occurs on albumin of patients with renal dysfunction (*ca.* 0.3).

The apparently irreversible changes in albumin that give rise to its fluorescent properties in uremia may contribute to the binding defect as well. Recently, it was shown that a column of nonionic resin (polystyrene-divinylbenzene copolymer) could be used in place of charcoal treatment to correct the binding defect of uremic serum [27]. A second, subsequent elution of the same column with ethanol yielded a poorly characterized substance (or substances) which when added to normal serum established the binding defect with phenytoin as the ligand. Interestingly, the column eluate fluoresced at excitation and emission maxima of 340 and 420 nm respectively.

Irreversible changes in albumin induced by other putative uremic toxins, such as the guanidino compounds, and their possible roles in the binding defect, require further investigation. Boumendil-Podevin *et al.* [28] have established that indoxyl sulfate and hippuric acid accumulate in uremic serum and promote uricosuria. Their effects on warfarin binding have not yet been investigated.

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