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A possible role for cyanate in the albumin binding defect of uremia

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The decrease in binding of numerous drugs by uremic albumin or plasma is now widely recognized. Sjöholm *et al.* [1] reported a decrease in binding and affinity constants for both salicylic acid and warfarin in uremic serum. The binding of phenytoin [2], diazoxide [3], furosemide, clofibrate and sulfonamides [4], penicillins [5], diazepam [6], and valproic acid [7] to albumin is also diminished in uremic serum.

The precise nature of the binding defect has not been elucidated, though evidence for at least three separate and perhaps complementary mechanisms has been published. Andreasen [8], on the basis of dialysis studies, has suggested that the binding defect in uremia is attributable to the presence of small molecules which reversibly alter ligand-binding characteristics of albumin and which accumulate in uremia. The dilution studies of others [1] using warfarin and salicylic acid in uremic and normal sera suggest, however, that the substances which mediate the uremic binding defect are irreversibly bound to albumin at normal pH. Craig *et al.* [5] and other investigators [9] have evaluated the possible contribution to the binding defect of small molecules such as urea, guanidinosuccinic acid, creatinine, *p*-aminohippuric acid, and other small molecules which accumulate during uremia. As yet, none have been found to create a binding defect in normal serum or to reinstate the defect in charcoal-treated uremic serum. Changes in the uremic albumin molecule have been proposed. Electrophoretic differences between normal serum albumin and albumin isolated from uremic sera have been reported [10]. Moreover, for uremic and normal serum albumin, a quantitative difference in the amino acid composition of the two albumin bands separated by isoelectric focusing has been demonstrated [11].

Cyanate, a substance which is capable of reacting with albumin via carbamoylation of free amino groups, is a reasonable suspect as a potential contributor to the albumin binding defect. While values for cyanate concentrations in either normal or uremic serum have not, to our knowledge, been reported, cyanate is believed to circulate at excessively high levels in uremia [12]. We undertook a series of experiments to determine the impact of cyanate upon albumin and its warfarin-binding characteristics.

Methods

Derivatization (carbamoylation) of albumin. Bovine serum albumin (BSA, fraction V, electrophoretically pure, CalBiochem, San Diego, CA) was prepared as a 4% solution in a physiologic electrolyte solution (Normosol-R, Abbott Laboratories, North Chicago, IL)* with pH adjusted to 7.3. BSA was incubated with various concentrations of potassium cyanate (200, 250, 500, 1000 and 2000 mg/l) for 4, 24 or 48 hr. All solutions were sterilized by filtration through 0.45 μm membranes (Nalge). Incubations were performed in air under sterile conditions at 37° using a Dubnoff metabolic shaking incubator oscillating at 120 cycles/min. Derivatized albumin was dialyzed for 24 hr against running water at 22° and then lyophilized. Lyophilized samples were reconstituted with distilled water to a 0.4% concentration and electrophoresed on cellulose acet-

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

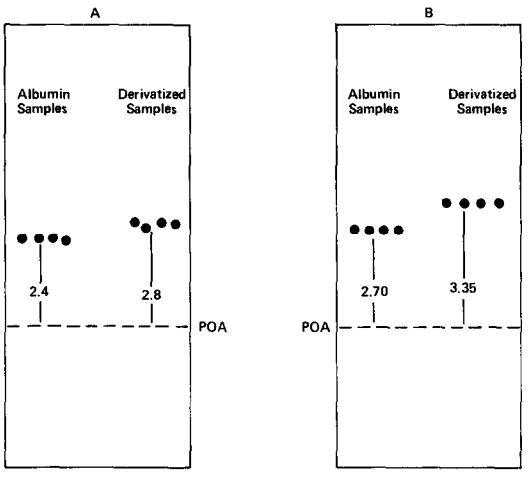


Fig. 1. Electrophoretic mobility of normal BSA and of BSA after 100 mg/dl CNO incubation. Numbers represent migration distance in cm. Panel A: 24-hr incubation; and panel B: 48-hr incubation. Current: 2–3 mA. POA; point of application.

ate strips (Sepraphore III, Gelman Instrument Co., Ann Arbor, MI) using a Gelman model 38206 power source. Strips were immersed in Tris–barbital buffer (0.05 M, pH 8.8), and current was maintained at 2–3 mA for 80 min. Normal BSA (0.4%) was electrophoresed alongside the derivatized samples. Cellulose acetate strips were stained with Ponceau stain [13] (Gelman Instrument Co.) for 8 min and decolorized by three successive washings with 5% acetic acid. Migration patterns of normal and derivatized BSA were compared.

Binding of [^{14}C]*cyanate to BSA.* [^{14}C]Potassium cyanate (57 mCi/mmole, 98 per cent radiochemically pure, Amersham/Searle, Arlington Heights, IL) was added to 4% BSA at a concentration of 1.125 μCi/ml. Samples were incubated at 37° for 24 hr as described above.

Two ml aliquots were ultrafiltered through dialysis tubing with 20 Å porosity, as described previously [14]. [^{14}C]Cyanate in 0.2 ml aliquots of ultrafiltrate and retentate were placed in 5 ml of PCS solubilizer (Amersham/Searle) and quantitated by liquid scintillation spectrometry using a Beckman LS 133 spectrometer. The extent of binding was estimated as:

$$\left(1 - \frac{\text{ultrafiltrate d.p.m.}}{\text{retentate d.p.m.}}\right).$$

Membrane binding was evaluated by ultrafiltering aqueous, protein-free solutions of [^{14}C]cyanate.

Binding of [^{14}C]*warfarin to normal and derivatized BSA.* [^{14}C]Warfarin (51 mCi/mmole, 99 per cent radiochemically pure, Amersham/Searle) was diluted with 0.1 N NaOH to obtain a concentration of 0.3 μg/μl. [^{14}C]Warfarin was incubated for 15 min at ambient temperature with 4% solutions of derivatized and normal BSA in the following concentrations: 0.3, 0.6, 1.2, 2.4 and 4.8 μg/ml. All samples were ultrafiltered through dialysis membranes, and the extent of warfarin binding to both normal and derivatized BSA was estimated as described previously [9]. Differences in warfarin binding, as a function of concentration incubated or type of albumin, were assessed using a two-way analysis of variance, and considered significant at $P < 0.05$.

Covalent [^{14}C]*cyanate binding to normal and uremic plasma.* Plasma obtained from normal subjects and a uremic patient (BUN, 91 mg/dl; serum creatinine, 16.2 mg/dl) was adjusted to a constant 0.4% albumin concentration by dilution. Albumin was assayed colorimetrically using the bromcresol green method [15] (Spec Tru BCG, Pierce Chemical Co., Rockford, IL). Samples were incubated with [^{14}C]cyanate (1.125 μCi/ml) at a total cyanate concentration of 1000 mg/l at 37° for 24 hr. The extent of [^{14}C]cyanate binding was determined by ultrafiltration and quantitated by liquid scintillation spectrometry as described above.

Results and Discussion

The incubation of potassium cyanate for 4–48 hr with BSA gave rise to a derivatized albumin, as determined electrophoretically after extensive dialysis. Concentrations of cyanate as low as 200 mg/l yielded a different electrophoretic pattern than that which was produced by normal BSA. Extent of carbamoylation, as demonstrated by differences between normal and cyanate-treated albumin, increased both as a function of the length of BSA and cyanate incubation as well as the concentration of potassium cyanate with which BSA was incubated. Increasing either the duration of incubation of BSA with cyanate or the

Table 1. Electrophoretic migration of normal and derivatized albumin (BSA)

Incubation (hr)	Cyanate conc (mg/l)	Migration distance of normal BSA*	Migration distance of cyanate-derivatized BSA	Differences in migration distances†	P‡
4	1000	24.0 ± 0.04	26.0 ± 0.45	2.0	< 0.1
	2000	25.8 ± 0.13	30.0 ± 0.33	4.2	< 0.01
24	200	33.0 ± 0.17	34.0 ± 0.58	1.0	< 0.2
	250	21.0 ± 0.20	22.5 ± 0.41	1.5	< 0.1
	500	25.5 ± 0.43	29.0 ± 0.48	3.5	< 0.01
	1000	24.0 ± 0.33	28.0 ± 0.58	4.0	< 0.01
	2000	28.0 ± 0.14	37.0 ± 0.38	9.0	< 0.001
48	500	21.0 ± 0.20	25.5 ± 0.24	4.5	< 0.001
	1000	27.0 ± 0.29	33.5 ± 0.24	6.5	< 0.001
	2000	25.5 ± 0.33	38.0 ± 0.20	12.5	< 0.001

* Migration distance from point of application (mm). Values are means ± S.E.M. N = 4.

† Distance beyond normal BSA which derivatized BSA migrated.

‡ Differences between mean migration distances for normal BSA and derivatized BSA were assessed via Student's two-tailed *t*-test.

Table 2. [^{14}C]Warfarin binding to normal and derivatized albumin (BSA)

Cyanate conc (mg/l)	Duration of incubation (hr)	N	% Bound (β)	P*
—	24	4	99.00 ± 0.001†	
1000	24	4	97.58 ± 0.01	< 0.01
2000	24	4	95.40 ± 0.01	< 0.01

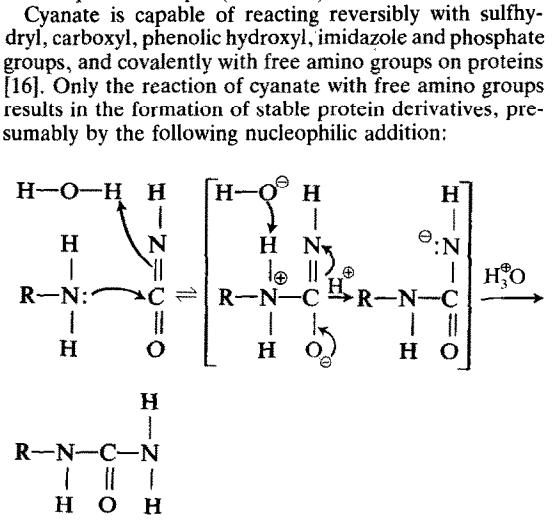
* Student's two-tailed *t*-test.
† Represents ± S.E.M.

concentration of cyanate incubated gave rise to protein which migrated increasingly faster than normal BSA. A characteristic example of the electrophoresis pattern of normal BSA and cyanate-incubated BSA (1000 mg/l cyanate for 24 and 48 hr) is shown in Fig. 1. The impact of duration of incubation and concentration of cyanate incubated with BSA is shown in Table 1. The extent of covalent cyanate binding to BSA determined after the incubation of [^{14}C]cyanate (1.125 μCi/ml) for 24 hr was estimated to be 92.8 per cent after correcting for membrane-binding of cyanate (*ca.* 12 per cent).

The binding of [^{14}C]warfarin to cyanate-treated BSA was less extensive than to normal BSA for warfarin concentrations ranging from 0.3 μg/ml to 4.8 μg/ml. The extent of [^{14}C]warfarin binding did not change as a function of warfarin concentration ($0.1 < P < 0.5$), but binding differences between normal and derivatized albumin were detected ($P < 0.01$) by a two-way analysis of variance. The mean values for fraction bound (β) were 98.88 and 97.82 per cent for normal and cyanate-incubated (1000 mg/l for 24 hr) BSA, respectively. Furthermore, warfarin binding was even less extensive for derivatized albumin which was even more extensively carbamoylated (i.e. BSA treated with 2000 mg/l potassium cyanate for 24 hr), as shown in Table 2. A plot of $\frac{1}{r}$ vs $\frac{1}{D}$ in which D is the [^{14}C]warfarin concentration in ultrafiltrates (d.p.m./0.2 ml) is shown in Fig. 2. The slope, which represents $1/nK_a$, is greater for derivatized albumin, signifying a smaller value for nK_a, where n represents the number of primary binding sites, and K_a the corresponding binding affinity constant. Using the method of Odar-Cederloff and Borga [2], the values for nK_a for normal and derivatized BSA were 1.52 and 0.77×10^5 l/mole, respectively.

The mean (± S.E.M.) bound: free ratio for [^{14}C]cyanate was 1.21 ± 0.04 for normal plasma and 0.65 ± 0.06 for the uremic plasma sample ($P < 0.025$).

Cyanate is capable of reacting reversibly with sulfhydryl, carboxyl, phenolic hydroxyl, imidazole and phosphate groups, and covalently with free amino groups on proteins [16]. Only the reaction of cyanate with free amino groups results in the formation of stable protein derivatives, presumably by the following nucleophilic addition:



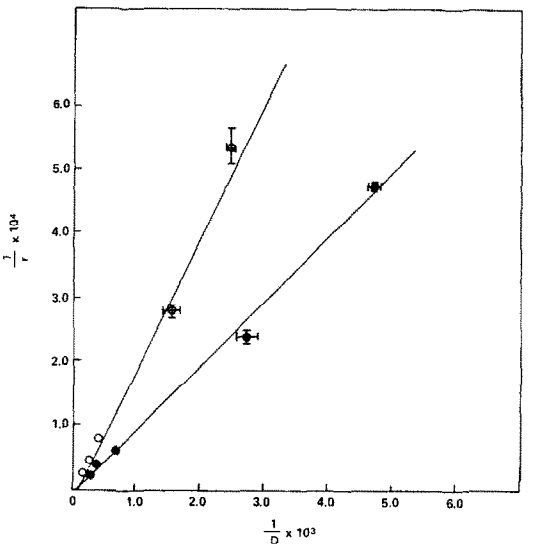


Fig. 2. Double reciprocal plot for computed values of r and D. Bars denote S.E.M. D is computed in d.p.m. Open symbols are derivatized BSA; closed symbols represent normal BSA.

The rate of carbamoylation is dependent upon both the pK_a of the reacting amino group as well as the pH of the medium [16]. It appears as though the carbamoylation of the amino terminal valine of the β chain of hemoglobin by cyanate confers antisickling activity upon cyanate [17].

The foregoing data suggest that cyanate is capable of carbamoylating albumin under physiologic conditions. Moreover, the extent of carbamoylation can be increased either by extending the duration of BSA incubation with cyanate or by increasing the concentration of cyanate incubated. More importantly, the extent of warfarin binding appears to be reduced significantly when warfarin is incubated with derivatized BSA. It is interesting to note that for warfarin the estimated value for nK_a for normal BSA is more than twice as great as for derivatized BSA. Both Sjöholm *et al.* [1] and others [9] have reported an approximately 50 per cent decrease for nK_a for warfarin in uremic human serum or plasma relative to normal human serum or plasma. Similarly, nearly a 2-fold increase in the warfarin-free fraction has been reported for uremic serum or plasma [1, 18]. The free fraction for warfarin added to normal BSA in our experiments was 1.00–1.12 per cent compared to 2.18–2.42 per cent when added to BSA derivatized with 1000 mg/l cyanate for 24 hr.

The incubation of [^{14}C]cyanate with normal and uremic plasma gave rise to a significantly higher bound to free ratio of cyanate in normal than in uremic plasma. This is consistent with the existence of a greater number of preexisting carbamoylated amino acids in uremic than in normal plasma.

Virtually all of the cyanate added to 4% BSA reacts with the protein insofar as only 7.1 per cent of added cyanate was recoverable in ultrafiltrates. We did not determine whether the recovery of cyanate in ultrafiltrates could be increased by increasing the concentration of cyanate added. However, based upon electrophoresis it appears that an increase in added cyanate simply gives rise to more extensively derivatized protein. It should also be noted that if one assumes 7.1 per cent of the added cyanate does not react with protein, then the concentration of dissolved cyanate will be 0.89 mM. Zelman *et al.* [19] reported that cyanate arises from urea both spontaneously and by an

enzymatic pathway, and suggested that BUN levels of 100–300 mg/dl would be accompanied by cyanate concentrations of 0.75–1.40 mM.

Although our findings for warfarin binding to cyanate-derivatized BSA are consistent with the notion that cyanate is a mediator of the albumin binding defect for warfarin in uremia, more conclusive evidence remains to be reported. In that connection either an absolute or relative difference in carbamoyl substitutions on free amino groups of albumin isolated from uremic and normal serum would be compelling. Preliminary data recently reported by Erill *et al.* [20] suggest that carbamoylated human serum albumin binds salicylate and sulfadiazine less extensively than normal albumin, and describes a correlation between the magnitude of the free fraction of salicylate and the extent of carbamoylation. The synthesis of a qualitatively different type of albumin, binding inhibition effected by endogenous reversible binding inhibitors, and irreversible changes in albumin arising from covalent interactions with other uremic toxins, such as the guanidino compounds, may also contribute to the uremic binding defect.

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The dose-dependent effect of warfarin on vitamin K_1 metabolism and clotting factor synthesis in the rabbit

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It has been postulated that warfarin produces its anticoagulant effect by inhibiting the enzyme vitamin K_1 epoxide reductase which is responsible for the regeneration of vitamin K_1 from its biologically inactive metabolite vitamin K_1 epoxide [1]. In keeping with this hypothesis, we found that a series of 4-hydroxycoumarins, including warfarin, produced an accumulation of [^{3}H] vitamin K_1 epoxide in the plasma of rabbits dosed intravenously with [^{3}H] vitamin K_1 [2]. However, there was no correlation between the increase in [^{3}H] vitamin K_1 epoxide : [^{3}H] vitamin K_1 plasma concentration ratios and anticoagulant potency, in line with results obtained by other workers using the rat [3, 4]. One possible explanation for this lack of correlation is that the anticoagulants have different modes of action. We have investigated, therefore, the effect of several doses of one anticoagulant, warfarin, on the relationship between [^{3}H] vitamin K_1 metabolism and clotting factor activity in the rabbit.

Before each experiment the control prothrombin time was determined for each animal. Male New Zealand White rabbits (3.0–3.5 kg) were dosed with warfarin (0.16, 0.63, 2.5 and 10 mg/kg) intravenously into the marginal ear vein. One hour later, [^{3}H] vitamin K_1 (100 μCi; 2.1 μg) was injected intravenously into the same vein. Blood samples (4 ml) were taken from the other marginal ear vein 1, 2, 3, 4, 5 and 6 hr after administration of [^{3}H] vitamin K_1 for determination of [^{3}H] vitamin K_1 and [^{3}H] vitamin K_1 epoxide, as described previously [2]. The accuracy of the method was confirmed using reversed-phase chromatography on a partisil-10 ODS column using the solvent system acetonitrile–water (97:3, v/v). From 6 hr after injection of [^{3}H] vitamin K_1, blood samples (0.9 ml) were taken every four hours for determination of prothrombin complex activity (P.C.A.) by the method of Quick [5], as described previously [2].

The effect of warfarin on the [^{3}H] vitamin K_1 epoxide : [^{3}H] vitamin K_1 plasma concentration ratio is shown in Fig. 1. Figure 2 contains the corresponding prothrombin complex activity data.

Shearer *et al.* [5] have shown that there is a log-dose relationship between plasma warfarin concentrations and increases in plasma [^{3}H] vitamin K_1 2,3-epoxide concentrations in volunteers dosed with [^{3}H] vitamin K_1, but the corresponding changes in prothrombin complex activity were not measured. From Fig. 2 it can be seen that 0.63, 2.5 and 10 mg/kg of warfarin produced the same maximum rate of decay of P.C.A., suggesting that 0.63 mg/kg is sufficient to block clotting factor synthesis completely in