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# DRUG BINDING DEFECT OF URAEMIC PLASMA: UNLIKELY INVOLVEMENT OF CARBAMOYLATED ALBUMIN

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**Abstract**—Carbamoylation of bovine and human albumin *in vitro* decreased the binding of methyl red and salicylic acid. Charcoal extraction of the carbamoylated albumin under acid conditions produced some decrease in the degree of carbamoylation, but did not substantially improve the binding of methyl red and salicylate. Albumin from rats with glycerol-induced acute renal failure showed no significant degree of carbamoylation compared to controls. Carbamoylation is not responsible for the binding defect of uraemic rat plasma, nor is it likely to be involved in the case of human uraemic plasma.

A characteristic feature of the drug binding defect of both human and rat uraemic plasma is the ability of several extraction procedures to restore binding to more or less normal. Charcoal [1–4], ion exchange resins [5, 6],1-chlorobutane [7] and affinity chromatography [8] have all been found to remove the binding defect and substantially or completely restore binding. Both an abnormal albumin with diminished binding properties and/or accumulated endogenous inhibitors of binding may be responsible for the binding defect, but its extractability points to inhibitors as the major cause.

Recent work with human plasma [9], however, suggested that carbamoylation of albumin *in vivo*, due to prolonged exposure to cyanate derived from plasma urea, may play a role in the binding defect. Bachmann *et al.* [10, 11] found that carbamoylation of human albumin *in vitro* reduced the binding of warfarin, primarily because of a decrease in affinity. Although the binding of warfarin to plasma from uraemic patients was decreased the extent of carbamoylation of albumin from these patients was low [11], and so it seemed doubtful whether carbamoylation was responsible for the binding defect of uraemic plasma.

We have studied the effect of carbamoylation *in vitro* on the binding of the ligands methyl red and salicylic acid, two commonly used markers for the binding defect, to both human and bovine albumin. The albumin from rats with glycerol-induced acute renal failure has also been examined to see if carbamoylation occurs *in vivo*.

## MATERIALS AND METHODS

*Albumin.* Bovine serum albumin (BSA) and human serum albumin (HSA) both fraction V, were obtained from Sigma Chemical Co. (Poole, U.K.). Albumin from 6 control rats and 6 rats with glycerol-induced acute renal failure [12] was isolated from plasma by precipitation with ethanol as described by Erill *et al.* [9]. Samples of plasma (0.5 ml) were mixed with 5 ml of ethanol and centrifuged at 1,000 *g* for 10 min. The precipitate was then washed with 95% ethanol (6 × 5 ml) and finally redissolved in 1.5 ml saline.

*Derivatization of albumin.* BSA and HSA were carbamoylated by incubation of a 4% solution of albumin in 0.05 M sodium phosphate–chloride buffer, pH 7.4, with potassium cyanate (200 mg/100 ml) at 37° for 24 hr. Derivatized albumin was dialysed against running water for 24 hr and then freeze-dried [10]. The degree of carbamoylation, expressed as the homocitrulline : albumin ratio, was measured by the method of Hunninghake and Grisolia [13].

*Binding of* [$^{14}$C]*salicylic acid and methyl red to albumin and plasma.* [Carboxyl-$^{14}$C]salicylic acid, (specific radioactivity = 59 mCi/mmole, radiochemical purity > 98%) was purchased from the Radiochemical Centre (Amersham, U.K.) and the ortho-isomer of methyl red was obtained from Koch Light Laboratories (Colnbrook, U.K.). Binding to solutions (1 g/100 ml) of normal and derivatized HSA and BSA and to normal and uraemic plasma, was determined by equilibrium dialysis at 37° against an isotonic sodium phosphate–chloride buffer, pH 7.4 [12]. Binding measurements were also made after treatment of albumin and plasma with activated charcoal (50 mg/ml) at pH 3.0 by the method of Chen [14].

Results are expressed as mean ± S.D. and statistical comparison was made with the Student's *t*-test.

## RESULTS

*Carbamoylation.* Measurement of the degree of carbamoylation (expressed as the homocitrulline : albumin ratio) by the method which was used by Bachmann *et al.* [11] gave a similar ratio to that found by those authors. However, this assay produced a precipitate which was felt to be unsatisfactory and so the method of Hunninghake and Grisolia [13] was used. The homocitrulline : albumin

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Table 1. Carbamoylation of albumin *in vitro* and *in vivo*

| | Homocitrulline : albumin ratios | | | |
|---|---|---|---|---|
| | Before charcoal | | After charcoal | |
| Albumin | Control | Carbamoylated* | Control | Carbamoylated |
| Bovine | 0.46 ± 0.07 | 2.3 ± 0.14 | 0.56 ± 0.09 | 1.87 ± 0.08† |
| Human | 0.43 ± 0.06 | 3.21 ± 0.29 | 0.63 ± 0.08 | 2.14 ± 0.31† |
| Rat‡ | 0.16 ± 0.02 | 0.15 ± 0.01 | | |

Values are means ± S.D. of five or six experiments.
* Albumin exposed to cyanate (200 mg/100 ml) at 37° for 24 hr.
† $P < 0.001$ relative to control value after charcoal extraction.
‡ Albumin isolated from male rat plasma obtained 24 hr after an i.m. injection of glycerol. Plasma urea and creatinine concentrations (mg/100 ml) were 41 ± 5 and 0.72 ± 0.15 respectively for controls and 245 ± 13 and 3.49 ± 0.2 for those with renal failure.

Table 2. Effect of charcoal extraction on the binding of methyl red and salicylate to carbamoylated albumin

| | % Unbound | | | |
|---|---|---|---|---|
| | Before charcoal | | After charcoal | |
| Albumin and ligand | Control | Carbamoylated | Control | Carbamoylated |
| Bovine | | | | |
| Methyl red | 9.1 ± 0.5 | 11.7 ± 0.9 | 13.9 ± 0.8 | 16.8 ± 1.5* |
| Salicylic acid | 22.8 ± 0.5 | 29.6 ± 0.8 | 29.4 ± 2.2 | 34.8 ± 4.0* |
| Human | | | | |
| Methyl red | 9.2 ± 0.4 | 11.7 ± 2.4 | 6.5 ± 1.3 | 11.9 ± 1.3* |
| Salicylic acid | 24.2 ± 0.9 | 30.3 ± 1.0 | 14.1 ± 0.3 | 27.9 ± 1.8* |

* $P < 0.01$ relative to control value after charcoal extraction.

Table 3. Binding of methyl red and salicylic acid to male rat plasma

| | % Unbound | | | |
|---|---|---|---|---|
| | Before charcoal | | After charcoal | |
| Ligand | Control | Uraemic | Control | Uraemic |
| Methyl red* | 3.1 ± 0.5 | 12.2 ± 3.3 | 3.0 ± 0.6 | 5.9 ± 1.2‡ |
| Salicylic acid† | 11.8 ± 2.1 | 26.6 ± 6.2 | 10.8 ± 1.3 | 10.3 ± 1.3 |

Each result is the mean of three or four rats.
* Plasma (1 vol.) diluted with buffer (2 vol.) prior to measurement of binding.
† Binding measured in undiluted plasma.
‡ $P < 0.001$ relative to uraemic value before charcoal extraction and $P < 0.05$ relative to charcoal extracted controls.

ratios found after exposure to cyanate were somewhat lower than those obtained by Bachmann *et al.* [11] and control albumin solutions exhibited an apparent homocitrulline : albumin ratio of about 0.45. This was not unexpected in view of the substances which are known to be detectable by this method [13].

There were significant ($P < 0.001$) increases of more than 5- and 7-fold in the homocitrulline : albumin ratios of the derivatized samples of bovine and human albumin respectively (Table 1). Although charcoal extraction significantly decreased by 19–33%, the homocitrulline : albumin ratio of both bovine ($P < 0.001$) and human ($P < 0.05$) albumin, a substantial degree of carbamoylation remained and these ratios were still significantly raised by more than 3-fold (Table 1) over the post-extraction controls. There were no differences in the low homocitrulline : albumin ratios of albumin isolated from control and uraemic rat plasma. Thus the binding defect, which develops within 24 hr of the onset of experimental renal failure (Table 3; Refs. 4 and 12), is not dependent upon the presence of carbamoylated albumin.

*Binding to derivatized albumin.* Exposure to cyanate significantly decreased the binding of methyl red ($P < 0.02$) and salicylate ($P < 0.01$) to both bovine and human albumin (Table 2) as shown by the increase in percentage of unbound ligand. Charcoal extraction (see Table 2) produced some significant effects upon binding to control albumin : an increase in the unbound fraction with bovine albumin ($P < 0.001$ for both ligands) and a decrease in human albumin ($P < 0.01$ for methyl red and $P < 0.001$ for salicylate). The reason for these effects are not entirely clear, but the impact of fatty acid removal on ligand binding to both bovine and human albumin depends upon the ligand and the ratio of fatty acid to albumin [15–17]. The main finding, however, was that charcoal extraction did not abolish the significantly diminished binding ability of the carbamoylated albumin. Charcoal extraction did produce a slight relative diminution of the difference in extent of binding to control and carbamoylated bovine albumin (Table 2). This was not the case with human albumin, where the difference in binding by control and carbamoylated albumin was exacerbated by charcoal extraction.

The unbound fractions of methyl red and salicylate were significantly ($P < 0.01$) increased in plasma from rats with glycerol-induced renal failure (Table 3). Charcoal extraction fully restored the ability of the uraemic rat plasma to bind salicylate and considerably improved the binding of methyl red and these results agree with earlier work [4].

## DISCUSSION

Urea is well known as a protein denaturant and carbamoylation of protein can occur in the presence of high (1–8 M) concentrations of urea [18] and smaller (0.2–1 M) concentrations decrease the affinity of methyl orange for bovine albumin [19, 20]. A considerable amount of evidence, however, has shown that when pathological concentrations (say 1–10 mM) of urea are added *in vitro* to either plasma or albumin there is no effect on drug or dye binding [2, 21–27]. Furthermore, exposure of human albumin to urea for 48 hr at 2° prior to equilibrium dialysis with phenytoin for 24 hr at 37° significantly increased the binding of this drug [27]. A urea-induced increase in the binding of other drugs has also been observed [28]. The observations of Erill *et al.* [9] were therefore somewhat surprising and prompted this investigation of the possible effects of carbamoylation *in vitro* and *in vivo*.

The binding of both methyl red and salicylate to bovine and human albumin which had been exposed to cyanate was decreased. Charcoal extraction of the carbamoylated albumin samples however, did not restore binding to normal or even nearly normal. This contrasts with the substantial, or complete restoration of binding which can be produced by the charcoal extraction of uraemic serum or plasma from both patients [1–3] and rats (Table 3 and Ref. 4).

Bachmann *et al.* [11] found that carbamoylation of human albumin *in vitro* decreased its ability to bind warfarin. Charcoal treatment of the carbamoylated albumin increased the percentage of bound warfarin from 92.7 to 96.3, although it was not made clear whether this represented a statistically significant improvement in binding. Charcoal extraction also decreased the molar ratio of homocitrulline : human albumin by an average of about 26% [11] and we found similar decreases of about 19 and 33% for bovine and human albumin respectively. Erill *et al.* [9] stated that charcoal treatment of human plasma, which had been previously carbamoylated *in vitro*, neither modified the degree of carbamoylated nor improved the binding defect for salicylate. This conflicts with the marked improvement in salicylate binding after the charcoal extraction of human and rat uraemic plasma samples.

The slight degree of carbamoylation of albumin from uraemic patients was insufficient to account for the decreased binding of warfarin to uraemic plasma [11]. Acute experimental renal failure caused the characteristic drug binding defect in rat plasma (Table 3) but produced no detectable carbamoylation of rat plasma albumin (Table 1). Our findings both *in vitro* and *in vivo* are therefore in broad agreement with those of Bachmann *et al.* [11].

Carbamoylation of bovine and human albumin *in vitro* can decrease drug binding, but this defect is different from that of renal failure because binding cannot be restored by charcoal extraction. The binding defect of uraemic rat plasma was not accompanied by carbamoylation of the plasma albumin and so it seems unlikely that carbamoylation is involved to any significant extent in the binding defect of uraemic plasma.

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