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DECREASED BINDING OF DRUGS AND DYES TO PLASMA PROTEINS FROM RATS WITH ACUTE RENAL FAILURE: EFFECTS OF URETER LIGATION AND INTRAMUSCULAR INJECTION OF GLYCEROL

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1 The decreased binding of drugs and dyes to plasma proteins from male and female rats with acute renal failure has been investigated using equilibrium dialysis at 37°C.

2 Acute renal failure induced by bilateral ligation of the ureters produced a greater than threefold increase in the unbound fraction of *o*-methyl red relative to normal rat plasma. Unbound dye concentration in plasma from sham-operated control rats was also significantly increased but to a lesser extent.

3 Glycerol-induced acute renal failure produced a significant increase in the unbound fractions of *o*-methyl red, methyl orange, bromocresol green (BCG), 2-(4'-hydroxybenzeneazo) benzoic acid (HABA), phenytoin and salicylic acid. A marginally significant increase in unbound warfarin concentration was observed.

4 Glycerol-induced renal failure had no effect on total plasma protein concentration and experiments with *o*-methyl red and salicylic acid indicated that a direct effect of glycerol was not responsible for the diminution of binding.

5 Glycerol-induced acute renal failure, which produced decreases in drug and dye binding similar to those reported for human uraemic plasma, provides a convenient non-surgical animal model for the investigation of this phenomenon.

Introduction

The binding of anionic drugs and dyes to plasma proteins from patients with acute and chronic renal failure is decreased (Reidenberg, 1977). This can occur despite a normal concentration of plasma albumin but even if hypoalbuminaemia occurs this often does not fully account for the decrease.

The relatively few investigations of this problem in experimental animals have used mainly surgically induced acute renal failure. Renal failure in the dog (Baker, 1951), rabbit (Taylor, Richards, Davin & Asher, 1954), rat (Ghoneim, Pandya, Kelley, Fischer & Corry, 1976) and goat (Baggot & Davis, 1973) produced a decrease in the plasma protein binding of several anionic drugs. The one study where acute renal failure was produced by intravenous injection of a nephrotoxic agent (uranyl nitrate) also demonstrated a decrease in drug binding in the rat, rabbit, guinea-pig, cat and dog (Belpaire, Bogaert & Mussche, 1977).

Investigation of the drug binding defect in uraemic human plasma is complicated by several factors. The underlying renal disease may differ in aetiology and severity between patients and the use of heparin during the dialysis procedure may cause release of non-esterified fatty acids which are inhibitors of drug and dye binding (Erich & Eggstein, 1972; Dromgoole, 1973; Spector, 1975). Human plasma samples may also contain a mixture of drugs and drug metabolites which may contribute to the binding defect.

We have therefore sought an animal model for acute renal failure which would produce binding changes for anionic compounds similar to those observed in patients. Bilateral ligation of the ureters and intramuscular injection of glycerol (Bowmer & Lindup, 1978a) are the two methods which have been studied. Azo dyes such as *o*-methyl red and methyl orange (Breyer & Radcliff, 1954; Campion, 1973; Dromgoole, 1974) were among the first type of ligand for which defective plasma protein binding was observed in patients with renal failure. Therefore, these two dyes together with 2-(4'-hydroxybenzeneazo) benzoic acid (HABA), bromocresol green (BCG) and the drugs phenytoin, salicylic acid and warfarin were used.

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Methods

Renal failure induced by bilateral ligation of ureters

Acute renal failure was produced in male and female Wistar albino rats (204 to 280 g) by bilateral ligation of the ureters under ether anaesthesia. Control rats (sham-operated) received the same surgical treatment during which both ureters were manipulated. Rats were fed on Oxoid Laboratories Animal Breeding Diet. Forty-eight hours after surgery, rats were killed by exsanguination (cardiac puncture) under ether anaesthesia. Blood was collected into lithium–heparin tubes (G.D. Searle Ltd.) and centrifuged to separate the plasma. The haematocrit (PCV) was measured by centrifugation at 12,000 *g* for 5 min in a micro-haematocrit centrifuge (Gelman Hawksley Ltd.).

Glycerol-induced acute renal failure

Acute renal failure was also produced by intramuscular injection of glycerol by a method similar to that of Thiel, Wilson, Arce & Oken (1967). Wistar albino rats (154 to 378 g) were deprived of drinking water for 20 h but allowed food *ad lib.* An intramuscular injection of 50% v/v glycerol in water, 10 ml/kg body wt., was then administered under ether anaesthesia in divided doses to two sites in each of the two hind limbs. The drinking water was immediately restored and rats were killed as before, 24 h after the glycerol injection. Control rats received sham injections into the hind limbs.

Total plasma protein, urea and creatinine

Standard spectrophotometric assays were used: total plasma protein by the biuret method (human albumin as standard), urea by reaction with diacetylmonoxime and creatinine by reaction with alkaline picrate solution (Henry, Cannon & Winkelman, 1974).

Measurement of plasma protein binding

Equilibrium dialysis. Rat plasma was used within 60 min of collection for all binding experiments except those with BCG when the plasma was stored frozen for 24 h before use. An isotonic sodium phosphate–chloride buffer pH 7.4 (5.75 g Na_2HPO_4, 1.5 g $NaH_2PO_4.2H_2O$ and 2.075 g NaCl per litre) was used for all experiments. Solutions of *o*-methyl red (0.40 mM), methyl orange, HABA and BCG (all 0.45 mM) in buffer were prepared and equal volumes (0.3 ml) of dye solution, buffer and fresh rat plasma were mixed and incubated at room temperature for 20 min. An aliquot (0.85 ml) was dialysed against an equal volume of buffer for measurement of binding. Various dilutions of rat plasma were obtained by alteration of the relative amounts of buffer and plasma.

[^{3}H]-phenytoin and unlabelled phenytoin were dissolved in ethanol and 10 µl (50,000 d/min) added to undiluted rat plasma (1 ml) to produce a total phenytoin concentration of 10 µg/ml (39.6 µM). After 20 min incubation as before, aliquots (0.9 ml) were dialysed against an equal volume of buffer.

Concentrated stock solutions of salicylic acid and warfarin sodium in buffer were diluted to 20 ml with buffer and then 100 µl (1 µCi) of [^{14}C]-salicylic acid (in $C_2H_5OH \cdot H_2O$; 2:8 by vol.) or 50 µl (1.25 µCi) of [^{14}C]-warfarin (in C_2H_5OH) were added. Drug solution (0.85 ml) was then dialysed against an equal volume of rat plasma.

The equilibrium times for each ligand were determined and the following equilibrium dialysis times were used: *o*-methyl red (2 h), methyl orange, HABA and salicylic acid (3 h), phenytoin and warfarin (4 h), BCG (5 h). Equilibrium dialysis at 37°C was carried out as previously described (Bowmer & Lindup, 1978b).

Determination of dye and drug concentrations

Unbound *o*-methyl red in the buffer compartment was determined spectrophotometrically at 525 nm after dilution with an equal volume of 0.1 M HCl. Methyl orange, HABA and BCG were measured directly at 464, 350 and 618 nm respectively. All measurements were made with a Cecil CE 272 linear readout u.v. spectrophotometer. Bound dye concentrations were calculated by difference. Mean (±s.d.) percentage recoveries for each dye were: *o*-methyl red 94.4 ± 2.5 (n = 7), methyl orange 97.0 ± 0.8 (n = 6), HABA 100.2 ± 0.6 (n = 10) and BCG 96.7 ± 2.3 (n = 10).

When radioactive drugs were studied, unbound and total drug concentrations were determined by liquid scintillation counting. Samples (0.25 ml) from both plasma and buffer compartments containing radiolabelled drugs were mixed with 2.5 ml scintillator ('Multisol II', Intertechnique Ltd.) in plastic insert vials (Sterilin Ltd) and counted with an Intertechnique SL33 liquid scintillation counter. Quenching was assessed by the use of either [^{14}C]- or [^{3}H]-*n*-hexadecane (Radiochemical Centre, Amersham) as internal standard or by an external standard channels ratio quench curve constructed with haemolysed rat plasma. Mean (±s.d.) percentage recoveries for each drug were: [^{3}H]-phenytoin 100 ± 5.5 (n = 20), [^{14}C]-salicylic acid 96.5 ± 5.0 (n = 11) and [^{14}C]-warfarin 99.2 ± 0.6 (n = 11).

Analysis of results

For the radiolabelled drugs: % drug unbound =

$D_u/D_t \times 100$ where D_u and D_t are the concentrations of unbound ligand and total ligand respectively. For the dyes: % unbound = $[D_u/(D_{t(o)} - D_u)] \times 100$ where $D_{t(o)}$ is the starting concentration of ligand in the protein compartment. Results are expressed as the mean ± s.d. and statistical comparison was made by the non-paired Student's *t* test unless otherwise stated.

Drugs, dyes and other reagents

Phenytoin was purchased from Aldrich Chemical Co. and warfarin sodium (B.P. grade) from Ward Blenkinsop & Co. *o*-Methyl red was obtained from Hopkin & Williams Ltd. and its purity checked (Bowmer & Lindup, 1978b). Salicylic acid ('Analar' grade), HABA, methyl orange, buffer salts, glycerol, reagents for the urea assay and other chemicals were obtained from B.D.H. Ltd. Methyl orange was recrystallized from hot water. HABA was recrystallized from methanol (Baxter, 1964) and found to migrate as one band ($R_F = 0.66$) on cellulose thin-layer plates (cellulose with fluorescent indicator, Eastman Kodak) with 25% v/v CH_3COOH in toluene as solvent. BCG as monosodium salt (Koch Light pure A.R. grade) was used as supplied. Thin-layer chromatography of BCG on cellulose plates using ethyl acetate:pyridine:water (6:3:1 by vol.) as solvent ($R_F = 0.53$) revealed a u.v. absorbing impurity ($R_F = 0.64$) similar to that found by Yap & Schaffer (1977). Human serum albumin (fraction V, lot no. 116CO 1582) and reagents for the assay of total plasma protein were obtained from Sigma Chemical Co. Chemicals for the measurement of creatinine were obtained as a 'Creatinine II rapid stat kit', Pierce & Warriner (U.K.) Ltd.

Radiolabelled drugs

4-[^{3}H]-phenytoin (sp. act. = 51 Ci/mmol), [carboxyl-^{14}C]-salicylic acid (59 mCi/mmol) and racemic [^{14}C]-warfarin (51 mCi/mmol) all of stated radiochemical purity >98% were purchased from the Radiochemical Centre, Amersham, and used as supplied. The radiochemical purity of [^{3}H]-phenytoin was checked as previously described (Bowmer & Lindup, 1978b) and found to be >98%.

Results

Bilateral ligation of ureters

Severe renal failure ensued in the ligated male rats which had a mean (±s.d.) plasma urea concentration of 586 ± 41 mg/100 ml ($n = 5$) compared to values of 36 ± 6 ($n = 8$) and 36 ± 10 ($n = 3$) for sham-operated and normal rats, respectively. The percentage of unbound *o*-methyl red was 4.6 ± 0.6 ($n = 3$) in normal rats and this was significantly ($P < 0.001$) increased by renal failure to 18.5 ± 4.3% ($n = 5$). The unbound concentration of *o*-methyl red was also significantly increased ($P < 0.05$) to 7.1 ± 2.3% ($n = 7$) in sham-operated rats, which indicates that the surgical procedure itself decreased binding. Preliminary results were similar for female rats.

Glycerol-induced acute renal failure

Table 1 lists the results for blood and plasma analysis of control and glycerol injected rats. The PCV of males and females was significantly reduced by acute renal failure and an orange-brown colouration in one or more samples from uraemic rats was regularly observed. Total plasma protein concentration, which was significantly lower ($P < 0.01$) in both groups of male rats compared to females, was not affected by glycerol treatment. Mean plasma urea levels were significantly increased though not so markedly as in ligated male rats.

The effect of glycerol itself on binding was tested with *o*-methyl red and [^{14}C]-salicylic acid. The dye was 90.7 ± 0.0% ($n = 3$) bound to 1% w/v human serum albumin in a control experiment and in the presence of 0.46 M glycerol this was decreased ($P < 0.01$) to 90.4 ± 0.1% ($n = 3$). Table 2 shows that

Table 1 The packed cell volume (PCV), total plasma protein, urea and creatinine concentrations in the rat 24 h after glycerol-induced acute renal failure

Group	*PCV* (%)	*Protein* (*g/100 ml*)	*Urea* (*mg/100 ml*)	*Creatinine* (*mg/100 ml*)
Control male	43 ± 2 (6)	6.7 ± 0.6 (40)	39 ± 9 (43)	ND
Control female	38 ± 3 (13)	7.3 ± 0.8 (28)	33 ± 9 (24)	0.62 ± 0.07 (15)
Uraemic male	39 ± 3 (7)**	6.4 ± 0.9 (40)	312 ± 76 (43)***	ND
Uraemic female	34 ± 3 (12)***	7.8 ± 0.7 (27)	278 ± 59 (27)***	4.81 ± 0.52 (15)***

ND = not determined.
** $P < 0.05$; *** $P < 0.001$ relative to respective control group.

glycerol at 0.2 and 0.45 M produced a very slight increase in the binding of salicylic acid to whole rat plasma. The highest concentration of glycerol used was equivalent to 2.5 ml 50% glycerol + 10 ml of rat plasma, i.e. the maximum concentration of glycerol which could be achieved *in vivo* assuming that the glycerol was non-metabolized and restricted solely to the plasma compartment. Since neither of these assumptions would be expected to be true, it appears that any effect of glycerol itself on binding must be negligible.

Decreased drug and dye binding

The unbound concentration of *o*-methyl red was increased more than threefold in uraemic rat plasma (Table 3) and this increase was similar to that observed for the ligated rats, although in the latter the plasma urea levels were considerably higher. The unbound concentration of methyl orange was increased about twofold by renal failure (Table 3).

Table 4 shows that the binding of HABA and BCG was significantly decreased with increases in unbound dye concentrations of between two and threefold. The binding of phenytoin, salicylic acid and warfarin to undiluted rat plasma at total drug concentrations within the therapeutic range was diminished also. A small decrease in binding occurred with warfarin; the difference between groups was not significantly different by the *t* test but was significant ($P = 0.01$) by the Wilcoxon two-sample test. There are pronounced interindividual differences in the plasma protein binding of warfarin in rats (Yacobi & Levy, 1975) and this may account for the marginally significant effect of acute renal failure on the binding of this drug.

Effect of plasma dilution

Figure 1 illustrates the degree of dilution of normal plasma needed with *o*-methyl red, methyl orange and phenytoin to produce a decrease in fraction bound similar to that found in uraemic plasma. If albumin is the main binding protein for these three ligands then plasma albumin concentration must fall by about 50% to account for the decreases seen. In ureter ligation experiments (Nilsen, Fremstad & Jacobsen, 1975) neither plasma albumin nor total plasma protein were significantly decreased and the latter result agrees with our findings. Similarly, bilateral nephrectomy in the rat had no effect on either total plasma protein or albumin (Ghoneim *et al.*, 1976). Renal failure in the rabbit (Taylor *et al.*, 1954; Belpaire *et al.*, 1977) and dog (Baker, 1951; Markievicz, Walasek, Trznadel & Lutz, 1974) also did not affect plasma albumin concentration. The likelihood that certain methods may underestimate the albumin content of

Table 2 Binding of salicylic acid to undiluted rat plasma at 37°C in the presence of glycerol

Glycerol concentration (mM)	*0*	*25*	*50*	*100*	*200*	*450*
% Bound	89.9 ± 0.1	89.9 ± 0.1	89.7 ± 0.2	90.2 ± 0.2	90.2 ± 0.1*	90.2 ± 0.1*

* $P < 0.05$.
Pooled female rat plasma was dialysed against buffer containing salicylic acid (146 μM) and glycerol (0 to 450 mM). Equilibrium dialysis was carried out in triplicate for each concentration of glycerol

Table 3 Binding of *o*-methyl red and methyl orange at 37°C to plasma from rats 24 h after glycerol-induced acute renal failure

	% unbound			
	o-Methyl red (133 μM)†		*Methyl orange (150 μM)†*	
Group	*Male*	*Female*	*Male*	*Female*
Control	4.7 ± 1.7 (29) (2.4–8.3)‡	4.8 ± 1.7 (21) (3.0–9.9)	4.8 ± 0.6 (8) (4.2–5.9)	4.7 ± 0.5 (13) (4.0–5.9)
Uraemic	16.3 ± 7.4 (27)*** (5.6–36.7)	17.9 ± 5.6 (21)*** (11.4–35.6)	11.0 ± 2.2 (9)*** (8.4–15.0)	9.6 ± 1.9 (12)*** (6.4–13.0)

† Initial total dye concentration in plasma compartment. ‡ Range of unbound dye concentrations.
*** $P < 0.001$ relative to control group.

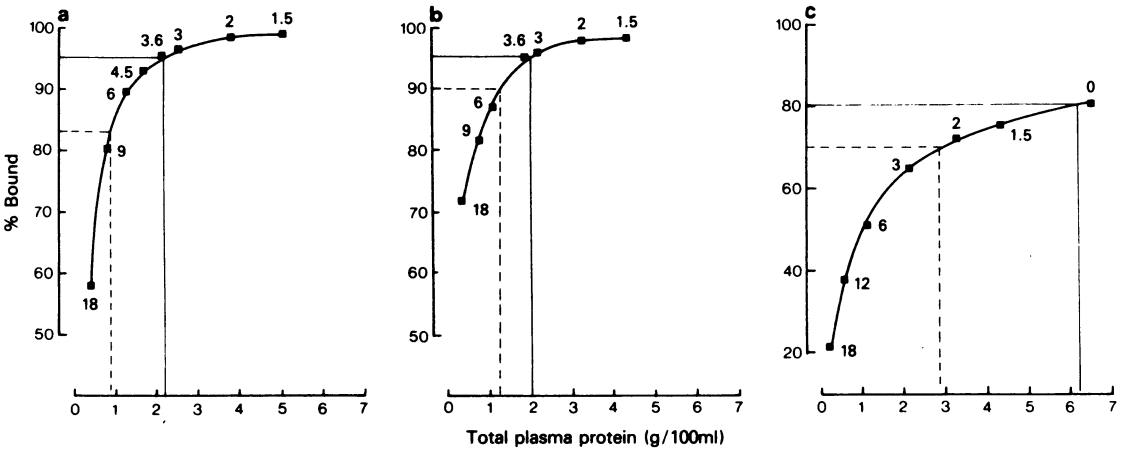

Figure 1 Effect of dilution on the plasma protein binding of (a) *o*-methyl red (133 μM), (b) methyl orange (150 μM) and (c) phenytoin (39.6 μM) to normal male rat plasma at 37°C. This illustrates the degree of dilution of normal plasma needed to produce a decrease in % bound, similar to that found in uraemic plasma. Pooled plasma from two normal rats was used for each ligand and each point is the mean of 3 determinations. The numerals show the degree of dilution at the point indicated, e.g. 18 = 1 vol. plasma + 17 vol. buffer. Extrapolations from the values for % bound in control and uraemic rat plasma (see Tables 3 and 4) are shown by solid and broken lines respectively.

uraemic plasma will be discussed later. Although plasma albumin concentrations were not measured in this present work it seems likely that a 50% fall in plasma albumin concentration would have been reflected by a fall in total plasma protein concentration, and this was not observed. Hypoalbuminaemia is unlikely therefore to be the major cause of the binding defect in plasma from rats with acute renal failure.

Discussion

Bilateral ligation of the ureters has been used previously to produce a model for the effect of acute renal failure on the binding of drugs in the dog (Baker, 1951) and rat (Nilsen *et al.*, 1975; Fremstad, 1977). However, surgery has several disadvantages: it is time consuming and, in addition to the effect of very severe renal failure, the animal is exposed to the risk of infection. The surgery itself produced a significant decrease in the binding of *o*-methyl red. This effect does not appear to have been reported previously in animal experiments but a post-operative decrease in binding may occur for phenytoin in humans (Fremstad, Bergerud, Haffner & Lunde, 1976). Since a component of the decrease was due to the operative procedure, binding to normal rat plasma has also to be measured for comparative purposes.

Table 4 Binding of drugs and dyes at 37°C to plasma from male or female rats 24 h after glycerol-induced acute renal failure

			% *unbound*		
Group	*BCG* (*150 μM; 5F*)†	*HABA* (*150 μM; 5F*)	*Phenytoin* (*39.6 μM; 10M*)	*Salicylic acid* (*134.8 μM; 5F*)	*Warfarin* (*4.3 μM; 5F*)
Control	0.5 ± 0.1	13.0 ± 1.0	19.8 ± 1.7	12.6 ± 2.3	0.96 ± 0.31
Uraemic	1.8 ± 0.8**	24.2 ± 4.0***	30.2 ± 2.3**	39.8 ± 5.1***	3.20 ± 2.78‡

The binding of phenytoin, salicylic acid and warfarin was measured in undiluted plasma. The binding of bromocresol green (BCG) and of 2-(4'-hydroxybenzeneazo) benzoic acid (HABA) was measured in plasma (1 vol.) diluted with buffer (2 vol.).

† Total initial ligand concentration in plasma compartment; number of rats and sex.

** $P < 0.01$; *** $P < 0.001$; ‡ $P < 0.02$ by t test, $P = 0.01$ by Wilcoxon two-sample test.

Glycerol-induced acute renal failure

Intramuscular injection of glycerol produces an animal model for renal failure which is analogous in many ways to the crush syndrome seen after certain types of trauma in man (Stein, Lifschitz & Barnes, 1978). This, therefore, seemed an appropriate model to investigate, particularly since it did not involve administration of a foreign compound which may affect drug binding and metabolism. Previous investigators who have studied some of the same drugs and dyes with human plasma have not necessarily used similar experimental conditions with regard to technique, temperature and plasma dilution and so no detailed comparison, in terms of percentage changes in binding, will be made.

The results for phenytoin agree with the extensive earlier work with plasma samples from patients with renal impairment (Reidenberg, Odar-Cederlöf, von Bahr, Borgå & Sjöqvist, 1971; Reidenberg, 1977). Accumulation of drug metabolites in human uraemic plasma samples as a factor which may potentiate decreased drug binding has largely been ignored. Competition between phenytoin and its accumulated metabolite *p*-hydroxydiphenylhydantoin (HPPH) was suggested as a reason for decreased phenytoin binding in the plasma of patients with renal insufficiency (Letteri, Mellk, Louis, Kutt, Durante & Glazko, 1971). Direct experimental evidence was lacking and we have found in experiments with phenytoin (10 μg/ml; 39.6 μM) in normal human plasma at 37°C that HPPH did not inhibit the binding of phenytoin. Phenytoin was 87.9 ± 1.1% ($n = 3$) bound (control); 88.1 ± 0.6% ($n = 3$) and 87.9 ± 0.5% ($n = 3$) bound in the presence of 20 and 30 μg/ml of HPPH respectively. The results with uraemic rat plasma, where possible interference from drug metabolites is excluded, further indicate that renal failure itself is sufficient to cause the decrease.

The binding of salicylic acid and warfarin is decreased in uraemic patients (Farrell, Grib, Fry, Popovich, Broviac & Babb, 1972; Bachmann, Shapiro & Mackiewicz, 1976; Sjöholm, Kober, Odar-Cederlöf & Borgå, 1976) and the findings were similar with glycerol-induced acute renal failure. The binding of warfarin to plasma from rabbits with uranyl nitrate-induced renal failure was decreased to a greater extent than with three patients with acute tubular necrosis (Belpaire *et al.*, 1977). Glycerol-induced acute renal failure produced a statistically marginal decrease in warfarin binding which was more akin to the human results of Belpaire *et al.* (1977) than to their findings for the rabbit.

Dye binding and uraemia

Ehrström (1937) was one of the earlier workers to find that dye binding was decreased in uraemic plasma and the azo dyes *o*-methyl red (Campion, 1973) and methyl orange (Breyer & Radcliff, 1954; Dromgoole, 1974) have been used to study this phenomenon in some detail with human uraemic plasma. The four dyes investigated either have been, or still are, used in dye-binding assays for the routine measurement of plasma albumin concentrations and BCG in particular is widely used (Henry *et al.*, 1974). A decrease in dye binding due to uraemia would diminish the fraction of dye–albumin complex formed and thereby lead to underestimation of albumin concentration. Comparison of the HABA method with the biuret method has shown this to be the case and there was no correlation of the extent of underestimation with plasma urea concentration, although any effect of urea on the biuret assay was not determined (Bergeron, Talbot, Gauvin, Loiselle & Page, 1977). Urea is unlikely to be the cause of the dye binding defect in uraemic plasma because it did not affect the binding of *o*-methyl red to human albumin (Bowmer & Lindup, 1976). Bergeron *et al.* (1977) suggested that dye-binding assays for albumin should not be used for uraemic plasma. Our results for BCG with rat plasma suggest that the same problem will occur with this dye and preliminary equilibrium dialysis studies with BCG and uraemic human plasma support this. Qualitative analysis of plasma proteins, by techniques which utilize dye staining may also be affected.

The results indicate that the glycerol model for acute renal failure is convenient and suitable for production of the binding defect in rat plasma. Further investigations are in progress to determine the mechanism by which drug binding is affected.

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