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Dibromosulphophthalein: its pharmacokinetics and binding to hepatic cytosol proteins in rats with acute renal failure

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1 The pharmacokinetics, biliary excretion and binding of dibromosulphophthalein (DBSP) to plasma proteins and hepatic cytosol proteins have been studied in male rats with glycerol-induced acute renal failure (ARF).

2 The rate constants for hepatic uptake, efflux from liver to plasma and excretion into bile were all significantly decreased in rats with ARF. Furthermore, the plasma clearance of DBSP was also reduced.

3 The initial (0–10 min) and maximum biliary excretion rates of DBSP were both diminished in animals with ARF. The maximum excretion rate occurred between 5–10 min in control rats and 10–15 min in rats with ARF. However, there was no statistically significant change in the percentage dose recovered from bile after 30 min.

4 The plasma-protein binding of DBSP was decreased in rats with ARF and this change was due to a significant reduction in the association constant for the primary binding sites.

5 The binding of DBSP to ligandin (Y protein) was reduced by about 38% in rats with ARF but no change was noted in binding to Z protein. Reduced binding to ligandin was accompanied by decreased total liver glutathione S-transferase (GST) activity and a 36% reduction in the GST activity of ligandin.

6 The results support the contention that altered hepatic handling of cholephilic dyes in rats with ARF may be due to reduced binding to ligandin.

Introduction

The liver plays a major role in the elimination of most xenobiotics from the body and its function in this respect is compromised in renal failure. In the rat, the metabolism of many xenobiotics is decreased in this disease state (Leber & Schütterle, 1972; Black *et al.*, 1977) and, in addition, the activity of other processes involved in elimination have also been shown to be diminished. *In vitro* studies using isolated perfused livers of the rat have shown decreased hepatic uptake of *p*-aminobenzoic acid (Howie & Bourke, 1979), propranolol (Terao & Shen, 1985; Hori *et al.*, 1985) and sodium cromoglycate (Barber *et al.*, 1987). *In vivo*, Lin *et al.* (1985) observed reduced biliary clearance of the anti-inflammatory drug sulindac and they suggested that this change was brought about by impaired biliary excretion of sulindac in rats with renal failure.

The phthalein dye bromosulphophthalein (BSP) and the tricarbocyanine dye indocyanine green (ICG) have been used by us to investigate the effect of acute renal failure (ARF) on hepatic excretory function (Bowmer *et al.*, 1982; Bowmer & Yates, 1984). These *in vivo* studies showed that the uptake of BSP and ICG by the liver was reduced in rats with ARF. Moreover, this change was accompanied by a delay in the appearance of dye in bile (Bowmer *et al.*, 1983; Bowmer & Yates, 1984). Scharschmidt *et al.* (1975) and Schwenk *et al.* (1976) have provided evidence that BSP and ICG share the same plasma to bile transport route, so it is likely that the hepatic handling of other substances which also share this pathway would be altered in renal failure. One such substance is dibromosulphophthalein (DBSP). This dye is the 3,6-dibromo analogue of BSP and Klaassen & Plaa (1968) have shown that its hepatic disposition is similar to that of BSP. In common with both BSP and ICG, DBSP probably interacts with the same hepatic cytosol proteins (Meijer *et al.*, 1977) and is avidly bound by albumin (Meijer *et al.*,

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1983a). However, the major advantage of DBSP over BSP is that it appears not to be metabolized in the rat (Javitt, 1964). Consequently, it is possible with DBSP to assess hepatic uptake and biliary excretion independently of effects that might arise because of altered metabolism. By contrast to ICG, DBSP is stable in aqueous solution in the absence of protein which makes it relatively easy to assay spectrophotometrically. These two properties of DBSP make it potentially a more useful tool, than BSP and ICG, with which to assess the impact of ARF on excretion of cholephilic anions by the liver.

As an extension to our earlier work, we have examined the effect of ARF on the pharmacokinetics and biliary excretion of DBSP in the rat. In addition, the binding of this phthalein dye to both plasma and liver cytosol proteins has also been investigated. These latter experiments were done to test a previous suggestion that decreased binding to cytosol proteins may explain altered hepatic handling of BSP and ICG in ARF (Bowmer & Yates, 1984). A preliminary account of this work has been published (Bowmer *et al.*, 1986).

Methods

Induction of renal failure

ARF was induced according to the method developed by Thiel *et al.* (1967). Male Wistar albino rats (240–320 g) were dehydrated for 24 h and ARF was produced by intramuscular injection of 50% v/v glycerol in sterile saline (0.9% w/v NaCl), $10\,ml\,kg^{-1}$. Control rats were injected with saline ($10\,ml\,kg^{-1}$) and both groups of animals were studied 48 h after dosing.

Experimental protocol

Rats were anaesthetized with sodium pentobarbitone (Sagatal, $60\,mg\,kg^{-1}$ i.p.) and a cannula was inserted into the trachea to allow artificial ventilation. Cannulae were also placed in the left jugular vein, right carotid artery and common bile duct. Body temperature (rectal) was kept constant at 37°C with the aid of a heat lamp.

DBSP was dissolved in isotonic saline ($25\,mg\,ml^{-1}$) and injected i.v. as a bolus dose of $25\,mg\,kg^{-1}$. Heparinised arterial blood samples (0.1 ml) were removed 1, 2, 3, 4, 6, 8, 10, 15, 20, 25 and 30 min after injection of dye and bile was collected over 5 min intervals for 30 min. Bile volume was measured gravimetrically assuming a density of 1.0.

Urinary excretion of DBSP was estimated in separate experiments in which the jugular vein and bladder were cannulated. A solution of mannitol (4% w/v in saline) was infused, using a Braun Perfusor IV syringe pump, via the jugular vein at a rate of $2.3\,mg\,min^{-1}$ and, when a constant diuresis was achieved at about 60 min, a bolus dose of DBSP ($25\,mg\,kg^{-1}$) was given to three control rats and three rats with ARF. Urine was collected for 30 min and 0.2 ml samples were assayed for DBSP.

DBSP was assayed spectrophotometrically. Bile and plasma samples (50 μl) and aliquots (0.2 ml) of urine were suitably diluted with 0.1 M NaOH and optical density at 575 nm was measured. The limit of detection was about $1.5\,\mu g\,ml^{-1}$ in biological fluid.

Thin layer chromatography of bile

Control rats were injected with DBSP ($25\,mg\,kg^{-1}$) and bile was collected over 30 min. Aliquots (5 μl) of bile were chromatographed according to the methods of Whelan & Plaa (1963) and Whelan *et al.* (1970). Three solvent systems were used: system A was the organic phase of butan-1-ol:glacial acetic acid:water (4:1:5, v:v); system B consisted of propan-1-ol:water:glacial acetic acid (10:5:1) and system C was acetone:water:ammonia (81:15:4). Silica gel (Kieselgel 60, 0.2 mm thick) and cellulose (0.1 mm thick) plates were used with systems A and B, and only cellulose plates were used with C. Plates were developed by exposure to ammonia vapour.

Binding of dibromosulphophthalein to plasma-proteins

Rats were anaesthetized with ether and blood samples (8–10 ml) were withdrawn by cardiac puncture. Blood was placed in tubes containing lithium heparin and centrifuged at 1500 *g* for 5 min. Plasma was stored in polyethylene tubes at −20°C until used and total plasma protein was measured by the Biuret method using bovine albumin as the standard.

The binding of DBSP was measured using a 'Dianorm' equilibrium dialysis apparatus (Diachema, Zurich). Rat plasma (0.3 ml) was diluted with 0.6 ml of DBSP dissolved in 0.1 M sodium phosphate buffer, pH 7.4. This dilution was used to minimize potential osmotic effects. The final concentration of dye was 0.1 to 1.0 mM. Aliquots (0.85 ml) of this solution were dialysed against an equal volume of 0.1 M sodium phosphate buffer. Dialysis was carried out at 37°C for 2.5 h and the recovery of DBSP in the absence of protein was 99.3 ± 0.4% ($n = 30$). Unbound DBSP in the diffusate was determined after dilution with 0.1 M NaOH at 575 nm. The limit of sensitivity was about 0.5 μM in diffusate.

The concentration of DBSP bound to plasma-protein, [b], was calculated from:

$$Co = 2[u] + [b]$$

where Co is the initial concentration of dye in the DBSP – protein mixture and [u] is the unbound concentration in the diffusate. The ratios [b]/[u] were plotted against [u] (Rosenthal, 1967) and, because such plots were curvi-linear, these data were fitted by non-linear least squares regression analysis, using the BMDP statistical software package (Ralston, 1983), to the following equation:

$$[b] = \frac{K_1[u]C_1}{1 + K_1[u]} + \frac{K_2[u]C_2}{1 + K_2[u]}$$

where K_1 and K_2 are the association constants of the primary and secondary classes of binding sites and C_1 and C_2 are the capacities of these two classes of sites.

Binding of dibromosulphophthalein to cytosol proteins

Rats were killed by cervical dislocation, a cannula was placed in the portal vein and the liver perfused *in situ* with 15 ml of ice-cold 0.25 M sucrose-0.05 M sodium phosphate buffer, pH 7.4. The liver was removed, rinsed with ice-cold saline and a 25% w/v homogenate was prepared in sucrose-phosphate buffer. The homogenate was centrifuged at 9000 *g* for 20 min at 4°C followed by centrifugation of the supernatant at 105,000 *g* for 1 h at 4°C. The supernatant was carefully separated from surface lipid and the pellet and stored at −20°C until used.

The method of Levi *et al.* (1969) was used to separate cytosol binding proteins. DBSP (10 mg, 14.7 μmol) was added to 5 ml of cytosol. A 2 ml aliquot was applied to a Sephadex G-75 column (2.6 × 27 to 31 cm) and proteins were eluted with 0.05 M sodium phosphate buffer, pH 7.4, using an upward flow rate of 18 ml h^{-1} at 4°C. About 120 ml of eluant were collected as 3 ml fractions. Protein concentration was monitored at 280 nm and DBSP was measured at 575 nm in each fraction after dilution with 0.1 M NaOH. DBSP bound by Y and Z protein peaks was estimated from the areas under these peaks (Levi *et al.*, 1969).

Protein concentration of cytosols was determined by the method of Lowry *et al.* (1951). Sephadex columns were calibrated with the following globular proteins: cytochrome C (horse heart, type VI; mol. wt.: 12,500); α-chymotrypsinogen A (bovine pancreas, type II; mol. wt.: 25,000); ovalbumin (grade V; mol. wt.: 43,000) and bovine albumin (fraction V; mol. wt.: 67,000). This enabled the molecular weights of the X, Y and Z fractions to be estimated.

Separation of glutathione S-transferases (GSTs) in hepatic cytosol

GSTs were separated by ion-exchange chromatography (Hayes *et al.*, 1979). Cytosols were prepared as described using sucrose-phosphate buffer. This buffer was exchanged for 0.01 M sodium phosphate buffer, pH 6.8, using a PD-10 disposable column packed with Sephadex G-25. A 5 ml sample of cytosol was eluted at 4°C and upward flow rate of 35 ml h^{-1} from a column of CM-Sepharose CL-6B (2.6 × 17 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. After 67.5 ml had been collected, GSTs retained by the column were eluted with a continuous 0–150 mM NaCl gradient in 0.01 M sodium phosphate buffer. Fractions (4.5 ml) were collected and assayed for enzyme activity and Na^+ content.

GST activities of liver cytosols and column fractions were assayed by the method of Habig *et al.* (1974a) using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate for conjugation with glutathione. Na^+ was measured with a Corning 450 flame photometer.

Pharmacokinetic calculations

Plasma concentration-time data were fitted to a bi-exponential equation by non-linear least squares regression analysis using BMDP (Ralston, 1983). The model and equations devised by Richards *et al.*, (1959) were used to estimate: k_{12} the first order rate constant for hepatic uptake, k_{21} the rate constant for efflux back into plasma and k_{23} the rate constant for excretion into bile. Plasma clearance (Cl_p) and apparent volume of distribution at steady state (Vd_{ss}) were calculated from standard equations.

Materials

DBSP was purchased from Société d'Études et de Recherches Biologiques (SERB, Paris) and CDNB was obtained from Aldrich Chemical Co. Thin layer chromatography plates (20 × 20 cm) were supplied by BDH Ltd. Sephadex G-75, CM-Sepharose CL-6B and the PD-10 columns were purchased from Pharmacia. Molecular weight markers for gel chromatography were obtained from Sigma. All other materials were of analytical grade and were bought from the usual laboratory suppliers.

Statistical analyses

Values are given as mean ± s.e.mean and statistical comparisons were made by use of Student's unpaired *t* test.

Results

Induction of renal failure

Glycerol-injected rats had body weights (296 ± 6 g; $n = 46$) and liver weights (3.7 ± 0.07 g 100 g^{-1} body

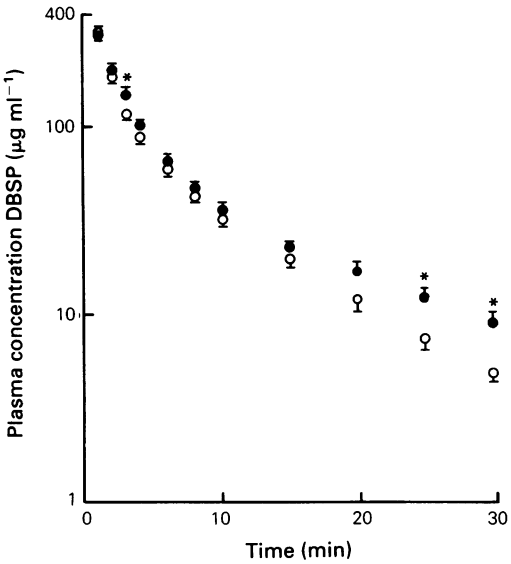

Figure 1 Plasma concentrations of dibromosulphophthalein (DBSP; 25 mg kg^{-1} i.v.) in control rats (○) and rats with acute renal failure (●). Values are mean ($n = 8$); s.e.mean shown by vertical lines. Significantly different from control values: $^{*}P < 0.05$.

Table 1 Effect of glycerol-induced acute renal failure (ARF) on the pharmacokinetics of dibromosulphophthalein (DBSP, 25 mg kg^{-1} i.v.)

Pharmacokinetic parameters	*Control rats* ($n = 8$)	*Rats with ARF* ($n = 8$)
A (μg ml^{-1})	488 ± 43	442 ± 23
α (min^{-1})	0.75 ± 0.030	0.58 ± 0.029**
B (μg ml^{-1})	87 ± 9.2	67 ± 6.7
β (min^{-1})	0.097 ± 0.0050	0.077 ± 0.0040**
k_{12} (min^{-1})	0.65 ± 0.025	0.52 ± 0.029**
k_{21} (min^{-1})	0.084 ± 0.0080	0.055 ± 0.0050**
k_{23} (min^{-1})	0.11 ± 0.007	0.087 ± 0.0050*
Vd_{ss} (ml 100 g^{-1} body wt.)	20 ± 2.0	24 ± 1.9
Cl_p (ml min^{-1} 100 g^{-1} body wt.)	1.8 ± 0.070	1.5 ± 0.090*

Results are given as mean ± s.e.mean of n rats.
$^{*}P < 0.05$; $^{**}P < 0.01$ relative to respective control group.
A and α = intercept at t = 0 and slope of initial exponential phase, respectively; B and β = intercept at t = 0 and slope of terminal exponential phase; k_{12} = rate constant for transfer from plasma to liver; k_{21} = rate constant for transfer from liver to plasma; k_{23} = rate constant for transfer from liver to bile; Vd_{ss} = apparent volume of distribution at steady state and Cl_p = plasma clearance.

wt.) which were not significantly different ($P > 0.05$) from control rats (306 ± 6 g and 3.9 ± 0.07 g 100 g^{-1} body wt. respectively, $n = 46$). By contrast, kidneys removed from glycerol-injected rats (0.61 ± 0.02 g 100 g^{-1} body wt.) were about 60% heavier ($P < 0.001$) than those removed from controls (0.36 ± 0.01 g 100 g^{-1} body wt.). There was also a six fold increase ($P < 0.001$) in plasma urea levels in glycerol-injected rats (278 ± 35 mg 100 ml^{-1}; $n = 46$) compared to controls (44 ± 2 mg 100 ml^{-1}; $n = 46$). Packed cell volume (PCV) was also recorded for some animals and PCV in glycerol-injected rats (42 ± 1%; $n = 20$) was about 12% lower ($P < 0.01$) than the value noted for control rats (48 ± 1%; $n = 20$). These data are similar to those obtained previously (Bowmer *et al.*, 1982) and show that glycerol-injected rats had pronounced ARF.

Kinetics of dibromosulphophthalein

Curves for the disappearance of DBSP (25 mg kg^{-1}) from plasma in controls and rats with ARF are shown in Figure 1. Mean plasma concentrations of dye were significantly greater ($P < 0.05$) in rats with ARF at 3, 25 and 30 min after the injection of DBSP when compared to control values. Both curves contained two exponential components with slopes α and β and intercepts at zero time of A and B, respectively. Table 1 shows that there were no significant differences ($P > 0.05$) in A and B between the two groups of rats, but α and β were both decreased by about 20% in rats with ARF ($P < 0.01$). Furthermore, the inter-compartmental rate constants governing transfer of DBSP from plasma to liver, k_{12}, liver to plasma, k_{21}, and liver to bile, k_{23}, were all reduced ($P < 0.05$) in these animals. Decreases in these rate constants were also accompanied by a small, but significant ($P < 0.05$) reduction of about 17% in the plasma clearance of dye. The apparent volume of distribution at steady state, Vd_{ss}, was increased by 20% in the ARF group, but this change was not statistically significant ($P > 0.05$).

Biliary excretion of dibromosulphophthalein

The rapid initial removal of DBSP from plasma was reflected by prompt appearance of dye in bile. Figure 2 shows that initial biliary excretion rates over the 0–5 and 5–10 min intervals were lower ($P < 0.001$) in rats with ARF than in controls. Moreover, when these two values were combined, the excretion rate over the first 10 min after dye administration was reduced by about 37% ($P < 0.001$) in the ARF group (329 ± 19 μg min^{-1} kg^{-1}; $n = 8$) compared to controls (523 ± 31 μg min^{-1} kg^{-1}; $n = 8$). Bile flow rates in rats with ARF during the 0–5 and 5–10 min

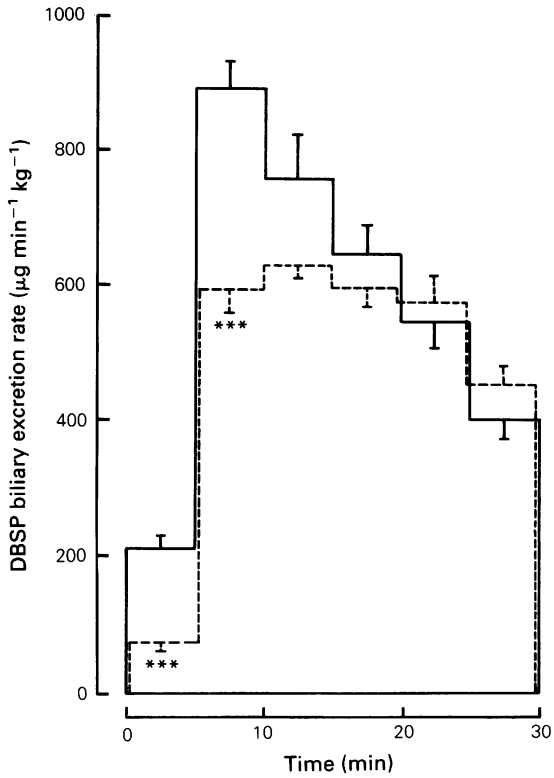

Figure 2 Biliary excretion profile of dibromosulphophthalein (DBSP, 25 mg kg^{-1} i.v.) in control rats (unbroken line) and rats with acute renal failure (broken line). Values are mean ($n = 8$); s.e. mean shown by vertical lines. Significantly different from control values: *** $P < 0.001$.

intervals (8.0 ± 0.3 and 8.1 ± 0.4 μl min^{-1} 100 g^{-1}, respectively; $n = 8$) were not, however, significantly different ($P > 0.05$) from the corresponding values in control rats (8.4 ± 0.3 and 8.6 ± 0.3 μl min^{-1} 100 g^{-1}, respectively; $n = 8$).

The maximum biliary excretion rate was also lower ($P < 0.001$) in rats with ARF (625 ± 16 μg min^{-1} kg^{-1}; $n = 8$) than in control rats (887 ± 43 μg min^{-1} kg^{-1}; $n = 8$). The biliary excretion profile was displaced to the right and so, the maximum excretion rate occurred between 5–10 min in control rats and between 10–15 min in rats with ARF. However, after 10 min there were no significant differences ($P > 0.05$) in biliary excretion rates between these groups of animals (Figure 2). In animals with ARF the % recovery of DBSP from bile after 30 min ($58 \pm 2.3\%$; $n = 8$) and mean bile flow rate (8.0 ± 0.4 μl min^{-1} 100 g^{-1}; $n = 8$) were not significantly changed ($P > 0.05$) from the same

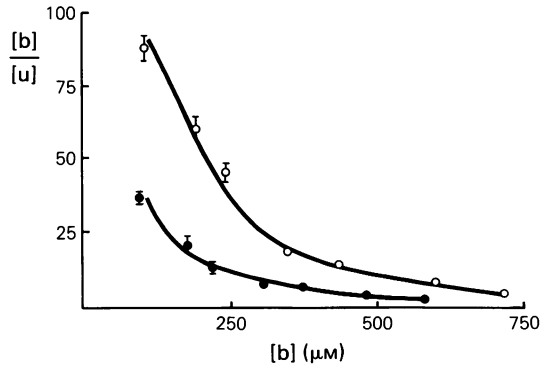

Figure 3 Binding of dibromosulphophthalein (DBSP) to plasma from six control rats (○) and six rats with acute renal failure (●). The ordinate scale shows the ratio of bound [b] to unbound [u] DBSP and the abscissa scale is the bound concentration. Values are mean; s.e.mean shown by vertical lines unless too small to show.

variables in control rats ($68 \pm 4.2\%$ and 8.7 ± 0.6 μl min^{-1} 100 g^{-1}, respectively; $n = 8$).

Chromatography of bile and renal excretion

When samples of bile from rats ($n = 3$) that had received DBSP (25 mg kg^{-1} i.v.) were chromatographed, single spots with R_F values of 0.44, 0.63 and 0.92 were obtained with cellulose plates and solvent systems A, B and C, respectively. Under the same conditions, DBSP, dissolved in rat bile, gave R_F values of 0.45, 0.63, and 0.92. With silica gel plates and systems A and B spots with R_F values of 0.22 and 0.60 were found for bile from rats given DBSP, whereas R_F values for DBSP dissolved in bile were 0.23 and 0.62, respectively.

No DBSP could be detected by spectrophotometry in urine collected for up to 30 min after injection of dye in either control or ARF rats.

Binding of dibromosulphophthalein to plasma protein

Equilibrium dialysis experiments showed that the plasma-protein binding of DBSP was substantially decreased in rats with ARF. The unbound concentrations of DBSP, [u], in control rats at total dye concentrations of 0.1 and 1.0 mM were 1.12 ± 0.06 ($n = 6$) and 142 ± 24 μM ($n = 6$), respectively. For rats with ARF, the corresponding values of [u] were 2.65 ± 0.13 ($n = 6$; $P < 0.001$) and 222 ± 8 μM ($n = 6$; $P < 0.05$). Figure 3 illustrates binding data plotted by the method of Rosenthal (1967). Plots for

Table 2 Parameters for the binding of dibromosulphophthalein (DBSP) to plasma proteins from control rats and rats with glycerol-induced acute renal failure (ARF)

Parameter	*Control rats* ($n = 6$)	*Rats with ARF* ($n = 6$)
K_1 ($M^{-1} \times 10^{-5}$)	6.7 ± 0.10	3.7 ± 0.06*
K_2 ($M^{-1} \times 10^{-3}$)	9.9 ± 2.3	6.0 ± 1.0
C_1 ($M \times 10^{-4}$)	2.5 ± 0.20	2.0 ± 0.20
C_2 ($M \times 10^{-3}$)	1.5 ± 0.69	0.75 ± 0.15

Results are given as mean ± s.e.mean of n rats.
K_1 and K_2 are the association constants for the primary and secondary binding sites and C_1 and C_2 are the capacities of the primary and secondary binding sites, respectively.
* $P < 0.05$.

plasma from controls and rats with ARF were curved which suggests binding of dye to at least two classes of sites. Analyses of these data using a two site model gave the estimates of association constants (K) and capacities (C) listed in Table 2. There was a significant decrease ($P < 0.05$) of about 45% in K_1, the association constant for the primary binding sites, in rats with ARF when compared to K_1 for controls. However, although K_2, C_1 and C_2 were all lower for diluted plasma from rats with ARF, these changes were not statistically significant ($P > 0.05$).

Total plasma protein levels in control plasma samples (6.7 ± 0.5 g $100\,ml^{-1}$; $n = 6$) were not significantly different ($P > 0.05$) from those in plasma from ARF rats (6.3 ± 0.6 g $100\,ml^{-1}$; $n = 6$).

Binding of dibromosulphophthalein to cytosol proteins

The mean elution profiles obtained after Sephadex G-75 gel filtration of cytosols from control rats ($n = 8$) and rats with ARF ($n = 8$), with DBSP added *in vitro*, are displayed in Figure 4a and b, respectively. It is clear from the pattern of absorbance at 575 nm that DBSP adsorbs to three groups of macromolecules which probably correspond to the X, Y and Z groups of proteins described by Levi *et al.* (1969). DBSP was bound mainly to Y and Z proteins with only a small amount to X (Figure 4). The X peak had the smallest elution volume, about 30 ml, and the elution volume of the fraction with the greatest optical density at 575 nm was that expected of a globular protein of molecular weight 69,000. Similarly the elution volume of the fraction in the Y peak with maximum absorbance corresponded to a molecular weight of 45,000, whilst that of the Z peak gave a molecular weight of 13,500. The estimated molecular weights of the X, Y and Z proteins are in good agreement with the values obtained by Levi *et al.* (1969). Unbound DBSP eluted at fraction 56 and is not shown in Figure 4.

In cytosols from animals with ARF there was a significant decrease ($P < 0.01$) of 38% in the amount of dye bound to the Y peak: 0.71 ± 0.06 nmol mg^{-1} cytosol protein ($n = 8$) compared to 1.15 ± 0.10 nmol mg^{-1} cytosol protein ($n = 8$) in controls. However, no significant difference ($P > 0.05$) in the quantity of dye bound to Z protein was found between control (1.07 ± 0.07 nmol mg^{-1} cytosol protein; $n = 8$) and ARF groups (1.00 ± 0.07 nmol mg^{-1} cytosol protein; $n = 8$).

Protein content of cytosols and pooled Y peak fractions

There was no significant difference ($P > 0.05$) in total cytosol protein level in homogenates from livers of control rats (27.7 ± 2.6 mg ml^{-1}; $n = 8$) and rats with ARF (29.2 ± 1.9 mg ml^{-1}; $n = 8$). Cytosols to which no DBSP had been added were eluted from the Sephadex G-75 column and assay of all the fractions that constituted the Y peak indicated that the protein concentration of this peak was similar in control ($972 \pm 160\,\mu g\,ml^{-1}$; $n = 8$) and ARF rats ($917 \pm 110\,\mu g\,ml^{-1}$; $n = 8$). These latter results agreed with the absorbance of the eluant at 280 nm (Figure 4).

Glutathione S-transferase activity

Mean GST activity in cytosols from rats with ARF was $0.72 \pm 0.04\,\mu mol\,min^{-1}\,mg^{-1}$ cytosol protein ($n = 12$) and this value was about 35% less than mean enzyme activity in control cytosols which was $1.1 \pm 0.10\,\mu mol\,min^{-1}\,mg^{-1}$ protein ($n = 12$; $P < 0.01$).

Figure 5 shows that five peaks of enzyme activity were discernible in cytosols from both groups of rats. These peaks were designated I to V. Peak I (10–45 ml) was eluted with the void volume. Peaks II and III were not well resolved, but required 38 and 47 mM Na^+ respectively for displacement from the gel. Peak IV was eluted at 62 mM Na^+ and together with II and III accounted for about 70% of total enzyme activity present in the cytosol. Peak V contained enzymes most strongly bound to the gel and required 88 mM Na^+ for elution. Total recovery of cytosol enzyme activity from the column was about 80%.

It is clear from Figure 5 that the profiles of enzyme activity obtained after elution of cytosols from rats with ARF were similar to those from the control group. However, the height of peak III ($1.02 \pm 0.08\,\mu mol\,min^{-1}\,ml^{-1}$; $n = 8$) was significantly reduced ($P < 0.001$) when compared with the corresponding peak in the control group ($1.60 \pm 0.06\,\mu mol\,min^{-1}\,ml^{-1}$; $n = 8$). This suggests

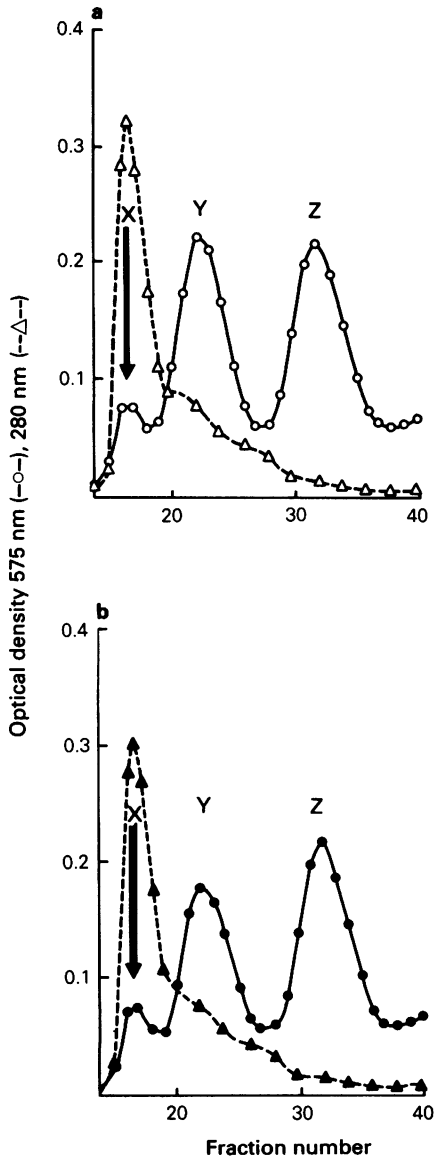

Figure 4 Mean elution profiles of dibromosulphophthalein (DBSP) from a Sephadex G-75 column after the addition of 2 ml of liver cytosol, containing 5.9 μmol of DBSP, from (a) control rats ($n = 8$) and (b) rats with acute renal failure ($n = 8$). Flow rate was 18 ml h^{-1} using 0.05 M sodium phosphate buffer, pH 7.4. Fractions were 3.0 ml. Optical density at 280 nm (broken line) indicates protein and at 575 nm (unbroken line) after dilution with 0.1 M NaOH indicates DBSP. X, Y and Z are fractions of cytosolic protein which bind DBSP. These fractions have been labelled after the nomenclature of Levi *et al.* (1969).

that the GST activity of peak III from rats with ARF was reduced.

Discussion

In control rats DBSP was rapidly removed from plasma and extensively excreted into bile. The half-lives of the initial (α) and terminal (β) exponential phases were about 1 and 7 min, respectively. Furthermore, some 68% of the dose was recovered from bile after just 30 min and we, like others (Javitt, 1964; Klaassen & Plaa, 1968; Vonk *et al.*, 1978), found no evidence to suggest that this dye is metabolised before its appearance in bile. By contrast to the rat, about 2% of the dose is converted to ninhydrin-reacting substances in rabbit and dog (Klaassen & Plaa, 1968). Meijer *et al.* (1983b) observed that up to 25% of a single dose of DBSP is conjugated with glutathione in man. These workers also found that significant renal excretion of dye occurs in man. About 6% of the dose was recovered from urine of volunteers, whereas 16% was present in patients with cholecystectomy (Meijer *et al.*, 1983a). In both groups DBSP appeared in urine as unchanged dye. However, no dye could be detected in urine collected for 30 min from controls and rats with ARF. This suggests that in the rat, plasma clearance (Cl_p) largely reflects the efficiency with which dye is removed by the liver.

The two compartment model used in this study was first proposed by Richards *et al.* (1959) to describe the elimination of BSP from dog plasma. This model was used because it affords both experimental and mathematical simplicity (Forker & Luxon, 1978). Its use to analyse curves for the disappearance of DBSP from plasma revealed that the intercompartmental rate constants k_{12}, k_{21}, and k_{23} together with Cl_p were all decreased in rats with ARF. These changes were not accompanied by a significant alteration of Vd_{ss} and it is unlikely that such changes were due to reduced liver blood flow because blood flow to this organ is significantly increased 48 h post glycerol injection (Hiley *et al.*, 1980). Thus the decrease in Cl_p probably reflected a reduction in the ability of liver to extract DBSP. Support for this is also provided by previous work which showed that isolated perfused livers from rats with ARF eliminated ICG more slowly than their control counterparts (Yates *et al.*, 1984).

In common with a number of organic anions, the plasma-protein binding of DBSP was decreased in rats with ARF. This binding defect is probably the result of occupation of binding sites by endogenous metabolites retained in the plasma of rats with ARF. The nature of these inhibitory metabolites is not known, but furanoid acids have been implicated as putative binding inhibitors (Lindup *et al.*, 1986).

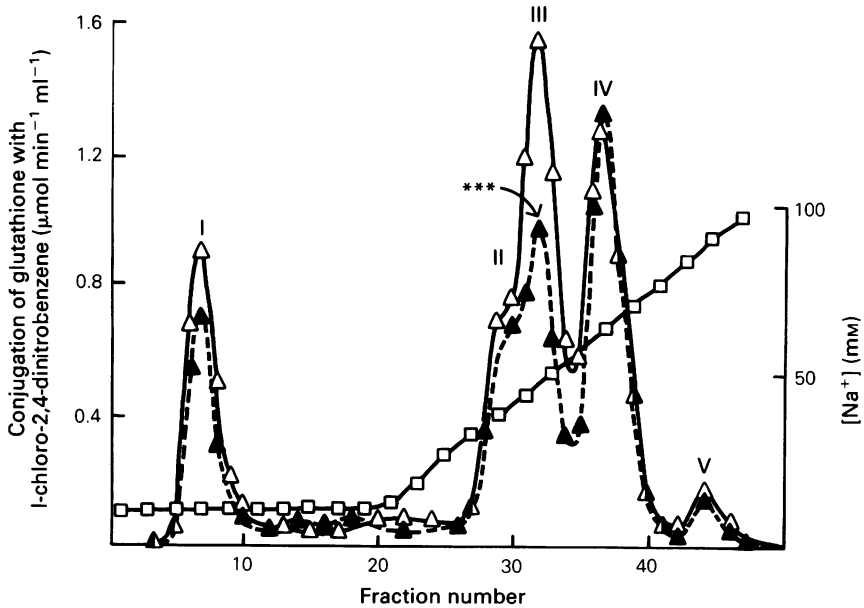

Figure 5 Mean elution profiles from a CM-Sepharose CL-6B column of glutathione S-transferase activity (GST) after application of hepatic cytosol samples from control rats ($n = 8$; △—△) and rats with acute renal failure ($n = 8$; ▲---▲). About 90 mg of protein were applied to the column and elution was performed with 0.01 M sodium phosphate buffer, pH 6.8 at a flow rate of 35 ml h^{-1}. Fractions (4.5 ml) were collected and the Na^+ concentration measured (□). GST activity was assessed using 1-chloro-2,4-dinitrobenzene as the substrate. Significantly different from peak height in control cytosol: *** $P < 0.001$.

There is evidence that the hepatic uptake of DBSP is dependent on the fraction bound to albumin (Meijer *et al.*, 1977). If uptake were solely restricted to the bound fraction, then decreased binding in ARF could contribute to the reduced Cl_p of dye in this disease. Recent work by Van der Sluijs *et al.* (1987) has provided evidence that the uptake of DBSP by rat liver is limited by its rate of dissociation from albumin. In view of this it would seem unlikely that an increase in the unbound fraction of dye was responsible for the altered kinetics of DBSP noted in rats with ARF.

Altered biliary excretion accompanied the changes in DBSP kinetics. The recovery of dye from bile in rats with ARF was some 15% less than that recovered from control animals. This difference, however, was not statistically significant, but both initial biliary excretion (0–10 min) and maximum biliary excretion rate were significantly diminished in rats with ARF. These results are similar to those obtained in earlier studies with BSP (Bowmer & Yates, 1984) and ICG (Bowmer *et al.*, 1983). Biliary excretion of DBSP is dependent on bile flow rate (Vonk *et al.*, 1978), but since there was no change in this variable in rats with ARF, reduced bile flow could not account for the delayed appearance of dye in bile of this group of animals. We have previously suggested that reduced hepatic uptake and subsequent delay in accumulation of dye in cytosol could explain the early changes in biliary excretion rate (Bowmer *et al.*, 1983). However, the possibility that the alteration in hepato-biliary transport may extend to processes involved in translocation across the hepatocyte or into the cannaliculus cannot be excluded.

In rats with ARF there was a 38% decrease in the binding capacity of the Y-fraction for DBSP. The reason for this change is not clear, but it is unlikely to be due to reduced content of binding proteins because there was no difference in either cytosol or Y-fraction protein levels between control and ARF rats. About 80% of the binding capacity of the Y fraction is due to a single protein called ligandin (Arias *et al.*, 1980). Ligandin is capable of binding a large number of structurally diverse ligands (Litwack *et al.*, 1971; Ketley *et al.*, 1975; Magos *et al.*, 1985) and in this respect it is similar to albumin. By analogy to albumin, it is possible that endogenous metabolites may also inhibit binding to ligandin.

The diminished binding capacity of the Y-fraction is of interest in another respect. We have mentioned previously (Bowmer & Yates, 1984) that reduced binding to liver cytosol proteins may account for the altered uptake of dyes such as DBSP in ARF. Ligan-

din is a major intracellular binding protein (Arias *et al.*, 1980) and it may aid net hepatic uptake by restricting efflux of its ligands from the hepatocyte into plasma (Wolkoff *et al.*, 1979a). Thus a decrease in the binding capacity of this protein could facilitate loss of dye from the cell, so reducing net uptake. However, if this were so then it would be expected that the rate constant for efflux (k_{21}) would be significantly increased in rats with ARF. In fact k_{21} was substantially decreased in such rats and the ratio of k_{12} to k_{21} increased from 7.7 to 9.4 in rats with ARF. Clearly these data are not consistent with the above hypothesis. It must be borne in mind, however, that both efflux and influx of DBSP were not measured directly in these experiments and the model used to quantitate the micro-rate constants is a simple representation of events *in vivo* (Forker & Luxon, 1978).

In addition to its ability to adsorb solutes, ligandin appears to be identical to GST B (Habig *et al.*, 1974b) which consists of two isoenzymes: GSTs 1-1 and 1-2 (Jacoby *et al.*, 1984). GST B is the major GST of rat liver and it accounts for about 50% of total liver cytosol GST activity (Wolkoff *et al.*, 1979b). Assay of cytosol GST activity using CDNB as substrate revealed a 35% reduction of enzyme activity in cytosols from rats with ARF. We have also observed decreased GST activity using BSP as the substrate for conjugation with glutathione (Bowmer & Yates, 1984). It is not possible from these data to deduce whether or not enzyme function as well as binding capacity are diminished in ARF, because both CDNB and BSP are substrates for GSTs other than GST B (Wolkoff *et al.*, 1979b). However, ion-exchange chromatography showed that the activity of peak III was reduced in cytosols from rats with ARF. The elution profiles obtained in this study were similar to those of both Hayes *et al.* (1979) and Habig & Jakoby (1981). These workers showed that the major peak of GST activity, peak III in our study, was due to GSTs 1-1 and 1-2. Thus it is likely that peak III represented the summed activity of GSTs 1-1 and 1-2, so it would seem that enzyme activity of ligandin as well as its ability to bind the non-substrate ligand DBSP is reduced in ARF.

In sum, the kinetics and initial biliary excretion of the phthalein dye DBSP were altered in rats with glycerol-induced ARF and these changes were similar to those found previously with BSP and ICG (Bowmer *et al.*, 1982; 1983; Bowmer & Yates, 1984). The binding of DBSP to plasma and liver cytosol proteins was decreased. Reduced binding to ligandin may be responsible for the altered hepatic handling of this dye in rats with ARF. If this is the case, then it would be expected that the binding of BSP and ICG to ligandin should also be diminished. In addition, where reduced binding to ligandin occurs, altered hepatic uptake should also be evident. These possibilities are currently under investigation.

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