Biochemical Pharmacology, Vol. 32, No. 17, pp. 2467–2472, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

DRUG–PROTEIN CONJUGATES—IV

THE EFFECT OF ACUTE RENAL FAILURE ON THE DISPOSITION OF [^{14}C]CAPTOPRIL IN THE RAT

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(*Received* 23 *December* 1982; *accepted* 14 *February* 1983)

Abstract—The effect of acute renal failure (ARF) on the metabolism and covalent binding to plasma proteins (PP) of [^{14}C]captopril ([^{14}C]CP) was investigated in the Wistar rat *in vivo* and *in vitro* using human and rat plasma. In the rat, ARF was induced by parenteral administration of glycerol. Glycerol-induced ARF markedly reduced the renal excretion of [^{14}C]CP, the major route of elimination of the drug in control rats, but did not alter the plasma clearance of [^{14}C]CP. However, there was a significant increase in the plasma concentrations of [^{14}C]CP mixed disulphides with glutathione, cysteine and PP. The increase in mixed disulphide formation did not result in an increase in the concentration of radioactivity in the lung, liver, kidney, spleen or bile. Thus, control rats excreted 9.64 ± 4.24% of the dose into bile in 3 hr while rats with ARF excreted 7.14 ± 2.46%. *In vitro*, [^{14}C]CP reacted rapidly with human or rat plasma to form mixed disulphides with endogenous thiols and PP. With uraemic plasma, there was a significant decrease in the amount of [^{14}C]CP covalently bound to PP from both man and the rat.

Captopril [D-3-mercapto-2-methyl-propanoyl-L-proline (CP)*] is a specific and potent inhibitor of ACE [1, 2], used in the treatment of various forms of hypertension and congestive heart failure [3, 4]. A number of adverse reactions to the drug have been reported, including skin rash, fever, loss of taste, proteinuria, ulcers, leucopenia, agranulocytosis and nephrotic syndrome [3, 5–10]. Although the mechanism(s) of such adverse reactions remain to be established, the incidence of certain side-effects appears to increase in patients with renal impairment and in patients receiving high doses [11]. Furthermore, the inhibitory effect of CP on ACE is substantially prolonged in patients with renal insufficiency, indicating a reduced rate of clearance of CP from its site of action [12]. The major route of elimination of CP and its metabolites is renal elimination [13, 14]. We have therefore investigated the effect of experimentally induced ARF on the disposition of CP in the rat *in vivo*. The glycerol model of ARF was used in this study because it has been found to be superior to other models of uraemia in drug metabolism studies [15, 16]. Intramuscular administration of glycerol produces myohaemoglobinuria which leads to renal ischaemia and thus resembles the "crush syndrome" in man [15]. In addition, the effect of uraemia on the interaction of CP with rat and human plasma proteins was studied *in vitro*.

* Abbreviations: ARF, acute renal failure; CP, captopril; NEM, *N*-ethylmaleimide; PP, plasma proteins; ACE, angiotensin converting enzyme; CP–NEM, *N*-ethylmaleimide derivative of captopril; CPD, captopril mixed disulphide; CP–CYS, captopril–cysteine mixed disulphide; CP–GSH, captopril–glutathione mixed disulphide.

MATERIALS

The [^{14}C]CP used was labelled in the amide carbonyl group (sp. act. 4.66 μCi mg^{-1}) and was a gift from Dr B. H. Migdalof of the Squibb Institute, New Brunswick, NJ, who also supplied authentic samples of CP disulphide, and CP mixed disulphides of cysteine and glutathione. Silica gel thin-layer chromatographic plates (20 × 20 × 0.2 cm) were obtained from British Drug House (Poole, U.K.) and scintillant (NE 260) and NCS tissue solubiliser from Nuclear Enterprises. The radioactive content of all samples was determined using an Intertechnique SL30 liquid scintillation spectrometer; counting efficiency was determined with automatic external standardisation and previously prepared quench curves. Other reagents were obtained from British Drug House and Sigma Chemical Co. (London, U.K.). All solvents were redistilled before use.

METHODS

Glycerol-induced ARF. ARF was produced by intramuscular injection of glycerol [17]. Male Wistar rats (200–300 g, from Bantin & Kingman, U.K.) were deprived of drinking water for 24 hr while food was allowed *ad lib*. Fifty per cent (v/v) glycerol/water was injected intramuscularly (10 ml kg^{-1}) under ether anaesthesia, in divided doses to two sites in each of the hind limbs. The drinking water was immediately restored. Control rats received sham injections into the hind limbs.

Determination of PP, urea and creatinine. Standard spectrophotometric assays were used: total PP was measured by the method of Lowry *et al.* [18], urea by reaction with diacetylmonoxime and plasma

creatinine concentration by reaction with alkaline picrate solution [19].

Determination of [^{14}C]CP *metabolites and* [^{14}C]CP–PP *conjugates in plasma.* Plasma from *in vivo* and *in vitro* experiments was mixed immediately with NEM (3 mg ml^{-1}) at room temperature to derivatise free CP [14]. Plasma was obtained by centrifugation and then extracted with 3 vol. of methanol. The protein precipitate was further extracted (twice) with methanol (3 vol.) and the extracts combined. An aliquot of the pooled extract was taken to determine total methanol extractable radioactivity; the remainder was concentrated under a stream of nitrogen and then separated by TLC together with authentic standards of CP–NEM, CP disulphide and CP mixed disulphides with cysteine and glutathione. The relative proportions of CP and its metabolites were determined essentially as previously described [14, 20]. The amount of [^{14}C]CP covalently bound to PP was assessed by the method of Sun and Dent [21] with some modifications as previously described [22].

The effect of ARF on the metabolism of [^{14}C]CP *with human and rat plasma* in vitro. Human blood was obtained from normal volunteers and patients with renal failure from the Royal Liverpool Hospital. Blood was drawn from the antecubital vein into heparinised containers. Plasma was then obtained by centrifugation and stored at 0–4° prior to use. Rat blood was obtained by heart puncture while under ether anaesthesia.

Incubation of [^{14}C]CP with plasma (human or rat) was carried out as previously described [22]. Briefly, a 0.2 mg ml^{-1} stock solution of [^{14}C]CP in methanol was prepared, 0.05 ml (*ca.* 0.05 μCi) of the stock solution was evaporated under a stream of nitrogen. Plasma (1 ml) was added and incubated at 37°. Immediately after mixing (0 min), and after 15, 30 and 60 min NEM (3 mg ml^{-1}) was added. [^{14}C]CP metabolites and CP covalently bound to PP were then determined as described earlier.

The effect of glycerol-induced ARF on [^{14}C]CP *disposition in the rat.* This part of the experiment was carried out 24 hr after the glycerol injection. Male Wistar rats (200–300 g) were anaesthetised with urethane [14% (w/v) in saline, 10 ml kg^{-1} i.p.]. The jugular vein, carotid artery and the trachea were cannulated with cannuli of the appropriate sizes. A small aliquot of blood (0.5 ml) was obtained before dosing, to determine PP, urea and creatinine concentrations. After heparin (100 units) was given, 4 mg kg^{-1} of [^{14}C]CP (4.66 μCi mg^{-1}) in saline was administered via the jugular vein. Blood samples (0.5 ml) were collected at 5, 30, 60, 120 and 180 min, centrifuged (1 min) and then added immediately to NEM (3 mg ml^{-1}). The volume of blood taken out was replaced by an equal volume of saline. [^{14}C]CP metabolites and [^{14}C]CP covalently bound to PP were determined as described earlier.

At 3 hr, the rats were killed and the liver, kidneys, lung and spleen removed to determine radioactive content as previously described [22]. All the urine in the bladder was aspirated at 3 hr into tubes containing NEM. The relative amounts of [^{14}C]CP metabolites in 0.1 ml aliquots were determined by liquid scintillation spectrometry after separation by TLC, as described for the plasma samples.

Biliary excretion of [^{14}C]CP *metabolites in rats with glycerol-induced ARF.* This experiment was also carried out 24 hr after glycerol injection. Male Wistar

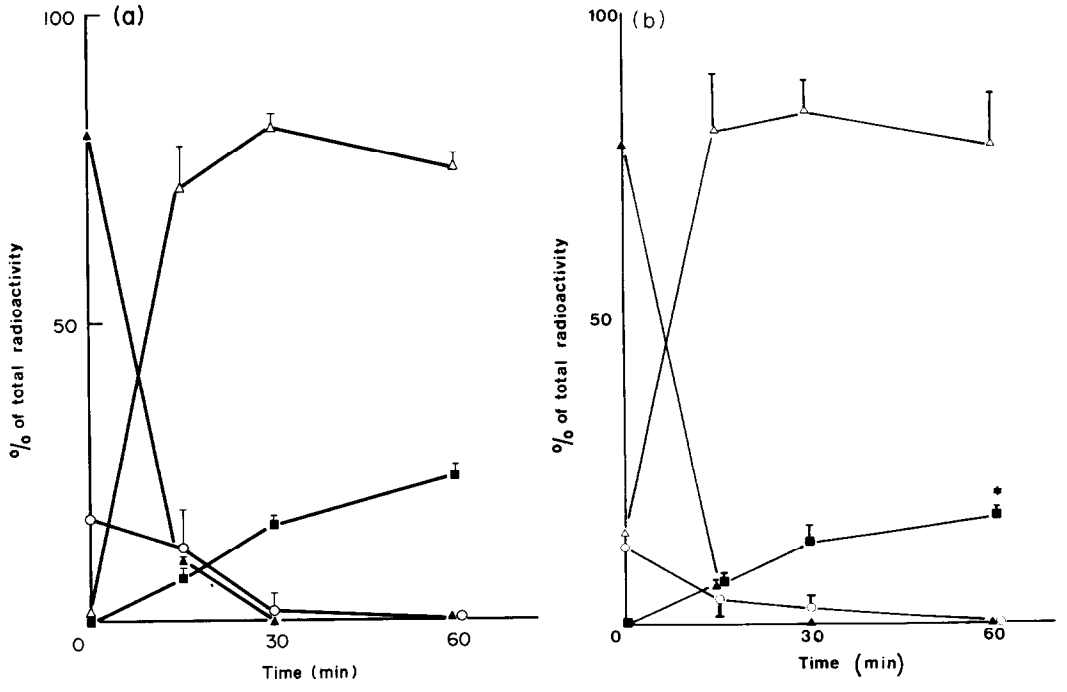

Fig. 1. The relative concentrations of [^{14}C]CP (▲) [^{14}C]CP disulphide (○), [^{14}C]CP covalently bound to plasma proteins (■), and polar metabolites of [^{14}C]CP (△), after incubation of [^{14}C]CP with: (a) normal human plasma (N = 4), and (b) uraemic human plasma (N = 6) *in vitro*. The results are means ± S.D. *P < 0.05 between control and uraemic plasma using Student's *t*-test.

rats (200–300 g) were anaesthetised with urethane [14% (w/v) saline, 10 ml kg^{-1} i.p.]. The jugular vein, carotid artery and the bile duct were cannulated with a cannula of the appropriate size. An aliquot of blood was taken before dosing to determine PP, urea and creatinine concentrations. [^{14}C]CP (4 mg kg^{-1}) in saline was administered via the jugular vein. Bile was collected into pre-weighed vials for 180 min at 30-min intervals. The amount of bile for each interval was recorded and aliquots of the bile samples were taken for quantitation of radioactivity. Aliquots of the remainder were separated by TLC to determine the relative amounts of [^{14}C]CP metabolites present in bile.

RESULTS

The effect of uraemia on the metabolism of [^{14}C]CP *with human and rat plasma* in vitro

The time-courses for the reactions of [^{14}C]CP with control and uraemic human plasma are shown in Fig. 1(a) and (b) respectively. The patients showed varying degrees of uraemia, with creatinine concentrations in plasma ranging from 146 to 1334 μmoles $l.^{-1}$ (normal range 60–110 μmoles $l.^{-1}$). In both sets of incubations the major products were polar metabolites of CP [predominantly CP–CYS (54.6–57.5%) and a smaller amount of CP–GSH (0–29%)]. The amount of [^{14}C]CP covalently bound to PP at 60 min was significantly less ($P < 0.05$) in the incubations with uraemic plasma (18.1 ± 1.81%) compared to controls (24.1 ± 1.90%). The results obtained with control plasma are similar to those reported previously [22].

The time-courses for the reactions of [^{14}C]CP with control and uraemic rat plasma are shown in Fig. 2(a) and (b) respectively. As reported previously [22], the amount of [^{14}C]CP which binds covalently to PP is greater with rat plasma than with human plasma. The amount of [^{14}C]CP covalently bound to PP was significantly less ($P < 0.02$) with uraemic rat plasma (36.3 ± 2.3%) than for control plasma (42.5 ± 2.9%). Uraemia had no effect on the rate of disappearance of CP. Polar metabolites achieved maximum concentration after 30 min, and then declined.

Glycerol-induced ARF

Plasma urea and creatinine concentrations were used to measure the degree of ARF induced by glycerol injection [16]. The total PP, urea and creatinine concentrations are shown in Table 1. Glycerol-induced ARF resulted in significant increases in urea and creatinine concentrations but no significant difference in PP concentrations, results which are similar to those reported previously for glycerol-induced ARF [16]. The radioactivity recovered in urine may slightly underestimate renal excretion of radioactivity because urine was obtained simply by terminal aspiration of the bladder. However, urine collected from uraemic rats [0.3 ± 0.13 ml (mean ± S.D.)] was less ($P < 0.005$) than from the controls (0.43 ± 0.16 ml).

The effect of glycerol-induced ARF on [^{14}C]CP *disposition in the rat*

The excretion in urine of total radioactivity over

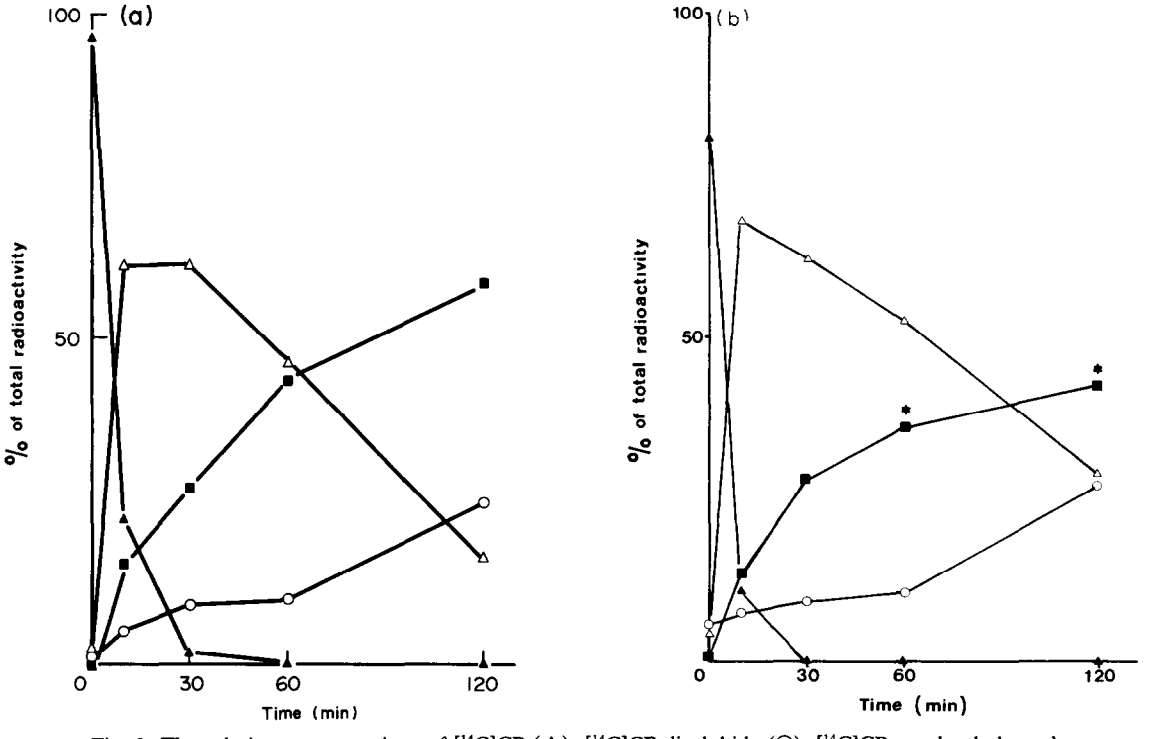

Fig. 2. The relative concentrations of [^{14}C]CP (▲), [^{14}C]CP disulphide (○), [^{14}C]CP covalently bound to PP (■) and polar metabolites of [^{14}C]CP (△), after incubation of [^{14}C]CP with rat plasma *in vitro* (a) control and (b) glycerol-induced ARF. The results are means of three individual experiments. S.D. < 10%. *$P < 0.05$ between control and uraemic plasma using Student's *t*-test.

Table 1. The plasma, creatinine, urea and protein concentrations of rats 24 hr after intramuscular administration of glycerol/water [50% (w/v)] and immediately before [^{14}C]CP was given

	Creatinine (μmoles l.$^{-1}$)	Urea (mg/100 ml)	PP (mg ml^{-1})
Control (sham injection)	79.3 ± 29.8	52.9 ± 13.7	64.7 ± 4.6
Glycerol/water treated	407.4 ± 80.2*	325.3 ± 27.0*	67.8 ± 8.4

Results are means ± S.D. of at least eight determinations.
* $P < 0.001$ relative to control.

Table 2. Metabolites of [^{14}C]CP present in urine 3 hr after [^{14}C]CP was administered (i.v.) to male Wistar rats with glycerol-induced ARF

		% relative distribution of radioactivity		
	% of dose in urine	CP	CPD	CP–CYS CP–GSH
Control (sham injection)	25.63 ± 2.5	79.97 ± 1.89	7.31 ± 1.7	12.30 ± 3.4
Glycerol-induced ARF	1.12 ± 0.04*	76.99 ± 13.9	0.02 ± 0.02†	23.03 ± 13.9

N = three or four determinations ± S.D.
* $P < 0.01$.
† $P < 0.05$.

3 hr was reduced by 95% in rats with ARF compared with control rats. In both groups, unchanged [^{14}C]CP was the major radioactive component in urine (Table 2). However, there was no significant difference in the amount of radioactivity excreted into bile over 3 hr between uraemic (7.1 ± 2.5% of dose) and control rats (9.6 ± 4.2% of dose). The amount of bile excreted by uraemic rats (1.69 ± 0.48 g) was significantly ($P < 0.05$) less than that excreted by control rats (2.77 ± 0.37 g). The major radioactive components in bile were [^{14}C]CP disulphide and [^{14}C]CP mixed disulphides with cysteine and glutathione.

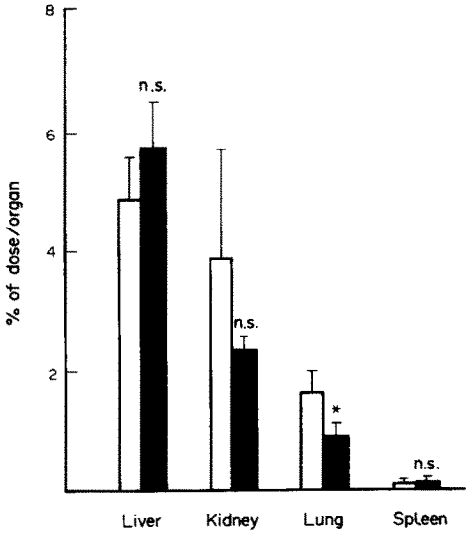

Fig. 3. Tissue distribution of radioactivity 3 hr after administration of [^{14}C]CP to control rats (□) and rats with glycerol-induced ARF (■). The results are means ± S.D. of four animals. *$P < 0.05$ using Student's t-test.

There was no significant difference in the accumulation of radioactivity 3 hr after administration of [^{14}C]CP in the liver, kidney or spleen between control and uraemic rats (Fig. 3), while there was a significant difference of radioactivity in the lung. The plasma concentration of radioactivity was significantly greater ($P < 0.05$) from 5 to 180 min after administration of [^{14}C]CP in uraemic rats compared to the controls [Fig. 4(a) and (b)] respectively. Thus, at 180 min, plasma from uraemic rats contained 1.5% of dose ml^{-1} compared to 1.05% of dose ml^{-1} in control rats. The increase in total plasma radioactivity in uraemic rats represented a two-fold increase in [^{14}C]CP covalently bound to PP and a one-and-a-half-fold increase in the amount of polar metabolites of [^{14}C]CP [Fig. 4(a) and (b)]. However, there was no difference between the plasma concentration–time curves of [^{14}C]CP for control and uraemic rats. The proportion of [^{14}C]CP covalently bound to PP at 180 min was not significantly different (30.09 ± 4.90% in uraemic rats and 23.59 ± 6.03% in the controls). However, the amount of [^{14}C]CP–GSH present in plasma *in vivo* (24.5–35.8%) was greater than that present *in vitro* (0–2.9%) while the amounts of [^{14}C]CP–CYS were 31.1–42.2% *in vivo* and 54.6–57.7% *in vitro*.

DISCUSSION

The influence of renal failure on the disposition of a wide range of drugs has been studied [23–26]. It is usually necessary to reduce the doses of drugs

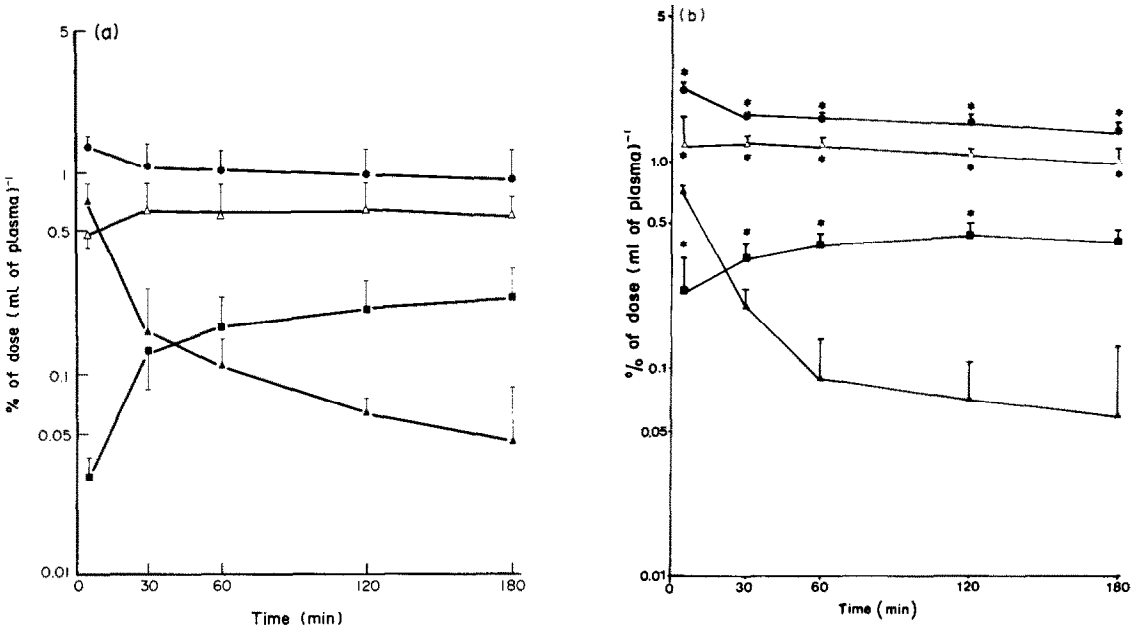

Fig. 4. Concentrations in plasma of [^{14}C]CP (▲), polar metabolites of [^{14}C]CP (△), [^{14}C]CP covalently bound to PP (■), and total radioactivity (●), after i.v. administration of [^{14}C]CP to: (a) control rats and (b) rats with glycerol-induced ARF. Results are means ± S.D. of four experiments. *$P < 0.05$ between control and rat with glycerol-induced ARF by Student's *t*-test.

which are excreted predominantly unchanged by the kidney in order to avoid excessive accumulation. It was found in this, and previous studies, that CP is eliminated mainly by direct renal excretion in man, the rat and the dog [14, 22, 27].

The urinary excretion of [^{14}C]CP was markedly reduced in rats with experimentally induced ARF compared to controls. This, however, did not lead to an increased plasma concentration of free [^{14}C]CP, but did produce a two-fold increase in [^{14}C]CP covalently bound to PP and a one-and-a-half-fold increase in [^{14}C]CP mixed disulphides with cysteine and glutathione. However, the increased concentration of [^{14}C]CP mixed disulphides in plasma did not appear to alter either the disposition or route of excretion of CP and its metabolites. There was no significant increase in the radioactive content of liver, kidney or spleen, the tissues in which [^{14}C]CP mixed disulphides accumulate [14, 22], nor was there any increase in the radioactivity excreted in bile in this study.

The mixed disulphides of CP with endogenous thiols may be formed by either spontaneous thiol–disulphide interchange in blood, or by interaction with glutathione and hepatic thioltransferase [28]. To distinguish the role of those processes, we investigated the reaction of [^{14}C]CP with rat and human plasma *in vitro*.

CP reacted rapidly with control and uraemic plasma to form mixed disulphides. With both human and rat plasma, there was a small but significant decrease in the proportion of CP–PP mixed disulphides. This may reflect a reduction in the affinity of uraemic plasma proteins for CP, or the presence of drug binding inhibitors, as has been reported for a number of drugs [29].

In vitro mixed disulphide formation with cysteine preceded mixed disulphide formation with PP. Circulating cysteine (220 and 186 μM in human and rat plasma, respectively) is known to be 50% covalently bound to PP via a disulphide linkage [30]. It is, therefore, possible that CP forms a mixed disulphide with cysteine via the following equilibrium reactions in plasma:

$$\text{CP–SH} + \text{PP–S–S–CYS} \rightleftharpoons \text{PP–SH} + \text{CP–S–S–CYS}$$

$$\text{CP–S–S–CYS} + \text{PP–SH} \rightleftharpoons \text{CYS–SH} + \text{CP–S–S–PP}$$

In vivo, it was found that there was a significantly greater amount of CP–GSH formed than *in vitro*. The plasma concentration of glutathione in the rat is 5 μM compared to 186 μM for cysteine. Therefore, CP–GSH is probably formed mainly in the liver as suggested previously by *in vitro* work [28], while CP–CYS may be derived from plasma as described, and as a hydrolytic product of CP–GSH in the kidney. Thus, this may explain why there is a different proportion of CP mixed sulphides of cysteine and glutathione present in plasma from *in vivo* and *in vitro* experiments.

The accumulation of mixed disulphides (with PP, cysteine and glutathione) that occurs in ARF may provide a source of free captopril *in vivo* [22]. This may explain why renal failure is associated with an increased duration, but not necessarily amplitude, of action of the drug [12]. Therefore, the increased incidence of CP toxicity in patients with renal failure would be due to an accumulation of the drug secondary to diminished renal clearance. It is unlikely that thiol–disulphide reactions of CP with albumin produce toxicity directly, because this pro-

cess occurs in plasma with endogenous thiols such as cysteine [30].

Acknowledgements—This work was supported by E. R. Squibb & Sons Ltd. We would like to thank Mrs K. A. Bishop for inducing experimental ARF in the animals used for this study.

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