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THE EFFECT OF ZINC AND COPPER PRETREATMENT ON THE BINDING OF GOLD (I) TO HEPATIC AND RENAL METALLOTHIONEINS

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Abstract—In the first part of this study, single s.c. injections of gold (I) sodium thiomalate were given to male Wistar rats at various dose levels and sacrificed either at 48 hr or 4 days later. The results indicate that though metallothioneins play a significant role in the sequestration of renal gold, the ability of gold itself to induce synthesis of the metalloprotein was limited in the kidneys and practically insignificant in the liver. Pre-injection with zinc however, (4 mg/kg body wt), significantly ($P < 0.01$) enhanced the uptake of gold into the metallothioneins both, in the liver as well as the kidneys. The pretreatment increased the proportion of cytosolic gold incorporated into the thioneins from 29% to 48% in the kidneys and from 22% to 47% in the liver. Concurrent increases in the uptake of zinc and copper into the thioneins was also observed. Pre-injection with copper induced a similar effect in the kidneys only. There was no significant difference in the overall uptake of gold into the cytosol itself between the control animals (injected with gold only) and those pretreated with either zinc or copper. This indicated that the increased uptake of gold into the thioneins in the pretreated animals were due to enhanced synthesis of metallothioneins and subsequent increased binding of gold to thioneins rather than increased levels of intracellular gold itself. It is suggested that increased protection against the deposition and possible toxicity of gold in the cell organelles of the liver and kidney tissues may be provided by an increased binding of gold to metallothioneins through pretreatment with zinc, and to a lesser extent copper.

At least four controlled studies have demonstrated the effectiveness of chrysotherapy for the treatment of rheumatoid arthritis [1–4]. The administration of gold, however, can be followed by severe toxic side effects, mainly disturbances of the haemopoietic system, dermatitis and nephrosis [5]. Treatment of gold toxicity is primarily supportive, and the role of corticosteroids and chelating agents remains controversial. The pathophysiologic mechanism of these adverse effects appear to be immunologic, although there are conflicting data [6]. Experiments on the subcellular distribution of antiarthritic gold (I) complexes [7, 8] have shown that gold was present in all the organelles of the tissues studied, indicating that gold could affect a variety of cellular functions and that a similar distribution in humans could be responsible for both the therapeutic and adverse effects. Recent studies with chloroauric acid [9] demonstrated that gold (III) induced biosynthesis of low molecular weight gold-binding proteins in the rat kidney cytosol. In our previous report [10] we have shown that a significant proportion of the cytosolic gold, administered as gold (I) sodium thiomalate, was also bound to the metal-binding proteins in the rat liver and kidney tissues. It was suggested that the low molecular weight metalloprotein may play an important role in the sequestration and localisation of gold, particularly in the kidneys. Although various interpretations have been reported, it is clear that the low molecular weight gold-binding protein is metallothionein [11, 12].

The detoxification function of metallothionein against exposure to heavy metals such as Cd^{2+} and Hg^{2+} has been confirmed by a number of investigators [13–15]. Pretreatment of experimental animals with a low dose of Cd^{2+}, or Zn^{2+}, is known to protect them against subsequent, toxic doses of Cd^{2+}. As both cations stimulate the synthesis of the corresponding metallothionein, protection against such acute effect of Cd^{2+} also has been attributed to these metalloproteins [16, 17]. It was suggested that protection against the toxicity of Au^{+} might similarly be conferred by pretreatment with low doses of gold and/or other metallothionein biosynthesis stimulating agents such as zinc [10].

In the present study we have investigated the effect of pretreatment with zinc and copper on the binding of gold (I) to metallothioneins in the rat liver and kidney tissues. The results show a significant enhancement in the incorporation of gold into the metallothioneins following pretreatment with zinc and to a lesser extent copper.

MATERIALS AND METHODS

Gold dose and protein binding. Two series of experiments were carried out to determine the extent of gold binding to the cytosolic proteins as a function of dose. In the first series male Wistar rats weighing between 145 and 180 g, received a single s.c. injection of either 0.1, 0.2, 0.4, 0.6, 1.0, 1.5, 2.0, 2.5 or 3.0 mg Au/kg body wt as Myocrisin (May & Baker Ltd., Dagenham, U.K.). For each dose two groups of three rats were used (a total of 54 rats). The animals

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were killed 48 hr later by exsanguination. In the second series the rats were similarly dosed with Myocrisin but killed 4 days later. The rest of the procedure was identical for the two series of experiments. The liver and kidneys were removed and either fractionated immediately or frozen and analysed later. Freezing tissues may rupture some organelles, however, in this study only a few duplicate samples were frozen before fractionation and the results obtained from these were not significantly different from the results of the corresponding fresh samples. Furthermore, approximately 95% of the gold in the organelles are attached to the organelle membrane or membrane adsorbed material and do not redistribute into the cytosol in the sucrose medium used for the fractionation (unpublished data). Equal weights of the liver and kidney tissues within each group were pooled and homogenised at 4° in 25% (w/v) 0.25 M sucrose solution. The homogenates were differentially centrifuged at 10,000 *g* (av.) for 30 min and then the supernatant centrifuged further at 100,000 *g* (av.) for 1 hr in a Beckman L-5 ultracentrifuge to obtain the cytosol. A portion of the cytosol was chromatographed on a Sephadex G-75 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column (2.5 × 75 cm), using 0.1 M ammonium formate solution containing 8 mM Tris–HCl as the eluant at pH 8.0. The cytosol eluates were analysed for gold by flameless atomic absorption spectroscopy. A deuterium continuum source was used for simultaneous background correction. The standard addition calibration method was used for the determination of gold concentrations in the samples. The experiment was done in duplicate and the values expressed in results are an average of the two. Details of the experimental procedure and the criteria used for the characterisation of the metallothioneins have been described in an earlier report [10].

Pretreatment with zinc and copper. Nine groups of three rats were used in this experiment. The first group received 1 mg Au/kg body wt as Myocrisin. A second group of rats initially received 4 mg Zn/kg body wt as zinc acetate (Hopkin & Williams Ltd., Essex, U.K.) followed by 1 mg Au/kg body wt 24 hr later. Similarly, a third group received 4 mg Cu as copper acetate (Hopkin & Williams Ltd., Essex, U.K.) followed by 1 mg Au. The animals were killed 48 hr following the gold injection. In each case (Au only, Zn + Au and Cu + Au) the experiment was repeated twice to obtain samples of liver and kidney tissues in triplicates. The tissues were fractionated, the cytosol obtained and chromatographed as described above. To help maintain the stability of the low molecular weight metal binding proteins the eluant was de-aerated and the chromatography was carried out using a refrigerated fraction collector. The concentration of gold in the eluates were determined as described above. Analysis for zinc and copper was performed using air/acetylene flame atomic absorption spectroscopy. The standard addition calibration method was used for the determination of zinc and copper in the samples. The average recoveries of Au, Zn and Cu from the Sephadex G-75 columns were 99.4 ± 1.2, 98.6 ± 1.8 and 98.9 ± 1.4% respectively.

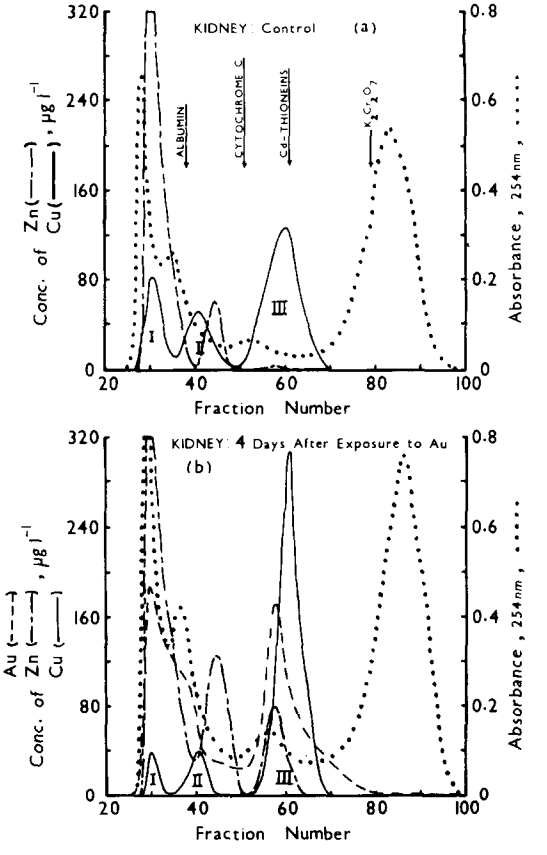

Fig. 1. Sephadex G-75 profiles of Au, Zn, Cu and the cytosolic proteins of the rat kidney. (a) Profile of samples from control rats that were not treated with any metals. (b) Profiles of samples from rats treated with gold only.

RESULTS

The Sephadex G-75 profiles of Au, Zn, Cu and the cytosolic proteins are illustrated in Fig. 1. The high molecular weight (H.M.W.) protein fractions, as discussed in this paper, represents the sum of all proteins eluted between the void volume and the metallothionein fractions (Peak III). Similar profiles of the metals and the proteins were obtained for rat liver and the definition for the cytosolic proteins apply equally to the liver fractions. The metal–protein interactions of Au, Zn and Cu with the various H.M.W. proteins have been described in detail elsewhere [24].

The binding of Au to the renal cytosolic proteins at various doses of gold (I) sodium thiomalate is illustrated in Fig. 2. In the kidney tissue samples obtained at 48 hr after the injection of Au, the incorporation of the metal into the metallothioneins increased with the increase in the Au dose from 0.1 mg to 0.5 mg/kg body wt. At dose levels between 0.5 mg and 1.5 mg Au, there was little increase in the binding to the metallothioneins. However, when the Au dose was increased even further, the uptake into the thioneins showed significant increases. A similar pattern of Au incorporation into the corresponding H.M.W. proteins was also observed at 48 hr. Thus in the 48 hr samples, over the lower dose

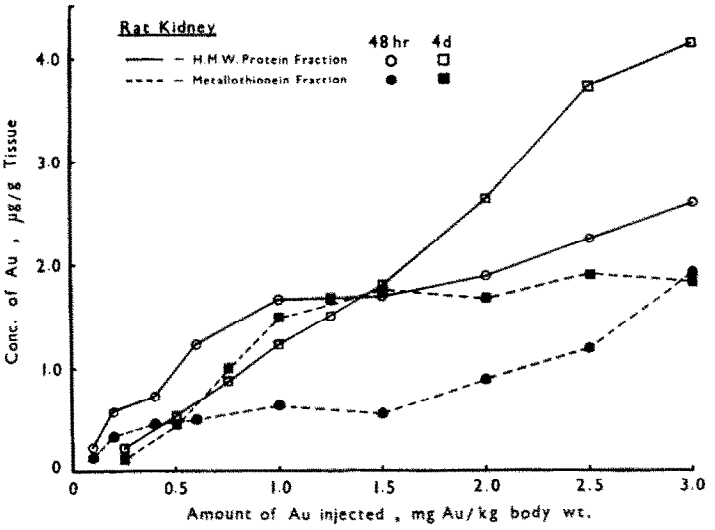

Fig. 2. Binding of gold to rat kidney cytosolic proteins at various dose levels following single s.c. injections of gold sodium thiomalate.

range (0.1 mg to 1.5 mg), the proportion of Au bound to thioneins relative to H.M.W. proteins decreased, from 38% to 24%. At the higher dose levels (1.5 mg to 3.0 mg) the proportion increased (24–42%). In fact the increase in the binding of Au to the renal metallothioneins (from about 0.5 μg to 2.0 μg/g tissue) over the higher dose range, was significantly greater at 48 hr than that for the corresponding H.M.W. proteins (from about 1.7 μg to 2.6 μg/g tissue).

In our previous study [10] it was shown that the binding of Au to the renal H.M.W. proteins was rapid, achieving maximum incorporation within 24 hr. Uptake of Au into the metallothioneins, however, was not maximal until 4 days after the injection of gold (I) sodium thiomalate. From the results of the present experiments, shown in Fig. 2, it is apparent that the gold–protein interactions vary markedly, not only with time but with dose.

At 4 days the binding of Au to the metallothioneins in the kidney increased sharply over the dose range 0.25 mg to 1.25 mg/kg, but thereafter, in contrast to the corresponding 48 hr samples reached a plateau. The increase in the uptake of Au into the H.M.W. proteins however was linear over the whole dose range.

In the liver cytosol (Fig. 3) the concentration of Au in the H.M.W. proteins showed an approximately linear rise with the corresponding increase in Au dose from 0.1 mg to 3.0 mg/kg body wt. This observation was true for data obtained from the 48 hr as well as 4 day samples. In fact there was no significant difference in the concentrations of Au in the corresponding cytosolic protein fractions in the liver between the 48 hr and the 4 day samples. While the increases in the uptake of Au into the H.M.W. proteins were large, those into the metallothioneins were relatively insignificant. It must also be noted that in the liver, the concentrations of Au in the H.M.W. proteins were on an average one tenth, and those in the metallothionein fraction were one twelfth of those in the corresponding kidney samples.

Effects of Zn *and* Cu *pretreatment.* Table 1 shows the concentrations of Au, Zn and Cu in the metallothionein and the H.M.W. protein fractions of the rat kidney cytosol at 48 hr following the injection of

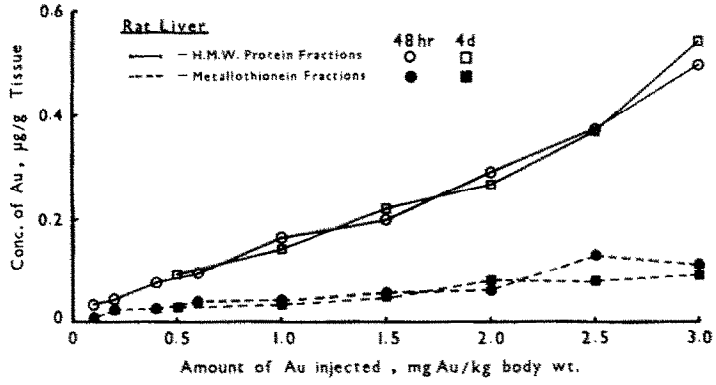

Fig. 3. Binding of gold to rat liver cytosolic proteins at various dose levels following single s.c. injections of gold sodium thiomalate.

Table 1. Effect of zinc and copper pretreatment on the incorporation of gold into renal metallothioneins

	μg metal/g wet tissue					
	Metallothioneins			H.M.W. Proteins		
Dose	Au	Zn	Cu	Au	Zn	Cu
Au only	0.962	1.095	3.541	2.288	21.240	10.656
	(29)	(4)	(21)	(69)	(85)	(64)
Zn^{2+}-pretreated	1.615*	4.092*	16.089*	1.789*	23.043	11.844
	(48)	(14)	(47)	(52)	(81)	(35)
Cu^{2+}-pretreated	1.471*	6.005*	21.034*	1.599*	26.618	10.921
	(48)	(16)	(59)	(52)	(71)	(31)

The values for the concentration of the metal are an average of three separate analysis with an average RSD of ±6.0%.

Asterisks denote the incorporation of the metal as significantly ($P < 0.01$) different from those treated with gold only (Student's *t*-test).

The values in parentheses are expressed as a percentage of the total metal in the cytosol.

Au only (the control values) or following pretreatment with Zn or Cu prior to the injection of Au. The asterisks denote changes that were statistically significant ($P < 0.01$) in the uptake of metal.

In the animals that were injected with Au only, 29% of the cytosolic Au was incorporated into the renal metallothioneins. However, in the animals in which the synthesis of the binding protein had been stimulated by Zn, Au accumulated more rapidly and to a significantly higher level than in the kidneys of control rats. The proportion of cytosolic Au bound to the metallothioneins was increased to 48%. It was also noted that the uptake into the corresponding H.M.W. proteins was significantly decreased. As expected, pretreatment with Zn resulted in significant increases in the uptake of Zn and Cu as well, into the metallothionein fractions. The binding of these metal ions to the H.M.W. proteins remained virtually unchanged.

Pretreatment with Cu also induced a significant increase in the incorporation of Au into the renal metallothioneins (Table 1). Although the concentrations of Au in the metallothionein fractions was slightly lower in the animals pretreated with Cu than in those pretreated with Zn, the proportion of cytosolic Au bound to the metalloproteins was remarkably similar to that in the Zn-pretreated rats, an average of 48%. The uptake of Au into the H.M.W. proteins, however, decreased significantly in rats pretreated with Cu. Pretreatment with Cu not only increased the incorporation of Cu itself (as one might expect), but also increased the incorporation of Zn into the renal metallothioneins (an approx. 6-fold increase). In fact the concentrations of Zn in the metallothionein fractions of the Cu-pretreated rats were significantly higher than those pretreated with Zn itself. Cu-pretreatment did not alter the extent of Zn and Cu binding to the H.M.W. proteins, except perhaps for a small increase in the binding of Zn.

The effect of pretreatment with Zn and Cu on the binding of Au, Zn and Cu to the hepatic proteins is shown in Table 2. It was evident that even though all the three metals studied were present in the metallothionein fractions, the concentrations of these metals were considerably lower than those determined in the corresponding renal fractions. As in the kidneys, pretreatment with Zn stimulated the incorporation of Au into the metallothionein fractions and significantly reduced the binding to the H.M.W. proteins. In the control animals (injected with Au only), 22% of the cytosolic Au was incorporated into the hepatic metallothioneins. Whereas in the Zn pretreated animals, this proportion increased to about 47%. The uptake of Cu, however, decreased significantly, both in the metallothionein as well as the H.M.W. protein fractions. Surprisingly the pretreatment of the animals with Zn had little effect on the uptake of Zn itself into the H.M.W. proteins.

Cu-pretreatment had virtually no effect on the incorporation of Au either into the metallothioneins or the H.M.W. proteins in the rat liver cytosol. As one might expect, the binding of Cu to the cytosolic proteins increased significantly in the Cu-pretreated animals. While the pretreatment had little effect on the uptake of Zn into the hepatic metallothioneins, the uptake into the H.M.W. proteins was significantly increased.

Concentration of Au, Zn *and* Cu *in kidney and liver cytosol.* The concentrations of Au, Zn and Cu in the liver and kidney cytosol of the control (treated with Au only) animals and those pretreated with Zn or Cu was also determined (Table 3). It was evident that the pretreatment with Zn or Cu had no sig-

Table 2. Effect of zine and copper pretreatment on the incorporation of gold into hepatic metallothioneins

	μg Metal/g wet tissue					
	Metallothioneins			H.M.W. Proteins		
Dose	Au	Zn	Cu	Au	Zn	Cu
Au only	0.067	0.302	1.434	0.249	20.280	6.759
	(21)	(1)	(17)	(79)	(94)	(83)
Zn^{2+}-pretreated	0.148*	0.542*	0.799*	0.170*	24.959	5.062*
	(47)	(2)	(14)	(53)	(98)	(86)
Cu^{2+}-pretreated	0.074	0.271	3.398*	0.260	26.980*	8.790*
	(22)	(1)	(28)	(78)	(99)	(72)

The values for the concentration of the metals are an average of three separate analysis with an average RSD of ±8.4%.

Asterisks denote the incorporation of the metal as significantly ($P < 0.01$) different from those treated with gold only (Student's *t*-test).

The values in parentheses are expressed as a percentage of the total metal in the cytosol.

nificant effect on the overall uptake of Au into the cytosol, in either the liver or kidney tissues. Zn-pretreatment showed little effect on the level of Zn itself in the liver or kidney cytosol. However, it had a marked effect on the concentrations of Cu. While the level of Cu in the liver cytosol was significantly decreased, that in the kidney increased to a level approximately twice that in the control animals injected with Au only.

Cu-pretreatment also showed no effect on the overall concentrations of Au in the rat liver or kidney cytosol. However, the effect on the level of Zn and Cu itself was significant. Zn and Cu levels both increased in the liver as well as the kidney cytosol. As one might expect, the effect on the Cu levels were far more dramatic than that on Zn.

DISCUSSION

The usual adverse effects of Au (I) salts are well known [18] and include hematological changes, lesions in the skin, liver and kidney damage. Recently serious toxic reactions in the lungs [19, 20] resulting in diffuse pulmonary injury associated with chrysotherapy have also been described. Because currently used Au (I) compounds are heavy metal mercaptides, it has been frequently suggested that their biological activity is caused by reactivity of Au

Table 3. Concentrations of gold, zinc and copper in the rat liver and kidney tissue cytosol

	μg metal/g wet tissue					
	Kidney			Liver		
Dose	Au	Zn	Cu	Au	Zn	Cu
Au only	3.28	25.00	16.67	0.316	21.48	8.19
Zn^{2+}-pretreated	3.40	28.38	34.25*	0.318	25.50	5.86*
Cu^{2+}-pretreated	3.07	37.44*	35.63*	0.334	27.25*	12.19*

Asterisks denote the change in the concentration of the metal as being significantly different ($P < 0.01$) from the control, injected with gold only (Student's *t*-test).

with sulfhydryl groups. Several studies [21–23] have shown that the sulfhydryl reactivity of Au may in part explain not only its therapeutic efficacy but also the adverse effects of the metal. Previous investigations [9, 10] have shown that a significant proportion of the cytosolic Au may be incorporated into the low molecular weight cysteine-rich protein, metallothionein, particularly in the kidneys. It is clear from the present experiments that the proportion of cytosolic Au bound to the thioneins, is dependent on the Au dose, the time elapsed following the exposure to the metal and the presence of other metals, such as Zn and Cu.

In the rat kidney (Fig. 2) the results show that at 48 hr and in dose levels of up to 1.5 mg Au/kg, a significantly smaller proportion of the cytosolic Au was incorporated into the metallothioneins (24–38%) than in that at 4 days (37–55%). In our previous study [10], a time lag of about 4 days was reported for the maximum incorporation of Au into the renal metallothionein. This time lag factor may be responsible for the relatively lower levels of Au–thionein found in the 48 hr samples.

In the 48 hr samples, at dose levels from 2.0–3.0 mg Au/kg, there was a significant increase in the proportion of cytosolic Au bound to the renal metallothioneins (24–42%). In the corresponding 4 day samples however the uptake plateaued.

With the increased exposure to Au (I or III) the level of Cu in the kidney cytosol increases markedly [11, 24]. It is also known that Cu itself induces the synthesis of metallothioneins in the kidney and the liver tissues [25, 26]. Therefore, it is probable that at the higher doses of Au, the increased levels of Cu in the kidney cytosol further enhanced the synthesis of the thioneins, which subsequently incorporated a greater proportion of the cytosolic Au. This mechanism is also supported by the fact that pretreatment with Cu prior to the injection of Au, significantly increases the incorporation of Au into the renal metallothioneins (Table 2).

Although Au (I) has a high affinity for the sulfhydryl binding sites on the thioneins [12], the results of the present experiments indicate that in both kidney (Fig. 2) and liver (Fig. 3), Au has a limited capacity to stimulate metallothionein biosynthesis as a response to acute exposure to the metal.

Cortell and Richards [27] have previously reported that tolerance develops to the toxic effects of Au if rats were pretreated with small doses of the metal every other day for 3 weeks. It is also known [28] that patients receiving small doses of Au (I) salts or oily suspensions of the drug rather than the fast absorbing aqueous solution, show a lower susceptibility to Au toxicity. More recently, Eiseman and Avares [23] have shown that gold (I) sodium thiomalate, administered acutely (37 mg Au/kg) has an inhibitory effect on the heme biosynthetic pathway and cytochrome P-450-dependent monooxygenases in the liver, kidney and erythrocytes. In contrast, chronic administration of the Au (I) salt at a low dose (3.7 mg Au/kg) did not cause any significant changes in the various parameters of heme metabolism. The incorporation of higher proportions of Au (up to 55%) into thioneins at low doses observed in the present experiments and the reports described above, suggests that pretreatment with low doses of Au itself may sufficiently stimulate thionein synthesis and enhance the uptake of any subsequent higher doses of the metal, thereby providing further protection against the possible adverse effects of the toxic metal ions.

The present results also demonstrate that pretreatment with Zn, significantly enhanced the uptake of Au into the hepatic as well as the renal metallothioneins, thereby immobilising a greater proportion of the intracellular Au. Pretreatment with Cu induced a similar effect in the kidneys only. The mechanism by which the increases were achieved, in the incorporation of Au into the metallothioneins is not clear. The concurrent increases in the uptake of Zn and Cu into the thioneins suggests that the accumulation of Au may not occur by replacement of Zn or Cu ions in the presynthesised metalloprotein by Au, but through an enhanced increase in the synthesis of the binding protein following the subsequent injection of gold (I) sodium thiomalate. In the pretreatment of rats with Zn against cadmium toxicity, Webb [16] postulated that after induction by Zn, the synthesis of the metallothionein is stimulated readily and without lag by the subsequent injection of Cd. In view of the time lag in the complete uptake of Au into the thioneins reported previously [10], together with the results of the present experiments described above, it is possible that a similar mechanism may also explain the increased uptake of Au in the Zn- or Cu-pretreated animals.

It was also observed that in all the instances where the incorporation of Au into the metallothionein was increased, either in the liver or kidney cytosol, there was no significant change in the overall uptake of the metal into the cytosol itself. This further supports the suggestion that pretreatment with Zn or Cu may have increased the capacity of Au to induce the synthesis of metallothionein, rather than increase the availability of Au itself in the cytosol, thereby increasing the uptake of the metal into the metalloprotein.

The fact that the uptake of Zn and Cu into the H.M.W. proteins showed no significant increase in the Zn or Cu pretreated animals (except in the liver of Cu pretreated animals) and that the uptake of Au was decreased (the Au-binding sites on the H.M.W. proteins do not appear to be saturated at the dose level given; Figs. 2 and 3), suggests that once the biosynthesis of the metallothionein was accelerated by pretreatment and the availability of the metalloproteins increased, Au was preferentially incorporated into the thioneins. It was also evident from the results that while Zn pretreatment stimulated the uptake of Au, Zn and Cu into the metallothioneins, in both the liver and kidney cytosol, in the case of Cu it was limited to the kidneys only. In the liver of Cu pretreated animals, only the uptake of Cu into the metallothioneins was increased. The differences were probably due to the varying degrees of affinity the metals have for the metal-binding sites on the protein in differing chemical environment.

Following the injection, Au is systemically transported to the tissues and rapidly taken up into the cells, in particular the cells of the reticuloendothelial systems [7, 8]. Studies on the intracellular distribu-

tion of Au has shown [7, 8] that in the liver and kidney tissues, Au was initially localised in the cytosol and then gradually redistributed into the organelles, particularly the lysosomes. While the results of the present experiments show no direct evidence that may suggest a role for metallothionein in the intracellular translocation of Au, it is clear however, that in the Zn/Cu pretreated animals (Zn in particular, since it significantly increased the uptake of Au into the metallothioneins, in both the liver and the kidneys), metallothioneins could play an important role in the immediate sequestration, storage and regulation of cytosolic Au, thus reducing the interactions of Au with processes with important biologic functions [23, 29]. It has been shown for Hg-induced renal damage, that after treatment with spironolactone, the amount of Hg bound to the kidney metallothionein fractions increased and thereby the deposition of Hg to cell organelles was markedly decreased and, in turn, injury to the tubular cells of the kidney was prevented [30]. In chrysotherapy, the kidneys play a major role in the systemic depositioning of Au [12]. In the experimental animals the relative contribution of the metal complex with thioneins to the total metal content of the cytosol fraction has a rising tendency following multiple exposure, reaching up to 55% in the kidneys [10, 11]. The results described here indicate that protection from the deposition of Au in the cell organelles of the liver and kidney tissues may similarly be provided by an increased binding of Au to metallothioneins through pretreatment with Zn, and to a lesser extent Cu.

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