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BINDING OF COPPER AND ZINC BY THE ANTITUMOUR AGENT L-ALANOSINE

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Abstract—L-Alanosine [L-2-amino-3(hydroxynitrosamino)proprionic acid], an anticancer agent recently introduced into clinical trial, formed a 1:1 complex with Cu(II) with an effective stability constant (log K_{eff}) at pH 7.2, determined by the method of competing equilibria, of 22.7 and with Zn(II) of 17.2. The intravenous administration of L-alanosine to rabbits at a dose of 30 mg/kg resulted in an immediate decrease in total plasma copper of up to 17 per cent. Plasma copper levels returned to normal within 24 hr. There was no increase in urinary copper excretion and a 4-fold increase in urinary zinc excretion. Cancer patients receiving L-alanosine also had a transient decrease in plasma copper (up to 30 per cent) and an increase in urinary zinc excretion. There was no significant difference in the antitumor effects of L-alanosine against intraperitoneally transplated P-388 leukemia in normal, copper-deficient, or copper-loaded mice. The Cu(II) complex of L-alanosine lacked antitumor activity at doses that were tolerated by the mice. The increased toxicity of the Cu(II) complex of L-alanosine compared to L-alanosine was attributable to the Cu(II).

L-Alanosine [L-2-amino-3(hydroxynitrosamino) propionic acid] (Fig. 1) is an antibiotic produced by *Streptomyces alanosinicus* nov. sp. (ATCC-15710) which exhibits antiviral activity [1] and immunosuppressive properties [2] and, in addition, antitumor activity against SV-40 virus-induced fibrosarcoma in hamsters and several tumors in mice, including P-388 and L-1210 leukemias and CD8F mammary tumor [1, 3]. The sodium salt of L-alanosine (NSC 153353) has been placed in clinical trial for antitumor activity. Alanosine inhibits cellular conversion of IMP to AMP, with a decrease in ATP levels and an increase in GTP levels. There is a decreased synthesis of deoxynucleotides and DNA [4]. Although the site of inhibition appears to be adenylosuccinate synthetase, alanosine itself has no effect on enzyme activity [5]. A metabolite, possibly the alanosine analog of *N*-succinylcarboxamide-aminoimidazole ribotide, is believed to be the inhibitor [6, 7]. Attempts to provide unequivocal identification of the inhibitory metabolite have not been successful [7].

The hydroxynitrosamino functional group of alanosine is not a common group. It is found as the ammonium salt in cupferron (*N*-hydroxy-*N*-nitrosobenzenamine ammonium salt), a copper chelating agent [8]. This suggested to us that L-alanosine might also be a chelating agent. Because of the antitumor activity and other biological activities of copper chelating agents [9], we studied the copper binding activity of L-alanosine *in vitro* and *in vivo* and the effect of copper upon the biological activity of L-alanosine.

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MATERIALS AND METHODS

The association between Cu(II) and L-alanosine was measured spectrophotometrically as a decrease in the absorption of the hydroxynitrosamino chromophore at 250 nm. Binding studies were conducted at room temperature with 50 μM L-alanosine in 5 mM potassium phosphate buffer, pH 7.2. Cu(II) and Zn(II) were added as the acetate or sulfate salts. The ratio of Cu(II) to L-alanosine in the complex was determined by the method of continuous variation [10]. The binding affinity of Cu(II) and Zn(II) for L-alanosine was measured by the method of competing equilibria [11] with ethylenediamine and histidine as competing ligands. The equilibrium is described by

$$MA + 2E^- \overset{K}{\rightleftarrows} ME_2 + A^{2-}$$

where M is the metal ion, A^{2-} L-alanosine and E^- the competing ligand (in this case ethylenediamine). The equilibrium constant for this system is given by

$$K = \frac{[ME_2]\,[A^{2-}]}{[MA]\,[E^-]^2} = \frac{\beta_1}{\beta_2}$$

where β_1 and β_2 are the equilibrium constants for the formation of the metal complexes of ethylenediamine and L-alanosine. L-Alanosine and $CuSO_4$ or $ZnSO_4$ were maintained at 50 μM, and ethylenediamine was varied between 0.1 and 0.5 mM, and

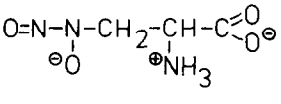

Fig. 1. Structure of L-alanosine.

histidine between 0.5 and 10 mM. Because of the high concentrations of competing ligand, it is assumed that the metal ligand complex exists entirely as ME_2 [11]. The Cu(II) complex of L-alanosine was prepared by mixing equimolar amounts of L-alanosine and cupric acetate. The light blue precipitate was washed extensively with ice-cold water and dried. The copper complex was much less soluble than L-alanosine and was suspended in 0·3% hydroxypropyl cellulose (Klucel, Hercules Inc., Wilmington, DE) for antitumor testing. Antitumor activity was determined *in vivo* using murine leukemia P-388 in BDF_1 mice according to National Cancer Institute protocols [12]. Compounds were administered intraperitoneally to six mice at each dose level, starting 1 day after the intraperitoneal implant of 10^6 viable tumor cells. The median lethal dose (LD_{50}) was determined by injecting BDF_1 mice intraperitoneally (four mice at each level) with doses of compound that increased geometrically by a factor of 1.26. Deaths were recorded for 30 days and the LD_{50} and confidence limits were calculated by the method of Weil [13]. Male BDF_1 mice, weighing between 20 and 30 g, were housed in plastic and stainless steel cages. Control mice were maintained on a normal diet (4% mouse and rat diet, Teklad, Winfield, IA) and tap water. Copper-deficient mice were maintained on a copper-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH) and deionized water for 25 days prior to and during the antitumor study. Copper-loaded mice were maintained on a normal diet, and their drinking water was replaced with 0.025% cupric acetate 4 days before and during the antitumor study. The 24-hr urinary excretion of copper (N = three groups of six mice, ± S.E.M.) in control mice was 1.77 ± 0.41 μg/six mice, in copper-deprived mice was 0.02 ± 0.01 μg/six mice ($P < 0.05$), thus confirming their copper deficiency, and in 4-day copper-loaded mice was 1.82 ± 1.10 μg/six mice ($P > 0.05$ compared to control), suggesting that the excess dietary copper was accumulating in the tissues. L-Alanosine was administered (into an ear vein) to male Dutch white rabbits at a dose of 30 mg/kg, and 3 ml blood samples were collected from the other ear vein into heparinized tubes at various times. Urine was collected over 24 hr, before and after administering the drug, with the rabbit in a metabolism cage and allowed free access to food and water. The cage and containers used to collect the urine were acid washed. L-Alanosine in the plasma was determined as described previously [14]. Total copper and zinc were determined by atomic absorption spectroscopy. Urine and blood samples were also collected from patients with advanced cancer receiving L-alanosine as part of a Phase I study.* Superoxide dismutase activity was determined at pH 7.2 by the method of Salin and McCord [15]. L-Alanosine was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

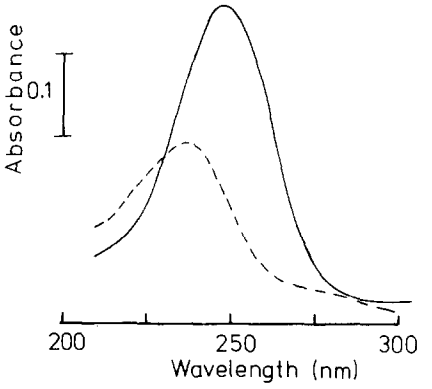

Fig. 2. Spectral change produced by the binding of Cu^{2+} to L-alanosine. Key: (solid line) spectrum of 50 μM L-alanosine in 5 mM potassium phosphate buffer, pH 7.2; and (dashed line), following the addition of 50 μM cupric acetate.

RESULTS

Binding of Cu(II) *and* Zn(II) *to alanosine.* The addition of Cu(II) to a solution of L-alanosine resulted in a bathochromic shift in the ultraviolet adsorption due to the hydroxynitrosamino chromophore (Fig. 2), which is similar to the shift observed at low pH [16]. Maximum complex formation was

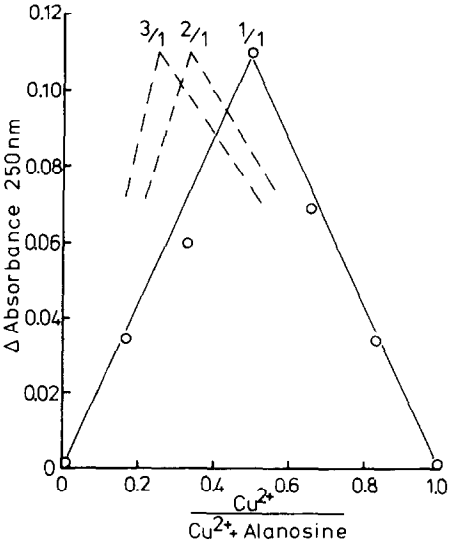

Fig. 3. Stoichiometry of copper alanosine complex formation. Complex formation was measured at 250 nm by mixing different proportions of solutions of 50 μM L-alanosine and 50 μM cupric acetate, both in 5 mM potassium phosphate, pH 7.2 ΔAbsorbance is the difference in absorbance between the L-alanosine–cupric acetate mixture and a solution of L-alanosine at the same concentration. The dashed lines represent theoretical plots for copper ligand complexes of 3:1 and 2:1. The experimental data correspond to a copper ligand complex of 1:1 shown by the continuous line.

* A Phase I protocol is a research study designed to determine the maximal dose of a new drug that can be given with safety and to look for any evidence of therapeutic effect in humans with advanced cancer who have failed all standard forms of therapy. All patients are fully informed as to the purpose of the study and sign an informed consent form. This protocol was approved by our Human Studies Committee.

Table 1. Effective stability constants of Zn and Cu–alanosine at pH 7.2*

Ligand added (μm)	Zn Zn(A) (μm)	E (μm)	log K_{eff}	Ligand added (μM)	Cu Cu(A) (μM)	E (μM)	log K_{eff}
Ethylenediamine				Ethylenediamine			
200	40	60	16.8	100	39	78	23.0
300	34	268	17.6	150	29	108	22.6
400	26	352	17.4	200	25	150	22.7
500	8	416	16.5	250	20	189	22.6
		Mean	17.1			Mean	22.7
Histidine				Histidine			
1,000	31	963	17.4	500	27	454	22.3
2,000	17	1,933	17.2	1,000	17	935	22.4
			—	10,000	3	9,905	23.3
		Mean	17.3			Mean	22.7

* Stability constants were determined at pH 7.2. No correction was made for competitive equilibria with H^+. The possible formation of mixed complexes of Cu, L-alanosine, and competing ligand has been ignored. ZnA and CuA represent the Zn(II) and Cu(II) chelates of L-alanosine; E stands for unbound ligand. The log cumulative stability constants of the competing ligands were [Zn (ethylenediamine)$_2^{2+}$]= 13.65; [Zn (histidine)$_2^{2+}$)= 12.44; [Cu (ethylenediamine)$_2^{2+}$]= 22.72; and [Cu (histidine)$_2^{2+}$] = 22.68 [17].

observed at a Cu(II) to L-alanosine ratio of 1 : 1 (Fig. 3). This was determined by the method of continuous variation where, at a constant total concentration of metal and chelating agent, the concentration of the chelate is the greatest when the metal and chelating agent are brought together in the same ratio in which they exist in the chelate [10]. The effective stability constants of the Cu(II) and Zn(II) complexes of L-alanosine at pH 7.2 are shown in Table 1. For comparison, log K Cu(II)EDTA is 18.8 and Zn(II)EDTA is 16.6 [17].

Biological activity of Cu(II) *alanosine.* The LD_{50} for L-alanosine in mice is 1497 mg/kg. [3]. The copper complex was much more toxic, with an LD_{50} of 10.1 mg/kg (95 per cent confidence interval limits 9.5 to 12.9 mg/kg). If it is assumed, however, that toxicity is due solely to Cu(II), the LD_{50} of Cu(II) in the chelate is 3.0 mg/kg which is similar to the toxicity of free Cu(II) (administered as cupric acetate), LD_{50} 2.5 mg/kg (95 per cent confidence interval limits 2.1 to 3.0 mg/kg). In both cases mice died within 2–5 days of administration of the compound.

L-Alanosine was administered on days 1, 5 and 9 to control, copper-deficient, and copper-loaded mice bearing leukemia P-388. There were no significant differences between dose–response curves to L-alanosine, measured as the increase in survival times, of the three groups of mice (Fig. 4). A repeat experiment with fewer intermediate dose levels gave the same results (not shown). The daily intake of copper by even the copper–loaded mice (no more than 1 m-equiv./kg) was less than some of the doses of L-alanosine (0.2 to 3.0 m-equiv./kg), and it is possible that insufficient free copper was available *in vivo* to form appreciable levels of the copper chelate of L-alanosine. A direct comparison was made, therefore, of the antitumor effects of the copper complex of L-alanosine and L-alanosine sodium. The copper complex of L-alanosine had no measurable antitumor activity (Table 2). Repeat experiments gave the same result.

Superoxide dismutase activity. Copper is commonly found to have square planar or tetragonally distorted octahedral geometry, although some copper chelates exist in the square pyramidal, trigonal bipyramid, or distorted tetrahedral geometry. Regardless of the geometry, L-alanosine can accommodate a maximum of three of the minimum of four ligand positions of copper. This means that the L-alanosine–copper complex must possess at least one open coordination position. It has been shown that

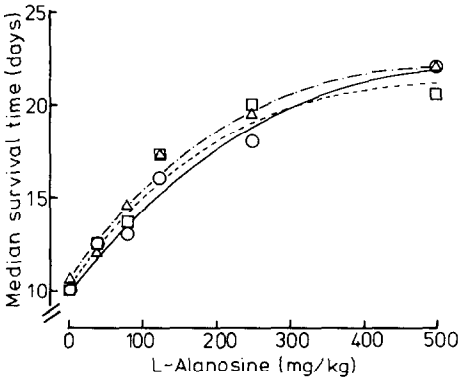

Fig. 4. Antitumor activity of L-alanosine against murine leukemia P-388. Key: (○) control mice, (△) copper-deficient mice, and (□) copper-loaded mice. Mice were treated as described in the text. There were six mice in each group. L-Alanosine was administered on days 1, 5, and 9 at the dose shown, commencing 24 hr after the intraperitoneal implantation of 10^6 P-388 cells. There were no nontumored animals in the control group. No drug toxicities were observed (more than a 4 g loss of weight per animal between days 0 and 5). For example, the weight changes between days 0 and 5 observed at the highest dose of L-alanosine (500 mg/kg) were: control mice + 0.2 per cent, copper-deficient mice − 5.2 per cent and copper-loaded mice − 0.4 per cent, compared to weight changes of tumored, untreated mice of + 10.0 per cent + 7.1 per cent and + 13.4 per cent respectively. Groups of data were analyzed by the Mann Whitney test with correction for ties [18]. There was no significant difference between the median survival in any of the groups of mice receiving the same dose of L-alanosine.

Table 2. Antitumor activity of copper alanosine against murine leukemia P-388*

	Dose (mg/kg)	Schedule	No.	Weight change (%)	Median life span (days)	T/C (%)
Control			12	+7.6	10.0	
Copper alanosine	2.5	Q4D	6	+18.1	9.5	95
	5.0	Q4D	6	−1.6	10.0	100
	1.0	Q1D	6	+8.8	10.0	100
Alanosine	2.5	Q4D	6	+15.4	9.5	95
	5.0	Q4D	6	+16.7	14.0	140†
	1.0	Q1D	6	+17.8	10.0	100
Cupric acetate	5.0	Q4D	6	+7.1	9.5	95

* Compounds were administered daily for 9 days (Q1D) or on days 1, 5, and 9 (Q4D) at the dose shown, commencing 24 hr after the intraperiotoneal implantation of 10^6 P-388 cells. There were no nontumored animals in the control group. Weight changes were measured between days 0 and 5. Survival curves were analyzed by the Mann Whitney test with correction for ties [18].

† $P < 0.05$, compared to the control survival curve.

an open coordination position is essential for copper complexes to show superoxide dismutase activity [19], and copper complexes formed by amino acids and several other compounds display superoxide dismutase activity [20]. The copper complex of L-alanosine, however, did not exhibit superoxide dismutase activity, and L-alanosine even inhibited the dismutation of superoxide caused by free Cu(II) (results not shown).

Plasma and urinary divalent ion levels. Intravenously administered L-alanosine disappeared rapidly from the plasma in the rabbit in biphasic manner (Fig. 5). The distributive half-life ($T_{\frac{1}{2}}^{\alpha}$) was 4.3 ± 0.1 min (± S.E.M., N = 3), the postdistributive half-life ($T_{\frac{1}{2}}^{\beta}$) was 22.0 ± 1.7 min, and the steady-state volume of distribution of the drug (V_{ss}) was 62.6 ± 15 ml/kg body weight. There was an almost immediate decrease in plasma copper, maximal at 17 per cent by 3 hr; by 24 hr plasma copper had returned to normal. Plasma zinc exhibited a transient decrease at 1 hr and had returned to normal by 2 hr. There was a significant decrease in plasma Zn at 24 hr. There was a transient decrease in plasma unionized calcium, from 6.3 mg/dl to 5.4 mg/dl by 5 min, which had returned to normal by 30 min (results not shown). L-Alanosine had little effect upon the 24-hr urinary excretion of copper. Before treatment it was 14.0 ± 1.9 μg/kg and after the drug 16.7 ± 3.9 μg/kg (± S.E.M., N = 3). The 24-hr urinary excretion of zinc was increased from 8.8 ± 0.9 to 37.0 ± 2.7 μg/kg. The relatively small effect of L-alanosine upon urinary copper excretion was confirmed in mice using higher doses of L-alanosine. With an intraperitoneal dose of 1000 mg L-alanosine/kg body weight the 24-hr urinary excretion of copper increased from 22.9 μg/kg in controls to 60 μg/kg.

In the patients studied, L-alanosine was administered in 0·9% sodium chloride (10 mg L-alanosine/ml) at 1000 mg/m² per hour for 3 or 4 hr (a total dose of 3000 mg/m² and 4000 mg/²m respectively). Serum copper was not significantly decreased after a 3-hr infusion but showed a significant decrease (30 per cent) after 4-hr infusion (Table 3). Plasma copper concentrations 24 hr after the infusion were similar to pretreatment concentrations. Plasma zinc concentrations were relatively unaffected by L-alanosine administration. Total serum calcium was not significantly decreased after the 4-hr infusion: control (± S.E.M., N = 3), 9.6 ± 0.8 mg/kl; after L-alanosine, 8.4 ± 0.7 mg/dl ($P > 0.05$, paired Student's *t*-test). The 24-hr urinary excretion of copper was not altered by L-alanosine, but there was over a 2-fold increase in the 24-hr urinary excretion of zinc. The 24-hr urinary excretion of calcium was not affected by L-alanosine (results not shown). Two patients with normal renal function prior to treatment received L-alanosine as a 5-hr intravenous infusion at 1000 mg/m² per hr. Both of these patients developed acute renal failure. Urine collected before the

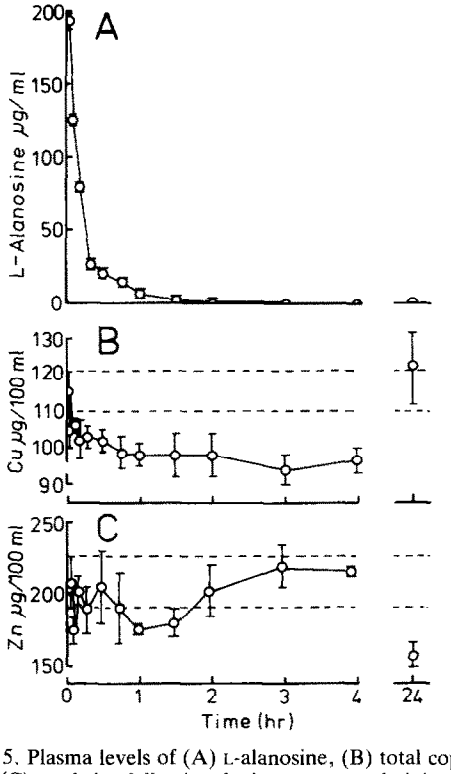

Fig. 5. Plasma levels of (A) L-alanosine, (B) total copper, and (C) total zinc following the intravenous administration of L-alanosine at a dose of 30 mg/kg to three rabbits. Bars are the S.E. of the means. The dotted lines show the ranges of the control values for plasma copper and zinc.

Table 3. Urinary excretion and plasma copper and zinc in patients receiving L-alanosine*

L-Alanosine (mg/m^2)	Metal	Plasma concentration			Urinary excretion	
		Before (μg/100 ml)	End of infusion (μg/100 ml)	24 hr (μg/100 ml)	Before (μg/24 hr)	After (μg/24 hr)
3000	Cu(II)	128 ± 13	115 ± 18	133 ± 26	31 ± 5	52 ± 30
	Zn(II)	55 ± 7	57 ± 3	65 ± 4	982 ± 560	2770 ± 738†
4000	Cu(II)	127 ± 15	89 ± 21†	100 ± 28		60 ± 11
	Zn(II)	64 ± 4	64 ± 6	70 ± 4†		2410 ± 110‡

* Patients received L-alanosine as an intravenous infusion at a rate of 1000 mg/m^2 per hr. Total plasma Cu and Zn were measured immediately prior to the infusion, at the end of the infusion, and 24 hr after commencing the infusion. Urinary excretion of Cu and Zn was measured over a 24-hr period prior to giving L-alanosine and for 24 hr after commencing infusion of the drug. Values are the mean (± S.E.M.) of three patients at each dose level.

† $P < 0.05$ by Student's paired t-test.

‡Control values for uninary Cu and Zn excretion were not obtained in these patients; values were therefore compared by Student's non-paired t-test to control values in the other group of patients, $P < 0.05$.

patients became anuric showed no change in the levels of copper but a large increase in the concentration of zinc as seen in patients treated at the lower dose of L-alanosine. None of the patients treated with the lower doses of L-alanosine showed impairment of renal function.

When purified human ceruloplasmin at 1 mg/ml, a concentration similar to that in plasma, was dialyzed for 20 hr against 40 vol. of L-alanosine (140 μg/ml in 5 mM sodium phosphate buffer, pH 7.4) there was an 11 per cent decrease in copper content [from 2.34 ± 0.04 μg/mg protein to 2.09 ± 0.08 μg/mg protein ($N = 5$, ± S.E.M., $P < 0.05$)].

DISCUSSION

The precise nature of the complex formed between copper and L-alanosine is unknown. It could take the form of pure chelate, an inner complex salt (a chelate in which both ionic and chelate bonds are present), or even a simple salt through the hydroxyls of the carboxyl and hydroxynitrosamino groups of L-alanosine. Because of the close association between copper and L-alanosine, we have chosen to regard the complex as a chelate or inner complex salt in which dissociation of the copper from alanosine is retarded when compared to the sodium salt. Dreiding molecular models were used to study possible configurations of an L-alanosine copper chelate. Under no circumstances could L-alanosine be contorted into a position where it could act as a tetradentate ligand, although mono-, di-, and tri-dentate complexes could readily made, depending upon the geometry chosen for the complex. Based on molecular models, it would appear that the interaction of alanosine with copper is through the hydroxyls of the hydroxynitrosamino and carboxyl groups and, in addition, could involve a coordinate bond formed through the nitrogen of the amino group. Depending upon whether two or three binding sites are involved, the L-alanosine copper complex could accommodate a square, distorted octahedral, or perhaps tetrahedral geometry. Square pyramidal geometry, which is known to occur with the copper complex of some amino acids [21, 22], is also possible. These studies provide no evidence to suggest which of these might be the preferred geometry.

The observation that several classes of cystostatic agents are able to chelate metal ions [23] has prompted interest in metal chelates and chelating agents as potential antineoplastic agents. An important group of chelating agents with antitumor activity is the bis(thiosemicarbazones), first reported by French and Freedlander [24]. The extensive studies of Petering and Petering [9] have established that the action of the bis(thiosemicarbazones) is due to the formation of Cu(II) chelates *in vivo*. The compound H_2KTS (3-ethoxy-2-oxobutyraldehyde-bis-thiosemicarbazone) was found to be devoid of antitumor activity when administered to animals fed a semi-purified diet low in copper. When copper was added to the drinking water at doses that had no effect upon tumor growth, H_2KTS inhibited tumor growth in proportion to the amount of copper in the drinking water. In some studies, H_2KTS had no antitumor activity in animals fed a normal diet and was active only when administered with Cu(II) [25]. The antitumor activity of Cu(II)KTS is probably due to the lipophilicity of the complex that facilitates the transport of Cu(II) into the cell [26]. Within the cell, dissociation of the chelate produces Cu(II) poisoning of DNA synthesis and oxidative phosphorylation [18, 26].

L-Alanosine forms a strong 1:1 association with Cu(II). We found no evidence, however, that copper was involved in the antitumor activity of L-alanosine. There were no differences in the antitumor effects of L-alanosine against leukemia P-388 in control, copper-deficient, and copper-loaded mice, and the copper complex of L-alanosine had no appreciable antitumor activity. The acute toxicity of the copper complex of L-alanosine, measured as the LD_{50}, is similar to that of the free cupric ion. This suggests that the complex may be broken down *in vivo*. Free L-alanosine is metabolized mainly by deamination [27], which would be expected to reduce the ability to bind Cu(II). The available deaminated metabolites were insufficient to conduct binding studies. L-Alanosine administration had little effect upon the urinary excretion of copper, which is not surprising

since little free L-alanosine is excreted in the urine. In rabbit less than 16 per cent and in man less than 6 per cent of the dose of L-alanosine is excreted in the urine in 24 hr ([14], whereas deaminated metabolites account for almost 60 per cent of the dose (G. Powis, M. M. Ames and J. S. Kovach, unpublished observations). The decrease in plasma copper concentrations following L-alanosine administration in rabbit and man is consistent with the binding of copper by the parent drug with redistribution of copper into tissues, and the reappearance of copper in plasma as L-alanosine is metabolized. Redistribution of metal ions, including copper and zinc, is one of several mechanisms currently being considered as the cause of the acute renal failure seen in two patients receiving 5000 mg/m^2 of L-alanosine.

Over ninety five per cent of the copper in plasma is bound in the relatively inert metalloprotein ceruloplasmin that is not in equilibrium with exchangeable copper bound to serum alumin [28]. The magnitude of the decrease in plasma copper produced by L-alanosine (17 per cent in the rabbit and up to 30 per cent in man), is greater than can be accounted for by exchangeable copper, suggesting that some copper was removed from ceruloplasmin. Dialysis experiments demonstrated that, at concentrations approximating those found under physiological conditions, L-alanosine could remove copper bound to ceruloplasmin. *In vitro* studies have shown that copper chelating agents can remove copper from ceruloplasmin in proportion to their copper-binding abilities, the most efficient compound being triethylenetetramine [29]. The stability constant of Cu(II) for triethylenetetramine is 20.6 [11]. Administration of triethylenetetramine to man does not produce an immediate decrease in serum copper but does induce cupruresis [30]. D-Penicillamine, a drug used in the treatment of Wilson's disease [30], produces a much larger cupruresis that does triethylenetetramine. D-Penicillamine does not remove copper from ceruloplasmin [29] and, in man, it has no acute effect on plasma copper levels [30]. L-Alanosine appears to be unusual among copper complexing agents in causing an immediate decrease in plasma copper with little increase in urinary copper excretion. The reason for the increased urinary zinc excretion is not known. Although L-alanosine will bind both copper and zinc, the copper complex of L-alanosine appears to play no part in antitumor activity.

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