Binding of Copper(II) and Other Metal Ions by Lysinoalanine and Related Compounds and Its Significance for Food Safety

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Acid–base equilibrium constants for the five ionizable groups and metal ion (Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}) binding constants of N^{ϵ}-(2-amino-2-carboxyethyl)-L-lysine (lysinoalanine, LAL) have been determined at 25 °C and 0.16 M ionic strength by potentiometric titration. Less extensive data are reported for the related compounds DL-2,3-diaminopropanoic acid (DAPA), 3-[(2-phenylethyl)amino]-DL-alanine (PEAA), and L-lanthionine (LAN), three other unnatural amino acids also formed during food processing. These unnatural amino acids are sufficiently strong chelators to influence copper transport by histidine in vivo at plasma levels of 49 μM LAL, 23 μM DAPA, 243 μM PEAA, and 511 μM LAN. Relatively high concentrations of these compounds are calculated to be necessary for competitive binding of essential zinc ions and inactivation of carboxypeptidase A and other enzymes. Possible mechanisms for kidney damage by these dehydroalanine-derived copper chelators are discussed.

INTRODUCTION

Lysinoalanine (LAL), which is present in many processed foods (Antila et al., 1987; Hasegawa et al., 1987; Maga, 1984), may be produced when protein-containing foods are heated, especially at alkaline pH. It has been postulated that highly reactive dehydroalanine (DHA) is formed by hydroxide ion induced β-elimination reactions involving cysteine, serine, and threonine residues in the protein. The DHA then reacts with a nearby lysine residue, forming LAL and a protein cross-link. The protein-bound LAL is presumed to be an equimolar mixture of L-lysino-L-alanine and L-lysino-D-alanine (LL-LAL and LD-LAL, respectively) (Finot, 1983; Friedman et al., 1984; Maga, 1984).

LAL formation in foods results in a loss of the essential amino acid lysine and of methionine-sparing cysteine. LAL formation also reduces digestibility because of cross-linking. However, the chief concern regarding LAL is the effect on the kidney found when this abnormal amino acid is fed to rats. LD-LAL in the free form, and to a lesser extent in the protein-bound form, can cause enlargement of the nuclei and cytoplasm of the proximal renal tubular cells (nephrocytomegaly). The other stereoisomers of LAL have much less effect (Feron et al., 1977; Karayiannis et al., 1979). LAL absorbed by the rat is eliminated in the urine both as N-acetylated derivatives and more slowly as free LAL. Free LAL accumulates in the part of the kidney containing the proximal tubules (Finot et al., 1977; Struthers et al., 1980). LAL is probably acetylated in the kidney. A metabolite of LAL, which may be due to the action of L-amino acid oxidase in the presence of catalase, has been reported in rat urine (Leegwater and Tas, 1980). Moreover, a recent report that extracts from human kidneys are less effective in metabolizing LAL than corresponding extracts from several animal species implies that human kidney cells may be more susceptible to LAL than those from rats or other animals tested (Kawamura and Hayashi, 1987).

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[1]Tenure as a Foreign Research Associate at WRRC during 1986/1987 was supported by the New Zealand Dairy Research Institute, Palmerston North, New Zealand, which is his current address.

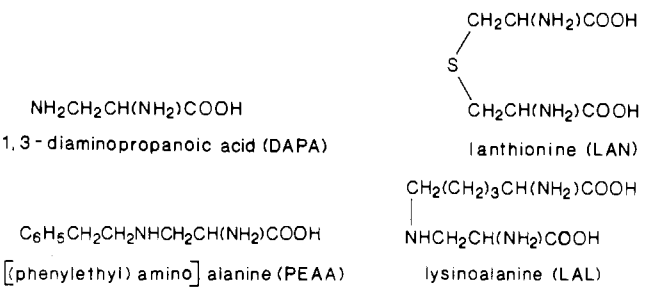
The mechanism of the effect of LAL on the kidney is unknown, but it has been suggested that LAL may act as a metal ion chelator (Friedman, 1977). The healthy kidney is rich in copper and zinc and contains appreciable amounts of the metal-binding protein metallothionein (Bremner et al., 1981; Brady and Webb, 1981). The metallothionein is located within the tubular cells and extracellularly in the tubules of the rat kidney (Danielson et al., 1982). The protein-bound copper is in the cuprous state [Cu(I)] and is readily oxidized to the cupric state [Cu(II)] (Geller and Winge, 1982; Hartmann et al., 1984). Oxidation of copper(I) thionein facilitates removal of the copper by chelating agents such as ethylenediaminetetraacetic acid (EDTA) and apoenzymes. LAL has been shown to chelate copper(II), cobalt(II), and zinc(II) ions and to inactivate metalloenzymes. Its structure is expected to allow it to act as a polydentate ligand (Hayashi, 1982; Friedman et al., 1985, 1986).

Because of its reactive nature, DHA is expected to react with other protein amino acid residues in addition to lysine and with other food components such as biogenic amines. The extent of these competing reactions depends in part on the concentrations of the potential reactants and their stereochemical accessibility (Friedman, 1977). The stability of products so formed will also affect their concentration in foods.

The reaction product of DHA and ammonia, DL-2,3-diaminopropanoic acid (DAPA), is a well-known and much-studied bidentate metal ion chelator. The D stereoisomer of this compound damages the rat kidney in apparently the same way as LD-LAL (Kaltenbach et al., 1982). Again, the mechanism is unknown. DAPA has been found in food (Fritsch and Klostermeyer, 1981) and has been produced in small amounts by heating alkaline solutions of pure proteins in the laboratory (Nashef et al., 1977; Asquith and Otterburn, 1977). Maillard browning products also induce nephrocytomegaly in rat kidneys (von Wagenheim et al., 1984; Finot and Furniss, 1986).

Other compounds that occur in foods and are expected to have metal-binding properties include lanthionine (LAN), the reaction product of DHA and cysteine (Asquith and Otterburn, 1977; Watanabe and Klostermeyer, 1977; Scharf and Weder, 1983), and 3-[(2-phenylethyl)amino]-DL-alanine (PEAA), formed by reaction of DHA with phenylethylamine (Jones et al., 1981, 1987; Tucker et al., 1983; Rivett et al., 1983; Friedman and Noma, 1986; Friedman et al., 1986).

The chemical structures of the DHA derivatives are as follows:



L-Serine residues in proteins racemize faster to the D isomer under the influence of alkali and heat than any other amino acid (Friedman and Liardon, 1985). Free D-serine causes damage to the rat kidney in much the same way as the other compounds mentioned above (Kaltenbach et al., 1982). The rat appears to be uniquely sensitive to kidney damage by D-serine, D-DAPA, PEAA, and LD-LAL.

The present work, involving pH titrations and spectrophotometric studies of the test substances in the absence and presence of various metal ions, was undertaken to obtain information on the acid–base equilibria and metal ion binding properties of LAL and related compounds. Such information may aid in the discovery of the mechanism of LAL-related (or induced) kidney damage and the possible effects of LAL and related compounds in animal and human nutrition.

EXPERIMENTAL SECTION

Reagents. Water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) and had a conductivity of less than 0.2 μS/cm. Lysinoalanine and 2,3-diaminopropanoic acid were obtained from Sigma Chemical Co., St Louis, MO. L-Lanthionine was obtained from Serva, Heidelberg, GFR. The [(phenylethyl)-amino]alanine was the same preparation as used in previous studies (Friedman et al., 1986). All other reagents were of analytical reagent grade.

pH Titrations. Potentiometric titrations were carried out in a water-jacketed vessel at 25.0 °C with constant stirring under a blanket of moist nitrogen. Carbonate-free potassium hydroxide solution (typically 0.25 M) was added to the titration mixture from a micrometer-driven glass syringe fitted with a fine-bore stainless steel needle (Gauge 22S). A Radiometer pH meter (Type PHM84) fitted with a Schott type H combined glass/3.5 M KCl Thalamid reference electrode was used to measure the hydrogen ion concentration. The meter and electrodes were calibrated by titrating 5 mM HCl with standard KOH solution. The negative logarithm of the ionization constant for water at 25 °C in 0.16 M KCl solution was assumed to be 13.767 (Harned and Owen, 1958). Potassium hydrogen phthalate was used as the primary acidimetry standard. Gran plots (Gran, 1952) were used to determine equivalence points. The titration mixture (2 or 4 mL) consisted of the test ligand (1–5 mM), the test metal ion (0 or 1–10 mM), and sufficient KCl to bring the ionic strength to 0.16 M at the beginning of the titration. The titration data were processed with the computer program SUPERQUAD (Gans et al., 1985) modified to run on a personal computer.

SUPERQUAD is a Fortran program of about 2000 statements. In the executable or compiled form it occupies 210 000 bytes and is conveniently stored on a 5.25-in. diskette. This compiled form can be run on a personal computer without the need for additional software. SUPERQUAD allows two, three, or four reactants (e.g., copper, proton, LD-LAL, and LL-LAL) and evaluation of up to 18 formation constants. The concentrations of the reactants can, if necessary, be included among the parameters to be refined. Preliminary estimates must be obtained for all formation constants; these are then refined iteratively.

Acidity and Stability Constants for Metal Complexes. In the present work, the acidities of various ligands and metal complexes are reported as the negative logarithms of stepwise dissociation constants. Thus, for the reaction

$$M_pH_qL_r \rightleftharpoons M_pH_{q-1}L_r + H$$

$$K = [M_pH_{q-1}L_r][H]/[M_pH_qL_r] \quad (1)$$

where M stands for the metal ion, H the proton, and L the fully deprotonated ligand. Charges are omitted for ease of notation. Brackets denote concentrations. All results are for 25 °C and an ionic strength of 0.16 M with KCl as the supporting electrolyte.

The stabilities of metal ion complexes are reported as the logarithms of overall formation constants. Thus, for the reaction

$$pM + qH + rL \rightleftharpoons M_pH_qL_r$$

$$\beta_{pqr} = [M_pH_qL_r]/[M]^p/[H]^q/[L]^r \quad (2)$$

Optical Rotations. The optical rotations of solutions of LAL in 2 M HCl were measured on a Perkin-Elmer 241 polarimeter fitted with a 10-cm path length, thermostated (25 °C), water-jacketed cuvette having a capacity of 1 mL. The wavelengths available were 589, 578, 546, 436, and 365 nm.

Absorbance Measurements. Electronic absorbance spectra were obtained and spectrophotometric titrations were carried out on a Cary Model 14 double-beam recording spectrophotometer and a matching pair of quartz cuvettes (generally 1-cm path length) at ambient temperature.

RESULTS AND DISCUSSION

Stereoisomeric Purity of Lysinoalanine. Chemical synthesis of LAL from L-lysine is expected to produce an equimolar mixture of the LD-LAL and LL-LAL diastereomers. Okuda and Zahn (1965) synthesized LAL from a L-lysine derivative and reported a specific rotation of +15°, which corresponds to a molecular rotation of +35°, assuming a molecular mass of 233 Da. In the present work, molecular rotations of +36° and +37° were obtained for LAL and L-lysine, respectively (589 nm, 25 °C, 2 M HCl, and concentrations of 1.2% and 2.0%, respectively). The optical rotatory dispersion results for the LAL are conveniently summarized by the single-term Drude equation

$$\phi = [1074 \pm 8] \times 10^4/(\lambda^2 - [233 \pm 1]^2) \quad (3)$$

where ϕ is the molecular rotation and λ the wavelength (nm). The corresponding equation found for L-lysine was

$$\phi = [1104 \pm 2] \times 10^4/(\lambda^2 - [229 \pm 1]^2) \quad (4)$$

Tas and Kleipool (1976), who isolated the four stereoisomers of LAL using the difference in solubility of the diastereomers, reported molecular rotations of −14.8° and +100.7° for LD-LAL and LL-LAL, respectively. The calculated molecular rotation of an equimolar mixture of the diastereomers with their values is +43°, while for a 56:44 molar ratio the calculated molar rotation is +36°. In the current work, an equimolar mixture has been assumed and no attempt was made to resolve the diastereomers.

Stoichiometry of the Complexes by the Spectrophotometric Titration Method. Spectrophotometric

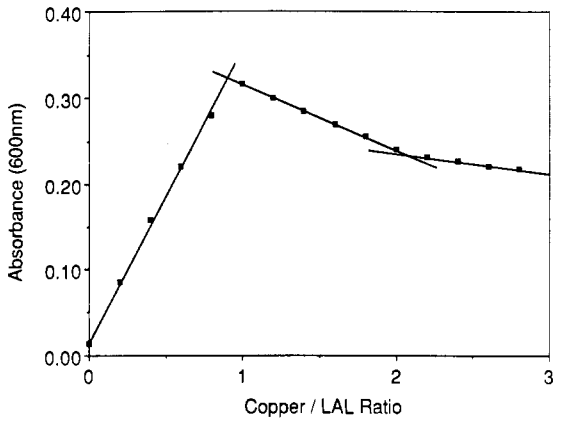


Figure 1. Spectrophotometric titration of 5.0 mM lysinoalanine with 100 mM $CuCl_2$ solution at pH 6.0 and 0.16 M ionic strength (wavelength, 600 nm; path length, 1 cm).

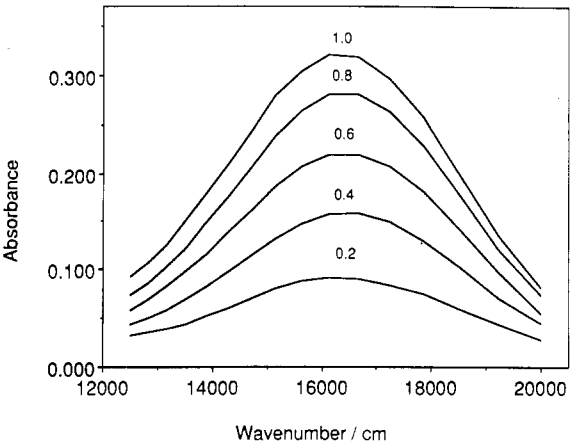


Figure 2. Absorbance spectra for copper(II)/lysinoalanine (LAL) mixtures. The Cu:LAL ratios are as marked on the individual curves. Other details are as in Figure 1.

titrations of solutions of LAL with copper(II) ions were carried out at various pH values to determine the stoichiometry of the complexes formed. The buffer, 2-(*N*-morpholino)ethanesulfonic acid (MES), which is expected to form only weak complexes with copper(II) ions, was used to maintain pH 6.0. In another experiment, borax was used to maintain pH 9.0 [cf. Hayashi (1982)]. Titrations were also carried out at pH 3.7 and 5.0 in the absence of buffers, by adding KOH as required to maintain constant pH during the titration with copper(II) ions.

At pH 6.2, the titration curve showed a sharp break in slope at a Cu:LAL ratio of about 1.0 and a second break at about Cu:LAL = 2.0 (Figure 1). At pH 5.0 and 9.0, only a single break at Cu:LAL = 1.0 was observed. In the solution containing borax, precipitation occurred as soon as Cu:LAL exceeded 1.1. At low pH (3.7) a curve lacking clear breaks was obtained.

The LAL consisted of an (almost) equimolar mixture of the LL-LAL and LD-LAL diastereomers. These data indicate that both diastereomers can chelate copper to form 1:1 complexes. The complexes are sufficiently strong over the range pH 5–9 to bind essentially all of the copper ions.

The visible absorbance spectrum of copper(II) ions is sensitive to the nature of the coordinating ligand donor atoms. Replacing water molecules by ligands containing nitrogen or oxygen donor atoms shifts the absorbance maximum to shorter wavelengths. Each coordinating carboxylate oxygen and amine nitrogen causes a characteristic and cumulative shift in the wavenumber of maximum absorbance (Billo, 1974; Sigel and Martin, 1982).

LAL potentially contains two coordinating carboxylate groups and three amine nitrogens. When LAL was present in excess during the early part of the spectrophotometric titration with copper(II) at pH 6.0, the wavelength of maximum absorbance (610 nm) corresponded to chelation involving two carboxylate and two amino groups. Neither the wavelength of the maximum absorbance, nor the shape of the absorbance band changed as the amount of copper increased up to a Cu:LAL ratio of 1.0 (Figure 2). This suggests that both diastereomers of LAL were chelating the copper in a similar way. This is contrary to the suggestion by Hayashi (1982), based on a comparison of molecular models, that only the LD diastereomer was likely to form tetradentate complexes with copper. At pH 9.0, when no protonated complexes of copper are present (Figure 5), the wavelength of maximum absorbance was 590 nm. This suggests that the third amino group of LAL may also be involved in coordination to copper.

As Cu:LAL ratios increased above 1.0, there was a progressive increase in the wavelength of maximum absorbance and an increase in the width of the absorbance band. This suggests a decrease in the ligand field strength and the presence of more than one colored species. For Cu:LAL ratios between 1.2 and 2.2, a single isosbestic point was observed at a wavelength of 645 nm. Resolution of overlapping bands was not possible, but it seems likely that an LAL molecule can bind more than one copper ion, possibly by acting as a double bidentate ligand. Polynuclear complexes are also possible.

Table I. Logarithms of Formation Constants for Various Lysinoalanine Metal Ion Complexes at 25 °C and 0.16 M Ionic Strength[a]

metal	β_{101}	β_{111}	β_{121}
copper(II)	15.27 ± 0.01	20.83 ± 0.01	25.40 ± 0.01
nickel(II)	10.56 ± 0.01	17.52 ± 0.01	23.33 ± 0.01
mercury(II)	8.54 ± 0.01	16.14 ± 0.01	21.62 ± 0.05
zinc(II)	7.90 ± 0.01	15.61 ± 0.01	22.53 ± 0.01
cobalt(II)	7.56 ± 0.01	15.55 ± 0.01	22.31 ± 0.01
cadmium(II)	7.00 ± 0.02	15.06 ± 0.02	21.81 ± 0.05
iron(II)	6.46 ± 0.03	15.07 ± 0.02	22.15 ± 0.04
copper(I)	5.5 ± 0.2	14.5 ± 0.1	22.6 ± 0.1
manganese(II)	3.84 ± 0.04	12.8 ± 0.1	
calcium(II)	1.51 ± 0.04	11.32 ± 0.07	20.06 ± 0.09
β_{011} = 10.13 ± 0.01		β_{031} = 25.80 ± 0.015	
β_{021} = 19.21 ± 0.01		β_{041} = 28.01 ± 0.02	

[a] Subscripts denote the number of metal ions, protons, and LAL molecules in the complex, respectively. Standard deviations for the constants are given.

In the absence of air only copper(II) formed complexes with a color sufficiently intense for convenient spectrophotometric study at the concentrations used in the present work. In contrast, when LAL was titrated with cobalt(II) in the presence of atmospheric oxygen at pH 7.5 [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer], an apparently stable yellow color (λ = 407 nm, plus very strong ultraviolet bands) formed. This was presumably due to the formation of a cobalt(III)/LAL/dioxygen complex (Neiderhoffer et al., 1984). The titration curve showed a break in slope at a Co:LAL molar ratio of about 2:3. Titrations of LAL with cobalt(II) at pH 6.0 (MES buffer) resulted in only very weak absorbance indistinguishable from that for an aqueous solution of cobalt(II).

Acidity and Stability Constants for LAL and Its Metal Complexes. Table I contains a summary of the results for LAL derived from potentiometric titration curves, examples of which are shown in Figure 3. The standard deviation of the unweighted experimental points from the titration curves, calculated from the derived constants (β_{pqr}), was generally less than 0.5 mV (or 0.01

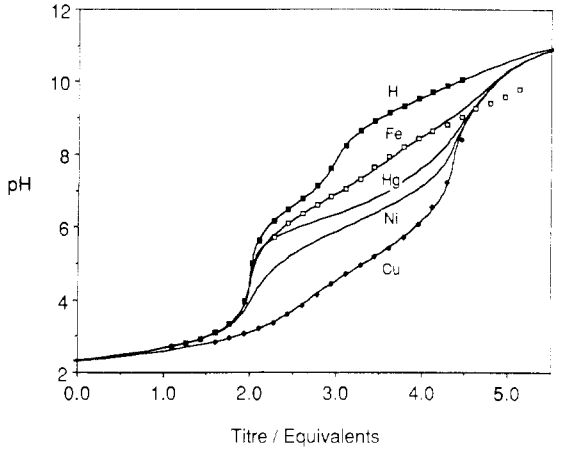


Figure 3. Potentiometric titration curves for lysinoalanine in the presence and absence of various divalent metal ions at 25 °C and 0.16 M ionic strength. The continuous lines have been calculated from the acidity and stability constants determined from the titrations and are given in Table I. Not all the experimental data points are shown to avoid clutter. Initial concentrations: lysinoalanine, 2.95 mM; metal ion, 2 mM.

pH unit). The β_{0q1} values correspond to macroscopic stepwise acidity constants of $pK_2 = 2.21$, $pK_3 = 6.59$, $pK_4 = 9.08$, and $pK_5 = 10.13$. Bohak (1964) reported corresponding values of 2.2, 6.5, 8.8, and 9.9 for an unspecified temperature and ionic strength. He also reported $pK_1 < 1.5$, estimated from a pH titration in 70% ethanol solution. No value for this constant could be obtained in the present work because of the low LAL concentrations used.

When LAL was titrated with KOH solution in the presence of a small excess of insoluble cuprous iodide crystals, the titration curve was displaced relative to that obtained in the absence of the cuprous iodide. This displacement indicates the formation of complexes. The formation constants obtained from the titration data by SUPERQUAD had rather large standard deviations (0.13–0.23 log unit) as did the cuprous ion concentration (11%). [The latter quantity was included as one of the parameters to be refined by SUPERQUAD.] The amount of copper in solution probably varied during the titration from initially negligible to a maximum at the end of the titration. The calculated Cu:LAL ratio was 0.65. Consequently the formation constants obtained can only give a preliminary indication of the strength of the complexes.

The cuprous LAL complexes are very much weaker than the cupric LAL complexes (Table I), and at physiological pH, only the diprotonated, positively charged cuprous complex can exist in appreciable amounts. Relatively little work on the chelation of cuprous ions in aqueous solution has been reported in the literature (Martell and Smith, 1974, 1982), presumably in part because of the difficulty of studying the unstable colorless ion. However, in general the cuprous ion is bound much less strongly than the cupric ion. A notable exception is metallothionein (Geller and Winge, 1982).

The order of stability of the LAL metal ion complexes (Table I) is similar to the Irving-Williams series Cu > Ni > Co > Zn > Cd > Fe > Mn > Ca, with zinc and cobalt complexes having essentially the same stability, as does cadmium and iron. The electrically neutral CuLAL complex is comparable in strength with the copper(II) complex formed by the tetradentate ligand ethylenediamine-*N*,*N*′-diacetic acid (EDDA; $\log \beta_{101} = 15.9$; Martell and Smith, 1974, 1982). The other metal ions form weaker complexes with LAL than with EDDA (Figure 4). Cadmium is the only metal studied that lies off the otherwise smooth curve.

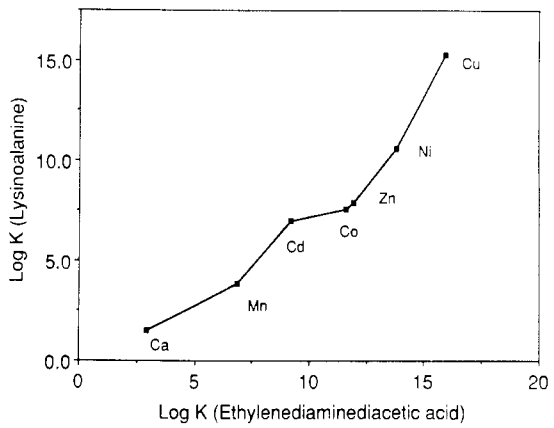


Figure 4. Comparison of the stability constants for lysinoalanine and ethylenediaminediacetic acid (EDDA) complexes of divalent metal ions. The data for EDDA are from Martell and Smith (1974, 1982), Gualtieri et al. (1979), and Ewin and Hill (1983).

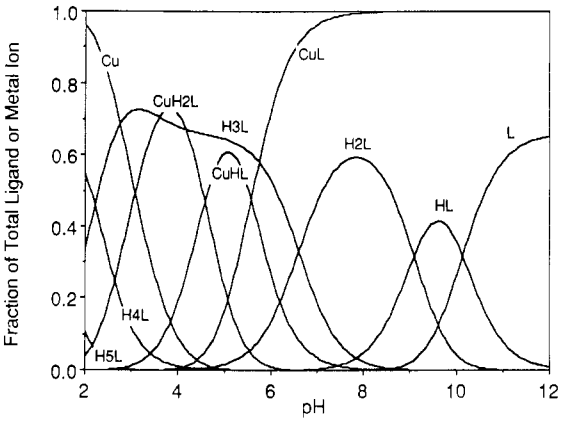


Figure 5. Relative proportions of the various species present in a mixture of 2.95 mM lysinoalanine and 1.0 mM copper(II) ions at 25 °C and 0.16 M ionic strength as a function of hydrogen ion concentration. Fully deprotonated lysinoalanine is denoted by L in the chemical formulas.

This plot can be used to predict LAL binding constants for other metal ions from known values for EDDA. For example, from the known value of the binding constant for $Pb^{II}EDDA$ ($\log \beta_{101} = 10.66$; Gualtieri et al., 1979), a value of $\log \beta_{101} = 6.9$ for $Pb^{II}LAL$ is predicted from Figure 4. Similarly, for $Cr^{II}EDDA$ ($\log \beta_{101} = 9.1$; Martell and Smith, 1982), Figure 4 predicts $\log \beta_{101} = 5.3$ for $Cr^{II}LAL$.

Figure 5 shows the relative concentrations of the different species present in a mixture of excess LAL and $CuCl_2$, as a function of pH, calculated from the acidity and stability constants reported in Table I. Analogous plots for Cu/DAPA, Cu/PEAA, Cu/EDTA, Co/LAL, and Cu/LAN are shown in Figures 6–10 for comparison. The protonated copper(II) complexes have acidity constants (negative logarithms) of $\log \beta_{121} - \log \beta_{111} = 4.57$, and $\log \beta_{111} - \log \beta_{101} = 5.56$. The major species at pH 7 are $CuLAL^0$ and H_2LAL^0, with a smaller amount of H_3LAL^+ and trace amounts of other species. The protonated complexes become progressively more important at neutral pH for successive metals in the Irving-Williams series. Thus, for cobalt(II), the dominant species in pH 7.4 is $CoHLAL^+$ ($pK_a = 7.99$). The very weak complex $CaLAL^0$ only becomes the dominant calcium species when pH exceeds 9.81.

Under the conditions of concentration and ionic strength used in the present work, the titration curves were adequately described by assuming that the two diastereomers of LAL behaved identically in their acidity and metal ion binding properties. Attempts to treat the LAL as an

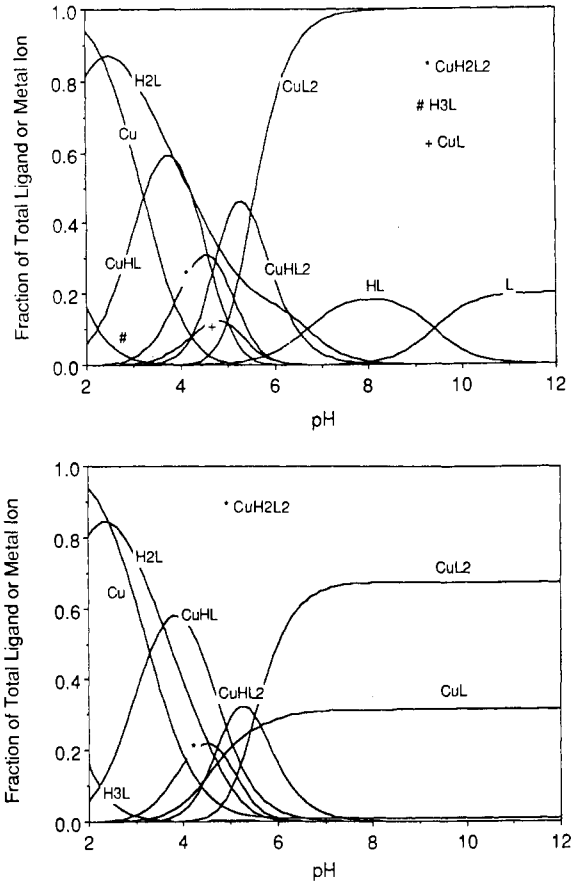


Figure 6. Relative proportions of the various species present in mixtures of copper(II) and 2.5 mM 2,3-diaminopropanoic acid at 25 °C and 0.16 M ionic strength: A (top), 1.0 mM $CuCl_2$; B (bottom), 1.5 mM $CuCl_2$. L denotes the fully deprotonated ligand.

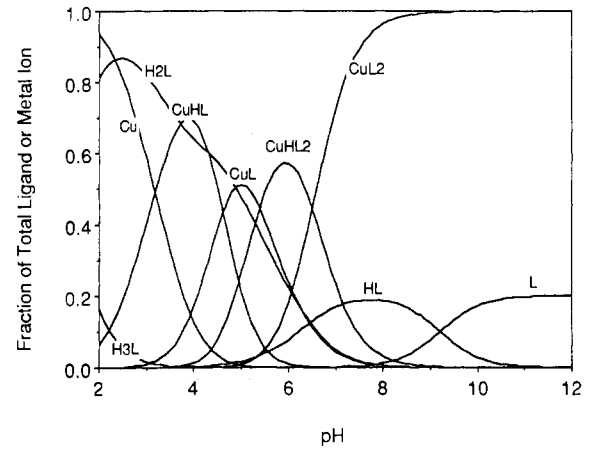


Figure 7. Relative proportions of the various species present in a mixture of 1.0 mM $CuCl_2$ and 2.5 mM [(phenylethyl)amino]-propanoic acid at 25 °C and 0.16 M ionic strength. L denotes the fully deprotonated ligand.

equimolar mixture of two distinct compounds resulted in mathematically ill-defined constants being obtained. The spectrophotometric titration data for copper also suggest that both LL-LAL and LD-LAL chelate similarly, contrary to predictions made with molecular models (Hayashi, 1982). The possibility that copper(II) and other metal ions might cause the inversion of LL-LAL was considered. However, prior exposure to copper(II) at neutral pH did not alter the molecular rotation of a sample of LAL from that expected for an equimolar mixture of the diasteromers (data not shown). Further work with the individual LAL stereoisomers may clarify the situation.

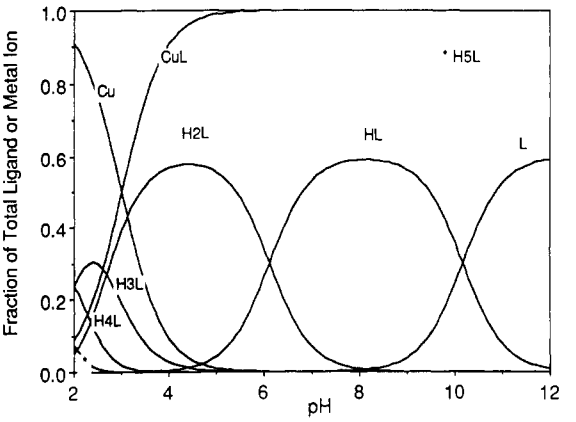


Figure 8. Relative proportions of the various species present in a mixture of 1.0 mM $CuCl_2$ and 2.5 mM ethylenediaminetetraacetic acid (EDTA) at 25 °C and 0.16 M ionic strength. L denotes the fully deprotonated ligand.

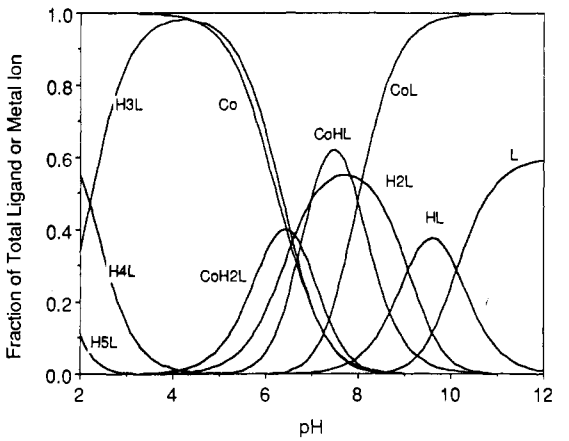


Figure 9. Relative proportions of the various species present in a mixture of 1.0 mM $CoCl_2$ and 2.5 mM lysinoalanine at 25 °C and 0.16 M ionic strength. L denotes the fully deprotonated ligand.

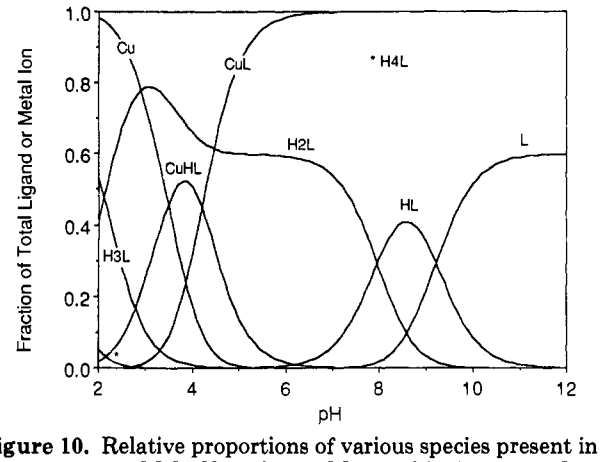


Figure 10. Relative proportions of various species present in a mixture of 1.0 mM $CuCl_2$ and 2.5 mM L-lanthionine at 25 °C and 0.16 M ionic strength. L denotes the fully deprotonated ligand.

Hydrolysis of the manganese, iron, and zinc LAL complexes was clearly apparent at alkaline pH, as evidenced by increased buffering and displacement of the titration curves. No attempt was made to determine either the stoichiometry or the formation constants for the hydrolyzed complexes, and the high-pH titration data were excluded from the calculations made with SUPERQUAD. Hydrolyzed complexes are not expected to be important under physiological conditions. As only a limited range of metal ion and ligand concentrations have been studied, the ex-

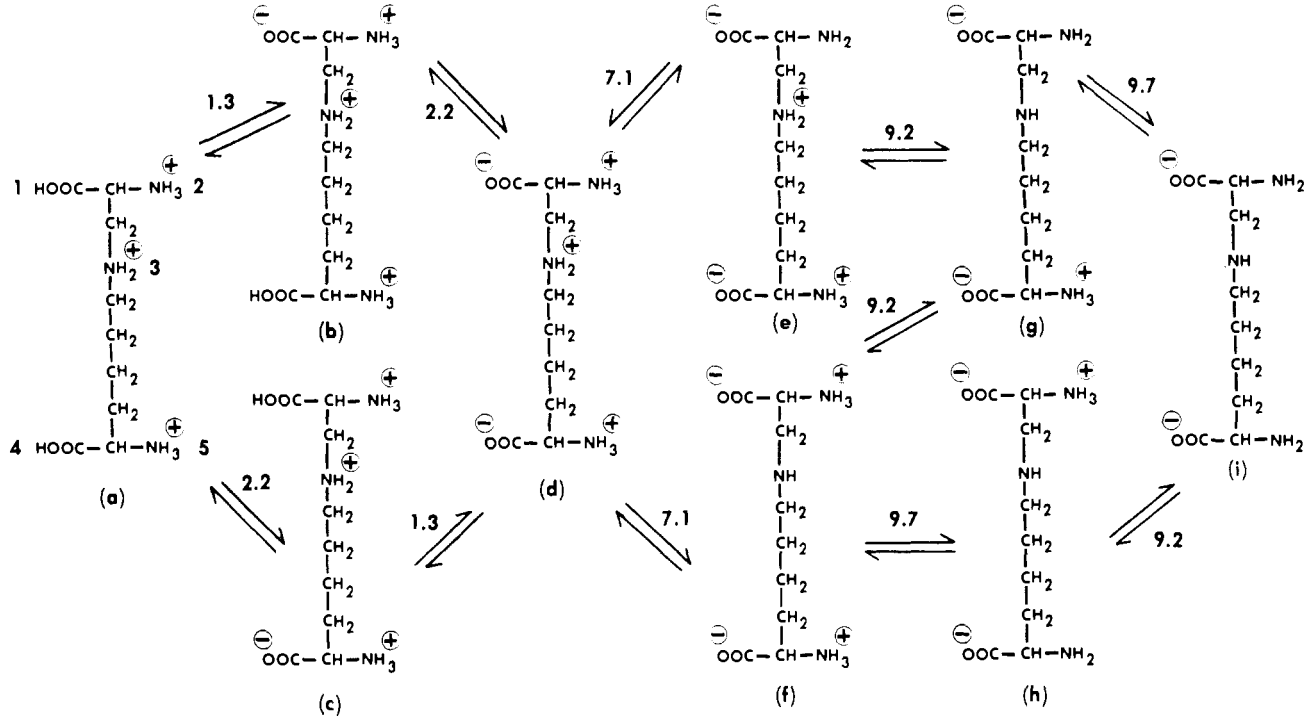


Figure 11. Proton ionization scheme for lysinoalanine. The chemical structure of lysinoalanine is given at the left together with a symbolic representation of the fully protonated molecule. The numbers in the figure are the negative logarithms of the microscopic acidity constants for the equilibria, as shown. The macroscopic acidity constants calculated from the microscopic constants are pK_1 = 1.16, pK_2 = 2.25, pK_3 = 6.8, pK_4 = 9.4, and pK_5 = 9.8. (See the Appendix for the derived relationship between the macroscopic and microscopic acidity constants.)

Table II. Acidity and Zinc and Copper Binding Constants for Various Bidentate Ligands at 25 °C and 0.16 M Ionic Strength[a]

ligand	diaminopropanoic acid[b]	[(phenylethyl)amino]alanine[c]	histidine[d]	L-lanthionine[e]	
		Proton			
pK_1	1.31	nd[f]	1.72	0.95	nd
pK_2	6.38	6.46 ± 0.01	6.05	2.05	2.12 ± 0.03[c]
pK_3	9.40	9.21 ± 0.01	9.11	8.17	7.95 ● 0.02[c]
pK_4	*g*	*g*	*g*	9.05	9.22 ± 0.01[c]
		Zinc(II)			
log β_{101}	6.38 ± 0.01[c]	5.65 ± 0.04	6.51	nd	6.72 ± 0.03[c]
log β_{111}	12.61 ± 0.01[c]	12.31 ± 0.04	*g*	nd	13.3 ± 0.01[c]
log β_{102}	11.48 ± 0.015[c]	9.7 ± 0.1	12.01		
		Copper(II)			
log β_{101}	10.56	10.59 ± 0.04	10.16	13.49	12.21 ± 0.01[c]
log β_{111}	15.57	15.20 ± 0.04	14.11	17.53	16.42 ± 0.01[c]
log β_{102}	19.82	17.4 ± 0.1	18.11	20.95	*g*
log β_{112}	25.33	23.9 ± 0.1	23.81	*g*	*g*
log β_{122}	30.08	*g*	27.2	24.48	*g*

[a] Standard deviations for the constants are given. [b] Martell and Smith, 1974, 1982. [c] This work. [d] Pettit, 1984. [e] Stapleton and Weber, 1971; Weber, 1971. [f] nd = not determined. [g] Complex not detected.

istence of complexes in addition to those listed in the table, and the hydrolyzed complexes mentioned above, cannot be excluded. However, as metal ion and LAL concentrations are likely to be low in vivo, it is unlikely that these other complexes will be important.

Assignment of Microscopic Acidity Constants to Specific Acidic Groups on Lysinoalanine. LAL has five distinct acidic groups (two carboxyls, one secondary and two primary amino groups). The ionization scheme is, therefore, characterized by 80 ($n \times 2^{n-1}$, n = 5) microscopic acidity constants (denoted by lower case k in the present work) (King, 1965). However, as these acidity constants vary greatly in size, only a few will be significant during pH titrations of LAL and only some of the 32 (2^n, n = 5) distinct ionic species will ever be present in more than trace amounts. In general, it is not possible to assign macroscopic acidity constants (denoted here by upper case K), such as obtained in the present work, to specific acidic groups. Some simplifying assumptions and analogy to model compounds allow estimates to be obtained for the more important microscopic acidity constants.

The ionization scheme for LAL deduced by a procedure given in the Appendix is shown in Figure 11, together with the macroscopic acidity constants calculated from the microscopic constants. The agreement with the experimental values is satisfactory considering the assumption necessary in the assignments. In principle, it would be possible to determine the microscopic acidity constants by NMR or other techniques to determine the concentrations of the individual species, as a function of pH [e.g. Sayer and Rabenstein (1976)]. At physiological pH, the two diprotonated LAL species shown in the figure will be present in approximately equal amounts. The relationships between macroscopic and microscopic acidity con-

stants for LAL are described in the Appendix.

Acidity and Metal Ion Binding Constants for DAPA, PEAA, and LAN. Literature data for DAPA and histidine and data obtained in the present work for DAPA and PEAA are summarized in Table II. The three compounds have rather similar acidity and metal ion binding constants. Substitution of the α-amino group of DAPA with a phenylethyl group is expected to increase the acidity of the amino group due to inductive effects. This is reflected in the smaller pK_2 and pK_3 values for PEAA relative to those for DAPA (Table II). The zinc and copper ion binding constants are also reduced, particularly in the bis complexes. Possibly, there is steric interference involving the bulky phenylethyl groups, or the benzene ring interacts with d orbitals on the metal ion. Since the metal ion complexes of PEAA and DAPA differ in hydrophobicity and size, they are expected to differ also in their biological effects.

In the case of L-lanthionine, there was only moderate agreement between the acidity constants obtained in the present work and those reported by Stapleton and Weber (1971; Table III). For copper binding constants, the agreement with the literature was unsatisfactory, with markedly lower values being obtained in the present case. Under the limited range of conditions studied (2.5 mM LAN, 1.0 and 2.0 mM $CuCl_2$), no bis complexes were detected.

Inactivation of Metalloenzymes by LAL. Both free LAL and proteins containing bound LAL have been reported to inactivate copper- and zinc-containing enzymes (Hayashi, 1982; Friedman et al., 1985, 1986). In some cases, the inactivation could be prevented by addition of excess copper and zinc ions. This suggested that LAL might be acting as a metal ion chelator by removing or binding to the metal ions essential for activity of the enzymes.

Bovine carboxypeptidase A (CPA) requires one zinc ion per molecule of protein for activity and, at 25 °C in 1 M NaCl solution, has a zinc binding constant given by $\log \beta_{11} = 2.5 + \text{pH}$ (Billo et al., 1978). The corresponding formation constant for $ZnHLAL^+$, the predominant LAL complex of zinc at physiological pH, is $\log K_{ZnHLAL} = \log \beta_{111} - \log \beta_{011} = 5.48$ (Table I). The enzyme binds zinc more strongly than LAL over the range pH 6–8. It can be shown by calculation that an LAL concentration of about 400 mM is required to inactivate 50% of the enzyme in a 1 μM solution of CPA, by the process of lowering the free metal ion concentration and thus causing the zinc to dissociate from the enzyme. (See the Appendix.) In contrast, EDTA is a much stronger zinc chelator at neutral pH ($\log \beta_{101} = 16.4$, $\log \beta_{011} = 10.17$, $\log \beta_{021} = 16.28$; Martell and Smith, 1982) and readily takes up zinc ions released by the enzyme. DAPA is expected to inactivate 50% of the enzyme in a 1 μM CPA solution, by chelating free zinc ions, at a concentration of about 13 mM. PEAA is a somewhat weaker zinc chelator than DAPA, and a concentration of about 65 mM is calculated to be necessary for 50% inactivation of 1 μM CPA. The corresponding concentration for LAN is 200 mM.

The dissociation of zinc from CPA is a slow process, first order in enzyme concentration, with a half-life of about 45 min at pH 7 (Billo et al., 1978). The inactivation of CPA by EDTA is also slow compared with the inactivation of the enzyme by LAL. The latter is substantially complete in about 5 min at a LAL concentration of 4 mM (Friedman et al., 1986). It is, therefore, likely that LAL reacts directly with the enzyme. Further studies are required to elucidate the mechanism. However, it has been found that commercially available LAL·2HCl is a sufficiently strong acid to cause acid inactivation of CPA in poorly buffered solutions such as used in the earlier studies (data not shown). Both 13 mM DAPA and 65 mM PEAA would be expected to be slow acting, by analogy to EDTA, if they chelate zinc dissociating from the enzyme. Additional studies are needed to verify these predictions.

Possible Influence of Various Ligands on Copper Transport and Retention in Vivo. Most of the labile copper in blood is bound to the serum albumin, while $CuHis_2$ is the major labile low molecular weight species (Mason, 1979). Ternary copper/histidine/serine and copper/histidine/threonine complexes also occur (Berthon et al., 1984). With use of the appropriate copper binding constants, it is possible to predict the equilibria in vivo between histidine and chelators such as LAL and DAPA originating from the ingestion of alkali-treated proteins. (See the Appendix.)

When more DAPA than copper is present, the dominant species at pH 7.4 are HDAPA and $CuDAPA_2$. For the equilibrium

$$CuHis_2 + 2HDAPA \rightleftharpoons CuDAPA_2 + 2HHis$$

$$\log K = \log \beta_{102}^{DAPA} - 2 \log \beta_{011}^{DAPA} + 2 \log \beta_{011}^{His} - \log \beta_{102}^{His} = +1.1 \text{ (Table II)} \quad (5)$$

Thus, when DAPA concentration exceeds 23 μM, the histidine (typically 85 μM in human blood serum) will be displaced as the dominant low molecular weight copper-chelating species. Similarly, concentrations of PEAA exceeding 243 μM are calculated to displace histidine as the chief low molecular weight copper carrier. For LAN the corresponding concentration is 511 μM.

The major LAL species in the presence of copper at pH 7.4 are H_2LAL and CuLAL (Figure 5). For the equilibrium

$$CuHis_2 + H_2LAL \rightleftharpoons CuLAL + 2HHis$$

$$\log K = \log \beta_{101}^{LAL} - \log \beta_{021}^{LAL} + 2 \log \beta_{011}^{His} - \log \beta_{102}^{His} = -3.8 \text{ (Tables I and II)} \quad (6)$$

and concentrations of LAL exceeding about 49 μM are calculated to cause displacement of histidine as the major copper carrier in vivo. Lower concentrations of LAL will carry proportionally less copper. The above calculations of ligand concentrations are sensitive to small errors in log values and should be considered as indicative rather than exact.

Bile rather than urine is the method by which small excesses of copper are eliminated in healthy animals, and urine normally contains little copper (Mason, 1979). Thus, either $CuHis_2$ (the chief low molecular weight carrier of labile copper in the plasma) is not excreted by the glomerulus of the kidney, which is unlikely, or it is excreted but is reabsorbed in the proximal tubules, probably by specific mediated processes. Reabsorption probably occurs in the convoluted proximal tubules where the bulk of the amino acids are absorbed (Silbernagl, 1981). This part of the kidney is damaged when rats are fed high levels of copper (Haywood et al., 1985). $CuHis_2$, $CuDAPA_2$, $CuPEAA_2$, and CuLAL are electrically neutral species at physiological pH, but they differ in chemical character, size, and stereochemistry. Chelation of copper by DAPA, PEAA, LAL, and perhaps D-serine may interfere with the mechanism by which the rat kidney conserves copper because of the specificity of the amino acid transport system in the tubules. If this is the case, prolonged dietary exposure to these compounds would be expected to cause copper deficiency in animals with a marginal copper intake.

Possible Mechanism for Kidney Damage in the Rat. The following biochemical and cellular events in the straight portion of the proximal renal tubules may be responsible for the LAL-, DAPA-, PEAA-, and D-serine-induced kidney damage: (a) the presence of high-affinity L-amino acid transport systems with the ability to transport D-amino acids in the absence of natural substrates (Kragh-Hansen et al., 1984); (b) high *lumen* concentrations of those solutes not absorbed in the preceding convoluted portion of the tubules, due to reabsorption of water (Hook, 1984); (c) accumulation of LAL within the epithelial cells (Finot et al., 1977; Struthers et al., 1980); (d) the presence of D-amino acid oxidases and catalases (Le Hir and Dubach, 1981); (e) high concentrations of copper(I) bound to metallothionein, especially in adult animals (Haywood et al., 1985; Brady and Webb, 1981; Bremner et al., 1981).

It is known that hydrogen peroxide produced by the action of amino acid oxidases can oxidize copper(I) thionein and thus could facilitate dissociation of copper(II) from metallothionein in vivo (Hartman et al., 1984; Geller and Winge, 1982). These considerations suggest the possibility that the damage observed in the proximal renal tubules in rats fed LAL, DAPA, PEAA, or D-serine arises from interaction of these compounds with copper(II) within the epithelial cells. LAL and DAPA might exert their effect in part by inactivating the metalloenzyme catalase and thus potentiate peroxide formation and the liberation of copper(II). If this is indeed the case, then it can be predicted that copper nutritional status, water intake, and urine volume would influence the incidence and severity of lesions and, in turn, these four compounds will alter copper retention and redistribution, and thus affect copper nutritional status.

It is also noteworthy that the amino acid penicillamine (D-dimethylcysteine), which is used to treat patients suffering from excess copper retention (Wilson's disease), does not remove copper from plasma (Laurie and Prime, 1979). It is likely therefore that both penicillamine and LAL interact with copper in the kidneys. Additional studies are needed to determine possible therapeutic value of LAL and related compounds for Wilson's and related diseases.

ACKNOWLEDGMENT

We are grateful for assistance with computing from the U.S. Naval Weapons Station, Concord, CA, and A. E. Pavlath, L. C. Whitehand, and other colleagues at WRRC.

APPENDIX

Relationship between the Macroscopic and Microscopic Acidity Constants for Lysinoalanine. The major species present in a solution of lysinoalanine at various pH values are shown in Figure 11. These species are labeled a–i. The microscopic acidity constants (i.e. the acidity constants for these individual species) are denoted by lower case k. Subscripts indicate the positions of the protons lost from a species, and the final subscript indicates the position of the proton undergoing dissociation. (The proton positions are labeled from the top left to bottom right on the chemical formula for fully protonated lysinoalanine at the left of Figure 11.) Upper case K values denote the macroscopic acidity constants (i.e. the constants for the overall proton dissociations, e.g. the pentahydrogen to tetrahydrogen dissociation, K_1). [a], [b], ..., are used to denote the concentrations of the respective species while [H] denotes the proton concentration. In the following derivations the conventional equations for the macroscopic acidity constants are expanded and written in terms of the microscopic constants.

$$K_1 = [H_4LAL][H]/[H_5LAL] = ([b] + [c])[H]/[a] = [b][H]/[a] + [c][H]/[a] = k_1 + k_4 = 10^{-1.3} + 10^{-2.2} = 10^{-1.25} \text{ (data from Figure 11)}$$

$$pK_1 = 1.25$$

$$1/K_2 = [H_4LAL]/[H][H_3LAL] = ([b] + [c])/[H][d] = [b]/[H][d] + [c]/[H][d] = 1/k_{14} + 1/k_{41} = 10^{+1.3} + 10^{+2.2} = 10^{+2.25}$$

$$pK_2 = 2.25$$

$$K_3 = [H_2LAL][H]/[H_3LAL] = ([e] + [f])[H]/[d] = [e][H]/[d] + [f][H]/[d] = k_{142} + k_{143} = 10^{-7.1} + 10^{-7.1} = 10^{-6.8}$$

$$pK_3 = 6.8$$

$$K_4 = [HLAL][H]/[H_2LAL] = ([g] + [h]) \times [H]/([e] + [f]) = ([g][H] + [h][H])/([e] + [f]) = ([f]k_{1342} + [f]k_{1345})/([g][H]/k_{1243} + [g][H]/k_{1342}) = [f](k_{1342} + k_{1345})/[(1/k_{1243} + 1/k_{1342})[f]k_{1342}] = (k_{1342} + k_{1345})/[(1/k_{1243} + 1/k_{1342})k_{1342}] = (10^{-9.2} + 10^{-9.7})/[(10^{+9.2} + 10^{+9.2}) \times 10^{-9.2}] = 10^{-9.4}$$

$$pK_4 = 9.4$$

$$1/K_5 = [HLAL]/[H][LAL] = ([g] + [h])/[H][i] = [g]/[H][i] + [h]/[H][i] = 1/k_{12345} + 1/k_{13452} = 10^{+9.7} + 10^{+9.2} = 10^{+9.8}$$

$$pK_5 = 9.8$$

Assignment of Microscopic Acidity Constants to Specific Acidic Groups on Lysinoalanine. When different acidic groups on a polybasic molecule differ greatly in acidity, the groups can be considered separately. Thus, the two carbonyl groups on LAL are very acidic in comparison with the amino groups, and their acidities can be related exclusively with pK_1 and pK_2 for LAL. The two ends of the LAL molecule are separated by a chain of four methylene groups and can, as an approximation, be considered independent. Inductive effects will be very weak, and intermolecular ionic interactions lessened by the ions from the salts present at 0.16 M. Under such circumstances, and by analogy to DAPA ($pK_1 = pk_1 = 1.3$; Table II) and alanine ($pK_1 = pk_1 = 2.2$; Martell and Smith, 1974, 1982), the carboxyl groups on the alanine and lysine moieties of LAL are assigned $pk = 1.3$ and $pk = 2.2$, respectively, for both fully protonated and tetraprotonated LAL. These values are the same as the macroscopic constants for LAL and reflect the essential independence of the ends of the LAL molecule from each other.

Similarly, by analogy to alanine ($pK_2 = pk_{12} = 9.7$), the primary amino group on the lysine moiety is assigned $pk = 9.7$. For the primary and secondary amino groups on the alanine moiety, the ^{13}C NMR literature values for the microscopic acidity constants for DAPA (Sayer and Rabenstein, 1976; Griffith et al., 1979) are applicable. The two amino groups have similar acidities, with $pk = 7.1$ and $pk = 9.2$ for the ionization states in which the second amino group is protonated and unprotonated, respectively. The ionization scheme deduced in this way is shown in Figure 11, together with the macroscopic acidity constants calculated from the microscopic constants. The agreement with the experimental values is satisfactory considering the assumptions necessary in the assignments. In principle, it would be possible to determine the microscopic acidity constants by NMR or other techniques to determine the concentrations of the individual species, as a function of pH [e.g. Sayer and Rabenstein (1976)]. At

physiological pH, the two diprotonated LAL species shown in the figure will be present in approximately equal amounts. The relationships between macroscopic and microscopic acidity constants for LAL are described above.

Calculation of Degree of Enzyme Inhibition by Metal Ion Chelators. Consider an equilibrium mixture of a metalloenzyme and a metal ion chelating ligand. The ligand is assumed to interact only with free metal ions and not with either the native or apoenzyme. The apoenzyme has no enzymic activity while the native enzyme is fully active.

The following equilibrium constants apply. For the chelator: $\beta_{011} = [HL]/[H][L]$; $\beta_{021} = [H_2L]/[H]^2[L]$; etc. $\beta_{101} = [ML]/[M][L]$; $\beta_{111} = [MHL]/[M][H][L]$; etc. $\beta_{102} = [ML_2]/[M][L]^2$; $\beta_{112} = [MHL_2]/[M][H][L]^2$; etc. For metal ion binding by the apoenzyme: $K_E = [ME]/[M][E]$, where ME denotes the native (active) enzyme and E the apoenzyme and the other symbols are as defined in the text of the paper. K_E may be either a constant or a known function of pH.

Mass balance of the enzyme requires

$$E_T = [E] + [ME] = [E](1 + K_E[M])$$

Solving for [ME]

$$[ME] = K_E[M][E] = K_E[M]E_T/(1 + K_E[M])$$

Mass balance of ligand requires

$$L_T = [L] + [HL] + [H_2L] + ... + [ML] + [MHL] + ... + 2[ML_2] + 2[MHL_2] + ... = [L][1 + \beta_{011}[H] + \beta_{021}[H]^2 + ... + [M](\beta_{101} + \beta_{111}[H] + ...) + 2[L][M](\beta_{102} + \beta_{112}[H] + ...)] = [L](1 + C + [M]B + 2[L][M]D) \quad (A)$$

where $C = \beta_{011}[H] + \beta_{021}[H]^2 + ...$, $B = \beta_{101} + \beta_{111}[H] + ...$, and $D = \beta_{102} + \beta_{112}[H] + ...$ Solving for [M]

$$[M] = (L_T/[L] - 1 - C)/(B + 2[L]D) \quad (B)$$

Differentiating with respect to [L]

$$d[M]/d[L] = M1 = -L_T/([L]^2Q1) - 2D(L_T/[L] - 1 - C)/Q1^2 \quad (C)$$

where $Q1 = B + 2[L]D$.

Mass balance of the metal ion requires

$$M_T = [M] + [ML] + [MHL] + ... + [ML_2] + [MHL_2] + ... + [ME] = [M][1 + [L]B + [L]^2D + K_EE_T/(1 + K_E[M])] \quad (D)$$

In many cases $M_T = E_T$, but this is not essential for the derivation.

Equation D can be solved for [L] by the Newton–Raphson method. Let

$$Y = [M][1 + [L]B + [L]^2D + K_EE_T/(1 + K_E[M])] - M_T$$

Substituting for [M], using eq B

$$Y = (L_T/[L] - 1 - C) \times [1 + Q + K_EE_T/(1 + K_E[M])]/Q1 - M_T$$

where $Q = [L]B + [L]^2D$. (Note that $Q1 = dQ/d[L]$.) Differentiating Y with respect to [L] gives

$$dY/d[L] = Y1 = M1[1 + Q + K_EE_T/(1 + K_E[M])] - [M][1 + Q1 - K_E^2E_T/(1 + K_E[M])^2M1]$$

An initial estimate of [L], denoted by $[L]_1$, is calculated from eq A assuming [M] = 0: $[L]_1 = L_T/(1 + C)$. Subsequent values of [L] are calculated from $[L]_{I+1} = [L]_I - Y/Y1$. When the change in [L] becomes sufficiently small, the iteration is terminated. The degree of enzyme inactivation, caused by the competitive binding of the essential metal ion by the ligand, is calculated from $I = (E_T - [ME])/E_T$. A simple computer program has been written in BASIC to carry out the above calculations. The program contains safeguards against nonconvergence of the iteration.

Effect of Lysinoalanine on Copper Transport in Vivo. From a consideration of the acidity and metal ion binding constants given in Tables I and II (and from plots such as Figure 5) it is apparent that at pH 7.4 the major species present in a mixture of copper(II) and excess lysinoalanine and histidine are H_2LAL^0, $CuLAL^0$, $HHis^0$, and $CuHis_2^0$. The concentrations of these four species are determined by the equilibrium reaction

$$CuHis_2 + H_2LAL \rightleftharpoons CuLAL + 2HHis$$

$$K = \frac{[CuLAL][HHis]^2}{[CuHis_2][H_2LAL]}$$

$$K = \frac{\beta_{101}^{LAL}[Cu][LAL]}{\beta_{102}^{His}[Cu][His]^2} \frac{(\beta_{011}^{His})^2[H]^2[His]^2}{\beta_{021}^{LAL}[H]^2[LAL]}$$

$$K = \frac{\beta_{101}^{LAL}(\beta_{011}^{His})^2}{\beta_{102}^{His}\beta_{021}^{LAL}}$$

$$\log K = \log \beta_{101}^{LAL} - \log \beta_{021}^{LAL} + 2 \log \beta_{011}^{His} - \log \beta_{102}^{His} = 15.27 - 19.21 + (2 \times 9.11) - 18.11 = -3.83 \text{ (data from Tables I and II)}$$

When [CuLAL] = [$CuHis_2$], i.e. LAL is equally important as histidine as a carrier of copper in vivo, [H_2LAL] = $[HHis]^2/K = (85 \times 10^{-6})^2/10^{-3.83} = 49$ μM. This calculated concentration is sensitive to errors in the log β values used in its calculation. The total concentration of lysinoalanine will only be a little greater than [H_2LAL] in vivo as the other LAL species occur in only very small amounts at pH 7.4.

Effect of DAPA, PEAA, and LAN on Copper Transport in Vivo. For the equilibrium reaction

$$CuHis_2 + 2HDAPA \rightleftharpoons CuDAPA_2 + 2HHis$$

$$K = \frac{[CuDAPA_2][HHis]^2}{[CuHis_2][HDAPA]^2}$$

$$K = \frac{\beta_{102}^{DAPA}[Cu][DAPA]^2(\beta_{011}^{His})^2\,[H]^2[His]^2}{\beta_{102}^{His}[Cu][His]^2(\beta_{011}^{DAPA})^2[H]^2[DAPA]^2}$$

$$\log K = \log \beta_{102}^{DAPA} - 2 \log \beta_{011}^{DAPA} + 2 \log \beta_{011}^{His} - \log \beta_{102}^{His} = 19.82 - (2 \times 9.40) + (2 \times 9.11) - 18.11 = 1.13 \text{ (Table II)}$$

When [$CuDAPA_2$] = [$CuHis_2$], [HDAPA] = $[HHis]/K = 85 \times 10^{-6}/(10^{1.13})^{1/2} = 23$ μM. Similarly for PEAA, log $K = -0.91$ and [HPEAA] = 243 μM when [$CuHis_2$] = [$CuPEAA_2$]. For LAN, log $K = -4.85$ and [H_2LAN] = 511 μM when [$CuHis_2$] = [CuLAN].

Registry No. LAL, 18810-04-3; DAPA, 6018-54-8; PEAA, 80267-23-8; LAN, 922-55-4; Ca, 7440-70-2; Mn, 7439-96-5; Fe, 7439-89-6; Cu(I), 17493-86-6; Ni, 7440-02-0; Co, 7440-48-4; Zn, 7440-66-6; Cd, 7440-43-9; Hg, 7439-97-6; Cu(II), 15158-11-9; Cr, 7440-47-3; carboxypeptidase, 9031-98-5.

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Received for review March 31, 1987. Accepted February 2, 1988.

Stereomeric Composition of Urinary Lysinoalanine after Ingestion of Free or Protein-Bound Lysinoalanine in Rats

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Alkali-treated or heated casein and whey protein containing various amounts of bound lysinoalanine (LAL) were fed to various groups of rats. An additional group was given a diet containing synthetic free LAL. The total amount of urinary LAL excreted during the feeding period and the proportions of LL- and LD-LAL diastereomers were determined by GC/SIM-MS. Among the groups of rats, total urinary LAL varied from 2 to 13% of the intake and about 60% of it was excreted in the free form, independent of the administered form. When synthetic LAL was given to the rats, the isomeric composition of excreted free LAL was identical with that of the administered product, indicating the absence of stereospecific excretion at the kidney level. On the other hand, free LAL found in the urine of rats fed protein-bound LAL consisted mainly of LL isomer (80–95%), independent of the type of treatment applied to the proteins. This result could be due to a reduced capacity for intestinal proteolytic enzymes to cleave peptide bonds involving D-amino acids, resulting in a lower absorption rate for LD-LAL. This lower absorption would also explain the lesser nephrocytomegaly-inducing capacity of bound LAL as compared with free LAL.

Lysinoalanine (LAL, I), a compound formed in food proteins under alkaline or heat conditions (Sternberg et al., 1975), has been found to induce in the rat a renal lesion called nephrocytomegaly. This nephrotoxicity is more pronounced with free LAL than with LAL covalently bound to protein: 15–25 times more according to the Food Protein Council for the Codex Alimentarium Commission (Report CX/VP 82/5) and 80–100 times more according to Struthers et al. (1977). The possible implication of nutritional imbalances in the formation of nephrocytomegalia has also been mentioned by Newberne and Young (1966), Feron et al. (1978), Gould and McGregor (1977), and Karayiannis et al. (1979). However, the biological significance of nephrocytomegaly is not yet understood.

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More recently the chelating ability of LAL has been demonstrated in vitro on Zn-dependent enzymes (Hayashi, 1982) and confirmed in vivo in rats that excreted more urinary zinc when fed free LAL (Furniss et al., 1985). A possible relationship between the capacity of LAL to induce nephrocytomegaly and its chelating ability remains to be established.

Other important observations concern the effect of LAL stereochemistry. Feron et al. (1978) found that the capacity to induce nephrocytomegaly of the LD diastereomer of LAL (LD-LAL) was about 10 times higher than that of the LL diastereomer (LL-LAL). More recently, Hayashi (1982) suggested that LD-LAL might be a stronger metal chelator than LL-LAL. Although such a difference between the two isomers would appear rather surprising, it might be considered as a possible explanation for LD-LAL's higher toxicity.

On the basis of these considerations, it appeared that knowledge of the isomeric composition of excreted LAL

0021-8561/88/1436-0717$01.50/0