Copper(II) and Cobalt(II) Affinities of LL- and LD-Lysinoalanine Diastereomers: Implications for Food Safety and Nutrition

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L-N^6-(2-Amino-2-carboxyethyl)-L-lysine (LL-lysinoalanine, LL-LAL) and D-N^6-(2-amino-2-carboxyethyl)-L-lysine (LD-lysinoalanine, LD-LAL), which are found in many processed foods, have been studied by spectrophotometric and potentiometric techniques. Molar optical rotation data were used to calculate the stereochemical composition of a synthetic mixture of both compounds as 56:44 LD to LL. The two diastereomers have similar acid ionization constants but differ somewhat in their affinities for copper(II) ions, as measured by their binding constants. This difference may be related to the reported differences in the ability of the isomers to induce kidney damage in rats. These results support a mechanism for the biological effects of LAL based on its ability to strongly chelate copper ions. Limited studies on the binding of LAL to cobalt(II) ions suggest that they form a yellow dioxygen complex with a possible structure of $Co_2(LAL)_3O_2$. The possible significance of these findings for food safety and nutrition is discussed.

Food proteins are often exposed to high pH in several processes such as production of spun-textured vegetable proteins, the preparation of sodium caseinate, peeling of fruits and vegetables, and detoxification of foods and feeds. Such exposure, especially in the presence of heat, induces the formation of the unnatural amino acid lysinoalanine (LAL). Lysinoalanine is present in a wide variety of foods containing milk, meat, soy, and wheat proteins (Sternberg and Kim, 1977; Maga, 1984; Pfaff, 1984; Cuq and Cheftel, 1985; Antila et al., 1987; Hasegawa et al., 1987).

Lysinoalanine causes histological changes in rat kidney cells characterized by enlargement of the nuclei and cytoplasm, increased nucleoprotein content, and disturbances in DNA synthesis and mitosis (Woodard et al., 1975; Gould and MacGregor, 1977; Karayiannis et al., 1979). The lesions (nephrocytomegaly) affect epithelial cells of the Pars recta or straight portion of the proximal renal tubules of rats. This is also the site of high-affinity amino acid reabsorption and of LAL accumulation and reabsorption (Finot et al., 1977; Gould and MacGregor, 1977). A report (Kawamura and Hayashi, 1987) that extracts from human kidneys are less effective in metabolizing lysinoalanine than are corresponding extracts from kidneys of pigs, cattle, rats, mice, rabbits, chickens, and quail implies that lysinoalanine may reside longer in the kidneys of humans than in the other species and may, therefore, be more toxic for humans than for the other listed species. Although derived from lysine, LAL is only marginally used by mice as a nutritional source of lysine (Friedman et al., 1982).

These observations cause some concern about the safety of foods containing LAL. For example, Pfaff (1984) expresses concern about possible adverse effects of lysinoalanine to newborn children at the levels found in infant formulas. It is not known by what mechanism LAL and related dehydroalanine-derived amino acids such as 2,3-diaminopropanoic acid (Kaltenbach et al., 1982) and 3-[*N*-(phenylethyl)amino]alanine (Jones et al., 1987) as well as D-serine (Kaltenbach et al., 1982) and Maillard browning reaction products (Finot and Furniss, 1986; von Wagenheim et al., 1984) damage the rat kidney. Therefore, it is difficult to assess the risk to human health caused by their presence in the diet. To obtain evidence for a possible mechanism for the toxicological manifestations of LAL and related compounds, we previously measured the affinity of a mixture of LL-LAL and LD-LAL diastereomers for copper(II), iron(II), cobalt(II), and other metal ions (Pearce and Friedman, 1988). On the basis of the observed strong binding of copper(II) to LAL, we suggested a possible mechanism for kidney damage in the rat involving (1) interference with the kidney's retention of blood plasma copper, and/or (2) the possible interaction of LAL with copper within the epithelial cells of the proximal tubules.

Since LAL has two asymmetric carbon atoms, four separate diastereomers occur: L-lysino-L-alanine, L-lysino-D-alanine, D-lysino-L-alanine, and D-lysino-D-alanine. The first two are derived from L-lysine and the second two from D-lysine (Friedman, 1977a). Since L-lysine is the natural amino acid present in proteins, most of the LAL formed during food processing is probably a mixture of LL and LD isomers. However, since exposure of food proteins to heat and alkali may concurrently racemize a small fraction of L-lysine to D-lysine (Friedman and Liardon, 1985), treated food proteins may also contain small amounts of the DL and DD isomers.

The objective of this study was to measure the stability constants of copper(II) complexes of individual LL and LD isomers of LAL and to compare the observed values to the reported toxicities of the two compounds.

EXPERIMENTAL SECTION

Materials. A mixture of the LL and LD isomers of LAL (mixed LAL) was obtained from Sigma, St. Louis, MO. The individual LL and LD isomers are the same preparations synthesized and resolved by Tas and Kleipool (1976). The $CoCl_2$ and $CuCl_2$ were of analytical reagent grade and were obtained from J. T. Baker Co., Phillipsburg, NJ. All other compounds were obtained from Sigma.

Amino Acid Analysis. Amino acid analyses were carried out in a single-column Durrum amino acid analyzer as previously described (Friedman et al., 1984).

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.

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Table I. Properties of Lysinoalanine Diastereomers

property	LL isomer	LD isomer
appearance	off-white granular powder	fine, fluffy white crystals
mol wt[a]	246.4	232.1
acidic protons/mol of LAL, mol	2.01	2.00
water/mol of LAL,[b] mol	0.73	0
Lys content[c]	0	0

[a] Calculated from potentiometric titration data with the computer program SUPERQUAD. Calculated molecular weights: $C_9H_{19}N_3O_4$ free base, 233.27; monohydrochloride, 269.73; dihydrochloride 306.19. [b] Assuming the high molecular weight is due to water as discussed in the text. [c] From amino acid analysis.

Copper Ion Spectrophotometric Titrations. Successive 60-μL samples of 0.1 M $CuCl_2$ solution were added to 3 mL of 10 mM LAL solution in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 6.0, containing 0.1 M KCl (ionic strength ~0.16 M). The absorbance spectrum was recorded between 850 and 450 nm in a 1-cm cuvette on a Perkin-Elmer Lambda 4C UV/vis spectrophotometer. The cuvette was maintained at 25 °C by means of a thermostat. The absorbances were subsequently corrected for the dilution caused by the additions of $CuCl_2$ solution.

Cobalt Ion Spectrophotometric Titrations. Successive 30-μL samples of 25 mM $CoCl_2$ solution were added to 3 mL of 2.5 mM LAL solution in 0.1 M tris(hydroxymethyl)methylamine (Tris) buffer (pH 8.0) containing 0.11 M KCl, in a 25 °C thermostated 1-cm path length quartz cuvette. The absorbance spectrum was recorded between 300 and 500 nm after each addition of $CoCl_2$ solution. The absorbance at 410 nm was corrected for the dilution caused by the addition of $CoCl_2$ and was plotted against the Co:LAL molar ratio.

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 the stepwise dissociation constants. Thus, for the reaction

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

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

where M stands for the metal ion, H the proton, L the fully deprotonated ligand, and K the equilibrium constant. Charges are omitted for ease of notation. The square 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 the overall formation constants (β). Thus, for the reaction

$$pM + qH + rL \leftrightarrow M_pH_qL_r$$

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

Potentiometric titrations were carried out with use of a glass electrode calibrated to read hydrogen ion concentrations and standard carbonate-free KOH solution in a syringe buret, as described previously (Pearce and Friedman, 1988). The computer program SUPERQUAD (Gans et al., 1985) was used to calculate the acidity and stability constants from the titration data.

RESULTS AND DISCUSSION

Properties of the Lysinoalanine Diastereomers. Both LL-LAL and LD-LAL were shown to be chromatographically pure by ion-exchange chromatography (amino acid analysis). Both compounds had a similar number of acidic protons, as determined by potentiometric titration (Table I). The molecular weight of both compounds was calculated from the known mass concentration used in the potentiometric titrations, and the refined molar concentration was determined from the titration data by SUPERQUAD. Because the proton contents equal those expected for free base, it was assumed that the higher calculated molecular weight obtained for the LL isomer is due to the presence of H_2O. If this assumption is correct, then the calculated value corresponds to 0.73 mol of H_2O/mol of LL-LAL (Table I). The LD-LAL appears to be anhydrous.

Table II. Molar Rotations (deg M^{-1} m^{-1}) of LL- and LD-Lysinoalanine Diastereomers, of a Mixture of Both Diastereomers, and of L-Lysine[a]

λ, nm	LL-LAL	LD-LAL	(LL + LD)/2	mixed LAL[b,c]	L-Lys[d]
589	98.6	−13.6	42.5	36.2	35.7
578	102.9	−14.1	44.4	38.3	37.3
546	117.4	−15.8	50.8	44.2	42.9
436	204.8	−23.6	90.6	79.6	77.5
365	336.9	−29.8	153.6	136.3	133.7

[a] Conditions of measurement: 1.6% solutions in 2 M HCl at 25 °C. [b] Data from Pearce and Friedman (1988). [c] The composition of the mixture was calculated as $X = (\phi_{mixture} - \phi_{LL})/(\phi_{LD} - \phi_{LL}) = (36.2 - 98.6)/(-13.6 - 98.6) = 0.556$, where ϕ is the molar rotation, X the mole fraction of LD-LAL, and $1 - X$ the mole fraction of LL-LAL. [d] Values calculated from Drude parameters given by Katzin and Gulyas (1964).

Table III. Logarithms of Acidity and Copper(II) Ion Binding Constants for Diastereomers of Lysinoalanine at 25 °C and an Ionic Strength of 0.16 M

constant	LL-LAL	LD-LAL	mixed LAL[a]
β_{011}	10.06 (1.01)[b]	10.07 (0.01)	10.13 (0.01)
β_{021}	19.16 (0.01)	19.16 (0.01)	19.21 (0.01)
β_{031}	25.74 (0.01)	25.75 (0.02)	25.80 (0.015)
β_{041}	27.89 (0.01)	27.94 (0.02)	28.01 (0.02)
β_{101}	14.96 (0.02)	15.52 (0.02)	15.27 (0.01)
β_{111}	20.70 (0.02)	21.13 (0.02)	20.83 (0.01)
β_{121}	25.16 (0.02)	25.47 (0.02)	25.40 (0.01)

[a] Data from Pearce and Friedman (1988). [b] Values in parentheses are the standard deviations of the estimate calculated by the computer program SUPERQUAD.

Stereoisomeric Purity of Lysinoalanine. Tas and Kleipool (1976) reported molar rotations at a wavelength of 589 nm of 100.7° for the LL-LAL isomer and −14.8° for the LD isomer. The corresponding values found in this study for the same compounds are 98.6° and −13.6°, respectively (Table II). The two sets of values are probably identical within experimental error.

The mixed LAL had a molar rotation consistent with a 55.6:44.4 molar ratio of LD- to LL-LAL. Probably there is a greater loss of the more soluble LL-LAL than LD-LAL during the purification and crystallization of the synthetic equimolar mixture. The optical rotation of this mixture is similar to that of lysine itself. Presumably, in accord with the principle of linear superposition, the contributions of the alanine asymmetric centers are largely self-canceling while the alanine moiety has little effect on the rotation due to the lysine asymmetric center.

Copper(II) Binding to Lysinoalanine Isomers. L and D isomers of amino acids containing one or more asymmetric centers may chelate copper ions stereospecifically (Pettit and Hefford, 1979). Such stereospecificity is expected for strong ligands such as LAL, containing two asymmetric centers. An objective of the present work was to determine the difference in acidity and copper binding affinities of the LAL isomers as these may relate to the relative toxicity in rats.

Table IV. Acidity and Copper Binding Equilibrium Constants for Lysinoalanine Diastereomers at 25 °C, 0.16 M Ionic Strength[a]

	LD-LAL	LL-LAL	mixed LAL[b]
pK_2	2.19	2.15	2.21
pK_3	6.59	6.58	6.59
pK_4	9.09	9.10	9.08
pK_5	10.07	10.06	10.13
log K_{CuL}	14.96	15.52	15.27
log K_{CuHL}	10.61	11.06	10.70
log K_{CuH_2L}	6.00	6.31	6.19
pK_{CuHL}	5.74	5.62	5.56
pK_{CuH_2L}	4.46	4.34	4.57

[a] Calculated from log β_{0n1} values given in Table III. The pK_1 value was too small to be determined (Pearce and Friedman, 1988). [b] Data from Pearce and Friedman (1988).

Lysinoalanine ($HOOCCH(NH_3^+)CH_2NH_2^+$-$CH_2CH_2CH_2CH_2CH(NH_3^+)COOH$) has five ionizable groups that can participate in equilibria with protons and copper(II) ions as illustrated in the equations below, where H stands for the proton (hydrogen ion), H_5L for the completely protonated form of LAL, H_4L, H_3L, H_2L, HL, and L for partly to completely unprotonated forms of LAL, and K's for acidity or metal ion binding equilibrium constants.

$$H_5L \leftrightarrow H + H_4L \quad pK_1 = \log \beta_{051} - \log \beta_{041} \quad (3)$$

$$H_4L \leftrightarrow H + H_3L \quad pK_2 = \log \beta_{041} - \log \beta_{031} \quad (4)$$

$$H_3L \leftrightarrow H + H_2L \quad pK_3 = \log \beta_{031} - \log \beta_{021} \quad (5)$$

$$H_2L \leftrightarrow H + HL \quad pK_4 = \log \beta_{021} - \log \beta_{011} \quad (6)$$

$$HL \leftrightarrow H + L \quad pK_5 = \log \beta_{011} \quad (7)$$

$$L + Cu \leftrightarrow CuL \quad \log K_{CuL} = \log \beta_{101} \quad (8)$$

$$HL + Cu \leftrightarrow CuHL \quad \log K_{CuHL} = \log \beta_{111} - \log \beta_{011} \quad (9)$$

$$H_2L + Cu \leftrightarrow CuH_2L \quad \log K_{CuH_2L} = \log \beta_{121} - \log \beta_{021} \quad (10)$$

$$CuHL \leftrightarrow H + CuL \quad pK_{CuHL} = \log \beta_{101} - \log \beta_{111} \quad (11)$$

$$CuH_2L \leftrightarrow H + CuHL \quad pK_{CuH_2L} = \log \beta_{111} - \log \beta_{121} \quad (12)$$

Table III summarizes the results for the overall acidity and copper(II) binding constants of LL-LAL, LD-LAL, and the mixture of both. Table IV shows the corresponding stepwise constants calculated from these values using the equations given above. The acidity constants for the two isomers are rather similar and in good agreement with the values previously obtained for the mixture (Pearce and Friedman, 1988).

Spectrophotometric Titration with Copper(II) Chloride. Previous studies (Pearce and Friedman, 1988) on the affinity of the mixture of LL-LAL and LD-LAL for copper as measured by spectrophotometric titrations showed that, at pH 6.0, the titration curve had breaks in slope at Cu to LAL molar ratios of about 1.0 and 2.0. At pH 5.0 and 9.0, only a single break was observed at Cu:LAL = 1.0.

Similar studies were carried out with the individual LAL isomers at pH 6.0 (Figures 1 and 2). These show that LL-LAL and LD-LAL have very similar spectrophotometric titration curves, with a marked break at a Cu:LAL = 1.0 and a second break at Cu:LAL = 2.0. Precipitation occurred for Cu:LAL > 3.0 and when $CuCl_2$ was added to the buffer in the absence of LAL. The corresponding spectra for the diastereomers were very similar. For Cu:LAL = 1.0–2.0, there was a single isosbestic point at a wavelength of 645 nm for both compounds. The wavelength of maximum absorbance increased from about 599 nm to about 610 nm as the Cu to LAL ratio increased from 0.2 to 1.0. There was a much more marked increase in wavelength of the maximum as the Cu to LAL ratio increased above 1.0. These changes in wavelength indicate a reduction in the number of ligand coordinating groups interacting with each cupric ion (Billo, 1974).

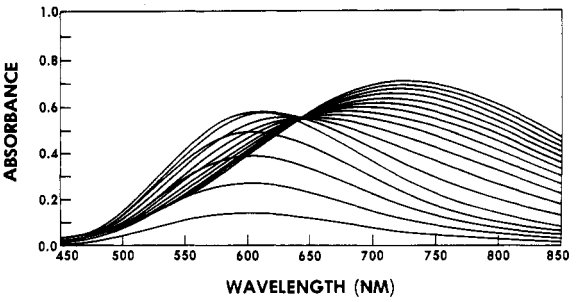

Figure 1. Absorbance spectra for successive additions of $CuCl_2$ solution to 10 mM LL-lysinoalanine at pH 6.0, approximately 0.16 M ionic strength. The curves correspond to increasing Cu to LAL molar ratios ranging from 0.2 (the lowest curve) to 3.4.

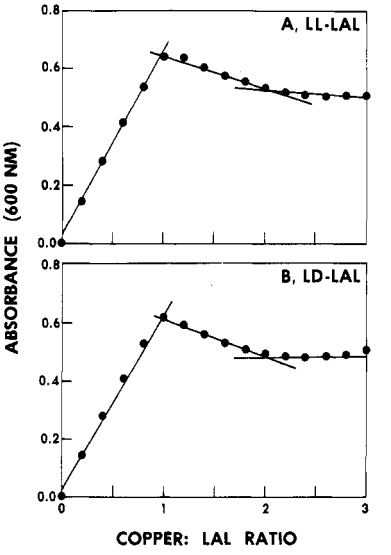

Figure 2. Spectrophotometric titration curves for the addition of $CuCl_2$ to 10 mM lysinoalanine, pH 6.0, approximately 0.16 M ionic strength: A, LL-lysinoalanine; B, LD-lysinoalanine.

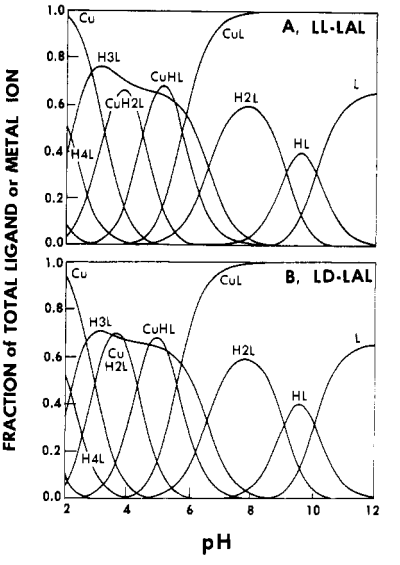

Figure 3. Relative proportions of various lysinoalanine species in a mixture of 10 mM lysinoalanine and 100 mM $CuCl_2$ as a function of pH at 25 °C and 0.16 M ionic strength: A, LL-lysinoalanine; B, LD-lysinoalanine.

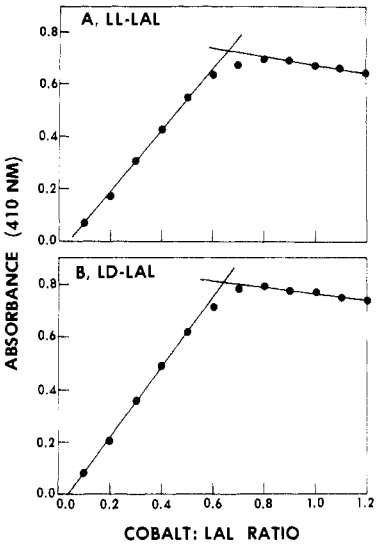

Figure 4. Spectrophotometric titration curves for the addition of $CoCl_2$ to 2.5 mM lysinoalanine, pH 8.0, approximately 0.16 M ionic strength: A, LL-lysinoalanine; B, LD-lysinoalanine.

The predicted computer-calculated distribution of the relative proportions of the various diastereomeric LAL species as a function of pH in the presence of $CuCl_2$ is shown in Figure 3. Only slight differences for the two isomers are apparent from a comparison of the top and bottom plots.

These data indicate that both diastereomers form 1:1 complexes with Cu^{2+} and that these complexes probably involve coordination of the metal ion by two carboxylate and two amine groups, as proposed previously (Pearce and Friedman, 1988). At Cu:LAL > 1, additional copper can be chelated. The data suggest that each LAL molecule can bind up to three copper ions, since no precipitation occurs until Cu:LAL > 3, although precipitation does occur when only copper(II) is added to buffer. No evidence for Cu_2LAL or Cu_3LAL complexes was found when SUPERQUAD was applied to potentiometric data for Cu:LAL < 1.0. However, potentiometric titration data for 10 mM Cu and 2.95 mM LAL were also investigated with SUPERQUAD. The constants found for Cu:LAL < 1.0 were assumed applicable, and a search was made for di- and tricopper complexes. Cu_2HLAL was found to be a major species with log β_{211} = 23.5. No other species were detected. Ratios Cu:LAL > 1.0 are unlikely to be important in vivo.

Spectrophotometric Titration with Cobalt(II) Chloride. We reported previously that the mixed diastereomers of LAL showed a break in the spectrophotometric titration curve at a Co:LAL ≈ 0.67 in the presence of atmospheric oxygen (Pearce and Friedman, 1988). This indicated that a complex such as $Co_2(LAL)_3O_2$ might be formed. SUPERQUAD treatment of potentiometric data subsequently found evidence for the formation of $Co(LAL)_2$ at high pH, when LAL is present in excess (log β_{102} = 10.4 ± 0.1).

In the current experiments, spectrophotometric titrations were carried out on LL-LAL and LD-LAL (Figure 4). Both diastereomers gave similar yellow solutions in the presence of atmospheric oxygen, indicating that both form dioxygen complexes with cobalt. Isosbestic points occurred at about 380 nm and above 500 nm for Co:LAL > 1.0. There was a break in the titration curve near Co:LAL = 0.65. An equimolar mixture of CoLAL and $Co(LAL)_2$ would have a Co:LAL = 0.667. However, the maximum concentration of $Co(LAL)_2$ (and $Co(LAL)_2O_2$) occurs at Co:LAL = 0.5. The maximum yellow intensity would be expected at this ratio, if this dioxygen species is responsible for the color. Therefore, a yellow $Co_2(LAL)_3O_2$ species probably is formed.

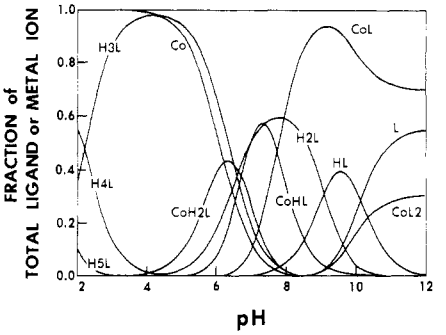

Figure 5. Relative proportions of various lysinoalanine species in a mixture of 2.5 mM LD-LAL and 25 mM $CoCl_2$ as a function of pH. The results are for 25 °C and 0.16 M ionic strength.

The computer-calculated distribution of the relative proportions of various LD-LAL species as a function of pH in the presence of $CoCl_2$ is shown in Figure 5. The shapes of the curves do not differ significantly from those previously described for the LL–LD mixture (Pearce and Friedman, 1988).

Species concentrations during cobalt addition to LAL solution were calculated, ignoring dioxygen complex formation (since the formation constant is not known) using the formation and acidity constants found for the mixed LAL. Only trace amounts of $Co(LAL)_2$ were predicted to exist at pH 8. The major species present were Co:LAL < 1.0, H_2LAL, CoHLAL, CoLAL; and Co:LAL > 1.0, CoHLAL, CoLAL, Co. Since the spectrophotometric studies previously reported with the mixed LAL (Pearce and Friedman, 1988) were generally similar to the results reported here for the individual isomers, stereochemical factors evidently do not have a major effect on the affinity of LAL for cobalt(II) ions.

Significance of Metal Ion Binding Properties for Nutrition and Food Safety. Copper, an essential metal, is carried in the blood in a variety of forms. Ceruloplasmin and serum albumin are the principle protein carriers of nonlabile and labile copper, respectively, while histidine is the major low molecular weight carrier of labile copper. Although the amount of copper bound to histidine is always much less than the amount bound to the ceruloplasmin, histidine is believed to be important in mediating the transfer of copper between proteins (Gordon et al., 1987; Ettinger et al., 1986). Metallothionein, which occurs in the liver, kidney, and elsewhere, is the major copper storage protein in the body (Mason, 1979).

We suggested earlier that LAL may interfere with the mechanism by which the kidney conserves copper, by displacing histidine as the major low molecular weight carrier of copper in vivo (Pearce and Friedman, 1988). As described in detail elsewhere, it is possible to predict the equilibria between histidine and competing chelating agents such as LAL (Pearce and Friedman, 1988). Applying this previously derived mathematical analysis to the isomeric LAL samples gives the following equilibrium constants for the reaction:

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

$$K = [CuLAL][HHis]^2/[CuHis_2][H_2LAL] \quad (13)$$

Substitution of the appropriate values into eq 13 gives log K = −3.58 for LD-LAL, −4.14 for LL-LAL, and −3.83 for mixed LAL. These values make it possible to calculate the LAL plasma concentrations needed to displace histidine as the major low molecular weight copper carrier in

vivo. The calculated values are 27 μM for LD-LAL, 100 μM for LL-LAL, and 49 μM for the mixture of both.

CONCLUSIONS

The above considerations suggest that LD-LAL would be a better competitor for copper(II) in vivo than the LL isomer. Thus, it should take about one-fourth as much LD-LAL as LL-LAL to displace the same amount of copper from copper-containing histidine complex. This difference could explain the greater observed toxicity of LD-LAL (Feron et al., 1977), although stereospecificity of transport mechanisms and metabolism differences for the two isomers in the rat kidney cannot be ruled out. The results also imply that, because of its strong affinity for copper ions, lysinoalanine may influence the nutritional availability and utilization of this essential trace element. Because cobalt(II) shows a much weaker affinity for lysinoalanine, corresponding effects on cobalt ions, which are present in both storage proteins such as metallothioneins and as part of the active site of vitamin B_{12} (Smith, 1987), may be less important. These considerations suggest that changing the mineral content of the diet may alter lysinoalanine and Maillard browning product induced kidney damage. Additional studies are needed to demonstrate this possibility and the possible value of LAL, both free and in protein digests, to treat patients suffering from excess copper retention (Wilson's disease). Although penicillamine is widely used for this purpose (Friedman, 1977b), it has undesirable side reactions.

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Registry No. LL-LAL, 23250-50-2; LD-LAL, 63121-95-9; Cu, 7440-50-8; Co, 7440-48-4.

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