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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

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could contribute to a better understanding of mechanism of action of this compound. For this reason, we developed a method for the separation and quantification of LAL isomers by capillary GC/MS. This method was then applied to determine LAL concentrations and isomeric composition in the urine of rats fed different diets containing free or protein-bound LAL.

EXPERIMENTAL SECTION

Synthesis of Reference Compounds. Lysinoalanine (LAL) dihydrochloride salt was prepared as described by Pintauro et al. (1985). This sample was characterized by a molecular rotation $[M]^{20}_D$ of 34.7° (*c* 2, 2 N HCl) corresponding to a mixture of about 40% LL-LAL and 60% LD-LAL (Tas and Kleipool, 1976) as confirmed by GC/MS (39.9% LL-LAL). The discrepancy with respect to the expected theoretical composition (50% LL-LAL) was probably due to the final crystallization step. Indeed, Tas and Kleipool (1976) have shown that the two isomers differ in their solubility in water, and they used this property to isolate pure LAL diastereomers.

Following the same procedure, a fraction of the above product was used to prepare a sample enriched in LD-LAL, which was used to determine the elution order of the two diastereomers in the GC separation. This sample contained 97% LD-LAL and was characterized by a molecular rotation $[M]^{23}_D$ of −11.85° (*c* 2, 2 N HCl).

Test Proteins. The following proteins were used in the two rat assays:

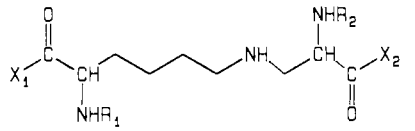
Assay I. Control whey protein (WP): ultrafiltrated lactoserum, Guigoz S. A., Vuadens, Switzerland; LAL content, 70 mg/kg of protein. Alkali-treated whey protein (AL-WP): obtained by heating 5% WP in 0.1 N NaOH at 60 °C for 5 min, followed by precipitation at pH 3.9, washing of the precipitate with water, and freeze-drying; LAL content, 10 540 mg/kg of protein. Control casein (CAS): Kliba, Kaiseraugst, Switzerland; LAL content, 173 mg/kg of protein. Severely alkali-treated casein (AL-CAS-S): obtained by heating 5% CAS in 0.1 N NaOH at 80 °C for 2 h, followed by neutralization and freeze-drying; LAL content, 14 800 mg/kg of protein.

Assay II. Moderately alkali-treated casein (AL-CAS-M): obtained by heating 5% CAS in 0.05 N NaOH (adjusted to pH 9.75) at 60 °C for 4 h, followed by precipitation at pH 4.6, washing with water, and freeze-drying; LAL content, 779 mg/kg of protein. Heated casein (H-CAS): obtained by precipitation at pH 4.6 of skim milk sterilized in can at 115 °C for 55 min, followed by washing with water and freeze-drying; LAL content, 1544 mg/kg of protein. Whey protein with added free LAL (WP + LAL): LAL content, 1300 mg/kg of protein.

Animal Assays. Two different trials were carried out with male Sprague–Dawley rats (Süddeutsche Versuchstierfarm GmbH, Tuttlingen, FRG) weighing about 100 g and fed ad libitum. In the first trial the protein level (N × 6.25) was 20%. In the second trial this level was raised to 40% to obtain higher urinary LAL level leading to more accurate measurements. Besides protein, each diet contained in grams/100 g the following: sucrose, 25; corn oil, 10; mineral mix (USPXVII), 5; cellulose, 2; vitamin mix (Peret et al., 1973), 1.25; starch, to 100. The animals were placed in individual metabolic cages for urine collection.

In general, urine was collected for 3 days following a 4-day (assay I) or 2-day (assay II) adaptation period. Alternatively, the urine of rats fed diets containing AL-WP or AL-CAS-S (see below) was collected for 1 day after 3-day adaptation. Urine was collected in 1 mL of 0.5 N HCl and 1 mL of toluene, and 24-h batches were frozen separately to prevent bacterial growth. During the collection period, the food intake of each rat was determined and the volume of collected urine was measured.

Chart I



	free LAL	protein-bound LAL
X_1, X_2	OH	Prot-NH-
R_1, R_2	H	Prot-C(=O)-

Analyses of LAL in Rat Urines. *Sample Preparation.* One milliliter aliquots were taken from each urine sample, centrifuged, and adjusted to acid pH 3–4 with concentrated HCl. After the addition of 5 μg of N-methyllysine (NML) to serve as an internal standard, the samples were applied to 1 mL of Dowex 50 (H^+) cartridges and washed with 10 mL of distilled water. Amino acids were then displaced with 1 mL of 3 N NH_4OH followed by 1 mL of water into conical reaction vials and dried under nitrogen flow.

For the determination of total LAL excretion, this procedure was preceded by urine sample acid hydrolysis. This was done by combining 1 mL of urine aliquots with 1 mL of concentrated HCl and heating them for 24 h at 110 °C in evacuated hydrolysis ampules.

Amino acids recovered from Dowex cartridges were converted into *N*(*O*,*S*) perfluoropropionylisopropyl esters in a two-step reaction: (1) esterification in 2-propanol/acetyl chloride followed by (2) acylation with perfluoropropionic acid anhydride (PFPA). The details of the procedure have been described elsewhere (Liardon et al., 1981). After the excess reagent was evaporated, the amino acid derivatives were redissolved in 0.5–1 mL of hexane.

Sample Analysis. The analyses of the derivatized samples were performed on a HP 5995 bench-top GC/MS system with a 30 m × 0.3 mm (i.d.) DB-5 fused silica capillary column. Sample introduction was made by on-column injection. Column temperature was programmed from 60 to 250 °C at 6 °C/min. Ion source temperature was set at 250 °C, and the ionizing voltage was 70 eV. The mass spectrometer was run in the selected ion monitoring mode (SIM). Quantification was obtained by measuring the abundance of ion *m*/*z* 434 for NML and *m*/*z* 451 or 626 for LAL, at the retention times measured for authentic samples of the two compounds.

RESULTS AND DISCUSSION

LAL Determination in Urine. Unlike amino acid enantiomers, lysinoalanine diastereomers (LL-LAL, LD-LAL) (I) have different chemical properties (Chart I). The differences, however, are small, and capillary GC was found to be the only separation technique with sufficient resolution power to distinguish these two compounds after being converted into volatile *N*-PFP *O*-isopropyl esters. Because of the risk of interference with other minor constituents of urine, selected ion monitoring (SIM) mass spectrometry was used as a specific detection technique. On the basis of the electron impact mass spectrum of the LAL derivative (Figure 1), two characteristic fragments ions, *m*/*z* 451 and 626, were selected and monitored during the chromatographic separation. A typical mass chromatogram of the two LAL diastereomers is shown in Figure 2.

The calibration of the quantification procedure was made by running control urine samples with added known amounts of synthetic free LAL. LAL-measured concen-

Table I. Levels and Isomeric Composition of LAL Excreted in the Urine of Rats Fed Protein-Bound and Free LAL

	dietary LAL, mg/kg		urinary LAL		
			free		
	protein	diet	% of intake	isomeric ratio[a]	total: % of intake
			Assay I		
WP	70	14	not detectable		
AL-WP	10500	2100	6.2	93.0	
			(2.1)[c]	(0.9)	
CAS	173	35	1.5	80–85	
			(0.4)		
AL-CAS-S	14800	2960	2.1	85	
			(1.1)		
			Assay II		
AL-CAS-M	779	312	7.6	90.2	11.1
			(1.2)	(2.3)	(2.7)
WP + LAL	1300 (free)	520	8.5	40.5[b]	13.1
	70 (bound)	14	(5.3)	(2.2)	(6.6)
H-CAS	1544	618	1.7	82.3	3.1
			(0.3)	(1.7)	(1.2)

[a] [LL-LAL]/([LL-LAL] + [LD-LAL]). [b] Synthetic LAL administered in this assay contained 39.9% L isomer. [c] Values in parentheses are standard deviations.

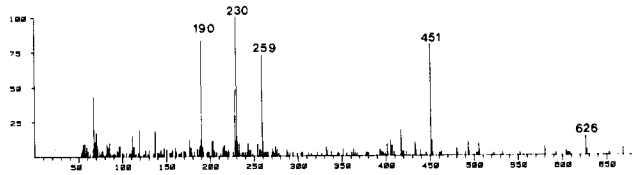


Figure 1. Electron impact (EI) mass spectrum of *N,N',N''*-tris-PFP-*O*-isopropyllysinoalanine.

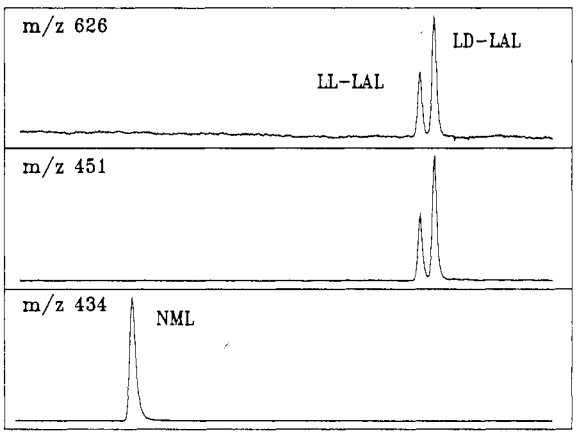


Figure 2. Mass chromatogram obtained for a standard sample containing 15 μg/mL LAL isomers and 5 μg/mL NML (internal standard).

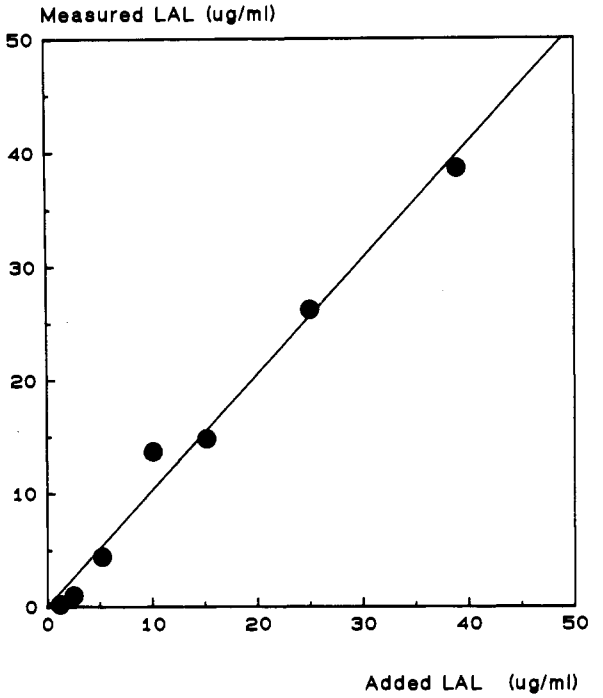


Figure 3. Calibration curve for free LAL total urinary excretion: measured LAL concentration versus added concentration.

tration was calculated from the cumulated peak surface areas of the two isomers relative to that of the added internal standard, *N*-methyllysine. The results of this calibration run are illustrated in Figure 3. The detection limit of the method ranged between 0.2 and 0.5 μg of LAL/mL of urine for each isomer.

The distribution of LAL isomers in each sample was simply derived from the corresponding peak areas. This distribution was expressed as the ratio

$$\frac{[\text{LL-LAL}]}{[\text{LL-LAL}] + [\text{LD-LAL}]} \times 100$$

Free LAL Urinary Excretion. The level of LAL excreted in the urine as free LAL and expressed in percent of ingested protein was found to vary from 1.5 to 8.5% (Table I). For similar LAL levels in proteins exposed to severe alkaline treatment, LAL relative excretion was 3 times higher with whey proteins (AL-WP) than with casein (AL-CAS-S). Similarly, moderately alkali-treated casein (AL-CAS-M) induced a relative excretion level 3.6 times higher than severely treated casein (AL-CAS-S). A significant difference in excretion level could also be observed when casein exposed to moderate alkaline (Al-CAS-M) was compared with that exposed to heat treatment (H-CAS).

Abe et al. (1981) have shown that protein-bound LAL residues are mainly absorbed and transported in the blood in the form of peptides. This suggests that LAL absorption should depend on the size of the peptides liberated by intestinal enzymes, which in turn must be influenced by the capacity of amino acids surrounding LAL in the protein chains to be released enzymatically. This probably explains the differences in LAL excretion level depending on the nature of the protein or the type of treatment. Proteins exposed to alkaline treatments could be expected to have suffered modifications like amino acid racemization, formation of new cross-linkings [LAL (Bohak, 1964), lanthionine (Horn et al., 1941), ornithinoalanine (Ziegler et al., 1967)], or destruction of arginine (Geschwind and

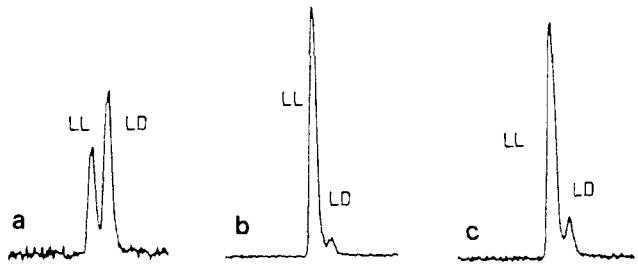


Figure 4. Typical profile of free LAL isomers in the urine of rats fed different diets: (a) WP + LAL; (b) AL-CAS-S; (c) AL-WP.

Li, 1964). On the other hand, heated milk casein contained not only some LAL but also Maillard reaction products: lactoselysine and advanced Maillard reaction products. All these chemical modifications, known to reduce the overall nitrogen digestibility, can be expected to diversely affect the absorption of LAL-containing peptides.

When LAL was administered in the free form (diet WP + LAL), the average excretion level expressed in percent of LAL intake was 8.5%. [In this calculation the small amount of protein-bound LAL (70 mg/kg) present as an impurity in the control WP was not taken into consideration, as being negligible in regard to the added free LAL (1300 mg/kg).] This value was lower than expected. In previous studies based on radioactively labeled LAL, Finot et al. (1977) and Struthers et al. (1980) found excretion rates ranging respectively from 26 to 64% and 50 to 58%. The discrepancy with the present results might be due to a difference in the mode of administration: incorporation of LAL in the diet for the present study against stomach tubing in fasting animals for the previous studies.

Free versus Total Urinary LAL. Urine samples from assay II were analyzed for both free and total urinary LAL. Total LAL values were obtained by submitting the urine samples to acid hydrolysis prior to analysis. Results are reported in Table I. For each rat, the total LAL value was always slightly higher than the free LAL value. This indicated that LAL had partly been excreted in a combined form. A similar observation had already been made by Finot et al. (1977) with [^{14}C]LAL and by Karayiannis et al. (1979) with protein-bound LAL. Until now, the nature of LAL combined forms could not be determined. They might consist of peptides not hydrolyzed in the kidneys. This hypothesis, however, seems to be ruled out by the present results showing that the total over free urinary LAL ration was about the same for diets containing protein-bound or added free LAL. On the other hand, such results might be expected for acetylated conjugates as proposed by Finot et al. (1977).

Isomeric Composition of Urinary LAL. The isomeric composition of LAL excreted in the urine of the different groups of rats is reported in Table I. Examples of the corresponding mass chromatograms are shown in Figure 4. These data led to the following observations:

In the case of rats fed a diet containing free LAL, the isomeric composition of excreted LAL was not significantly different from that of the administered compounds (Figure 4a). This result indicates that for free LAL there was no difference in absorption or excretion between the LL and LD diastereomers.

On the other hand, for all diets containing protein-bound LAL the urinary excretion was characterized by a much higher proportion of LL-LAL, ranging from 80 to 93%, irrespective of the type of treatment (alkaline or heat) or of the nature of LAL precursor [cyst(e)ine in whey protein, serine phosphate in casein] (Figure 4b,c). This original observation needs some comment.

As a first possible explanation, the high proportion of urinary LL-LAL may reflect the actual isomeric composition of bound LAL in the proteins. However, the generally accepted two-step mechanism for alkali-induced formation of LAL is not expected to favor one isomer over the other. Therefore, alkali-treated proteins are expected to contain approximately equal amounts of LL- and LD-LAL. Only a one-step substitution mechanism, as proposed for the reaction of lysine with serine phosphate in heated milk casein would result in the specific formation of LL-LAL (Friedman, 1977). Until now, the direct determination of LAL isomeric composition in processed proteins had not been possible due to the fact that the alanine moiety of LAL completely racemizes during acid hydrolysis (Tas and Kleipool, 1976). Recently, however, we developed a new approach to this analytical problem, allowing us to confirm the presence of the two isomers in about equal proportion in all proteins used in this study (Liardon, unpublished results).

Another hypothesis was that the observed LAL composition could result from LL-LAL selective excretion by the kidney, due for instance to specific reabsorption in the proximal tubule or to a specific degradation of LD-LAL in the rat tissues. However, the results obtained when a mixture of free LAL isomers (diet WP + LAL) was administered rule out this possibility.

Actually, the discrimination between LAL diastereomers is more likely to take place at the level of intestinal absorption. As discussed before, LAL is mainly liberated and absorbed in the form of peptides, the size of which should depend on the ability of proteolytic enzymes to liberate the amino acids surrounding LAL residues. On the basis of digestibility studies performed on racemized proteins (Masters and Friedman, 1980; Bunjapamai et al., 1982; Tovar and Schwass, 1983), it can be inferred that proteases have a reduced capacity for cleaving peptide bonds including D-amino acid residues. In particular, this would apply to the D-Ala moiety of LD-LAL. Consequently, it can be assumed that the average size of LD-LAL-containing peptides is larger than that of LL-LAL-containing peptides and that this results in lesser absorption of the former diastereomer.

Interestingly, this conclusion would also explain the difference in nephrocytomegaly-inducing activity between protein-bound and free LAL. Keeping in mind that LD-LAL is much more active than LL-LAL (Struthers et al., 1977), the lesser activity of bound LAL might simply result from the nonabsorption of bound LD-LAL. On the other hand, the mechanism involved in LAL induction of nephrocytomegaly still remains to be found.

CONCLUSION

Independent of the type and severity of the treatments applied to the test proteins, urinary free LAL relative to dietary free or protein-bound LAL ranged from 1.5 to 8.5%. A smaller proportion of ingested LAL was excreted as combined forms, regenerating free LAL upon acid hydrolysis. When rats were fed a diet containing free LAL, the isomeric composition of the urinary free LAL did not differ from that of the administered compound. For rats fed a diet containing protein-bound LAL, urinary free LAL consisted primarily of the LL diastereomer (80–93%).

The most likely explanation of this observation lies in the reduced ability for intestinal proteolytic enzymes to cleave peptide bonds involving D-amino acids. LD-LAL-containing peptides liberated from dietary proteins enzymes are expected to have a larger size than LL-LAL-containing peptides and to be less readily absorbed. This difference in absorption rate can also explain the lesser

nephrocytomegaly-inducing capacity of protein-bound LAL as compared with free LAL.

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Registry No. LAL, 18810-04-3; LL-LAL, 23250-50-2; LD-LAL, 63121-95-9.

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