Identification of N^{ϵ}-Carboxymethyllysine: A New Maillard Reaction Product, in Rat Urine

Rémy Liardon,* Danièle de Weck-Gaudard, Georges Philippossian, and Paul-André Finot

N^{ϵ}-Carboxymethyllysine (CML), an unusual amino acid, was identified in urine samples from various groups of rats on the basis of mass spectral and capillary GC retention data. CML urinary concentration varied widely among the rat groups, apparently in relation with the composition of the diets. Analyses of the dietary proteins revealed the presence of CML in all samples in concentrations ranging from trace level (about 100 ppm) in control proteins to 1000–2000 ppm in heated milk casein or alkali-treated whey protein. This compound probably originates from the oxidative degradation of fructoselysine (FL), a Maillard reaction product formed during protein treatment. Among the various groups of rats, CML urinary excretion ranged from 4 to 19% of the intake, validating the hypothesis that urinary CML is mainly of exogenous origin.

In recent independent nutritional studies on the rat, capillary GC analyses of urinary amino acids unexpectedly revealed the presence of an unknown amino acid. The concentration of this compound eluting between lysine and lysinoalanine varied widely, apparently in relation with the dietary proteins. A detailed investigation led to its identification as N^{ϵ}-carboxymethyllysine (CML, I).

The presence of this new amino acid in the urine led to the question of its endogenous or exogenous origin. During processing and storage of food proteins, some amino acids may be chemically modified, forming new species that are partially absorbed and metabolized or excreted as such in the urine (Finot, 1982). Lysine is particularly sensitive to such chemical modifications. Conditions of formation and the metabolism of two of its derivatives, fructoselysine (FL, II) and lysinoalanine (LAL, III), have been extensively studied. Fructoselysine results from the reaction of lysine with reducing sugars and is excreted unmodified in the urine (Finot and Magnenat, 1980). This compound has also been reported in serum albumin (Schleicher and Wieland, 1981), nails, and hair (Oimomi et al., 1986), with larger amounts in diabetics than in healthy subjects. These findings indicate that the formation of fructoselysine can take place in vivo. Lysinoalanine is produced during protein alkaline or heat treatment (Sternberg et al., 1975) and is partially metabolized into several catabolites that are found in the urine along with unmodified lysinoalanine (Finot et al., 1977).

In view of these examples, the possible origin of CML appeared to be (i) process-induced formation in dietary protein, (ii) metabolism of some other process-induced derivative of lysine, and (iii) in vivo formation. As it turned out, analysis of data coming from earlier rat assays, not originally designed to test the origin of CML, suggested that this compound was mainly of exogenous origin.

This paper describes the identification of N^{ϵ}-carboxymethyllysine and discusses its origin.

EXPERIMENTAL SECTION

Synthesis of Reference Compounds. N^{ϵ}-Carboxymethyllysine (CML, I) was prepared by reductive alkylation of the ϵ-amino group of N^{α}-Boc-L-lysine with glyoxylic acid, followed by removal of the protecting group. This method was more convenient and gave a much higher yield than alkylation with bromoacetic acid (Matsutani et al., 1979). A solution of N^{α}-Boc-L-lysine (122.5 mg, 0.50 mmol) and of sodium glyoxylate (85.0 mg, 0.75 mmol) in citrate buffer (1 mL; Merck No. 9437) at pH 6 was reacted at room temperature under a hydrogen atmosphere in the presence of 10% Pd/C (10 mg). The reaction was monitored by TLC (silica gel, phenol–water (4:1), ninhydrin) and reached completion in about 24 h. The reaction mixture was filtered and evaporated to dryness. The residue was dissolved in methanol (20 mL) and passed through a short column packed with 3 g of cellulose to remove the citrate salt and the excess sodium glyoxylate. The eluate was evaporated to dryness, yielding by NMR pure N^{α}-Boc-N^{ϵ}-CML as a colorless crystalline residue. This was subjected to acid hydrolysis in 1 N HCl (2 mL) at 70 °C for 1 h. After evaporation of the solution, the residue was crystallized from water–ethanol: yield 110 mg (80%); mp 175–177 °C dec. Analyses of the product (^{1}H NMR, AA analyzer, GC/MS of N-PFP isopropyl ester) indicated a purity of about 95%. N^{ϵ},N^{ϵ}-dicarboxymethyllysine was identified as a minor side product.

N-Fructoselysine (FL, II) was prepared by the method of Finot and Mauron (1972) by reacting N^{α}-formyl-L-lysine with glucose in methanol. In view of the large amounts of product needed for the animal tests, the workup of the reaction product was simplified as follows. The solvent was evaporated and the residue taken up in water and applied in a Dowex 50X4 (H^+ form) column. After unreacted glucose was removed with water, N^{α}-formyl-N^{ϵ}-fructosyl-L-lysine was eluted with 0.5 N aqueous ammonia. A few fractions, containing L-lysine and N^{α}-formyl-L-lysine contaminants, were discarded. The remaining fractions were pooled and evaporated to dryness, and the residue was deformylated in 2 N HCl at 80 °C for 2 h. After HCl was removed with Dowex 1X4 (CH_3OO^- form), the colored solution was treated with active charcoal. N^{ϵ}-Fructosyl-L-lysine was obtained by precipitation as a slightly brownish material, which was shown to contain small amounts of N^{ϵ},N^{ϵ}-difructosyl-L-lysine.

Lysinoalanine (LAL, III) was prepared as the dihydrochloride salt as previously described (Pintauro et al., 1985).

Animal Assays. Data reported here were obtained from three independent rat trials. These trials were carried out with the common objective of studying the nutritional effects of proteins treated with alkali or heated in the presence of reducing sugars. Dietary proteins contained different levels of protein-bound or free LAL and/or FL. Results corresponding to the original objectives of these assays will be reported separately.

The same general pattern was applied for the three assays: All rats, male Sprague–Dawley strain, were fed ad

Nestle Research Center, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.

0021-8561/87/1435-0427$01.50/0

libitum. Besides protein, each diet contained (g/100 g) the following: sucrose, 25; glucose, 20 (assays II and III); corn oil, 10; mineral mix (USP XVII), 5; cellulose, 2; vitamin mix (Peret et al., 1973), 1.25; starch, to 100. After being fed 2 days on a commercial diet (Nafag No. 850, Gossau, Switzerland), rats were fed on the experimental diets and later placed in metabolic cages for urine collection. The cages were made of plastic material, except for the bottom grids that were of stainless steel. Urine was collected in vessels containing 1 mL of 0.5 N HCl to keep the pH below 3 and 1 mL of toluene for preventing bacterial contamination. The urine samples collected were frozen at the end of each day. With these precautions the catalytic oxidation and bacteriological modification of urine constituents were minimized.

In assay I, the rats (six per group) weighed 100 g at the beginning of the experiment and were fed 20% protein diets. They received the experimental diet for 3 days before urine collection that lasted for 3 days, except for groups 2 and 6 in which the animals received the experimental diet only for 2 days before the urine was collected over a 24-h period. All animals received the experimental diet throughout the urine collection period.

In assay II, the rats (six per group) weighed 45 g at the beginning of the experiments and were fed 18% protein diets. They received the experimental diet for 10 days before urine was collected over 2 days.

In assay III, the rats (eight per group) weighed 80 g at the beginning of the experiment and were fed diets containing 17% protein. They received the experimental diet for 8 days before urine was collected over 5 days.

A description of the protein fraction of the various diets is given below.

Assay I: group 1, control whey protein (WP) ultrafiltrated lactoserum, Vuadens, Switzerland; group 2, alkali-treated WP (AL-WP), obtained by heating 5% WP in 0.1 N NaOH at 60 °C for 5 min followed by neutralization at pH 3.9, washing of the precipate with water, and freeze-drying; group 3, AL-WP mixed with control WP, final proportions in the diet AL-WP 1.3%, WP 18.7%; group 4, control casein (CAS), Kliba, Kaiseraugst, Switzerland; group 5, CAS enriched with free LAL (5 mg/100-g diet); group 6, alkali-treated casein (AL-CAS), obtained by heating 5% CAS (according to Hammerstein, Merck, Darmstadt, FRG) in 0.1 N NaOH at 80 °C for 2 h, followed by neutralization and freeze-drying; group 7, AL-CAS mixed with control CAS, final proportion in the diets AL-CAS 0.95%, CAS 19.05%; group 8, heated milk casein (H-CAS), obtained by precipitation at pH 4.6 of skim milk sterilized in can at 115 °C for 50 min, followed by washing with water and freezing-drying, H-CAS mixed in the diet with control CAS in the proportions H-CAS 11.7%, CAS 8.3%.

Assays II and III: group 9, control casein (CAS), same as for group 4; group 10, Maillard casein (4 days), prepared by incubation at 37 °C for 4 days of a mixture of casein, glucose, and water (100:100:30, w/w), followed by freeze-drying, level of lysine blocked as fructoselysine 1.67 g/16 g N, as measured by the furosine method (Finot et al., 1980); group 11, mixed control proteins, whey gluten 8.3%, zein 3.7%, control casein 5.35%, percentage values are protein levels in the diet; group 12, same mixture as for group 11, except control casein replaced by Maillard casein (15 days) prepared in the same manner a for group 10, incubation time 15 days, level of lysine blocked as fructoselysine 4.24 g/16 g n, 0.227% lysine added to the diet to compensate for blocked lysine; group 13, same mixture as for group 12, but enriched with free fructoselysine acetate to provide 95.7 mg of fructoselysine equivalent in 100-g diet.

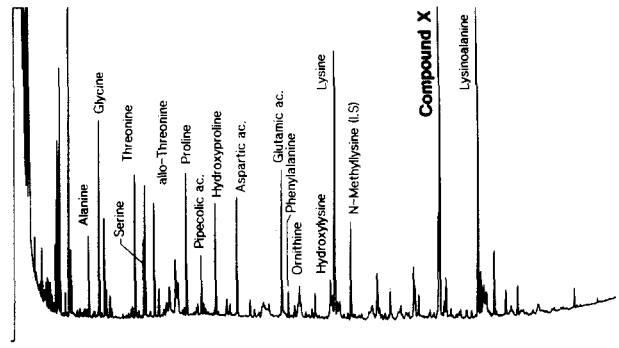


Figure 1. Capillary GC profile of free amino acids in the urine of a rat fed alkali-treated whey protein (AL-WP) (X = unknown amino acid).

Sample Preparation for Analysis. One-milliliter aliquots were taken from each urine sample and adjusted to acid pH (3–4) with concentrated HCl. After the addition of 5 μg of N^{ϵ}-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. The same procedure was applied to samples of the different proteins used in the diets after 24-h hydrolysis in 6 N HCl.

The recovered amino acids were converted into perfluoropropionyl isopropyl 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 by gas chromatography using a 30 m × 0.3 mm (i.d.) DB-5 fused silica capillary column installed in a HP 5995 bench-top GC/MS system. Sample introduction was made by on-column injection. Column temperature was programmed from 60 to 250 °C at 6 °C/min. The mass spectrometer was run alternatively in scan mode for compound identification or selected ion monitoring for quantification. In scan mode, mass spectra were recorded in both the electron impact and chemical ionization (CH_4) modes on the HP 5995 spectrometer.

N^{ϵ}-Carboxymethyllysine and lysinoalanine in proteins and urine were quantified by selected ion monitoring mass spectrometry. Fructoselysine in proteins was measured by the furosine method (Finot et al., 1980).

RESULTS AND DISCUSSION

Identification of N^{ϵ}-Carboxymethyllysine (I). The free amino acids excreted in the urine of rats receiving the different processed food proteins described in the Experimental Section included an unknown amino acid. In the aminograms obtained by capillary GC, this amino acid eluted between lysine and lysinoalanine (compound X in Figure 1). This compound was observed in all urine samples, in concentrations strongly dependent on the nature of the dietary proteins (Table I, column 1).

The CI (CH_4) mass spectrum of compound X reproduced in Figure 2 showed a pseudomolecular ion (MH^+) at m/e 581 and two major fragment ions corresponding to successive losses of C_3H_6 molecules. Comparison with the CI spectra of usual amino acids revealed that this fragmentation pattern was specific to species containing two

Table I. CML Excretory Levels in Rat Assays

	gp dietary protein	protein intake, ppm	CML excretion relative to intake of CML,[a] %	intake of FL,[a] %	LAL,[a] %
	Assay I (20% Protein)				
1	control whey protein (WP)	12 (3)[b]	3.6 (0.8)	ND[c]	ND
2	alkali-treated WP (AL-WP)	250 (25)	11.6 (1.2)	18[b]	2.7 (0.3)
3	AL-WP (1.3%) in WP (18.7%)	18 (2)	13.1 (1.6)	12[b]	2.6 (0.3)
4	control casein (CAS)	7 (2)	9 (2)	ND	4.5 (1.1)
5	CAS + free LAL	12 (3)	15 (3)	ND	3.2 (0.8)
6	alkali-treated casein (AL-CAS)	8 (3)	5 (2)	ND	0.06 (0.02)
7	AL-CAS (0.95%) in CAS (19.05%)	5 (2)	8 (2)	ND	0.6 (0.2)
8	heated milk casein (H-CAS) (11.7%) in CAS (8.3%)	79 (21)	15 (4)	0.6 (0.2)	12 (3)
	Assay II (18% Protein)				
9	CAS	12 (1)	19.0 (1.4)	ND	10 (2)
10	Maillard casein I (4 days)	84 (13)	5.3 (0.9)	1.3 (1.0)	ND
	Assay III (17% Protein)				
11	mixed control proteins	6 (2)	10 (4)	ND	13 (5)
12	Maillard casein II (15 days) (45%) + control proteins (55%)	79 (12)	13 (2)	0.4 (0.1)	170 (25)
13	control proteins + free FL	23 (8)	40 (18)	0.6 (0.2)	50 (17)

[a] Mole to mole ratio. [b] Numbers in parentheses are standard deviations. [c] ND = not detected.

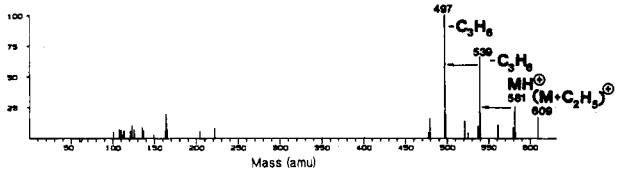


Figure 2. Chemical ionization (CH_4) mass spectrum of compound X.

isopropyl-substituted carboxylic groups. Furthermore, only the presence of two perfluoroacylated amino groups could account for the high molecular weight of compound X.

The EI spectrum of compound X, shown in Figure 3, was too complex for a complete interpretation. However, this spectrum exhibited a striking resemblance to those of N^{ϵ}-methyllysine and lysinoalanine derivatives.

In particular, fragment ions at m/e 451, 434, 259, 230, and 190 were common to the spectra of the three compounds.

On the basis of these observations, the most probable candidate for compound X appeared to be the N^{α},N^{ϵ}-diPFP isopropyl ester of N^{ϵ}-carboxymethyllysine (CML, I).

$$HO_2CCH_2NH(CH_2)_4CH(NH_2)CO_2H$$

I

An authentic sample of this compound was synthesized and submitted to derivatization and GC/MS analysis. Both mass spectra and GC retention time of the reference sample confirmed the identify of compound X with CML (Figures 4 and 5).

To our knowledge, this is the first report of the presence of CML in animal urinary excretion. However, this compound has been previously identified in the urine of various hospital patients (Wadman et al., 1975).

Origin of N^{ϵ}-Carboxymethyllysine (CML). Assuming CML to be an abnormal metabolite, Wadman et al. (1975) attempted to link its appearance in the urine of patients to some health disorder, ruling out the possibility of a dietary origin. However, their results did not permit them to relate CML excretion to any pathology.

Without ruling out the possibility that some CML was formed endogenously, the results of our assays on rats showed that the level of urinary CML was essentially related to the type of dietary protein (Table I). Some of these proteins had been exposed to an alkaline treatment and therefore contained lysinoalanine (LAL, II) or to a

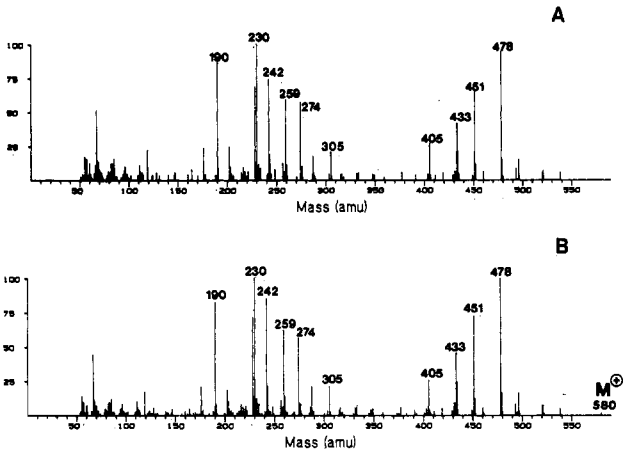
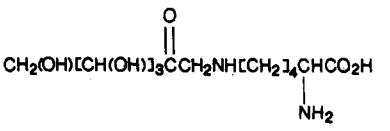
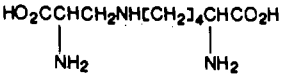


Figure 3. Electron impact mass spectra of (A) compound X and (B) reference N^{ϵ}-carboxymethyllysine (CML) diPFP isopropyl ester.



II



III

Maillard-type reaction and contained the lysine Amadori compound N^{ϵ}-fructoselysine (FL, III). Therefore, it was postulated that urinary CML could have essentially three different origins: (i) presence of this compound in the proteins as a result of the processing; (*ii*) in vivo formation through an oxidative splitting of FL sugar moiety; (iii) oxidative deamination of LAL alanine moiety followed by decarboxylation.

On the basis of data reported in Table I, column 4, it appeared that CML urinary excretion was not related to

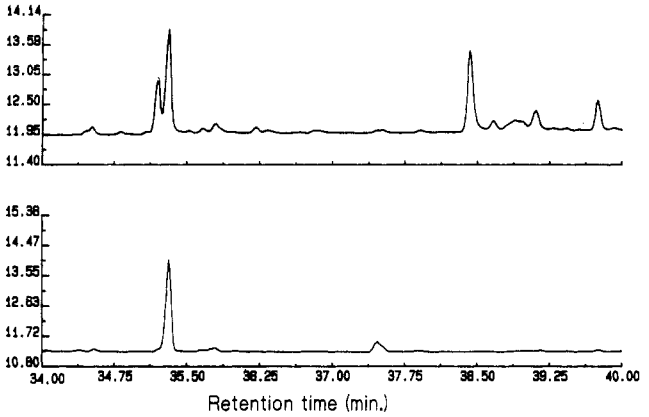


Figure 4. Partial capillary gas chromatograms: top trace, analysis of rat urine (enlarged from Figure 1); bottom trace, analysis of reference CML diPFP isopropyl ester.

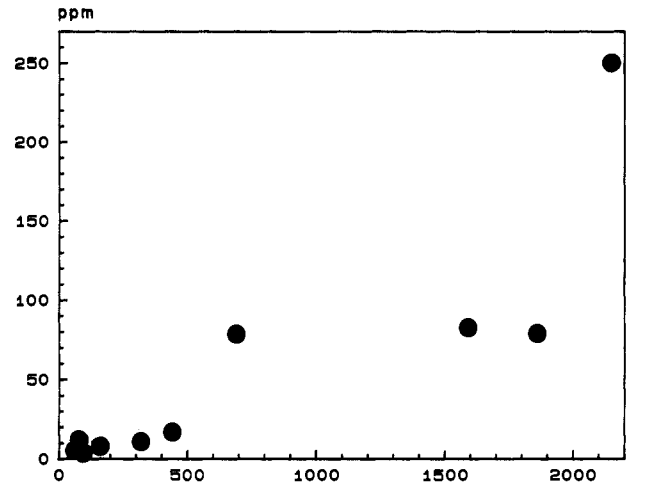


Figure 5. Relationship between the amount of excreted CML normalized to protein intake and CML content in the dietary protein.

LAL levels in the diets. Therefore, CML was probably not one of the 10 different catabolites of LAL detected by Finot et al. (1977). In the same way, there was no apparent relationship between FL ingestion and CML excretion (Table I, column 3).

Recently, Baynes et al. (1986) reported the formation of CML by the oxidative decomposition of FL. The half-life for CML formation from FL under physiological conditions (pH 7.4, 37 °C) and in the presence of air was approximately 10 days. These authors also reported an increase of CML formation rate with pH and temperature. Samples of the different proteins used in the rat assays were analyzed for their possible content in CML. The results of these determinations are reported in Table II. Indeed, CML was found in all proteins in concentration ranging from trace level (about 100 ppm) in control samples and in alkali-treated casein to 1000–2000 ppm in heated milk casein and alkali-treated lactalbumin. Interestingly, the CML to FL ratio presented considerable variations among the proteins. In agreement with the observation of Baynes et al. (1986), CML appeared to be more readily formed under alkaline conditions.

A plot of the CML excreted against the CML ingested appeared to show some kind of relationship, maybe nonlinear, but with a definite trend (Figure 5). For groups 1–12, excreted CML ranged from 4 to 19% of the intake (Table I, column 3). These results appeared to validate the hypothesis that urinary CML was mainly of exogenous origin. The variations in the excretion rate was assumed

Table II. N^{ϵ}-Carboxymethyllysine (CML), Fructosyllysine (FL), and Lysinoalanine (LAL) Contents in the Proteins Used in Rat Assays[a]

protein	CML	FL	LAL	CML/FL[b]
control casein (CAS)	80	ND[c]	170	
control whey protoen (WP)	320	ND	70	
control gluten	60	ND	0	
control zein	20	ND	0	
heated milk casein (H-CAS)	1115	19400[d]	1190	0.09
alkali-treated casein (AL-CAS)	160	ND	14800	
alkali-treated whey protein (AL-WP)	2150	2110	10540	1.55
Maillard casein (4 days)	1590	35200	170	0.07
Maillard casein (15 days)	1860	89500	170	0.03

[a] Contents are given in mg/kg or ppm. [b] Mole to mole ratio. [c] ND = not detected (detection limit 1500 ppm). [d] Lactoselysine expressed as FL equivalent.

to be due to differences in CML absorption rate depending on the nature and extent of process-induced modifications of the proteins.

The results of this study do not yet permit us to confirm or rule out the possibility of CML formation in vivo. The only indication that such a process might occur is given by the results obtained with group 13 (Table I, columns 1 and 2). This group was given control proteins enriched with free FL. A control of the synthetic FL used for this assay showed that it did not contain any CML. Yet the CML excretion level for this group was significantly higher than for group 11 that had received the same proteins. A possible explanation is that part of the absorbed FL was metabolized into CML. Alternatively, FL decomposition into CML might have occurred in the diet mix before consumption. Current investigations of the kinetics of this reaction might help in clarifying this point.

ACKNOWLEDGMENT

We are grateful to D. Furniss and C. Mettraux for their help in organizing the rat assays, to S. Ledermann for her skillful assistance in the analytical work, and R. Fumeaux for the synthesis of the reference compounds. We also thank I. Horman for a helpful reviewing of this manuscript.

LITERATURE CITED

Baynes, J. W.; Ahmed, M. U.; Fisher, C. I.; Hull, C. I.; Lehman, T. A.; Watkins, N. G.; Thorpe, S. R. In *Amino-Carbonyl Reactions in Food and Biological Systems*; Fujimaki, M., Namiki, M., Kato, H., Eds.; Elsevier: Amsterdam, 1986; p 921.

Finot, P. A. In *Modification of Proteins: Food Nutritional and Pharmacological Aspects*; Feeney, R. E., Whitaker, J. R., Eds.; American Chemical Society: Washington DC, 1982; p 91.

Finot, P. A.; Mauron, J. *Helv. Chim. Acta* **1972**, *99*, 1153.

Finot, P. A.; Magnenat, E. In *Maillard Reactions in Food: Chemical, Physiological and Technical Aspects*; Ericksson, C., Ed.; Pergamon: Oxford, 1980; p 193.

Finot, P. A.; Bujard, E.; Arnaud, M. In *Protein Crosslinking: Nutritional and Medical Consequences*; Friedman, M., Ed.; Plenum: London, 1977; p 51.

Finot, P. A.; Deutsch, R.; Bujard, E. In *Maillard Reactions in Food: Chemical, Physiological and Technical Aspects*; Ericksson, C., Ed.; Pergamon: Oxford, 1980; p 369.

Liardon, R.; Ledermann, S.; Ott, U. *J. Chromatogr.* **1981**, *203*, 385.

Matsutani, H.; Kusumoto, S.; Koizumi, R.; Shiba, T. *Phytochemistry* **1979**, *18*, 661.

Oimomi, M.; Kitamura, Y.; Nishimoto, S.; Matsumoto, S.; Hatenaka, H.; Ishikawa, K.; Baba, S. In *Amino-Carbonyl Reactions in Food and Biological Systems*; Fujimaki, M., Namiki, M., Kato, H., Eds.; Elsevier: Amsterdam, 1986; p 475.

Peret, J.; Macaire, F.; Chanez, M. *J. Nutr.* **1973**, *103*, 866.

Pintauro, S. J.; Philippossian, G.; Finot, P. A.; Lee, T. C. *Food Chem. Toxicol.* **1985**, *23*, 763.

Schleicher, E.; Wieland, O. H. *J. Clin. Chem. Biochem.* 1981, *19*, 81.
Sternberg, M.; Kim, C. Y.; Schwende, F. J. *Science (Washington, D.C.)* 1975, *190*, 992.
Wadman, S. K.; De Bree, P. K.; Van Sprang, F. J.; Kamerling, J. P.; Haverkamp, J.; Vliegenthart, J. F. G. *Clin. Chim. Acta* 1975, *59*, 313.

Received for review June 18, 1986. Revised manuscript received January 27, 1987. Accepted February 27, 1987.

Identification and Quantitation of the Alkaloids of *Lupinus latifolius*

James E. Meeker* and Wendell W. Kilgore

Lupinus latifolius is the species of lupine implicated as being responsible for a human deformity with the alkaloid anagyrine speculated to be the teratogen. Alkaloids were extracted in the present study from *L. latifolius* utilizing Soxhlet extraction. This modification proved less time consuming and significantly more efficient ($p < 0.01$) than the original method. Capillary gas chromatographic and mass spectrometric analysis of the alkaloid extract revealed six components. Anagyrine represented 86% of the total alkaloids and 1.14% of the plant dry weight. Lupanine, aphylline, and 5,6-dehydrolupanine accounted for 6.5, 3.9, and 1.9% of the total alkaloids, respectively. Aphyllidine and 4-hydroxylupanine were also identified in small quantities.

Ingestion of various species of lupine has resulted in outbreaks of acute poisoning of sheep and cattle. Couch (1926) postulated that the poisonous properties of lupines were due primarily to alkaloids. Wagnon (1960) reported that congenital crooked calf disease was suspected by California ranchers to be due to maternal ingestion of *Lupinus laxiflorus*. This congenital deformity is characterized by twisted or bowed limbs (arthrogryposis), curvature of the spine (scoliosis), twisted neck (torticollis), and/or cleft palate (Keeler et al., 1977). Shupe et al., (1967) proved that ingestion of certain species of lupines by pregnant cows resulted in crooked calf disease. Detailed studies were performed later by Keeler (1973a,b, 1976) and Keeler et al. (1976) to identify the compound responsible for the teratogenic effects of lupines. Keeler's efforts implicated the quinolizidine alkaloid anagyrine as the probable teratogen.

Kilgore et al. (1981) reported that in September 1980, a baby boy born in the mountainous back-country of northwestern California (Trinity County) was brought to the U.C. Medical Center in Sacramento with severe bilateral deformities of the distal thoracic limbs. The deformities included bilateral radial hypoplasia, curvature of the ulna, absent thumbs, and webbed fingers on one hand. On the basis of circumstantial evidence it was proposed that the child's deformities resulted from in utero exposure to anagyrine from maternal ingestion of milk from goats that had foraged on teratogenic lupines. Subsequently, Lazerson and Ortega (1984) determined that the child also suffered from red cell aplasia thought to be an anagyrine-induced stem cell defect.

The area where the goats had regularly foraged showed the presence of the perennial lupine *Lupinus latifolius*. This investigation was undertaken to identify and quantify the alkaloids present in this species of lupine.

EXPERIMENTAL SECTION

Plant Material—Collection and Preparation. Approximately 25 kg of lupine plants (*L. latifolius*) in the early preflower state was collected over an 8-h period during May 1983 in Trinity County, CA. The whole plants were spread out over a plastic tarp for 24 h prior to drying. Three portions were successively air-dried in a dehydrator at 98 °F for 24 h over a 3-day period. After air-drying, the plants were placed in large plastic bags and stored at room temperature for up to 1 week at which time they were ground with a Wiley mill containing a 2.0-mm mesh screen. The ground lupine plants were placed in plastic storage containers and stored at 6 °C.

Alkaloid Extraction. Triplicate samples of ground plant material were treated in the following manner. A 25-mL sample of 5% NH_4OH in methanol was mixed into 10 g of each sample. After air-drying, individual samples were placed in a Soxhlet extractor containing 350 mL of CH_2Cl_2. After overnight extraction the solvent containing the alkaloids was evaporated to ca. 10 mL on a rotary vacuum evaporator at 40 °C. The alkaloids were then partitioned into 150 mL of 1% H_2SO_4. The CH_2Cl_2 was discarded, and the aqueous phase was alkalinized to pH 9.5 with concentrated NH_4OH. The alkaloids were then partitioned back into 50 mL of CH_2Cl_2 followed by two additional 20-mL partitions with CH_2Cl_2. The combined CH_2Cl_2 layers were evaporated to near dryness on a rotary vacuum evaporator and transferred to test tubes for drying with a nitrogen evaporator at 40 °C. The residue was taken up in ethanol for gas chromatographic analysis.

In order to ascertain the efficiency of alkaloid extraction with the Soxhlet method, triplicate samples of ground plant material (10 g each) were also treated as described by Keeler (1973a). The only modification was that the alkaloids were repartitioned with 50 mL of $CHCl_3$ followed by two additional 20-mL partitions rather than 10 and 5 mL, respectively.

Concentration of Anagyrine Standard. Anagyrine was concentrated on a Varian Model 920 GC equipped with a 6 ft × $^1/_4$ in. (o.d.) packed stainless-steel column (10% OV-101) and a thermal conductivity detector. After injection of the plant extract, a 30-cm capillary tube, inside a Pasteur pipet, was placed in contact with the column exit port when the peak corresponding to anagyrine was recorded, for collection of the anagyrine standard. The operating conditions consisted of an injector temperature of 210 °C, column temperature of 235 °C, detector temper-

Department of Environmental Toxicology, University of California, Davis, California 95616.

0021-8561/87/1435-0431$01.50/0