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# Bound Malondialdehyde in Foods: Bioavailability of N,N′-Di-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine

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Reactions between lipid peroxidation products and proteins in foods have detrimental nutritional effects, most importantly, losses of essential amino acids. One of the major products of the reaction of malondialdehyde and alkanals with amino groups are 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde derivatives. The product of the reaction of lysine with malondialdehyde and acetaldehyde, N,N′-di-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine (MDDL), has been synthesized and used for in vitro and in vivo bioavailability studies. Release of free lysine did not occur in incubations of MDDL with tissue homogenates. After oral administration of radioactively labeled MDDL, radioactivity was only recovered in feces. Radioactivity was not incorporated into hepatic microsomes after intraperitoneal administration, which would have indicated release of available lysine. These results show that MDDL is a form of unavailable lysine, because it is not metabolized to free lysine and cannot be absorbed from the gut. Thus, formation of this derivative in foods would result in loss of available lysine.

**KEYWORDS: Food proteins; available lysine; aldehydes; malondialdehyde; alkanals; 1,4-dihydropyridine-3,5-dicarbaldehyde, lipid peroxidation**

## INTRODUCTION

The peroxidative decomposition of polyunsaturated lipids in foods has detrimental effects on the nutritional and functional properties of foods (*1*−*5*). Among the deleterious effects of lipid peroxidation is the loss of essential amino acids caused by reactions between proteins and lipid peroxidation products. Losses of available lysine due to oxidative lipid spoilage have been determined by both chemical and in vivo methods (*6*−*10*), but the chemical nature of the structures produced by reaction between proteins and lipid peroxidation products that are responsible for these losses remains to be determined.

Malondialdehyde (MDA) is one of the products of lipid peroxidation that has been more extensively studied (*11*−*15*). This aldehyde readily reacts with functional groups present in proteins, nucleic acids, and phospholipids, especially amino groups (*16*−*23*) and has been found to be a toxic, carcinogenic, and mutagenic agent (*17*, *24*−*26*). Reaction of MDA with amino groups leads to the formation of iminopropene Schiff bases (*27*), but formation of these structures is of no nutritional concern, because they are hydrolyzed at the acidic pH of the stomach. N-2-propenals are also formed by reaction of MDA with amino groups in neutral or acidic aqueous media (*20*, *28*). A third type of structures, 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde derivatives of amines, is formed when alkanals are also present and react with MDA and an amino compound such as lysine (*29*, *30*). Thus, iminopropene Schiff bases, N-2-propenals, and 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde structures are all forms of "bound" MDA that may be present in foods and tissues.

In experiments in which the presence of free and bound forms of MDA was investigated in enzymatic hydrolysates of foods of animal origin, very little free aldehyde was found, and the bound form of MDA, N-$\epsilon$-(2-propenal)lysine, was found instead (*31*). In the presence of alkanals, the reaction of MDA with amino groups to form 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde structures occurs very easily, so that these structures are most likely formed in foods as well. 4-Methyl-1,4-dihydropyridine-3,5-dicarbaldehyde is formed in the reaction of bovine serum albumin or polylysine with MDA and acetaldehyde (*32*).

We have previously reported that the bioavailability of the N-2-propenals of lysine is very low at best (*33*). We now report the investigation of the in vitro and in vivo availability of N,N′-di-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)-lysine (MDDL). Radioactive labeling was used to assess excretion and incorporation into tissues after oral administration. The derivative was also administered intraperitoneally, and incorporation of the radioactive label into liver microsomes was determined. In these experiments, incorporation and excretion of the radioactive label similar to the incorporation and excretion

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that is observed after administration of free lysine would suggest release of lysine from the administered lysine derivative. Alternatively, patterns of incorporation and excretion of radioactivity different than the ones corresponding to free lysine would indicate that the derivative is not hydrolyzed in vivo and is not a source of free lysine. In addition, the availability of lysine from MDDL has been investigated in vitro by carrying out incubations with tissue homogenates.

## MATERIALS AND METHODS

**Materials and Animals.** Diethylethoxymethylenemalonate and ϵ-N-L-lysyl-L-lysine dihydrochloride were obtained from Sigma (St. Louis, MO). MN-Kieselgel 60 for column chromatography and Allugram analytical plates with fluorescent indicator for thin-layer chromatography were purchased from Macherey and Nagel (Duren, Germany). PD-10 disposable columns were purchased from Pharmacia (Uppsala, Sweden). Ready Safe liquid scintillation fluid and BTS 450 tissue solubilizer were purchased from Beckman (Palo Alto, CA). [1,2-$^{14}$C] acetaldehyde and L-[4,5-$^{3}$H]lysine monohydrochloride were purchased from Pharmacia. The sodium salt of MDA was prepared as described by Kikugawa and Ido (*29*) using 1,1,3,3-tetramethoxypropane and Dowex 50W-X8 resin obtained from Fluka (Buchs, Switzerland).

Varian XL-200 and Bruker AC 300 instruments with tetramethylsilane as internal standard were used for nuclear magnetic resonance spectroscopy. A Bomem MB-120 instrument was used for infrared spectroscopy.

Male Wistar rats were obtained from IFFA CREDO (Lyon, France) and kept in standard conditions for at least a week before experiments.

**Preparation of MDDL.** Reaction of L-lysine monohydrochloride (1.5 mmol), MDA sodium salt (3 mmol) and acetaldehyde (2 mmol) in phosphate buffer 20 mM pH 7 (*29*) was allowed to proceed for 40 h at 37 °C. At this time, the peaks of absorbance characteristic of the 4-substituted-1,4-dihydropyridine-3,5-dicarbaldehyde structure (238, 267, and 395 nm) reached a maximum. The products of the reaction were resolved by analytical thin-layer chromatography into three bands, a major one with $R_f$ 0.65 and two minor ones with $R_f$ 0.53 and 0.43 (*n*-propanol/water (8:2) as developing solvent). These three products were fluorescent with maximum of excitation at 356 nm and absorption at 254 nm and were also revealed by the 2-thiobarbituric acid spray reagent for malondialdehyde derivatives (*31*). The product with $R_f$ 0.65 was obtained by chromatography in a MN-Kieselgel 60 column using a chloroform/methanol stepwise elution gradient (3:2, 1:1, 2:3, 1:2, 1:3; 200 mL each, per 1.5 mmol of the reagents, 11% yield). This product was identified as MDDL by $^{1}$H nuclear magnetic resonance, UV and IR spectroscopy, and by elemental analysis. $^{1}$H NMR (dimethyl sulfoxide-d6) $\delta$: 0.86 (dd, 2.58, 6.47, 6H), 1.2−2.0 (m, 6H), 3.5−3.7 (m, 4H), 3.91 (m, H), 7.33 (m, 4H), 9.19 (m, 4H). UV (ethanol) $\lambda_{max}$: 236 nm log $\epsilon$ 4.51, 265 nm log $\epsilon$ 4.23, 391 nm log $\epsilon$ 4.21. IR (KBr) $\nu$: 1661 cm$^{-1}$ (C═O aldehyde), 1568 cm$^{-1}$ (C═C). Elemental analysis expected for $C_{22}H_{25}O_6N_2Na \times 2.5H_2O$: C, 54.87; H, 6.28; N, 5.82. Found: C, 55.19; H, 5.69; N, 5.68. Melting point 172 °C. The two compounds with lower $R_f$, presumably N-α-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine and N-ϵ-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine, were found to be more unstable, and attempts to obtain them by column chromatography as described above failed.

To obtain radioactively labeled products L-[4,5-$^{3}$H]lysine monohydrochloride and [1,2-$^{14}$C]acetaldehyde were added to the reaction mixtures. Radiochemical purity of the isolated product was assessed by radioactivity counting using a TLC plate scanner. The radiochemical purity of the preparations that were administered to the experimental animals was 98% or better.

**In Vivo Studies.** Wistar rats weighing approximately 180 g were fasted overnight before the experiments and kept in individual metabolic cages for urine and feces collection. Gastric intubation of the samples diluted in 1.5 mL of water was carried out using an 18 G round-tip needle. After anesthesia with diethyl ether, blood was collected from the heart for plasma preparation and organs and tissues were removed. Intestine contents were collected by perfusion with saline. For intraperitoneal injection, the samples were diluted in 0.4 mL of saline. Microsomal fractions of fresh livers were prepared by differential centrifugation (*34*, *35*).

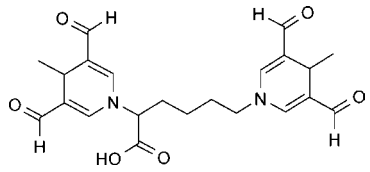

**Figure 1.** MDDL.

Samples were prepared for liquid scintillation counting according to the recommendations of the scintillation liquid manufacturer (Beckman) for the specific type of biological sample. This included treatment with hydrogen peroxide and acidification to prevent chemiluminescence. Samples of tissue, feces, plasma, digestive tract content and microsomal fractions were predigested using a quaternary base solubilizer (BTS 450 tissue solubilizer). The accuracy of the liquid scintillation measurements was assured in selected samples by repeating the determination after adding an internal standard.

**In Vitro Incubations with Tissue Homogenates.** The preparation of homogenates and the determination of lysine, lysine dipeptide, and MDDL was carried out following the same basic procedure previously described for incubations of N-ϵ-(2-propenal)lysine (*36*). Tissues for preparation of homogenates were taken from male Wistar rats weighing 200−250 g after overnight fasting. Livers and kidneys were homogenized in 10 mM Na phosphate buffer pH 7.4 containing 1 mM EDTA and $2 \times 10^{-2}$ mM butylated hydroxytoluene (*37*). Scrapings of small intestine mucosa were homogenized in 200 mM Tris-HCl buffer pH 7.6 containing 1 mM EDTA and $2 \times 10^{-2}$ mM butylated hydroxytoluene (*37*). Incubation mixtures contained 290 μL of homogenate, to which 850 nmol MDDL, lysine, or lysine dipeptide dissolved in homogenization buffer were added. Incubations were performed at 37 °C for 30 min with gentle shaking. Lysine, lysine dipeptide, and MDDL were quantified by reverse-phase HPLC after derivatization with dyethylethoxymethylenemalonate (*36*). MDDL, having both amino groups unavailable, was not modified by the precolumn derivatization with dyethylethoxymethylenemalonate and was eluted with a retention time of 15.5 min.

## RESULTS

**Incubation of MDDL with Tissue Homogenates.** MDDL (**Figure 1**) was incubated with homogenized liver, kidney, and intestinal mucosa in order to determine whether it can be hydrolyzed or somehow degraded by the action of enzymes contained in these metabolically very active organs. A procedure described by Finot et al. to study in vitro the availabiltity of diverse N-substituted lysine derivatives was used for this purpose (*37*). **Figure 2** shows the amount of free lysine that was released to the medium from MDDL and the lysine dipeptide positive control (**Figure 2A**). It also shows the amount of MDDL and free lysine and lysine dipeptide controls that remained in the homogenates after incubation (**Figure 2B**). The lysine dipeptide is completely hydrolyzed under these conditions, which serves as a positive control for release of lysine. A negative control, consisting of incubation in homogenization buffer alone, was included in these experiments as well (**Figure 2B**). The concentration of MDDL decreased by up to 35% during incubation, but this did not result in release of lysine.

**Excretion and Tissue Incorporation After Oral Administration.** MDDL was synthesized using L-[4,5-$^{3}$H]lysine monohydrochloride and [1,2-$^{14}$C]acetaldehyde in order to follow the distribution of the radioactive labels after administration to experimental animals. Using a $^{3}$H label for lysine and a $^{14}$C label for acetaldehyde would make it easier to determine hydrolysis of MDDL, which was administered by intubation into the stomach and not by mixing it into the diet to avoid

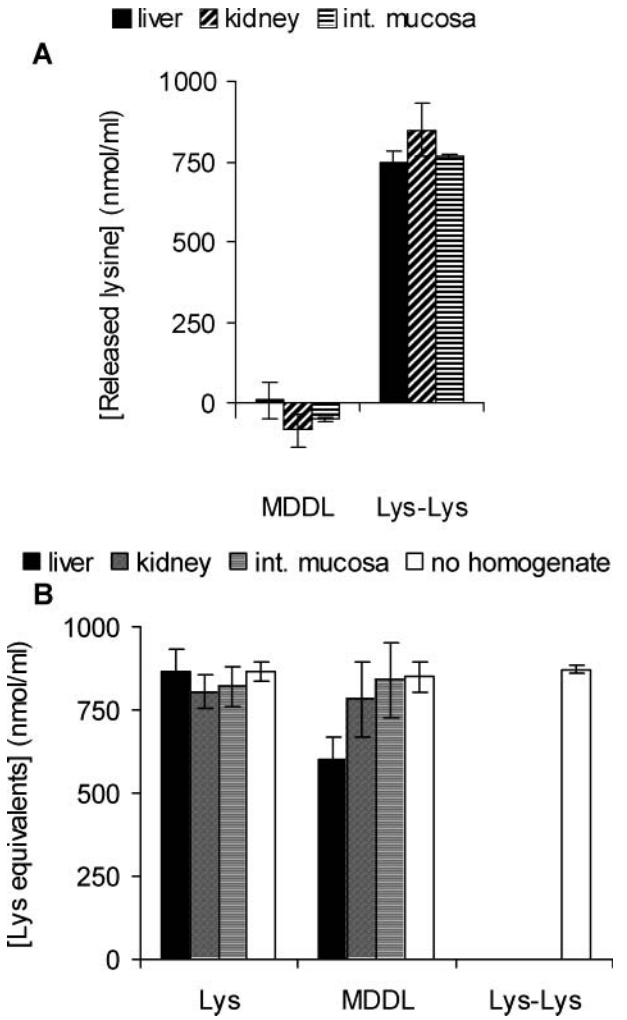


**Figure 2.** Incubations of MDDL with tissue homogenates: (**A**) Lysine released from MDDL and lysine dipeptide. (**B**) MDDL, lysine, and lysine dipeptide remaining after incubations. MDDL (850 nmol), free lysine (850 nmol), and lysine dipeptide (425 nmol) were incubated with tissue homogenates (1 mL) for 30 min at 37 °C. MDDL, lysine, and lysine dipeptide were analyzed by reverse-phase HPLC after derivatization with ethoxymethylenemalonate. Endogenous lysine was determined in blank incubations prepared using the corresponding tissue homogenates, and these values were subtracted from the total before plotting results. Data correspond to two independent experiments and the corresponding averages.

reaction with other components of the diet and to ensure consistent administration. The distribution of radioactivity in urine, feces, and tissues after administration of unaltered lysine or MDDL is shown in **Figure 3** as a percentage of administered dpm. While 32% of the radioactivity administered as lysine was recovered in tissues, 11% in urine, and 20% in feces, all the radioactivity recovered after administration of MDDL was found in feces. Recovery of the $^{14}C$ label was lower than recovery of the $^{3}H$ label, indicating that the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde moieties may have been altered to some extent, although not enough for regeneration of any form of lysine that could be absorbed from the digestive tract.

**Incorporation into Liver Microsomes After Intraperitoneal Injection.** To determine whether free lysine available for protein synthesis can be released from MDDL, radioactivity in the hepatic microsomal fraction of rats injected intraperitoneally with radiolabeled MDDL or free lysine was determined. Radioactivity in plasma and urine was determined as well

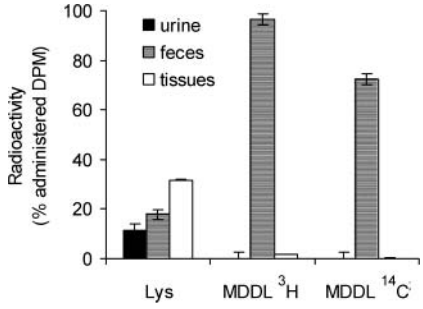


**Figure 3.** Distribution of radioactivity in urine, feces, and tissues after administration of unaltered lysine or MDDL. Radioactivity has been expressed as a percentage of the radioactivity that was administered. Lysine (12.5 $\mu$mol, $1.92 \times 10^6$ dpm) or MDDL ($3.2 \times 106$ dpm $^{3}H$, $1.2 \times 10^6$ dpm $^{14}C$) were administered by gastric intubation. Feces and urine were collected in metabolic cages for 24 h, and rats were sacrificed for tissue sampling. Data represent mean ± SEM ($n = 3$).

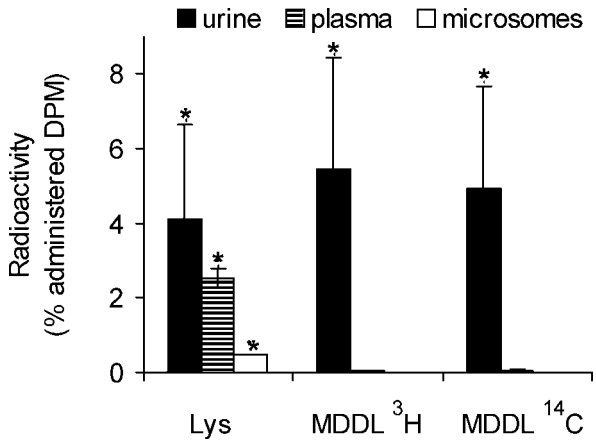


**Figure 4.** Distribution of radioactivity in urine, plasma, and microsomes after intraperitoneal injection of unaltered lysine or MDDL. Radioactivity has been expressed as a percentage of the radioactivity that was administered. Lysine (6.25 $\mu$mol, $0.96 \times 10^6$ dpm) or MDDL ($1.59 \times 106$ dpm $^{3}H$, $0.95 \times 10^6$ dpm $^{14}C$) were administered. Feces and urine were collected for 7 h, after which rats were sacrificed for extraction of liver and plasma. The whole hepatic microsomal fractions were counted. Values for plasma correspond to total plasma and were calculated assuming 3.5 mL plasma/100 g of body weight. Data represent mean ± SEM ($n = 3$). * = Statistically different than no incorporation ($p < 0.05$, one-way ANOVA followed by Tukey test).

(**Figure 4**). Administration of free lysine resulted in incorporation of 0.5% of the radioactive label in the hepatic microsomal fraction 7 h later. No radioactivity was found in the microsomes from rats injected with MDDL. Only plasma from the rats injected with free lysine presented radioactivity as well. Radioactivity in urine was the same in the two treatment groups.

## DISCUSSION

A study of the bioavailability of the N-2-propenals of lysine was recently published (*33*). N-2-propenals are derivatives of lysine that are formed in foods and in vivo and represent a bound form of MDA. The bioavailability of these derivatives in vivo and in vitro was found to be zero or very low at best, because they are readily excreted in urine (*38*−*40*) and are not hydrolyzed to release free lysine (*33*, *36*). In the present report, the bioavailability of another product of the reaction between MDA and lysine, MDDL, has been investigated. In contrast to the N-2-propenals, the presence of alkanals together with MDA and an amino compound is necessary for formation of 4-sub-

stituted-2,6-dihydropiridine-3,5-dicarbaldehyde derivatives. Although the reaction can proceed in the absence of alkanals due to formation of acetaldehyde by hydrolysis of MDA (*41*), the reaction in the absence of an alkanal proceeds very slowly. Thus, although alkanals do not react as easily as MDA with amino groups by themselves, they play an important role in the interactions of lipid peroxidation products with proteins by modulating the reactivity of MDA.

Oral administration of radiolabeled MDDL resulted in excretion of radioactive label in feces, with no radioactivity appearing in urine or tissues. Thus, MDDL was not absorbed from the gut of the experimental animals, while free lysine was absorbed and incorporated into tissues and excreted in urine. The $^{3}H$ radioactivity that was found in feces after administration of MDDL represented 96% of the administered radioactivity, but the $^{14}C$ radioactivity recovered represented only 72% of the radioactivity administered. The $^{14}C$ label correspondsto the acetaldehyde groups in the dihydropiridine rings in MDDL, while the $^{3}H$ label corresponds to the lysine moiety. Thus, a decrease in the $^{14}C$ label as compared to the $^{3}H$ label represents a loss of acetaldehyde groups. This could be due to alteration of MDDL by the intestinal microflora. Our findings indicating the stability in vivo of MDDL are consistent with the study by Yoden et al. (*42*), in which the metabolism of a series of compounds containing the 4-substituted-2,6-dihydropiridine-3,5-dicarbaldehyde group was investigated. Their results indicate that although the most hydrophobic of these compounds could be hydrolyzed to water soluble forms in incubations with rat microsomes, the 4-substituted-2,6-dihydropiridine-3,5-dicarbaldehyde groups remained unaltered.

The data presented here show that MDDL, besides not being absorbed from the gut, cannot be hydrolyzed in tissues to free lysine. This is demonstrated by the experiments in which MDDL was incubated with tissue homogenates obtained from liver, kidney and intestinal mucosa, and by experiments in which MDDL was injected intraperitoneally to bypass the gut barrier. A small amount of MDDL was lost during incubation with tissue homogenates, although this loss did not represent hydrolysis with release of free lysine. This loss is most likely explained by reaction of MDDL with amino groups present in the homogenates, belonging mostly to proteins. The 4-substituted-1,4-dihydropyridine-3,5-dicarbaldehyde groups have two aldehyde groups that can react with other amino groups, causing cross-linking of proteins (*43*). Thus, enzymatic activities present in tissues do not hydrolyze MDDL to free lysine or to any other form of available lysine. These experiments are of interest because, in addition to the likely presence of 4-substituted-1,4-dihydropiridine-3,5-dicarbaldehyde derivatives of lysine in foods, this type of structures is probably formed in vivo as well (*43*). It has been shown that 4-methyl-1,4-dihydropiridine-3,5-dicarbaldehyde derivatives of proteins are formed during excessive ethanol consumption and that this structure is highly immunogenic and toxic (*44*). Our results indicate that the 4-substituted-1,4-dihydropiridine-3,5-dicarbaldehyde structure is very stable in vivo. Thus, modification of proteins by formation of these structures is most likely irreversible and would have to be repaired by recycling of the affected proteins. The same conclusion was drawn for the modification of amino groups in proteins by formation of N-2-propenals (*33*).

Despite causing a loss of available lysine, formation of 1-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde structures in foods might have beneficial effects. Thus, this type of structure can inhibit the peroxidative decomposition of polyunsaturated lipids in foods as demonstrated in fish microsomes (*45*) and oil (*46*) oxidation systems. In these studies, protection was achieved against lipid peroxidation and protein oxidation induced by a variety of systems.

## ABBREVIATIONS USED

MDA, malondialdehyde; MDDL, N′-di-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine.

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