Effect of Sterilization of Milk on Vitamin B-6 Composition and Bioavailability†

Arunabala V. Pingali and Paula R. Trumbo*

Department of Foods and Nutrition, Purdue University, West Lafayette, Indiana 47907

To assess the effect of heat sterilization of milk on vitamin B-6 composition and bioavailability, rats were orally administered [^{3}H]pyridoxine (PN), [^{3}H]-4-pyridoxic acid (4-PA) or [^{3}H]pyridoxal (PL) in water, or [^{3}H]PL in fresh milk with or without subsequent sterilization at 120 °C for 20 min. [^{3}H]PL in sterilized milk underwent partial conversion to pyridoxamine (PM) and 4-PA, as well as irreversible binding to protein. [^{3}H]PL in water and unheated milk was well absorbed and similar to [^{3}H]PN. The intestinal absorption of ^{3}H from sterilized milk and [^{3}H]4-PA was significantly reduced compared to [^{3}H]PN or [^{3}H]PL. The results of this study indicate that heat sterilization of milk reduces vitamin B-6 activity by irreversible binding to protein, as well as by partial formation of 4-PA, a nonbiologically active form of vitamin B-6.

In the early 1950s, there were reports suggesting that infants suffering from convulsive seizures after consuming a sterilized liquid milk formula were cured upon pyridoxine supplementation (Coursin, 1954; Gyorgy, 1954; Snyderman et al., 1953). Although no significant losses of vitamin B-6 in milk have been observed as a result of pasteurization, ultra high temperature sterilization, or spray drying, a marked reduction in total vitamin B-6 has been demonstrated in heat sterilized milk products and liquid food model systems (Hassinen et al., 1954; Gregory and Hiner, 1983; Gregory et al., 1986). This loss in vitamin B-6 activity can continue up to 10 days after sterilization (Hassinen et al., 1954). Furthermore, reduced weight gain has been observed when rats were fed sterilized milk compared to fresh and spray dried milk (Tomarelli et al., 1955).

Reduced vitamin B-6 activity was suggested to be the result of the formation of an unknown form of the vitamin in milk which demonstrated minimal vitamin B-6 activity based upon microbiological growth (Hodson, 1956; Gregory, 1959). Pyridoxal (PL), the major form of vitamin B-6 in milk, was reported to bind to cysteine (Bernhart et al., 1960; Srncova and Davidek, 1972) and lysine residues (Gregory and Kirk, 1977) in heated milk or liquid food model systems. Pyridoxyllysine complexes were shown to possess 50% molar vitamin B-6 activity based on rat bioassays (Gregory, 1980). It has been suggested that the aldehyde moiety of vitamin B-6 may interact with amino acids in the intestinal lumen resulting in reduced absorption of the vitamin (Middleton, 1990). Recently, it has been reported that 4-pyridoxic acid (4-PA) is the second most abundant vitamin B-6 compound in milk (Bitsch and Moellar, 1989). 4-PA, which can be produced by the oxidation of PL, does not exhibit vitamin B-6 activity based on various microbiological growth assays (Hodson, 1956).

There is limited information regarding the effect of heating on the bioavailability of vitamin B-6 in milk which is of nutritional significance. In addition, further knowledge of the causes of reduced vitamin B-6 activity after sterilization of milk can provide information for the development of processing procedures for nutrient- and shelf-stable milk products. The present research employed [^{3}H]PL to directly assess the effect of sterilization of milk on the intestinal absorption and metabolic utilization of vitamin B-6.

MATERIALS AND METHODS

Reagents. [^{3}H]Pyridoxine (PN) hydrochloride (3.75 Ci/mmol) was purchased from Amersham Inc. (Arlington Heights, IL). The distribution of tritium reported by the manufacturer was as follows: methyl, 61.9%; 5-methylene, 2.0%; 4-methylene, 21.9%; C-6, 11.9%.

Preparation of [^{3}H]PL and [^{3}H]4-PA. [^{3}H]PL and [^{3}H]4-PA were produced by reacting [^{3}H]PN with MnO_2 in KH_2PO_4 buffer, pH 5.5 for 0.5 and 4 h, respectively, and purified by reverse-phase HPLC (Trumbo and Raidl, 1991).

Test Sample Preparation. Test samples to be orally administered were [^{3}H]PN, [^{3}H]PL, or [^{3}H]4-PA diluted in H_2O (1.5 μCi/mL) and [^{3}H]PL diluted in milk (1.5 μCi/mL), with or without subsequent heating at 121 °C for 20 min. After a 30-min waiting period for cooling of the chamber to 100 °C, the milk sample was cooled at 4 °C.

Animals and Diets. Twenty male rats weighing approximately 50 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All rats were housed individually in stainless steel metabolism cages and fed ad libitum a nonpurified diet (Ralston Purina Rodent Laboratory Chow 5001, St. Louis, MO) for 2 days for acclimatization. After an overnight fast, rats were orally administered one of the five test samples (1.4 ± 0.2 μCi). A 48-h fecal collection and two 24-h urine collections were conducted. Rats were euthanized 48 h after administration of the isotope. Muscle, liver, brain, and the remaining carcass were collected and stored at −20 °C until later analysis.

Analytical Methods. Chromatographic analyses were conducted with a Rainin HP drive module (Rainin Instrument Co., Woburn, MA), sample injection valve (Rheodyne, Model 7125), a fluorescence detector (Model LS 40, Perkin-Elmer, Norwalk, CT), a Dynamax HPLC Method Manager Integration Software Program, and a MacIntosh SE computer.

Urine samples were deproteinated by ultrafiltration with micropartition tubes and YMT membrane filters (Amicon, Danvers, MA). Urine 4-pyridoxic acid was isolated and collected by reversed-phase HPLC employing a mobile phase consisting of 0.1 N formic acid and a Partisil ODS-3 column (Whatman) (Trumbo and Gregory, 1988). The wavelengths for fluorometric detection were 295 nm for excitation and 405 nm for emission.

The portion of ^{3}H which was irreversibly bound to protein was determined (Gregory et al., 1986). An equal volume of trichloroacetic acid (TCA) (14%) was added to the milk samples. After centrifugation, the pellet was washed twice with TCA and dissolved in Scintigest (Fisher Scientific Co.). Aliquots of the protein digests and the supernatants were counted to determine radioactivity.

* Author to whom correspondence should be addressed.

† Journal Paper 13321 of the Agricultural Experiment Station, Purdue University, West Lafayette, IN 47907.

0021-8561/92/1440-1860$03.00/0

Table I. Distribution of ^{3}H-Labeled Vitamin B-6 Compounds in Test Samples Orally Administered to Rats

sample	treatment, °C	% distribution PN	PL	PM	4-PA	other[a]	% bound to protein
PN/water	25	98				2.0	ND[b]
PL/water	25		100				ND
PL/milk	25		98.8	1.2			
PL/milk	121		59.1	23.7	11.3	5.9	16.9
4-PA/water	25			0.4	97.6		ND

[a] Unidentified peaks which eluted prior to the vitamin B-6 compounds. [b] ND = not determined.

For HPLC analysis, milk test samples and feces were homogenized with deionized water. An equal volume of TCA (14%) was added, followed by centrifugation for protein precipitation. The supernatant was extracted twice with an equal volume of ethyl ether. Vitamin B-6 analysis of the feces and test samples was conducted using a gradient elution method (Trumbo and Raidl, 1991) consisting of two mobile phases and a C_{18}-IP Ultrasphere column (Beckman). Mobile phase A consisted of 8 mM octanesulfonic acid and 0.033 M phosphoric acid, pH 2.2. Mobile phase B consisted of 17% 2-propanol and 0.033 M phosphoric acid, pH 2.2.

For measurement of total radioactivity, fecal and tissue samples were homogenized in water and the total homogenate volumes were recorded. Fecal and tissue homogenates, milk samples, and urine 4-PA, fecal, and test sample HPLC fractions were measured for radioactivity in scintillation fluid employing a liquid scintillation spectrophotometer (Beckman LS 1800). A quench curve for tritium was used to correct for quenching. Radioactivity of tissue samples was expressed as percent of oral dose administered. Urinary [^{3}H]4-PA was expressed as percent of total radioactivity in urine. Distribution of radioactivity in the fecal and test samples was expressed as the percent distribution of ^{3}H-labeled vitamin B-6 compounds or percent of ^{3}H irreversibly bound to milk protein.

Statistical Analysis. Mean values were analyzed by one-way analysis of variance and the Student–Newman–Keuls multiple comparisons (SAS Institute, Inc., 1988).

RESULTS AND DISCUSSION

Distribution of Radioactivity in Test Samples. Pyridoxine (PN) is well absorbed by a nonsaturable process (Henderson, 1985); therefore, PN was used as a reference form of vitamin B-6 in this study. Because PL is the major form of vitamin B-6 in milk, milk was extrinsically labeled with [^{3}H]PL for direct monitoring of the conversion to other vitamin B-6 compounds, as well as bioavailability. The sterilization procedure employed in this study is similar to that used in batch sterilization and in-can sterilization of milk (Rolls, 1982). As shown in Table I, the purity of [^{3}H]PN and [^{3}H]PL was 98% and 100%, respectively, when orally administered in water. There was slight conversion of [^{3}H]PL to [^{3}H]pyridoxamine ([^{3}H]-PM) in milk at 25 °C. Heat sterilization of milk resulted in significant formation of [^{3}H]PM. PM formation can occur in milk because transamination between PL and amino acids is increased with heating (Gregory, 1959). Similar to our findings, when PL was added to milk, 40% of PL was converted to PM after heat sterilization (Gregory, 1959). Furthermore, when a liquid food model system was fortified with PL, a 20% increase in PM was observed within 40 min of heating at 118 °C (Gregory and Hiner, 1983). PM has been reported to be well absorbed (Henderson, 1985) and to exhibit similar biological activity compared to PL (Nguyen et al., 1983). Therefore, the formation of PM after heat sterilization of milk should not contribute to reduced vitamin B-6 activity.

After heat sterilization of milk, approximately 11% of the total radioactivity was 4-PA (Table I). PL can be chemically oxidized to 4-PA in the presence of an oxidizing agent and is accelerated in an acidic environment (Coburn et al., 1982). The pH of milk is reduced with heating (Walstra and Jenness, 1984). The extent of PL oxidation to 4-PA in milk may differ as a result of variations in sterilization procedures, oxygen head space, and shelf life. Such factors have been suggested to influence reported differences in the loss of vitamin B-6 after sterilization (Chapman et al., 1957). Recently, it was reported that 4-PA accounted for as much as 26% of the total vitamin B-6 in raw milk; however, the level of 4-PA decreased after heat sterilization (Bitsch and Moellar, 1989). It is possible that 4-PA can be converted to 4-PA lactone upon heat sterilization because the lactone is formed from 4-PA at high temperatures and in an acidic environment. In the present study, [^{3}H]4-PA lactone was not detected. Past studies have typically employed microbiological growth assays for quantifying the level of vitamin B-6 in milk. 4-PA does not exhibit vitamin B-6 activity based on microbiological growth assays (Hodson, 1956). Thus, the formation of 4-PA may contribute, in part, to the reduced vitamin B-6 activity that has consistently been observed in sterilized milk.

Various researchers have suggested that heat-induced interactions of PL or pyridoxal phosphate (PLP) with food proteins or amino acids may contribute to reduced vitamin B-6 bioavailability (Hassinen et al., 1954; Tomarelli et al., 1955; Srncova and Davidek, 1972; Gregory and Kirk, 1977; Gregory et al., 1986). Pyridoxyllysine complexes were shown to possess 50% molar vitamin B-6 activity based on rat bioassays (Gregory, 1980). In our study, approximately 17% of ^{3}H was irreversibly bound to milk protein after heat sterilization (Table I). Gregory et al. (1986) reported 22.5% binding of ^{3}H to protein after heat

Table II. Recovery and Isotopic Retention of ^{3}H in Tissues and Excreta[a-c]

	sample (temp) PN/water (25 °C)	PL/water (25 °C)	PL/milk (25 °C)	PL/milk (120 °C)	4-PA/water (25 °C)
	Percent of Oral Dose				
feces	3.9 ± 1.1*	3.4 ± 0.9*	4.3 ± 0.6*	18.1 ± 4.9	35.2 ± 8.1
brain	0.3 ± 0.3*	0.4 ± 0.1*	0.4 ± 0.3*	0.1 ± 0.1*	ND
muscle	3.2 ± 0.1*	2.7 ± 0.2*	2.9 ± 0.4*	2.0 ± 0.5	ND
liver	4.4 ± 2.6*	4.6 ± 1.4*	6.9 ± 0.9*	4.1 ± 1.9*	ND
carcass	50.4 ± 8.5*	46.9 ± 18.3*	41.8 ± 19.3*	26.9 ± 9.6	ND
urine					
24 h	6.7 ± 2.6*	9.5 ± 1.5*	7.4 ± 2.6*	8.1 ± 2.4*	6.6 ± 2.3*
48 h	3.4 ± 0.8*	3.2 ± 1.5*	2.0 ± 0.5*	2.7 ± 1.2*	0.6 ± 0.2*
	Percent of Urinary Radioactivity Excreted as [^{3}H]4-PA				
24 h 4-PA	9.2 ± 3.6*	12.7 ± 1.7*	12.6 ± 3.8*	13.3 ± 4.4*	ND

[a] Values are means ± SD of five to seven rats. [b] Values within each row followed by an aterisk were not significantly different ($P > 0.05$). [c] ND = not determined.

Table III. Relative Concentration of Radiolabeled Forms of Vitamin B-6 in Feces of Rats Orally Administered Sterilized Milk Containing [^{3}H]PL[a]

percent distribution		
4-PA	PL	PM
49.1 ± 9.5	23.8 ± 14.3	17.8 ± 8.3

[a] Values are means ± SD of five rats.

sterilization of evaporated milk extrinsically labeled with [^{3}H]PN, and the loss of total vitamin B-6 was 57.8%.

Intestinal Absorption of Radiolabeled Test Samples. Based on the level of radioactivity excreted in the feces 48 h after administration of the tritiated test samples (Table II), PL in water was well absorbed (>95% of oral dose) and similar to that of PN. PN and PL were shown to be well absorbed based on rat intestinal perfusion assays and employing [^{3}H]PL and [^{3}H]PN (Henderson, 1985).

The intestinal absorption of pyridoxal phosphate (PLP) was reported to be impaired when rats were intestinally perfused increasing concentrations of amino acids (Middleton, 1990). Based on this observation, it was suggested that conjugation of the aldehyde moiety of PLP with amino acids in the intestine may be a cause for reduced intestinal absorption of PLP. In the present study, [^{3}H]PL in unheated milk was well absorbed (≥95%) and similar to [^{3}H]PN (Table II). Thus, the absorption of PL is not impaired by the presence of other nutrients in unheated milk.

The intestinal absorption of [^{3}H]vitamin B-6 was reduced by approximately 14% when milk was heat sterilized (Table II). The distribution of radioactivity in the feces from rats administered the heated milk samples was analyzed to determine which vitamin B-6 compounds were poorly absorbed (Table III). 4-PA was the major form (49%) of [^{3}H]vitamin B-6 present in the fecal material, suggesting that this form was not well absorbed. To confirm that 4-PA was poorly absorbed, rats were administered [^{3}H]4-PA. Based on the level of radioactivity secreted in the feces of rats orally administered [^{3}H]4-PA, only 65% of the oral dose was absorbed, which was significantly lower compared to PN and PL (Table II).

Because [^{3}H]PL was shown to be well absorbed in water, the presence of [^{3}H]PL in the feces from rats administered heat-sterilized milk (Table III) may possibly be a result of complex formation with other nutrients such as cysteine and lysine which were disrupted during extraction with TCA for HPLC analysis. PL and PLP can complex with protein in either a reversible or an irreversible manner (Gregory et al., 1986). Schiff base formation between the carbonyl group of PL or PLP and an amino group can occur at neutral and alkaline environments (Metzler, 1957). This bond is unstable under acidic conditions unless further converted to an acid-resistant pyridoxylamino complex (Snell and Rabinowitz, 1948).

[^{3}H]PM was also detected in the feces of rats administered heat-sterilized milk (Table III). Because PM has been shown to be well absorbed (Henderson, 1985), the amine moiety of PM may react with carbonyl-containing compounds to form conjugates which may be poorly absorbed.

Radioactivity in Tissues and Urine. Regardless of the test sample that was administered, the carcass contained the majority of radioactivity. Although heat sterilization of milk reduced the intestinal absorption of [^{3}H]vitamin B-6, there was no difference in the level of radioactivity present in the muscle, brain, or liver from rats administered the different test samples. Reduced intestinal absorption of radioactivity from the heated milk sample resulted in reduced radioactivity in the carcass. The lack of significant difference with respect to carcass radioactivity is due to experimental imprecision rather than similar in vivo retention.

Approximately 10% of the oral dose absorbed was excreted in the urine 48 h after administration of the test samples (Table II). There was no treatment effect on the extent of urinary excretion of the isotope. 4-PA is the irreversible product of vitamin B-6 metabolism; therefore, urinary 4-PA can be used for assessment of vitamin B-6 metabolic utilization. Approximately 9–13% of the radioactivity in urine was detected as 4-PA for all treatment groups measured, indicating no difference in the metabolic utilization of absorbed [^{3}H]PL and [^{3}H]PN. The lack of difference in urinary excretion of radioactivity in rats administered [^{3}H]4-PA compared to the other test samples was surprising. Because 4-PA is not metabolically utilized or converted to biologically active vitamin B-6 compounds, it was expected to be rapidly excreted in the urine. The urinary [^{3}H]4-PA data from rats administered heat-sterilized milk are difficult to interpret because the portion of the [^{3}H]4-PA that was absorbed from the milk sample would be expected in the urine without participation in vitamin B-6 metabolism.

Conclusion. Results of this study demonstrate that heat sterilization of milk induced the oxidative formation of 4-PA from PL, as well as the irreversible binding to protein. The formation of 4-PA during sterilization of milk is of nutritional significance because 4-PA exhibits no vitamin B-6 activity. In addition, heat sterilization of milk resulted in reduced intestinal absorption of vitamin B-6. Further research is being conducted to determine the effect of heat pasteurization of milk on vitamin B-6 composition and utilization.

LITERATURE CITED

Bernhart, F. W.; D'Amato, E.; Tomarelli, R. M. The vitamin B-6 activity of heat-sterilized milk. *Arch. Biochem. Biophys.* **1960**, *88*, 267–269.

Bitsch, R.; Moellar, J. Evaluation of the vitamin B-6 content in foods by HPLC analysis. In *Nutrient Availability: Chemical & Biological Aspects*; Southgate, D., Johnson, I., Fenwick, G. R., Eds.; Royal Society of Chemistry: Cambridge, 1989; No. 72, Part 3.

Chapman, H. R.; Ford, J. E.; Kon, S. K.; Thompson, S. Y.; Rowland, J. Further studies of the effect of processing on some vitamins of the B-complex in milk. *J. Dairy Res.* **1957**, *24*, 191–197.

Coburn, S. P.; Lin, C. C.; Schaltenbrand, W. E.; Mahuren, J. D. Synthesis of deuterated vitamin B-6 compounds. *J. Labelled Compd. Radiopharm.* **1982**, *19*, 703–716.

Coursin, D. B. Convulsive seizures in infants with pyridoxine deficient diet. *J. Am. Med. Assoc.* **1954**, *154*, 406–408.

Gregory, J. F. Effects of pyridoxyllysine bound to dietary protein on the vitamin B-6 status of rats. *J. Nutr.* **1980**, *110*, 995–1005.

Gregory, J. F.; Kirk, J. R. Interaction of pyridoxal and pyridoxal phosphate with peptides in a food model system during thermal processing. *J. Food Sci.* **1977**, *42*, 1554–1558.

Gregory, J. F.; Hiner, M. E. Thermal stability of vitamin B6 compounds in food liquid model systems. *J. Food Sci.* **1983**, *48*, 1323–1327.

Gregory, J. F.; Ink, S. L.; Sartain, D. B. Degradation and binding to food proteins of vitamin B-6 compounds during thermal processing. *J. Food Sci.* **1986**, *51*, 1345–1351.

Gregory, M. E. The effect of heat on the vitamin B-6 in milk. I. Microbiological tests. *J. Dairy Res.* **1959**, *26*, 203–214.

Gyorgy, P. Vitamin B-6 in human nutrition. *J. Clin. Nutr.* **1954**, *2*, 44–46.

Hassinen, J. B.; Durbin, G. T.; Bernhart, F. W. The vitamin B-6 content of milk products. *J. Nutr.* **1954**, *53*, 249–257.

Henderson, L. M. Intestinal absorption of B-6 vitamers. In *Vitamin B-6: Its Role in Health and Disease*; Leklem, J. E., Reynolds, R. D., Eds.; Liss: New York, 1985.

Hodson, A. Z. Vitamin B-6 in sterilized milk and other milk products. *J. Agric. Food Chem.* **1956**, *4*, 876–881.

Metzler, D. E. Equilibria between pyridoxal and amino acids and their imines. *J. Am. Chem. Soc.* **1957**, *79*, 485–490.

Middleton, H. M. Intestinal hydrolysis of pyridoxal 5-phosphate in vitro and in vivo in the rat. *Dig. Dis. Sci.* **1990**, *35*, 113–120.

Nguyen, L. B.; Hiner, M. E.; Litherland, S. A.; Gregory, J. F. Relative biological activity of nonphosphorylated vitamin B-6 compounds in the rat. *J. Agric. Food Chem.* **1983**, *31*, 1282–1287.

Rolls, B. A. Effect of processing on nutritive value of food: Milk and milk products. In *Handbook of Nutritive Value of Processed Food*; Rechcigl, M., Ed.; CRC Press: Boca Raton, FL, 1982; Vol. 1.

SAS Institute Inc. The ANOVA procedure. In *SAS User's Guide*; SAS Institute: Cary, NC, 1985; pp 113–135.

Snell, E. E.; Rabinowitz, J. C. The microbiological activity of pyridoxylamino acids. *J. Am. Chem. Soc.* **1948**, *70*, 3432–3434.

Snyderman, S. E.; Holt, L. E.; Carretero, R.; Jacobs, K. Pyridoxine deficiency in the human infant. *J. Clin. Nutr.* **1953**, *1*, 200–207.

Srncova, V.; Davidek, J. Reaction of pyridoxal with milk serum proteins. *J. Food Sci.* **1972**, *37*, 310–312.

Tomarelli, R. M.; Bernhart, F. W. Biological availability of vitamin B-6 of heated milk. *J. Agric. Food Chem.* **1955**, *3*, 338–341.

Trumbo, P. R.; Gregory, J. F. Metabolic utilization of pyridoxine-β-glucoside in rats: Influence of vitamin B-6 status and route of administration. *J. Nutr.* **1988**, *118*, 1336–1342.

Trumbo, P. R.; Raidl, M. A. Effect of sulfasalazine on vitamin B-6 status and metabolism in vitamin B-6 adequate and deficient rats. *Nutr. Res.* **1991**, *11*, 53–60.

Walstra, P.; Jenness, R. H. Heating. In *Dairy Chemistry and Physics*; Wiley: New York, 1984; pp 162–184.

Received for review February 13, 1992. Revised manuscript received June 11, 1992. Accepted July 2, 1992.

Registry No. 4-PA, 82-82-6; PL, 66-72-8; PM, 85-87-0; vitamin B-6, 8059-24-3.