Biological Availability of Vitamin B_6 of Heated Milk

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Heat sterilization of liquid milk products results in a loss of vitamin B_6 as determined by a microbiological assay. A rat growth procedure was adapted for analysis of milk samples by the use of a semisynthetic basal diet with a composition simulating milk. The biological assay of heat-sterilized liquid milk yielded vitamin B_6 values that were lower than those obtained by the microbiological method. The bioassay and the microbiological method were in agreement in the assay of spray-dried milk.

The occurrence of vitamin B_6 deficiency in humans has only recently been established. Deficiencies have been produced experimentally by the feeding of restricted diets to infants (*18*) and by the administration of a vitamin B_6 antimetabolite to adults (*20*). A biochemically demonstrable vitamin B_6 deficiency has been found in pregnant women (*21*). Hunt and coworkers (*9*) have described an infant with a severe convulsive disorder which could be entirely controlled by pyridoxine administration. A convulsive syndrome that occurred in a small percentage of infants fed a commercial sterilized liquid milk formula was promptly alleviated by pyridoxine administration (*4, 6*). This syndrome was never observed in infants fed the same formula marketed as a spray-dried powder (*6*).

A survey of the vitamin B_6 content of processed milks by microbiological assay disclosed that the heat treatment employed in the food industry for the sterilization of canned liquid milk products destroyed vitamin B_6 to an extent hitherto unappreciated (*7*). A rat growth assay for the vitamin B_6 content of an infant food formula revealed that while the values obtained for the spray-dried product agreed with the results obtained by microbiological assay, the values for the sterilized liquid product were definitely lower. This discrepancy led to the experiments, herein reported, on the effect of heat on the biological availability of the vitamin B_6 of milk. That the vitamin B_6 content of processed food is not adequately assessed by a microbiological assay has been previously indicated by the studies of Register and coworkers (*13*) and Tappan and coworkers (*19*).

Experimental Methods and Results

Bioassay Procedure

The assay procedure was patterned after that of Sarma, Snell, and Elvehjem (*14*). Weanling rats were partially depleted of vitamin B_6 by feeding a basal diet of purified ingredients for a 2-week period. The weight gain during the depletion period averaged about 45 grams. The more typical rats on the basis of weight gains were divided into groups. One group continued on the basal diet; other groups received varying amounts of vitamin B_6 in the form of pyridoxine hydrochloride or heated or unheated milk added to the basal diet. A satisfactory growth response curve was obtained with concentrations of 25 to 100 γ of pyridoxine per 100 grams of diet.

Sprague-Dawley weanling male rats were used in all experiments. They were housed individually in screen-bottomed cages in a constant temperature animal room. Food and water were given ad libitum. The rats were weighed twice weekly and diet consumption per group was recorded.

Composition of Basal Diet

Investigations on the influence of dietary carbohydrate on the growth of rats (*14*) and chicks (*22*) fed diets containing suboptimal levels of vitamin B_6 have revealed that dextrin and lactose support a greater rate of gain than do the more soluble sugars, glucose or sucrose. Pyridoxal and pyridoxamine, the forms of the vitamin present in milk (*12*), are not so active as pyridoxine, when incorporated into diets containing soluble sugars, presumably because of a preferential utilization of the former compounds by intestinal bacteria (*10*). The three forms of the vitamin were of equal activity when mixed into a diet containing dextrin (*14*). To avoid the possible complication which might result from the high percentage of lactose in the milk products tested, the basal diet of the present study was formulated to approximate the composition of cow's milk; thus the incorporation of the test samples would effect only slight qualitative or quantitative changes in the gross composition. The composition of the vitamin B_6-low basal diet is presented in Table I. The salt mixture was based on the data of Macy and coworkers (*11*) with the further addition of iron, copper, and manganese salts. The fat-soluble vitamins were supplied by a corn oil solution containing 15,000 units of vitamin A, 2200 units of vitamin D, and 15 mg. of α-tocopherol per gram; each rat received 2 drops twice weekly.

All diets were fed as dry powders. The liquid milk samples were either spray- or roll-dried, treatments which cause only a slight loss of vitamin B_6

Table I. Composition of Basal Diet

	Grams		Grams
Lactose, U.S.P.	375	$CaHPO_4$	13.51
Casein, vit. test[a]	258	$MgHPO_4$	7.94
Butter oil, washed	297	K_2HPO_4	17.77
	Mg.		
Thiamine HCl	5	K citrate.H_2O	4.28
Riboflavin	5	Na citrate.$2H_2O$	19.61
Calcium pantothenate	50	$CaCl_2$	9.20
Niacin	50	Ca lactate.$5H_2O$	20.77
Inositol	100	$FeSO_4.7H_2O$	0.2
p-Aminobenzoic acid	100	$CuSO_4.5H_2O$	0.06
Choline chloride	1000	$MnSO_4.H_2O$	0.06
2-Methylnaphthoquinone	5		
Folic acid	2		
Biotin	0.5		
Vitamin B_{12}	0.05		

[a] General Biochemicals Inc.

(5, 7). The unheated milk samples were taken from a large pool of fresh milk. The heat-sterilized milk samples were commercial evaporated milk purchased at a local market. Commercial practice in the sterilization of liquid milk products employs temperatures of 240° to 250° F. for periods of 5 to 30 minutes. The vitamin B_6 contents of the milk samples and the various batches of the basal diet were determined by at least seven repeated microbiological assays using the *S. carlsbergensis* method of Atkin and coworkers (1, 2). All values are expressed as micrograms of pyridoxine hydrochloride.

Student's t test was used for the calculation of statistical significance of the differences in weight gain (15); a probability value of 0.05 or lower was considered necessary for significance.

Experiment 1. The results of this bioassay are presented in Table II. The heat-sterilized milk sample consisted of canned evaporated milk that had been stored one year at room temperature. The supplements were mixed into the diet to supply, per 100 grams, 25, 50, 75, and 100 γ of vitamin B_6 activity as determined by the microbiological assay. The growth response data of the groups fed pyridoxine hydrochloride, pyridoxal phosphate, and unheated milk were essentially similar. The response produced by the heat-sterilized milk was inferior to that of the other three supplements, especially at the lower concentration of vitamin B_6 intake. The microbiological and biological assays were in agreement in the assay of the unheated milk; the biological method yielded a lower value than the microbiological for the heat-sterilized milk sample. The difference in weight gains between the heat-sterilized milk groups and either the pyridoxine hydrochloride or pyridoxal phosphate groups was statistically significant at levels of 25 and 75 γ but not at 50 or 100 γ per 100 grams of diet. Considerable variation was found in the weight gains of the rats within a group; in subsequent assays the number of rats fed the depletion diet was increased from 10% to 30% in excess of that required for the assay, and eight rats per assay group were used instead of seven.

With the particular diet composition used in this study, pyridoxal phosphate and pyridoxine hydrochloride were of equal activity, thus justifying the use of the latter for a standard in the assay of milk samples.

The data of Table II also show that with increased amounts of vitamin B_6 in the diet there was an increase in both food consumption and food efficiency—i.e., grams of growth per gram of food.

Experiment 2. The comparison of heat-sterilized and unheated milk as sources of biologically available vitamin B_6 was repeated. A second brand of evaporated milk was purchased locally, and not subjected to further storage. Heat-sterilized liquid and spray-dried varieties of commercial infant formula which had been fortified with pyridoxine hydrochloride were also assayed in this experiment. Samples were fed at levels of 30 and 60 γ of vitamin B_6 activity per 100 grams of diet. The growth gains and food consumption data of the various groups are presented in Table III.

Growth gains that were in agreement with those predicted by microbiological assay were obtained with the samples of unheated milk and the heat-sterilized liquid and spray-dried powder varieties of the infant food fortified with pyridoxine hydrochloride. In contrast, heat-sterilized milk yielded growth gains inferior to that of pyridoxine hydrochloride; the differences in weight gains between appropriate groups were of statistical significance. The group receiving 30 γ as the heat-sterilized milk supplement grew no better than the unsupplemented control, and the group receiving 60 γ grew at a rate equal to that of the group receiving 30 γ of activity in the form of pyridoxine hydrochloride or unheated milk. The heat-sterilized liquid infant food fortified with pyridoxine yielded growth gains equal to those obtained with equivalent activity supplied as pyridoxine hydrochloride.

Experiment 3. To investigate the possibility that only vitamin B_6 utilization is involved in the low growth rate of rats fed sterilized milk samples, two groups of weanling rats were fed the two diets of Experiment 2 that supplied 60 γ of vitamin B_6 in the form of unheated and heat-sterilized milk; in addition, both the diets were fortified with an excess of pyridoxine hydrochloride 500 γ per 100 grams of diet. The growth results of these two groups of rats (Table IV), clearly indicate that with ample vitamin B_6 present, the diet containing heated milk will support optimal growth.

Experiment 4. The results of this assay, presented in Table V, again demonstrate a biological activity for heat-sterilized milk that is lower than expected on the basis of microbiological assay. Statistical significance was found in the differences between the weight gains of the heated milk groups and the corresponding groups receiving pyri-

Table II. Bioassay of Heat-Sterilized and Unheated Milk

(Experiment 1)

Diet Supplement	Vit. B_6, γ/G. Supplement[a]	Vitamin B_6 in Added Supplement, γ/100 G. Diet: 0	25	50	75	100
		Average weight gain ± standard error[b], g.				
Basal diet	0.12	81 ± 7.4 (261)[c]	...	...	...	...
Pyridoxine HCl	..	...	123 ± 6.1 (397)	130 ± 7.5 (394)	152 ± 4.6 (422)	157 ± 7.4 (425)
Pyridoxal PO_4	..	...	124 ± 6.4 (400)	135 ± 6.3 (409)	148 ± 3.4 (423)	151 ± 4.0 (397)
Unheated milk	4.1	...	128 ± 8.1 (410)	137 ± 6.0 (392)	150 ± 4.8 (428)	162 ± 4.8 (428)
Heat-sterilized milk	2.9	...	105 ± 5.4 (339)	125 ± 7.6 (406)	139 ± 1.9 (408)	143 ± 3.8 (380)

[a] Microbiological assay.
[b] 7 rats per group, initial weight 93 grams; 31 days' assay.
[c] Grams average food consumption in parentheses.

Table III. Bioassay of Milk and Infant Food

(Experiment 2)

Diet Supplement	Vit. B_6, γ/G.[a] Supplement	Vit. B_6 in Added Supplement, γ/100 G. Diet: 0	30	60
		Average weight gain ± standard error[b], g.		
Basal diet	0.07	105 ± 14.7 (367)[c]	...	...
Pyridoxine HCl	..	...	118 ± 5.5 (344)	140 ± 4.3 (374)
Unheated milk	3.8	...	119 ± 7.6 (349)	126 ± 7.8 (351)
Heat-sterilized liquid infant food, pyridoxine HCl fortified[d]	6.3	...	116 ± 9.6 (334)	134 ± 5.0 (369)
Spray-dried powder infant food, pyridoxine HCl fortified[d]	5.3	...	119 ± 4.1 (344)	129 ± 4.3 (345)
Heat-sterilized milk	2.7	...	97 ± 6.9 (312)	116 ± 3.2 (335)

[a] Microbiological assay.
[b] 8 rats per group, initial weight 90 g., 30-day assay.
[c] Grams average food consumption in parentheses.
[d] SMA concentrated liquid and powder, Wyeth Laboratories Inc.

Table IV. Growth of Weanling Rats Fed Pyridoxine HCl-Fortified Diets Containing Heated and Unheated Milks

(Experiment 3)

Supplement to Basal Diet with Excess Pyridoxine HCl	Average Weight ± Standard Error[a], G.			Food Consumed, G.
	Initial	Final	Gain	
15.8% unheated milk[b]	48	175	127 ± 2.3	219
22.2% heated milk[b]	48	179	131 ± 3.1	210

[a] 12 rats per group, 30-day experiment.
[b] Amounts that supply 60 γ of vitamin B_6, microbiological activity.

doxine hydrochloride. The good growth rate of the group receiving heat-treated milk plus an excess of pyridoxine hydrochloride was closely comparable to the groups of Experiment 3. This is further evidence that the inferior response to heated milk in a vitamin B_6 bioassay is not the result of destruction of other known or unknown nutrients.

Discussion

The heat processing necessary for the sterilization of canned liquid milk products not only destroys a considerable amount of the vitamin B_6 as measured microbiologically but also decreases the biological response of the remaining vitamin. Sterilized liquid milk products have been found to contain 33 to 64% of the vitamin B_6 activity of fresh milk (*7*) as measured by the *S. carlsbergensis* assay. As indicated by the growth tests herein reported, only about half of this activity is biologically available; thus the over-all effect of heat sterilization on the vitamin B_6 of milk results in a product which appears to contain biological activity from one third to one sixth the original content. This is well illustrated in the data of the second and fourth experiments of the present report; 22 and 23 grams of heated milk solids (2.7 and 2.6 γ per gram) supplying 60 γ of microbiologically active vitamin B_6, had a biological activity equivalent to 30 γ of pyridoxine hydrochloride, or 1.35 and 1.30 γ per gram, respectively. The spray-dried fresh milk used in these two experiments contained 3.8 and 4.1 γ per gram by microbiological assay, a value in agreement with the results of the bioassay.

It does not appear likely that the inferior growth response of the assay groups fed heat-sterilized milk could involve nutritional factors other than vitamin B_6. The basal diet is adequately fortified with all the known vitamins. Only a slight loss in protein efficiency has been reported to occur as a result of sterilization of evaporated milk (*3, 8*). In such studies, the milk protein was fed at a level of 9% and as the sole protein of the diet. In the vitamin B_6 assay reported here the diets with heat-sterilized milk contained 26% protein, with only 10 to 30% of it as protein from the heated milk. The rat growth test of Experiment 3 showed no difference in nutritional quality of diets containing heat-sterilized or unheated milk when fortified with an excess of pyridoxine hydrochloride.

The biological assay and the microbiological assay agreed in the determination of the vitamin B_6 content of the sterilized liquid infant food fortified with pyridoxine hydrochloride. This agreement, not found with heat-sterilized milk, may be attributed to the fact that whereas all of the vitamin of heat-sterilized milk is present as pyridoxal and pyridoxamine, 80% of the vitamin activity of the infant food is present as pyridoxine hydrochloride. Pyridoxine is more stable, both to heat (*7*) and to destruction by intestinal bacteria (*10, 14*).

Formulas fed to the majority of bottle-fed infants contain cow's milk that has been subjected to heat sterilization. In addition to a lowered intake as the result of sterilization loss, the vitamin B_6 concentration, in relation to calories, is usually further lowered by addition of carbohydrate to the milk, either by the manufacturer of commercial infant foods or by the mother preparing an evaporated milk formula. The formula in most cases receives a second heat treatment in the terminal sterilization practiced in the hospital nursery. Whether the sterilized liquid milk products when used as the only food contain adequate amounts of vitamin B_6 for optimum nutrition is not known. The vitamin B_6 content of human milk averages about 0.15 mg. per liter (1.2 γ per gram of solids), with values ranging from 0.04 to 0.22 mg. per liter (*7, 11*). Infant formulas prepared from diluted and heat-sterilized cow's milk contain between 0.10 and 0.23 mg. per liter of vitamin B_6 as measured by microbiological assay (*7*); thus, they appear to contain levels of vitamin B_6 equal to that of human milk. However, if only part of this microbiological activity is utilizable by the infant, the margin of safety based on a comparison of the vitamin B_6 content of human milk may be less than has been supposed.

The mechanism responsible for the decrease in the biological activity of the vitamin B_6 of milk as a result of heat sterilization can only be surmised. The vitamin may be converted to a form that, while still active for the microorganism, is not active for the animal. It may be rendered less available by the formation of a nondigestible complex, such as occurs with certain amino acids when proteins are heated in the presence of sugar. Heat-induced changes in diet ingredients other than vitamin B_6 may increase the animal's requirement for this vitamin, either directly or through the intermediation of intestinal bacteria. The participation of pyridoxal phosphate as a coenzyme in numerous reactions of amino acid metabolism (*16*) suggests that alterations in the amino acids of the heated protein may increase the need for the vitamin. Tappan and coworkers (*19*) have found that highly processed protein appears to be involved in the increased requirement for vitamin B_6 by rats fed combat rations. The ability of dietary lactose to spare vitamin B_6 (*22*) may be lessened as a result of heat treatment. The stimulation of the growth of various organisms by autoclaved sugar has been demonstrated in test tube experiments (*17*). If the heat treatment of lactose favors the propagation of vitamin B_6-consuming bacteria in the intestinal tract, a smaller share of the vitamin of the diet is available to the rat.

The only manifestation of vitamin B_6 deficiency observed in these experiments has been growth retardation; the conclusions in regard to the biological activity of heated milk do not necessarily apply to the more specific symptoms of

Table V. Bioassay of Heat-Sterilized Milk

(Experiment 4)

Diet Supplement	Vit. B_6, γ/G. Supplement[a]	Vitamin B_6 in Added Supplement, γ/100 G. Diet			
		0	30	60	360
		Average weight gain ± standard error[b], g.			
Basal diet	0.10	67 ± 6.9 (250)[c]	...	...	...
Pyridoxine HCl	..	...	96 ± 6.2 (294)	112 ± 5.1 (326)	...
Heat-sterilized milk	2.68	...	77 ± 5.3 (287)	90 ± 4.5 (278)	...
Heat-sterilized milk + pyridoxine HCl	..	...	...	...	135 ± 8.0 (378)

[a] Microbiological assay.
[b] 8 rats per group, initial weight 92 grams, 30-day assay.
[c] Grams average food consumption in parentheses.

vitamin B_6 deficiency, such as acrodynia and convulsions.

The increased requirement for vitamin B_6 by the rat and monkey fed highly processed army combat ration (*13, 19*), and by the rat fed heat-sterilized milk, as herein reported, suggests that this effect of food processing is of a general nature. Fortification of heat-treated food may be deemed advisable for the proper nutrition of infants, combat men, and other humans required to live for extended periods on diets comprised solely of processed food.

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NUTRITIONAL VALUES OF CROPS

Amino Acid Content of West Indies Sugar Cane

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Although the nitrogen content of sugar cane has an important bearing not only on plant nutrition but also on the sugar manufacturing process, little information on this subject is available. These studies are an attempt to remedy this situation. By chromatographic techniques the presence of 11 amino acids has been demonstrated in sugar cane juice. Their distribution in the cane stalk and their quantitative variation with age of the plant and climatic conditions have been examined. Different varieties of cane have essentially the same amino acid picture, although there are quantitative differences. Cane leaves contain the same amino acids as the juice, but have the majority of their nitrogen in protein form. Although under normal conditions the amino acid content of cane juice decreases with age of cane, under drought conditions the amino acid content increases spectacularly. The high amino acid level of drought cane may be related to the difficulties of processing such material in a sugar factory. Cane juices of abnormally high amino acid content are characterized by the formation, on lime-heat clarification, of very small sized flocs which settle extremely slowly and are extremely difficult to deal with in the factory.

IN SPITE of the fundamental importance of amino acids for the growth, behavior, and properties of plants, little information regarding the amino acid status of sugar cane is available. The authors have undertaken to examine the amino acid relationships of typical West Indies sugar cane varieties during growth.

A preliminary report by Pratt and Wiggins (*4*) described the separation of aspartic, glutamic, and γ-aminobutyric acids, glycine, alanine, asparagine, glutamine, lysine, serine, leucine, and valine from the juice of sugar cane variety B.34104 (Barbados Cane Breeding Station selection No. 104 of 1934), using the two-dimensional paper chromatogram technique of Consden, Gordon, and Martin (*2*). Subsequently (*6*) it was found that single-dimensional "strip" chromatograms could be made to give adequate separation of all the amino acids occurring in significant amount, and to show up the presence of the isomeric leucines.

Further studies (*7*) indicated that