Comparison of Vitamin B_6 Values of Selected Food Samples by Bioassay and Microbiological Assay

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Free and total vitamin B_6 values of a few selected foods determined microbiologically were compared to vitamin B_6 values obtained by rat bioassay. Free and total vitamin B_6 values were obtained on extracts chromatographically separated into pyridoxine, pyridoxal, and pyridoxamine using a Dowex 50 resin column, as well as on extracts not chromatographed. Bioassay values agree with values obtained microbiologically on the chromatographed extracts of these samples.

Vitamin B_6, as it occurs naturally in foods, consists of free and combined forms of pyridoxine, pyridoxal, and pyridoxamine. For the determination of vitamin B_6 in the low concentrations in which it occurs in many foods, the microbiological assay with *Saccharomyces carlsbergensis* has been most commonly used (*9*). Bioassays, such as the response in rat growth to increments of food supplements to a basal diet (*5*), measure biological availability of the vitamin B_6 in the food, but there has been no information relating the bioassay value to amounts of the different forms of vitamin B_6 in the food. *S. carlsbergensis* has been found to respond in growth to all three free forms of vitamin B_6, but to a lesser degree (*4*) to pyridoxamine than to pyridoxal and pyridoxine. It responds almost equally to pyridoxal and pyridoxine.

The chromatographic separation of food extracts into pyridoxine, pyridoxal, and pyridoxamine fractions, using an ion exchange column with Dowex 50 resin, has made it possible to assay for these three factors individually and to calculate the total vitamin B_6 content of the food (*3, 6, 7*).

The purpose of this paper is to compare vitamin B_6 values obtained by the rat bioassay of Richardson *et al.* (*5*) and free and total vitamin B_6 values of a few selected food samples assayed by the yeast microbiological procedure (*6*) on nonfractionated extracts and on the individual fractions of pyridoxine, pyridoxal, and pyridoxamine obtained by chromatographing the extracts.

Experimental

Beef muscle, nonfat dry milk solids, Lima beans, and whole wheat flour were selected to represent different kinds of foods which could be prepared in a dry, reasonably divided state capable of being used as the supplements to the diet in the rat assay and for sampling for the microbiological assays. The foods were prepared by the Texas Agricultural Experiment Station laboratory. Lean muscle tissue of beef round was trimmed of excess fat, freshly ground, frozen, and lyophylized. The dry material was ground to pass a 0.5-mm. sieve, placed in polyethylene bags, and sealed in tin cans for storage at −20° C. The dried Lima beans, nonfat dry milk solids, and whole wheat flour samples were ground if necessary, and similarly stored without further drying.

Analyses for proximate composition were made according to procedures of the AOAC (*1*) with minor modifications. Moisture determinations were made using the vacuum oven at 50° C. For the fat analyses, the dried beef sample was extracted in a Soxhlet apparatus with petroleum ether. The Lima bean and flour samples were acid hydrolyzed, and the fat was extracted in a Röhrig tube with alcohol and mixed ethers. The nonfat dry milk solids were analyzed for fat using the Roese-Gottlieb procedure. Nitrogen was determined by the micro-Kjeldahl procedure using 0.02N HCl in place of the boric acid as collectant in the distillation, and titrating with 0.02N NaOH using methyl red indicator.

The procedure for the rat bioassay was that described by Richardson *et al.* (*5*). Weanling, 21-day old rats of the Texas A and M strain were placed on the basal diet for a 14-day depletion period. Five males and five females constituted an assay group. Supplements for the standard were 0, 1, 3, 5, 10, and 15 μg. of pyridoxine as pyridoxine hydrochloride per rat per day. The nonfat dry milk solids supplements were 0.25, 0.50, 0.80, and 1.25 grams per rat per day; Lima beans, 0.25, 0.50, 0.80, and 1.50 grams; beef, 0.15, 0.25, 0.5, and 0.8 grams; and whole wheat flour, 0.3, 0.6, 1.0, and 2.0 grams. Average weekly weight gains were obtained for each animal over a 4-week assay period. A linear relationship existed between weight gains for the individual animals (Y) and the logarithms of the daily supplement (X), which were coded to give proper numerical distribution. The data were calculated according to the equations of Bliss (*2, 8*) to obtain the values of the means with their 95% confidence limits.

The microbiological assays were made according to the procedures of Toepfer and Lehmann (*6*) using *S. carlsbergensis* as the test organism and the media and techniques there described. Microbiological assays were carried out in a darkened laboratory. For the free forms of vitamin B_6, extractions of 1-gram samples were made by suspending the sample in 200 ml. of distilled water, adjusting to pH 6.0, and autoclaving for 15 minutes at 15 pounds steam pressure. When cool, the suspensions were adjusted to pH 4.5 with HCl, made to 250 ml., and filtered through Whatman No. 40 filter paper. For the total forms, extractions of the samples were made by autoclaving 5 hours at 15 pounds steam pressure with 200 ml. of 0.055N HCl for the nonfat dry milk solids and the dried ground lean beef, and 200 ml. of 0.44N HCl for the Lima beans and whole wheat flour. The extractions were adjusted to pH 4.5, made to 250 ml., and filtered.

Microbiological assays were carried

out on these unchromatographed extracts for free and total vitamin B_6. Extracts were diluted to known volumes containing approximately the equivalent of 1 mμg. of pyridoxine per ml. Triplicate tubes at five levels, from 1 to 5 mμg., were used for the assay, with pyridoxine as the standard. Results were expressed as micrograms of pyridoxine per gram of sample.

Assays for the individual forms of vitamin B_6, both free and total, were made on the filtered extracts at pH 4.5 and were chromatographically separated into pyridoxine, pyridoxal, and pyridoxamine fractions on Dowex Ag 50WX8 ion exchange resin columns (*6*). The resin was specially prepared in the potassium form, resulting in a wet slurry of which 30 ml. were used in the column. In a 19-mm. i.d. tube the depth of the resin was approximately 11 cm. The amount of the extract taken for chromatographing depended on the amounts of vitamin B_6 factors present, and ranged from 50 to 125 ml. for these samples. After the extract had passed through the column, it was washed with 100 ml. of warm (75° C.) 0.02*M* potassium acetate, pH 5.5. The pyridoxal fraction was eluted with two 50-ml. portions of boiling 0.04*M* potassium acetate at pH 6.0; the pyridoxine fraction was similarly eluted with boiling 0.1*M* potassium acetate, pH 7.0; and the pyridoxamine fraction, with boiling 1.0*M* KCL–0.1*M* K_2HPO_4, pH 8.0. The fractions were assayed microbiologically using pyridoxine, pyridoxal, and pyridoxamine as standards. The data from these assays were also used for calculations according to the Bliss equations to give mean values for the vitamin B_6 factors and their 95% confidence limits.

Results and Discussion

The proximate composition data for the samples are given in Table I. Prior to drying the beef sample by lyophylization, the moisture content was 72.3%.

Results of the rat bioassays are shown in Table II. The calculation of 95% confidence limits for the mean value included the variation of the data for the standard and for the sample. Theoretically, the standard curve may be taken as an indication of absolute values. However, the actual data show as much variation in response to increments of the standard as to those of the sample being assayed. To apply the Bliss equations, a linear relationship is required between growth responses and increments of the factor being measured, and the standard and sample curves must be parallel. A factor was used to make micrograms of the standard mathematically equivalent to grams of the sample. Linear relationships and parallelism were established by use of logarithms. The resulting data were translated into terms giving values for the means, in micrograms of the factor per gram of sample, and high and low values within which 95% of the data would occur assuming the same variation. The 95% confidence limits were about ±25% of the mean values.

Free and total microbiologically determined vitamin B_6 values are also included in Table II. The values of free vitamin B_6 were approximately one third of the total vitamin B_6 with the exception of the milk sample in which the free vitamin B_6 was approximately 90% of the total vitamin B_6. The rat bioassay values agree with the total vitamin B_6 values obtained microbiologically on chromatographed extracts. The 95% confidence limits for these values overlap. There appear to be differences at this level of comparison between bioassay values and those obtained microbiologically on unfractionated extracts of the nonfat dry milk solids and the whole wheat flour, the microbiological assay value being somewhat greater. There is also a difference indicated between the microbiological assays on whole wheat flour between chromatographed and not chromatographed extracts. Vitamin B_6 was present in Lima beans and whole wheat

Table I. Proximate Composition of Samples

Sample	Moisture, %	Fat, %	Nitrogen, %	Protein,[a] %	Ash, %
Beef, lean, dried	5.0	19.9	11.46	71.6	3.7
Lima beans, dry	11.7	1.1	3.82	23.9	4.4
Milk solids, nonfat dry	11.9	2.0	2.51	14.6	1.7
Whole wheat flour	3.8	0.1	5.79	37.0	8.1

[a] N × 6.25 for beef and Lima beans; N × 6.38 for milk; N × 5.83 for whole wheat.

Table II. Vitamin B_6 Values of Selected Food Samples by Bioassay and Microbiological Assay Procedures

	Bioassay			Microbiological Assay									
				Not Fractionated Extract		Fractionated Extract							
		Vitamn B_6 value, μG./Gram			Vitamin B_6 Value	Sum of Vitamin B_6 Components		Pyridoxine		Pyridoxal		Pyridoxamine	
Sample	Number of animals	Mean	CL[a]	Number of Assays	Mean	CL	Mean	CL	Mean	CL	Mean	CL	Mean	CL
						VITAMIN B_6—FREE, μG./GRAM								
Beef, lean, dried				3	6.27		5.04		0.47		3.39		1.18	
Lima beans, dry				4	2.60		1.93		0.65		0.90		0.38	
Milk solids, nonfat dry				4	3.72		3.66		0.10		2.84		0.72	
Whole wheat flour				3	1.22		1.17		0.69		0.31		0.17	
						VITAMIN B_6 FROM HYDROLYZED SAMPLES, μG./GRAM								
Beef, lean, dried		13.25			14.33		15.93		0.80		6.40		8.73	
	77		16.91	4		15.96		17.31		0.96		7.07		9.28
			10.37			12.70		14.55		0.64		5.73		8.18
Lima beans, dry		7.13			7.42		6.72		5.05		0.97		0.70	
	80		8.79	7		8.07		7.33		5.43		1.16		0.74
			5.78			6.77		6.11		4.67		0.78		0.66
Milk solids, nonfat dry		3.16			4.53		4.06		0.12		2.50		1.44	
	80		3.80	5		4.81		4.39		0.15		2.70		1.54
			2.64			4.25		3.73		0.09		2.30		1.34
Whole wheat flour		2.94			4.30		3.50		2.49		0.57		0.44	
	75		3.59	6		4.60		3.73		2.64		0.62		0.47
			2.40			4.00		3.27		2.34		0.52		0.41

[a] 95% confidence limits, high and low, respectively.

flour mostly as pyridoxine, 75 and 71%, respectively. Over 90% of the vitamin B_6 values of lean beef and milk solids were pyridoxal and pyridoxamine—40% pyridoxal and 55% pyridoxamine in the lean beef, and 62% pyridoxal and 36% pyridoxamine in the milk solids. The Texas laboratory also assayed these samples microbiologically using the procedures, including fractionation, developed at Beltsville. Results for the total vitamin B_6 values were as follows: dried lean beef, 14.91 μg. per gram; dried Lima beans, 6.99; nonfat dry milk solids, 3.68; whole wheat flour, 3.51. Agreement between the data obtained by two laboratories working independently was strong evidence that the chromatographic fractionations of extracts and microbiological procedures were reproducible and should be applicable routinely for the determination of vitamin B_6 in foods.

From these data, it was concluded that the bioassay measures free and combined forms of vitamin B_6 and that the bioassay values agree with the values obtained microbiologically on chromatographed, fractionated extracts of these samples.

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STORAGE EFFECTS ON BEEF

The Effect of Three Years of Freezer Storage on the Thiamine, Riboflavin, and Niacin Content of Ripened and Unripened Beef

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The change in thiamine, riboflavin, and niacin content of longissimus dorsi and semimembranosus muscles of eight Hereford steers was determined after 3 years of freezer storage. Samples were ground and frozen on the day of slaughter and after 21 days of ripening at 34° F. During 3 years of storage at 0° F., thiamine increased significantly in unripened beef but was unchanged in ripened beef. Riboflavin increased slightly in both ripened and unripened beef. Niacin decreased significantly in unripened beef but was unchanged in ripened meat. Although all changes were statistically significant, none was of such magnitude as to be important nutritionally.

THEORETICALLY, vitamins of the B-complex should be stable to freezer storage, especially in foods packaged to minimize oxidative changes and treated to reduce enzyme activity. However, in raw meat there is the possibility of enzymatic changes occurring during freezer storage. Several reports as to the stability of thiamine, riboflavin, and niacin in animal tissues during freezer storage have been published (*2, 4–8, 11–13*). Detailed comparison of results of these studies is complicated by variations in the storage times and temperatures employed, as well as by variations in the amount of aging of the meat prior to freezing.

Generally, reported losses of thiamine were slight (*7, 8*) and nonsignificant (*5, 6, 13*) in red meats and poultry (*2, 11*). Results of tests for the stability of riboflavin were less consistent. Reported changes ranged from a decrease of 31% (*7*) to an increase of 22 to 42% (*13*) in pork loins, and decreases up to 46% were reported for beef rib steaks (*5*). Kotschevar (*4*) obtained greater losses of thiamine and riboflavin in sliced calf liver than in ground calf liver stored in an atmosphere of carbon dioxide, suggesting that oxidation during freezer storage could be a factor in losses of these two vitamins.

The amount of aging prior to freezing was reported to have an effect on niacin retention in pork (*12*). Niacin decreased 18% in 32 weeks in loins aged for 1 day before freezing, but no loss occurred when loins were aged for 3 or 7 days. Others have reported nonsignificant changes in the niacin content of beef rib steaks (*5*), pork chops (*6, 7*). and beef liver (*4*) during freezer storage.

Beef may be ripened (aged) for several days or weeks prior to freezing. No data were found on how this practice affects the retention of B vitamins during subsequent freezer storage. Therefore, the present study was designed to investigate the effects of ripening prior to freezing and of long-time storage on the thiamine, riboflavin, and niacin content of beef.

Experimental Procedure

Meat for this study was procured from four pairs of Hereford steers. Each pair consisted of a grain-finished and grass-finished animal. Thiamine, riboflavin,