Vitamin B_6 in Sterilized Milk and Other Milk Products

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Two microbiological assay methods for vitamin B_6 have been slightly modified to conform to the newer knowledge of the vitamin and the metabolic requirements of the assay organisms. The modified methods give similar but not necessarily identical results when applied to fresh or pasteurized milk or nonfat dry milk solids, but different results when applied to stored evaporated milk. Experimental work suggests that pyridoxal of fresh milk is changed progressively to pyridoxamine and then to an unknown form of vitamin B_6, during processing and storage of evaporated milk. The unknown form of the vitamin is not equally active for the assay organisms.

THE PYRIDOXINE (VITAMIN B_6) CONTENT of fresh and processed milk was investigated in this laboratory about 10 years ago (*5*). At that time the chemistry and physiology of pyridoxine seemed to be reasonably well worked out, but a short time later other members of the vitamin B_6 group (pyridoxamine, pyridoxal, and pyridoxic acid) were discovered and synthesized (*11, 12*). Since then knowledge of the chemistry and physiology of vitamin B_6 has increased. In addition to the three forms of the vitamin, there are a number of phosphorylated and conjugated forms that are difficult to estimate quantitatively. As is mentioned by Snell (*14*), two well established methods of microbiological assay give results that usually fall in the same range. Sometimes these methods are not precise enough for the study of small losses. Both methods have been used in this laboratory.

In 1952, 1953, and 1954 a manufacturer of canned sterilized infant formula encountered a problem in infant nutrition which appeared to be at least partially due to destruction of vitamin B_6 during processing of the product. Hassinen, Durbin, and Bernhart, using the *Saccharomyces carlsbergensis* microbiological method for determining vitamin B_6 (*3*), found that two samples of evaporated milk and a number of samples of canned sterilized infant formula had much less vitamin B_6 than would be predicted from their milk solids content and the amount of vitamin B_6 found in fresh milk. Further work by Tomorelli, Spence, and Bernhart in the same laboratory (*18*) indicates that sterilized milk products have even less activity for the rat than for *Saccharomyces carlsbergensis*. These investigations suggested the need for additional data on the vitamin B_6 content of evaporated milk, although this milk has been widely used for many years as the principal ingredient of infant formulas without reports of convulsions or other symptoms of vitamin B_6 deficiency.

Table I. Vitamin B_6 Content of Evaporated Milk by Unmodified *Neurospora* Method

Sample No.	Vitamin B_6 as Pyridoxine, Mg./Liter Reconstituted Milk	
	Nonster.	Ster.
1	0.61	0.54
2	0.66	0.59
3	0.65	0.60
4	0.52	0.59
5	0.54	0.56
6	0.62	0.56
7	0.54	0.60
8	0.61	0.57
9	0.50	0.47
10	0.45	0.55
11	0.52	0.55
Av.	0.57	0.56

In initial work an attempt was made to verify earlier observations, using the method proposed by Stokes, Larsen, Woodward, and Foster (*17*). With *Neurospora sitophila* 299 "pyridoxineless" as the test organism, the method was used to compare the vitamin B_6 content of nonsterilized and sterilized evaporated milk. The data in Table I in general confirm the earlier observations (*5*) obtained by the same method, suggesting that there is no large loss of vitamin B_6 during the sterilization of evaporated milk. Although somewhat lower, they are also in general agreement with published values for fresh, reconstituted irradiated evaporated, reconstituted dry skim, and reconstituted dry whole milk—0.67, 0.73, 0.66, and 0.67 mg. per liter, respectively. Because in this comparison of nonsterilized and sterilized milk the results obtained were highly variable, attempts were made to improve the method by utilizing some of the newer facts on *Neurospora* metabolism.

Modifications of Method

The destruction of thiamine has always been a critical step in the *Neurospora* method for vitamin B_6. Harris (*2*) suggested avoiding this critical step by addition of thiamine to the medium. This results in a more sensitive and probably more specific method. The bisulfite reaction as originally proposed is not carried out under optimum conditions and thiamine destruction may not always be complete. Harris also suggested that cleavage products have stimulatory activity in the pyridoxine assay. Morris, Herwig, and Jones (*6*) suggested that the sulfite cleavage destroys pyridoxine and proposed autoclaving the samples with sodium hydroxide to destroy thiamine. The latter procedure produces an annoying quantity of soap with high fat products and appears not much more convenient than the original method.

The addition of 50 mg. of thiamine per liter of double-strength medium results in a five- to tenfold increase in sensitivity. The assay curve is plotted over a range of 0 to 0.20 γ of pyridoxine per 10 ml. of medium rather than 0 to 1.0 γ. Pyridoxal and pyridoxine give similar assay curves. Pyridoxamine added to the medium in the absence of pyridoxine or pyridoxal shows a definite lag for the lower levels of additions (Table III). At higher levels or in the presence of pyridoxal or pyridoxine its activity is similar to that of pyridoxine. This lack of response to pure pyridoxamine at low levels has not yet been encountered in assays of natural products, as results seem usually to be higher, not lower, than those found by the *Saccharomyces carlsbergensis* and other

Table II. Need for Hydrolysis in *N.S.* 299 Vitamin B_6 Assay of Evaporated Milk

(Milligrams of B_6 per liter,[a] reconstituted basis)

	Nonhydrolyzed Sample		Hydrolyzed Sample	
	Nonster.	Ster.	Nonster.	Ster.
	1*N* Hydrochloric Acid			
	0.395	0.336	0.428	0.346
	0.392	0.338	0.345	0.329
	0.388	0.299	0.370	0.319
	0.379	0.297	0.350	0.332
	0.364	0.351	0.369	0.350
Av.	0.383	0.324	0.372	0.335
	0.055*N* Sulfuric Acid			
		0.30		0.30
		0.32		0.27
		0.32		0.29
		0.30		0.30
		0.27		0.29
		0.29		0.26
		0.27		0.32
Av.		0.30		0.29

[a] In terms of pyridoxine, not pyridoxine hydrochloride.

methods. The possibility of error from this source must be kept in mind, however. The poor response of *Neurospora sitophila* 299 to low levels of pyridoxamine in the presence of thiamine differs from the response in the absence of thiamine. Under the latter conditions the growth responses to the three forms of the vitamin are similar. This has been shown by Snell and Rannefeld (*15*), and confirmed experimentally.

Another modification of the Stokes method was elimination of the acid hydrolysis step. If the method is applied to milk products, there appears to be little need for hydrolysis. The milk may be simply diluted to the proper strength and added to the assay flask (Table II). The average data do not show an increase in vitamin B_6 content after hydrolysis. The Stokes method uses hydrolysis with 1*N* hydrochloric acid, while the yeast method of Atkin, Schultz, Williams, and Frey (*1*) uses 0.055*N* sulfuric acid. Both methods were tested. Later it was shown that the *N.S.* 299 mutant utilized pyridoxamine phosphate and pyridoxal phosphate as well as the nonphosphorylated forms (Table III). The modified method as outlined was used for the present study.

Comparison of Two Methods

The *Saccharomyces carlsbergensis* 4228 method of Atkin, Schultz, Williams, and Frey (*1*) was chosen for comparison with the *N.S.* 299 method. This is probably the most widely used microbiological method and was the one that gave results in conflict with those secured on evaporated milk with *N.S.* 299. The method was modified by addition of niacin to the medium, as suggested by Rabinowitz and Snell (*10*) and by the use of a 50-ml. Erlenmeyer flask in place of a tube for growing the yeast, as proposed by Snyder and Wender (*16*). Satisfactory growth is secured in the flasks without continuous shaking. However, the flasks were shaken at the beginning of the assay and at least once during the assay. All flasks in any one assay were treated alike. Incubation was at 30° C. for about 20 hours.

The *Saccharomyces carlsbergensis* assay is a comparison of rates of growth. As such it is subject to some errors not found in microbiological assays, in which growth reaches or approaches completion. There are many factors affecting growth rates, most of which are controlled at least between standard and sample. Others, unfortunately, are not recognized or cannot be controlled: addition of extraneous material with the sample, an unknown and varying ratio of the forms of the vitamin in the unknown, the buffer capacity of the unknown, and its salt content. Usually it is not within the scope of the study to determine for each sample whether such unknown conditions are present and their importance. In the data presented below and in Tables I, II, and III there is ample illustration that both of the methods used in this study give variable results.

Light destruction (particularly ultraviolet) of vitamin B_6 compounds must be guarded against. In the present study all work was carried out in yellow light, and samples and standards were covered when not actually being manipulated. Usually the total exposure to a bare 150-watt yellow light at not less than 4 feet was less than 15 minutes. Two-hour exposure at 2 feet with the sample on a white background destroyed 28% of pyridoxine and 22% of pyridoxal in standard solutions of 0.005 γ per ml. The use of red instead of yellow light would have been preferable, but in view of the large number of pipettings required, yellow light of the intensity used was held to be the absolute minimum for accuracy. The light was not sufficient to avoid discomfort from eye strain.

The *Saccharomyces carlsbergensis* 4228 and the *Neurospora sitophila* 299 "pyridoxineless" vitamin B_6 methods were applied to the determination of the vitamin in fresh milk. There is good agreement between averages, but on any single sample the two methods may give variable results (Table IV). The results by *Saccharomyces carlsbergensis* method are the more variable, as other investigators have also found. Hassinen, Durbin, and Bernhart (*3*) report variable results with milk products.

Both methods were also applied to a number of samples of instant-type nonfat dry milk solids. Average values by the *Saccharomyces carlsbergensis* and *Neurospora*

Table IV. Vitamin B_6[a] Content of Greenville, Ill., Fresh Milk

(December 1954)

Sample No.	*Saccharomyces carlsbergensis* Assay, Mg. B_6/Liter	Sample No.	*N.S. 299* Assay, Mg. B_6/Liter
	Unpaired Data		
1	0.22	1	0.34
2	0.48	2	0.37
3	0.31	3	0.36
4	0.31	4	0.34
5	0.14	5	0.43
6	0.43	6	0.47
7	0.31	7	0.33
8	0.29	8	0.35
9	0.53	9	0.33
10	0.52	10	0.34
Av.	0.35		0.37
	Paired Data		
1	0.35	1	0.35
2	0.36	2	0.31
3	0.47	3	0.36
4	0.27	4	0.30
5	0.15	5	0.30
6	0.30	6	0.32
7	0.23	7	0.33
8	0.19	8	0.31
9	0.30	9	0.35
10	0.38	10	0.33
Av. 10	0.30		0.33
Av. 20	0.33		0.35

[a] In terms of pyridoxine.

Table III. Comparative Vitamin B_6 Activity of Pyridoxine, Pyridoxal, Pyridoxal Phosphate, Pyridoxamine, and Pyridoxamine Phosphate and Mixtures for *Neurospora sitophila* 299

	Per Cent Recovery of Equimolar Amounts					
Pyridoxine, γ/10 Ml. Medium	Pyridoxal	Pyridoxal phosphate	Pyridoxamine	1:1 pyridoxamine and pyridoxal	Pyridoxamine phosphate	1:1 pyridoxamine phosphate and pyridoxal
0.04	118	115	5	69	16	95
0.08	111	114	2	87	30	92
0.12	110	117	5	126	38	105
0.16	...	104	38	125	87	116
0.20	...	81	100	100	105	93
Av.	113	106	30	100	55	100

Table V. Vitamin B_6 Content of Nonsterilized and Sterilized Evaporated Milk

Sample No.	B_6[a] (as Pyridoxine), Mg./Liter Reconstituted Milk				Sterilization Retention, %	
	Saccharomyces carlsbergensis Assay		*Neurospora sitophila* 299 Assay			
	Nonster.	Ster.[b]	Nonster.	Ster.[b]	*Saccharomyces carlsbergensis* assay	*Neurospora sitophila* 299 assay
1	0.340	0.301	0.411	0.324	88	79
2	0.404	0.405	0.369	0.338	100	92
3	0.370	0.360	0.363	0.342	97	94
4	0.334	0.341	0.364	0.319	102	88
5	0.362	0.399	0.380	0.343	110	90
6	0.307	0.240	0.395	0.336	78	85
7	0.278	0.250	0.392	0.338	90	86
8	0.323	0.364	0.388	0.299	112	77
9	0.376	0.295	0.379	0.297	78	78
10	0.348	0.325	0.364	0.351	93	96
11	0.395	0.375	0.360	0.309	95	86
Av.	0.349	0.332	0.378	0.325	95	86

[a] In terms of pyridoxine (free base, not hydrochloride).
[b] Sterilization range 242–3.5° F. for 14.5 minutes.

Table VI. Vitamin B_6 Content of Stored Evaporated and Filled Milks

Sample No.	Approx. Storage Period, Months	Vitamin B_6[a], Mg./Liter Reconstituted Milk	
		Saccharomyces carlsbergensis 4228 assay	N.S. 299 assay
1	5	0.13	0.34
2	5	0.12	0.30
3	6 or more	0.33	0.31
4	6 or more	0.18	0.35
5	5	0.11	0.35
6	5	0.08	0.31
7[b]	Unknown	0.06	0.29
8[b]	Unknown	0.11	0.34
9	1 week	0.07	0.29
10	1 week	0.07	0.32
Av. 10		0.13	0.32

[a] In terms of pyridoxine (free base, not hydrochloride).
[b] Filled milk.

sitophila 299 methods were, respectively, 0.35 and 0.32 mg. per liter on a reconstituted basis. Individual analytical values on nonfat dry milk samples (*4*) show a reasonably good agreement between the two methods. The data also agree with those for fresh milk (Table IV). In assays on five regular-type and five instant-type samples of nonfat dry milk solids which had been stored over a year at room temperature, average values were 0.36 mg. per liter of reconstituted milk by the *Saccharomyces carlsbergensis* method and 0.38 by the *N.S.* 299 method. These data suggest that no major storage losses of vitamin B_6 occur at room temperature in nonfat dry milk solids.

Evaporated Milk

Because of the report of large sterilization losses, the investigation of the vitamin B_6 content of evaporated milk was of special interest. It was possible to secure data on the milk before and after sterilization in a commercial continuous-type sterilizer; all samples were, in fact, taken from commercial production. The average data are again in general agreement (Table V), and indicate small average sterilization losses, which are larger when measured by the *N.S.* 299 method than by the *Saccharomyces carlsbergensis* method. As Hassinen and coworkers (*3*) report a continued loss after sterilization, a fact confirmed by Table VI, variations in handling the samples from the sterilizer probably account for the differences in results. The samples in the present study, both nonsterilized and sterilized, were held refrigerated until the assays were started. The data of Hassinen, Durbin, and Bernhart (*3*) and those reported in this paper are in agreement that evaporated milk samples available on the retail market contain less vitamin B_6 as measured by the *Saccharomyces carlsbergensis* method than fresh milk samples on the same solids basis. The *Neurospora sitophila* method fails to show a major loss during processing or storage. On a reconstituted basis even stored samples show only slightly less vitamin B_6 than fresh milk. When data by the two methods for various types of milk are compared, there is reasonable general agreement except on samples of stored evaporated milk.

Evaporated milk is extensively used for infant feeding. Preparation of an infant formula by terminal heating procedure might conceivably further destroy vitamin B_6. This problem was investigated using both methods of assay (Table VII). Half of the samples were assayed immediately after terminal heating, and half were held 24 hours in the refrigerator before assay, as infant formula is often stored briefly before consumption. Two different carbohydrates were used in the preparation of the formula. None of these treatments appeared to affect the results and only minor losses are indicated. However, because evaporated milk was used, the results by the *Saccharomyces carlsbergensis* method are appreciably lower than those by the *N.S.* 299 method.

An explanation of the discrepancy when the two methods are applied to stored evaporated milk was sought. This developed into an extensive study, reported in part here.

One of the first investigations was of the possibility that pyridoxal was oxidized to 4-pyridoxic acid and that this compound was active for *N. S.* 299 but not active for *Saccharomyces carlsbergensis.* Other biologically active compounds such as ascorbic acid are oxidatively destroyed during the sterilization of evaporated milk, and this destruction continues during the early storage period, at least until the head space

Table VII. Vitamin B_6 Content of Infant Formula Prepared from Evaporated Milk

(Mg. of B_6[a] per liter of formula)

Sample No.	*Saccharomyces carlsbergensis* 4229 Assay			*Neurospora sitophila* Pyridoxineless 299 Assay		
		Heated Formula			Heated Formula	
	Untreated formula	Low pressure terminal	High pressure terminal	Unheated formula	Low pressure terminal	High pressure terminal
1	0.131	0.131	0.132	0.213	0.200	0.198
2	0.136	0.119	0.099	0.199	0.126	0 119
3	0.083	0.090	0.091	0.160	0.160	0.155
4	0.128	0.127	0.135	0.229	0.193	0.192
5	0.185	0.157	0.149	0.209	0.185	0.191
6	0.126	0.140	0.137	0.213	0.192	0.191
7	0.142	0.151	0.150	0.259	0.256	0.252
8	0.157	0.160	0.170	0.260	0.242	0.248
9	0.126	0.127	0.123	0.248	0.243	0.242
10	0.166	0.166	0.169	0.281	0.280	0.249
11	0.172	0.174	0.174	0.333	0.329	0.314
12	0.173	0.174	0.177	0.364	0.342	0.352
Av.	0.144	0.143	0.142	0.241	0.229	0.225
% of untreated		99	99		95	93
Av. on fresh milk basis	0.221	0.220	0.219	0.371	0.353	0.346

[a] In terms of pyridoxine (free base, not hydrochloride).

Table VIII. Determination of Pyridoxal by *L. casei* Assay

Sample No.	Type of Milk	*L. casei* Assay for Pyridoxal, Mg./Liter	Assay for Total B_6[a], Mg./Liter	
			Saccharomyces carlsbergensis	*N.S.* 299 *Pyridoxineless*
1	Fresh	0.30	0.29	0.37
2	Fresh	0.36	0.27	0.33
3	Nonster. evap.	0.35	0.36	0.37
4	Ster. evap.	0.24	0.31	0.37
5	Stored evap.	0.13	0.17	0.29
6	Stored evap.	0.15	0.18	0.29
7	Stored evap.	0.16	0.15	0.31
8	Stored evap.	0.19	0.17	0.32

[a] In terms of pyridoxine (free base, not hydrochloride).

Table IX. Determination of Pyridoxal by Alkali-Acetone Treatment

Type of Milk	Vitamin B_6, Mg./Liter, Fresh Basis			
	Total vitamin B_6[a]	Nonpyridoxal[a]	Pyridoxal	% pyridoxal
Fresh	0.367	0.027	0.340	93
Pasteurized	0.342	0.046	0.294	86
Nonster. evap.	0.401	0.113	0.288	72
Ster. evap.	0.377	0.207	0.177	47
Nonster. evap.	0.341	0.086	0.255	75
Ster. evap.	0.275	0.136	0.139	51
Stored evap.	0.303	0.228	0.085	29
	0.320	0.325	0.005	0
	0.360	0.295	0.055	15
	0.372	0.372	0.000	0

[a] In terms of pyridoxine (free base, not hydrochloride).

Table X. Pyridoxal and Pyridoxamine Content of Evaporated Milk

(Mg. per liter[a])

Sample No.	Type of Milk	Total Vitamin B_6[b]		Pyridoxal	Pyridoxamine
		Assay 1	Assay 2		
1	Nonster. evap.	0.34	0.30	0.25	0.12
2	Ster. evap.	0.27	0.40	0.13	0.28
3	Stored evap. 20 months	0.32	0.32	0.00	0.15
4	Stored evap. 20 months	0.36	0.30	0.05	0.17
5	Stored evap. 20 months	0.29	0.27	0.00	0.07

[a] All assays by *Saccharomyces carlsbergensis* method.
[b] In terms of pyridoxine (free base, not hydrochloride).

Table XI. Comparative Vitamin B_6 Activity of Pyridoxine, Pyridoxal, Pyridoxal Phosphate, Pyridoxamine, and Pyridoxamine Phosphate for *Saccharomyces carlsbergensis*

Pyridoxine, γ	Per Cent Recovery of Equimolar Amounts of			
	Pyridoxal	Pyridoxal phosphate	Pyridoxamine	Pyridoxamine phosphate
0.0025	80	88	80	108
	80	88	80	108
0.0050	84	96	86	94
	100	94	86	96
0.0100	93	98	87	95
	93	96	89	94
0.0150	95	96	91	95
	95	103	91	95
0.0200	91	96	89	89
	92	97	89	85
Av.	90	95	87	96

oxygen is consumed. No activity for either organism was shown by 4-pyridoxic acid. No growth stimulation for either organism was noted when 4-pyridoxic acid was added in equimolar amounts to pyridoxine, pyridoxal, or pyridoxamine.

Rabinowitz and Snell (*10*) indicate that the vitamin B_6 activity of fresh milk is due largely to pyridoxal. This was shown by assays with *L. casei* (*8*), as pyridoxine and pyridoxamine have little activity for this organism. Assays on fresh, nonsterilized evaporated, sterilized evaporated, and stored evaporated milk have been made and the pyridoxal content has been calculated (Table VIII). All or nearly all of the vitamin B_6 of fresh milk and nonsterilized evaporated milk appears to be pyridoxal. After sterilization only part of the vitamin B_6 activity of the milk results from pyridoxal content. Upon storage the vitamin B_6 activity of evaporated milk for *L. casei* drops to approximately the same level as that shown for *Saccharomyces carlsbergensis*. This is considerably lower than the activity shown for *N.S.* 299. Unsuccessful simultaneous attempts were also made to assay for pyridoxamine by use of *S. faecalis*. This assay has given trouble in other laboratories, as noted by Snell and Rannefeld (*15*) and Rabinowitz and Snell (*9*). One of the problems appears to be autoclaving the basal portion (nonvitamin B_6) of the media.

Another method of determining pyridoxal was based on the work of Snell (*11*, *13*), showing that pyridoxal, but not pyridoxine or pyridoxamine, is destroyed by alkali and acetone. To 1 ml. of fresh or evaporated milk, 3 ml. of water, 2 ml. of 1*N* sodium hydroxide, and 1 ml. of acetone were added and the mixture was allowed to stand in the dark at 25° C. for 4 hours. Then 160 ml. of water were added, the mixture was neutralized, and 1 ml. of 10*N* sulfuric acid was added. The samples were hydrolyzed and the assays completed by the *Saccharomyces carlsbergensis* procedure. Results were compared with other assays carried out without the alkali-acetone treatment (Table IX). The difference represents pyridoxal. The vitamin B_6 activity of fresh milk appears to be largely from pyridoxal, which is converted by mild heat treatment to some other compound, probably pyridoxamine, by reaction with amino groups. Sterilization further reduces the pyridoxal content. Most stored samples of evaporated milk contain little pyridoxal as measured by the alkali-acetone method. Assays by the *L. casei* method suggested that somewhat more was present.

An attempt was also made to measure pyridoxamine by destruction with nitrous acid, as proposed by Snell (*11*, *13*). The nitriting solution consisted of 1 gram of sodium nitrite, 12 ml. of glacial acetic acid, and sufficient water to make a total of 50 ml. One milliliter of milk was treated with 2.5 ml. of nitriting solution and shaken occasionally for 30 minutes, then 100 mg. of urea was added, and the sample was again shaken occasionally for 30 minutes. The mixture was diluted to 180 ml. (acidity was then about 0.055*N* from glacial acetic acid added) and the assays were completed by the *Saccharomyces carlsbergensis* method. The nitrous acid treatment does not completely destroy the biological activity of pyridoxamine. Probably there is some conversion to pyridoxine. Recovery experiments indicated that 25.6% of the activity remained. This factor was used in calculating results, but only semiquantitative results were obtained. Table X shows that pyridoxal predominates in nonsterilized milk and pyridoxamine in sterilized milk. Neither seems to account for the activity in stored evaporated milk. This fact is of special interest. Unfortunately, the samples chosen for the pyridoxal and pyridoxamine studies were not those which show minimum values by the *Saccharomyces carlsbergensis* method; nevertheless they fail to show appreciable pyridoxal or pyridoxamine.

Table XII. Recovery of Pyridoxine Added to Milk

(*Saccharomyces carlsbergensis* 4228 method)

Sample No.	Type of Milk	Mg. per Liter: Pyridoxine added	Vitamin B_6 found	Pyridoxine recovery	Recovery, %
1	Pasteurized	0	0.328	...	...
1R	Pasteurized + pyridoxine	1	1.480	1.152	115
2	Nonster. evap.	0	0.400	...	...
2R	Nonster. evap. + pyridoxine	0.5	1.085	0.685	137
3	Ster. evap.	0	0.317	...	...
3R	Ster. evap. + pyridoxine	0.5	0.806	0.489	97
4	Stored evap.	0	0.226	...	...
4R	Stored evap. + pyridoxine	0.5	0.777	0.551	110
5	Stored evap.	0	0.240	...	...
5R	Stored evap. + pyridoxine	0.5	0.738	0.498	100
	Av. recovery				112

Another possible source of error in the microbiological assays is failure to hydrolyze pyridoxal phosphate and pyridoxamine phosphate. The problem of suitable hydrolysis procedures is discussed by Snell (*14*). Probably acid autoclaving is of value in freeing other combinations than phosphates and in disintegrating certain samples as well as hydrolyzing the phosphate forms of pyridoxal and pyridoxamine. Increasing the hydrolysis time or acid strength did not increase the vitamin B_6 values of stored evaporated milk found by the *Saccharomyces carlsbergensis* method. Samples of pyridoxal phosphate and pyridoxamine phosphate were obtained from Bios Laboratories and tested by both methods. Results of the *N.S.* 299 assays have been presented in Table III. As expected the phosphates behave like the free compounds. Results with *Saccharomyces carlsbergensis* were surprising. They were in agreement both before and after hydrolysis. Moreover, the unhydrolyzed phosphate forms showed activity equivalent to pyridoxal, pyridoxamine, or pyridoxine (Table XI). The sample preparation for the *Saccharomyces carlsbergensis* assay includes filtration to clarify the sample for the eventual turbidimetric measurement as well as hydrolysis. The possibility that vitamin B_6 might be lost in one of these steps and therefore the *Saccharomyces carlsbergensis* method would give lower results on certain samples than the *N.S.* 299 method was checked by preparing the stored evaporated milk samples as for the *Saccharomyces carlsbergensis* assay, but completing the assay by the *N. S.* 299 method and comparing the results with those from nonhydrolyzed and nonclarified samples (Table II). It was concluded that the discrepancy in results did not originate in the hydrolysis step and that the hydrolysis procedure for the *Saccharomyces carlsbergensis* method was satisfactory for the samples and conditions under consideration. The hydrolysis step cannot be abandoned for milk samples, as it is a definite aid in clarification, which is necessary for the turbidimetric measurement.

The possibility that a growth-inhibiting substance may be present in stored evaporated milk was investigated by addition of pyridoxine to milk before assay (Table XII). The recoveries are variable, but there is no indication of the presence of an inhibitor.

As judged by any one assay, the *Saccharomyces carlsbergensis* method appears capable of giving uniform results. Nevertheless, recovery and other experiments led to the conclusion that this was not true, as has been shown by Hassinen, Durbin, and Bernhart (*3*) and confirmed by the data presented in Table XIII. The variability on the stored evaporated milk may not be entirely due to the method. Different cans were opened for the different assays and there is no absolute assurance that the rate of loss in all cans was identical, even though the milk, cans, and conditions of storage were alike.

Table XIII. Agreement between Replicate Assays for Vitamin B_6[a] *Saccharomyces carlsbergensis* 4228 Method

Sample No.	Type of Milk	Mg. per Liter Reconstituted Milk: Standard Assays, 20–24-Hour Incubation: 1	2	3	Paired Experimental Assay Incubation: 16 hours	40 hours	N.S. 299 method
1	Fresh	..	..	..	0.23	0.34	0.34
2	Fresh	..	..	..	0.37	0.55	0.46
3	Nonster. evap.	0.34	0.30	..	0.29	0.46	0.41
4	Ster. evap.	0.27	0.40	..	0.26	0.32	0.37
5	Stored evap. 20 mo.	0.32	0.32	..	0.21	0.27	0.32
6	Stored evap. 20 mo.	0.36	0.34	..	0.21	0.22	0.29
7	Stored evap. 20 mo.	0.29	0.27	..	0.20	0.18	0.30
8	Stored evap. 9 mo.	0.23	0.17	0.23	0.20	0.19	0.32
9	Stored evap. 9 mo.	0.30	0.18	0.24	0.18	0.20	0.36
10	Evap. 1 week	..	..	..	0.20	0.18	0.36

[a] In terms of pyridoxine (free base, not a hydrochloride).

Discussion

After most of the work reported in the present paper was completed, Parrish, Loy, and Kline (*7*) published a paper on the yeast method for determining vitamin B_6. These authors have carefully developed a number of steps in the procedure to give optimum results but the paper is largely confined to use of the assay with pure compounds. The curves shown for pyridoxine, pyridoxal, and pyridoxamine are similar. However, if certain points on the pyridoxamine curve were chosen for interpretation on the pyridoxine curve, variations would be much wider than those reported in Table XI. It is doubtful if enough information is at hand to permit complete standardization of a method for vitamin B_6. Much useful, albeit puzzling, information may still be obtained by minor changes in the method. A less standardized method may make better use of available equipment in a laboratory not specifically equipped for vitamin B_6 assays by the method in question.

Another paper, which aids greatly in interpreting the present work (*18*), points out that the vitamin B_6 activity of sterilized (and stored) milk is less for the rat than for *Saccharomyces carlsbergensis*.

The combined investigations furnish a basis for theories explaining the data, but final proof of their validity is lacking. The vitamin B_6 activity of fresh milk is largely due to pyridoxal. Extensive heat treatment, such as sterilization, in the presence of amino acids and proteins converts part of the pyridoxal to pyridoxamine. This conversion may continue during storage of the sterilized product. Storage of a sterilized product, sometimes even for only a few days, results in conversion of the pyridoxamine to another unknown form of vitamin B_6, which has highly different biological activity for various test organisms. This may be a new form of vitamin B_6, but probably it is a simple derivative of pyridoxamine or possibly pyridoxal or pyridoxine. This unknown form of vitamin B_6 has activity equal to pyridoxine for *Neurospora sitophila* 299 but much less activity for *Saccharomyces carlsbergensis*, and even less activity for the rat. Its activity for *L. casei* compared

to a pyridoxal standard appears to be equal to or slightly less than that for *Saccharomyces carlsbergensis*. What activity for humans the unknown form of vitamin B_6 may possess is not known. That it has considerable is suggested by the fact that evaporated milk has been used in infant formulas for many years without the appearance of deficiency symptoms and thus appears to possess adequate vitamin B_6 activity to meet the requirement of the human infant.

Summary

Modifications of the *Neurospora sitophila* 299 and *Saccharomyces carlsbergensis* methods of assay for vitamin B_6 give similar but not identical results when applied to fresh or pasteurized milk or to nonfat dry milk solids, but different results when applied to evaporated milk, particularly after storage. The *Neurospora sitophila* method gives the higher results and indicates a vitamin B_6 content similar to that of fresh milk. The *Saccharomyces carlsbergensis* method usually gives lower results, which suggests loss or change of the vitamin B_6 content.

In the sterilization of evaporated milk, pyridoxal is partially converted to pyridoxamine. This conversion probably continues during storage.

During the storage of evaporated milk a part of its vitamin B_6 activity appears to be changed to an unknown form.

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Literature Cited

(1) Atkin, L., Schultz, A. S., Williams, W. L., Frey, C. N., *Ind. Eng. Chem., Anal. Ed.* **15,** 141 (1943).
(2) Harris, D. L., *Arch. Biochem. Biophys.* **41,** 294 (1952).
(3) Hassinen, J. B., Durbin, G. T., Bernhart, F. W., *J. Nutrition* **53,** 249 (1954).
(4) Hodson, A. Z., *Food Technol.* **10,** 221 (1956).
(5) Hodson, A. Z., *J. Nutrition* **27,** 415 (1944).
(6) Morris, S., Herwig, G., Jones, A. *Analyst* **74,** 37 (1949).
(7) Parrish, W. P., Loy, H. W., Kline, O. L., *J. Assoc. Offic. Agr. Chemists* **38,** 506 (1955).
(8) Rabinowitz, J. C., Mondy, N. I., Snell, E. E., *J. Biol. Chem.* **175,** 147 (1948).
(9) Rabinowitz, J. C., Snell, E. E., *Ibid.*, **169,** 631 (1947).
(10) *Ibid.*, **176,** 1157 (1948).
(11) Snell, E. E., *J. Am. Chem. Soc.* **66,** 2082 (1944).
(12) Snell, E. E., *J. Biol. Chem.* **154,** 313 (1944).
(13) *Ibid.*, **157,** 491 (1945).
(14) Snell, E. E., "The Vitamins," vol. **III,** ed. by W. H. Sebrell and R. S. Harris, pp. 253–5, Academic Press, New York, 1954.
(15) Snell, E. E., Rannefeld, A. N., *J. Biol. Chem.* **157,** 475 (1945).
(16) Snyder, J., Wender, S. H., *Arch. Biochem. Biophys.* **46,** 465 (1953).
(17) Stokes, J. T., Larsen, A., Woodward, C. R., Jr., Foster, J. W., *J. Biol. Chem.* **150,** 17 (1943).
(18) Tomorelli, R. M., Spence, E. R., Bernhart, F. W., J. AGR. FOOD CHEM. **3,** 388 (1955).

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FOOD IRRADIATION

Sulfides Released from Gamma-Irradiated Meat as Estimated by Condensation with *N,N*-Dimethyl-*p*-phenylenediamine

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The reaction of hydrogen sulfide with *N,N*-dimethyl-*p*-phenylenediamine to form methylene blue has been adapted to determine small quantities of hydrogen sulfide from gamma irradiated meat. The odorous vapors from irradiated meat are carried with a stream of nitrogen into a trapping tube containing cadmium hydroxide and sodium hydroxide. The color is then developed by adding a mixture of the acid amine solution and Reissner's solution to a cup in the trapping tube, closing, and shaking. The intensity of the methylene blue color developed is then measured in a photoelectric colorimeter at 665 mμ. The method can be used for the quantitative estimation of 2 to 16 γ of hydrogen sulfide. Experiments show that free hydrogen sulfide probably is not present in gamma irradiated meat but that cadmium sulfide is formed in the trapping solution from some volatile sulfur-containing complex.

BEFORE APPLYING IONIZING RADIATIONS for the cold sterilization of certain meats, a method to prevent the development of undesirable odor and flavor must be found. Batzer and Doty (*2*) reported that hydrogen sulfide was probably one of the components of the undesirable odor developed in meat during irradiation. To study the effect of various conditions and methods of treatment on the development of hydrogen sulfide during gamma irradiation of meat, a selective, sensitive method for the estimation of small quantities of hydrogen sulfide was devised.

The classical reaction between hydrogen sulfide and *N,N*-dimethyl-*p*-phenylenediamine to form methylene blue, first proposed by Emil Fischer (*6*) in 1883, was the basis for the improved quantitative method. Since then several