Vitamin B_6 in Milk: Review of Literature*

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I. Introduction

Although the existence of vitamin B_6 was definitely established in 1936, it was not known until some time thereafter that it was needed by human beings. Now, in contrast, one is confronted with such a multiplicity of pyridoxine-dependent reactions that those in the field of research find it difficult to keep up with them. Of prime importance has been the discovery of the relationship of vitamin B_6 to infant nutrition and the tragic results which can follow a deficiency.

With the recognition that vitamin B_6 deficiency can result in convulsive seizures and associated symptoms has come a renewed interest in the vitamin B_6 content of milk and milk products used for infant feeding. May (27) and Nelson (29) have given complete reports of the incidents which led to the discovery in 1952 and 1953 that certain infants fed the proprietary product, liquid SMA, suffered repeated convulsions, and that these seizures were relieved with a change of diet or with dietary supplements. Later it was demonstrated that the administration of pyridoxine hydrochloride brought about almost immediate relief. Bessey, *et al.* (5) performed a number of tests to determine the extent to which B_6 deficiency was responsible and also to establish a requirement of the vitamin for infant nutrition.

Harris (17) suggests that the average American diet may not meet our vitamin B_6 needs as well as had been assumed. Although the daily requirement has not been definitely established, a figure of 1.5 to 2 mg per day has been rather widely accepted; however, Harris refers to Greenberg's findings that 4 mg per day is more nearly justifiable. Even though milk is not rich in vitamin B_6, it is such an important dietary constituent for many people as to be a factor when total daily intake of the vitamin is estimated. Harris contends that milk may be deficient in vitamin B_6 as well as in certain other vitamins because of seasonal variations and processing losses. These factors must be taken into consideration by those responsible for establishing dietary requirements.

II. Early Work on Vitamin B_6 in Milk

Most of the early work on the pyridoxine content of milk had been coincidental either with the estimation of the vitamin content of foods in general or with the development of methods of analysis. The values obtained (Table 1) varied considerably, but the consensus was that milk is relatively low in this vitamin. It is worthy of note that at the time of these early estimations, among the now known forms of vitamin B_6, only pyridoxine had been isolated. All other forms were referred to as "pseudopyridoxine" (49). Since all the organisms used in those early studies respond to all three forms (and the hydrolytic processes used would have released the phosphates), the "pyridoxine," as reported then, would be comparable to the "total B_6" of more recent investigations.

One of the most comprehensive studies dealing entirely with vitamin B_6 in milk was that of Hodson (20), who tested not only fresh and pasteurized whole milk but also evaporated and dried whole milk and dried skim milk. He was concerned about the possible effect of heat treatment, since the data reported by Atkin, *et al.* (2) gave a much lower value for evaporated milk on a reconstituted basis than for fresh milk. Hodson's analyses were made with the mold *Neurospora sitophila* 299 "pyridoxineless," which responds about equally to all forms of vita-

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min B_6, and were the first extensive milk studies made after the recognition of pyridoxal and pyridoxamine as other isotels of the B_6 group. The results showed good agreement in all the forms of milk tested, ranging from 0.65 mmg per ml for pasteurized whole milk and dry whole milk (on a reconstituted basis) to 0.73 mmg per ml for irradiated evaporated milk (on a reconstituted basis). This did not indicate a loss through heat sterilization of the evaporated product and is in contrast to the findings of Atkin and his associates (2).

Shortly before this, Siegel, *et al.* (35) conducted a series of experiments to determine how much of the vitamin B_6 in certain biological materials was in a free state and how much was in the bound form. Determinations were made on some aliquots before hydrolysis to estimate the free B_6 and on others after hydrolysis to estimate the total vitamin B_6; the difference indicated the amount of the vitamin which was in the bound form.

Although the existence of the three forms had been established for several years, the work of Rabinowitz and Snell on the distribution of pyridoxal, pyridoxamine, and pyridoxine in some natural products was the first significant report (1948 (31)). Their report is still the main reference in that field.

III. Later Work and Special Studies

Since the SMA incidents and the discoveries which followed, several extensive investigations have been made to determine as much as possible about the effect of various methods of treatment on the vitamin B_6 content of milk and milk products, particularly those used for infant feeding. The results of these investigations may be found in Table 1. The data of other workers who included milk among the foods they analyzed for vitamin B_6 may be found in the same table.

Two of these projects were carried out in the research department of the Wyeth Laboratories. Hassinen and his associates (18) were primarily interested in determining the vitamin B_6 content of milk products. They investigated the vitamin B_6 content of processed milk, including commercial infant formulas; the effect of heat sterilization and storage; and the heat stability of pyridoxal, pyridoxamine, and pyridoxine in milk. Tomarelli and his coworkers (48) attempted to ascertain the effect of heat on the biological availability of vitamin B_6 in milk. By means of bioassay they compared unheated and heated milk samples, with and without added vitamin B_6, as well as fortified liquid and dried infant foods.

A. Z. Hodson (21) of the research laboratories of the Pet Milk Company, as a continuation and extension of his earlier work, tested the effect of processing, heat treatment, and storage on the total vitamin B_6 in milk, as well as on the different components of the vitamin.

The investigations of Garrett and associates (13) of the M. and R. Dietetic Laboratories have dealt with the stability of vitamin B_6 in modified milk products.

A number of papers have come from the National Institute for Research in Dairying at Reading, England. The report of 1957 by Chapman, *et al.* (6) was concerned with the effect of heat processing on several vitamins of the B complex, including vitamin B_6. They compared the B_6 content of raw milk with that of pasteurized, sterilized, evaporated, sweetened condensed, roller-dried, and spray-dried milk. Gregory, *et al.* (15) investigated the B-vitamin content of milk in relation to breed of cow and the stage of lactation. The most recent papers from this Institute were published in June 1959 by Gregory (14) and Davies, *et al.* (10) and were concerned with the effect of heat on the vitamin B_6 content of milk.

Methodology

A. Biological

Biological methods of estimation have been in use since the early work with vitamin B_6. The fact that no hydrolytic or extractive procedures are required not only simplifies the process but also eliminates a common source of error inherent in such procedures. Animal assays measure "available" vitamin B_6, not the total amount present. This is both an advantage and a disadvantage, for although nutritionists especially are primarily

interested in the amount which can be used by the body rather than the amount present, it still remains that not all biological systems are the same. One needs to know how much the rat, the chick, and the human being have in common insofar as utilization of the vitamin is concerned.

One of the earliest biological tests was the cure of rat acrodynia, but it has been replaced largely by the rat growth test, in which the vitamin content is determined by growth resulting from the addition of graded doses of B_6 to a diet deficient in that vitamin. The method of Sarma, *et al.* (32), a modification of that of Conger and Elvehjem (9) is the one mainly in use today. Sarma and his associates developed a basal ration to permit minimum growth without vitamin B_6 and maximum growth with optimum amounts of B_6. Biological materials to be assayed are mixed in the diet, usually at two levels, and weight gain is recorded for four weeks. The values thus obtained are compared to a standard curve. It has been established that pyridoxal and pyridoxamine incorporated in the diet give slightly lower values than when fed as a supplement with a medicine dropper or injected intraperitoneally. This presumably accounts for the somewhat lower values usually obtained by bioassay methods than by yeast growth (34). Chick assays are similar but are used less frequently than those employing rats.

Henderson, *et al.* (19) and Sarma, *et al.* (32) made use of the rat growth method in their estimation of the vitamin B_6 content of various food products. Tomarelli and his staff (48) employed this method in their efforts to determine the biological availability of vitamin B_6 of heated milk products, but they did not use the bioassay as a means of estimating total vitamin B_6 in milk. They made certain modifications in the sugar content of the basal diet in an attempt to secure a form of sugar with which pyridoxal and pyridoxamine were equal in activity to pyridoxine and yet avoid the complications which might arise from too high a proportion of lactose in the milk products to be tested. Davies, *et al.* (10) used both rat and chick assays in their tests of the effect of heat on vitamin B_6 in milk.

B. Microbiological

The microbiological method is used far more than any other in the analysis of biological materials for vitamin B_6. It is very sensitive and less subject to interference from other compounds. Although pyridoxal, pyridoxamine, and pyridoxine and their phosphates can all be measured by bioassay, the bound forms are not active for most microorganisms and the assay materials must therefore be hydrolyzed. Recent work by Hodson (21) indicates that *N. sitophila* will respond to milk equally well with or without preliminary hydrolysis, but for any method which measures turbidity, hydrolysis apparently is necessary for the purpose of clarification.

Some microorganisms (*Saccharomyces cerevisiae* G. M., *N. sitophila* 299, and *Saccharomyces carlsbergensis* 4228) respond approximately equally to all three forms (1) and thus are used to measure total vitamin B_6. Any of the forms may be used as the standard, but the usual practice is to express the results in terms of pyridoxine, either the free base or the hydrochloride. On the other hand, certain microorganisms do not respond to some of the forms. *Lactobacillus casei* shows activity only for pyridoxal while *Streptococcus faecalis* R and *Streptococcus faecium* Ø 51 are activated by both pyridoxal and pyridoxamine.

Most of the assays for total B_6 are now done with *N. sitophila* 299 (43) or *S. carlsbergensis* (2). These methods were developed about the same time and have been used with simple modifications by various workers since then. Both give good results, and the choice of method may depend somewhat upon the available laboratory facilities (1). The yeast method (2) in which turbidity is measured, is quicker, simpler, and more convenient, although there has been some question as to its reliability with milk (21). With the mold mutant (43) the growth is measured gravimetrically, a process which is favored by some analysts. If, as reported by Hodson (21), *N. sitophila* utilizes the phosphates without hydrolysis, the consequent elimination of this sometimes difficult step may also be a further recommendation.

S. cerevisiae was used in some of the earlier work, but it has not been particularly popular as an assay organism in recent years. The method is similar to the one using *S. carlsbergensis* in that it involves the measurement of turbidity. Assay values agree quite well with those of other methods reported in the literature. *S. cerevisiae* shows only 40 to 46% as much activity for pyridoxamine or pyridoxal as it does for pyridoxine. Therefore if most of the B_6 in the product being tested is either pyridoxamine or pyridoxal, the values obtained will be unduly low if measured against a pyridoxine standard (38). Since it has been demonstrated (32) that when fed in the ration for bioassay, pyridoxal and pyridoxamine are also less active than pyridoxine, it may follow that such a method of measurement may more accurately reflect the nutritionally available form of B_6 than the other methods currently in use.

In the differential assay certain problems are encountered. There is no known organism which will measure pyridoxine alone. *Lactobacillus casei* is specific for pyridoxal, *Streptococcus faecalis* measures both pyridoxamine and pyridoxal, and *Saccharomyces carlsbergensis* measures all three forms; thus a differential determination may be made by calculation, i.e., total B_6 − (pyridoxal + pyridoxamine) = pyridoxine (31). The accuracy of the results obtained by this means is open to question, particularly those for pyridoxine, since the final value is dependent upon three other values and any errors which might be present could be cumulative. At the time this differential assay was proposed, *S. carlsbergensis* was giving equal response to all three forms of the vitamin but later work has indicated that most strains of the organism are not so sensitive to pyridoxamine as they are to pyridoxine and pyridoxal (30, 4). However, there is no better method of approximating pyridoxine microbiologically at this time.

Inasmuch as milk apparently contains practically no pyridoxine, relatively satisfactory differential determinations can be made by using *L. casei* and *S. faecalis*. The *S. faecalis* method has not been considered entirely satisfactory by either its originators or Hodson, but further refinements in the method may reduce the margin of error.

Gregory, in her microbiological tests of the effect of heat on vitamin B_6 (14), compared *S. faecalis* with *Streptococcus faecium* Ø 51 for measuring pyridoxal plus pyridoxamine. She reported that the differential assay technique is improved by the use of *S. faecium* which is more specific than *S. faecalis* since it does not respond to alanine alone.

Snell (36, 37) reported that pyridoxal, but not pyridoxamine or pyridoxine, is destroyed by alkali and acetone. In the same references Snell proposed determining pyridoxamine by measuring the response of *S. carlsbergensis* to the assay material before and after treatment with nitrous acid. Hodson used these methods for comparison with the figures obtained by *L. casei* analysis. For a summary of the methods see page 78.

Good summaries of the methods currently in use and their comparative advantages and disadvantages are given by Snell (38, 40); however, milk is not mentioned specifically. Probably the most complete comparison in recent literature is that made by Hodson in 1956 (21). His concern was with milk and milk products as his only assay material and with finding the best way of determining total B_6 as well as the specific members of the B_6 group. He compared the use of *N. sitophila* and *S. carlsbergensis* with modifications of both methods; tested the need for hydrolysis and the effect of different hydrolytic procedures; compared the activity of pyridoxal and pyridoxamine and their phosphates for *N. sitophila* and *S. carlsbergensis;* checked the effect of sterilization, evaporation, and storage on milk; and tested the use of three different methods of determining pyridoxal in milk.

Little work has been done in determining the free and bound forms of vitamin B_6 in milk; in fact, with the exception of the report of Siegel, *et al.* in 1943 (35), no values are available which compare the amount of B_6 before and after hydrolysis. None could be found dealing with conjugated or unconjugated forms of pyridoxal, pyridoxamine, and pyridoxine. It has been assumed that most vitamin B_6 in nature is in the bound

state but the analysis of milk referred to above indicates only 14% in the bound form.

C. Chemical

One of the earliest reports on the B_6 content of various foodstuffs is that of Swaminathan in 1940 (44, 45). He used a chemical method of analysis, making use of a color reaction between pyridoxine and diazotized sulfanilic acid as a basis for estimation. However, the complete removal of interfering substances is such an involved and difficult procedure that the method has not been widely used. A number of people have attempted to modify it so that it would be more satisfactory. The only one of these who has included milk in his studies is Fujita (12) who, with his colleagues, has done extensive work with chemical methods in recent years, making use of ion exchange resins to remove interfering substances. As a result, Fujita and his coworkers have estimated total B_6, pyridoxal, pyridoxamine, and pyridoxic acid and have made some contributions in the field of comparing hydrolytic methods.

D. Hydrolytic Processes

Hydrolysis has been a problem since the early days of developing methods of estimation. Williams, Cheldelin, and Mitchell (50) used only enzymatic hydrolysis in their milk analyses and their results, determined microbiologically, were very low. On the other hand Swaminathan (44, 45) used a combination of pepsin digestion and acid hydrolysis before chemical determination, and his results were quite high. Just how much of this variation is due to the hydrolytic process is certainly open to question but it may be an important factor. Siegel, *et al.* (35) suggest that the value obtained by Williams, *et al.* (50) represents only free vitamin B_6. Most of the other methods use either H_2SO_4 or HCl varying in concentration from 0.055*N* to 2.0*N*. Methods differ also in the time and temperature of autoclaving. For example, Rabinowitz and Snell (31) adapted the method of Atkin, *et al.* (2) for *S. carlsbergensis* by increasing the time from 1 hour to 5 hours, the pressure from 15 lb. to 20 lb., and changing the acid from H_2SO_4 to HCl. After trying several methods, Fujita, *et al.* (12) concluded that pyridoxine and pyridoxamine could be extracted most successfully by preheating the homogenate at pH 4.5 at 80° C for 15 minutes, then making the supernatant 0.6*N* in H_2SO_4 and autoclaving at 130° C for one hour. On the other hand, better results were obtained for pyridoxal and 4-pyridoxic acid when the homogenate was heated directly in 0.1*N* H_2SO_4 at 130° C for one hour.

Hopkins and Pennington (24) reported that the dried milk gave low results when 2.0*N* H_2SO_4 was used as had been suggested by Siegel, *et al.* unless the sample had been treated previously with 0.1*N* H_2SO_4; they suggested that the vitamin in the bound state was more readily destroyed by the stronger concentration of acid.

Gregory compared several extraction procedures (14). She autoclaved the samples in 0.055*N* HCl at 120° C for various periods of time, viz., 30 minutes, 1 hour, 2 hours, and 4 hours. She also tried heating the samples in 0.055*N* HCl with steam for 30 min. The results after the steam treatment were similar to those obtained by autoclaving; she therefore adopted this process for extraction of the samples.

E. Further Modifications of Methods

Other modifications in the treatment of the samples and in the various assay methods may well be summarized. Siegel, *et al.* (35) modified the method of Williams (49) using *S. cerevisiae* by adding Lloyd's reagent to remove all the pyridoxine from the medium. This necessitated an increase in the biotin and casein hydrolysate content of the medium and the addition of tryptophan, but it resulted in much greater response.

The Stokes, *et al.* method for *N. sitophila* required the destruction of thiamine in the sample by means of bisulfite. Tatum, *et al.* (47) suggested adding thiamine in excess to the medium so as to eliminate the necessity of destroying it in the sample and at the same time increase the sensitivity of the assay, but these suggestions apparently were not tested to any extent. Morris, Herwig, and Jones (28), thinking that the sulfite cleavage might destroy pyridoxine, proposed

autoclaving the samples with sodium hydroxide to destroy the thiamine; but since such a procedure produced an annoying amount of soap in high-fat products without any appreciable increase in convenience, the method did not become popular (21). Harris (16) also suggested avoiding the cleavage problem, with its possible stimulatory action, by the addition of thiamine to the sample. Hodson (21) followed this method and secured highly increased sensitivity. He also modified the method by eliminating the acid hydrolysis entirely after testing samples of sterilized and nonsterilized milk which were nonhydrolyzed, hydrolyzed with 1.0*N* HCl, and hydrolyzed with 0.055*N* H_2SO_4.

The Atkin, *et al.* method using *S. carlsbergensis* was modified by Rabinowitz and Snell (31) by the addition of niacin to the medium. This has been followed in most laboratories since then. Cheslock (8) suggested the use of tryptophan in the medium used for blood analysis but there is no report of its being used for milk. Hopkins and Pennington (24) added nicotinic acid and ammonium phosphate. Snyder and Wender (42) advocated the use of 50 ml Erlenmeyer flasks for growing the yeast in order to avoid the continuous shaking.

F. Effect of Light on Vitamin B_6

It has long been known that pyridoxine and its related compounds were light labile. For that reason, every effort is made during analyses to avoid light, particularly ultraviolet light. Suggestions vary all the way from working with drawn shades in the laboratory to the use of a dark room with darkroom lights. Microbiological methods require many pipettings, so provision must be made for adequate visibility without vitamin destruction.

G. Reporting of Results

Although not literally a part of the method, the matter of reporting results has almost as many variations, yet it is of prime importance. A study of the literature will soon reveal that some authors report their findings in terms of pyridoxine hydrochloride, since this form is generally used for a standard. Others specifically state that their results are in terms of the free base, which is 0.82 times the pyridoxine hydrochloride. In some instances it is not clear how the values were expressed. Furthermore, results for fresh milk are usually reported in terms of micrograms per milliliter (or milligrams per liter), and for evaporated milk in the same way on a reconstituted basis. Values for dried milk may be given either as micrograms per milliliter on a reconstituted basis or micrograms per gram for the dry form. Some authors report values for liquid milk (fresh or evaporated) in micrograms per gram without being explicit as to whether this means grams of liquid milk or dry solids.

V. Discussion

Examination of the data reported by various authors will reveal a wide range of results and some unexplained discrepancies. (See Tables 1 and 2.) Some of these may be attributed to differences in technique; others may result from too few samples being tested. Doubtless, as methods of estimation have been improved, some errors have been eliminated; consequently, more recently recorded values may differ from those reported earlier. For example, most of the values reported for liquid milk before 1950 were between 0.50 and 0.70 mmg per ml; in fact, Hodson reported 0.56 mmg per ml (free base) for nonsterilized evaporated milk when assayed with his unmodified *N. sitophila* method in 1956 (21), but after modifying the method by the addition of thiamine to the medium he reported 0.38 mmg per ml for the same product.

In spite of these variations we may draw some definite conclusions. Since recent years have brought a great increase in the amount of dried milk products on the retail market, the effect of such processing on the vitamin content has been studied. There is apparent agreement on the part of all those who have tested these products that there is no appreciable loss in the drying process. Chapman and associates (6) reported three methods of drying milk, one of which used higher than usual temperatures. The B_6 content of the milk so treated was approximately 10%

Table 1. Total vitamin B_6 content of milk

Sample	Product	Total B_6 mmg/g	Total B_6 mmg/ml	Method	Treatment of sample[a]	Reference
			Whole Milk, Fresh			
1		1.7[c]		chemical	acid, enzyme (1a)	Swaminathan, 1940 (44, 45)
2		1.3[c]		rat growth		Henderson, *et al.*, 1941 (19)
3			0.06[c]	*S. cer.*	enzyme (1b)	Williams, *et al.*, 1942 (50)
4	Pasteurized		0.54[a]	*S. carls.*	acid (2a)	Atkin, *et al.*, 1943 (2)
5			0.58[b]	*N. sito.*	acid (2b)	Stokes, *et al.*, 1943 (43)
6		0.51[c,d]		*S. cer.*	acid (2c)	Seigel, *et al.*, 1943 (35)
7			0.67[b]	*N. sito.*	acid (2b)	Hodson, 1944 (20)
8	Pasteurized		0.65[b]	*N. sito.*	acid (2b)	Hodson, 1944 (20)
9			0.30[b]	*S. carls.*	acid (2a) mod.	Snell & Keevil, 1954 (41)
10			0.51[a]	not stated	not stated	Macy, 1953 (26)
11			0.58[a]	*S. carls.*	acid (2a)	Hassinen, *et al*,. 1954 (18)
12			0.48[c]	not stated	not stated	NRC Bulletin 1953 (11)
13	Raw	0.22[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
14	Pasteurized	0.20[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
15	Sterilized	0.22[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
16		6.5[c]		*N. sito.*	alkali (2f)	Morris, *et al.*, 1949 (28)
17		0.36[a]		chemical	acid (2e)	Fujita, *et al.*, 1955 (12)
18		0.57[a]		chemical	acid (2e)	Yano and Fujita, 1956 (52)
19			0.33[b]	*S. carls.*	acid (2a)	Hodson, 1956 (21)
20			0.35[b]	*N. sito.*	acid (2b)	Hodson, 1956 (21)
21			0.31[a]	*S. carls.*	acid (2a) mod.	Gregory, *et al.*, 1958 (15)
22	Freeze-dried	3.38[b]		*S. carls.*	acid (2a) mod.	Gregory, 1959 (14)
	Freeze-dried	3.2[b]		chick growth		Davies, *et al.*, 1959 (10)
	Freeze-dried	4.9[b]		rat growth		Davies, *et al.*, 1959 (10)

Table 1 (*Continued*)

Sample	Product	Total B_6 mmg/g	Total B_6 mmg/ml	Method	Treatment of sample[a]	Reference
			Whole Milk, Dry			
1		5.5[c]		*S. carls.*	acid (2d)	Hopkins, *et al.*, 1947 (24)
2	Spray-dried	3.3[c]		*S. carls.*	acid (2a)	Sharp, *et al.*, 1945 (33)
3		8.2[c]		*N. sito.*	acid (2b)	Barton-Wright, 1945 (3)
4		7.0[c]		*N. sito.*	alkali (2f)	Morris, *et al.*, 1949 (28)
5		5.0[b]	0.65[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1944 (20)
6	Spray-dried		0.47[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
7	Roller-dried	1.8[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
8	Spray-dried	1.8[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
9	Spray-dried UHT	1.5[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
			Whole Milk, Sweetened, Condensed			
1			0.43[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
2		0.52[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
3	(Before condensation)	0.17[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
			Whole Milk, Evaporated			
1			0.62[a]	*S. carls.*	acid (2a)	Atkin, *et al.*, 1943 (2)
2	Irradiated		0.73[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1944 (20)
3			0.28[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
			0.35[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
4	Nonsterilized		0.57[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1956 (21)
5	Sterilized		0.56[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1956 (21)
6	Sterilized		0.33[b] (reconst.)	*S. carls.*	acid (2a)	Hodson, 1956 (21)
7	Sterilized		0.33[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1956 (21)
8	Nonsterilized		0.35[b] (reconst.)	*S. carls.*	acid (2a)	Hodson, 1956 (21)

Table 1 (*Continued*)

Sample	Product	Total B_6 mmg/g	Total B_6 mmg/ml	Method	Treatment of sample[a]	Reference
			Whole Milk, Evaporated (*Continued*)			
9	Nonsterilized		0.38[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1956 (21)
10	Sterilized		0.32[b] (reconst.)	*N. sito.*	nonhydrolyzed	Hodson, 1956 (21)
11	Sterilized		0.34[b] (reconst.)	*N. sito.*	1 *N* HCl	Hodson, 1956 (21)
12	Sterilized		0.29[b] (reconst.)	*N. sito.*	0.055 *N* H_2SO_4	Hodson, 1956 (21)
13	Nonsterilized		0.38[b] (reconst.)	*N. sito.*	nonhydrolyzed	Hodson, 1956 (21)
14	Nonsterilized		0.37[b] (reconst.)	*N. sito.*	1 *N* HCl	Hodson, 1956 (21)
15	Nonsterilized		0.30[b] (reconst.)	*N. sito.*	0.055 *N* H_2SO_4	Hodson, 1956 (21)
16	Stored		0.13[b] (reconst.)	*S. carls.*	acid (2a)	Hodson, 1956 (21)
17	Stored		0.32[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1956 (21)
18	Nonsterilized	0.57[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
19	Sterilized	0.61[c]		*S. carls.*	acid (2a) mod.	Chapman, *et al.*, 1957 (6)
20	Freeze-dried	1.0[b]		*S. carls.*	acid (2a) mod.	Gregory, 1959 (14)
	Freeze-dried	2.1[b]		chick growth		Davies, *et al.*, 1959 (10)
	Freeze-dried	2.7[b]		rat growth		Davies, *et al.*, 1959 (10)
21	Stored, freeze-dried	0.63[b]		*S. carls.*	acid (2a) mod.	Gregory, 1959 (14)
	Stored, freeze-dried	1.4[b]		chick growth		Davies, *et al.*, 1959 (10)
	Stored, freeze-dried	2.0[b]		rat growth		Davies, *et al.*, 1959 (10)
			Skim Milk, Fresh			
1	Pasteurized		0.427[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
2	Pasteurized		0.403[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
			Skim Milk, Dry			
1		5.5[a]		*S. carls.*	acid (2a)	Atkin, *et al.*, 1943 (2)
2		6.8[b]	0.66[b] (reconst.)	*N. sito.*	acid (2b)	Hodson, 1944 (20)

Table 1 (*Continued*)

Sample	Product	Total B_6 mmg/g	Total B_6 mmg/ml	Method	Treatment of sample[e]	Reference
			Skim Milk, Dry (*Continued*)			
3	Spray-dried		0.52[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
4		3.8[a]		rat growth		Sarma, *et al.*, 1947 (32)
5		5.7[a]		*S. carls.*	acid (2g)	Hoff-Jorgensen, 1952 (23)
6			0.38[b]	*S. carls.*	acid (2a)	Hodson, 1956 (22)
			0.33[b]	*N. sito.*	acid (2b)	Hodson, 1956 (22)
7			0.33[b]	*S. carls.*	acid (2a)	Hodson, 1956 (22)
			0.32[b]	*N. sito.*	acid (2b)	Hodson, 1956 (22)
			Infant Formulas, Sterilized Liquid			
1			0.17[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
2			0.19[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
3			0.10[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
4			0.22[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
5			0.13[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
6	(Fortified with B_6)		0.61[a]	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
7	(Fortified with B_6)	6.3[a]		*S. carls.*	acid (2a)	Tomarelli, *et al.*, 1955 (48)
8	Before sterilization		0.494[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
9	After sterilization		0.367[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
10	Storage, 6 mo. R. T.		0.367[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
11	Storage, 1 yr. R. T.		0.365[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
12	Storage, 3 mo. 100°F.		0.351[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)
13	Storage, 6 mo. 100°F.		0.345[a]	*S. carls.*	acid (2a) mod.	Garrett, *et al.*, 1958 (13)

Table 1 (*Continued*)

Sample	Product	Total B_6		Method	Treatment of sample[e]	Reference
		mmg/g	mmg/ml			
Infant Formulas, Spray-Dried						
1	(Fortified with B_6)	5.3[a]	0.61[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
2	(Fortified with B_6)			*S. carls.*	acid (2a)	Tomarelli, *et al.*, 1955 (48)
3			0.26[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
4			0.24[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)
5			0.31[a] (reconst.)	*S. carls.*	acid (2a)	Hassinen, *et al.*, 1954 (18)

[a] Pyridoxine hydrochloride.
[b] Pyridoxine (free base).
[c] Form of B_6 standard not stated.
[d] Free, .44; bound, .07.
[e] Numbers in parentheses in this column refer to method of hydrolytic procedure as described on p. 78.

less than that of the other two. Reported values for dry skim milk and dry whole milk are usually the same. Storage, even for a year, did not affect the values (22).

Hodson (21) is the first investigator to report on nonsterilized evaporated milk. Although he did not compare the same lots of milk before and after evaporation, a number of nonsterilized evaporated samples were tested both with *S. carlsbergensis* and *N. sitophila*. Both organisms gave similar values for fresh milk and nonsterilized evaporated milk.

Although the early investigations of Hodson (20) indicated no loss of vitamin B_6 from heating, such as is necessary for sterilization, the later work of Hassinen and associates (18) differed in this respect, showing that canned milk products had a B_6 content of from 33 to 64% that of the fresh milk. In Hodson's later investigation (21) he found that he obtained a significant difference in the results after heating, particularly upon storage. (See Table 3.) The response differed according to the organism used for the assay, as the heat- and storage-produced loss was more apparent with *S. carlsbergensis* than with *N. sitophila*. Chapman and associates (6) also concluded that the heat stability of vitamin B_6 may have been overestimated, even though their own tests showed no appreciable loss. That the loss which took place was due to the heating process itself was demonstrated by the tests made on evaporated milk before and after the sterilization process (21). But the loss in the milk tested immediately after sterilization was not so significant as that in milk which had been stored for even a short time. Gregory's tests (14) indicated that the major loss of pyridoxal occurred within the first two weeks of storage. (See Table 4.) This may be an important factor, particularly in view of the increasing use of canned evaporated milk for infant feeding.

Hassinen, *et al.* (18) indicated that a loss in vitamin B_6 took place, but most of the reported data are from Hodson's study (21). He measured not only total B_6, but also pyridoxal and pyridoxamine, in stored milk. As a result of his work and that of others in the field. Hodson has postulated certain

Table 2. Components of vitamin B_6 in fresh milk

	Whole Milk, Sample No.	Pyridoxal	Pyridoxamine	Pyridoxine	Pyridoxic Acid	Total B_6	Method	Reference
1	Fresh (mmg/ml)	0.32[a]	0.09[a]	−0.02[a]			microbiological	Rabinowitz & Snell, 1948 (31)
2	Fresh (mmg/g)	0.25[a]			0.45[a]		fluorometric	Fujita, *et al.*, 1955 (12)
3	Fresh (mmg/g)	0.25[a]	0.11[a]			0.36[a]	fluorometric	Fujita, *et al.*, 1955 (12)
4	Fresh (mmg/g)	0.47[a]	0.12[a]			0.57[a]	fluorometric	Yano & Fujita, 1956 (52)
5	Fresh (mmg/ml)	0.30[b]				0.29, 0.37[b]	microbiological	Hodson, 1956 (21)
6	Fresh (mmg/ml)	0.36[b]				0.27[b], 0.33[b]	microbiological	Hodson, 1956 (21)
7	Fresh (mmg/ml)	0.340[b]				0.367[b]	microbiological[c]	Hodson, 1956 (21)
8	Pasteurized (mmg/ml)	0.294[b]				0.342[b]	microbiological[c]	Hodson, 1956 (21)
9	Raw (freeze-dried) (mmg/g)	3.20[b]	0.60[b]	−0.13[b]		3.38[b]	microbiological	Gregory, 1959 (14)

[a] Hydrochloride.
[b] Free base.
[c] Preceded by alkali-acetone treatment for pyridoxal determination. See page 79.

theories regarding the effect of heat and storage but agrees that definite proof of their validity is lacking at this time. It is generally accepted (14,21,31) that the vitamin B_6 activity of fresh milk is due largely to pyridoxal. Extensive heat-treatment apparently changes part of the pyridoxal to pyridoxamine. This process of conversion may continue during storage. Hodson (21) and Tomarelli, *et al.* (31) both suggest that storage, even for a few days, may result in conversion of the pyridoxamine to another unknown form which has different activity for various organisms. From their work it appears that this unknown form has activity equal to pyridoxine for *N. sitophila,* less for *S. carlsbergensis* and still less for the rat. The work of Davies, *et al.* (10) does not confirm Tomarelli's findings that B_6 activity in stored evaporated milk is less for the rat than for *S. carlsbergensis;* in fact, they found greater activity for both the rat and chick than for yeast.

It has been suggested (14) that the residual activity in stored milk products may be due to pyridoxine, but since the differential assay method of Rabinowitz and Snell (31) cannot be considered as accurate for pyridoxine as for pyridoxal, this contention cannot be established definitely at this time. Just how much of the vitamin is available for the human is not known, but since there has been no evidence of deficiency from the use of stored evaporated milk, the human infant may be able to utilize this form or else sufficient vitamin B_6 remains to meet his needs.

The work of Gregory and her associates (15) indicates that there is no more variation in the B_6 content of the milk from breed to breed than there is from one cow to another, but that the B_6 content does change during the stages of lactation. The values which she reported for "mature" milk ranged from 0.03 to 0.68 mmg per ml with an average of 0.31 mmg per ml; there was a steady rise in values from the 7th to the 14th day, after which there was a gradual decrease until the 5th month. From that time on changes in vitamin B_6 content were not significant.

Since commercial infant foods and home-prepared formulas contain added sugars, the type of carbohydrate which is added may have physiological significance. Sarma, *et al.* (32), Tomarelli, *et al.* (48), and Parrish, *et al.* (30) have noted an interrelationship of sugars, amino acids, and B_6 which is not fully understood. Also, these formulas frequently receive a second heat treatment in the form of terminal sterilization. It may be possible that some complex is formed which cannot be assimilated readily. Snell (39) suggests that alteration in the amino acids of heat-treated proteins may increase the need for pyridoxal phosphate as the coenzyme. It was demonstrated by Tappan, *et al.* (46) that highly processed protein appeared to result in an increased need for B_6. Biological availability tests have been made with the rat and chick. In such studies the intestinal synthesis of vitamin B_6 must be considered. What has not yet been determined is whether the heat treatment of

lactose favors the propagation of the intestinal flora which use B_6, thus decreasing the amount available to the rat or chick, or if some other factor is responsible for the lowered growth response.

As a result of the evidence that the sterilized liquid infant food, SMA, definitely contained less B_6 (18), pyridoxine hydrochloride was added as a supplement. Further tests revealed that no more B_6 was lost from the fortified product than from the unfortified ones, indicating a greater heat stability for synthetic pyridoxine hydrochloride than for natural pyridoxal. In fact, when pyridoxal and pyridoxamine were used as supplements, they were destroyed by autoclaving in approximately the same degree as were natural pyridoxal and pyridoxamine. This was in agreement with the findings of Rabinowitz and Snell (31).

A still more recent report by Garrett and associates (13) from the M. and R. Dietetic Laboratories confirms the loss of B_6 during the commercial processing of milk and milk

Table 3. Pyridoxal, pyridoxamine, and total B_6 in evaporated milk; effects of sterilization and storage[a]

Evaporated Whole Milk, Sample No.	Pyridoxal mmg/ml	Pyridoxamine, mmg/ml	Total B_6, mmg/ml	Assay Organisms	Auxiliary Treatment
			Nonsterilized		
1	0.35		0.36, 0.37	*L. casei, S. carls., N. sito.*	none
2	0.288		0.401	*S. carls.*	alkali-acetone
3	0.255		0.341	*S. carls.*	alkali-acetone
4	0.25	0.12	0.34, 0.30	*S. carls.*	nitrous acid
			Sterilized		
5	0.24		0.31, 0.37	*L. casei, S. carls., N. sito.*	none
6	0.177		0.377	*S. carls.*	alkali-acetone
7	0.139		0.275	*S. carls.*	alkali-acetone
8	0.13	0.28	0.27, 0.40	*S. carls.*	nitrous acid
			Stored		
9	0.13		0.17, 0.29	*L. casei, S. carls., N. sito.*	none
10	0.15		0.18, 0.29	*L. casei, S. carls., N. sito.*	none
11	0.16		0.15, 0.31	*L casei, S. carls., N. sito.*	none
12	0.19		0.17, 0.32	*L. casei, S. carls., N. sito.*	none
13	0.085		0.303	*S. carls.*	alkali-acetone
14	0.005		0.32	*S. carls.*	alkali-acetone
15	0.055		0.36	*S. carls.*	alkali-acetone
16	0.00		0.372	*S. carls.*	alkali-acetone
			Stored 20 Months		
17	0.00	0.15	0.32	*S. carls.*	nitrous acid
18	0.05	0.17	0.36, 0.30	*S. carls.*	nitrous acid
19	0.00	0.07	0.29, 0.27	*S. carls.*	nitrous acid

[a] All values expressed as free base. Reference Hodson, 1956 (21).

Table 4. Pyridoxal, pyridoxamine, and pyridoxine in milk; effect of heat and storage[a]

Sample No.	Milk Product	Pyridoxal	Pyridoxamine	Pyridoxine	Assay Organisms
1	Raw, freeze-dried, mmg/g	3.20	1.73	−0.71	*L. casei, S. faecalis*
		3.20	0.60	−0.13	*L. casei, S. faecium*
2	Evaporated, fresh, freeze-dried, mmg/g	0.46	3.34	−1.22	*L. casei, S. faecalis*
		0.46	0.93	0.05	*L. casei, S. faecium*
3	Evaporated, stored, freeze-dried, mmg/g	0.05	2.05	−0.31	*L. casei, S. faecalis*
		0.05	0.65	0.30	*L. casei, S. faecium*
4	Sterilized, fresh, mmg/ml	0.13	0.10	0.02	*L. casei, S. faecium*
5	Sterilized, stored, mmg/ml	0.02	0.05	0.0	*L. casei, S. faecium*
6	Evaporated, fresh, mmg/g	0.08	0.18	0.04	*L. casei, S. faecium*
7	Evaporated, fresh, mmg/g	0.04	0.19	0.12	*L. casei, S. faecium*
8	Evaporated, stored, mmg/g	0.01	0.09	0.26	*L. casei, S. faecium*
9	Evaporated, stored, mmg/g	0.01	0.11	0.28	*L. casei, S. faecium*
10	Evaporated, stored, mmg/g	0.01	0.10	0.29	*L. casei, S. faecium*
11	Evaporated, stored, mmg/g	0.01	0.08	0.35	*L. casei, S. faecium*
12	Condensed, fresh, mmg/g	0.45	0.22	0.01	*L. casei, S. faecium*
13	Condensed, fresh, mmg/g	0.14	0.25	0.03	*L. casei, S. faecium*

[a] All values expressed as free base. Reference Gregory, 1959 (14).

products. For the dried product (infant food) there was a loss of 23% of the vitamin B_6 from the amount present at the pasteurized milk stage, 11% of which occurred prior to the drying step. For the liquid product, there was a loss of 14% prior to sterilization and a total loss of 25%. When synthetic pyridoxine hydrochloride was added to the liquid product and processed in the same way, the loss of the synthetic form was only 10%. These findings concerning the relative heat stability of the natural and synthetic forms are in agreement with Hassinen, *et al.* (18). The M. and R. Laboratory report indicates that as a result of terminal autoclaving, the destruction of vitamin B_6 is arithmetically proportional to the length of heating time and semi-logarithmically proportional to temperature; i.e., losses at 250° F for 5 minutes and 230° F for 10 minutes were comparable. An additional factor was that of the type of cans used; tests showed 5 to 10% greater loss of B_6 in epoxy resin enamel or "corn" enamel than with hot-dipped or electrolytically-deposited tin cans. Storage of the liquid milk products did not appreciably affect the B_6 content, although there was slight loss if the temperature was high (100° F). Can surface was more important than time of storage as a factor in loss.

Other recent tests (13) indicate no correlation between the type of fat and the stability of vitamin B_6 during sterilization; however, palm oil was not included in the fats tested. Coconut oil had been substituted for palm oil in the SMA formula shortly before the incidents of 1952 and was subsequently replaced by palm oil.

A few studies have been made on the vitamin B_6 content of milk of various species of animals. (See Table 5.)

VI. Summary

By way of summary there are two main divisions. First, what has been learned about the vitamin B_6 content of milk and milk products; and second, what should be the

course of future investigations. In view of recently reported data one may conclude the following:

1. Fresh cow's milk ranges in value from 0.40 to 0.58 mmg per ml.
2. Pasteurization, drying, and evaporation *per se* do not appreciably change the vitamin B_6 content.
3. Heat-treatment, such as sterilization, may cause lowered B_6 values, perhaps because pyridoxal and pyridoxamine, the B_6 forms present in milk, are more heat labile than pyridoxine.
4. Storage, such as is practiced with commercial evaporated milk products, may further decrease the B_6 content, possibly by changing it to a less readily available or less measurable form.

What, then, is indicated for further work? Certain paths are already obvious. One of the first would be toward more reliable methods of estimation of the three members of the B_6 group and their phosphates. There is now no satisfactory method for the direct measurement of pyridoxine. *S. faecalis* cannot be completely relied upon to give satisfactory results for pyridoxal and pyridoxamine, at least with milk. In addition to the problem created by the stimulation of *S. faecalis* by alanine, there is evidence that the phosphorylated forms of pyridoxamine and pyridoxal also are active for *S. faecalis* (31, 4). Therefore, any analysis of unhydrolyzed samples will reflect the response of this organism to the free plus the phosphorylated forms of pyridoxamine and pyridoxal. Gregory's use of *S. faecium* Ø 51 may offer a partial solution, since it is apparently more specific than *S. faecalis*. There is still uncertainty about the response of some of the other organisms to the bound forms (21). *N. sitophila* apparently responds approximately the same to unhydrolyzed samples as to hydrolyzed ones. Also, pyridoxal and pyridoxamine and their phosphates were approximately equivalent in activity for *S. carlsbergensis* in Hodson's tests, but such response has not been reported from other sources.

The newer knowledge of the response of *S. cerevisiae* to the different members of the B_6 group and its similarity to the response of the rat to these same forms may be worthy of study.

An effort is being made to improve chemical methods of estimation, either to supplant or to supplement microbiological methods. Refinements in the field of chromatography and ionophoresis offer encouragement in this direction.

Further modifications of methods of hydrolysis may result in greater accuracy. This may well be one factor contributing to the lack of reproducibility noted in some assays.

Table 5. Total vitamin B_6 content of milk of various species[a]

Species	B_6, mmg/ml	No. of Specimens	Assay Organism	Reference
Goat (Sanaan, Nubian, Toggenberg)	0.067	4	*S. cer.*	Williams, *et al.*, 1942 (50)
Goat	0.48	bulk sample	*S. carls.*	Gregory, 1959 (14)
Dog (English Bull)	0.084	1	*S. cer.*	Williams, *et al.*, 1942 (50)
Mare (Thoroughbred)	0.014	1	*S. cer.*	Williams, *et al.*, 1942 (50)
Mouse (albino)	0.14	3	*S. cer.*	Williams, *et al.*, 1942 (50)
Sow	0.27	bulk sample	*S. carls.*	Gregory, 1959 (14)
Cow (Jersey, Guernsey)	0.06	14	*S. cer.*	Williams, *et al.*, 1942 (50)
Cow	0.25	bulk sample	*S. carls.*	Gregory, 1959 (14)
Human	0.04	5	*S. cer.*	Williams, *et al.*, 1942 (50)
Human	0.18	not stated	not stated	Macy, 1949 (25)
Human	0.13	not stated	*S. carls.*	Hassinen, *et al.*, 1954 (18)
Human	0.06	14	*S. carls.*	Wolf, 1958 (51)
Human	0.008	bulk sample	*S. carls.*	Gregory, 1959 (14)

[a] Method: microbiological. Form of B_6 used as standard not stated, except for the data of Gregory (14) whose values are expressed in terms of pyridoxine, free base.

It might be helpful to have further studies on the percentage of bound and free B_6 in milk. The data reported by Siegel, *et al.* in 1943 (35) are all that are available in the literature to date. Although the phosphorylated forms predominate in most biological products, the above data indicate that only about 14% of the B_6 in milk is in the bound form. No data have been reported on bound and free forms of pyridoxal and pyridoxamine in milk.

Since the question has been raised as to whether or not all of the vitamin B_6 is available to the organism, microbiological or biological, there is undoubtedly room for further exploration in this field. There should be more evidence as to what actually happens during the heat treatment and storage of the products and to the possible interrelationships of B_6 and additives such as carbohydrates, fatty acids, etc., and the treatment processes, and intestinal flora.

May (27) presents the possibility of certain "sparing effects" of other vitamins on B_6, about which little is known. He suggests that a possible loss of vitamin B_{12} during processing of milk may have been a contributory factor in the SMA-caused convulsions. Chapman, *et al.* (6) reported a definite loss of B_{12} during the heat treatment. This, then, opens still another field for investigation.

APPENDIX

Hydrolytic Treatment

(1) Enzyme

(a) *Swaminathan (44,45).*—The sample was heated for 30 minutes at 80° C in 0.1*N* H_2SO_4, then subjected to pepsin digestion for 24 hours at 38°. Tungstic acid was added to remove protein and protein derivatives.

(b) *Cheldelin, et al. (7).*—The sample was incubated with takadiastase and papain for 24 hours at 37° C, then heated in flowing steam for 30 minutes.

(2) Acid or Alkali

(a) *Atkin, et al. (2).*—The sample was autoclaved at 15 lb. pressure for 1 hour with 0.055*N* H_2SO_4. Rabinowitz and Snell (31) modified this method, autoclaving the samples at 20 lb. for 5 hours with 0.055*N* HCl. Garrett, *et al.* (13) used the original method but increased the time of autoclaving to 16 hours. Still another modification was that of Gregory (14) who heated the sample in 0.055*N* HCl in steam for 30 minutes.

(b) *Stokes, et al. (43).*—The sample was autoclaved at 15 lb. pressure for 1 hour with 1.0*N* HCl.

(c) *Siegel, Melnick, and Oser (35).*—The sample was autoclaved for 30 minutes at 15 lb. with 2.0*N* H_2SO_4.

(d) *Hopkins and Pennington (24).*—The method of Siegel, Melnick, and Oser (35) was modified by pretreatment with 0.1*N* H_2SO_4 for 2 hours at 15 lb., followed by autoclaving for 1 hour at 15 lb. with 2.0*N* H_2SO_4.

(e) *Fujita, Fujita, and Fujino (12).*—For the determination of pyridoxine and pyridoxamine the sample was heated at 80° for 15 minutes at pH 4.5; the supernatant was then autoclaved at 130° for 1 hour with 0.6*N* H_2SO_4.

For the determination of pyridoxal and pyridoxic acid the sample was autoclaved at 130° for 1 hour with 0.1*N* H_2SO_4 without pretreatment.

(f) *Morris, Herwig, and Jones (28).*—The sample was autoclaved with 1.0*N* NaOH at 15 lb. for 1 hour.

(g) *Hoff-Jørgensen, et al. (23).*—The sample was autoclaved at 120° for 1 hour with 1.0*M* H_2SO_4.

Organisms Used for Microbiological Methods

(1) Neurospora sitophila 299 "Pyridoxineless"

(Stokes, *et al.* (43); Hodson (20,21); Barton-Wright (3); Tatum, *et al.* (47); Morris, *et al.* (28))

Total B_6 may be measured by this organism, as it responds about equally to all three forms (41). According to Hodson (21) *N. sitophila* utilizes pyridoxal phosphate and pyridoxamine phosphate as well as the nonphosphorylated forms, eliminating the need for hydrolysis. The reliability of the assay has been improved by the destruction of thiamine in the sample (43, 3) or by the addition of thiamine to the assay medium (21, 47, 28).

(2) Saccharomyces carlsbergensis 4228

(Atkin, *et al.* (2); Hopkins and Pennington (24); Rabinowitz and Snell (31); Association of Vitamin Chemists (1); Hassinen, *et al.* (18); Chapman, *et al.* (6); Gregory, *et al.* (15); Gregory (14))

S. carlsbergensis is used most frequently to determine total B_6. According to Snell (41)

all three forms are approximately equal in activity, but others report pyridoxamine to be less active than pyridoxine and pyridoxal (14, 4). This turbidimetric method is quicker than the gravimetric process used for *N. sitophila*. Work by Hodson (21) and Tomarelli, *et al.* (48) indicates less response by *S. carlsbergensis* to stored sterilized milk products than by *N. sitophila* and still less by the rat. Nonsterilized milk products seem about equal in activity for *S. carlsbergensis* and *N. sitophila*. The findings of Gregory were not in agreement (14).

(3) Saccharomyces cerevisiae G. M.

(Williams, *et al.* (50); Siegel, *et al.* (35))

This yeast also measures total B_6 but all forms are not equal in activity (38).

(4) Lactobacillus casei ATCC 7469

(Rabinowitz and Snell (31))

This organism is specific for pyridoxal.

(5) Streptococcus faecalis R ATCC 8043

(Rabinowitz and Snell (31); Gregory (14))

Both pyridoxal and pyridoxamine are measured by *S. faecalis*. It shows activity also for the phosphate forms, pyridoxamine phosphate stimulating even greater response than pyridoxamine alone. Pyridoxal phosphate and pyridoxal show about equal activity. D-alanine and L-alanine may replace pyridoxal or pyridoxamine as a growth factor (14).

(6) Streptococcus faecium Ø 51

(Gregory (14))

S. faecium is active for both pyridoxal and pyridoxamine. It may be stimulated by alanine but cannot substitute alanine for vitamin B_6.

Other Methods

(1) Alkali Acetone

(Hodson (21); Snell (36, 37))

This method, which is used for determining pyridoxal, is based on the work of Snell showing that pyridoxal, but not pyridoxamine or pyridoxine, is destroyed by alkali and acetone. To 1 ml of fresh or evaporated milk, 3 ml of water, 2 ml of 1*N* NaOH, and 1 ml of acetone were added and the mixture was allowed to stand in the dark at 25° for 4 hours. Then 160 ml of water was added, the mixture was neutralized, and 1 ml of 10.0*N* H_2SO_4 was added. The samples were then hydrolyzed, and the assay was completed by the *S. carlsbergensis* procedure. Results were compared with other assays carried out without the alkali-acetone treatment. The difference represented pyridoxal.

(2) Nitrous Acid

(Hodson (21); Snell (36, 37))

This method is used for determining pyridoxamine by destruction with nitrous acid. The nitriting solution consists of 1 g sodium nitrite, 12 ml glacial acetic acid, and sufficient water to make a total of 50 ml. Next 1 ml milk was treated with 2.5 ml nitriting solution, and the solution was shaken occasionally for 30 min. Then 100 mg urea was added and again the solution was shaken occasionally. The mixture was diluted to 180 ml (acidity was then about 0.055*N* as a result of the glacial acetic acid added) and the assays were completed by the *S. carlsbergensis* method. The nitrous acid does not completely destroy the biological activity of pyridoxamine. There is probably some conversion to pyridoxine. Recovery experiments by Hodson indicated that about 25% of the activity remained, and he used this factor in calculating but considered the results only semiquantitative.

(3) Fluorometric

(Fujita, *et al.* (12); Yano and Fujita (52)).

Pyridoxamine, pyridoxal, pyridoxine, and 4-pyridoxic acid were determined chemically; total B_6 was calculated from the values for the three components. The interfering substances in the sample were removed by adsorption on resins. Pyridoxine, pyridoxal, and pyridoxamine each were converted to 4-pyridoxic acid by oxidation, heated with acid to form the lactone, and the fluorescence measured.

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