B-VITAMIN CONTENT
OF
CORN MEAL DURING
NATURAL LACTIC ACID FERMENTATION

A Thesis

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by

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CHAPTER I

INTRODUCTION

Cereal grains and food legumes provide 70% of the calories and protein for the people of Asia, Africa, and Latin America (Johnson et al., 1978). Corn is a main dietary staple of Latin America and Africa. In Africa essentially all of the corn grown is for food (Inglett, 1970a). In the United States, about 75% of the corn produced is for feed and about 20% is for export (Johnson et al., 1978). The production of corn is larger than that of any other crop in the United States. Corn is the major feed used in hog production in the United States. About 40% of the corn grain fed to livestock during the period 1965-1968 was fed to hogs (Moore and Dwoskin, 1970).

A major portion of some human diets is corn. However, present day varieties of corn cannot qualify as a complete food source for humans or animals such as swine which are similar in nutritional requirements to man. The quality of the endosperm protein is low, and there are deficiencies of several vitamins and minerals (Mertz, 1970). In particular, pellegra is associated with corn staple diets (Dyke, 1965). Fortification, blending with legumes, and genetic experimentation, are among the methods that are used to improve corn and corn products nutritionally. Opaque-2-corn, a genetic variant with improved protein quality, is used to only a limited extent because its agronomic characters are inferior to those of normal corn (Johnson et al., 1978). Another method that is being explored for nutritional improvement is fermentation, either by pure cultures or by the natural flora.

According to Frazier (1967), a wet mash of grains or of the meals

undergoes a lactic acid fermentation, chiefly by the lactic acid bacteria and coliforms normally present on plant surfaces. Hesseltine (1979) reported that many traditional main course dishes of Mid-Asia, Africa, and the Mideast are produced by natural lactic acid fermentations of the grains, legumes, and milk, either singly or in various combinations. Hesseltine (1979) stated that many of these fermented foods, as compared to the nonfermented ingredients, had enhanced digestability, higher amounts of some vitamins, and longer shelf life due to the acid produced and the lowered pH.

Concerning both traditional lactic acid fermented foods and fungal fermented foods, such as tempeh, Van Veen and Steinkraus (1970) stated that digestability was increased, but the nutritive quality of the protein was not increased by the process. The riboflavin content of these foods was generally increased or remained constant, but the contents of other vitamins were variable. Increases in riboflavin in these foods were significant because, according to Platt (1964), the lack of riboflavin is the most common vitamin deficiency among the people of the world.

Hamad and Fields (1979) reported that a natural lactic acid fermentation of cereal grains improved the protein quality. Using the method of Tetrahymena pyriformis to determine the percent Relative Nutritive Value (% RNV) as the index of protein quality, Hamad and Fields (1979) found significant ($P<0.05$) increases in barley, wheat, and rice. The improvements in % RNV of corn and millet were significant ($P<0.01$). They also evaluated available lysine in these products and found a significant ($P<0.05$) increase. Available lysine, and tryptophan, and % RNV increased significantly ($P<0.05$) in natural lactic

acid fermented corn meal (Tongnual et al., 1981). Zamora and Fields (1979a) found similar increases in % RNV and in limiting amino acids in fermented cowpeas and chickpeas.

The conditions of fermentation found to increase % RNV varied, but generally a 1:4 w/v mixture of meal and tapwater was incubated at temperatures between 25° and 37°C for four to six days. The amounts of riboflavin, niacin, and thiamin in these fermented products were also evaluated after four or five days of fermentation. Riboflavin usually increased or remained constant, while niacin and thiamin usually decreased or remained constant.

Tongnual et al. (1981) evaluated the changes in % RNV during a seven day natural lactic acid fermentation of corn meal and found that the % RNV increased most during the first 48 hr of fermentation and continued to increase until the fourth day but was unchanged afterward. Lopez (1982) also found that the greatest decrease in phytate occurred during the early stages of the fermentation. Similarly, it was the purpose of this research to evaluate the effect of natural lactic acid fermentation of corn on vitamins of the B-complex. Thiamin, niacin, riboflavin, folacin, vitamin B_{12}, pantothenic acid, pyridoxine, biotin, and choline contents of whole nonfermented corn meal and samples fermented either one, two, three, or four days were assayed in order to determine the time of fermentation required to give maximum levels of all of these vitamins.

CHAPTER II

REVIEW OF LITERATURE

Composition and Nutritive Quality of Corn

Corn is mainly composed of starch, protein, and lipids. Fiber, sugar, vitamins, and minerals are also present (Inglett, 1970b). The proximate analysis of corn is listed in Table 1.

Carbohydrate

Starch is the main carbohydrate in corn, and most of it is present in the endosperm. Total sugars of the corn kernel range between 1 and 3%. Sucrose is the major sugar, with glucose, fructose, and raffinose also present (Inglett, 1970b). The sugar content of the corn germ is about 11%. The crude fiber of corn is mainly cellulose and hemicellulose (Kent, 1975).

Protein

Corn seed averages 9-10% protein. On a quantitative basis, 27% is present in the germ and 73% is in the endosperm. The germ has a high percentage of protein that has excellent nutritional quality for monogastric animals. The endosperm protein is of relatively poor nutritional quality and is deficient in the amino acids lysine and tryptophan (Johnson et al., 1978). In normal whole corn, the first limiting amino acid is lysine, the second is trytophan (Howe et al., 1965). The essential amino acid pattern of corn, as compared to the recommended pattern, is presented in Table 2.

The superiority of proteins from animal sources, as compared to

Table 1. Proximate analysis of whole corn.

Component	Percent of whole corn
Moisture	12.0
Protein	9.2
Fat	3.9
Total Carbohydrate	73.7
Fiber	1.6
Ash	1.2

Brockington (1970).

Table 2. Essential amino acid pattern of whole corn compared to the FAO/WHO standard.[1]

Amino Acid	FAO/WHO amino acid pattern g/16g N[2]	Whole corn g/16g N[1]
Lysine	4.2	3.0
Tryptophan	1.4	0.7
Cystine	2.0	1.7
Methionine	2.2	2.6
Threonine	2.8	3.6
Valine	4.2	5.0
Isoleucine	4.2	3.7
Leucine	4.8	12.8
Tyrosine	2.8	4.9
Phenylalanine	2.8	5.1

[1]Cluskey *et al.* (1978).

[2]FAO/WHO (1965).

plants, is usually attributed to the better ratios of the essential amino acids relative to each other in animal proteins. Another contributing factor is the fact that the essential amino acids, plus arginine, histidine, cystine, and tyrosine (the semi-essential amino acids) form a higher proportion of the total amino acids in animal protein. In egg proteins, the essential plus semi-essential amino acids comprise 62% of the total nitrogen, while in corn the same amino acids comprise only 48% (Swendseid et al., 1969). Robinson (1978) stated that the semi-essential amino acids are those that reduce the requirement for an essential amino acid. Robinson, however, listed histidine as essential, arginine as nonessential, and cystine and tyrosine as semi-essential.

Lipid

About 85% of the lipids of corn occur in the germ, mainly as triglycerides of fatty acids. Linoleic acid comprises 59% of the fatty acids in the germ, and whole corn satisfies the essential fatty acid requirements of growing animals when used as a major feed component (Mertz, 1970).

B-vitamins

In this section, corn as a source of B-vitamins is discussed. A more thorough discussion of some other aspects of the B-vitamins follows.

Mertz (1970) stated that adult humans and animals could probably be maintained indefinitely on a diet of Opaque-2-corn supplemented with certain vitamins and minerals. These vitamins and minerals included riboflavin, vitamin B_{12}, pantothenic acid, niacin, calcium, phosphorous, iron, copper, manganese, and zinc.

Mertz (1970) also calculated that 770g of normal hybrid corn provided the total caloric requirements (2800 kcal) for a man, age 22, weighing 70kg, and living in a temperate climate. However, based on data provided in Table 3, if the reference man was obtaining his total caloric requirements from corn, he would not consume sufficient amounts of riboflavin, vitamin B_{12}, pantothenic acid, or folacin. The amount of niacin also would be inadequate.

Corn is a relatively good source of niacin, but the major fraction of niacin in corn is in a bound form that is unavailable unless subjected to alkaline treatment (Christianson et al., 1968). Niacin requirements can be spared, however, by adding tryptophan; sixty mg tryptophan is considered equivalent to one mg niacin (National Academy of Science/National Research Council, 1974). This explains the observations of Krehl et al. (1945) that tryptophan added to the diet of corn fed rats alleviated symptoms of niacin deficiency. Pellegra is the disease associated with niacin deficiency in man. The high incidence of pellegra, where corn is a staple diet, is attributed to the combined effects of unavailable niacin and low tryptophan levels.

Corn does not contain any vitamin B_{12}. Rosenthal (1968) stated that vitamin B_{12} is not present to any extent in higher plants and that small amounts present are assumed to be due to microbial action or contamination.

Microbiology of Natural Lactic Acid Fermentations

Natural flora of grains

Bacterial numbers on grain at harvest range from a few thousand

Table 3. Vitamin content of whole corn compared to recommended daily allowances.

Vitamin	Whole corn		RDA[3]
	mg/100g[1]	mg/770g[2]	mg
Thiamin	0.45	3.47	1.40
Riboflavin	0.09	0.69	1.68
Nicotinic acid	2.30	17.70	18.48
Pantothenic acid	0.46	3.54	5.00-10.00
Biotin	0.01	0.08	*
Pyridoxine	0.69	5.30	2.00
Folacin	0.02	0.12	0.40
Choline	45.00	347.00	*
Vitamin B_{12}	0.00	0.00	0.003

[1]Kent (1975).

[2]Mertz (1970). 770g is sufficient to supply the total caloric requirements of the reference man, age 22, weighing 70 kg, living in a temperate climate, and requiring 2800 kcal per day.

[3]National Academy of Science/National Research Council (1974). Thiamin, riboflavin, and niacin calculated as based on 2800 kcal.

*RDA not determined.

to millions/g. They are mostly from the families, Pseudomonadaceae, Micrococcaceae, Lactobacillaceae, and Bacillaceae (Frazier, 1967). As listed by Elliot (1980), certain bacterial types nearly always present on grains include psychrotrophic bacteria from 10^4 to greater than 10^5/g, and aerobic sporulating bacteria, 10^0 to 10^5/g (catalase negative and aerobic sporeformers are irregularly present).

Natural lactic acid fermentation of whole corn meal

According to Fields et al. (1981) when whole corn was ground in a Wiley mill using a 1-mm screen, mixed with water 1:4 w/v, and incubated at 37°C, the following changes occurred. During the first day of fermentation, coliforms rose from an average initial count of 9.7×10^1/ml to 5.9×10^5/ml, with heavy gassing, a large drop in Eh, and a pH drop from 6.0 to 4.0. The coliforms disappeared by two days of fermentation. Lactic acid bacteria predominated all four days of the fermentation. Total lactic counts increased from an initial value of 5×10^5/ml to 10^8/ml after one day of fermentation and remained constant until the fourth day when fermentation was complete. After two days, the pH was 3.8 and after three days, 3.7, where it remained. No acetic acid was detected, but volatile acids accounted for 5.5% of total acidity. The remaining portion was assumed to be lactic acid, since a test for lactic acid was positive. The final acidity, after four days, was 1.12 expressed as % lactic acid. Bacteriological studies showed that rods predominated after one day, and some of the rods were motile. Motile, Gram negative rods did not increase after the second day. Very short Gram positive rods, mainly in pairs, and a few thick rods were also observed. As the fermentation progressed, cocci dominated. Total

aerobic yeast and mold counts were the highest initially, and steadily declined during the first two days of fermentation.

In another study of corn meal fermentation, under similar conditions except that a temperature of 34°C was used, Tongnual et al. (1981) evaluated the proteolytic microflora. Total proteolytic counts rose from 2×10^2/ml to 2×10^8/ml after one day. The count remained the same after two days, declined to 10^4 after three days and no proteolytic bacteria were found after four days. The organisms which were predominately aerobic, Gram negative rods, did not grow at pH 4.7 or below.

Isolates from natural lactic acid fermentations

Fields et al. (1981) isolated Lactobacillus cellobiosus, Lactobacillus fermentum, and Pediococcus acidilactici from corn meal fermentation at 37°C. Zamora and Fields (1979a) isolated Lactobacillus casei, Lactobacillus leichmannii, Lactobacillus plantarum, Pedioccus acidilactici, and Pediococcus pentosaceus from fermenting cowpeas at 25°C. In addition to these organisms Lactobacillus helveticus was isolated from chickpeas. No Bacillus cereus, Staphylococcus aureus, Salmonella, or Shigella were found in the beans (Zamora and Fields, 1979b). In the corn fermentation at 37°C, heterofermentative lactobacilli were isolated, while in the bean fermentations at 25°C homofermentative organisms were isolated.

Tongnual et al. (1981) identified twenty one isolates of proteolytic Gram negative rods from corn fermentation as being primarily Pseudomonadaceae. Nanson and Fields (1982) identified three proteolytic organisms involved in corn fermentation as Pseudomonas maltophilia,

Bacillus cereus, and Bacillus subtilis.

Coliform bacteria were not identified but it would be expected that the coliforms would be those typical of plant coliforms. According to Frazier (1967), Enterobacter aerogenes is a coliform bacterium commonly of plant origin. This probably was the type occurring in these fermentations.

B-Vitamins

The B-vitamins have a nearly ubiquitous occurrence in living cells. With the exception of choline, which has no known coenzyme function, the other B-vitamins discussed in this study are coenzymes for essential metabolic reactions (Lehninger, 1975). With the exception of choline, none of these vitamins are synthesized by the body, hence an external source is required. This external source has proved to be either food or the intestinal flora (Dyke, 1965).

Occurrence and function

Thiamin is found in biological materials in the free form, as the mono-, di-, and triphosphoric esters, and as the mono-, and disulfide. In plant tissues, the most abundant form is free thiamin while in animals the predominate form is the diphosphate also known as cocarboxylase (Lamden, 1972). Hydroxyethyl thiamin diphosphate (active acetaldehyde) is another naturally occurring form, and it was found to comprise 60% of the thiamin in Escherichia coli. Active acetaldehyde was also found in Saccharomyces cerevisiae, and in other bacteria, but was present in a lower proportion in these than in E. coli (Carlson

and Brown, 1961). The biological activity of thiamin is due mainly to its diphosphate ester which is the coenzyme cocarboxylase. In fermentation, cocarboxylase is involved in the conversion of pyruvate to acetaldehyde, and in respiration it is involved in oxidative decarboxylation of pyruvate and α-oxoglutaric acid. It is also the coenzyme of transketolase and, in cases of thiamin deficiency, carbohydrate metabolism is disturbed (Dyke, 1965).

Riboflavin, riboflavin-5-phosphate (FMN), and flavine adenine dinucleotide (FAD), are the only naturally occurring flavins to have vitamin B_2 activity, and they are all equally effective in promoting the growth of rats and Lactobacillus casei (Horwitt, 1972). Free riboflavin is a relatively rare natural compound, occurring in significant amounts only in the culture media of a restricted number of microorganisms and in the retina, urine, and occasionally in the milk and seminal fluids of animals (Goodwin, 1963). The riboflavin coenzymes, FAD and FMN, undergo reversible redox reactions to $FADH_2$ and $FMNH_2$ and function in the oxidative degradation of pyruvate, fatty acids, and amino acids, and in electron transport (Lehninger, 1975).

Niacin is a generic term for both nicotinic acid and nicotinamide, the molecular weights of which differ by less than 1% (National Academy of Science/National Research Council, 1974). Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are the two coenzyme forms of the vitamin. NAD^+ and $NADP^+$ are reversibly reduced to NADH and NADPH when hydrogen is enzymatically removed from specific substrates (Lehninger, 1975). As mentioned previously, most of the niacin in corn is present in a bound form (Christianson et al., 1968).

Pyridoxine is the commonly used name for the vitamin B_6 complex which also includes pyridoxal and pyridoxamine. In this thesis, pyridoxine refers to the whole complex. Pyridoxine itself is the alcohol form, pyridoxol. All three forms have equal activity for humans, higher animals, and Saccharomyces carlsbergensis. The various forms in which it occurs in foods include pyridoxal, pyridoxol, pyridoxamine, pyridoxal-5-phosphate, and pyridoxamine-5-phosphate (Brubacher and Wiss, 1968). After acid hydrolysis of the phosphate and bound forms, pyridoxal accounts for 50%, and pyridoxamine 30% of the vitamin B_6 activity in corn (Polansky et al., 1964). Pyridoxal phosphate functions in all known non-oxidative enzymic amino acid transformations such as decarboxylation, transamination, and racemization (Wagner and Folkers, 1966).

Pantothenic acid, as it occurs naturally, is either in the free form or bound as a component of Coenzyme A (CoA) with exceptions such as in E. coli grown under certain conditions (Bird and Thompson, 1967). The structure of the pantothenic acid modecule is specific, in that analogues that are simple esters, which would be expected to be hydrolyzed easily, are inactive in bacteria (Dyke, 1965). CoA is involved in a variety of transacetylation reactions such as condensation of Acetyl-CoA with oxaloacetic acid in the Krebs cycle to produce citrate (Wagner and Folkers, 1966).

Biotin in animal and yeast products is mainly in a bound water insoluble form, but in plants and vegetables it is mainly in a water soluble form (Wagner and Folkers, 1966). Free biotin and biocytin (ε-N-biotinyl-L-lysine) are both water soluble. As a coenzyme, it functions in a bound form. In several enzymes, biotin is bound to a

lysine residue in the form of biocytin (György and Langer, 1968). Biotin functions in enzymatic transfers or incorporations of CO_2 in its bound form, biocytin (Lehninger, 1975).

Folacin is the collective name for compounds qualitatively exhibiting the biological activity of folic acid, pteroylmonoglutamic acid. These compounds are folic acid glutamates or polyglutamates, which differ in the number of glutamic acid residues (National Academy of Science/National Research Council, 1974). In addition to the polyglutamate forms, there are different metabolically active forms such as N^5-formyltetrahydrofolic acid (citrovorum factor), N^{10}-formyltetrahydrofolic acid and others (Stokstad and Koch, 1967). In all foods examined by Santini et al. (1964), N^{10}-formylpteroylglutamic acid and citrovorum factor were the main components of the folacin activity. They made up 57% and 21%, respectively, of the folacin activity in rice, while folic acid made up 29%. Folic acid coenzymes function in the transfer of one carbon units at the oxidation levels of formate, formaldehyde, and methanol, and in their transformation from one oxidation state to another. Folic acid coenzymes function in the synthesis of purines, pyrimidines, methionine, and serine. The methyl groups that are synthesized by folic acid enzyme systems provide the source of labile methyl groups for transmethylations such as to choline (Stokstad and Koch, 1967).

Vitamin B_{12} is synthesized by a wide range of bacteria and streptomycetes but not to any extent by yeasts or fungi. There is no convincing evidence of synthesis by plants or animals, and it seems probable that the only primary source of vitamin B_{12} in nature is the activity of microorganisms (Smith, 1951). Most of the vitamin B_{12}

activity is retained within the cells and is released by heating, cyanide, or other treatment (Wuest and Perlman, 1968). For many other B-vitamins, a significant portion of the total activity produced during bacterial growth is excreted into the media (Thompson, 1942). In its coenzyme forms in man, vitamin B_{12} is concerned with a wide variety of metabolic processes including transmethylation and synthesis of amino acids, purines, and pyrimidines. Its function in nucleoprotein synthesis is closely linked to that of folacin (Riesner, 1968). The functions of vitamin B_{12} and folacin are similarly related in microbes (Koser, 1968).

Choline can be synthesized in the body and has no known coenzyme function. Biosynthesis of choline seems to be universal in nature, an exception being Pneumonococci which require it for growth. The reason for including choline as a vitamin is because the synthesis of choline is dependent on adequate amounts of methionine, betaine, folacin, and vitamin B_{12}. Choline is produced by transferring a methyl from methionine via reactions involving vitamin B_{12} and folacin coenzymes. Betaine may also provide labile methyl. When vitamin B_{12}, folic acid, and methionine are present in adequate amounts, they can make up for a lack of choline, but when they are not present in adequate amounts, choline content of the diet becomes more important. Choline, however, cannot be converted to methionine in humans. Choline and its derivatives are important as structural components of tissue and as intermediates in metabolic reactions (Griffith and Nyc, 1971). Choline occurs in plant and animal cells as either free choline, acetylcholine, or in phospholipids such as lecitin (Wagner and Folkers, 1966).

B-vitamin content of fermented foods

A variety of substrates have been fermented by procedures similar to those described for corn by Fields et al. (1981). The contents of riboflavin, niacin, and thiamin were evaluated and the results and conditions of fermentation are summarized in Table 4. Overall riboflavin was increased in five samples and unchanged in three, with no instances of a decrease reported. Thiamin was increased in one, unchanged in two, and decreased in four products. Niacin was increased once, decreased twice, and unchanged twice. Chompreeda (1982) evaluated eight B-complex vitamins in corn meal after four days of fermentation at 32°C. These results are presented in Table 5. There were significant increases in riboflavin, vitamin B_{12}, and in pantothenic acid, and decreases in thiamin and pyridoxine with no other significant differences.

The B-vitamin content of some traditionally used natural lactic acid fermented foods has also been evaluated. Akinrele (1970) evaluated corn Ogi, an important food of Nigeria. Thiamin and niacin were increased by the traditional process but pantothenate was decreased as compared to nonfermented Ogi. Lactobacillus plantarum and Aerobacter cloacae were identified as the predominate organisms during souring and were tested in pure culture Ogi for abilities to alter vitamin content (Table 6). A. cloacae fermentations of Ogi were higher in riboflavin and niacin than other Ogi. The increase in thiamin found in the traditional process was not duplicated in pure culture. Saccharomyces cerevisiae was also isolated from Ogi, but was not tested in pure culture. Overall, because of pre-fermentation removal of much bran and germ, the vitamin content of all of the Ogi was lower than that of whole corn.

Table 4. Vitamin content of natural lactic acid fermented meals compared to nonfermented controls.

Substrate	Vitamin mg/100g			Conditions		Reference
	Riboflavin	Thiamin	Niacin	°C	Days	
Rice with hulls	0.15a[1]	0.035a	4.44a	25	5	A
Control	0.09b	0.060b	6.59b			
Rice without hulls	0.14a	0.035a	4.16a	25	5	A
Control	0.08b	0.051b	6.31b			
Whole sorghum	0.13a	0.471a	4.13c[2]	30	5	B
Control	0.09b	0.202b	3.79d			
Whole sorghum	0.13a	0.318a	7.09a	25	4	C
Whole sorghum	0.14a	0.387b	7.09a	35	4	
Control	0.13a	0.366ab	6.84a			
Whole wheat	0.22c*					D
Control	0.14d*					
Whole cowpeas	0.29a	1.53a	3.84a	25	4	E
Control	0.24b	1.58a	4.56b			
Whole chickpeas	0.22a	0.52a	3.01a	25	4	E
Control	0.19a	0.69b	4.00b			

[1] a,b; Differing subscripts within each set (vitamin for a product) denote significant ($P<0.05$) differences.

[2] c,d; Denote significant ($P<0.01$) differences.

A) Tongnual and Fields (1979).

B) Kazanas and Fields (1981).

C) Au and Fields (1981).

D) Hamad and Fields (1979).

E) Zamora and Fields (1979a).

*Reported as mg/g N.

Table 5. Vitamin content of natural lactic acid fermented corn meal compared to nonfermented control.

Vitamin	Whole corn meal	
	Nonfermented control	Fermented 4 days at 32°C
Thiamin, mg/100g	0.41a[1]	0.29b
Riboflavin, mg/100g	0.11a	0.30b
Niacin, mg/100g	2.04a	2.11a
Pyridoxine, mg/100g	0.37a	0.19b
Folacin, mg/100g	0.01a	0.02a
Biotin, µg/100g	5.03a	3.81a
Pantothenic acid, mg/100g	0.91a	1.63b
Vitamin B_{12}, µg/100g	0.22a	1.45b

[1] a,b; Differing subscripts within each set (vitamin for a product) denote significant ($P<0.05$) differences.

Chompreeda (1982).

Table 6. Effect of type of fermentation on vitamin content of corn Ogi compared to whole corn.

Type of Ogi fermentation[1]	Vitamins mg/100g (dry weight)				
	Thiamin	Riboflavin	Niacin	Folacin	Pantothenic Acid
Nonfermented Ogi	0.06	0.07	0.68	0.05	0.04
Traditionally fermented	0.11	0.08	0.85	0.05	0.01
Lactobacillus plantarum	0.04	0.04	0.64	0.05	0.03
Aerobacter cloacae	0.04	0.15	1.25	0.05	0.01
L. plantarum + *A. cloacae*	0.04	0.14	1.15	0.05	0.03
Whole corn nonfermented[2]	0.45	0.09	2.30	0.02	0.46

[1] Akinrele (1970).

[2] Kent (1975).

Ofosu (1971) reported that Kenkey, another traditional food of Africa, produced in this case from corn, was increased in niacin content by the fermentation. Overall, however, niacin levels were lower than in whole corn. The values in Table 4 and Table 5, on the other hand, refer to the whole grain or legume, as compared to the fermented sample.

Idli is an Indian leavened product produced by natural lactic acid fermentation of rice and black gram, usually in a 1:3 ratio. Leuconostoc mesenteroides, Streptococcus faecalis, and Pediococcus cerevisiae are the most important organisms involved in this fermentation. Riboflavin was reported to decrease from 0.137 mg/100g in the raw nonfermented product to 0.075 mg/100g in the cooked fermented Idli. Cooked, but nonfermented Idli, contained 0.077 mg/100g (Van Veen et al., 1967). Rajalakshmi and Vanaja (1967), however, reported that riboflavin increased in Idli from 0.25 mg/100g in the nonfermented to 0.54 mg/100g in the fermented Idli. Thiamin was also increased from 0.21 mg/100g to 0.58 mg/100g and phytate was decreased. Rao (1961) reported that Idli was increased in folacin and choline contents, but vitamin B_{12} was decreased by fermentation.

Many types of cultured dairy products are produced by using Lactobacillus sp. and Streptococcus sp.. Today, these products are not natural lactic acid fermentations, but are controlled fermentations. The B-vitamin content of cultured dairy products (Table 7) is frequently higher than that of milk or the directly acidified counterparts (Shahani and Chandon, 1979). Folacin was consistently increased in all of the cultured products listed in Table 7 but other vitamins varied. Cottage cheese starter culture actively synthesized folacin and vitamin B_{12}. Folacin synthesis was increased by increasing the level of calcium

Table 7. Vitamin content of milk, cultured milk products, and their directly acidified counterparts.[1]

Product	Vitamin µg/100g or ml					
	Folacin	Biotin	Niacin	Pantothenic acid	B_6	B_{12}
Milk	.13-.73	2.9-4.9	71-96	330-460	17-40	.27-.57
Cheddar cheese[2]	4-21	.65-2.5	13-212	111-711	49-147	---
Yogurt,						
cultured	3.9*	4.0-5.1	130-141	280-381	---	.35-.52
acidified	4.3	4.2	131	427	---	.42
Cottage cheese,						
cultured	2.3-5.0	3.2	70-257	463	24-56	.8-2.1
acidified	.1	2.8	42.5	375	30	.42
Sour cream,						
cultured	10.8	2.6	11-67	320-360	16	.3-.4
acidified	3.1	3.1	64	330	17	.3

[1] Shahani and Chandon (1979).

[2] Ayebo and Shahani (1980).

* Seaweed stabilizer contained it.

(Reif et al., 1976). The B-vitamin content of cultured milk products was affected by various factors including the amount and type of innoculum, temperature and time of incubation, and by further processing (Ayebo and Shahani, 1980).

Synthesis and utilization by microorganisms

An important generalization concerning the relationship between microorganisms and B-vitamins was expressed by Pederson (1979). Vitamins of the B-complex are coenzymes which may serve as a part of several enzyme systems. The inability of a microorganisms to synthesize one of the B-vitamins indicates that it requires this vitamin and obtains the vitamin from the substrate in which it is growing. In other words, with exceptions, the nearly ubiquitous occurrence of B-vitamins in microorganisms implies that if the microorganism does not require a specific B-vitamin, it is synthesizing it. Exceptions to this generalization are particularly prominent among lactic acid bacteria. For example, Nurmikko (1954) reported that Lactobacillus arabinosus, which normally required pyridoxine did not when supplied with nineteen amino acids. Another example is the relationship of fungi and vitamin B_{12}. According to Brock (1970), fungi never require and do not synthesize vitamin B_{12}. Instead, the reactions that involve vitamin B_{12} in bacteria are carried out by different pathways in fungi.

With exceptions such as these noted, however, it can be assumed that bacteria involved in a natural lactic acid fermentation that do not require a B-vitamin from substrate sources are synthesizing the B-vitamin. According to Koser (1968), most Pseudomonas, Bacillus, and coliforms require no vitamins from substrate sources. Lactic acid

bacteria, on the other hand, frequently require several vitamins. Nearly all Lactobacillus and Pediococcus require nicotinic acid, pantothenic acid, and biotin, but requirements for the other vitamins are variable. As mentioned previously, a requirement for choline is very rare and all these organisms probably synthesize it.

The fact that a microorganism does not require a vitamin does not necessarily mean that the microorganism can increase the vitamin content of a food. Akinrele (1970), for example, found that Aerobacter cloacae increased two of the five vitamins measured in a pure culture Ogi. However, if this strain of A. cloacae has vitamin requirements of typical coliforms, then it could synthesize the other three vitamins as well (Table 6).

In order for a microorganism to increase the vitamin content of a food, it must, obviously, synthesize more than it utilizes. According to McIlwain (1946a), the rates measured for synthesis of vitamin-like compounds by bacteria may actually be the results of a balance between vitamin production and breakdown. For example, in E. coli and Pseudomonas aeruginosa, the synthesis of pantothenic acid normally precedes at rates comparable to its inactivation. This imples that the breakdown of a vitamin is part of normal cell metabolism.

Thompson (1942) studied several species of bacteria that did not require B-vitamins from substrate sources for growth. Thompson found that after growth in vitamin free medium the amount of thiamin, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, and folacin found in the cells was relatively constant. The amount found in the media, however, varied widely. The amount of these vitamins found in the growth media of Aerobacter aerogenes ranged from 20-90% of the

total vitamin content, and the amount found in the media was not attributed to autolysis. Thompson concluded that, although the intracellular concentrations of vitamin required by bacteria are about the same, the synthetic powers vary widely, and some of that synthesized is excreted into the media. Wilson and Pardee (1962) stated that riboflavin and several other vitamins were excreted into the media by bacteria in proportionally larger quantities than other metabolites. For all bacteria and conditions they tested, the ratio of flavins excreted to flavins retained within the cell ranged from 0.8 to 8.0. They stated that the equivalent ratio for amino acids is usually about 0.01. They concluded that the control mechanisms of the pathway are too weak to prevent the accumulation of vitamin residue, but that bacteria would benefit little by stricter controls (such as are present for amino acids) because the waste of energy and materials is small.

Van Lanen and Tanner (1948) stated, that within the limits of concentration usually found in media, the presence of a vitamin does not affect its synthesis. This helps to explain why, when a food may already contain sufficient vitamins for microbial growth, the vitamin content could be increased. Van Lanen and Tanner (1948) summarized the factors involved in the vitamin metabolism of microorganisms. Inorganic elements, cultural conditions, and the species of organism all have an effect on the rate and extent to which vitamins are synthesized, absorbed, excreted, and utilized by microorganisms.

Stability to environmental factors

Thiamin is one of the least stable vitamins. The most important factors determining its destruction are time, temperature, and pH.

As pH, and time and temperature of heating increase, thiamin destruction increases. It is generally recognized that thiamin is very stable in acid solution, especially when stored at refrigeration temperatures (Farrer, 1955). The percent moisture can have an effect on storage stability. Bookwalter et al. (1968) found that when a corn-soy-milk mixture was kept at 10% moisture or less there was no loss of thiamin after 182 days at 100°F. At 13½% moisture 50% was lost.

Riboflavin is very sensitive to light, and the rate of destruction increases as the pH and temperature increase. It is stable to heat in the dry form or in acid medium (Harris, 1960).

Niacin is one of the most stable vitamins. Nicotinamide is partially hydrolysed by acid or alkali, but the resulting nicotinic acid has the same biological activity and is generally stable to air, light, heat, acids, and alkali (Harris, 1960).

Pyridoxol is stable to heat, strong alkali, or acid. Pyridoxal and pyridoxamine are rapidly destroyed by exposure to air and heat. All three forms are destroyed by light, especially UV, at neutral and alkaline pH's (Harris, 1960).

Pantothenic acid is most stable in the pH range 5.5-7.0 and is rapidly destroyed under stronger acid or alkaline conditions. It is unstable to light, air, and oxygen (Harris, 1960).

Biotin is relatively stable in air and oxygen, or when exposed to UV light, it is inactivated by agents which oxidize the sulfur atom and by strong acids and alkalis (Harris, 1960). For extraction, H_2SO_4 is used for hydrolysis and should not be replaced by HCl because HCl is more destructive to biotin (György and Langer, 1968).

Folacin differs from the other vitamins in terms of stability. It is more stable in alkaline than acid solutions. Folacin is also unstable to light, air, oxygen, and heat (Harris, 1960). In milk, oxygen seems to be the most important factor. About 20% was lost after ultra high temperature pasteurization, but losses during storage depended on the oxygen content. In the absence of oxygen, no further loss occurred after storage at 15-19°C for ninety days (Ford et al., 1969).

The cobalamin coenzymes and cyanocobalamin are decomposed slowly by UV light, visible light, and alkaline conditions (Rosenthal, 1968). Aqueous solutions of vitamin B_{12} are most stable in the pH range 4-7 at normal temperatures. Vitamin B_{12} is thermally unstable and significant decomposition occurs when autoclaved at 115°C for thirty min (Moore and Folkers, 1968). Harris (1960) reported that vitamin B_{12}, if pure, is stable to heat in neutral solutions but is rapidly destroyed by acid or alkaline conditions in foodstuffs, and that it is unstable to air, oxygen, heat, and light.

Choline is stable to heat in dilute aqueous solutions but concentrated solutions give off trimethylamine when boiled (Griffith and Nyc, 1971). Choline is strongly alkaline, and slightly unstable to air and oxygen when in solution, but is otherwise stable (Harris, 1960).

CHAPTER III

MATERIALS AND METHODS

Sample Preparation

Corn meal preparation

Five bags of corn (Zea mays) were purchased from a local grain distributor and were stored at 4°C until used. The grain was sifted using a 5.66-mm seive (The W.S. Tyler Co., Cleveland, Ohio) to separate broken kernels and undesirable debri. The cleaned seeds were then ground in a Wiley Laboratory Mill (Model 4, Arthur H. Thomas Co., Philadelphia, Pennsylvania) using a 1-mm screen. One portion of the whole corn meal served as control and was stored in gallon jars at room temperature (20-25°C) until assayed for each vitamin. The other portion of corn meal was used in the fermentation studies.

Fermentation procedures

Five 100g samples of meal from each bag of corn were weighed. This constituted five replicate samples (Fig. 1). Each sample was placed in a 600-ml beaker, labeled according to the bag from which it was taken, and 400 ml of tap water, pH 7.3, was added. One sample from each bag was used to measure initial pH and titratable acidity. The remainder was covered with foil and placed in a 30°C incubator. After either 24, 48, 72, or 96 hr of fermentation, one sample from each bag of corn was removed and the pH and titratable acidity were measured. The samples were then transferred to aluminum pans (for increased surface area) and were placed in an air flow oven (Freas Model 835) at 50-60°C for 48 hr. The dried samples were reground through a 1-mm

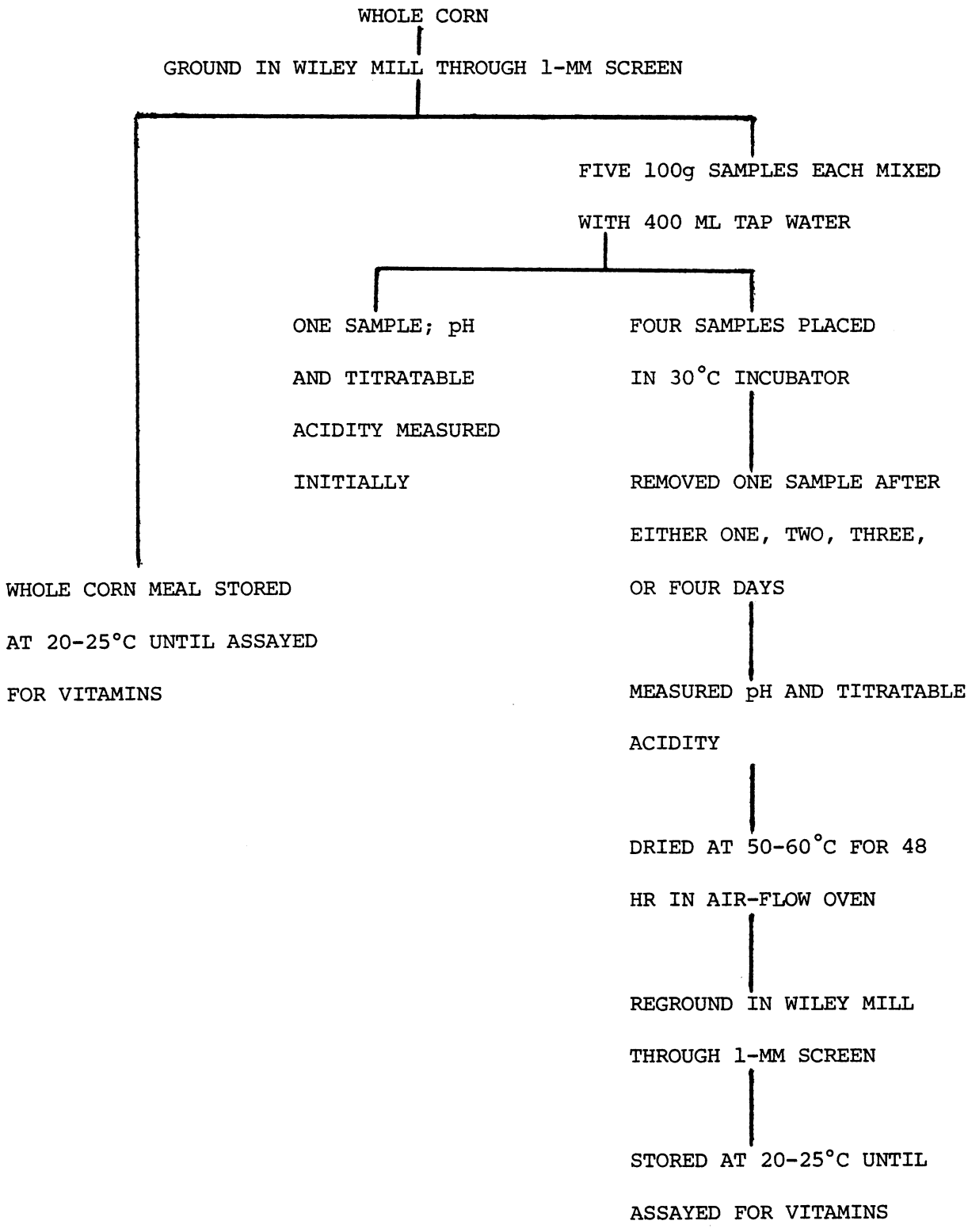

Fig. 1. Flow chart of fermentation procedures for one replicate.

screen in the same Wiley Mill previously mentioned. The samples were placed in pint Mason jars, covered, and stored at room temperature (20-25°C) until assayed for each vitamin.

Quantitative Analyses

Titratable acidity and pH determinations

Titratable acidity, calculated as lactic acid, was determined by titration of 10 ml of the corn suspension with 0.089N NaOH to pH 8.6 according to the formula:

$$\% \text{ titratable acidity} = \frac{\text{ml NaOH} \times \text{N NaOH} \times .09}{\text{ml sample}} \times 100$$

The pH was measured with a Beckman zeromatic pH meter.

Moisture determinations

The moisture content of the samples was determined by the air-oven method (AOAC, 1975) according to the formula:

$$\% \text{ moisture} = \frac{\text{weight before drying-weight after drying}}{\text{weight before drying}} \times 100$$

The percent moisture of each fermented sample and of the nonfermented control sample was determined in triplicate, at the beginning, middle, and end of the period during which vitamin analyses were made. The mean percent moisture of the three replicates was then used to calculate vitamin contents on a dry weight basis.

Vitamin analyses

All vitamins were determined by microbiological assay. The microorganisms used and method of measuring growth response to each

vitamin are listed in Table 8. The microorganisms used in the assays were those suggested by Freed (1966) except for the thiamin and choline assays. The microorganism used for thiamin determination was recommended by Sarrett and Cheldelin (1944) and that for choline by Horowitz and Beadle (1943). The method of measuring growth response was recommended by Difco (1977). When the option of titrimetric or turbidimetric measurement was given, titrimetric methods were used because factors such as unequal tube temperatures cause greater error in short incubation times than in 72 hr titrimetric methods (Freed, 1966). A Spectronic 20 Spectrophotometer was used to measure turbidity. Titrimetric measurements were made with 0.1N NaOH, titrating to pH 7.0. Gravimetric measurements were made with a Sartorius Analytical Balance.

Procedures for preparation of standard solutions, standard curves, assay tubes, inoculum, and culture maintenance were according to Difco (1977). Media used was that recommended by Difco (1977).

Extractions of niacin, biotin, pyridoxine, riboflavin, vitamin B_{12}, pantothenic acid, and folacin were according to methods described by Freed (1966). Extraction of niacin was performed using the 1N H_2SO_4 method. Biotin was extracted using 6N H_2SO_4. Pyridoxine was extracted using 0.055N HCl. Riboflavin was extracted using 0.1N HCl. Vitamin B_{12} was extracted using the acetate buffer-NaCN method. Pantothenic acid was extracted using the alkaline phosphatase-chicken liver extract method. Folacin was extracted using the phosphate buffer-chicken pancreas conjugase method. Thiamin was extracted according to Sarrett and Cheldelin (1944). Choline was extracted according to the original method of Horowitz and Beadle (1943) using modifications developed by Hodson (1945) and Luecke and Pearson (1944). The original

Table 8. Microorganisms and methods of measurement used in the vitamin assays.

Vitamin	Assay organism ATCC number	Method
Vitamin B_{12}	*Lactobacillus leichmannii* 7830	Titrimetric
Riboflavin	*Lactobacillus casei* 7469	Titrimetric
Folacin	*Streptococcus faecalis* 8043	Turbidimetric
Pantothenic acid	*Lactobacillus plantarum* 8014	Titrimetric
Pyridoxine	*Saccharomyces carlsbergensis* 9080	Turbidimetric
Thiamin	*Lactobacillus fermentum* 9338	Turbidimetric
Choline	*Neurospora crassa* 9277	Gravimetric
Niacin	*Lactobacillus plantarum* 8014	Titrimetric
Biotin	*Lactobacillus plantarum* 8014	Titrimetric

method called for extraction of samples in 3N H_2SO_4 followed by permutit extraction. Luecke and Pearson found 3N HCL and neutralization with NaOH as effective as the H_2SO_4 extraction, and this was used in this study. Hodson found that permutit extraction was unnecessary if only the steep portion of the standard curve was utilized in interpolating values. Therefore, the permutit extraction was not done and values were taken from the steep portion of the curve only. The exact method used for choline is outlined in Appendix Table 2.

Calculation of Results

Calculation of vitamin contents

Turbidimetric measurements were obtained as % transmission and converted to absorbance according to the formula:

absorbance = 2- log % transmission

Titrimetric and gravimetric measurements were graphed and interpolated without modification. Standard curves obtained during the assays are presented in Appendix Figs. 1 through 9.

The average growth response value of duplicate tubes at a particular level of added extract was used for interpolation of vitamin contents from the standard curve. The value obtained was averaged with values obtained at differing levels of added extract and an average amount per ml was determined. In all cases values from at least three different levels of added extract were used to calculate the average amount per ml. Vitamin contents on a dry weight basis were then calculated according to the formula:

$$\frac{\text{vitamin}}{\text{ml extract}} \times \frac{\text{total dilution factor}}{\text{g(s) sample}} \times \frac{\text{g(s) sample}}{\text{g(s) solids}} = \frac{\text{vitamin}}{\text{g dry wt}}.$$

The following example calculation illustrates the procedure used. The niacin content of the extract is the mean value of eight total assay tubes at four levels of added extract.

$$\frac{0.038\ \mu\text{g niacin}}{\text{ml extract}} \times \frac{500}{\text{1g sample}} \times \frac{\text{1g sample}}{0.959\text{g solids}} = \frac{19.81\ \mu\text{g niacin}}{\text{g dry wt}}.$$

Statistical analyses

The experimental design was a completely randomized assignment of treatment to well mixed samples of corn meal from five replicate bags. This design is explained by Cochran and Cox (1957).

Data obtained from the vitamin assays were analyzed using the Analysis of Variance (Snedecor and Cochran, 1976). When statistically significant F-values were obtained, Duncan's (1955) Multiple Range Test was applied to determine the statistical probability of observed differences in mean values.

CHAPTER IV

RESULTS AND DISCUSSION

Titratable Acidity and pH Determinations

Data in Fig. 2 show the changes that occurred during the four day fermentation period. Titratable acidity and pH changed most rapidly during the first day of fermentation. The amount of acid continued to increase until the third day. The samples fermented four days had a higher pH and lower titratable acidity than the samples fermented three days. A similar overall pattern was observed by Fields et al. (1981) and Zamora and Fields (1979a). This pattern is characteristic of a natural lactic acid fermentation.

Moisture Determinations

Data in Table 9 show the mean percent moisture of whole corn meal and of the fermented and dried samples. The difference in the percent moisture for the fermented samples is not large but the moisture retained by the samples seemed to increase with fermentation time. Factors such as pH and titratable acidity might affect the stability of the vitamins during drying, but the pH and titratable acidity range among samples was relatively narrow when the stability of vitamins in varying pH units is considered. It was, therefore, concluded that the drying process was probably not responsible for the differences observed in the vitamin content among the fermented samples. In the case of pyridoxine, however, the differences between whole corn and the fermented samples may be related to drying.

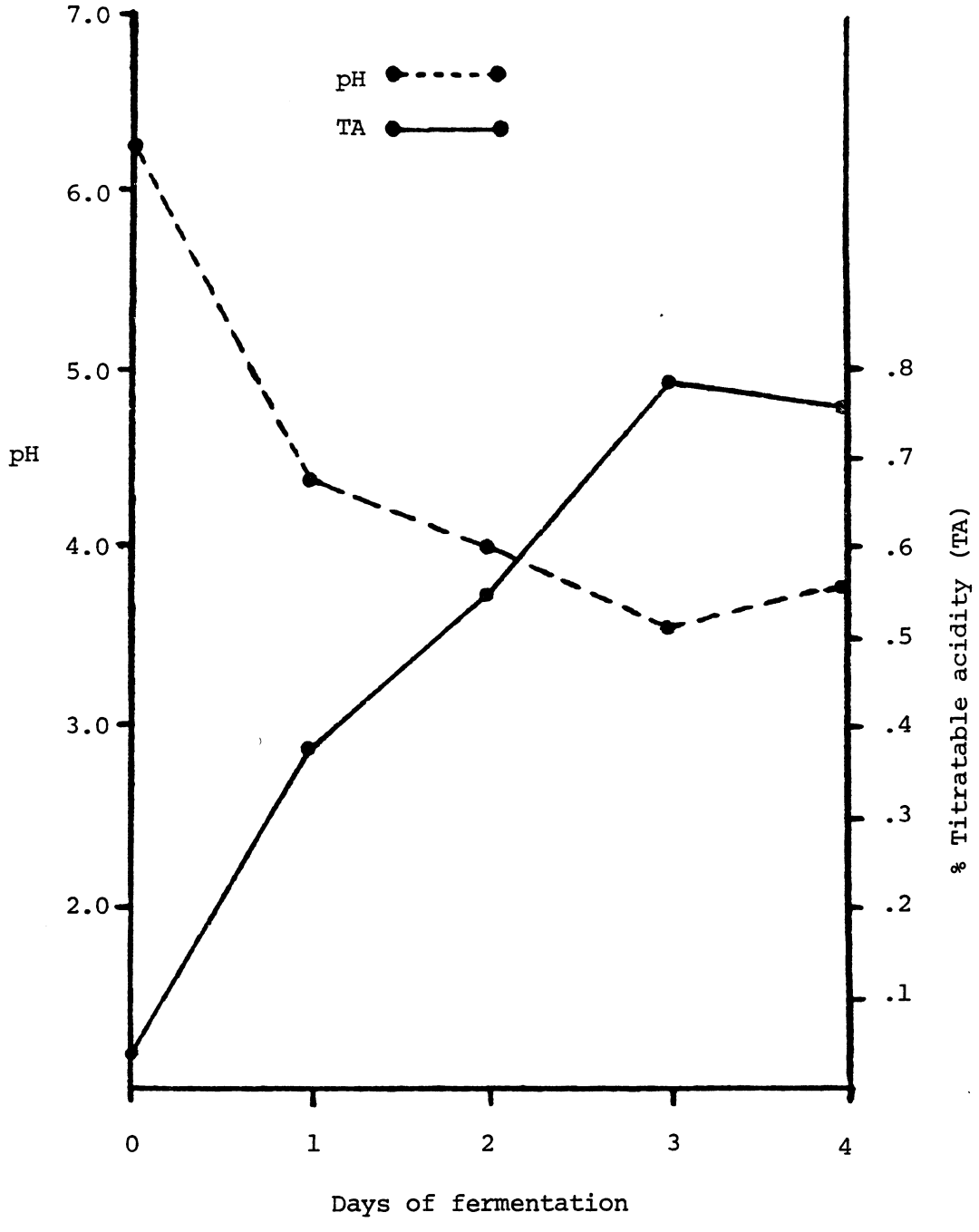

Fig. 2. pH and titratable acidity during whole corn meal fermentation at 30°C.

Table 9. Means[1] of moisture content of fermented and nonfermented whole corn meal.

Sample	% Moisture
Whole corn meal	13.35
Whole corn meal fermented one day	4.74
Whole corn meal fermented two days	5.06
Whole corn meal fermented three days	5.21
Whole corn meal fermented four days	5.38

[1] N=5.

Vitamin Analyses

Overall, four vitamins increased, two were unchanged, and three decreased at some point during the fermentation. Data in Table 10 indicate the vitamin content of whole corn meal and the fermented samples. Values with differing subscripts are significantly ($P<0.05$) different, as determined by Duncan's Multiple Range Test (Duncan, 1955). A summary of the analysis of variance on data for vitamin content is given in Appendix Table 1. All references to statistical significance of values measured in this study refer to the values in Table 10. Fig. 3 shows the relative changes that occurred in vitamin content during the fermentation period based on whole corn meal as 100%.

Comparison to literature values

One can compare the vitamin levels measured in this study for whole corn to those listed by Kent (1975) and Chompreeda (1982) in Table 11. With the exception of folacin and choline, the values measured in this study seem to be in good agreement with one or both of these author's values. Folacin content in this study was higher than that indicated by both authors. On the other hand, Freed (1966) gave the range of values for folacin in yellow corn as 13 µg/100g to 36.5 µg/100g, and folacin values in this study are at the top of this range. Chompreeda (1982) did not measure choline, but the value from this study is higher than Kent's and higher than the 54 mg/100g that was given by Mertz (1970). This might be due to the presence of stimulatory substances for the assay organism since a permutit extraction of the samples was not done. It might also be simply the result of variation in corn. Glick (1945)

Table 10. Means[1] of vitamin content of nonfermented and fermented whole corn meal.

Vitamin	Whole corn meal				
	Nonfermented Control	Days of sample fermentation			
		1	2	3	4
Vitamin B_{12}, µg/100g	0.06d[2]	2.55bc	3.00b	2.31c	3.51a
Riboflavin, mg/100g	0.14c	0.29b	0.40a	0.28b	0.42a
Folacin, µg/100g	37.20b	104.80a	99.80a	124.70a	100.30a
Pantothenic acid, mg/100g	0.93b	1.05ab	1.20ab	1.11ab	1.34a
Pyridoxine, mg/100g	0.38a	0.30b	0.31b	0.26b	0.26b
Thiamin (HCl), mg/100g	0.36a	0.26b	0.39a	0.39a	0.37a
Choline, mg/100g	87.00a	54.00b	80.00a	84.00a	86.00a
Niacin, mg/100g	1.93a	1.90a	2.00a	1.87a	2.00a
Biotin, µg/100g	4.82a	4.62a	5.07a	5.52a	5.19a

[1] N=5. Values calculated on a dry basis.

[2] a,b,c,d; Differing subscripts within each set (vitamin for a product) denote significant ($P<0.05$) differences as determined by Duncans Multiple Range Test (Duncan, 1955).

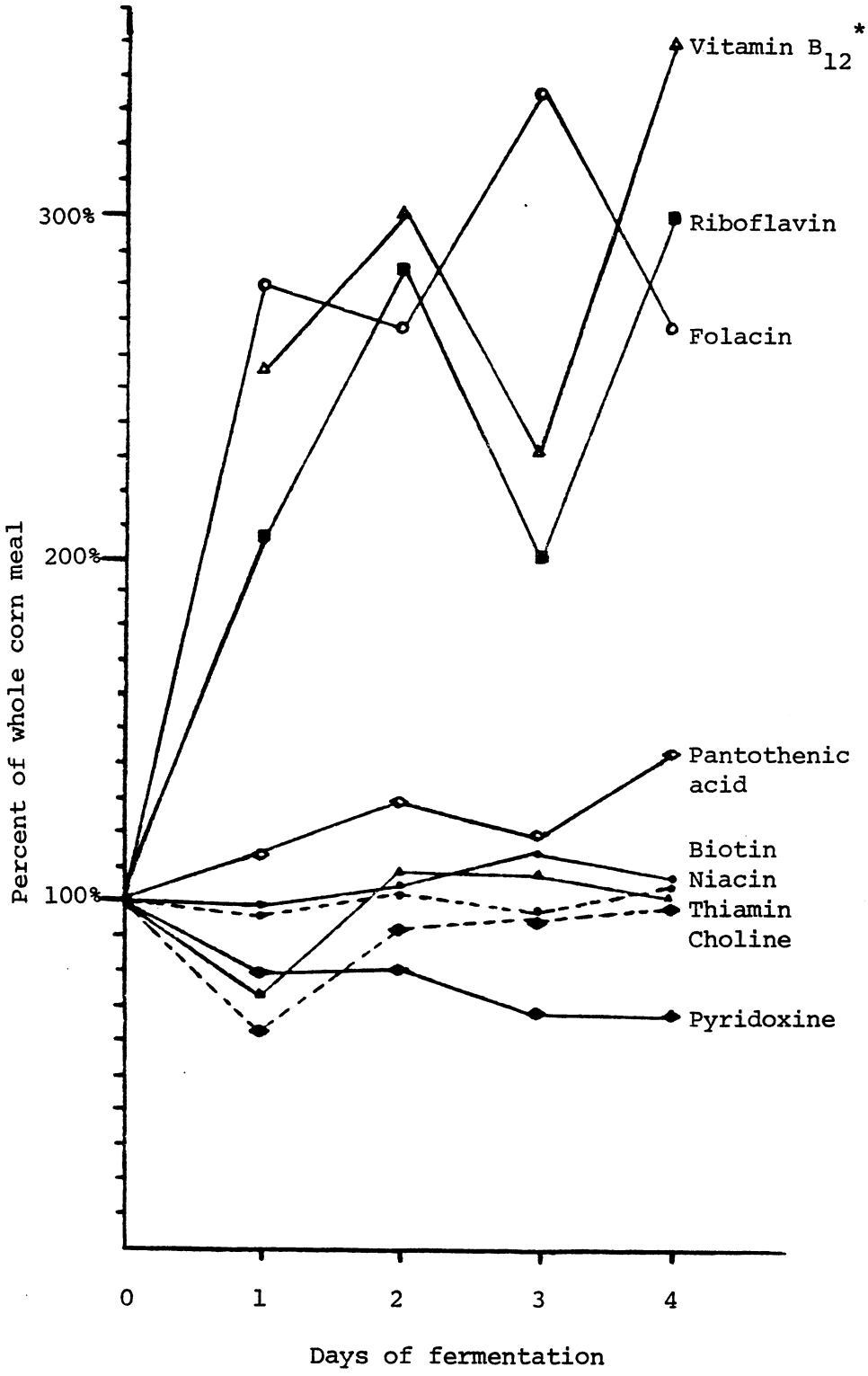

Fig. 3. Relative changes in vitamin content as a percent of whole corn meal.
*Vitamin B_{12} graphed as 200%=2.0 µg/100g.

Table 11. Vitamin content of whole corn meal compared to literature values.

Vitamin	Whole corn	Whole corn[1]	Whole corn[2]
Vitamin B_{12}, µg/100g	0.06	0.215	0.00
Riboflavin, mg/100g	0.14	0.11	0.09
Folacin, µg/100g	37.20	12.00	16.00
Pantothenic acid, mg/100g	0.93	0.91	0.46
Pyridoxine, mg/100g	0.38	0.37	0.69
Thiamin, mg/100g	0.36	0.40	0.45
Choline, mg/100g	87.00	---	45.00
Niacin, mg/100g	1.93	2.04	2.30
Biotin, µg/100g	4.82	5.03	10.00

[1]Chompreeda (1982).

[2]Kent (1975).

did not evaluate corn, but found that some grains varied considerably in choline content while others were relatively consistent. Hard winter wheats ranged from 58 mg/100g to 96 mg/100g (as choline Cl), for example.

Chompreeda (1982) measured the B-vitamin content in whole corn meal fermented four days at 32°C (Table 5). On an increase-decrease basis, the results seem consistent with values from this study. Folacin values in this study were significantly higher in fermented corn compared to whole corn. Although folacin also seemed to increase in Chompreeda's study, the increase was not statistically significant ($P<0.05$). Thiamin was decreased in Chompreeda's study ($P<0.05$), but in this study there was no significant difference between samples fermented four days and whole corn. The other vitamins measured in this study were comparable to those of Chompreeda when considered on an increase-decrease basis.

Vitamins increased by fermentation

Riboflavin, vitamin B_{12}, folacin, and pantothenic acid were increased after fermentation. Pantothenic acid had a statistically significant increase of 40% as compared to whole corn, after four days of fermentation. For the other three vitamins, an increase of greater than 100% occurred after one day of fermentation.

The percentage increase in vitamin B_{12} was the largest of the vitamins mainly because the whole corn value was low. All fermented samples had significantly higher amounts than whole corn. A fluctuating pattern was observed among the fermented samples. Samples fermented three days contained significantly less vitamin B_{12} than samples fermented two or four days, but were not significantly different from

samples fermented one day. The highest value was obtained after four days of fermentation and it was significantly higher than all other samples. When the natural occurrence of vitamin B_{12} is considered, these large increases by bacterial fermentation of a plant material are not surprising. Vitamin B_{12} is not found to any extent in plant materials (Rosenthal, 1968). Bacteria and some other microbes are the only known source of vitamin B_{12} in nature (Smith, 1951). However, due to the analytical method used, the vitamin B_{12} activity as measured may not be a true indication of the vitamin B_{12} activity for humans and animals.

Lactobacillus leichmannii, the assay organism used, may respond to other factors present in the fermented product and thus, give a false high measure of the amount of vitamin B_{12}. True vitamin B_{12} is 5:6-dimethylbenziminazole cobamide, but bacteria also produce analogues differing in the nucleotide part of the molecule such as pseudovitamin B_{12} which is adenine cobamide. Pseudovitamin B_{12} has about 50% of the activity of true vitamin B_{12} for L. leichmannii but little or no activity for higher animals (Smith, 1965). Thymidine and other deoxyribonucleosides can replace vitamin B_{12} in the nutrition of L. leichmannii and, if present, can also lead to false high measures of vitamin B_{12} activity. These breakdown products of DNA are found particularly in autolysed samples (Rosenthal, 1968). The possible presence of both analogues and deoxyribonucleosides may be responsible for all, a part, or none of the vitamin B_{12} activity measured. Probably, there was some true vitamin B_{12} and also some of the other factors. Assay with an organism such as Ochromonas malhamensis, which is reported to respond only to true vitamin B_{12} (Rosenthal, 1968), should be performed

to confirm this activity.

Folacin also increased as a result of fermentation. All fermented samples had significantly more folacin than nonfermented control corn. There were no significant differences among the fermented samples. The folacin assay with Streptococcus faecalis, however, may also give false high measures of the activity for animals.

Similar to vitamin B_{12}, the breakdown products of DNA may replace folic acid in the nutrition of the assay organism. In this case, the presence of thymine and purines could spare the requirements of the assay organism for folacin (Stokstad and Koch, 1967). S. faecalis can also respond to pteroic acid which is naturally present in some foods, but is inactive in higher animals including humans. L. casei is also employed for microbiological assays of folacin. Although L. casei responds to thymine and purines, it does not respond to pteroic acid (Bird and Thompson, 1967). Assay with this organism is suggested to confirm the activity measured in this study.

According to Koser (1968), folacin is not required by most Bacillus, Pseudomonas, coliforms, or by Lactobacillus fermentum, L. cellobiosus, or Pediococcus acidilactici. The requirement for folacin is variable among the lactics as a group, however. Synthesis of folacin by any or all of these microorganisms is probably responsible for the increases noted.

Riboflavin, like vitamin B_{12} and folacin, also increased most during the first day of fermentation. Samples fermented one day had significantly more riboflavin than the nonfermented whole corn control, and samples fermented two days were significantly higher than samples fermented one day. A fluctuating pattern similar to that for vitamin

B_{12} was observed. Samples fermented three days contained significantly less riboflavin than samples fermented two or four days. The samples fermented four days had the highest value, but this value was not significantly different from the value obtained after two days fermentation. Increases in riboflavin were the most often reported vitamin increases due to natural lactic acid fermentation (Table 4). The data presented here conforms to these observations.

Although the lactic acid bacteria may also contribute to the increased riboflavin content, most of the increase can probably be attributed to other members of the flora. According to Wilson and Pardee (1962), production of large quantities of flavins is a widespread phenomena, occurring in three families, Bacillaceae, Pseudomonadaceae, and Enterobacteriaceae. Flavins are produced by these organisms during exponential growth, but flavin synthesis also continues after growth has ceased.

The three vitamins that increased after one day of fermentation were shown to be related to one another, either in terms of their synthesis or their function. The pattern observed for vitamin B_{12} and for riboflavin was similar; and, if it is assumed that the actual amount of vitamin measured is the result of the competing effects of synthesis versus degradation, the observations of Wooley (1950) may provide an explanation. Wooley found that 1,2,-dichloro-4,5,-diaminobenzene at sublethal concentrations inhibited synthesis of riboflavin and vitamin B_{12} by Bacillus megaterium. The compound had previously been shown to inhibit growth of organisms that could synthesize vitamin B_{12}, riboflavin, or both. The compound, 1,2,-dimethyl-4,5,-diaminobenzene, was postulated to be a common precursor of riboflavin and vitamin

B_{12}, and it stimulated synthesis of both vitamins in media without the analogue. Wooley concluded that it was the common precursor. The decreased amounts of vitamin B_{12} and riboflavin on the third day of fermentation may have been the result of decreased synthesis via this common pathway, and values on the fourth day the result of reactivation of this pathway.

The synthetic pathway for riboflavin has also been related to that of folacin. According to Koser (1968), much information has accumulated that shows that the biosynthetic pathways of purines, riboflavin, and pteridines are closely linked. Goodwin (1963) stated that it is generally accepted that folic acid is constructed stepwise from p-aminobenzoic acid, 2-amino-4 hydroxy pteridine, and joined by a methylene bridge. Goodwin (1963) stated that all available evidence points to the pathway of pteridene synthesis being similar to that involved in purine and riboflavin synthesis.

Folacin and vitamin B_{12} coenzymes were shown to be closely related in terms of function. Both are involved in metabolism of one carbon units in the synthesis of purines, pyrimidines, methionine, and serine. Vitamin B_{12}, folacin, and riboflavin are all involved in the synthesis of methionine in some microorganisms (Koser, 1968).

Pantothenic acid was also significantly increased by fermentation, but not until the fourth day, and the magnitude of increase was much less than those of vitamin B_{12}, riboflavin, and folacin. Whole corn meal was not significantly different from samples fermented one, two, or three days, nor were samples fermented four days significantly different from other fermented samples. A slow increase occurred as the fermentation progressed.

When the vitamin requirements of the organisms involved are considered, this slow increase cannot be attributed to the lactic acid bacteria which predominate especially in the later stages of fermentation. According to Koser (1968), almost all Lactobacillus and Pediococcus require pantothenic acid. The organisms responsible for the increase may be the pseudomonads. Tongnual et al., (1981) found that total counts of proteolytic microorganisms increased the first day, were about the same the second day, were decreased on the third day, and none were found on the fourth day. These organisms, identified as predominately Pseudomonadaceae apparently died out between the third and fourth day, but they still may have been responsible for the increase. McIlwain (1946b) found that when either E. coli or Pseudomonas aeruginosa was placed in a nitrogen free media to stop growth, the synthesis and excretion of pantothenic acid continued at the same rate as that of the growing bacteria. Although not growing, the pseudomonads may still have been synthesizing pantothenic acid and this may be responsible for the gradual increase observed.

Vitamins decreased by fermentation

The first day of fermentation was important in terms of vitamins decreased as well as those that were increased. All vitamins that decreased did so during the first day of fermentation.

All fermented samples contained significantly less pyridoxine than nonfermented whole corn. A downward trend in pyridoxine content occurred as fermentation time increased, but the fermented samples were not significantly different from each other.

According to Koser (1968), the requirement for pyridoxine is

variable among Pediococcus, but few, if any, of the other organisms involved in fermentation, require it. This implies that the organisms could synthesize pyridoxine, but apparently destroyed more than they produced during the fermentation. One of the main functions of pyridoxine coenzymes is transamination (Wagner and Folkers, 1966). It may have been via these transamination reactions that pyridoxine was degraded. Tongnual et al. (1981) hypothesized that transamination of amino acids may have been responsible for increases in % RNV observed in fermented corn meal. There may be a correlation between the increased % RNV and the decreased pyridoxine. The pattern observed for decreased pyridoxine roughly paralleled the increases in % RNV.

Drying is another possible reason for the decreases observed. All the fermented samples were dried, while whole corn meal was not. According to Polansky et al. (1964), after hydrolysis, pyridoxal and pyridoxamine account for 80% of the pyridoxine activity in corn. According to Harris (1960), pyridoxal and pyridoxamine are rapidly destroyed by heat and air.

Thiamin and choline followed a similar pattern, both were found significantly decreased after one day of fermentation, as compared to either whole corn or other fermented samples. There were no other significant differences.

According to Fields et al. (1981), the first day of fermentation is the period of active bacterial growth. Bunker (1963) stated that in many microorganisms the thiamin content depends on factors such as the age of the culture and the nature of the substrate. Dividing algal cells, which synthesize their own thiamin, contain more thiamin than non-dividing cells. This implies there is an increased need for

thiamin during rapid growth. According to Koser (1968), the heterofermentative lactobacilli are the only types of organisms involved in natural lactic acid fermentation that commonly require thiamin. Other microflora can synthesize it. Lactic acid bacteria dominate throughout the fermentation. At first, rods, and as the fermentation progresses, cocci dominate. Destruction of thiamin by heterofermentative lactobacilli followed by synthesis by non-thiamin requiring Pediococcus, or pseudomonads that also may still be present, may explain the pattern observed. The pattern did not conform to the observations of Van Lanen and Tanner (1948) who stated that, upon continued incubation, microorganisms destroy thiamin both in cells and in the medium. In this study, however, there was no change in thiamin contents after two days of fermentation.

Another possible explanation for the pattern observed for thiamin is the presence of active acetaldehyde. Krampitz et al. (1958), using the method of Sarrett and Cheldelin for thiamin assay, found that active acetaldehyde possessed 80% of the activity of thiamin for L. fermentum. Carlson and Brown (1961) found that E. coli grown 20 hr at 30°C had 60% of its total thiamin as active acetaldehyde. If active acetaldehyde also made up a high percentage of the thiamin in the microorganisms involved in fermentation, then this could have caused the apparent decrease in thiamin measured. If, on the other days of fermentation, thiamin was found in a different form, this could explain the apparent increase.

Still another possibility is the presence of thiaminase. According to Lamden (1972), the type of thiaminase produced by most bacteria cleaves thiamin into its pyrimidine and thiazole components. Some heterofermentative lactobacilli which require thiamin may also be able

to utilize the components of the molecule by linking them together (Koser, 1968). The strain of L. fermentum used in thiamin assay does not respond to the thiazole and pyrimidine components during 18 hr incubation, but can utilize them thereafter (Sarrett and Cheldelin, 1944). Thiaminase produced early in the fermentation could have cleaved thiamin into its pyrimidine and thiazole components followed, after sufficient incubation, by the relinking of the components by heterofermentative lactobacilli.

Although the pattern for choline resembles that of thiamin, the two vitamins do not seem to have a particularly close relationship in terms of function or synthesis. Choline, as a source of labile methyl, or produced as the result of a transfer of labile methyl, would seem more closely related to vitamin B_{12} and folacin than to thiamin. Choline and its derivatives are important as intermediates in metabolic reactions, but choline has no known coenzyme function (Griffith and Nyc, 1971). Since all the organisms in the fermentation can probably synthesize choline, the decreased amount on the first day may simply be the result of its conversion to other compounds during the growth phase of the microorganisms, followed by a build up of choline after growth ceased.

Vitamins unchanged by fermentation

There were no significant differences in values measured for niacin and biotin as a result of fermentation. Apparently synthesis equaled degradation and there was no net change.

Because of the previously mentioned pellegra problem in corn staple diets, it would have been desirable to increase niacin levels.

Tongnual et al. (1981) found that available tryptophan increased in natural lactic acid fermentation, but an increase in available niacin would also be desirable. According to Freed (1966), the 1N. H_2SO_4 extraction of niacin that was used in this study is as effective as weak base for extraction of niacin from cereal grains. Christianson et al. (1968) reported that weak base treatment frees the unavailable niacin in corn. Therefore, the method used for niacin extraction in this study yields a measure of the total niacin. Since nearly all Lactobacillus and Pediococcus require niacin (Koser, 1968), the lactics must have obtained their niacin either from corn, or from the other bacteria. In order to obtain niacin from corn, the lactics might possess an enzyme capable of freeing bound niacin. For this reason, although total niacin might be the same, the available niacin might be increased. Further research should be done to determine if available niacin is increased. A microbiological method using Lactobacillus casei for estimation of bound versus free niacin is given by Clegg (1963).

Nutritive value of fermented corn meal

One can project which B-vitamins would not be consumed in adequate amounts if corn were used as the only source of calories (Table 3). Niacin, pantothenic acid, riboflavin, vitamin B_{12}, and folacin were deficient in corn on this basis. When the values measured for nonfermented whole corn meal in this study are compared similarly, the same vitamins would be deficient in corn except possibly pantothenic acid which falls within the range listed for the recommended daily allowance (RDA), Table 12.

Table 12. Vitamin content of whole corn meal and corn meal fermented two days compared to recommended daily allowances.

Vitamin	Whole corn meal		RDA[2]
	Nonfermented mg/770g[1]	Fermented two days mg/770g	mg
Vitamin B_{12}	0.0005	0.0231	0.003
Riboflavin	1.0800	3.0800	1.680
Niacin	14.8600	15.4000	18.480
Pantothenic acid	7.1600	9.2400	5.000-10.000
Biotin	0.0370	0.0390	*
Pyridoxine	2.9300	2.3900	2.000
Folacin	0.2900	0.7700	0.400
Choline	669.0000	616.0000	*
Thiamin	2.7700	3.0000	1.400

[1]Mertz (1970). 770g is sufficient to supply the total caloric requirements of the reference man, age 22, weighing 70 kg, living in a temperate climate, and requiring 2800 kcal per day.

[2]National Academy of Science/National Research Council (1974). Thiamin, riboflavin, and niacin calculated as based on 2800 kcal.

*RDA not established.

Most of the increases in B-vitamin content occurred during the first day of fermentation and increases after that were relatively small. Although the highest overall vitamin content in fermented corn was found on the fourth day of fermentation, corn meal fermented two days had values almost as high. Tongnual et al. (1981) found that most of the increase in % RNV occurred during the first two days of fermentation, and Lopez (1982) found that decreases in phytate were also greatest early in the fermentation. For these reasons, two days of fermentation is probably the best time of fermentation in terms of time expended versus nutritional value gained.

One can evaluate corn meal fermented two days as whole corn by consulting Table 12. If the reference man was consuming fermented corn meal as his only source of calories then he would not consume enough niacin, and possibly not enough pantothenic acid. He would, however, consume more than the RDA of riboflavin, vitamin B_{12}, and folacin. This assumes that the caloric content of fermented corn meal is equal to that of whole corn, although it is probably lower due to carbohydrates utilized during fermentation. The pantothenic acid value obtained for fermented corn and nonfermented corn was higher than the value of Kent (1975), and the amount of increase was only 40% after four days fermentation. For this reason, pantothenic acid would probably not be present in adequate amounts, if corn with a low initial value were fermented. The folacin value was also higher than the value given by Kent (1975), but if corn containing the folacin value of Kent underwent the same magnitude of increase (170%), folacin would still be present in adequate amounts for the reference man. Although pyridoxine was decreased by fermentation, the reference man would still consume enough pyridoxine.

It would be unlikely, of course, for anyone to consume corn as their only source of calories. A more realistic figure for a staple diet might be 50% of the total calories obtained from corn. If the reference man consumed fermented corn as 50% of his total caloric intake, he would consume the RDA for thiamin and vitamin B_{12}, and nearly the RDA for riboflavin and folacin. If the reference man obtained 50% of his calories from corn, he would obtain the RDA for thiamin, and probably the RDA for pyridoxine depending on whether the values from this study or from Kent (1975) were used. He would, however, not obtain enough vitamin B_{12}, riboflavin, or folacin. If the overall diet of a person on a corn staple diet was deficient in riboflavin, vitamin B_{12}, and folacin, then fermentation of corn meal would be beneficial. If, however, the diet was low in pyridoxine, fermentation would not be beneficial.

Another way to approach the evaluation of vitamin content is to compare the amounts required to provide the RDA. Using values from this study, the reference man would have to consume 1200g of nonfermented corn to obtain sufficient riboflavin, but only 420g of corn meal fermented two days would be required. Similarly, the values for vitamin B_{12} would be 5000g vs 100g, for folacin 1075g vs 400g, and pyridoxine 526g vs 645g. The other vitamins were not significantly different in whole corn compared to corn fermented two days.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objective of this study was to evaluate the effect of length of fermentation on the B-vitamin content of natural lactic acid fermented corn meal. The B-vitamin content of the whole corn meal control and of samples fermented either one, two, three, or four days at 30°C was determined.

The greatest changes in vitamin content occurred during the first day of fermentation. As compared to the control, all fermented samples had significantly ($P<0.05$) higher amounts of riboflavin, vitamin B_{12}, and folacin. All fermented samples contained significantly ($P<0.05$) less pyridoxine than the control. Samples fermented for four days had significantly ($P<0.05$) more pantothenic acid than the control, but other fermented samples were not significantly ($P<0.05$) different from the control, nor were they significantly ($P<0.05$) different from samples fermented four days. Thiamin and choline levels were significantly ($P<0.05$) lower in samples fermented for one day than in the control or in the other fermented samples. There were no other significant ($P<0.05$) differences in thiamin and choline contents. There were no significant ($P<0.05$) differences in niacin and biotin contents. A fluctuating and parallel pattern was observed in vitamin B_{12} and riboflavin contents among the fermented samples. A fluctuating and parallel pattern was also observed in thiamin and choline contents. There were no significant ($P<0.05$) differences among the fermented samples in folacin and pyridoxine contents.

Future research should include animal assay and/or use of other microorganisms to confirm the vitamin B_{12} and folacin activity. The

effect of natural lactic acid fermentation on bound niacin should also be evaluated.

Despite decreases in pyridoxine, the overall B-vitamin content of corn was improved by fermentation. With the exception of niacin, the B-vitamins reported to be present in inadequate amounts in corn, were increased by fermentation. When other factors such as % RNV increases, phytate decreases, and development of sufficient pH to inhibit pathogens are considered, a fermentation time of from two to four days is recommended. Fermentation for four days produced the highest B-vitamin content, but the differences among fermented samples were slight as compared to the differences between fermented and nonfermented corn meal samples.

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APPENDIX

Table 1. Summary of analysis of variance for vitamin content.

DF	Error 20	Model 4	Treatment 4	Total corrected 24
		Mean Square		
	Error	Model	F value	
Vitamin B_{12}	14.437	879.559	60.92	
Folacin	47004	544795	11.59	
Riboflavin	0.71749	6.33789	8.83	
Pantothenic acid	6.87257	11.9174	1.73	
Pyridoxine	0.18203	1.11867	6.15	
Thiamin (HCl)	0.18161	1.50814	8.30	
Choline (Cl)	19108	121087	6.34	
Niacin	5.7664	1.5812	0.27	
Biotin	75.342	59.441	0.79	

Table 2. Method of choline determination.

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1) 1g sample mixed with 50 ml 3N HCl and autoclaved at 15 lb for 2 hr.
2) Cooled and neutralized to pH 5.5 with 20% NaOH.
3) Diluted to 200 ml and filtered through Whatman #1 filter paper.
4) 1, 2, and 3 ml added to 125 ml duplicate flasks containing 10 ml double strength media and water to make 20 ml.
5) Autoclaved 10 min, cooled, innoculated with spore suspension.
6) Incubated at 30°C for five days.
7) Mycelial mat filtered onto preweighed Whatman #1 filter paper and dried in air oven at 50°C for 12 hr.
8) Allowed mats to cool to room temperature and weighed.

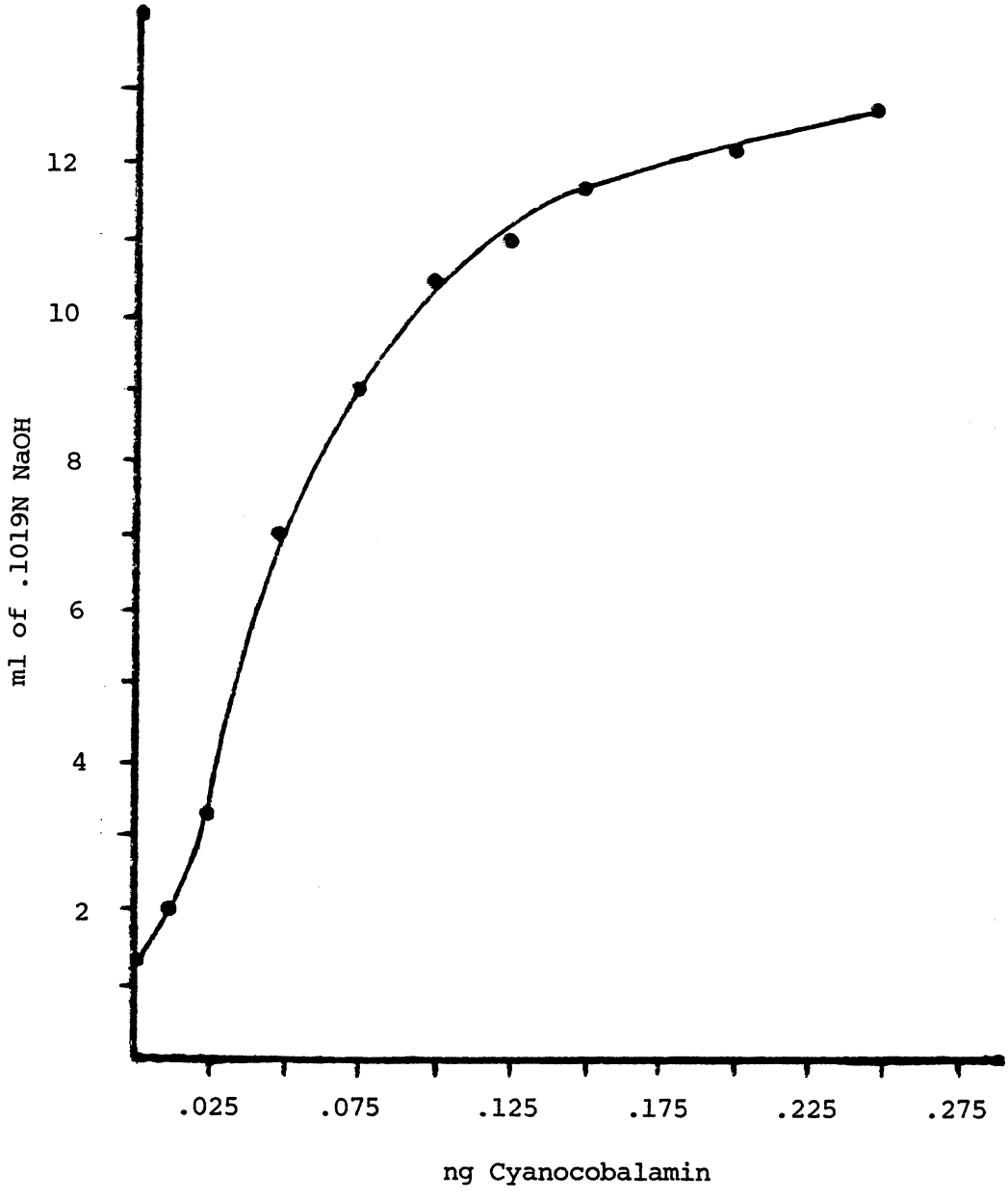

Fig. 1. Standard curve for vitamin B_{12}.

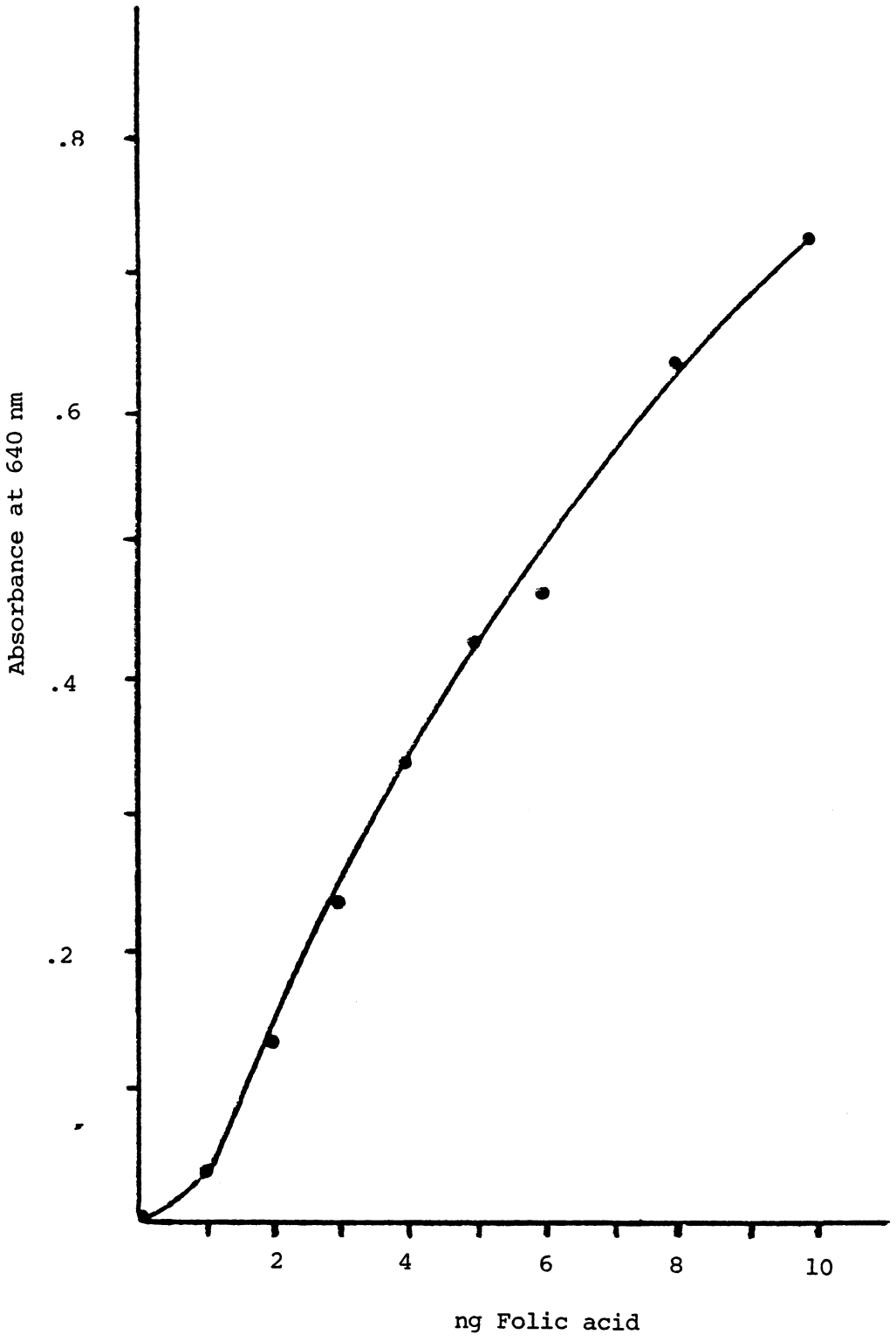

Fig. 2. Standard curve for folacin.

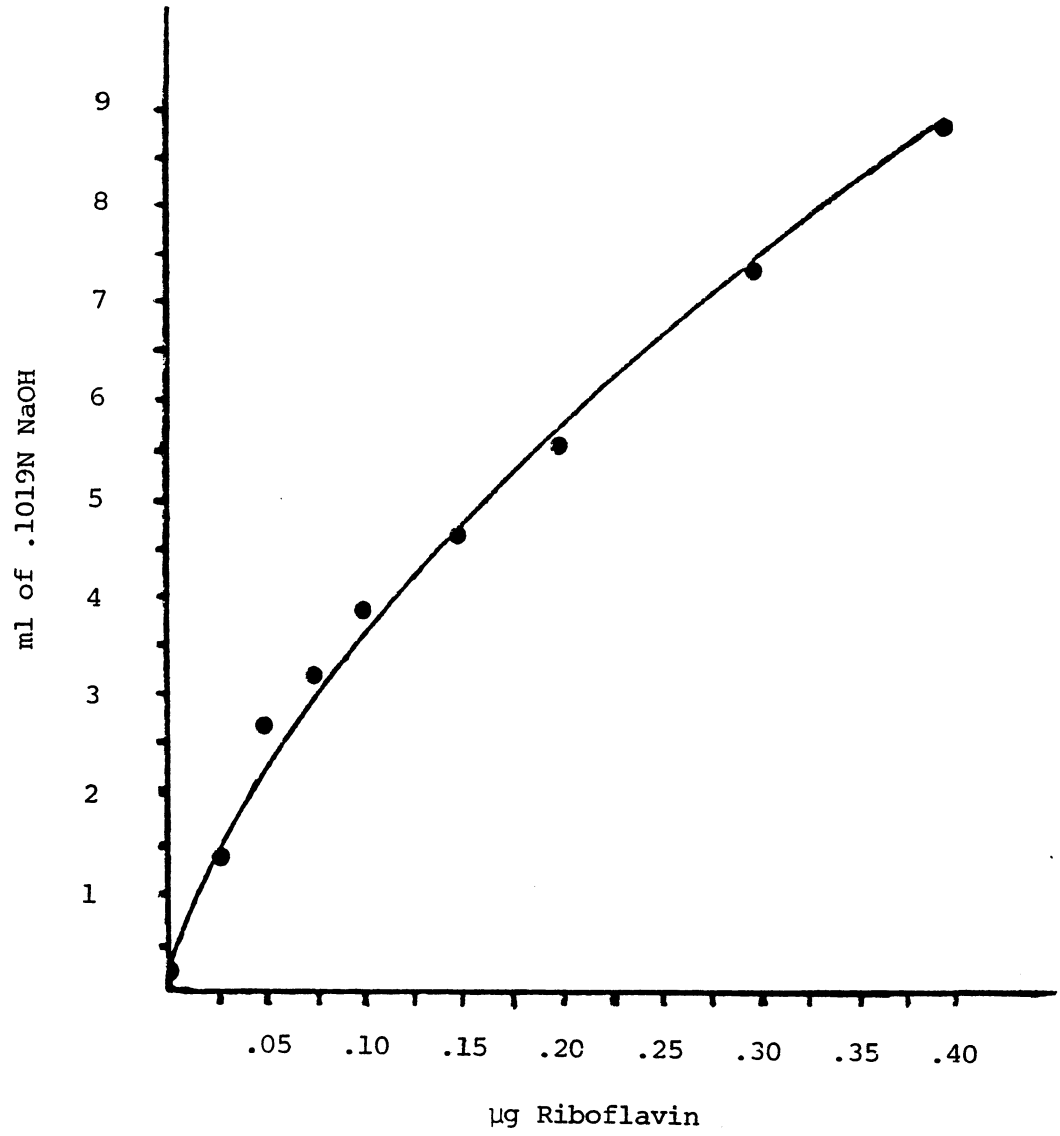

Fig. 3. Standard curve for riboflavin.

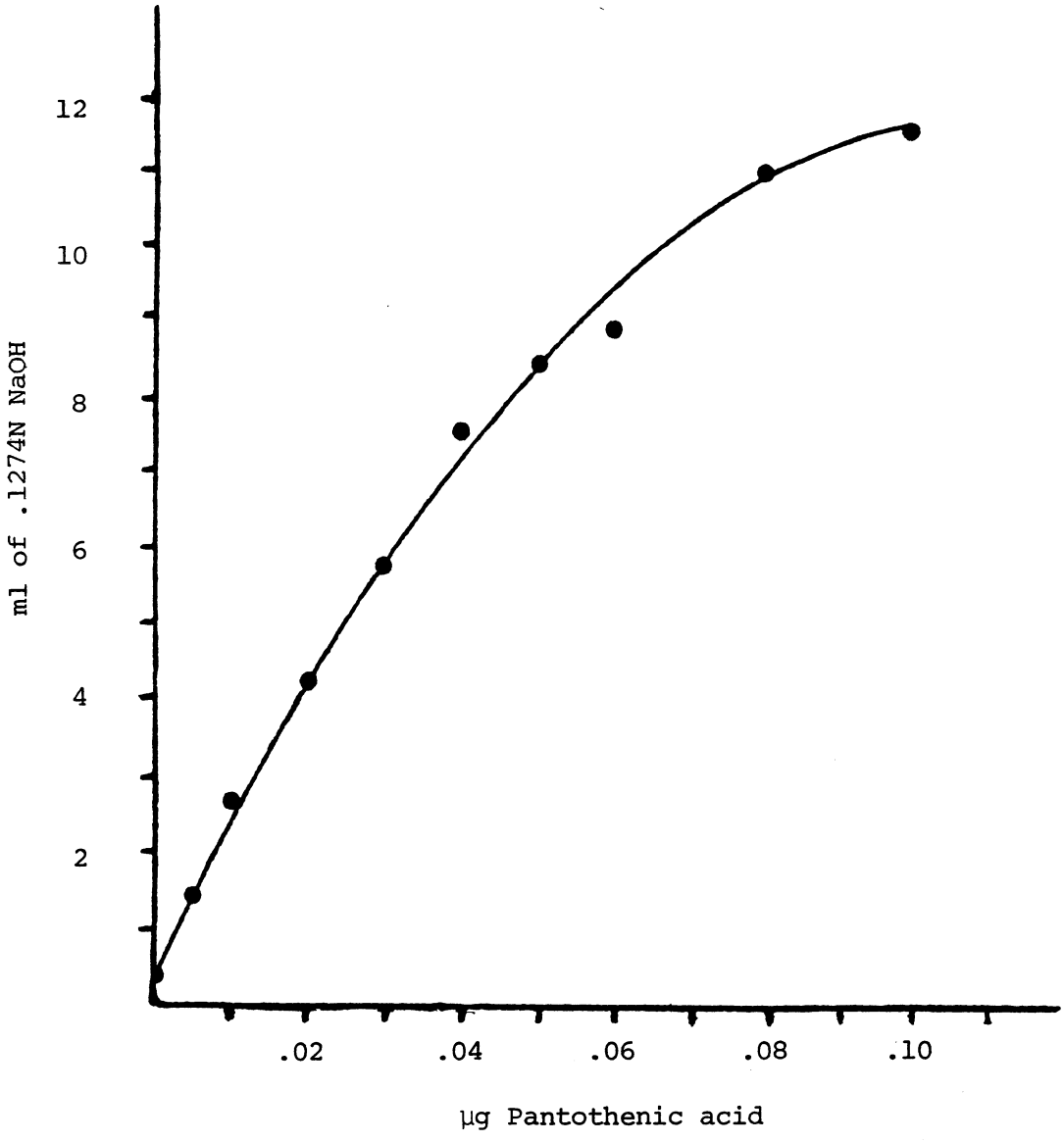

Fig. 4. Standard curve for pantothenic acid.

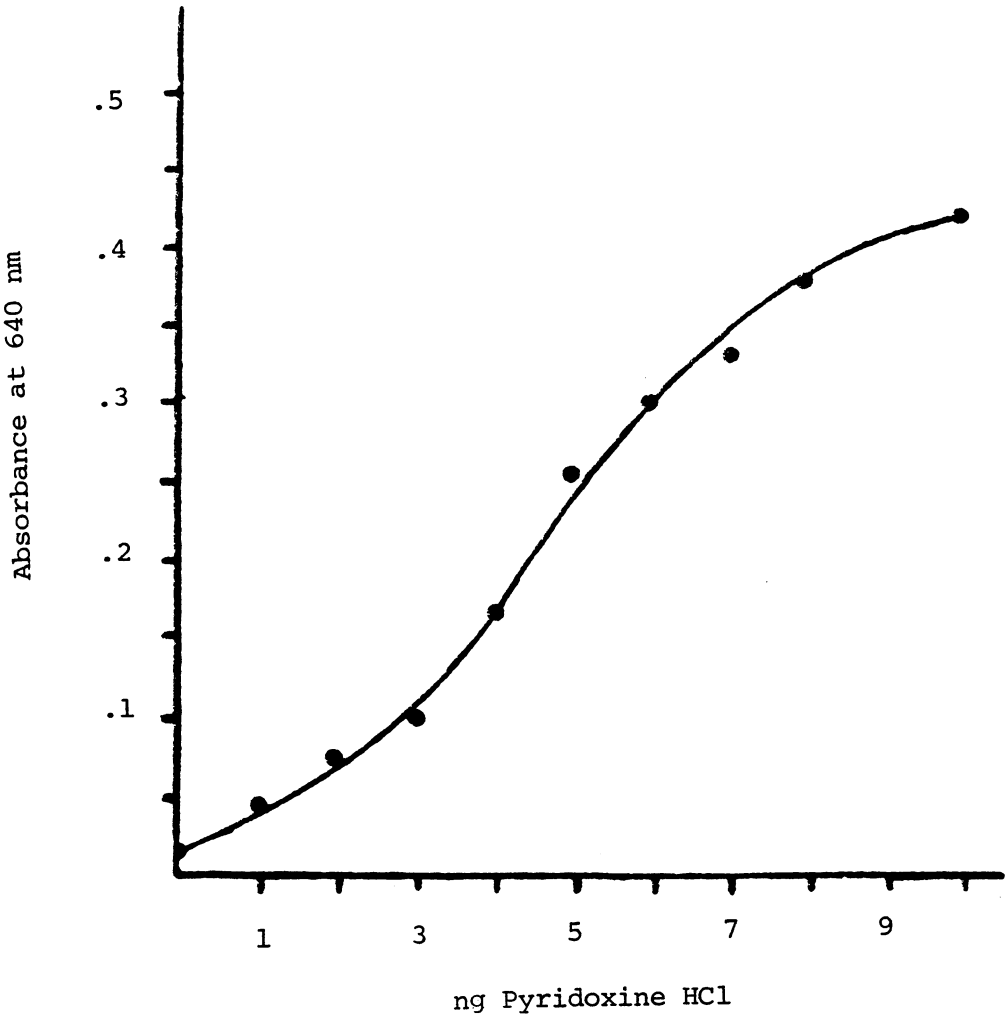

Fig. 5. Standard curve for pyridoxine.

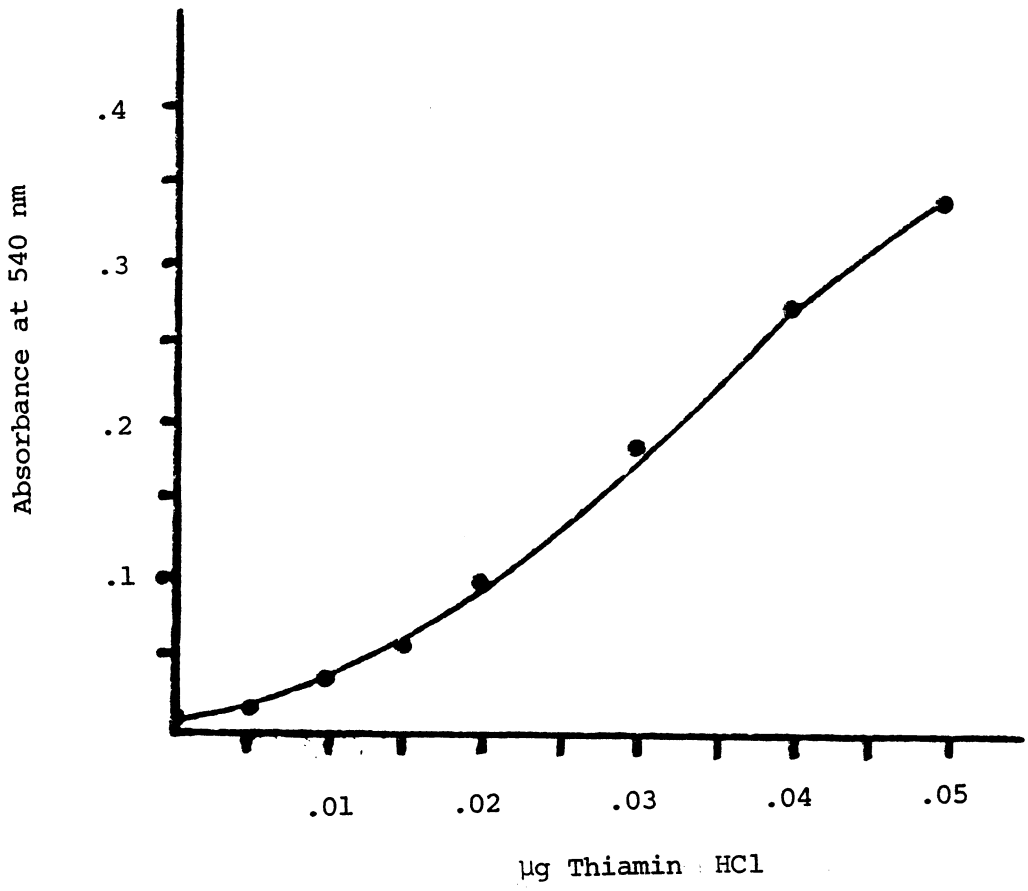

Fig. 6. Standard curve for thiamin.

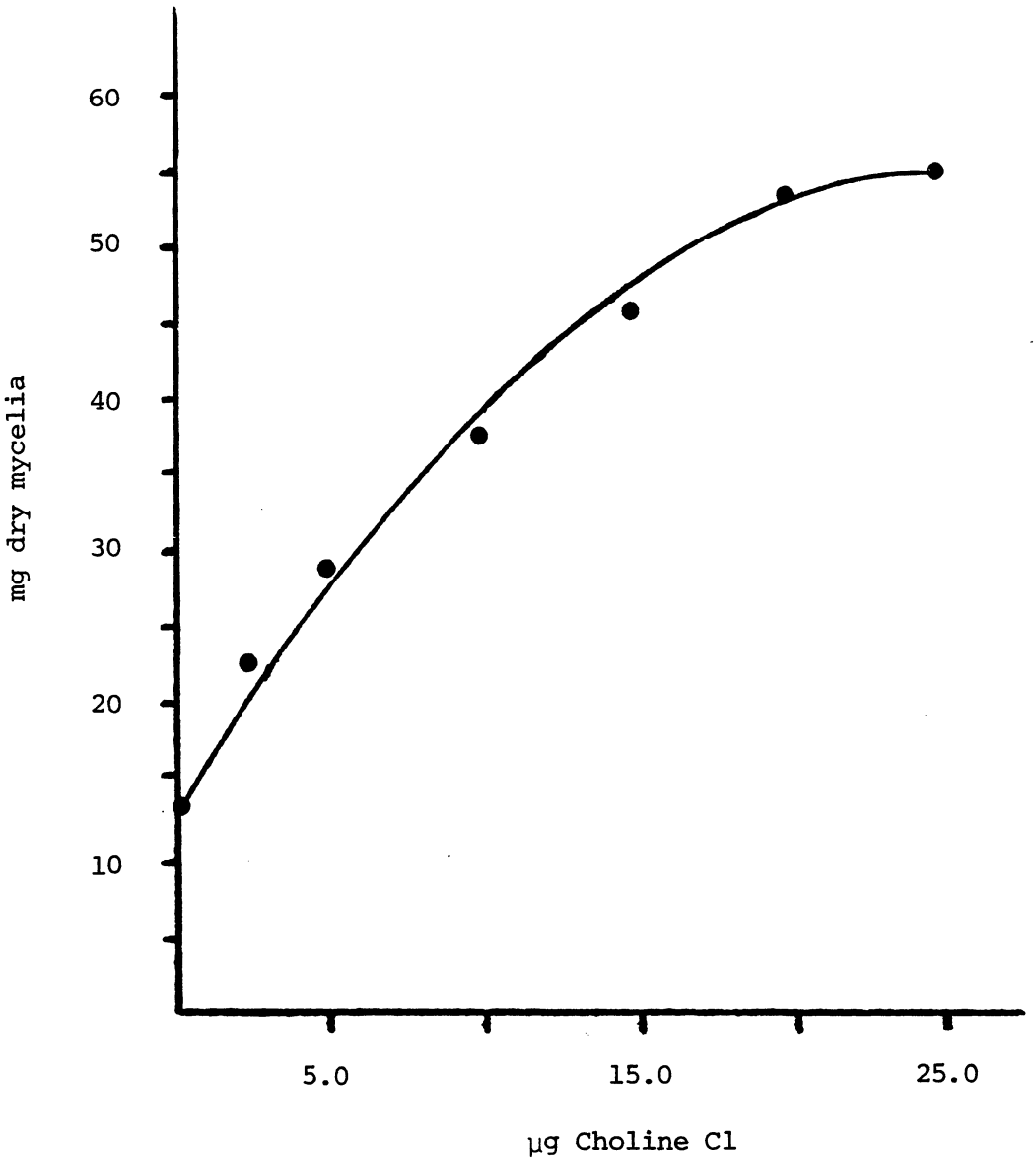

Fig. 7. Standard curve for choline.

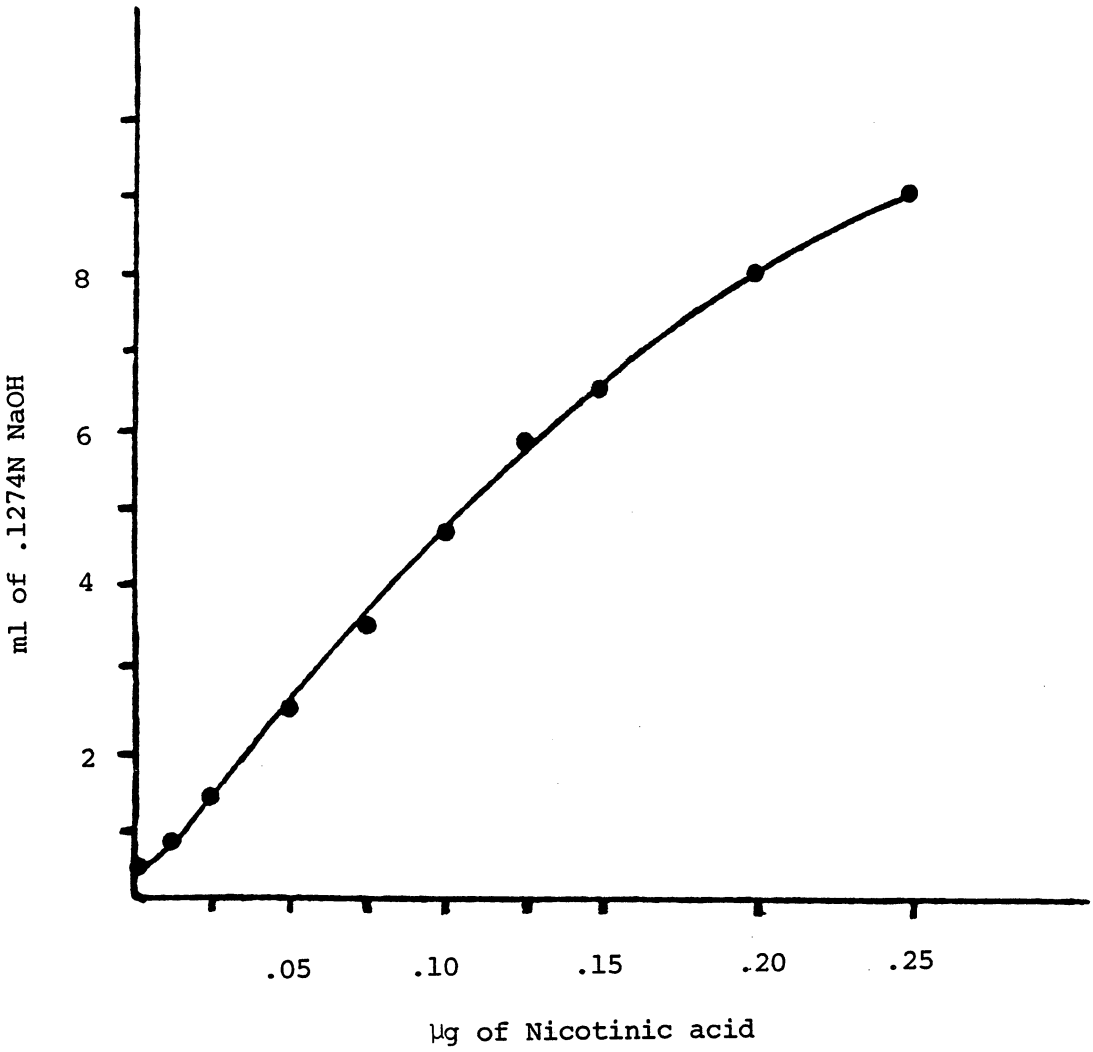

Fig. 8. Standard curve for niacin.

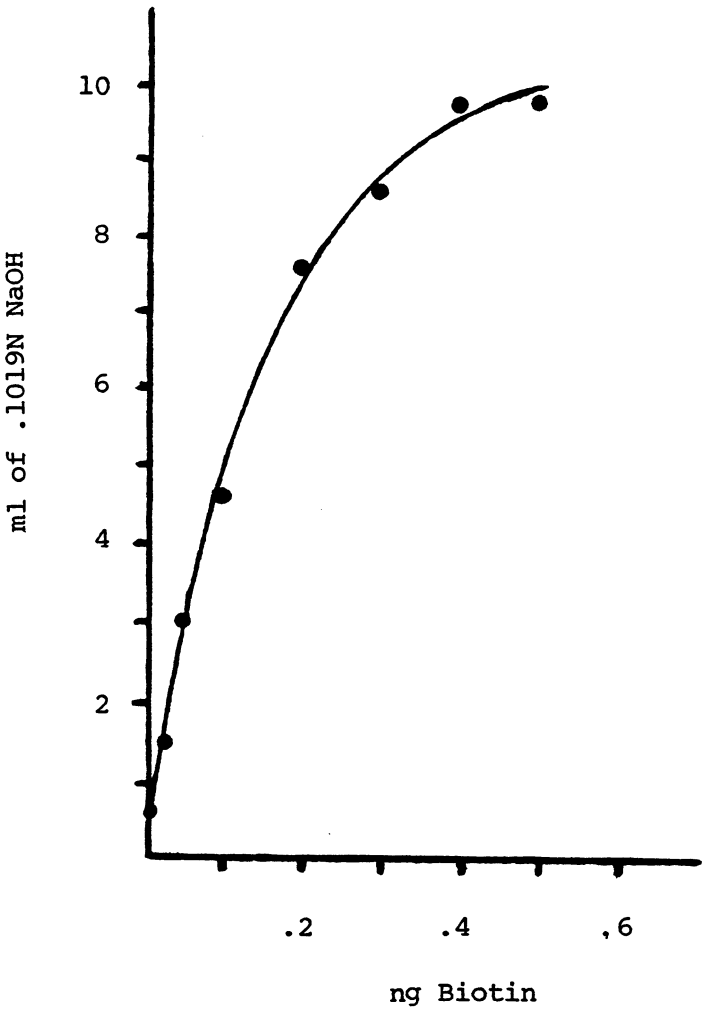

Fig. 9. Standard curve for biotin.

The undersigned, appointed by the Dean of the Graduate Faculty, have examined a thesis entitled

"B-Vitamin Content of Corn Meal During Natural Lactic Acid Fermentation"

presented by Fred Murdock

a candidate for the degree of Master of Science

and hereby certify that in their opinion it is worthy of acceptance.

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