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BIOLOGICAL AVAILABILITY OF VITAMIN B_6 IN LIQUID AND DEHYDRATED MODEL FOOD SYSTEMS AFTER THERMAL PROCESSING AND STORAGE

By

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ABSTRACT

BIOLOGICAL AVAILABILITY OF VITAMIN B_6 IN LIQUID AND DEHYDRATED MODEL FOOD SYSTEMS AFTER THERMAL PROCESSING AND STORAGE

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Liquid and dehydrated model food systems were employed to study the behavior of the B_6 vitamers with respect to the retention of vitamin B_6 activity during thermal processing and storage.

A liquid model system comprised of a neutral solution of heat stable peptides was utilized to examine the extent and manner of binding of pyridoxal (PL) and pyridoxal phosphate (PLP) to peptides and proteins during retort processing. Isolation and spectrophotometric examination of the model system peptides after processing for 20 min at 121°C revealed that about 21% of the PLP in the model system was peptide bound. Characterization of the PLP-peptide complexes indicated that about 10% of the PLP in the model system was bound as a pyridoxylamino complex, potentially limiting the bioavailability. The presence of glucose and ascorbic acid in the model system solutions appeared to weakly inhibit the binding.

The effects of glucose and ascorbic acid on the retention of available pyridoxamine (PM) were studied using a similar liquid model system which was fortified with PM. While losses were low during 20 min treatment at 121°C in the absence of glucose or ascorbate, 21% of the total B_6 was degraded during processing in the presence of glucose. Ascorbic acid promoted only a slight loss, and its effect was not additive with that of glucose. Gel filtration chromatographic examination of thermally induced interactions between PM and glucose, ascorbate, or their degradation products revealed no detectable binding.

Dehydrated model food systems of composition simulating breakfast cereals were utilized to examine the relative stability and bioavailability of the B_6 vitamers. Roasting and storage conditions were selected to permit the estimation of the maximum vitamin B_6 degradation encountered in food processing and storage.

Roasting of the dehydrated model systems for 25 min at 180°C resulted in a loss of 50-70% of each of the B_6 vitamers. During the storage of similar dehydrated systems at 37°C after equilibration to a water activity (a_w) of ≈0.6, varying stability of the B_6 vitamers was observed by liquid chromatographic (HPLC) analysis. When packaged in enameled metal cans with a small headspace (TDT cans), the dehydrated model systems exhibited higher rates of nonenzymatic browning and

degradation of each of the B_6 vitamers than the rates observed with storage in cans with a large headspace. Half-lives of the vitamers in model systems stored in TDT cans at 37°C, ≈0.6 a_w, were 35-46 days for PM, PL, and PLP, and 141 days for pyridoxine (PN). The degradation of each of the B_6 vitamers during storage followed first order kinetics.

Microbiological and HPLC assays for total vitamin B_6 correlated closely with rat bioassay estimates of available B_6 in the dehydrated model systems which had been roasted 25 min at 180°C or stored for 128 days at 37°C. These results demonstrated that the vitamin B_6 remaining after roasting or storage retained full bioavailability. Vitamin B_6 assay by the semiautomated fluorometric method was not accurate in estimating available B_6 in the dehydrated model systems.

The only significant mechanism identified for the loss of vitamin B_6 activity during storage was the binding of PLP by the proteins of the dehydrated model systems in the form of ε-pyridoxyllysine. Tests of the vitamin B_6 activity of protein bound pyridoxyllysine revealed only 50% of the molar potency of PN. In addition, this complex inhibited the utilization of about 55% of the free PN added to rat diets.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	x
INTRODUCTION	1
LITERATURE REVIEW	3
Vitamin B_6 History	3
Determination of Vitamin B_6	5
Extraction Methods	6
Microbiological Methods	12
Preparative Chromatography	15
Bioassay Methods	18
Evaluation of Vitamin B_6 Bioassays	20
Chemical Methods for Determination of Total Vitamin B_6	22
Spectroscopic Methods	22
Analytical Chromatographic Methods	25
Vitamin B_6 Chemistry	26
Formation of Complexes	26
Nonenzymatic Interconversions and Catalysis by Vitamin B_6	32
Vitamin B_6 Stability and Bioavailability	34
EXPERIMENTAL METHODS	44
Preparative Methods	44
Heat Stable Peptide Mixture	44
ε-Pyridoxyl-L-lysine Synthesis	44
Phosphopyridoxyl-Bovine Serum Albumin	44
Chemical Methods	44

Page

Protein Determination 44
Amino Groups 45
Sulfhydryl Groups 45
Fluorometric Determination of Vitamin B_6 Compounds 45
Erythrocyte Aspartate Aminotransferase Assay . 46
Hemoglobin Determination 47

Physical Methods 47

Spectrophotometry 47
High Performance Liquid Chromatographic Assay of Vitamin B_6 47
High Performance Liquid Chromatographic Assay of Pyridoxyllysine 48
High Performance Liquid Chromatographic Assay of 4-Pyridoxic Acid 48
Discontinuous Polyacrylamide Gel Electrophoresis. 48
Thin Layer Peptide Mapping 49
Gel Filtration Chromatography 49

Biological Methods. 50

Microbiological Determination of Total Vitamin B_6 50
Rat Bioassay of Biologically Available Vitamin B_6 50

Experimental Design and Procedures 54

Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Food System during Thermal Processing. 54

Characterization of Peptides 55
Pyridoxal and Pyridoxal Phosphate Binding Studies 57

Interactions of Pyridoxamine in a Liquid Food System during Thermal Processing. 61

Model System Formulation and Treatment. . . 61
Evaluation of Thermal Effects. 63
Statistical Analysis. 64

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Roasting Effects 64

Page

Formulation and Treatment of the Model Systems 65
Analysis of Model Systems. 66
Statistical Analysis 67

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Storage Effects. 68

Formulation, Processing, and Storage of Model Systems 69
Analysis of Model Systems. 70

Biological Activity of Phosphopyridoxyl-Bovine Serum Albumin. 73

Analysis of Phosphopyridoxyl-Bovine Serum Albumin 74
Determination of Biological Activity 75

RESULTS 77

Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Model Food System during Thermal Processing 77

Preparation and Characterization of Heat Stable Peptides 77
Binding of PL and PLP in Liquid Model Systems. . 84

Interactions of Pyridoxamine in a Liquid Model Food System during Thermal Processing 93

Stability of Pyridoxamine 93
Pyridoxamine--Carbohydrate Complex Formation . . 93

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Roasting Effects 95

Comparison of Assay Methods for Total Vitamin B_6. 95
Interference in Semiautomated Fluorometric Vitamin B_6 Assay. 104
Biologically Available Vitamin B_6 in Roasted Dehydrated Model Systems 105

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Storage Effects. . . 110

Page

Moisture Content and Water Activity 110
Nonenzymatic Browning 112
Kinetics of Vitamin B_6 Degradation 117
Determination of 4-Pyridoxic Acid in Dehydrated Model Systems after Storage 127
Determination of Pyridoxyllysine in Dehydrated Model Systems after Storage 129
Determination of Total Vitamin B_6 in Dehydrated Model Systems after Storage 135
Biologically Available Vitamin B_6 in Dehydrated Model Systems after Storage 138

Biological Activity of Phosphopyridoxyl-Bovine Serum Albumin 144

Analysis of Phosphopyridoxyl-Bovine Serum Albumin. 144
Pyridoxylamino Group Utilization and Anti-vitamin Activity. 145

DISCUSSION. 149

Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Model Food System during Thermal Processing 149
Interactions of Pyridoxamine in a Liquid Model System during Thermal Processing 158
Vitamin B_6 Assay Methods 159
Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Roasting Effects . . 162
Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Storage Effects. . . 164

Water Activity and Nonenzymatic Browning . . . 164
Stability of Vitamin B_6 during Storage 168
Degradation Products of Vitamin B_6 170
Biological Availability of Vitamin B_6 in Dehydrated Model Systems after Storage . . . 171

Biological Activity of Protein Pyridoxylamino Compounds. 172

SUMMARY AND CONCLUSIONS 175

APPENDIX 180

SELECTED BIBLIOGRAPHY 198

LIST OF TABLES

Table		Page
1.	Composition of diets for rat bioassays . . .	51
2.	Formulation of liquid model systems for study of PL and PLP interactions	58
3.	Formulation of liquid model systems for study of pyridoxamine interactions. . . .	62
4.	Composition of dehydrated model system for determination of roasting effects	66
5.	Total peptide bound PL and PLP.	86
6.	Percent of model system PLP bound as a non-reducible complex (substituted aldamine or pyridoxylamino compound) at pH 7.0. . .	89
7.	Percent of PLP in liquid model systems which was bound as a pyridoxylamino complex. . .	90
8.	Sulfhydryl content of peptides in liquid model systems after thermal processing . .	92
9.	Vitamin B_6 in PM-fortified liquid model systems after thermal processing	94
10.	Calibration curves for the HPLC assay of vitamin B_6 using 280 nm absorption detection	98
11.	Total vitamin B_6 in control and roasted model systems determined by microbiological and HPLC methods	100
12.	Vitamin B_6 in control and roasted model systems, as determined by the semiautomated fluorometric assay procedure.	101
13.	Comparison of fluorometric and microbiological methods for determination of total vitamin B_6 in control and roasted model systems . .	103

Table | Page

14. Characteristics of the emission spectra of the fluorophores produced from vitamin B_6 standards and model system extracts during the fluorometric assay of vitamin B_6 106

15. Rat bioassay of roasted model systems (Bioassay 1). 107

16. Linear regression parameters of the dose-response curves. Bioassay 1, roasted model systems 108

17. Biologically available vitamin B_6 in roasted model systems. 109

18. Comparison of rat bioassay estimates of biologically available vitamin B_6 in roasted model systems with microbiological and semiautomated fluorometric assay results for total vitamin B_6 111

19. Moisture content and water activity of stored model systems 113

20. Comparison of browning levels in model systems stored in TDT and 303 cans for 128 days at 37°C 116

21. Kinetic data for the loss of the B_6 vitamers from dehydrated model systems during storage in TDT cans at 37°C. 126

22. Vitamin B_6 content in dehydrated model systems after storage for 128 days at 37°C in TDT and 303 cans 128

23. Determination of 4-pyridoxic acid in model systems stored for 128 days in TDT cans at 37°C 136

24. Determination of pyridoxyllysine in model systems from TDT and 303 cans pooled after storage for 128 days at 37°C . . . 136

25. Microbiological assay of vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C. 137

Table | Page

26. High performance liquid chromatographic assay of vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C 137

27. Semiautomated fluorometric determination of vitamin B_6 in dehydrated model systems pooled after storage for 128 days at 37°C in TDT and 303 cans 138

28. Rat Bioassay 2. Determination of available vitamin B_6 in model systems pooled after 128 days storage in TDT and 303 cans at 37°C, and analysis of biological activity of PP-BSA 139

29. Linear regression parameters of the dose-response curves. Bioassay 2. 141

30. Biologically available vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C 142

31. Comparison of semiautomated fluorometric, microbiological, HPLC, and rat bioassays for vitamin B_6 in model systems pooled after 128 days in storage in TDT and 303 cans at 37°C 143

32. Determination of free vitamin B_6 in dialyzed PP-BSA 146

33. Biological activity of pyridoxyllysine residues in PP-BSA 148

LIST OF FIGURES

Figure		Page
1.	Chemical forms of vitamin B_6 (free bases) . .	4
2.	Complexed forms of pyridoxal	27
3.	Polyacrylamide gel electropherogram of β-lactoglobulin which was used in isolation of heat stable peptides	79
4.	Thin layer peptide map of heat stable peptides	80
5.	Sephadex G-50 (fine) calibration curve . . .	82
6.	Sephadex G-50 chromatogram of heat stable peptide mixture	83
7.	Ultraviolet-visible difference absorption spectrum of peptides isolated from a PLP-fortified model system versus those from the nonfortified blank.	85
8.	Fluorescence emission spectra of peptides from liquid model systems with and without PLP fortification	88
9.	Sephadex G-10 chromatograms of PM-fortified liquid model systems after 20 min at 121°C	96
10.	HPLC chromatogram of standard vitamin B_6 free bases.	97
11.	Loss of free amino groups in soluble proteins of dehydrated model systems during storage in TDT cans at 37°C	114
12.	Formation of melanoidin pigments during storage of dehydrated model systems in TDT cans at 37°C.	115

Figure | Page

13. HPLC chromatograms of a recovery sample of a nonfortified model system stored for 97 days in a TDT can at 37°C 120

14. Degradation of PLP in dehydrated model systems stored in TDT cans at 37°C 122

15. Behavior of vitamin B_6 in a PM-fortified dehydrated model system stored in TDT cans at 37°C 123

16. Degradation of added PN and inherent PL in dehydrated model systems stored in TDT cans at 37°C 125

17. Absorption spectrum of pyridoxyllysine; difference spectrum of 1 mg/ml PP-BSA versus 1 mg/ml BSA 131

18. HPLC chromatograms of pyridoxyllysine standard and protein hydrolysate of PLP-fortified model system. 132

19. Fluorescence excitation and emission spectra of crystalline pyridoxyllysine standard and collected pyridoxyllysine chromatographic peak from PLP-fortified model system protein hydrolysate. . . . 134

INTRODUCTION

Vitamin B_6 serves as a cofactor in the metabolism of all plant and animal species, and, hence, is widespread in nature. Variation in the B_6 requirement among humans and the possible instability of certain forms of the vitamin in foods are factors which may contribute to the marginal nutriture of certain groups of the population.

The stability of vitamin B_6 in foods has not been extensively studied. Previous research has demonstrated that, under certain processing and storage conditions, losses of vitamin B_6 may occur. The precise behavior of the various chemical forms of the vitamin, their rates of degradation, and the identity of their degradation products are largely unknown.

The aldehyde forms of vitamin B_6, pyridoxal and pyridoxal phosphate, have the capability of forming several types of complexes with compounds containing free amino groups. Pyridoxamine may react with aldehydes to form analogous complexes. Potential reactions involving these vitamin B_6 compounds have never been investigated in food systems. Because these complexes vary

widely in their stability and bioavailability, their characterization and quantitation in foods would provide important information on nutritional quality.

Animal bioassay results have indicated that in certain instances, particularly in the thermal sterilization of dairy products, the content of biologically available B_6 may be less than that predicted by microbiological or chemical assay procedures. Whether this disparity represents an inherent difference in assay specificity or a true loss of bioavailability of the vitamin has not been established.

The purposes of this research were: (1) to investigate the interaction of several of the B_6 vitamers with food proteins and carbohydrates and determine the biological activity of the complexed forms of the vitamin; (2) to determine the accuracy and specificity of newly developed and existing methods for vitamin B_6 assay; (3) to study the stability of the B_6 vitamers under detrimental conditions of processing and storage, such that maximal losses encountered in foods could be estimated; and (4) to determine the effects of these severe thermal processing and storage conditions on the biological availability of vitamin B_6 in foods. It was anticipated that the results of these studies would further the understanding, with respect to vitamin B_6, of the nutritional quality of foods.

LITERATURE REVIEW

Vitamin B_6 History

The chemical and nutritional properties of vitamin B_6 were first recognized when Birch and Gyorgy (1936) identified a rat acrodynia-preventing factor in rice bran concentrates. Shortly thereafter, pyridoxine (PN) was isolated and crystallized independently in five laboratories (Lepkovsky, 1938; Keresztesy and Stevens, 1938; Gyorgy, 1938; Kuhn and Wendt, 1938; Ichiba and Michi, 1938). Coincidentally, this compound proved to be identical to the unidentified nitrogenous base which had been previously isolated by Ohadake (1932).

From studies with vitamin B_6-requiring microorganisms, Snell, et al. (1942), identified a non-pyridoxine factor or factors, termed "pseudopyridoxine," which possessed vitamin B_6 activity. These were subsequently identified as pyridoxal (PL) and pyridoxamine (PM), having aldehyde and amino groups at the C-4 position of the pyridine ring, respectively (Snell, 1942; Snell, 1944a,b; Harris, et al., 1944a,b). The structures of these three vitamers are illustrated in Figure 1.

CHEMICAL FORMS OF VITAMIN B_6

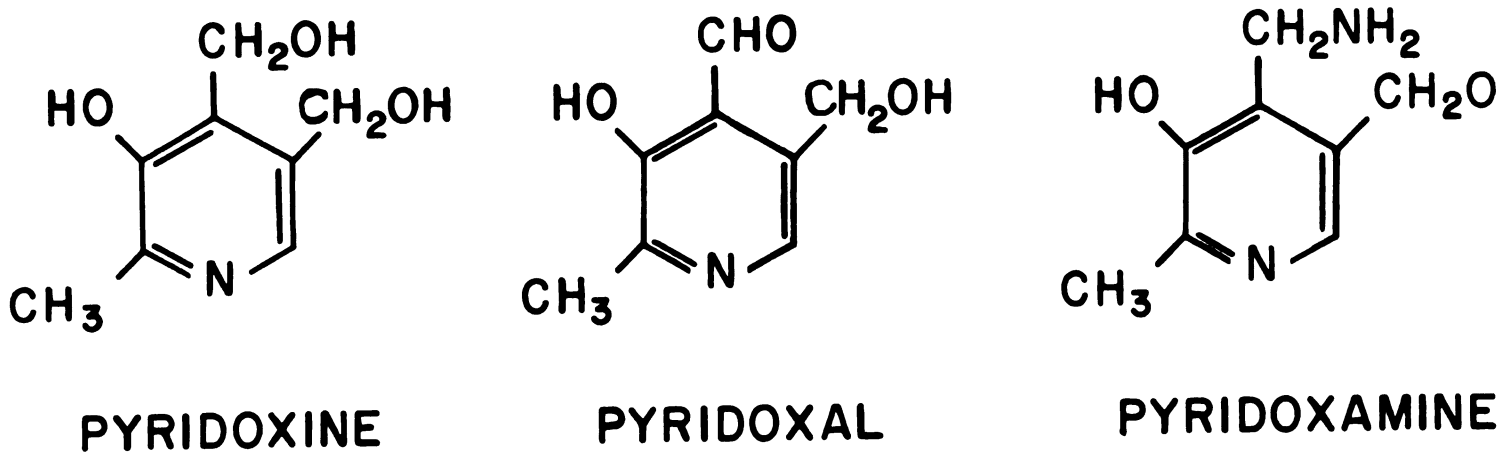


Figure 1. Chemical forms of vitamin B_6 (free bases).

Studies concerning microbial metabolism demonstrated the active coenzyme forms of vitamin B_6 to be phosphorylated derivatives of pyridoxal and pyridoxamine (Bellamy and Gunsalus, 1944; Gale and Epps, 1944; Gunsalus and Bellamy, 1944). Heyl, et al. (1951a,b), showed that the active coenzyme forms were the 5'-phosphates of pyridoxal (PLP) and pyridoxamine (PMP).

Determination of Vitamin B_6

For the accurate determination of vitamin B_6 in foods, all active forms of the vitamin, i.e. pyridoxal, pyridoxine, pyridoxamine, and their 5'-phosphates, must be measured. All of these vitamin B_6 compounds have been shown to possess approximately equal activity in mammalian and avian species.

Numerous assay procedures have been developed for the determination of the active forms of vitamin B_6, based upon chemical, physical, and microbiological methodologies. All of these analytical procedures require extraction of the B_6 vitamers from the sample matrix since they are based on determination of the vitamers in solution. In contrast, several animal bioassay methods have been developed for the determination of biologically available vitamin B_6. These bioassay procedures measure only biologically available vitamin B_6, as opposed to total B_6, because no extraction or hydrolysis procedures are employed.

Extraction Methods

In addition to releasing the free forms of the vitamin from the sample, the extraction procedure must recover any complexed forms of the vitamin which are biologically active for accurate quantitation of vitamin B_6 activity.

Initially, most of the assay procedures were based on microbial growth. This necessitated hydrolysis of the sample to cleave phosphate ester forms of vitamin B_6 because most of the organisms did not respond to the phosphorylated vitamers. Extraction of samples in strong acids at 121-130°C proved to be a fairly satisfactory method, because this treatment resulted in the disintegration of the sample and hydrolysis of the phosphate derivatives to their respective free base forms.

Atkin, et al. (1943), found that optimum extraction efficiency could be achieved by autoclaving most biological samples 1 hour at 20 psi in 0.055 N sulfuric acid. For the assay of cereals, the use of 0.44 N sulfuric acid provided a more complete extraction. In a rigorous study of extraction efficiency, Rubin, et al. (1947), found that for most samples, autoclaving in sulfuric acid within the range of pH 1.7 to 1.8 was most effective. This corresponds closely to 0.055 N sulfuric acid.

Extraction methods developed by Fujita, et al. (1955a,b,c), for the chemical assay of vitamin B_6 were

based on the use of H_2SO_4. For the determination of PN and PM, a sample homogenate adjusted to pH 4.5 was heated initially to 80°C for 15 minutes. The supernatants were then adjusted to 0.6 N with respect to H_2SO_4 and autoclaved 1 hour at 130°C. PL was extracted by direct autoclave treatment in 0.1 N H_2SO_4 for 1 hour at 130°C.

Studies of autoclave extraction methods by Rabinowitz and Snell (1947) demonstrated that 0.055 N HCl functioned as well as 0.055 N H_2SO_4 as an extraction medium for the assay of vitamin B_6 in most samples. Extraction in an autoclave at 20 psi for 5 hours resulted in the greatest hydrolysis and release of vitamin B_6 in most cases. Again, cereals required a more rigorous extraction treatment; autoclaving for 1 hour at 20 psi in 2 N HCl was found to be satisfactory. Storvick, et al. (1964), slightly modified the procedure of Rabinowitz and Snell (1947) by extracting with 0.055 N HCl at 15 psi for 5 hours. This was found to provide satisfactory extraction of vitamin B_6 in most samples. Toepfer, et al. (1963, 1970), adopted the 0.055 N HCl, 15 psi, 5 hour autoclave extraction method for their microbiological assay procedure. For plant products, including cereals, they determined that autoclaving in 0.44 N HCl at 15 psi for 2 hours was sufficient for complete extraction.

A method for the microbiological determination of vitamin B_6 in milk (Gregory, 1959) was based on extraction

with 0.55 N HCl under flowing steam, rather than autoclaving. Subsequently, Gregory and Mabbitt (1961) demonstrated the presence of pyridoxamine phosphate in milk and found that this vitamer required heat treatment for 3 hours at 125°C in 0.055 N HCl for complete hydrolysis of the phosphate ester. Under the same conditions, pyridoxal phosphate was totally hydrolyzed in only 30 minutes. Therefore, extraction of samples containing pyridoxamine phosphate for less than 3 hours at 125°C would result in erroneously low vitamin B_6 values by most assay procedures.

In studies concerning the effects of autoclaving time on apparent vitamin B_6 content of blood samples using several species of assay microorganisms, highly divergent results have been observed (Benson, *et al.*, 1964). Using blood samples which were extracted with 0.055 N HCl for time periods from 30 minutes to 72 hours, *Saccharomyces uvarum* (formerly *S. carlsbergensis*) assays showed a maximum value after 2 to 5 hours of autoclaving, followed by a sharp decline to almost nondetectable levels by 10 hours. In contrast, assays with *Lactobacillus casei* and *Streptococcus faecium* showed peak values after 10 to 24 hours, with little decline up to 72 hours. These anomalous results were later explained by the observations that both *L. casei* and *S. faecium* assays suffer interference from free and peptide bound D-alanine formed

by racemization during extensive autoclaving (Gregory, 1959; Haskell and Wallnofer, 1967; Raines and Haskell, 1968).

Further evidence against the use of long heat treatments for extraction of vitamin B_6 was obtained by Storvick and Peters (1964). Nonfat dry milk samples were hydrolyzed in 0.055 N HCl at 121°C for periods up to 72 hours, then assayed by an S. uvarum method. The apparent vitamin B_6 in the samples increased with increasing hydrolysis time to a maximum at approximately 5 hours, followed by a progressive decline. After 72 hours at 121°C, only 10-20% of the maximum apparent vitamin B_6 remained in the samples. Parallel treatments of pure vitamin B_6 standards, however, demonstrated very little degradation over the entire 72-hour period. Storvick and Peters (1964) suggested that extensive heat treatment during the extraction of biological samples could result in erroneously low values because of an irreversible binding of the vitamers to proteins. Extraction at 121°C for 5 hours in 0.055 N HCl provided maximal extraction and hydrolysis of all forms of the vitamin, yet minimized the possible losses by this mechanism.

Extraction with aqueous buffers, followed by enzymatic hydrolysis and chemical deproteination, were investigated in an effort to alleviate the numerous problems inherent in the autoclave extraction procedures

and to shorten the extraction time. Early methods of enzymatic hydrolysis using digestion with proteolytic and diastatic enzymes failed largely because of the high levels of vitamin B_6 in the enzyme preparations (Rabinowitz and Snell, 1947; Hopkins and Pennington, 1947).

A combination of the autoclave extraction procedure with enzymatic hydrolysis was performed by Barton-Wright (1971). Samples were autoclaved in 0.055 N HCl for 1 hour, followed by adjustment to pH 4.5 and treatment with takadiastase. This method was satisfactory for a variety of biological samples and foods, including cereals.

For analysis of vitamin B_6 in blood using *S. uvarum*, Storvick and Peters (1964) compared autoclave extraction with 0.055 N HCl to extraction by deproteination with trichloroacetic acid (TCA) without enzymatic treatment. Results with the autoclave extraction method were approximately 50% greater than those with the TCA method. These data demonstrated the need for cleavage of phosphate esters for the accurate determination of total vitamin B_6 using the *S. uvarum* method.

Numerous studies of vitamin B_6 metabolism have involved extraction of all forms of the vitamin from tissue samples, followed by individual analysis of the vitamers. Columbini and McCoy (1970) demonstrated 95-100% recovery of all B_6 vitamers by homogenization

of the tissue in cold water, followed by protein precipitation with TCA. Bain and Williams (1960) and Lyon, et al. (1962), developed a similar method employing deproteination with perchloric acid. This method provided extraction of at least 85% of the total vitamin B_6 in tissues (Johansson, et al., 1968).

Enzymatic hydrolysis of the vitamin B_6 phosphate esters was readily accomplished by Takanashi, et al. (1970a,b). Potato acid phosphatase treatment of sample homogenates in pH 4.0 acetate buffer provided quantitative hydrolysis. TCA was employed for subsequent protein precipitation. Recoveries of PL and PLP added to biological samples were 95-100% and 70-90%, respectively.

Recently, a biologically active β-glucoside and several other conjugates of pyridoxine have been found to occur naturally in certain cereals (Yasumoto, et al., 1976, 1977; Tsuji, et al., 1977). These derivatives, although found to be readily cleaved by autoclave treatment, were resistant to hydrolysis with takadiastase. Treatment of buffer extracts with a β-glucosidase preparation cleaved the linkages, rendering the pyridoxine available for microbiological or chromatographic analysis. In addition, these authors observed the presence of phosphorylated B_6 vitamers in the cereals examined. Therefore, determination of total vitamin B_6 in such a product would require treatment of aqueous extracts with phosphatase and β-glucosidase.

Microbiological Methods

Numerous methods for assaying vitamin B_6 have been developed employing microorganisms which require an exogenous source of the vitamin for growth. These methods are based on the stimulation of microbial growth by the vitamin B_6 in a sample extract added to a basal medium containing adequate levels of all other nutrients.

The procedure developed by Atkin, *et al*. (1943), employing the yeast *Saccharomyces uvarum*, has been the most widely applied of the microbiological methods. Numerous modifications of the method have been made to improve the response of the organism and increase the sensitivity (Hopkins and Pennington, 1947; Jones and Morris, 1950; Parrish, *et al*., 1955, 1956; Toepfer and Lehmann, 1961; Haskell and Snell, 1970).

The primary problem encountered with this procedure is the differential in growth response of *S. uvarum* to PM, PN, and PL. The response to PM has been found to be significantly lower than to PN or PL, with PM often exhibiting only 50-60% of the potency of the other vitamers (Rabinowitz and Snell, 1948; Parrish, *et al*., 1955; Gregory, 1959; Woodring and Storvick, 1960; Chin, 1975). The use of a vigorously growing yeast culture has been shown to minimize this difference in response (Sauberlich, 1970). Toepfer, *et al*. (1961, 1970), circumvented the problem by developing a method for *S. uvarum* assay of

the individual B_6 vitamers after separation by preparative ion exchange chromatography. This improved the accuracy of the determination of each vitamer by permitting the quantitation of each by comparison to its respective standard curve. Although the method is laborious and cumbersome, it alleviates the problem of erroneously low values for total vitamin B_6 in products containing high levels of pyridoxamine.

Another problem encountered in S. uvarum assays is the inhibition of yeast growth by high salt concentration in sample extracts (Rabinowitz and Snell, 1949). Presumably, this, too, would be alleviated by preparative ion exchange chromatography.

A method for the determination of total vitamin B_6 in foods using Neurospora sitophilia 299 was developed by Stokes, et al. (1943), and subsequently modified by Harris (1952) and Hodson (1956). In contrast to S. uvarum and most other assay organisms, N. sitophilia exhibits equal response on a molar basis to all forms of vitamin B_6, including PLP and PMP (Hodson, 1956). This assay method has not been widely used since it is complex and requires a 5-day growth period.

Recently, assay methods employing Kloeckera apiculata have shown considerable promise for the measurement of total vitamin B_6. Procedures using cup-plate (Barton-Wright, 1971) and turbidimetric (Daoud, 1973)

analysis have been developed. This organism shows equal response to PM, PL, and PN (Barton-Wright, 1971; Daoud, 1973), thus obviating the problems encountered with *S. uvarum*.

Methods for the assay of total vitamin B_6 using other organisms have been less successful. Baker and Sobotka (1962) reported a procedure based on stimulation of the growth of *Tetrahymena pyriformis*. While adequate for the assay of vitamin B_6 of tissues and physiological fluids containing mainly PM and PL, further application has been limited by the poor response to PN. A procedure using *Escherichia coli*, strain 154-59L, was developed by Diding (1955). Although quite simple, this method suffers from interference by certain amino acids and shows only a weak response to PM.

Differential methods for the determination of the individual vitamers have also been developed. These have been based on the specific requirements of certain microorganisms for the various forms of vitamin B_6. The procedure of Rabinowitz and Snell (1948) was based on the use of *S. uvarum* assays for total PN, PM, and PL, *Lactobacillus casei* assays for PL, and *Streptococcus faecalis* R for PL and PM. Because PN was determined by difference it was susceptible to high experimental uncertainty. Gregory (1959) modified this differential method by employing *Streptococcus faecium* Ø 51 for the determination

of PL and PM, since this organism was less sensitive to interference. As previously mentioned, however, D-alanine, L-alanine, and certain peptides were later shown to stimulate the growth of S. faecium Ø 51 (Haskell and Wallnofer, 1967; Raines and Haskell, 1968). This has made the differential method unsatisfactory for most analyses of vitamin B_6.

Preparative Chromatography

Numerous methods have been developed for the preparative chromatographic separation of the B_6 vitamers. These methods were developed to facilitate quantitative analysis of the individual B_6 compounds by microbiological, chemical, or radioisotopic counting methods. In addition, most of these methods served to remove potentially interfering compounds to some extent.

The ionogenic nature of the B_6 vitamers makes them well suited for separation by ion exchange chromatography. Peterson and Sober (1954) developed the first method for the separation of PN, PL, PM, and their respective phosphates. The separation was performed on a column using the weak cation exchange resin, Amberlite XE-64 (H^+). This procedure was never applied to extracts of foods or biological samples.

Fujita, et al. (1955a,b,c) developed a procedure for the preparative purification of the B_6 vitamers prior to fluorometric analysis. PL was determined following

elution from columns of Amberlite IRC-50 (H^+) or IR-112 (H^+). PN and PM, after deamination to PN, were determined after elution from Permutit columns. These procedures represented the first application of preparative chromatography to the quantitative analysis of vitamin B_6.

MacArthur and Lehmann (1959) successfully separated PN, PM, and PL on a single column containing Dowex AG 50W-X8 (K^+) by a stepwise elution system of boiling potassium acetate and citrate buffers. Because each of the vitamers eluted in a discrete fraction, the method was ideally suited as a preparative technique. Toepfer, et al. (1960), improved the chromatographic resolution of the free base vitamers by slightly modifying the elution buffers. Although this ion exchange procedure has been successfully applied to microbiological analysis of the B_6 vitamers (Toepfer and Lehmann, 1961; Toepfer and Polansky, 1970; Brin, 1970), the method is cumbersome because of the use of boiling buffers.

Because of the problems associated with the use of boiling buffers, attempts were made to develop a room temperature separation of PL, PN, and PM. Hedin (1963) proposed a method for the separation of the free base vitamers using an Amberlite IR-120 (Na^+) column, isocratically eluting with 0.1 M sodium phosphate, pH 6.5. The three phosphorylated B_6 compounds could be isocratically separated on columns containing the same resin by

elution with 0.1 M sodium acetate, pH 5.0. The stepwise elution methods of Toepfer, et al. (1960), had the advantage of eluting each vitamer as a discrete chromatographic fraction readily suited for analysis. The isocratic and continuous gradient methods were limited by the problem of collection and identification of the fractions containing the various vitamers. For this reason, neither the procedure of Hedin (1963) nor that of Storvick, et al. (1964), has received extensive application.

The separation of PN, PM, PL, and their phosphates was accomplished by Johansson, et al. (1968), using a single column of Dowex 50-X8 (NH_4^+) with an ammonium formate gradient. This procedure was modified slightly by Tiselius (1972) to provide better separation of PLP and PNP. Excellent resolution of all six vitamers by this method permitted the study of the metabolic distribution of vitamin B_6 in tissues.

In addition to the ion exchange chromatographic methods for vitamin B_6 separation, several thin layer techniques have been developed. Both thin layer electrophoretic (Ahrens and Korytnyk, 1970; Columbini and McCoy, 1970; Smith and Dietrich, 1971) and thin layer chromatographic (Smith and Dietrich, 1971) methods have been described for the preparative separative separation of all B_6 vitamers.

Bioassay Methods

Animal bioassay techniques for the determination of biologically available vitamin B_6 have the advantage that no extraction or hydrolysis of the sample is required. The animal bioassay is the ultimate method for comparison with microbiological and chemical assay procedures, serving as a check of their accuracy in the determination of the vitamin B_6 potency of foods. Limitations of the bioassay methods are: (1) a relatively large amount of the test substance is required, (2) the assays are time consuming and expensive, and (3) the precision of bioassay estimates is much poorer than that of the other analytical methods.

The predominant methods for bioassays with laboratory rats have been based on the procedures of Conger and Elvehjem (1941) and Clarke and Lechycka (1943). Sarma, et al. (1946), succeeded in developing a basal diet which permitted minimal growth in the absence of vitamin B_6, but allowed maximal growth with optimal levels of the vitamin. Using this basal diet, the bioassay was performed with a 2-week depletion period followed by a 4-week test period. During the test period, the rats were fed the basal diet fortified with graded levels of crystalline PN or the test substance. Rat growth over the 4-week period was used as an indicator of the dietary B_6 concentration. Numerous modifications

of the diet composition have been made, largely depending on the material to be assayed (Linkswiler, et al., 1951; Tomarelli, et al., 1955; Lushbough, et al., 1959; Davies, et al., 1959; Richardson, et al., 1961).

The chick has also been used in vitamin B_6 bioassays because of its high growth rate and sensitive nutrient requirements. The procedures of Ott (1946) and Coates, et al. (1950), with the modification of Davies, et al. (1959), provided sensitive assays for biologically available vitamin B_6. The recent method of Yen, et al. (1976), utilizing a chemically defined basal diet, permits bioassays in less than 2 weeks, thus markedly shortening the analysis time.

Problems encountered in the interpretation of the results of rat or chick bioassays have been mainly due to the role of the intestinal microflora in vitamin B_6 utilization. The use of dextrin or starch as the source of dietary carbohydrate has been shown to stimulate the intestinal synthesis of vitamin B_6, inducing greater growth on the nonfortified basal diet. Such growth markedly reduces the sensitivity of the bioassay. The presence of soluble sugars, such as sucrose, lactose, or glucose, in the basal diet was shown to provide much lower growth rates in the absence of dietary vitamin B_6 (Sarma, et al., 1946; Waibel, et al., 1952). The bioassay results of materials containing high levels of

complex carbohydrates would therefore be inaccurate if dietary replacement levels were sufficiently high to increase intestinal synthesis of vitamin B_6.

The relative activities of PN, PM, and PL have been shown to be approximately equal when administered orally by medicine dropper or intraperitoneally (Sarma, et al., 1946). In contrast, the activity of PN was found to be consistently greater than that of PM or PL when mixed in rat or chick diets (Sarma, et al., 1946; Waibel, et al., 1952; Davies, et al., 1959). Compared to PN, the apparent activities of PL and PM were found to be approximately 60-70% in rats (Sarma, et al., 1946) and 70-80% in chicks (Davies, et al., 1959). Equal activity of the three vitamers was observed when the animals were treated orally with aureomycin (Linkswiler, et al., 1952). These results suggested that under normal conditions the intestinal microbes preferentially utilize the PL and PM contained in the diet.

Evaluation of Vitamin B_6 Bioassays

Most bioassay methods have used animal growth as an indicator of the content of vitamin B_6 in diets containing suboptimal levels of the vitamin. Several functional indices of vitamin B_6 status have been discovered which would increase the specificity of bioassays if these were used in addition to growth data.

Numerous researchers have observed the correlation between aminotransferase activity of various tissues and blood fractions and the vitamin B_6 nutriture of the animal (Raica and Sauberlich, 1964; Beaton and Cheney, 1965; Brin and Thiele, 1967; Chen and Marlatt, 1975; Kirksey, et al., 1975; Lee, et al., 1976; Yen, et al., 1976). Aspartate aminotransferase (AspAT), also known as glutamate oxaloacetate transaminase, has been shown in all tissues to possess much higher activity than alanine aminotransferase (Beaton and Cheney, 1965; Brin and Thiele, 1967). Therefore, AspAT activity is generally considered to be the more sensitive indicator of vitamin B_6 status.

Raica and Sauberlich (1964) examined the AspAT activity of blood plasma, leukocytes, and erythrocytes of human subjects, and concluded that the enzyme activity was not sufficiently sensitive to be used as an indicator of vitamin B_6 nutriture in diverse human populations. However, in laboratory animals under a controlled dietary regimen, erythrocyte AspAT activity has been found to be a sensitive measure of vitamin B_6 status (Beaton and Cheney, 1965; Brin and Thiele, 1967; Kirksey, et al., 1975). Plasma AspAT activity was found to be more sensitive to changes in vitamin B_6 status than serum AspAT activity in chicks; however, growth was even more sensitive than either enzyme activity (Yen, et al., 1976).

The relationship between the stimulation of the aminotransferase enzymes by in vitro addition of the cofactor PLP and vitamin B_6 deficiency state was first noted by Raica and Sauberlich (1964). These authors observed that the extent of stimulation of erythrocyte AspAT by added PLP was a sensitive indicator of human vitamin B_6 nutriture. Recent detailed studies have confirmed the sensitivity and specificity of the in vitro stimulation method for the estimation of human vitamin B_6 status (Bayoumi and Rosalki, 1976; Kishi and Folkers, 1976). The PLP stimulation of the erythrocyte aminotransferases has also been used to monitor B_6 status in rat feeding studies (Chen and Marlatt, 1975; Kirksey, *et al.*, 1975).

Other indicators of vitamin B_6 nutriture, such as 4-pyridoxic acid excretion, tryptophan load tests, and the vitamin B_6 concentration of tissues, blood, and urine could potentially be used in animal bioassays. However, the laborious analytical methods tend to preclude the use of these indices because of the large numbers of animals generally employed.

Chemical Methods for Determination of Total Vitamin B_6

Spectroscopic Methods.--Several spectrophotometric methods have been developed for the determination of the various vitamin B_6 compounds (Wada and Snell,

1961; Soda, et al., 1969). These methods were of limited use in food analyses because of their lack of specificity and sensitivity.

The methods of Fujita, et al. (1955a,b,c), were the first application of fluorometry to the determination of vitamin B_6 in foods. These methods were based on the formation of the fluorophore, 4-pyridoxic acid lactone, by a complex series of reactions for each vitamer. Hennessy, et al. (1960), modified this method for the determination of PN in bread.

Basic studies by Bonavita (1960) demonstrated the feasibility of fluorometric analysis of PL by quantitation of the product of the reaction of potassium cyanide with PL. Detailed studies by Ohishi and Fukui (1968) confirmed that the reaction product of PL and cyanide was 4-pyridoxic acid lactone. The assay of PN and PM could be performed by their conversion to PL with manganese dioxide and glyoxylic acid, respectively, prior to treatment with potassium cyanide (Toepfer, et al., 1961; Polansky, et al., 1964). These reactions provided a much simpler method for the formation of the fluorophore than those employed by Fujita, et al. (1955a,b,c).

Numerous modifications of the 4-pyridoxic acid lactone method have been made for the analysis of foods, biological fluids, and tissues (Contractor and Shane, 1968; Loo and Badger, 1969; Takanashi, et al., 1970;

Takanashi and Tamura, 1970; Masukawa, et al., 1971; Fiedlerova and Davidek, 1974; Chin, 1975). Most of these methods involved preparative ion exchange column chromatography prior to fluorophore formation. Contractor and Shane (1968) observed that their fluorometric results correlated well with published microbiological data for total vitamin B_6 in blood, but were lower than the values obtained by Fujita, et al. (1955b). They suggested that the method of Fujita, et al., may be subject to interference from nonspecific fluorophores. Studies by Chin (1975) demonstrated fluorometric values significantly greater than microbiological results for most foods analyzed. It was suggested, based on these studies, that a possible source of interference was the extensive browning routinely observed following the autoclave extraction step (Chin, 1975). Notably, the procedure of Fujita, et al. (1955b), was based on an autoclave extraction, while that of Contractor and Shane (1968) used only TCA precipitation. Kraut and Imhoff (1967) developed a fluorometric method based on entirely different methods of 4-pyridoxic acid lactone formation. Here, the vitamers were isolated by paper chromatography, followed by combined chemical and enzymatic treatments. The determination of vitamin B_6 in foods by this flurometric also yielded results which were much greater than published microbiological data. No studies, to date,

have systematically and conclusively established the validity of the fluorometric assay procedures with respect to interfering compounds.

An innovative procedure for the fluorometric determination of PL and PLP in blood and tissues has been reported by Srivastava and Beutler (1973). Sample homogenates were treated with semicarbazide, forming the respective semicarbazones of PL and PLP. Each semicarbazone was identified and quantitated according to its characteristic fluorescence spectra. The advantage of this technique was the ability of the semicarbazide to react with and release protein bound PL and PLP, thereby permitting direct fluorometric quantitation after protein precipitation. This procedure has not been applied to the assay of the other B_6 vitamers and has not been used with food systems.

Analytical Chromatographic Methods.--Several gas liquid chromatographic methods have been developed for the quantitative analysis of vitamin B_6 compounds in relatively pure mixtures, such as pharmaceutical preparations (Sheppard and Prosser, 1970; Korytnyk, 1970; Williams, 1974). The method of Williams is the most sensitive, employing electron capture detection of the heptafluorobutyryl derivatives. No successful applications of these methods have been made to foods or biological samples.

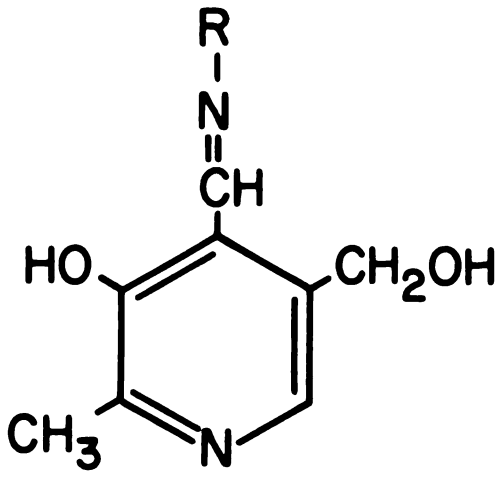
Williams and Cole (1975) proposed a high performance liquid chromatographic (HPLC) method for the separation of PN, PM, and PL. The vitamers were separated by gradient elution from a column of Aminex A-5, requiring 80 minutes per separation. The low specificity of the ultraviolet absorption detector employed prevented the application of this method to food systems. HPLC was applied to the determination of vitamin B_6 in foods by Yasumoto, et al. (1975). The specificity of the detection was heightened by continuously treating the effluent of the Aminex A-5 column with the diazide of 5-dichloroanaline 2,4-disulfonyl chloride, a chromogenic reagent for aromatic compounds. Although the separation requires 90 minutes and the instrumentation is complex, this method is the first successful chromatographic procedure for analysis of vitamin B_6 in foods.

Vitamin B_6 Chemistry

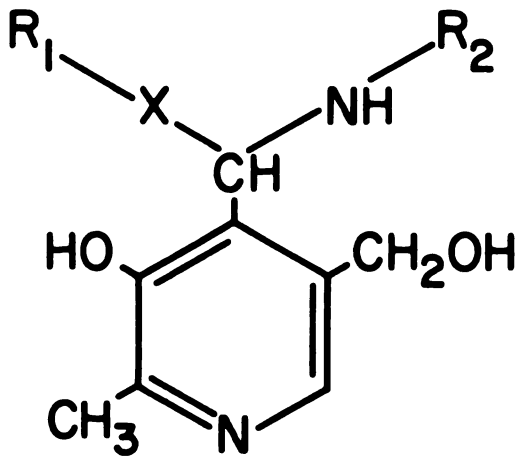
Formation of Complexes

The ability of PL and PLP to form several types of complexes with amino acids, amines, peptides, and proteins (Figure 2) has been widely studied. The most widely studied of these reactions has been the formation and behavior of the Schiff bases. Metzler (1957) and Lucas, et al. (1962), observed that Schiff base formation occurs most readily in neutral or alkaline media. Matsuo (1957a) observed that PL was much less reactive than PLP, by

1. Schiff base



2. Substituted aldamine (X=amino, sulfhydryl, or imidazole)



3. Pyridoxylamino compound

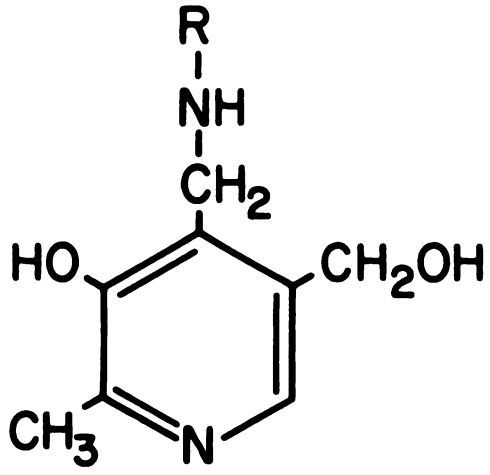


Figure 2. Complexed forms of pyridoxal. Pyridoxal phosphate forms analogous complexes.

virtue of the existence of PL predominantly in the form of an internal hemiacetal (Heyl, et al., 1951a). Schiff bases of PL and amino acids were shown to readily chelate a number of polyvalent metal ions (Matsuo, 1957a). In addition to the pH-dependence of the rate of formation and stability of the Schiff bases, several tautomeric forms have been found to exist in a pH-dependent equilibrium (Christensen, 1958; Arrio-Dupont, 1970).

When PLP or PL forms a Schiff base with a polyfunctional amine, amino acid, peptide, or protein, nucleophilic attack at the azomethine linkage can lead to the formation of a cyclic derivative, the substituted aldamine. This reaction has been extensively studied with amino acids and amines (Heyl, et al., 1948a; Buell and Hansen, 1960; Bergel and Harrap, 1961; Mackay and Shepherd, 1962; Mackay, 1963; Abbott and Martell, 1970; O'Leary, 1971; Kierska and Maslinski, 1971; Schonbeck, et al., 1975; Der Garabedian and Der Garabedian, 1976). Amino, sulfhydryl, and imidazole groups have been shown capable of reaction with a Schiff base to form the substituted aldamine. In acid media, these complexes are highly unstable and readily dissociate via the Schiff base intermediate (O'Leary, 1971; Kierska and Maslinski, 1971; Buell and Hansen, 1960; Bergel and Harrap, 1961).

Chemical reduction of the double bond of the azomethine linkage of the Schiff base by a sufficiently

strong reducing agent forms a pyridoxylamino compound. Methods for catalytic hydrogenation (Hey, et al., 1948a, 1952) and sodium borohydride reduction (Fischer, et al., 1958; Severin, et al., 1969) have been widely used in the synthesis of pyridoxylamino compounds. These complexes are highly stable, showing only slight degradation under hydrolytic conditions including incubation for 24 hours at 100°C in 6 N HCl (Dempsey and Snell, 1963; Ronchi, et al., 1969; Anderson, et al., 1971).

The reaction of PL and PLP with proteins occurs in a manner identical to the reactions with small molecules such as amino acids, amines, and peptides. The interaction of these vitamers with protein amino groups has been recently reviewed by Feeney, et al. (1975). Numerous enzymes have been shown to be inhibited in vitro by Schiff base formation between PLP and an active site amino group. Among them are: glutamate dehydrogenase (Anderson, et al., 1966), 6-phosphogluconate dehydrogenase (Rippa, et al., 1967), hexokinase (Grillo, 1968), fructose 1,6 diphosphate aldolase (Shapiro, et al., 1968), glyceraldehyde 3-phosphate dehydrogenase (Ronchi, et al., 1969), pyruvate kinase (Johnson and Deal, 1970), and ribonuclease A (Riquelme, et al., 1970).

The binding of PLP to serum albumin and glycogen phosphorylase probably represent the most thoroughly examined vitamin B_6-protein interactions. In the case

of serum albumin, PLP has been found to bind in strong preference to PL, with no binding of PN to the protein (Anderson, et al., 1974). Spectrophotometric evidence and resistance to sodium borohydride reduction suggested that PLP was bound as a substituted aldamine at the primary binding site at neutral pH (Dempsey and Christensen, 1962; Anderson, et al., 1971). However, Hilak, et al. (1975), demonstrated that the primary binding of PLP to serum albumin occurs by Schiff base formation at a specific apolar binding site. Similarly, initial studies with glycogen phosphorylase suggested that PLP was bound as a substituted aldamine complex at neutral pH (Kent, et al., 1958; Scheller and Will, 1973). The existence of a Schiff base at an apolar binding site was also demonstrated for glycogen phosphorylase (Shalteil and Cortijo, 1970; Cortijo, et al., 1976).

In addition to its binding as a cofactor in many enzymatic systems, PLP readily forms a Schiff base with the amino groups of many other proteins. Typical examples are studies in which PLP was used as a reagent for modifying collagen (Page, et al., 1968) and erythrocyte membrane proteins (Cabantchick, et al., 1975).

The bioavailability of the various complexed forms of vitamin B_6 has not been thoroughly studied. Presumably, the Schiff base and substituted aldamine forms would be totally available because of their

instability in acid environments, which would be encountered in the stomach. Pyridoxylamino acids have been found to possess low, but measurable, vitamin B_6 biological activity. Studies using the microbiological assay organisms, S. uvarum, N. sitophilia, S. faecalis, and L. casei demonstrated that pyridoxylamino acids possessed no more than 0.5% of the molar activity exhibited by PL; however, no significant antivitamin activity was observed (Snell and Rabinowitz, 1948). In contrast, the vitamin B_6 activity of numerous pyridoxylamines was found to range from 50 to 100% of that of PN for most of the compounds tested using rat bioassays (Heyl, et al., 1952). Contrary to the observed vitamin B_6 activity of the pyridoxylamines, several pyridoxyl-pressor amines were found to possess very low pressor activity in rats (Heyl, et al., 1952).

The antivitamin B_6 activity of numerous analogues and complexes has been widely studied and reviewed by Sauberlich (1968) and Klosterman (1974). Through the formation of a stable complex with PL or PLP, carbonyl reagents, such as semicarbazides and hydrazines, may effectively reduce the in vivo concentration of metabolically available vitamin B_6. McCormick and Snell (1961) have demonstrated that many of the stable complexes strongly inhibit pyridoxal phosphokinase, thereby inhibiting the in vivo formation of PLP. These complexes, whether formed in vivo or present in the diet,

would interfere with the utilization of dietary vitamin B_6. McCormick and Snell (1961) observed that many of the pyridoxylamino acids did not possess inhibitory activity, although the reduced complex, N, N'-bis(pyridoxyl)hydrazone, was itself a potent inhibitor.

Nonenzymatic Interconversions and Catalysis by Vitamin B_6

Transamination reactions between PL or PLP and amino acids were first shown to occur nonenzymatically by Metzler and Snell (1952), producing the respective α-ketoacid and PM or PMP. Matsuo (1957) reported that these reactions occurred via a Schiff base intermediate. In aqueous media, the presence of metal salts was required (Metzler and Snell, 1952), although the reaction proceded spontaneously with no metal catalyst in ethanol solution (Matsuo, 1957). PL and α-amino acid esters readily reacted in aqueous media in the absence of metal ions (Cennamo, 1964). These results suggested that the role of the metal ion was merely to mask the charged α-carboxyl group of the amino acid. Dixon (1966) reported that transamination reactions could occur between PL and the free amino groups of proteins in the presence of metal ions.

The occurrence of transamination reactions during the thermal processing of foods has been demonstrated. The formation of PM and PMP from PL and PLP have been

shown to occur during milk processing (Hodson, 1956; Gregory, 1959; Gregory and Mabbitt, 1961; Polansky and Toepfer, 1969). Transamination during the cooking of pork was suggested by the data of Polansky and Toepfer (1969), which indicated that fully cooked ham contained predominantly PM, while raw pork contained mainly PL.

PL has been shown to catalyze the oxidative deamination of PM and amino acids in the presence of metal ions (Ikawa and Snell, 1954; Hamilton and Revesv, 1966; Rotilio, et al., 1970; Hill, 1972). These oxygen-dependent reactions resulted in the variable oxidation of PL to 4-pyridoxic acid (Ikawa and Snell, 1954; Hamilton and Revesv, 1966; Rotilio, et al., 1970), and, therefore, represent a potential mechanism for the loss of vitamin B_6 activity in foods.

The catalytic cleavage of hydroxyamino acids (Metzler, et al., 1954b) and the β-elimination of serine and O-phosphoserine (Reiber, 1976) by PL and PLP have been described. The analogous catalysis of the non-enzymatic release of hydrogen sulfide and methyl mercaptan from cysteine and methionine, respectively, has also been observed (Metzler, et al., 1954a; Gruenwedel and Patnaik, 1971). These reactions have been found to require the presence of metal ions. Metzler, et al. (1954b), demonstrated that a side reaction involving glycine, formed from threonine cleavage, and PL formed

β-pyridoxylserine, a compound with no vitamin B_6 activity for S. uvarum. β-pyridoxylserine formation was observed to be responsible for a loss of up to 8% of the total PL in an equimolar mixture of PL and threonine at 100°C in the presence of metal ions. This compound was also found as a secondary product of the room temperature β-elimination reactions involving PLP (Reiber, 1976).

A mechanism for the nonenzymatic oxidation of PN to PL has been identified by Shane and Snell (1972). In the presence of ferrous ions and low concentrations of hydrogen peroxide, PN was found to be readily oxidized to PL at room temperature.

Vitamin B_6 Stability and Bioavailability

Vitamin B_6 in pure solution has been shown to retain its activity in the presence of heat, acid, and alkali, although all of the vitamers are readily destroyed by strong oxidizing agents (Keresztesy and Stevens, 1938; Cunningham and Snell, 1945). The irreversible degradation of vitamin B_6 by light, particularly ultraviolet wavelengths, has long been recognized (Hochelberg, et al., 1943; Cunningham and Snell, 1945). The oxygen-dependent, light catalyzed, oxidation of PLP to 4-pyridoxic acid phosphate was reported by Morrison and Long (1958) and Reiber (1972). Under the same conditions, PL was converted to 4-pyridoxic acid, while PMP was converted to an unidentified product (Reiber, 1972). The sensitivity

of vitamin B_6 in foods was demonstrated by Hellstrom (1960) by exposure of milk to sunlight for a 2-hour period, inducing the loss of 50% of the vitamin.

Although pure solutions of vitamin B_6 are resistant to thermal degradation, food processing has been shown to induce significant losses of biologically available B_6 in certain products. In most cases, the mechanism of loss has not been identified. The relative stability and behavior of the individual vitamers have not been well elucidated.

The roasting of many types of meat has been found to induce a loss of approximately 50% of the total vitamin B_6, as measured by S. uvarum or rat bioassay (Lushbough, et al., 1959). The rat bioassay data were observed to be approximately twice the microbiological values for all samples, raw and roasted. This discrepancy could not be satisfactorily explained.

Several studies concerning the effects of thermal processing during canning have indicated that losses of low levels of vitamin B_6 occur. Losses of no greater than 20% of the total vitamin B_6 have been observed during the retorting of several types of beans at 120°C for 30 to 45 minutes (Everson, et al., 1964; Raab, et al., 1973; Miller, et al., 1973; Daoud, et al., 1977). Similar losses were observed in the processing of canned beef, while there was no degradation of the vitamin B_6 in

canned tomato juice concentrate during retort processing (Everson, et al., 1964). No differences were observed between conventional and aseptic canning methods on the retention of vitamin B_6 (Everson, et al., 1964).

The effects of processing corn and soybean meal on the content of biologically available vitamin B_6 were studied by Yen, et al. (1976). Mild roasting treatments slightly increased the content of available B_6 in corn, while significant losses were induced by a more severe roasting treatment. The processing of soybean meal by autoclaving decreased the content of available vitamin B_6 by approximately 30%.

Although no systematic studies have been carried out on the storage stability of vitamin B_6 in foods, a number of experimental findings have been reported. Harding, et al. (1959), observed a 43% lower content of total vitamin B_6 in canned combat rations stored for 20 months at 38°C compared to those stored at 1°C. During the storage of canned beef and bean products for 270 days at room temperature, approximately 25% of the vitamin B_6 was degraded in each product, although the initial rate of loss was greater in the beef (Everson, et al., 1964). No loss was observed during the storage of tomato juice concentrate under the same conditions. These results suggested a pH effect on the rate of degradation of the vitamin. Analyzing by rat bioassay,

Richardson, et al. (1961), found little difference in the rate of loss of vitamin B_6 at room temperature in stored products processed by either retorting or gamma irradiation, while frozen storage generally retarded the degradation. The net retention of vitamin B_6 in products stored 20 months at room temperature after retort processing or irradiation was found to be only 40 to 60% of that of comparable products stored frozen.

Several researchers have examined the stability of PN used in the fortification of various cereal products. Bunting (1965) reported the retention of 90 to 100% of the PN added to corn meal and macaroni following storage for 1 year at 38°C and 50% relative humidity. Cort, et al. (1976), demonstrated no loss of PN in corn meal which had been fortified with salts of iron, zinc, magnesium, and calcium, when stored for 6 months at room temperature. Storage at 45°C for 12 weeks resulted in a loss of only 4% of the PN. Anderson, et al. (1976), observed no loss of PN in iron fortified breakfast cereals during storage for 3 months at 40°C or 6 months at 22°C.

The stability and bioavailability of vitamin B_6 has been studied more extensively in dairy products than in other foods. Most of this research was prompted by the occurrence of severe vitamin B_6 deficiency in a group of infants which were fed diets composed largely of

commercially sterilized infant formulas with no B_6 fortification (Coursin, 1954).

Pasteurization and cold storage of milk have been shown to have little effect on the content of total vitamin B_6 in milk, as measured by S. uvarum (Hassinen, et al., 1954). The manufacture of sweetened condensed milk and the spray drying of milk or infant formula have been shown to induce a loss of approximately 20% of the total vitamin B_6 (Hassinen, et al., 1954). Commercial sterilization of evaporated milk or infant formula was observed to be much more detrimental, resulting in losses of 40 to 60%, as measured by S. uvarum. However, using N. sitophilia as the assay organism, only a small loss of biologically active B_6 was observed (Hassinen, et al., 1954; Gregory, 1959). Hassinen, et al. (1954), demonstrated that after the sterilization of evaporated milk, a rapid loss of apparent vitamin B_6 (S. uvarum assay) proceeds for approximately 1 week, followed by a much slower rate of loss during subsequent storage. Hodson (1956) also observed this phenomenon, but found a much slower rate of apparent degradation when using the N. sitophilia assay. Storage of evaporated milk at room temperature for 6 months induced the loss of approximately 20% and 40-50% when assayed by N. sitophilia and S. uvarum, respectively (Hodson, 1956).

These unexpected results may be explained by the distribution of total vitamin B_6 among the various

vitamers in the milks. While over 80% of the vitamin B_6 in raw milk exists as PL and PLP, at least 60% of the total B_6 is in the form of PM and PMP in sterilized evaporated milk (Gregory, 1959; Polansky and Toepfer, 1969). Because S. uvarum exhibits somewhat limited response to PM, while N. sitophilia shows no such discrepancy, the difference was probably one of assay specificity.

Hassinen, et al. (1954), demonstrated that milk fortified with PN exhibited little loss of the added vitamin during sterilization and storage. PL and PM added in fortification were found to be degraded at the same rate as the B_6 occurring naturally in milk.

The biological availability of vitamin B_6 in sterilized evaporated milk and infant formula remains unclear after considerable investigation. Tomarelli, et al. (1955), determined the content of available B_6 in milk products by rat bioassay. They found excellent agreement between bioassay and S. uvarum microbiological data for raw milk and sterilized infant formula which had been fortified with PN. However, in nonfortified sterilized infant formula and sterilized milk, rat bioassay data indicated that only 50% of the total vitamin B_6 as measured microbiologically was available to the rats. In direct opposition is the data of Davies, et al. (1959). These authors observed that rat and chick

bioassays of evaporated milk, even after a 6-month storage period, gave apparent vitamin B_6 values which were significantly greater than S. uvarum data, although the three assay methods gave comparable results in the assay of raw milk. Therefore, Davies, et al. (1959), concluded that S. uvarum assays may serve effectively as an indicator of the maximum levels of degradation of vitamin B_6 in milk products.

In an attempt to explain the losses of available vitamin B_6 during the sterilization of milk, Bernhart, et al. (1960), postulated a reaction between the sulfhydryl groups of milk proteins and PL, forming bis-4-pyridoxyl disulfide (Wendt and Bernhart, 1960). This compound was synthesized by heating a concentrated mixture of cysteine and PL at neutral pH, and was identified in milk which had been fortified with high levels of PL prior to sterilization. Bernhart, et al. (1960), demonstrated that this compound possesses vitamin B_6 activity of 20% for S. uvarum, 20% for rats, and 65% for N. sitophilia, relative to the activity of PN. The formation of bis-4-pyridoxyl disulfide in thermally sterilized nonfortified milk or infant formula was never reported. Srncova and Davidek (1972) obtained evidence of the formation of a complex involving PL and milk protein sulfhydryl groups. However, calculations based on their data indicate the involvement of only 2 to 3% of the PL in their highly

fortified reaction mixtures. Furthermore, the chemical nature of these complexes was never identified. The possibility of substituted aldamine formation involving the sulfhydryl groups was not ruled out.

Few studies of vitamin B_6 biological availability have been carried out on nondairy food products. Sarma, et al. (1947), compared rat bioassay and S. uvarum microbiological assay data for a variety of natural materials. The rat bioassay values were lower than S. uvarum data in most cases. Most of the products showed a difference of 10 to 30% between the two assays, with a maximum difference of 34% for whole wheat. The authors suggested that the observed differences were due to the slightly lower activity of PL and PM when mixed in the diet of the rat.

In studies of the vitamin B_6 content of canned combat rations, the response of rats fed only the rations suggested that vitamin B_6 was limiting, although microbiological assays for total vitamin B_6 indicated the presence of sufficient levels of the vitamin (Register, et al., 1950; Tappan, et al., 1953). The addition of PN to the rations stimulated rat growth in these studies. As a possible explanation, Sauberlich (1961) observed that the addition of PN to a diet adequate in B_6, but limiting in an essential amino acid, would induce a growth stimulation. Therefore, the low protein quality

in the processed rations may have been responsible for the apparently low bioavailability of the vitamin.

The chick bioassay results of Yen, et al. (1976), suggested that the content of available B_6 in corn and soybean meal, either raw or thermally processed, is considerably less than predicted by published microbiological data. Similarly, Leklem (1977) demonstrated in a human study that the bioavailability of vitamin B_6 in whole wheat bread was significantly less than that of white bread. Toepfer, et al. (1963), observed a slight, but statistically significant, difference between rat and microbiological assays for whole wheat flour and nonfat dry milk, suggesting that the vitamin B_6 in these products may not be entirely available biologically. No differences were observed between rat and microbiological assays for dried beef and Lima beans.

Further evidence that naturally occurring vitamin B_6 may not be completely utilized biologically was found by Nelson, et al. (1976). The intestinal absorption of the vitamin B_6 in orange juice by humans was significantly less than that of the pure B_6 vitamers in saline solution. Under the experimental conditions, a 30 cm jejunal segment absorbed 65% of the vitamin B_6 in a saline solution, with a slightly enhanced uptake induced by the addition of glucose. In contrast, only 30% of the vitamin B_6 in diluted orange juice was absorbed. Whether these data

represented differences in rate of absorption or indicated true differences in absolute bioavailability could not be determined.

EXPERIMENTAL METHODS

Preparative Methods

Heat Stable Peptide Mixture

The isolation of heat stable peptides from a peptic hydrolysate of bovine β-lactoglobulin was performed as described in the Appendix.

ε-Pyridoxyl-L-lysine Synthesis

The synthesis of α-acetyl-ε-pyridoxyl-L-lysine was performed according to the method of Dempsey and Christensen (1962). ε-Pyridoxyllysine was formed by a modification of the procedure of Dempsey and Snell (1963). Details are presented in the Appendix.

Phosphopyridoxyl-Bovine Serum Albumin

The reductive linkage of pyridoxal phosphate to bovine serum albumin was performed according to the method of Dempsey and Christensen, procedure 2 (1962). Details are presented in the Appendix.

Chemical Methods

Protein Determination

The concentration of proteins and peptides in solution was determined by the method of Lowry, *et al*.

(1951). Crystalline β-lactoglobulin, 3X (United States Biochemical Corp.) was employed as the standard.

Amino Groups

Protein and peptide amino groups were determined by the method of Fields (1972) using 2, 4, 6-trinitrobenzenesulfonic acid dihydrate from Pierce Chemical Co. Molar absorptivity coefficients at 420 nm for the trinitrophenyl derivatives of heat stable peptides, β-lactoglobulin, and serum albumin were calculated to be 19,650, 18,700, and 18,980 $M^{-1}cm^{-1}$, respectively. These calculations were based on the published amino acid composition of β-lactoglobulin and bovine serum albumin and the reported molar absorptivity coefficients of TNP-α-amino, TNP-ε-amino, and TNP-thiol groups (Fields, 1972).

Sulfhydryl Groups

Peptide sulfhydryl groups were determined by the procedure of Ellman (1959) using the reported molar absorptivity coefficient of 13,600 $M^{-1}cm^{-1}$ at 412 nm for the 3-carboxy-4-nitrothiophenol anion. The 3, 3'-dithiobis(6-nitrobenzoic acid) was obtained from Eastman Organic Chemicals.

Fluorometric Determination of Vitamin B_6 Compounds

The fluorometric determination of PN, PM, and PL was performed by the semiautomated procedure of Gregory

and Kirk (1977a). All phosphorylated forms were determined as their free base. The fluorometric procedure is based on the conversion of each vitamer to the fluorophore, 4-pyridoxic acid lactone. Details are presented in the Appendix.

Erythrocyte Aspartate Aminotransferase Assay

The activity of erythrocyte AspAT was assayed, in the presence and absence of PLP added in vitro, by a slight modification of the automated serum glutamic-oxaloacetic transaminase method of Levine and Hill (1966). This procedure is based upon the coupling of malate dehydrogenase to the AspAT reaction, with fluorometric quantitation of the oxidation of reduced nicotinamide adenine dinucleotide. A standard curve for enzyme activity was determined by running serial dilutions of a hemolysate of high activity. The enzyme activity of the standard hemolysate was determined spectrophotometrically by the Calbiochem (1974) procedure. After calibration of the AutoAnalyzer, all unknown hemolysates were assayed by the modified automated procedure. PLP stimulation of AspAT activity was determined by adjusting 0.9 ml of each hemolysate to 0.820 mM with respect to added PLP by the addition of 0.1 ml of an 8.20 mM aqueous solution of PLP (Bayoumi and Rosalki, 1976). Following incubation for 30 minutes at room temperature, the hemolysates were

reassayed. The percent stimulation induced by the in vitro addition of PLP was calculated from the observed enzyme activity in the presence and absence of PLP.

Hemoglobin Determination

Determination of hemoglobin in the hemolysates was performed according to the automated colorimetric method described by Technicon (1969). Working standards were prepared by dissolving bovine hemoglobin (2X crystallized, Type I; Sigma Chemical Co.) in distilled water and diluting over a range of 4 to 20 mg/ml.

Physical Methods

Spectrophotometry

Ultraviolet and visible absorption spectra were determined using a Beckman DK-2A spectrophotometer. Single wavelength measurements were made using a Gilford Model 240 spectrophotometer. Fluorescence spectra were determined using an Aminco Bowman spectrophotofluorometer, equipped with a xenon lamp.

High Performance Liquid Chromatographic Assay of Vitamin B_6

Analyses were performed using an ALC/GPC-202 liquid chromatograph, equipped with a Model M-6000 pump, Model U6K septumless injector, and a Model 440 absorbance detector, which were manufactured by Waters Associates. Chromatographic separations were performed on a μBondapak

C_{18} column (4mm ID x 30 cm, Waters Associates). Fluorescence detection was accomplished using an Aminco Fluoro-Monitor equipped with an 18 μl flow cell. All B_6 vitamers were determined as their free base. The details of sample extraction, chromatography, and detection are presented in the Appendix.

High Performance Liquid Chromatographic Assay of Pyridoxyllysine

ε-Pyridoxyllysine in protein acid hydrolysates was determined by a procedure similar to the HPLC assay of vitamin B_6. Details are presented in the Appendix.

High Performance Liquid Chromatographic Assay of 4-Pyridoxic Acid

The determination of 4-pyridoxic acid in dehydrated model system extracts was performed by a modification of the HPLC method of Gregory and Kirk (1977b) for the determination of 4-pyridoxic acid in urine. The modifications and details of the procedure are presented in the Appendix.

Discontinuous Polyacrylamide Gel Electrophoresis

Gel electrophoresis of β-lactoglobulin was performed by a modification of the discontinuous method of Melachouris (1969). Details are presented in the Appendix.

Thin Layer Peptide Mapping

Peptide mapping was performed on cellulose MN-300 (Sigma Chemical Co.) plates by the procedure of McDonald, *et al*. (1976). Peptides (230 μg) were applied to the 20 x 20 cm plate with a cellulose support thickness of 0.50 mm. Electrophoresis was carried out for 2 hours in a Desaga Heidelberg thin layer electrophoresis apparatus at a field strength of 20 V/cm, 13 mA, in a pH 4.5 buffer (pyridine: acetic acid: water; 5:9:986), with running tap water as the coolant. The peptides were visualized with ninhydrin spray (von Arx and Neher, 1963).

Gel Filtration Chromatography

Gel filtration chromatography was used both in preparative and analytical applications. Columns of various dimensions were prepared with Sephadex G-10, G-25, and G-50 packings as described by the manufacturer (Pharmacia Fine Chemicals, 1974). Column effluents were continuously monitored at 254 or 280 nm with an ultraviolet absorbance detector (Instrumentation Specialties Co.). Columns were preserved during storage by equilibration in 0.1 M ammonium acetate, pH 7.0, containing 0.1% sodium azide, or 0.05 M KCl-HCl, pH 2.5.

Biological Methods

Microbiological Determination of Total Vitamin B_6

The method of Haskell and Snell (1970) was employed for the assay of total vitamin B_6 using *Saccharomyces uvarum* 4228 (ATCC 9080). Stock cultures were maintained on malt agar slants at 2°C. Sample extracts were added to the assay tubes in 0.05, 0.10, 0.15, and 0.20 ml aliquots and were assayed in triplicate. A dose-response curve using PN as the standard was prepared for each assay, covering the range of 0 to 10 ng/tube. The vitamin B_6 content of each sample was calculated from the mean value of ng total B_6/ml extract, as determined from the dose-response curve.

Rat Bioassay of Biologically Available Vitamin B_6

The concentration of biologically available vitamin B_6 in dehydrated model systems and phosphopyridoxyl-bovine serum albumin (PP-BSA) was determined by a modification of the methods of Sarma, *et al*. (1946) and Linkswiler, *et al*. (1951).

All diets were of the composition shown in Table 1 and were prepared by thoroughly mixing the diet ingredients in a Hobart mixer. The composition of the standard diets was identical to that of the basal, with the exception of the addition of a dry pyridoxine

Table 1. Composition of diets for rat bioassays.[a]

Ingredient	Basal, Standard, or PP-BSA Diets	Test Diets (model systems)	
		Stored	Roasted
Casein (vitamin free, test)[b]	19.80	18.86	18.85
DL-Methionine, N.R.C.[c]	0.20	0.20	0.20
Sucrose	60.50	57.44	57.45
Cellulose (Alphacel)	9.40	9.40	9.40
Salt mix[d]	4.00	4.00	4.00
Vitamin mix[e]	1.10	1.10	1.10
Corn oil	5.00	5.00	5.00
Model system	--	4.00	5.00

[a]Water added to all diets at a level of 52.1 ml per 3000 g to all diets in Bioassay 2. A 5 mg/ml solution of PP-BSA replaced the water in diets designated for PP-BSA additions.

[b]ICN Pharmaceuticals, Inc. (Bioassay 1) and Teklad Test Diets (Bioassay 2).

[c]Grand Island Biological Co.

[d]Teklad Test Diets. Wesson modified Osborne-Mendel. $ZnCO_3$ added to provide 15 ppm Zn in all diets.

[e]Vitamin mix (ICN Pharmaceuticals, Inc.) provided (per kg diet): vitamin A, 9900 units; vitamin C, 1100 units; α-tocopheral, 55 mg; choline chloride, 825 mg; menadione, 25 mg; niacin, 50 mg; riboflavin, 11 mg; calcium pantothenate, 33 mg; thiamin, 0.22 mg; folic acid, 0.99 mg; vitamin B_{12}, 0.015 mg.

hydrochloride premix (prepared in casein) to a level of 0.25, 0.50, 0.75, and 1.00 μg PN per gram diet. The test diets were prepared by blending finely powdered dehydrated model systems into the basal diet. In Bioassay 1, roasted model systems were incorporated at a level of 5% of the diet and in Bioassay 2, stored model systems were incorporated at a level of 4% of the diet. Replacement of the basal diet ingredients to maintain composition with addition of the test systems was based on the model system proximate composition. The formulation of test diets containing phosphopyridoxyl-bovine serum albumin was performed by substituting 52.1 ml of a 5 mg/ml solution of the conjugated protein for the 52.1 ml of water added to each of the other diets. All diets were stored in sealed containers at 2°C until fed.

Male weanling Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, MI.) were randomly assigned into groups of 8 to 9 rats each for each bioassay. The rats were individually housed in metal cages with raised wire mesh floors. Water was supplied ad libitum. The basal diet was fed to all rats for 14 days to induce a mild state of vitamin B_6 depletion. The rats were weighed at the start of the assay period and weekly thereafter. Standard (0.00, 0.25, 0.50, 0.75, and 1.00 μg PN/g diet) and test diets (containing model systems or PP-BSA) were fed to the respective groups ad

libitum for 21 days. Feed consumption was carefully measured. Spillage was found to be low, and, therefore, it was neglected.

After the 21-day assay period, the rats were killed by decapitation. Blood was collected into 16 x 100 mm screw cap test tubes containing several drops of a 100 U/ml aqueous solution of heparin (Grade 1, sodium salt; Sigma Chemical Co.). Immediately after collection, samples were gently mixed with the heparin solution and then centrifuged in a clinical centrifuge (Damon/IEC Division) at approximately 3000 rpm. The plasma of each sample was removed by aspiration, and the erythrocytes washed by suspension in cold 0.9% (w/v) saline solution and recentrifuged. After removal of the saline wash solution, the packed erythrocytes were stored at -25°C until assayed. On the day of the AspAT assays, the tubes containing the erythrocytes were thawed and 10 ml of distilled water added to each. The contents of each tube were then thoroughly mixed on a vortex mixer, and cell debris sedimented by centrifugation in the clinical centrifuge at 3000 rpm. The supernatants were assayed for AspAT activity and hemoglobin content without dilution.

The bioassay results were evaluated by comparison of the response of the animals on the test diets to the dose-response results of animals fed the standard diets.

Does-response curves were determined by linear regression techniques (Neter and Wasserman, 1974). The logarithm of the dietary PN concentration (μg/g) was plotted against rat growth, growth per gram feed consumed, and percent PLP stimulation of AspAT. The dietary PN concentration was coded to permit taking the log of 0.00 μg/g by the addition of 1 to each standard concentration, as described by Bliss and White (1967). Because of the nonlinearity of the log dose-response curves observed for the enzyme activity data, several other methods of plotting the data were evaluated. These included: linear dose versus activity, log dose versus log activity, and reciprocal dose versus reciprocal activity (Bliss and White, 1967). The enzyme activity curve providing the highest linear correlation coefficient was used for quantitation in each bioassay.

Experimental Design and Procedures

Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Food System during Thermal Processing

The behavior of PL and PLP in liquid model systems was examined to determine whether complexes may be formed which could affect the biological availability of the vitamin. The aldehyde forms of the vitamin were examined in this experiment because they have been shown to be the most reactive of the B_6 vitamers (Anderson, *et al.*, 1974). Peptides, rather than intact proteins,

were chosen on the basis of their thermal solubility properties which facilitated the stabilization of Schiff bases with sodium borohydride ($NaBH_4$), gel filtration chromatography to recover the peptides after heat treatment, and spectrophotometric evaluation of the peptide bound vitamin. The effects of glucose and ascorbic acid were also examined. Glucose, through its role in Maillard browning, may compete with PL or PLP for free amino groups, thus acting as a binding inhibitor. Ascorbic acid or certain browning intermediates may be sufficiently strong reducing agents to promote pyridoxylamino compound formation by reduction of Schiff base forms of the vitamin.

1. Characterization of Peptides.--The sulfhydryl content of the heat stable peptides was measured (Ellman, 1959) after denaturation with 8 M urea in 0.1 M potassium phosphate, pH 8.0. Free amino groups (Fields, 1972) and peptide (Lowry, *et al.*, 1951) concentrations were also determined.

The number of peptide species in the heat stable mixture was determined by peptide mapping, as previously described.

Gel filtration chromatography on Sephadex G-10, G-25, and G-50 columns was employed to estimate the molecular weight distribution of the peptide mixture. The Sephadex G-50 column (2.6 x 40 cm) was calibrated using blue dextran (Pharmacia Fine Chemicals) to

determine the void volume, and the following proteins: trypsin, 23,300 daltons (ICN Pharmaceuticals, Inc.); ribonuclease A, 13,700 daltons (Worthington Biochemical Corp.); insulin, monomer = 5,733 daltons (Sigma Chemical Co.); insulin B-chain, monomer = 3,378 daltons (Sigma Chemical Co.); and insulin A-chain, monomer = 2,360 daltons (Sigma Chemical Co.). All standards were chromatographed individually. Insulin undergoes a pH-dependent aggregation reaction, and, therefore, this protein could serve as a standard in several polymeric forms. Oncley, et al. (1952), observed that, at neutral pH, insulin exists as a hexamer, while at pH 2, the dimer predominates. Hence, when dissolved and chromatographed in 0.01 N HCl, insulin was used as a molecular weight standard of 11,466 daltons, where in 0.1 M ammonium acetate, pH 7.0, insulin was used as a 34,398 dalton standard. The A- and B-chains of insulin were chromatographed in 0.1 M ammonium acetate, pH 7.0, containing 0.1% 2-mercaptoethanol, maintaining the peptides in their reduced state. Under these conditions, both assumed the dimer state. Attempts to dissociate intact insulin and the two subunits to their respective monomer states by chromatography in a pH 10.3 carbonate buffer (Fredericq, 1953), even with 8 M urea, failed. Contamination of the samples with small amounts of zinc, as discussed by Klostermeyer and Humbel (1966), may have

been responsible for the persistent aggregation. Trypsin, ribonuclease, and the heat stable peptides were dissolved and chromatographed in 0.1 M ammonium acetate, pH 7.0. The ratio of the elution volume to the void volume was plotted against the log of the molecular weight for each standard. Pyridoxal, L-tryptophan, and sodium azide were the low molecular weight compounds chromatographed individually to determine the internal volume of the Sephadex G-50 column. The molecular weight range of the peptide mixture was determined by comparison with the calibration curve.

2. Pyridoxal and Pyridoxal Phosphate Binding Studies.--

a. Stock Solutions.--Stock solutions of pyridoxal hydrochloride and pyridoxal phosphate monohydrate (ICN Pharmaceuticals, Inc.), L-ascorbic acid (Sigma Chemical Co.), and D-glucose (Matheson, Coleman, and Bell) were prepared in 0.1 M ammonium acetate and adjusted to pH 7.0 with ammonium hydroxide, as follows:

1. Pyridoxal, 500 μg free base/ml (2.93 mM), was prepared by dissolving 15.1 mg pyridoxal HCl in 25 ml.

2. Pyridoxal phosphate, 670 μg free base/ml (2.93 mM), was prepared by dissolving 18.1 mg pyridoxal phosphate monohydrate in 25 ml.

3. L-ascorbic acid, 1.50 mg/ml, was prepared by dissolving 37.5 mg in 25 ml.

4. D-glucose, 100 mg/ml, was prepared by dissolving 5 g in 50 ml.

All stock solutions were stored at 2°C. Vitamin stock solutions were protected from light and kept no longer than one week.

b. Liquid Model System Preparation and Treatment.--Model system mixtures were prepared in 16 x 100 mm screw cap test tubes by the addition of 0.1 ml of each appropriate stock solution to 0.7 ml of the 1.57 mg/ml peptide solution, as shown in Table 2.

Table 2. Formulation of liquid model systems for study of PL and PLP interactions.

Treatment	ml Stock Solution			
	Peptides	Ascorbate	Glucose	PL or PLP
Control	0.7	0	0	0.1
Ascorbate	0.7	0.1	0	0.1
Glucose	0.7	0	0.1	0.1
Glucose & Ascorbate	0.7	0.1	0.1	0.1

Final concentrations of components, when present, were: peptides, 1.10 mg/ml; PL, 50 μg/ml (0.293 mM); PLP 67 μg/ml (0.293 mM); glucose, 10 mg/ml, ascorbate, 150 μg/ml. All model systems were adjusted to 1.0 ml

total volume with 0.1 M ammonium acetate. After mixing, the samples were autoclaved 20 minutes at 121°C, and upon removal from the autoclave, cooled to 2°C. A corresponding blank containing no added PL or PLP was prepared for each sample and was treated in a manner identical to the sample. Each model system was prepared, treated, and analyzed in duplicate.

c. Evaluation of Binding to Peptides.--After heating at 121°C for 20 minutes, for the determination of total peptide bound PL or PLP all model systems were treated with 3 mg solid $NaBH_4$ at 2°C. This stabilized the labile Schiff base forms by conversion to pyridoxylamino compounds. Thus, the total bound forms of the vitamin would represent these reduced Schiff base forms in addition to any nonreducible substituted aldamine or pyridoxylamino complexes present. Determination of PL or PLP bound solely by nonreducible linkages was carried out by omitting the $NaBH_4$ treatment. All samples were held at 2°C until chromatographed as described below.

Peptides were isolated from the treated model system solutions by gel filtration chromatography on a Sephadex G-10 column (1.6 x 30 cm, V_o = 28 ml). The entire 1 ml model system solution was chromatographed using 0.1 M ammonium acetate, pH 7.0. The column effluent was monitored at 254 nm and the peptides were recovered from the void volume of the column.

The bioavailability of the nonreducible complexes was estimated by determination of PLP which remained bound following acidification of the model systems using the following procedure: after autoclaving the model systems, they were cooled and adjusted to pH 2.5 with 3 N HCl. Following overnight incubation at 2°C, the peptides were isolated using a Sephadex G-10 column (1.6 x 30 cm) equilibrated and eluted with 0.05 M KCl-HCl, pH 2.5.

Browning in the model systems was quantitated by measuring the free amino groups in the peptides collected from the Sephadex column. The concentration of free sulfhydryl groups was determined (Ellman, 1959) before and after gel filtration isolation of the peptides from the model systems.

The concentration of PL or PLP bound to the peptides was determined spectrophotometrically using the molar absorptivity coefficient (5800 $M^{-1}cm^{-1}$) at 323 nm for bound pyridoxyl groups (Dempsey and Christensen, 1962). Correction for blank absorbance was made using an equivalent peptide concentration. Peptide concentrations were determined according to Lowry, et al. (1951).

To minimize photochemical destruction of PL and PLP, all experimental procedures were run in the absence of incandescent light and direct sunlight. Two parallel trials of each binding study were performed. The data

were analyzed by analysis of variance employing 2 x 2 and 3 x 2 factorial designs (Neter and Wasserman, 1974).

Interactions of Pyridoxamine in a Liquid Food System during Thermal Processing

Using the initial carbonyl-amine condensation reactions of nonenzymatic browning as a theoretical model, it was postulated that PM may condense with reducing sugars to form Schiff bases. Thermal rearrangement of the sugar-PM complexes could affect the biological availability of the PM.

This experiment was designed to study the behavior of PM in a liquid model system at neutral pH. The stability of PM and its participation of transamination or the formation of stable complexes during thermal processing were investigated. The effects of glucose and ascorbic acid on the vitamin were examined, as in the previous experiment. Peptides were omitted from these model systems to facilitate analysis by high performance liquid chromatography.

1. Model System Formulation and Treatment.--Pyridoxamine dihydrochloride (ICN Pharmaceuticals, Inc.), L-ascorbic acid, and D-glucose stock solutions were prepared in 0.1 M ammonium acetate and adjusted to pH 7.0 with ammonium hydroxide. Pyridoxamine, 500 μg free base/ml (2.93 mM), was prepared by dissolving 17.8 mg

in 25 ml. L-ascorbic acid (1.50 mg/ml) and D-glucose (100 mg/ml) stock solutions were prepared as previously described.

Duplicate model system mixtures were prepared in 0.1 M ammonium acetate, as shown in Table 3.

Table 3. Formulation of liquid model systems for study of pyridoxamine interactions.

Treatment	ml Stock Solution		
	Glucose	Ascorbate	Pyridoxamine
Control	0	0	0.4
Glucose	0.4	0	0.4
Ascorbate	0	0.4	0.4
Glucose & Ascorbate	0.4	0.4	0.4

Final concentrations of the components, when present, were: PM, 50 μg/ml (0.293 mM); glucose, 10 mg/ml; and ascorbate, 150 μg/ml. All model systems were adjusted to 4.0 ml total volume with 0.1 M ammonium acetate. Corresponding blanks containing no PM were prepared for each sample and treated in an identical manner. Each model system was autoclaved 20 minutes at 121°C, then cooled to 2°C. To induce dissociation of any acid labile complexes, the autoclaved solutions were adjusted to pH 2.5 with 3 N HCl, followed by incubation overnight at 2°C.

2. Evaluation of Thermal Effects.--

a. Stability of Pyridoxamine.--The concentration of PM and the other B_6 vitamers in the autoclaved model systems was determined by the HPLC method for the quantitative analysis of the B_6 vitamers, as described in the Appendix. The HPLC analysis was performed as described, with the exception of the omission of the extraction and enzymatic hydrolysis steps. Aliquots of the liquid model systems were injected directly into the HPLC; no TCA was added to these samples. It was found that the absence of TCA shortened the retention time of the vitamers. Therefore, a standard curve was prepared for the B_6 vitamers under the conditions employed for the analysis of the liquid model system samples. Using this HPLC procedure, the possible conversion of PM to PN or PL could be readily detected.

b. Gel Filtration Chromatography.--Two ml aliquots of the PM-fortified liquid model systems and their blanks were chromatographed on a Sephadex G-10 column (2.6 x 34 cm; V_o = 63 ml). The column was equilibrated and the samples chromatographed with 0.1 M ammonium acetate, pH 7.0. The absorbance of the effluents was monitored at 254 nm. Changes in the molecular size distribution of the model system components versus blanks would provide evidence of the formation of acid stable complexes.

3. Statistical Analysis.--The significance of the effects of glucose and ascorbic acid on the thermal stability of PM was determined by two-way factorial analysis of variance. Differences between treatment means were determined by the Tukey procedure for pair-wise comparisons (Neter and Wasserman, 1974).

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Roasting Effects

The results of Yen, *et al*. (1976), have indicated that roasting of shelled corn during the production of animal feeds may reduce the amount of biologically available vitamin B_6. Few other studies have examined the stability of vitamin B_6 in low moisture foods during roasting processes.

The purposes of this experiment were: (1) to determine the extent of destruction and relative stability of the B_6 vitamers during roasting, (2) to compare the results of microbiological, HPLC, and fluorometric methods of vitamin B_6 determination, and (3) to determine the bioavailability of vitamin B_6 in the roasted model systems. The severe time-temperature conditions employed in the processing of the model systems were selected in order to quantitate the maximum effect that could be encountered during the commercial roasting of foods and feeds.

1. Formulation and Treatment of the Model Systems.--The dehydrated model system, formulated to simulate breakfast cereal composition, was a modification of that designed by Kirk, *et al*. (1977). The composition is shown in Table 4. Four model systems were prepared, differing only in vitamin B_6 fortification. Model systems were fortified with: pyridoxine hydrochloride, pyridoxamine dihydrochloride, or pyridoxal phosphate monohydrate. A nonfortified blank was also prepared. Each model system was homogenized as a slurry of 40% solids by mixing all ingredients in distilled water in a Waring blender. Vitamin B_6 fortification was performed by thoroughly mixing aqueous solutions of the respective vitamers with the slurry. Fortification was to a level of approximately 25% NAS/NRC recommended dietary allowance (0.5 mg) per ounce.

The slurries were transferred to shallow trays, cooled to -40°C, and dried in a Virtis Model FFD 42 WS Freeze-Dryer at a platen temperature of 110°F to a constant absolute pressure of 5 μm Hg. After drying, the model systems were finely powdered in a Waring blender and layered on stainless steel sheets at a thickness of approximately 3 mm. The trays were placed in an oven at 180°C and held for 25 minutes, after which the model systems had a golden brown appearance. The roasted model systems, and aliquots of each taken prior to roasting,

were stored in stoppered glass containers at 2°C until assayed for their vitamin B_6 contents.

Table 4. Composition of dehydrated model system for determination of roasting effects.

Component	Percentage by Weight
Casein, vitamin free test[a]	5
Corn syrup solids[b]	37
Starch[c]	50
Cellulose (Alphacel)[d]	7
Sodium chloride	1

[a]General Biochemicals, Inc.

[b]Malto-Dextrin (D. E. = 15). Provided approximately 5% reducing sugar. American Maize.

[c]Food grade powdered starch. A. E. Staley Mfg. Co.

[d]ICN Pharmaceuticals, Inc.

2. Analysis of Model Systems.--The effects of roasting on the stability of vitamin B_6 in the model systems was determined by comparison of assay results from roasted and unroasted system samples. In order to compare the specificity of the vitamin B_6 assay procedures, the model systems were assayed by microbiological (Haskell and Snell, 1970), fluorometric (Gregory and Kirk, 1977a; Appendix), and HPLC (Appendix) methods.

Biologically available vitamin B_6 in the roasted model systems was determined by rat bioassay. Rat growth, growth per gram feed consumed, erythrocyte AspAT activity, and PLP stimulation of AspAT were employed as indicators of vitamin B_6 status. Each roasted model system was incorporated into the test diet at a level of 5% by weight (Table 1).

3. Statistical Analysis.--The presence of significant differences among the rat bioassay estimates of available vitamin B_6 using the various criteria of B_6 status was tested by analysis of variance, employing a randomized complete block design.

For each model system, completely randomized distribution analysis of variance (ANOVA) was used to compare estimates by any one of the bioassay criteria with the microbiological and fluorometric assay data. An ANOVA was run repeatedly for each model system in which the microbiological and fluorometric assay data were tested against the bioassay data based on each of the response parameters. This repeated analysis for each model system was necessitated because of the lack of independence between the bioassay estimates; that is, for each model system, each of the four bioassay estimates was derived from the response of the same group of rats. Therefore, to achieve a family error rate of $\alpha = 0.05$, the critical probability employed in each of the four

ANOVAs for each model system was $\alpha/4 = 0.0125$. All statistical procedures were described by Neter and Wasserman (1974).

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Storage Effects

Previous research has demonstrated that naturally occurring vitamin B_6 in foods may undergo degradation during prolonged storage (Harding, *et al.*, 1959; Richardson, *et al.*, 1961; Everson, *et al.*, 1964). The storage stability of vitamin B_6 in PN fortified cereal products has also been examined (Bunting, 1965; Cort, *et al.*, 1976; Anderson, *et al.*, 1976). No studies to date have quantitatively examined the relative stability of PN, PM, and PLP in foods. Although several studies have demonstrated the occurrence of transamination reactions during thermal processing, the possible occurrence of interconversions among the B_6 vitamers during storage has not been examined. Similarly, the products of vitamin B_6 degradation in foods have not been identified.

In this study, the stability of various forms of vitamin B_6 was examined in dehydrated model food systems. These systems were equilibrated to a water activity of approximately 0.65 and stored in metal cans at 37°C: conditions which have been previously shown by numerous researchers to produce high rates of vitamin degradation and nonenzymatic browning. Storage under these conditions

should give maximum rates of B_6 degradation that may be encountered in food systems during storage. The experimental objectives were: (1) to determine the kinetics of degradation and/or interconversion of PL, PM, PLP, and PN during storage, (2) to relate the vitamin degradation to the rate of nonenzymatic browning, (3) to determine the effect of headspace oxygen content in the container on the stability of the B_6 vitamers, (4) to identify the degradation products of the B_6 vitamers, (5) to compare fluorometric, microbiological, and HPLC assay results for vitamin B_6 in the model systems, and (6) to determine the bioavailability of vitamin B_6 in the model systems after the storage period of 128 days.

1. Formulation, Processing, and Storage of Model Systems.--The dehydrated model food system was identical in composition to that previously employed (Table 4), with the exception that bovine serum albumin (essentially fatty acid-free; Sigma Chemical Co.) and bovine β-lactoglobulin (United States Biochemical Corp.) were substituted for the casein at levels of 4% and 1% of the total solids, respectively. These proteins were selected on the basis of their high solubility which facilitated their isolation from the model systems after storage. This permitted determination of protein bound forms of vitamin B_6. Maillard browning pigments

and losses of free amino groups in the soluble proteins served as convenient indicators of nonenzymatic browning in the systems.

The model systems were formulated, fortified, and freeze dried as previously described. Equilibration was performed by the method of Palnitkar and Heldman (1971), in which air, conditioned to a relative humidity of 65% by an Aminco-Aire unit, was forced for 24 hours over pieces of the freeze dried model systems of approximately 1 cm thickness. The systems were equilibrated on the adsorption branch of the water sorption hysteresis loop.

After equilibration, aliquots of the model systems were quickly packaged in 208 x 006 thermal death time (TDT) and 303 x 406 (303) enameled cans to prevent transfer of water vapor and oxygen into or out of the container (Kirk, et al., 1977). Each can, irrespective of size, contained approximately 10 to 12 g of the appropriately fortified model system. The use of large (303) and small volume containers permitted storage under atmospheres differing greatly in total oxygen content. All canned samples were stored in a constant temperature incubator at 37 ± 1°C.

2. Analysis of Model Systems.--

a. Physical Measurements.--The moisture contents of the equilibrated model systems were determined by the method of Kirk, et al. (1977). Samples were dried

in a vacuum oven at a vacuum of 28 inches Hg at 37°C. The use of a cold trap between the oven and the vacuum pump facilitated water vapor transfer from the samples. Displacement of water vapor from the oven was accelerated by slow admission of air at a rate of approximately 20 ml/min after drying by bubbling through sulfuric acid. The moisture content, expressed on a dry weight basis, was determined by sample weight loss after each had reached constant weight.

The water activity of each system was measured directly by the vapor pressure manometric method of Taylor (1961), as described by Sood and Heldman (1974).

b. Determination of Nonenzymatic Browning.-- The progression of nonenzymatic browning during storage was evaluated by monitoring losses of protein free amino groups and formation of protein bound melanoidin pigments. The soluble protein was extracted by mixing 1 g of each model system with 15 ml 0.05 M KCl-HCl, pH 2.5, and sonicating 30 minutes in a 65 watt ultrasonic cleaning bath (Fisher Scientific Co.). The mixtures were centrifuged 20 minutes at 6000 rpm in a Beckman Model J-21C centrifuge, rotor JA-20. Ten ml aliquots of the supernatants were placed in 0.39 inch diameter dialysis tubing and dialyzed 3 days against 4 l of 0.05 M KCl-HCl, pH 2.5, with two changes of the buffer. To remove any fine particulates, the dialyzed extracts were filtered through Whatman 42

filter paper. The protein concentrations were determined (Lowry, et al., 1951), and the absorbance of each extract was measured against a buffer reference at 420 nm. The absorbance at 420 nm per mg protein/ml was used as an index of nonenzymatic browning. The free amino group concentrations were determined in the soluble proteins (Fields, 1972) to permit evaluation of the loss of a reactant in the browning reaction.

c. Rate of Vitamin B_6 Degradation.--The concentration of vitamin B_6 in the dehydrated model systems was determined at time = 0 (1 day after canning) and at subsequent intervals by the HPLC method previously described. Model systems which were stored in TDT cans were assayed approximately every 3 weeks over the 128-day storage period, while the model systems packaged in 303 cans were assayed at the end of storage.

d. Vitamin B_6 Degradation Products.--The postulated degradation products, 4-pyridoxic acid and pyridoxyllysine, were determined by the HPLC methods described in the Appendix. Analysis was performed at the end of the 128-day storage period.

e. Bioavailability of Model System Vitamin B_6.--Prior to incorporation into the bioassay test diets, the model systems which were stored in TDT and 303 cans were blended, yielding a mixture comprised of approximately 60% 303 and 40% TDT can stored model systems. These

mixtures were assayed for their vitamin B_6 contents by microbiological (Haskell and Snell, 1970), fluorometric (Gregory and Kirk, 1977a; Appendix), and HPLC methods previously described. The biologically available vitamin B_6 in the model systems was determined by the rat bioassay methods as previously described.

f. Statistical Methods.--In these studies, vitamin B_6 degradation rate constants and their errors were determined by linear regression methods. The significance of differences between assay methods for vitamin B_6 was tested by analysis of variance. Statistical analysis of the rat bioassay data was performed as described for the bioassay of the roasted model systems (c. f. pages 67-68). All procedures were described by Neter and Wasserman (1974).

Biological Activity of Phosphopyridoxyl-Bovine Serum Albumin

Although previous research has demonstrated varying vitamin B_6 activities for several pyridoxylamino compounds, the activity of protein bound derivatives has never been investigated. No antivitamin activity has ever been reported for the pyridoxylamino acids examined. McCormick and Snell (1961), in an extensive study of pyridoxal phosphokinase, reported that while most pyridoxylamino acids had no inhibitory activity, other pyridoxylamino compounds were potent inhibitors. Because

pyridoxal phosphokinase is essential for the conversion of dietary vitamin B_6 to the metabolically active phosphorylated derivatives, their data suggested that certain pyridoxyamino compounds may act as B_6 antivitamins.

The purposes of this experiment were: (1) to determine the apparent vitamin B_6 content of phosphopyridoxyl-bovine serum albumin (PP-BSA) using the microbiological and fluorometric assay procedures based on autoclave extraction, (2) to determine the availability of protein bound pyridoxylamino groups for utilization by the rat, and (3) to determine whether the presence of PP-BSA in the rat diet affects the utilization of dietary pyridoxine.

1. Analysis of Phosphopyridoxyl-Bovine Serum Albumin.--PP-BSA was prepared as previously described. Pyridoxylamino residues were quantitatively determined from the ultraviolet-visible absorption difference spectrum of a 1 mg/ml solution of PP-BSA in 0.1 M ammonium acetate, pH 7.0, against a 1 mg/ml solution of bovine serum albumin (Sigma Chemical Co.) in the same buffer. Calculations were based on the molar absorptivity coefficient at 323 nm for protein bound pyridoxyl groups of 5800 $M^{-1}cm^{-1}$ (Dempsey and Christensen, 1962). Pyridoxylamino groups were also quantitated using the HPLC method for the determination of pyridoxyllysine in protein hydrolysates, as previously described.

The PP-BSA preparation was examined for the presence of free vitamin B_6 contaminants by gel filtration chromatography over a Sephadex G-25 column (2.6 x 36 cm, V_o = 78 ml) equilibrated and eluted with 0.1 M ammonium acetate pH 7.0. The column effluents were monitored at 254 nm with an ultraviolet absorption detector. Trace contamination of free vitamin B_6 in the PP-BSA was quantitated by the HPLC method for vitamin B_6 free bases. The assays were performed as previously described, with the exception that the sample size of PP-BSA was 25 mg.

Samples of the PP-BSA preparation were analyzed by microbiological (Haskell and Snell, 1970) and fluorometric (Gregory and Kirk, 1977a) methods to determine the extent of release of the protein bound pyridoxyl groups during the autoclave extraction. Apparent vitamin B_6 levels were corrected by subtracting the low level of free vitamin B_6 in the PP-BSA, as determined by HPLC (buffer extraction, rather than autoclave extraction methods).

2. Determination of Biological Activity.--The rat bioassay for biologically available vitamin B_6 was performed as previously described. The bioassay was run parallel to the assay of stored model systems (Bioassay 2), and utilized the same dose-response curves for quantitation. The biological availability of the bound

vitamin B_6 in the PP-BSA was determined from the response of rats fed a test diet with PP-BSA as the sole source of vitamin B_6. Antivitamin B_6 activity of the PP-BSA was determined from the response of rats fed diets containing the same level of PP-BSA plus 0.25 μg pyridoxine free base per gram. The extent of utilization of the free PN was found by comparing the response to the diet containing the added PN to the response expected from the dose-response curves. In each diet, the estimated vitamin B_6 content of the PP-BSA was corrected for free B_6 contaminants by subtraction of the value determined by HPLC vitamin B_6 assay.

RESULTS

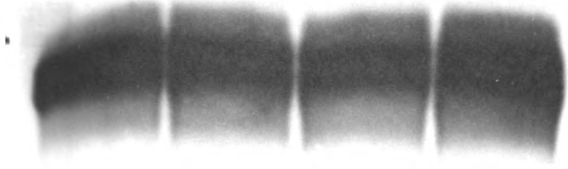
Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Model Food System during Thermal Processing

Preparation and Characterization of Heat Stable Peptides

The commercially obtained preparation of β-lactoglobulin, when examined by discontinuous polyacrylamide gel electrophoresis (Melachouris, 1969), was found to contain several minor contaminants (Figure 3). Upon denaturation with 8 M urea, the protein exhibited 1.53 moles of free sulfhydryl (SH) groups per mole of β-lactoglobulin dimer (36,000 daltons). In the absence of urea, 0.10 moles SH per mole dimer were detected. For the preparation of heat stable peptides, this protein was used without further purification.

Heat stable peptides were isolated from a peptic hydrolysate of β-lactoglobulin with a yield of approximately 5%. Analysis of the isolated peptides revealed 1.26 μmoles of free amino groups per mg. No free SH groups could be detected in the presence or absence of urea. Mapping of the peptide mixture revealed a high degree of heterogeneity; at least 17 peptide species were detected (Figure 4).

Figure 3. Polyacrylamide gel electropherogram of β-lactoglobulin which was used in isolation of heat stable peptides. Slots 1-4 contained 75, 100, 125, and 150 μl of a 5 mg/ml protein solution.



1 2 3 4

Figure 3

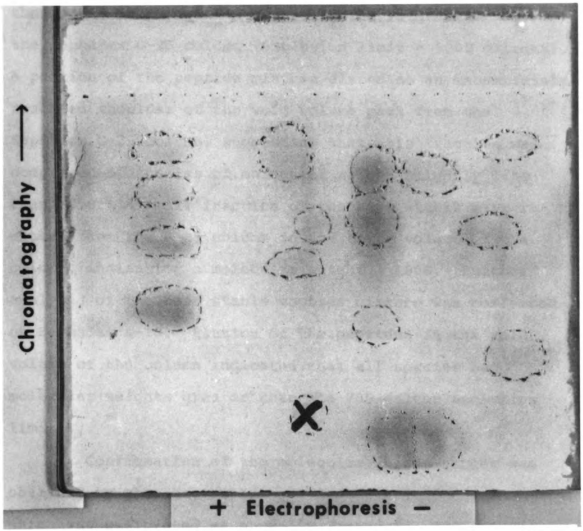


Figure 4. Thin layer peptide map of heat stable peptides. The origin is designated by X.

The molecular weight range of the peptide mixture was determined by gel filtration chromatography on Sephadex G-10, G-25, and G-50. The largest fraction of the heat stable peptides eluted in the void volume of the Sephadex G-25 column (exclusion limit = 5000 daltons). A portion of the peptide mixture eluted as an incompletely resolved shoulder of the void volume peak from the Sephadex G-25 column, suggesting that this fraction was comprised of species of molecular weight slightly less than 5000. A small fraction of the heat stable mixture eluted from the G-25 column in the total volume of the column, indicating a molecular weight $\leq$ 1000. Further analysis of the heat stable peptide mixture was performed on Sephadex G-10. Elution of the peptides in the void volume of the column indicated that all species had molecular weights greater than the 700 dalton exclusion limit.

Confirmation of the molecular weight ranges was obtained by chromatography on a Sephadex G-50 column which was calibrated as shown in Figure 5. The chromatogram of the heat stable peptide mixture (Figure 6) indicated that the most prevalent peptide species were those of approximately 4,500 and 12,500 daltons.

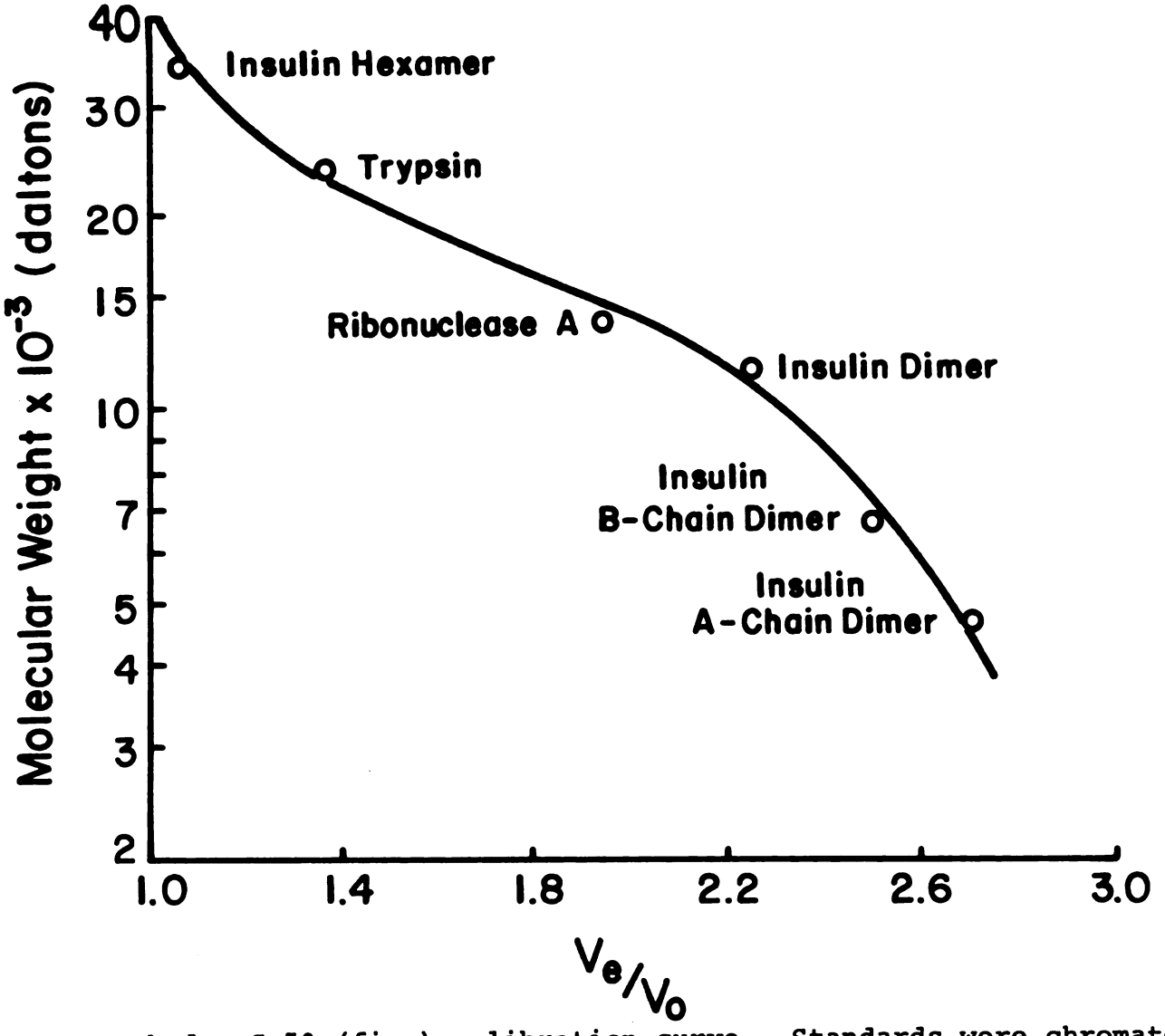


Figure 5. Sephadex G-50 (fine) calibration curve. Standards were chromatographed under conditions described in text.

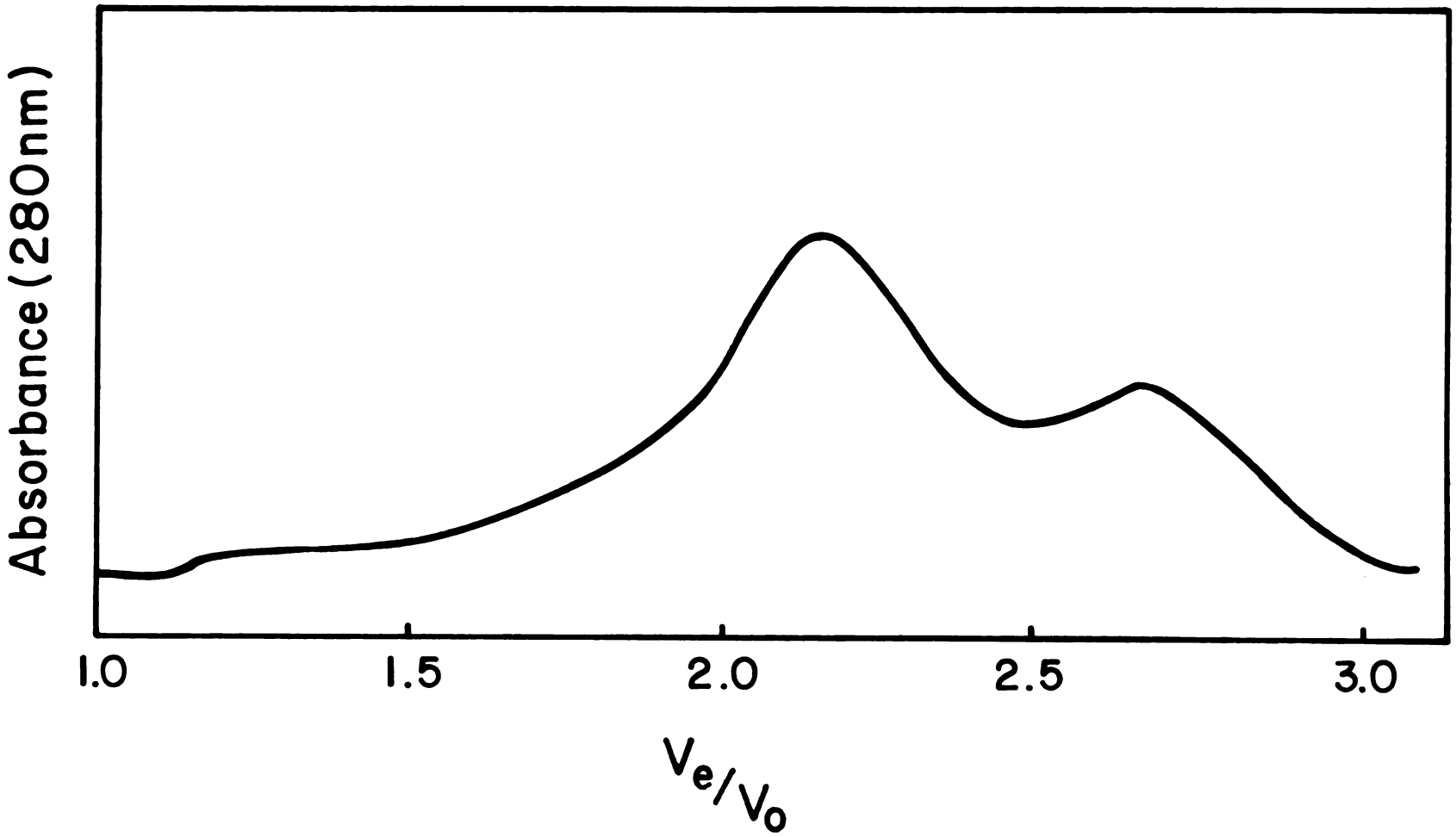


Figure 6. Sephadex G-50 chromatogram of heat stable peptide mixture.

Binding of PL and PLP in Liquid Model Systems

Determination of total bound PL and PLP was carried out using liquid model systems which were treated with sodium borohydride after autoclave processing for 20 minutes at 121°C. Peptide bound forms of vitamin B_6 would thus represent borohydride-reduced Schiff base derivatives plus nonreducible complexes such as substituted aldamines and pyridoxylamino compounds. The peptides were isolated from the autoclaved liquid model systems by chromatography on a column of Sephadex G-10. The presence of peptide bound PL or PLP was demonstrated by the UV-visible difference absorption spectra of each peptide fraction versus its respective blank. Figure 7 is a difference spectrum recording typical of those observed for all model systems. The absorption maxima at 323-325 and 253-254 nm are characteristic of the absorption spectra of pyridoxylamino and substituted aldamine derivatives (Dempsey and Christensen, 1962; Kierska and Maslinski, 1971; O'Leary, 1971) and demonstrated the presence of peptide bound vitamin B_6 in the heat treated liquid model systems. Quantitation of the levels of total peptide bound vitamin B_6 (Table 5) indicated that PLP was much more reactive than PL ($P < .001$), as had been previously reported. Factorial analysis of the data showed that glucose, ascorbic acid, and their interaction did not significantly affect the

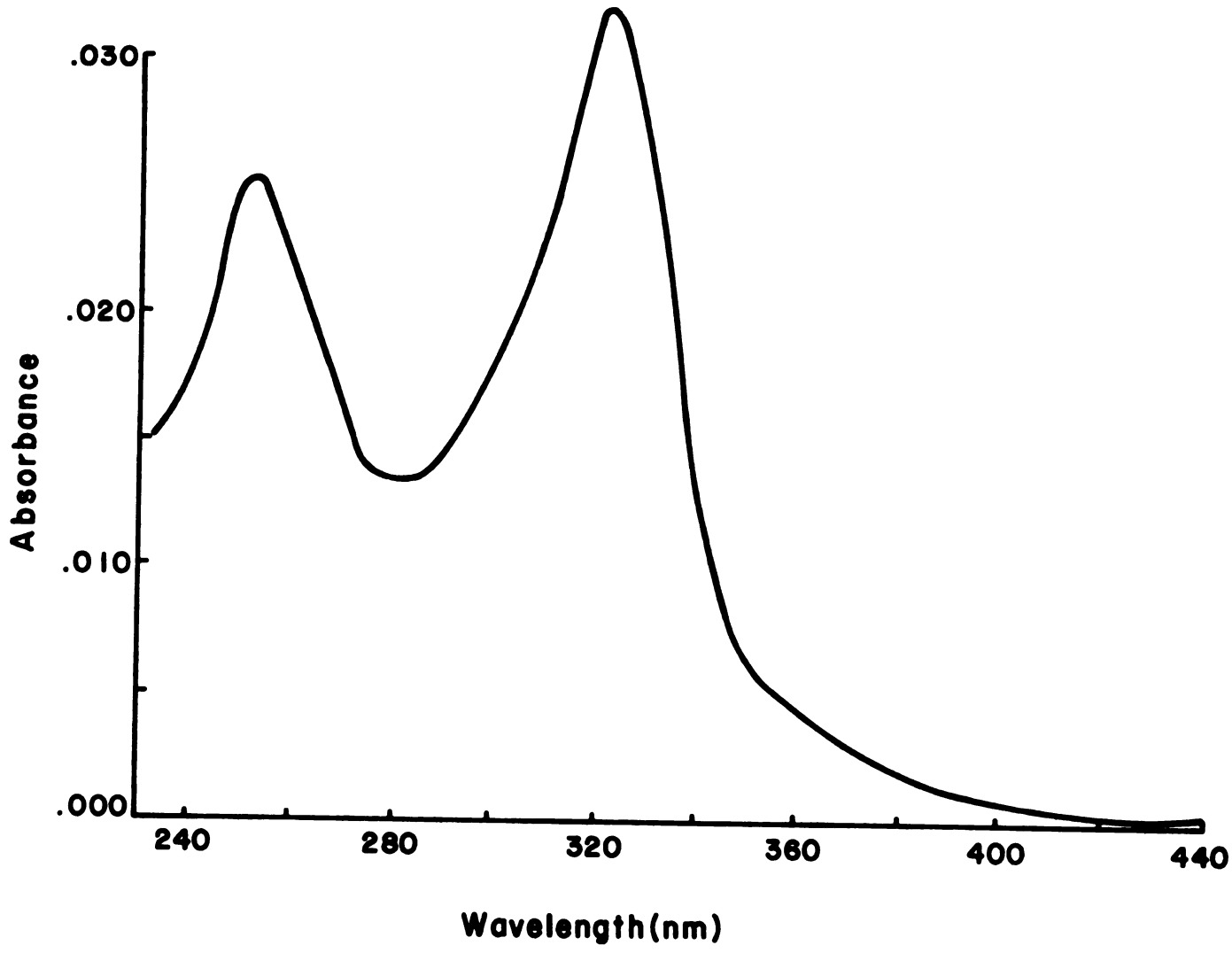


Figure 7. Ultraviolet-visible difference absorption spectrum of peptides isolated from a PLP-fortified liquid model system versus those from the non-fortified blank. Buffer = 0.1 M ammonium acetate, pH 7.0.

levels of bound PL or PLP ($P < .05$). Inspection of these data suggests, however, that the presence of ascorbate may have had a slight inhibitory effect on the net binding of PL and PLP.

Table 5. Total peptide bound PL and PLP. Data represents percent of model system B_6 present as a Schiff base, substituted aldamine, or pyridoxylamino complex at pH 7.0.

Treatment[a]	PL		PLP[b]	
	1	2	1	2
Control	8.1	3.0	27.8	20.0
Ascorbate	3.0	3.9	15.1	9.5
Glucose	8.9	6.1	23.9	22.3
Glucose & Ascorbate	10.4	9.9	26.3	15.7

[a]Effects of glucose, ascorbate, and their interaction were not significant ($P < .05$).

[b]PLP was significantly more reactive ($P < .001$).

Nonreducible complexed forms of vitamin B_6, substituted aldamine and pyridoxylamino derivatives, were determined spectrophotometrically after isolation of the peptides from heated liquid model systems which were not treated with borohydride. It was presumed that the labile Schiff base forms would dissociate during peptide isolation, leaving only the stable nonreducible complexes. In determining the nonreducible complexes, only PLP was studied because of the low reactivity observed with PL in the previous studies. UV-visible difference

spectra were determined on the peptide solutions from the autoclaved model systems which had not undergone $NaBH_4$ reduction. In all cases, the observed spectra were qualitatively identical to Figure 7, indicating the presence of peptide bound nonreducible complexes. The absence of an absorption maximum at 410-430 nm indicated that little or no PLP remained bound as a Schiff base (Kent, et al., 1958). Further evidence for the presence of peptide bound PLP was found in the fluorescence emission spectra of the recovered peptides when excited at 280 nm. Churchich (1965) observed that the emission of protein tryptophan residues is markedly quenched by bound PLP through a radiationless energy transfer process. The emission spectra of the peptides of each model system and the respective blanks exhibited this quenching effect. Typical emission spectra are shown in Figure 8. These spectrophotometric results confirmed the formation of nonreducible peptide bound vitamin B_6 complexes during thermal processing.

Quantitation of the peptide bound nonreducible complexes at neutral pH (Table 6) demonstrated that they comprised approximately 12% of the total model system PLP, or about 60% of the total bound PLP. The degree of binding was not significantly affected by the presence of glucose and/or ascorbate ($P < .05$), although the variability of the data may have precluded the

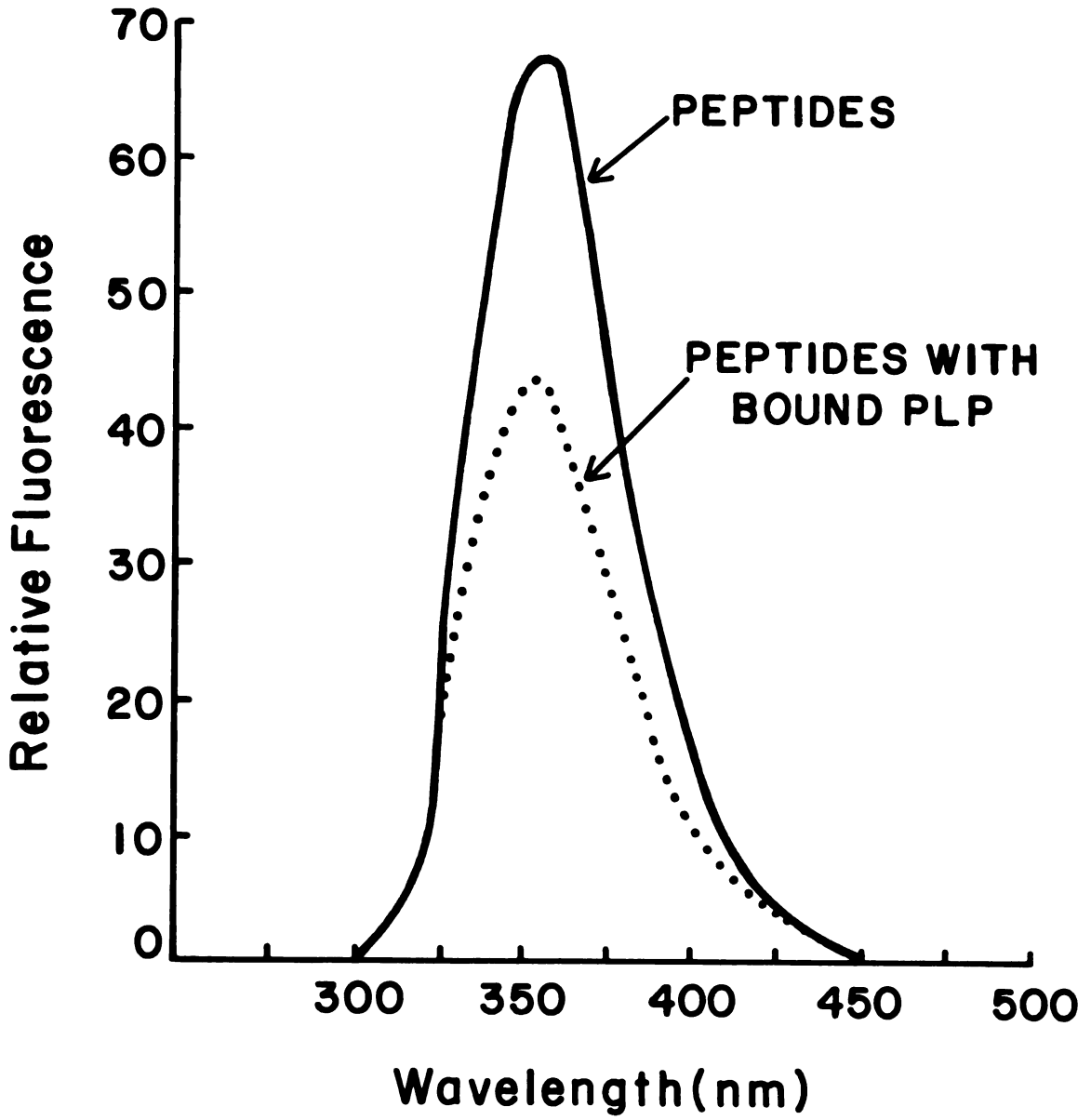


Figure 8. Fluorescence emission spectra of peptides from liquid model systems with and without PLP fortification. Buffer = 0.1 M ammonium acetate, pH 7.0. Excitation wavelength = 280 nm.

detection of any slight effects of these factors. Because of the similarity of the UV-visible spectra of the non-reducible complexes, these data could not be used to differentiate between the acid labile substituted aldamine and the acid stable pyridoxylamino derivatives (Dempsey and Christensen, 1962; Anderson, et al., 1971; O'Leary, 1971).

Table 6. Percent of model system PLP bound as a non-reducible complex (substituted aldamine or pyridoxylamino compound) at pH 7.0.

Treatment[a]	Trial	
	1	2
Control	12.3	16.1
Ascorbate	11.1	7.0
Glucose	10.7	17.1
Glucose & Ascorbate	17.5	2.9

[a]Effects of glucose, ascorbate, and their interaction were not significant ($P < .05$).

Direct measurement of pyridoxylamino complexes was accomplished after peptide isolation under conditions which would induce dissociation of the acid labile complexes. Each model system was adjusted to pH 2.5 after autoclaving, and the peptides were isolated by gel filtration chromatography using 0.05 M KCl-HCl, pH 2.5, as the eluent. In this acidic medium, the absorption maximum of the pyridoxylamino groups shifted from 323-325 nm

to 295 nm. Therefore, prior to quantitation, the pH of the peptide fractions was adjusted to 7.0 with 0.4 M K_2HPO_4. At pH 7.0, the UV-visible difference spectra of peptides from PLP-fortified systems versus those from the corresponding blanks were qualitatively identical to that of Figure 7. These results confirmed that PLP was bound to the peptides during the autoclave treatment as a highly stable pyridoxylamino complex. Quantitation of the pyridoxylamino groups (Table 7) indicated a mean value of 9.7 ± 6.4% of the PLP in the model systems was bound in a state which may affect the bioavailability. These results indicated that essentially all of the PLP isolated as nonreducible derivatives at pH 7.0 was comprised of acid stable pyridoxylamino complexes.

Table 7. Percent of PLP in liquid model systems which was bound as a pyridoxylamino complex.

Treatment[a]	Trial	
	1	2
Control	15.9	19.7
Ascorbate	12.6	7.6
Glucose	10.4	2.4
Glucose & Ascorbate	1.1	8.0

[a]Effects of glucose, ascorbate, and their interaction were not significant ($P < .05$).

The presence of added glucose and/or ascorbic acid had no significant effect on the extent of

pyridoxylamino complex formation ($P < .05$), although variability in the data may have masked any slight effects of these factors.

The data suggest that the presence of glucose and/or ascorbate may partially retard the formation of pyridoxylamino complexes (Table 7) through browning reactions which may compete for free amino groups. As a measure of the extent of browning beyond the initial carbonyl-amine condensation complexes, free amino groups were measured (Fields, 1972) in the peptides recovered at pH 2.5. Mean values of 0.93 ± 0.06 and 1.26 ± 0.07 μmoles of free amino groups per mg peptide were found in the presence and absence of glucose, respectively, indicating a 26% loss. The presence of ascorbic acid showed no effect on the concentration of free amino groups.

Because of the slight inhibitory effect observed for ascorbic acid on the levels of total bound vitamin B_6 (Table 5), and the previously reported involvement of SH groups in a possible binding mechanism of PL or PLP to proteins (Bernhart, et al., 1960), the levels of free SH groups in the various liquid model systems were examined. The content of free SH groups in the peptides of the heated model systems was determined directly on the model system solutions, and on the peptides recovered after Sephadex G-10 gel filtration. In this manner, the

reaction of the Ellman reagent with nonpeptide SH compounds, such as H_2S released from the peptides during heat treatment, could be detected by comparison. The results of Table 8 demonstrated that the presence of ascorbic acid significantly increased the levels of peptide free SH groups ($P < .01$), probably by reduction of intramolecular disulfide bonds and hindrance of their reoxidation. By comparison, the effect of glucose on SH levels was weak, although significant in the case of the isolated peptides ($P < .01$). The consistent differences between direct analysis and analysis of the isolated peptides indicated that a DTNB-reactive small molecule, probably H_2S, was released from the peptides during thermal processing.

Table 8. Sulfhydryl content of peptides in liquid model systems after thermal processing. Analysis either performed directly or after peptide isolation by gel filtration chromatography.[a]

Treatment	Direct Analysis[b]		Sephadex G-10 Isolation[c]	
	1	2	1	2
Control	7.7	10.1	2.1	1.0
Ascorbate	27.5	31.5	8.6	10.2
Glucose	5.7	4.5	4.8	7.7
Glucose & Ascorbate	24.8	27.0	16.6	15.8

[a]nmoles SH/mg peptides.

[b]Effect of ascorbate was significant ($P < .01$).

[c]Effects of glucose and ascorbate were significant ($P < .01$). The interaction in either analysis was not significant ($P < .05$).

Interactions of Pyridoxamine in a Liquid Model Food System during Thermal Processing

Stability of Pyridoxamine

The concentration of the B_6 vitamers in liquid PM-fortified model systems after thermal processing 20 minutes at 121°C was performed by the HPLC method. Losses of PM during thermal processing were quite small, except in the case of the model systems containing glucose (Table 9). The presence of glucose in the model systems resulted in significantly lower levels of PM after the heat treatment ($P < .01$), with an observed decrease of 24% of the PM added in fortification. No effect of the presence of ascorbic acid was detected. Partial conversion of PM to PL could be detected, accounting for 1 to 3% of the added PM in the model systems containing glucose and/or ascorbate. No conversion of PM to PN was observed. The results of this study indicated that the losses of total vitamin B_6 in the PM-fortified liquid model systems were generally low, with the maximum being about 21%.

Pyridoxamine--Carbohydrate Complex Formation

Gel filtration chromatography on Sephadex G-10 revealed no detectable changes in the molecular size of the fractions containing PM in the PM-fortified model systems, as indicated by a constant elution volume

Table 9. Vitamin B_6 in PM-fortified liquid model systems after thermal processing. Fortification prior to processing was at a level of 50 μg/ml.[a,b]

Treatment	μg B_6/ml			Percentage Loss
	PM	PL	Total	
Control	48.5	ND	48.5	3.0
Ascorbate	45.6	0.6	46.2	7.6
Glucose	38.0	1.5	39.5	21.0
Glucose & Ascorbate	41.8	1.6	43.3	13.2

[a]Means of duplicate determinations. ND = not detectable.

[b]Effect of glucose on lowering PM and increasing PL was significant ($P < .01$). Ascorbate significantly increased PL levels, but did not affect the concentration of PM ($P < .05$). The glucose x ascorbate interactions were not significant ($P < .05$).

(Figure 9). Thus, complexes containing PM were not observed by this procedure.

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Food Systems: Roasting Effects

Comparison of Assay Methods for Total Vitamin B_6

The assay dehydrated roasted and nonroasted model systems provided the first application of the HPLC method for determination of vitamin B_6 in complex systems containing low levels of the vitamin. The chromatographic procedure, based on the methods of Horvath, *et al*. (1977), involved the separation of the B_6 vitamers by reversible "solvophobic" interaction with an octadecylsilica stationary phase. A chromatogram of the vitamin B_6 standards is shown in Figure 10. The theoretical plate counts for PM, PL, and PN were approximately 6850, 3850, and 3000, respectively. The linearity of response with the 280 nm absorbance detector, reproducibility of the retention times, and high sensitivity of the method is demonstrated by the calibration data of Table 10. Typical recovery values for the B_6 vitamers added to the dehydrated model systems prior to extraction were 75% for PM and PLP and 95% for PN.

The HPLC assay of vitamin B_6 in the nonroasted model systems demonstrated excellent precision and correlated well with the expected concentrations based on

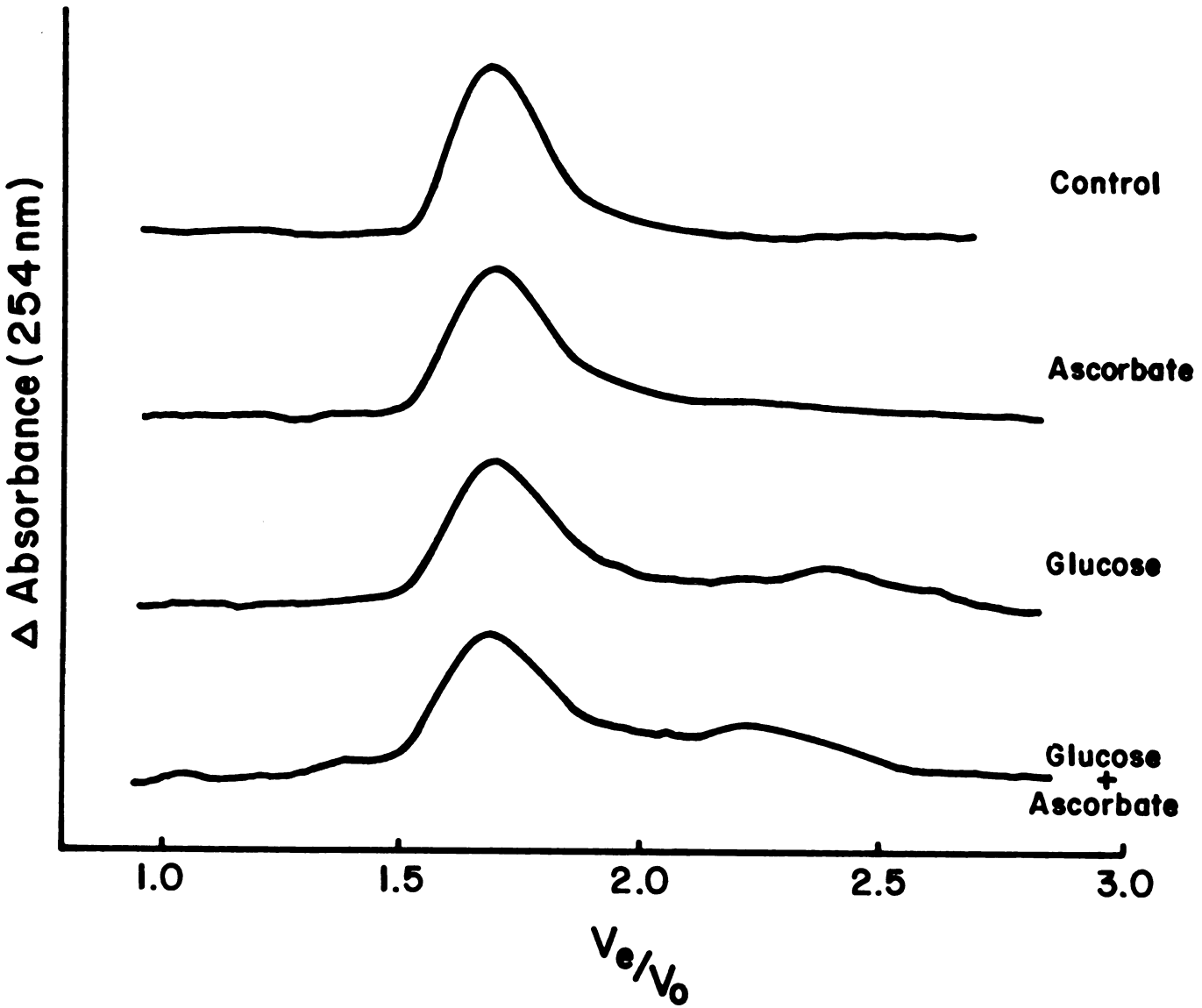


Figure 9. Sephadex G-10 chromatograms of PM-fortified liquid model systems after 20 min at 121°C. The peak at V_e/V_o = 1.7 was PM. Buffer = 0.1 M ammonium acetate, pH 7.0.

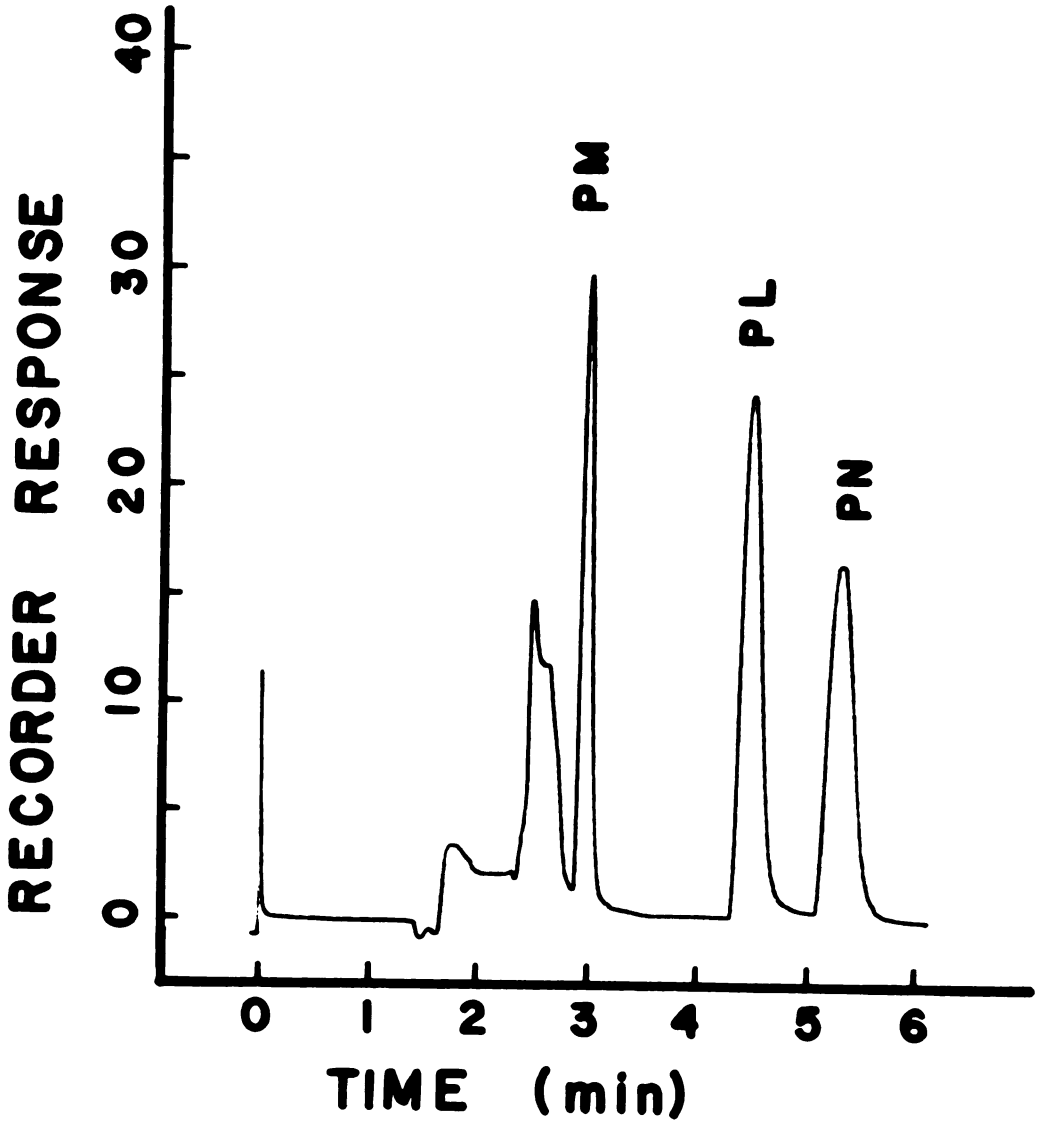


Figure 10. HPLC chromatogram of standard vitamin B_6 free bases. Detection = 280 nm absorption; flow = 2.0 ml/min; 1700 psig; injection = 50 µl containing 0.100 µg of each vitamer in 0.2 M potassium acetate in 5% TCA, as performed with model system extracts.

Table 10. Calibration curves for the HPLC assay of vitamin B_6 using 280 nm absorption detection. Flow = 2.0 ml/min, 1700 psig, 0.02 AUFS. Pk ht = peak height; t_r = retention time.

	PM		PL		PN	
µg/50 µl	t_r (min)	pk ht (mm)	t_r (min)	pk ht (mm)	t_r (min)	pk ht (mm)
0.125	2.85	223	4.35	196	5.20	116
0.100	2.95	181	4.45	159	5.30	93
0.075	2.90	144	4.35	125	5.20	73
0.050	2.90	103	4.40	88	5.25	54
0.025	2.85	60	4.35	51	5.20	33
0.0125	2.90	29	4.45	22	5.30	15
Mean	2.89		4.39		5.24	
Std. Dev.	0.04		0.05		0.05	
Slope (mm/µg/50µl)	1683 ± 49		1505 ± 45		861 ± 31	
Y-intercept (mm)	14.7 ± 3.7		9.5 ± 3.4		8.4 ± 2.3	
Correlation Coefficient	0.9983		0.9982		0.9975	

the 25% RDA per ounce (15 μg/g) fortification (Table 11). Attempts to determine the vitamin B_6 in the roasted model systems were unsuccessful because the many UV-absorbing peaks from soluble browning compounds obscured the chromatographic peaks of the B_6 vitamers. Comparison of the HPLC and microbiological results for vitamin B_6 in the nonroasted model systems (Table 11) exhibited excellent correlation, with the exception of the markedly lower microbiological value observed for the PM-fortified model system. The low activity of PM for the assay organism, S. uvarum (Rabinowitz and Snell, 1948; Parrish, et al., 1955; Gregory, 1959; Woodring and Storvick, 1960; Chin, 1975), was presumably responsible for this discrepancy.

The data from the semiautomated fluorometric determination of vitamin B_6 are presented in Table 12. Inspection of these fluorometric data for the individual vitamers indicated reasonably good agreement with the HPLC results for PN and PL in the nonroasted model systems (Table 11). By contrast, semiautomated fluorometric values for PM in the nonroasted systems (Table 12) were much higher than those determined by HPLC (Table 11). A comparison of the data for the nonroasted and roasted model systems indicated that the roasting process degraded all forms of the vitamin (Table 12). The observed decrease in PM and corresponding increase in PL in the

Table 11. Total vitamin B_6 in control and roasted model systems determined by microbiological and HPLC methods.[a,b,c]

Fortification	Control		Roasted
	HPLC	Micro.	Micro.
Nonfortified	< 1.0	1.1 ± 0.3	1.0 ± 0.4
PN	18.7 ± 1.5	19.6 ± 3.6	9.3 ± 0.8
PM	19.8 ± 0.1	9.7 ± 2.4	6.2 ± 1.6
PLP	14.4 ± 0.1	13.6 ± 0.8	5.1 ± 0.5

[a]μg total B_6/g model system.

[b]Mean and standard deviations. Duplicate determinations.

[c]The HPLC assay was performed with 280 nm absorption detection. This could not be applied to the roasted model systems because of the extensive browning of the sample and resulting complexity of the chromatograms.

Table 12. Vitamin B_6 in control and roasted model systems, as determined by the semiautomated fluorometric assay procedure.[a,b]

Fortification	PN	PM	PL	Total
Control				
Nonfortified	1.4 ± 1.6	15.1 ± 3.0	2.5 ± 0.7	19.0 ± 3.5
PN	18.6 ± 1.1	13.9 ± 4.5	2.0 ± 1.3	34.5 ± 4.8
PM	9.7 ± 1.8	24.5 ± 4.2	1.6 ± 0.9	35.2 ± 4.7
PLP	2.1 ± 0.6	8.1 ± 2.6	15.1 ± 0.3	25.3 ± 2.7
Roasted				
Nonfortified	1.2 ± 1.1	2.8 ± 1.4	1.2 ± 0.0	5.2 ± 1.8
PN	4.4 ± 0.4	2.1 ± 0.5	7.9 ± 1.2	14.4 ± 1.4
PM	2.8 ± 1.2	2.1 ± 0.3	5.6 ± 1.2	10.5 ± 1.7
PLP	3.7 ± 0.4	1.5 ± 1.1	5.8 ± 0.4	11.0 ± 1.2

[a] μg/g model system.

[b] Mean and standard deviation, triplicate determinations.

PM-fortified model system suggested the occurrence of a thermally induced transamination reaction.

Comparison of semiautomated fluorometric and microbiological assay data for total B_6 in nonroasted and roasted model systems (Table 13) indicated a similar percent loss by both assay methods. Observed losses during the roasting process, as assessed by either assay method, ranged from 53 to 73%, except in the case of the microbiological analysis of the PM-fortified system. As previously mentioned, the possible inaccuracy of the microbiological assay for PM may have been responsible for the observed difference. The apparent total vitamin B_6 contents of all roasted and nonroasted model systems were consistently higher when assayed by semiautomated fluorometric, rather than microbiological, methods. The nonroasted and roasted model food systems fortified with PN and PLP and the roasted PM-fortified systems exhibited a ratio of fluorometric to microbiological values for total B_6 ranging from 1.65 to 2.16. The ratio rose to 3.63 in the case of the nonroasted PM-fortified system. The poorest correlation between semiautomated fluorometric and microbiological assays was observed in the case of the nonfortified model systems, in which the observed fluorometric to microbiological ratios were 17.3 and 5.2 for nonroasted and roasted systems, respectively. The mean difference between semiautomated

Table 13. Comparison of fluorometric and microbiological methods for determination of total vitamin B_6 in control and roasted model systems.[a,b]

Model System Fortification	Fluorometric			Microbiological		
	Control	Roasted	% Loss	Control	Roasted	% Loss
Nonfortified	19.0	5.2	73	1.1	1.0	---
PN	34.5	14.4	58	19.6	9.3	53
PM	35.2	10.5	70	9.7	6.2	36
PLP	25.3	11.0	57	13.6	5.1	63

[a] μg total B_6/g model system.

[b] Mean of triplicate determinations for fluorometric and duplicate determinations for microbiological assays.

fluorometric and microbiological data for all nonroasted model systems was 17.5 ± 5.9 μg/g, while the mean difference for roasted systems was 4.9 ± 0.8 μg/g.

Interference in Semiautomated Fluorometric Vitamin B_6 Assay

Thin layer chromatographic studies performed on the products of the reactions employed to form 4-pyridoxic acid lactone in the semiautomated fluorometric vitamin B_6 assay (Gregory and Kirk, 1977a; Appendix) indicated that the preparative ion exchange chromatography was effective in removing potentially interfering compounds. Since the poor correlation of data from semiautomated fluorometric assays with microbiological and HPLC values suggested the presence of interfering fluorophores, further studies were undertaken to identify these interfering compounds. The fluorescence emission spectra of sample extracts treated for fluorometric analysis, and their assay blanks, were examined in an effort to detect these interfering compounds. All samples were adjusted to pH 10.2 with 0.4 M sodium carbonate, 0.2% Brij 35, prior to spectral analysis. The emission spectrum of the fluorophore, 4-pyridoxic acid lactone, was calculated manually by subtraction of the emission spectra of the assay blanks from those of chemically treated samples. All samples and blanks were prepared in a manner identical to that followed in the semiautomated fluorometric

assay. The excitation wavelength was 355 nm. The fluorophore emission spectra determined for all model system extracts were found to be highly similar to that of 4-pyridoxic acid lactone formed from B_6 standards (Table 14). No significant differences were observed in the emission maxima, and differences in peak width (full width at half maximum, FWHM) were not considered to be significant, in view of the imprecision in the determination of these spectra. Reagent blank fluorescence was observed in the case of the PL and PM determinations.

Biologically Available Vitamin B_6 in Roasted Dehydrated Model Systems

The results of the rat bioassay (Bioassay 1) for biologically available vitamin B_6 in the roasted model systems are presented in Table 15. The responses of rats fed the standard diets containing 0 to 1.0 μg PN/g were used to generate the dose-response curves used in calculation of available vitamin B_6 in the test diets containing the roasted model systems. The dose-response curves of best linear fit were determined, and their regression parameters shown in Table 16.

The estimated concentration of biologically available vitamin B_6 in each of the roasted model systems is shown in Table 17. Comparison of the data based on each of the four response parameters indicated no significant differences among the estimates for each model

Table 14. Characteristics of the emission spectra of the fluorophores produced from vitamin B_6 standards and model system extracts during the fluorometric assay of vitamin B_6. Excitation wavelength = 355 nm. Wavelength values are approximately ± 5 nm for maxima and ± 10 nm for full width at half maximum (FWHM).

Vitamer Assayed	Sample[a]	Relative Fluorescence	λ max[b] (nm)	FWHM (nm)
Pyridoxamine				
	PM standard	74	430	78
	Nonfortified-C	18	430 (450 shld)	95
	Nonfortified-R	13	430 (450 shld)	95
	PM-C	73	430	71
	PM-R	38	430	73
	Reagent blank	3	450	85
Pyridoxal				
	PL standard	65	430	75
	Nonfortified-C	14	430	77
	Nonfortified-R	12	430	78
	PLP-C	80	430	66
	PLP-R	43	430	68
	Reagent blank	8	425	105
Pyridoxine				
	PM standard	76	430	68
	PN-C	50	430	71
	PN-R	9	430	60
	Reagent blank	0	---	--

[a]Sample designation: C and R refer to control and roasted model systems.

[b]Shld refers to the approximate maximum of an emission peak shoulder.

Table 15. Rat bioassay of roasted model systems (Bioassay 1).[a,b]

Diet	Rat Growth	Feed Consumed	Growth (g) / g Feed Consumed	Erythrocyte AspAT	
				Activity	PLP Stimulation
	(g)	(g)	(g/g)	(mU/mg Hb)	(%)
Standard					
0.00 μg PN/g	44 ± 3	179 ± 5	0.243 ± 0.009	7.00 ± 0.68	61.0 ± 5.7
0.25 "	78 ± 4	227 ± 4	0.346 ± 0.018	7.18 ± 0.72	59.4 ± 8.9
0.50 "	102 ± 6	261 ± 10	0.387 ± 0.012	9.52 ± 1.52	53.0 ± 3.8
0.75 "	134 ± 5	333 ± 9	0.401 ± 0.008	9.27 ± 0.95	43.5 ± 2.5
1.00 "	140 ± 5	347 ± 14	0.406 ± 0.010	14.17 ± 1.60	23.8 ± 3.3
5% Roasted Model Systems					
Nonfortified	51 ± 6	211 ± 14	0.236 ± 0.017	6.45 ± 0.51	65.4 ± 5.8
PN	84 ± 4	248 ± 9	0.338 ± 0.005	9.20 ± 1.07	56.0 ± 4.4
PM	77 ± 7	241 ± 5	0.305 ± 0.029	7.48 ± 1.31	57.9 ± 5.4
PLP	90 ± 5	258 ± 9	0.351 ± 0.014	7.91 ± 0.62	54.1 ± 4.2

[a]Mean and standard error, 8 rats per dietary group.

[b]Growth, feed consumption, and growth/g feed consumed were for the 21-day test period following a 2-week depletion.

Table 16. Linear regression parameters of the dose-response curves.[a] Bioassay 1, roasted model systems.

X[b]	Y	Y-intercept	Slope	Correlation Coefficient
log (PN + 1)	Growth (g)	44.9 ± 3.7	334 ± 19	+.9425
log (PN + 1)	Growth (g)/g Feed	0.270 ± 0.012	0.530 ± 0.059	+.8235
(PN)	AspAT Activity	6.15 ± 0.92	6.57 ± 1.50	+.5778
(PN)	AspAT PLP Stimulation	66.3 ± 3.8	-36.7 ± 5.6	-.7660

[a]These dose-response curves represent functions which provided the least variability about a linear relationship, i.e., those which provided the highest correlation coefficients for each response criterion.

[b]All X data represents the dietary concentration of added pyridoxine. When the log was taken, the concentrations were coded by the addition of 1 to each value.

Table 17. Biologically available vitamin B_6 in roasted model systems.[a] Data represent μg available B_6/g model system.

Model System Fortification	Response Criterion[b,c]			
	Growth	Growth (g) / g Feed Consumed	Erythrocyte AspAT	
			Activity	PLP Stimulation
Nonfortified	1.4 ± 0.8	-2.4 ± 1.2	0.9 ± 1.6	0.5 ± 3.2
PN	6.3 ± 0.7	7.0 ± 0.6	2.8 ± 2.5	5.6 ± 2.4
PM	5.2 ± 1.2	4.7 ± 3.6	1.5 ± 2.7	5.1 ± 2.9
PLP	7.5 ± 0.8	8.8 ± 1.8	4.5 ± 1.9	4.6 ± 2.0

[a]Mean and standard error, 8 rats per assay group.

[b]Response criterion refers to the index of B_6 nutritional status used in calculation of the available vitamin B_6 in the model system.

[c]Estimates of available vitamin B_6 by each of the response criteria were not significantly different for each of the model systems ($P < .05$).

system ($P < .05$). Inspection of the standard errors of each estimate indicated that rat growth generally provided the greatest precision. This was also suggested by the high correlation coefficient of the rat growth dose-response curve.

Statistical comparison of the microbiological assay data for total vitamin B_6 with each of the rat bioassay estimates (Table 18) indicated no significant differences ($P < .05$). The microbiological data correlated well with the bioassay estimates based on growth, growth per gram feed consumed, and PLP percent stimulation of AspAT. The bioassay estimates based on AspAT activity exhibited a slightly poorer correlation with the microbiological data. In strong contrast, the semiautomated fluorometric assay results were consistently higher than all bioassay estimates, with a mean difference of 6.3 ± 1.9 μg vitamin B_6/g.

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Storage Effects

Moisture Content and Water Activity

The dehydrated model systems were equilibrated to the predetermined water activity (a_w) under forced air which had been conditioned to 65% relative humidity. The precise moisture equilibrium for each fortified model system stored in TDT and 303 cans was determined by measuring the a_w and moisture content of the systems

Table 18. Comparison of rat bioassay estimates of biologically available vitamin B_6 in roasted model systems with microbiological and semiautomated fluorometric assay results for total vitamin B_6.[a,b]

Model System Fortification	Response Criterion					
	Growth	Growth (g) / g Feed Consumed	AspAT		Microbiol.	Fluorom.
			Activity	PLP Stimulation		
Nonfortified	1.4	-2.4	0.9	0.5	1.0	5.2
PN	6.3	7.0	2.8	5.6	9.3	14.4
PM	5.2	4.7	1.5	5.1	6.2	10.5
PLP	7.5	8.8	4.5	4.6	5.1	11.0

[a] μg total B_6/g model system.

[b] No significant differences between microbiological and rat bioassay estimates; fluorometric data were significantly greater than microbiological and rat bioassay ($P < .05$).

after 128 days at 37°C (Table 19). No significant differences in moisture content or a_w were observed between fortified model systems after storage in either container. Therefore, the headspace volume did not affect the moisture equilibria. Slight differences in the moisture data were observed for the model systems fortified with the different B_6 vitamers. These differences were not statistically significant ($P < .05$). The analysis of the water activity data revealed several significant differences among the various model systems. The a_w of the non-fortified and PM-fortified systems was found to be significantly less than that of the PN- and PLP-fortified systems ($P < .05$).

Nonenzymatic Browning

Browning in the model systems was determined by measuring the levels of free amino groups and browning pigments in the soluble protein fraction. Plots of the time-dependent loss of amino groups (Figure 11) and the concomitant formation of melanoidins (Figure 12) demonstrate that nonenzymatic browning is not a linear function, indicative of the complex kinetics of the reaction. The data in Table 20 show the concentration of free amino groups and browning pigments associated with the soluble protein fraction of the dehydrated model systems stored in TDT and 303 cans for 128 days. These data demonstrate a large difference in the concentration of both amino

Table 19. Moisture content and water activity of stored model systems. Model systems analyzed after storage for 128 days.[a,b,c]

Model System Fortification	TDT Cans		303 Cans	
	Moisture (%)	a_w	Moisture (%)	a_w
Nonfortified	11.12	0.584	10.88	0.582
PN	12.08	0.621	11.37	0.617
PM	11.48	0.571	11.19	0.589
PLP	11.53	0.626	---	---

[a]All moisture and water activity determinations were performed in duplicate. Data represent the mean. PLP-fortified system stored in 303 cans not analyzed due to insufficient supply. Moisture expressed on a dry weight basis.

[b]No significant differences between the moisture content or water activity of equivalent model systems whether stored in TDT or 303 cans ($P < .05$).

[c]Moisture contents not significantly different among model systems of different fortification. Water activity of nonfortified and PM-fortified systems was significantly less than that of PN- and PLP-fortified systems ($P < .05$).

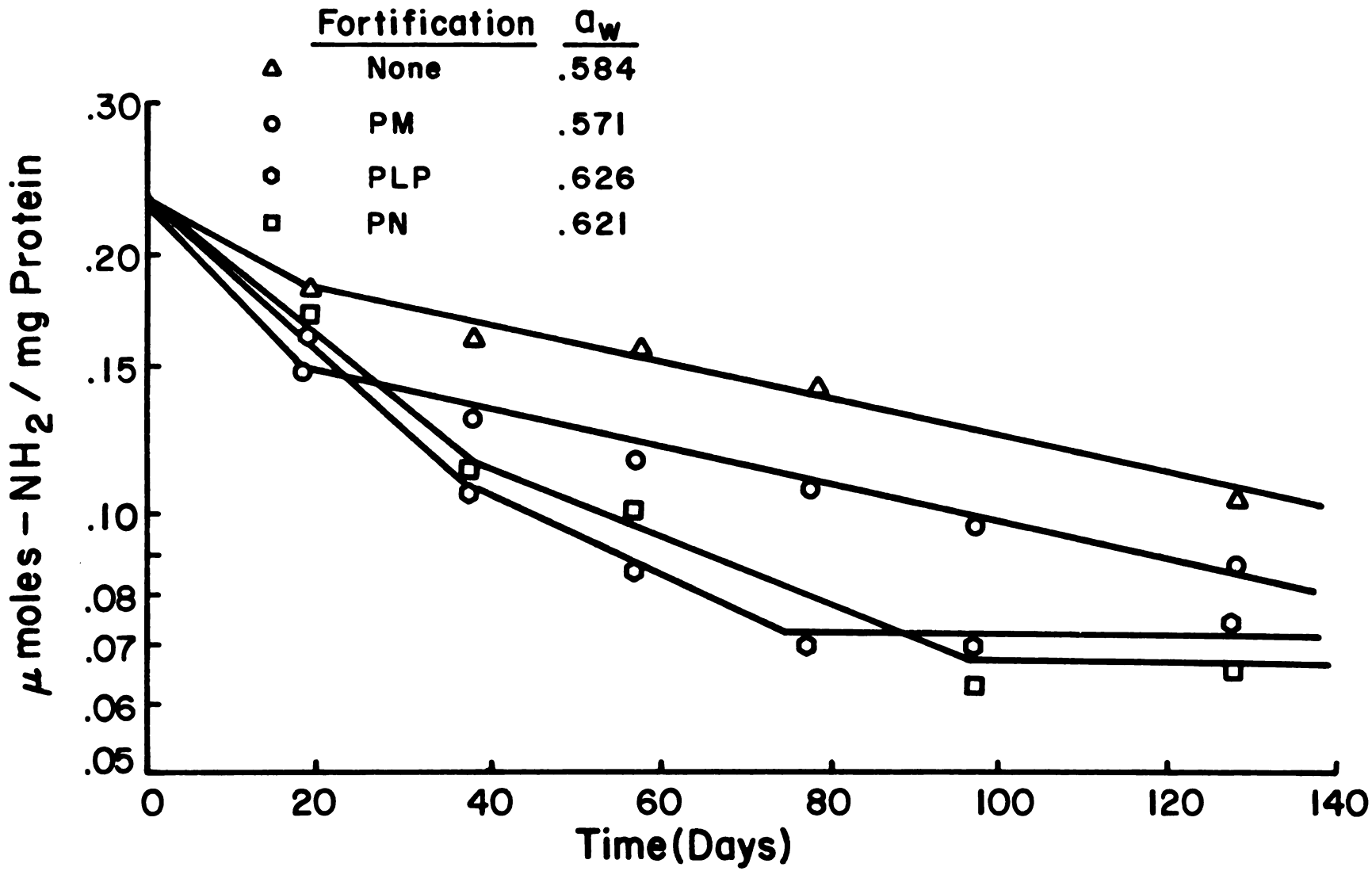


Figure 11. Loss of free amino groups in soluble proteins of dehydrated model systems during storage in TDT cans at 37°C. Proteins were extracted with 0.05 M KCl-HCl, pH 2.5.

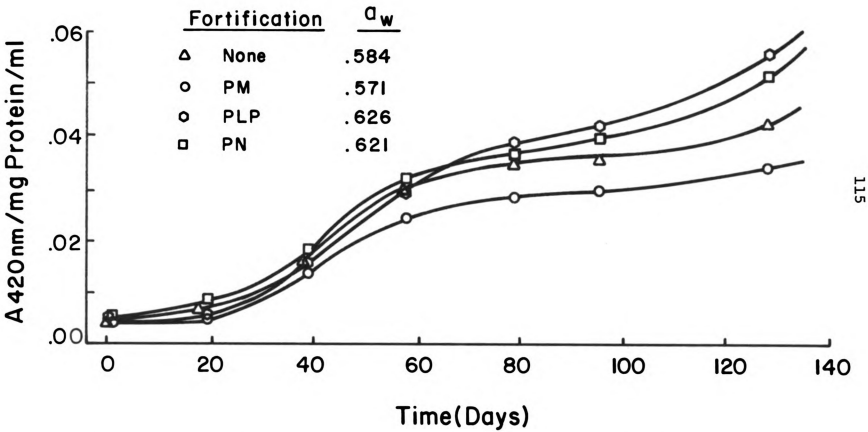


Figure 12. Formation of melanoidin pigments during storage of dehydrated model systems in TDT cans at 37°C.

Table 20. Comparison of browning levels in model systems stored in TDT and 303 cans for 128 days at 37°C.

Model System Fortification	Free Amino Groups[a]		Browning Pigments[b]	
	TDT	303	TDT	303
Nonfortified	0.108	0.111	0.0422	0.0082
PN	0.066	0.103	0.0523	0.0114
PM	0.088	0.117	0.0331	0.0071
PLP	0.075	0.099	0.0527	0.0095

[a]Mean of triplicate determinations. Data = μmoles/mg protein.

[b]Mean of triplicate determinations. Data = $A_{420\ nm}$/mg protein/ml.

groups and browning pigments, indicating that the rate of browning was much greater in the model systems stored in TDT cans. The data indicate that the model systems stored in TDT cans browned 4 to 6 times faster than identical systems packaged in 303 cans. A comparison of the data for the browning pigment levels and amino group concentration for each of the fortified model systems indicated that the difference in free amino groups between the TDT and 303 can systems was much less than the difference in pigment formation.

Within model systems of each storage container (TDT or 303 metal can), the loss of free amino groups and formation of pigments was strongly a function of the water activity of the system. The data suggest that maximum browning would occur at an a_w greater than 0.62 in this model system. Numerous investigators have reported similar values for the dependence of browning on water activity (Lea and Hannan, 1949; Loncin, et al., 1968; Warmbier, et al., 1976).

Kinetics of Vitamin B_6 Degradation

The time-dependent loss of the B_6 vitamers used in fortification of the dehydrated model systems was examined to determine the degradation kinetics during the relatively severe storage conditions of 37°C at an a_w of about 0.6. In order to determine whether the

headspace atmosphere of the storage container affected the rate of degradation, the model systems stored in 303 cans were assayed at the end of the 128-day storage period and compared with the TDT can data.

All vitamin B_6 analyses in this study were performed by the HPLC method. Initially, all assays were performed using absorption detection at 280 nm. During the later stages (after 78 days) of the storage period, the extensive browning of the model systems precluded accurate quantitation by this procedure. Final assays were made using HPLC separation and fluorescence detection. The HPLC fluorescence calibration curves were prepared as previously described, and were found to be linear over the entire range employed. The specificity of the fluorescence detection method permitted quantitation of the vitamin B_6 in all dehydrated model system samples (Figure 13).

The degradation of all B_6 vitamers could be described by the first order equation:

$$-\frac{dC}{dt} = -kt$$

where k represents the first order rate constant, C represents reactant concentration, and $-\frac{dC}{dt}$ is the rate of change of reactant concentration (Moore, 1962).

Pyridoxal phosphate was found to degrade at a high rate during storage of the dehydrated model system

Figure 13. HPLC chromatograms of a recovery sample of a nonfortified model system stored for 97 days in a TDT can at 37°C. Detection = 280 nm absorbance or fluorescence.

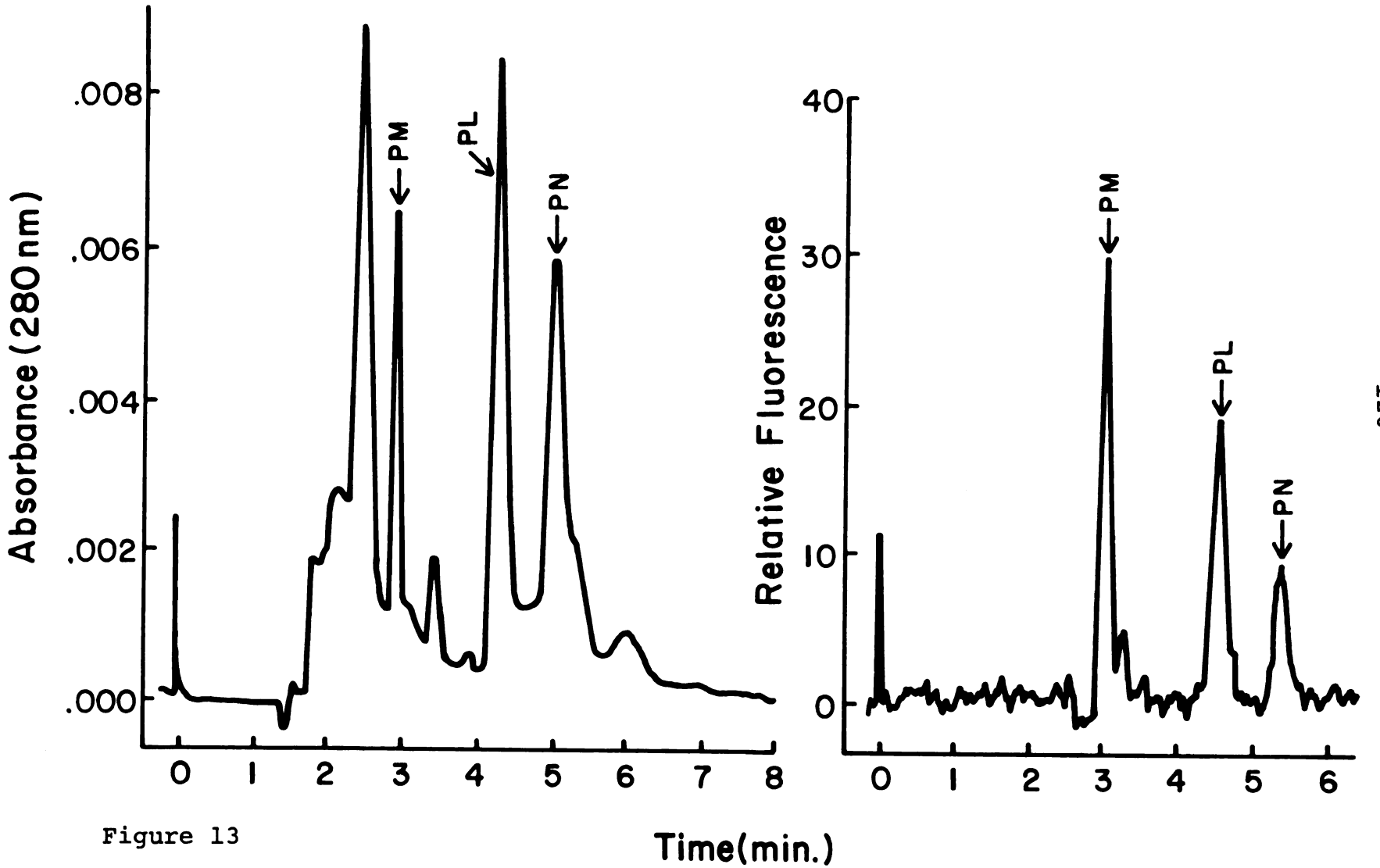
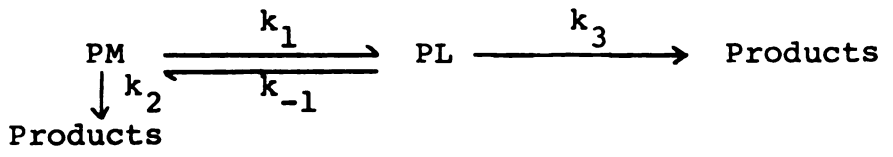

Absorbance (280 nm)
.008
.006
.004
.002
.000
PM
PL
PN
0
1
2
3
4
5
6
7
8
Relative Fluorescence
40
30
20
10
0
PM
PL
PN
0
1
2
3
4
5
6
Time(min.)

Figure 13

in a TDT can at 37°C, with the process following first order kinetics (Figure 14). No conversion of PLP to PM (PMP) was detected.

The greatest complexity in vitamin B_6 degradation was observed in the PM-fortified system (Figure 15). The disappearance of PM was rapid and could be described by first order kinetics. The coincident rapid increase in PL concentration during the first several weeks of storage was evidence for a transamination process. No evidence for a reverse reaction could be obtained. Therefore, under these conditions, the conversion of PM to PL was unidirectional. The PL curve (Figure 15), showing a maximum PL concentration after approximately 35 days of storage, suggests that the rate of PL formation exceeded that of PL degradation. Therefore, the rate limiting step in the loss of vitamin B_6 in the PM-fortified model system was the degradation of PL. Accurate determination of the rate of conversion of PM to PL could not be made because of the limited number of observations during the phase of rapid conversion. As a result, the rate of degradation of PM directly to inactive products could not be determined. The behavior of the vitamin in the PM-fortified system is most readily described by the following model:



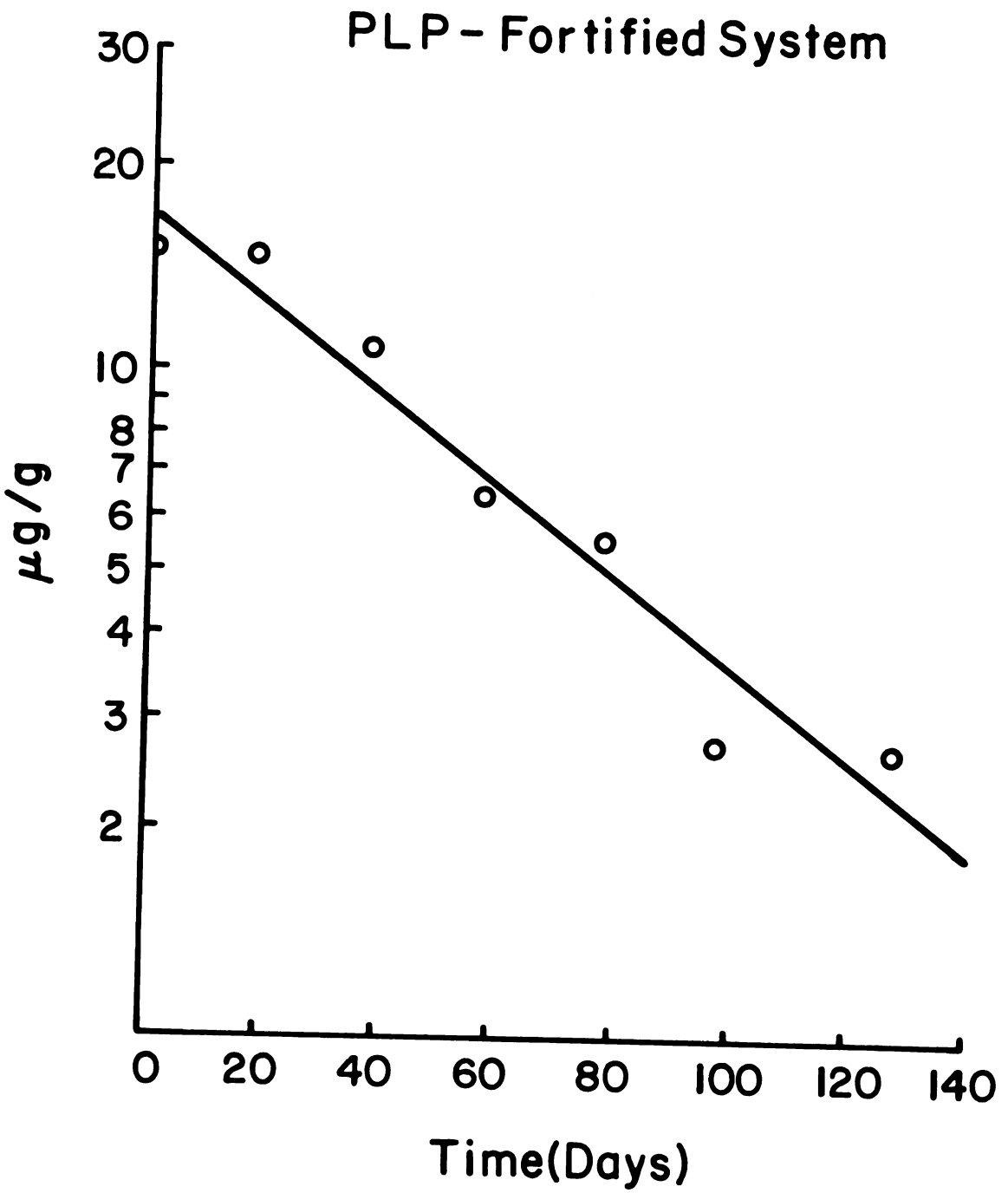


Figure 14. Degradation of PLP in dehydrated model systems stored in TDT cans at 37°C.

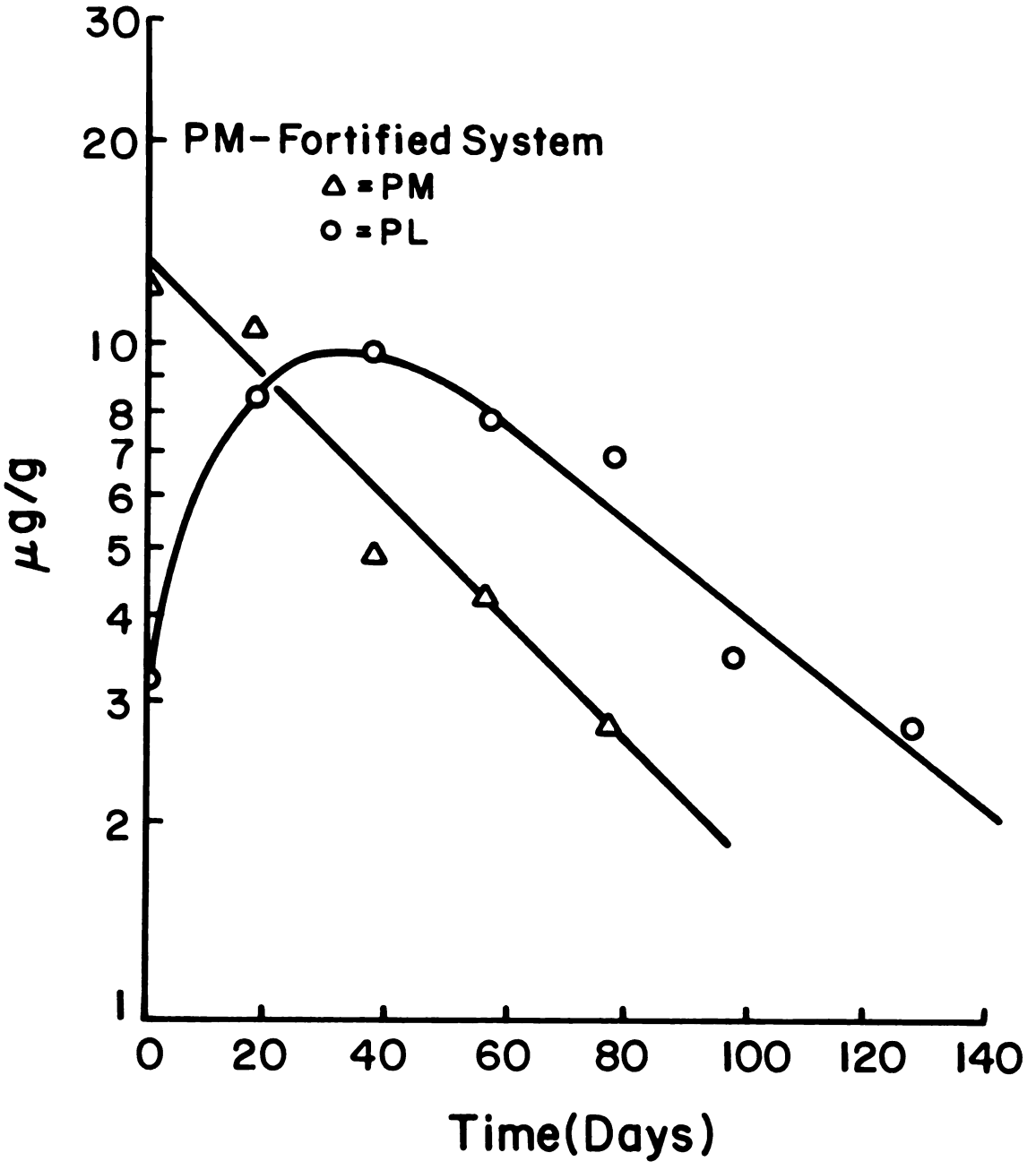


Figure 15. Behavior of vitamin B_6 in a PM-fortified dehydrated model system stored in TDT cans at 37°C.

During storage of the model system at 37°C at an a_w of about 0.6, k_{-1} was much smaller than k_1. Thus the rate of loss of PM from the model system was represented by the sum of the rates of degradation (k_2) and conversion to PL (k_1). Calculation of k_1 or k_2 cannot be performed from these data.

Of the three vitamers used in the fortification studies, PN was the most stable. No loss was observed for the first 58 days of storage, followed by a very slow degradation which followed first order kinetics (Figure 16). The rate of loss of PL inherent in the ingredients of the PN-fortified and nonfortified model systems (Figure 16) was found to be much more rapid. During storage of the PN-fortified model system, no formation of PL or PM could be detected, indicating that the losses were not due to conversion to the more labile vitamers.

Linear regression analysis of the PL, PM, PLP, and PN degradation data confirmed that all of the observed reactions were compatible with description by first order kinetics. All first order plots of the data exhibited correlation coefficients greater than -0.93 (Table 21). Zero and second order rate functions did not provide better fit for these data. The observed rate constants for the loss of PL and PLP were similar (0.0155 and 0.0158 day^{-1}, respectively), while that for the net

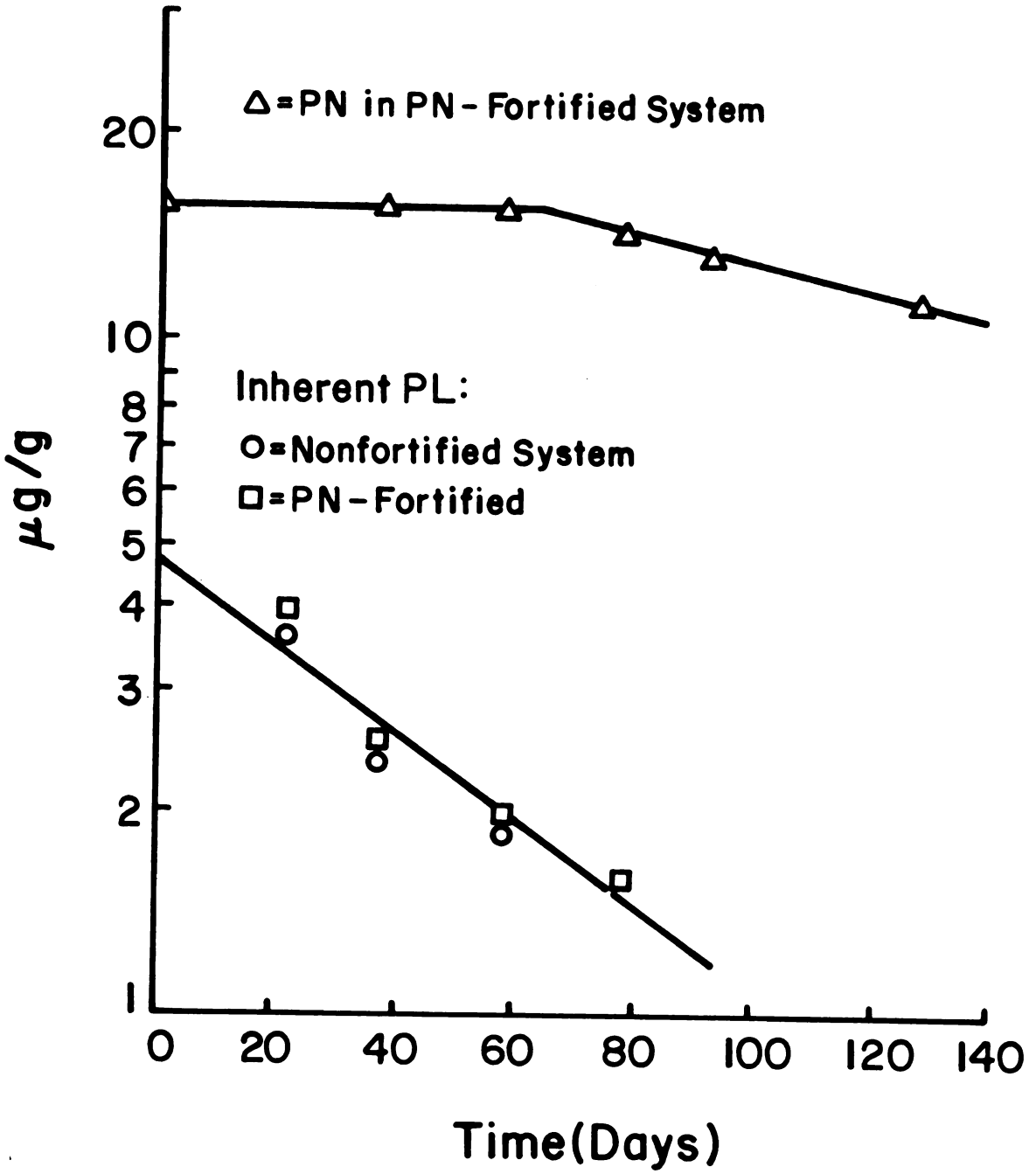


Figure 16. Degradation of added PN and inherent PL in dehydrated model systems stored in TDT cans at 37°C.

Table 21. Kinetic data for the loss of the B_6 vitamers from dehydrated model systems during storage in TDT cans at 37°C. Vitamin B_6 assays performed by the HPLC procedure.

Model System Fortification	Reaction	k^a (days^{-1})	Correlation Coefficient	$t_{1/2}^b$ (days)
Nonfortified				
	Loss of inherent PL	.0170 ± .0027	-.9876	41
Pyridoxine				
	Loss of inherent PL	.0151 ± .0020	-.9826	46
	Loss of PN (> 58 days)	.0049 ± .0005	-.9889	141
Pyridoxal Phosphate				
	Loss of PL	.0158 ± .0019	-.9667	44
Pyridoxamine				
	Loss of PM	.0200 ± .0026	-.9753	35
	Loss of formed PL	.0165 ± .0040	-.9667	42
	Loss of total PM + PL	.0165 ± .0027	-.9377	42

[a]First order rate constant ± standard deviation. Determined by linear regression analysis of semilogarithmic plots.

[b]Half life.

disappearance of PM was slightly higher (0.0200 day^{-1}). The rate constant for the first order degradation of PN was observed to be 0.0049 day^{-1}.

The analysis of the vitamin B_6 contents of the model systems packaged in 303 cans and stored for 128 days at 37°C revealed significantly greater stability of the vitamin in these containers than in the TDT cans (Table 22). The detection of PM after 128 days storage in the 303 cans was indicative that the rate of conversion of PM to PL was slower in storage containers with the large headspace.

Determination of 4-Pyridoxic Acid in Dehydrated Model Systems after Storage

The slow oxidation of PL and PLP to 4-pyridoxic acid and its 5'-phosphate has been observed in various liquid nonenzymatic systems (Ikawa and Snell, 1954; Hamilton and Revesv, 1966; Rotilio, *et al.*, 1970). The formation of 4-PA during the storage of the dehydrated model systems at 37°C was monitored in these studies. After 128 days of storage, the model systems stored in TDT cans were analyzed by HPLC for the presence of 4-PA. 4-Pyridoxic acid was detected only in the PLP-fortified model system. Only 2.6% of the initially added PLP was found as 4-PA (or its 5'-phosphate). These results indicated that 4-PA was a minor degradation product of PLP, since 88% of the PLP added to the dehydrated

Table 22. Vitamin B_6 content in dehydrated model systems after storage for 128 days at 37°C in TDT and 303 cans. HPLC analysis, fluorescence detection.[a,b]

Model System Fortification	TDT Cans				303 Cans			
	PN	PM	PL	Total	PN	PM	PL	Total
Nonfortified	ND	ND	tr	<0.5	ND	ND	tr	<0.5
PN	10.8	ND	tr	10.8	14.2	ND	tr	14.2
PM	ND	ND	2.7	2.7	ND	4.3	4.7	9.0
PLP	ND	ND	2.7	2.7	ND	ND	4.8	4.8

[a]μg/g model system. Means of duplicate determinations.

[b]ND = not detectable; tr = trace, too low to quantitate. Trace data not used in calculation of total B_6.

model system was lost during the 128-day storage period. The presence of 4-PA as its lactone could not be determined by the HPLC assay procedure because this compound does not fluoresce in the acid environment of the HPLC mobile phase.

Determination of Pyridoxyllysine in Dehydrated Model Systems after Storage

In order to determine the biologically available vitamin B_6 in the model systems by the rat bioassay, the systems in the TDT and 303 cans were pooled after storage for 128 days at 37°C. As previously discussed, researchers have suggested that vitamin B_6 derivatives, such as certain pyridoxylamino compounds, may possess only partial B_6 activity or may act as antivitamins (Snell and Rabinowitz, 1948; Heyl, *et al.*, 1952; McCormick and Snell, 1961). In order to evaluate the effect of pyridoxylamino compound formation on the biological availability of vitamin B_6, model system protein hydrolysates were quantitatively examined for ε-pyridoxyllysine. The hydrolysates were analyzed solely for ε-pyridoxyllysine since numerous researchers have observed that essentially all of the PLP bound to proteins by reductive coupling was attached to the ε-amino group of lysine (Dempsey and Christensen, 1962; Dempsey and Snell, 1963; Anderson, *et al.*, 1966; Page, *et al.*, 1968; Ronchi, *et al.*, 1969; Anderson, *et al.*, 1971).

The synthesized pyridoxyllysine was examined for purity by several methods. The UV-visible absorption spectrum of pyridoxyllysine in 0.1 M ammonium acetate, pH 7.0, demonstrated the characteristic maxima at 323-325 and 253-254 nm (Figure 17). Thin layer chromatography on cellulose MN-300 gave a single ninhydrin reactive spot which exhibited a strong blue fluorescence when irradiated with ultraviolet light at 254 or 366 nm. Analysis by HPLC demonstrated a single major peak and two minor contaminants (Figure 18). The crystalline pyridoxyllysine was used as a standard without further purification.

In the analysis of the hydrolysates of the proteins from the pooled TDT and 303 can model systems, only the PLP-fortified system exhibited detectable levels of pyridoxyllysine (Figure 18). The identity of the pyridoxyllysine chromatographic peak from the PLP-fortified model system was confirmed by comparison of the excitation and emission spectra of the collected peak with those of the prepared pyridoxyllysine standard (Figure 19). Although the collected fraction from the model system exhibited a shoulder on the excitation peak at approximately 340 nm, probably the result of incomplete resolution on the chromatogram, the basic spectral features confirmed the identity of the peak as pyridoxyllysine. Quantitation of the chromatogram revealed that 4.17 μg PL/g of the PLP-fortified model system participated in

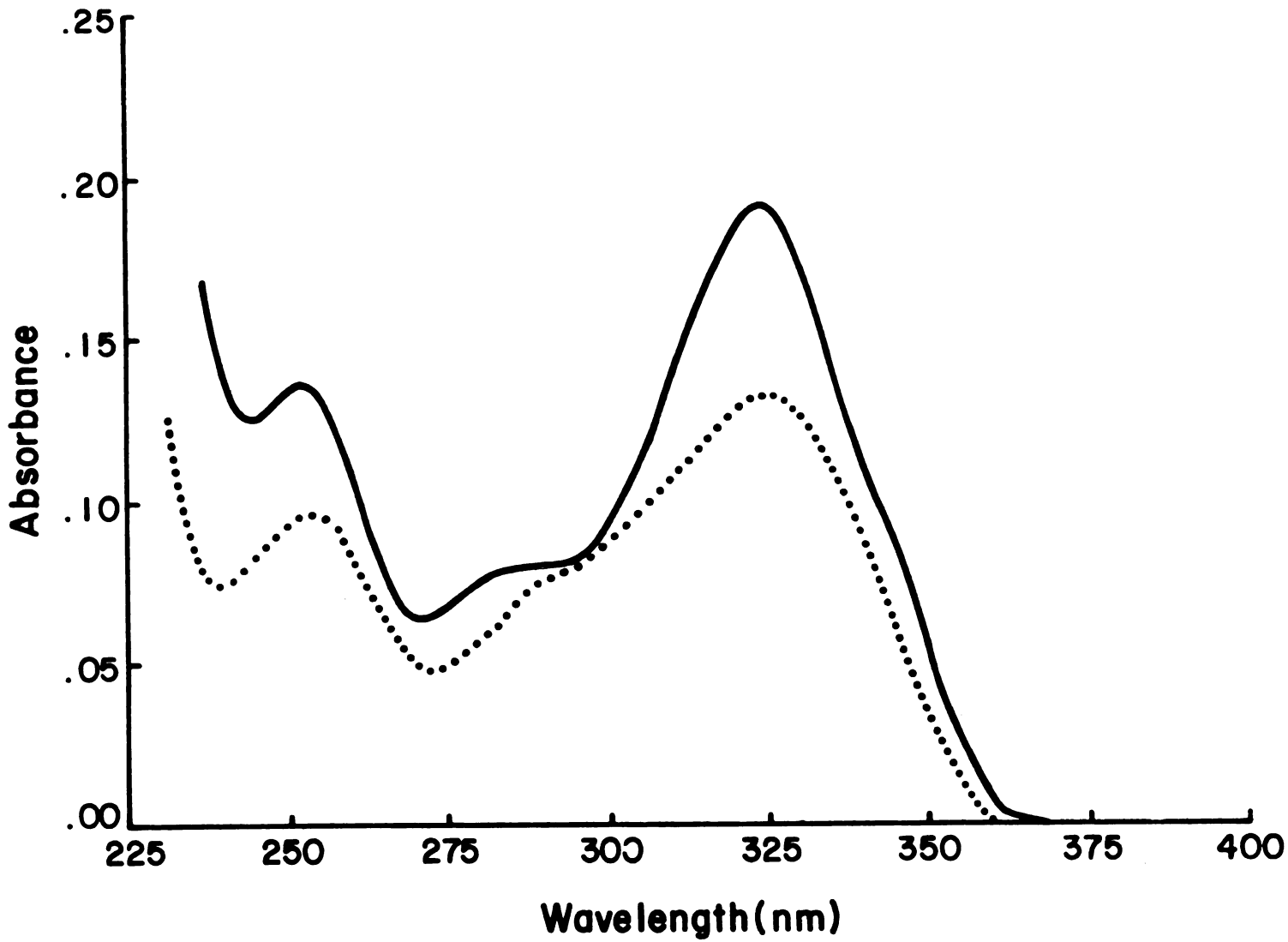


Figure 17. Absorption spectrum of pyridoxyllysine (·····); difference spectrum of 1 mg/ml PP-BSA versus 1 mg/ml BSA (———). Buffer of both spectra was 0.1 M ammonium acetate, pH 7.0.

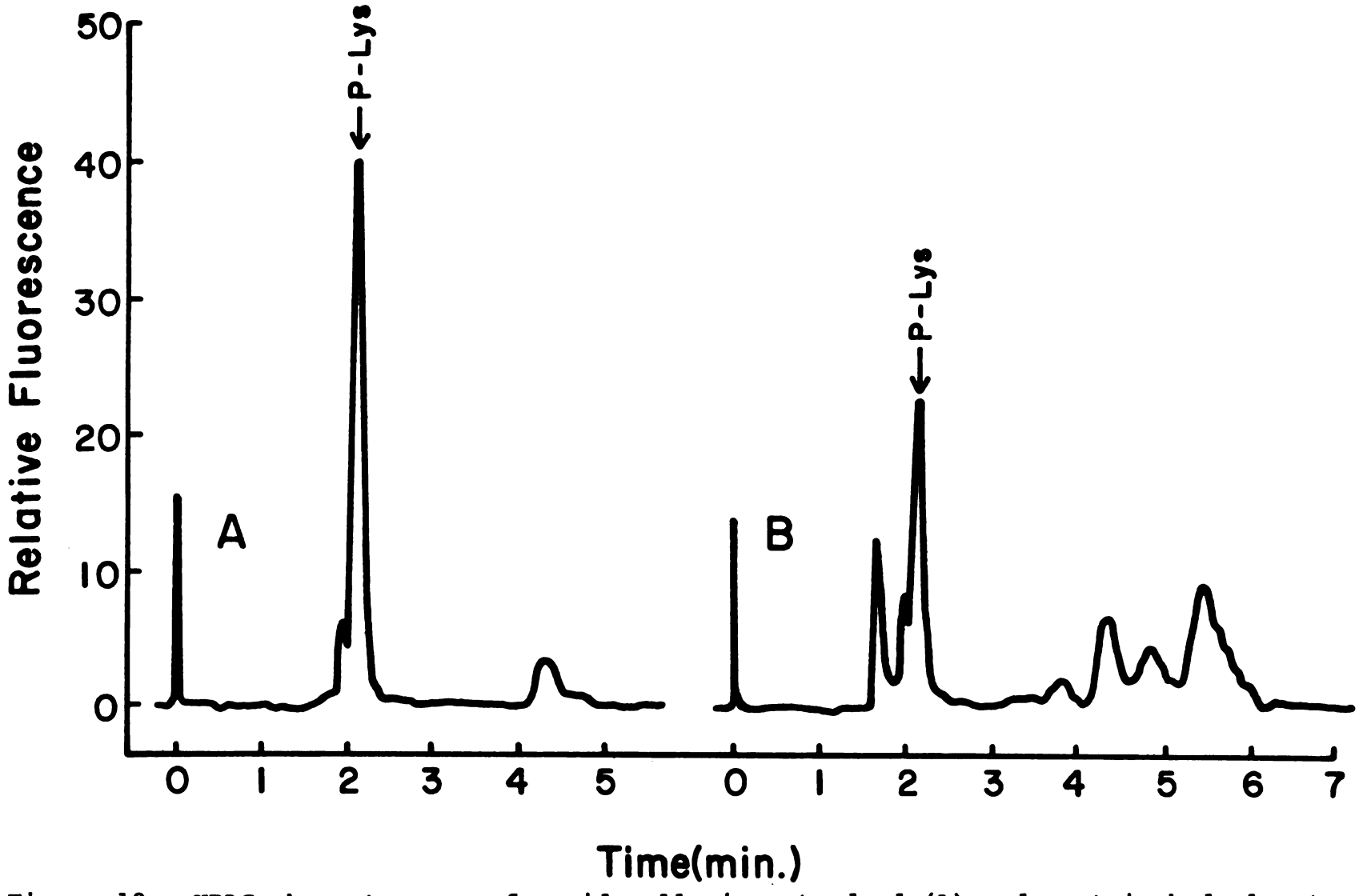


Figure 18. HPLC chromatograms of pyridoxyllysine standard (A) and protein hydrolysate of PLP-fortified model system (B). P-Lys = pyridoxyllysine.

Figure 19. Fluorescence excitation (Ex.) and emission (Em.) spectra of crystalline pyridoxyllysine standard (————) and collected pyridoxyllysine chromatographic peak from PLP-fortified model system protein hydrolysate (·····). Buffer = pH 2.2 HPLC mobile phase.

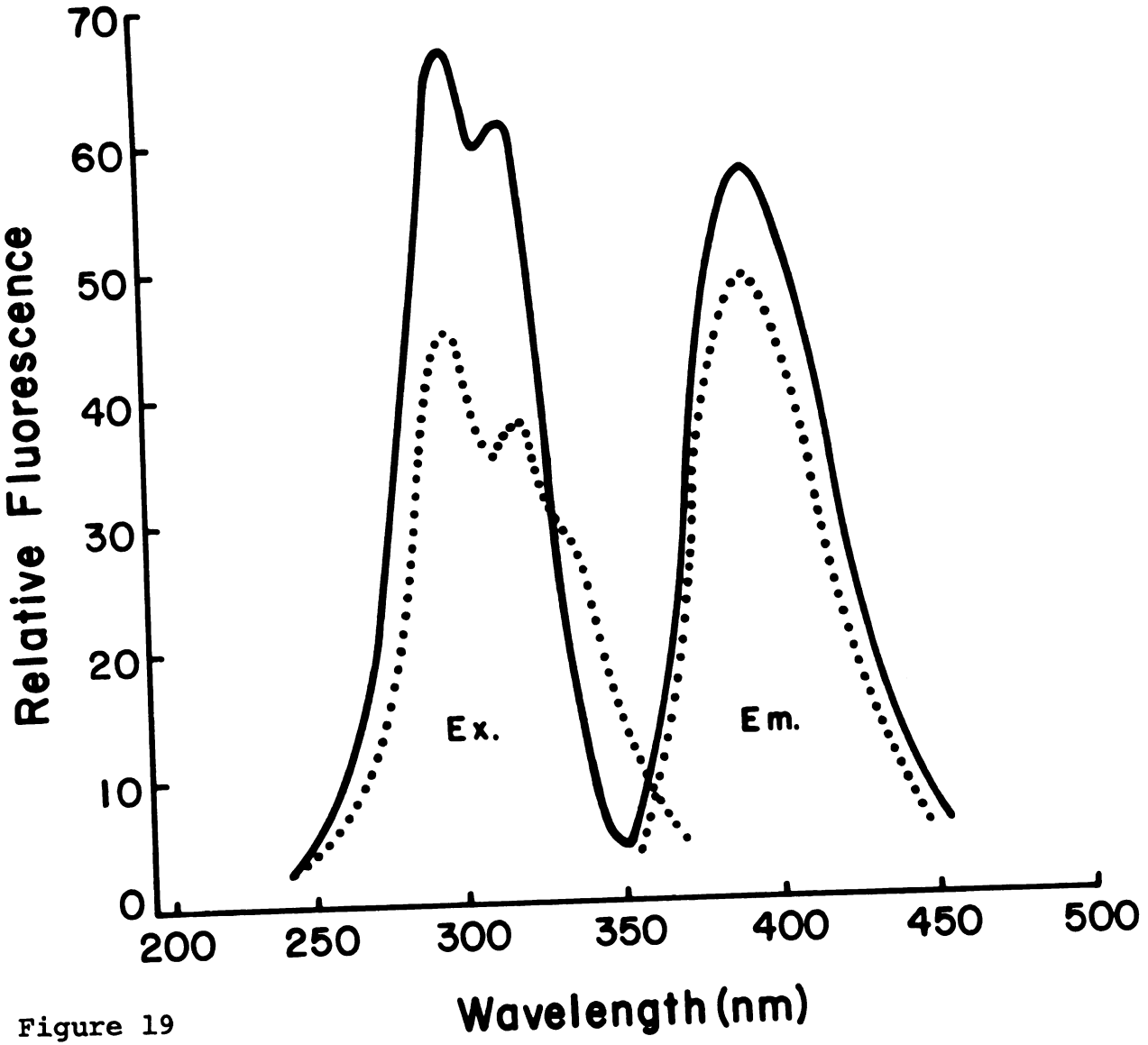

Relative Fluorescence
70
60
50
40
30
20
10
0
200
250
300
350
400
450
500
Wavelength (nm)
Ex.
Em.

Figure 19

the formation of pyridoxyllysine (Table 24). Therefore, the reductive coupling of PLP to protein amino groups represented a significant mechanism for the loss of biologically available PLP from the model systems during storage at 37°C at a water activity of about 0.6.

Determination of Total Vitamin B_6 in Dehydrated Model Systems after Storage

After pooling the model systems stored for 128 days in TDT and 303 cans at 37°C, total vitamin B_6 was determined by microbiological, semiautomated fluorometric, and HPLC methods. The vitamin B_6 data obtained by microbiological (Table 25) and HPLC (Table 26) methods were in good agreement. This was not true for the results of the semiautomated fluorometric assay (Table 27) which did not correlate well with either HPLC or microbiological data. As observed in the study concerning the effects of roasting on vitamin B_6, the semiautomated fluorometric values were consistently higher than those of either of the other methods. Analysis of the data for total B_6 in the fortified dehydrated model systems (PN, PM, and PLP) revealed discrepancies in the semiautomated fluorometric data ranging from 2.0 to 4.6 fold relative to the results from the microbiological assay and 1.9 to 3.2 fold relative to the HPLC assay data. As previously observed, the greatest relative differences were observed in the analysis

Table 23. Determination of 4-pyridoxic acid in model systems stored for 128 days in TDT cans at 37°C.

Model System Fortification	μg 4-PA/g[a,b,c]	Percentage of Initial B_6
Nonfortified	ND	---
PN	ND	---
PM	ND	---
PLP	0.39 ± 0.02	2.6 ± 0.1

[a]Mean and standard deviation. Duplicate determinations.

[b]Detection limit (signal/noise = 3) approximately 3 ng/50 μl injection, corresponding to 0.08 μg/g model system.

[c]ND = not detectable.

Table 24. Determination of pyridoxyllysine in model systems from TDT and 303 cans pooled after storage for 128 days at 37°C. Data expressed as bound pyridoxal/g model system.

Model System Fortification	μg Bound PL/g[a,b,c]
Nonfortified	ND
PN	ND
PM	ND
PLP	4.17 ± 0.03

[a]Mean and standard deviation. Duplicate determinations.

[b]Detection limit (signal/noise = 3) approximately 6 ng/50 μl injection, corresponding to 12 ng/mg protein or 0.6 μg/g model system.

[c]ND = not detectable.

Table 25. Microbiological assay of vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C.[a]

Model System Fortification	μg Total B_6/g
Nonfortified	<0.5
PN	11.9 ± 2.1
PM	6.3 ± 0.1
PLP	2.9 ± 0.6

[a]Mean and standard deviation. Duplicate determinations.

Table 26. High performance liquid chromatographic assay of vitamin B6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C.[a,b,c]

Model System Fortification	PN	PM	PL	Total
Nonfortified	ND	ND	tr	<0.5
PN	12.8 ± 0.1	ND	ND	12.8 ± 0.1
PM	ND	3.3 ± 0.4	4.2 ± 0.4	7.5 ± 0.6
PLP	ND	ND	4.2 ± 0.3	4.2 ± 0.3

[a]μg/g model system. Mean and standard deviation of duplicate determinations.

[b]ND = not detectable; tr = trace, too low to quantitate.

Table 23. Determination of 4-pyridoxic acid in model systems stored for 128 days in TDT cans at 37°C.

Model System Fortification	μg 4-PA/g[a,b,c]	Percentage of Initial B_6
Nonfortified	ND	---
PN	ND	---
PM	ND	---
PLP	0.39 ± 0.02	2.6 ± 0.1

[a]Mean and standard deviation. Duplicate determinations.

[b]Detection limit (signal/noise = 3) approximately 3 ng/50 μl injection, corresponding to 0.08 μg/g model system.

[c]ND = not detectable.

Table 24. Determination of pyridoxyllysine in model systems from TDT and 303 cans pooled after storage for 128 days at 37°C. Data expressed as bound pyridoxal/g model system.

Model System Fortification	μg Bound PL/g[a,b,c]
Nonfortified	ND
PN	ND
PM	ND
PLP	4.17 ± 0.03

[a]Mean and standard deviation. Duplicate determinations.

[b]Detection limit (signal/noise = 3) approximately 6 ng/50 μl injection, corresponding to 12 ng/mg protein or 0.6 μg/g model system.

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Table 25. Microbiological assay of vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C.[a]

Model System Fortification	μg Total B_6/g
Nonfortified	<0.5
PN	11.9 ± 2.1
PM	6.3 ± 0.1
PLP	2.9 ± 0.6

[a]Mean and standard deviation. Duplicate determinations.

Table 26. High performance liquid chromatographic assay of vitamin B6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C.[a,b,c]

Model System Fortification	PN	PM	PL	Total
Nonfortified	ND	ND	tr	<0.5
PN	12.8 ± 0.1	ND	ND	12.8 ± 0.1
PM	ND	3.3 ± 0.4	4.2 ± 0.4	7.5 ± 0.6
PLP	ND	ND	4.2 ± 0.3	4.2 ± 0.3

[a]μg/g model system. Mean and standard deviation of duplicate determinations.

[b]ND = not detectable; tr = trace, too low to quantitate.

of the nonfortified systems. Comparing the data for the semiautomated fluorometric and HPLC assays for the individual vitamers, the greatest difference was consistently observed in the PM fraction, while the PL and PN data correlated more closely.

Table 27. Semiautomated fluorometric determination of vitamin B_6 in dehydrated model systems pooled after storage for 128 days at 37°C in TDT and 303 cans.[a,b]

Model System Fortification	PN	PM	PL	Total
Nonfortified	3.8 ± 0.3	8.0 ± 1.5	1.7 ± 0.3	13.5 ± 1.6
PN	12.9 ± 0.8	7.5 ± 1.6	3.7 ± 0.3	24.0 ± 1.8
PM	0.6 ± 0.5	9.7 ± 1.1	6.1 ± 1.0	16.3 ± 1.6
PLP	0.5 ± 0.7	8.5 ± 1.8	4.4 ± 1.8	13.4 ± 2.6

[a]μg/g model system.

[b]Mean and standard deviation of triplicate analyses.

Biologically Available Vitamin B_6 in Dehydrated Model Systems after Storage

The results of the rat bioassay for available vitamin B_6 in the dehydrated model systems which were pooled after 128 days storage in TDT and 303 cans at 37°C (Bioassay 2) are presented in Table 28. (The data for the diets containing PP-BSA will be discussed in the next section.) The dose-response curves based on the response of rats fed the 0-1.0 μg PN/g standard

Table 28. Rat Bioassay 2. Determination of available vitamin B_6 in model systems pooled after 128 days storage in TDT and 303 cans at 37°C, and analysis of biological activity of PP-BSA. Mean and standard error, 8-9 rats per group.

Diet	Growth	Feed Consumed	Growth (g) / g Feed Consumed	Erythrocyte AspAT	
				Activity	PLP Stimulation
	(g)	(g)	(g/g)	(mU/mg Hb)	(%)
Standard					
0.00 µg PN/g	48 ± 2	190 ± 6	0.254 ± 0.022	3.17 ± 0.14	148.2 ± 5.7
0.25 "	75 ± 4	229 ± 11	0.329 ± 0.019	5.00 ± 0.45	119.4 ± 4.0
0.50 "	99 ± 5	254 ± 10	0.390 ± 0.025	5.15 ± 0.70	99.8 ± 7.2
0.75 "	117 ± 5	278 ± 11	0.422 ± 0.015	5.31 ± 0.42	80.8 ± 2.0
1.00 "	131 ± 5	294 ± 8	0.443 ± 0.025	5.85 ± 0.38	58.8 ± 2.4
4% Stored Model Systems					
Nonfortified	46 ± 3	193 ± 8	0.255 ± 0.044	3.28 ± 0.21	150.2 ± 7.3
PN	113 ± 3	279 ± 12	0.407 ± 0.009	5.00 ± 0.39	98.5 ± 7.3
PM	69 ± 2	231 ± 6	0.299 ± 0.012	4.27 ± 0.33	105.8 ± 5.7
PLP	67 ± 5	217 ± 8	0.295 ± 0.013	3.51 ± 0.13	140.8 ± 12.8
PP-BSA (0.5 g bound PL/g diet)					
PP-BSA only	88 ± 3	248 ± 7	0.345 ± 0.013	4.35 ± 0.20	108.4 ± 6.0
PP-BSA + 0.25 µg PN/g	100 ± 5	265 ± 12	0.375 ± 0.011	4.92 ± 0.38	102.4 ± 5.9

diets were calculated, and their regression parameters are presented in Table 29. Log dose-response functions provided the best linear fit for the growth, growth per gram feed, and PLP stimulation of AspAT data, while a double reciprocal plot was employed for the erythrocyte AspAT activity data.

The bioassay estimates of biologically available vitamin B_6 in the model systems which had been stored 128 days at 37°C are presented in Table 30. The estimates of available B_6 by each of the response parameters were not significantly different in the case of the non-fortified and PN- and PLP-fortified model systems ($P < .05$). However, for the PM-fortified system, the estimates based on growth and growth per gram feed were significantly less than those determined by PLP stimulation of AspAT, and the growth-based estimate was significantly less than the estimate based on AspAT activity ($P < .05$). As previously observed, the precision of the estimates was generally highest when employing growth and growth per gram feed as the response criteria.

Generally excellent correlation was observed between the rat bioassay, microbiological, and HPLC methods for determination of vitamin B_6 in the dehydrated model systems (Table 31). No significant differences were observed between the total vitamin B_6 in the model systems, as determined by the microbiological and HPLC

Table 29. Linear regression parameters of the dose-response curves. Bioassay 2: available B_6 in stored model systems and biological activity of PP-BSA.[a]

X[b]	Y	Y-intercept	Slope	Correlation Coefficient
log (PN + 1)	Growth (g)	48.9 ± 3.2	276.3 ± 16.6	+.9377
log (PN + 1)	Growth (g)/g Feed	0.236 ± 0.007	0.640 ± 0.034	+.9496
log (PN + 1)	AspAT PLP Stimulation	148.7 ± 3.7	-289.3 ± 19.1	-.9259
$(PN + 1)^{-1}$	$(\text{AspAT Activity})^{-1}$	0.042 ± 0.039	0.260 ± 0.053	+.6196

[a]These dose-response curves represent functions which provided the least variability about a linear relationship, i.e., those which provided the highest correlation coefficients for each response criterion.

[b]All X data represent the dietary concentration of added pyridoxine. When the log was taken, the concentrations were coded by the addition of 1 to each value.

Table 30. Biologically available vitamin B_6 in model systems pooled after storage for 128 days in TDT and 303 cans at 37°C. Data represent μg available B_6/g model system.[a]

Model System Fortification	Response Criterion[b,c]			
	Growth	Growth (g) / g Feed Consumed	Erythrocyte AspAT	
			Activity	PLP Stimulation
Nonfortified	-0.5 ± 0.7	-0.7 ± 0.3	-1.2 ± 2.0	-0.3 ± 1.2
PN	17.7 ± 1.1	17.2 ± 1.4	16.8 ± 4.6	12.3 ± 2.1
PM	4.5 ± 0.5	3.7 ± 1.2	9.1 ± 3.2	12.2 ± 1.4
PLP	4.2 ± 1.2	3.2 ± 1.3	1.8 ± 1.2	1.6 ± 2.9

[a]Mean and standard error, 8 rats per assay group.

[b]Response criterion refers to the index of B_6 nutritional status used in calculation of the available vitamin B_6 in the model system.

[c]Estimates of available vitamin B_6 by each of the response criteria not significantly different in nonfortified, PN-, and PLP-fortified systems. In the PM-fortified system, estimates by growth and growth/g feed were significantly less than those by PLP stimulation, while the growth estimate was significantly less than that based on AspAT activity ($P < .05$).

Table 31. Comparison of semiautomated fluorometric, microbiological, HPLC, and rat bioassays for vitamin B_6 in model systems pooled after 128 days storage in TDT and 303 cans at 37°C.[a,b]

Model System Fortification	Response Criterion				Microbiol.	HPLC	Fluorom.
	Growth	Growth (g) / g Feed Consumed	AspAT				
			Activity	PLP Stimulation			
Nonfortified	-0.5	-0.7	-1.2	-0.3	0.5	0.5	13.5
PN	17.7	17.2	16.8	12.3	11.9	12.8	24.0
PM	4.5	3.7	9.1	10.2	6.3	7.5	16.3
PLP	4.2	3.2	1.8	1.6	2.9	4.2	13.4

[a] μg total vitamin B_6/g model system.

[b] Fluorometric data significantly greater than all other estimates. No significant differences between microbiological and HPLC values. Microbiological and HPLC values were not significantly different from the bioassay estimates for each model system ($P < .05$).

methods, and any of the rat bioassay estimates, indicating no significant loss of bioavailability during the storage period ($P < .05$). That is, in spite of the losses of vitamin B_6 during storage of the dehydrated model systems, the remaining B_6 exhibited complete bioavailability. In contrast to the correlation exhibited between the microbiological, HPLC, and rat bioassay data, the results of the semiautomated fluorometric assay were observed to be consistently higher than the rat bioassay results. For all model systems, semiautomated fluorometric values were greater by a mean of 10.6 ± 3.0 μg vitamin B_6/g.

Biological Activity of Phosphopyridoxyl-Bovine Serum Albumin

Analysis of Phosphopyridoxyl-Bovine Serum Albumin

The dialyzed, freeze dried, PP-BSA was examined to determine the concentration of pyridoxylamino residues prior to analysis of their biological activity by rat bioassay. The ultraviolet absorption difference spectrum of PP-BSA against a solution of bovine serum albumin at neutral pH (Figure 16) permitted measurement of the pyridoxylamino groups using their molar absorptivity at 323 nm (Dempsey and Christensen, 1962). Employing this spectrophotometric method, 2.32 moles of pyridoxylamino groups were detected per mole of serum albumin (68,000 daltons) in the PP-BSA preparation. This was equivalent to 7.81 μg bound PLP/mg protein or 5.76 μg bound PL/mg

protein. Measurement of pyridoxyllysine residues in a PP-BSA acid hydrolysate by HPLC confirmed this value, and indicated that essentially all of the pyridoxylamino groups of the PP-BSA were in the form of ε-pyridoxyllysine residues.

Chromatography of the PP-BSA on Sephadex G-25 revealed a low concentration of UV-absorbing compounds, suggesting trace contamination with free vitamin B_6. Identification and quantitation of the contaminants was performed by semiautomated fluorometric and HPLC assays for free vitamin B_6 (Table 32). Both fluorometric and HPLC procedures revealed approximately 1 μg total free B_6/mg protein. In view of the greater precision of the HPLC analysis, the value of 1.06 μg/mg was used in subsequent calculations to quantitate the free vitamin B_6 contributed by PP-BSA when added to the rat diets. Microbiological assay of the PP-BSA revealed an apparent value of only 0.46 μg/mg (Table 32). These results suggest incomplete utilization of the free B_6 by the assay organism, possibly as a result of inhibition by the PP-BSA pyridoxylamino groups.

Pyridoxylamino Group Utilization and Antivitamin Activity

The vitamin B_6 activity of protein bound pyridoxylamino groups was determined by rat bioassay of diets with PP-BSA as the sole source of vitamin B_6. The

Table 32. Determination of free vitamin B_6 in dialyzed PP-BSA.[a,b]

Analytical Method	PN	PM	PL	Total
HPLC	0.79 ± 0.06	ND	0.27 ± 0.03	1.06 ± 0.07
Fluorometric	0.46 ± 0.04	0.29 ± 0.13	0.21 ± 0.04	0.96 ± 0.19
Microbiological	---	---	---	0.46 ± 0.07

[a] μg vitamin B_6/mg protein.

[b] Mean and standard deviation of triplicate determinations.

addition of PP-BSA to the diets was calculated to provide 0.5 μg bound PL/g diet. In the same study, the effects of dietary protein bound pyridoxylamino groups on the utilization of free pyridoxine were determined by rat bioassay of diets containing PP-BSA at the same level, as well as an additional 0.25 μg PN/g diet. The results of the rat bioassay (Bioassay 2) are shown in Table 28. The vitamin B_6 activity of the diets was determined by comparison of the responses of the rats of each dietary group to the dose-response curves (Table 29).

The apparent vitamin B_6 content of diets containing PP-BSA and PP-BSA fortified with free PN are shown in Table 33. These data indicated that approximately 60% of the pyridoxylamino group B_6 in PP-BSA was available for utilization as vitamin B_6 by the rats. Each estimate was corrected for the contribution of the free B_6 in the PP-BSA by subtraction of 0.092 μg/g diet. No significant differences were observed between the bioassay estimates based on the various response parameters for either diet ($P < .05$).

Comparison of the observed response of the rats to the 0.25 μg PN/g diet with the response predicted by the dose-response curves was used to determine the extent of utilization of free PN added to the diet. The results indicated a mean utilization of only 44.5% of the free PN (Table 33).

Table 33. Biological activity of pyridoxyllysine residues in PP-BSA. PP-BSA was added to both diets to provide 0.50 μg bound PL/g diet.[a,b]

Response Criterion	PP-BSA Only		PP-BSA + 0.25 μg PN/g Diet	
	μg/g Diet	% Activity[c]	μg/g Diet	% PN Utilized[d]
Growth	0.30 ± 0.03	60	0.45 ± 0.07	58
Growth (g)/g Feed	0.31 ± 0.04	62	0.41 ± 0.06	40
AspAT Activity	0.30 ± 0.08	59	0.43 ± 0.13	53
AspAT PLP Stimulation	0.29 ± 0.06	57	0.35 ± 0.05	27

[a]Mean and standard error, 9 rats per group.

[b]μg/g data corrected for the 0.092 g free B_6/g diet contributed by the trace contaminants in the PP-BSA (as determined by HPLC).

[c]Percent activity refers to the activity of protein bound PL relative to that of free PN.

[d]Percent PN utilized refers to the comparison of the observed response to the added 0.25 μg PN/g to that expected from the dose-response curves.

DISCUSSION

Model food systems were employed to study the stability and bioavailability of vitamin B_6 as a function of selected processing and storage conditions. The model system approach was chosen to permit: (1) ready isolation of individual system components for analysis, (2) determination of the effects of various ingredients by varying the model system composition, and (3) direct study of the stability and bioavailability of the individual vitamers because of the low levels of vitamin B_6 inherently present. The results obtained in these studies could readily serve as a basis for further investigations of the effects of ingredients, processing, and storage conditions on the stability of vitamin B_6 in the model systems.

Interaction of Pyridoxal and Pyridoxal Phosphate with Peptides in a Liquid Model Food System during Thermal Processing

Thermal stability of the peptide mixture was required to facilitate the reduction of Schiff bases with sodium borohydride, isolation of the model system peptides by gel filtration chromatography, and analysis

of peptide bound vitamin B_6 by spectrophotometric methods. The heterogeneity of the peptide mixture, as indicated by the peptide mapping results and molecular weight range (700-16,500), was considered to be an advantage of these peptides as a model for complex mixtures of food proteins.

The UV-visible absorption difference spectra of each peptide fraction versus its respective nonfortified blank demonstrated the presence of bound PL and PLP. The absorption maxima at 323-325 nm and 253-254 nm (Figure 7) were identical to those observed for pyridoxylamino and substituted aldamine complexes (Dempsey and Christensen, 1962; Kierska and Maslinski, 1971; O'Leary, 1971). These difference spectra were also identical to those exhibited by a wide variety of proteins with PLP bound by reduced linkages (Dempsey and Christensen, 1962; Anderson, et al., 1966; Page, et al., 1968; Ronchi, et al., 1969; Johnson and Deal, 1970). Thus, the total bound forms of the vitamin represented the sum of borohydride-reduced Schiff base derivatives plus nonreducible complexes.

The examination of peptides recovered at neutral pH without prior borohydride reduction revealed difference spectra identical to those obtained in the analysis of total bound PL and PLP. These results confirmed that nonreducible B_6 complexes were formed during the thermal processing of all liquid model systems. The absence of an absorption maximum at 410-430 nm indicates

that none of the bound PLP derivatives were present as a Schiff base (Kent, et al., 1958). This observation supports the hypothesis that the labile Schiff base complexes would dissociate during the gel filtration isolation of the peptides, leaving only the stable nonreducible forms.

Several researchers have demonstrated that PLP bound as a Schiff base in specific hydrophobic sites of glycogen phosphorylase and serum albumin exhibits absorption spectra similar to those of the nonreducible complexes. In addition, these forms of bound PLP are resistant to $NaBH_4$ reduction (Shaltiel and Cortijo, 1970; Hilak, et al., 1975; Cortijo, et al., 1976). However, in the thermally denatured peptides used in this study, the existence of such specific apolar binding sites is highly unlikely.

Further evidence for the formation of nonreducible complexes was observed in the fluorescence spectra of the isolated peptides upon excitation at 280 nm. Proteins with reduced pyridoxylamino groups have been shown to exhibit a decrease in the native fluorescence of tryptophan residues as a result of the radiationless transfer of electronic energy to the bound B_6 chromophores (Churchich, 1965). The fluorescence emission spectra of the peptides from PLP-fortified model systems, when compared to the spectra of peptides from nonfortified

blanks, demonstrated this quenching effect (Figure 8). Thus the presence of nonreducible PLP-peptide conjugates was confirmed by two independent spectrophotometric methods.

In the determination of peptide bound pyridoxylamino compounds, the peptides were isolated from the model systems at pH 2.5 to induce dissociation of the acid labile substituted aldamine and Schiff base complexes. Upon readjustment of the recovered peptide solutions to pH 7.0, the difference spectra confirmed the presence of peptide bound PLP as pyridoxylamino derivatives in each of the liquid model systems, regardless of treatment with glucose and/or ascorbic acid.

Quantitation of levels of total bound vitamin B_6 (Table 5) indicated that PLP was significantly more reactive than PL ($P < .001$). The difference in reactivity, previously observed in many coenzyme-apoenzyme binding studies, has been attributed to the inhibition of internal hemiacetal formation by the 5'-phosphate group of PLP (Heyl, et al., 1951). Because of this blocking effect, the concentration of free aldehyde is much greater in solutions of PLP than in PL solutions of comparable molar concentration. Presumably, electrostatic effects between the phosphate group and cationic sites on the peptides may also play a role in the greater affinity of PLP. A

mean of 20% of the total model system PLP was peptide bound, compared to only 6.7% of the total PL.

Comparison of the mean value of the data of Table 5 for total bound PLP (Schiff base, substituted aldamine, and pyridoxylamino) with that of Table 6 for PLP bound through nonreducible linkages indicated that, at neutral pH, approximately 60% of the total bound PLP existed as a nonreducible complex. These data (Table 6) suggested that losses of biologically available B_6 may occur through complex formation. The direct determination of the pyridoxylamino complexes as acid stable, non-reducible conjugates (Table 7) confirmed the existence of bound PLP in a form which has limited bioavailability. Binding in this manner would not represent a major mechanism for the loss of available vitamin B_6, as indicated by the fact that only 9.7 ± 6.4% of the total PLP in the systems was involved in these complexes.

The analysis of the effects of glucose and ascorbic acid on the binding of PL and PLP may have been hampered by the precision of the data. Statistically, neither factor along nor in combination significantly affected the extent or manner of PL or PLP binding ($P < .05$).

Several qualitative effects were apparent which correlated with chemical analysis of the peptides. Data shown in Table 5 for the total bound vitamin B_6 indicate

that, in systems treated only with ascorbic acid, the extent of binding was partially depressed. Tables 6 and 7 also exhibited this trend. This mechanism, although unclear, may involve the competition between ascorbate degradation products and PL or PLP for peptide free amino groups. Free SH levels in the peptides in all ascorbate-treated model systems were significantly higher than in nontreated systems (Table 8). The experimentally determined SH levels in the heat treated peptides, which were comparable to those of denatured milk serum proteins (Srncova and Davidek, 1972), indicated a reduction of disulfide bonds during the heat treatment. These SH data, when correlated with the levels of total bound PL and PLP (Table 5), suggest that the binding of these B_6 vitamers was wholely independent of the concentration of peptide SH groups. This conclusion is supported by the kinetic results of Schonbeck, et al. (1975), which demonstrated that the affinity of amino groups toward PLP was much greater than that of SH groups. Since the concentration of peptide amino groups was approximately 50 fold greater than the SH concentration, the reaction of PL and PLP with amino groups would be greatly favored in most food systems. In total, these observations suggest that SH groups would not significantly participate in the mechanism of binding by proteins or peptides.

The reaction between PL and milk protein SH groups, which was proposed to explain the losses of biologically available vitamin B_6 in thermally sterilized milk (Bernhart, et al., 1960; Wendt and Bernhart, 1960), was not supported by the results of the present study and the findings of Schonbeck, et al. (1975). The data of Srncova and Davidek (1972) demonstrated that, although SH groups may be involved in the binding of vitamin B_6 to milk proteins, they only react with a small fraction of the total B_6 in the system. In addition, studies of SH reactions have never conclusively demonstrated the formation of bis-4-pyridoxyl disulfide in milk products, as postulated by Wendt and Bernhart (1960), except in milk experimentally fortified with unrealistically high levels of PL. The findings of Srncova and Davidek, which demonstrated a small loss of protein free SH groups when heated in the presence of PL, could be interpreted as indicating low levels of substituted aldamine formation, with SH groups acting as the nucleophile attacking the Schiff base linkage.

Inspection of the data of Tables 6 and 7 suggests that the presence of glucose may have a weak effect in inhibiting the formation of nonreducible complexes, probably by competing for free amino groups. Chemical analysis of the peptides after heat treatment of the liquid model systems indicated that the presence of

glucose induced a 26% loss of free amino groups. Such levels of browning in food processing have been widely reported; for example, Mauron (1960) found a 20% loss of available lysine in evaporated milk after retort sterilization. Thus, the level of browning routinely encountered in food as a result of thermal processing may retard the loss of available vitamin B_6 by reaction with free amino groups.

At the onset of these studies, it was postulated that ascorbic acid or reductones formed in browning reactions during processing may promote the reduction of Schiff bases to pyridoxylamino complexes. The experimental data, as discussed, contradict this hypothesis. The data of Table 7 demonstrate that the highest levels of pyridoxylamino compound formation occurred in control model systems containing no added ascorbate or glucose. Based on these experimental results, the mechanism of Schiff base reduction remains unclear. Conceivably, the reducing equivalents may originate with the peptides per se, possibly during the thermally induced degradation of certain amino acids.

The levels of pyridoxylamino compound formation (9.7 ± 6.4% of the total PLP) observed in these studies indicate that, under the conditions employed, losses of available vitamin B_6 by complex formation are of little nutritional significance. Therefore, these data do not

support the thermally induced 50% reduction of vitamin B_6 bioavailability in sterilized milk products reported by Tomarelli, et al. (1955). Davies, et al. (1959), observed an apparent thermal destruction of vitamin B_6 in milk during sterilization; however, they were unable to demonstrate any losses of bioavailability of the B_6 remaining after processing. In fact, they observed higher values for available B_6 as measured by rat or chick bioassays than were obtained for apparent total B_6 in microbiological (S. uvarum) analyses. Similar results, i.e. higher rat bioassay than S. uvarum results, were obtained by Lushbough, et al. (1959), in a study involving cooked meats. When taken in total, the present experimental results, along with those of Davies, et al. (1959), and Lushbough, et al. (1959), suggest that the specificity of the microbiological assay method employed by Tomarelli, et al. (1955), may have artifactually indicated losses of biological availability of the vitamin B_6 in the sterilized milk products.

As previously discussed, PLP has been found to react with proteins to a much greater extent than PL, PM, or PN (Anderson, et al., 1974). Since the losses of bioavailability due to the binding of PLP were quite low, it may be inferred that losses of the other vitamers by binding to proteins during food processing would be minimal.

Interactions of Pyridoxamine in a Liquid Model System during Thermal Processing

Although the losses of available vitamin B_6 by the binding of PL and PLP to peptides and proteins were found to be low, the reaction of PM with low molecular weight carbonyl compounds was postulated as another possible mechanism for the loss of available B_6. Following a thermal process of 20 minutes at 121°C, the vitamin B_6 concentration of PM-fortified liquid model systems was determined. Peptides were omitted from these systems to permit HPLC analysis of vitamin B_6. To ensure measurement of all biologically available forms of the vitamin, the model systems were adjusted to pH 2.5 after autoclaving to induce the dissociation of any acid labile complexes. The data of Table 9 indicate that, while glucose was involved in the partial conversion of PM to PL, it also participated in the degradation of PM. Ascorbic acid had a similar effect, although much less pronounced. These results suggest that the observed losses of PM proceded through a reaction involving the carbonyl groups of glucose, ascorbate, or their thermal degradation products. Efforts to detect PM-containing complexes by gel filtration chromatography were not successful. The low levels of PM degraded, in addition to the poor resolving power of the chromatographic method, may have precluded their detection.

The extent of conversion of PM to PL in these liquid model systems was very low. Similar results were obtained by Metzler and Snell (1952) in their findings that metal ions were required for the catalysis of transamination in aqueous solution.

In summary, the results of this study have demonstrated the involvement of glucose and ascorbic acid in the loss of PM during thermal processing. The losses of total vitamin B_6 in the PM-fortified liquid model systems under these conditions were quite low. Therefore, the thermally induced degradation and/or binding of PM probably does not represent an important pathway for the loss of biologically available vitamin B_6 during the retort processing of foods.

Vitamin B_6 Assay Methods

Comparison of the vitamin B_6 analyses of the dehydrated model system studies (Tables 11, 13, 18, and 31) provided an opportunity to determine the accuracy of the various assay methods (microbiological, HPLC, semiautomated fluorometric, and rat bioassay). The data indicate that, in general, the microbiological and HPLC methods correlated very well with each other, and were in close agreement with rat bioassay estimates based on growth and growth per gram feed consumed. Model systems used in these studies exhibited no significant losses of vitamin B_6 bioavailability during processing

or storage, therefore, the rat bioassay data served to confirm the validity of the microbiological and HPLC methods as estimators of biologically available vitamin B_6 in the dehydrated model systems. Unless prior animal bioassays were performed on other food products, the accuracy of the microbiological and HPLC methods in predicting biologically available vitamin B_6 could not be assumed.

In the case of all dehydrated model systems assayed, the semiautomated fluorometric assay data was significantly higher than HPLC, microbiological, and rat bioassay results. The greatest differential between HPLC and semiautomated fluorometric results was observed in the PM determination. This was unexpected because of the theoretically adequate blank to provide correction for non-B_6 fluorophores. In thin layer chromatographic (Gregory and Kirk, 1977a) and fluorescence spectrophotometric studies (Table 14), no non-B_6 interfering compounds could be detected. The initial studies of Gregory and Kirk (1977a) demonstrated that previous fluorometric methods based on 4-pyridoxic acid lactone formation were subject to interference by cyanide-reactive browning compounds. The present ion exchange preparative procedure was developed to alleviate this problem. However, the observed poor correlation with other assay methods suggests that the semiautomated

fluorometric assay is still subject to interference. The data of Table 13 indicate that the interfering compound(s) possesses heat sensitivity quite similar to that of PN, PM, and PLP. The fact that the fluorescence emission spectra of all model system fractions were identical to that of pure 4-pyridoxic acid lactone suggests that the interfering compound is converted to 4-pyridoxic acid lactone or a very similar product during the formation of the fluorophore. The heat sensitivity and fluorescence spectra indicate that the interfering compound may be a vitamin B_6 derivative which possesses little or no biological activity. Further research would be required to identify this compound and alleviate the problem of lack of specificity of the semiautomated fluorometric assay.

The results of Bioassays 1 and 2 (Tables 15 and 28) demonstrate dose-response relationships similar to those previously reported (Sarma, et al., 1946; Linkswiler, et al., 1951; Lushbough, et al., 1959; Beaton and Cheney, 1965; Brin and Thiele, 1967). The positive correlation between feed consumption and dietary vitamin B_6 concentration, when using an ad libitum feeding regimen, was previously observed by Beaton and Cheney (1965) and Yen, et al. (1976). Thiele and Brin (1966) observed that rats receiving adequate levels of PN exhibited a much greater growth response when fed ad libitum than

when pair-fed to rats receiving a nonfortified diet. The growth rate of pair-fed rats was only slightly greater than that of vitamin B_6 deficient rats in their study. Thus, for quantitation of a bioassay using growth as a response criterion, an ad libitum design must be employed. In such a feeding regimen, the results of the assay could be biased by any preference for or avoidance of the test diets by the rats. In the bioassays of this study, the high correlation between estimates of available B_6 based on growth and growth per gram feed consumed indicated that this was not a problem (Tables 17 and 30).

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Roasting Effects

The relative stability of the B_6 vitamers used in fortifying the model systems was determined by comparison of the total vitamin B_6 data of microbiological and semiautomated fluorometric analyses of control and roasted model systems. In spite of the high values obtained by the semiautomated fluorometric assay of the dehydrated model systems for total vitamin B_6, the percent loss calculated from the fluorometric assay correlated well with that determined microbiologically. Therefore, for relative measurements, the fluorometric assay appears to be valid. The data of Table 13 indicated that, within the limits of experimental error, no

differences in the stability of PN, PM, and PLP could be detected. All three underwent approximately 50 to 70% degradation as a result of the 25-minute roasting at 180°C. The apparent greater stability of PM as determined microbiologically was presumably due to the lower response of the organism to the high level of PM in the nonroasted system, which was lowered somewhat by partial conversion to PL during the roasting process (Table 12).

The levels of thermal degradation of vitamin B_6 in the roasted model systems were comparable to those observed for the loss of total B_6 in the retort sterilization of evaporated milk, as monitored by _S. uvarum_ assays (Hassinen, _et al._, 1954). Hassinen, _et al._ (1954), also observed that added PN was much more resistant to thermal degradation than added PL or PM in milk. The equivalent levels of destruction of PM, PN, and PLP during the roasting of the model systems, as compared to the thermal destruction in fluid milk, may have been due to the fact that moisture levels were much lower in the dehydrated model system.

The fact that all rat bioassay estimates of available vitamin B_6 were not significantly different from the microbiological data for total B_6 indicates that the roasting process did not affect the bioavailability of the vitamin (Table 18). As previously

discussed, the data indicate that the semiautomated fluorometric assay procedure was not an accurate measure of the biologically available vitamin B_6 in the model systems.

Stability and Bioavailability of Vitamin B_6 in Dehydrated Model Systems: Storage Effects

Water Activity and Nonenzymatic Browning

The observation that the water activity of all model systems was slightly less than that predicted (Table 19) suggests that moisture uptake from the air (65% relative humidity) during the 24-hour equilibration period was partially incomplete. The slight differences in water activity of the model systems fortified with different B_6 vitamers may have been a result of differences in the rate of moisture adsorption from the air, possibly due to differences in model system position in the equilibration chamber.

The slight differences in water activity between the model systems containing various B_6 vitamers strongly affected the rate of nonenzymatic browning. The high sensitivity of browning rate to changes in water activity has been previously demonstrated (Lea and Hannan, 1949; Labuza, 1975; Warmbier, *et al.*, 1975). The correlation observed between the browning rate and water activity of the model systems stored in TDT or 303 cans (Tables 19

and 20) demonstrated the sensitivity of the browning in these systems to changes in water activity.

The rate and extent of browning was examined in these model systems primarily to provide an index of quality in addition to vitamin B_6 content. Browning has been found to be the major organoleptic defect appearing during the storage of many intermediate moisture foods (Waletzko and Labuza, 1976). The composition of low and intermediate moisture food systems, particularly the type of protein, its ratio to reducing sugars, the pH of the system, and the presence of humectants have been found to be important factors affecting the rate of nonenzymatic browning (Lea and Hannan, 1949; Warmbier, et al., 1976; Schnickels, et al., 1976). The direct applicability of these browning data to food systems of different composition is of limited value. Similarly, reaction kinetic parameters describing the degradation reactions of the B_6 vitamers are not known (energy of activation, Q_{10}, etc.). Thus, the rate constants for browning and vitamin B_6 loss in these studies cannot be directly applied to the prediction of losses of nutritional and organoleptic quality under other storage conditions. The storage conditions used in this study were selected to provide rates of vitamin degradation and browning near the maximum for dehydrated low and intermediate moisture foods.

The kinetics of the browning reaction for model systems stored in TDT cans were examined. The loss of free amino groups in the various dehydrated model systems during storage appeared to follow a very complex overall kinetic function. Plotting the data as a first order function (Figure 11), several linear processes were apparent. These results suggest that the progressive phases of the reaction could each be described by first order kinetics. Similar results, indicating several first order processes involved in the disappearance of browning reactants, have been observed by Warmbier, et al. (1976), and Warren and Labuza (1977). The water activity of the model systems appeared to affect time required for transition between the various first order phases of the loss of free amino groups in this study. The kinetics observed for the formation of browning pigments in the TDT can model systems were contrary to those previously reported (Waletzko and Labuza, 1976; Warmbier, et al., 1976; Warren and Labuza, 1977). After an initial induction period of approximately 20 days, the formation of browning pigments followed complex kinetics (Figure 12). The zero order rate function reported for pigment formation in low and intermediate moisture food systems (Waletzko and Labuza, 1976; Warmbier, et al., 1976; Warren and Labuza, 1977) was not observed. This difference cannot be readily explained.

The large differences observed between systems stored in TDT and 303 cans in the extent of browning during the 128-day storage period (Table 20) was unexpected. The model systems fortified with various B_6 vitamers were not found to vary in water activity between the TDT and 303 cans. These data indicate that slight shifts in moisture equilibria were not responsible for the difference in browning rates. The only storage variable between the two systems was the volume of the container headspace. This would imply that the larger supply of gaseous oxygen in the 303 cans may have played a role in the inhibition of pigment production. The extent of browning pigment in the model systems in TDT cans was 4.6-5.5 times that of the 303 can model systems, while the difference in final amino group concentration ranged from only 1.03 to 1.50 fold. This small difference in free amino groups, compared to the large disparity in pigment formation, suggests that the locus of the oxygen effect was in the intermediate stages of the sequence of browning reactions. This effect of headspace atmosphere on sugar-amine browning has not been previously reported. Recently, Waletzko and Labuza (1976) demonstrated that in an oxygen-free environment, an intermediate moisture food, which had been highly fortified with ascorbic acid, exhibited a slightly lower rate of browning due to the increased

stability of ascorbic acid in the absence of oxygen. Ascorbic acid degradation products readily participate in the browning mechanism at neutral pH. These data are not applicable to the model system used in the present study because of the absence of ascorbic acid fortification.

Stability of Vitamin B_6 during Storage

The kinetics of the degradation of all forms of vitamin B_6 in the model systems stored in TDT cans were satisfactorily described by the first order rate function (Table 21). The kinetics of vitamin B_6 degradation in food systems have not been previously reported. The stability observed for PN was in agreement with several studies concerning the storage stability of PN in low moisture cereal products (Bunting, 1965; Anderson, *et al.*, 1976; Cort, *et al.*, 1976). The induction period observed prior to the slow degradation of PN (Figure 16) suggested that the PN degradation was dependent upon another reaction occurring in the model system during storage, or that an activated state must be reached. Coincident with the initiation of PN degradation, the most rapid period of melanoidin formation was observed (Figure 12), indicating a possible link between the formation of melanoidins and the degradation of PN.

PM, PL, and PLP were much less stable than PN during storage of the dehydrated model systems at 37°C (Table 21). The magnitude of the stability differential between PN and the other vitamers was comparable to the differential reported for the thermal destruction of these vitamers during retort processing of milk products (Hassinen, et al., 1954). In contrast, the dry roasting process employed in these studies induced a fairly uniform destruction of all of the B_6 vitamers. Because the thermodynamic parameters for the degradation of vitamin B_6 were not measured in this study, this difference in relative stability cannot be explained.

The stabilizing effect of storage in the 303 cans (Table 22), which had a large headspace volume, cannot be explained. The fact that nonenzymatic browning pigment formation was retarded in the 303 cans, in addition to the slower rate of destruction of the B_6 vitamers, is evidence that the degradation of vitamin B_6 during storage may be intimately related to browning. The greater stability of the B_6 vitamers is in contrast to the results obtained for ascorbic acid and riboflavin (Dennison, et al., 1977; Kirk, et al., 1977). Both ascorbic acid and riboflavin exhibited greater rates of degradation when the oxygen concentration of the storage environment was greater than or equivalent to atmospheric conditions, as opposed to oxygen concentrations less

than 0.2 atmospheres. Because of the severe nature of the storage conditions of 37°C at approximately 0.6 a_w, several predictions concerning the storage stability of vitamin B_6 in low to intermediate moisture foods can be made. Pyridoxine, whether added or naturally occurring, is highly stable. The data from this study suggest that foods fortified with PN would retain high vitamin B_6 activity for long periods of time, provided light was excluded. Foods in which the vitamin B_6 was present as PM, PL, PLP, or PMP occurring naturally would be subject to a rapid loss of vitamin B_6 activity. Fortification with pyridoxine hydrochloride would be required to maintain the nutritional quality of such foods, particularly in products such as breakfast cereals and those designed for meal replacement.

Degradation Products of Vitamin B_6

Little progress was made in identifying the degradation products of the B_6 vitamers used to fortify the dehydrated model systems. A very small fraction of the model system PLP was found to be oxidized to 4-PA (Table 23). None of the other vitamers were converted to 4-PA to any detectable extent. As mentioned previously, 4-pyridoxic acid lactone could not be measured by the HPLC method employed for 4-PA analysis; therefore, the lactone cannot be ruled out as a possible degradation

product. The absence of significant levels of 4-PA suggests that oxidative degradation to 4-pyridoxic acid lactone was not a major mechanism for the loss of active vitamin B_6 during storage.

The detection of significant levels of pyridoxyllysine associated with the proteins of the stored PLP-fortified model systems of TDT and 303 cans which were pooled prior to bioassay (Table 24) indicates that binding was a major mechanism for the loss of biologically available B_6. Since pyridoxyllysine could not be detected in hydrolysates of systems fortified with PN or PM, binding of these vitamers as pyridoxylamino compounds was not responsible for the losses of available PN, PM, or PL.

Biological Availability of Vitamin B_6 in Dehydrated Model Systems after Storage

The absence of significant differences between HPLC and microbiological assays for vitamin B_6 and the rat bioassays for biologically available B_6 model systems after storage for 128 days at 37°C indicated that the vitamin B_6 remaining in the stored model system possessed full bioavailability. In spite of the numerous reactions occurring during the storage of the food systems, including nonenzymatic browning and the binding of the vitamers to other food components, the potency of the remaining B_6 was not affected under these experimental conditions.

Biological Activity of Protein Pyridoxylamino Compounds

The pyridoxylamino residues of phosphopyridoxyl-bovine serum albumin were observed to possess approximately 60% of the activity of free PN. In addition, these pyridoxylamino residues inhibited the utilization of approximately 55% of the free PN which was added to the rat diet (Table 33). These data indicate that protein bound pyridoxyllysine, an apparent degradation product of vitamin B_6, possessed both pro- and anti-vitamin B_6 activities. Under the conditions of the bioassay of PP-BSA, the inhibition of utilization of the free PN was counteracted by the partial vitamin B_6 activity of the pyridoxyllysine residues.

Whether the negation of pro- and antivitamin B_6 activities would occur in food systems which had undergone formation of protein bound pyridoxyllysine cannot be determined. The results of the bioassay of the PLP-fortified dehydrated model system after 128 days storage at 37°C provide some evidence for this effect. The PLP-fortified model system contained approximately equal molar quantities of free PLP and protein bound pyridoxyllysine (Tables 24 and 26) following storage. The fact that full apparent bioavailability of the vitamin B_6 was observed suggests that the partial availability of

B_6 in the pyridoxyllysine residues compensated for the partially inhibited utilization of free PLP in the model system.

Since the antivitamin B_6 activity of the protein bound pyridoxyllysine residues was not thoroughly studied at more than one dosage level, the possible dose-dependence of its effects cannot be predicted. Although the antivitamin activity of these complexes was observed to be rather weak in comparison to other B_6 antimetabolites, the nutritional effects of higher levels of the pyridoxylamino compounds should be investigated. Higher dosage levels of pyridoxyllysine could conceivably induce a more severe inhibition of vitamin B_6 utilization and/or metabolic function.

Comparison of the chemical assays for the concentration of free vitamin B_6 in the PP-BSA preparation (Table 32) revealed no significant differences between the results of semiautomated fluorometric assay using the autoclave extraction technique and the HPLC assay, which employed aqueous buffer extraction. These data indicate that the pyridoxylamino compounds were not hydrolyzed to a significant extent by the autoclave extraction procedure. These results suggest that most vitamin B_6 assay methods would not suffer interference due to hydrolysis of pyridoxylamino compounds. Thus, the formation of pyridoxylamino complexes may be

responsible for the loss of available B_6 in a food product, but their presence would probably not cause a discrepancy between the total B_6 assay values and animal bioassay values for biologically available vitamin B_6. This would indicate that the apparent difference between biologically available B_6 and total B_6 of 50% in sterilized milk products (Tomarelli, et al., 1955), probably cannot be explained on the basis of pyridoxylamino complexes.

SUMMARY AND CONCLUSIONS

The stability and bioavailability of vitamin B_6, as affected by thermal processing and storage, was investigated using liquid and dehydrated model food systems.

Autoclave treatments simulating the retort processing of foods were performed on liquid model systems. In a liquid system of heat stable peptides at pH 7.0, PLP was found to partially bind to free amino groups, forming pyridoxylamino complexes of limited bioavailability. The presence of glucose and/or ascorbic acid weakly inhibited the formation of these complexes. A mean of approximately 10% of the PLP in the liquid model system was spectrophotometrically demonstrated to bind as a pyridoxylamino complex, representing a nutritional effect of relatively minor consequence. In a similar neutral solution, PM was found to interact with glucose and/or ascorbic acid in a manner which lowered its biological availability. Maximum losses of PM in the liquid model system were approximately 20%. These results suggest that in a system containing both the aldehyde

and amine forms of vitamin B_6, an additive loss may occur. Therefore, losses of available B_6 could approach 30% of the total vitamin B_6 during the retort processing of a food system with physical-chemical characteristics similar to the liquid model system and containing equimolar amounts of PLP and PM.

The study of vitamin B_6 stability in dehydrated model food systems during roasting and storage provided an opportunity to correlate microbiological, semiautomated fluorometric, and HPLC methods for the quantitation of vitamin B_6. The HPLC and microbiological methods were found to correlate well in all systems except those containing predominantly PM. In samples containing high levels of PM, the HPLC method provided greater accuracy. Although no direct evidence for the presence of interfering compounds could be found, the semiautomated fluorometric assay procedure yielded apparent vitamin B_6 values for all dehydrated model systems which were significantly higher than those of the other methods.

The dry roasting of dehydrated model systems at 180°C for 25 minutes, a relatively severe treatment, was designed to permit an approximation of the maximum levels of vitamin B_6 which could be affected by a roasting process. The three forms of the vitamin examined, PN, PM, and PLP, were found to undergo between 50 and 70% degradation. Rat bioassay of these roasted systems

demonstrated excellent correlation with the microbiological assay employed for the determination of total B_6 in the systems. The results indicated that the roasting process induced no losses of bioavailability in the remaining B_6. Semiautomated fluorometric analysis of the roasted model systems did not give an accurate estimation of biologically available vitamin B_6.

Studies were designed to estimate the maximum effects of storage on the vitamin B_6 in a dehydrated food system. Storage conditions were selected on the basis of previous literature data to maximize browning and vitamin degradation in a normal storage environment. The degradation of PN, PM, PL, and PLP in model systems at a water activity of approximately 0.6 at 37°C followed first order kinetics. A rapid unidirectional conversion of PM to PL was observed. Calculated half lives were 141 days for PN, and 35-46 days for PM, PL, and PLP when stored in containers having a limited headspace. Storage in containers providing a large headspace significantly lowered the rates of degradation of all B_6 vitamers and nonenzymatic browning.

4-Pyridoxic acid, postulated to be a degradation product of vitamin B_6, was found at very low levels in PLP-fortified dehydrated model systems. It was not found in model systems fortified with the other B_6 vitamers. Oxidation to 4-PA was not a significant

pathway for the loss of vitamin B_6 activity during storage. Approximately 50% of the total PLP which was lost from the model systems during storage was found to be bound to lysine residues of proteins as the pyridoxylamino compound, pyridoxyllysine. This complex could only be detected in PLP-fortified model systems. Other degradation products of the B_6 vitamers were not identified.

Rat bioassay of the dehydrated model systems after 128 days at 37°C indicated that the remaining B_6, as measured microbiologically or by HPLC assay, was biologically available. The fluorometric values for total B_6 were significantly greater and were not an accurate estimation of available vitamin B_6.

To determine the biological activity of protein bound pyridoxyllysine, a rat bioassay was performed on diets containing added phosphopyridoxyl-bovine serum albumin. Approximately 60% of the complexed B_6 was found to be available to the rat, while the remaining pyridoxyllysine exerted a partial inhibitory effect on the utilization of free PN added to the diet. The fact that full bioavailability was observed for vitamin B_6 in the stored PLP-fortified dehydrated model system, which was shown to contain approximately equal molar amounts of protein bound pyridoxyllysine and free PLP, was evidence that the pro- and antivitamin B_6 activities of pyridoxyllysine may counteract each other.

In conclusion, the results of this study have indicated that, under the several processing and storage conditions examined, varying losses of biologically available vitamin B_6 may occur. The nutritional consequences of such losses can be monitored accurately by microbiological assay procedures. The data also suggest that the HPLC vitamin B_6 assay method could be applied to accurately determine the content of available B_6 in foods. Fortification with pyridoxine hydrochloride, the most stable form of the vitamin, would assure minimum losses of biologically active vitamin B_6 in food products subject to extensive nutrient degradation in processing or storage.

APPENDIX

APPENDIX

Heat Stable Peptides

Formation of Peptides

Crystalline bovine β-lactoglobulin (3X) was obtained from United States Biochemical Corporation. Although discontinuous polyacrylamide gel electrophoresis revealed several minor contaminants, for this study, this preparation was used without further purification.

β-Lactoglobulin (1.5 g) was dissolved in 20 ml 0.1 M ammonium acetate to yield a concentration of 75 mg/ml. Concentrated hydrochloric acid was added to adjust the pH to 2.0, followed by the addition of 15 mg porcine pepsin (3X, Nutritional Biochemicals Corp.). The mixture was incubated at 37°C for 6 hours with gentle agitation. The reaction was terminated by the adjustment of the mixture to pH 7.0 with 30% ammonium hydroxide.

Isolation of Peptides

The peptic hydrolysate was divided into two 10 ml portions and each chromatographed separately on a Sephadex G-50 (fine) column of 2.6 x 40 cm (V_o = 81 ml)

using 0.1 M ammonium acetate, pH 7.0, at 1.0 ml/minute. The column effluent was monitored at 254 nm using an ultraviolet absorption detector and collected in 10 ml fractions. Studies indicated that all material eluting after the void volume was heat stable, i.e. not precipitable by autoclaving 20 minutes at 121°C in the presence of 10 mg/ml glucose. Based on this information, the heat stable fractions were pooled and lyophilized.

Further purification of the heat stable peptides was carried out on a Sephadex G-10 column (2.6 x 4.0 cm; V_o = 78 ml) to remove free amino acids and peptides less than 700 daltons. The lyophilized peptides were dissolved in 20 ml 0.1 M ammonium acetate, pH 7.0, and 10 ml aliquots were chromatographed. The void volume peaks were collected, mixed, and aliquots stored at -25°C until used.

Synthesis of ε-Pyridoxyl-L-Lysine

α-Acetyl-ε-pyridoxyl-L-lysine was synthesized by the method of Dempsey and Christensen (1962). In 30 ml anhydrous methanol (Burdick and Jackson), 392 mg potassium hydroxide, 1.00 g α-acetyl-L-lysine (Sigma Chemical Co.), and 1.14 g pyridoxal hydrochloride (ICN Pharmaceuticals, Inc.) were stirred for 15 minutes in the dark at room temperature. After undissolved solids were removed by filtration through Whatman 1 filter paper, 50 mg of platinum dioxide catalyst (ICN Pharmaceuticals, Inc.)

was added. The mixture was hydrogenated in a 50 ml Erlenmeyer flask at atmospheric pressure by bubbling hydrogen gas for 1 hour, with periodic swirling of the flask. The catalyst was removed by filtration through Whatman 1 filter paper. The mixture was adjusted to approximately pH 6 with dry methanolic HCl using moistened indicator paper. The dry HCl in methanol was prepared by bubbling HCl gas (technical; Matheson Gas Products) through anhydrous methanol. After pH adjustment to 6, the mixture was concentrated to one-third volume in a rotary evaporator at 50°C. The precipitated KCl was removed by filtration. The filtrate was adjusted with dry methanolic HCl to pH 4.4, monitoring directly with a pH meter. A yellowish precipitate of α-acetyl-pyridoxyl-L-lysine appeared at this point and was removed by filtration. To maximize the recovery, a second crop of crystals was obtained by evaporating the filtrate to dryness under reduced pressure.

The crystals of α-acetyl-pyridoxyl-L-lysine were dissolved in 6 N HCl and autoclaved 45 minutes at 121°C to hydrolyze the α-acetyl moiety (Dempsey and Snell, 1963). After drying in a rotary evaporator, the product was dissolved in 10 ml distilled water. Salts and free lysine and PL were removed by chromatography of 3 ml portions on a column of Sephadex G-10 (2.6 x 34 cm, V_o = 63 ml). The void volume fractions were pooled,

dried under reduced pressure at 50°C and dissolved in 10 ml absolute ethanol. Pale yellow crystals of ε-pyridoxyl-L-lysine slowly formed upon storage at 2°C, as described by Dempsey and Snell (1963).

Preparation of Phosphopyridoxyl-Bovine Serum Albumin

Phosphopyridoxyl-bovine serum albumin was prepared by procedure 2 of Dempsey and Christensen (1962). Fourteen grams of bovine serum albumin (essentially fatty acid-free; Sigma Chemical Co.) was dissolved in 560 ml of 0.1 M potassium phosphate, pH 7.5. Pyridoxal phosphate monohydrate (2.4 mg; ICN Pharmaceuticals, Inc.) was dissolved in a small volume of water and added to the albumin solution in an ice bath. The mixture was stirred at 1 to 2°C for 1 hour. Sodium borohydride was added as a 1% aqueous solution by injection under the surface of the solution. To ensure complete reduction, a 15 fold molar excess of $NaBH_4$ was added relative to PLP, with small additions to minimize foaming. The pH of the solution was maintained between 7.5 and 7.7 during the reduction by the periodic addition of 6 N sodium hydroxide.

After storage overnight at 2°C, the protein solution was transferred to several dialysis bags of 1 1/8 inch diameter (Fisher Scientific Co.) and dialyzed in a five-gallon stainless steel container against

distilled water at 2°C. Dialysis continued for 1 week, with six changes of the cold distilled water. The dialyzed protein was freeze dried in a shallow tray in a Virtis Model FFD 42WS Freeze-Dryer. Drying was performed at a platen temperature of 110°F to an absolute pressure of 5 μm Hg.

High Performance Liquid Chromatographic Determination of Vitamin B_6

Materials

1. Extraction buffer. Potassium acetate (39.26 g) was dissolved in about 1800 ml distilled water, adjusted to pH 4.5 with glacial acetic acid, and diluted to 2 l, providing a 0.2 M acetate buffer.

2. Trichloroacetic acid, 20% (w/v), was prepared by dissolving 20 g crystalline TCA in distilled water and diluting to 100 ml. TCA solutions were prepared fresh daily prior to analysis.

3. HPLC mobile phase buffer, 0.033 M potassium phosphate, pH 2.2, was prepared by adding 4.6 ml concentrated phosphoric acid to about 1800 ml distilled water, adjusting the pH with 6 N potassium hydroxide, and diluting to 2 l. Prior to use, the buffer was filtered through a 0.45 μm filter (Gelman Instrument Co.) under vacuum in order to remove small particulate matter. This step provided satisfactory degassing of the solution.

4. Potato acid phosphatase (2 U/mg) was obtained from Sigma Chemical Co. A 1.0 mg/ml solution was prepared (2 U/ml) by dissolving 50 mg of the dry enzyme preparation in 50 ml of cold 0.2 M potassium acetate, pH 4.5. This solution was prepared immediately prior to use.

5. Vitamin B_6 standard stock mixture was prepared at a concentration of 10 μg free base/ml with respect to PM, PN, and PL by dissolving 14.2 mg pyridoxamine dihydrochloride (ICN Pharmaceuticals, Inc.), 12.2 mg pyridoxal hydrochloride (ICN Pharmaceuticals, Inc.), and 12.2 mg pyridoxine hydrochloride (Sigma Chemical Co.) in 1 l 0.2 M potassium acetate, pH 4.5. Working standards were prepared over the range of 0.25 to 2.5 μg free base/ml by transferring appropriate volumes of the stock to 25 ml volumetric flasks, adding 8.33 ml 20% TCA, and diluting to 25 ml with 0.2 M potassium acetate, pH 4.5.

6. Recovery standard mixtures were prepared at a concentration of 30 μg free base/ml for PM and PN, and 41.1 μg/ml for PLP (equivalent to 30 μg PL/ml). Pyridoxal phosphate monohydrate (22.2 mg; ICN Pharmaceuticals, Inc.), pyridoxine hydrochloride (18.2 mg), and pyridoxamine dihydrochloride (21.5 mg) were dissolved in 500 ml 0.2 M potassium acetate, pH 4.5.

Sample Extraction

Finely ground model system (about 3 g) was accurately weighed and transferred into a 25 ml volumetric flask. Fifteen ml of 0.2 M potassium acetate, pH 4.5, was added and the sample thoroughly agitated to ensure complete wetting. For recovery samples, 1.0 ml of the recovery mixture was added at this point. To promote the extraction, all flasks were sonicated 30 minutes in a 65 watt ultrasonic cleaning bath (Fisher Scientific Co.) at room temperature. After sonication, the contents of each flask were diluted to 25 ml with the pH 4.5 acetate buffer. Insoluble solids were removed by centrifugation of the samples at 6000 rpm (4340 x g max) in a Beckman Model J-21C centrifuge at 25°C for 20 minutes (JA-20 rotor).

Ten ml of each of the supernatants was transferred into a clean 40 ml screw cap centrifuge tube. Phosphorylated forms of the vitamin were hydrolyzed with potato acid phosphatase (Takanashi, et al., 1970). Two ml of the 1.0 mg/ml enzyme solution was added to each 10 ml aliquot of the sample supernatants, followed by incubation in a 37°C water bath for 1 hour. Preliminary studies showed that this enzyme treatment quantitatively hydrolyzed PLP in the extracts. The reaction was terminated and protein precipitated by the addition of 4 ml of 20% TCA, followed by incubation in a 50°C water bath

for 15 minutes. After cooling to room temperature, the samples were recentrifuged at 6000 rpm for 20 minutes at 25°C. Aliquots (50 µl) of each supernatant were taken for direct injection into the chromatograph.

Chromatographic Analysis

Chromatographic separations were performed using a µBondapak C_{18} reverse phase column (4mm ID x 30 cm; Waters Associates). The mobile phase was pumped for at least 1 hour prior to analysis to maximize the stability of the system. Chromatography of the standards and samples was carried out at a flow rate of 2 ml/minute. The absorbance of the column effluents was monitored at 280 nm, 0.02 AUFS. The fluorescence of the effluents was monitored using an Aminco Fluoro Monitor equipped with a Germicidal lamp (General Electric Corp.), a 295 nm interference excitation filter (American Instrument Co.), and a Corning 7-51 (370 nm narrow pass) emission filter. Fluorescence spectra of the B_6 vitamers in the mobile phase buffer exhibited excitation and emission maxima at approximately 290 and 395 nm, respectively.

A standard curve was prepared by injecting 50 µl of each calibration standard mixture and plotting peak height, either relative fluorescence or absorbance, against micrograms for each vitamer. The concentration

of the B_6 vitamers in the sample extracts was determined by comparison of peak height with the appropriate standard curve.

Following analysis, the HPLC column was washed with approximately 20 ml distilled water, then 30 ml methanol. The column was stored in a 33% methanol:67% water mixture.

High Performance Liquid Chromatographic Determination of ε-Pyridoxyl-L-lysine

Model System Protein Extraction and Hydrolysis

Model systems (5 g) were finely powdered and suspended in 15 ml 0.05 M KCl-HCl, pH 2.5. The mixtures were sonicated 30 minutes and centrifuged at 6000 rpm in a Beckman Model J-21C centrifuge with a JA-21 rotor. The supernatants were dialyzed in 0.39 inch diameter tubing (Fisher Scientific Co.) against 4 l of 0.05 M KCl-HCl, pH 2.5, with three changes of the buffer over a 4-day period. After dialysis, the protein concentrations were determined by the procedure of Lowry, *et al.* (1951). Solutions of bovine serum albumin and phosphopyridoxyl-bovine serum albumin were prepared in the same pH 2.5 buffer at a concentration of 10 mg/ml.

A volume equivalent to 10 mg of protein of each sample was pipetted into duplicate 10 ml glass ampules, adjusted to 6 N in constant boiling HCl, and diluted to 5 ml total volume. The ampules were frozen in a dry

ice-ethanol mixture, followed by slow thawing under vacuum to degas the solutions. The ampules were then refrozen and sealed under vacuum.

The proteins were hydrolyzed by incubation of the ampules in a 105°C oven for 24 hours. This procedure also hydrolyzed the phosphate esters of the bound B_6 compounds. Upon cooling, the hydrolysates were quantitatively transferred to 100 ml round bottom flasks and evaporated to dryness in a rotary evaporator at approximately 50°C. Small portions of water were added to each flask several times, followed by redrying, to ensure complete removal of the HCl. Each hydrolysate was dissolved in 10 ml 0.2 M potassium acetate, pH 4.5, and filtered through a 5 μm membrane (Type HA, Millipore Corporation) prior to chromatography.

Chromatographic Analysis of ε-Pyridoxyllysine

The chromatographic conditions for the determination of pyridoxyllysine were identical to those described for the HPLC determination of vitamin B_6 in model system extracts. The fluorescence of the column effluents was monitored with an Aminco Fluoro Monitor using a Germicidal lamp (General Electric Corp.), a 295 nm interference excitation filter (American Instrument Co.), and a Corning 7-51 (370 nm narrow pass) emission filter.

Crystalline ε-pyridoxyl-L-lysine was dissolved in 0.2 M potassium acetate, pH 4.5, and its concentration was determined using the molar absorptivity at 323 nm of 5800 $M^{-1}cm^{-1}$ (Dempsey and Christensen, 1962). Injections of 5 to 40 μl portions of the standard solution provided a standard curve range of 0.087 to 0.689 μg ε-pyridoxyllysine. A standard curve was prepared by plotting peak height against μg bound pyridoxal, determined using the gravimetric factor of 0.563 μg bound PL/μg pyridoxyllysine.

High Performance Liquid Chromatographic Determination of 4-Pyridoxic Acid

Materials

1. HPLC mobile phase buffer was prepared by adding 100 ml anhydrous methanol (Burdick and Jackson) and 4.6 ml concentrated phosphoric acid to approximately 1800 ml distilled water. The pH was adjusted to 2.2 with 6 N potassium hydroxide, and the solution was diluted to 2 l with distilled water. This provided a final concentration of 0.033 M phosphate and 5% (v/v) methanol. The mobile phase buffer was filtered through a 0.45 μm filter (Gelman Instrument Co.) prior to use.

2. 4-Pyridoxic acid standards were prepared as follows. Crystalline 4-pyridoxic acid (4-PA) was obtained from Sigma Chemical Co. A working stock solution was prepared immediately before use by

dissolving 20 mg 4-PA in 1 l 0.2 M potassium acetate, pH 4.5. Calibration standards were prepared over the range of 0.05 to 6.0 μg/ml. To 25 ml volumetric flasks containing 5 ml of the pH 4.5 acetate buffer, appropriate volumes of the 4-PA stock were added, followed by 8.33 ml 20% TCA. The contents were diluted to 25 ml with the acetate buffer.

Chromatographic Analysis of 4-Pyridoxic Acid

The extraction and enzymatic hydrolysis of the model systems were performed as described for the HPLC analysis of vitamin B_6. Therefore, any 4-pyridoxic acid phosphate formed in PLP-fortified systems would be determined as 4-PA.

All extracts and standard solutions were held in an ice bath until chromatographed. Chromatographic conditions were identical to those employed for the HPLC analysis of vitamin B_6, with the exception of 5% methanol added to the mobile phase to shorten the retention time of 4-PA. The fluorescence of the column effluents was monitored using an Aminco Fluoro Monitor with a Corning 7-60 (355 nm narrow pass) excitation filter and a Wratten 47B (436 nm narrow pass) emission filter. The concentration of 4-PA in model system extracts was determined from a standard curve of fluorescence peak height against μg 4-PA injected.

Discontinuous Polyacrylamide Gel Electrophoresis

Gel electrophoresis of β-lactoglobulin was performed by a modification of the discontinuous method of Melachouris (1969). The running gel solution was prepared by dissolving 22.5 g Cyanogum 41 (E-C Apparatus Corp.) in 0.380 M Tris-HCl buffer, pH 8.9, and diluting to 250 ml, giving a total gel concentration of 9%. To this gel solution, 0.25 ml N, N, N', N'-tetramethylethylenediamine was added. Polymerization of the gel was initiated by the addition of 2 ml of fresh 10% aqueous ammonium persulfate solution to 190 ml of the gel solution. The mixture was quickly poured into a vertical slab gel apparatus (E-C Apparatus Corp.) in its horizontal position, and the sample slot former positioned. Thirty minutes were allowed for gel polymerization. No spacer gel was employed.

Excess gel was cut away from the sample slot former. The electrophoresis apparatus was then placed in its vertical position. Electrophoresis chamber buffer, 0.046 M Tris-glycine, pH 8.3, was gently loaded into the upper and lower buffer chambers of the apparatus. After filling the buffer chambers, the sample slot former was carefully removed.

The protein samples were dissolved at a concentration of 5 mg/ml in 0.062 M Tris-HCl, pH 6.7. Sucrose was added to the sample solution to provide a greater

density than the chamber buffer, thus facilitating layering of the protein solutions in the sample slots. Bromphenol blue was added to the protein solutions to serve as a tracking dye.

Electrophoresis was performed at approximately 17°C at a constant voltage of 130 V for two and one-half hours (Heathkit Power Supply). During this time, the tracking dye migrated 10 cm.

Following electrophoresis, the gels were carefully removed from the apparatus and fixed overnight by soaking in 15% (w/v) TCA. The gels were stained with a solution of 0.1% Coomassie brilliant blue in 13.75% TCA for 2 hours. Destaining was accomplished by several washes with 15% TCA.

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IMPROVED CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETERMINATION OF VITAMIN B_6 COMPOUNDS IN FOODS

ABSTRACT

Several fluorometric procedures have been previously developed for the determination of vitamin B_6 compounds in foods. These have mainly been based on the separation of pyridoxal (PAL), pyridoxine (PIN), and pyridoxamine (PAM) by column chromatography on Dowex AG 50W-X8, chemical conversion of PIN and PAM to PAL, and formation of the 4-pyridoxic acid lactone fluorophore by reaction with KCN. These procedures were limited by a cumbersome chromatographic procedure utilizing boiling potassium acetate buffers, and interfering compounds in the PAL fraction often precluded accurate measurement. In this study, Maillard browning pigments resulting from the autoclave extraction procedure were shown to interfere by reacting with KCN to form highly fluorescent products. To alleviate the interference problem, an alternative ion exchange chromatographic method was developed which eliminated the KCN-reactive browning pigments from the PAL fraction. After the ion exchange clean up steps, PAL was determined directly by reaction with KCN, whereas PIN and PAM were first converted to PAL by reaction with MnO_2 and sodium glyoxylate, respectively. The effectiveness of the procedure was demonstrated on casein in the presence or absence of glucose, and on selected food samples.

INTRODUCTION

THE DEVELOPMENT of an adequate chemical procedure for the determination of biologically active forms of vitamin B_6 in foods has been a complex and enigmatic problem. Basic studies by Bonavita (1960), Toepfer et al. (1961), and Polansky et al. (1964) have demonstrated the feasibility of fluorometric measurement of pyridoxal (PAL), pyridoxamine (PAM), and pyrodixine (PIN) by conversion to PAL, and reaction with KCN, forming the fluorophore, 4-pyridoxic acid lactone. Various fluorometric methods have been applied to vitamin B_6 compounds in biological materials (Fujita et al., 1955; Contractor and Shane, 1968; Loo and Badger, 1969; Takanashi et al., 1970a, b; Fiedlerova and Davidek, 1974; Chin, 1975). The recent results of Chin (1975) have suggested that interfering compounds may be present in the PAL fraction after column chromatographic separation of the B_6 analogs by the procedure of Toepfer and Lehmann (1961). This possibility has not been previously investigated.

In this study, certain Maillard browning pigments formed during the standard autoclave extraction procedure were shown to react with KCN to form highly fluorescent products. Since these pigments appeared in the PAL chromatographic fraction using the ion exchange method of Toepfer and Lehmann (1961), erroneously high PAL values would be obtained with all fluorometric assay procedures based on autoclave extraction and this chromatographic separation.

To alleviate the problem, various ion exchange chromatographic procedures were examined to develop a method which would eliminate these interfering compounds from the PAL fraction. The fluorometric procedure of Chin (1975) was modified to be compatible with this chromatographic method, providing a more accurate chemical assay for B_6 compounds in foods.

MATERIALS & METHODS

1. Vitamin B_6 stock standards were individually prepared at 10 μg free base per ml in 1N HCl (Toepfer and Polansky, 1970) using pyridoxal-HCl and pyridoxamine-2 HCL (ICN Pharmaceuticals, Inc.) and pyridoxine-HCl (Sigma Chemical Co.).
   Calibration standards were prepared over the range of 0.02–0.10 μg free base per ml by dilution of the stock standards with the appropriate buffer.
2. Extraction and hydrolysis of the samples were performed by the procedure of Toepfer and Polansky (1970). Finely ground sample (0.5–2.0g) was weighed into a 250 ml Erlenmeyer flask. To plant products, 180 ml 0.44N HCl was added and autoclaved for 2 hr at 121°C; animal products required 180 ml 0.055N HCl and autoclaving for 5 hr at 121°C. Recovery standards were prepared by adding 1 ml of stock solution (10 μg/ml) of each form of vitamin B_6 to an aliquot of the sample prior to autoclaving. After cooling to ambient temperature, the pH of the samples was adjusted to 4.5 with 6N KOH. Each sample was diluted to a final volume of 250 ml with distilled water and filtered through Whatman No. 42 filter paper. These filtrates were applied to the ion exchange columns.
3. Chromatographic procedures–All ion exchange fractionations of the food extracts were performed on Dowex AG 50W-X8, 100–200 mesh, K^+-form (Bio-Rad Laboratories). Separation of PAL was done with a 0.7 × 20 cm column packed with 5.5 ml of resin, while PAM and PIN were eluted in a single fraction from a separate 1.8 × 40 cm column packed with 30 ml of resin. The surfaces of the resin beds of the large columns were protected by discs of Whatman No. 1 filter paper. The elution procedures for PAL, PIN and PAM are shown in Table 1. The PAL fraction was eluted into a 25 ml volumetric flask, and diluted to 25 ml with the pH 6.30 phosphate buffer. The PAM and PIN fraction was eluted from the larger column into a 200 ml volumetric flask. All separations were performed at room temperature. After three to four separations, the column resins were regenerated according to the procedure of Toepfer and Polansky (1970).
4. Conversion of the B_6 analogs to 4-pyridoxic acid lactone was based on the procedure of Chin (1975). Reagents: 0.5N sodium glyoxylate (Sigma Chemical Co.), manganese dioxide prepared according to Polansky et al. (1964), and 1M KCN (Mallinckrodt Chemical Works).

Pyridoxal. Two ml of PAL calibration standard or column eluate was pipetted into each of duplicate 15 ml screw cap test tubes labeled A and B (A = sample, B = blank). Each was diluted by the addition of 8 ml 0.4M K_2HPO_4 pH 7.5. For conversion to 4-pyridoxic acid lactone, 0.4 ml 1M KCN was added to A, while 0.4 ml distilled water was added to B, with incubation 2 hr at 50°C.

Pyridoxamine. Duplicate 2-ml aliquots of PAM calibration standard or eluate was pipetted into 15 ml screw cap test tubes, labeled as above. Each was diluted by the addition of 8 ml 0.4M K_2HPO_4 pH 7.5. For conversion to pyridoxal, 0.4 ml 0.5M sodium glyoxylate was added to A, 0.4 ml distilled water was added to B. Following the addition of glyoxylate or water the samples were incubated for 15 min in a boiling water bath. After cooling the tubes to room temperature in running water, 0.4 ml 1M KCN was added to each tube followed by incubation 2 hr at 50°C to convert PAL to 4-pyridoxic acid lactone.

Pyridoxine. A 20-ml aliquot of sample eluate or calibration standard was transferred to separate 125 ml Erlenmeyer flasks and the pH of

Table 1—Ion exchange column elution procedures

	Buffer type	
	Potassium acetate	Potassium phosphate
PAL Column		
(1) Equilibration	10 ml 0.2M pH 4.50	–
(2) Sample applied (10–15 ml)	–	–
(3) Wash	–	18 ml 0.1M pH 5.70
(4) PAL elution		18 ml 0.15M pH 6.30 (dilute collected fraction to 25 ml using this buffer)
(5) Wash before re-equilibration	35 ml 0.5M pH 8.00	–
PIN and PAM Column		
(1) Equilibration	100 ml 0.2M pH 4.50	–
(2) Sample applied (50–100 ml)	–	–
(3) Wash	100 ml 0.15M pH 6.20	–
(4) PIN and PAM elution	200 ml 0.5M pH 8.00	–

each flask adjusted to 5.5 with 1N HCl. Oxidation of PIN to PAL was carried out by adding 0.1g MnO_2 and gently mixing at room temperature for 30 min. Addition of MnO_2 was omitted for the PIN blanks. The pH of the samples was then adjusted to 7.2 with 0.4M K_2HPO_4 (about 14 ml), followed by filtration using quantitative technique through Whatman No. 42 filter paper directly into a 100 ml volumetric flask. The filtrates were diluted to 100 ml with distilled water. Ten ml aliquots were then transferred to screw cap test tubes, 0.4 ml 1M KCN added, and the tubes were held at 50°C for 2 hr to convert the PAL to 4-pyridoxic acid lactone.

Table 2—Typical calibration curves for fluorometric determinations. Free base concentration 0.02–0.10 μg/ml

B_6 analog	Intercept (Fluor. units)	Slope (Fluor. units/ μg/ml)	Correlation coefficient
PAL	24.3	673.8	+.9977
PIN	2.3	612.5	+.9989
PAM	11.1	572.5	+.9984

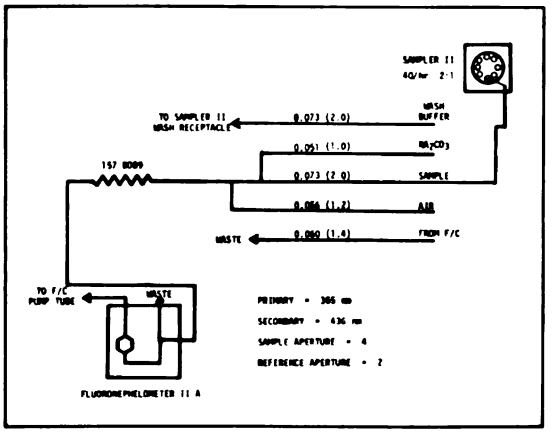

Fig. 1—Continuous flow analysis system for determination of vitamin B_6 as 4-pyridoxic acid lactone (modified from Chin, 1975). Figures in parentheses represent flow rate in ml/min.

5. Fluorometric procedures were based on those of Chin (1975) for automated analysis, with the following modifications:
   a. The higher ionic strength buffers employed necessitated the use of wash buffers of similar composition and ionic strength. The following buffers were used as wash solutions between samples: PAL: 1 part 0.15M phosphate, pH 6.30; 4 parts 0.4M phosphate, pH 7.4. PIN: 1 part part 0.5M acetate, pH 8.00; 4 parts 0.4M phosphate, pH 7.4.
   b. Fluorometric apertures were set at a reference value of 2 and a sample value of 4.
   c. The flow pattern through the Technicon AutoAnalyzer was improved by the addition of 2 ml of 30% Brij 35 (Fisher Scientific Co.) per liter of 0.4M Na_2CO_3.
   d. Due to the differences in sample buffers and, hence, buffer capacity, slight adjustments in fluorometer calibration were required between determinations of the three forms of the vitamin. Typical calibration curve data appear in Table 2.
6. Studies on interfering compounds.

Various food samples and a model system (described below) were extracted, chromatographed according to Toepfer and Lehmann (1961), and examined for the presence of interfering compounds. The PAL ion exchange column effluents for sample extracts and PAL standards were evaporated to dryness under vacuum at 50°C in a rotary evaporator and reconstituted with water to give a tenfold concentration. Aliquots (2 ml) of this concentrate were treated with 0.1 ml 1M KCN (0.1 ml distilled water for blanks) and held at 50°C for 2 hr. These samples were examined qualitatively for fluorescing compounds by thin-layer chromatography. Seventy-five microliters of each sample were spotted on a silica gel G precoated plate (Analtech, Inc.) and developed with a 50:50 mixture (v/v) of methanol and water. Fluorescence was observed upon excitation at 366 nm. Differences in the pattern of fluorescing compounds between KCN-treated and blank samples were indicative of the presence of KCN-reactive interfering substances.

For evaluation of the effectiveness of various column chromatography procedures in removing interfering compounds from the PAL fraction, extracts from autoclaved model food systems were employed. The model systems consisted of 5g of "vitamin free" test casein (General Biochemicals, Inc.), either with or without 5g of D-glucose ('Baker Analyzed Reagent', J.T. Baker Chemical Co.) to induce Maillard browning during autoclaving. The ratio of apparent μg of PAL/g casein

Table 3—Efficiency of ion exchange column removal of interfering compounds from PAL fraction[a,b]

	Procedure	Apparent µg PAL/g Casein: Casein Only	Casein + Glucose	Ratio[b]
1.	Boiling acetate buffers (Toepfer and Lehmann, 1961)	1.20 ± 0.08	2.11 ± 0.06	0.57 ± 0.05
2.	Room temperature acetate buffers PAL eluant = 0.17M acetate pH 6.25 Preceeding wash: 0.1M acetate pH 5.70 Volume			
	100 ml	0.91 ± 0.06	1.26 ± 0.04	0.72 ± 0.07
	150 ml	1.00 ± 0.06	1.13 ± 0.11	0.88 ± 0.14
	200 ml	0.59 ± 0.04	0.85 ± 0.01	0.69 ± 0.05
3.	Room temperature, mixed. Wash=100 ml 0.01M phosphate pH 5.70 PAL eluant=100 ml 0.17M acetate pH 6.25	1.08 ± 0.21	1.44 ± 0.06	0.75 ± 0.18
4.	Room temperature phosphate buffers Wash=100 ml 0.1M phosphate pH 5.70 PAL eluant=100 ml 0.15M phosphate pH 6.30	0.81 ± 0.10	0.78 ± 0.08	1.04 ± 0.23

[a] Thirty ml resin bed columns, duplicate separations and PAL determinations. Values are mean ± standard deviation.
[b] A ratio of one would indicate complete removal of interfering compounds.

in the casein plus glucose system to µg PAL/g casein in the casein without glucose system was used as a quantitative indicator of column cleanup efficiency

RESULTS & DISCUSSION

STUDIES undertaken to investigate the possibility of interfering compounds in the PAL fraction following ion exchange chromatography confirmed the presence of non-PAL, KCN-reactive substances. Qualitative differences were observed in the thin-layer chromatographic pattern of KCN treated PAL fractions of food extracts and their corresponding blanks. As this qualitative difference was also observed with model systems of casein plus glucose, but not casein alone (Fig. 2, plate 1), it was felt that the interfering compounds could be Maillard browning pigments formed during the autoclave extraction procedure. Analysis of these compounds by gel filtration chromatography and ultraviolet spectrophotometry suggested a strong similarity to the Maillard browning pigments previously characterized (Clark and Tannenbaum, 1970). Therefore, the ratio of PAL/g casein values of model systems with or without glucose was employed as a quantitative indicator of cleanup efficiency in the development of an ion exchange chromatographic procedure to eliminate these interfering compounds. After evaluating several ion exchange systems, a room temperature potassium phosphate wash and elution procedure was developed which provided adequate cleanup of the PAL fraction. As shown in Table 3, interference was found with all other procedures tested, the most noted example being the boiling acetate elution method. The absence of any qualitative differences in the thin-layer chromatographic patterns exhibited by the casein and casein plus glucose samples shown on Figure 2, plate 2, was further evidence for removal of interfering compounds from the PAL fraction by the phosphate elution system. Similar thin-layer chromatographic results were observed with all food extracts tested.

The results of Chin (1975) for the determinations of PIN and PAM suggested little, if any, problem with interfering compounds. Studies utilizing casein and casein plus glucose model systems confirmed this observation. Values of apparent PIN or PAM/g casein were not affected by the presence of glucose in the sampling indicating that browning pigments did not interfere with these determinations. Such specificity may be attributed to the selective reactions for converting PIN and PAM to PAL, in addition to blank determinations which, in

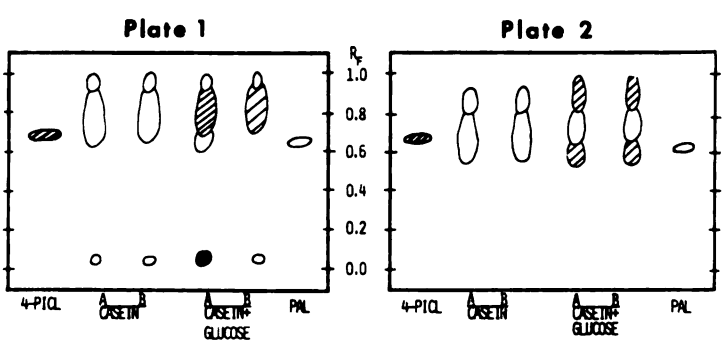

Fig. 2—Thin layer chromatograms of PAL column eluates. Excitation = 366 nm, visual analysis. Extent of hatching proportional to fluorescence. Designation: A = KCN treated; B = Blank; PICL = 4-pyridoxic acid lactone. Plate 1, boiling acetate wash and elution. Plate 2, room temperature phosphate wash and elution.

Table 4—Recovery of B_6 standards from ion exchange columns[a,b]

B_6 analog	µg Applied	Recovery (%)
PAL	5.0	95.2 ± 3.1
PIN	10.0	103.2 ± 1.9
PAM	10.0	106.4 ± 4.3

[a] Vitamin B_6 solutions were prepared in 0.2M pH 4.50 potassium acetate buffer.
[b] Mean and standard deviation of four separations

Table 5—Vitamin B_6 content of selected food samples[a]

	µg free base/g		
Sample	PAL	PIN	PAM
Corn flakes	7.3 ± 0.9	17.8 ± 2.0	8.6 ± 3.1
Infant formula (powdered)	1.8 ± 0.6	0.6 ± 0.5	11.0 ± 2.4
Cured ham	1.7 ± 0.8	2.6 ± 1.4	9.2 ± 1.4

[a] Mean and standard deviation of triplicate determinations

Table 6—Recovery of PAL, PAM, and PIN added to food samples[a,b]

	Percentage recovery		
Sample	PAL	PIN	PAM
Corn flakes	78.3 ± 5.8	80.0 ± 8.7	105.0 ± 13.2
Infant formula (powdered)	72.9 ± 3.6	123.3 ± 5.8	110.0 ± 14.1
Cured ham	84.4 ± 4.4	100.0 ± 10.0	115.0 ± 21.2

[a] Mean and standard deviation of triplicate determinations
[b] 10 µg of each analog added prior to autoclaving.

contrast to the PAL procedure, provide a measure of extraneous KCN-reactive compounds.

Whereas the elution of PIN and PAM was readily accomplished from the PAL column using the room temperature phosphate system, several problems in the determination of PIN precluded the use of a single column for separation and purification of the other B_6 analogs. First, the oxidation of PIN to PAL by MnO_2 was found to be inhibited by the phosphate buffer. This could possibly be alleviated by using another oxidizing agent. Second, attempts to reequilibrate the column with 0.15M potassium acetate, pH 6.30, caused exceptionally high losses of PIN from the column. Therefore, the two-column procedure was adopted. For convenience, the PAL elution procedure was scaled down to permit the use of a smaller column. The cleanup efficiency of the column was not affected by this change. The recovery data (Table 4) indicate quantitative and reproducible elution of the three B_6 analogs from the Dowex AG 50W-X8 columns. Studies concerning the oxidation of PIN to PAL in a phosphate buffer are in progress and will be completed in the near future.

Selected food samples were assayed for total B_6 compounds by the fluorometric procedure (Table 5). For all forms of the vitamin in these individual samples, the chemically determined values were considerably higher than published results based on microbiological assay (Polansky and Toepfer, 1969). By direct comparison of chemical and microbiological procedures, Chin (1975) observed similar large differences in apparent vitamin B_6 in foods. Using a chemical assay based on totally different sample cleanup and fluorophore formation procedures, Kraut and Imhoff (1967) also obtained results which were significantly greater than published microbiological values. These independent results suggest that either the chemical assay may be measuring forms of the vitamin which are not available microbiologically or that factors in the food extract may inhibit microbial growth, thereby affecting the accuracy of the microbiological procedure. Further research will be required to resolve this difference.

The recovery of B_6 compounds added to food samples prior to autoclaving was observed to be somewhat variable (Table 6), with net recovery and precision being dependent of the food sample. The variability could not be satisfactorily explained, although certain heat-induced reactions of the added B_6 vitamins with various food components may be involved. Similar results were observed by Chin (1975). Because of this variability, duplicate recovery samples should be run with each product assayed to assure accuracy.

The precision of this method is slightly less than that of other recently published semi-automated methods of vitamin analysis for B complex vitamins, which is probably due to the complexity of the analysis. However, the sensitivity and specificity of the procedure have been demonstrated. Therefore, this assay method provides a satisfactory means for the chemical complexity of the analysis. However, the sensitivity and specificity of the procedure have been demonstrated. Therefore, this assay method provides a satisfactory means for the chemical determination of vitamin B_6 in foods and biological materials.

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