INFLUENCE OF VITAMIN B-6 INTAKE ON VITAMIN B-6 STATUS OF LACTATING WOMEN AND ON THE VITAMER CONTENT OF THEIR MILK: ENZYMATIC, MICROBIOLOGICAL, AND HPLC TECHNIQUES

by

Leslie A. Morrison

Thesis submitted to the Faculty of
the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Human Nutrition and Foods

APPROVED:

J. A. Driskell, Chairman

H. M. McNair

S. J. Ritchey

October, 1982
Blacksburg, Virginia

ACKNOWLEDGEMENTS

The author wishes to express her appreciation for the guidance and support given by her major professor, Judy Driskell.

The author would also like to express her gratitude to committee members Harold McNair, for his valuable advice with HPLC analyses, and S. J. Ritchey for his support of the thesis.

Special recognition is also extended to the following people:

The subjects who participated in the research and Caryl Grey, President of the New River Valley Childbirth Education Association, who assisted in the recruitment of the subjects; Barbara Chrisley, Registered Medical Technologist, for her technical advice and for her continuing encouragement; Cynthia Gross, for her assistance with the microbiological assay; Miriam Saltmarch, for her advice and encouragement; Terry Hefferan, Libby Sutker, and Jay Pierotti, fellow graduate students and special friends, for their assistance, support, and friendship; my friends, especially Dreama Montague, and to my family; special thanks and gratitude is given to my parents who gave me endless support and encouragement throughout my graduate program.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS ii

LIST OF FIGURES v

LIST OF TABLES vi

LIST OF APPENDICES vii

CHAPTER

Introduction 1

Review of Literature 4

Experimental Procedures 9

A. Recruitment of Subjects 9
B. Description of Subjects 9
C. Collection of Dietary Data 10
D. Collection and Preparation of Samples 10
1. Milk Samples 10
2. Blood Samples 11
E. EALAT Activity 12
F. Microbiological Assay for B-6 Vitamer Concentrations in Milk 15
G. HPLC Assay for B-6 Vitamer Concentrations in Milk 20
H. Statistical Analyses 26

Results and Discussions 28

A. Coenzyme Stimulation of EALAT Data 29
B. Age and Anthropometric Data 31
C. Demographic Data 31
D. Dietary Data 34
E. RPLP Data 38
F. Milk B-6 Vitamer Concentrations as Measured by Microbiological Assay 40
G. Milk B-6 Vitamer Concentrations as Measured by HPLC Assay 45
H. Correlations Between Data Obtained by Dietary and Biochemical Measurements 48
I. Distribution of B-6 Vitamers in Human Milk . . . 51

CONCLUSIONS 54

Table of Contents (cont) | Page

REFERENCES 56

APPENDICES 60

VITA 91

ABSTRACT

LIST OF FIGURES

Figure		Page
1	The Structures of the B-6 Vitamers	2
2	The Structure of DPN	24

LIST OF TABLES

Table		Page
1	Daily Vitamin B-6 Intakes and EALAT Values of Subjects	30
2	Age, Height, and Weight Measurements of Subjects . . .	32
3	EALAT Values of Subjects Catagorized According to Demographic Data	33
4	Comparison of Daily Nutrient Intakes According to Technique of Data Collection	35
5	Daily Dietary Intakes of Subjects	37
6	RPLP Values of Subjects	39
7	Daily Vitamin B-6 Intakes and RPLP Values of Subjects	41
8	Microbiological Assay Values of Subjects	42
9	Daily Vitamin B-6 Intakes and Microbiological Assay Values of Subjects	44
10	HPLC Assay Values of Subjects	46
11	Daily Vitamin B-6 Intakes and HPLC Assay Values of Subjects	47
12	Significant Correlations Between Data Obtained by Dietary and Biochemical Measurements	49
13	Distribution of B-6 Vitamers in Human Milk	52
14	Microbiological and HPLC Assay Values of Subjects . .	53

LIST OF APPENDICES

Appendix — Page

A. Letter of recruitment 61

B. Consent form 62

C. Letter of approval for research 63

D. Subject background information questionnaire 64

E. Cross checklist for 24 h recall 66

F. Four day food record form 68

G. Instructions for nutrition study participants 69

H. Reagents used for erythrocyte preparation 70

I Chemicals and vendors 71

J. EALAT calibration curve 72

K. Preparation of reagents for EALAT assay 73

L. Preparation of exchange resin for microbiological assay 74

M. PL calibration curve for microbiological assay . . . 75

N. PM calibration curve for microbiological assay . . . 76

O. PN calibration curve for microbiological assay 77

P. Preparation of reagents for microbiological assay . . . 78

Q. Calibration curve for HPLC analyses 79

R. Chromatogram of human milk sample 80

S. Chromatogram of human milk sample "spiked" with PL, PN, DPN, and PM 81

T. Preparation of reagents for HPLC assay 82

U. EALAT and RPLP values of subjects 83

List of Appendices (cont) Page

V. Age, height, and weight measurements of subjects . . . 84

W. Demographic data of subjects 85

X. Daily vitamin B-6 intakes of subjects 86

Y. Daily protein and energy intakes of subjects 87

Z. Microbiological assay values of subjects 88

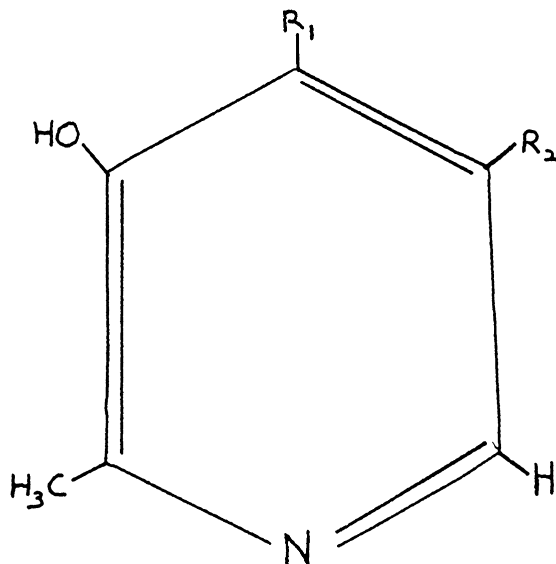
AA. HPLC assay values of subjects 89

BB. B-6 vitamer distribution of subjects 90

Introduction

In recent years there has been increasing concern regarding the nutritional status of lactating women and nutrient composition of human breast milk. It has been shown from human studies that a low dietary intake of vitamin B-6, less than the Recommended Dietary Allowance (RDA), significantly lowers the content of the vitamin in milk (1). It has been suggested that the content of vitamin B-6 in milk reflects the mothers' nutritional status in regard to the vitamin (1). Felice and Kirksey (2) investigated the effects of vitamin B-6 deficiency during lactation on the content of the vitamin in milk, liver, and muscle of rats. The milk was affected sooner than the other tissues, thus being an earlier indicator of impending vitamin B-6 deficiency. Previous studies (1,2) have measured the total vitamin B-6 composition of milk by *Saccharomyces uvarum* (ATCC 9080) microbiological assay. However, applications of various types of chromatographic separations enables one to determine not only total vitamin B-6 composition but also the various vitamin B-6 components. Separation and quantitation of the B-6 vitamers may be achieved via a microbiological assay (3) and by high performance liquid chromotography, HPLC (4-7). The B-6 vitamers are pyridoxal (PL), the aldehyde; pyridoxamine (PM), the amine; and pyridoxine (PN, also referred to as pyridoxol), the alcohol; and the 5'-phosphate forms (PLP, PMP, PNP) of each of the above (Figure 1).

The objectives of this research project included the following:



B-6 Vitamer	R Group Constituents
PL	R_1 = CHO; R_2 = CH_2OH
PM	R_1 = CH_2NH_2; R_2 = CH_2OH
PN	R_1 = CH_2OH; R_2 = CH_2OH
PLP	R_1 = CHO; R_2 = $CH_2OPO_3H_2$
PMP	R_1 = CH_2NH_2; R_2 = $CH_2OPO_3H_2$
PNP	R_1 = CH_2OH; R_2 = $CH_2OPO_3H_2$

Figure 1. The Structures of the B-6 Vitamers.

1. To determine the vitamin B-6 intakes of a group of healthy lactating women.
2. To determine the vitamin B-6 status of the lactating women using coenzyme stimulation of alanine aminotransferase activity in erythrocytes, EALAT (EC 2.6.1.2; L-Alanine: 2-oxoglutarate aminotransferase), and by the radioisotopic measurement of plasma PLP (RPLP) levels.
3. To determine the B-6 vitamer (PL, PM, PN) content of the milk of the lactating women by the established *Saccharomyces ivarum* microbiological assay and by HPLC techniques.
4. To analyze the relationships between the above mentioned parameters.

Review of Literature

It has been established through several studies that vitamin B-6 deficiency in the young can cause aberrations in the central nervous system; Pang and Kirksey (8) demonstrated that rats fed a diet deficient in vitamin B-6 had a low content of the vitamin, cerebrosides, cholesterol, and protein in the brain. Coursin (9) observed that a lack of vitamin B-6 in the maternal diet resulted in a number of abnormalities of the central nervous system. Results of an experiment by Williamson and Coniglio (10) indicated that progeny of dams fed a diet deficient in vitamin B-6 during lactation showed a significantly lower amount of sphingomyelin and PN content in the brain when compared to progeny of PN supplemented dams. Alton-Mackey and Walker (11) found that the progeny of dams receiving 0-100% (0 mg/kg diet - 0.4 mg/kg diet) of the National Research Council's recommendation for PN during lactation exhibited inferior performance of skills requiring advanced neuromotor coordination when compared to progeny of dams receiving 400% (1.6 mg/kg diet) of the recommendation. Driskell and Foshee (12) found that rats receiving a suboptimal, 15 μg, level of vitamin B-6 and progeny whose dams were fed a suboptimal level but were switched to a control diet, 90 μg, at weaning tended to have lower activity scores, significantly lower curiosity scores, fewer correct tone discrimination responses and significantly different distributions of timing responses than rats receiving control levels or offspring whose dams were vitamin B-6 repleted 2 weeks after conception, at parturition, or 1 week after parturition.

The deleterious effects of low vitamin B-6 intakes in the young have also been shown in humans. A deficiency in the human infant may result in irritability and epileptiform-type seizures (13). West and Kirksey (1) observed that one lactating woman with a low level of vitamin B-6 in her milk had an infant who experienced seizures after birth. Mothers with low levels of vitamin B-6 in their milk had infants with unsatisfactory Apgar scores (<7) at 1 min (14).

There has been some evidence that the dietary intakes of vitamin B-6 of lactating women influence the concentration of the vitamin in their milk. West and Kirksey (1) presented evidence that women who consumed < RDA of 2.5 mg (15) had significantly lower amounts of vitamin B-6/l milk than women who consumed $\geq$ 2.5 mg vitamin B-6/day. Kirksey and Susten (16) found that a dietary deficiency of PN in rats resulted in a marked reduction in the vitamin B-6 content of milk on day 12 of lactation. Thomas et al. (17) observed that the vitamin B-6 level in human breast milk of an unsupplemented group of mothers was significantly lower than a B-6 supplemented group of women at 5-7 days postpartum but not at 43-45 days postpartum. In another study, Thomas et al. (18) reported that milk concentrations of vitamin B-6 did not differ significantly between vitamin B-6 supplemented and unsupplemented groups at 6 months postpartum.

Hegsted (19) reported that an inadequate intake of vitamin B-6 (and magnesium, zinc, and folic acid) may be more prevalent than many of the inadequacies of the nutrients that are typically included in surveys. Roepke and Kirksey (14) and Coursin and Brown (20) have re-

ported vitamin B-6 intakes <RDA by women during pregnancy and lactation. The concentration of vitamin B-6 in the milk of lactating women appears to reflect their nutritional state in regard to the vitamin (15). The vitamin B-6 concentration in human milk is approximately 0.01-0.02 mg/l during the first days of lactation and gradually increases to 0.10-0.25 mg/l (15); these data are from microbiological assay measuring total vitamin B-6.

PLP is the vitamer that serves as the coenzyme, although PMP can also activate a number of vitamin B-6 dependent enzymes. The other B-6 vitamers are converted to the coenzyme form, PLP, via enzymatic reactions (21); however, there is no information on the relative biological activity of the B-6 vitamers in man. In rats, these forms are equally active if given parenterally (15).

Many methods have been employed to determine vitamin B-6 status from physiological samples. One indicator that has been used is the coenzyme stimulation of EALAT. Cheney et al. (22) investigated the effect of depletion and repletion of vitamin B-6 in rats and found that this erythrocyte enzyme reflected the state of vitamin B-6 nutrition most accurately and was not affected by other factors. Dirige and Beaton (23) demonstrated a significant correlation between vitamin B-6 intake and EALAT activity in rats. Cinnamon and Beaton (24) studied several parameters that indicate vitamin B-6 status including urinary excretion of xanthurenic acid after a tryptophan load, and the activites of erythrocyte aspartate aminotransferase (EC 2.6.1.1; L-Aspartate: 2-oxoglutarate aminotransferase), and EALAT. They concluded that EALAT

appeared to be the most sensitive index of vitamin B-6 adequacy in man.

Another method of determining vitamin B-6 status is the measurement of PLP in plasma based on the amount of $^{14}CO_2$ evolved from the decarboxylation of L-tyrosine-^{14}C by tyrosine decarboxylase (EC 4.1.1.25; L-Tyrosine carboxy-lyase). Modifications of the method described by Chabner and Livingston (25) have been made by Reinken (26). Sauberlich (27) reported that this method appears to possess the sensitivity necessary to measure the low levels of PLP in plasma. Hamfelt (28) did a comparison of determination of PLP in plasma by the decarboxylation of L-tyrosine-^{14}C and by a tryptophan load test. He found that the enzymatic RPLP method was sensitive and specific making it possible to analyze samples containing low levels of PLP. Russ et al. (29) reported a significant correlation between values obtained by the RPLP assay and coenzyme stimulation of EALAT.

Much of the previous work done on the vitamin B-6 content of milk has been done using the Saccharomyces uvarum assay, the Association of Official Analytical Chemists (AOAC) method 43.159 for vitamin B-6 determination in foods (3). However, this analysis is quite cumbersome and time consuming.

Recently, methods have been described using HPLC to analyze foods and biological fluids for their B-6 vitamers. This analytical technique may hold promise for the analyses of B-6 vitamers in human milk as a simpler and more rapid assay than the microbiological assay, Gregory (30) compared HPLC and S. uvarum assays for total vitamin B-6 on 5 cereal samples; however, the microbiological assay showed evidence of growth inhibi-

tion in 4 of the 5 samples. Results from the remaining sample, based on the mean response of 3 levels of extract addition to the assay tubes, correlated well with HPLC results.

Previous research has related dietary vitamin B-6 intakes of lactating women to the total vitamin B-6 content present in their milk; however, to the knowledge of this researcher, the concentrations of the B-6 vitamers (PL, PM, and PN) in human breast milk have not been measured, not has the relationships between these measurements and dietary vitamin B-6 intake and mother's nutritional status been investigated.

Experimental Procedures

A. Recruitment of Subjects

Names and addresses of women who had recently attended childbirth education classes were acquired from the New River Valley Childbirth Education Association. Letters of recruitment (Appendix A) containing an explanation of the study, the requirements, and the payment were sent to 120 women. The letter also included a stamped card that they were requested to mail back indicating whether or not they were interested in participating in the study.

Volunteers signed a consent form (Appendix B) for the study which had previously been approved by Virginia Polytechnic Institute and State University's Institutional Review Board for Research Involving Human Subjects (Appendix C).

B. Description of Subjects

The 21 volunteer subjects were in apparent good health, between the ages of 21 to 35 years, caucasian, and 3 to 7 months postpartum. All subjects completed a questionnaire (Appendix D) to supply background information. Questionnaires included questions related to problems during lactation, medications, nutrient supplements, use of oral contraceptives, smoking, drinking, and exercise habits.

C. Collection of Dietary Data

Five consecutive days of food intake were collected from each subject. A 24 h recall using an interview technique with cross list checking (Appendix E) and food models were employed to obtain food intake from a Sunday. The subjects were then instructed to keep a food record for the next 4 days using the forms supplied (Appendix F).

D. Collection and Preparation of Samples

1. Milk Samples

Subjects were given oral and written instructions (Appendix G) regarding collection of milk samples. They were each supplied with 3 20 ml polyethylene vials marked with a fill line of 15 ml, a plastic freezer container, and a styrofoam ice chest. Subjects were instructed to fill a vial with their milk for 3 consecutive mornings in the absence of direct light. They were then told to put the vial in the supplied freezer container filled with ice and place in the freezer.

The subjects were instructed to transfer the frozen milk samples from the freezer container to the styrofoam ice chest on the fourth morning and bring it directly to the laboratory so that the milk samples would not thaw. The samples were checked upon arrival and all were frozen.

Milk samples from the 3 consecutive mornings were pooled and redistributed into vials with approximately 5 ml in each.

2. Blood Samples

A registered medical technologist from Virginia Polytechnic Institute and State University obtained approximately 20 ml of nonfasting blood from each subject on the fifth morning of the study. Venous blood samples were collected in vacutainer tubes containing sodium ethylenediamine tetraacetic acid, EDTA. Samples were placed in crushed ice and covered to protect them from light. Whole blood samples were centrifuged in a refrigerated rotor at 3000 x g and 5°C for 10 min.

Plasma was removed with a Pasteur pipet and placed into a polypropylene tube. The plasma samples were placed in containers for protection from light and frozen at -20°C until analyses (25).

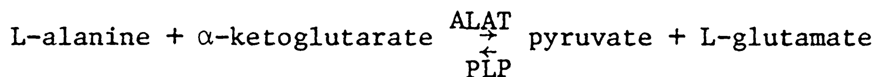
The buffy coat and any remaining plasma was removed from the erythrocytes. The erythrocytes were prepared for EALAT analysis via the method of Heddle et al. (31). The erythrocytes were washed with twice their volume of cold saline. These samples were inverted and centrifuged for 10 min at 3000 x g and 5°C. The saline was removed and 0.5 ml of the remaining erythrocytes were placed in a polypropylene tube; 4.5 ml cold phosphate buffer (pH 7.4) were added to the red cells and the mixture was mixed by inversion. The buffered erythro-

cytes were then stored at -20°C until analyses.

Reagents are listed in Appendix H. Chemical and vendors are listed in Appendix I.

E. EALAT Activity

EALAT activity was determined using the method of Tonhazy et al. (32) as modified by Heddle et al. (31). The procedure of Racia and Sauberlich (33) was used to determine the coenzyme stimulation of enzymatic activity. The assay was based upon the transamination reaction between L-alanine and α-ketoglutarate to form pyruvate and L-glutamate. PLP served as the carrier of amino groups.



$$\text{L-alanine} + \alpha\text{-ketoglutarate} \underset{\text{PLP}}{\overset{\text{ALAT}}{\rightleftarrows}} \text{pyruvate} + \text{L-glutamate}$$

Steps 1-8 of the following procedure were carried out in a room with no direct light:

1. Each sample was run in duplicate using 2 tubes for zero activity (zero activity tubes), 2 tubes for basal activity (basal-activity tubes), and 2 tubes for measurement with additional PLP (stimulation tubes). In addition, for each run, 2 tubes were used as procedure blanks and 2 tubes each were used for standards with concentrations representing 25 μg/0.5 ml and 50 μg/0.5 ml. All samples were kept in an ice brine at 0°-4°C until the 10 min incubation period in order to inhibit enzyme activity.

2. Ten μl PLP were added to each of the stimulation tubes. Next, 0.5 ml cold buffered erythrocytes was placed into each of the zero, basal, and stimulation tubes.
3. The contents of the stimulation tubes were mixed by vortex for 5 s then placed in a 37°C shaker water bath for exactly 20 min.
4. During the incubation of the stimulation tubes, the remaining tubes were kept in the ice brine. To each of the procedure blanks 0.5 ml deionized water was added, and 0.5 ml of pyruvate standard was added to the standard tubes.
5. To the zero activity tubes 3 drops of 100% trichloroacetic acid (TCA) were added. The tubes were then covered with parafilm and their contents were mixed by vortex for 5 s. TCA was added to stop the enzymatic activity since it denatures the protein of the enzyme. It was necessary to take a zero time measurement since some pyruvate is normally present in erythrocytes. Basal samples were used to measure the amount of pyruvate produced in the sample during the incubation period. PLP was added to the stimulation tubes to determine % PLP stimulation.
6. Following the 20 min incubation period of the stimulation tubes, these tubes were returned to the ice brine, and 0.5 ml alanine reagent was added to all of the tubes. Each tube was covered with parafilm and mixed by vortex for 5 s. Alanine

functioned as a substrate to facilitate the production of pyruvate.

7. All tubes were placed in a 37° shaker water bath for exactly 10 min. The tubes were returned to the ice brine following the incubation period and 3 drops 100% TCA were added to each of the tubes except the zero activity tubes. The samples were immediately mixed by vortex for 5 s; 100% TCA was added to stop further enzymatic activity.
8. The tubes were removed from the ice brine and allowed to stand at room temperature for at least 20 min. If necessary, they were frozen at this point as previous experiments indicated that this does not affect the measurements.
9. To each tube, 1 ml dinitrophenyl hydrazine (DNP) solution was added, mixed by vortex for 15 s, and allowed to stand at room temperature for exactly 5 min DNP converted the pyruvate to diphenyl pyruvate hydrazone.
10. Two ml toluene were added to each tube exactly 5 min. after adding the DNP. The tubes were covered with plastic wrap and shaken vigorously for 20 s. All tubes were centrifuged for 10 min at 3000 x g, and 5°C.
11. One ml of the top toluene layer, which contained the diphenyl pyruvate hydrazone, was removed sequentially from each tube at 1 min. intervals and placed in a spectrophotometer tube.
12. Five ml alcoholic potassium hydroxide (KOH) and 1 ml deionized water were added to each spectrophotometer tube, covered

with plastic wrap, and inverted 5 times. Treatment of the toluene with alcoholic KOH produced a colored compound which could be measured spectrophotometrically.

13. Absorbance of each tube was read on a Bausch and Lomb Spectronic 20 at 430 nm. The procedure blanks were used to "zero" the instrument.

14. A standard calibration curve was obtained from pyruvate standards containing concentrations of 12.5, 25, and 50 µg pyruvic acid/ 0.5 ml (Appendix J) After subtracting the absorbance values obtained from the zero activity tubes from the values obtained from the basal and stimulation tubes, a comparison of the absorbance values of the basal and stimulation tubes to the calibration curve reflected the amount of end product, pyruvate, in each tube. Results were expressed as µg pyruvate/ml erythrocytes/h (34). Percent stimulation was calculated as follows:

    [(µg pyruvate/ml erythrocytes/h with added PLP - µg pyruvate ml erythrocytes/h without added PLP) ÷ µg pyruvate/ml erythrocytes/h without added PLP] x 100.

Reagents used in this method are listed in Appendix K. Chemicals and vendors are listed in Appendix I.

F. Microbiological Assay for B-6 Vitamer Concentrations in Milk

A modification of the AOAC procedure (3) for analyzing vitamin B-6 in foods was used.

1. Assay Inoculum

Saccharomyces uvarum cells were incubated on Difco Bacto Y M agar 24 h at 28°C before use. A liquid broth culture tube was prepared by putting 10 ml Pyridoxine Y media (1.3g/50 ml) and 2 4mm glass beads into a pyrex tube with a tight fitting plastic cap and steamed at 121°C for 10 min. Cells were transferred under aseptic conditions from the agar slant to the liquid broth culture tube. The cells were incubated in a shaker water bath at 28°C for 20 h. The resulting inoculum was then washed with a sterile saline solution by centrifuging the inoculated media for 1-2 min at 2500 x g, decanting the liquid and resuspending the cells in 10 ml saline solution under aseptic conditions. This was done 3 times. The cells suspended in the third 10 ml inoculum saline rinse were used as the assay inoculum.

2. Preparation of Sample

Two ml milk and 0.10 g sulfosalicylic acid (SSA) were mixed by vortex and allowed to sit 3 min. SSA was used to denature and precipitate the protein, thereby, releasing the vitamin B-6 (35). After centrifuging for 20 min at 3000 x g and 4°C, the liquid layer was decanted and filtered through a 0.45 μm filter; the precipitant was discarded. Six ml 0.2N HCl (hydrochloric acid) were added to the filtered super-

natant and placed in a boiling water bath for 1 h. This was done in order to cleave the phosphate groups from the B-6 vitamers. The sample was allowed to cool to room temperature and the pH was adjusted to 4.5 with 10% KOH.

3. Preparation of Ion Exchange Column

A Kontes size 222 glass column (17 mm OD, 14.5 mm ID, 250 mm length, 250 ml reservoir) was used for the separation of PL, PM, and PN. Hartz fibre floss was used to plug the bottom of the column. Then, 20 ml activated resin (Appendix L) suspended in distilled H_2O was poured into the column. After the resin had settled into the column another fibre floss plug was placed on top of resin. Next, the column was rinsed with 50 ml hot distilled H_2O, followed by 2 50 ml portions of hot 0.01M potassium acetate, KOAc (pH 4.5).

4. Column Chromotography of B-6 Vitamers

The prepared sample of milk was placed on the column followed by a rinsing of the beaker with deionized water. The sample was allowed to pass completely through the column. The column was then washed with 2 50 ml portions of hot 0.02M KOAc (pH 5.5). PL was eluted with 2 48 ml portions boiling 0.04M KOAc (pH 6.0) using a 100 ml volumetric flask as the receiver. Next, PN was eluted with a 48 ml portion of boiling 0.1M KOAc (pH 7.0) using a 50 ml volumetric flask as the

receiver. PM was eluted next with a 48 ml portion of boiling potassium chloride-phosphate, $KCl-K_2HPO_4$ (pH 8.0) into a 50 ml volumetric flask. Each collected solution was adjusted to pH 4.5 with 10% KOH. Column eluates were brought to volume in the volumetric flasks with deionized water.

5. Microbiological Assay

Tubes used for the assay were cleaned in a 2% microwash solution. Two sterile 4 mm glass beads were placed into each pyrex test tube. Six ml Pyridoxine Y media (19.9 g/450 ml) were put into each tube. Standards were run in duplicate at concentrations representing 1,2,3,4 and 5 ng/tube for vitamers PL and PN, while PM standards were run at concentrations representing 2,4,6,8 and 10 ng/tube. Total volume in each tube was 10 ml. A set of blanks was also prepared which were not to be inoculated; 2 tubes contained 10 ml media and 1 tube contained 10 ml water. Sample eluates were set up at 2 different levels, each in duplicate in the following manner:

Vitamer	Level 1			Level 2		
	Media	Column eluate	H_2O	Media	Column eluate	H_2O
	ml			ml		
PL	6	1	3	6	2	2
PM	6	2	2	6	4	0
PN	6	2	2	6	4	0

All tubes (standards, blanks, samples) were then covered with plastic caps and steamed for 10 min at 121°C. Contents of the tubes were cooled to room temperature. Next the contents of the tubes, omitting the blanks, were inoculated with 1 drop of the prepared *S. uvarum* inoculum using a Pasteur pipet under aseptic conditions. The tubes were then incubated in a 28°C shaker water bath for 22 h. At the end of the incubation period, tubes were steamed for 5 min at 121°C in order to prevent further microbial growth. The contents of the tubes were then cooled to room temperature. Each tube was mixed by vortex and the contents were poured into a spectrophotometer tube. Absorbance at 550 nm using a Bausch and Lamb Spectronic 20 was read. The uninoculated blanks were used to zero the instrument.

Readings of duplicate tubes of standards were averaged and standard calibration curves of absorbance against concentration were prepared for each vitamer - PL, PM, PN (Appendices M, N, O). Readings of sample tubes were averaged and PL, PM, and PN concentrations were calculated by interpolation and reported in terms of ng/ml sample.

Recoveries of PL, PM, and PN were determined by adding 100 ng/ml sample of each standard at the beginning of the preparation of the sample. Recoveries were calculated to be as follows:

B-6 vitamer	Recovery %
PL	93.3
PM	87.1
PN	84.0

Reagents used in this method are listed in Appendix P. Chemicals and vendors are listed in Appendix I.

G. HPLC Assay for B-6 Vitamer Concentrations in Milk

Several methods of extracting the B-6 vitamers from milk for HPLC analyses were tried. The first method tried was similar to that used in the microbiological assay; SSA was used to deproteinate the sample and 0.2N HCL was used to dephosphorylate the B-6 vitamers. However, the recoveries for PLP and PMP were only in the 50-60% range.

Another attempt included treating the sample, with perchloric acid to precipitate the protein (36) followed by a 5 h autoclaving in 0.055N HCL to dephosphorylate the B-6 vitamers (3). This extraction seemed to be suitable for cow's milk; however, when it was used on human milk there were many peaks on the chromatogram which were not B-6 vitamer peaks. The B-6 vitamer peaks could not be identified by "spiking" with standards because of these extra peaks.

A third method of preparing the sample for HPLC analyses involved using a phosphatase for dephosphorylating the B-6 vitamers and TCA for precipitating the protein (37). Several variations of this procedure were attempted in order to make the dilution factor as small as possible.

The best method developed by this researcher for preparing the sample is given in detail below.

1. Preparation of milk sample

One hundred μl of a "spiking" solution containing the internal standard, deoxpyridoxine (DPN), was added to 3 ml milk sample. The sample was covered with parafilm and mixed by vortex for 15 s. Next 0.6 ml of a 10 ng/ml solution of 2U/ml potato acid phosphatase (EC 3.1.3.2; Orthophosphoric-monoester phosphohydrolase) in 0.2M KOAc, pH 4.5, was added to the sample in order to hydrolyze the phosphate esters of the B-6 vitamers. Sample tubes were mixed by vortex for 15 s and incubated for 1 h in a 37°C shaker water bath. The protein was then precipitated by adding 0.25 ml 100% TCA. The sample tube was mixed by vortex for 15 s and incubated for 15 min in a 50°C water bath. Three ml methylene chloride (MeCl) were added to the samples and shaken vigorously in order to remove the lipids. The samples were then centrifuged for 15 min at 4°C and 4000 x g. The resulting supernatants were adjusted to pH 5.2 with 33% sodium hydroxide (NaOH) and dilute HOAc solution and filtered through a 0.45 μm filter with a syringe attachment.

Ion-pair chromatographic conditions for separating B-6 vitamer standards were determined in this laboratory. Retention times of each vitamer were determined by injecting each standard separately. Many solvent gradients were attempted for this particular sample before the optimum conditions using this system were determined. The chromatographic conditions are given below in detail.

2. HPLC Analyses

Vitamin B-6 assays were performed using a Waters Associates liquid chromatograph equipped with the following components:

a. Data module; model 730

b. System controllor; model 720

c. Two Solvent delivery systems; Model 45

d. Universal chromatograph injector; Model U6K

e. Flourescent detector; Model 420E, equipped with a mercury lamp, a 300 nm excitation filter and a 375 nm emission filter.

The separations and quantitations of B-6 vitamers were performed by using the following conditions:

a. Column; μ Bondapak C_{18} (3.9 mm x 30 cm)

b. Mobile phase; 85% methanol (MeOH) and H_2O/PIC B-7 reagent.

c. Gradient

Time Min	Flow rate ml/min	Mobile phase	Curve[a]
Initial	1.5	0% MeOH 100% H_2O/PIC B-7	-
12	1.5	40% MeOH 60% H_2O/PIC B-7	5
16	1.5	40% MEOH 60% H_2O/PIC B-7	5
20	1.5	0% MeOH 100% H_2O/PIC B-7	6

[a]Curve 5 represents a rapid curvilinear increase in solvent strength; while, curve 6 represents a linear increase in solvent strength.

d. Injection volume; 250 μl

3. Quantitation of B-6 Vitamers

The method of internal standard quantitation was used for determining the concentration of each vitamer in the samples. DPN (Figure 2) met the requirements for use as an internal standard as defined by Johnson and Stevenson (38). A calibration curve was obtained by injecting a calibration solution containing the following components:

PL 50.00 ng/250 μl

PM 6.25 ng/250 μl

PN 6.25 ng/250 μl

DPN 118.75 ng/250 μl

These specific concentrations of each standard (PL, PM, and PN) were chosen since they represent amounts close to the expected concentrations (determined from the microbiological assay of the samples used in this research) of the corresponding vitamer in the samples following sample preparation and injection. The concentration of DPN in the calibration solution was equal to the amount of DPN in the sample following sample preparation and injection. The peak areas of the in-

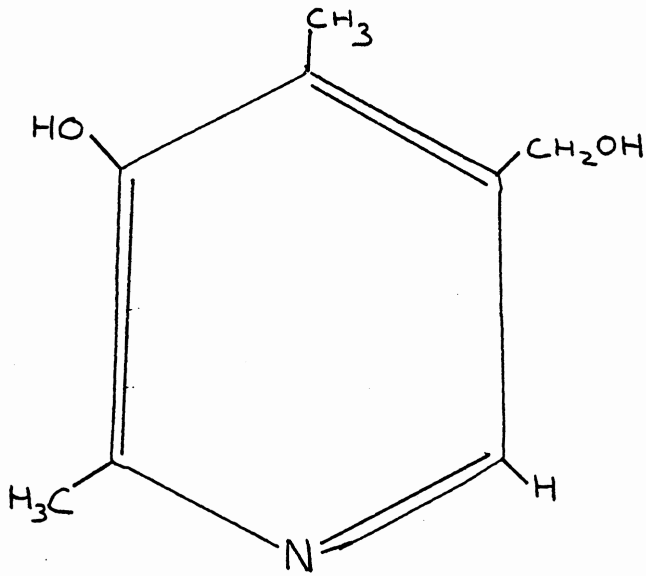


Figure 2. The Structure of DPN

ternal standard did not vary more than 2 standard deviations between samples.

Calculations were performed using the following equation (39):

$$\text{Amount injected} = \frac{\text{Response factor x Area of vitamer peak}}{1000}$$

$$\times \frac{\text{Area of internal standard in calibration solution}}{\text{Area of internal standard in sample}}$$

The concentration of each vitamer was expressed in terms of ng/ml sample. Typical chromatograms of a calibration curve, a sample, and a sample "spiked" with standards are shown in Appendices Q, R, and S.

Recoveries of each vitamer were determined by adding 1000 ng to 3 ml sample prior to sample preparation. The areas of the B-6 vitamer peaks in the "unspiked" sample were subtracted from the areas of the respective B-6 vitamer peaks in the "spiked" samples. A ratio between these areas and the areas of peaks in a calibration solution with corresponding B-6 vitamer concentrations was calculated. Recoveries were determined to be as follows:

B-6 vitamer	Recovery %	
PL	85.8	
PM	105.0	
PN	82.5	
DPN	94.8	
PLP	95.7	(dephosphorylated to PL)
PMP	70.2	(dephosphorylated to PM)
PNP	82.5	(dephosphorylated to PN)

Approximate minimal detectable levels of the B-6 vitamers were as follows: 3 ng for PL which corresponds to 18 ng/ml sample (6 fold dilution) and 1 ng for PM and PN which corresponds to 6 ng/ml sample. Many of the sample PM and PN values were not detectable. In an attempt to remedy this phenomenom, 500 μl, was injected. However, this did not significantly affect the PM and PN peaks and tended to broaden the PL and DPN peaks.

Reagents used in this procedure are listed in Appendix T. Chemicals and vendors are listed in Appendix I.

H. Statistical Analyses

Food intakes were coded and analyzed by the computer system at the Department of Experimental Statistics, Louisiana State University, Baton Rouge, LA, 70803. Vitamin B-6, kilocalorie, and protein intake values (40,41) were determined. Nutrient intakes were expressed in terms of intake/day and as % RDA (15).

For the purpose of discussing the results of the data gathered during the course of this research, the following modes of classifying the subjects were used:

1. EALAT activity
    a. Adequate status; 0-16% stimulation (stim)
    b. Inadequate status; > 16% stim (42)
2. Vitamin B-6 supplementation
    a. Supplemented subjects
    b. Nonsupplemented subjects

3. Daily vitamin B-6 intake (including supplementation)

   a. < 2.5 mg [2.5 mg is the RDA (15)]

   b. 2.5-10.0 mg

   c. > 10.0 mg.

All data were evaluated according to the first and second modes of grouping; the biochemical measurements were evaluated according to all 3 modes of grouping. Analysis of variance (43) and Duncan's multiple range test (33) were used on all data in order to determine differences between values in the various groups. Means ($\bar{x}$) and standard deviations (SD) were also calculated. Pearson r correlation coefficients (45) between data obtained for the various parameters were also determined.

Results and Discussions

Upon separating the subjects into the groups that have been described, it was found that the subjects with adequate status were the same subjects taking vitamin B-6 supplements and that those with inadequate status were the subjects not taking vitamin B-6 supplements. Therefore, the data are discussed according to the subjects' vitamin B-6 status since this mode of grouping is identical to the grouping of subjects according to vitamin B-6 supplementation.

The number of subjects in each classification mode is as follows:

1. EALAT activity
   a. Adequate status; n = 14
   b. Inadequate status; n = 7.
2. Daily vitamin B-6 intake
   a. < 2.5 mg; n = 8
   b. 2.5 - 10.0 mg; n = 8
   c. > 10.0 mg; n = 5 .

In the Appendices the 100 series numbers designate subjects with adequate vitamin B-6 status and the 200 series numbers represent those with inadequate status. The second digit of the subject numbers represent daily vitamin B-6 intake groups; 1, 2, and 3 designate subjects with < 2.5, 2.5-10.0, > 10.0 mg respectively.

A. Coenzyme Stimulation of EALAT Data

EALAT values for individual subjects are presented in Appen-

dix U. Fourteen (66.6%) of the subjects had adequate status, while 7 (33.3%) of the subjects had inadequate status. The $\bar{x} \pm$ SD for the subjects in the former group was 5.4% $\pm$ 6.4 and 34.9% $\pm$ 8.8 for those in the latter group. All the subjects with an adequate vitamin B-6 status reported taking vitamin B-6 supplements; while, the subjects with an inadequate status were not taking supplements.

EALAT values in relation to total vitamin B-6 intake are summarized in Table 1. Subjects who consumed < RDA of 2.5 mg (15) had significantly higher ($p<0.01$) values than those who consumed > 2.5 mg. Subjects with vitamin B-6 intakes of 2.5-10 mg had slightly higher EALAT values than those with intakes > 10 mg; however, this difference was not significant.

Thomas et al. (18) measured the stimulation of EALAT using a slightly different procedure than the method used in this research. The $\bar{x}$ vitamin B-6 intake of unsupplemented subjects at 6 months postpartum was 1.13 mg/day, while the $\bar{x}$ vitamin B-6 intake of supplemented subjects was 5.34 mg/day. The $\bar{x}$ stimulation of the coenzyme was 17% for the nonsupplemented subjects and 33% for the supplemented subjects. This indicates that the vitamin B-6 supplemented subjects had less adequate vitamin B-6 status than the nonsupplemented subjects. Thomas et al. did not offer any explanations of this finding. These findings did not concur with those of this researcher.

Table 1. Daily Vitamin B-6 Intakes and EALAT Values of Subjects

Group Vitamin B-6 mg	Vitamin B-6 mg	EALAT % Stim
< 2.5	1.04[a] ± 0.38	30.5[c] ± 14.8
2.5-10.0	5.01[b] ± 1.12	6.9 ± 6.9
> 10.0	23.13[a,b] ±24.79	4.0 ± 6.0

Values represent $\bar{x} \pm$ SD.

[a]Significant difference at $p<0.01$

[b]Significant difference at $p<0.05$.

[c]Significantly different from other groups at $p<0.01$.

B. Age and Anthropometric Data

Age, height, and weight measurements of individual subjects are presented in Appendix V. These measurements of subjects having adequate and inadequate status are summarized in Table 2. There was not a significant difference in age, height, or weight measurements between groups.

Published measurements of height and weights of lactating women have not been reported to the knowledge of this researcher; therefore, published measurements for heights and weights of adult women were used for comparison purposes. Height and weight measurements for the majority of the subjects corresponded to published values for adult women in the HANES Study (46). Two of the subjects' height measurements were higher than the 95th percentile. One subject's weight measurement fell below the 5th percentile; she also had an inadequate vitamin B-6 status; however, she reportedly consumed only 46% of the RDA for vitamin B-6.

C. Demographic Data

Demographic data of individual subjects are found in Appendix W. The data are summarized in Table 3. Subjects who had previously used oral contraceptives had slightly higher EALAT values as did nonsmokers; however, large SD were noted. Ilcohol consumption, exercise, education, and income levels did not appear to affect vitamin B-6 status. Statistics were not performed due to the low n values of many of the groups and due to the large variances.

Table 2. Age, Height, and Weight Measurements of Subjects

Group	Age yr	Height cm	Weight kg
Adequate Status	26.7 ± 2.5	164.4 ± 7.7	58.4 ± 9.5
Inadequate Status	27.9 ± 5.2	164.4 ± 6.0	59.1 ± 7.1

Values represent $\bar{x} \pm$ SD.

Table 3. EALAT Values of Subjects Categorized According to Demographic Data

Subject Description	n	EALAT % Stim
Oral Contraceptive Users	8	17.6 ± 18.4
Oral Contraceptive Nonusers	13	13.7 ± 14.7
Cigarette Smokers	3	9.5 ± 16.5
Cigarette Nonsmokers	18	16.2 ± 16.1
Alcohol Consumption (drinks/week)		
0	3	14.4 ± 13.7
1	6	12.4 ± 11.1
2	7	9.7 ± 15.8
3	3	30.3 ± 5.5
4	1	0
5	1	42.5
Exercise Level		
Light	1	36.6
Moderate	13	9.0 ± 12.6
Strenuous	5	22.1 ± 20.1
Very Strenuous	2	27.9 ± 0.9
Education Level		
High School	3	27.1 ± 16.4
College	10	13.1 ± 13.1
Graduate School	8	13.4 ± 8.8
Income Level		
< $ 8,000	3	9.1 ± 15.8
$ 8,000-$16,000	7	14.9 ± 13.7
$17,000-$24,000	7	19.2 ± 18.0
> $24,000	4	13.3 ± 20.3

EALAT values represent $\bar{x} \pm$ SD.

Past studies have shown a lower vitamin B-6 status in women using oral contraceptives (15); however, other investigations have indicated that the requirement for vitamin B-6 is approximately the same for oral contraceptive users as for nonusers (15). Roepke and Kirksey (47) investigated the effect of long-term use of oral contraceptives on the vitamin B-6 nutriture during pregnancy and lactation. They found that the long-term use of oral contraceptives resulted in low levels of vitamin B-6 in maternal serum at 5 months gestation and at delivery and in milk compared to values for short-term and nonuser or oral contraceptives.

Evidence of vitamin B-6 deficiency in chronic alcoholic patients has been reported (21); however, the subjects in this investigation did not consume large amounts of alcohol. To the knowledge of this writer, there have been no investigations on the effects of smoking, exercise, or education and income levels on the vitamin B-6 status of lactating women.

D. Dietary Data

Dietary intake information was collected by 2 techniques: a 24 h recall and a 4 day diet record. Diets were analyzed for vitamin B-6, protein, and energy intakes. For the nutrients calculated, there was not a significant difference between the values obtained from the 24 h recall and from the average daily intake from the 4 day diet record (Table 4). Therefore, average daily intakes over the 5 day period of data collection were calculated for vitamin B-6, protein, and energy.

Table 4. Comparison of Daily Nutrient Intakes According to Technique of Data Collection.

Method of Collection	Vitamin B-6[a] mg	Protein g	Energy kcal
24 h recall	0.95 ± 0.35	74.9 ± 20.1	2009 ± 477
4 day record	1.13 ± 0.30	79.5 ± 19.0	2031 ± 451

Values represent $\bar{x} \pm$ SD.

[a]Vitamin B-6 values represent intakes from food sources only.

Fourteen (66.6%) of the subject reported taking vitamin B-6 supplements. The amount of supplementation was added to the vitamin B-6 intake from food for a total vitamin B-6 intake. These values were also expressed in terms of % RDA of 2.5 mg. Dietary information for the individual subjects regarding vitamin B-6 is presented in Appendix X. Dietary information regarding protein and energy intakes of individual subjects appears in Appendix Y.

Not one of the subjects met the RDA for vitamin B-6 for lactating women from their diet excluding supplementation. The $\bar{x}$ intake of all the subjects was 44% of the RDA. Coursin and Brown (20) reported vitamin B-6 intakes of lactating women to be 60% of the RDA,; Roepke and Kirksey (14) published vitamin B-6 intakes of 50% of the RDA; Thomas et al. (18) reported vitamin B-6 intakes of 45-55% of the RDA.

All supplemented subjects had adequate vitamin B-6 status (Table 5). The $\bar{x}$ vitamin B-6 intake of these subjects was 10.88 mg/day (435% RDA). It was noted that one of the supplemented subjects consumed $<$ RDA even with the supplement added; however, she still had an adequate vitamin B-6 status.

The $\bar{x}$ vitamin B-6 intake of the unsupplemented subjects was 0.95 mg (38% of the RDA), and all of these subjects had inadequate vitamin B-6 status (Table 5). There was a significant difference ($p<0.05$) between groups with regard to food + supplement vitamin B-6 intake. There was not a significant difference between groups when considering vitamin B-6 intake from food sources alone (Table 5).

Table 5. Daily Dietary Intakes of Subjects

Group	Food Vitamin B-6 mg	Food + Supplement Vitamin B-6 mg	RDA %	Protein g	Energy kcal
Adequate Status	1.17 ± 0.25	10.88[a] ± 16.32	435[b] ± 653	82.6 ± 17.5	2161[c] ± 446
Inadequate Status	0.95 ± 0.32	0.95[a] ± 0.32	38[b] ± 13	70.4 ± 13.1	1757[c] ± 154

Values represent $\bar{x} \pm$ SD.

[a,b]Significant difference at $p < 0.05$.

[c]Significant difference at $p < 0.01$.

Protein intakes for both adequate and inadequate subjects exceeded the RDA for lactating women of 64 g(15). There was not a significant difference between groups in regard to protein intake (Table 5). West and Kirksey (1) and Thomas et al. (48) reported protein intakes of lactating women what met or exceeded the RDA.

The recommendation for energy intake depends on height, weight, and activity level (15). The range the National Research Council recommends for lactating women with heights of 163 cm and weights of 55 kg is 2100-2900 kcal (15). Subjects with adequate vitamin B-6 status ($\bar{x}$ height-164 cm; $\bar{x}$ weight-58.6 kg) had an energy intake within this range (2161 kcal). Subjects with inadequate vitamin B-6 status ($\bar{x}$ height-164 cm; $\bar{x}$ weight-58.6 kg) had an energy intake below this range (1757 kcal). The energy intake values of the subjects with adequate status were significantly higher ($p < 0.01$) than the subjects with inadequate status (Table 5). Thomas et al. (48) reported energy intakes of 2270 $\pm$ 699 kcal/day (x $\pm$ SD) for lactating women (42-45 days postpartum). Blacksburn and Calloway (49) found energy intakes to be 1800 $\pm$ 454 kcal/day ($\bar{x} \pm$ SD) for lactating women at 8-12 weeks postpartum.

E. RPLP Data

RPLP levels found in plasma of individual subjects are found in Appendix U. The values of subjects with adequate status were significantly higher ($p<0.001$) than those with inadequate status (Table 6). Rose et al. (50) suggested that a RPLP level of 8.5

Table 6. RPLP Values of Subjects

Group	RPLP ng/ml
Adequate Status	39.45[a] ± 18.06
Inadequate Status	15.34[a] ± 5.92

Values represent $\bar{x} \pm$ SD.

[a]Significantly different at $p<0.001$.

ng/ml or less represented inadequate vitamin B-6 status after evaluating a group of men. All RPLP values determined in this investigation exceeded 8.5 ng/ml. In the present research, subjects with an inadequate vitamin B-6 status according to EALAT activity had a RPLP value of 15.34 ng/ml $\pm$ 5.92, $\bar{x} \pm$ SD (Table 6).

When evaluating RPLP values in relation to daily vitamin B-6 intakes, results indicated that those who consumed $>$ 10 ng vitamin B-6/day had significantly higher ($p<0.01$) values than those who consumed $<$ 10 mg/day. Subjects with vitamin B-6 intakes from 2.5-10 mg/day had values higher than those with intakes of $<$ 2.5 mg/day, but this difference was not significant (Table 7).

To the knowledge of this researcher, RPLP values of lactating women have not been previously measured. In the present investigation, lactating women taking vitamin B-6 supplements had significantly higher ($p<0.001$) RPLP values than women not taking vitamin B-6 supplements.

F. Milk B-6 Vitamer Concentrations as Measured by Microbiological Assay

The microbiological assay was used to determine the concentrations of PL (MPL), PM (MPM), and PN (MPN) in the subjects' milk. Totals (MTL) were calculated based on the sum of the vitamers. Data for individual subjects are presented in Appendix Z.

Data are summarized according to EALAT activity in Table 8. Values for each vitamer were higher in the adequate status group than in the inadequate status group. However, only the MPL and

Table 7. Daily Vitamin B-6 Intakes and RPLP Values of Subjects

Group Vitamin B-6 mg	Vitamin B-6 mg	RPLP ng/ml
< 2.5	1.04[a] ± 0.38	19.5 ± 13.0
2.5-10.0	5.01[b] ± 1.12	29.8 ± 11.4
> 10.0	22.13[a,b] ± 24.79	53.1[c] ± 19.8

Values represent $\bar{x} \pm$ SD.

[a]Significant difference at $p < 0.01$.

[b]Significant difference at $p < 0.05$.

[c]Significantly different from other groups at $p < 0.01$.

Table 8. Microbiological Assay Values of Subjects

Group	MPL	MPM	MPN	MTL
	ng/ml			
Adequate Status	137.07[a] ± 14.14	11.07 ± 2.60	11.51 ± 5.41	159.65[b] ± 16.34
Inadequate Status	107.86[a] ± 9.50	9.13 ± 1.83	9.50 ± 4.07	126.48[b] ± 11.12

Values represent $\bar{x}$ ± SD.

[a,b]Significant difference at $p < 0.0001$.

MTL values of the adequate status group were significantly higher ($p<0.0001$) than the corresponding values of the inadequate status group.

The values of the B-6 vitamer concentrations in milk according to daily vitamin B-6 intake are found in Table 9. MPL values of those who consumed > 2.5 mg vitamin B-6/day had significantly higher ($p<0.01$) values than those with a vitamin B-6 intake of < 2.5 mg/day. The MPM values of subjects with vitamin B-6 intakes > 10 mg/day were significantly higher ($p<0.05$) than those with intakes of < 2.5 mg/day. Subjects with vitamin B-6 intakes of > 2.5 mg/day had significantly higher ($p<0.01$) MTL values than those with intakes of < 2.5 mg/day. MTL values of subjects who consumed > 10 mg/day approached being significantly different ($p<0.10$) when compared to those with 2.5-10 mg vitamin B-6 intakes.

Results of this investigation concurred with the results published by West and Kirksey (1). They also found that subjects with a vitamin B-6 intake of > 2.5 mg/day had significantly higher ($p<0.01$) total vitamin B-6 values in their milk than subjects with vitamin B-6 intakes of < 2.5 mg/day. Thomas et al. (18), however, did not find a significant difference in the vitamin B-6 content of milk in subjects with a $\bar{x}$ intake of 1.13 mg vitamin B-6/day as opposed to subjects with $\bar{x}$ vitamin B-6 intakes of 5.34 mg/day.

West and Kirksey (1) determined total vitamin B-6 in milk microbiologically. They reported $\bar{x}$ values of 129 ng/ml when subjects had intakes of < 2.5 mg/day and 239 ng/ml when subjects' in-

Table 9. Daily Vitamin B-6 Intakes and Microbiological Assay Values of Subjects

Group Vitamin B-6 mg	Vitamin B-6 mg	MPL ng/ml	MPM ng/ml	MPN ng/ml	MTL ng/ml
< 2.5	1.04[a] ± 0.38	109[c] ± 9	9.1[d] + 1.7	9.4 ± 3.8	127.2[e] ± 10.5
2.5-10.0	5.01[b] ± 1.12	135 ± 12	10.7 ± 3.1	11.0 ± 5.0	156.7[f] ± 14.9
> 10.0	22.13[a,b] ±24.79	145 ± 14	12.1[d] ± 1.3	12.6 ± 6.8	169.7[f] ± 12.2

Values represent $\bar{x} \pm$ SD.

[a]Significant difference at $p < 0.01$

[b, d]Significant difference at $p < 0.05$

[c,e]Significantly different from other groups at $p < 0.01$.

[f]Approaches significant difference at $p < 0.10$.

takes were 2.5-5.0 mg/day. Mean MTL values determined in this research were 127 ng/ml when subjects had vitamin B-6 intakes of < 2.5 mg/day and 156 ng/ml when subjects had 2.5-10.0 mg vitamin B-6/day.

The current writer found no published reports on the concentrations of the individual B-6 vitamers in human milk. The results of the present research indicated that PL was the predominant vitamer in human milk. Results also indicated that B-6 vitamer concentrations were higher in subjects taking vitamin B-6 supplements than in subjects not taking supplements; MPL and MTL values of subjects taking vitamin B-6 supplements were significantly higher ($p < 0.0001$) than MPL and MTL values of subjects not taking supplements.

G. Milk B-6 Vitamer Concentrations as Measured by HPLC Assay

HPLC was used to determine the levels of PL (HPL), PM (HPM), and PN (HPN) in the subjects' milk. Total vitamin B-6 (HTL) was calculated as the sum of HPL, HPM, and HPN. HPLC B-6 vitamer values for individual subjects are presented in Appendix AA.

HPLC data are summarized according to EALAT activity in Table 10. HPL and HTL values were significantly higher ($p < 0.001$) for those subjects with adequate status than for those with inadequate status.

HPLC data in relation to vitamin B-6 intakes are found in Table 11. Subjects with vitamin B-6 intakes > 10 mg/day had significantly higher ($p < 0.01$) HPL values than those with intakes < 2.5

Table 10. HPLC Assay Values of Subjects

Group	HPL	HPM		HPN		HTL
	ng/ml					
		nd omited	nd = 0	nd omitted	nd = 0	
Adequate Status	117.29[a] ± 66.12	19.4 ± 12.7	12.49[b] ± 13.91	13.1 ± 7.1	2.80 ± 6.21	132.58[c] ± 74.66
Inadequate Status	31.83[a] ± 11.56	12.1 ± 0	1.73[b] ± 4.57	nd	0	33.56[c] ± 10.08

Values represent $\bar{x} \pm$ SD.

[a,c]Significant difference at $p < 0.001$.

[b]Significant difference at $p < 0.05$.

Table 11. Daily Vitamin B-6 Intakes and HPLC Assay Values of Subjects

Group Vitamin B-6 mg	Vitamin B-6 mg	HPL	HPM[a]	HPN[a]	HTL
		ng/ml			
< 2.5	1.04[b] ± 0.38	45.9[d] ± 41.2	1.5[f] ± 4.3	nd	47.4[g] ± 40.3
2.5-10.0	5.01[c] ± 1.12	89.1 + 42.4	11.4 ± 15.6	4.1 + 7.8	104.6[h] ± 58.0
> 10.0	22.13[b,c] ± 24.79	157.0[d,e] ± 85.5	16.8[f] ± 11.7	1.2 ± 2.8	175.1[g,h] ± 91.4

Values represent $\bar{x} \pm$ SD.

[a]Calculations include nd values as 0.

[b,d,g]Significant difference at $p < 0.01$.

[c,f]Significant difference at $p < 0.05$.

[e]Significantly different from other groups at $p < 0.05$.

[h]Approaches significant difference at $p < 0.10$.

mg/day and significantly higher ($p < 0.05$) than those with intakes < 10 mg/day. HPM values were significantly higher ($p < 0.05$) for subjects with daily vitamin B-6 intakes > 10 mg than for those with intakes < 2.5 mg. Subjects with vitamin B-6 intakes > 10 mg had significantly higher ($p < 0.01$) HTL values than the subjects with intakes of < 2.5 mg/day. Differences in HTL values approached significance ($p < 0.10$) between subjects who consumed > 10 mg/day and subjects with vitamin B-6 intakes 2.5-10 mg/day.

This researcher found no published reports regarding individual B-6 vitamer concentrations or total vitamin B-6 content in human milk as determined by HPLC. Results of previous research reporting total vitamin B-6 concentration in human milk as determined by microbiological assay have been discussed previously. Results of the present investigation indicated that B-6 vitamer content in milk was higher in subjects taking vitamin B-6 supplements than in subjects not taking vitamin B-6 supplements. HPL and HTL values of vitamin B-6 supplemented subjects were significantly higher ($p < 0.001$) than the corresponding values of nonsupplemented subjects.

H. Correlations Between Data Obtained by Dietary and Biochemical Measurements

Significant Pearson r correlation coefficients are presented in Table 12. All other parameters measured were not found to have significant correlations.

Results indicated that vitamin B-6 intake was significantly

Table 12. Significant Correlations Between Data Obtained by Dietary and Biochemical Measurements

Measurements	r	p value
B-6 intake: EALAT	-.38	0.10[a]
B-6 intake: RPLP	.42	0.10[a]
B-6 intake: MPL	.42	0.10[a]
B-6 intake: MPN	.57	0.01
B-6 intake: MTL	.52	0.05
B-6 intake: HPL	.72	0.001
B-6 intake: HTL	.70	0.001
EALAT: RPLP	-.59	0.01
EALAT: MPL	-.65	0.01
EALAT: MPM	-.48	0.05
EALAT: MPN	-.40	0.10[a]
EALAT: MTL	-.71	0.001
EALAT: HPL	-.64	0.01
EALAT: HTL	-.63	0.01
RPLP: MPL	.59	0.01
RPLP: MTL	.56	0.01
RPLP: HPL	.75	0.0001
RPLP: HTL	.71	0.001
MPL: MTL	.96	0.0001
MPM: MTL	.51	0.05
MPN: MTL	.43	0.05
HPL: HTL	.98	0.0001
HPM: HTL	.64	0.01
HPN: HTL	.48	0.05
MPL: HPL	.62	0.01
MTL: HTL	.60	0.01

[a]Values approached being significant.

correlated with several of the biochemical measurements. Vitamin B-6 intakes approached being significantly correlated ($p < 0.10$) with EALAT values. Vitamin B-6 intakes and RPLP values in plasma approached having a significant correlation ($p < 0.10$). RPLP values in plasma of lactating women have not been previously published to the knowledge of this researcher. Significant correlations were found to exist between vitamin B-6 intake and PL as well as total vitamin B-6 values in milk as measured by both microbiological assay and HPLC assay. As previously discussed, Thomas et al. (18) did not find that vitamin B-6 intake affected vitamin B-6 concentration in milk, while, West and Kirksey (1) did find that vitamin B-6 content in milk was affected by vitamin B-6 intake; correlation coefficients were not determined in that investigation.

A significant ($p < 0.01$) correlation coefficient of -.59 existed between the measurements obtained by the status indicators EALAT and RPLP in the current research. Russ et al. (29) reported a significant ($p < 0.001$) correlation coefficient of -.60 between these measurements.

Significant ($p < 0.01$) correlation coefficients existed between values obtained by the methods of analysis, microbiological assay and HPLC assay, used to determine B-6 vitamer content in milk for PL and for total vitamin B-6 values. As previously stated, the current writer has found no published values indicating B-6 vitamer concentrations in human milk.

Both EALAT and RPLP measurement were found to be significantly

correlated with MPL, MTL, HPL, and HTL measurements. These results strongly suggest that a mother with adequate vitamin B-6 status will have more vitamin B-6 in her milk than a mother with inadequate vitamin B-6 status.

I. Distribution of B-6 Vitamers in Human Milk

The distribution of B-6 vitamers in the milk of individual subjects is presented in Appendix BB. The data are summarized according to vitamin B-6 status in Table 13. The PL vitamer is the predominant form. Upon evaluating the microbiological data, it appears that the distribution of vitamers remains constant despite vitamin B-6 status. When evaluating the data from HPLC analyses one can see that distribution changes slightly for the subjects with inadequate status. The B-6 vitamer measurements in milk when classified in relation to EALAT activity (Table 14) showed that the microbiological data are similar to the HPLC data for those with adequate status but not for subjects with inadequate status. The numerous nondetectable values for HPM and HPN may contribute to to this difference. In an attempt to explain the large difference between the MPL and HPL values for subjects with inadequate status, HPL was tested for linearity due to the hypothesis that values may not be linear close to the minimal detectable values. However, amounts of PL ranging from 5 ng to 50 ng were found to be linear; thus, the hypothesis was not proven to be valid. The current researcher does not know of any published reports indicating the distribution of B-6 vitamers in human milk.

Table 13. Distribution of B-6 vitamers in Human Milk

Group	MPL	HPL	MPM	HPM	MPN	HPN
			%			
All subjects	85.7 ± 3.3	91.5 ±12.3	7.0 ±1.5	7.5 ±11.3	7.2 ±2.8	1.0 ±2.9
Subjects with adequate status	85.9 ± 3.6	90.0 ±11.2	6.9 ±1.5	8.5 ±9.6	7.2 +3.0	1.5 ±3.4
Subjects with inadequate status	85.3 ± 2.9	94.4 ±14.8	7.3 ±1.7	5.6 ±14.8	7.4 ±2.7	nd

Values represent $\bar{x} \pm$ SD.

Table 14. Microbiological and HPLC Assay Values of Subjects

Group	MPL	HPL	MPM	HPM[a]	MPN	HPN[a]	MTL	HTL
	ng/ml							
Adequate Status	137.07 ± 14.14	117.29 ± 66.12	11.07 ± 2.60	19.4 ±12.7	11.51 ± 5.41	13.1 ± 7.1	159.65 ± 16.32	132.59 ± 74.66
Inadequate Status	107.86 ± 9.50	31.83 ± 11.56	9.13 ± 1.83	12.1 ± 0	9.50 ± 4.07	nd	126.49 ± 11.12	33.58 ± 10.88

Values represent $\bar{x} \pm$ SD.

[a]Calculations include detectable values only.

CONCLUSIONS

No significant difference in age, height, and weight measurements existed between vitamin B-6 status groups. Previous use of oral contraceptives, smoking and drinking habits, and exercise, education, and income levels of the subjects did not appear to affect their vitamin B-6 status.

None of the subject met the RDA for lactating women when considering vitamin B-6 intakes from food sources. All subjects taking nutrient supplements had adequate vitamin B-6 status; while all subjects not taking supplements had inadequate vitamin B-6 status.

RPLP values were significantly higher ($p < 0.001$) for subjects with adequate vitamin B-6 status than for subjects with inadequate status. A significant ($p < 0.01$) Pearson r correlation coefficient, -.59, existed between values obtained for EALAT and RPLP measurements.

Subjects taking vitamin B-6 supplements not only had adequate vitamin B-6 status but also had higher vitamin B-6 concentrations in their milk as indicated by both microbiological and HPLC analyses. PL values and total vitamin B-6 values were significantly higher ($p < 0.0001$ for microbiological assay, $p < 0.001$ for HPLC assay) for the subjects taking vitamin B-6 supplements than for those not taking supplements. Subjects with higher vitamin B-6 intakes had higher concentrations of the vitamers in their milk.

PL is the predominant B-6 vitamer in human milk as indicated by both microbiological and HPLC analyses. Distribution of PL, PM, and PN in human milk appeared to stay relatively constant between subjects de-

spite vitamin B-6 status.

REFERENCES

1. West KD, Kirksey A. Influence of vitamin B-6 intake on the content of the vitamin in human milk. Am J Clin Nutr 1976;29:961-9.

2. Felice JH, Kirksey A. Effects of vitamin B-6 deficiency during lactation on the vitamin B-6 content of milk, liver and muscle of rats. J Nutr 1981;111;610-7.

3. Horwitz W, ed. Official Methods of Analysis of the Association of Official Analytic Chemists. 13th ed. Menash, WI: George Banta Company, 1980.

4. Williams AK, Cole PO. Vitamin B-6 ion exchange chromatography of pyridoxal, pyridoxol, and pyridoxamine. J Agr Food Chem 1975; 23: 915-6.

5. Wong, FF. Analyses of vitamin B_6 in extractives of food materials by high performance liquid chromotography. J Agr Food Chem 1978; 26:1444-6.

6. Vanderslice JT, Stewart KK, Yarmas MM. Liquid chromatographic separation and quantification of B_6 vitamers and their metabolite, pyridoxic acid. J Chrom 1979;176:280-5.

7. Lim KL, Young RW, Driskell JA. Separation of vitamin B-6 components by high performance liquid chromatography. J Chrom 1980;188:285-8.

8. Pang RL, Kirksey A. Early postnatal changes in brain composition in progeny of rats fed different levels of dietary pyridoxine. J Nutr 1974;104:11-7.

9. Coursin DB. Vitamin B-6 and brain function in animals and man. In: Krauss M, Ed. Vitamin B-6 in metabolism of the nervous system. Ann NY Acad Sci 1969;166:7-28.

10. Williamson B, Coniglio JG. The effects of pyridoxine deficiency of caloric restriction on lipids in the developing rat brain. J Neuro Chem 1971;18:267-76.

11. Alton-Mackey MG, Walker BL. The physical and neuromotor development of progeny of female rats fed graded levels of pyridoxine during lactation. Am J Clin Nutr 1978;31:76-81.

12. Driskell JA, Foshee DP. Behavioral patterns and brain nucleic acid and pyridoxal phosphate contents of male rat progeny from vitamin B-6 repleted dams. J Nutr 1974;104:810-8.

13. Coursin DB. Vitamin B-6 metabolism in infants and children. Vit and Horm 1964;22:755-86.

14. Roepke JLB, Kirksey A. Vitamin B-6 nutriture during pregnancy and lactation. I. Vitamin B-6 intake, levels of the vitamin in biological fluids, and condition of the infant at birth. Am J Clin Nutr 1979;32:2249-56.

15. National Academy of Sciences. Recommended dietary allowances. 9th ed. Washington, DC: National Academy of Sciences, 1980.

16. Kirksey A, Susten SS. Influences of different levels of dietary pyridoxine on milk in the rat. J Nutr 1978;108:104-12.

17. Thomas RM, Kawamoto J, Sneed SMS, Eakin R. The effects of vitamin C, vitamin B-6, and vitamin B-12 supplementation on the breast milk and maternal status of well-nourished women. Am J Clin Nutr 1979; 32:1679-85.

18. Thomas MR, Sneed SM, Wei C, Nail PA, Wilson M, Sprinkle EE. The effects of vitamin C, vitamin B-6, vitamin B-12, folic acid, riboflavin, and thiamin on the breast milk and maternal status of well-nourished women at 6 months postpartum. Am J Clin Nutr 1980;33: 2151-6.

19. Hegsted DM. Priorities in nutrition in the United States. J Am Diet Assoc 1977;71:9-13.

20. Coursin DB, Brown VC. Changes in vitamin B-6 during pregnancy. Am J Obstet Gynecol 1961;82:1207-11.

21. Sauberlich HE, Canham JE. Vitamin B-6 In: Goodhart RS, Shils ME, eds. Modern nutrition in health and disease. Philadelphia, PA: Lea and Febiger, 1980:216-29.

22. Cheney MC, Sabry ZI, Beaton GH. Blood transaminase activities in vitamin B-6 deficiency: Effect of depletion and repletion on the erythrocyte enzymes. Can J Physiol Pharmacol 1967;45:343-51.

23. Dirige OV, Beaton JR. Factors affecting vitamin B-6 requirement in the rat as determined by erythrocyte transaminase activity. J Nutr 1969;97:109-16.

24. Cinnamon AD, Beaton JR. Biochemical assessment of vitamin B_6 status in man. Am J Clin Nutr 1970;23:696-702.

25. Chabner B, Livingston D. A simple enzymatic assay for pyridoxal phosphate. Anal Biochem 1970;34:413-23.

26. Reinken L. Eine mikrom ethode zer bestimm ung von pyridoxal-5-phosphate in serum mittels decarboxylierung von 1-tryosin-1-^{14}C. Int J Vit Nutr Res 1972;42:476-481.

27. Sauberlich HE, Skala JH, Dowdy RP. Vitamin B-6. In: Sauberlich HE, Skala JH, Dowdy RP, eds. Laboratory tests for the assessment of nutritional status. Cleveland, OH: CRC Press, 1974:37-44.

28. Hamfelt A. Enzymatic determination of pyridoxal phosphate in plasma by decarboxylation of L-tyrosine-^{14}(CU) and a comparison with the tryptophan load test. Scand J Clin Lab Invest 1967;20:1-10.

29. Russ, CS, Hendricks TA, Chrisley BM, Kalin NH, Driskell JD. Vitamin B-6 status of depressed and obsessive-compulsive patients. Submitted for publication to Am J Clin Nutr, Aug 26, 1982.

30. Gregory JF. Comparison of high-performance liquid chromotagraphic and Saccharomyces uvarum methods for the determination of vitamin B-6 in fortified breakfast cereals. J Agric Food Chem 1980; 28: 486-9.

31. Heddle JG, McHenry EW, Beaton GH. Penicillamine and vitamin B-6 interrelationships in the rat. Can J Biochem Physiol 1963:17:1215-22.

32. Tonhazy NE, White NG, Umbreit WW. A rapid method for the estimation of the glutamic aspartic transaminase in tissues and its application to radiation sickness. Arch Biochem 1950;28:36-42.

33. Racia N, Sauberlich, HE. Blood cell transaminase activity in human vitamin B-6 deficiency. Am J Clin Nutr 1964:15:67-72.

34. Woodring M, Storvick C. Effects of pyridoxine supplementation on glutamic pyruvic transaminase and in vitro stimulation in erythrocytes of normal women. Am J Clin Nutr 1970;23:1385-95.

35. Vanderslice JT, Marie CE, Doherty RF, Beecher GR. Sulfosalicylic acid as an extraction agent for vitamin B-6 in food. J Agric Food Chem 1980;28:1145-9.

36. Gregory JF. Determination of pyridoxal 5'-phosphate as the semicarbazone derivative using high-performance liquid chromotography. Anal Biochem 1980; 102:374-9.

37. Gregory JF, Kirk JR. Assessment of storage effects of vitamin B-6 stability and bioavailability in dehydrated food systems. J Food Sci 1978;43:1801-8.

38. Johnson EL, Stevenson R. Basic liquid chromotography. Palo Alto, CA: Varian Associates, Inc., 1978.

39. Waters Associates. Data module model 730 operator's manual. Milford, MA: 1981.

40. Orr ML. Pantothenic acid, vitamin B-6 and vitamin B-12 in foods. Washington, DC: United States Department of Agriculture, 1969. (Home Economics Research Report No. 36).

41. Watt BK, Merrill AL. Composition of foods. Washington, DC; United States Department of Agriculture, 1963. (Agricultural Handbook No. 8).

42. Kirksey A, Keaton K, Abernathy PR, Greger JL. Vitamin B-6 nutritional status of a group of female adolescents. Am J Clin Nutr 1978;31:946-54.

43. Sokal RR, Rohlf FJ. Biometry. San Francisco, CA: WH Freeman, 1969.

44. Steel RGD, Torrie JH. Principles and Procedures in Statistics. New York, NY: McGraw-Hill, 1960.

45. Downie NM, Heath RW, Basic Statistical Methods. 2nd ed. New York, NY: Harper & Row, 1965.

46. National Center for Health Statistics. Height and weight of adults 18-74 years of age in the United States. Rockville, MD: National Center for Health Statistics, 1976. (Vital and health statistics. no. 31) [DHEW publication no. (HRA) 77-1250].

47. Roepke JL, Kirksey A. Vitamin B-6 nutriture during pregnancy and lactation. II. The effects of long-term use of oral contraceptives. Am J Clin Nutr 1979;32:2257-64.

48. Thomas MR, Kawamoto J. Dietary evaluation of lactating women with or without vitamin and mineral supplementation. J Am Diet Assoc 1979;74:669-72.

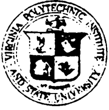
49. Blackburn MW, Calloway DH. Energy expenditure and consumption of mature, pregnant and lactating women. J Am Diet Assoc 1976;69:29-37.

50. Rose CS, György P, Butler M, et al. Age differences in vitamin B-6 status of 617 men. Am J Clin Nutr 1976;29:847-53.

APPENDICES

APPENDIX A

Letter of recruitment



COLLEGE OF HUMAN RESOURCES

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Blacksburg, Virginia 24061

DEPARTMENT OF HUMAN NUTRITION AND FOODS

Dear :

I am a graduate student in the Human Nutrition and Foods Department at Virginia Tech. I received your name from Caryl Gray, President of the Childbirth Education Association. I am conducting a nutrition study in our department and could use your help if you are presently breastfeeding your baby.

In order to participate all you need to do is keep a diet record for five days, collect a small sample of breast milk for three mornings, and have one blood sample taken by a Registered Medical Technologist.

You will receive $5.00 for each milk sample and $10.00 for a blood sample for a total of $25.00. You will also receive a computer analysis of your diet and results of the study which includes the vitamin B-6 content of your milk and blood samples.

Please consider participating. It will take a minimal amount of time and the knowledge gained will be useful to both of us. This study has been approved by the Institutional Review Board for Research and is being conducted under the supervision of Dr. Judy Driskell who has extensive experience in vitamin research. Please send back the enclosed card.

Thank you,

Leslie Morrison

st
enclosure

APPENDIX B

Consent Form

Project for Determination of Vitamin B-6 Composition in Human Breast Milk

I have received written and oral explanations of the study and understand the following:

The study will endure for five days. The subjects will keep diet records for five days. The last three days the subjects will collect 15 ml of breast milk per day. The last day the subject will come to Wallace Hall, and a Registered Medical Technologist will take 20 ml blood from the subject.

The identity of the subject will be held confidential in all reports of this research. Subjects are at essentially no risk by participating in the project. It is the subject's responsibility to advise the investigators of any medical problems that arise in the course of the experiment. No compensation is available if injury is suffered as a result of this research. However, information regarding the subject's vitamin B-6 status will be available. Consent for participation may be withdrawn at any time. Questions regarding this project may be answered by any of the investigators listed below.

I understand the above and agree to participate in the nutrition study at times arranged with us between September 1, 1981 and February 1, 1982.

____
Subject

____
Date

____
Code Number

Investigators:
Dr. Judy Driskell
Leslie A. Morrison
Theresa Hefferan

Chairman, Institution Review Board:
Dr. M. P. Stombler

APPENDIX C

Letter of approval for research

RESEARCH DIVISION

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Blacksburg, Virginia 24061

OFFICE OF THE DEAN (703) 961-5281

August 20, 1981

Ms. Leslie Morrison
Graduate Student
Human Nutrition and Foods
Campus

Dear Ms. Morrison:

Your study entitled "Determination of B-6 Vitamers in Human Breast Milk" has been approved for your use of human subjects via our expedited approval process.

Please do not hesitate to contact me if additional information is needed.

Yours truly,

M. P. Stombler, Chairman
Institutional Review Board
for Research Involving Human
Subjects

MPS:bsb

APPENDIX D

Subject background information questionnaire

Subject Code Number ____________________ Address ______________________________

Phone ______________________________________ Date ______________________________

1. What day was your baby born? ____________________

2. Are you breastfeeding? ____________________

3. How often do you breastfeed? ____________________

4. Are you having any problems breastfeeding? ________________

   If so, please explain. ____

5. Did you have any illnesses during your pregnancy? ________________

   If so, please explain. ____

6. Have you had any illnesses since the birth of your baby? ____________

   If so, please explain. ____

7. Have you had children before? __________ If yes, give dates. __________

8. Have you breastfed before? ______________

9. Are you taking any medications or other drugs? ______ If so, give

   names ____

   How often? ____

10. Do you take vitamin pills or mineral supplements? ______ If so, give

    brand name. ____________________ How often? ________________

11. Did you take vitamin pills or mineral supplements during your pregnancy? _________ If so, give brand name. ____________ How often? ________

12. Did you take oral contraceptives prior to becoming pregnant? ________

    If so, for how long? ________________ Please give brand name and dose

    level ____

    How long had you discontinued using them before becoming pregnant?

    ____

13. Do you smoke? __________ If yes, how many packs weekly? _________ Did you smoke during your pregnancy? _____ How long have you been smoking? ____

14. Do you consume alcoholic beverages? _____ If yes, what type of

    alcohol? ________________ How many drinks weekly? ____________________

APPENDIX D con't.

Did you drink during pregnancy?______How many drinks weekly?________

How long have you been drinking?____________________________________

15. Check the amount of exercise you do on a normal day.

____Light: typing, teaching, labwork, some walking but no strenuous exercise.

____Moderate: walking, housework, gardening, little sitting.

____Strenuous: unskilled labor, skating, outdoor games, dancing, jogging.

____Very strenuous: tennis, swimming, running.

16. Years of education completed.

___a. 6 years;____b. High school; ___c. College; ___d. graduate.

17. What approximately is your family income?

___a. under $8000; ___b. $8000-$16,000; ___c. $17,000-$24,000;

___d. over $24,000

18. Do you consider your eating habits ___a. excellent; ___b. good;

____c. fair; ___d. poor.

All information obtained from this questionnaire will be held confidential.

APPENDIX E

Cross checklist for 24 h recall

Did you eat any of the following foods yesterday?

Cereal:	Dry cereal products such as cornflakes?
	Cooked cereal products such as oatmeal and grits?
Bread:	Bread products such as toast.
	Doughnuts
	Sweet Rolls
	Danish pastry
	Pancakes or waffles
Sweet:	James or jelly
	Honey or syrup
Milk:	Milk products, whole milk with cereal or after.
	Skim milk or low fat milk.
	Powdered milk, such as non-fat dry milk.
	Evaporated milk.
Flavor:	Chocolate or other kind?
	Instant breadfast products, which kind?
	Cheese, what kind?
Beverages:	Coffee or tea
	Hot chocalate or cocoa.
	Milk shake, what kind?
	Coke or other soft drink,
	Low calorie beverages.
	Fruit juices, what kind?
	Beer, what kind or brand.
	Wine, what kind or brand.
Eggs:	How many, and how were they prepared?
Breakfast Meats:	Bacon, ham, sausage or other kind:
Fruits:	Oranges, bananas or other fresh fruit?
	Peaches, pears or other canned fruit?
Soups:	What kind and how much.
Sandwiches:	Roast beef.
	Tuna fish.
	Egg salad or other meat-type salad?
	Peanut butter.
	Hamburgers.
	Hot dogs.
Dressing:	Catsup, mustard or mayonnaise?
	Pickles, relish, onions.
Raw Vegetables:	such as avocado, carrots, celery, tomatoes, other?
Salads:	What kind, how prepared, how much?
	What kind of salad dressing?
Cooked Vegetables:	Such as beans, peas, squash, corn, others?
	Any dressing such as butter, margarine, other sauce?

APPENDIX E CROSS CHECK FOR 24-HOUR RECALL Con't

Meat: such as Beef, what type, how prepared?
Chicken, how prepared?
Pork
Fish or sea food
Veal
Liver

Starches: such as potatoes, how prepared?
Rice
Noodles, macaroni, how prepared?

Desserts: such as pie, what kind?
Cake
Cookies
Pudding
Jello, custard, etc.
Ice cream or ice milk.
Sherbert

Miscellaneous foods such as potato chips?

French fries
Fritos
Popcorn, with butter or margarine?
Onion rings
Pizza, what kind, how much, how prepared?
Cheese crackers
Biscuits or cornbread
Candy, waht kind?
Sugar, cream, lemon with your coffee or tea?
Marshmallows

Nuts such as Peanuts?

Pecans
Brazil nuts
Walnuts
Sunflower seeds

Any other foods or drinks not listed, such as health foods?

APPENDIX F

Four day food record form

Date of Record:__________________ Subject Code Number__________

Day of Week Taken: M T W Th F Sat Sun (circle)

Food and Beverage Consumed Today

	Items	Cooking Method	Quantity
Breakfast:			
Lunch:			
Snack:			
Dinner:			
Any other time:			

APPENDIX G

Instructions for nutrition study participants

1. Keep complete diet records Monday-Thursday,

2. Fill freezer container with ice. Salt it thoroughly.

3. Tues, Wed., & Thurs. Fill one vial to marked line each day. Please do this in as subdued light as possible. Close curtains and turn out lights. Put in freezer container and cover with ice. Place in freezer. Please do not reopen vials as salt may get in them.

4. Friday morning. Fill ice chest with ice. Salt it thoroughly. Transfer vials from freezer container to ice chest. Please do this in the <u>dark</u>. Please cover with aluminum foil before transferring.

5. Bring in diet records, freezer container, and ice chest with sample vials. Please come directly to Wallace Hall since we cannot use any samples that thaw. Come to room 301. Bring questionnaire also.

Thank you,

Leslie A. Morrison

APPENDIX H

Reagents used for erythrocyte preparation

1. Saline

   0.9 NaCl/dl

2. Phosphate buffer, pH 7.0, 0.067 M

   9.08 g KH_2PO_4/1

   11.61 g K_2HPO_4/1

Add 804 ml of the K_2 HPO_4 solution to 196 ml of the KH_2PO_4 solution. Check pH.

APPENDIX I

Chemicals and vendors

American Type Culture Collection (Rockville, MD)
    Saccharomyces uvarum 9080

Bio-Rad Laboratories (Richmond, CA)
    Dowex AG 50w-x8 resin 142-1441

Difco Laboratories (Detroit, MI)
    Bacto Y M Agar 0712-01
    Pyridoxine Y Medium 0951-15-2

ICN Nutritional Biochemicals (Cleveland, OH)
    Pyridoxine phosphate 102778

Pierce Chemical Co. (Rockford, ILL)
    5-Sulfosalicylic acid dihydrate 27800

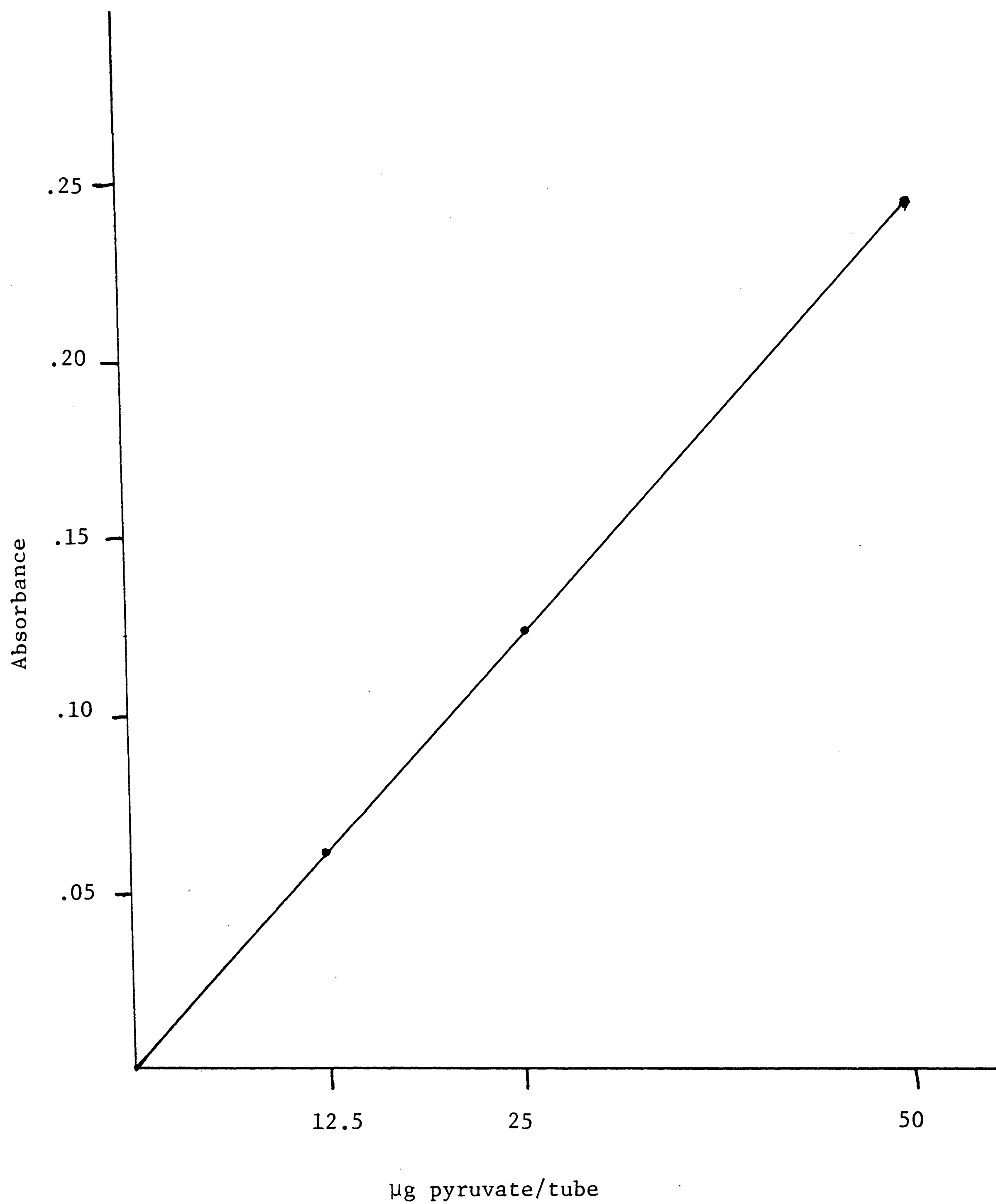
Sigma Chemical Co. (St. Louis, MO)
    Pyridoxal hydrochloride P 9130
    Pyridoxal 5-phosphate P 9255
    Pyridoxamine dihydrochloride P 9380
    Pyridoxamine 5-phosphate hydrochloride P 9505
    Pyridoxine hydrochloride P 9755
    Type IV-S acid phosphotase P 1146

United States Biochemical Corp. (Cleveland, OH)
    4-deoxypyridoxine hydrochloride 14215

Waters Associates (Milford, MA)
    PIC B-7 85103

APPENDIX J

EALAT calibration curve



APPENDIX K

Preparation of reagents for EALAT assay

1. PLP

   0.05 g PLP/10 ml
   Store at 5°C protected from light.

2. Pyruvate standards

   Stock solution
   1.01 g 99% pyruvic acid/1
   Store at 5°C

   Standard pyruvate solutions
   12.5 μg/0.5 ml
   25 μg/0.5 ml
   50 μg/0.5 ml

3. Alanine reagent

   4.5 g D-L-Alanine, 5.0 g KH_2PO_4, and
   1.5 α-Ketoglutaric acid/dl

   Adjust pH to 7.4 using 10% KOH.
   Store at 5°C

4. DNP reagent

   0.2 g DNP and 40 ml concentrated HCl/2dl.
   Store at 5°C. Stable 10 days. Use at room temperature.

5. Alcoholic KOH

   25 g KOH/1 95% ethanol.

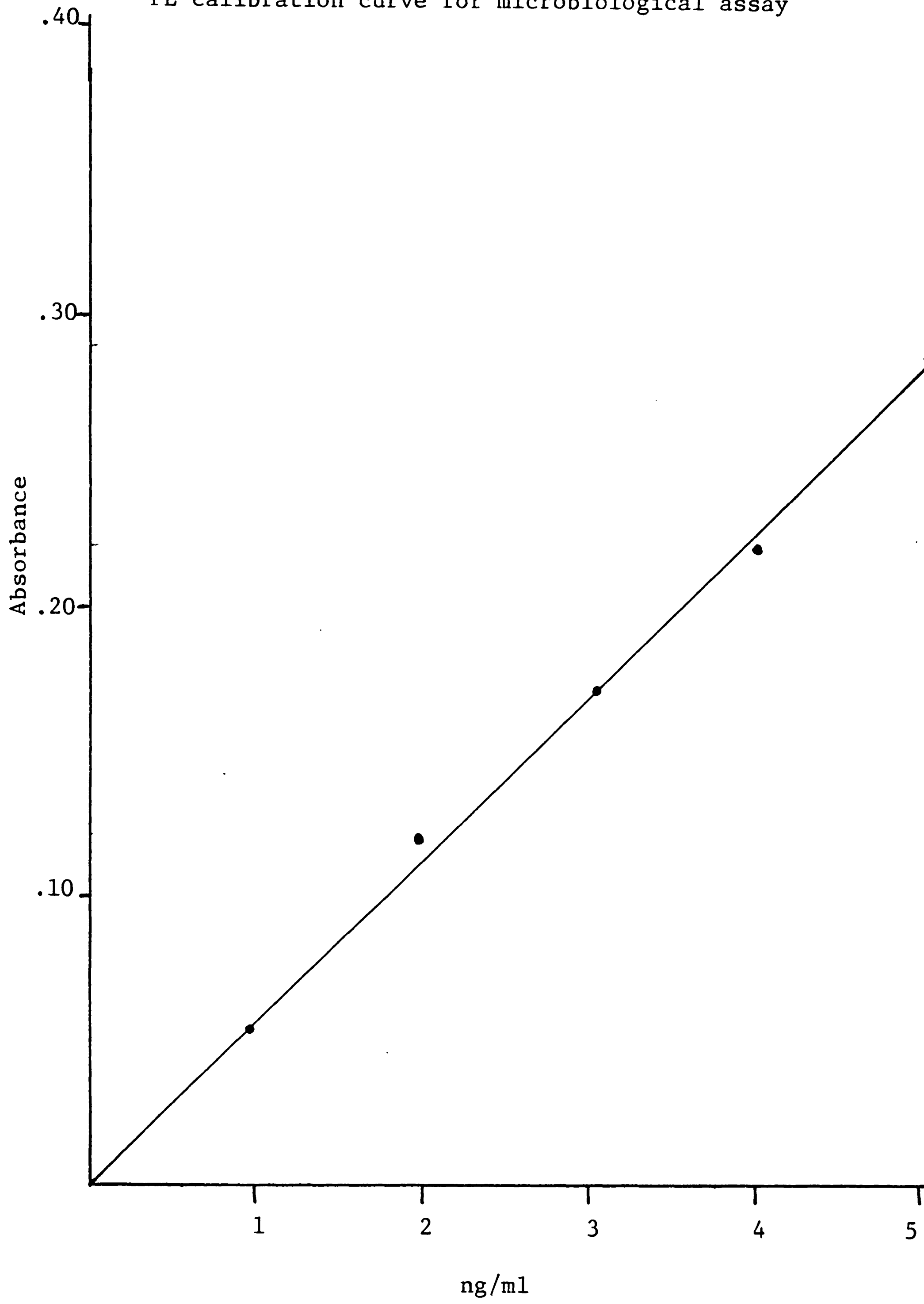
APPENDIX L

Preparation of exchange resin for microbiological assay

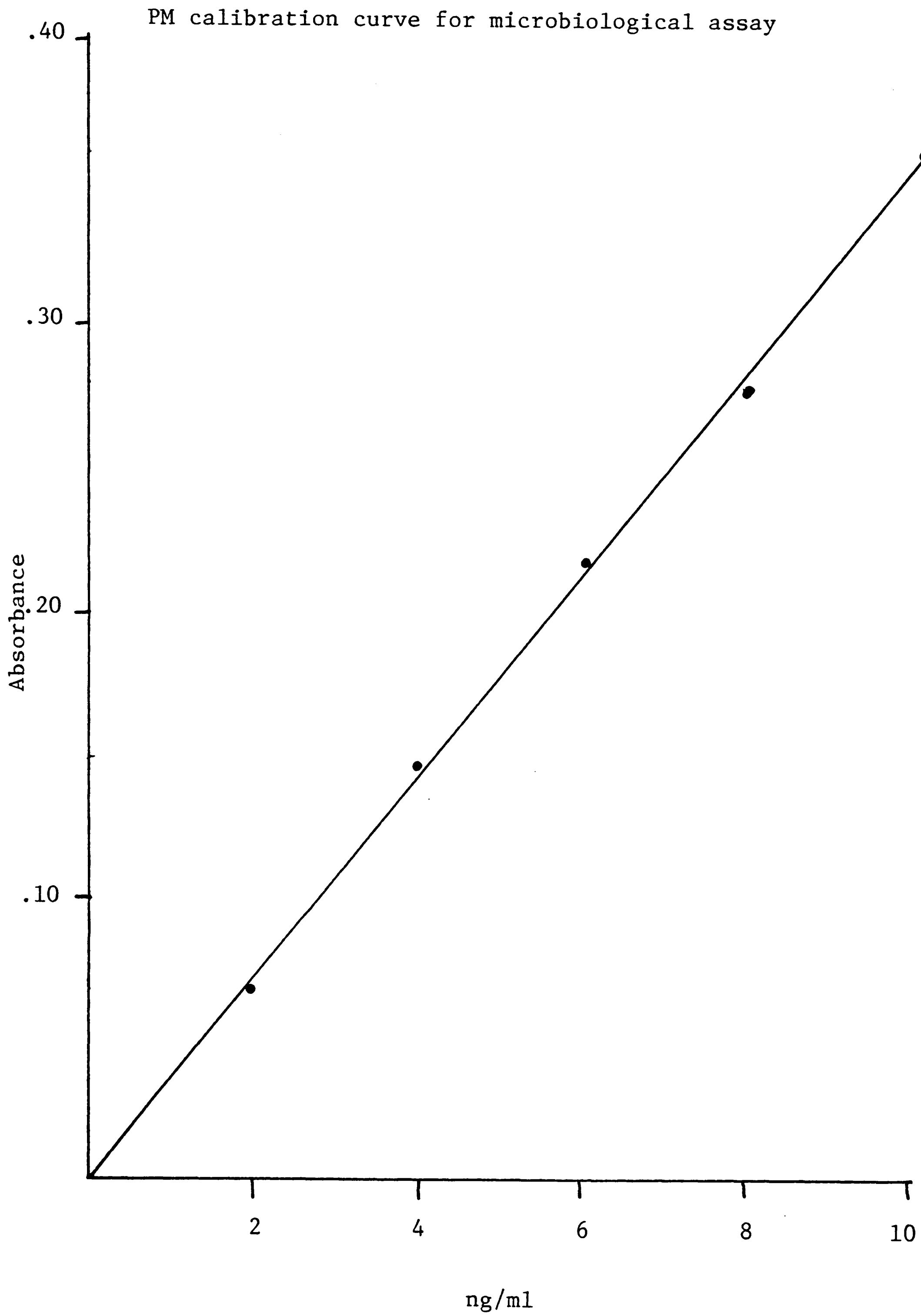
1. Place 1 lb Dowex AG 50w-x8 in hydrogen form resin in flask.
2. Wash with distilled water 6 times.
3. Cover with 6 N KOH.
4. Let settle; decant, rinse with distilled water until supernate is clear.
5. Add 1200 ml 3 N HCl; stir and heat in boiling water bath for 0.5 h.
6. Decant and repeat step 5 twice.
7. Rinse resin until rinse water is neutral.
8. Add 6 N KOH until pH is strongly basic.
9. Stir 1 h.
10. Rinse until rinse water is neutral.
11. Suspend in 2 M KOAc and store at 5°C.
12. Just prior to use, measure out amount needed, cover with distilled water, invert, let settle, and decant water. Repeat this step 5-6 times.

APPENDIX M

PL calibration curve for microbiological assay

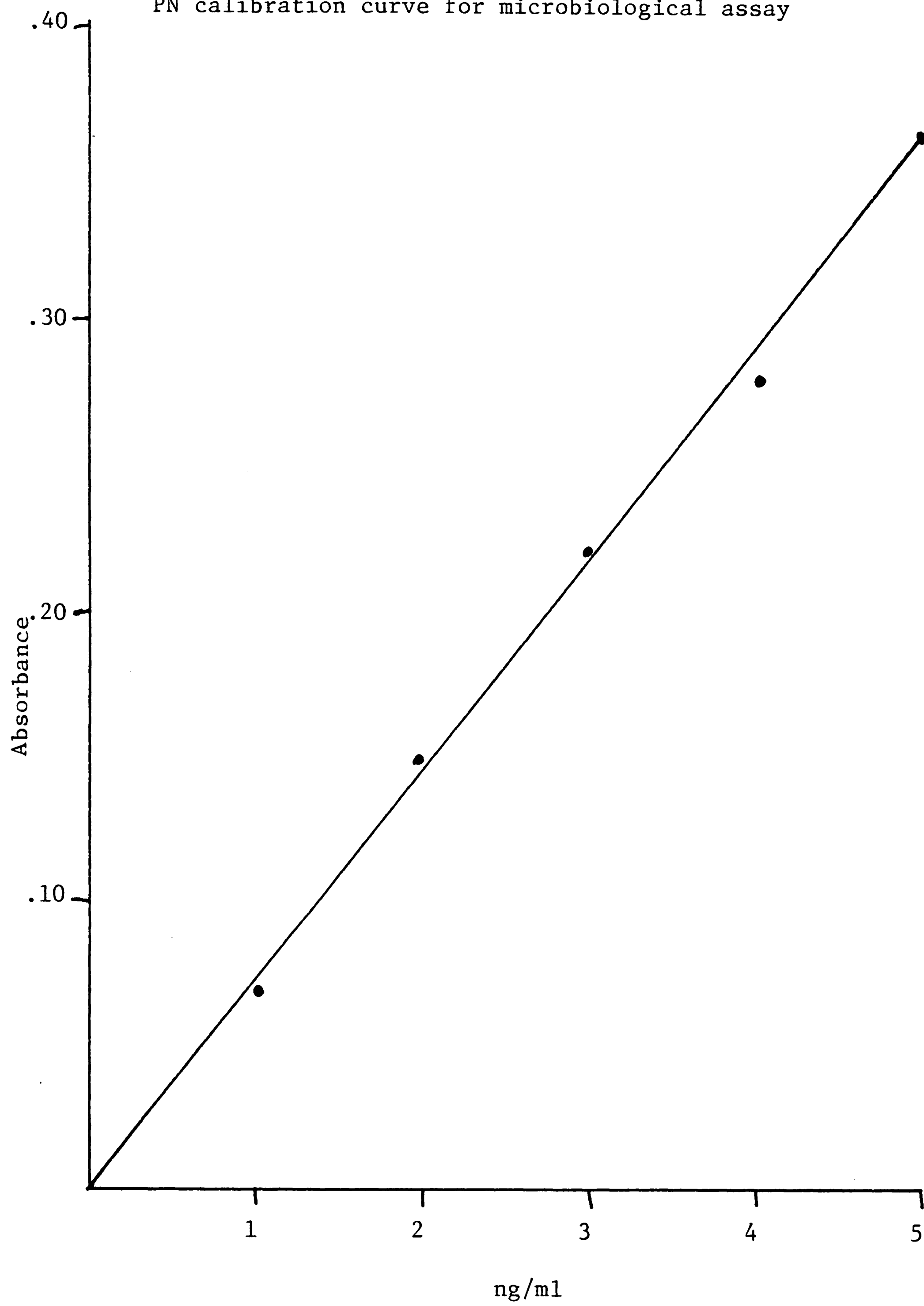


APPENDIX N



APPENDIX O

PN calibration curve for microbiological assay



APPENDIX P

Preparation of reagents for microbiological assay

1. Agar Slants

   41 g/1
   Place 5 ml in each screw cap tube.
   Boil while shaking constantly.
   Autoclave for 15 min at 121°C, 15 psi.
   Cool in slant position at room temperature.
   Store at 5°C

2. Saline

   0.9 g NaCl/dl

3. Buffers

   KOAc buffers

   a. 0.01 M, pH 4.5
      0.981 g/1
      Adjust pH with HOAc.

   b. 0.02 M, pH 5.5
      1.96 g/1
      Adjust pH with HOAc.

   c. 0.04 M, pH 6.0
      3.92 g/1
      Adjust pH with HOAc.

   d. 0.10 M, pH 7.0
      9.815 g/1
      Adjust pH with HOAc.

   KCL-K_2HPO_4 buffer

   74.6 g KCL and 17.4 g K_2HPO_4/1600 ml.
   Adjust pH with HOAc. Dilute to 2 1.

4. Standards

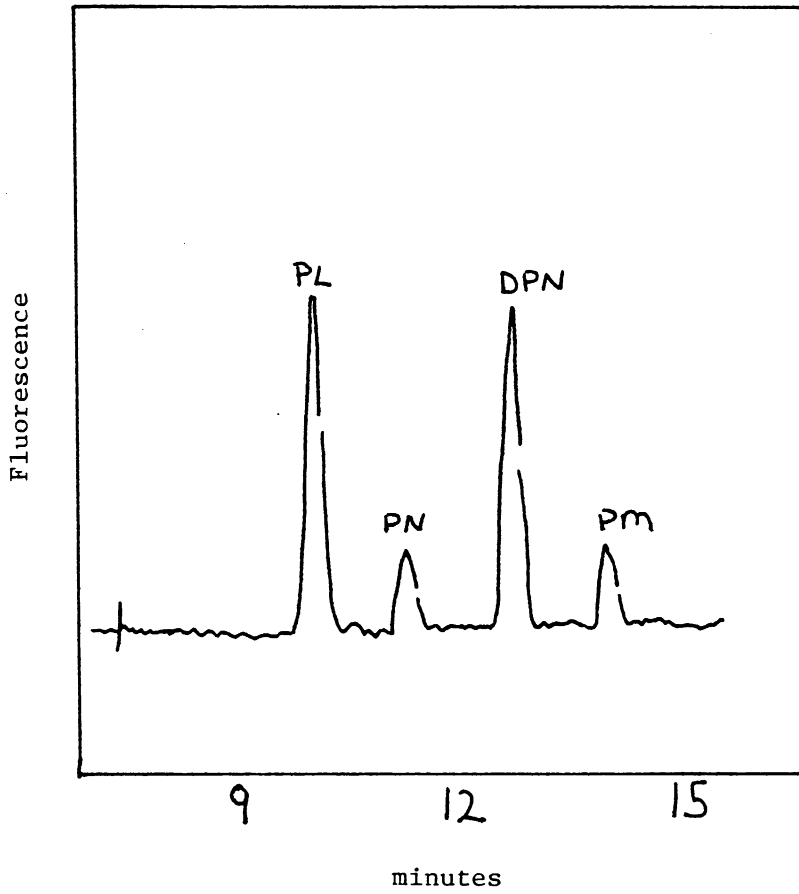
   Stock solutions
   50 μg/ml 25% ethanol for each vitamer
   Store at 5°C protected from light.

Working standards

PL and PN - 1, 2, 3, 4, and 5 ng/ml
PM - 2, 4, 6, 8, and 10 ng/ml
Store protected from light at 5°C. Stable for 10 days.

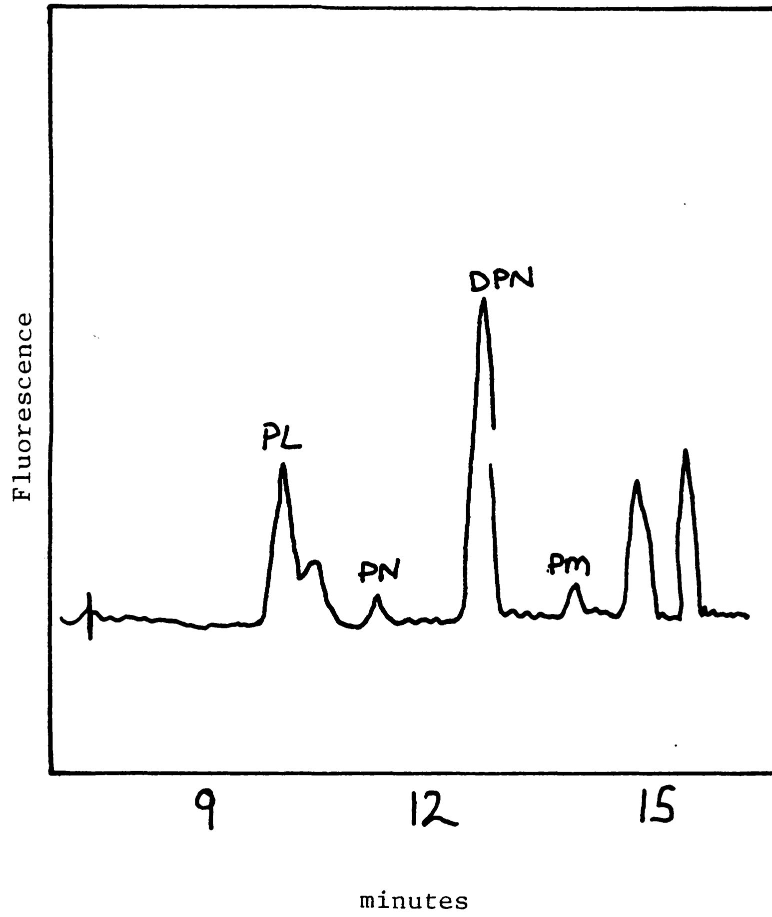
APPENDIX Q

Calibration curve for HPLC analyses



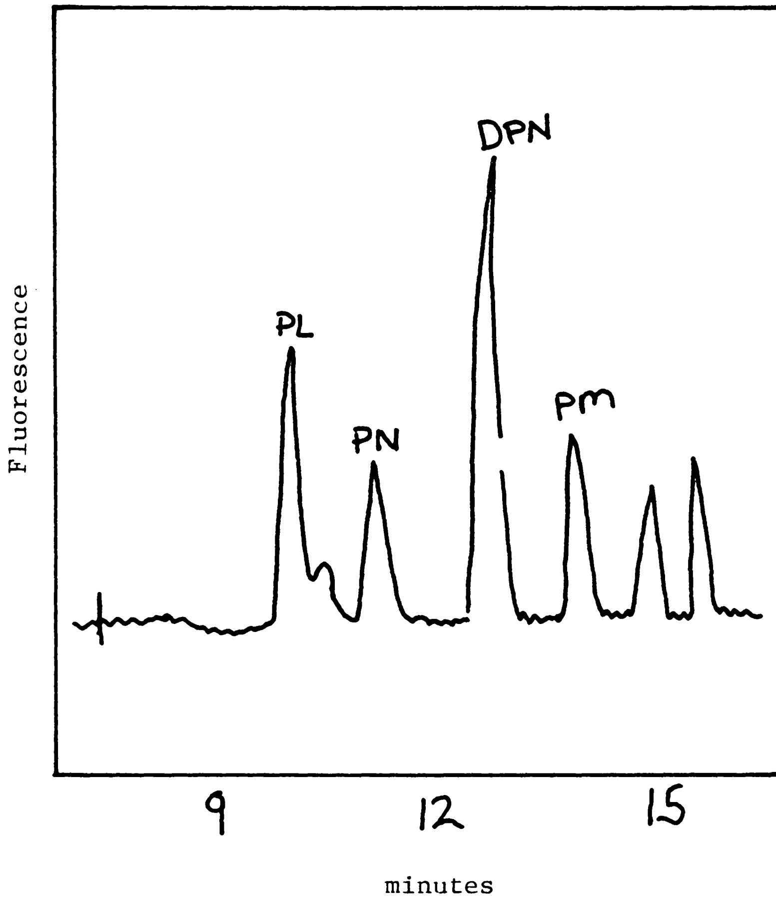
APPENDIX R

Chromatogram of human milk sample



APPENDIX S

Chromatogram of human milk sample "spiked" with PL, PN, DPN, and PM



APPENDIX T

Preparation of reagents for HPLC assay

1. DPN spiking solution

   20,000 ng/ml

2. Potato acid phosphotase solution

   10 mg/0.2 M KOAc, pH 4.5
   Make fresh daily.

3. 85% MeOH

   850 ml HPLC grade MeOH/150 ml HPLC water
   Filter and sonicate.

4. H_2O/PIC B-7

   1 bottle PIC B-7 reagent/1 HPLC grade water
   Filter and sonicate.

5. Calibration solution

   PL - 100 ng/ml
   PM - 25 ng/ml
   PN - 25 ng/ml
   DPN - 475 ng/ml

   Store at 5°C protected from light.

APPENDIX U

EALAT and RPLP values of subjects

Subject	EALAT % Stim	RPLP ng/ml
111	0	48.6
121	15.4	27.0
122	3.7	30.3
123	0	43.8
124	10.0	15.7
125	10.3	22.6
126	0	16.3
127	0	42.4
128	16.0	40.1
131	13.3	68.5
132	0	54.9
133	0	51.3
134	6.9	70.0
135	0	20.8
211	22.2	24.2
212	42.8	13.0
213	36.6	21.3
214	42.5	11.1
215	44.0	9.6
216	28.6	9.8
217	27.3	18.4

APPENDIX V

Age, height, and weight measurements of subjects

Subject	Age yr	Height cm	Weight kg
111	25	160	49.5
121	29	166	55.9
122	28	160	47.7
123	22	165	54.2
124	27	155	52.0
125	30	165	74.3
126	23	163	54.5
127	26	160	63.3
128	26	164	46.0
131	28	166	52.7
132	28	163	58.1
133	30	183	64.5
134	24	178	72.0
135	28	155	72.7
211	32	160	49.5
212	35	155	73.6
213	25	173	63.3
214	27	165	59.0
215	32	162	54.0
216	21	165	58.5
217	23	170	55.9

APPENDIX W

Demographic data of subjects

Subject	O.C.[a]	Cigarettes[b]	Alcohol[c]	Exercise[d]	Education[e]	Income[f]
111	-	+	2	2	2	2
121	+	-	2	2	2	3
122	+	-	1	2	3	3
123	-	-	1	2	2	1
124	-	-	2	2	1	2
125	-	-	3	3	3	4
126	-	-	0	2	2	1
127	+	-	2	2	3	4
128	-	-	0	2	2	2
131	-	-	1	3	2	2
132	+	+	2	2	2	2
133	-	-	4	3	3	3
134	+	-	1	2	3	3
135	-	-	2	2	3	2
211	-	-	1	2	2	3
212	-	-	2	3	1	4
213	-	-	3	1	2	2
214	+	-	5	2	3	3
216	+	+	1	4	1	2
217	-	-	0	4	2	1

[a]Previous use of oral contraceptives: +(yes); - (no).

[b]+(yes); -(no).

[c]Number of drinks/week.

[d]1(light); 2(Moderate); 3(Strenous); 4(Very strenous).

[e]1(High school); 2(College); 3(Graduate school).

[f]1(<$8,000); 2($8000-16,000); 3($17,000-24,000); 4(>$24,000).

APPENDIX X

Daily vitamin B-6 intakes of subjects

Subject	24 h recall	4 day[a] record	5 day average	Supplementation	Total	RDA
			mg			%
111	0.60	0.66	0.65	1	1.65	66
121	1.55	1.55	1.55	3	4.55	182
122	1.30	1.40	1.38	3	4.38	175
123	0.91	1.31	1.23	4	5.23	209
124	0.65	0.88	0.83	2	2.83	113
125	0.95	1.28	1.21	4	5.21	208
126	1.47	1.34	1.37	5	6.37	255
127	1.05	1.31	1.26	4	5.26	210
128	0.92	1.30	1.22	5	6.22	249
131	0.84	1.24	1.16	10	11.16	446
132	1.09	1.09	1.09	10	11.09	444
133	1.57	1.44	1.47	65	66.47	2659
134	1.08	1.07	1.07	10	11.07	443
135	0.79	0.87	0.85	10	10.85	434
211	1.15	1.70	1.59	-	1.59	64
212	0.85	0.59	0.64	-	0.64	26
213	0.92	1.04	1.02	-	1.02	41
214	0.97	0.91	0.92	-	0.92	37
215	0.13	1.12	0.92	-	0.92	37
216	0.56	0.68	0.66	-	0.66	26
217	0.63	1.00	0.93	-	0.93	37

[a]Values represent $\bar{x}$ of the 4 days.

APPENDIX Y

Daily protein and energy intakes of subjects

Subject	Protein (g) 24 h recall	Protein (g) 4 day[a] record	Protein (g) 5 day average	Energy (kcal) 24 h recall	Energy (kcal) 4 day[a] record	Energy (kcal) 5 day average
111	92.9	59.3	66.0	1720	1789	1775
121	108.2	90.0	93.6	3051	2667	2744
122	92.8	72.2	76.3	1944	1125	1289
123	66.2	81.9	78.8	1807	1945	1917
124	82.2	84.2	83.8	1947	2129	2093
125	75.1	69.9	70.9	1427	1902	1807
126	101.4	135.7	128.8	2336	2953	2830
127	77.7	65.7	68.1	2410	1807	1928
128	69.0	89.7	85.6	1936	2336	2256
131	106.9	86.6	90.7	1879	2185	2124
132	79.9	91.9	89.5	2314	2045	2099
133	57.2	87.5	81.4	2609	2890	2834
134	89.6	90.6	90.4	2800	2490	2552
135	56.1	52.4	53.1	2212	1956	2007
211	86.5	72.9	75.6	2156	1818	1886
212	63.3	87.1	82.3	1793	1713	1729
213	65.3	78.7	76.0	1494	1872	1796
214	73.0	43.3	49.2	1969	1347	1471
215	34.4	80.6	71.2	1029	1930	1750
216	53.9	90.1	82.9	1495	2070	1955
217	42.4	59.0	55.7	1864	1676	1714

[a]Values represent $\bar{x}$ of the 4 days.

APPENDIX Z

Microbiological assay values of subjects

Subject	MPL	MPM	MPN	MTL
	ng/ml			
111	115	8.7	8.7	132.4
121	140	10.0	8.0	158.0
122	147	11.3	16.7	175.0
123	135	11.5	5.8	152.3
124	115	11.3	8.8	135.1
125	150	15.0	8.3	173.3
126	123	11.3	16.7	151.0
127	140	11.7	17.5	169.2
128	130	3.8	6.3	140.1
131	160	12.5	6.5	179.0
132	155	10.6	7.0	172.6
133	147	11.0	23.2	181.2
134	125	12.5	13.8	151.3
135	137	13.8	13.8	164.6
211	100	11.8	8.6	120.4
212	125	7.5	8.8	141.3
213	105	11.3	7.5	123.8
214	100	8.8	6.3	115.1
215	100	7.5	5.8	113.3
216	115	7.5	12.0	134.5
217	110	9.5	17.5	137.0

APPENDIX AA

HPLC assay values of subjects

Subject	HPL	HPM	HPN	HTL
	ng/ml			
111	144.3	nd	nd	144.3
121	73.0	6.0	nd	79.0
122	75.3	17.9	nd	93.2
123	120.4	nd	nd	120.4
124	35.8	nd	nd	35.8
125	26.6	nd	nd	26.6
126	120.3	11.1	12.7	144.1
127	128.2	9.4	nd	137.6
128	133.0	46.6	20.3	199.9
131	185.2	9.1	nd	194.3
132	193.9	26.3	nd	220.2
133	265.8	22.1	6.2	294.1
134	78.9	nd	nd	78.9
135	61.4	26.4	nd	87.8
211	47.6	nd	nd	47.6
212	32.3	nd	nd	33.3
213	18.7	12.1	nd	30.8
214	23.9	nd	nd	23.9
215	38.8	nd	nd	38.8
216	19.2	nd	nd	19.2
217	42.3	nd	nd	42.3

APPENDIX BB

B-6 vitamer distribution of subjects

Subject	MPL	MPM	MPN	HPL	HPM	HPM
	% of MTL			% of HTL		
111	86.8	6.6	6.6	100	nd	nd
121	88.6	6.3	5.1	92.4	7.6	nd
122	84.0	6.5	9.5	80.8	19.2	nd
123	88.6	7.6	3.8	100	nd	nd
124	85.1	8.4	6.5	100	nd	nd
125	86.6	8.6	4.8	100	nd	nd
126	81.5	7.5	11.0	83.5	7.7	8.8
127	82.7	6.9	10.3	93.2	6.8	nd
128	92.8	2.7	4.5	66.5	23.3	10.2
131	89.4	7.0	3.6	95.3	4.7	nd
132	89.8	6.1	4.1	88.1	11.9	nd
133	81.1	6.1	12.8	90.4	7.5	2.1
134	82.6	8.3	9.1	100	nd	nd
135	83.2	8.4	8.4	69.9	30.1	nd
211	83.1	9.8	7.1	100	nd	nd
212	88.5	5.3	6.2	100	nd	nd
213	84.8	9.1	6.1	60.7	39.3	nd
214	86.9	7.6	5.5	100	nd	nd
215	88.3	6.6	5.1	100	nd	nd
216	85.5	5.6	8.9	100	nd	nd
217	80.3	6.9	12.8	100	nd	nd

The vita has been removed from the scanned document

INFLUENCE OF VITAMIN B-6 INTAKE ON VITAMIN B-6 STATUS OF LACTATING WOMEN AND ON THE VITAMER CONTENT OF THEIR MILK

by

Leslie A. Morrison

(ABSTRACT)

The influence of vitamin B-6 intake on vitamin B-6 status and the concentration of B-6 vitamers in milk of 21 white lactating women (21 to 35 years) was examined at 3 to 7 months postpartum. None of the women met the RDA for lactating women of 2.5 mg/day when considering vitamin B-6 intakes from food sources alone. All subjects taking vitamin B-6 supplements had adequate vitamin B-6 status as determined by coenzyme stimulation of erythrocyte alanine aminotransferase activity; all subjects not taking vitamin B-6 supplements had inadequate vitamin B-6 status. Plasma pyridoxal 5-phosphate values were significantly higher for subjects in the supplemented than in the nonsupplemented group. Pyridoxal, pyridoxamine, pyridoxine, and total vitamin B-6 concentrations in milk were higher, sometimes significantly, in the supplemented than in the unsupplemented group as determined by microbiological assay and HPLC. There were significant correlations between data obtained by the microbiological and HPLC analyses for pyridoxal and total vitamin B-6 concentrations. Pyridoxal was the predominant B-6 vitamer found in human milk. Distribution of the B-6 vitamers appeared to stay relatively constant despite vitamin B-6 status.