THIAMIN, RIBOFLAVIN, AND PYRIDOXINE LEVELS IN DIABETIC WOMEN

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

COLLEGE OF NUTRITION, TEXTILES, AND

HUMAN DEVELOPMENT

BY

CARYL J. ANTALIS, B. A.

DENTON, TEXAS

DECEMBER, 1979

TABLE OF CONTENTS

LIST OF TABLES

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Cutberto Garza for providing the laboratory space, equiptment, and supplies needed to carry out this study, and for his invaluable guidance and encouragement.

Gratitude is also extended to Dr. James Solar, chairman of the thesis committee, for directing the study, and to the other members of the committee, Dr. William Duer and Dr. Valerie Knotts, for significant contributions to the professional education and development of the author.

A special thanks is due to David Noran for his patience, encouragement, and support throughout the course of the author's graduate studies.

I. INTRODUCTION

Diabetes mellitus is a disease that affects approximately four million Americans, of which an estimated two to three million are now known, and one to two million remain to be diagnosed. About 389,000 new cases are reported each year (1). Diabetes is a chronic, incurable disease, but with modern treatment the patient has a much better prognosis for relief of symptoms and a longer life.

The treatment of diabetes usually includes a well-balanced calorie-controlled diet used alone or in conjunction with insulin or a hypoglycemic drug (2). A Special Report from the American Diabetes Association stated that the goals of the diabetic diet are to avoid deleterious metabolic derangements of the diabetic state, to reduce the known risk factors associated with atherosclerosis, and to reduce the risk of other specific complications (3). Albrink and Davidson feel that the most important single goal of the diabetic diet is to attain ideal weight, since 80% of diabetic patients are obese, and the "most important fact that emerges from clinical and epidemiological studies is the deleterious effect of obesity on diabetes (4)."

Renold et al. state that 60% of adult diabetic patients are obese and 60% of obese patients have impaired glucose

tolerance (5). They claim that obesity and adult-onset diabetes share the following characteristics: increased insulin response to glucose, relative ineffectiveness of endogenous or exogenous insulin, prevalance in times of economic prosperity, and association with increased morbidity and decreased longevity from vascular disease.

It is usually assumed that a well-controlled diabetic patient on a balanced diet is getting the amounts of vitamins recommended by the Food and Nutrition Board, and that these are adequate. However, there are several factors which might predispose a diabetic person to vitamin deficiencies. These factors would be especially important in those who were also growing or elderly, not well-controlled, on marginally adequate or subadequate intakes, or who had infections or other illnesses. Diabetics are more vulnerable than nondiabetics to infections (6), which may precipitate a clinical deficiency in a person who was only marginally deficient (7). Also, control is difficult to maintain in some diabetic patients, as is strict adherence to a diet plan, and this must be considered when studying nutritional needs.

The first factor that might predispose a diabetic person to vitamin deficiencies is that in diabetes there is an increased catabolism of body protein and fat for energy needs. Insulin is among the chief regulators of the metabolic state, and the impairment of the insulin function

leads to a decreased insulin/glucagon ratio and to an increase in the catabolic over the anabolic state. A relative or absolute hyperglucagonemia in diabetes also contributes to this (8). Other catabolic states such as fever, trauma, and starvation are known to be associated with a wastage of nitrogen, vitamins and other nutrients (9, 10).

The second factor is that varying degrees of hypercemia exist in diabetes. Even a symptomless diabetic can have blood sugar levels near or above the renal threshold (11), leading to glycosuria and polyuria. Any marked diuresis or even small amounts of diuresis over a long period of time might be expected to deplete the body of water-soluble vitamins.

A third factor is that several alterations in metabolic pathways have been observed in diabetes which may be due to the hyperglycemia, or the hormonal imbalance, or both. There is increased activity of the polyol pathway, where glucose is converted to sorbitol and then to fructose, in nerve tissue. The sorbitol and fructose do not easily diffuse out of the nerve sheath, and have been implicated in the etiology of diabetic neuropathy (12). There is also increased synthesis of glycoprotein, as evidenced by increased levels of total serum protein-bound carbohydrate and thickening of the basement membrane of small blood vessels (13, 14). This may be related to the microangiopathy of diabetes. Hemoglobins A_{1a}, A_{1b}, and A_{1c}, glycosylated hemoglobins

which are found in higher proportions in diabetic patients, are being used as indicators of diabetic control (15).

The exact relationship between diabetes and lipid metabolism is not known, but persisting elevations in blood ketones, triglycerides, and cholesterol have been noted in the "controlled" diabetic patient (16). Protein metabolism is also altered in diabetes. The differences are observed in both the fasted and the protein-fed state. In the fasted state there are decreased levels of alanine in the circulation, due to increased hepatic uptake of alanine for gluconeogenesis. There is a concommitant increase in the oxidation of the branched-chain amino acids in muscle to provide the nitrogen and possibly the carbon skeletons for alanine synthesis. In the protein-fed state the oxidation of amino acids in muscle is not reduced, and alanine output continues, in contrast to the situation in normal metabolism. There is a transient increase in hepatic glycose production and a resulting hyperglycemia. Thus the diabetic person is protein as well as glucose intolerant (17).

The extent to which the three factors described above operate to alter the metabolic milieu depends upon how closely normal metabolism, or the insulin-dependent utilization of glucose, is supported in the individual diabetic patient. But in the face of these and possibly other derangements of metabolism in diabetes, it seems reasonable to expect a difference in the requirements for the B-complex

vitamins, the cofactors of metabolism. It is possible that diabetic patients, as a subgroup of the general population, have increased nutritional needs because of their disease.

II. STATEMENT OF THE PROBLEM

If it can be shown that diabetics are more prone to deficiencies of the water-soluble vitamins, particularly the B-complex vitamins, perhaps these vitamins will find a useful place in the clinical treatment of diabetes and its complications. A deficiency of a nutrient is usually related to a decreased intake of that nutrient. In the diabetic person, it may be due to an increased need for, or a decreased utilization of, that nutrient.

The purpose of this study was to survey a group of diabetics for their status of three B-complex vitamins, thiamin, riboflavin, and pyridoxine, and to compare them to a group of nondiabetics drawn from the same population. The variables of age, ethnic group, duration of diabetes, and obesity were studied for possible effects on the B-vitamin status. The following problems were addressed:

Can the diabetic group be significantly discriminated from the control group using three discriminating variables, the tissue statuses of thiamin, riboflavin, and pyridoxine?

Is there a significant relationship between the age of the patient and the tissue statuses of the vitamins tested?

Is there a significant difference between the ethnic groups with respect to the tissue statuses of the vitamins

tested, and is there a significant interaction between group and ethnic group on the tissue statuses of the vitamins?

Is there a significant relationship between duration of diabetes in years and the tissue statuses of the vitamins tested?

Is there a significant relationship between obesity and tissue status of these vitamins?

III. HISTORICAL PERSPECTIVE

The clinical similarity between diabetic neuropathy and thiamin deficiency - loss of peripheral reflexes, hyperesthesia, paresthesia, disappearance of the vibration sense (18) - led to many investigations of the thiamin status of diabetic patients and of the effect of large doses of thiamin in the treatment of diabetic neuropathy. Even before thiamin was isolated in 1910 there were reports of a beneficial effect of liver on diabetes (19, 20). There were many reports between 1940 and 1954 of improvements in neuropathies, general health, and glucose tolerance in diabetic patients due to the administration of various preparations of B-complex vitamins.

Fein, Ralli, and Jolliffe in 1940, and Rudy and Epstein in 1945 claimed clinical improvement of diabetic neuropathies in patients given thiamin (21, 22). In 1945 Gaebler and Cizewski (23) gave three depancreatized dogs maintained on minumum insulin a supplement of yeast or a mixture of thiamin, riboflavin, niacin, inositol, pantothenate, and PABA. They found that removal of the supplement precipitated diabetic symptoms, and readministration caused withdrawal of the symptoms. Collens et al. in 1950 (24) gave large doses of crude liver extract, B-complex vitamins, and supplemental thiamin chloride and niacinamide to their patients with diab-

etic peripheral neuropathies. They saw an improvement in vibration sense and subjective relief in 72% of the cases. They also noted some improvement in carbohydrate tolerance.

Collens et al. in 1952 (25) isolated a new liver extract from pregnant mammalian liver which they found to effect an improvement in the neuropathies of all twelve patients to whom it was given. Rabinowitch (26) tested the same extract in seven of his patients with diabetic neuropathies and also found improvement in all cases. No improvement in glucose tolerance was seen in this study.

Contradicting reports have also been submitted. Rundles in 1945 (27) and Martin in 1953 (28) found that even massive doses of thiamin given intramuscularly failed to produce any improvement in diabetic neuropathies. Shuman and Gilpin in 1954 (29) tried the pregnant mammalian liver extract on 15 patients with neuropathies with no encouraging results. When they treated six patients with thiamin and ATP they found subjective improvement in two patients but no objective change.

No recent studies of this type were found in the literature. However, Moorhouse (30) in 1976 mentioned that thiamin chloride and cyanocobalamin are often used and are helpful in the clinical treatment of diabetic neuropathy, and that "investigations of this point seem appropriate." He wondered "whether the prevailing disorders of fatty acid and of pyruvate metabolism might somehow lower the thres-

holds for the manifestations of cofactor depletion, and whether the administration of thiamin and cyanocobalamin may in fact have a useful therapeutic place on this basis."

The status of the B-complex vitamins in diabetics has also been examined over the years. In 1951 Lossy, Goldsmith, and Sarrett (31) reported an increased excretion of a test dose of thiamin and riboflavin in diabetic patients over normal patients, which they thought might indicate an abnormality in utilization of these vitamins. They also noted that 11 of the 22 diabetic patients showed clinical evidence of a B-complex deficiency even though their diets were judged as good as those of the control patients.

Beidleman (32) claimed that there were two groups of diabetics where clinical vitamin deficiencies were seen: those on low calorie, low fat diets, and those who were poorly controlled. For the first group, he hypothesized that (a) 900-1500 calorie diets would not contain the minumum daily requirement of the B vitamins, and (b) the carbohydrate metabolic cycle might require more vitamins per gram in diabetic patients.

Ninety-four diabetics were examined by Biskind and Schreirer (33) in 1954. All showed signs and symptoms of deficiencies of factors of the B-complex. After treatment with large daily doses of the B vitamins, 16 required no more insulin. Thirty-seven showed improvement in general health and often in glucose tolerance.

Some investigators did not find deficiencies. Goodhart and Sinclair (34) in 1940 estimated the levels in the blood of thiamin pyrophosphate in five patients with diabetic neuropathy. They found normal levels. Robinson, Melnick, and Field (35) in 1940 and Needles (36) in 1943 studied the urinary excretion of thiamin before and after a test dose in diabetic patients with and without neuropathy. Again, normal values were found.

Field et al. (37) in 1957 attempted to resolve the question of the B vitamin status of diabetics. They studied the urinary excretion following a test dose of B-complex vitamins in three groups of seven males: healthy normals, diabetics without complications, and diabetics with complications. The ages were from 20 to 49 years, and most of the diabetics were of the juvenile type. They found differences with thiamin, niacin, and pantothenic acid but no pattern emerged and they were not able to draw any conclusions.

A study was done in 1976 by Davis, Calder, and Curnow (38) who used an automated microbiological system to measure the pyridoxal and folate concentrations in the serum of 518 diabetics and 371 controls. The levels of pyridoxal were significantly lower in the diabetics, and 25% had levels below the lower limit of the normal range. The pyridoxal levels were not related to type of treatment, duration of disease, or the age of the patients. Only 20 of the 518 diabetics had a reduced level of folate.

In a follow-up study this same group found that administration of pyridoxine decreased the insulin requirement in diabetic patients (39). This may be related to the fact that in a deficiency of pyridoxine the tryptophan metabolite xanthurenic acid accumulates. Kotake and Murakami (40) in 1971 reported that xanthurenic acid was able to bind to insulin and reduce its ability to lower blood glucose levels.

It had recently been discovered that there is a relationship between vitamin B_6 deficiency and diabetic neuropathy. McCann and Davis in 1978 (41) reported lower serum pyridoxal concentrations in diabetic patients with neuropathy when compared with randomly selected diabetic patients without evidence of neuropathy. Jones and Gonzales in 1978 (42) found abnormally high excretions of kynurenic and xanthurenic acids in the urine of 10 insulin-dependent diabetic patients with symptoms of neuropathy, when compared with 10 insulin-depencent diabetics without neuropathy and 10 controls. Six weeks of supplementation with pyridoxine resulted in the elimination of these high excretions, improvement of the symptoms of neuropathy, and in two patients, improvement of glucose tolerance.

Dietary pyridoxine, pyridoxal, and pyridoxamine are converted to pyridoxal phosphate, the active form of the vitamin, in the red blood cell (43). It is not known if serum pyridoxal accurately reflects dietary intake or tissue saturation of the vitamin. Tests have now been devel-

oped which reveal the cellular metabolic needs for a vitamin more precisely than serum levels or urinary excretion. These tests measure cofactor stimulation of enzyme activity. The reactions are specific and the active form of the vitamin is used. Erythrocytes are used because blood is an easy tissue to obtain, and red blood cells contain larger amounts of these enzymes than plasma (44).

These tests potentially allow for the identification of the primary biochemical lesion in cellular function caused by a vitamin deficiency that precedes clinical symptoms. They are being used with increasing frequency in research, but have not been used extensively in studies of diabetes.

The effects of catabolism on nutrient balance have been studied during starvation and trauma of surgery. Felig (45) says that diabetes can be compared to short term starvation because of similar blood levels of free fatty acids, keto-acids, and amino acids. Also, in both, insulin levels are reduced and glucagon levels are raised. Consolazio et al. (10) studied the effects of a 10-day fast on six adult males. They found that the daily excretions of thiamin and pyridoxine were in the low-to-deficient range, indicating rapid depletion of body stores. Riboflavin excretion was in the acceptable-to-high range, indicating adequate reserves or a retrieval of the vitamin from the catabolism of tissue for energy.

Pollack and Bookman (46) related riboflavin excretion to nitrogen balance in pre- and post-surgery patients. They found that urinary riboflavin was high (50% of intake) when the nitrogen balance was negative, and that when balance was restored the riboflavin was retained. When they withdrew insulin from a dependent diabetic, the nitrogen balance became very negative, and the riboflavin excretion became very high (independent of glycosuria).

The results cited here suggested that the question of the B-complex vitamins in diabetes was still open, and should be explored again in the light of the current knowledge of diabetes and nutritional assessment.

IV. HYPOTHESIS

This study reexamined the problem of the B-complex vitamins in diabetes using new biochemical methodologies to determine intracellular vitamin adequacy. Vitamin stimulation of coenzyme activity was used to measure the tissue statuses of thiamin, riboflavin, and pyridoxine in the red blood cells of diabetic subjects and matched controls. The potential of the vitamin levels to separate the two groups was assessed. The effects of age, ethnic group, duration of disease and obesity were considered.

The hypotheses tested in this study, stated in their null forms, are:

1. The diabetic group cannot be significantly discriminated from the control group using three discriminating variables, the activity coefficients for thiamin, riboflavin, and pyridoxine, measured by coenzyme stimulation in erythrocyte hemolysates.

2. There is no significant relationship between the age of the patient and the activity coefficients of the vitamins tested.

3. There is no significant difference between the ethnic groups with respect to the tissue statuses of the vitamins tested. There is no significant interaction between group and ethnic group on the tissue statuses of the vitamins.

4. There is no significant relationship between the duration of diabetes and the activity coefficients for the vitamins tested.

5. There is no significant relationship between obesity and the activity coefficients for the vitamins tested.

A probability level equal to or less than .05 was required for the rejection of the above hypotheses.

V. DEFINITIONS

1. Diabetes mellitus (DM) - a disease of metabolism in which there is an inadequate supply of insulin, characterized by disturbances in carbohydrate, fat, and protein homeostasis and macroangiopathic, microangiopathic, and neuropathic changes (2).
2. Adult-onset diabetes mellitus (AODM) - DM which has its onset after the age of 15 years (47). Marble (48) states that the dividing line between the two types of diabetes is not sharp. Though insulin production ceases in the juvenile-onset type and continues to some degree in the adult-onset, both types of idiopathic diabetes are characterized by an effective deficiency of insulin. Both can lead to the same complications, though these seem to develop more rapidly in the juvenile-onset type.
3. Duration of diabetes - defined here as years since diagnosis. It is recognized that this is only an estimate, since the condition might have existed for some time prior to diagnosis.
4. Obesity - usually 20-30% over average weight for age, sex, and height. In this paper, the term refers to percent over this average weight.

VI. METHODS AND PROCEDURES

Sampling

Subjects for the study were drawn from the population of patients visiting the outpatient clinics at Ben Taub General Hospital (n = 35) and Casa de Amigos Clinic (n = 4) in Houston, Texas. The sampling was not random, due to practical restrictions, but the sample obtained was assumed to be representative of the population. Twenty diabetic subjects and 19 control subjects were selected according to the criteria below:

1. All of the subjects were female, not by design, but because of the infrequency of male diabetics visiting the Diabetic Clinic. It was decided to simplify the study by eliminating the sex variable.
2. All were adult-onset diabetics, chosen to include a spread of years of duration of disease.
3. An arbitrary age limit of 66 and weight limit of 250 pounds was set to eliminate possible intervening variables. Also, diabetic patients with diseases unrelated to diabetes were not admitted into the study.
4. A representation of three ethnic groups, black, Spanish surname, and white, was sought.

5. Control subjects were healthy nondiabetics matched for age and ethnic group with the diabetic subjects. The major complaint of most control subjects was hypertension.

All participants were from a lower socioeconomic class, because Ben Taub and Casa de Amigos clinics draw most of their patients from this class. This factor is important because it affects education, attitudes, and resources, which in turn affect eating patterns (49). Nutritional deficiencies are more common among the lower socioeconomic class. Matching for ethnic group was thought to be important because this factor also affects eating patterns.

Methodology

The medical charts of possible participants were screened as the patients came into the clinics for a routine visit. Selected patients were then interviewed, and the study was explained to them as they waited to see the doctor. To provide motivation, it was emphasized that they would be able to find out, free of charge, it they were even slightly deficient in thiamin, riboflavin, or pyridoxine. If so, they could then discuss the possibility of taking vitamins with their doctor, for the improvement of their general health. To follow up on this, a form letter giving their personal results and comments was sent to each participant upon completion of the study (see Appendix A).

A medical and dietary history was taken on all diabetic patients who chose to participate in the study (see Appendix B). Control subjects were asked what medication and supplements they were taking, if any. A small blood sample (3-10 ml) was drawn in heparin and immediately put on ice. The subjects did not have to be fasting the day the blood was drawn because the assays measured tissue levels of the vitamins which would not fluctuate with day-to-day intake.

That same day, using a refrigerated centrifuge, the red blood cells from each blood sample were collected and washed several times with cold saline. The washed cells were aliquotted into small plastic vials and frozen at -20°C.

The vitamin assays were done over the next three months.

Vitamin status was measured indirectly by a laboratory determination of the vitamin-dependent red cell enzymatic activity with and without the added vitamin. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmole of NADH or NADPH per minute. The activity coefficient is defined:

$$AC = \frac{\text{units/mmole Hb with vitamin}}{\text{units/mmole Hb without vitamin}}$$

An activity coefficient (AC) of 1.00 for a vitamin indicated tissue saturation with that vitamin. A higher value represents a greater deficiency of the vitamin.

For thiamin, transketolase activity with and without added thiamin pyrophosphate was measured. For riboflavin, glutathione reductase activity with and without added flavin adenine dinucleotide was measured. Pyridoxine status was assessed by measuring the activity of glutamate-oxaloacetate transaminase with and without pyridoxal phosphate. The methods for these assays were from Williams (50), with modifications made by the researcher so that the assays could be run with the equipment available (see Appendix C). Hemoglobin was measured by the cyanmethemoglobin method (51).

All laboratory work was done in the Nutrition and Gastroenterology Laboratory of the Baylor College of Medicine, Houston, Texas. Equipment and supplies needed for this study were provided by that department.

Research Design

In this study the subjects were not manipulated in any way but were divided into groups on the basis of a diagnosis of AODM. The investigation sought to determine if a deficiency state of B-complex vitamins existed in the subgroup, diabetic women, of the population of women outpatients at Ben Taub General Hospital and Casa de Amigos Clinic. It also sought to determine if the vitamin levels found might be related to ethnic group, the age of the patient, duration of diabetes, or obesity. The sample was not randomly selected, but was systematically selected to allow for the evaluation of the above given variables. Because of the small sample size, this could not be left to chance. For example, most of the patients who came to the clinics were black, and if the researcher had not applied a bias to the selection of subjects to include white and Spanish surname, the effect of ethnic group could not have been evaluated.

This method of sample selection precludes the generalization of the results of the study to the whole population from which the sample was drawn, but it has the advantage of yielding more useful data with which to test the hypotheses.

VII. RESULTS AND DISCUSSION

The study was completed in two parts. The first 18 diabetic subjects were interviewed and tested from January to May, 1978. Because of circumstances beyond control, the last two diabetic subjects and 19 control subjects were not interviewed and tested until a year later, from January to May, 1979. Obtaining suitable subjects and matched controls was the most time-consuming part of the study.

The raw data from the survey, which were of nominal, interval, and ratio level, are listed in tables 1 and 2. These data were subjected to the following statistical procedures: discriminant analysis, the t-test of independent means, the Pearson r coefficient of correlation, and 2-way factorial analysis of variance. The independent variables were group (diabetic, control), ethnicity (black, Spanish, white), age, duration of diabetes, and obesity (the last two classifications were eveluated in diabetic subjects only). The dependent variables were the activity coefficients (AC's) for thiamin, riboflavin, and pyridoxine, and the enzyme activities (EA's) for transketolase, glutathione reductase, and aspartate aminotransferase.

The first hypothesis, that the diabetic group could not be discriminated from the control group, using the vitamin AC's as discriminating variables (DV's), was tested using

TABLE 1

ETHNIC GROUP, AGE, DURATION OF DIABETES, WEIGHT, AND VITAMIN ACTIVITY COEFFICIENTS FOR SUBJECTS IN DIABETIC GROUP

I.D.	Ethnic[a] Group	Age	Years[b] DM	Weight	AC's for vitamins[c] B_1	B_2	B_6
001	S	56	8	153	1.09	1.76*	1.45*
002	W	60	16	149	1.12	1.10	1.12
004	B	57	12	224	1.06	1.04	1.68*
005	S	65	28	145	1.02	0.99	1.15
006	B	57	9	187	1.04	0.96	1.05
007	S	43	9	187	0.89	1.03	1.33*
008	B	47	10	181	1.10	1.03	1.06
009	B	50	20	200	1.16	1.07	1.61*
010	B	61	2	199	1.01	1.07	1.15
011	W	65	8	175	0.98	1.07	1.21*
012	W	66	9	114	1.04	1.02	1.14
013	W	61	14	180	1.17	1.04	1.42*
014	S	62	?	164	1.05	1.10	1.31*
015	B	54	6	148	1.09	1.21	1.09
016	B	62	4	179	1.09	1.19	1.20
017	S	56	16	178	1.13	1.02	1.25*
018	B	51	3	153	1.13	1.19	1.42*
019	S	51	8	153	1.16	1.01	1.22*
020	B	62	13	224	1.22	1.10	1.17
Mean		57.1	10.5	170	1.08	1.10	1.26

(a) B is black, S is Spanish, W is white
(b) Duration of diabetes in years
(c) Normal range for AC's: B_1, 1.00-1.30; B_2, 1.00-1.25; B_6, 1.00-1.20 (50)
(*) Above normal range

TABLE 2

ETHNIC GROUP, AGE, WEIGHT, AND VITAMIN ACTIVITY COEFFICIENTS FOR SUBJECTS IN CONTROL GROUP

I.D.	Ethnic[a] Group	Age	Weight	AC's for vitamins[b] B_1	B_2	B_6
101	B	59	?	1.09	1.13	1.16
102	B	49	144	1.23	1.11	1.32*
103	B	65	162	1.16	1.12	1.30*
104	W	63	142	1.15	1.03	1.29*
105	B	63	183	1.19	1.05	1.17
106	B	47	160	1.13	1.10	1.37*
107	W	57	204	1.08	0.96	1.28
108	B	59	90	1.33*	1.27*	1.42*
109	B	64	247	1.15	1.09	1.37*
110	B	59	174	1.18	1.23	1.19
111	W	64	172	1.12	1.09	1.13
112	B	56	182	1.12	1.19	1.17
113	S	48	140	1.12	1.19	1.16
114	W	64	126	1.15	1.02	1.19
115	W	57	113	1.10	1.13	1.31*
116	S	57	182	1.13	1.07	1.31*
117	S	65	153	1.13	1.03	0.88
118	S	65	171	1.22	1.20	1.10
119	S	50	177	1.14	1.03	1.16
Mean		58.5	162	1.15	1.11	1.23

(a) B is black, S is Spanish, W is white
(b) Normal range for AC's: B_1, 1.00-1.30; B_2, 1.00-1.25; B_6, 1.00-1.20 (50)
(*) Above normal range

discriminant analysis. Subprogram DISCRIMINANT, from the Statistical Package for the Social Sciences (52), (a) weighted and linearly combined the DV's in such a fashion that the groups were forced to be as distinct as possible (discriminant function), (b) measured the success with which the DV's actually discriminated between the two groups when combined into the discriminant function, and (c) selected the single best DV, then the second best DV as best able to improve the value of the discriminating criterion in combination with the first variable, etc. This resulted in a ranking of the three vitamins in order of importance in diabetes. A t-test of independent means was used to substantiate the findings. The results are summarized in table 3. The discriminating function was derived:

$$Y = 1.14\ X_1 - 0.034\ X_2 - 0.432\ X_3$$

where X_1 is thiamin, X_2 is riboflavin, and X_3 is pyridoxine. This is interpreted to mean that the two groups can be significantly ($p = .009$) discriminated, with thiamin being the strongest DV, pyridoxine the second strongest, and riboflavin the weakest DV. The t-test also showed that the two groups are distinct ($p = .002$) with respect to thiamin, but not with respect to riboflavin or pyridoxine.

Thus the null hypothesis must be rejected. The hypothesis which is then accepted is contradictory because it is the control group which is indicated to have the greater deficiency of thiamin. This result was not due to the diab-

TABLE 3

MEANS AND STANDARD DEVIATIONS OF VITAMIN ACTIVITY COEFFICIENTS AND DISCRIMINATING FUNCTION COEFFICIENTS FOR THIAMIN, RIBOFLAVIN, AND PYRIDOXINE

Vitamin	Mean and SD of AC's[a]		sig. of t	DFC's[b]	sig. Wilks
	Diabetic	Control			
Thiamin	1.08 ± .08	1.15 ± .06	.002	1.14	.009
Riboflavin	1.10 ± .17	1.11 ± .08		-0.034	
Pyridoxine	1.26 ± .18	1.23 ± .13		-0.432	

(a) SD is standard deviation, AC is activity coefficient
(b) DFC is discriminating function coefficient

etic group taking vitamin supplements because an equal number of subjects in both groups (five) were taking supplements that contained the vitamins in question at the time of the survey. Explanations for this result may lie in (a) the small sample, (b) the method of sampling, (c) dietary factors, or (d) underlying differences in enzyme levels or activities. Kjosin et al. (53) have noted that diabetic patients seem to have lower levels of transketolase than normal. If this were the case in the diabetics tested here, it would be expected that the activity could not be stimulated beyond a certain extent with exogenous vitamin. But when the unstimulated enzyme activities were evaluated (table 4), the diabetic group had significantly higher levels.

Another possible explanation is that the diabetics were more "diet conscious," having been instructed in the proper diet to follow for control of their disease. In tabulating the results from the medical and dietary history (Appendix B), it was found that of the 20 diabetics, 15 claimed to know something of their diet, and 10 claimed to follow the diet regularly. It was not undertaken to evaluate in any more depth their understanding of their diet. However, it is the subjective feeling of the author that with two or three exceptions the group was not extremely "diet conscious."

Since previous investigations cited in this paper (38, 42) found significant evidence of pyridoxine deficiency in diabetics compared with control groups, it was surprising

TABLE 4

BASAL ENZYME ACTIVITY MEANS AND STANDARD DEVIATIONS FOR TRANSKETOLASE, GLUTATHIONE REDUCTASE, AND ASPARTATE AMINO-TRANSFERASE IN DIABETIC AND CONTROL GROUPS AND COMPARISON WITH NORMAL VALUES

Enzyme	Activity in I. U.'s		Normal
	Diabetic	Control	from Wms. (50)
transketolase	0.72 ± 0.15	0.53 ± 0.12	0.82 ± 0.4
glutathione reductase	5.91 ± 1.14	6.23 ± 1.05	6.4 ± 3.0
aspartate amino-transferase	2.45 ± 0.91	2.99 ± 1.23	4.2 ± 2.2

I. U. is international units (units/g Hb)
*significance of t = .001

that this study did not confirm these findings. No significant difference was seen in the AC's for pyridoxine or the basal aminotransferase activities between the two groups (tables 3, 4). It was interesting that of the 39 patients tested 20 were deficient in the vitamin using the normal range established by Williams (50). This is in contrast to the findings for thiamin and riboflavin where only one and two, respectively, were deficient. It was perhaps because of this apparently widespread deficiency of pyridoxine that the difference between the two groups was not significant.

Jones and Gonzales (42) suggested that a deficiency of pyridoxine in diabetes was related to the administration of insulin. In this study eight of the diabetics on insulin were deficient in pyridoxine, seven were not; three of the diabetics on oral hypoglycemic drugs were deficient, two were not. Thus, type of therapy did not seem to influence the status of pyridoxine as reflected by the AC's.

Table 5 gives the means, medians, ranges, and percentage above normal range of the AC's for pyridoxine for the diabetic group and the control group. These consistently show the diabetic group to be more deficient than the control group. Also, five diabetic subjects had AC's above 1.40, while only one control subject had an AC in that range. These data suggest that if the study were repeated with a larger sample size, the differences might become significant. In order to determine how large a sample would be needed,

TABLE 5

DIABETIC AND CONTROL GROUP MEANS AND STANDARD DEVIATIONS, MEDIANS, RANGES, AND PERCENTAGES ABOVE NORMAL RANGE OF ACTIVITY COEFFICIENTS FOR PYRIDOXINE

Group	N	Mean and S. D.	Median	Range	% Above Normal*
Diabetic	20	1.26 ± 0.18	1.22	1.05 - 1.68	55
Control	19	1.23 ± 0.13	1.19	0.88 - 1.42	47

* Normal range = 1.00 - 1.20 (50)

the following formula was used:

$$N = \left[\frac{z_{1-\frac{1}{2}\alpha}\,\sigma}{d}\right]^2 \qquad (54)$$

where z is obtained from table A-4 in Dixon and Massey (54), d (the precision desired) is set at 0.05, and σ is the standard deviation, taken from the data. Thus to obtain a more precise mean, a sample of 50 diabetics and 26 controls would be needed.

It can be seen from examining table 4 that the basal activities of all three enzymes are below the normal means in both groups. This could reflect the fact that all of the subjects were from the lower socioeconomic class where nutritional deficiencies are more prevalent. The aspartate aminotransferase levels were especially low. Vir and Love (55) relate that in the early stages of vitamin B_6 deficiency diminished transaminase levels probably indicate decreased saturation of the apoenzyme. In chronic deficiency, decreased basal transaminase levels may be a result of repression of synthesis through lack of coenzyme or a direct effect of deficiency on protein synthesis.

Pearson r coefficients were computed to correlate the basal EA's and the AC's for each vitamin in both groups. Strong negative correlations ($p = .019$ to $.001$) were found in all cases except for the thiamin data in the diabetic group. Thus, when the basal EA was low, the AC was high. This makes sense in terms of the above explanation for an

early stage deficiency.

Table 6 is included for the sake of comparison. Data from other labs on the EA's and AC's for the three vitamins and coenzymes evaluated in this paper are given. When different methods are used and the raw data are not available for comparison, it is difficult to explain the discrepancies that exist.

To test the second hypothesis, that there was no significant relationship between the AC's for the three vitamins and the age of the patient, Pearson r coefficients of correlation were computed (pairwise deletion for missing data was in effect for all Pearson correlations). The age range of the subjects in the diabetic group was 43 to 66 years, the mean being 57.1 years. For the control group, the range was 47 to 65 years, the mean, 58.5 years. No significant relationship was found and the null hypothesis was accepted.

To determine a relationship between the duration of diabetes and the AC's for the three vitamins, the Pearson r coefficients were computed with these variables. The range of duration of disease for 19 diabetics was 2 to 28 years, and the mean was 10.5 years (for one patient the duration was unknown). No significant relationship was found and the null hypothesis was accepted. These evaluations of the effects of age and duration of diabetes on AC's corroborate the findings of Davis, Calder, and Curnow (38), that these two factors were not related to a deficiency of serum pyri-

TABLE 6

COMPARISON OF NORMAL VALUES FOR ACTIVITY COEFFICIENTS AND ENZYME ACTIVITIES FROM DIFFERENT LABORATORIES

	This Study	Williams (50)	Hoorn (56)	Bayouni & Rosalki (57)
T - EA	0.53±0.12	0.82±0.4		852±125
AC	1.15±0.06	1.00-1.30	1.09±0.09	
GR - EA	6.23±1.05	6.4 ±3.0		10.5±2.2
AC	1.11±0.08	1.00-1.25	1.14±.075	
AA - EA	2.99±1.23	4.2 ±2.2		1.82±0.50
AC	1.23±0.13	1.00-1.20	1.58±1.14	

T is transketolase; GR is glutathione reductase; AA is aspartate aminotransferase; EA is enzyme activity; AC is activity coefficient

doxal in diabetics.

The third hypothesis concerned the effect of ethnic group on the vitamin levels. A 2 X 3 factorial analysis of variance was done to look for an interaction between group (diabetic, control) and ethnic group (black, Spanish, white). This was repeated for each of the vitamins. The results are given in table 7. The ratio of black; Spanish: white was 9:6:5 for the diabetic group, and 9:5:5 for the control group. The only significant F-value that was found was that for the 1-way interaction of group on thiamin levels. This result was discussed earlier in the paper. The lack of a significant F-value for the 1-way interaction of ethnic group on vitamin levels and the 2-way interaction between group and ethnic group on vitamin levels leads to an acceptance of the null hypothesis. What this may show is that other factors, such as age and socioeconomic class, override the differences in diet between ethnic groups.

The fifth hypothesis concerned the relationship between obesity and vitamin levels. Obesity was defined as percent over average weight, as given in the "Average Weights of Adults" table in the Ciba-Geigy Scientific Tables (58). Two inches were added to the height data obtained from the from the subjects because the height in shoes is required in the table. If the person was under average weight, the variable was equal to zero. For two diabetic patients the height data was not available, and it was not available for

TABLE 7

F-VALUES AND MEANS FROM ANALYSIS OF VARIANCE FOR INTERACTION BETWEEN GROUP (DIABETIC, CONTROL) AND ETHNIC GROUP (BLACK, SPANISH, WHITE) ON ACTIVITY COEFFICIENTS FOR VITAMINS B_1, B_2, AND B_6

Vitamin	Group	Means of AC's for vitamins			F-values		
		Black	Span.	White	1-way (Group)	1-way (Ethnic)	2-way
B_1	DB	1.09	1.06	1.11	11.2*	.488	1.27
	C	1.18	1.15	1.12			
B_2	DB	1.09	1.15	1.07	.051	1.23	.452
	C	1.14	1.10	1.05			
B_6	DB	1.28	1.29	1.21	.706	.785	1.25
	C	1.27	1.12	1.24			

*significant of F = .002

any of the control patients. A Pearson r coefficient was then computed for the 18 diabetic patients using percent overweight with vitamin levels (AC's). The mean percent overweight was 15.5 % ± 15.5 %. A weak but nonsignificant ($p = .082$) relationship was found between percent overweight and thiamin levels (AC's increased with percent overweight), but no relationship at all was seem with the riboflavin or pyridoxine levels.

Since it is known that thiamin needs increase with carbohydrate intake, the first (weak) relationship might be explained partially on this basis. The lack of a relationship with pyridoxine is interesting because some of the diabetics were clearly deficient in this vitamin. If obesity is an indicator of severity or lack of control of diabetes, it must be assumed that these factors and pyridoxine deficiency are not related. In another paper (41) diabetic neuropathy, which was associated with pyridoxine deficiency, was found to be unrelated to severity of diabetes.

VIII. CONCLUSIONS

The results of this study did not support the hypothesis that diabetic women are more deficient in the B-complex vitamins than a control group drawn from the same population. The major limitation of the survey were the small number of patients tested, and the non-random method of sample selection. A large scale survey could obtain sample groups that were more representative of the population. The data from such a survey might be significant, especially in the case of pyridoxine. The diabetic group was found to have higher levels of thiamin than the control group, and this finding was not explained.

The most important result to emerge from this study was that more than half (51%) of the patients tested were deficient in pyridoxine. This deficiency was not related to age or ethnic group. In the diabetic patients, it was not related to duration of disease, insulin intake, or obesity. Possible explanations for the deficiency are (a) the age range of the sample, 43 to 66 years, (b) the lower socioeconomic class from which the sample was drawn, and (c) the fact that most flour products are not enriched with pyridoxine, as they are with riboflavin and thiamin, resulting in a lower intake of this vitamin.

IX. IMPLICATIONS FOR FUTURE RESEARCH

Technical weaknesses of this study included: (a) the span of eight months between the completion of the first part of the study and the start of the second, and (b) the length of time that elapsed between the drawing of the blood samples and tha analysis, which was as long as three months in some cases. Although Williams (50) says that the red cells may be kept forzen at -20°C "until needed," Bayoumi and Rosalki (57) found that 3-4 weeks was the maximum time that the cells could be kept frozen without some loss of enzyme activity. If this study were to be repeated, the samples should be analyzed more quickly or a systematic testing for loss of activity should be done. As for (a) above, ideally the study should be done all at one time for confidence in making comparisons between the two groups. The fact that it was not detracts from one of the strengths of the study, which is the care with which the two groups were matched.

Because of the recently discovered findings about the importance of pyridoxine in diabetes, it might be valuable to do a larger scale survey of pyridoxine status with 50 or more patients in each group, to try and establish a definite difference between diabetics and nondiabetics and to quantitate this difference. Since this study did not find a cor-

relation between pyridoxine levels and age, duration of disease, use of insulin, or obesity, it would be interesting to try to relate it to intake of the vitamin or degree of control of diabetes (perhaps through the measurement of hemoglobin A_1).

Since 51% of the women in this survey were found to be deficient in pyridoxine, it would also be important to explore the area of pyridoxine deficiency or adequacy in a non-diabetic population. For example, is the deficiency as wideapread in men as it appears to be in women? Is it related to age or socioeconomic status? How is it related to intake and can it be reversed with supplementation?

These questions would be very important in the formulation of a national nutrition policy and making sure that all segments of the population have access to all the nutrients they need for good health.

LIST OF REFERENCES

(1) Knowles, H. C., Meinert, C. L., and Prout, T. E.: Diabetes: The overall problem and its impact on the public. In Diabetes Mellitus. DHEW Publication No. (NIH) 76-854, 1976.

(2) Friedman, G. J.: Diet in the treatment of diabetes mellitus. In Modern Nutrition in Health and Disease. Ed. by R. S. Goodhart and M. E. Shils. 5th Ed. Philadelphia: Lea and Febiger, 1973.

(3) American Diabetes Association Special Report: Principles of nutrition and dietary recommendations for patients with diabetes mellitus. Diabetes 20:633, 1971.

(4) Albrink, M. J., and Davidson, P. C.: Dietary therapy and prophylaxis of vascular disease in diabetes. Med. Clin. N. Amer. 55:877, 1971.

(5) Renold, A. E., Stauffacher, W., Cahill, G. F.: Diabetes mellitus. In The Metabolic Basis of Inherited Disease. Ed. by J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson. 3rd Ed. New York: McGraw-Hill, 1972.

(6) Younger, D. and Hadley, W. B.: Infection and diabetes. In Joslin's Diabetes Mellitus. 11th Ed. Philadelphia: Lea and Febiger, 1971.

(7) Scrimshaw, N. S., Taylor, C. E., and Gordon, J. E.: Interactions of Nutrition and Infection, WHO Monograph Series No. 57. Geneva: WHO, 1968.

(8) Unger, R. H.: Alpha- and beta-cell interrelationships in health and disease. Metab. 23:581, 1974.

(9) Beisel, W. R.: Nutrient wastage during infection. Proc. 9th Int. Congr. Nutrition 2:160, 1972.

(10) Consolazio, C. F., Johnson, H. L., Kryzwicki, H. J., Daws, R. A., and Banhart, R. A.: Thiamin, riboflavin, and pyridoxine excretion during acute starvation and calorie restriction. Am. J. Clin. Nutr. 24:1060, 1971.

(11) Spiro, R. G.: Glycoproteins and diabetic microangiopathy. In Joslin's Diabetes Mellitus. 11th Ed. Philadelphia: Lea and Febiger, 1971.

(12) Keen, H., and Jarrett, J., eds: Complications of Diabetes. Chicago: Edward Arnold Ltd., 1975.

(13) Winegrad, A. I. and Clements, R. S.: Demonstrable metabolic abnormalities in diabetes. In Diabetes Mellitus. DHEW Publication No. (NIH) 76-854, 1976.

(14) Koenig, R. J. and Cerami, A.: Synthesis of hemoglobin A_{1c} in normal and diabetic mice: potential model of basement membrane thickening. Proc. Nat. Acad. Sci. 72:3687, 1975.

(15) Gabbay, K. H.: Blood test may start new era in diabetes therapy evaluation. JAMA 237:847, 1977.

(16) Bradley, R. F.: Cardiovascular disease. In Joslin's Diabetes Mellitus. 11th Ed. Philadelphia: Lea and Febiger, 1971.

(17) Felig, P., Wahren, J., Sherwin, R., and Palaiologos, G.: Amino acid and protein metabolism in diabetes mellitus. Arch. Intern. Med. 137:507, 1977.

(18) Root, H. F.: The nervous system and diabetes. In The Treatment of Diabetes. 10th Ed. Philadelphia: Lea and Febiger, 1959.

(19) Gilbert, A. and Carnot, P.: Semaine Med. 16:513, 1896.

(20) Gilbert, A. and Carnot, P.: Semaine Med. 17:189, 1897.

(21) Fein, H. D., Ralli, E. P. and Jolliffe, N.: Peripheral neuropathy due to B-1 deficiency in diabetes mellitus. J. Amer. Med. Assoc. 115:1973, 1940.

(22) Rudy, A. and Epstein, S. H.: Review of one hundred cases of "diabetic neuropathy" followed from one to ten years. J. Clin. Endocrin. 5:92, 1945.

(23) Gaebler, O. H. and Cizeivski, W. E.: Effects of yeast on water soluble vitamins in experimental pancreatic diabetes. Exper. Med. and Surg. 3:299, 1945.

(24) Collens, W. S., Rabiner, A. M., Zilinsky, J. S., Boas, L. C., and Greenwald, J. J.: The treatment of peripheral neutopathy in diabetes mellitus. Am. J. Med. Sci. 219:482, 1950.

(25) Collens, W. S., Zilinsky, J. S., Greenwald, J. J., and Stern, A. B.: A new liver extract derived from pregnant mammalian liver. Am. J. Med. 12:53, 1952.

(26) Rabinowitch, M.: Experiences with a new liver extract for the treatment of diabetic neuropathy. Am. J. Med. 12:59, 1952.

(27) Rundles, R. W.: Diabetic neuropathy. Medicine 24:111, 1945.

(28) Martin, M. M.: Diabetic neuropathy. Brain 76:594, 1953.

(29) Shuman, C. R. and Gilpin, S. F.: Diabetic neuropathy: controlled therapeutic trials. Am. J. Med. Sci. 227:612, 1954.

(30) Moorhouse, J. A.: Diabetic peripheral neuropathy. In Diabetes Mellitus. DHEW Publication No. (NIH) 76-854, 1976.

(31) Lossy, F. T., Goldsmith, G. A., Sarett, H. P.: A study of the test dose excretion of five B-complex vitamins. J. Nutr. 45:213, 1951.

(32) Beidleman, B.: Clinical vitamin deficiencies in patients with diabetes. J. Clin. Nutr. 1:119, 1953.

(33) Biskind, M. S., and Schreirer, J.: Nutritional deficiencies in diabetes. Exp. Med. and Surg. 3:299, 1954.

(34) Goodhart, R. and Sinclair, H. M.: Deficiency of vitamin B-1 in man as determined by the blood cocarboxylase. J. Biol. Chem. 132:11, 1940.

(35) Robinson, W. D., Melnick, D., and Field, H.: Urinary excretion of thiamin in clinical diseases. J. Clin. Invest. 19:399, 1940.

(36) Needles, W. : Vitamin B-1 therapy in diabetic neuritis. J. Amer. Med. Assoc. 121:914, 1943.

(37) Field, J. B., Federman, D. D., McDaniel, E., and Bakerson, H.: Urinary excretion patterns of some B-vitamins in diabetes. Diabetes 6:508, 1957.

(38) Davis, R. E., Calder, J. S., and Curnow, D. H.: Serum pyridoxal and folate concentrations in diabetics. Pathology 8:151, 1976.

(39) Davis, R. E., Calder, J. S., and Curnow, D. H.: The effect of pyridoxal on carbohydrate tolerance in patients with diabetes mellitus. Report to NHMRC, 1977.

(40) Kotake, Y. and Murakami, E.: A possible diabetogenic role for tryptophan metabolites and effects of xanthurenic acid on insulin. Am. J. Clin. Nutr. 24:826, 1971.

(41) McCann, V. J. and Davis, R. E.: Serum pyridoxal concentrations in patients with diabetic neuropathy. Aust. N. Z. J. Med. 8:259, 1978.

(42) Jones, C. L. and Gonzales, V.: Pyridoxine deficiency: a new factor in diabetic neuropathy. J. Am. Pod. Assoc. 68:646, 1978.

(43) Conversion of vitamin B-6 compounds in human red blood cells. Nutr. Rev. 30:119, 1972.

(44) Pike, R. L. and Brown, N. L.: Nutrition: An Integrated Approach. 2nd Ed. New York: John Wiley and Sons, 1975.

(45) Felig, P.: Amino acid metabolism in man. Ann. Rev. Biochem., 1975, p. 933.

(46) Pollack, H., and Bookman, J. J.: Riboflavin excretion as a function of protein metabolism in the normal, catabolic, and diabetic human being. J. Lab. Clin. Med. 38:561, 1951.

(47) Thomas, C. L.: Taber's Cyclopedic Medical Dictionary. Philadelphia: F. A. Davis, 1973.

(48) Marble, A.: Current concepts of diabetes. In Joslin's Diabetes Mellitus. 11th Ed. Philadelphia: Lea and Febiger, 1971.

(49) Williams, S. R.: Nutrition and Diet Therapy. 2nd Ed. St. Louis: The C. V. Mosby Co., 1973.

(50) Williams, D. G.: Methods for the estimation of three vitamin dependent red cell enzymes. Clin. Biochem. 9:252, 1976.

(51) Henry, R. J., Cannon, D. C., and Winkelman, J. W.: Clinical Chemistry: Principles and Techniques. 2nd Ed. Hagerstown: Harper and Row, 1974.

(52) Nie, N. H., Hull, C. H., Jenkins, J. G., Steinbrenner, K., and Bent, D. H.: Statistical Package for the Social Sciences. 2nd Ed. New York: McGraw-Hill, 1975.

(53) Kjosin, B. and Seim, S. H.: The transketolase assay of thiamine in some diseases. Am. J. Clin. Nutr. 30:1591, 1977.

(54) Dixon, W. J. and Massey, F. J.: Introduction to Statistical Analysis. 3rd Ed. New York: McGraw-Hill, 1969.

(55) Vir, S. C., and Love, A. H. G.: Vitamin B_6 status of the hospitalized aged. Am. J. Clin. Nutr. 31: 1383, 1978.

(56) Hoorn, R. K.: Vitamin B_1, B_2, and B_6 deficiencies in geriatric patients, measured by coenzyme stimulation of enzyme activities. Clin. Chim. Acta. 61: 151, 1975.

(57) Bayoumi, A. and Rosalki, S. B.: Evaluation of methods of coenzyme activation of erythrocyte enzymes for detection of deficiency of vitamins B_1, B_2, and B_6. Clin. Chem. 22:327, 1976.

(58) Documenta Geigy Scientific Tables. Ed. by K. Diem and C. Lentner. 7th Ed. Basle: Ciba-Geigy Ltd., 1970.

APPENDIX A

June 20, 1979

Dear _____,

You agreed to participate in a study called "Thiamin, Riboflavin, and Pyridoxine Levels in Diabetes Mellitus" when you were in the clinic at _____ on _____. At that time, a sample of your blood was drawn for the measurement of vitamins B_1, B_2, and B_6. The results of your blood sample were as follows:

Vitamin B_1 (thiamin) _____

Vitamin B_2 (riboflavin) _____

Vitamin B_6 (pyridoxine) _____

Comments: _____

_____

_____

The results of the study showed that nearly everyone had normal levels of B_1 and B_2, but half of the people tested were deficient in B_6.

Thank you again for being a part of this study. I hope the information on your vitamin levels will be useful to you.

Sincerely,

Caryl J. Antalis, B. A.
Texas Woman's University

APPENDIX B

MEDICAL AND DIETARY HISTORY QUESTIONNAIRE date:

Name_____ ID no._____

Address_____ Occupation_____

_____ Date of birth_____

Physician_____ Age_____

Ethnic background_____

Alcoholic beverage consumption_____

1. About how old were you when a doctor first told you that you had diabetes? Age_____

2. Have you ever been hospitalized for...
   ...diabetic coma? Yes____No____
   ...insulin reaction? Yes____No____
   ...gangrene? Yes____No____
   ...regulation? Yes____No____

3. Have you ever taken insulin injections? Yes____No____
   If yes, how many years? Years_____

4. Are you now taking insulin injections? Yes____No____

5. When do you usually take your insulin? _____

6. What kinds of insulin are you now using? (regular, semi-lente, globin, NPH, protamine zinc, ultra-lente, lente, other)

7. What strength insulin are you now using?_____

8. Have you ever taken diabetes pills? Yes____No____
   If yes, how many years? Years_____

9. Are you now taking diabetes pills? Yes____No____
   What kind?_____

10. How many pills do you take each day? No._____

11. When do you usually take your pills?_____

12. Do you test your urine for sugar? Yes____No____
    What test do you use?_____

13. How many times did you test your urine last week? No._____

14. When was the last time you tested it?_____

15. Do you write down any of the results of these tests?_____

16. About how tall are you? Feet_____Inches_____

17. About how much do you weigh? Pounds_____

18. What is the most you have weighed during the past 12 months?
Pounds_____

19. What is the least you have weighed during the past 12 months?
Pounds_____

20. Have you been given a diet for your diabetes? Yes____No____

21. How many calories a day are you allowed?_____

22. Who taught you how to use this diet?_____

23. How long have you had this diet?_____

24. Do you follow this diet? Yes____No____
If no, why?_____

25. Who prepares most of your meals? (self, spouse, other_____)

26. Does the person who fixes your meals use any special recipes prepared for persons with diabetes? Yes____No____

27. Is the diet list used as a guide in the preparation of your meals?
Yes____No____
If no, when did you last look at your diet list?_____

28. Does your diet give the size of food portions? Yes____No____
Do you weigh, measure, or estimate the portions?_____

29. Do you have to follow your diet carefully in order to feel well?
Yes____No____

30. Do you ever eat away from home? Yes____No____
Do you have trouble following your diet when eating away from home?
Yes____No____

31. Do you take any vitamin supplements? Yes____No____
If yes, what kind and how much?_____

_____

Signature of Interviewer Date

APPENDIX C

August, 1979

ENZYME ASSAYS FOR THE ASSESSMENT OF THIAMIN, RIBOFLAVIN, AND PYRIDOXINE STATUS IN HUMANS

Reference

The methods for all three assays are taken from the following reference, with a few modifications:

> Williams, D. G.: Methods for the estimation of three vitamin dependent red cell enzymes. Clin. Biochem. 9(6):2521, 1976.

A recording spectrophotometer with a temperature-controlled chamber was not available, so the procedure was modified to be run without these. Also, the concentrations were changed so that all three assays could be run using 3 ml cuvettes with 10 mm path lengths.

Sample Collection and Preparation

Three ml of blood is more than adequate for all three assays. The blood is collected in heparin and immediately put on ice. A refridgerated centrifuge is used to spin down the red cells and wash them 2-3 times with cold saline. All the white cells should be removed in this process. The packed red cells are then aliquotted into 3 or more small vials and frozen at -20° until use.

Use of Spectrophotometer

The Beckman DU spectrophotometer is used. The machine should be allowed to warm up for about 30 minutes (see manual for lighting of the UV lamp). Cuvettes chosen must be suitable for use with UV light. The slit adjustment is set on "manual", and the slit is opened 3 full turns.

The readings are taken in % transmittance and converted to absorbance according to the equation

$$A = -\log (\%T/100).$$

Sometimes the needle jumps around quite a bit when the reading is being taken. This cannot be avoided, and judgement must be used in obtaining the best reading. If the duplicates are more than 10% off, the assay should be repeated.

Calculations

An estimate of the vitamin status of the patient can be gained by the activity coefficient (AC):

$$AC = \frac{A \text{ with vitamin}}{A \text{ without vitamin}}$$

Calculations (con't)

where A is the change in absorbance per minute.

Enzyme activity and vitamin effect can be calculated by the following equations:

$$\text{Activity} = \frac{1000 \times A \times V_c}{A_{NAD} \times V_H \times Hb}$$

where V_c = volume in cuvette
V_H = volume of hemolysate used
A_{NAD} = absorbance of 1 µmol of NADH/NADPH at 340 nm (6.22 for 10 mm light path)
Hb = hemoglobin concentration in g/l

$$\text{Vitamin effect} = \frac{E_{sat} - E}{E_{sat}} \times 100\%$$

where E_{sat} = enzyme activity with vitamin added

E = enzyme activity without vitamin added

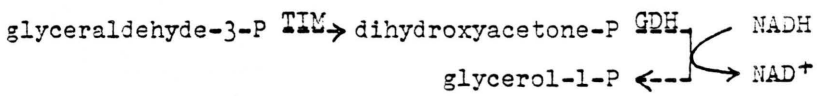
THIAMIN ASSESSMENT: measurement of erythrocyte transketolase activity with and without added thiamin pyrophosphate

Principle: Transketolase, which requires TPP for its activity, catalyzes the following reactions:

xylulose-5-P + ribose-5-P --> sedoheptulose-7-P + glyceraldehyde-3-P

xylulose-5-P + erythrose-4-P --> fructose-6-P + glyceraldehyde-3-P

The glyceraldehyde-3-P formed can be utilized as follows:



TIM = triosephosphate isomerase
GDH = glycerolphosphate dehydrogenase

NADH (and NADPH) absorb maximally at 340 nm while NAD^+ (and $NADP^+$) absorb maximally at 260 nm. Thus the reaction can be monitored by measuring the decrease in absorbance at 340 nm. Under the ideal conditions of this assay, transketolase activity is directly related to the change in absorbance. When the reaction is repeated with the addition of exogenous TPP, a greater change in absorbance is seen if the patient has low stores of the vitamin.

Reagents: 1. Tris buffer - 100 mmol/l, pH 7.6

2. Substrate - 1g ribose-5-phosphate, barium salt, was suspended in a small amount of water, and 1 N HCl was added dropwise to dissolve. Barium ions were precipitated with saturated sodium sulphate added dropwise. The solution was centrifuged and the supernatant decanted into another tube. Further sodium sulfate was added, and the procedure repeated until no more barium precipitated (6 or 7 times). The final supernatant containing the sodium ribose-5-P was made up to 190 ml with tris buffer, giving a substrate concentration of 15 mmol/l. The final pH of the solution was checked and adjusted to 7.6 if necessary, and the solution was stored in 10 ml amounts at -20°.

3. NADH disodium salt - 3.9 mg were dissolved in 0.5 ml buffer giving a concentration of 10 mmol/l. This was freshly prepared for each run.

4. Thiamin pyrophosphate chloride - 98 mg were dissolved in 20 ml buffer giving a concentration of 10 mmol/l. This was stored in 1 ml amounts at -20°.

5. GDH/TIM - 2 mg/ml suspension

Procedure: To prepare the hemolysate, the contents of one vial of thawed packed red cells is transferred to a tube and diluted with an equal volume of water. This is mixed by pipetting up and down a Pasteur pipette until clear. The hemoglobin concentration is determined using the cyanmethemoglobin method, and adjusted to 3.5 g hemoglobin/100 ml with water. Care must be taken to keep the hemolysate cold during manipulations. (Note: if the packed red cells have not been frozen at least overnight, then they should be frozen and and thawed several times using an ethanol/dry ice bath to ensure complete lysis.)

To run in duplicate, four tubes are set up for each sample to be assayed. A blank is also set up for each sample. The tubes are prepared as follows:

Reagent	tubes 1&2	tubes 3&4	blank
Buffer	1.5 ml	1.5 ml	3.0 ml
Substrate	1.5	1.5	--
Hemolysate	0.05	0.05	0.05
NADH	0.02	0.02	--
TPP	--	0.05	--
water	0.05	--	--

The tubes and all reagents are kept on ice. The spectrophotometer is set at 340 nm, and both cuvettes are filled with the "blank" and put in the chamber. The machine is adjusted to 100% transmittance. Then the cuvette in the sample beam is replaced with the opaque block and the machine is adjusted to 0% transmittance.

To assay, the first tube is placed in the 37° water bath and the stopwatch is started. At 15 min. the contents of the tube are transferred to the cuvette and a reading is taken. The reaction is started by adding 0.01 ml GDH/TIM suspension and mixing by inverting the cuvette with parafilm over the top. (Note: the tiny opening of the pipet tends to clog with this suspension. This can be avoided by rinsing the pipet with water between uses, and keeping it in water rather than in the suspension.)
After mixing, the solution is poured back into the original tube and returned to the water bath. At 40 min. a second reading is taken and the solution discarded.

Up to 8 tubes can be added to the water bath in 3 min. intervals with plenty of time for readings and manipulations, as shown in the example below (two samples are assayed in 61 minutes). With practice, tubes can be added to the water bath in 2 min. intervals (three samples are assayed in 62 minutes). The cuvette is rinsed with water between uses, and the "blank" in the reference cuvette is changed with each sample.

Since the reaction rate reamins constant for 30 min., the length of time between the first and second reading is not crucial as long as the time is noted so that it can be figured in the calculations.

Example: Samples A, B, and C - tubes are prepared as described above and designated A1-4, B1-4, and C1-4.

Timetable I (minutes on stopwatch)

tube #	into bath	1st reading & rx started	2nd reading
A1	0	15	40
A2	3	18	43
A3	6	21	46
A4	9	24	49
B1	12	27	52
B2	15	30	55
B3	18	33	58
B4	21	36	61

Reaction time is 24 min., allowing one min. for manipulations.

Timetable II (minutes on stopwatch)

tube #	into bath	1st reading & rx started	2nd reading
A1	0	16	40
A2	2	18	42
A3	4	20	44
A4	6	22	46
B1	8	24	48
B2	10	26	50
B3	12	28	52
B4	14	30	54
C1	16	32	56
C2	18	34	58
C3	20	36	60
C4	22	38	62

Reaction time is 23 min., allowing one min. for manipulations.

Data and calculations:

tube #	$\%T_{340}$ 0'	$\%T_{340}$ 24'	A_{340} 0'	A_{340} 24'	ΔA (ave.)	Activity Coeff.
A1	57.6	66.4	.240	.178	.064	1.08
A2	57.3	66.5	.242	.177		
A3	56.2	65.8	.250	.182	.069	
A4	54.8	64.2	.261	.192		
B1	60.3	67.5	.220	.171	.051	1.15
B2	60.8	68.5	.216	.164		
B3	55.3	63.0	.257	.201	.060	
B4	57.3	65.7	.242	.182		

Calculation of enzyme activity (see pg. 2):

$$\text{Activity} = \frac{1000 \times A \times 2.63}{6.22 \times 0.05 \times Hb} = 8457 \times \frac{A}{Hb}$$

Since the hemoglobin concentration was adjusted to 3.5 g/100 ml, this reduces to

Activity = 242 A

Sample A

Activity without added TPP = 242 X .064/24 = .645 U/g Hb

Activity with added TPP = 242 X .069/24 = .696 U/g Hb

$$\text{Vitamin effect} = \frac{.696 - .645}{.696} \times 100\% = 7.3\%$$

Sample B

Activity without added TPP = 242 X .051/24 = .514

Activity with added TPP = 242 X .060/24 = .605

$$\text{Vitamin effect} = \frac{.605 - .514}{.605} \times 100\% = 15.0\%$$

RIBOFLAVIN ASSESSMENT: measurement of erythrocyte glutathione reductase with and without added flavin adenine dinucleotide

Principle: Glutathione reductase, which requires FAD for its activity, catalyzes the following reaction:

This reaction can be monitored by measuring the decrease in absorbance at 340 nm. Enzyme activity is directly to the change in absorbance. When the reaction is repeated with the addition of exogenous FAD, a greater change in absorbance is seen if the patient has low stores of the vitamin.

Reagents:

1. Potassium phosphate buffer - 100 mmol/l. 16.41g K_2HPO_4 and 0.79g KH_2PO_4 are dissolved in 800 ml water, which is adjusted to pH 7.4 and diluted to 1 liter.

2. NADPH, tetrasodium salt - 4.15 mg are dissolved in 2.5 ml of a 1% (w/v) solution of sodium bicarbonate giving a concentration of 2.0 mmol/l.

3. Flavin adenine dinucleotide, monodosium salt (FAD) - 2.4 mg are dissolved in 10 ml water giving a concentration of 250 µmol/l.

4. Dipotassium ethylene diamine tetracetate (K_2EDTA) - 1.6g of the dihydrate are dissolved in 50 ml water to give a solution of concentration 80 mmol/l.

5. Oxidized glutathione (GSSG) - 11.5 mg are dissolved in 2.48 ml water to which is added 0.025 ml 1 molar NaOH, giving a concentration of 7.5 mmol/l.

Solutions 2, 3, and 5 are prepared fresh each day and kept on ice.

Procedure: The hemolysate is prepared by taking 100 µl of the thawed packed red cells and diluting with 1.9 ml water (a capillary-type micropipet is necessary to pipet these cells). This solution can be mixed by pipetting up and down a Pasteur pipette until clear.

To run in duplicate, four tubes are set up for each sample to be assayed. A blank is also set up for each sample. The tubes are prepared as follows:

Reagent	tubes 1&2	tubes 3&4	blank
Buffer	2.0 ml	2.0 ml	2.5 ml
NADPH	0.1	0.1	--
Hemolysate	0.1	0.1	0.1
K_2EDTA	0.05	0.05	--
FAD	--	0.1	--
water	0.1	--	--

The spectrophotometer is set at 340 nm and "blanked" as previously described.

To assay, the first tube is placed in the 37° water bath and the stopwatch is started. At 15 min. the contents of the tube are transferred to the cuvette and a reading is taken. The reaction is started by adding 0.1 ml GSSG solution and mixing by inverting the cuvette with parafilm over the top. The time in seconds is noted when the GSSG is added. The cuvette is placed back into the chamber and three minutes later a second reading is taken. The solution is then discarded and cuvette rinsed out.

If the tubes are added to the water bath in 5 min. intervals, assays may be run continuously as described above. The "blank" in the reference cuvette is changed with each sample.

Example: Samples A and B - tubes are prepared as described above and designated A1-4 and B1-4.

Data and calculations:

tube #	$\%T_{340}$ 0'	$\%T_{340}$ 3'	A_{340} 0'	A_{340} 3'	Δ A (ave.)	Activity Coeff.
A1	43.2	51.4	.365	.289	.079	1.03
A2	41.5	50.0	.382	.301		
A3	38.7	46.5	.412	.333	.081	
A4	38.3	46.3	.417	.334		
B1	44.1	50.8	.356	.294	.063	1.20
B2	43.8	50.7	.359	.295		
B3	39.1	46.6	.408	.332	.076	
B4	39.0	46.3	.409	.334		

Calculation of enzyme activity (see pg. 2):

$$\text{Activity} = \frac{1000 \times A \times 2.45}{6.22 \times 0.1 \times Hb} = 3940 \times \frac{A}{Hb}$$

The hemoglobin concentration of each hemolysate is measured by the cyanmethemoglobin method.

Sample A

Activity without added FAD = $3940 \times \frac{.079/3}{14.6} = 7.01$ U/g Hb

Activity with added FAD = $3940 \times \frac{.081/3}{14.8} = 7.19$ U/g Hb

Vitamin effect = $\frac{7.19 - 7.01}{7.19} \times 100\% = 2.5\%$

Sample B

Activity without added FAD = $3940 \times \frac{.063/3}{14.4} = 5.75$

Activity with added FAD = $3940 \times \frac{.076/3}{14.4} = 6.93$

Vitamin effect = $\frac{6.93 - 5.75}{5.75} \times 100\% = 17.0\%$

PYRIDOXINE ASSESSMENT: measurement of aspartate aminotransferase activity with and without added pyridoxal-5-phosphate

Principle: Aspartate aminotransferase, which requires pyridoxal-5-phosphate for its activity, catalyzes the following reaction:

aspartate + α-ketoglutarate ---> oxaloacetate + glutamic acid

Malate dehydrogenase then catalyzes the following:

oxaloacetate + NADH ---- malate + NAD^+

The reactions can be monitored by measuring the decrease in absorbance at 340 nm. Aminotransferase activity is directly to the change in absorbance. When the reaction is repeated with the addition of exogenous pyridoxal-5-P, a greater change in absorbance is seen if the patient has low stores of the vitamin.

Reagents:

1. Potassium phosphate buffer - 100 mmol/l. 16.41g K_2HPO_4 and 0.79g KH_2PO_4 are dissolved in 800 ml water, which is adjusted to pH 7.4 and diluted to 1 liter.

2. Pyridoxal-5-phosphate (PAL-P) - 400 µmol/l. 4.95 mg are dissolved in 50 ml water (vigorous shaking or stirring is necessary to dissolve).

3. GOT optimized kit (Boehringer Cat. No. 124419) -
   a. Buffer/substrate
      100 mM phosphate buffer, pH 7.4
      250 mM L-(+)-aspartate
   b. NADH, 13.5 mM
   c. MDH/LDH, 48 U/ml MDH, 96 U/ml LDH
   d. α-ketoglutarate, 445 mM

It is convenient to combine (a), (b), and (c) above in the amounts necessary for that day's assays, to have only one solution to pipet. For 20 assays, 21 ml (a) would be combined with .35 ml each (b) and (c). This would give a buffer/substrate-plus (B/S-plus) with final concentrations of 97 mM phosphate buffer, 213 mM L-aspartate, 0.18 mM NADH, 0.7 U/ml MDH, and 1.4 U/ml LDH.

Procedure: The hemolysate is prepared by taking 100 µl of the thawed packed red cells and diluting with 1.9 ml water (a capillary-type micropipet is necessary to pipet these cells). This solution can be mixed by pipetting up and down a Pasteur pipette until clear.

To run in duplicate, four tubes are set up for each sample to be assayed. A blank is also set up for each sample. The tubes are prepared as follows:

Reagent	tubes 1&2	tubes 3&4	blank
Buffer	1.0 ml	1.0 ml	2.0 ml
B/S-plus	1.0	1.0	--
Hemolysate	0.04	0.04	0.04
PAL-P	--	0.05	--
water	0.05	--	--

The spectrophotometer is set at 340 nm and "blanked" as previously described.

To assay, the first tube is placed in the 37° water bath and the stopwatch is started. At 15 min. the contents of the tube are transferred to the cuvette and a reading is taken. The reaction is started by adding 0.025 ml solution (d) (α-ketoglutatate) and mixing by inverting the cuvette with parafilm over the top. After mixing, the solution is poured back into the original tube and returned to the water bath. At 36 min. a second reading is taken and the solution is discarded.

IMPORTANT: each day that assays are done, a control must be run substituting water for the hemolysate. The control is run induplicate with and without PAL-P, just as for each sample. This is because there will be a ΔA even without hemolysate, and this must be subtracted from the readings to have meaningful results.

If tubes are added to the water bath in 2 min. intervals, 2 samples can be assayed in 50 minutes. If a second batch of tubes are added to the water bath as the second reading is being taken on the first batch, then 4 samples can be assayed in 86 minutes as shown in the example below.

Example: Samples A, B, C and control - tubes are prepared as described above and designated A1-4, B1-4, C1-4 and X1-4.

Timetable (minutes on stopwatch)

tube #	into bath	1st reading	2nd reading	tube #	into bath	1st reading	2nd reading
A1	0	15	36	C1	36	51	72
A2	2	17	38	C2	38	53	74
A3	4	19	40	C3	40	55	76
A4	6	21	42	C4	42	57	78
B1	8	23	44	X1	44	59	80
B2	10	25	46	X2	46	61	82
B3	12	27	48	X3	48	63	84
B4	14	29	50	X4	50	65	86

Reaction time is 20 min., allowing one min. for manipulations.

Data and calculations:

tube #	$\%T_{340}$ 0'	$\%T_{340}$ 20'	A_{340} 0'	A_{340} 20'	ΔA (ave.)
A1	46.2	52.2	.335	.282	.052
A2	48.2	54.2	.317	.266	
A3	46.0	61.6	.337	.210	.129
A4	44.9	60.5	.348	.218	
B1	49.7	49.9	.304	.302	.002
B2	51.1	51.4	.291	.289	
B3	47.2	56.2	.326	.250	.078
B4	47.3	56.9	.325	.245	
X1	43.7	40.0	.360	.398	+ .039
X2	43.8	40.0	.359	.398	
X3	41.3	43.2	.384	.365	- .020
X4	41.0	43.1	.387	.366	

Calculation of activity coefficient:

Sample A $\quad \frac{.129 - .020}{.052 + .039} = \frac{.109}{.091} = 1.19$

Sample B $\quad \frac{.078 - .020}{.002 + .039} = \frac{.058}{.041} = 1.42$

Calculation of enzyme activity—(see pg. 2):

$$\text{Activity} = \frac{1000 \times A \times 2.09}{6.22 \times .04 \times Hb} = 8394 \times \frac{A}{Hb}$$

The hemoglobin concentration of each hemolysate is measured by the cyanmethemoglobin method.

Sample A

Activity without added PAL-P = $8394 \times \frac{.091/20}{15.9} = 2.40$ U/g Hb

Activity with added PAL-P = $8394 \times \frac{.109/20}{15.9} = 2.88$ U/g Hb

Vitamin effect = $\frac{2.88 - 2.40}{2.88} \times 100\% = 16.7\%$

Sample B

Activity without added PAL-P = $8394 \times \frac{.041/20}{15.2} = 1.13$

Activity with added PAL-P = $8394 \times \frac{.058/20}{15.2} = 1.60$

Vitamin effect = $\frac{1.60 - 1.13}{1.60} \times 100\% = 29.4\%$