THE EFFECT OF DIFFERENT LEVELS OF THIAMINE INTAKE ON THE URINARY EXCRETION OF THIAMINE

by

JEANNE LINA PERKINS

A THESIS

submitted to the

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1943

APPROVED:

Associate Professor of Foods and Nutrition
In Charge of Major

Head of Department of Foods and Nutrition

Chairman of School Graduate Committee

Chairman of State College Graduate Council

ACKNOWLEDGMENTS

The writer wishes to express her gratitude and appreciation for the advice and kind assistance given to her by Dr. Margaret L. Fincke, Associate Professor of Foods and Nutrition, during the course of this investigation.

She would also like to acknowledge the loyal cooperation of her experimental subjects, and the interest and support of Mrs. Jessamine C. Williams, Head of the Department of Foods and Nutrition.

TABLE OF CONTENTS

	Page
CHAPTER I. INTRODUCTION	1
Historical Background	1
The Distribution of Thiamine in Foods	3
Chemical Nature and Properties of Thiamine	4
Physiology of Thiamine	5
The Role of Thiamine in Metabolism	7
Methods of Thiamine Assay	10
1. Biological	10
2. Chemical	13
Methods of assessing the level of thiamine nutrition	16
Human Requirement for Thiamine	22
CHAPTER II. PURPOSE OF THIS INVESTIGATION	28
CHAPTER III. EXPERIMENTAL	29
General Outline of Procedure for the Determination of Thiamine	29
Determination of Thiamine in Urine	31
Determination of Thiamine in Foods	32
The Thiochrome Method of Assay for Thiamine	34
Solutions and Equipment Used	35
Description of Subjects	38
The Experimental Diet	38
Caloric Record	41
CHAPTER IV. RESULTS AND DISCUSSION	42
CHAPTER V. SUMMARY AND CONCLUSIONS	49
BIBLIOGRAPHY	51

LIST OF TABLES AND CHARTS

	Page
Table I. The Experimental Diet	40
Table II. Daily Excretion of Thiamine During a Constant Diet Supplemented With Different Amounts of Thiamine	43
Table III. Average Daily Excretions of Thiamine on Different Levels of Intake	45
Table IV. Excretion of Thiamine in Response to Test Dose After Different Levels of Intake .	47
Chart A. Average Daily Excretions of Thiamine on Different Levels of Intake	43-a

THE EFFECT OF DIFFERENT LEVELS OF THIAMINE INTAKE ON THE URINARY EXCRETION OF THIAMINE

CHAPTER I

INTRODUCTION

Historical Background

Beri-beri, a disease common in the Orient, has been shown to be caused by a lack of adequate thiamine in the diet. The disease was known in China as early as 2600 B. C., and was first demonstrated to be of dietary origin by Takaki, a high medical officer in the Japanese navy. For several years twenty to forty per cent of the men in the Japanese navy were sick at some time during the year with beri-beri. Takaki received permission to change the diet on board ship. By decreasing the rice, increasing the barley, and adding vegetables, meat and fish, he practically eliminated beri-beri among the men. He reported the results of this large-scale experiment in 1882, attributing the eradication of the disease to the dietary changes, but failing to give satisfactory reasons. The disease continued to be regarded as infectious in origin, and many people believed that improvement in sanitary conditions accounted for the decrease in cases of beri-beri which Takaki had reported. (25).

In 1897 Eijkman first produced polyneuritis in fowls by limiting them to a diet of polished rice, and cured the symptoms by feeding rice polishings. He reported his work in such a way that the "antineuritic substance" which he postulated was thought to be a medicine, and not a constituent of all normal diets. It remained for Grijns (1901) to extend these experiments with fowl and advance a theory which, clearly nutritional, attributes beri-beri to a lack of a substance needed by the central nervous system, from the diet.

American and British workers in the Orient at about the same time were discovering the importance of diet in the prevention and cure of beri-beri. The realization of the importance of certain foodstuffs stimulated the search for the substance in them that possessed antineuritic properties. The substance was discovered to be water-soluble and non-protein before 1910, and work continued in many laboratories, in Java, Maylaya, the Dutch East Indies, the Philippines, and Japan, as well as in Europe and America, to isolate and identify it.

Funk, in 1912, introduced the "vitamine theory," after he had worked with extracts of rice polishings, and had carefully studied the literature available concerning scurvy, pellagra, and rickets. It was not until 1926 that Jansen and Donath isolated the substance, and another

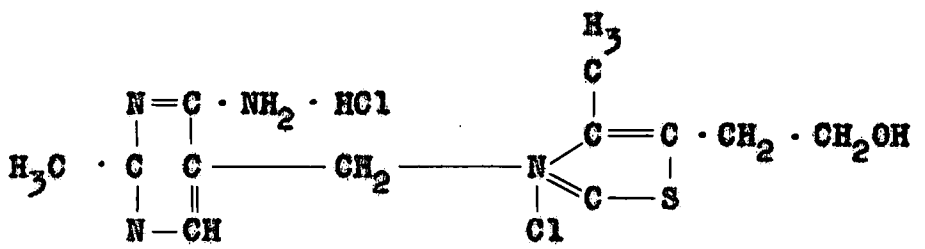
ten years of research followed before Williams had separated enough of the pure substance to permit the determination of the full molecular structure and chemical properties of thiamine hydrochloride itself.

The Distribution of Thiamine in Foods

Thiamine occurs in small amounts in almost all of the plant or animal tissues which are used as foods. None of the foods are highly potent, although pork muscle, the germ and bran of cereals, and the legumes, furnish amounts sufficient to be important in the human dietary. Yeast is also a good source of thiamine, and some high vitamin strains have been used to enrich bread made from refined flour. Only highly refined foods, such as commercial sweets, fats, and unenriched white flour are practically devoid of thiamine. When these foods assume an undue importance in the diet, lack of dietary thiamine may become a real problem. When fruits and vegetables, however, are eaten in quantities large enough to provide adequate amounts of other dietary constituents, they will also provide ample thiamine. The widespread choice of whole-wheat or the recently introduced enriched bread, will also help to ensure adequate thiamine in the diet.

Chemical Nature and Properties of Thiamine

The white crystals of thiamine hydrochloride are hygroscopic and readily soluble in water, slightly soluble in alcohol, and insoluble in ether, chloroform and benzene. Thiamine hydrochloride is dializable, and will not precipitate with any of the salts of heavy metals which precipitate protein. The dry crystals are stable to both air and light, and heating at 100°C for twenty-four hours in air causes no loss in potency, (51). Acid solutions are stable for long periods, but the vitamin is sensitive to alkali, especially when heated. Mild oxidation with alkaline ferricyanide results in a change to thiochrome, a yellow dye which emits a strong blue-violet fluorescence in ultra-violet light, and which is biologically inactive. The compound thiamine hydrochloride has been assigned the following structural formula:



Thiamine Hydrochloride

Physiology of Thiamine

The longest-known functions of thiamine are the promotion of growth and the prevention of polyneuritis (66).

The lack of thiamine in the animal diet has been observed to cause a marked anorexia (63), which appears rather shortly after the vitamin is removed from the diet. Return of thiamine causes a return of normal appetite, even when it is given separately from the food. Williams, Mason, Wilder and Smith (76) reported that the feeding of thiamine to women who had been fed a diet very low in the vitamin for some time caused them to return willingly and even eagerly to food which they had formerly been unable to eat.

The accumulation of intermediary products of carbohydrate metabolism may account for the failure of appetite and the general decline in gastrointestinal activity which has been often observed in thiamine-deficient animals and in man (66). The nervous system, which can apparently use only energy derived from the burning of glucose, also shows early signs of thiamine deficiency.

Symptoms of a serious thiamine deficiency, which was produced in normal women fed a diet almost devoid of thiamine, were reported by Williams, Mason, Wilder and Smith (76). They listed depressed mental states, general-

ized weakness, dizziness, backache, soreness of muscles, palpitation, dyspnea, and percordial distress on exertion, insomnia, anorexia, nausea, vomiting, loss of weight, atony of muscles, and slight roughness of the skin. The symptoms could be relieved by the administration of thiamine, and then produced again when it was withheld. In another study from the same laboratory (74) a less marked deficiency was produced by limiting thiamine intake to 0.45 mg/day, which approximates that in the "poor" American diet. After 8-12 weeks on the restricted thiamine intake, there were "gross changes in behavior, marked changes of attitude, diminished inclination to perform accustomed tasks, and progressive decrease of ability to make social adjustments within the group." Complaints, weakness, nausea, and periodic involvements of the digestive tract were also noted.

This diet, which represented a sub-optimal intake of thiamine over a long period, produced symptoms which are commonly observed in many groups. During the study, the symptoms were removed by the administration of thiamine, but much more slowly than those caused by an extreme deficiency which had resulted in more rapid appearance of the symptoms.

Thiamine, therefore, appears to be necessary to the normal functioning of the body; its lack causes symptoms of malfunction, and its return restores normal function.

The symptoms are varied and widespread because the production of energy from the metabolism of carbohydrates is intimately associated with life processes in every cell of the body.

The Role of Thiamine in Metabolism

In the living organism, thiamine functions as the pyrophosphate, called cocarboxylase (4). This substance acts as a coenzyme in carbohydrate metabolism. The exact mechanism for this metabolism, and the role or roles which thiamine may perform, remain unknown. Research, however, has thrown considerable light at different points in the complicated series of reactions.

The normally functioning body can completely break glucose to carbon dioxide and water, deriving energy from the process. Glucose is first phosphorylated and split to phosphoglyceric acid (11); then pyruvic acid is formed from this product. Several things may happen to the pyruvic acid in normal metabolism. The concentration of lactic acid in the blood during and following strenuous exercise probably indicates that the reduction of pyruvic to lactic acid takes place with ready production of energy. The pyruvic acid under other conditions may be oxidized, dismuted, or condensed. Barron and Lyman (5) have demonstrated the possibility of conversion of

pyruvic acid to glucose in the presence of added thiamine. Thiamine can be readily converted to the phosphorylated form, and the phosphate can be easily split off again. Phosphorylation takes place at many points in the metabolic chain of reactions, and there is evidence that thiamine, in its active form, cocarboxylase, functions as a phosphate carrier (5, 38). Other compounds which seem to be necessary for carbohydrate metabolism may also function as phosphate carriers, as adenylic acid probably does (34). Ochoa (54) has suggested a system of reactions during which pyruvic acid is oxidized, glucose phosphorylated, and adenylic acid changed to adenosine triphosphate and back to adenylic acid. Lipton and Elvehjem (38) have suggested that the adenosine triphosphate may donate its phosphorus, or part of it, to thiamine when cocarboxylase is produced.

It is evident that pyruvate metabolism may proceed in several different ways. Primary, we presume, will be the direct breakdown to carbon dioxide and water, with production of energy for use in further phosphorylation processes, for heat, or for other bodily functions. Secondary, but necessary, reactions may be the resynthesis of carbohydrate in the liver, the formation of lactic acid, and the formation of the catalytic four-carbon acids. For all of these reactions, it has been shown

that thiamine, as cocarboxylase, is needed (11, 54, 38, 71, 5).

During the repeated reactions in which thiamine takes part, the molecule may be split to its component pyrimidine and thiazole rings. This reaction occurs readily in the test tube and probably occurs readily in the body also; the animal body is unable to use the fractions. Although only a small quantity of thiamine will catalyze many oxidation reactions, (55), there does seem to be a loss of the vitamin sufficient to make a daily replenishing of the supply necessary.

Because the burning of fat yields energy, less energy will have to be produced from the burning of carbohydrate in a high fat diet, therefore thiamine will take part in fewer reactions, and be destroyed less quickly than when carbohydrate alone is utilized. Cahill (7), however, could find no change in thiamine excretion when the fat content of the diet was markedly increased, on either adequate or inadequate levels of thiamine intake. Wang and Yudkin (73) report a similar lack of urinary indication of thiamine-sparing action when they added to the diet quantities of fat sufficient to markedly change the fat to carbohydrate ratio.

Methods of Thiamine Assay

1. Biological. Several methods have been proposed for the determination of thiamine in tissues and in foodstuffs. Biological methods have included work with pigeons and more extensive work with rats.

The rat-growth method has been used for the assay of many substances for thiamine. It involves feeding healthy young rats a diet devoid of thiamine, but adequate in all other dietary factors essential for growth, until the body stores of thiamine have been depleted. The animals are then fed supplements of the unknown during a period of from ten days (63) to eight weeks. Several levels of the test food are fed, and supplements of crystalline thiamine are given to litter-mate rats which serve as controls. The growth responses of animals on the various levels of the test food are compared to those of the animals fed the standard thiamine solution for estimation of the thiamine content of the test food. Knott (36) has used this method for thiamine determinations on foods and excreta in a balance study with children. Adsorbates on acid clay were prepared for feeding to the animals.

The disadvantages of the rat-growth method are the length of time involved, since a test period of three or four weeks is advisable, and the large number of animals

needed to ensure precision.

A shorter method has been adopted by the U. S. Pharmacopoeia (56) for the assay of concentrates containing thiamine. This is called the rat curative method, and was earlier used with pigeons. The animals are fed a diet low in thiamine, but adequate in other dietary essentials. When the animals develop polyneuritis, they are cured with known doses of thiamine, and the length of time before the onset of the next attack of polyneuritis noted. They are then cured by feeding a weighed amount of a test food, and the length of cure compared with the standard for estimation of thiamine content of the food. This method is sensitive, specific, and accurate for determination of the thiamine content of foods rich in thiamine.

A third method using rats has been widely employed in England (26, 79). It was first described in detail by Birch and Harris (6), and is called the rat bradycardia method. Rats fed a diet low in thiamine develop a slow heart rate, which may be measured using the electrocardiograph. When the rat has been depleted, the heart rate may fall as low as 300 beats per minute from a normal rate of 500 to 600 beats per minute. Between 300 and 400 beats per minute, as determined by frequent tracings of the rat's heart beat, or electrocardiograms, the rat

is fed pure thiamine or a test food. The heart rate increases at once; both the increase in rate and the time before it returns to its former low level, are proportional to the amount of thiamine fed. Harris and Leong (26) have employed this method for assay of urine in an experimental study of human thiamine deficiency. Robertson and Doyle (60) report lack of success with this method: they failed to obtain consistent results in the responses to graded doses, and in the response of the same animal, or different animals, to the same dose of thiamine. There is some question about the ability of a depleted rat to fully utilize thiamine when it is adsorbed on acid clay. Harris and Leong (26), however, reported an error of 15 to 20 per cent with the bradycardia method.

Microbiological methods have also been proposed for the determination of thiamine. Meiklejohn (44) described a method using Phycomyces Blakesleeanus, a mold which is stimulated to produce more mycelia by the addition of thiamine to a medium adequate in all other substances necessary for growth. He proposed the determination of total thiamine in blood by this method, since the mold could use cocarboxylase as well as thiamine. Sinclair and coworkers criticized the method as non-specific (68) and suggested modifications (21, 19).

Yeasts have been used for the determination of thiamine in two ways. Goodhart and Sinclair (20), and Schultz, Atkin and Frey (64) used fermentation methods which measure the carbon dioxide evolved by yeasts when stimulated by thiamine. Modifications to include sulfite cleavage of thiamine, to separate the effect of the thiamine alone from the effects of its degradation products, were adopted by Schultz, Atkin, Frey, and Williams (65).

Williams, McMahan, and Eakin (78), introduced, in 1941, a yeast growth method which is sensitive for very small amounts of thiamine. Turbidity of the yeast suspension, determined colorimetrically, reflects the growth of yeast when different amounts of thiamine in the crystalline form or in a natural substance, have been added to a suitable medium inoculated with the "old process" strain of Saccharomyces cerevisiae.

2. Chemical. Two methods have been most widely adopted for the chemical determination of thiamine.

Probluda and McCollum (58) discovered the quantitative conversion of the thiamine to a purplish-red compound when it was treated with p-amino acetanilid or methyl-p-aminophenyl ketone with nitrous acid. Melnick and Field (46) using diazotized p-amino acetophenone, have

developed a quantitative technique for thiamine assay of foods and urine which measures the intensity of the red color formed by thiamine and the acetophenone, when the dye has been extracted with xylene. This method is satisfactory for foods rich in thiamine, and for concentrates prepares by adsorption techniques.

The second method to be used extensively for chemical determinations is called the thiochrome method. After Peters (55) discovered the blue-fluorescing thiochrome, the fluorometric method was developed by Jansen (31) in 1936. In this method, the blue fluorescence of thiochrome, which results from the alkaline oxidation of thiamine, is measured, and checked against the standard fluorescence of quinine sulfate. Many modifications have been proposed. Enzymatic separation of thiamine from its protein and phosphorus compounds has been used by many workers (1, 2, 15, 28), because the thiochrome determination measures only thiamine in its free form.

The need for concentrating thiamine extracted from foods of low potency, and the presence of interfering fluorescent materials in many foods, and in urine, brought about research which led to the announcement in 1938 of a base-exchange method, by Hennessy and Cerecedo (28). The artificial zeolite decalso was used to adsorb the thiamine.

The zeolite could be washed with water to remove interfering substances, and then potassium chloride, when added in excess, eluted the vitamin in pure form. The purification process may be omitted when foods are high in thiamine and low in interfering substances, but it has been widely and successfully used in determining the thiamine content of urine (16, 17, 18, 33, 43, 50, 76). Wang and Harris (72) found that the disadvantages of the base-exchange purification outweighed its advantages, and they remove interfering materials from urine by a preliminary washing with isobutanol.

Several modifications of Hennessy and Cerecedo's method have been suggested by Egaña and Meiklejohn (16), Ferrebee and Carden (18), Jowett (33), Elsom and co-workers (17), and others. Minor modifications as suggested by the laboratories of Merck and Company (50) were adopted as best suited to this laboratory, and are described in detail in Chapter III. Lane, Johnson, and Williams (37), using similar modifications of the thiochrome method, found that their chemical determinations checked closely with those by the rat curative method, for foods containing 1 mcg or more of thiamine per gram. Moyer and Tressler (52) found that the thiamine content of three vegetables--spinach, corn, and asparagus--agreed well when determined by the rat growth and thiochrome methods,

but that the values for five-peas, cauliflower, broccoli, lima beans, and snap beans--when determined by the thiochrome method were appreciably lower than values derived from rat growth studies. They also report that the fermentation method, using sulfite cleavage (65) gives results agreeing with those using the thiochrome method. In unpublished work from this laboratory, it has been found that for most of the vegetables assayed, the thiochrome method gave lower thiamine values than did the rat curative technique.

Methods of Assessing the Level of Thiamine Nutrition

The difference between radiant health and the presence of symptoms listed in the discussion of the physiology of thiamine can be easily detected by a physician, but during the state of suboptimal health when no clinical evidence of deficiency is seen, the doctor can only suspect a thiamine deficiency. The doctor needs some reliable test to aid in diagnosing these subclinical deficiencies; some way of measuring tissue state in regard to thiamine must be found.

Several ways are open: the tissues themselves, the blood or any of its parts, or the feces or urine could be analyzed for thiamine, or for some other substance related to it in the body.

The thiamine content of tissues has been determined by Carleen (9), but it is too new to be a widely used method of proved merit.

Cowgill (14) states that the thiamine of the feces, except during diarrhea, is found in the bodies of the bacteria present in the large intestine. Since thiamine is water-soluble, he suggests, it will be generally absorbed from the food, and excreted in the urine. Knott (36) assayed both urine and feces in a balance study with children. She found that the thiamine content of the feces did not increase proportionately to increases in food intake, and that there was some tendency for the feces to be low in thiamine while urine was high, and vice versa.

Thiamine in the blood has proved difficult to determine. Sinclair (69) reported that the thiamine in blood may appear in at least five different forms: (1) as free thiamine, (2) as free cocarboxylase, (3) as a compound of protein with thiamine or (4) protein with cocarboxylase, from which the thiamine or cocarboxylase could be split by heating at pH 2, or (5) as a thiamine-protein compound which could be split by pepsin but not by heating. Meiklejohn (44) proposed the method of determining the thiamine found in the first four of the compounds listed by Sinclair, but Sinclair, in another publication (68) criticized Meiklejohn's method as

lacking specificity. Goodhart and Sinclair's fermentation method (21), later modified by Goodhart (19), determined the blood cocarboxylase, and "normal" values for the cocarboxylase content of the blood of men and boys were reported.

During thiamine deficieney, an excess of pyruvate and other metabolites is accumulated in the tissues. Lu (39) proposed a method for determining blood pyruvate, using the color developed by the 2:4-dinitrophenylhydrazone of pyruvic acid. She also studied the relation of blood pyruvate to bradycardia in rats (40), but found that levels of blood pyruvate were easily influenced by exercise, and did not reflect just the level of the body's thiamine nutrition. The method of Lu could determine not the pyruvate content of the blood alone, but certain other substances, which are called bisulfite-binding substances or B. B. S. by Clift and Cook (10), who proposed a method for determining them. A later evaluation of the determination of bisulfite-binding substances in blood was made by Robinson, Melnick and Field (61) in 1940. They kept a subject on a diet grossly deficient in thiamine, and found that no elevation of the B. B. S. value occurred during the period, "despite a fall in urinary thiamin excretion to very low levels which persisted after thiamin supplementations, and de-

spite the development of early manifestations of a clinical thiamin deficiency." They concluded that the value for bisulfite-binding substances in blood "lacked specificity and sensitivity as a means of detecting latent or mild chronic forms of thiamin deficiency."

Determinations of B. B. S. in urine, using rats, (40) revealed that while the excretion of the B. B. S. reached levels proportional to the content of the vitamin in the diet, it was necessary to check also the urine's thiamine content, to determine whether the abnormal carbohydrate tolerance was a result of specific thiamine deficiency.

The indication of thiamine nutrition most widely used today is the excretion of thiamine in urine. Both Melnick and Field's colorimetric method (46) and the thiochrome method have been widely used. Melnick and Field (47) reported that thiamine in urine is always found in the free form, and is apparently unaffected by the administration of many drugs. The excretion of thiamine has been found in many laboratories (26, 32, 73, 79) to be roughly proportional to the intake of the vitamin, under normal conditions (41, 49). In order to establish a normal range to aid in interpreting routine clinical analyses of urine for thiamine, many laboratories (8, 7, 29, 33, 62, 79, 48, 49, 70, 72) have reported the values

of the daily thiamine excretions of normal individuals. For example, Robinson, Melnick and Field (62) reported that males with previously adequate dietary intake of thiamine excreted 90 mcg or more per day, and females who had had adequate diets excreted 60 mcg or more per day. Mason and Williams (43) state that excretion of 100 ± 10 mcg or more daily probably indicates adequate nutrition. The depressing effects of some pathological states on the urinary excretion of thiamine have been reported by Machella and Elsom (41), and by Pollack and co-workers (57).

Measurement of tissue status using shorter periods than twenty-four hours for urine collection are proposed by Smith, Burlinson and Spector (70), and by Holt and Najjar (30).

The test dose of thiamine has also been suggested as a means of detecting subclinical deficiencies or of confirming diagnoses of deficiency, as well as in attempts to determine human requirement for thiamine. Oral doses, usually of 5.0 mg of thiamine given with a meal (49) have the advantage of being subjected to the same absorption processes as the thiamine of food; Melnick, Field and Robinson (49) report that test doses given in other ways may mask the effect of deficiency by temporarily flooding the organism with thiamine. Injections of 1.0 or 0.5 mg

of thiamine have been recommended as test doses by Melnick and Field (48), Williams, Mason and Wilder (75), and Carden, Province and Ferrebee (8). An elaborate scheme for evaluating the results by determining 24-hour excretion and the test dose excretion of thiamine has been formulated by Melnick, Field and Robinson (49). For instance, an excretion by an adult female of 60-70 mcg per day, with 7 to 10 per cent return of the test dose, is said to indicate that the subject is borderline in thiamine status, but consuming an adequate diet at the time of the test.

Response to the test dose indicating normal nutrition has been said to be 7 to 20 per cent (49) of a 5.0 mg oral or 1.0 mg injected dose. Ten to 40 per cent of a 0.5 mg subcutaneous dose is reported for normal males by Carden, Province and Ferrebee (8). Mason and Williams (43) recommend a test dose of 1.0 mg of thiamine given intravenously, with a diet containing about 800 mcg per day, and expect 20 ± 2 per cent of this test dose to be excreted by individuals with normal tissue state. Smith, Burlinson and Spector (70) report that excretion of 7 per cent or more of a 5.0 mg oral test dose indicates good nutritional status with regard to thiamine.

Human Requirement for Thiamine

As soon as it was realized that thiamine was a necessary factor in normal nutrition, the search for facts about human requirements began. Cowgill (13) reported in 1938 a formula for calculating the vitamin: calorie ratio; which he had developed after experimenting with many species of animals:

$$\frac{\text{Vitamin in International Units}}{\text{Calories}} = 0.00426 \times \text{weight in kilograms}$$

This ratio has been used to determine the minimum requirement for the prevention of beri-beri. Requirement for thiamine, since this vitamin functions in carbohydrate metabolism, will be increased in any situation which increases metabolism in general, for example: during growth, exercise (22), fevers, hyperthyroidism, pregnancy, and lactation. Diuresis and diarrhea also increase the need (13).

Using the techniques developed for measuring the status of nutrition, experiments have been carried on to determine human requirements. Elsom and co-workers (17) using a plan similar to those used in many other laboratories, kept six apparently normal women on a constant diet "representative" of the American dietary but low in thiamine, for 28 to 120 days. They report that three subjects receiving the smallest amount needed

according to Cowgill's formula, began to show signs of deficiency before the end of the period. They state that 651 mcg per day, with a thiamine: calorie ratio of 0.35 probably met the minimum requirements of these women, but that this amount might prove inadequate over a longer period of time.

Melnick (45) has summarized the results of many objective thiamine balance studies carried on by several workers in his laboratories. He reports that thiamine added to a diet containing inadequate thiamine will not immediately cause an increase in urinary excretion, but that a subject who has previously taken an adequate diet will respond promptly to changes in the amount of dietary thiamine. When six normal individuals were kept on a diet containing 1070 mcg of thiamine per day, the daily excretion of thiamine and the responses to test dose were well within normal range. Probably it may be concluded from this that one milligram per day will be adequate for the minimal demands of the adult. The adequacy of this intake was tested further by giving the same subjects ten milligrams of extra thiamine daily. After one week, 4 mg of thiamine were being excreted in excess of the excretion on the diet alone; this level remained until the end of the high-thiamine period. After saturation, the subjects were left on the basal diet

alone for one week, during which the excretion dropped to a level still much above the basal level before saturation. A test dose, however, resulted in increases in excretion, above the new basal level, that were approximately the same as increases noted when the test dose had been given before saturation. This would seem to indicate, Melnick reports, that the subjects had been in a state of adequate thiamine nutrition before saturation, on 1070 mcg per day. Previous studies had shown that men receiving one milligram of thiamine per day excreted about 200 mcg while women receiving 700 mcg excreted 90 mcg daily. This is not a proportionate drop in excretion, so it may be assumed that the body is conserving thiamine at this 700 mcg level, which may be borderline for normal requirement. The responses to test dose for both men and women were 13 per cent after this basal level; it may be assumed that adequate stores had been maintained on each level. This intake may be called the conservation-of-test-dose level, which is slightly higher than Cowgill's 600-900 mcg per 2000-3000 calories, which should just prevent beri-beri, or frank deficiency symptoms. The level resulting in conservation of test dose is about 350 mcg per thousand calories, or, adding 50 per cent for a margin of safety, about 500 mcg per 1000 calories.

The requirement of thiamine for normal nutrition is probably higher than that contained in the "average" diet before enriched flour was introduced, which Lane, Johnson and Williams (37) reported to be 320 mcg per 1000 calories. Many clinical observations, correlated by Melnick, Field and Robinson (49) with 24-hour excretion and response to test doses of thiamine, showed that 27 per cent of the subjects who had been classified as normal by physicians failed to meet previously formulated standards of normal thiamine excretion.

Williams, Mason, and Wilder (75) have summarized the findings about thiamine requirement which result from studies carried on in their laboratories. They state that minimum requirement figures should be based on the prevention of minimum abnormality, as determined by long-time studies, and recommend the detection of a biochemical defect in carbohydrate metabolism as the best measure of this minimum abnormality. Subjects should be kept on a known intake of thiamine, they believe, for several months without the development of biochemical defect if the intake tested shall be regarded as adequate. Elevation of the blood lactic and pyruvic acid levels, with decreased glucose tolerance, should indicate a fault in carbohycrate metabolism which should result from barely sub-optimal intakes of thiamine.

Urinary excretion of thiamine, and response to the test dose, were correlated with blood lactic and pyruvic acid levels, since these may rise from causes other (40) than the biochemical defect associated with thiamine deficiency. Williams, Mason and Wilder report (75) that an intake of 0.45 mg per 1000 calories was associated in three of five subjects with some depletion of tissue cocarboxylase stores, and "in four of the five with slow development of a mild degree of biochemical defect." They state that probably 0.45 mg per 1000 calories could be taken as a minimal requirement, and that since proportions of fat to carbohydrate in diets may vary, probably 0.6 mg per 1000 calories is none too high.

The difference between inadequate and adequate thiamine nutrition has been hard to detect, but the difference between adequate and optimal nutrition may be even harder. Less work has been done to determine optimal needs, probably because of lack of criteria for judging what is optimal. Williams, Mason, Wilder and Smith (76) reported that, while 0.95 mg daily prevented deficiency symptoms, an intake of two milligrams of thiamine daily resulted in the best performances as measured in terms of physical work done by normal women. Keys and Henschel (35) have reported that when large supplements of thiamine

were added to diets containing 430 mcg of thiamine per thousand calories, no improvement in ability to do physical work could be detected.

CHAPTER II

PURPOSE OF THIS INVESTIGATION

Since thiamine requirements vary from person to person, and may be influenced by many different factors, extensive study would have to be carried on to determine optimal requirements, which would meet the minimal needs of the body, allow a margin of safety, and prevent waste through excessive excretion.

The purpose of this study is to determine the thiamine requirements of three subjects, by measuring daily urinary excretion, and the urinary response to a test dose of thiamine, with different levels of thiamine supplementation to a constant diet.

CHAPTER III

EXPERIMENTAL

Plan of Experiment

A constant diet low in thiamine was fed to two subjects for twenty-four days, and to one for twenty-three days. This time was divided into three periods of eight days each, which differed only in the amount of thiamine given as a supplement to the diet. For the first seven days a supplement of 1.0 mg thiamine chloride was given, and on the eighth a test dose of 5.0 mg replaced the supplement. The next level fed for seven days was 1.5 mg, daily, with a 5.0 mg test dose on the eighth day. The third supplement of thiamine was 0.5 mg daily, followed by a test dose of 5.0 mg for two subjects on the eighth day, and for the third (AG) on the seventh. Twenty-four hour collections of urine were made on the last five days of a supplement-level, and during the day of the test dose. These were analyzed for thiamine.

General Outline of Procedure for the Determination of Thiamine

Foods to be assayed were finely ground with acid, and the material buffered to pH 4.5, which is optimal

for digestion with two enzymes: taka-diastase, which splits cocarboxylase, leaving free thiamine, and papain, which liberates thiamine from any of its compounds which include protein. Since the thiochrome method determines only free thiamine, the compounds which bind it must be split. Thiamine in urine seems to be present only in free form (47), so no digestion is needed.

The thiamine of urine must be separated from interfering fluorescent materials. Urine was buffered to pH 4.5, and passed through an artificial zeolite, decalso, which exchanged the potassium chloride with which it had previously been activated, for thiamine as an adsorbate. When a strong solution of potassium chloride was later introduced, the thiamine was displaced, or eluted, and passed out with the excess potassium chloride, free from impurities.

In the thiochrome procedure, the thiamine was oxidized to thiochrome by ferricyanide in an alkaline solution, and this was extracted with isobutanol. The strength of fluorescence from the thiochrome varies with its concentration in the isobutanol. This fluorescence was measured by the photofluorometer, which had been calibrated against the readings produced by solutions of known thiamine concentration oxidized by the same procedure.

Determination of thiamine in urine

Urine was collected from about 6:45 A.M. to the same time the next morning. The 24-hour collection was thoroughly mixed, the volume noted, and the pH determined using brom cresol green as an outside indicator. Three 25 cc aliquots were taken from each collection of urine, except on the day during which the test dose was given for the last two levels, when 10 cc aliquots were taken. During the first two experimental periods, 10 cc of sodium acetate-acetic acid buffer at pH 4.5 was added to each aliquot, without a subsequent check of pH. During these periods the recoveries of added thiamine averaged about 50 per cent, and variation between samples often resulted in the necessity for repeating. During the last period, the pH was checked after addition of buffer. Buffer at pH 5.0 was added in varying amounts to insure a pH of 4.5, which Hennessy (27) reports optimum for adsorption of thiamine on decalso. Average recoveries of added thiamine for this period were much higher: 80 per cent or over for all subjects.

Two of the buffered aliquots were allowed to pass through a decalso bed; to the third was added one cc of a solution containing 5.0 mcg of thiamine per cc, to test the recovery of added thiamine from the decalso; when the level of liquid was from one inch to one-fourth

inch above the decalso, 10 cc of water buffered to pH 4.5 was added. Two more washings followed, and when the last washing was nearly through, a graduated cylinder was placed under the capillary opening, and about 15 cc of a solution 25 per cent KCl in .1 N HCl was added to the exchange tube. This solution eluted the adsorbed thiamine. KCl was poured into the tube until the collected eluate reached the 25 cc mark. This eluate contained, theoretically, all of the thiamine which was present in the original buffered urine. The eluates were assayed by the thiochrome method.

Determination of thiamine in foods

A sample of the food which would contain 14-16 mcg of thiamine was placed in the Waring Blendor with 90 cc of 2 per cent acetic acid, and ground for one minute. Then 10 cc of acid were used to wash the sides of the Blendor, and the grinding continued for one or two minutes. Immediately after grinding, one half of the material was measured in a graduated cylinder, and poured into a 125 cc erlenmeyer flask. Five cc of each of the enzymes, takadiastase and papain, in the buffer at pH 4.5 were added to wash the material from the cylinder. Three or four samples were treated in this way. One of these was used to test the recovery of added thiamine: 2 cc of a

solution containing 5 mcg thiamine per cc replaced 2 cc of the 2 per cent acetic acid, and the material taken from the Blendor was tested to determine whether it contained 5 mcg of thiamine beside the thiamine of the food.

After addition of the buffered enzymes, the flasks were incubated for two hours at about 40°C, to allow digestion of the thiamine-containing compounds. Then the material was brought to a boil, cooled under running cold water, and filtered through dry qualitative grade filter paper into dry beakers. Aliquots of the filtrates were passed through decalso, following the procedure described for urine, if the residual or "blank" fluorescence gave a reading of above twenty divisions, or if the solution needed to be concentrated in order to measure the thiamine. Rice filtrates were subjected to this procedure because of their low thiamine content; an aliquot taken for adsorption was greater than the volume of eluate collected. Aliquots of the filtrates were assayed directly by the thiochrome method if the "blank" fluorescence was low, and the thiamine concentration at least 0.1 mcg in 5 cc. The procedure outlined above was used for all of the foods in the diet except milk and grapefruit juice. The milk was incubated with enzymes in water, and buffered after incubation, as suggested by Halliday and Deuel (23). Grapefruit juice

was incubated with takadiastase alone, and treated with decalso, as suggested by Bailey and Thomas (2). Recovery of thiamine added to foods ranged around 100 per cent, with less variation than with urines. The recovery percentages were not used to correct the values for thiamine content, as they were all within the limits of the error for the method.

The Thiochrome Method of Assay for Thiamine

An aliquot of the solution to be assayed which would contain 0.2-1.0 mcg of thiamine was made up to 5 cc with buffered water, in a graduated cylinder equipped with a ground glass stopper. Three drops of 1 per cent $K_3Fe(CN)_6$ were added, and then 3 cc of 15 per cent NaOH, followed immediately by 13 cc of isobutanol delivered from a fast-flowing pipette. This mixture was shaken vigorously for one and one-half minutes, and the layers allowed to separate in a separatory funnel, with or without previous centrifuging. Aqueous solutions of thiamine, or eluates, treated with isobutanol, usually separated quickly without centrifuging. The aqueous layer was drawn off and discarded, and the isobutanol layer poured into a centrifuge tube. From 1 to 4 grams of anhydrous sodium sulfate were added, with stirring or shaking, until there was a clearing of the cloudy liquid.

The tube was then spun in a centrifuge to throw down the particles of sodium sulfate, with the water which they had removed from the solution. The brilliantly clear isobutanol was then poured into a dry cuvette, and its fluorescence measured. A blank, which duplicated the procedure with the omission of ferricyanide, served to correct the reading of the fluorometer for non-thiochrome fluorescence.

Distilled water and blank KCl eluates usually gave readings from 6.0 to 8.5 divisions on the galvanometer scale. The blank was subtracted from the determination reading, and the thiamine content read from a chart upon which corrected readings for known thiamine solutions were plotted. The thiamine content of the aliquot used was thus estimated, and the day's excretion of thiamine, or the thiamine content of the foods, calculated.

Solutions and Equipment Used

A. Standard solutions

1. Quinine sulfate solution A

0.0054 grams of quinine sulfate in one liter of 0.1 N H_2SO_4.

2. Quinine sulfate working standard

Dilution of solution A, using one volume of A and 39 volumes of 0.1 N H_2SO_4. The fluorometer was set to 70.0 with this solution in a tested cuvette before each reading of an unknown.

3. Thiamine solution A

   25.0 milligrams of thiamine hydrochloride crystals (Merck Betabion) dissolved in 500 cc 20 per cent ethanol, adjusted to pH about 4.0 with 0.1 N HCl.

4. Thiamine intermediate solution

   5.0 cc Solution A diluted to 50 cc with water adjusted to pH 4.5 with acetate buffer. 1 cc = 5 mcg thiamine.

B. Reagents

1. 15 per cent sodium hydroxide

2. 1 per cent potassium ferricyanide

3. Isobutyl alcohol (isobutanol). Distilled before using, to insure fluorescence of 5.0 to 6.0 divisions, equivalent to the fluorescence of distilled water.

4. Anhydrous sodium sulfate. Used to remove water from, and clear, the isobutanol phase before fluorometric measurements.

5. 25 per cent potassium chloride in 0.1 N hydrochloric acid.

6. Decâlso (Zeolite, Permutit). No. 80 mesh obtained from the Permutit Company, 330 W. 42nd St., New York, N. Y.

   Prepared by soaking with several successive portions of 2 per cent acetic acid, then twenty minutes with 25 per cent KCl in 0.1 N HCl followed by two washings with 2 per cent acetic acid, and three washings with distilled water before drying. One gram was introduced into the exchange tube.

7. pH Indicator. Brom cresol green, pH 3.8-5.4, for use with a spot-plate.

8. Enzyme solutions for food analyses.

   a. 5 per cent Taka-diastase made up just before use, in acetate buffer at pH 4.5.

b. 5 per cent papain made up freshly with buffer at pH 4.5.

9. 20 per cent acetic acid, diluted as needed to 2 per cent acetic acid for food analyses.

10. Buffers.

a. pH 4.5 : 55 cc glacial acetic acid and 110.5 grams $NaC_2H_3O_2 \cdot 3H_2O$ made up to one liter with distilled water, according to directions of Conner and Straub (12).

b. pH 5.0 : Used for adjusting pH of urine during last experimental level: 30 cc glacial acetic acid and 168 grams $NaC_2H_3O_2 \cdot 3H_2O$ made up to one liter with distilled water.

C. Equipment

1. Coleman electronic photofluorometer with filters for determination of vitamin B_1.

The thiochrome solutions are exposed to ultra-violet light from a mercury vapor lamp; the fluorescence which results is amplified, and measured by a photoelectric cell equipped with a galvanometer. Sensitivity of the instrument was checked before each reading with the standard quinine sulfate solution.

2. Exchange tubes.

Described by Merck and Company (50). A tube of one centimeter diameter is widened at one end to hold about 25 cc liquid, and a capillary tube at the other regulates flow to about 1 cc per minute. A plug of glass wool supports the bed of one gram of decalso for base exchange.

3. Centrifuge

Accommodating 50 cc centrifuge tubes.

4. Incubator oven, maintained at about 40 degrees centigrade.

Note: No rubber or cork was allowed to come in contact with the isobutanol used in the procedure, as they caused the formation of interfering fluorescent materials. No lubricants except glycerol for the separatory funnels were used. All joints were ground glass except for foil-covered rubber stoppers used in the isobutanol distillation apparatus.

Description of Subjects (AG, JP, and JF)

Three women served as subjects. Two were graduate students and the third a staff member. They recorded their weights daily before lunch. AG varied between 138 1/2 and 141 pounds, JP between 103 1/2 and 104 1/2, and JF weighed 154 at the beginning and 151 at the end of the experimental period; these were extremes in her weight variations. Heights were 5'5", 5'4" and 5'7" respectively. All subjects were apparently normal, moderately active, and in good health.

The Experimental Diet

The diet given below (Table I), believed to be adequate except in thiamine, riboflavin, and iron was fed. Experimental supplements of thiamine, and of 4.0 mg iron and 1.0 mg riboflavin, were given to each subject with the morning meal. Each food was analyzed for thiamine; three determinations, including a test for recovery of added thiamine, were considered sufficient unless the

results varied considerably. At least three cans of every food were used for sampling; each of the four lots of cheese used was sampled, and cake and biscuits from three different lots. All cakes and biscuits were made from one lot of unenriched white flour supplied through the courtesy of Crown Mills. Meat taken from each lot purchased was mixed and sampled before cooking. Analyses for thiamine were done on the cooked meat patties. Samples for analysis were taken from two different cooked patties. Celery from two stalks was analyzed. The thiamine content, expressed as the average of the several determinations on each food, is presented in Table I.

To check the adequacy of the diet in vitamin C, the plasma ascorbic acid levels were determined in this laboratory. After four days on the experimental diet, subject JP had a level of 0.68 mg of ascorbic acid per 100 cc of blood plasma. Tested again on the twelfth day of the diet, her level was 0.69 mg per 100 cc of plasma. Many investigators consider that a plasma ascorbic acid level of about 0.6 mg per 100 cc indicates an adequate state of ascorbic acid nutrition. Subjects JP and AG, perhaps because they were under somewhat less nervous tension, after 6 days on the diet showed levels of 0.98 and 1.2 mg per 100 cc of plasma, which are accepted as indicating a state of tissue saturation.

Table I

The Experimental Diet

Food	Quantity gms		Total Calories	Non-fat Calories	Thiamine Present mcg
Rice	100		93	93	8.3
Ground round steak, beef	100		156	85	81.0
Celery	25		6	6	5.0
Cheddar cheese	60		247	61	24.3
Evaporated milk	60		85	40	28.3
Canned string beans	80		38	33	31.8
Canned carrots	100		45	44	22.7
Canned peaches Juice from peaches	80 20		62	62	13.0
Canned grapefruit juice	140		59	58	62.8
Angel food cake =1 egg white, about	56		149	149	3.6
Baking power biscuits, about 8 biscuits		(AG) (JF and JP)	584 621	368 391	37.2
Butter, about	50	(AG) (JF and JP)	450 441	2	--
Tea, ad lib.					
Soda crackers, ave.	8		136	100	9.0
Cooking fat, 1 tsp.			30	--	--
Totals:		(AG) (JF and JP)	2138 2166	1422 1445	328.0

Ratios:

AG Thiamine in I.U.: calories = .46

JF and JP Thiamine in I.U.: calories = .45

AG Thiamine: non-fat calories = .69

JF and JP Thiamine: non-fat calories = .68

These tests seemed to indicate that the experimental diet furnished adequate ascorbic acid for these subjects.

Caloric Record

The number of biscuits, of crackers, and the number of grams of butter which each subject ate were recorded daily. With the exception of the first day, the caloric intake of subject AG varied only with the caloric content of biscuits from different lots. The amount of flour in the lot of 24-29 biscuits, varied between 320 and 400 grams; an average is taken for calculation of calories and thiamine content. A slight increase in caloric intake over the first period was noted in the last two periods for JP and JP, but this was not as great as the variation from day to day, so the total average caloric intake is used for calculation. Tables in Sherman and Lanford's Essentials of Nutrition (67) furnished data for calculation of total and non-fat calories, as shown in Table I; the measured total thiamine content of the foods in the diet was 0.328 mg for all subjects. Subject AG took slightly fewer calories than did the other two, whose intake was almost identical throughout the investigation.

CHAPTER IV

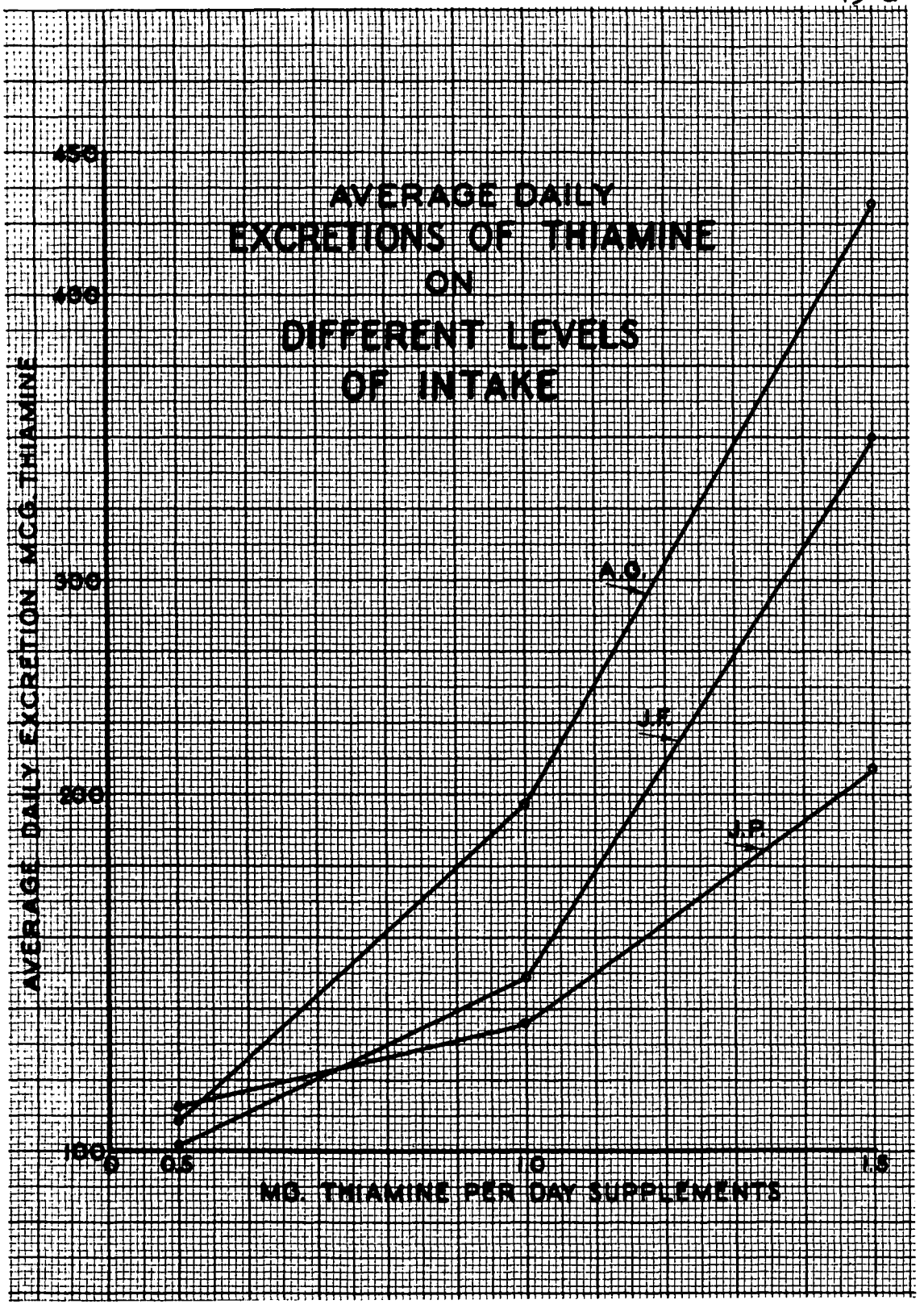
RESULTS AND DISCUSSION

Data collected during the investigation from determinations of thiamine in urine are presented in Table II. As will be seen in this table, the daily excretion of thiamine, even with a constant intake of the vitamin, and relatively constant caloric intake, varies somewhat. During the first two periods reported in this table, the recoveries of added thiamine were low, and themselves varied greatly. During these two periods, checks on the pH of the urine after buffering were omitted. During the last period, when the pH of the uriniferous solution was more carefully adjusted, with the addition of buffer at pH 5.0, and pH determined with brom cresol green, the percentages of recovery of added thiamine increased. Chart A represents the average excretion during each period for each subject, corrected by the average per cent of recovery of added thiamine for that period. Percentage of recovery of added thiamine has been used to check the thiochrome method in several laboratories. Jowett (33) reports a constant recovery of 68 per cent. Egana and Meiklejohn (16) found that 75 per cent of thiamine added to the urines of deficient subjects could be recovered from those of normal subjects. Elsom and

Table II

Daily Excretion of Thiamine During a Constant Diet Supplemented With Different Amounts of Thiamine

Subject / Supplement	Date 1942	AG Thiamine excreted mcg	AG Recovery of added thiamine %	JF Thiamine excreted mcg	JF Recovery of added thiamine %	JP Thiamine excreted mcg	JP Recovery of added thiamine %
1.0 mg thiamine	10/17	115.0	52	65.0	45	59.7	29
	10/18	118.3	50	34.8	34	48.8	50
	10/19	101.3	43	80.5	55	89.5	49
	10/20	772.9	44	97.9	59	75.8	66
	10/21	104.6	41	91.5	39	94.5	61
5.0 mg test dose	10/22	206.3	60	208.1	47	204.4	--
1.5 mg thiamine	10/25	158.7	15	102.0	34	104.0	54
	10/26	142.8	43	118.3	25	97.8	39
	10/27	147.0	66	136.6	27	123.2	72
	10/28	160.8	20	142.7	39	147.2	52
	10/29	273.6	59	183.0	71	157.4	51
5.0 mg test dose	10/30	545.6	89	432.9	81	436.2	84
0.5 mg thiamine	11/1	121.2	93				
0.5 mg thiamine	11/2	116.7	63	67.8	81	104.0	95
	11/3	59.8	57	69.0	64	80.0	74
	11/4	73.8	87	92.9	96	110.2	65
	11/5	77.6	108	94.6	81	85.3	93
	11/6	750.3	104	97.4	94	80.2	74
5.0 mg test dose	11/7	(test dose)		492.8	101	655.7	91


AVERAGE DAILY EXCRETIONS OF THIAMINE ON DIFFERENT LEVELS OF INTAKE
AVERAGE DAILY EXCRETION MCG. THIAMINE
400
300
200
100
0
A.Q.
J.K.
J.P.
0
0.5
1.0
1.5
MG. THIAMINE PER DAY SUPPLEMENTS

co-workers (17) and Hennessy and Cerecedo (28) report recoveries of added thiamine near the theoretical values.

Widely varying excretions of thiamine on a constant diet have been reported by Jolliffe and co-workers (32): variations of several hundred per cent were noted in the same subject on the same diet. Wang and Yudkin (73) decreased the daily variation by limiting their subjects to highly refined foods, which were not only lower in total thiamine but probably varied less in thiamine content from one day to the next. When the intake of thiamine was increased, the variations in excretion were greater.

The diet used in this investigation, and in that of Jolliffe and his co-workers (32) aimed to follow the American dietary pattern, to include two fruits, two vegetables, milk or its equivalent, and meat, as suggested by the Bureau of Home Economics. Some variations in the food sources are unavoidable, even when conditions are carefully controlled. Variation in activity of the subjects from day to day, and in caloric intake, may account for some of the fluctuations in excretion.

It will be seen in Table II that on the supplement-level of 1.0 mg per day, the excretions during the last three days for all subjects fall close to each other, with

no definite trend toward higher or lower excretion. During the supplement-level of 1.5 mg daily, excretions start at the final point of the former period, or above, and show a marked trend toward higher excretion, but recoveries of added thiamine for the period show no similar trend.

Table III and Chart A show the average recovery of added thiamine for that period, to make clear the general differences in excretion on the three levels of thiamine intake. No average excretion fell below 100 mcg per day, while AG, on the highest intake level, averaged over 400 mcg per day. Since excretion of 100 mcg of thiamine or more per day probably indicates adequate nutrition (49, 43), all three of the levels of intake seem to have been adequate for these subjects. Adding to the supplement the thiamine content of the food, which, allowing for the error of the chemical method as compared to the biological, probably was about 0.5 mg per day, the total intake of thiamine on each of the three levels was: 1.0 mg, 1.5 mg, and 2.0 mg. This represents an intake of 0.5 mg, 0.75 mg, and 1.0 mg per 1000 calories.

While excretions of all subjects increased with increased intake of thiamine, the increase was less for JP than for JF or AG. The curves plotted in Chart A make a sharper slope between supplement-levels of 1.0 and

Table III

Average Daily Excretions of Thiamine on Different Levels of Intake

Subject	Daily Thiamine Supplement	Average Excretion of Thiamine	Average Recovery of Added Thiamine	Corrected Values for Excretion
	mg	mcg/day	per cent	mcg
AG	0.5	90	82	110
	1.0	102	46	222
	1.5	177	41	432
JF	0.5	84	83	101
	1.0	74	46	161
	1.5	137	39	350
JP	0.5	92	80	115
	1.0	74	51	145
	1.5	126	54	233

1.5 mg than they do between 0.5 and 1.0 mg. This may be important in evaluating the results of the investigation, and gives some indication of the thiamine requirement of these subjects. Melnick and Field (48) had found that men and women given comparable diets excreted comparable amounts of thiamine. When men were given 1.0 mg of thiamine daily, they excreted 200 mcg, and women on 0.7 mg daily excreted 90 mcg per day. Melnick (45) reported these

results, and stated that since this is not a proportionate drop in excretion, he assumes that the body is conserving thiamine at the 0.7 mg level. Both levels of intake were followed by a 13 per cent response to test dose, so both groups were assumed to be maintaining body stores of thiamine, but since the body was conserving thiamine on the lower level, that may be on the borderline for normal requirement.

Table IV shows the responses to test doses after different levels of thiamine intake. No correlation can be seen between excretion after the test dose and the level which preceded the test dose. The corrected excretions are all nearly 10 per cent of the test dose, or more, which response is considered indication of an adequate state of nutrition with respect to thiamine (45, 70). As a means of differentiating between the adequacies of the supplement-levels, the test dose in this experiment had little value. It may have been so large that differences in the tissue state with respect to thiamine, existing before the dose, were masked, or the responses may mean that all levels were sufficient for the subject, and that similar fractions of the test dose were excreted because no deficiencies existed before an excess of thiamine was given.

Table IV

Excretion of Thiamine in Response to Test Dose After Different Levels of Intake

Subject	AG			JF			JP		
5.0 mg thiamine given after the supplement-level of:	Measured	Recovery of added thiamine	Corrected	Measured	Recovery of added thiamine	Corrected	Measured	Recovery of added thiamine	Corrected
	mcg	%	mcg	mcg	%	mcg	mcg	%	mcg
0.5 mg thiamine	750	104	721	493	101	488	656	91	721
1.0 mg thiamine	206	60	343	208	47	443	204	—	—
1.5 mg thiamine	546	89	613	433	81	534	436	84	519

The lowest total intake of thiamine in this investigation, 0.8-1.0 mg of thiamine, probably was adequate for these subjects, since at least 100 mcg were being excreted daily, and the test dose responses were satisfactory. Thiamine probably was being conserved by the body at this level, and also at the 1.0 mg supplement or 1.3-1.5 mg level of total intake. The optimal level which should be sought in the daily diet would provide thiamine adequate to meet the daily needs for the subject, and maintain tissue stores, but not waste the vitamin through excessive excretion. The level of 1.3-1.5 mg of total thiamine per day should provide a margin of safety over the lower level, and yet the thiamine seems to have been efficiently utilized, since the subjects reached an equilibrium. On the highest level of intake, the excretion values were still increasing when the test dose was given; the thiamine intake probably exceeded the optimal level. Some intake between 1.5 and 2.0 mg may have been closer to optimal than either of these tested levels.

There is need to extend this investigation, by lengthening the experimental periods, by trying other levels of supplementation, and by increasing the number of subjects studied. There is also the more general need for education in nutrition, to bring the average intake of thiamine closer to the optimal for health and efficiency.

CHAPTER V

SUMMARY AND CONCLUSIONS

Three subjects were given a constant diet containing 0.3-0.5 mg of thiamine. Three different supplements of thiamine were given with this diet, in different 7-day periods. A supplement of 0.5 mg resulted in average excretions of 100 mcg thiamine or more per day. A supplement of 1.0 mg resulted in average excretions of about 150 mcg for two subjects and over 200 mcg for the third. The 1.5 mg daily supplement resulted in considerably higher average excretions for all subjects, with a trend toward higher daily excretions for all subjects at the end of this experimental period. The urinary responses to oral test doses for all subjects after all experimental periods were nearly 10 per cent, or more, of the 5.0 mg of thiamine given. This response to test dose indicates adequate tissue stores of thiamine, and daily excretion of 100 mcg of thiamine, or more, indicates adequate dietary intake. All three levels seemed to be adequate for these subjects, but the supplement of 1.0 mg, or the total intake of 1.3-1.5 mg would provide a margin of safety, without the waste of vitamin which apparently occurred when the 1.5 mg supplement was given. The optimal level found in this study is considerable higher than the amount of thiamine found by Lane, Johnson and

Williams (37) in the average American diet, but is equal to the standard proposed by the National Research Council for moderately active women.

BIBLIOGRAPHY

1. Andrews, John S., and Nordgren, Robert. The application of the thiochrome method to the thiamine analysis of cereals and cereal products. Cereal Chem. 18:686-695, 1941.

2. Bailey, M.I., and Thomas, Arthur W. The thiamine and riboflavin contents of citrus fruits. Jour. Nutrition 24:85-92, 1942.

3. Banerji, G.C., and Harris, Leslie J. Methods for assessing the level of nutrition. A carbohydrate tolerance test for vitamin B1. 1. Experiments with rats. Biochem. Jour. 33:1346-1355, 1939.

4. Banga, I., Ochoa, S., and Peters, R.A. Pyruvate oxidation in brain. VI. The active form of vitamin B_1 and the role of C_4 dicarbonylic acids. Biochem. Jour. 33:1109-1121, 1939.

5. Barron, E.S.G., and Lyman, C.M. Studies on biological oxidations. XI. The metabolism of pyruvic acid by animal tissues and bacteria. Jour. Biol. Chem. 127:143-161, 1939.

6. Birch, Thomas William, and Harris, Leslie J. Bradycardia in the vitamin B_1-deficient rat and its use in vitamin B_1 determinations. Biochem. Jour. 28:602-621, 1934.

7. Cahill, William A. Urinary excretion of thiamine on high fat and high carbohydrate diets. Jour. Nutrition 21:411-418, 1941.

8. Carden, George A., Province, William D., and Ferrebee, Joseph W. Clinical experiences with the measurement of the urinary excretion of vitamin B_1. Proc. Soc. Exp. Biol. Med. 45:1-5, 1940.

9. Carleen, Mildred H., Weissman, Norman, Owen, Philip S., and Ferrebee, Joseph W. Subclinical vitamin deficiency. Science 97:47-49, 1943.

10. Clift, Francis Paul, and Cook, Robert P. A method of determination of some biologically important aldehydes and ketones, with special reference to pyruvic acid and methylglyoxal. Biochem. Jour. 26:1788-1799, 1932.

11. Colowick, S. P., Kalckar, H. M., and Cori, C. F. Glucose phosphorylation and oxidation in cell-free tissue extracts. Jour. Biol. Chem. 137: 343-356, 1941.

12. Conner, R.T., and Straub, G.J. Determination of thiamin by the thiochrome reaction. Ind. Eng. Chem., Anal. Ed. 13:380-384, 1941.

13. Cowgill, George R. Human requirements for vitamin B_1. Jour. Amer. Med. Assoc. 111:1009-1016, 1938.

14. Cowgill, George R. The physiology of vitamin B_1. Jour. Amer. Med. Assoc. 110:805-812, 1938.

15. Dawson, E.R., and Martin, G.W. Vitamin B_1. Estimation in yeast and bread and stability during bread-making. Jour. Soc. Chem. Industry 60: 241-245, 1941.

16. Egana, Enrique, and Melklejohn, Arnold P. The estimation of thiamine in urine. Jour. Biol. Chem. 141:859-870, 1941.

17. Elsom, K.O., Reingold, J.G., Nicholson, J.T L., and Chornock, Charlotte. Studies of the B vitamins in the human subject. V. The normal requirement for thiamine; some factors influencing its utilization and excretion. Amer. Jour. Med. Sci. 203:569-577, 1942.

18. Farrebee, Joseph W., and Carden, G.A. A procedure for the routine determination of vitamin B_1 in urine. Jour. Lab. Clin. Med. 25:1320-1324, 1940.

19. Goodhart, Robert. A revaluation of the method described by Goodhart and Sinclair for the determination of blood cocarboxylase values. Jour. Biol. Chem. 135:77-84, 1940.

20. Goodhart, Robert, and Sinclair, H. M. Deficiency of vitamin B_1 in man as determined by the blood cocarboxylase. Jour. Biol. Chem. 132:11-21, 1940.

21. Goodhart, Robert, and Sinclair, H. M. The estimation of cocarboxylase (vitamin B_1 diphosphate ester) in blood. Biochem. Jour. 33:1099-1108, 1939.

22. Guerrant, N. B., and Dutcher, R. Adams. The influence of exercise on the growing rat in the presence and absence of vitamin B_1. Jour. Nutrition 20: 589-598, 1940.

23. Halliday, N., and Deuel, H.J., Jr. The presence of free and combined thiamine in milk. Jour. Biol. Chem. 140:555-561, 1941.

24. Harper, Harold A., and Deuel, H. J., Jr. The urinary pyruvate in thiamine deficiency. Jour. Biol. Chem. 137:233-238, 1941.

25. Harris, Leslie J. Vitamins and vitamin deficiencies. Volume 1. London, J. and A. Churchill, Ltd., 1938.

26. Harris, L. J. and Leong, P.C. Vitamins in human nutrition: the excretion of vitamin B_1 in human urine and its dependence on the dietary intake. Lancet 1936 I:886-894. 1936.

27. Hennessy, D. J. Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal. Ed. 13:216-218, 1941.

28. Hennessy, D. J. and Cerecedo, L. R. The determination of free and phosphorylated thiamin by a modified thiochrome assay. Jour. Amer. Chem. Soc. 61: 179-183, 1939.

29. Hills, George M. The thiochrome test for aneurine (vitamin B_1) in urine as an index of nutritional level. Biochem. Jour. 33: 1966-1979, 1939.

30. Holt, L. Emmett, Jr., and Najjar, Victor A. A simple method for the laboratory diagnosis of subclinical deficiencies of thiamine, riboflavin, and nicotinic acid. Bull. Johns Hopkins Hospital 70:329-330, 1942.

31. Jansen, B. C. P. A chemical determination of aneurin (vitamin B_1) by the thiochrome reaction. Rec. Trav. Chim. 55:1046-1052, 1936.

32. Jolliffe, Norman, Goodhart, Robert, Gennis, Joseph, and Cline, J. K The experimental production of vitamin B_1 deficiency in normal subjects. The dependence of the urinary excretion of thiamine on the dietary intake of vitamin B_1. Amer. Jour. Med Sci. 198:198-211, 1939.

33. Jowett, Maurice. The estimation of vitamin B_1 in urine. Biochem. Jour. 34:1348-1353, 1940.

34. Kalckar, H. M. The nature of phosphoric esters formed in kidney extracts. Biochem. Jour. 33: 631-641, 1939.

35. Keys, Ancel, and Henschel, Austin F. Vitamin supplementation of U. S. Army rations in relation to fatigue and the ability to do muscular work. Jour. Nutrition 23:259-269, 1942.

36. Knott, Elizabeth M. A quantitative study of the utilization and retention of vitamin B by young children. Jour. Nutrition 12:597-611, 1936.

37. Lane, Robert L., Johnson, Elizabeth, and Williams, R. R. Studies of the average American diet 1. Thiamine content. Jour. Nutrition 23:613-624, 1942.

38. Lipton, M. A., and Elvehjem, Conrad A., Mechanism of the enzymatic phosphorylation of thiamin. Nature 145:226-227, 1940.

39. Lu, G. D. Studies on the metabolism of pyruvic acid in normal and vitamin B_1-deficient states. I. A rapid, specific, and sensitive method for the estimation of blood pyruvate. Biochem. Jour. 33:249-254, 1939.

40. Lu, G. D. Studies on the metabolism of pyruvic acid in normal and vitamin B_1-deficient states. II. Blood pyruvate levels in the rat, pigeon, rabbit, and man. III. The relation of blood pyruvate to cardiac changes. Biochem. Jour. 33:774-786, 1939.

41. Machella, Thomas E., and Elsom, K. O. Studies of the B vitamins in the human subject. II. Urinary excretion of ingested thiamin in patients with chronic hepatic disease. Amer. Jour. Med. Sci. 202:512-516, 1941.

42. Mason, H. L., and Williams, Ray D. The effect of ingestion of nicotinic acid on the determination of thiamine in urine by the thiochrome method. Jour. Biol. Chem. 140:417-422, 1941.

43. Mason, H. L., and Williams, R. D. The urinary excretion of thiamine as an index of the nutritional level: Assessment of the value of a test dose. Jour. Clin. Investigation 21:247-255, 1942.

44. Meiklejohn, Arnold P. The estimation of vitamin B_1 in blood by a modification of Schopfer's test. Biochem. Jour. 31:1441-1451, 1937.

45. Melnick, Daniel. Vitamin B_1 (thiamine) requirement of man. Jour. Nutrition 24:139-152, 1942.

46. Melnick, Daniel, and Field, Henry, Jr. Chemical determination of vitamin B_1. II. Method for estimation of the thiamine content of biological materials with the diazotized p-amino-acetophenone reagent. Jour. Biol. Chem. 127:515-540, 1939.

47. Melnick, Daniel, and Field, Henry, Jr. Chemical determination, stability, and form of thiamine in urine. Jour. Biol. Chem. 130:97-107, 1939.

48. Melnick, Daniel, and Field, Henry, Jr. Thiamine clearance as an index of nutritional status. Jour. Nutrition 24:131-138, 1942.

49. Melnick, Daniel, Field, Henry, Jr., and Robinson, William D. A quantitative chemical study of the urinary excretion of thiamine by normal individuals. Jour. Nutrition 18:593-610, 1939.

50. Merck and Co., Rahway, N. J. Determination of thiamin chloride by fluorometric method, 1941.

51. Merck Service Bulletin, Vitamin B_1 Merck, 1940. Merck and Company.

52. Moyer, James C., and Tressler, D. K. Determination of thiamine in vegetables. Ind. Eng. Chem., Anal. Ed. 14:788-790, 1942.

53. Munsell, Hazel E. Vitamin B_1. Methods of assay and food sources. Jour. Amer. Med. Assoc. 111: 927-934, 1938.

54. Ochoa, S. Nature of oxidative phosphorylation in brain tissue. Nature 146:267, 1940.

55. Peters, R. A. Cocarboxylase. Nature 146:387-391, 1940.

56. Pharmacopoeia of the United States of America, twelfth revision, Easton, Pa., Mack Printing Company, by authority of the U. S. Pharmaceutical Convention, 1940.

57. Pollack, Herbert, Dolger, Henry, Ellenberg, Max, and Cohen, Sanford. A test proposed to measure vitamin B_1 saturation in humans. Proc. Soc. Exp. Biol. Med. 44:98-100, 1940.

58. Prebluda, H. J., and McCollum, E. V. A chemical reagent for the detection and estimation of vitamin B_1. Science 84:488, 1936.

59. Pyke, Magnus. The distribution of vitamin B_1 in foods as determined by chemical analysis. Jour. Soc. Chem. Industry 58:338-340, 1939.

60. Robertson, Elizabeth Chant, and Doyle, M. Elizabeth, Difficulties in the use of the brachycardia method of assaying vitamin B_1. Proc. Soc. Exp. Biol. Med. 37:139-140, 1937.

61. Robinson, William D., Melnick, Daniel, and Field, Henry, Jr. Correlation between the concentration of bisulfite binding substances in the blood and the urinary thiamin excretion. Jour. Clin. Investigation 19:483-488, 1940.

62. Robinson, W. D., Melnick, Daniel, and Field, Henry, Jr. Urinary excretion of thiamin in clinical cases and the value of such analyses in the diagnosis of thiamin deficiency. Jour. Clin. Investigation 19:399-408, 1940.

63. Schlutz, Frederic W., and Knott, Elizabeth M. The use of a 10-day period for the assay of vitamin B by rat growth technic. Jour. Nutrition 12: 583-595, 1936.

64. Schultz, Alfred S., Atkin, Lawrence, and Frey, Charles N. A fermentation test for vitamin B. Jour. Amer. Chem. Soc. 59:2457-2460, 1937.

65. Schultz, Alfred S., Atkin, Lawrence, Frey, Charles N., and Williams, R. R. Application of the sulfite cleavage of thiamine to the yeast fermentation method. Jour. Amer. Chem. Soc. 63:632-633, 1941.

66. Sherman, Henry C, Chemistry of food and nutrition. New York, The Macmillan Co., 1941.

67. Sherman, Henry C., and Lanford, Caroline Sherman. Essentials of nutrition, second edition. New York, The Macmillan Co., 1943.

68. Sinclair, H. M. The estimation of vitamin B_1 in blood. Biochem. Jour. 32:2185-2199, 1938.

69. Sinclair, H. M. Metabolism of vitamin B_1. Jour. of Physiol. 96:55P-56P, 1939.

70. Smith, M. C., Burlinson, L. O., and Spector, H. The significance of thiamin excretion levels after a test dose. Jour. Home Econ. 33:601-602, 1941.

71. Smyth, D. A. The oxidation of pyruvate by heart muscle. Biochem. Jour. 34:1046-1056, 1940.

72. Wang, Y. L., and Harris, Leslie J. Methods for assessing the level of nutrition of the human subject. Estimation of vitamin B_1 in urine by the thiochrome test. Biochem. Jour. 33: 1356-1369, 1939.

73. Wang, Y. L., and Yudkin, John. Assessment of the level of nutrition. Urinary excretion of aneurin at varying levels of intake. Biochem. Jour. 34:343-352, 1940.

74. Williams, Ray D., Mason, H. L., Smith, Benjamin F., and Wilder, Russell M. Induced thiamine (vitamin B_1) deficiency and the thiamine requirement of man. Further observations. Arch. Internal. Med. 69:721-738, 1942.

75. Williams, R. D., Mason, H. L., and Wilder, Russell M. The minimum daily requirement of thiamine of man. Jour. Nutrition 25:71-97, 1943.

76. Williams, R. D., Mason, H. L., Wilder, R. M., and Smith, B. F. Observations on induced thiamine (vitamin B_1) deficiency in man. Arch. Internal Med. 66:785-799, 1940.

77. Williams, Robert R., and Spies, T. D. Vitamin B_1 and its use in medicine. New York, The Macmillan Company, 1938.

78. Williams, Roger J., McMahan, J. Raymond, and Eakin, Robert E. Assay method for thiamine. The Univ. of Texas Pub. No. 4137: 31-35, 1941.

79. Wright, Margaret D., and Baker, Audrey Z. Vitamin B_1 excretion on a varied intake. Jour. of Hygiene 39:638-642, 1939.