INTERRELATIONSHIPS OF THIAMINE AND PROTEIN

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

A. Elaine Simpson

1966

ABSTRACT

INTERRELATIONSHIPS OF THIAMINE
AND PROTEIN

by A. Elaine Simpson

Thiamine is involved in the metabolism of carbohydrate. Thus, the need for thiamine is determined, in part, by the carbohydrate content of the diet. The bulk of the experimental evidence has indicated that fat spares thiamine. However, the relationship between thiamine and protein has not been established. The beneficial effects which resulted when the protein content of the diet was increased at the expense of carbohydrate in a thiamine-deficient diet may be explained either by a decrease in the dietary carbohydrate or by a sparing action of protein on thiamine.

The purpose of this study was to investigate the influence of dietary protein levels upon thiamine concentration in rat tissue and the urinary excretion of this vitamin.

Rats were fed diets containing 0, 6 and 20% casein and 90.6, 84.6 and 70.6% sucrose respectively. In the first experiment rats were fed, on an ad libitum basis,

the above diets containing 4 mcg of thiamine/gram. The thiamine concentration, expressed as mcg/gm of tissue on a wet weight basis, in the liver and thigh muscle of these rats was the same despite wide variations in thiamine intake, food consumption and body and tissue weight. The rats fed the higher levels of casein excreted more thiamine than animals fed diets containing lower quantities of casein.

In the second experiment, rats were pair-fed diets containing 6, 12 or 24% casein with rats fed 6% protein as the control. The urinary excretion of thiamine was the same for all animals which consumed more casein, the urinary excretion of thiamine by rats fed the protein-free diet was only half that excreted by the animals fed the protein-containing ration.

A second group of rats were fed, on an ad libitum basis, diets containing 6, 6 or 24% casein. The thiamine content of these diets was adjusted so that all rats consumed similar amounts of this vitamin daily. Although the rats consumed like amounts of thiamine the urinary thiamine excretion varied directly with the level of dietary casein. The amount of ingested thiamine which could not be accounted for by urinary excretion was similar in all animals despite the wide variation in food consumption and body size. Possible explanations for this observation were discussed.

The results of these experiments were discussed and it was concluded that the level of casein in the diet did not influence the thiamine content of rat tissue when "normal" thiamine levels were used, and that the daily excretion of thiamine was not directly related to the dietary casein level but possibly to the caloric intake of the animal.

INTERRELATIONSHIPS OF VITAMIN

AND PROTEIN

by

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Foods and Nutrition
East Lansing, Michigan

April, 1965

ACKNOWLEDGMENT

The author wishes to thank Dr. C. Nicholson for his valuable assistance and criticism of this work, and also to thank her husband, Ilmo, for his patience and understanding.

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INTRODUCTION

This study stemmed from an interest in the possible harmful effects of large intakes of vitamins. This concern was based on the adverse effects resulting from the ingestion of excessive amounts of fat soluble vitamins. A recent illustration of this was the fortification of infant foods with vitamin D in Great Britain which resulted in numerous cases of idiopathic hypercalcemia. The extra vitamin D in the diet brought the total daily ingestion to a level which caused hypervitaminosis D in the first year of life (Anonymous editorial, 1964). Because the intake of only moderately high levels of this fat soluble vitamin produced signs of hypervitaminosis and because the availability of vitamin preparations has encouraged self-medication to an increasing extent, there is a possibility that adverse manifestations might accrue from an excessive intake of the water soluble B vitamins.

A former student (Brown, 1963) in this department studied the effects produced by feeding rats high intakes of thiamine. To evaluate the effects of the high vitamin intake in conjunction with "poor" diets, she used rations containing protein levels ranging from 0 to 20%. During

the course of this work she observed a relationship between the level of protein in the ration and the concentration of thiamine in the tissues and urine. The results of her work suggested that the current theories concerning the influence of protein on thiamine metabolism needed re-evaluation. Although this study was limited to a consideration of the relationship of thiamine to dietary protein, the influence of carbohydrate and fat are discussed briefly.

The purpose of this study was to determine whether the level of dietary protein has any specific effect on the concentration of thiamine in the tissues of weanling rats and secondly to investigate the influence of dietary protein on urinary excretion of this vitamin.

REVIEW OF THE LITERATURE

A. The Interrelationship of Thiamine with Other Food Components

1) Carbohydrate and Fat

Studies with experimental animals and humans suggest that as the dietary caloric and especially the carbohydrate levels in the diet increase there is an increased requirement for thiamine (Arnold et al, 1939; Holt, 1944). The phosphorylated form of thiamine, thiamine pyrophosphate (TPP), is required for the following enzyme systems: pyruvic decarboxylase, pyruvic dehydrogenase, alphaketoglutaric dehydrogenase, and transketolase (Kaper and Fellers, 1969).

The earliest suggestion that the need for thiamine was related to the carbohydrate content of the diet was in 1914 (Funk, 1914). Pigeons fed a high carbohydrate diet developed beriberi earlier than pigeons fed a diet containing a high percentage of either fat or protein.

Cowgill (1934) published a summary of studies on the thiamine requirement of man and animals as influenced by the caloric intake. He related the thiamine requirement to the total caloric intake, but as will be discussed, more recent studies have indicated that the thiamine requirement is more precisely related to the non-fat calories provided by the diet.

Early work (Evans and Lepkovsky, (1929), Salmon and Goodman, (1937)) concerning the interrelationship of thiamine and fat did not clearly indicate that fat spared thiamine, for as the fat content of the diet decreased the carbohydrate content increased. As thiamine is required for the metabolism of carbohydrate, the results could be explained by a decrease in the carbohydrate content of the diet when the fat level was raised. However later work (Stirn and co-workers, 1939) demonstrated that fat does spare thiamine.

In 1929, Evans and Lepkovsky (1929) demonstrated that the thiamine requirement decreases when the fat content of the diet is increased. Rats consuming thiamine-free diets containing 50% fat and no "commercial" sugar lived longer and grew faster than rats consuming thiamine-free diets containing a lower level of fat and 60% "commercial" sugar.

Salmon and Goodman (1937) fed weanling rats thiamine-free diets with varying levels of several different fats to determine if the addition of fat increased the weight gain or the time for the animals to develop beriberi. The diet containing 23% coconut oil and 54% sucrose did not delay the onset of beriberi. Rats fed this diet developed beriberi and died in the same number of days as rats fed the fat-free diet; however the initial weight gain was greater for those animals fed the former diet than for animals fed the fat-free diet. A diet containing 59.2%

of this oil and no carbohydrate not only increased the growth rate over that of the rats fed the fat-free or 2% fat ration, but also inhibited the symptoms of beriberi for a period of 4.1 weeks at which time the experiment was terminated. A comparison was made of the ability of the diets containing 40% coconut oil, cottonseed oil, pecan oil, lard or butter to prevent the onset of beriberi. These diets contained 2% sucrose. Coconut oil was the most effective natural fat tested in delaying the development of thiamine-deficiency symptoms. Cottonseed oil and pecan oil also prevented the onset of the thiamine deficiency syndrome. Lard and butter promoted growth of rats fed a thiamine-deficient diet but were less effective than other fats in delaying the appearance of deficiency symptoms. No explanation for the effects of the different fats was offered. From an examination of the fat content of the feces, these workers concluded that there was no impairment of fat digestion as a result of the vitamin deficiency.

Stirn and co-workers (1939) provided evidence to indicate that fat does spare the need for thiamine. Weanling rats were restricted to thiamine-deficient diets containing 60% sucrose until symptoms of polyneuritis appeared. The isocaloric substitution of coconut oil for all the carbohydrate in the thiamine-free diet relieved the polyneuritis and resulted in a growth rate of 20.1 gm. per week for a

period of six weeks for the male rats and 14.5 gm. per week for the female rats. When two-thirds of the carbohydrate was replaced with the oil, the results were similar to those obtained with complete substitution of the fat for carbohydrate. The replacement of one-third of the carbohydrate by the oil produced a prolonged recovery period and poor growth of only 5 gm. per week.

2) Protein

Protein may increase, have no effect upon or reduce the thiamine requirement. If protein does reduce the thiamine requirement, this reduction could be due to a decrease in the carbohydrate content of the diet when the protein level is increased or protein may completely spare the thiamine requirement as fat does. In all investigations which have been reported, the carbohydrate level of the diet was decreased when the protein content of the diet was increased. No report appears in the literature in which the investigators have increased the protein level of the diet at the expense of a non-nutritive substance in the diet.

No-one has replaced all the carbohydrate in a thiamine-deficient diet with protein and fed this diet to thiamine-deficient animals to observe if this diet alleviated the deficiency symptoms. An experiment such as this would indicate if protein spares thiamine. Stirn and co-workers

(1939) carried out a similar experiment replacing all the carbohydrate with fat, on an isocaloric basis, and concluded that fat spared thiamine. Any increase in protein at the expense of carbohydrate in the diet would be an isocaloric substitution as protein and carbohydrate provide the same number of calories per gram.

The following is a review of the work that has been concerned with the relationship of protein and thiamine.

a) Thiamine sparing effect

Workers have employed three criteria to determine if protein spares thiamine; growth response, the time required for deficiency symptoms to develop and kinices of thiamine metabolism.

Growth criterion

Scott and Griffith (1937) studied the effect of graded daily intakes of 0.2 - 5.0 mcg. of thiamine in the rat. At the higher levels of thiamine intake the rats grew at a faster rate. In fact there was a straight line relationship between the change in body weight and the thiamine intake of the rats.

Simultaneously Scott and Griffith (1937) attempted to compare the effect of various thiamine sparing agents in the rat. The sparing action is described as "the thiamine intake for equivalent growth". From the growth curves of rats fed graded levels of thiamine, they determined what

level of thiamine was necessary to cause a weight change of the same magnitude as rats fed one of the sparing agents under study. For rats fed the 5% protein diet they stated that the thiamine intake for equivalent growth was 2.3 mcg./rat/day. Because the rats fed the high protein diet received 0.5 mcg. of thiamine daily in their diet, the sparing action of protein was calculated to be 1.8 mcg. However in attempting to verify the calculations using the data presented in their article, we estimated from their graphs and records that 2.7 mcg. of thiamine would result in the same weight change as reported for rats fed the high protein diet. Hence the sparing action for this protein diet would be 2.7 - 0.5 = 2.2 mcg. Similarly our calculations did not agree with the values reported for the sparing action of fat, penicillin or ascorbic acid.

Scott and Griffith also reported that the sparing effect of thiamine sparing agents and thiamine were additive.

Waine (1942) investigated the relationship between the rat's requirement for thiamine and the protein content of the diet. He obtained a 37% decrease in the rat's requirement for thiamine when he used a 64% protein diet as compared to a 6% sucrose ration. Waine suggested that the requirement for thiamine may be reduced by the high protein diet since only certain amino acids are converted to glucose; the metabolism of the non-glycogenic amino

acids would presumably not require thiamine and thus reduce the requirement for this vitamin.

Sang (1952) working with drosophila reached the opposite conclusion to that reported by Naino (1943) for rats. Sang cultured germ-free drosophila with diets containing either 4 or 7% casein. Using growth as the criterion, he found that the requirements for thiamine and riboflavin, expressed as mcg./5 ml. of medium, were not affected by these protein levels, and that the requirements of all other B vitamins were significantly increased under the experimental conditions he used. The apparent contradiction between the observations of Naino and Sang may be because of the different species studied.

The results of these studies indicate that for the rat an increase in the protein content of the diet at the expense of carbohydrate does reduce the animal's need for thiamine as indicated by growth response. However the question of whether this effect is related to a decrease in the carbohydrate content of the diet or a sparing action of protein remains unanswered.

Delay in appearance of beriberi symptoms

Evans, Lepkovsky and Murphy (1934) extended the studies done by Evans and Lepkovsky (1929). They showed that when rats were fed a thiamine-deficient ration containing 20% protein, they developed beriberi in less time than rats fed similar rations containing 37% protein.

Aptekar (1939) reported that an increase of protein at the expense of carbohydrate in the diet inhibited clinical manifestations of a thiamine deficiency and increased survival time of pigeons. The pigeons in the control group were fed barley rations while those in the experimental groups consumed diets in which either 10 or 40% of the total calories came from casein; barley was the source of the remaining calories. The pigeons fed the diets containing casein did not develop outward symptoms of thiamine deficiency until two-three weeks after these signs appeared in the control group. The average survival time for the pigeons receiving 10 and 40% of their total calories as protein was 23 and 64 days respectively; the control group survived only 6-15 days. The pyruvic acid content of the blood increased continuously in all groups indicating that carbohydrate metabolism was equally impaired. Aptekar also showed that an increase in dietary fat at the expense of carbohydrate in the diet retarded the onset of deficiency symptoms and increased longevity only if 11% of the calories came from protein.

Thiamine deficiency symptoms did not appear as quickly when rats were fed a high level of protein compared to when rats were fed a lower level of protein. As before, there is the question of whether or not protein played a role over and above the effect produced by a decrease in the

carbohydrate in the diet, when the protein was increased.

Indices of thiamine metabolism

Banerji (1941) fed young male rats either a 20% or an 80% casein diet. Rats fed the basal thiamine-deficient diet lost weight after two weeks on the diet and developed bradycardia and died in about four weeks; those rats on the high protein, thiamine-deficient, diet gained weight and survived. In examining the thiamine content of the casein used, Banerji found that the casein contained 0.112 mcg. of thiamine per gram. The amount of extra thiamine which the rats fed the high protein diet would have received from this source could not have accounted for the results obtained. These rats fed the 20% protein, thiamine-deficient diet excreted a large amount of bisulphite-binding substances. Large amounts of protein in the diet prevented the development of bradycardia and the loss of weight but did not prevent the increased excretion of bisulphite-binding substances. In previous experiments he found that the addition of fat to a thiamine-deficient diet did prevent the increased excretion of these substances. Intraperitoneal injections of various levels of thiamine had the usual accelerating effect on growth. The level of bisulphite-binding substances in the urine varied inversely with the amount of the vitamin injected. For each level of thiamine injected the rate of growth was higher in those rats fed the 80% protein diet compared with those fed the 20% protein ration.

Banerji and Yudkin (1942) determined the "catatorulin effect"[1] of kidney slices from rats fed thiamine-deficient diets high in fat or protein in the previous report by this worker. Although outward symptoms of a thiamine deficiency did not appear in rats fed thiamine-deficient diets containing 80% protein, defective oxidation of pyruvate was shown in kidney slices obtained from these animals. This evidence supported the theory that protein reduces the thiamine requirement because animals on the high protein, thiamine-deficient diet did not exhibit outward deficiency symptoms although the metabolism of carbohydrate, as shown by the catatorulin test, was impaired.

No definite conclusion can be reached from the results of the work cited above. Protein appears to reduce the requirement for thiamine but this reduction may be related to a decrease in the carbohydrate content of the diet when the level of protein is increased. As Kaino (1942) has suggested, protein may reduce the thiamine requirement only to the extent of the non-glycogenic amino acids in the diet; some amino acids are converted to glucose and the metabolism of glucose from these amino acids would require thiamine. It may be possible to measure the "glycogen ability of protein" but under some circumstances certain amino acids are glycogenic and under other circumstances they are ketogenic

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1 The catatorulin test has been used to measure thiamine. The assay depends upon the uptake of oxygen by tissues immersed in a pyruvate solution (Michelsen and Yamamoto, 1933).

(Holster, 1940). Hence the investigator may not be certain that an amino acid is glycogenic under the experimental conditions he uses.

b) Thiamine in protein metabolism

Richter has conducted a number of studies on the "self-selection" of individual foodstuffs by rats. In his experiments rats were offered casein, sucrose and yeast in separate food dishes and olive oil, cod liver oil, solutions of minerals and tap water in separate drinking bottles. Rats offered such a variety of foodstuffs made selections which resulted in normal growth and reproduction (Richter, Holt and Barelaire, 1938). The growth and reproduction of these rats was compared with that of rats fed the standard McCollum diet.

Richter assumed that the rat's appetite for various foodstuffs was a reflection of the animal's ability to metabolize that food. Hence, if an animal increased or decreased its consumption of a certain item in response to a dietary change, that dietary change had brought about an increase or decrease in the rat's ability to metabolize that substance. "Just as glandular deficient animals exhibit an increased appetite for those substances which are necessary to life, and just as normal animals show appetites for substances in proportion to their needs, so vitamin deficient animals seem to reflect their needs in their appetites" (Richter, Holt, Barelaire and Hawkes,

1933). This was the assumption on which Richter based all his conclusions.

Richter, Holt, Barelaire and Hawkes (1933) fed nine animals, each weighing approximately 140 gm., the above foodstuffs with the omission of yeast. As the deficiency of B vitamins progressed the animals lost weight and became inactive. The total calorie intake dropped 43% during the deficiency period. Before the deficiency was established the rats consumed 23% (1.1 gm.), 59% (3.3 gm.) and 26% (1.3 gm.) of their total daily calories as fat, carbohydrate and protein respectively. During the deficiency period the rats consumed 44% (2.7 gm.) of their daily calories as fat, 11% (0.4 gm.) as carbohydrate and 5% (0.2 gm.) as protein. When the deficient animals were given access to yeast, the animals consumed it with the result that there was an increase in body weight, total food consumption and an alteration in the fat, carbohydrate and protein consumption to that of the pre-deficiency period. The authors concluded that "these appetite changes (during the deficiency period) are as characteristic signs of vitamin B deficiency as the anorexia, weight loss, activity increase (and) bradycardia".

Because all the water soluble B vitamins were absent from the deficient diet one cannot conclude that these results were solely the result of a thiamine deficiency.

In a subsequent experiment (Richter and Landes, 1941) rats were given access to food in the same manner as previously indicated. These rats were 65 days old at the beginning of the experiment, which lasted 40 days. A negative control group was not offered yeast while a positive control group was allowed yeast in addition to the other foodstuffs. Other experimental animals were given access to the same foodstuffs as the first group with the addition of another solution of thiamine hydrochloride, riboflavin, pyridoxine, or pantothenic acid either singly or in combination. The fat, carbohydrate and protein appetites were determined from the average intake of olive oil, sucrose and vitamin-free casein respectively. The average calories consumed from fat, carbohydrate and protein are presented:

Average caloric intake for last 20 days on diet
(Richter and Landes, 1941)

Vitamin(s) Offered	Calorie %		
	Carbohydrate	Fat	Protein
pyridoxine	34.1	59.1	6.8
no yeast	24.9	67.6	7.5
riboflavin, pyridoxine	41.1	53.0	5.2
riboflavin	14.3	82.4	3.3
nicotinic acid	30.6	66.1	3.3
thiamine	65.3	21.6	13.1
thiamine, riboflavin + pyridoxine	51.2	33.0	14.3
thiamine, riboflavin	35.4	49.6	15.0
thiamine, pyridoxine	61.2	35.6	3.2
all four vitamins	62.1	15.2	22.0
yeast	55.3	19.4	25.3

When thiamine was present the percent of calories consumed from fat decreased, and the percent of calories from carbohydrate increased over that of comparable groups not receiving thiamine. The inclusion of thiamine increased the percent of calories consumed from protein except when thiamine was provided in combination with pyridoxine. When all four vitamins were given to the rat, the percent of calories from fat was similar to that when yeast was the source of the B vitamins; the percent of calories from protein was not as great as when yeast was provided. As the investigators suggested, pantothenic acid and other B vitamins, which were not studied, could exert an effect upon the rat's appetite for carbohydrate and protein. By assuming that the rat's appetite for protein indicated its ability to metabolize protein, these workers concluded that thiamine plays an important role in protein utilization. A similar conclusion may be reached for carbohydrate.

Richter and Rice (1944) in an extension of studies on "self-selection" of food studied the effect of thiamine on survival of rats offered either vitamin-free casein or dextrose. When a solution of thiamine HCl, in addition to one of the nutrients, was placed in the cage, the rats lived longer than those offered only the individual nutrient. The control rats fed only the vitamin-free casein survived for 33 days; thiamine supplementation increased survival by 67%; for dextrose the corresponding values were 37 days and

97%. Since the inclusion of thiamine prolonged the life of rats fed casein as well as those fed dextrose, the investigators concluded that this vitamin plays an important role in the utilization of protein as well as carbohydrate. Thiamine was more effective in prolonging life when dextrose was the sole source of food.

If one accepts the assumption that, in lower animals, there is a physiological relationship between the ability to use protein and the appetite for protein, the results of these studies indicate that thiamine is required for protein metabolism. This conclusion is contradictory to the work reported in the previous section. Perhaps thiamine is required to metabolize only a part of the protein consumed, for example the glycogenic amino acids. Then the non-glycogenic amino acids would spare thiamine while the glycogenic amino acids increase the animal's need for this vitamin.

c) Thiamine and N metabolism

Several studies have been reported on the influence of thiamine upon nitrogen metabolism.

Sure and Ford (1942) restricted animals fed an adequate basal ration to the same food intake as eaten by rats fed a thiamine-deficient diet. By using the technique of paired-feeding, they attempted to eliminate the alterations associated with the increased appetite of thiamine supplemented animals. They observed that in mild deficiencies

of thiamine associated with small losses of body weight there were large increases in total urinary N.P.N and urea N over the levels of these substances excreted by the control rats.

These workers failed to indicate if this increase in urinary N was more than could be accounted for by the weight loss. In their report they did not provide the data necessary to answer this question.

Rats fed 10% casein diets with thiamine supplements varying from 0 to 30 mcg. daily were used to study the effects of this vitamin upon the utilization of casein. (Mayfield and Hedrick, 1949). The rats were fed a thiamine-free diet until depleted of this vitamin as indicated by a constant weight for three days. Then the thiamine supplements were given. After a five day stabilization period, urine and feces were collected for the following seven days. The excreta were analyzed for nitrogen. The digestability[1] of casein was 97-100% for all groups although the daily intake of thiamine ranged from 0 to 30 mcg. The biological value[2] of the protein was significantly affected by the amount of thiamine in the supplement only at levels of less than 5 mcg. of thiamine daily. When

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1. % digestability = $\frac{\text{food N} - (\text{fecal N} - \text{metabolic N})}{\text{food N}}$

2. Biological value =

$$\frac{\text{food N} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N})}{\text{food N} - (\text{fecal N} - \text{metabolic N})} \times 100$$

1.25 mcg. of thiamine were administered the biological value of the casein was 59%. This value rose to 7[illegible] at the 2.50 mcg. level of supplementation. At thiamine levels of 5 to 50 mcg. there was no significant change in the biological value of the protein. At these higher intakes of thiamine the biological value of casein was 91-95%.

B. The Excretion of Thiamine

1) The effect of the thiamine intake

Salcedo and co-workers (1943) studied the urinary excretion of thiamine and tissue thiamine concentration of rats fed a thiamine-deficient diet for 24 days. Pooled urine samples were analysed daily for thiamine. At three day intervals four rats were sacrificed; the tissues of these rats were analysed for thiamine. The workers plotted the thiamine content of the tissues in mcg. of thiamine/gm. of dry tissue and the thiamine excretion against the number of days the animals were fed the thiamine-deficient diets. The urinary excretion of thiamine fell sharply to a low constant level; however the concentration of thiamine in the tissues fell steadily in a curve which was of a logarithmic nature.

Lowry (1942) using the data of Salcedo and co-workers (1943) calculated the percent of the initial level of thiamine present in the tissues and urine during the course of

the development of thiamine deficiency. His calculations took into account the weight loss which became increasingly rapid as the deficiency progressed. He concluded that in the initial stages of a thiamine deficiency, the thiamine required for metabolism came from the tissue stores and resulted in a fall in tissue thiamine concentration. As the deficiency progressed, the animals lost weight. As the weight loss occurred the tissue mass became smaller and the thiamine concentration per gram of tissue did not alter appreciably. The thiamine required by the body came from the breakdown of body tissue.

Light and co-workers (1944) studied the excretion of thiamine in the urine and feces of rats fed 15 to 515 mcg. of thiamine daily. The excretion of the vitamin in both the urine and the feces was determined when the animal was in equilibrium. As soon as equilibrium was established at any one level of thiamine intake, the supplement was increased. Their data showed that increasing intakes of thiamine from 40 to 515 mcg. daily caused a proportionate increase in urinary and fecal thiamine. The relationship between thiamine intake and excretion was essentially a straight line relationship. About half the ingested vitamin appeared in the excreta of rats and was about equally divided between the urine and feces. The fermentation method which these workers used to determine thiamine

concentrations also determines the pyrimidine excretion. Pyramin is a metabolic breakdown product of thiamine which appears in the urine. Thus the values that these authors report for the thiamine content in the urine are slightly higher than those values reported by other workers (Caster, 1943; Brown, 1943) who employed methods which determine only the thiamine content of the sample.

In the years 1942 to 1947 Nickelsen, Caster and Keys (1947) investigated the urinary thiamine excretion of 22 young men. This work involved prolonged maintenance of the subjects on thiamine intakes from 0 to 16 mg. daily.[1] Thiamine intakes from 0.7 mg. daily and up produced substantially a straight-line relationship between urinary excretion and intake. Below a thiamine intake of about 0.7 mg. daily thiamine excretion in the urine approached zero. These workers found that the urinary excretion of thiamine was highly characteristic of the individual. It was found that with increasing thiamine intake the percentage of thiamine excreted in the urine increased but reached a plateau at about 13% of the intake when intakes were 1.5 mg. daily and above. At higher intakes of thiamine there was no increase in the percentage of the ingested thiamine which could be accounted for in the urine.

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1. The National Research Council (1964) recommends an intake of approximately 1.2 mg. daily for the "average" man. It will be helpful to bear this value in mind when reading the remainder of this section dealing with thiamine excretion.

Friedeman and co-workers (1950) similarly showed that the daily excretion of riboflavin and thiamine increased linearly with increasing intakes of these vitamins. They increased the vitamin intake of seven healthy young men at 5-9 day intervals over a total experimental period of 5-7 months. When the daily intake of thiamine exceeded 1.45 mg., the slope of the curve relating urinary to dietary thiamine became steeper indicating that above this level of thiamine a greater percentage of the intake was excreted. When the men consumed 1.45 mg. of thiamine daily, 15% of this amount appeared in the urine. This value agreed with the work of Nickelsen and co-workers (1947).

The most recent work on thiamine excretion as related to thiamine intake is reported by Sydenstricker and co-workers (1963). They restricted the thiamine intakes of eight men to 0.11-0.13 mg. of thiamine daily. Clinical signs of thiamine deficiency appeared as early as the ninth day. By the sixth day of the restriction, there was less than 50 mcg. of thiamine in the urine per day and the level was reduced to undetectable amounts by the eighteenth day. After 30 days of deficient diet, the diet was supplemented to bring the daily total ingestion to 0.54-0.61 mg. of thiamine. This level of thiamine during the repletion period did not produce measurable amounts of thiamine in the urine until the ninth to twelfth day.

2. The effect of the fat, carbohydrate and protein in the diet

Cahill (1941) fed 10 young men diets consisting of bread plus 1000 Calories of sucrose for a three day period. In the following three day period, the sucrose was replaced isocalorically with butter. On the third day of each period urine collections were made and analyzed for thiamine. The ratio of fat calories to carbohydrate calories in the first or high carbohydrate period averaged 1:19.23 and in the second or high-fat period averaged 1:1.04. These calculations included the calories received from the fat and carbohydrate in the bread. The experiment was conducted twice. In the first trial, all subjects were given a yeast supplement such that the daily thiamine intake averaged 13.6 mg.; in the second trial the supplement was not given, the subjects received an average of 1.6 mg. of thiamine daily. In both trials an increase in fat at the expense of carbohydrate in the diet did not significantly alter the urinary thiamine excretion. However the three day periods used in this study may not be of sufficient duration to allow the body to come into equilibrium with the dietary changes.

Reinhold and co-workers (1944) also examined the relationship between the level of urinary thiamine and the proportions of fat and carbohydrate in the diet. They measured the urinary thiamine excretion of six young women receiving in varying sequence a basal diet, a diet high in

fat and a diet high in carbohydrate. Each diet was fed for two weeks. The percent of total calories provided by fat averaged 34.7, 50.3 and 6.5% for the basal diet, the high fat diet and the high carbohydrate diet respectively. Alterations in the fat content of the diet were at the expense of carbohydrate; the total calories, the level of protein in the diet and the thiamine intake of the six subjects remained constant. The urinary excretion of thiamine decreased when the diet was changed from the basal to high carbohydrate or from the high fat to high carbohydrate diet. Since the thiamine excretion was the same when the basal and high fat diets were fed, these authors concluded that there was no sparing action of fat on thiamine and that the results of previous workers (Stirn et al, 1939; Peters and Yudkin, 1940; Salmon and Cockburn, 1937, Farrell and Fivoltjes, 1939) could be explained by decreases in the carbohydrate content of the diet.

The evidence presented in the literature especially the work of Stirn and co-workers (1939) indicates that fat spares thiamine. The diets used by Leichsenring contained an adequate amount of thiamine. Perhaps the change in fat calories (15.6%) was not large enough to effect a change in urinary thiamine excretion during the period of the study. Stirn and co-workers (1939) showed that the isocaloric substitution of fat for all the carbohydrate in

the thiamine-deficient diet alleviated thiamine-deficiency symptoms. Other workers (Banerji and Vaidkin, 1942; Aptekar, 1959) have shown that symptoms of a thiamine deficiency did not appear in rats fed a thiamine deficient diet which was high in fat although the metabolism of carbohydrate was impaired. Hence fat does spare the need for thiamine.

C. Dietary Protein and Tissue Thiamine Levels

Scott and Griffith (1957) analyzed the livers of rats for thiamine. At daily thiamine intakes of 2.2-6.3 mcg. the concentration of thiamine in the liver was similar; the range was 0.82-0.99 mcg. of thiamine/gm. of tissue. When only 0.2 mcg. of thiamine daily was fed to the rats, the liver concentration of thiamine was 0.46 mcg./gm. A comparison of the thiamine concentration of the livers of rats fed an 8% casein diet with livers from rats fed the basal 22% casein diet revealed that increasing the protein content of the diet did not increase the thiamine concentration in the liver. The animals fed the higher protein level grew more than those animals fed the lower level of protein; hence the livers of the former were larger than those in the latter group. Protein did increase the total thiamine content of the liver; however this increase was a function of the increased liver size and not due to an increased concentration of thiamine in the liver tissue.

Carpenter and co-workers (1953) determined the thiamine, riboflavin and niacin levels in fresh ham samples from pigs fed rations containing 14.3, 17.6 or 20.9% protein. As Carpenter wished to observe if antibiotics had any effect upon the tissue concentration of these vitamins at these protein levels, one-half of the pigs, fed each level of protein, received aureomycin; the remaining pigs were not fed the antibiotics. The thiamine values ranged from 11.61-12.73 mcg./gm. of tissue for pigs fed the antibiotic supplemented ration and 11.59-13.61 mcg./gm. for pigs receiving no antibiotics. Although these workers did not indicate the significance of these values, it would appear that the level of dietary protein did not affect the concentration of thiamine in ham samples whether or not antibiotics were provided.

Brown (1963) fed rats diets containing protein at the 20, 6 or 0% level. The following table presents the values which she reported for the thiamine concentration in the liver, muscle and urine of these rats.

Thiamine concentration in liver, muscle and urine of rats fed different protein levels (Brown, 1963)

% Protein in Diet	Thiamine		
	mcg./gm. tissue		mcg./day
	Liver	Muscle	Urine
20	4.17	1.02	14.9
6	3.42	0.72	7.1
0	20.30	1.32	2.4

Although she also studied the effects of various levels of thiamine in each of the diets, the values reported here are for those rats receiving 4 mcg. of thiamine/gm. of ration. It was with these groups of rats that differences in the thiamine content of these tissues were significant.

The present study was initiated in order to check these results using the same experimental procedure that she reported.

EXPERIMENTAL PROCEDURE

Series 1: A lifetime feeding of diets containing the same [illegible] but different protein levels

This series of experiments was carried out to duplicate the experimental design used by Brown (1960). The composition of the various diets, the duration of the experiment, the storage of the samples and the analytical techniques were the same as she used. A duplicate experiment was conducted six months after the first experiment.

Weanling rats were fed diets containing three levels of protein and an adequate level of thiamine. After 24 days of feeding, urine collections were made for three successive days. The animals were then sacrificed and the required tissues removed. The thiamine content of the urine and tissues was measured.

In this series of experiments 30 male weanling rats of the Sprague Dawley strain[1] were divided into three groups of 10 so that the total weight of each group did not differ by more than one gram. The three groups were designated[2] 1-C, 1-L and 1-H. Table 2 presents the composition of the diets used. These rats received rations containing

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1. Obtained from Spartan Laboratories, Haslett, Michigan.

2. The first number in the code refers to the series and the second number refers to the percent protein in the ration.

either 0, 6 or 20% vitamin-free casein. The remainder of the ration contained all the other nutrients required by rats. Sucrose made up for the differences in casein level. Each ration contained 4 mcg. of thiamine/gram diet. In Table 2 the composition of the vitamin mixture is presented.

Table 1: Composition of diets--Series 1

Group Code	1-0	1-6	1-20
Sucrose g	90.60	84.60	70.60
Vitamin-free casein g	0.00	6.00	20.00
Corn Oil* g	5.00	5.00	5.00
Hegsen Salts g	4.00	4.00	4.00
Vitamin Mix g	0.25	0.25	0.25
Choline Chloride g	0.15	0.15	0.15

* Corn Oil: 10 ml. of enriched corn oil which contained 75 mg. of tocopherol, brought to 50 gm. with unenriched corn oil.

Table 2: Concentration of vitamins in rations used in series 1.

Ingredient	Mg./Kg. Ration
Vitamin A	1.0
Calciferol	1.0
Riboflavin	8.0
Niacin	5.0
Pyridoxine HCl	2.5
Calcium Pantothenate	20.5
Inositol	10.0
Folic Acid	0.2
B_{12}*	0.02
Biotin	0.1
p- amino benzoic acid	2.0
Menadione	4.0
Thiamine HCl	4.0

The above vitamins were mixed with sucrose to make the total weight 2.5 gm. To insure accurate weight of such small quantities of vitamins 100 times each quantity was weighed.

*20 mg. of mannitol containing 0.1% Vitamin B_{12}.

The animals were housed individually in wire mesh cages equipped with individual water bottles and food containers. During the final three days of the experimental period the animals were placed in metabolism cages. Urine for this three-day period was collected under toluene in flasks which contained HCl to provide a stable acidic medium for the thiamine excreted. Food intake and body weight records were kept throughout the study.

At the end of the experimental period the animals were sacrificed by means of a sharp blow on the head. The liver and thigh muscle were removed and weighed. The tissues were frozen and stored for a period of up to three weeks in which time the analyses were completed.

The tissues were prepared in the Potter-Elvehjem homogenizer prior to analysis by the thiochrome technique (Association of Vitamin Chemists, 1951). The thiochrome technique determines the amount of intact thiamine present and does not detect the thiamine breakdown products, such as thialdo and pyramin which appear in the urine.

The urines were brought to a constant volume of 100 mls.; each flask was checked to determine that the final volume was acidic and then stored under refrigeration until analyzed by the thiochrome technique.

The thiamine content of the liver and thigh muscle was calculated as mcg. of thiamine/gram tissue on a wet

weight basis. Urinary thiamine was expressed as mcg. of thiamine excreted daily.

All results were statistically analyzed using the t test. A short method for calculating the standard errors of the means was employed (Mantel, 1951). This method is satisfactory for sample sizes of less than 15.

Series 2

A second series of experiments was conducted in order to rule out two of the variables—the different levels of caloric intake and the different levels of vitamin intake—in the previous experiment. The technique of paired-feeding was employed to eliminate the variations in caloric intake among the groups fed rations containing the different levels of protein. To rule out the second variable, the thiamine levels in the rations were adjusted so that the daily thiamine intake of the groups would be equal, if the rats in each group ate the same amount of feed as those in the corresponding group in Series 1.

a) Paired-feeding of diets with the same thiamine concentration but different protein levels

The duplicate trial of these groups of rats was conducted three months after the original trial.

Twenty rats were obtained from the same source as those used in series 1. These rats were divided into four groups which were fed the following rations:

Method of feeding for rats fed different levels of protein but restricted in food intake to the amount of food consumed by rats fed a protein-free ration

Group Code	% Protein in Diet	Type of Feeding
2-0cpf	0	ad libitum
2-6pf	6	pair-fed with 2-0cpf
2-12pf	12	pair-fed with 2-0cpf
2-20pf	20	pair-fed with 2-0cpf

The code used here is similar to that used in the previous experiment; pf signifies that the rats were pair-fed; cpf stands for control for pair-fed rats. With the exception of group 2-12pf, the diets used were the same as those fed to comparable groups in series 1. Group 2-12pf was fed a diet containing 12% casein and 73.6% sucrose, the remainder of the diet was the same as that of the other rations. All these rations contained 4 mcg. of thiamine/gm. of diet.

These rats received the same care as those in the previous experiment. After 29 days, three day urine collections were obtained and then the rats were sacrificed. No tissues were retained for analysis. The urine samples were treated and analysed as in the previous experiment.

b) Ad libitum feeding of diets with different protein levels and thiamine levels adjusted to provide each animal with approximately the same thiamine intake.

An attempt was made to adjust the thiamine content of the rations containing different levels of protein so that

the intake of this vitamin would be the same for all groups despite the great disparities of food intake. Since the tissue stores of thiamine are not maintained over long periods of time ([illegible]) the urinary excretion depends largely on the intake just prior to and during the collection period. For this reason it appeared desirable to adjust the thiamine intake so that it would be the same for all groups at the time of the urine collection. In general the food intake for some days prior to the urine collection (23–25 days of the experiment) was very similar to that during the collection period. For this purpose, the food consumption data for the last three days of series 1 were used in adjusting the thiamine content of the rations to be used.

All diets in series 2 contained 4 mcg. of thiamine/gram of ration. For the rats consuming the 6 and 6% protein rations, the levels of thiamine were increased to 18.46 and 8.96 mcg./gram of ration respectively.[1] Thus, if the rats ate the same amount of food as those in the

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1. In series 1 (Table 6) the rats receiving the 20% protein ration consumed 63.6/28.4 = 2.24 times as much thiamine as those receiving the 6% casein ration; therefore, the 4 mcg/gm. in the latter ration was increased by a factor of 2.24—2.24 x 4 = 8.96 mcg. of thiamine/gm. ration. Similarly, the thiamine content of the 6% protein ration was increased by a factor of 63.3/13.73 = 4.615—4.615 x 4 = 18.46 mcg. of thiamine/gm. ration.

corresponding group in series 1, they would obtain the same thiamine intake despite differences in food consumption.[2]

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2. If the rats in series 1 had received diets containing these adjusted thiamine levels, the thiamine intake by these groups would have been equal during the time when the urine collections were made. Also, it was calculated that if these levels of thiamine had been incorporated in the diets of series 1, the thiamine intake of the three groups of rats, at the midpoint of the experiment, would not have been significantly different.

Table 3: Vitamin content of rations used in series 2b

Ingredient	A	B	C
	(mg./kg. ration)		
Vitamin A	1.0	1.0	1.0
Riboflavin	3.0	3.0	3.0
Calciferol	1.0	1.0	1.0
Niacin	5.0	5.0	5.0
Pyridoxine HCl	2.5	2.5	2.5
Calcium pantothenate	20.0	20.0	20.0
Inositol	10.0	10.0	10.0
Folic Acid	0.2	0.2	0.2
B12*	0.02	0.02	0.02
Biotin	0.1	0.1	0.1
Para amino benzoic acid	2.0	2.0	2.0
Menadione	4.0	4.0	4.0
Thiamine HCl	4.0	8.96	16.96

The above vitamins were mixed with enough sucrose to make the total 2.5 gm. To insure accurate weight of such small quantities of vitamins 100 times each quantity was weighed.

*20 mg. of menhicol containing 0.1% Vitamin B12

Table 4: Composition of diets in which the concentration of thiamine was adjusted in an attempt to provide an uniform intake of this vitamin when rations containing different levels of casein were fed on an ad libitum basis

Group Code	2-20Cal	2-6Cal	2-0Cal
Sucrose g		74.1	90.00
Vitamin-free casein g	same	6.00	0.00
Corn Oil g	as	1.00	5.00
Salts g	diet	4.00	4.00
Vitamin Mix A* (1 mg. thiamine/ gm. diet)	1-2	-	-
Vitamin Mix B* (4 mg. thiamine/ gm. diet)		0.25	-
Vitamin Mix C* (16 mg. thiamine/ gm. diet)		-	0.25
Choline chloride g		0.15	0.15

1% ml of corn oil, in which 75 mg. of alpha tocopherol had been dissolved, brought to 50 gm. with corn oil.

*See Table 3 for composition of vitamin mixes.

Table 3 indicates the vitamin content of the rations incorporating these levels of thiamine. The composition of the diets as outlined in Table 4 was the same as the diets of comparable protein content in series 1 except that the vitamin mix was altered for the groups receiving 0 and 6% protein.

These diets were fed to three groups each composed of five weanling rats. The group code is similar to that used previously; al refers to al libitum feeding. The rats were assembled, housed and otherwise treated as in the previous experiment. After 21 days, three-day urine collections were obtained and analyzed for thiamine. The data obtained for the urinary excretion of thiamine were treated as previously indicated.

RESULTS

The results of the original and duplicate runs were similar and the data reported are a composite of both trials.

Series 1: Ad libitum feeding of diets containing the same thiamine content but different protein levels.

As would be expected the animals fed the diet devoid of protein lost weight; those fed the 6% level of protein gained slightly; those fed the 20% level of protein tripled their initial weight (Table 5).

Table 6 presents the daily food consumption and daily thiamine intake by these rats for the last three days of the experiment. Since these animals were fed on an ad libitum basis, the daily food intake was greatest with the animals receiving the 20% protein diet and least for those fed the protein-free diet. Thiamine intake ranged from 13.3 to 63.6 mcg. per day per rat during the last three days of the experiment.

The thiamine concentration in the liver was significantly lower for the rats receiving 6% protein when compared with rats receiving the 20% protein ration (Table 7). Although this difference appears to be large it is significant

only at the 5% level. The thiamine concentration in the livers of the rats fed the 0% protein diet was not significantly different from the thiamine concentration in the livers of the rats fed either the 6 or 20% protein diet. This difference in the thiamine concentration in the livers of the rats fed the 6% and 20% casein diets is probably an arithmetic difference due to the low standard errors and not of biological significance. For this reason one can conclude that the thiamine concentration in liver tissue was not affected by the level of protein in the diet. It is of interest to note that the level of thiamine in the thigh muscle of the group fed the 6% protein diet was also lower, but not significantly lower, than the values for the rats fed either the 0 or 20% protein ration. The differences in the thiamine concentration in thigh muscle for the three groups of animals are not significant.

The values for the daily urinary excretion of thiamine during the last three days of the experiment are shown in Table 9 for rats fed 0, 6 and 20% protein diets containing 4 mcg. of thiamine/gm. of diet. The actual thiamine excretion was related to one or more of these factors: food consumption, thiamine consumption, dietary protein level and dietary carbohydrate level. The animals receiving higher levels of protein ate more food, received more thiamine and excreted more thiamine than did animals which were

Table 5: Average initial and final weights of rats fed, on an ad libitum basis, rations containing varying levels of dietary protein and the thiamine level at 4 mcg./gm. of diet.

Group No.	% Protein in Diet	Average Initial Wt. (6)	Average Final Wt. (4 Wks) (6)
1-0	0	60.4	40.4 ± 1.2
1-6	6	60.4	73.4 ± 0.9
1-20	20	60.4	159.6 ± 4.9

Table 6: Food and thiamine intake of rats fed, on an ad libitum basis, rations containing different levels of protein. Values are for the last three days of the experiment. Thiamine concentration in each ration was 4 mcg./gm. of diet.

Group Code	% Casein in Diet	Ave. Food Consumption gm./day/rat	Ave. Thiamine Intake mcg./day/rat
1-0	0	3.45 ± 0.1	13.80 ± 0.4
1-6	6	7.10 ± 0.3	28.40 ± 1.2
1-20	20	15.90 ± 0.6	63.60 ± 2.4

Table 7: Thiamine concentration of livers and thigh muscles of rats fed, on an ad libitum basis, rations containing different concentrations of protein. All rations contained 4 mcg. of thiamine/gm.

Group Code	% Protein in Diet	Thiamine mcg./gm. of tissue, wet weight	
		Liver	Muscle
1-0	0	7.25 ± 0.79	1.72 ± 0.09
1-6	6	6.12 ± 0.23	1.54 ± 0.13
1-20	20	8.24 ± 0.09	1.67 ± 0.11

Table 9: Daily urinary excretion of thiamine for rats in series 1--urine collected for last three days of experiment. Thiamine content of rations was 4 mcg./gm. of diet. For daily thiamine intake during this period see Table 6.

Group Code	% Protein in Diet	Thiamine Excreted mcg./day/rat	% of Ingested Thiamine Excreted
1-0	0	0.3 ± 0.03	2.8
1-6	6	2.2 ± 0.2	7.3
1-20	20	20.4 ± 2.0	22.1

maintained on diets containing a lower level of protein. The rats fed the higher levels of protein also excreted a greater percentage of the thiamine intake than did rats fed diets containing lower levels of protein.

Series 3

a) Pair-feeding of diets with the same thiamine concentration but different protein levels

Table 9 presents the average initial and final weights of the animals which were pair-fed with group 2-Ocpf serving as the control. Two sets of weights are shown for the rats in groups 2-6pf and 2-20pf. When the repeat trial was begun only rats of less than 50 grams were available, therefore data pertaining to both runs are presented. The group 2-Ocpf was not repeated as this group was essentially the same as group 1-O and the data obtained from both these groups are not significantly different. In the repeat trial, the groups were pair-fed using food consumption records of 2-Ocpf in the first trial as the control.

Two groups of rats were fed a 12% protein ration during the second run. The 12% protein level was included since the protein levels used in the first series and in the first trial of series 2a were extremes with a large gap between the 6 and 20% levels. Since pair-feeding markedly restricts the feed intake of the animals fed the higher levels of protein, it appeared desirable to investigate the

influence of a ration containing an intermediate protein level.

The rats on the protein-free diet lost approximately one-third of their initial weight. Although the animals in the other groups were restricted to the same amount of food, these rats either maintained their weight or gained slightly. The rats in the second run which were smaller at the beginning of the experimental period gained more weight than the larger animals in the first trial; however the final weights of the animals in both trials were approximately the same (Table 9).

The urinary excretion of thiamine for these rats is shown in Table 10. The rats fed the protein-free diet excreted only half as much thiamine as those animals fed diets containing protein. The animals receiving protein in the ration excreted 17.0-17.7% of the thiamine ingested regardless of what level of protein was provided. During the time in which the urine was collected each rat consumed 14.67 mcg. of thiamine daily in the diet.

b) At libitum feeding of diets with different protein levels and thiamine intake to provide each animal with approximately the same thiamine intake.

The average initial and final weights for the animals fed different levels of protein, ad libitum, but whose daily intake of thiamine was similar are presented in Table 11. The rats fed the protein-free diet lost approximately

Table 9: Average initial and final weight for rats fed rations containing 0, 12 and 20% protein diets pair-fed with rats fed, on an ad libitum basis, 6% protein diets as the control.

Group Code	% Protein in Diet	Ave. Initial Wt.* g		Ave. Final Wt.* (4 wks) g	
2-0pf	0	60.4		50.0 ± 0.3	
2-6pf	6	60.4	49.2	59.6 ± 1.6	55.3 ± 1.0
2-12pf	12		49.2		64.7 ± 1.1
2-20pf	20	60.4	49.2	64.3 ± 1.4	66.2 ± 1.2

*the reason for the two sets of data is explained in the results.

Table 10: Urinary thiamine excretion of rats fed different levels of protein but restricted in food intake to the amount of food consumed by rats fed a protein-free ration. Rations contained 4 mcg. of thiamine/gm.

Group Code	% Protein in Diet	Urinary Thiamine mcg./day/rat	% of Ingested Thiamine Excreted*
2-0pf	0	1.2 ± 0.1	8.2
2-6pf	6	2.5 ± 0.2	17.7
2-12pf	12	2.6 ± 0.2	17.7
2-20pf	20	2.5 ± 0.1	17.0

*these animals consumed 14.67 mcg. of thiamine daily during the time the urine was collected.

one-third of their initial body weight while the rats fed the 6% protein diet gained slightly and those animals receiving the 20% level of dietary protein more than tripled their initial weights.

The thiamine intakes of the three groups differed but these differences are not significant (Table 12). The initial weights of the animals in this trial and series 1 were the same; however this trial was conducted six months later. Due to biological variability, the food consumption of rats in both experiments was not exactly the same. Although other methods to assure equal thiamine intake--for example limiting the food consumption or giving separate daily thiamine supplements--could have been employed, these procedures would tend to introduce other complications.

The values for the urinary excretion of thiamine (Table 13) indicate significant difference in urinary excretion of this vitamin. The rats fed the 6% protein diet excreted less thiamine than rats fed the 20% protein diet (group 2-6a1 vs group 2-20a1); this difference is significant at the 5% level. The group of rats fed the 0% protein ration excreted less thiamine than rats fed either 6 or 20% protein rations (group 2-0a1 vs groups 2-6a1 and 2-20a1) and these differences are significant at the 1% level or less.

The urinary excretion values were subtracted from the thiamine intake of rats fed 0, 6 and 20% protein

Table 11. Average initial and final weights for rats fed rations containing three levels of protein. Thiamine level in diet was adjusted in an attempt to provide rats in all groups with the same daily intake. All rats were fed on an ad libitum basis.

Group Code	% Protein in Diet	Ave. Initial Wt. g	Ave. Final Wt. (4 wks) g
2-Cal	0	60.2	39.5 ± 1.3
2-Cal	6	60.2	79.4 ± 0.6
2-2Cal	20	60.2	220.0 ± 6.0

Table 12: Daily food consumption and thiamine intake during final three days of study of rats fed diets containing three levels of protein. Thiamine level in diet was adjusted in an attempt to provide rats in all groups with the same daily intake. All rats were fed on an ad libitum basis.

Group Code	% Protein in Diet	Thiamine in Diet mcg./gm.	Food Consumption gm./day/rat	Thiamine Intake mcg./day/rat*
2-Cal	0	13.46	3.2 ± 0.2	42.1 ± 3.7
2-Cal	6	8.96	7.2 ± 0.3	64.3 ± 7.1
2-2Cal	20	4.00	17.9 ± 2.2	71.6 ± 8.9

*The differences in the thiamine intakes are not significant. Due to biological variability the animals in this series did not consume the same amount of food as animals in series 1. Food records for the rats in series 1 were used to determine the level of thiamine which was used in the 3 diets.

Table 13: Urinary excretion of thiamine for rats fed, on an ad libitum basis, diets containing three levels of protein. Thiamine levels in diets were adjusted in an attempt to provide rats in all groups with the same daily intake. For the thiamine intake of these animals, see Table 12.

Group Code	% Protein in Diet	Urinary Thiamine mcg./day/rat	% of Ingested Thiamine Excreted
2-Cal	0	5.7 ± 0.6	9.6
2-6al	6	10.5 ± 0.4	18.4
2-2(al	20	16.9 ± 1.3	23.2

diets; 53.4, 53.9 and 54.7 mcg. of thiamine per day respectively were not accounted for in the urine (Tables 12 and 13).

There were no symptoms of any dietary deficiencies nor disease in the animals except, of course, the smaller weight of the animals receiving inadequate levels of protein.

DISCUSSION

In this study the level of thiamine considered adequate for normal growth was 4 mcg. of thiamine/gram of diet. This is the level suggested by Brown and Sturtevant (1949) for the growing rat. Lower levels are recommended by the Committee on Animal Nutrition, National Research Council (1952); however a level of 4 mcg. of thiamine/gram of diet was used to assure an adequate intake.

Starvation resulting from inadequate dietary protein and as represented by severe loss of body weight apparently did not deplete the liver or muscle of thiamine. The evidence for this statement is shown in Table 7. Although the animals receiving the protein-free diet lost approximately one-third of their initial body weight, the tissue concentration of thiamine in these animals was essentially the same as in the rats which tripled their weight. The latter animals were fed diets that provided 20% casein.

In an animal which is losing weight thiamine behaves differently from riboflavin. Bro-Rasmussen (1951) stated that when experimental animals are in negative nitrogen balance, the riboflavin content of the organs decreases and the riboflavin level in the urine increases. Bro and

Ford (1942) observed high riboflavin excretion values in connection with negative nitrogen balances in rats suffering from a chronic deficiency of thiamine. Pollack and Lockman (1951) observed a correlation of negative nitrogen balance with increased riboflavin excretion in humans. Clarke and Guggenheim (1940) demonstrated poor utilization of riboflavin in rats fed low protein diets. When rats were fed diets providing 11% of the total calories as protein compared to 20 or 34% of the calories as protein, the riboflavin concentration in the tissues fell. This was associated with an increased excretion of riboflavin. In these respects thiamine differs from riboflavin.

In this study rats fed lower levels of dietary protein excreted considerably less thiamine than rats fed, on an ad-libitum basis, diets containing higher levels of protein (Table 8). The tissue concentration of thamine was the same regardless of the protein content of the diet (Table 7).

What appears to be contradictory results to the above work with riboflavin was observed in an experiment with humans conducted by Keys and co-workers (1950). Measurements were made of the average urinary excretion of riboflavin and thiamine at the mid-point and end of a twenty-four week period of semi-starvation. The urinary thiamine excretion was slightly, but not significantly, higher than that predicted from normal intake-excretion

relationships. From the amount of active body tissue which the men lost during the semi-starvation phase, it was calculated that a total of 6.0 mg. of thiamine could have been mobilized from the body tissues. These workers suggested that perhaps some of this liberated thiamine appeared in the urine accounting for the slight increase in excretion over the value which was anticipated. The excretion of riboflavin differed from that of thiamine. During semi-starvation the excretion of riboflavin was exactly what would have been expected from the level of riboflavin intake. The riboflavin liberated from the breakdown of body tissues did not appear in the urine.

The divergent results between the work of Keys and co-workers (1950) and the studies reported by Bro-Rasmussen (1951) concerning riboflavin may be related to the duration of the experimental period. Most of the studies reported by Bro-Rasmussen were of short duration. The urinary excretion of riboflavin increased in these studies. However the study conducted by Keys and co-workers was extended over a twenty-four week period of semi-starvation and the first urine collection was made at the twelfth week. By the twelfth week most of the weight loss had occurred and the riboflavin excretion values were not elevated over those levels that were expected from the riboflavin intake. Possibly the rapid increase in riboflavin excretion reported

by inflammation occurs only in the beginning of the period of negative nitrogen balance and corresponds to a liberation of "reserve" riboflavin in the tissues. This liberation of "reserve" riboflavin could be comparable to the liberation of "labile N stores" when individuals are subjected to stress conditions (Pollack and Bowman, 1941).

The level of dietary protein does not influence the concentration of thiamine in the liver or thigh muscle (Table 8). The data of this study do not agree with those reported by Brown[1] (1943), but they do agree with the findings of Scott and Griffith (1937). Mitchell and Isbell (1941) reported that the thiamine concentration in micrograms per gram of moist tissue for normal male rats was 7.3 mcg. in the liver and 1.3 mcg. in muscle tissue. The data reported in Table 7 for the liver is in agreement with their work; however the values for the muscle tissue are slightly greater than the levels they found. The difference between the values reported by Mitchell and Isbell and those reported in Table 7 could be explained by the fact that their work was done in the early 1940's using microbiological assays whereas the data reported in Table 7 were obtained using a chemical method.

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1. Dr. Brown has indicated in a personal communication that she is unable to duplicate her original results.

The major portion of the thiamine present in the body is found in the liver and muscle. For the purpose of the present discussion it was assumed that the other tissues in the body act in a manner similar to muscle and liver tissue in the retention of thiamine. The amount of thiamine present in the entire liver and muscle mass is tabulated in Table 14. As the protein content of the diet increased, the total thiamine content of these tissues became greater; however this increase was related to the larger tissue mass of the animals receiving higher levels of protein, not to the thiamine concentration per gram of tissue. The thiamine concentration in the tissues was essentially the same despite a fivefold difference in the total body weight of the animals. The thiamine retained in the liver and muscle mass when expressed as a percentage of the thiamine consumed is in the range of 11.1-13.4%. Despite the wide variation in body mass and thiamine intake among these groups it would appear that under the conditions used in this study a relatively constant percentage of the thiamine ingested by rats was stored in these two tissues.

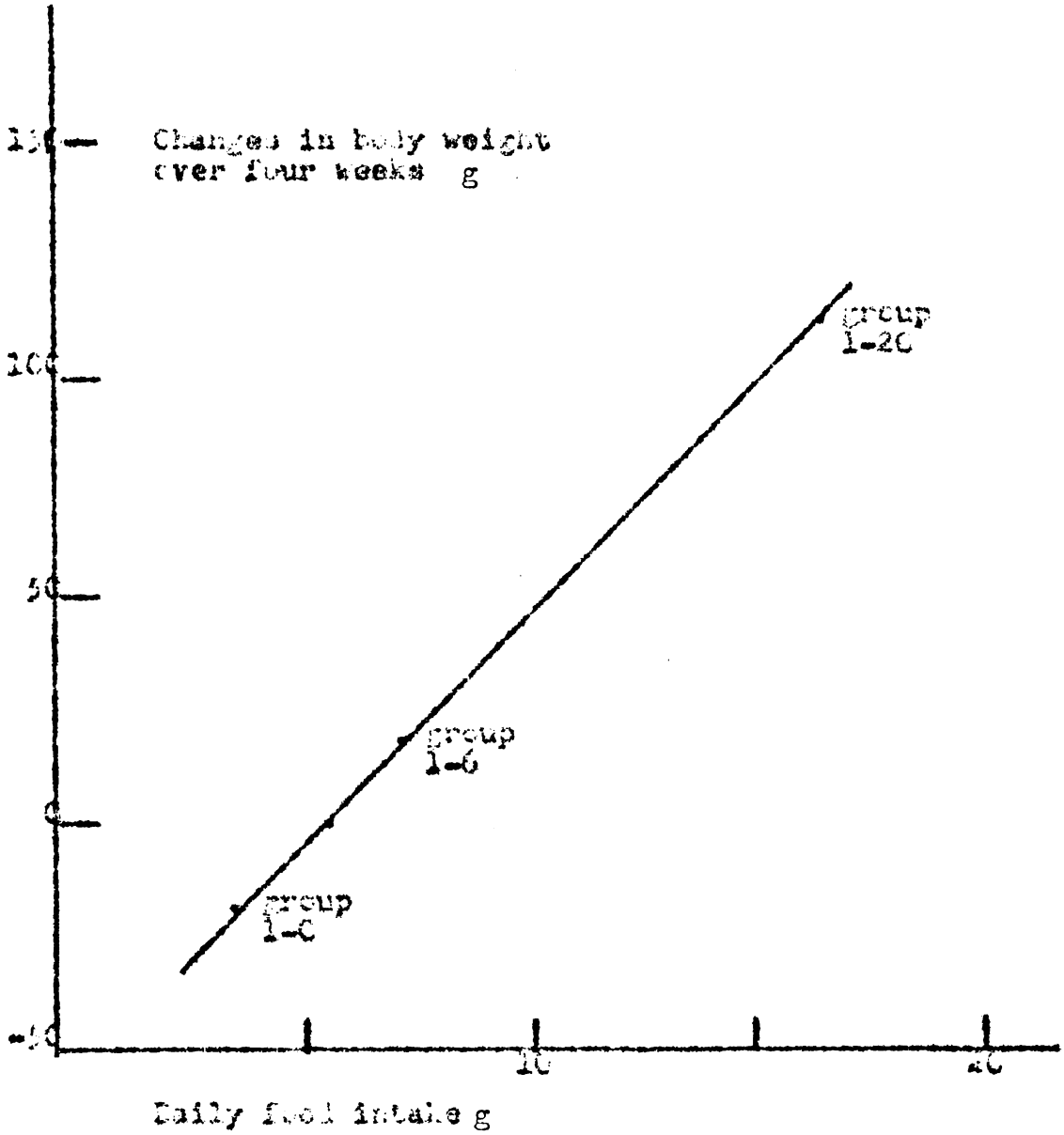
In figure 1 the change in body weight during the entire experiment is plotted versus the daily food consumption for the rats in series 1; a straight line relationship appears. This graph suggests that weanling rats could be maintained with no change in total body weight if fed, each day, 5.6 gms. of a diet containing 3.7% casein and 4 mcg.

Table 14: Total thiamine and percent retention in liver and muscle for rats fed diets containing varying protein levels. Thiamine level in diet was 4 mcg./gm. The thiamine content of the liver and muscle was calculated from analytical values secured for these tissues and the weight of muscle as reported by Caster et al (1956).

Group Code	% Protein in Diet	Ave. Wt. Liver g	Total Liver Thiamine mcg.	Calculated* Muscle Mass g	Total Muscle Thiamine mcg.	Ave. Total Thiamine Intake mcg	% Retained in Muscle & Liver
1-0	0	2.1 ± .05	15.2 ± 1.56	18.2	32.6 ± 2.95	401.2	11.6
1-6	6	3.3 ± .07	21.0 ± 1.01	33.0	50.8 ± 6.50	648.8	11.1
1-20	20	8.5 ± .40	69.7 ±10.18	83.5	139.4 ±13.06	1356.8	15.4

*muscle mass was calculated as 45% of body weight (Caster et al, 1956)

Figure 1: Body Weight Changes and Daily Food Consumption for rats fed diets containing 4 mcg of thiamine/gram of diet at three different protein levels.



of thiamine/gm. of diet. This conclusion was not tested because it became obvious only when the final interpretation of the data was made.

The final weights of the animals as tabulated in Tables5 and 11 indicate that when rats are fed a protein-free diet (groups 1-C and 2-Ca1), four fold increases in the thiamine content of that diet did not result in increased weight gains. However at the 6% level of dietary protein, rats which consumed 3.96 mcg. of thiamine/gm. of diet (group 2-Ca1) gained significantly more weight than rats consuming 4 mcg. of thiamine/gm. of diet (group 1-C). These weight gains during the four week experimental period, i.e. 19.2 and 13.0 gm. respectively for the two groups fed the 6% protein ration, are significantly different at the 1% level (Tables 5 and 11). This statement agrees with the work of Scott and Griffith (1957) who reported that as the thiamine intake of rats increased from 0.2 to 6.3 mcg. daily, the growth rate of rats was enhanced. The diets these workers used contained protein at either the 22 or 86% level and, at both levels of protein, the growth rate of rats was greater when the rats consumed greater amounts of thiamine than when rats consumed lesser amounts. In the present study no comparison of the growth rate of rats fed different thiamine levels in the 20% protein diet can be made; in no instance was the level of thiamine in the 20% casein ration altered from 4 mcg./gm. of diet.

The food consumption as indicated in Table 6 and 12 was not significantly affected by the inclusion of additional amounts of thiamine in the diet. Hence it may be concluded that thiamine in excess of the requirement does not stimulate the appetite.

Many investigators (Cowgill, 1939; Holt, et al, 1943; Kipperin, et al, 1963) have claimed that any thiamine in excess of the amount required by the body is excreted in the urine, and furthermore, thiamine appears in the urine only when the tissue stores are adequate.

The differences in the urinary thiamine excretion of the groups receiving different levels of protein in a diet containing 4 mcg. of thiamine/gm. of ration (groups 1-0, 1-6 and 1-20) are highly significant (Table 8). The differences in the urinary thiamine excretion among these groups of rats could have been influenced by many variables. As the protein level in the diet increased so did the daily caloric intake and final weight of the animals as well as the daily thiamine intake. Also, the increase in the protein content of the diet was at the expense of dietary carbohydrate.

In series 1 the rats receiving the 20% protein diet consumed more than four times the amount of food and thiamine as did those animals receiving the protein-free diet (Table 6). Therefore an attempt was made to equalize the caloric intake by using the paired-feeding technique

and also to equalize the thiamine intake by adjusting the thiamine content of the rations.

The difference in the urinary excretion of rats fed 6 and 20% protein rations with the daily thiamine intake adjusted so that it would be approximately equal is significant at the 5% level (group 2-6al vs group 2-20al). The groups receiving the 6% protein ration excreted less thiamine than those fed either the 6 or 20% protein rations and these differences are significant at the 1% level or less (Table 13). These differences occurred despite the fact that the daily intake of this vitamin was essentially the same for all groups (Table 12). These results indicated a direct relationship between urinary thiamine and the level of dietary protein and/or caloric intake.

To eliminate the caloric intake as a factor in thiamine excretion, groups 2-6pf, 2-12pf and 2-20pf were pair-fed with group 2-6cpf as the control. As seen in Table 10 the urinary concentration of thiamine was not influenced by the protein level in the diet provided that some protein was included. Actually the urinary excretion of thiamine for the groups receiving protein was remarkably constant, despite a three-to-four fold difference in protein intakes.

In this study growing organisms, weanling rats, were studied. Stearns and co-workers (1950) studied the effects of various thiamine intakes of 0.27-2.33 mg. daily in growing

children and found that the urinary thiamine values increased with the increase in daily thiamine intake. Similarly in our study, rats receiving the higher levels of thiamine (13.40 mcg. of thiamine/gm. of diet at the C% level of dietary protein and 8.36 mcg. of thiamine/gm. of diet at the 6% level of dietary protein) excreted more thiamine daily than the group fed comparable levels of protein in diets containing 4 mcg. of thiamine/gm. of diet (Tables 10 and 13).

The thiamine, which was unaccounted for, was a constant value in rats consuming approximately equal daily intakes of thiamine but different levels of protein (Table 15). Despite variations in food intake and body weight, the amount of thiamine which could not be accounted for was similar, 33.4 - 34.7 mcg./day/rat. When the corresponding calculations were made for the rats receiving different levels of protein in diets containing 4 mcg. of thiamine/gram (groups 1-C, 1-6 and 1-20), 13.0-43.2 mcg. of thiamine were not accounted for by urinary excretion (Table 15). For these rats the values for the amount of thiamine, which was not accounted for, are not similar.

The diets consumed by groups 1-20 and 2-20al were identical. Differences in food consumption and urinary excretion may be explained by biological variability. Nine months passed between the time when these two series were conducted.

Additional studies are needed to indicate the fate of the thiamine which did not appear in the urine of rats fed 2-4 times the "normal" level of thiamine (groups 2-Cal and 2-Bal). As no reports in the literature have suggested that all of the thiamine which was injected was not absorbed, the similar thiamine retention suggested an increase in tissue storage or an increased metabolic requirement for thiamine by the smaller rats which consumed lesser quantities of protein. Thiamine is required for the metabolism of carbohydrate. As the dietary protein levels increased the dietary carbohydrate levels fell. The animals fed the 0, 6 and 20% levels of protein consumed an average of 2.1, 6.0 and 12.5 gm. of carbohydrate daily. Thus, as these animals were all receiving approximately the same daily intake of thiamine, the rats fed the 20% protein ration would have required more thiamine for carbohydrate metabolism and excreted less thiamine than animals fed lower levels of protein. Similarly if thiamine was related to body size and calorie intake (Sargill, 1934) the smaller animals eating less food presumably would have required less thiamine. Also if thiamine was required for protein metabolism (Richter and Hawley, 1941; Richter and Rice, 1944) the animals ingesting the protein-free diet would have required less thiamine. In either of these cases the urinary thiamine levels would have been greatest in the animals which were fed the lowest levels of protein. The observations

Table 17: Thiamine intake and urinary excretion of rats fed on an ad libitum basis diets containing different levels of protein

Group Code	% Protein in Diet	Thiamine in Diet mcg/gm	Thiamine Intake mcg/day/rat	Thiamine Excretion mcg/day/rat	Thiamine Unaccounted for mcg/day/rat
1-0	0	4.00	13.0	0.3	13.0
1-6	6	4.00	26.4	2.2	23.2
1-20	20	4.00	63.6	20.4	43.2
2-Cal	0	13.45	77.1	5.7	53.4
2-Cal	6	8.96	64.5	10.6	53.9
2-20Cal	20	4.00	71.6	16.9	54.7

of this experiment are in agreement with those of Caster (1943). She observed that larger animals excreted more thiamine than smaller animals even when the daily intake of thiamine was the same. The animals consuming the diets containing a higher percentage of protein were larger than those animals consuming lesser amounts of protein.

Similarities in the percent of thiamine ingested which appeared in the urine despite differences in thiamine intake may be noted for rats fed diets containing 0 and 6% protein (Tables 10 and 13). Although the rats fed the 0% protein rations consumed like amounts of feed, the thiamine content of the diets varied by a factor of four; nevertheless the rats fed the 0% protein diets excreted 8.2-9.6% of the thiamine intake. In series 1, the group fed the protein-free diet was identical in all respects to the group which served as the control for the paired-feeding in the second series. Although it appears that the percent urinary excretion of these groups is different (Tables 8 and 10) the absolute values on which the percents were based were not significantly different. At such low levels of feed intake small differences in urinary thiamine excretion appear to be greater when expressed as a percentage whereas at higher levels of feed intake, differences of the same magnitude do not appear to be as great.[1] In series 2,

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1. Between 1 and 1.5 there is a 50% difference; however between 10 and 10.5 there is only a 5% difference. In both cases the actual difference is only 0.5. In addition to this, analytical difficulties are involved in accurately determining low levels of thiamine. Hence differences at such low levels of thiamine were dubious unless significant.

the rats fed the 6% protein rations (groups 2-6al and 2-6pf) excreted 16.4-17.7% of the thiamine intake (Tables 10 and 13) inspite of a twofold difference in the level of thiamine ingested. However, the rats in series 1 (group 1-6), which received the same level of protein but which consumed a level of thiamine midway between the daily injection of these groups, excreted only 7.1% of the thiamine intake (Table 8). This observation suggests that when rats fed low protein diets are either restricted in their food consumption or fed thiamine in excess of the requirement, they excrete twice as much of the thiamine intake as do rats fed, ad libitum, a low protein diet containing "normal" levels of thiamine. No explanation for this phenomenon can be offered at this time.

Balakrishnan and De (1952) reported urinary excretion values of around 10% of the intake regardless of the level of protein in the diet. However the levels of supplementation used in his work were less than the daily intake of thiamine by the animals on even the most limited region in this study.

In this study the observation of Light and co-workers (1933) was confirmed; rats excreted in the urine 23.6% of the dietary thiamine when the animals were receiving an adequate level of protein (group 2-20al, Table 13). When the food intake decreased as a consequence of the diminished protein content of the diet (groups 1-0 and 1-6) the percent

of the ingested vitamin which was excreted in the urine decreased (Table 8). This decrease was observed even when the total daily ingestion of the vitamin was essentially equal (groups 2-Cal, 2-Cal, and 2-20Cal, Table 12).

This study has demonstrated that:

1. The level of protein in the diet did not influence the thiamine concentration in the tissues of weanling rats when the concentration of thiamine in the diet was uniform.
2. The urinary excretion of thiamine was not related to the protein content of the diet. This was shown when weanling rats fed different levels of protein were restricted in food intake by paired-feeding.
3. Animals which consumed more total and more protein calories excreted more thiamine in the urine than animals that consumed fewer total and protein calories even when the daily intakes of thiamine by these groups were similar.

Discussion

Thiamine analyses were carried out on tissue and urine samples from rats fed diets containing protein at the 0, 6 and 20% level. The thiochrome technique of thiamine analysis was employed.

In series 1, weanling rats were fed the above diets containing 4 mcg. of thiamine/gm. of diet. The protein level in the diet did not exert any influence upon the concentration of thiamine in the liver or thigh muscle. It was observed that there was an increase in urinary thiamine excretion with an increase in protein intake. This enhanced urinary thiamine excretion was a result of the greater food consumption of rats fed the higher levels of dietary protein.

In series 2a, rats were paired-fed diets containing 6, 12 and 20% protein with rats consuming a protein-free diet as the control. The thiamine content of these diets was 4 mcg./gm. of diet. The urinary excretion of thiamine did not vary in response to the various levels of dietary protein.

In series 2b, the daily thiamine intake of rats fed different levels of protein was equalized by adjusting the thiamine content of the various diets. The rats in this

experiment were fed ad libitum. The daily excretion of thiamine varied; those animals eating more protein and more calories each day excreted a greater amount of thiamine than those animals that consumed less protein and calories. Despite the wide variation in food consumption and dietary protein there was a similar amount of ingested thiamine which did not appear in the urine. The animals which consumed thiamine in excess of the recommended level of intake, expressed as mcg. of thiamine/gram of diet, did not excrete this excess.

The results of this study are taken to indicate that the level of protein in the diet does not directly influence the thiamine content of rat tissue when "normal" thiamine levels are used; the daily urinary excretion of thiamine is not related to the dietary protein level directly but to the caloric intake of the animal.

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