COCARBOXYLASE AND THIAMINE IN TISSUES OF RATS RECEIVING DIFFERENT CONCENTRATIONS OF THIAMINE IN THE DIET

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

John Hilbert Flokstra
1950

This is to certify that the

thesis entitled

Cocarboxylase and Thiamine in Tissues of Rats Receiving Different Concentrations of Thiamine in the Diet

presented by

John H. Flokstra

has been accepted towards fulfillment of the requirements for

M.S. degree in Biochemistry

R.H. Byerrum
Major professor

Date May 22, 1950

COCARBOXYLASE AND THIAMINE IN TISSUES OF RATS RECEIVING DIFFERENT CONCENTRATIONS OF THIAMINE IN THE DIET

By

John Hilbert Flokstra

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1950

ACKNOWLEDGMENT

The author wishes to express his thanks to Doctor Richard U. Byerrum for his helpful and encouraging suggestions given during the work on this problem and the preparation of this manuscript; also to Doctor Carl A. Hoppert and others who have aided in this work.

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INTRODUCTION

In recent years considerable work has been done to indicate the participation of the water soluble vitamins, as coenzymes, in enzyme systems. Lohmann and Schuster (1) observed in 1937 that the pyrophosphoric acid ester of thiamine, which they called cocarboxylase, was a coenzyme in the system which catalyzes the decarboxylation of pyruvic acid. Ochoa and Peters (2) showed that the cocarboxylase content of tissues decreased when thiamine-deficient diets of polished rice were fed to rats and pigeons. They also indicated that the activity of the pyruvate carboxylase enzyme system in yeast depended on the quantity of the cocarboxylase and also thiamine present.

Most of the investigation for ascertaining the animal requirement for thiamine has been based on the rate of growth of animals receiving different quantities of the vitamin in the diet. Since it is now recognized that thiamine functions as a coenzyme and activator for pyruvate carboxylase, a more accurate index of the animal thiamine requirement might be a measurement of the change in concentration of cocarboxylase and thiamine in the tissues of animals fed different quantities of vitamin B_1. The present investigation was begun to observe the effect of known concentrations of thiamine in purified diets on the quantity of cocarboxylase or thiamine in the tissues of rats, since no such systematic study has been made. In addition, a comparison of vitamin B_1 requirement, based on observed growth, with the tissue cocarboxylase and thiamine content was made.

HISTORICAL

There appears to be no doubt that thiamine is intimately related to carbohydrate metabolism in animals. In experimental animals having a thiamine deficiency, abnormally large amounts of pyruvic and lactic acids accumulate in tissues and body fluids. A more precise analysis of the relationship between thiamine and carbohydrate metabolism was made when Lohmann and Schuster (1) discovered that cocarboxylase, the coenzyme of the pyruvate carboxylase system, was the pyrophosphoric ester of thiamine. In accord with this finding Lipschitz, Potter and Elvehjem (3) indicated that a thiamine deficiency produced first a general disturbance of carbohydrate metabolism which later caused a gross pathological effect in which the animal developed lesions in the nervous system.

All the work done to determine the requirement of thiamine for animals has been based on the results of growth studies. Light and Cracas (4), studying the amount of thiamine required for normal growth of different strains of rats, reported a variation in requirements from one strain to another. Arnold and Elvehjem (5) produced thiamine deficiency symptoms in rats on a variety of low-vitamin B_1 diets. Initially the growth rate increased as the vitamin B_1 concentration of the diet was increased if the basal ration provided an adequate supply of the other essential factors. Rats receiving a synthetic low-vitamin B_1 ration plus an additional 80-100 micrograms of vitamin B_1 per 100 grams of ration grew at what was considered to be a normal rate. These

same workers concluded (6) that for most species of animals the requirement for thiamine, as based on growth, was approximately one microgram of vitamin B_1 per gram of dietary solids. Ordinarily, in what may be considered to be normal synthetic diets this concentration is doubled to assure a sufficient supply of thiamine.

It was demonstrated as early as 1932 that there was a relationship between dietary vitamin B_1 and the tissue concentration of both cocarboxylase and thiamine. Simola (7) showed that a vitamin B_1-free diet had little, if any, effect on the rat liver and brain cozymase activity (the enzyme systems involved in the conversion of glucose to pyruvate), but that the cocarboxylase content of these organs appeared to be decreased under these conditions. Westenbrink (8) reported a reduction in the vitamin content of rat tissues after feeding for five weeks on a vitamin B_1-free diet. Leong (9) and Schultz et al (10), studying the influence of the amount of vitamin B_1 ingested on the amount that could be stored in rat tissues, found that an intake of from 65-90 micrograms per day gave maximum storage.

Since apparently the main function of thiamine was that of a precursor of cocarboxylase, a more significant method of tissue analysis was to compare the cocarboxylase as well as the thiamine content of tissues of animals fed different diets. Westenbrink and Goudsmit (11), using the thiochrome method, were among the first to do this. They found that the free thiamine content of most tissues in a normal rat was small compared to the cocarboxylase content. Ochoa and Peters (12,2)

came to conclusions similar to those of Westenbrink and Goudsmit. Using the yeast enzyme (manometric) method of analysis, they showed that there is much less thiamine than cocarboxylase in the liver and brain of the normal rat. The concentration of cocarboxylase was reported to be reduced in all tissues in the avitaminous rat, especially in the brain. In contrast to this work Salcedo et al (13) pointed out that, unlike other tissues, the brain maintains its cocarboxylase concentration for a considerable period.

In view of the differences in results concerned with tissue concentrations of cocarboxylase and thiamine, it is apparent that a systematic study of the relations between the intake of vitamin B_1 in a purified diet and tissue concentration of thiamine and cocarboxylase would be desirable.

In addition, it appears that a comparison should be made between the growth rate and tissue concentrations of cocarboxylase and thiamine as an index of ascertaining vitamin B_1 requirements.

A solution of these two problems was the aim of the present study.

EXPERIMENTAL

Animals and Diets

Male and female albino rats of a strain developed in the Michigan State College Chemistry Department approximately 35 days old were used as the experimental animals. The rats, at the beginning of the feeding experiments, weighed between 50 and 75 grams. Initial and final weights were measured.

Purified diets which differed only in their thiamine content were fed these animals for a period of about four weeks. All of the experimental diets contained the following constituents: casein (vitamin free) 18%, dextrose 74%, corn oil (Mazola) 4%, and salt mixture (14) 4%. Vitamins dissolved in 70% ethyl alcohol were added by dripping the alcoholic solution from a pipet on the casein and allowing the casein to dry. The following vitamins were added per hundred grams of all the diets: riboflavin 0.5 mg., pyridoxine 0.2 mg., choline chloride 50.0 mg., calcium pantothenate 2.8 mg.

Four experimental diets were then devised each containing the above constituents and in addition a given amount of thiamine hydrochloride as shown on Table I. Vitamins A, D, E and K were supplied by feeding each animal two drops (approximately 0.1 ml.) of haliver oil per week.

These diets were fed in special cups to prevent spilling and the approximate weight of diet consumed by the experimental animals was measured. Distilled water was supplied for drinking.

TABLE I

EXPERIMENTAL DIETS CONTAINING DIFFERENT CONCENTRATIONS OF THIAMINE HYDROCHLORIDE

Diet Number	Thiamine Hydrochloride mg./100 g. diet
1	0.0
2	0.1
3	0.2
4	0.4

Manometric Techniques

The influence of cocarboxylase and thiamine concentrations upon yeast pyruvate carboxylase activity was measured, using the Warburg-Barcroft apparatus, by observing the volume of carbon dioxide liberated in the decarboxylation of a substrate of sodium pyruvate. The enzyme preparation was prepared by washing brewers yeast once with water, three times with a 0.13 M phosphate buffer of pH 7.9, and once again with redistilled water. A suspension (10 mg./ml.) of the washed yeast was made in 0.05 M phosphate buffer of pH 6.2 and one ml. was added to the flask. 0.1 ml. of $MnCl_2$-$MgCl_2$ solution containing 20 μg. of Mn^{++} and 100 μg. of Mg^{++} was then added. 0.057 mM. of the substrate, sodium pyruvate, was added in 0.2 ml. of water to the flask sidearm. Cocarboxylase and thiamine were added in the desired concentrations and buffer of pH 6.2 to give a total volume in the flask of 3.0 ml. After a ten minute thermal equilibration period at 30° C., zero time readings were made on the manometers and the reaction was initiated by tipping the flask to mix enzyme and substrate. Volume measurements were then made at convenient intervals during the time required for the reaction.

Analytical Method for Thiamine and Cocarboxylase

The thiamine and cocarboxylase concentrations in rat liver, muscle and brain were measured. Rats which had eaten the experimental diets for a specified period were decapitated and the liver, brain and muscle from a hind leg were removed immediately and frozen by placing on solid carbon dioxide. These tissues were kept frozen until they were ready to be analyzed.

Analyses were made, using a modification of the method of Hennessey and Cerecedo (15). This method involves measuring the fluorescence of the thiochrome produced from thiamine by oxidation with alkaline potassium ferricyanide. The thiochrome from free thiamine is soluble in isobutanol, phosphorylated thiochrome from cocarboxylase is not; thus a separation of the two can be made.

Free thiamine - The weighed tissue sample was homogenized using the Potter-Elvehjem technique (16). It was then extracted with about 19 ml. of 0.1 N sulfuric acid in a boiling water bath for 30 minutes. The suspension was cooled, diluted to 25 ml. with water, mixed thoroughly, and centrifuged.

Total thiamine - A weighed tissue sample was homogenized and extracted with 19 ml. of 0.1 N sulfuric acid for 30 minutes. The suspension was cooled and 0.1 g. of Clarase, a phosphatase preparation which hydrolyzes cocarboxylase to free thiamine, in 1.25 ml. of 2.5 M sodium acetate solution was added. This was diluted to 25 ml. with water and mixed, the final pH being about 4.7. The sample was then kept at 45-50° C. for at least two hours or at 40° C. overnight and centrifuged just before the analysis was to be made.

Determination of thiochrome - A 5 ml. portion of the solution obtained after following either procedure described above was put into each of three glass-stoppered reaction vessels: A, B and C. To A, the blank, was added 3 ml. of 15% sodium hydroxide; to B, the sample, was added 3 ml. of alkaline ferricyanide solution (1.5 ml. 1% potassium ferricyanide diluted to 50 ml. with 15% sodium hydroxide);

to C, the internal standard, was added 0.5 ml. of a solution of thiamine hydrochloride containing 0.5μ g. thiamine hydrochloride and 3 ml. of alkaline ferricyanide solution. Fifteen ml. of purified isobutanol were added to each vessel, they were stoppered and shaken vigorously for one and one-half minutes. The vessels were then centrifuged for about four minutes. The upper isobutanol layer containing the thiochrome from thiamine was then drawn off with a pipette and blown into a test tube containing 2 g. of anhydrous sodium sulfate. The fluorescence of the three tubes was then measured on a Coleman Electronic Photofluorometer. The difference in fluorescence between tubes B and A was due to the thiamine in the sample. The difference in fluorescence between tubes C and B was due to the 0.5μg. of thiamine added. Knowing these two values and the sample weight, the concentration of thiamine in the sample was calculated.

The difference between the free thiamine and total thiamine is due to phosphorylated thiamine, or cocarboxylase. The concentration of thiamine in each case was calculated and the difference was multiplied by the proper factor to obtain cocarboxylase concentration.

RESULTS

Enzyme Experiments

Cocarboxylase and Enzyme Activity - Some information about the effect of added cocarboxylase upon the activity of yeast pyruvate carboxylase was obtained by the Warburg technique. Figure 1 shows the effect of added cocarboxylase upon the activity of the carboxylase system found in washed brewers yeast. The amount of added cocarboxylase in micrograms is plotted against the reciprocal of the time in seconds required to obtain an evolution of 200 μl. of carbon dioxide in excess of the blank. A total evolution of 1272 μl. of carbon dioxide was theoretically possible. It is seen that, within the limits of concentration of cocarboxylase used, the activity of the enzyme system as measured by carbon dioxide evolution is proportional to the amount of cocarboxylase added.

Thiamine and Enzyme Activity - In Figure 2 the activating effect of added thiamine upon a yeast pyruvate carboxylase system containing a constant amount of cocarboxylase is shown. Micrograms of thiamine added are plotted against the reciprocal of the time in seconds required to obtain an evolution of 100 μl. of carbon dioxide. Thiamine increased the activity of the system only when cocarboxylase was added to the flask, otherwise no increase in carbon dioxide production above that of the blank was noted. A marked increase in activity was noted as thiamine was increased up to a concentration of about 10 μg. of added thiamine; more than this amount seemed to have little further activating effect.

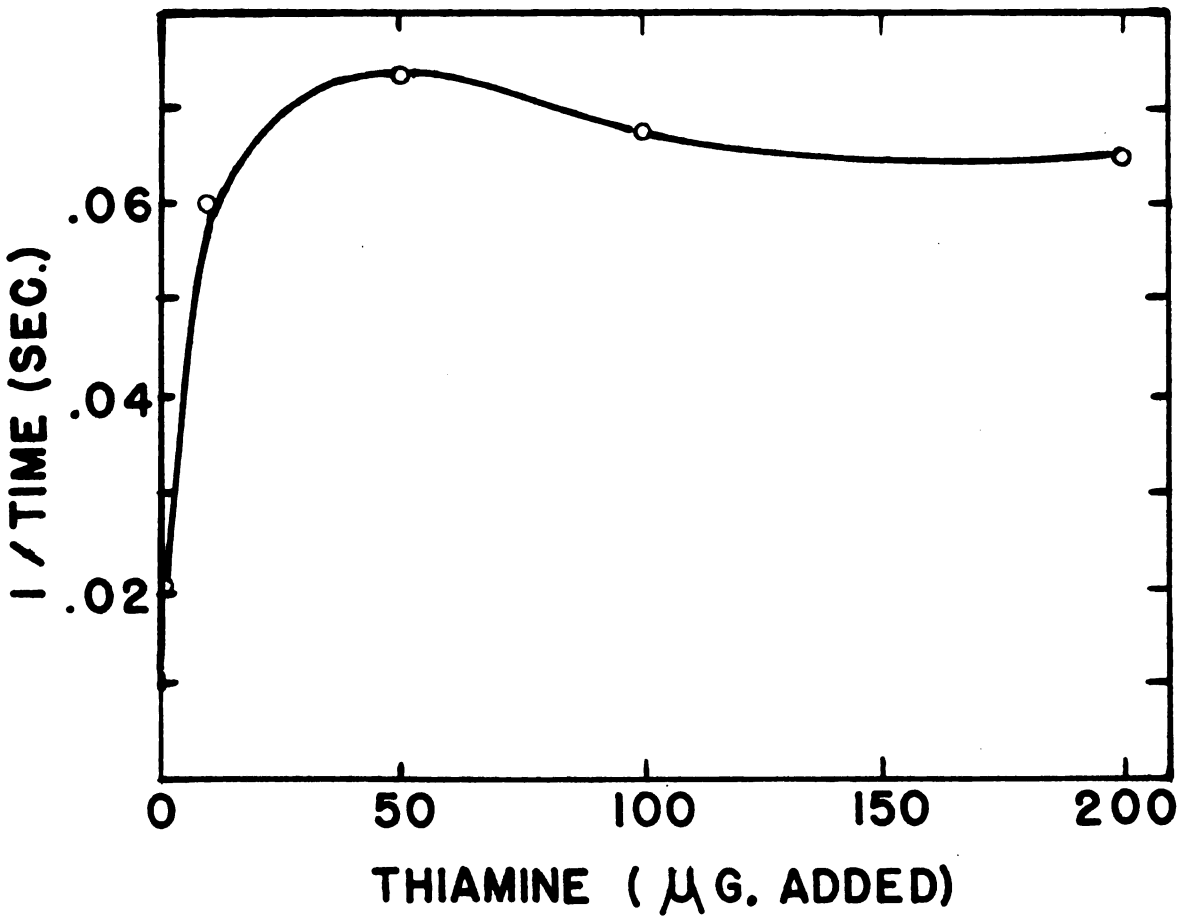

Figure 2. The Influence of Added Thiamine Upon Washed Yeast Pyruvate Carboxylase Activity.

Enzyme = 10 mg. washed yeast in 1 ml. 0.05 M phosphate buffer pH 6.2; substrate = 6.25 mg. sodium pyruvate added in 0.2 ml. H_2O (0.057 mM. sodium pyruvate); activators = 20 μg. Mn^{++} as $MnCl_2 \cdot 4H_2O$ in 0.1 ml. H_2O, 100 μg. Mg^{++} as $MgCl_2 \cdot 6H_2O$ in 0.1 ml. H_2O; cocarboxylase = 2 μg. added in 0.1 ml. H_2O; thiamine made up so that 0.2 ml. gave desired amount, 0.05 M phosphate buffer pH 6.2 to give a final volume of 3.0 ml. 1/time = reciprocal of time in seconds required for evolution of 100 μl. CO_2.

An attempt was made to use the manometric technique as an analytical method for thiamine and cocarboxylase. Due to difficulty in obtaining a source of yeast which gave consistent, duplicable results, this method was not found adaptable to such a quantitative measurement. However, the data presented in Figures 1 and 2 do indicate that the yeast enzyme activity is proportional to the amount of cocarboxylase present and also that thiamine in the presence of cocarboxylase exerts an activating influence upon the enzyme system.

Thiamine Intake and Growth of Rats Receiving Experimental Diets

Figure 3 shows the food intake of a typical rat receiving Diet No. 1 (thiamine free). The intake increased for about nine days, then fell sharply and gradually decreased until the end of the experimental period. Rats on this diet gained weight for just under two weeks, then lost weight, and at the end of the experiment the average weight was slightly less than the original weight. Thiamine deficiency symptoms appeared after about three weeks on a vitamin B_1-free diet.

The food intake of a typical rat fed Diet No. 2 (1 μg. thiamine hydrochloride/g. of diet) is given on Figure 4. A steady increase in food intake was observed. Rats on this dietary level gained weight steadily and no thiamine deficiency symptoms were apparent.

On Figure 5 is shown the daily food intake of a typical rat fed Diet No. 3 (containing 2 μg. thiamine hydrochloride/g. of ration). The food intake increased steadily and a gradual gain in weight was observed. Both average food intake and gain in weight were slightly

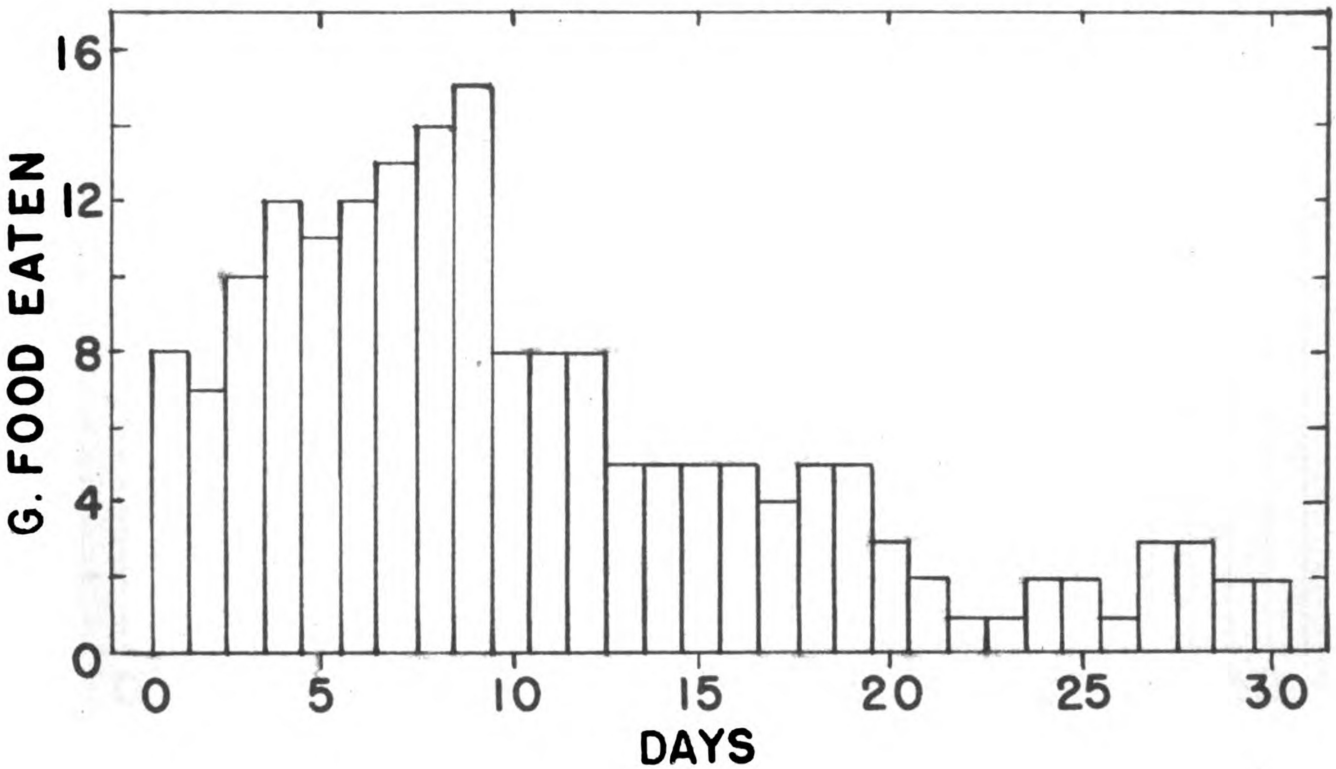

Figure 3. Daily Food Intake for a Typical Rat Receiving a Thiamine Free Diet (Diet No. 1)
Rat No. 2 Initial weight = 64 g. Final weight = 66 g. Average food intake / day = 6.2 g.

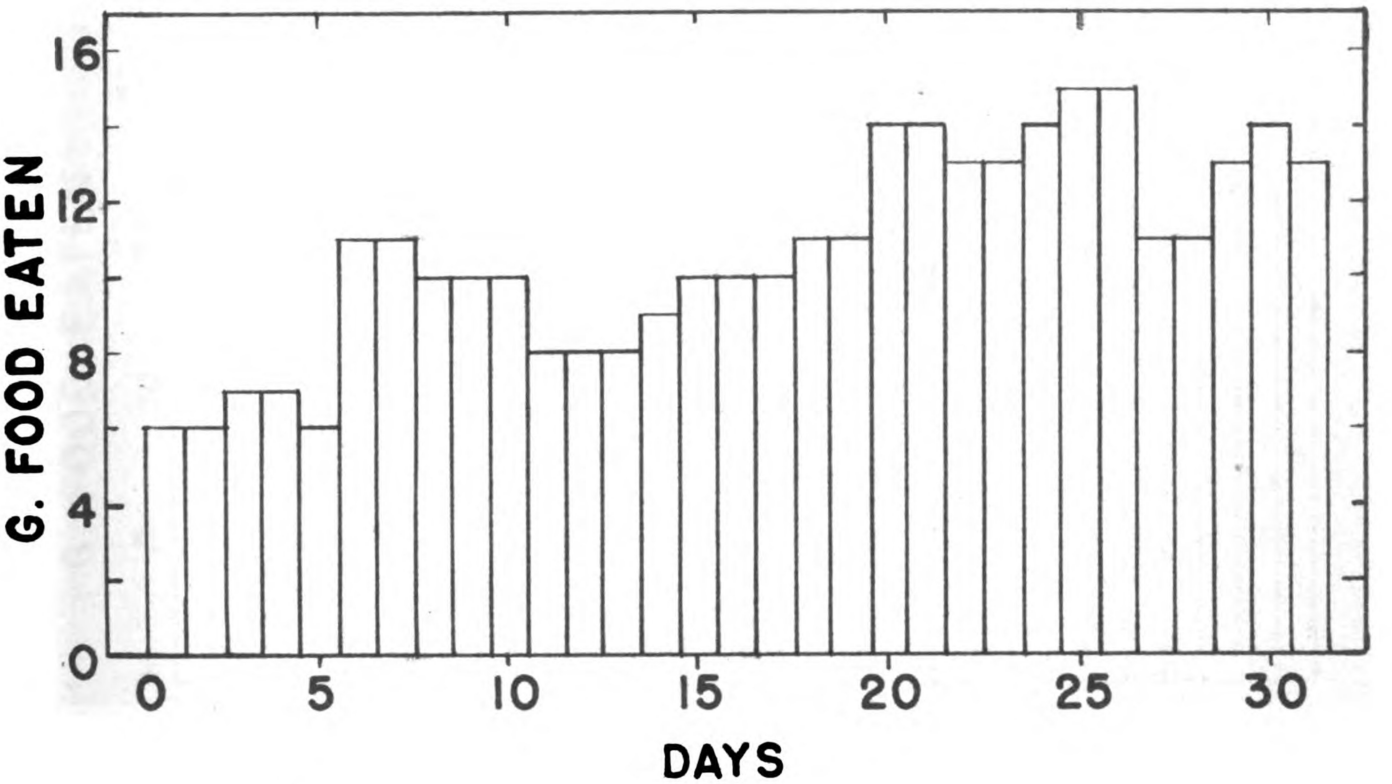

Figure 4. Daily Food Intake for a Typical Rat Receiving Diet No. 2 Rat No. 22 Initial weight = 76 g. Final weight = 178 g. Average food intake / day = 10.8 g. Average mg. vitamin B_1 hydrochloride / kg. body weight = .95

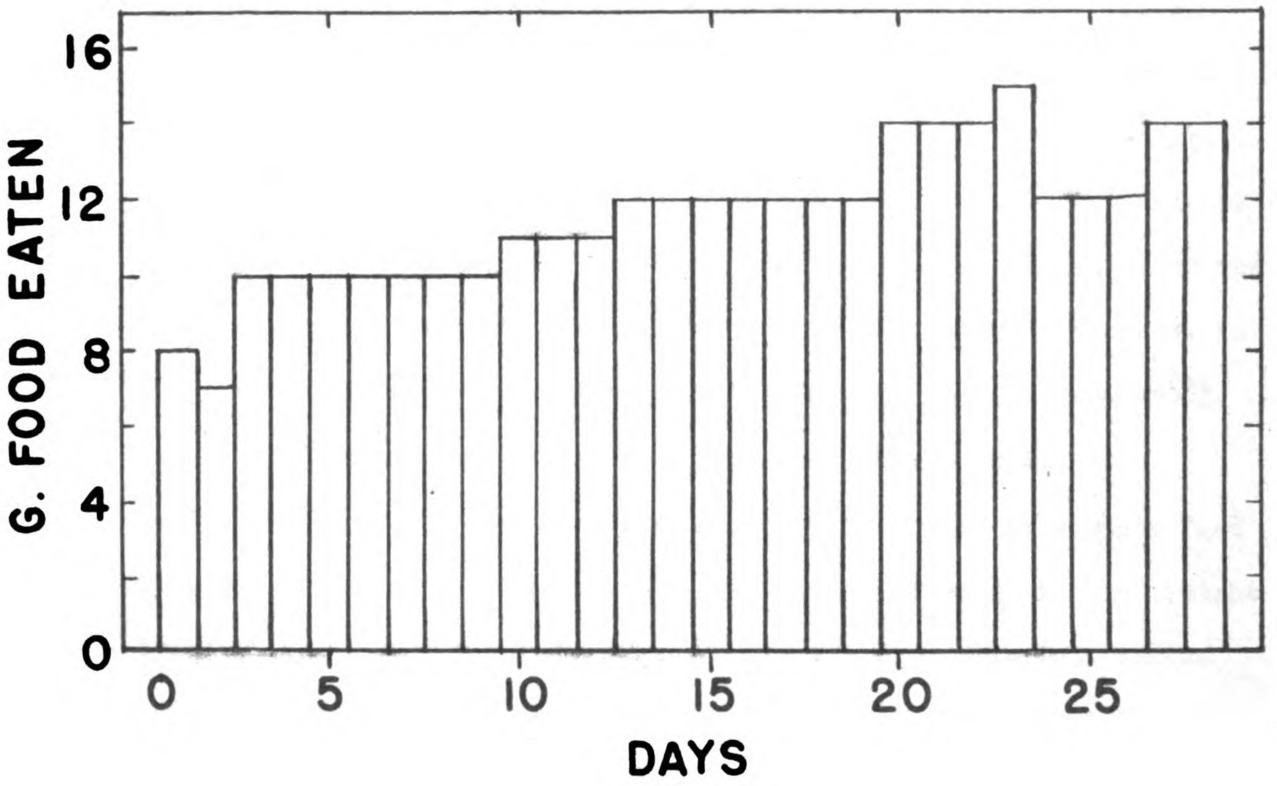

Figure 5. Daily Food Intake for a Typical Rat Receiving Diet No.3
Rat No. 12 Initial weight = 54 g. Final weight = 179 g.
Average food intake / day = 11.9 g. Average mg. vitamin B_1 hydrochloride / kg. body weight = 2.05

higher than in the case of rats receiving Diet No. 2. These rats appeared to be in excellent condition at the end of the experiment.

Figure 6 gives the daily food intake of a rat receiving 4 μg. thiamine hydrochloride/g. diet, Diet No. 4. The average daily intake is slightly lower than that of rats receiving Diet No. 3 and about the same as that of rats fed Diet No. 2. The gain in weight is steady and the average gain is slightly higher than that of rats on Diet No. 2, but slightly less than that of the rats receiving Diet No. 3.

Table II shows the average gain in weight and average daily food intake, comparing the four dietary levels. The average initial weight is given for purposes of comparison. The average daily food intake calculated on a per kilogram body weight basis shows very little difference between the four groups. In addition the average food intake on the last day is listed. That of the thiamine-free group was below its average daily intake, but those of the other groups, as might be expected, are higher than their average daily intakes, due to the increased weight of the rats.

Analysis of Tissues

Free Thiamine Content - In Table III are presented the data from analyses of three rat tissues for free thiamine. These data were calculated on a per gram fresh weight, per gram dry weight, and a per gram nitrogen basis. The free thiamine in the liver increased gradually as the amount of thiamine in the diet increased up to the level of Diet No. 3. A comparatively large amount of free thiamine was found in the

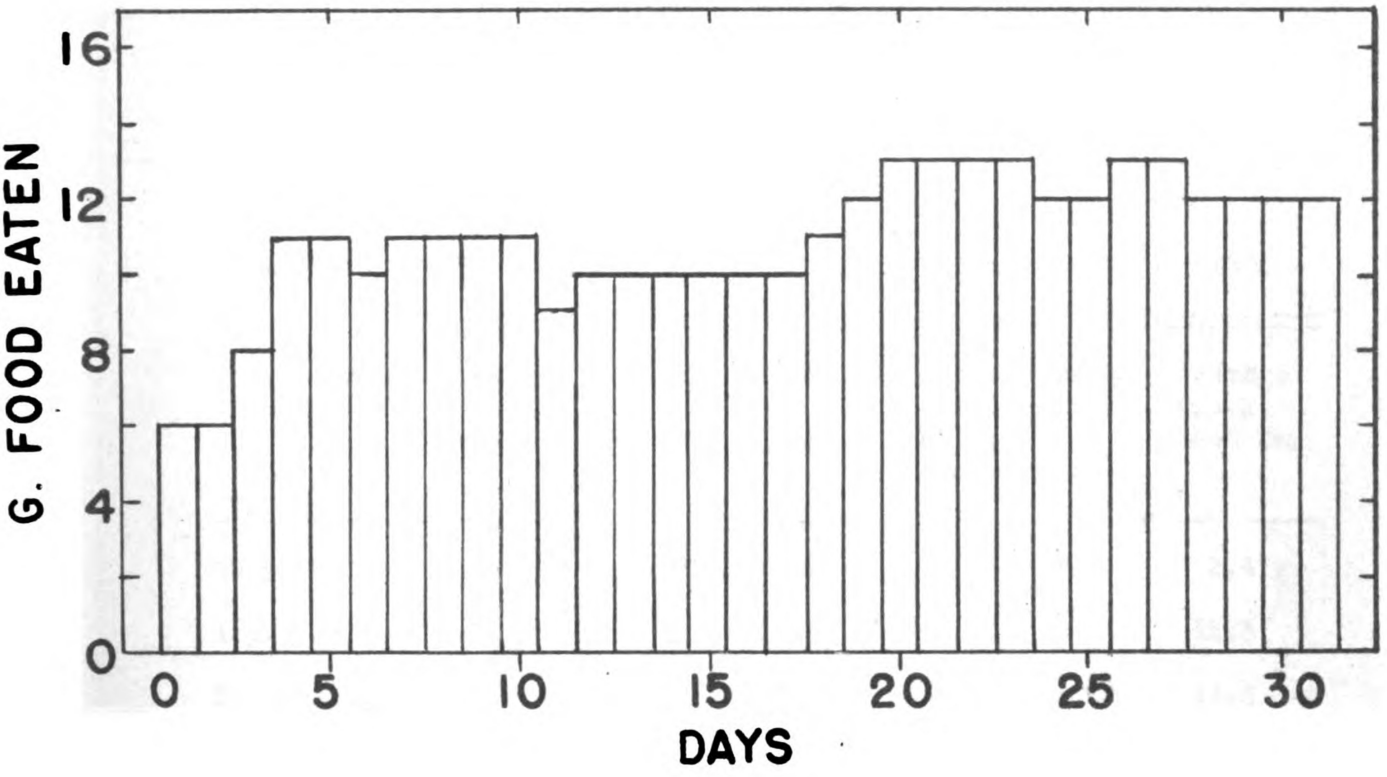

Figure 6. Daily Food Intake for a Typical Rat Receiving Diet No. 4. Rat No. 33 Initial weight = 69 g. Final weight = 170 g. Average food intake / day = 11.5 g. Average mg. vitamin B_1 hydrochloride / kg. body weight = 3.68

TABLE II

AVERAGE INTAKE AND WEIGHTS OF RATS RECEIVING EXPERIMENTAL DIETS

Thiamine Content of Diet	Average Initial Weight	Average Weight Gained	Average Daily Intake	Average Daily Intake per Kilogram Body Weight	Average Intake Last Day
Thiamine Free	68 g.	-1.9 g.	6.2 g.	93.0 g.	2.4 g.
1 μg./g.	65	96	10.7	97.5	12.3
2 μg./g.	57	115	11.6	94.7	14.5
4 μg./g.	64	102	10.6	92.2	13.0

TABLE III

AVERAGE TISSUE CONTENT OF FREE THIAMINE AND COCARBOXYLASE

Diet No.	Tissue	Free Thiamine				Cocarboxylase			
		μg./g. fresh weight		μg./g. dry weight	μg./g. N	μg./g. fresh weight		μg./g. dry weight	μg./g. N
1	liver	0.04*	±.06**	0.12	1.2	0.70*	±.16	2.20	21.4
1	muscle	0.02	±.04	0.08	0.6	0.45*	±.30	1.74	13.1
1	brain	0.10*	±.16	0.45	5.1	1.50*	±.38	6.77	76.0
2	liver	0.45	±.48	1.41	13.7	2.48*	±1.23	7.78	75.7
2	muscle	0.22	±.26	0.84	6.4	0.32*	±.33	1.27	9.3
2	brain	1.27	±.61	5.72	64.5	2.13*	±.62	9.61	108.
3	liver	0.88	±.50	2.76	26.4	6.27	±1.37	19.7	191.
3	muscle	0.07	±.23	0.27	2.4	1.43	±.60	5.48	41.6
3	brain	1.09	±.91	4.94	55.3	4.23	±1.71	19.1	214.
4	liver	2.94	±2.07	9.23	89.7	6.22	±2.23	19.5	189.
4	muscle	0.94	±.90	3.61	27.2	1.31	±1.02	5.03	38.1
4	brain	1.19	±1.01	5.37	60.4	3.38	±1.30	15.2	172.

* Indicates a significant difference from value listed for Diet No. 3 by observation or "t" test for significance.

** The ± is 1 standard deviation from a mean of 6 values (would be expected to include 67% of all values).

liver of animals receiving Diet No. 4. In muscle tissues the free thiamine content was quite low for rats fed Diet Nos. 1, 2 and 3. The experimental error in these cases may be large, since such a small amount of thiamine was present. A higher concentration, however, was noted in the muscle of animals fed Diet No. 4. In the brain of animals fed a vitamin B_1-free diet practically no free thiamine was found. The brain of rats fed the other dietary levels of thiamine had a higher thiamine concentration and the amount present was about the same for all dietary levels.

Calculations were made to determine whether the free thiamine content of tissues of rats fed Diet Nos. 1, 2 and 4 was statistically significantly different than that of rats fed Diet No. 3, the thiamine level which gave maximum growth. No significant difference was observed between the free thiamine content of liver, muscle, or brain of rats fed Diet Nos. 2 or 4 and those receiving Diet No. 3. The free thiamine content of both liver and brain of rats on Diet No. 1 was significantly different from that of rats on Diet No. 3. There was no significant difference between these two dietary levels in the free thiamine content of muscle tissue. These data are also presented in graphical form in Figure 7.

Cocarboxylase content - The concentrations of cocarboxylase on a per gram fresh weight, per gram dry weight and a per gram nitrogen basis are given for tissues from rats on all four dietary levels on Table III. With increasing amounts of thiamine in the diet, the tissue concentration of cocarboxylase increased more rapidly than that of

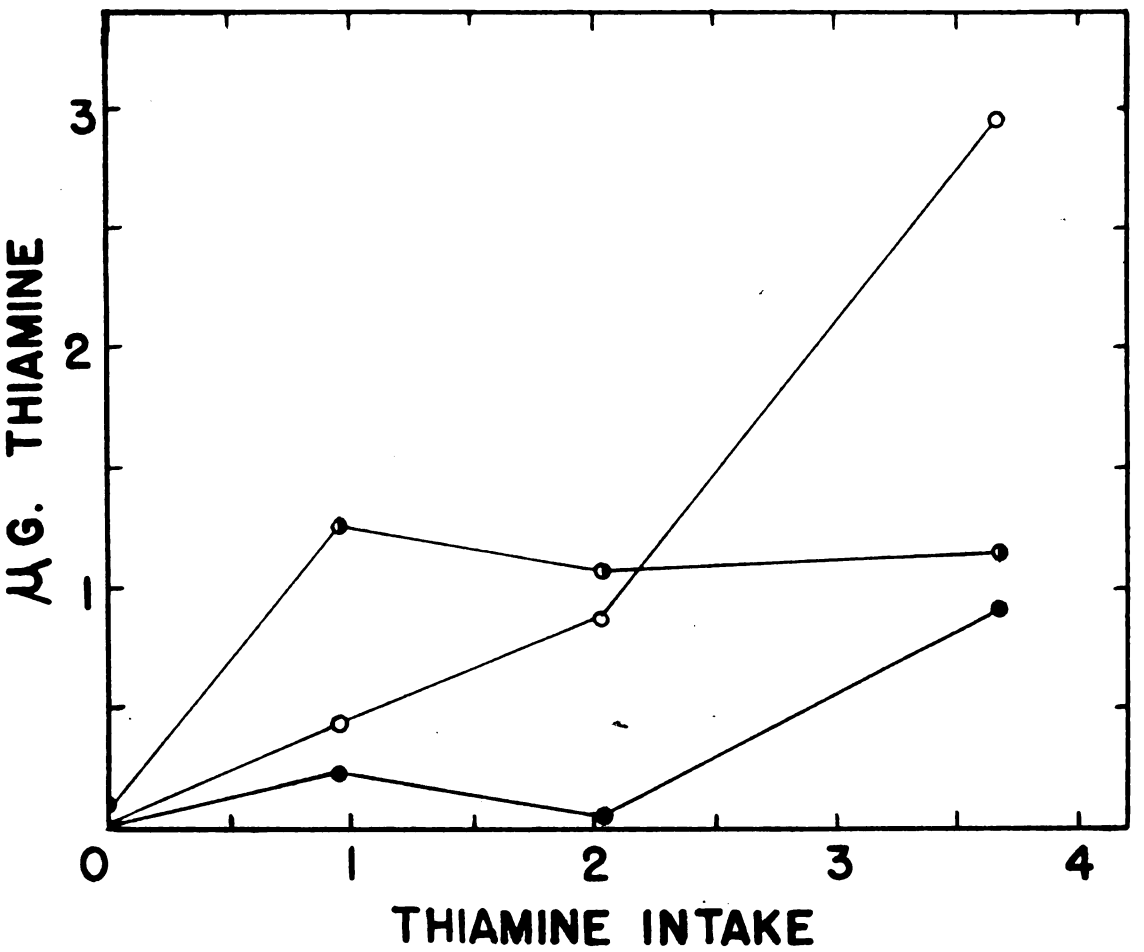

Figure 7. The Relation Between Dietary Thiamine and the Free Thiamine Content of Tissues.
Thiamine intake is expressed as mg. vitamin B_1 hydrochloride / kg. body weight / day (average). μg. thiamine is expressed as μg. vitamin B_1 hydrochloride / g. fresh weight of tissue (average). ○= liver. ●= muscle. ◑= brain.

free thiamine, up to a certain limit for each tissue. In the liver the cocarboxylase content increased with an increase in dietary thiamine up to about the Diet No. 3 level of thiamine. More dietary thiamine apparently gave no higher cocarboxylase concentration in the tissue. A similar observation was made with the muscle and brain tissues, although not such a high concentration of cocarboxylase was obtained in these tissues.

Significant differences were calculated, comparing the tissue cocarboxylase concentrations of rats receiving Diet No. 3 with those of the rats receiving other dietary levels of thiamine. The liver, muscle and brain cocarboxylase contents of rats fed Diet Nos. 1 and 2 were found to be significantly different than the tissue contents of rats fed Diet No. 3. Liver, muscle and brain cocarboxylase contents of the rats on Diet No. 4 were not significantly different than those of rats fed Diet No. 3. This information is also shown on Figure 8.

To check the validity of using frozen tissues in this work comparisons were made of the thiamine and cocarboxylase concentrations of fresh tissue and the same tissue after freezing. Results were the same for fresh and frozen tissue, within the limits of experimental error.

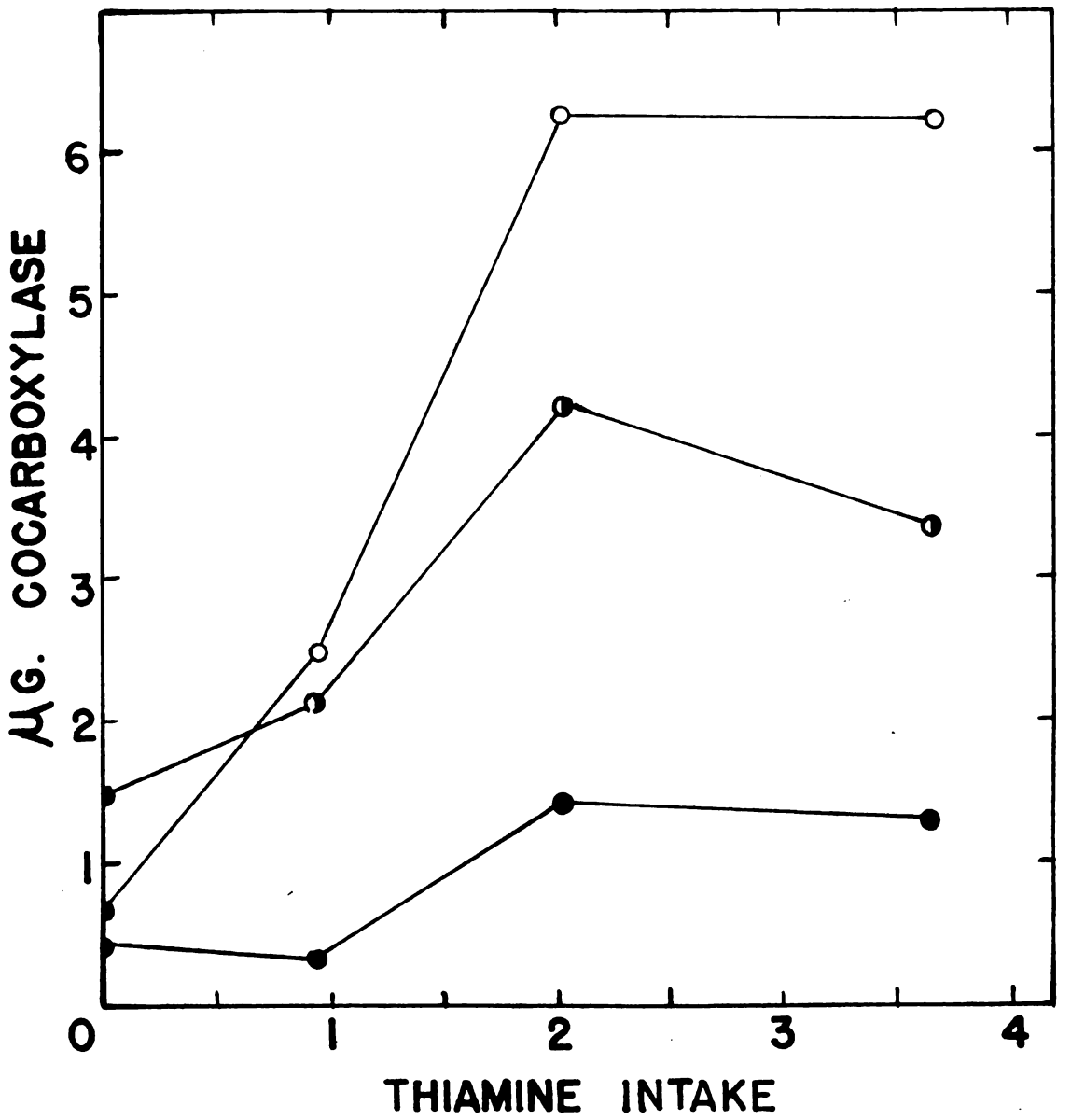

Figure 8. The Relation Between Dietary Thiamine and the Cocarboxylase Content of Tissues.
Thiamine intake is expressed as mg. vitamin B_1 hydrochloride / kg. body weight / day (average). μg. Cocarboxylase is expressed as μg. Cocarboxylase / g. fresh weight of tissue (average).
○= liver. ◎= muscle. ◑= brain.

CONCLUSIONS

An attempt was made to ascertain the effect of cocarboxylase and of thiamine on the activity of the pyruvic acid decarboxylase enzyme system found in yeast. It was noted that the activity of the enzyme system was proportional to the amount of cocarboxylase present when other variables were kept constant. At constant cocarboxylase concentration the addition of small amounts (up to 10 μg./10 mg. yeast) of thiamine caused further activation of the enzyme system. These results agree essentially with the findings of Ochoa and Peters (2).

In the thiamine feeding experiments it was shown that the average daily food intake was slightly greater for rats receiving two micrograms of thiamine hydrochloride per gram of diet than for those receiving greater or less concentrations of thiamine. A gain in weight, followed by a slightly larger loss was observed in rats receiving vitamin B_1-free diets. The largest average gain in weight was found in the group of animals receiving two micrograms of thiamine hydrochloride per gram of diet, although animals fed other dietary levels of thiamine also showed weight gains which were not significantly different from these. These data corroborate the findings of Arnold and Elvehjem (6), which indicate that one microgram of thiamine per gram of dietary solids provides "normal" growth for rats.

The concentration of free thiamine in tissues increased with ingestion of larger quantities of thiamine. In animals fed thiamine-free or low thiamine level diets, the brain retained a larger concentration

of free thiamine and cocarboxylase than the other tissues examined. This is in agreement with the work of Salcedo et al (13), but differs from the results obtained by Ochoa and Peters (2).

An increase in the tissue cocarboxylase concentration was not found in animals fed more than two micrograms of thiamine hydrochloride per gram of diet. However, if an analogy may be drawn from yeast experiments to the situation in rat tissue, it is apparent that increasing thiamine concentrations will increase the pyruvate carboxylase activity even if the cocarboxylase concentration is not increasing. A maximum concentration of cocarboxylase was found in tissues of rats fed two micrograms of thiamine hydrochloride per gram of diet. Since the main physiological function of thiamine is believed to be that of a precursor for cocarboxylase, it is significant that the amount of thiamine giving "normal" growth (1 μg./g. of diet) does not give the largest concentration of cocarboxylase in tissues. If the concentration of cocarboxylase in tissues is to be used as an index of the thiamine requirement, it is apparent that a minimum dietary level of two micrograms per gram of diet is necessary for an optimum coenzyme concentration.

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Cocarboxylase and thiamine in tissues of rats receiving different concentrations of thiamine in the diet.