The vitamin B1-sparing action of sorbitol

Gisela Eleanor Bethsold
Brigham Young University - Provo
THE VITAMIN B₁-SPARING ACTION OF SORBITOL

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INTRODUCTION

Historically, vitamin B₁ or thiamin was the first member of the B-complex vitamins to be isolated and discovered.¹ The term "vitamin" stems from the fact that in 1911, Casimir Funk obtained a crystalline substance from rice polishings with nutritional properties.² He coined for the substance the word "vitamine" - an amine essential for life. Later it was demonstrated that this substance had no antineuritic activity, and consequently it was not the desired substance to cure a deficiency termed "beriberi". Between 1884 and 1912, it was established that beriberi in man³ and polyneuritis in fowls⁴ and rats⁴ are deficiency diseases caused by the lack of some substance which is present only in certain foods, and it was shown that rice bran is a relatively rich source of this necessary substance.

The isolation of the anti-beriberi substance (which in European literature is known as aneurin and in the American literature is called

thiamin) presented many difficulties. In 1926, Jansen and Donath obtained a crystalline substance having a great antineuritic activity. Eijkman confirmed the antineuritic activity of this substance against avian polyneuritis by adsorbing the substance on fuller's earth and then testing the curative properties of the different fractions on small rice birds.

From 1926 to 1934, crystalline preparations of the vitamin approaching purity were obtained, and the empirical formula was established beyond reasonable doubt. In 1936, Williams and independently, Grewe showed that thiamin has the structure represented by the following formula:

\[
\begin{align*}
\text{thiamin} & \text{ H}_{3}C - \text{N} - \text{CH}_{2} & \text{CH}_{2} \text{CH}_{2} \text{OH} \\
\text{N} & \text{H}_{2} & \text{N} \text{CH}_{3} & \text{CH}_{2} \text{CH}_{2} \text{OH}
\end{align*}
\]

Williams proposed the name, thiamine, (or thiamine chloride or hydrochloride) after its chemical nature as an "amine essential for life" and its structure containing the important thiazole ring. At present the terms vitamin B\text{\textsubscript{1}} and thiamin(e) are used interchangeably for the antineuritic substance once called anti-beriberi vitamin(e) or vitamin B.

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7 Williams, R. R., 1936, "Structure of vitamin B\text{\textsubscript{1}}", J. Am. Chem. Soc., 58, 1063.

8 Grewe, R., 1936, "Constitution of aneurine (vitamin B\text{\textsubscript{1}})", Z. Physiol. Chem., 242, 89.
Synthesis of this compound was achieved by Williams and Cline in 1936⁹ and by Andersag and Westphal in 1937.¹⁰

Thiamin appears to participate in the form of a pyrophosphorylated ester (cocarboxylase) as a coenzyme in several enzyme systems.¹¹ It is established that thiamin is essential in the metabolism of carbohydrates. The importance of thiamin in carbohydrate metabolism has been seen by increasing the amount of dietary carbohydrates and noting a proportional decrease in time before the clinical deficiency symptoms appear. Even when the clinical symptoms of a thiamin deficiency are absent, the deficient animals have an impaired ability to oxidize certain alpha-keto acids (i.e. pyruvate and alpha-keto glutarate). Thiamin is an essential factor for pyruvate and alpha-ketoglutarate decarboxylation¹² and as a coenzyme of transketolase.¹³, ¹⁴


Gunsalus and others\textsuperscript{15} demonstrated the necessary function of thiamin in pyruvate decarboxylation. They found that when thiamin was added to brain slices of thiamin-deficient animals, an increase in oxygen uptake (catatorulxin effect) along with an increase in carbon dioxide output was noted. The accumulated alpha-keto acids were metabolized in the presence of thiamin. The symptoms of a thiamin-deficiency have been demonstrated to be partly due to these products of perverted carbohydrate metabolism. Animals on low carbohydrate diet have a decreased requirement for thiamin. The low carbohydrate diet appears to have a vitamin-sparing action for thiamin.\textsuperscript{16}

Experimental evidence indicates that among the B-complex vitamins one or more vitamins may have a number of structural forms.\textsuperscript{17} The biological activity of each form differs for some animal species. The requirement for a B-vitamin in a given species of animals cannot be met by a structural form that is without activity for that species regardless of

\begin{flushright}
Peters, R. A., 1933, Ibid. 27, 2031, "The Catatorulin Test for Vitamin B\textsubscript{1}".
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how high its activity may be for another species of animals.

Vitamin activity within the organism takes various forms: (a) immediate vitamin itself; (b) bound form of vitamin; (c) vitamin precursors; (d) functional forms (coenzymes); and (e) catabolic products of vitamin metabolism. The requirements of thiamin are greater than other B-vitamins due to certain factors. The first factor is that thiamin is not stored in the body. The second factor is that thiamin is unstable unless phosphorylated. The third factor is that thiamin may have other than a catalytic function in the body.

The vitamin action of vitamin $B_1$ seems to be connected with the specific structure of the molecule. The different vitamin salts, as the hydrochloride, hydrobromide, sulfate, etc., and the pyrophosphate ester, cocarboxylase, all have a corresponding biological vitamin activity. The vitamin $B_1$-disulfide is also as active as the vitamin. Certain structural alterations of the vitamin cause disappearance of the vitamin activity. Inactive forms include thiochrome, dihydrovitamin $B_1$, oxychlorothiamin, and the products obtained from the sulfite cleavage of the vitamin. So far as is known thiamin is the only substance with vitamin $B_1$ properties which occurs naturally.

As has been mentioned earlier, a decrease in the need for thiamin occurs in the presence of a low-carbohydrate diet. This fact was utilized by Morgan and Yudkin to clarify the role of sorbitol (a sugar

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alcohol related to fructose and glucose) as a metabolizable carbohydrate. The authors postulated that the action of sorbitol might be one of three possibilities or any combination of these: (1) Sorbitol increases or changes the microfloral activity of the intestine to produce more active vitamin B₁; (2) Sorbitol increases the absorption through the intestinal membrane of any vitamin B₁ present; and (3) Sorbitol decreases the B₁ requirement within the organism. The experiments of Morgan and Yudkin demonstrated that sorbitol exerted a "thiamin-sparing" action upon rats on a thiamin-deficient diet.

The experimental evidence indicating the vitamin-sparing action of sorbitol on carbohydrate metabolism raised the question as to whether sorbitol allows carbohydrates to be metabolized along the glycolytic sequence or whether some new pathway might be involved. If the breakdown of carbohydrates still followed the glycolytic sequence, that is, if alpha-ketoglutarate dehydrogenase and pyruvic dehydrogenase were both active in a thiamin-deficient animal with sorbitol included within its diet, an increased synthesis of thiamin must be attributed to the intestinal microflora since no more thiamin was added to the diet.

The present study was initiated to determine the effect which sorbitol has upon the amount of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities present within the liver mitochondria. In addition, the effect of varying the dietary sorbitol concentration on the development of an avitaminosis was studied. The antimetabolite,

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pyrithiamin, was used because it has such a completely inhibitory effect upon thiamin that any vitamin 
B₁ present in trace amounts in the diet would be unable to exert a nutritive effect upon the animal.
LITERATURE REVIEW

From the overall standpoint, the progress of an avitaminosis generally follows a rather set pattern, in which the course of the disease is first manifest in general feelings of ill health, and loss of appetite. As the symptoms progress, there is a gradual decrease in tissue vitamin levels until the point is reached where clinical deficiency signs and symptoms occur. Symptoms which have been noted in the rat include a loss in weight, backward twisting of the head, convulsions (especially when spun by the tail), brain and muscular lesions, loss of equilibrium, a decrease in the heart rate and testicular degeneration.

There are three known major causes of thiamin deficiency in the higher animals. Of prime importance is the lack of an adequate nutritional source of thiamin which results in beriberi in man. Secondly, there exists an increased or higher requirement of the vitamin within the body due to (a) physiological conditions connected with vitamin metabolism, (b) the action of toxic agents or pathological conditions, and (c) the effect of antithiamin compounds.

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22 R. R. Williams, et al., op. cit. p. 399.
A great many factors may be able to exert an influence upon the requirements of thiamin in animals. If the above-mentioned factors were critically considered, the following points might be noted:

(a)* Physiological Conditions Connected With Vitamin Metabolism:

Since thiamin plays such an important role in the metabolism of carbohydrates, the percentage of carbohydrate present within the diet is significant. It had been noted prior to 1937 that an individual deprived of fat had a lowered metabolic rate. Many individuals were therefore misled into thinking that a low-fat diet decreased the body requirement for thiamin. Later, Evans and Lepkovsky found what they termed the "thiamin-sparing" action of fats. The action was simply

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*These letters refer to preceding paragraph.
a lowering of the amount of vitamin which must be supplied in the food due to the presence of fats.\textsuperscript{29,30} This lowering was regarded as equivalent to supplying a certain amount of the vitamin as explained by the authors. The postulated reason for lowering the vitamin requirement mentioned was later shown to be due to a lower content of carbohydrates present within the diet which had a thiamin-sparing effect on the organism. This decrease in vitamin need was followed by a gain in weight and increase in the general well-being of the animal. If carbohydrate is replaced by fat in a thiamin-deficient diet, the amount of thiamin required to establish any definite level of growth is always less than is required when the fat content is normal or low.\textsuperscript{26,30,33} Individual fats differed in their ability to inhibit the onset of deficiency symp-

\textsuperscript{29}Banerji, G. G., 1940, "Effect of a high fat diet on excretion of bisulphite-binding substances in urine of rats deficient in vitamin B\textsubscript{1}", Biochem. J., 34, 1329.


\textsuperscript{32}Gruber, M., 1950, "Nature of the vitamin B\textsubscript{1}-sparing action of fat", Nature, 166, 78.

\textsuperscript{33}Guerrant, N. B., and R. A. Dutcher, 1934, "Some effects of the composition of the diet on the vitamin B and vitamin G requirements of the growing rat", J. Nutr. 8, 397.
toms, the optimal sparing effect being obtained with fats containing C₈-fatty acids. Kemmerer and Steenbock found that the vitamin B₁ contents of the tissues were the same whether the animals were fed a high carbohydrate thiamin-containing diet or a high fat thiamin-containing diet: 0.25 - 0.5 grams of rat liver contained about one unit of vitamin B₁. Longennecker et al. found that fat seemed to be synthesized from carbohydrate and from protein only when thiamin is present. Fat deposition seems to be suppressed by excessively high-and-low thiamin intakes.

Thiamin is not involved in the enzyme systems necessary for the

34 Evans, H. M., S. Lepkovsky, and E. A. Murphy, 1934, "The sparing action of fat on vitamin B, VI. The influence of the levels of protein and vitamin G", J. Biol. Chem., 107, 429.
40 R. R. Williams, et. al., op. cit., pp. 386-7.
metabolism of fats. When fats are metabolized in large quantities the amount of thiamin required is much less than when carbohydrates are metabolized. DeCaro and Rindi produced a state of thiaminosis in rats as noted by an increase in blood pyruvate level, by feeding them a thiamin-deficient diet containing glucose as the carbohydrate. Substituting olive oil (2% of diet) for the glucose, six hours after absorption the authors found that the blood pyruvic acid decreased to exactly a "normal" level. DeCaro and Rindi suggest that thiamin doesn't share in fermentative systems involved in the metabolism of fats, and that an excess of fat in the diet causes the metabolism of fat in preference to carbohydrates and proteins.

Proteins also appear to exert a thiamin-sparing action. In presenting the effect of diet composition upon the thiamin requirements of the organism, mention should be made to the effect of super-

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charging of other vitamins upon the thiamin requirements. No effect or benefit is seen from vitamin supercharging of B-complex vitamins in the presence of a thiamin deficient diet. Studies of B-vitamin inter-relationships in growing rats show that high levels of various individual B-vitamins do not sufficiently influence weight gains of various animals deficient in other individual B vitamins. On the other hand, thiamin is not any more effective than other B vitamins in increasing the total fat content of rats, even when rats are fed diets deficient in other B vitamins. Lack of vitamin B1 influences riboflavin metabolism as seen by a lower riboflavin content of the tissues when rats had been on a thiamin-deficient diet.


47 Sarett, H. P., and W. A. Perlzweig, 1943, "The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats", J. Nutr. 25, 173.


A brief discussion of the effect of dietary composition upon thiamin excretion from the organism under various conditions is presented. The level of thiamin in tissues can be correlated with the level of thiamin fed, however, when more than 65 micrograms of thiamin were fed, no increase in body thiamin concentration was observed.52

An increased dietary consumption would entail a greater loss of thiamin from the tissues, thus depleting the thiamin reserve present in the form of cocarboxylase.32,53,54 In all known cases, thiamin-deficient subjects tend to excrete less thiamin than normal subjects.55 Thiamin excretion decreases as a linear function of the dietary intake until a point of minimum excretion occurs.56 This point is close to where the clinical deficiency symptoms appear.

In conditions associated with thiamin deficiency, the fasting blood pyruvate is elevated, and the pyruvate curve after glucose ingestion


is abnormally elevated and prolonged. This was earlier thought to be due to a higher blood sugar test of the animals. However, it has now become accepted that the pyruvate formed from the glucose breakdown builds up and is not metabolized further.

(b) The Action of Toxic Agents or Pathological Conditions:

A subject which has been followed with a great deal of interest throughout the years is the state of the intestinal microflora in vitamin-deficient animals. Dann was able to maintain rats for over a year on a thiamin-free, carbohydrate-free synthetic diet. The production of thiamin within the gut by microorganisms (refection) seems to have produced enough thiamin for protein and fat metabolism in these animals.

Antibiotics seem to enhance the utilization of both protein and

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energy compounds within the organism. Aureomycin\textsuperscript{60-64} and penicillin\textsuperscript{44,63,65-68} have both been shown to have a thiamin sparing action in animals on a thiamin-deficient basal diet. Their effect may, in part, be due to a reduced number of intestinal microflora which compete for vitamin B\textsubscript{1},\textsuperscript{69} or may in part be due to an alternate action\textsuperscript{63} of micro-


\textsuperscript{62}Lih, H., and C. A. Baumann, 1951, "Effects of certain antibiotics on the growth of rats fed diets limiting in thiamin, riboflavin or pantothenic acid", J. Nutr. 45, 143.


organisms within the intestinal tract to synthesize more of the vitamin or a combination of the above two factors. If the diet is high in carbohydrate (in general), the intestinal microflora is predominantly acidophilic or a non-proteolyzing type. If the diet is high in protein, the intestinal microflora is of a proteolyzing type.

When a penicillin-treated animal is sacrificed and the weight of the intestinal tract is compared to the intestinal tract of a normal grown rat, it is seen that the former animal has a lighter intestinal tract. This has been postulated to mean that a more efficient absorption from the gut in the penicillin-treated animal exists as the food passes on through the tract instead of remaining as is noted by an increase in size and contents of the caecum. This was demonstrated by

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75 Draper, H. H., 1958, "Adsorption of radiolysine by the chick as effected by penicillin administration", J. Nutr. 64, 33.
measuring the rate of radiolysine uptake in a thiamin-deficient animal. The absorption was increased in a penicillin-treated animal; at least the radiolysine uptake was increased.

Elvehjem showed that intestinal bacteria are able to synthesize large quantities of certain B-vitamins. If these bacteria are able to synthesize the vitamins then absorption of these vitamins from the gastro-intestinal tract would be the limiting factor for a vitamin deficiency to occur. If no vitamin were absorbed upon the first passage of the food through the gut, the only way the animal could receive the vitamin is by coprophagy. It has already been noted that some antibiotics stimulate growth of rats fed diets limiting in certain B-vitamins. It has been shown that 75-85% of the feces were consumed by rats being fed antibiotics. Penicillin did not stimulate growth of rats fed a diet limiting in thiamin when coprophagy was prevented, while the usual growth-stimulating effect was obtained when the rats were given access to their feces. Emerson found that the feeding of feces of a thiamin-deficient rat (5 µg thiamin daily) to the same rat caused no alleviation of the vitamin deficiency. Also, thiamin synthesized by the intestinal micro-

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flora was not absorbed to any appreciable extent upon its first passage through the intestines and therefore the animal would have to obtain this thiamin by coprophagy.77

Mameesh and Johnson79 found that, by the use of C\textsuperscript{14} labelled thiamin in the two-position, the amount of available thiamin supplied by the intestinal microflora in rats given diets either adequate or limiting in the vitamin was increased in the presence of 50 mg of penicillin per kilogram of diet.

Another antibiotic, Terramycin, had no growth stimulating effect upon animals fed diets suboptimal in thiamin, riboflavin or pantothenic acid.62,80 When aureomycin or terramycin were present along with penicillin, the thiamin-sparing action of penicillin was prevented.80

(c) Effect of Anti-thiamin compounds:

The thiamin antimetabolite, pyrithiamin, depresses growth of the test animals\textsuperscript{44} and its action has been postulated to occur at the step of cocarboxylase synthesis.81,82 It fails, however, to inhibit combi-


nation of cocarboxylase with its apoenzyme. The variation it has with thiamin is that the pyridine ring has replaced the thiazole moiety. A structure of pyrithiamin is given below:

![Structure of pyrithiamin](image)

Five moles of pyrithiam nullify one mole of thiamin very effectively.\(^8\)

Another derivative of thiamin (oxythiamin) has no vitamin action in rats, but is an active inhibitor of the vitamin.\(^9\) 40 moles of oxythiamin nullified one mole of thiamin.\(^9\)

The inhibitory effect for thiamin depends upon the presence of a 4-aminopyrimidine group in the antivitamin. It is interesting to note the effect which variations upon the thiazole moiety have upon thiamin activity. Chlorooxythiamin and bromooxythiamin, where the hydroxyl in the thiazole moiety has been replaced by halogen, showed no thiamin or antithiamin activity when administered at very high levels.\(^9\)

This seems to show the importance of the alcoholic group as one of the factors determining the antagonistic effect which oxythiamin has upon the organism.\(^9\) In general, it appears that rats require the thiamin

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\(^8\) Williams, R. R., et al., op. cit., p. 697.

molecule in its entirety, while plants and micro-organisms can utilize a mixture of pyrimidine and thiazole components for the synthesis of thiamin.

Hegsted\textsuperscript{85} determined the minimum level of thiamin for the rat to be 300-400 gamma per 100 grams of diet. However, Brown and Sturtevant stated that 125 gamma thiamin per 100 grams of food supplies the minimum requirements.\textsuperscript{86} The factors considered by these latter authors included (1) intensity of metabolism, (2) temperature of environment and (3) composition of the diet. Later Byerrum and Flokstra\textsuperscript{11} determined thiamin and thiamin pyrophosphate content of liver, muscle, and brain of rats fed on different levels of thiamin. They found that as the level of thiamin was increased up to 200 gamma per 100 grams of food, the thiamin pyrophosphate contents of the tissues increased; beyond that level to approximately 400 gamma, no further increase was found. It is therefore recommended that, for maximal cocarboxylase or thiamin pyrophosphate content of these tissues, twice the amount is required as is needed for normal growth. Normal growth occurs at 100-150 gamma per 100 grams of dietary food.\textsuperscript{31}

When dietary carbohydrates are completely absent, rats are able to survive for many months.\textsuperscript{16} Morgan and Yudkin\textsuperscript{19} studied sorbitol,


due to its close relationship to glucose and fructose, to see whether or not it was metabolized as ordinary carbohydrate. Sorbitol is metabolized as ordinary carbohydrate. Sorbitol is metabolized to fructose, then phosphorylated to fructose-6-phosphate and undergoes an eventual oxidation to pyruvic acid. Sorbitol is slowly absorbed through the intestines over a period of about three hours. It appears to remain long enough in the intestines to favor any organism which would be able to utilize it when given orally. If sorbitol solutions were injected interperitonially, a rapid absorption occurs into the tissues. When sorbitol was present in the synthetic diet, it decreased the ketone bodies of rats present within the liver. An increase in acetoacetate and a decrease in beta-hydroxybutyrate were seen.

When Morgan and Yudkin gave sorbitol to rats at levels of up to 20% of a carbohydrate-free diet without thiamin, they found the animals survived for 30 weeks and were still gaining weight. The same amounts of glucose led to loss in weight, polyneuritis and death in 5-10 weeks. When they added sorbitol to diets containing up to 40% glucose, 10% sorbitol gave at least as good growth as when added to carbohydrate-free diets.

The possibility that the sorbitol produces a change in the intestinal flora of the rats so that thiamin is synthesized and is avail-


able to the animal was investigated. This may occur as the following points indicate: (1) Sorbitol has a marked effect upon intestinal function producing diarrhea for 3-4 days. (2) Rats receiving sorbitol in their diets develop enlarged caeca, as compared to normal rats. (3) The tissues of rats on thiamin-free diets with sorbitol contain more thiamin than tissues of rats near death on thiamin-free diets with glucose.

One group of animals was given 20% sorbitol with only choline and inositol of the B-complex group. A set of controls were given only choline and inositol, but no sorbitol. This latter group died in 4-5 weeks from thiamin deficiency.

A second set of animals were given choline, inositol and thiamin with no sorbitol. These survived for three months but didn't gain weight. The animals in the first set which were receiving 20% sorbitol, choline and inositol gained weight over this three-month period.

A third set of animals were placed upon a diet with sorbitol but none of the vitamins of the B-complex group, and they also continued to grow well for more than three months. The thiamin-sparing action of sorbitol as first noted by Morgan and Yudkin, could not be confirmed by Mehnert and Mehnert. Upon repeating the experiments of Morgan and Yudkin they found that, in the absence of vitamin B₁, inclusion of sorbitol in the diet, whether alone or with a sugar, had no power to avert the signs of thiaminosis or ultimate death. They did, however, find

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the increase in the size of the caeca in rats given sorbitol, but there was no change in the character of the intestinal microflora. The original article by Mehnert and Mehnert was not available to the author, so the procedure followed by them could not be checked.

Jones and Baumann\textsuperscript{90} ran a series of tests in which penicillin, sorbitol or both together were added to thiamin-deficient diets varying in carbohydrate and fat content. Both sorbitol and penicillin increased the growth and survival of the thiamin-deficient rats. These authors confirm the paper of Morgan and Yudkin that rats fed sorbitol grow in the absence of thiamin. It also appeared that penicillin and sorbitol both act to increase the intestinal synthesis of thiamin, although no direct proof was given.

Wharton, et al.,\textsuperscript{91} studied the effects of sorbitol upon chicks and rats. They found that in the absence of thiamin, deficiency symptoms and almost a complete lack of growth were apparent by the seventh day. Neither sucrose or sorbitol eliminated the need for thiamin in the chicks. However, the rats fed a thiamin-deficient, sorbitol diet gained at essentially a normal rate throughout an eight-week period. In the case of the chicks, the addition of penicillin had no appreciable effect upon the onset of polyneuritis.

\textsuperscript{90}Jones, J. D. and C. A. Baumann, 1958, "Growth of Thiamin-deficient rats fed sorbitol or antibiotics in rations of varying fat content", \textit{J. Nutr.} \textit{66}, 383.

\textsuperscript{91}Wharton, F. D., Jr., J. C. Fritz, and L. J. Classen, 1959, "Studies on the ability of sorbitol and various sugars to enable chicks and rats to survive dietary deficiencies of single vitamins", \textit{J. Nutr.} \textit{69}, 74.
RESEARCH DESIGN

Over a period of nine months, three separate animal experiments were conducted. During the course of the experiments, each animal was allowed to eat and drink ad libitum. Liver mitochondria were used in this study, because of the organ's high metabolic activity and since some previous exploratory work had already been done on the effects of thiamin deficiency and thiamin antagonists on enzyme activities in this tissue.

The experimental animals utilized were male albino rat litter mates of the Sprague-Dawley strain. The rats were housed individually in conventional cages equipped with wire-bottom floors. The animals were weighed daily on triple beam balances. Upon arrival, all animals were given tap water ad libitum and were fed basal diet (diet "A") for three days (for constituents of diet see below). On the fourth day each group was placed on its specific diet. This fourth day, taken as zero day in the weight tables, is the day the experiment officially began.

All components of the diet were mixed in five kilogram quantities,


94 C. J. Gubler, in preparation.
and a supply of diet was kept frozen at -17°C except for a small amount which was administered to the animals daily. The components of the thiamin-deficient or "A" basal diet are given below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>sucrose</td>
<td>68.5%</td>
<td>3425 grams</td>
</tr>
<tr>
<td>casein (vitamin free)</td>
<td>20.6%</td>
<td>1100 grams</td>
</tr>
<tr>
<td>corn oil</td>
<td>5.4%</td>
<td>250 grams</td>
</tr>
<tr>
<td>salt mixture IV (see below)</td>
<td>4.5%</td>
<td>225 grams</td>
</tr>
<tr>
<td>vitamin mixture (see below)</td>
<td>variable</td>
<td>11.11 grams</td>
</tr>
<tr>
<td>sorbitol</td>
<td>variable</td>
<td>20 grams</td>
</tr>
<tr>
<td>choline chloride</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During the first five days the animals on the sorbitol-containing diets all experienced a weight loss and diarrhea. However, after this period, the animal adjusted to the diet and no more diarrhea was noted during the course of the experiment.

In preparing the salt mixture, the following points were observed:
(a) to furnish all mineral requirements, (b) to use a minimum number of salts, (c) all the salts should be inorganic, (d) the mixture should be easily prepared, (e) the base-forming elements should be in slight excess of acid-forming elements. Constituents of the salt mixture IV are as follows:

- CaCO₃: 1200 grams
- K₂HPO₄: 1290 grams
- CaH₂PO₄ * 2 H₂O: 300 grams
- MgSO₄ * 7 H₂O: 408 grams
- NaCl: 670 grams
- Fe(C₆H₅O₇) * 6 H₂O: 110 grams
- KCl: 3.2 grams
- MnSO₄ * H₂O: 15.2 grams
27

\[
\begin{align*}
\text{ZnCl}_2 & & 1.0 \text{ gram} \\
\text{CuSO}_4 \cdot 5 \text{H}_2\text{O} & & 1.2 \text{ grams} \\
\text{TOTAL} & & 3998.6 \text{ grams}
\end{align*}
\]

Use 900 grams per 20 kilograms diet

Constituents of the vitamin mixture are as follows:

- inositol \hspace{1cm} 40 grams
- nicotinic acid \hspace{1cm} 0.800 grams
- pyridoxine HCl \hspace{1cm} 0.120 grams
- riboflavin \hspace{1cm} 0.240 grams
- biotin \hspace{1cm} 0.004 grams
- folic acid \hspace{1cm} 0.010 grams
- 2-methyl-1,4-naphthoquinone \hspace{1cm} 0.080 grams
- p-aminobenzoic acid \hspace{1cm} 2.000 grams
- calcium pantothenate \hspace{1cm} 1.200 grams

TOTAL \hspace{1cm} 44.464 grams for 20 kilograms of diet

Vitamins A and D were given in the form of fortified halibut liver oil in the amount of three drops per rat per week added to the food. Thiamin and thiamin antagonist supplements were administered daily subcutaneously in the amounts indicated.

Chemicals were obtained from the following sources: Pyridoxine hydrochloride, nicotinic acid and d-pantothenate from Eastman Organic Chemicals; pyrithiamin from Merck and Company, Inc; L-inositol, menadione, folic acid and vitamin-free casein from Nutritional Biochemicals Corporation; riboflavin (practical), d-biotin (U.S.P.), thiamin hydrochloride, d-sorbitol and choline chloride from the California Foundation for Biochemical Research; Hengar granules (selenized - BKH 51137) and
magnesium chloride from Van Waters and Rogers Company; adenosine-5'—tri-
phosphate (dibarium salt), alpha-ketoglutaric acid and ethylenediamine
tetraacetic acid (disodium salt) from Sigma Chemical Company; para-
amino benzoic acid from Brothers Chemical Company; Haliver Oil (forti-
ified halibut liver oil) from Parke Davis and Company; corn oil (mazola)
from Corn Products Company, New York; sucrose (U.S.P. grade) from U.
& I. Sugar Company; potassium ferricyanide (analytical), cupric sulfate
(anhydrous-analytical), calcium carbonate (U.S.P.) and hydrogen peroxide
30% (analytical) from Mallinckrodt Company; potassium sulfate, ferric
citrate (reagent), potassium phosphate (monobasic, reagent), and zinc
chloride from Fisher Scientific Company; sodium phosphate monobasic,
monohydrate (reagent), manganous sulfate, monohydrate, (reagent) and
ammonium sulfate (reagent) from Baker & Company; potassium iodide
(analytical) and potassium hydroxide (reagent) from Wasatch Chemical Com-
pany; iodine (reagent), magnesium sulfate and calcium phosphate (purified)
from General Chemical Division; sodium pyruvate from pyruvic acid,
April, 1957, prepared by Dr. Clark J. Gubler; triple-distilled mercury.

Other chemicals common to the laboratory were obtained from
commercial sources. Deionized distilled water was obtained by passing
distilled water through a mixed-bed ion-exchange column.

The first experiment was used primarily to determine the optimum
growth rates with varying concentrations of sorbitol. An outline of
this experiment is given in Table I.
### TABLE I

**Outline of First Experiment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Diet</th>
<th>vitamin supplements</th>
<th>time spent on diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>14</td>
<td>thiamin-deficient (basal)</td>
<td>None</td>
<td>18-32 days</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>basal with 5%* sorbitol</td>
<td>None</td>
<td>27-37 days</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>basal with 10% sorbitol</td>
<td>None</td>
<td>39-43 days</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>basal with 20% sorbitol</td>
<td>None</td>
<td>46-48 days</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>basal</td>
<td>10 µg thiamin/100 gm rat/day</td>
<td>18-34 days</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>basal with 20% sorbitol</td>
<td>10 µg thiamin/100 gm rat/day</td>
<td>26-46 days</td>
</tr>
</tbody>
</table>

*Sorbitol was added at the expense of the sucrose component.

The second and third experiments were conducted by dividing the rats into six new groups. For the sake of space, these two experiments will be combined as the groups in both cases were the same.

### TABLE II

**Outline of Second and Third Experiments**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Diet</th>
<th>vitamin supplements</th>
<th>time spent on diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>22</td>
<td>thiamin-deficient (basal)</td>
<td>None</td>
<td>16-28 days</td>
</tr>
<tr>
<td>II</td>
<td>19</td>
<td>thiamin-deficient (basal)</td>
<td>10 µg thiamin/100 gm rat/day</td>
<td>16-37 days</td>
</tr>
<tr>
<td>III</td>
<td>17</td>
<td>basal with 20% sorbitol</td>
<td>None</td>
<td>19-70 days</td>
</tr>
<tr>
<td>IV</td>
<td>18</td>
<td>basal with 20% sorbitol</td>
<td>50 µg pyrithiamin/100 gm rat/day</td>
<td>11-52 days</td>
</tr>
<tr>
<td>V</td>
<td>51</td>
<td>basal with 20% sorbitol</td>
<td>10 µg thiamin/100 gm rat/day</td>
<td>11-70 days</td>
</tr>
<tr>
<td>VI</td>
<td>16</td>
<td>basal with 20% sorbitol</td>
<td>10 µg thiamin/50 µg pyrithiamin/100 gm rat/day</td>
<td>18-60 days</td>
</tr>
</tbody>
</table>
No attempt was made to prevent coprophagy. It is recognized that the vitamin-deficient rat is capable of consuming his feces, although it was felt that the use of the wide-screen cages greatly lowered the percentage of feces consumed (50-60%) as reported by Barnes.95

**Methods**

**Preparation of Rat Liver Mitochondria**

The rats were sacrificed by decapitation after a blow on the head. The liver was rapidly removed and placed in a cold isotonic sucrose solution (0.25 M) immersed in ice. The liver was blotted to free it of excess fluid, and then one to ten grams of liver were taken for the study. The liver was homogenized in a cold 0.25 M sucrose solution with a Potter-Elvehjem homogenizer for about two minutes, during which time the homogenizer tube was immersed in cracked ice.

The liver homogenate was then transferred to a measuring cylinder or graduated centrifuge tube, and the volume made equal to ten times the weight of the liver used with 0.25 M sucrose. The homogenate was then placed in the Serval or high-speed International Centrifuge (IEC) attachment, using a lusteroid or other suitable tube and centrifuged for ten minutes at 600 x g. The supernate was carefully poured into a second tube. The sediment was resuspended in about 1/4 of the original volume of 0.25 M sucrose solution and recentrifuged for ten minutes at 600 x g.

---

as above. The washings were then carefully poured into the second tube containing the first supernate. The sediment of nuclei and debris was discarded.

The supernate and washings were combined and centrifuged at 8500 x g in the high speed head #295 or #296 of the IEC refrigerated centrifuge. The supernate was then carefully poured off and the sediment (mitochondria) resuspended in about one-fourth volume of 0.25M sucrose and centrifuged for ten minutes at 8500 x g. This washing may be repeated more than once. The washed mitochondria were then combined and homogenized lightly with a spatula after addition of a small portion of cold 0.25M sucrose. This process is repeated with shaking until the solution is uniform and the mitochondrial suspension is made up to a volume equal in milliliters to the original amount of liver utilized (i.e. if three grams of liver were originally taken and homogenized, the final volume of the mitochondrial suspension should be three milliliters).
Spectrophotometric Determination of Alpha-Keto Acid Oxidase Activity With Ferricyanide as Electron Acceptor

Reagents:

K₃Fe(CN)₆ - stock solution 0.333 M or 10.94 grams/100 ml. working solution: 0.0067 M or 1 ml diluted of stock solution to 50 ml. Use 0.5-0.7 ml per cuvette.

0.25 M sucrose: dissolve 85.6 grams sucrose with boiled, cooled, deionized distilled water and make to one liter.

0.2 M MgCl₂ - 4.93 grams per 100 ml deionized distilled water.

0.6% versene - 0.0161 M: dissolve 0.6 grams in 100 ml deionized distilled water.

Adenosine-5'-triphosphate - 0.06 M or 7.48 mg per 20 ml. Dissolve in 18.7 ml of deionized distilled water and add 1.3 ml 2 N KOH. This should give a pH of 7.4. Check. Use 0.1 ml per tube or 6 micromoles.

KOH - 2 N: 112.2 grams per liter in deionized distilled water.

Buffer: KH₂PO₄-K₂HPO₄ to a pH of 7.4. 0.15 M Prepare 0.15 M KH₂PO₄ or 20.4 grams per liter deionized distilled water and 0.15 M K₂HPO₄ or 34.3 grams per liter deionized distilled water. Add 192 ml KH₂PO₄ solution to 808 ml of K₂HPO₄ solution. pH should be 7.4. Check and adjust if necessary.

Substrates:

0.2M Pyruvate - sodium salt: Weight 4.40 mg sodium pyruvate and dissolve in circa 18 ml deionized distilled water. Check pH. If necessary, adjust pH to 7.0 with dilute NaOH. Make up to 20 ml. Use 0.1 ml per tube or 20 micromoles.

0.2 M alpha-ketoglutarate: Weight out 292 mg acid salt in 10 ml or 584 mg per 20 ml. Dissolve in circa 15 ml deionized distilled water, and titrate to pH 7.7.4 with 2N NaOH. Make to volume. Use 0.1 ml per tube or 20 micromoles.
Enzyme source: Liver mitochondria - from 1 gram of liver in 1 ml final volume, remove an aliquot and dilute with \( \frac{4}{4} \) volumes of cold 0.25 M sucrose to one volume of liver mitochondrial suspension.

The samples are prepared directly in the cuvettes as follows:

<table>
<thead>
<tr>
<th>Blank</th>
<th>micromoles</th>
<th>endogenous</th>
<th>sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer 0.15 M</td>
<td>75</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>MgCl(_2) (0.2 M)</td>
<td>20</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>ATP (0.06 M)</td>
<td>6</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Substrate (0.2 M)</td>
<td>20</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Versene (0.016 M)</td>
<td>1.6</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Enzyme suspension - 0.05 -</td>
<td>0.2 ml as required</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose (0.25 M) volume required to bring total volume to 2.3 ml (i.e. 1.35-1.20 ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferricyanide (0.0067 M)</td>
<td>4.6</td>
<td>0.7 ml</td>
<td>0.7 ml</td>
</tr>
</tbody>
</table>

All cuvettes are made up by additions in the order shown. After adding the ferricyanide and mixing the contents of the cuvette, readings are made in turn against a blank containing enzyme suspension, buffer, and water at 0, 5, 10, 15, 20, 25 and 30 minute intervals at 420 m\(\mu\) in the Beckman DU Spectrophotometer through the frosted side of the cuvette. The relative times between readings are kept constant for the respective cuvettes. The reading for each cuvette at zero time is subtracted from the reading at each time interval. This gives the changes in optical density (\(\Delta OD\)) at the given time interval. Then the blank \(\Delta OD\) is subtracted from the sample \(\Delta OD\) for each time period. Plot the \(\Delta OD\) on the ordinate against the time on the abscissa.

A sample of each enzyme suspension is saved for total nitrogen determinations so the enzyme activity can be reported in terms of total nitrogen content as the basis. Enzyme activity is reported as \(\Delta OD/0.1 \text{ mg N} / 30 \text{ minutes}\).
Determination of Total Nitrogen

**Reagents:** Concentrated Sulfuric Acid containing 0.5 grams copper selenite per liter.

CuSO₄ : K₂SO₄ (1:0) powdered mixture

Superoxol - 30% Hydrogen Peroxide

Ammonium Sulfate Standard: add 4.714 grams and dilute to one liter with deionized distilled water. Remove a 1.0 ml aliquot and dilute to 10 ml. This gives 0.1 mg N per 10 ml.

**Procedure:**

A sample containing from 1-5 mg nitrogen was pipetted accurately into a Kjeldahl microdigestion flask. Two ml digestion mixture (concentrated sulfuric acid) and a small pinch (0.5 gm) of CuSO₄:K₂SO₄ mixture was added to the sample. The solution was digested until clear or amber-colored. It was cooled and a drop or two of superoxol added and digested again until water-clear. If any amber remains, the superoxol treatment was repeated. Digestion was continued for one to four hours. The solution was cooled and transferred quantitatively to a 50 ml volumetric flask. The digested solution was made to volume with deionized distilled water.

The nitrogen was determined spectrophotometrically by direct nesslerization as follows: A 10 ml aliquot of sample was removed from the 50 ml volumetric flask and transferred to a 100 ml volumetric flask. The sample was diluted to about 60 ml with deionized distilled water. Ten ml of Nessler's reagent was added and the solution diluted to 100 ml with deionized distilled water. The mixture was allowed to stand five minutes. The absorbance of the sample was measured after five minutes in a spectrophotometer at 480 μ. A standard sample of ammonium
sulfate containing 1 mg nitrogen was carried through the same procedure.

Preparation of Nessler's reagent: To a solution of 22.5 grams of iodine in 20 ml. deionized distilled water, 30 grams KI followed by 30 grams of triple distilled mercury were added. The mixture was cooled under the tap with shaking until the supernatant liquid had lost its color due to iodine. It was then decanted and the supernate tested with a drop of 1% starch solution. The test should be positive. The supernate was diluted to 200 ml and well mixed and then added to 975 ml. of an exactly 10% NaOH solution. After mixing the reagent was allowed to stand until clear.
EXPERIMENT RESULTS

EXPERIMENT I

Table III and figure I show the effects of varying sorbitol concentrations upon growth rate as obtained from experiment I. The rats in group A, which received the basal thiamin-deficient diet, developed clinical deficiency symptoms and had almost ceased to grow by the ninth day. These animals experienced an initial increase in weight due to thiamin present in their tissues prior to the beginning of this experiment. These rats lost weight rapidly after the ninth day, and all animals had been sacrificed by the thirty-second day. The rats in group E, which received 10 µg thiamin per day, gained weight at a normal rate of about six grams per day throughout the experiment.

All rats which received the thiamin-deficient basal diet containing various levels of sorbitol (groups B, C, and D) continued to grow over a seven-week period at a rate similar to that of the control animals on the basal diet supplemented with thiamin (group E). There was a time lag in weight gain of two to five days with the rats fed diets containing the higher amounts of sorbitol. These animals all had diarrhea during the first week of the experiment. The rats receiving sorbitol developed greatly enlarged caeca; their contents were equally increased and were much more fluid than the control rats receiving no sorbitol in their diets but receiving thiamin daily.
The rats in group B, which were on the basal diet plus 5% sorbitol showed a good growth rate for the first ten days after which a levelling-off of the rate occurred. In the case of group C, which were on the basal diet plus 10% sorbitol, a good growth rate was observed for the first fifteen days. This was followed by a moderate decrease in rate for the next fifteen days. Group D rats, which were on the basal diet plus 20% sorbitol, had a growth rate comparable to that for group E rats (control).

The animals in group F, which were on the 20% sorbitol diet plus 10 µg thiamin injected daily, had a growth rate comparable to that shown by the animals in group E (control).

The growth curves for the groups in this experiment can only be interpreted accurately up to the day when the animals of the group began to be sacrificed. This occurred on the following days for the various groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>18</td>
</tr>
<tr>
<td>Group B</td>
<td>27</td>
</tr>
<tr>
<td>Group C</td>
<td>39</td>
</tr>
<tr>
<td>Group D</td>
<td>46</td>
</tr>
<tr>
<td>Group E</td>
<td>18</td>
</tr>
<tr>
<td>Group F</td>
<td>27</td>
</tr>
</tbody>
</table>

Interpretation of the curves after this date is uncertain. In some cases, the last readings from the tables were left off of the figures as they were too erratic to be included.
TABLE III
Average Weight Gained by Animals on Diets Containing Varying Amounts of Sorbitol with and without Vitamin Supplement: Experiment I

<table>
<thead>
<tr>
<th>Supplement</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol %</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Thiamin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10 µg/100 gm</td>
<td>10 µg/100 gm</td>
</tr>
</tbody>
</table>

Time in Days:

<table>
<thead>
<tr>
<th>Days</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>7</td>
<td>4</td>
<td>-2</td>
<td>5</td>
<td>3</td>
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<td>5</td>
<td>28</td>
<td>31</td>
<td>23</td>
<td>8</td>
<td>33</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>51</td>
<td>48</td>
<td>20</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>15</td>
<td>36</td>
<td>64</td>
<td>75</td>
<td>53</td>
<td>94</td>
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</tr>
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<td>23</td>
<td>63</td>
<td>91</td>
<td>78</td>
<td>119</td>
<td>112</td>
</tr>
<tr>
<td>25</td>
<td>7</td>
<td>62</td>
<td>97</td>
<td>96</td>
<td>130</td>
<td>125</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>74</td>
<td>109</td>
<td>114</td>
<td>152</td>
<td>142</td>
</tr>
<tr>
<td>35</td>
<td>-</td>
<td>80</td>
<td>114</td>
<td>132</td>
<td>-</td>
<td>160</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>-</td>
<td>113</td>
<td>145</td>
<td>-</td>
<td>171</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>156</td>
<td>-</td>
<td>174</td>
</tr>
</tbody>
</table>
KEY TO TABLE 1 - Group A: 0% sorbitol, thiamin-deficient diet; Group B: 5% sorbitol, no thiamin; Group C: 10% sorbitol; no thiamin; Group D: 20% sorbitol, no thiamin; Group E: 0% sorbitol, thiamin-deficient diet, 10 micrograms thiamin injected daily subcutaneously per 100 grams body weight; Group F: 20% sorbitol, 10 micrograms thiamin injected daily per 100 grams body weight.
EXPERIMENT II

The purpose for this second experiment was to determine the growth rate for animals on this optimum sorbitol concentration (20%) in the presence and absence of thiamin and the thiamin antagonist, pyrithiamin. Five moles of pyrithiamin effectively counteract one mole of thiamin. The duration of this study was ten weeks. The weight gains are summarized on Table IV and figure 2.

The growth rate for groups I and II are very similar to the growth rate of groups A and E, respectively, in the previous experiment, i.e. the groups on the thiamin-deficient basal diet without and with thiamin supplements, respectively.

Group III had a good continued growth rate to about thirty-five days. The drop in weight gain after this date may have been due to the fact that one of these animals was sacrificed each day in the order III-1, III-2, etc., at this time.

Group IV, which received 50 µg pyrithiamin per day along with 20% sorbitol, gained weight only slowly for the first seven days after which the growth rate exceeded that of the control group V. These animals continued at a good growth rate for the duration of the experiment. This is interesting in view of the results of a previous study on the effects of pyrithiamin on growth rate in rats. In this study, rats were fed a basal thiamin-deficient diet with supplements of 50 µg pyrithiamin and 10 µg thiamin injected daily, but received no sorbitol. These animals

96 Gubler, Clark J., in preparation.
showed a near normal growth rate for about five days, and then exhibited a rapid loss in weight followed quickly by convulsions and death. Rats receiving 50 µg pyrithiamin alone died sooner than those receiving 50 µg pyrithiamin with 10 µg of thiamin. In contrast to these earlier results, both groups in the present study which received pyrithiamin, but with 20% sorbitol in the diet, continued to grow well throughout the course of the experiment. In fact, two animals of this above-mentioned group IV lived fifty-two days while receiving 20% sorbitol diet and 50 µg pyrithiamin. These animals were gaining weight at a rate of about two grams daily when they were sacrificed.

According to figure 2, group V, which received 20% sorbitol and 10 µg thiamin per day, showed only a slightly lower than normal growth rate until the twenty-fifth day. At this time, some rats of this group were sacrificed. This may explain the apparent decrease in growth rate at this time. Subsequent to the twenty-fifth day, the remaining rats continued to grow at a normal rate.

The rats of group VI, which received 20% sorbitol, and 10 µg thiamin plus 50 µg pyrithiamin as supplements, showed the usual five-day lag in growth characteristic of sorbitol-fed rats, but from the fifth to thirtieth days, their growth rate was nearly normal. At this time some of the rats which began to lose weight were sacrificed. The remaining rats then continued on with a normal growth rate until they were sacrificed on the fifty-sixth day.
TABLE IV

Average Weight Gain of Animals Receiving
the Basal Diet with and without 20% Sorbitol
and with Vitamin and Vitamin-Antagonist Supplement:
Experiment II

<table>
<thead>
<tr>
<th>Supplement</th>
<th>AVERAGE WEIGHT GAIN IN GRAMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0</td>
</tr>
<tr>
<td>Thiamin</td>
<td>10 µg/100 gm</td>
</tr>
<tr>
<td>Pyrithiamin</td>
<td>50 µg/100 gm</td>
</tr>
</tbody>
</table>

Time in Days:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-2</td>
<td>10</td>
<td>3</td>
<td>9</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>23</td>
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</table>
Figure 2

KEY - Group I: 0% sorbitol, thiamin deficient diet; Group II: 0% sorbitol, thiamin deficient diet, 10 micrograms thiamin injected daily subcutaneously per 100 grams body weight; Group III: 20% sorbitol, no thiamin; Group IV: 20% sorbitol, 50 micrograms pyridoxine injected daily subcutaneously per 100 grams body weight; Group V: 20% sorbitol, 10 micrograms thiamin injected daily subcutaneously per 100 grams body weight; Group VI: 20% sorbitol, 10 micrograms thiamin and 50 micrograms pyridoxine injected daily subcutaneously per 100 grams body weight.
EXPERIMENT III

In the last study (experiment III), the animals were placed in groups and given the same diets as those with the corresponding roman numerals in experiment II. The results of experiment III are very similar to those found in experiment II as far as weight gains shown by the various groups (Table V and figure 3). Group I showed a normal weight gain up to the ninth day; thereafter a rapid loss in weight occurred. Group IV which received 50 µg pyrithiamin per day showed a longer time lag before normal weight gain began. However, after 15 days the growth rate for this group was comparable to that for the other groups receiving 20% sorbitol and for the control group. As was to be expected, in both studies (experiment II and III), the group receiving no sorbitol and 10 µg thiamin injected daily experienced the fastest rate of growth (about six grams per day). In this experiment, as in experiment II, all animals receiving 20% sorbitol showed a growth rate comparable to that for the control group V which received the sorbitol plus 10 µg thiamin injected daily.

In addition to the growth rates, the activities of the thiamin-independent enzymes, alpha-ketoglutarate and pyruvate dehydrogenases, were measured in liver mitochondria. These activities are summarized in Table VI below; in addition individual animals activities for these enzymes are presented in graph form in figures 4 and 5.

Animals which received no sorbitol on the thiamin-deficient basal diet (Group I) showed the lowest activities for both alpha-keto acid dehydrogenases as compared to all other groups tested.
TABLE V

Average Weight Gain of Animals Receiving a Basal with and without 20% Sorbitol Diet with Vitamin and Vitamin-Antagonist Supplement: Experiment III

<table>
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<tr>
<th>Supplement</th>
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<th>IV</th>
<th>V</th>
<th>VI</th>
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Time in Days:

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</table>
Figure 3

**KEY** - Group I: 0% sorbitol, thiamin deficient diet; Group II: 0% sorbitol, thiamin deficient diet, 10 micrograms thiamin injected daily subcutaneously per 100 grams body weight; Group III: 20% sorbitol, no thiamin; Group IV: 20% sorbitol, 50 micrograms pyritihiamin injected daily per 100 grams body weight; Group V: 20% sorbitol, 10 micrograms thiamin injected daily subcutaneously per 100 grams body weight; Group VI: 20% sorbitol, 10 micrograms thiamin and 50 micrograms pyritihiamin injected daily subcutaneously per 100 grams body weight.
TABLE VI

The oxidation of alpha-ketoglutarate and pyruvate by liver mitochondria from rats on various diets as carried out in experiment III. Ferricyanide as acceptor.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Percent Sorbitol</th>
<th>Vitamin Supplement µg/100 gm/day</th>
<th>α-ketoglutarate AOD/30 min/0.1 mg N</th>
<th>Pyruvate AOD/30 min/0.1 mg N</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>12</td>
<td>0</td>
<td>None</td>
<td>0.334 ± 0.0235</td>
<td>0.154 ± 0.0202</td>
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<tr>
<td>II</td>
<td>11</td>
<td>0</td>
<td>thiamin µg</td>
<td>0.474 ± 0.0285</td>
<td>0.239 ± 0.0174</td>
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<tr>
<td>III</td>
<td>9</td>
<td>20</td>
<td>thiamin µg</td>
<td>0.880 ± 0.0224</td>
<td>0.535 ± 0.0273</td>
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<tr>
<td>IV</td>
<td>8</td>
<td>20</td>
<td>pyrithiamin 50 µg</td>
<td>0.458 ± 0.0336</td>
<td>0.233 ± 0.0242</td>
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<tr>
<td>V</td>
<td>25</td>
<td>20</td>
<td>thiamin 10 µg</td>
<td>0.981 ± 0.0282</td>
<td>0.434 ± 0.0247</td>
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<tr>
<td>VI</td>
<td>7</td>
<td>20</td>
<td>thiamin 10 µg, pyrithiamin 50 µg</td>
<td>0.997 ± 0.0350</td>
<td>0.399 ± 0.0354</td>
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</table>

Group II, which received a sorbitol-free diet, and 10 µg thiamin daily, showed a significant increase in both enzyme activities as compared to Group I. In addition, the animals receiving no thiamin but 20% sorbitol in their diets (Group III) showed almost a three-fold increase in both dehydrogenase activities over the activities seen in Group I receiving no sorbitol or thiamin. When a 10 µg daily supplement of thiamin was given along with a diet containing 20% sorbitol, the animals in this group (Group V) showed a two-fold increase in both alpha-keto acid dehydrogenase activities as compared to Group II which received thiamin but no sorbitol. Those animals which received 10 µg of the thiamin antagonist, pyrithiamin, (Group IV) showed a significant decrease in enzyme activities on 20% sorbitol diet when compared to animals which received a 20% sorbitol diet but no pyrithiamin (Group III) and animals receiving
10 µg thiamin and a 20% sorbitol diet (Group V). When thiamin and pyri-thiamin were both injected, (Group VI) the animals on a 20% sorbitol diet showed an increase in enzyme activity over those animals receiving pyrithiamin alone (Group IV). In addition, the animals in Group VI, had enzyme activities quite comparable to those of Group V, which re-ceived thiamin but no pyrithiamin and were used primarily as controls.
Legend for Figure 4 - The oxidation of alpha-ketoglutarate by liver mitochondria of rats on various diets with Ferricyanide as electron acceptor. Reaction Mixture: Potassium phosphate buffer pH 7.4, 75 μ moles; MgSO₄, 20 μ moles; sodium versenate, 2 μ moles; ATP, 6 μ moles; alpha-keto acid, 20 μ moles; liver mitochondrial suspension (1:5) -0.1-0.4 ml, K₃Fe(CN)₆, 4.66 μ moles; and 0.25 M sucrose to 3.0 ml. A line is drawn for the average enzyme activity with its value for each group.
Legend for Figure 5 - The oxidation of pyruvate by liver mitochondria of rats on various diets with ferricyanide as electron acceptor.

Reaction Mixture: Potassium phosphate buffer pH 7.4, 75 µ moles; MgSO₄, 20 µ moles; sodium versenate, 2 µ moles; ATP, 6 µ moles; alpha-keto acid, 20 µ moles; liver mitochondrial suspension (1:5) -0.1-0.4 ml, K₂Fe(CN)₆, 4.66 µ moles; and 0.25 M sucrose to 3.0 ml. A line is drawn for the average enzyme activity with its value for each group.
DISCUSSION

The ability of dietary sorbitol to overcome the effects of thiamin deficiency on the growth rate of experimental animals, as demonstrated by others,\textsuperscript{19,90,91} has been adequately confirmed in the rat by the present study. It has been shown that the growth-promoting or thiamin-sparing activity is proportional to the sorbitol content in the diet, where the sorbitol content is increased at the expense of sucrose. A concentration of 20\% sorbitol was found to be optimal for growth. By merely cutting down on the sucrose content and replacing it by protein, or particularly by fat, a thiamin-sparing effect is evident, but this thiamin-sparing effect of sorbitol is much more pronounced than can be accounted for by a simple reduction in the dietary carbohydrate by an equivalent amount. One must then conclude that the thiamin-sparing activity of sorbitol is largely due to some more specific action.

There is some evidence that sorbitol may exert this effect by changing the intestinal microflora in some way so that more thiamin is produced or less consumed by these microorganisms with the net result that more thiamin is available in the feces.\textsuperscript{19,90} This would then necessitate coprophagy in order for the animal to obtain this added available thiamin. However, the proof for this as the sole or predominant mechanism for the thiamin-sparing action of sorbitol is not conclusive.

In order to explore some other possible mechanism for this action
of sorbitol, the effect of a thiamin antagonist (pyrithiamin) was studied in the presence of sorbitol. Also, in an attempt to determine whether sorbitol was acting by making more thiamin available to the tissues for the formation of thiamin-dependent enzymes (particularly alpha-keto-glutarate and pyruvate dehydrogenases) or by allowing the animal to utilize some alternative pathway of metabolism which didn't require thiamin (a truly thiamin-sparing action), the activities of these two enzymes were measured in the liver mitochondria. The results obtained are somewhat surprising. Instead of the activities of these enzymes being normal or low in thiamin-deficient liver from sorbitol-fed rats, as is found in uncomplicated thiamin deficiency in this study and a previous study by Gubler,97 these enzyme activities are about two to three times as great in sorbitol-fed (20%) animals as in straight thiamin-deficient animals. Even more striking is the finding that the levels are still twice as great as those found in thiamin-treated animals which were not fed sorbitol. In animals fed with 20% sorbitol diets, a slight enhancement of liver alpha-keto acid dehydrogenase activities was found after giving thiamin supplements. Pyrithiamin administration reduced the enzyme levels by half in 20% sorbitol-fed rats. The effects of pyrithiamin were completely reversed by 10 µg thiamin per day in animals on the 20% sorbitol diet.

In order to check the validity of the average enzyme values given

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In Table VI, it was felt advisable to calculate levels of "t" score to determine the significance of the difference between the means of the various groups. When the results between groups I and II on sorbitol-free diets were compared, a "t" value of 3.81 for alpha-ketoglutarate dehydrogenase and 3.21 for pyruvate dehydrogenase were calculated. Both results indicate that less than 1% of the time these values are arrived at by chance alone. When the alpha-keto acid dehydrogenase activities between the two control groups (II and V), which received thiamin, were compared, a "t" value of 11.08 for the alpha-ketoglutarate dehydrogenase was obtained. This corresponds to a level of less than 1% by chance. In this latter case of comparison, a "t" value of 2.48 for the pyruvate dehydrogenase was obtained, which corresponds to a level of 5% significance. When both thiamin-deficient groups (I and III) which received diets with and without sorbitol, were compared, "t" values for alpha-ketoglutarate and pyruvate dehydrogenases showed 16.17 and 13.04, respectively, indicating a level of less than 1% by chance alone. If group III, which received 20% sorbitol and no thiamin, and group V, which received 20% sorbitol and thiamin were compared for validity, "t" values of 2.10 and 2.31 were obtained for the alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase, respectively. These both show levels of significance of between 1 and 5% for the enzyme activities. When both groups receiving pyrithiamin and 20% sorbitol diets, with and without thiamin supplement, were evaluated, "t" values of 11.09 and 3.95 were found for alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase, respectively. Both values corresponded to levels of significance of less than 1%.
These results indicate that thiamin deficiency in rats causes a decrease in growth rate and significant reductions in the activities of alpha-ketoglutarate and pyruvate dehydrogenases in liver mitochondria. The inclusion of 20% sorbitol in the diet essentially raised the growth rate to normal and resulted in activities of the above enzymes which were double those in the controls without sorbitol. Pyrithiamin administration to sorbitol-fed rats without thiamin reduced the high enzyme levels to values corresponding to the normal control levels. However, inclusion of 10 µg of thiamin completely reversed this pyrithiamin effect.

These findings with respect to the enzyme activities were supported by the corresponding effects of these various conditions on the growth rates. The results of this study suggest that there is more to the mechanism of the thiamin-sparing action of sorbitol than is at present understood. The high thiamin enzyme activities in the tissues cannot be produced merely by increasing the available thiamin by dietary means or by injection. It suggests some sort of adaptive process in the presence of excess sorbitol, in addition to the making of more thiamin available. This aspect of the problem bears further study.

In making recommendation for further work in this area, the author would like to suggest the following:

1. A temperature-and humidity-controlled animal room for more uniform results and comparison between experiments. The reported experiments covered a period of nine months, that is during summer and winter climate conditions, so no uniform temperature control was present.
2. A comparison between the enzyme activities of animals, being injected with 10 µg thiamin and 50 µg pyrithiamin and also receiving 50 µg pyrithiamin, on basal thiamin-deficient diets and diets containing 20% sorbitol.

3. A better homogenization procedure in the last suspension of liver mitochondria in 0.25 M cold sucrose. It seems as though the use of a spatula to make a solution "uniform" does not adequately insure homogenity.

4. An animal room where undergraduate students are not allowed. This is felt to be necessary as these students do not seem to understand that nutritional experiments must not include candy bars, wrappers and chalk in the diets of the experimental animals. Animals on nutritional experiments should not be disturbed in any way.
SUMMARY AND CONCLUSIONS

The effect of sorbitol on the activities of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase were measured spectrophotometrically by following the disappearance of ferricyanide absorption at 420 nm in the Beckman DU Spectrophotometer. When 20% sorbitol was introduced in the diet at the expense of the carbohydrate concentration, a marked increase of activity in both enzyme assays was seen.

When the sorbitol concentration was present in increasing amounts, the growth rates of the animals increased in proportion to the percentage of sorbitol present within the diet.

A sorbitol-containing diet has a vitamin-sparing action for the rat. As the presence of trace amounts of thiamin would be the same in the case of the vitamin-deficient diet and for the 20% sorbitol diet, there could be no greater absorption of the vitamin through the intestinal wall from the diet.

It is concluded that sorbitol alters the intestinal microflora so that more thiamin is produced by the microflora or less thiamin is used by them. This increased thiamin production occurs along with an increase in alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities. However, proof of this as the sole mechanism for the thiamin-sparing action of sorbitol is not conclusive.
APPENDIX A

In order to compare each animal in a standard manner, it was necessary to run nitrogen determinations and establish a comparison between animals according to the activities demonstrated when a sample of 0.1 mg N was used. The following values were obtained in running an ammonium sulfate standard curve for nitrogen determination according to the nesslerization procedure.

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<tr>
<th>Mg N</th>
<th>Absorption at 480 m(\mu)</th>
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</thead>
<tbody>
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<td>0.1</td>
<td>0.071</td>
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<tr>
<td>0.2</td>
<td>0.156</td>
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<tr>
<td>0.3</td>
<td>0.246</td>
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<tr>
<td>0.4</td>
<td>0.319</td>
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<tr>
<td>0.5</td>
<td>0.402</td>
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</table>

A standard curve for these values is drawn as follows:
A tabulation of nitrogen/ml, alpha-ketoglutarate dehydrogenase activity and pyruvic dehydrogenase activity is given in Table VII for experiment III.
The nitrogen present per ml in rat liver mitochondria for animals in Experiment III. Also the oxidation of alpha-ketoglutarate and pyruvate by liver mitochondria with ferricyanide as electron acceptor are given. For reaction mixture of enzyme assay, see legend for Figure 5.

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<th>Animal</th>
<th>N/ml</th>
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<th>Pyruvate</th>
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<th>N/ml</th>
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THE VITAMIN B₁-SPARING
ACTION OF SORBITOL

An Abstract
Submitted to the
Department of Chemistry
Brigham Young University
Provo, Utah

In Partial Fulfillment
of the Requirements for the Degree of
Master of Arts

by
Gisela Eleanor Bethsold
June, 1960
ABSTRACT

The present study was initiated to determine the effect sorbitol has upon the enzymatic functions of thiamin; specifically, what effect sorbitol has upon the alpha-ketoglutarate and pyruvate dehydrogenases activities of rat liver mitochondria. In addition, the effect of varying the dietary sorbitol concentration on the development of an athia-minosis was studied, particularly the effect on growth rate.

The experimental animals utilized were male albino rat litter mates of the Sprague-Dawley strain. These animals, upon arrival, weighed about 100 grams and were divided into groups receiving varying concentrations of sorbitol, vitamin supplements and antivitamin supplements. No attempt was made to prevent coprophagy. It is recognized that the vitamin-deficient rat is capable of consuming his feces, although it was felt that the use of wide screen-bottom cages utilized in the study greatly lowered the percentage of feces consumed. The animals were sacrificed by decapitation, and the liver rapidly removed and placed in a cold isotonic solution of 0.25M sucrose. The liver was homogenized, and a rat liver mitochondrial suspension in 0.25 M sucrose was made up to a final volume equal in milliliters to the original amount of liver utilized. Enzyme activities are assayed spectrophotometrically at 420 mµ with ferricyanide as electron acceptor. Total nitrogen was determined spectrophotometrically at 480 mµ by the direct nesslerization technique.
During the first five days, the animals on the sorbitol-containing diets, all experienced a weight loss and diarrhea. However, after this period, the animals adjusted to the diet, and no more diarrhea was noted during the course of the experiment. Those animals receiving a thiamin-deficient (basal) diet (without sorbitol) gained weight to about the ninth day, and then a rapid loss in weight occurred. The group on the thiamin-deficient (basal) diet and receiving 10 µg thiamin injected daily subcutaneously, gained weight at a rate of six grams daily during the course of the experiment. For those groups on a sorbitol-containing diet, a growth rate occurred which corresponded to the amount of sorbitol present within the diets with an optimum growth rate at 20% sorbitol level. Those animals receiving a diet containing 20% sorbitol and no thiamin had a growth rate comparable to that seen by animals receiving thiamin injected daily subcutaneously. When the sorbitol concentration was present in increasing amounts, the growth rates of the animals increased in proportion to the percentage of sorbitol present within the diet up to 20%.

The enzyme activities of alpha-ketoglutarate and pyruvate dehydrogenases increased two-fold when sorbitol was introduced into a thiamin-deficient diet in the amount of 20%. This value was raised or lowered according to thiamin and/or antithiamin supplements injected into the animals.

It is concluded that sorbitol alters the intestinal microflora so that more thiamin is produced by the microflora or less thiamin is used by them. This increased thiamin production occurs along with an
increase in alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities, which suggests another action of sorbitol in addition to the increase in the available thiamin. However, proof of this as the sole mechanism for the thiamin-sparing action of sorbitol is not conclusive.
This Abstract of a Thesis by Gisela Eleanor Bethsold is accepted in its present form by the Department of Chemistry of the Brigham Young University as satisfying the thesis abstract requirements for the degree of Master of Arts.

Date