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The determination of thiamin and its derivatives in brain tissue of control, thiamin-deficient, oxythiamin- and pyrithiamin-treated rats

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THE DETERMINATION OF THIAMIN AND ITS DERIVATIVES IN BRAIN TISSUE OF CONTROL, THIAMIN-DEFICIENT, OXYTHIAMIN- AND PYRITHIAMIN-TREATED RATS

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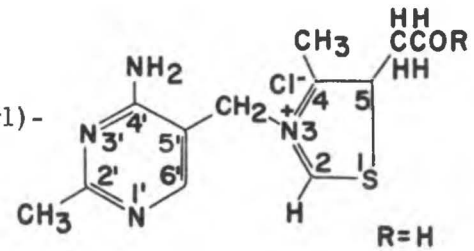
TABLE OF CONTENTS

LIST OF TABLES

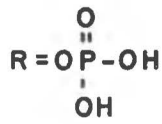
LIST OF FIGURES

STRUCTURES

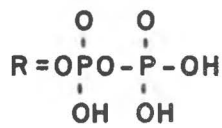
Thiamin chloride
3-[(4'-amino-2'-methyl-5'-pyrimidinyl)-methyl] -2 -(hydroxyethyl)-4-methylthiazolium chloride



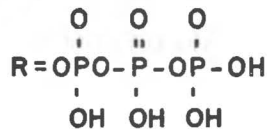
Thiamin chloride monophosphate = TMP· Cl



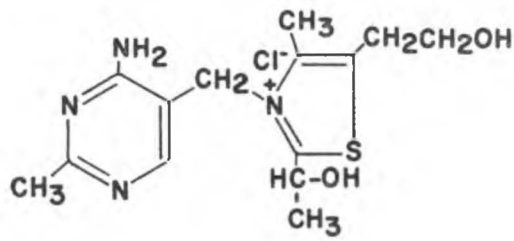
Thiamin chloride diphosphate = TDP· Cl



Thiamin chloride triphosphate = TTP· Cl

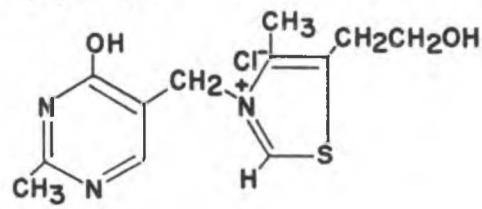


2(∝ -hydroxyethyl)-thiamin chloride = HET· Cl



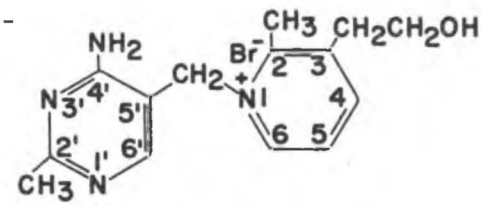
Oxythiamin = OTH· Cl

3-[(4'-hydroxy-2'-methyl-5'-pyrimidinyl)-methyl] 2-(hydroxy-ethyl)-4-methylthiazolium chloride



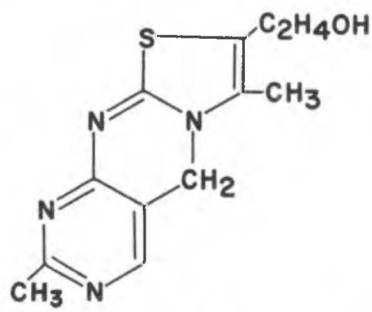
Pyrithiamin bromide = PTH· Br

1-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-3-(2-hydroxyethyl)-2-methyl-pyridinium bromide)

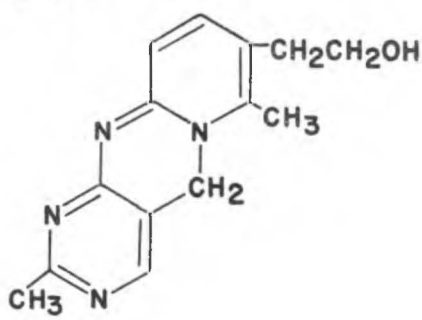


Thiochrome

2,7-dimethylthiachromine-8-ethanol



Pyrichrome



INTRODUCTION

Beriberi, a human thiamin deficiency disease, is associated mainly with populations such as in the Far East and South Asian countries that depend on over-milled rice for daily nourishment. Beriberi was shown to be due to nutritional factors by R. R. Williams and E. B. Vedder. In 1910, they prepared the earliest crude concentrated thiamin extract from rice bran which they used for treatment of beriberi in the Philippines [Williams (1961)].

A year after thiamin was synthesized by R. R. Williams, the diphosphate ester of the vitamin was identified as the cofactor for brewer's yeast pyruvate decarboxylase [Lohman and Schuster (1937)]. Lipmann (1939) and Silverman and Workman (1941) identified thiamin diphosphate (TDP) as a cofactor to bacterial pyruvate oxidative enzyme preparations. Gavrilescu and Peters (1931) reported that the brain slices of thiamin-deficient pigeons showed a loss of O_2 consumption. An intercerebral injection of thiamin extracts from yeast caused an increase to normal O_2 uptake. This injection of thiamin cured the condition of opisthotonus in the birds. Ochoa and Peters (1938), using an autoclaved yeast preparation free of TDP with pure TDP as the standard, enzymatically determined the TDP content of normal and thiamin-deficient pigeon tissues and found that the levels of TDP in

the deficient tissues were less than one-third of normal. Injection of thiamin concentrates corrected the avitaminotic symptoms and increased the tissue thiamin to normal levels.

Peters (1938) demonstrated that the rate of oxygen uptake by pigeon brain slices was directly proportional to the concentration of thiamin in the incubation media. He also showed that pyruvate utilization by the slices was increased by addition of thiamin. Green et al. (1942) isolated the pyruvate and α-ketoglutarate decarboxylation complexes from pig heart and found that TDP was a cofactor for these systems. The amount of pyruvate which was used up equalled the amount of acetoin and CO_2 formed in their system. The α-ketoglutarate was oxidized quantitatively to equal amounts of succinic semialdehyde and CO_2.

Gunsalus (1955) and Reed (1960) found that TDP was a cofactor for pyruvate dehydrogenase, one of the three enzymes of the pyruvate oxidase complex in mammalian tissues. Pyruvate is oxidized to acetylcoenzyme A (AcCoA) by this complex. The AcCoA is then utilized by the citric acid cycle, the main energy cycle of aerobic animal cells. The pyruvate dehydrogenase enzyme is therefore a key enzyme for the complete utilization of carbohydrate as a source of energy for the cell. Barron et al. (1941) and Debusk (1952) reported that TDP was a necessary cofactor for the oxidation of α-ketoglutarate to succinyl CoA in the citric acid cycle. The

requirement of TDP for the activity of transketolase was reported by Racker et al. (1953). The transketolase enzyme is the rate-limiting enzyme of the pentose phosphate shunt, which is found in the cytoplasmic portion of the cell.

The distribution of the various forms of thiamin in brain, heart, liver, and kidney of normal rats is similar. About 5% of the total thiamin in these tissues exists as free thiamin, 10% as thiamin monophosphate (TMP), 80% as the diphosphate and 5% as thiamin triphosphate (TTP) [Rindi and Guiseppe (1961)]. Other investigators have shown that a thiamin-deficient condition is associated with a decrease in the α-keto acid dehydrogenase activity in rat tissues [Gubler (1961), Gubler and Bethsold (1962), Bennett et al. (1966, 1969), McCandless and Schenker (1968), and Reinauer et al. (1968)]. Although only the diphosphate ester of thiamin presently is recognized to act as a coenzyme in mammalian systems, evidence is accumulating which indicates that TDP and/or TTP may play a necessary role in neuronal function [see Cooper and Pincus (1967) for review].

Tanaka and Cooper (1968) identified thiamin by a fluorescent technique in nerve tissue. Cooper (1968) reported that pyrithiamin (PTH), a thiamin antagonist, when added to rabbit vagus nerve homogenates, does not affect the α-keto acid dehydrogenase or transketolase activity in these tissues. Cooper also found that PTH displaces thiamin from the rabbit vagus nerve preparations and that thiamin was

localized mainly in the membrane structure of these tissues rather than in the axoplasm. Itokawa and Cooper (1969) found that neuroactive drugs such as acetylcholine, tetrodotoxin, ouabain, and K^+ displaced thiamin from frog spinal cord preparations. Thiamin diphosphatase was localized in the membrane portion of the nerves. Also, Itokawa and Cooper (1970) discovered that a significant percentage of thiamin was released from isolated rat nerve mitochondrial and microsomal fractions by acetylcholine, tetrodotoxin, snake venom and thiamin antagonists oxythiamin (OTH) and PTH.

"The experimental evidence in support of a role of thiamin in nerve conduction which is independent of its function as a coenzyme is strongly suggestive but far from equivocal." [Cooper and Pincus (1967).]

Recently, a specific thiamin triphosphatase has been isolated from rat brain. This enzyme is distinct from nucleotide triphosphatases already characterized [Barchi and Brown (1971)]. It has also been reported that a neurological condition that is due to a genetic defect is characterized by lack of TTP in the patient's brain. This neurological condition is similar to Wernick's encephalopathy, a severe form of beriberi. An inhibitor can be extracted from the brain tissue, blood, urine, and spinal fluid of these patients which prevents the formation of TTP in rat brain by TDP-adenosine triphosphate phosphotransferase enzyme preparation [Cooper et al. (1969)].

The most popular thiamin antagonists which have been used to study the condition of thiamin insufficiency are OTH and PTH because they produce different symptoms in the rat. In the terminal stages of treatment, the OTH-treated rat never develops convulsions [Gurtner (1961), Gubler (1961)], while the PTH rat almost always develops a polyneuritic condition [Cerecedo and Eich (1954), Gurtner (1961), Gubler (1961)]. Sometimes the terminal thiamin-deficient-treated rat experiences convulsions [Robertson et al. 1968)]. It is felt by some that the central nervous system is affected by thiamin deficiency and PTH treatment, although the particular biochemical impairment has not been clearly identified [McCandless and Schenker (1968), Dreyfus (1958), Robertson et al. (1968), Collins (1967), Rindi and Sciorelli (1970)].

The level and distribution of thiamin derivatives in the OTH-treated rat is probably normal since only a very small amount of OTH is able to cross the blood-brain barrier [Rindi et al. (1963)]. Also Gubler (1961) and Bennett et al. (1969) report that the α-keto acid dehydrogenase activity is normal in brain tissue of OTH-treated rats. PTH, however, accumulates in rat brain tissue as well as in other tissues [Morita et al. (1968b)] and displaces thiamin from the tissues rapidly [Rindi and Perri (1961), DeCaro et al. (1956)].

Some of the PTH in rat tissue is phosphorylated, perhaps to the diphosphate ester, and the remainder exists in a free form

[Morita et al. (1968b), Rindi and Perri (1961)]. Free PTH is a strong competitive inhibitor of purified thiamin pyrophosphokinase [Johnson and Gubler (1968)] while the diphosphate ester has been shown to weakly inhibit purified yeast pyruvate decarboxylase [Wittorf and Gubler (1971)].

It is not known which site of inhibition accounts for the major inhibitory effect of PTH on thiamin activity in rat tissue. Thiamin is actively transported into cells and accumulates in the cell as the diphosphate ester. When PTH is added to media which bathes brain cortex slices [Sharma and Quastel (1965)] or inverted rat intestine sacs [Ventura and Rindi (1965), Rindi and Ventura (1967), and Ventura et al. (1969)], the active transport of thiamin into the cells is inhibited. The inhibitory effect of PTH on the phosphorylation of thiamin in these studies accounted for almost all the inhibition of net transport of thiamin.

Free thiamin and TMP serve no recognized function in brain tissue and are considered hydrolysis products of TDP. Hydroxyethylthiamin (HET) has been identified as an intermediate in the conversion of pyruvate to AcCoA [Krampitz et al. (1958, 1959, 1961)] and has been found in rat, rabbit and dog tissues [Morita et al. (1968c)]. To date, the brain levels of free thiamin, the mono-, di-, and triphosphate esters of thiamin have been determined in normal [Rindi and Guiseppe (1961)] and in thiamin-deficient rats [DeCaro et al. (1961)]. There

have been no reports in the literature of HET levels in thiamin-deficient or thiamin-antagonized rats. The HET levels in the brain have been inadvertantly included in the thiamin assays, in which assays alkaline $K_3Fe(CN)_6$ was used as the oxidizing agents for thiamin [see Rindi and Guiseppe (1961), DeCaro et al. (1957), 1958, 1961), Rindi et al. (1963), Lewin and Wei (1966), Dreyfus (1961), Myint and Houser (1965), and McCandless and Schenker (1968)].

The rate of accumulation of PTH in the brain of PTH-treated rats and the simultaneous rate of displacement of thiamin from the brain of these PTH-treated rats, that receive appropriate daily doses of PTH or PTH plus thiamin which cause polyneuritic conditions to develop in the rat, has not been conclusively determined to date. A preliminary study of the effect of a wide range of doses of PTH over varying treatment periods in rats [DeCaro et al. (1954, 1958)] indicated that thiamin was displaced more rapidly from the brain in the PTH-treated rats than in the thiamin-deficient rats.

Using improved fluorometric techniques, Morita et al. (1968b) reported that a single intraperitoneal (i.p.) dose of PTH (1 mg/100 g rat) decreased the thiamin level in the brain of eighteen day thiamin-deficient and normal rats in just 24 hr after treatment, 33% and 67% respectively, of normal rat brain levels. The PTH was rapidly displaced from the brain by oral or i.p. administration of thiamin. Rindi and Perri (1961) did not determine the brain thiamin level in rats that

received 33 ug of thiamin and 210 ug of PTH orally, but the PTH levels in the brain after twenty days of treatment were close to the brain thiamin levels reported for normal rats [Rindi and Guiseppe (1961)]. Gurtner (1961) treated rats daily with higher levels of thiamin and PTH than used by Rindi and Perri (1961) and reported that the brain levels of TDP in the PTH-treated and thiamin-deficient rats after twenty-nine days of treatment were not significantly different, although only a small percentage of the thiamin-deficient rats did experience extreme neurological symptoms. All of the PTH-treated rats experienced extreme neurological symptoms. The level of PTH in these rats was not determined.

Rindi et al. (1963) reported that the TDP levels were not significantly affected in the brains of rats when treated with the same and higher levels of OTH plus thiamin as used by Gubler (1961).

The composition of the diet, level of thiamin in the diet, amount of antagonist, route of administration of the antagonist and thiamin, and age of the treated animals are factors which may significantly affect the level of thiamin or antagonist in animal tissue. For these reasons, it is difficult to compare directly results reported by different investigators of thiamin or thiamin-antagonist levels in tissue at a particular period of treatment. Therefore, in order to more clearly evaluate the effects of thiamin insufficiency on the central nervous system, a comparative analysis of the various forms of thiamin

in the brain of thiamin-deficient and thiamin-antagonized rats were determined at weekly intervals up to the terminal stages of treatment. Such a comprehensive investigation would allow one to compare directly the levels of thiamin and its derivatives in the brain of control rats to the corresponding level in the brain of thiamin-deficient and thiamin-antagonized rats on the same treatment day by the same oxidation procedure. Since no satisfactory method had been reported for the analysis of the various forms of thiamin in rat tissue, specific assays based on procedures reported by Morita et al. (1968a, 1969) were developed and were used for the determination of free thiamin, TDP + TTP[1], total thiamin[2], and HET in control, thiamin-deficient, OTH-, and PTH-treated rats. The PTH levels will be determined in the PTH-treated rats also.

From this study one can determine if there is any correlation between the physiological effects of treatment, i.e., weight loss and/or convulsions, and the respective levels of thiamin derivatives in the brain. Also, the levels of the thiamin derivatives, as well as the PTH levels in the case of the PTH-treated rats, should correlate with, and hence make more meaningful, the α-keto acid dehydrogenase activity

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[1]The di- and triphosphate esters of thiamin are not separated from each other by this assay.

[2]The total thiamin assay includes free thiamin, the mono-, di-, and the triphosphate esters of thiamin. HET is determined in a separate assay.

in the brain of thiamin-deficient and PTH-treated rats [see Gubler (1961), Bennett et al. (1966), McCandless and Schenker (1968)].

LITERATURE REVIEW

Thiamin, HET, OTH, and PTH Assays

The most sensitive, accurate, reproducible assay for thiamin in animal tissue is the thiochrome assay [Jansen (1936), see Metzler (1960) for review]. This assay is based on the fact that thiamin can be oxidized, under certain conditions, to thiochrome, a water soluble moiety that exhibits a rather unique fluorescent spectra. When thiochrome is extracted into isobutyl alcohol and subsequently excited with 365 nanometer (nm) light, an intense blue fluorescence can be monitored at 436 nm with a fluorometer. The extraction of the thiamin from an aqueous media into the alcohol is a purification step and also increases the intensity of the fluorescence. The limit of sensitivity of this method is about 0.02 ug thiamin per assay [Morita et al. (1968a, 1969)]. Since the phosphate esters of HET, OTH and PTH respond in a very similar fashion to the corresponding phosphate esters of thiamin, as far as chromatographic separation is concerned, it is necessary to use selective oxidizing agents when analyzing mixtures of thiamin, HET, and PTH. On a molar basis, the relative fluorescent intensities of thiamin, HET and PTH, when oxidized with alkaline $K_3Fe(CN)_6$ [Association of Vitamin Chemists (1951)] at the above

excitation and emission wavelengths, is 100, 100 [Morita et al. (1968)], and 14 [Sealock and White (1949)] respectively. However, cyanogen bromide (CNBr) and 1% $HgCl_2$ are very selective oxidizing agents and will oxidize thiamin to thiochrome but will not oxidize HET [Morita et al. (1968c, 1969)]. Also it has been found that the CNBr oxidation procedure is completely selective for thiamin in the presence of PTH [Fujiwara and Matsui (1953)]. It was found that in thiamin and HET mixtures, thiamin could be selectively destroyed by incubation of the mixture in base with a trace of $HgCl_2$ [Morita et al. (1969)], so that HET could then be oxidized with $K_3Fe(CN)_6$ to thiochrome. It has not been reported whether PTH interferes with this assay. The 1% $HgCl_2$ oxidant is comparable to the CNBr oxidant as far as selectivity is concerned [Morita et al. (1969)], but the 1% $HgCl_2$ is easier to prepare and the CNBr reagent is stable for only 3 hr at room temperature [Fujiwara and Matsui (1953)].

OTH does not form a thiochrome analogue or any fluorescent product when treated with alkaline $K_3Fe(CN)_6$ [Association of Vitamin Chemists (1951)] or with 1% $HgCl_2$ [unpublished work in Dr. Clark Gubler's lab]. The O atom in the 4' position of OTH will not react with the C-2 electron pair and allow a conjugated system to form as in thiochrome or pyrichrome[3]. The only successful assay for OTH in animal tissue that has been reported is a radioactive assay using

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[3]Pyrichrome is the fluorescent compound produced when PTH is oxidized with alkaline $K_3Fe(CN)_6$ [Fujita et al. (1952)].

^{35}S-OTH [Rindi et al. (1963)]. The labelled antagonist is extracted from the tissue, purified and chromatographically isolated according to the procedure described for thiamin [Rindi and Guiseppe (1961)].

Thiamin [Henessy and Cerecedo (1939), Association of Vitamin Chemists (1951), Rindi and Guiseppe (1961)], HET [Shiobara et al. (1965), Morita et al. (1969)], OTH [Rindi et al. (1963)] and PTH [Rindi and Perri (1961), Morita et al. (1968a, b)] are easily extracted from tissue with aqueous acids and can be recovered in excellent yield from ion exchange columns [Association of Vitamin Chemists (1951), Rindi and Guiseppe (1961), Morita et al. (1968a, 1969), Rindi et al. (1963)]. Thiamin monophosphate, TDP and TTP have been isolated in a quantitative manner [Rindi and Guiseppe (1961)] by selective elution from a Dowex 1 and an Amberlite CG-50 column. This procedure, however, requires large amounts of tissue and the purification step prior to column treatment requires a cholesterol stearate-treated charcoal column that is difficult to prepare [unpublished work in Dr. Clark Gubler's lab].

A quantitative paper chromatographic separation of thiamin and its phosphate esters has been reported [Lewin and Wei (1966)]. Itokawa and Cooper (1970b) reported that thiamin and its phosphate esters could be rapidly separated on Sepraphore strips by electrophoretic techniques. In both cases, after separation, the thiamin compounds were eluted from the support media and were then oxidized

to thiochrome with alkaline $K_3Fe(CN)_6$. However, both of the above techniques require purification steps prior to spotting the thiamin compounds on the paper or Sepraphore strips when the thiamin is extracted from tissue.

Thiamin Administration

Since the competitive nature of PTH and OTH with thiamin is so important in studying the effects of thiamin deficiency, Gubler (1961) reported a systematic injection procedure to study the effects of these inhibitors on the metabolism of pyruvate and α-ketoglutarate in rat tissues. Gubler (1961) used a 200/1 ratio of OTH/thiamin and a 5/1 ratio of PTH/thiamin in his inhibitor studies along with a thiamin-deprived group. All three groups were fed a thiamin-deficient diet. It was found that a subcutaneous (s. c.) injection of 10 ug of thiamin/100 g body weight causes the rats to grow at a maximum rate (see Figure 1) with a minimal effective amount of thiamin in the tissue stores and allows for a convenient and practical amount of antagonist to be used. The ratio of OTH to thiamin is such that the OTH effect on the rats can be achieved within about four weeks, and polyneuritis develops in the PTH-treated rats in about three weeks. Some investigators administer the thiamin and antagonists orally ad libitum. This becomes a significant additional variable, since the level of intake of thiamin and the antagonist would be affected by

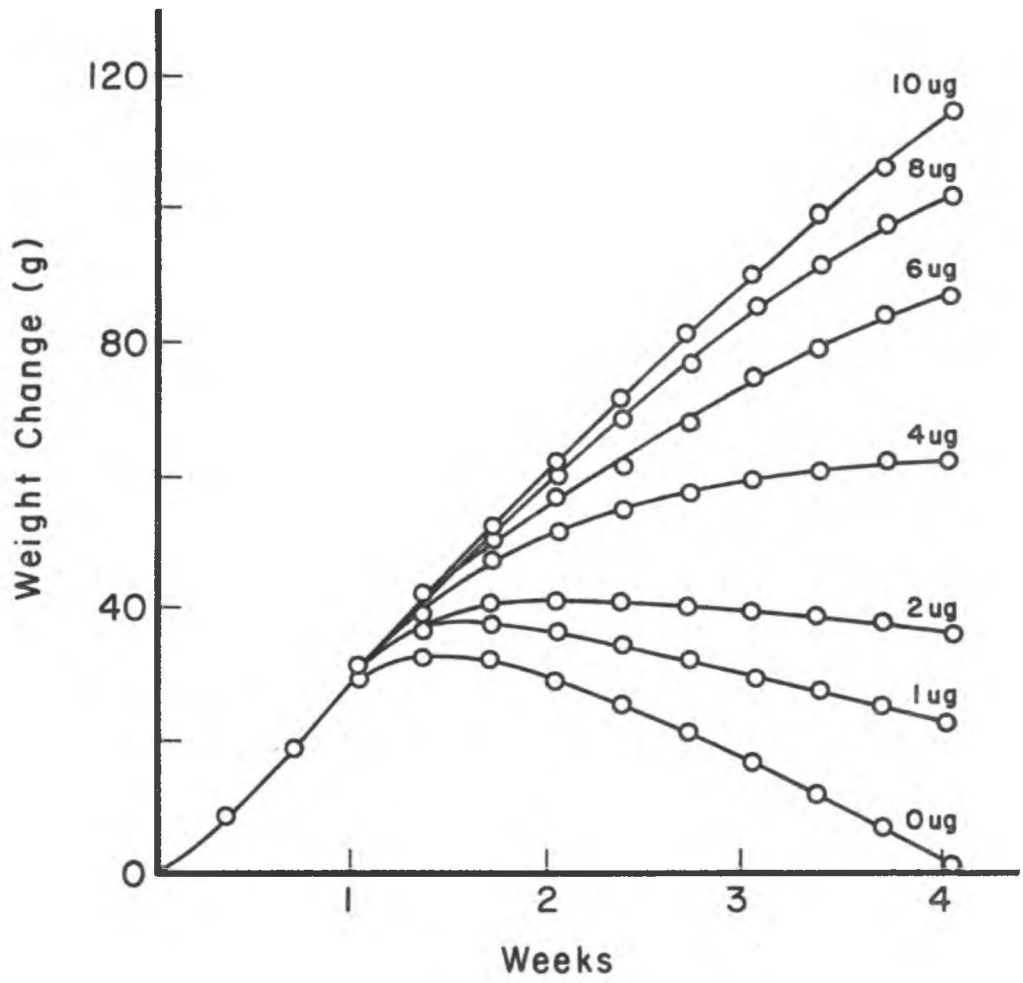
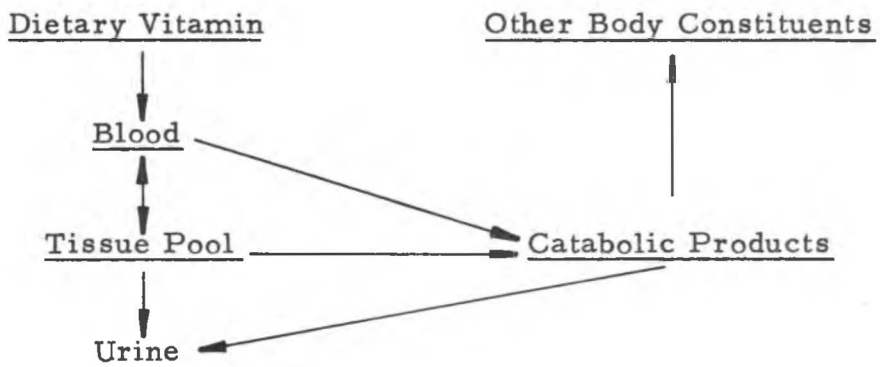


Fig. 1. --Minimum levels of thiamin required to maintain the growth rate of rats Scott and Griffith (1957) .

anorexia in all groups [Gubler and Bethsold (1962), Bitter et al. (1969), and Bai et al. (1971)].

Thiamin Distribution in Animals

Thiamin, taken in the diet, is distributed in the body in the manner indiacted in Figure 2. Dietary thiamin is stored in the tissue for further use or distributed in the blood to other areas in the body where the requirement for thiamin is greater or is excreted in the urine [Ziporin et al. (1964), Balaghi and Pearson (1966a, b)].



[Pearson (1967)]

Fig. 2. --Thiamin distribution in an animal

The distribution of thiamin in rats is about the same as in humans [Balaghi and Pearson (1966a), Ariaey-Nejad et al. (1970)]. About 85% of an oral dose is absorbed by the small intestine by active transport [Ventura et al. (1969), Pearson (1967)]. The vitamin is concentrated in and transported into the cells by phosphorylation according to Sharma and Quastel (1965) who used rat brain cortex.

artherosclerotic lesions as well as opisthotonus and ataxia. It is interesting to note that the transketolase activity of the brains of both strains did not decrease from the normal controls. The one strain showed no pathological symptoms.

As another example:

> We have failed to produce nerve degeneration in pigs by inducing thiamin deficiency. This does not mean that thiamin deficiency can under no circumstances cause nerve degeneration in the pigs nor that the results of these experiments necessarily apply to man. It has been shown repeatedly that various animals differ in their vitamin requirements. . .It should be pointed out that the most convincing studies indicating that deficiency leads to morphological alterations in the nervous system have been carried out in pigeons [Wintrobe (1942a)].

In the above paragraph Wintrobe refers to studies with several hundred pigs over a period of six years. The thiamin deficiency of the pigs was clinically determined by urine thiamin and blood pyruvate levels of the living animal and tissue thiamin levels at death. Forced exercise during the deficiency period brought on a cyanotic condition, collapse, brief convulsive movements of the hind quarters and finally death. The only overt signs of deficiency in the nonexercised pigs were recurrent vomitting and anorexia. The pigs were fed orally with crystalline vitamin preparations as opposed to yeast preparations used earlier [Wintrobe et al. (1942b)].

Rats fed a thiamin-deficient diet begin to lose weight in about two weeks of treatment due to a decreased food intake. After four weeks the rat is below his original weight, shows curvature of the

spine, is extremely weak and emaciated. Usually the thiamin-deficient rat dies within four to five weeks of treatment. Unless the rats survive for extended periods or are fed a low thiamin diet for extended periods, the thiamin-deficient rats usually do not show signs of polyneuritis [Gubler (1961), Dreyfus (1961), Park and Gubler (1968), Robertson et al. (1968), Bitter et al. (1969)]. The overt pathological symptoms associated with thiamin deficiency in experimental animals prompted Dreyfus (1958, 1961) to quantitate, histochemically, the thiamin distribution in the brain of normal as well as thiamin-deficient rats. His conclusion was that he could find "no correlation between brain tissue thiamin levels and the susceptibility of certain parts of the neuraxis to vitamin B_1 deprivation."

Electron microscopic studies on the thiamin-deficient rat brain are not conclusive [Collins (1967), Tellez and Terry (1968), and Robertson et al. (1968)] as far as the major site of tissue destruction is concerned. The earliest effects seem to occur in the glial cells in the brain stem. Robertson and Manz (1971) used the penetration of labelled albumin into brain tissue as a measure of tissue destruction in rats on a thiamin-deficient diet. They were able to show that during the early stage of thiamin deficiency when the glial cells of the brain stem were only swollen, no albumin penetrated the blood-brain barrier. Later, when frank necrosis was observed in the membranes, the dye-labelled albumin penetrated into the brain. The clinical symptoms of

thiamin deficiency in the rats correlated well with the histological examination of the brain stem. Estimation of the brain tissue damage correlated with the entrance of the dye into the brain tissue. When the animals showed signs of ataxia, tremors and loss of righting reflex, the necrosis of the brain stem membranes was observed, and at this time the fluorescent label was observed in the brain tissue.

It has not been shown that the transketolase activity of certain areas of the brain is a better index of thiamin depletion than is the pyruvate decarboxylase activity. The transketolase activity in thiamin-deficient rats in the brain stem, cerebral cortex, and cerebellum is decreased more than the pyruvate dehydrogenase activity in these regions [Dreyfus and Hauser (1965), Dreyfus 1965), McCandless and Schenker (1968)]. However, only the pyruvate dehydrogenase activity is restored toward normal values with thiamin repletion. The transketolase activity of the brain in thiamin-deficient rats is not increased by i.p. injections of 10 ug of thiamin in a four and a half to five week thiamin-deficient rat [McCandless and Schenker (1968)], although this treatment cured the ataxic condition of the rats. This fact suggests that the brain transketolase activity is not directly involved in the effects of thiamin deficiency.

The Effects of OTH and PTH on the Rat

Thiamin is distributed in rats by active transport across cell

membranes [Sharma and Quastel (1965)]. The effect of this transport process is to concentrate the thiamin in the cell as TDP against a concentration gradient, so that TDP will be in high enough concentration in the cell to activate its apoenzyme. The process of active transport of thiamin across the intestine was suggested by Polin et al. (1963). Sharma and Quastel (1965), using rat brain cortex slices, and Ventura and Rindi (1965), Rindi and Ventura (1967), Ventura et al. (1969), using inverted rat intestine sacs, reported that thiamin is actively transported into the cell where it accumulates in the phosphorylated form. The first four investigators showed that PTH, a thiamin analog, inhibits the accumulation of thiamin in the cell. Ventura and Rindi (1965) and Rindi and Ventura (1967) show that OTH does not inhibit this process at twice the concentration at which PTH inhibits the process. Sharma and Quastel (1964) and Rindi and Ventura (1967) found that PTH inhibited the phosphorylating action of thiamin pyrophosphokinase at the same concentrations at which inhibition of the active transport takes place. It had been determined that PTH inhibited purified thiaminokinase in rat liver [Mano and Tanaka (1960)], rat brain [Johnson and Gubler (1968)], pig brain [Peterson (1970)], and a purified liver thiaminokinase preparation [Koedam and Steyn-Parve' (1960), Eich and Cerecedo (1954)].

Wooley (1951) suggested that PTH may be phosphorylated to the diphosphate in vivo and that as the diphosphate may act as an

inhibitor of TDP enzymes either by inhibiting the union of TDP with its apoenzyme or by inhibiting the formation of TDP. Koedam and Steyn-Parve' (1960) showed that PTH acted as a very weak inhibitor of pigeon pyruvic dehydrogenase when competing with TDP for the apoenzyme. This same type of competition was also reported for yeast decarboxylase. Koedam and Steyn-Parve' found that PTH and OTH or their respective monophosphate esters do not inhibit yeast carboxylase activity, even if the preparation is treated with the antagonists before the TDP cofactor is added. However, PTH and OTH diphosphates can inhibit the yeast carboxylase if added to the preparation before the TDP cofactor. If TDP is added first, the antagonists do not exert any effect.

When the antagonists were tested as inhibitors of the pyruvate dehydrogenase activity of pigeon breast and heart muscle, it was found that free PTH and free OTH did not affect the amount of acetoin formed in this preparation [Koedam and Steyn-Parve' (1960)]. The diphosphates of OTH and PTH, however, did inhibit the enzymatic reaction even if the antagonists were added to the preparation after TDP. It was found that OTH diphosphate was a much stronger inhibitor of this pyruvate dehydrogenase activity than was PTH diphosphate. Wittorf and Gubler (1971) found that the diphosphate ester of PTH possessed one-third of the affinity of the diphosphate ester of OTH for yeast pyruvate decarboxylase. Morita et al. (1968b) reported

that 93, 90, 77, and 71% of the PTH content of the heart, skeletal muscle, liver and brain respectively of a rat injected with a large dose of PTH, was in the phosphorylated form and that this resulted in a rapid loss of thiamin from the tissue. Rindi and Perri (1961) indicated that less than 1% of the PTH in the brain of PTH-treated rats was not phosphorylated.

There have been a few reports in the literature concerning the effect of PTH treatment on the distribution of the thiamin phosphate esters and free thiamin in rat brain. Rindi and Sciorelli (1970) stated that PTH may exert its effect on the central nervous system of the rat because of the very rapid rate of depletion and extent of removal of thiamin phosphates from the brain. The initial per cent increase of free thiamin in the brains of rats receiving 0.6 u mole of PTH intracerebrally (i.c.) or when fed a thiamin-deficient diet, was not felt to be the cause of the neurological disturbances characteristically observed in terminal PTH-treated rats and which sometimes accompanied thiamin deficiency treatment. Rindi et al. (1970) and Rindi and Sciorelli (1970) found that an i.c. injection of 0.25 mg (0.6 u mole) of PTH caused a 40% decrease in total thiamin phosphates in the rat brain by the seventh day after the injection. Also the free thiamin and phosphorylated forms of thiamin increased in the brain when thiamin was orally administered seven days after the i.c. injection of PTH. Rindi et al. (1970) reported that seven days after

the single i. c. injection of 0.25 mg of PTH, the total phosphate level in the brain decreased to only 60% of the control levels.

Twenty-four hours after injecting (i. p.) 1 ug of OTH into rats that had been on a thiamin-deficient diet for fifteen days, the amount of TDP as well as the total thiamin level in the brain was not affected. These investigations showed that the highest total OTH levels in the brain of the rats was only one-seventh of the total thiamin levels [Rindi et al. (1963)]. Since OTH probably exerts its competitive inhibitory effects via the diphosphate ester [Johnson and Gubler (1968), Koedam and Steyn-Parve' (1960)], the injection of OTH alone, as Rindi et al. (1963) did, would have a greater inhibitory effect than would the injection of OTH plus thiamin. Rindi et al. (1970) found that only a 19% drop in three days of total thiamin phosphates was observed when 0.2 mg of OTH was injected directly into the brains of rats. This dose is 320 times the amount of OTH that is found in the brain 24 hrs after a 1 mg i. p. injection of OTH [Rindi et al. (1963)].

Rindi et al. (1963) showed that OTH, when injected into the rat, was phosphorylated to the diphosphate in liver, heart, and brain tissue. Since very little OTH entered the brain, only very small amounts of OTH diphosphate were found in that organ. This may explain why OTH-treated rats do not develop polyneuritis. The level of OTH in the tissues depended on the thiamin level already in the tissues and on the OTH/thiamin ratio in the dose administered. The rat is able to

eliminate OTH from the body tissues more rapidly than PTH; 24 hrs after a single i.p. injection of 1 mg of OTH into normal rats, the liver OTH level had decreased 98% and the heart OTH level had dropped by 75%.

The most striking characteristic of OTH-treated rats is the poor weight gain experienced at the beginning of the treatment. The OTH-treated rats usually gain less than 5 g the first week of the treatment [Gubler (1961), Gubler and Bethsold (1962), and Bitter et al. (1969)]. The thiamin-deficient and PTH rats show a weight gain about equal to or slightly less than the control rats for the first ten days of the treatment. Bai et al. (1971) correlated the inability of the OTH-treated rat to gain weight after the first day of treatment with a 50% decrease of transketolase activity in the gut of OTH-treated rats. The activity in the gut of thiamin-deficient and PTH-treated rats does decrease during treatment but does not decrease as much nor as consistently or as rapidly as in the OTH-treated rat. They concluded that "the anorexia would appear to be due to some local lesion in the gut. . ."

The extremely weak and emaciated condition of the terminal thiamin-deficient, OTH- and PTH-treated rats is not necessarily the reason for the death of the animals. Thiamin-deficient and PTH-treated rats that are force fed daily 8 g of thiamin-deficient diet show an almost normal weight gain up to three days before death. The

force fed OTH-treated rats gain weight at about one-half the rate of the control rats since they receive one-half (4 g) the amount of food. OTH-treated rats bloat and die at the beginning of treatment when force fed 8 g of diet per day. Although the PTH-treated rats that are force fed die at the same time as do rats fed ad libitum, the thiamin-deficient and OTH-treated rats die one-third sooner than the ad libitum fed rats [Bitter et al. (1969)]. The thiamin levels in the force fed rats that are terminal has not been determined. Therefore, the reason for the earlier death of the thiamin-deficient and OTH-treated rats is still speculative.

PTH and OTH treatment affect the heart rate in rats to a greater extent than thiamin-deficiency treatment does. The OTH and PTH treatment cause a 47 and 58% decrease respectively in the rat heart rate, while the thiamin-deficient treatment causes only a 28% decrease from the average control levels [Cheney, Gubler and Jaussi (1968)]. According to Gubler (1961) and Gubler and Bethsold (1962) the pyruvate metabolism in the rat heart is affected to a greater extent than is the α-ketoglutarate metabolism in thiamin-deficient, OTH- and PTH-treated rats (see Table 1).

In vitro enzymatic assays in this dissertation are intended to reflect the activity of the appropriate α-keto acid dehydrogenase complex in vivo. Cellular control mechanisms in the intact animal should be recognized in this type of a study so that the in vitro assay of the

TABLE 1

PER CENT DECREASE OF α-KETO ACID DEHYDROGENASE ACTIVITY, IN VITRO, OF TERMINAL THIAMIN-DEFICIENT, OTH- AND PTH-TREATED RATS

Tissue	Pyruvate Treatment			α-Ketoglutarate Treatment		
	Th Def	OTH	PTH	Th Def	OTH	PTH
Brain	25 (G) 30 (M) 72 (R)	0 (G) (1) 0 (Bb) (2) 0 (Bb)	50 (G) 30 (Ba) 75 (H)	0 (G)	0 (G) (1) 0 (Bb) (2) 0 (Bb)	45 (G) 60 (H)
Liver	60 (G) 60 (GB) 75 (R)	30 (G) 60 (GB) (1) 74 (Bb) (2) 51 (Bb)	50 (G) 60 (GB) 82 (H)	20 (G) 40 (GB)	0 (G) 0 (GB) (1) 28 (Bb) (2) 0 (Bb)	0 (G) 0 (GB) 54 (H)
Heart	30 (G) 30 (GB)	70 (G) 70 (GB)	60 (G) 60 (GB)	24 (G) 16 (GB)	36 (G) 30 (GB)	0 (G) 0 (GB)
Kidney	54 (G) 70 (GB)	50 (G) 50 (GB)	70 (G) 70 (GB)	56 (G) 50 (GB)	0 (G) 0 (GB)	40 (G) 40 (GB)

(G) = Gubler (1961)
(GB) = Gubler and Bethsold (1962)
(Ba) = Bennett et al. (1966)
(Bb) = Bennett et al. (1969)
(H) = Holowach et al. (1968) (mice)
(M) = McCandless and Schenker (1968)
(R) = Reinauer et al. (1968)

(1) = Low-fat diet
(2) = High-fat diet

enzymatic activity will be meaningful. Wieland et al. (1971) reported that pyruvate dehydrogenase from rat heart and kidney is activated by addition of Mg^{++}. The pyruvate dehydrogenase activity is controlled by a Mg^{++}-ATP dependent kinase and phosphatase enzymes. Phosphorylation of pyruvic dehydrogenase causes inactivation while higher levels of Mg^{++} activates the phosphatase enzyme and thus activates the pyruvate dehydrogenase enzyme. The pyruvate dehydrogenase complex from heart muscle, kidney, liver and brain are interconvertible. Fasting of rats for 24 hr causes the pyruvic dehydrogenase activity in the heart and kidneys to decrease to 15% of control rats. Refeeding of the fasted rats with glucose restores all the pyruvate dehydrogenase activity in 2 hr .

This Mg^{++}-ATP-dependent control system must be recognized in order to determine the state of the pyruvate dehydrogenase complex in vivo. Gubler (1961), Gubler and Bethsold (1962), Bennett et al. (1966), and Reinauer et al. (1968) used Mg^{++} salts in their assays and did not report the effect of withholding Mg^{++} from the assay, i.e., did not observe the extent to which pyruvate dehydrogenase exists in the inactivated form in tissue preparations from treated rats or mice. The above mentioned investigators probably were measuring the activity of the active form of the enzyme so would be observing only the effect of the TDP levels in the tissue on the activity of the pyruvate dehydrogenase.

Recent reports by Bennett et al. (1966) and Holowach et al. (1968) seem to confirm Gubler's results concerning the effect of PTH on rats as far as inhibition of pyruvate metabolism in the brain is concerned. The per cent inhibition by PTH of pyruvate dehydrogenase was 30, 75, and 50% inhibition according to Bennett et al. (1966), Holowach et al. (1968) and Gubler (1961) respectively (see Table 1). Holowach et al. (1968) found a normal pyruvate level in liver when the tissue showed a 75% decrease in pyruvate utilization, and concluded that the glucogenic pathway may utilize the extra pyruvate. The finding of an accumulation of α-ketoglutarate in the brain of PTH-treated mice associated with a 50% decrease in α-ketoglutarate dehydrogenase activity coincides with Guo's (1970) results, insofar as the accumulation of α-ketoglutarate is concerned. Guo (1970) reported that in the brain of thiamin-deficient, OTH- and PTH-treated rats, γ-aminobutyric acid, the intermediate moiety in the shunt pathway was not significantly altered. This pathway involves the conversion of glutamate $\longrightarrow$ γ-aminobutryic acid $\longrightarrow$ succinate and thus by-passes the α-ketoglutarate TDP-requiring oxidation pathway. Also the "glutamate-γ-aminobutyric acid-transaminase and succinic semialdehyde dehydrogenase activities in this pathway were not altered. . ." [Gubler (1968)] in the brain of thiamin-deficient, OTH- and PTH-treated rats.

Holowach et al. (1968) found that, although transketolase

activity in the brain and liver of PTH-treated mice decreased more than 50%, administration of 1 mg of thiamin to deficient animals 5 hr prior to killing did not cause the transketolase activity to rise to control levels. The administration of 1 mg of thiamin to the terminal thiamin-deficient mice did cause the α-keto acid dehydrogenase activities to increase 60% in the brain and the liver. A similar result was observed by McCandless and Schenker (1968) in the brain of terminal thiamin-deficient rats, as previously mentioned.

Effect of Thiamin Deficiency and Thiamin Antagonist Treatment on Adenosine Triphosphate Levels

In order to describe the effects of thiamin insufficiency in rats, a discussion of the level of adenosine triphosphate (ATP) is of great concern. If the drop in α-keto acid dehydrogenase activity during a thiamin insufficiency condition is of physiological importance, i.e., contributes to the death of the animal, one would expect to find a drop in the ATP levels in these tissues somewhat proportional to the decrease in α-keto acid dehydrogenase activity.

McCandless and Schenker (1968) and Inoue et al. (1970) reported that brain ATP levels are not below normal, even in severely ataxic thiamin-deficient rats, which suggests that the glycolytic pathway and tricarboxylic acid cycle are producing sufficient energy for the tissue. However, it should be noted that even in undernourished infant rats, which have lost 41% of their body weight, the brain ATP

levels are normal [Thurston et al. (1971)]. The brain normally utilizes glucose as the main energy source, but can utilize ketone bodies produced by the liver, in addition to the small amount of glucose supplied by the liver when the carbohydrate intake levels are insufficient [Cahill (1970)].

Schenker et al. (1971) reported an increase in plasma ketone bodies in pair fed and thiamin-deficient rats when compared to controls. The thiamin-deficient rats, however, maintained about the same level of plasma ketone bodies as did the pair fed animals. Holowach et al. (1968) found that, in PTH-treated mice, the brain ATP and creatine phosphate levels were normal. This condition is difficult to explain. The levels of ATP in the brain are about 3 nanomoles/g wet tissue [McCandless and Schenker (1968)]. According to Rindi and Guiseppe (1961) and DeCaro et al. (1961), thiamin levels in the normal rat brain are about 8 nanomoles/g wet tissue. The turnover of TDP in vivo is a constant significant drain on the ATP since thiamin diphosphate is constantly being formed by the following reaction:

$$\text{thiamin} \xrightarrow[\text{thiamin pyrophosphokinase}]{\text{ATP}} \text{TDP} + \text{adenosine monophosphate}$$

Mano and Tanaka (1960) . When the thiamin level is significantly reducedin tissue, less ATP is required for phosphorylation of thiamin, hence a correspondingly small deficit of ATP production in the cell might be masked by the ATP which is spared by the low thiamin levels.

Effects of High-Fat Diets

The effect of a high-fat diet in rats has been studied by Evans and Lepkousky (1929), Salmon and Goodman (1937), and Scott and Griffith (1957). Usually a high sucrose, low-fat diet contains 69% sucrose, 20% protein (casein), 5% salts, and 10% fat [Scott and Griffith (1957), Gubler (1961), Bennett et al. (1969)]. A high-fat, low-carbohydrate diet reverses this percentage of sugar and fat in the diet [Dr. Clark J. Gubler, unpublished data] or sucrose is left out of the diet and the fat is increased to over 40% [Scott and Griffith (1957)], or 30% [Jones (1962), Bennett et al. (1969)] of the diet by weight. These diets contain crystalline or pure vitamins and minerals, purified fats and proteins. It is reported that the high-fat diet, as opposed to a high-carbohydrate diet, prevented the normal thiamin deficiency response. Balaghi and Pearson (1966b) determined that the thiamin requirements of rats which receive adequate thiamin by injection and are fed a high-fat diet, are lower than those of rats fed a low-fat, high-carbohydrate diet. This conclusion was based on the following observations. The rats were injected with 50 ug of 2-^{14}C-thiamin daily. Half of the rats received a high-fat diet and half received a low-fat diet. The rats fed a high-fat diet expired 15% less $^{14}CO_2$ than did the rats fed a low-fat diet. Although the chromatographic pattern and level of radioactive metabolites was identical in both groups of rats, the level of urinary thiamin was 60% higher in the

rats fed a high-fat diet. The rate of loss of the 2-^{14}C-carbon of thiamin is a measure of the rate of catabolism of thiamin [Balaghi and Pearson (1966a)]. The fact that more thiamin is excreted in the urine of the rats fed a high-fat diet is an indication that the thiamin excreted represents excess thiamin not required for metabolism of pyruvate and α-ketoglutarate. A similar study has not been run on thiamin-deprived or antagonized rats.

Jones (1962), using a purified diet, reported that a high-fat diet produced a striking effect only on the OTH-treated rats and did not affect the PTH-treated and thiamin-deficient rats. The OTH-treated rats on a high-fat diet gained weight for ninety-eight days at the same rate as the control rats. The OTH group on a low-fat, high carbohydrate diet began losing weight on the second day, experienced diarrhea and died on treatment day 30. No enzymatic tests were performed on these rats.

Bennett, Jones, and Nelsen (1969) confirmed the above report but also determined enzymatic activities of OTH-treated rats fed a high- and low-fat diet. The enzymatic activities that were determined include pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase activity of brain and liver tissue. The transketolase activity of the blood was also determined. None of the mentioned enzymatic activities of the brain in the OTH-treated rats on a high- or low-fat diet were different from control rats. Pyruvate metabolism

in the OTH-treated rats on the high-fat diet was depressed 50% in the liver when compared to rats fed the same diet with no OTH-treatment, while the liver pyruvate dehydrogenase activity in OTH-treated rats fed a low-fat diet was depressed to 36% of the control values. The α-ketoglutarate dehydrogenase activity in the liver of OTH-treated rats fed the high-fat diet was not significantly different from the control rats fed the high-fat diet with no OTH. The same activity in the liver of OTH-treated rats fed a low-fat diet was 72% of the normal control values. The transketolase activity of the liver in the OTH-treated rats fed the high- and low-fat diet was depressed to 16% of the control values.

Datta and Racker (1961) reported that OTH diphosphate has a higher affinity for the transketolase apoenzyme than does TDP, a fact which may explain a significant loss of transketolase activity. Apparently the transketolase activity is not involved significantly in the normal maintenance of these tissues since it is lower in the blood of OTH-treated rats fed a high-fat diet than in the blood of OTH-treated rats fed a low-fat diet. Jones (1962) and Bennett et al. (1969) concluded that OTH-treatment symptoms are alleviated by AcCoA units made available to the citric acid cycle from the breakdown of fatty acid units, thus bypassing the pyruvate conversion step. "Outwardly it appeared as if the fat had completely substituted for the loss of thiamin pyrophosphate." If the more available supply of AcCoA units

in the high-fat diet is the reason why the OTH-treated rats are not affected, one might expect the thiamin-deficient and PTH-treated rats on a high-fat diet to respond in a similar fashion. Gubler (1969) and Jones (1962), found that a high-fat diet provided only a slight amount of thiamin-sparing action in the thiamin-deficient and PTH-treated rats.

This anomaly can be explained if it is assumed that the impaired α-keto acid dehydrogenase activity in the brain is not stimulated by the high-fat diet. It should be remembered that in the case of the thiamin-deficient and PTH-treated rat, the brain pyruvate dehydrogenase activity is decreased from 30 to 72% of normal (see Table 1). Also in the PTH-treated rat brain, the α-ketoglutarate dehydrogenase activity is decreased to 30 to 75% of the activity in the control rat. According to Gubler (1961) the α-keto acid metabolism in the brain of OTH-treated rats is not affected.

In normal rats fed a high sucrose diet and administered $^{35}SO_4$ by a stomach tube, the labelled sulfur was incorporated in situ by the microflora of the caecum into the thiamin molecule. No significant amount of the labelled thiamin was found in the heart and liver of the host, however [Wostmann and Knight (1961)]. They concluded that the thiamin produced by the bacteria in the caecum is not available to the host. The thiamin produced by the bacteria in the caecum seems to be bound to large soluble molecules, and, as such, is not

transported across the intestinal membrane. This type of tracer technique has not been used to study the absorption of thiamin from the intestine of rats fed a high-bulk or high-fat diet.

Meghal and Nath (1965) have shown that a high-fat diet does cause a several fold increase in the production of thiamin in the caecum of the rat as compared to rats fed a high-sucrose diet. It has been shown that the thiamin-sparing effect of orally administered sorbitol, a non-nutritive carbohydrate, is dependent on whether the rat is able to eat its feces [Morgan and Yudkin (1957, 1959)]. Similar studies with rats fed a high-fat diet have not been performed.

At the present time, investigators in Dr. Clark J. Gubler's laboratory are attempting to correlate the levels of the various forms of thiamin in the rat liver, heart, and brain to the α-keto acid dehydrogenase activity of these tissues in thiamin-deficient, OTH- and PTH-treated rats fed a high- and low-fat diet.

Discovery and Identification of HET

Mizuhara (1954) reported that in an aqueous solution of pH 8.4, thiamin would catalyze the decarboxylation of pyruvate and produce acetoin. Green et al. (1942) reported acetoin (acetylmethylcarbinol) was formed when acetaldehyde was incubated with pyruvate and pyruvate decarboxylase preparations isolated from pig heart. About 84% of the pyruvate utilized was accounted for as acetoin.

Breslow (1958) used deuterium exchange technique to show

that the 2 position in the thiazole ring contained a labile hydrogen. The $-NH_3^+$ moiety in the 4' position, the $-C_2H_4OH$ group in the 5 position and 1' pyrimidine nitrogen which exchanged D^+ atoms, had already been ruled out as active carbanionic sites in the 2 carbon transfer. The nucleophilic site was, thus, identified. Surprisingly enough, Bergel and Todd (1937) had already determined that a methyl radical in the 2 position of thiamin destroys its biological activity. Although Breslow felt that the 2 position in thiamin was sufficiently nucleophilic to react with benzaldehyde, pyruvate and acetaldehyde, his group was unable to isolate these postulated intermediates. Breslow, however, proposed the following scheme (see Figure 3).

Krampitz et al. (1958) synthesized an active aldehyde thiamin adduct and found that it was as active as thiamin in a microbiological assay. This thiamin adduct was given the trivial name of hydroxyethyl thiamin. Its systematic name is 3-(4-amino-2-methyl-5-α pyrimidyl)-methyl-5-(2-hydroxyethyl)-4-methyl-2(α-hydroxyethyl) thiazolium chloride (HET). When the synthetic compound was incubated with pyruvate at pH 8.4, acetoin was formed [Krampitz et al. (1959)]. This result confirmed earlier nonenzymatic models. Krampitz et al. (1961) isolated HET from mixtures of 1,2 ^{14}C acetaldehyde plus TDP at pH 8.5 at 50°C. The specific radioactivity of the labelled TDP-active aldehyde adduct was the same as the specific activity of the original aldehyde. The dephosphorylated adduct was isolated and

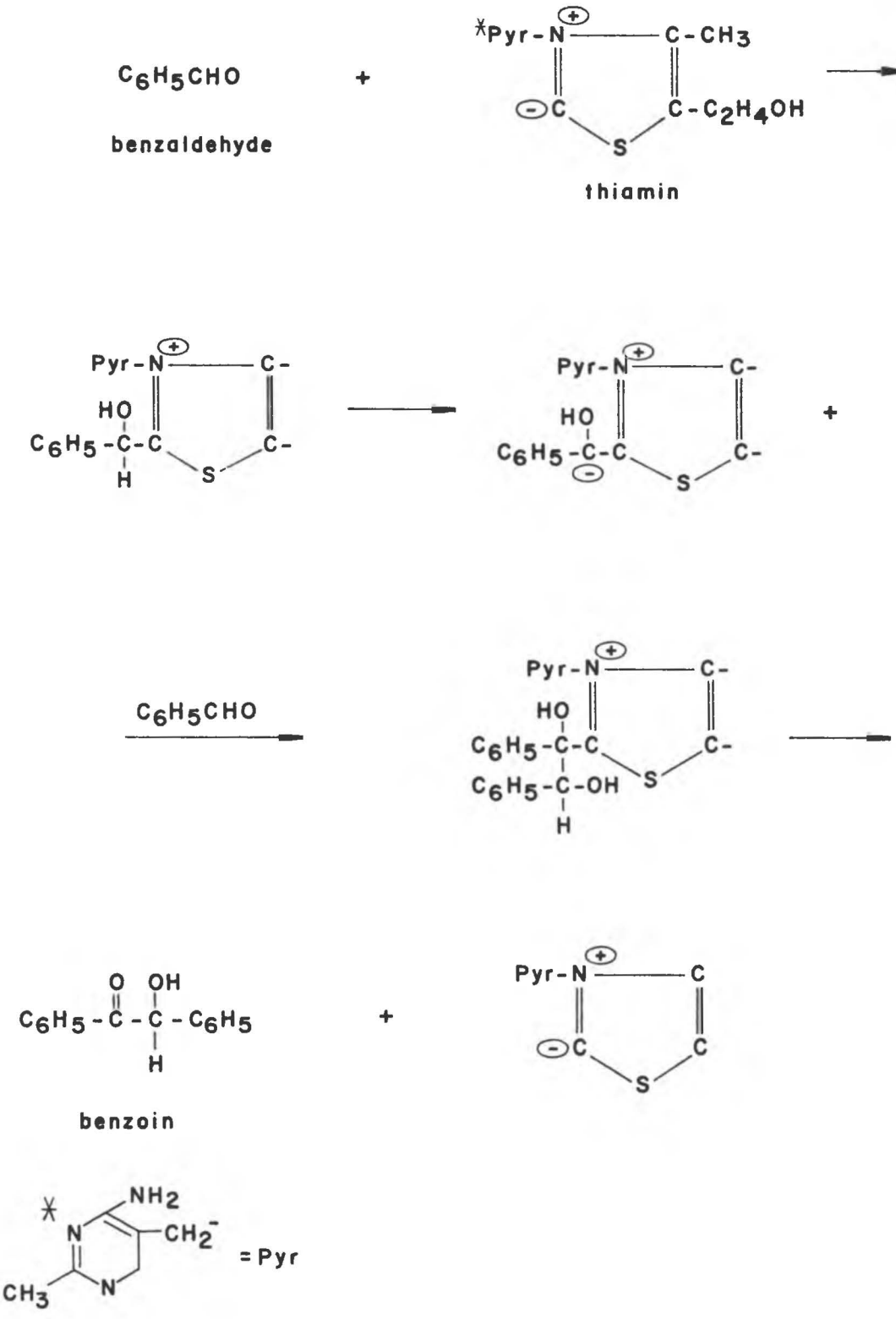


Fig. 3. --Mechanism of thiamin catalysis in the formation of benzoin.

was found to be identical to a synthetic HET preparation.

The benzoyl-thiamin adduct (HBT) that Breslow felt was an intermediate in the formation of benzoin, was isolated by Mieyal et al. (1971). They compared the acidity of HBT with that of HET. Mieyal (1967) had previously reported deuterium exchange of synthetic HET without the aid of an enzyme. When benzaldehyde, acetaldehyde and thiamin were incubated at pH 8.1, the principal products were benzoylmethylcarbinol and HET (see Figure 4). HBT possesses greater stability than HET and, therefore, is more reactive toward the carbonyl carbon of acetaldehyde than is HET. The carbonyl carbon of acyloins, therefore, must arise from the carbanion bonded to the 2 position of the thiazole ring. The greater acidity of HBT compared to HET is due to the well-known fact that the phenyl ring enhances the acidity of the substituted benzyl H.

When the pH and temperature are adjusted so that HBT does not show exchange with deuterium at the carbanionic site, the incubation of HBT with benzaldehyde will not react to form benzoin or any other product. The rate-limiting step of benzoin formation is the electrophilic attack of benzaldehyde on the HBT carbanion.

Holzer and Beauchamp (1959, 1961) have isolated the 2-(∝-hydroxy-∝-carboxyethyl) thiamin intermediate. When 1-^{14}C-pyruvate and TDP were incubated with purified pyruvate apodecarboxylase, the intermediate TDP-adduct was isolated and was found to be

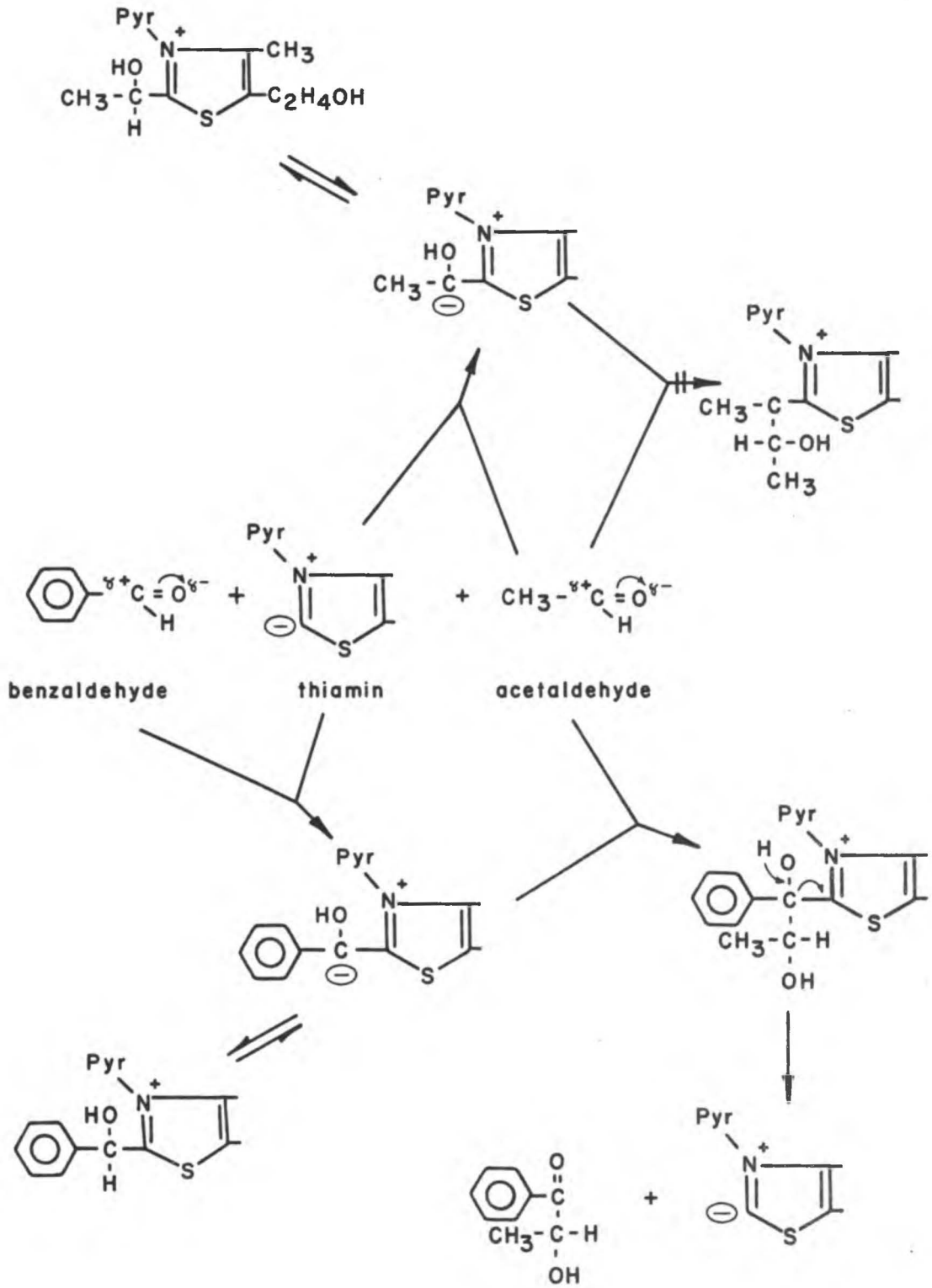


Fig. 4. --Competitive reaction between thiamin, acetaldehyde, and benzaldehyde [Mieyal et. al. (1971)].

radioactive. When this radioactive thiamin-adduct was incubated for longer periods with the pyruvate apodecarboxylase, radioactive CO_2 was produced. When 2-^{14}C-pyruvate and TDP were incubated with pyruvate apodecarboxylase, radioactive acetaldehyde was produced. If the 2-(∝-hydroxy-∝-carboxyethyl)-TDP, or 2-pyruvate TDP, isolated above, is reduced with HI, radioactive propionate is formed. These reactions can be summarized as follows:

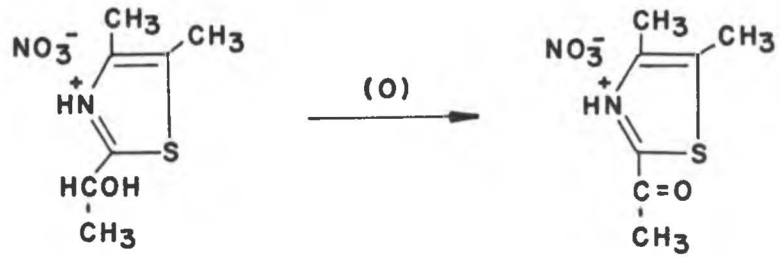
1-^{14}C-pyruvate + TDP-apoenzyme ⟶ 1-^{14}C-pyruvate-TDP-apoenzyme ⟶ $^{14}CO_2$

2-^{14}C-pyruvate + TDP-apoenzyme ⟶ 1-^{14}C-acetaldehyde + CO_2

1-^{14}C-pyruvate - TDP-apoenzyme $\xrightarrow{HI}$ 1-^{14}C-propionate

HET which has been extracted from mammalian tissues with acidic aqueous solutions [Morita et al. (1969)] is the protonated, stable form of the "active aldehyde"-thiamin adduct intermediate.

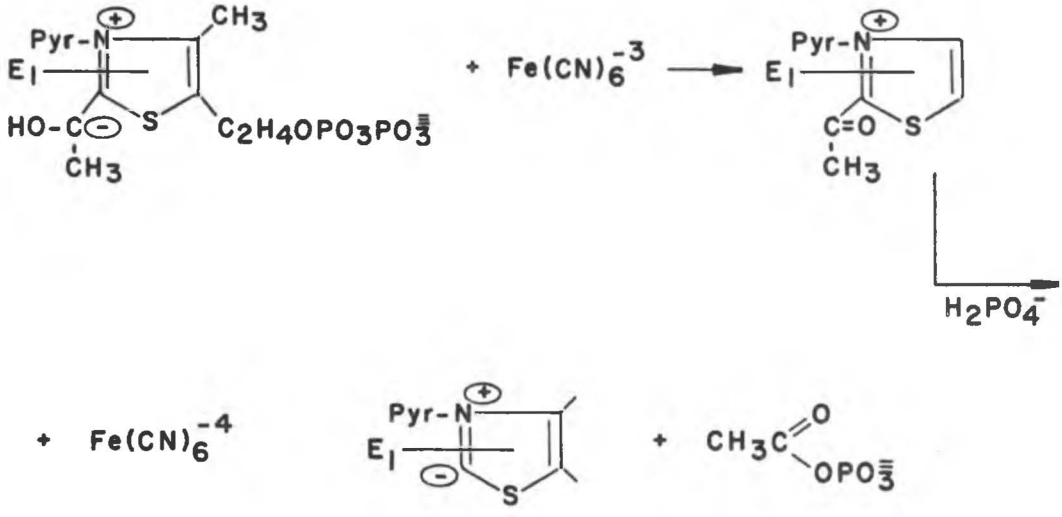
There is some evidence that indicates that HET may exist in vivo in an oxidized form as the acetyl-thiamin adduct. Spectral evidence indicated that the 2-∝-hydroxyethylthiazolium salt could be oxidized to the very unstable 2-acetylthiazolium salt [Breslow and McNeilis (1960)].



2(∝-hydroxyethyl) thiazolium salt

2-acetylthiazolium salt

Das et al. (1961) found the pyruvate decarboxylase (E_1) isolated from E. coli, in the absence of CoA and in the presence of pyruvate, ferricyanide ions, $H_2PO^-_4$ and TDP, will form CO_2 and acetylphosphate. The reaction postulated is as follows:



This fact suggests that in the complete enzyme system, in vivo, the HET carbanion is oxidized to the high energy acetyl-TDP compound prior to transfer of the acetyl group to reduced lipoic acid. The

acetyl-TDP adduct is thought to possess a high free energy of hydrolysis of -22 kcal/mole [Nash et al. (1958)]. This free energy of hydrolysis is higher than any other biological high-energy compound.

Holzer et al. (1962), using pyruvic oxidase isolated from pig heart muscle, reported that the amount of $^{14}CO_2$ produced in the reaction of 1-^{14}C-pyruvate, TDP and pyruvate oxidase, in the absence of CoA and NAD was directly proportional to the initial TDP levels in the reaction mixture. After the initial rapid reaction period, the rate of the formation of $^{14}CO_2$ decreased substantially and continued to a constant rate independent of the initial TDP levels in the reaction. These facts suggest that the initial rapid reaction involves the decarboxylation of pyruvate to HET diphosphate-apoenzyme plus $^{14}CO_2$ as follows:

$$1\text{-}^{14}\text{C-pyruvate} + \text{apoenzyme-TDP} \longrightarrow$$

$$^{14}CO_2 + \text{HET diphosphate-apoenzyme}$$

The pyruvate oxidase level is high enough so that an increase of TDP in the reaction will cause an increase in the reaction rate. The second part of the reaction must involve replacement of the HET diphosphate from the apoenzyme by free TDP so that another molecule of pyruvate can attack the 2 position of the TDP-apoenzyme complex and, thus, initiate another rapid decarboxylation reaction as follows:

$$\text{HET diphosphate-apoenzyme} \xrightarrow[\text{TDP}]{\text{slow}} \text{TDP-apoenzyme} + \text{HET diphosphate}$$

$$\text{TDP-apoenzyme} + 1\text{-}^{14}\text{C pyruvate} \xrightarrow{\text{rapid}} \text{HET diphosphate-apoenzyme} + {}^{14}CO_2$$

When HET diphosphate is incubated with CoA, NAD and pig heart pyruvate oxidase, the rate of formation of AcCoA was much slower than the rate observed when pyruvate and TDP is substituted for HET diphosphate in the above reaction [Holzer et al. (1962)]. Dr. Clark J. Gubler reported that his unpublished investigations indicate that the rate of formation of AcCoA from HET diphosphate in the presence of pyruvate dehydrogenase is 1/3000 of the formation of AcCoA formed from pyruvate plus TDP. Thus, the protonated "active aldehyde"-thiamin adduct seems to be a very unreactive species in in vitro enzymatic assays. In vivo utilization of HET has been verified, however.

Shiobara et al. (1965) studied the effect of the D and L optical isomers of HET using rats and rice birds. His group reported that both the D and the L form acted as effectively as did thiamin when fed to these deficient animals. Holzer and Beauchamp (1961) reported that the racemic mixture of HET, which they used in quantitatively determining its activity with pyruvic decarboxylase in vitro was only 43 to 47% as active as TDP. The rat and the rice bird must be able to transform the D or the L form to its optically active enantiomer. Shiobara et al. (1966) reported that the D and L isomer of HET, when fed orally to thiamin-deficient rats is readily converted to thiamin in the liver. The liver of the thiamin-deficient rats fed the DL isomers of HET was homogenized and was used as a source of thiamin for a

micro-organism culture. The culture grew at the same rate as one which received pure thiamin. That is, the animal was able to convert DL-HET to thiamin or the microorganisms were able to use the HET in place of thiamin. In order to clarify this situation, the same liver homogenate was assayed for thiamin. Cyanogen bromide was used as the oxidant in the assay; this oxidant is specific for thiamin. It will not oxidize HET to thiochrome. The assay showed that thiamin was increasing in the liver; therefore, DL-HET was being converted to thiamin in the liver of the rat.

MATERIALS AND EQUIPMENT

Materials

Thiamin chloride hydrochloride, oxythiamin chloride hydrochloride and pyrithiamin bromide hydrobromide were purchased from Sigma Chemical Company, St. Louis, Missouri. Minoru Morita of Japan donated α-hydroxyethylthiamin chloride hydrochloride as a gift.

Permutit T or Thiochrome Decalso, a silicate cation exchange resin, supplied by Fisher Scientific Company, Fair Lawn, New Jersey, was used to purify the dephosphorylated tissue homogenates. Amberlite CG-50, a polymethylacrylic acid cation exchange resin, chromatographic grade, made by Mallinkrodt Chemical, St. Louis, Missouri, was used to separate TDP and TTP from TMP and free thiamin.

Taka Diastase, a powdered preparation of Aspergillus oryzae enzymes, employed as a source of phosphatase activity, was manufactured by Parke Davis and Company, Detroit, Michigan.

The commercial thiamin-deficient diet mix was secured from Nutritional Biochemicals, Cleveland, Ohio. The rats also received a 68.5% sucrose diet, prepared according to Gubler (1961) (see Table 2). The composition of the diets are similar.

TABLE 2

THIAMIN-DEFICIENT DIET Gubler (1961)

Composition of Diet	Per cent
Sucrose	68.5
Vitamin-Free Casein	22.0
Corn Oil	5.0
Salt Mix	4.5
Vitamin Mix	0.22
Choline	0.40

Composition of Salt Mix	Grams
$CaCO_3$	600.0
K_2HPO_4	645.0
$CaHPO_4 . 2H_2O$	150.0
$MgSO_4 . 7H_2O$	204.0
NaCl	335.0
Ferric Citrate. $6H_2O$	55.0
KI	1.6
$MnSO_4 . H_2O$	7.6
$ZnCl_2$	0.5
$CuSO_4 . 5H_2O$	0.6

Composition of Vitamin Mix	
Inositol	40.000
Nicotinic Acid	0.800
Pyridoxine HCL (B_6)	0.120
Riboflavin (B_2)	0.240
Biotin	0.004
Folic Acid	0.010
2-Me-1,4-Naphtho-quinone	0.080
p-Amino Benzoic Acid	2.000
Calcium Pantothenate	1.200

Deionized water was used in all analytical steps. All other chemicals used in this study were reagent grade quality.

The male rats were obtained from Sprague-Dawley Inc., Madison, Wisconsin, and from Geneva Mott, Salt Lake City, Utah.

Equipment

The Turner Fluorometer, Model III, made by G. K. Turner Associates, Palo Alto, California, was used to determine the thiamin levels of rat brain. The Farrand Spectrofluorometer, made by Farrand Optical Company, New York, was used to establish the PTH levels in the rat tissue; the spectrofluorometer was equipped with a Honeywell Elektronic 15 recorder which was used to obtain fluorometric scans of thiochrome and pyrichrome.

A Corning Model 12 Research pH meter equipped with a combination electrode, made by Scientific Instruments, was employed in this study.

The motors used to drive a teflon pestle in the tissue homogenation step were made by Talboys Instrument Corporation, Emerson, New Jersey, a model 106 Con-Torque power unit, made by Eberback Corporation, Ann Arbor, Michigan, and a Vari Speed Stirrer, made by Precision Scientific. The teflon pestle and glass tissue grinder were both obtained from Arthur H. Thomas Company, Philadelphia, Pennsylvania.

Devices used to mix the aqueous solutions and the isobutyl

alcohol include a Rotary Evapo-mix shaking unit, made by Buchler Instruments, Fort Lee, New Jersey, and a Vortex Genie mixing device, supplied by Scientific Industries Inc., New York.

A Servall Refrigerated Automatic Centrifuge was used for the preparation of the homogenate. A 4.25 in. rotor was used.

The wet tissues were weighed on a Mettler microbalance made by Mettler Instrument Corporation, Highstown, New Jersey.

A Temptroll 152 waterbath, made by Precision Scientific Company, Chicago, Illinois, was used to provide a constant temperature of 48 to 50°C for incubation.

METHODS

Diet and Injection Schedule

The male Sprague-Dawley rats were placed in individual stainless steel cages in an air-conditioned room maintained at 72°C. All the rats had free access to water. The rats were allowed to eat Purina Rat Chow ad libitum for four days before introducing them to the particular diet and injections. Day zero was the day on which the rats were started on the thiamin-deficient diet and were injected with the appropriate supplements. The commercial thiamin-deficient diet, secured from Nutritional Biochemical or the diet prepared in our lab according to Gubler (1961) was used.

The rats were treated in four groups. Each group was fed thiamin-deficient diet ad libitum and given free access to water. Group I, the control group, was injected subcutaneously (s.c.) daily with 10 ug thiamin in 0.2 ml 0.9% NaCl/100 g body wt; Group II, the thiamin-deficient group, received daily s.c. injections of 0.2 ml of 0.9% NaCl/100 g body wt; Group III, the OTH group, was injected s.c. daily with 2000 ug of OTH plus 10 ug thiamin in 0.2 ml of 0.9% NaCl/100 g body wt; Group IV, the PTH-treated group, was injected s.c. daily with 50 ug PTH plus 10 ug thiamin in 0.2 ml of 0.9% NaCl/100 g body weight.

Sacrifice Schedule

On day zero, the rats were taken off the Purina Rat Chow and started on the thiamin-deficient diet. Rats from the four groups were sacrificed periodically for four to five weeks. Each group was split into two sets. The rats in Set II were started on the thiamin-deficient diet a day after Set I, but were treated the same as Set I otherwise. This staggered treatment was employed to allow sufficient time to process the tissues to a point where the tissues could be stored at -20°C for extended periods. Supplemental injections of thiamin or antagonists were not made on the day of sacrifice.

Extraction and Neutralization of Homogenate

The entire brain was removed from the rat immediately after decapitation, weighed and placed in 10 ml of ice cold 0.3M $HClO_4$ per g of wet tissue, and then homogenized for 3 min with a motorized teflon pestle which fits snugly into a reinforced glass container immersed in an ice bath. The homogenate was then centrifuged at 5900 xg at 0°C for 15 min. The pellet was rehomogenized in 2 ml of acid for 30 sec, centrifuged as before, and the supernatant extracts were combined. The tissue extracts were adjusted to pH 5-6 with 30% KOH, centrifuged to remove $KClO_4$ and then adjusted to pH 4-5 with 4N pH 4.5 acetate buffer. This solution will be referred to in the following text as the neutralized tissue extract.

Isolation of TDP and TTP

Approximately one-sixth of the neutralized tissue extract was placed in a glass column 1/4 in. in diameter with a 3 ml reservoir seated at the top. The column was filled 5 3/4 in. with Amberlite CG-50. This resin is equivalent to the Rexyn 102 used by Sharma and Quastel (1965). The resin was washed and prepared for use according to their procedure. After the aliquot had descended into the resin, 4 ml of distilled water were added to the column which eluted the anionic thiamin di- and tri-phosphates from the resin while the free thiamin and TMP were retained on the column. The samples were then prepared for dephosphorylation.

Dephosphorylation

One ml of 4N, pH 4.5 acetate buffer and 50 mg of dry Taka Diastase were added to each sample, the tubes were covered with parafilm and were then incubated at 48-50°C for 3 hr. The samples were then passed through a Decalso column as described below.

Decalso Purification

This procedure is a modification of the procedure reported by Morita et al. (1969). The Decalso resin was not washed, rinsed or sized before use. About 1.5 g of Decalso was allowed to settle into a glass column, 1/4 in. inner diameter, with a 20 ml reservoir on the top. The column was equipped with a glass stopcock.

After the column was washed with 25 ml of 0.5% acetic acid and 25 ml of water, the sample was poured into the column and allowed to drain into the resin. About 60 ml of boiling water was then poured through the column and the elute was discarded. The thiamin, HET or PTH was eluted from the column with 25 ml of hot 20% KCl in 0.1 N HCl. The 20% KCl eluent was drained into a 25 ml volumetric flask. The column material was not reused but was discarded after each run.

Preparation of Total Thiamin, HET and PTH Samples

About one-sixth of the original neutralized tissue extract was used for these determinations. This aliquot was not passed through the Amberlite CG-50 column but was prepared for dephosphorylation as previously described. This sample contains the free and phosphorylated species of thiamin, HET and PTH.

Preparation of Free Thiamin Samples

About two-thirds of the volume of neutralized tissue extract was used for the determination of free thiamin due to its low concentration in the tissue. This sample was not dephosphorylated and was placed directly onto the Decalso column for purification as previously described.

Calculations of Thiamin, HET and PTH Levels

Thiamin, HET and PTH were calculated as nanomoles of

thiamin chloride hydrochloride (mol. wt. 337), HET chloride hydrochloride (mol. wt. 381) and as PTH bromide hydrobromide (mol. wt. 420) respectively per g wet tissue. The formula is defined as follows:

$$\frac{\text{nanomoles}}{\text{g wet tissue}} = A \times B \times \frac{C}{D} \times \frac{E}{F \times G} \times 1000$$

A = fluorescent reading of samples minus blank

B = ug of pure thiamin, HET or PTH used as standards divided by the respective fluorescent reading of the standard minus blank

C = 25, which is the volume of the sample eluted from the Decalso column with 20% KCl in 0.1N HCl

D = aliquot of C used in the assay

E = total volume of neutralized tissue extract

F = aliquot of extract used per assay

G = appropriate molecular weight

Fluorescent Assay

The methods used to determine the levels of thiamin, HET and PTH in the tissue homogenates are based on the specific oxidation of each to strongly fluorescent compounds which can be selectively monitored at certain excitation and emission wavelengths. Appropriate aliquots, ranging up to 8 ml of the respective sample purified on the Decalso column, were used so that about 0.1 ug of thiamin or

HET and about 0.3 ug of PTH were fluorometrically assayed. Pure thiamin, HET and PTH were dissolved in 20% KCl in 0.1N HCl and appropriate aliquots of these standards were oxidized at the same time and in the same manner as in the respective assays of tissue samples.

Thiamin Assay. This procedure is based on the methods reported by Morita et al. (1969). An appropriate aliquot of the total thiamin sample which was purified on the Decalso column was placed in a 50 ml glass conical tube and mixed for 5 sec on a Genie vortex mixer with 0.3 ml of 1% $HgCl_2$. Two ml of 30% NaOH were added to each tube to complete the oxidation of thiamin to thiochrome. The samples were mixed for 5 sec. The blank received only 2 ml of 30% NaOH. Ten ml of distilled isobutyl alcohol were then added to each sample. The tubes were then mixed on a Rotary Evapo-mix shaking unit for 2 min in such a way that the liquid climbed the walls of the container and rotated rapidly around the tube. This mixing technique provided uniform and efficient extraction of the thiochrome from the aqueous layer into the alcohol layer. A similar extraction procedure was performed on the oxidized HET and PTH samples. The samples were centrifuged at 100 x g and about 4 to 5 ml of the alcohol layer were placed in 75 x 12 mm unmatched borosilicate or pyrex tubes. The samples were placed in the door compartment of a Model 111 Turner Fluorometer and assayed at an excitation wavelength of 365

nm and emission wavelength of 436 nm. These wavelengths were obtained using a #7-60 filter on the excitation light and a #2A and #47B filter on the emission light.

HET Assay. Another 5 to 8 ml aliquot of the total thiamin sample which was purified on the Decalso column was used for the HET determination. This oxidation procedure is also based on the assay reported by Morita et al. (1969). Two ml of 30% NaOH were added to the sample aliquot. The solution was mixed for 5 sec on the Vortex mixer, mixed again for 5 sec with two drops of 0.01% $HgCl_2$ and then briefly mixed with 0.3 ml of 1% $K_3Fe(CN)_6$ in water. The blank prepared for the thiamin sample assay served as a blank for the HET determination also. The sample and blank were then extracted with 10 ml of distilled isobutyl alcohol and fluorometrically assayed on the Turner Fluorometer exactly as described previously for the thiamin assay.

PTH Assay. This oxidation procedure is a modification of the procedure reported by Morita et al. (1968a). Two ml of 1% $K_3Fe(CN)_6$ in 10% NaOH were added to 3 to 8 ml of the total thiamin sample that had been purified on the Decalso column. The solution was mixed for about 5 sec after which the pyrichrome was extracted into 10 ml of distilled isobutyl alcohol as previously described.

Four to 5 ml of the alcohol were transferred to a cuvette

and the fluorescence was monitored on the Farrand Spectrofluorometer using 430 nm excitation wavelength and 460 nm emission wavelength.

RESULTS

Weight Gain of Rats

The control rats gained at the rate of 6.4 and 4.8 g per day in the 1970 and in the 1971 assays respectively for three weeks of treatment. At this time the average weight of the rats ranged from 240 to 250 g (see Figures 5 and 6).

The thiamin-deficient rats gained weight at the same rate as the controls for the first six and thirteen days respectively in the 1970 and in the 1971 analyses, after which time the thiamin-deficient rats then gained at a subnormal rate for four days. The rats then rapidly lost weight at the average rate of 5 to 6 g per day until the animals were sacrificed. The five thiamin-deficient rats in the 1970 analysis that were sacrificed after twenty-seven days of treatment were about 7 g below starting weight. In the 1971 treatment period, the ten thiamin-deficient rats sacrificed on treatment day 25 averaged 5 g above their starting weight. The four thiamin-deficient rats in this group that were allowed to live for up to thirty-two days of treatment averaged 30 g below starting weight. The weight gain was 53 and 58 g above the starting weight in the 1970 and the 1971 analyses respectively (see Figures 5 and 6).

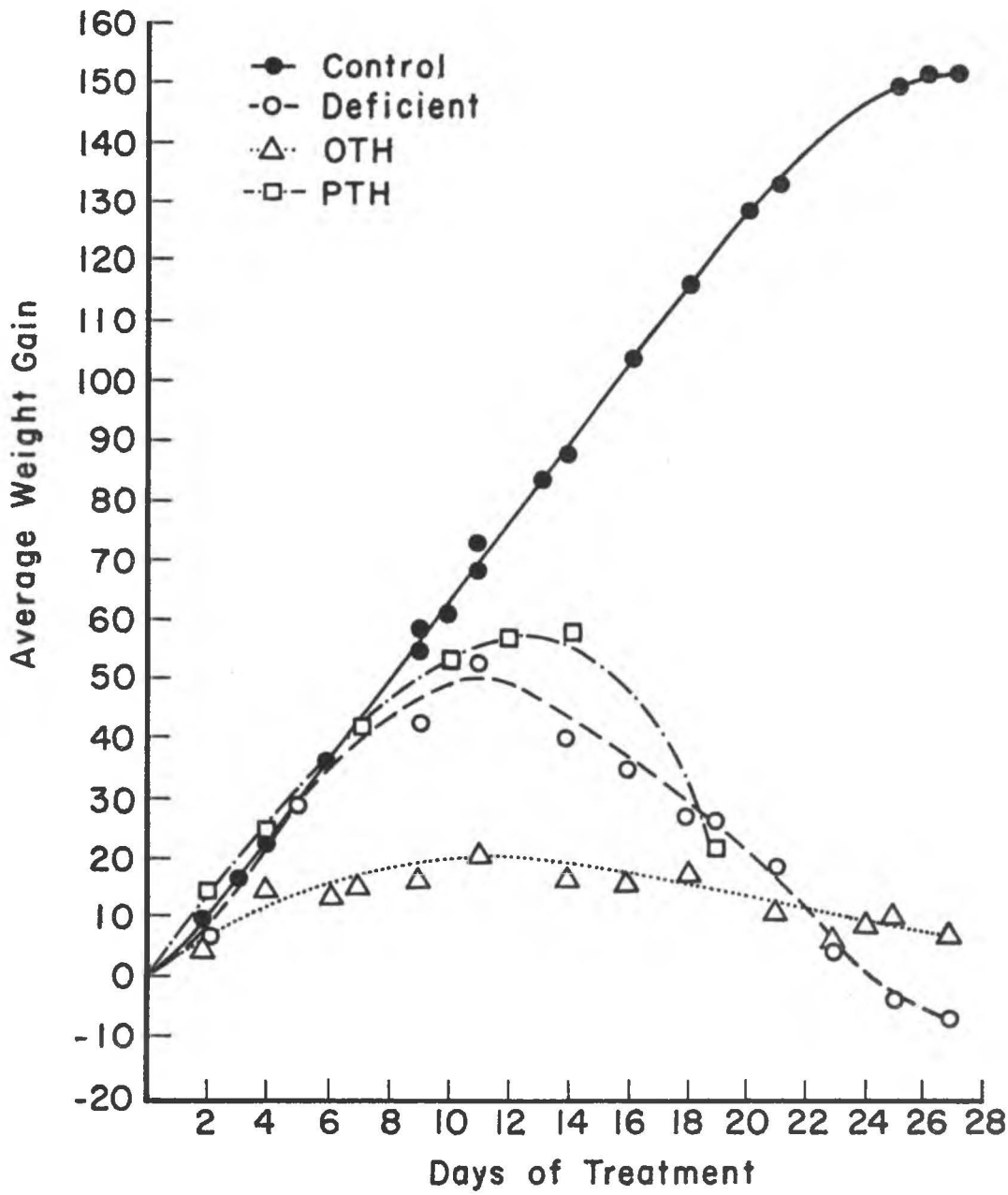


Fig. 5.--Average weight gain of control, thiamin-deficient, OTH- and PTH-treated rats--1970 analysis.

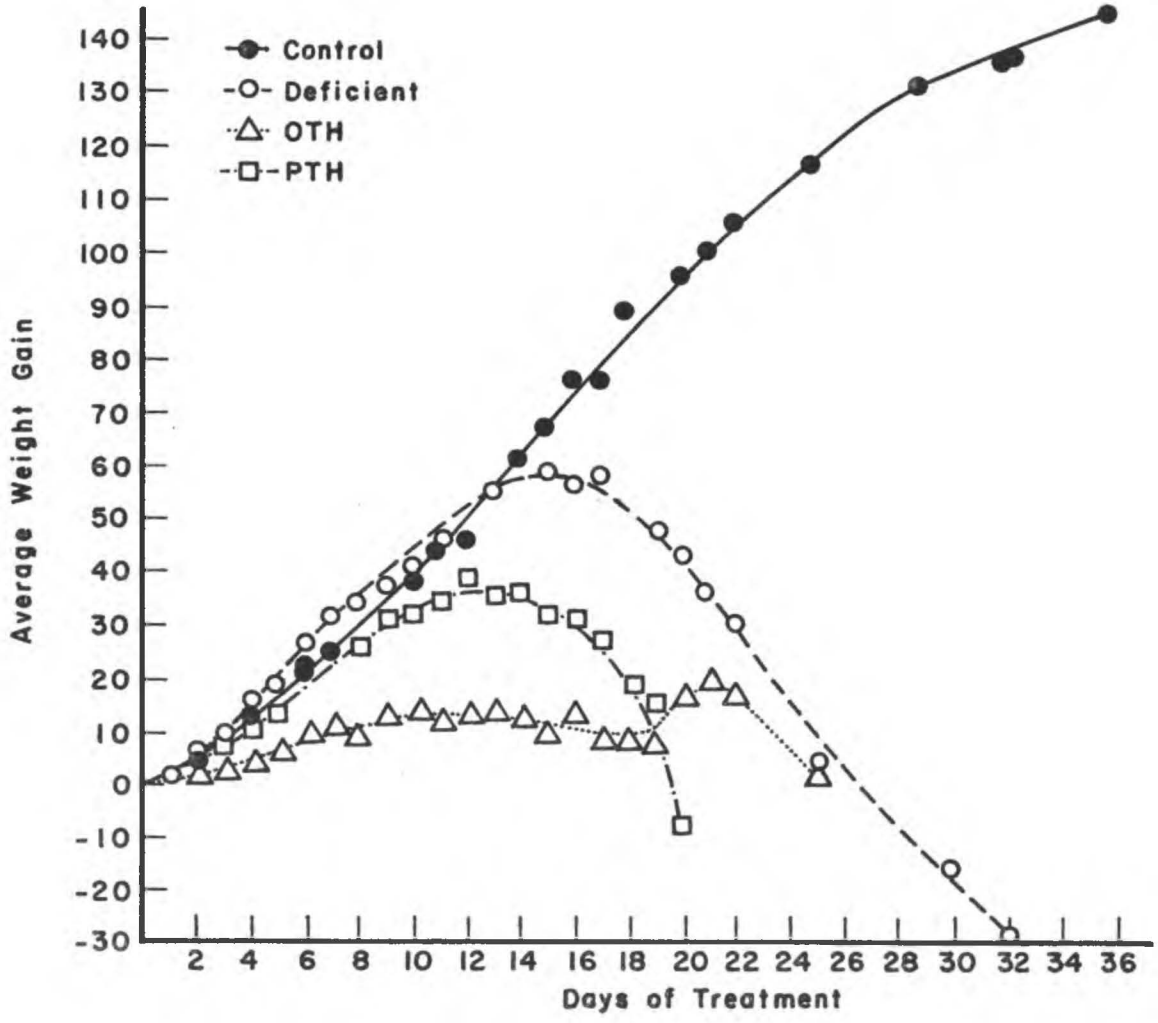


Fig. 6. --Average weight gain of control, thiamin-deficient, OTH- and PTH-treated rats--1971 analysis.

The OTH-treated rats, according to the 1970 and 1971 analyses, responded in a similar fashion and did not gain more than 20 g above their starting weights during the entire treatment period of twenty-seven and twenty-five days respectively.

The PTH-treated rats used in the 1970 and 1971 analyses, for seven to nine days, grew at the same rate as the controls. After the time the peak weight of the rat was reached, the PTH-treated rats lost on the average of 3 and 7 g per day respectively.

Thiamin and HET Assays

The major steps in the extraction of thiamin and HET from the rat brain include homogenation and deproteination, dephosphorylation by incubation at 48°C for 3 hr and finally absorption and elution from the Decalso column. The overall recovery of thiamin and HET was determined as well as the recovery at each major step of the analysis. It was found that 99.6 and 98.5% of the total thiamin and total HET respectively was extracted from the brain tissue by the procedure described in Methods. The recovery of thiamin and HET from dephosphorylated, deproteinated brain homogenate was 95 to 102% and 96.5% respectively (see Tables 3 and 4). When thiamin was added to deproteinated brain homogenate and incubated at 48°C for 3 hr and subsequently purified on the Decalso column, 97% was recovered.

When HET was treated in this way, only 84% of the HET was

TABLE 3

ELUTION OF THIAMIN FROM DECALSO COLUMN

Readings on Turner Fluorometer ± Standard Error

Solution A (Column-treated homogenate + pure thiamin)	Solution B (Column-treated homogenate)	Solution C (Pure thiamin solution - no column treatment)
40.5 ± 0.7 (5 samples)	2.0 (2 samples)	40.5 ± 0.33 (6 samples)

$$\% \text{ Recovery} = \frac{40.5 - 2}{40.5} \times 100 = 95\%$$

Solution A: To 8 ml of dephosphorylated deproteinated brain homogenate is added 0.1 ml of thiamin solution containing 207 ug/ml.

Solution B: The dephosphorylated brain homogenate.

Solution C: 0.1 ml of thiamin solution containing 207 ug/ml + 8 ml of 0.1M acetate buffer, pH 4.5.

1 ml of Solutions A and B is placed on a Decalso column. The thiamin is eluted with 25 ml of 20% KCl/0.1N HCl. One ml of Solution C is placed in a 25 ml volumetric and is diluted with 20% KCl/0.1N HCl to the mark. One ml of each of the above solutions is assayed according to the thiamin assay.

TABLE 4

ELUTION OF THIAMIN PLUS HET FROM DECALSO COLUMN

Readings on Turner ± Standard Error

Solution A (Column treated homogenate + thiamin + HET		Solution B (Column treated homogenate)		Solution C (Pure thiamin + HET)	
Thiamin (6 samples)	HET	Thiamin (6 samples)	HET	Thiamin (6 samples)	HET
54 ± 0.4	58 ± 1.4	7.0	3.0	46 ± 0.4	57 ± 0.9

$$\% \text{ Recovery of thiamin} = \frac{54 - 7}{46} \times 100 = 102\%$$

$$\% \text{ Recovery of HET} = \frac{58 - 3}{57} \times 100 = 96.5\%$$

Solution A: Exactly 0.1 ml containing 19.8 ug of thiamin and 0.1 ml containing 20.3 ug of HET is diluted to 25 ml with dephosphorylated and deproteinated brain homogenate.

Solution B: Dephosphorylated brain homogenate.

Solution C: 25 ml of 0.1M acetate buffer, pH 4.5, containing 19.8 ug of thiamin and 20.3 ug of HET.

Four ml of Solutions A and B are purified on the Decalso column as previously described. Four ml of Solution C is diluted with 20% KCl/0.1N HCl. One ml of each of the 20% KCl/0.1N HCl solutions is assayed for thiamin and for HET according to the thiamin and HET assay, respectively.

recovered (see Table 5). Since all the thiamin and HET was recovered from Decalso column treatment (see Tables 3, 4, and 6), 16% of the HET was destroyed by the 3 hr incubation at 48°C. The average per cent error associated with the thiamin and HET assay using pure thiamin and HET samples is about 1% (see Table 7).

The oxidation of thiamin to thiochrome by 1% $HgCl_2$ was found to be specific for thiamin in the presence of HET, as reported by Morita et al. (1969) (see Table 8), and in the presence of excess PTH (see Table 9). The 1% $HgCl_2$ oxidation procedure does not oxidize the PTH to pyrichrome. The fluorescent scan--with blank subtracted--of the oxidation product of pure thiamin and thiamin that was extracted from the brain of control and twenty-one day PTH-treated rats are similar (Figure 7) at high and at low concentrations in the assay.

It was determined that the total thiamin, as well as the TDP plus TTP levels in deproteinated, dephosphorylated rat brain homogenate were not significantly altered by freezing at -20°C for ten days (see Table 10). The homogenate was prepared as described in Methods. TDP could be recovered completely from brain homogenate as the diphosphate. That is, the diphosphate was not dephosphorylated by phosphatases in the homogenate and was not lost by binding to the insoluble fraction.

TABLE 5

RECOVERY OF THIAMIN AND HET FROM DEPROTEINATED BRAIN HOMOGENATE AFTER INCUBATION AT 48°C FOR THREE HOURS

Readings on Fluorometer ± Standard Error

Solution A (Incubated column-treated homogenate + thiamin + HET)	Solution B (Incubated column-treated homogenate)	Solution C (Pure thiamin + HET)
Thiamin assay[a]	Thiamin assay	Thiamin assay[c]
HET assay[b]	HET assay	HET assay[d]
72 ± 0.9^{a}	0	74 ± 1.6^{c}
51 ± 1.5^{b}	0	61 ± 0.5^{d}
(8 samples)		(4 samples)

$$\% \text{ Recovery of thiamin} = \frac{72 - 0}{74} \times 100 = 97\%$$

$$\% \text{ Recovery of HET} = \frac{51 - 0}{61} \times 100 = 84\%$$

Solution A: 4 ml of deproteinated brain homogenate is placed in 10 ml test tubes containing 40.5 ug HET and 39.6 ug thiamin. To this solution is added 1 ml of 4N acetate buffer, pH 4.5.

Solution B: 4 ml of brain homogenate only.

Solution C: 40.5 ug HET and 39.6 ug thiamin in 4 ml of water plus 1 ml of pH 4.5, 4M acetate buffer.

Solution C is stored at 4°C while Solutions A and B are incubated with 50 mg of Taka Diastase at 48°C for 3 hr. After incubation the solutions are purified on the Decalso column. All of Solution C is placed in a 25 ml volumetric and is diluted to the mark with 20% KCl/0.1N HCl. Exactly 0.1 ml of each of the 20% KCl/0.1N HCl solutions is analyzed according to the thiamin and HET assay.

TABLE 6

ELUTION OF HET FROM DECALSO COLUMN

Readings on the Fluorometer ± Standard Error

Solution A (Column-treated homogenate + HET)	Solution B (Column-treated homogenate)	Solution C (Pure HET)
33 ± 0.7 (6 samples)	1 (4 samples)	33 ± 0.7 (6 samples)

$$\% \text{ Recovery} = \frac{33 - 1}{33} \times 100 = 97\%$$

Solution A: Exactly 20.2 ug HET is placed in 8 ml of dephosphorylated and deproteinated brain homogenate.

Solution B: Dephosphorylated brain homogenate.

Solution C: 20.2 ug of HET is placed in 8 ml of 0.1M acetate buffer.

One ml of Solutions A and B is placed on a Decalso column and is eluted with 25 ml of 20% KCl/0.1N HCl. One ml of Solution C is added to a 25 ml volumetric and is diluted to the mark with 20% KCl/0.1N HCl. Exactly 1.5 ml of each of the above 20% KCl/0.1N HCl solutions is analyzed for HET according to the HET assay.

TABLE 7

AVERAGE PER CENT ERROR ENCOUNTERED IN THE THIAMIN, HET AND PTH ASSAY

Thiamin Assay		HET Assay		PTH Assay	
Mean ± Std Error*	% Error	Mean ± Std Error	% Error	Mean ± Std Error*	% Error
68 ± 0.4	0.6	58 ± 0.8	1.4	31 ± 0.4	1.3
70 ± 0.4	0.6	39 ± 0.2	0.5	40 ± 0.7	1.8
67 ± 0.5	0.7	40 ± 0.5	1.3	31 ± 0.3	1.0
74 ± 0.4	0.5	41 ± 0.2	0.5		
59 ± 0.3	0.5	47 ± 0.3	0.6		
64 ± 0.7	0.9	47 ± 0.6	1.3		
63 ± 0.4	0.6	44 ± 0.4	1.1		
61 ± 0.3	0.5				
56 ± 0.3	0.5				
65 ± 0.3	0.5				
Average % error	0.7		0.9		1.3

*Six aliquots are used to determine each mean. The mean is the average reading on the Turner fluorometer scale of 100 units.

TABLE 8

THIAMIN AND HET ASSAY STUDIES

Assay	ug Thiamin	ug HET	Other Treatment	Number Determinations	Fluorometer reading ± std. error
HET	0.10	0.00	---	5	0.0
	0.10	0.00	0.01% $HgCl_2$ was not added	3	1 - 4
	0.00	0.10	---	3	43 ± 0.4
	0.10	0.10	---	3	43 ± 0.4
Thiamin	0.10	---	---	7	64 ± 0.6
	0.00	0.20	---	5	0.0
	0.10	0.05	---	3	63 ± 0.6

TABLE 9

LACK OF INTERFERENCE OF PTH IN THE THIAMIN ASSAY

ug of thiamin added to assay	0.06	0.06	0.06
ug of PTH added to assay	4.96	2.48	0.00
Ratio of ug thiamin/ug PTH	1/83	1/41	1/0
Fluorometric* reading of solutions expressed as percent of reading of pure thiamin. Each value represents the mean of three determinations.	100.00	100.00	100.00

*Turner Fluorometer was used.

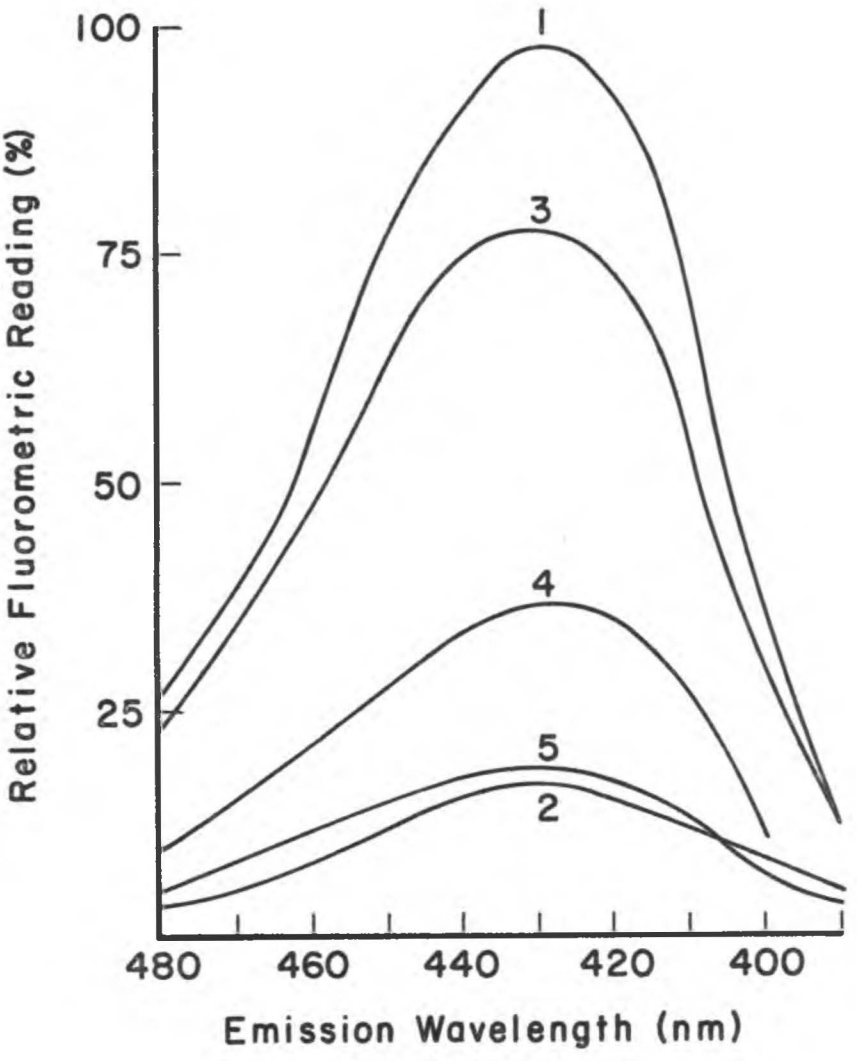


Fig. 7. --Fluorometric scan of thiochrome formed in the thiamin assay using (1) 5 ml of purified brain extract from control rat, (2) 5 ml of purified brain extract from PTH-treated rat, (3) 0.12 ug of pure thiamin per assay, (4) 0.05 ug of pure thiamin per assay, and (5) 0.02 ug of pure thiamin per assay. The thiochrome produced in these assays was scanned on the Farrand Fluorometer using 360 nm excitation wavelength. The blank reading was subtracted from each sample reading.

TABLE 10

THIAMIN LEVELS IN FRESH AND FROZEN BRAIN TISSUE HOMOGENATES

	Fresh tissue homogenate	The same homogenate that was stored at -20°C for 10 days
Total thiamin	6.5 ± 0.05	6.7 ± 0.08
TDP + TTP	5.3 ± 0.08	5.3 ± 0.06

There is no significant change in the thiamin levels in fresh and stored homogenates.

Values are expressed as the mean of four rat brains as nanomoles thiamin·HCl/g wet tissue ± standard error.

Thiamin does not interfere with the HET assay because it is destroyed prior to the alkaline potassium ferricyanide oxidation step. When thiamin and HET are rapidly mixed on a vortex mixer in a solution containing 5 ml of 20% KCl in 0.1N HCl, 2 ml of 30% NaOH and 2 drops of 0.01% $HgCl_2$, the thiamin is completely destroyed in a few seconds and only 6% of the HET is destroyed. If the 0.01% $HgCl_2$ reagent is not added to the above solution, 1 to 4% of the thiamin is not destroyed (see Table 9). This report is not in complete agreement with the assay used by Morita et al. (1969). They incubated the above solution for 5 min in order to destroy all the thiamin and only 20% of the HET. When attempting to duplicate their 5 min incubation technique, a 40% loss of HET was observed (see Figure 8). It should be noted from Figure 8 that the standard deviation of the HET assay

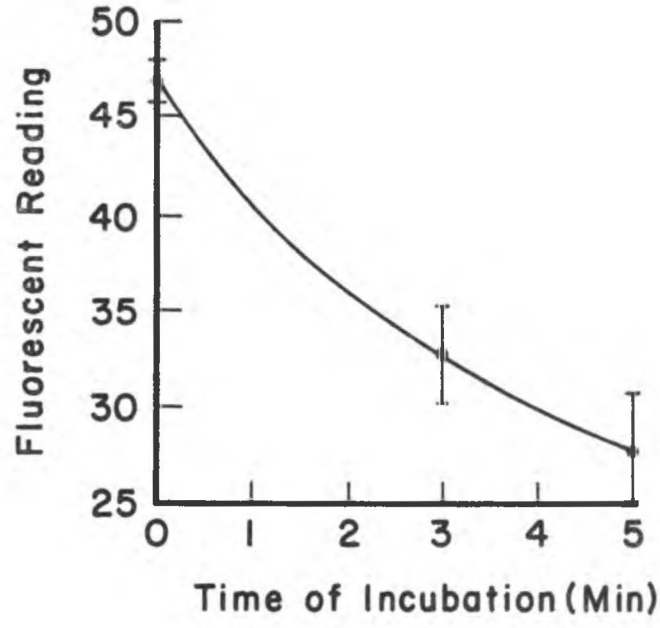


Fig. 8. --Rate of breakdown of HET in 2 ml of 30% NaOH plus 0.1 ml of 0.01% $HgCl_2$ for up to 5 min of incubation. Each point represents the mean of 4 samples $\pm$ standard deviation. The HET was oxidized to thiochrome by 0.3 ml of 1% $K_3Fe(CN)_6$. The thiochrome was then extracted in 10 ml of isobutyl alcohol. The fluorescence was read on the Turner fluorometer, with excitation γ of 365 nm with an emission γ of 436 nm.

TABLE 11

INTERFERENCE OF PTH IN THE HET ASSAY

ug of HET added to assay	0.06	0.06	0.06	0.06	0.06
ug of PTH added to assay	4.96	1.24	0.62	0.37	0.06
Ratio of ug HET/ug PTH	1/83	1/20	1/10	1/6	1/1
Fluorometric* reading of solutions, expressed as per cent of reading of pure HET. Each value represents the mean of three determinations.	175.00	116.00	108.00	103.00	100.00

*Turner Fluorometer was used.

increases several fold as the incubation time is extended from 5 sec up to 5 min.

Since PTH is sensitive to alkaline $K_3Fe(CN)_6$ when the PTH concentration is greater than the HET concentration in the HET assay, a significant amount of PTH is assayed as HET. However, until the ratio of ug PTH/ug HET is greater than 20/1, the error in the HET assay is less than 16% and increases to 75% when the ratio of ug of PTH/ug of HET increases to 83/1 (see Table 11). The fluorescent scan of the oxidation product of pure HET and of HET extracted from control rat brain are similar (see Figure 9) at high and low concentrations.

A sample of the HET received from Dr. Morita as a gift could be separated from pure thiamin by paper chromatography, using an ascending n-butanol-ethanol-water solvent system in a ratio of 3.1:1.5:1.5 (see Figure 10). Morita et al. (1968c) reported similar results using thin layer chromatography. The lowest level of thiamin or HET that can be detected is about 20 nanograms/assay. The tissue blank in all the assays is identical to the blank obtained using pure 20% KCl in 0.1N HCl.

Pure PTH, when mixed with deproteinated, dephosphorylated brain homogenate, was completely recovered from the Decalso column (see Table 12). The average per cent error associated with the PTH assay was 1% (see Table 7). Thiamin and HET do not

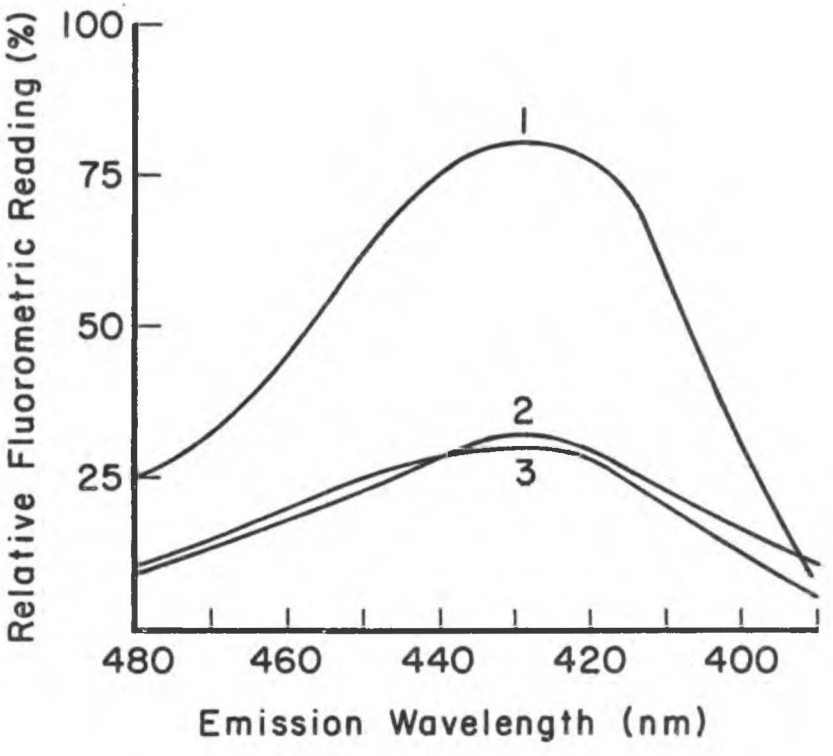


Fig. 9. --Fluorometric scan of thiochrome formed in the HET assay, using (1) 0.12 ug of pure HET per assay, (2) 5 ml of purified extract from control rat brain, and (3) 0.05 ug of pure HET per assay. The thiochrome produced in these assays was scanned on the Farrand Fluorometer using 360 nm excitation wavelength. The blank reading was subtracted from each sample reading.

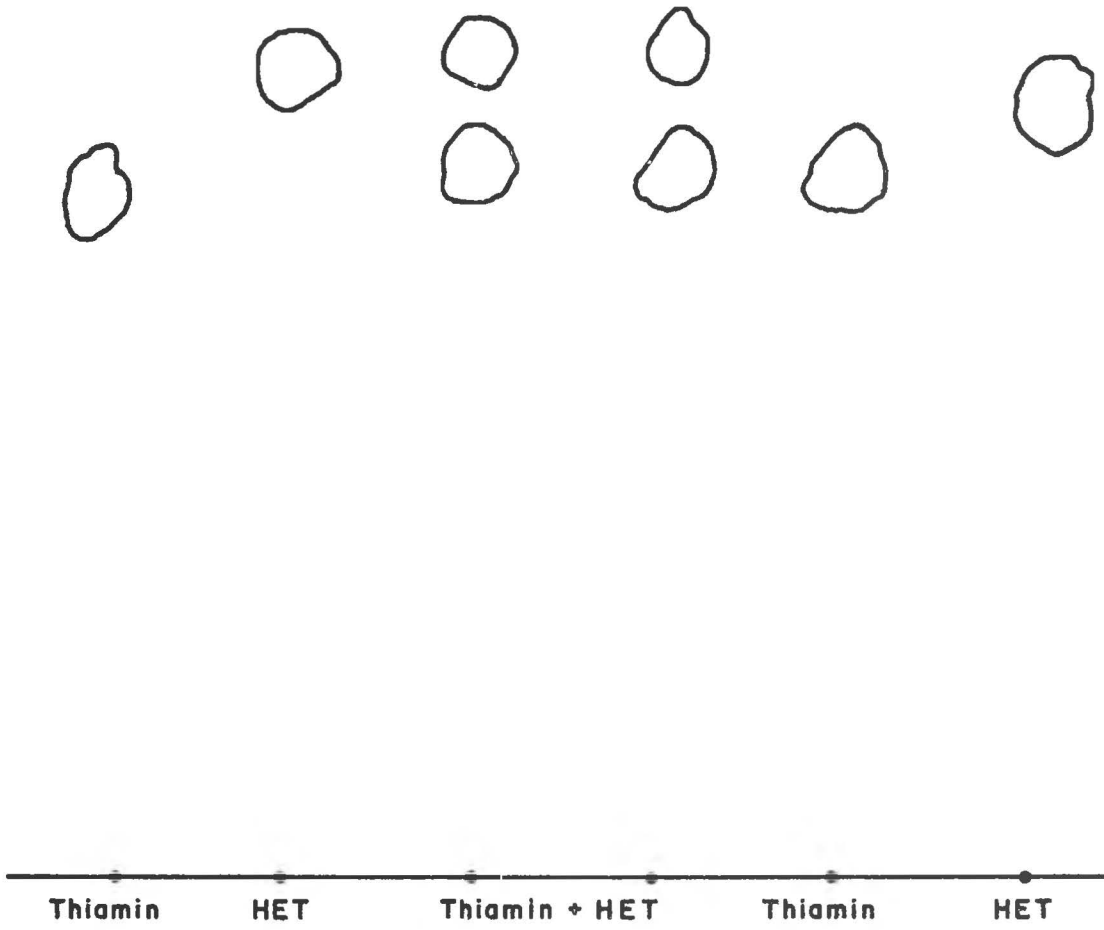


Fig. 10. --Chromatographic separation of thiamin and HET using an ascending n-butanol-ethanol-H_2O (3. 1:1. 5:1. 5) solvent system. The R_f values of thiamin and HET are 0. 51 and 0. 59 respectively. Two ul of solutions containing 200 ug thiamin/ml and 100 ug of HET/ml were spotted on Whatman #1 paper.

TABLE 12

ELUTION OF PTH FROM DECALSO COLUMN

Readings on Farrand Spectrofluorometer ± Standard Error

Solution A (Column-treated homogenate + PTH)	Solution B (Column-treated homogenate)	Solution C (Pure PTH-No column treatment)
31 ± 0.3 (6 samples)	0	31.0 ± 0.4 (6 samples)

$$\% \text{ Recovery} = \frac{31 - 0}{31} \times 100 = 100\%$$

Solution A: To 8 ml of dephosphorylated and deproteinated brain homogenate is added 0.1 ml containing 27 ug PTH.

Solution B: Dephosphorylated brain homogenate.

Solution C: 27 ug of PTH in 0.1 ml solution is added to 8 ml of 0.1M acetate buffer, pH 4.5.

Two ml of Solutions A and B are placed on a Decalso column and the PTH is eluted from the column with 25 ml of 20% KCl/9.1N HCl as previously described. Two ml of Solution C is placed in a 25 ml volumetric and is diluted to the mark with 25 ml of 20% KCl/0.1N HCl. Exactly 0.5 ml of each of the above 20% KCl/0.1N HCl solutions is assayed according to the PTH analysis.

interfere or contribute significantly to the PTH assay until the ratio of ug of thiamin plus HET/ug of PTH was 6/1. The error in the PTH assay was only 21% when the ratio of ug of thiamin and HET/ug of PTH was 60/1 (see Table 13). The fluorescent spectra of PTH in the purified brain homogenate is similar to the fluorescent spectra produced by oxidation of pure PTH samples by basic $K_3Fe(CN)_6$ (see Figure 11).

Total Thiamin Levels in Control, Thiamin-Deficient, OTH- and PTH-treated Rats

The data presented in this dissertation represents two different groups of rats which were analyzed during the summer of 1970 and again on another group of rats during the summer of 1971. The procedures described in this paper cover the 1971 analysis. The methods used for the two analyses were similar, with the exception that in the 1970 analysis, the homogenate was not purified on the Decalso column.

There is no significant difference between the brain total thiamin levels of the control rats and the OTH-treated rats in the 1970 assays even with $\alpha = 0.1$ in a two-tailed test.[4] In the 1971 assays, there were no significant differences between the brain total thiamin levels in the control and the OTH-treated rats. However, there is a

[4]Unless otherwise stated the student t test for significance is used in a two-tailed test.

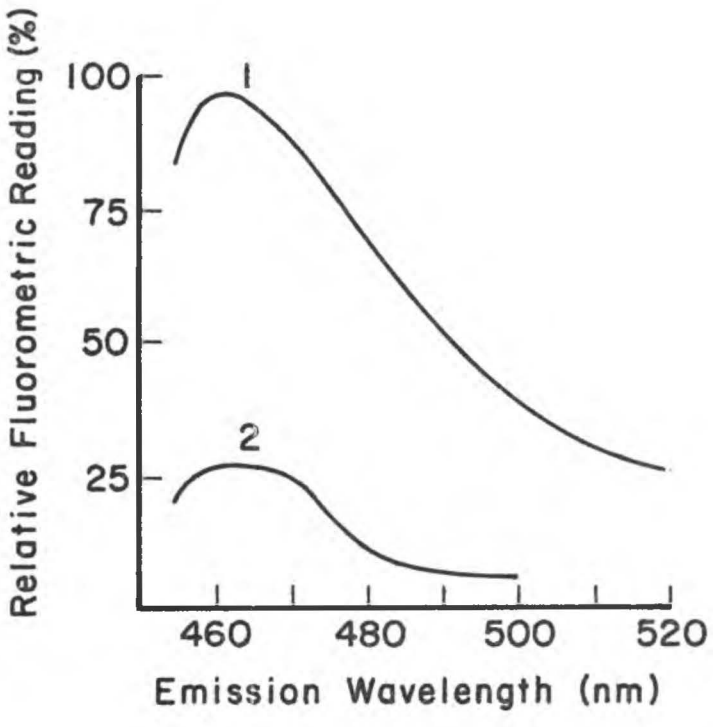


Fig. 11. --Fluorometric scan of pyrichrome formed in the PTH assay using (1) 0.37 ug of pure PTH per assay and (2) 5 ml of purified extract from brain tissue of a PTH-treated rat. The pyrichrome produced in these assays was scanned on the Farrand Fluorometer using 430 nm excitation wavelength. The blank reading was subtracted from each sample reading.

TABLE 13

INTERFERENCE OF THIAMIN AND HET IN THE PTH ASSAY

ug of PTH added to assay	0.37	0.37	0.37	0.37	0.37	0.37	0.37
ug of thiamin added to assay	0.36	0.72	1.8	7.2	12	10	---
ug of HET added to assay	0.12	0.24	0.36	1.2	12	---	10
Ratio of ug of thiamin/ug of HET/ug of PTH	1/0.3/1	2/0.8/1	5/1/1	20/3/1	30/30/1	27/0/1	0/27/1
Fluorometric* reading of solutions, expressed as per cent of reading of pure PTH solution. Each value represents a mean of three determinations.	100	100	106	110	121	118	119

*Farrand Fluorometer was used.

significant difference between the brain total thiamin levels in the control rats assayed in 1970 and in 1971 (see Tables 14 and 15, Figure 12). The reason for the difference between the two sets of assays has not been recognized. The rat brains assayed in the 1970 assays were extracted in a slightly different manner than used in the 1971 assay. The rats sacrificed in the 1970 analysis were lightly anesthetized with ether before they were decapitated and the brain was then quickly removed and placed in a vial kept in an ice bath until it was homogenized in 0.3M $HClO_4$. This slight difference in technique was not felt to be a meaningful factor since only the rats in the control and OTH-treated group seem to be affected. The total thiamin levels in the 1970 assays in the brains of the control and OTH-treated rats were 50% higher than those observed in the 1971 assay.

The brain total thiamin levels in the thiamin-deficient rats in the 1970 analysis were significantly lower than the brain levels in the control rats by treatment day 2 ($\alpha = 0.005$) and decreased to 24% of the control levels on treatment day 27. The brain total thiamin levels in the thiamin-deficient rats in the 1971 analysis were not significantly different from the brain levels in the control rats until treatment day 9 ($\alpha > 0.05$) and steadily decreased to 36% of the control levels on treatment day 36, the terminal day. It should be noted that in the PTH assay, since the ratio ug of thiamin and HET/ug of PTH was less than 4/1 during the treatment period, there was no error in the

TABLE 14

BRAIN LEVELS OF TOTAL THIAMIN, FREE THIAMIN, TMP AND TDP + TTP IN CONTROL, THIAMIN-DEFICIENT, OTH- AND PTH-TREATED RATS AND PTH LEVELS IN PTH-TREATED RATS--1970 ANALYSIS

	Treatment day	No. Rats	Treatment		
			Con[a]	% of Total Th	Deficient[a]
Total Th Levels	2	5	8.2 ± 0.34	100	5.9 ± 0.39[b]
	7	5	6.2 ± 0.13	100	3.7 ± 0.34[b]
	14	5	5.9 ± 0.12	100	2.3 ± 0.09[b]
	21	5	6.1 ± 0.23	100	1.6 ± 0.10[b]
	27	5	5.8 ± 0.20	100	1.4 ± 0.05[b]
TDP + TTP	7	5	4.6 ± 0.24	75	3.1 ± 0.24[b]
	14	5	4.7 ± 0.13	79	1.8 ± 0.18[b]
	21	5	5.1 ± 0.13	84	1.6 ± 0.11[b]
	27	5	4.3 ± 0.17	74	1.7 ± 0.09[b]
Free Th Levels	2	5	0.50 ± 0.02	6	0.33 ± 0.02
	7	5	0.10 ± 0.09	2	0.10 ± 0.09
	14	5	0.10 ± 0.09	2	0.04 ± 0.09
	21	5	0.10 ± 0.05	1	---
	27	5	0.10 ± 0.09	1	0
TMP Levels	7	5	1.4	23	0.6
	14	5	0.1	14	0.5
	21	5	0.9	15	0.0
	27	5	1.5	25	0

PTH Levels in PTH-treated Rats (5 rats/determination)

Day of Treatment	Mean ± Standard Error	
2	1.5	0.17
7	5.3	0.44
14	4.8	0.54
19	5.1	0.36

[a]Means expressed as nanomoles/g wet tissue ± standard error.

[b]Significant difference at $\alpha = 0.005$ in a two-tailed t test.

TABLE 14--Continued

Treatment					
% of Total Th	OTH[a]		% of Total Th	PTH[a]	% of Total Th
100	8.8 ± 0.23		100	5.8 ± 0.36[b]	100
100	5.8 ± 0.28		100	1.8 ± 0.25[b]	100
100	6.7 ± 0.06		100	0.7 ± 0.09[b]	100
100	---		--- day 19	0.7 ± 0.12[b]	100
100	5.8 ± 0.25		100	---	---
83	5.2 ± 0.18		89	1.6 ± 0.21[b]	90
78	5.5 ± 0.23		82	0.5 ± 0.04[b]	76
98	---		-- day 19	0.4 ± 0.12[b]	66
--	5.0 ± 0.31		87	---	--
6	0.71 ± 0.06		8	---	--
1	0.14 ± 0.03		2	0.1 ± 0.01	3
2	0.14 ± 0.02		2	0	0
--	---		-- day 19	0.01 ± 0	6
--	0.1 ± 0.01		1	---	--
16	0.5		9	0.1	6
21	1.1		16	0.2	24
25	---		--	0.2	28
--	0.7		12	---	--

TABLE 15

BRAIN LEVELS OF TOTAL THIAMIN, TDP + TTP AND HET IN CONTROL, THIAMIN-DEFICIENT, OTH- AND PTH-TREATED RATS AND PTH LEVELS IN PTH-TREATED RATS--1971 ANALYSIS

	Treatment day	No. Rats	Treatment Con[a]	% of Total Th	Treatment day	No. Rats	Treatment Deficient[a]
Total Th Levels	0	10	5.6 ± 0.17	100	--	--	---
	4	8	5.0 ± 0.09	100	4	10	4.9 ± 0.12
	9	8	4.2 ± 0.13	100	9	9	3.2 ± 0.11[c]
	18	4	4.1 ± 0.26	100	18	9	3.2 ± 0.16[b]
	25	10	3.8 ± 0.17	100	25	10	1.8 ± 0.16[c]
	33	3	3.6 ± 0.16	100	32	3	1.3 ± 0.25[c]
	36	7	4.4 ± 0.18	100	33	4	1.7 ± 0.21[c]
TDP + TTP Levels	0	10	4.8 ± 0.24	85	--	--	---
	4	8	4.2 ± 0.12	85	4	10	4.1 ± 0.12
	9	7	3.9 ± 0.28	94	9	9	3.5 ± 0.18
	18	5	2.9 ± 0.20	72	18	8	2.5 ± 0.13
	25	10	3.1 ± 0.14	82	25	10	1.5 ± 0.14[c]
	32	3	3.3 ± 0.17	92	32	4	1.2 ± 0.19[c]
	36	7	3.4 ± 0.11	76	33	5	1.4 ± 0.25[c]
HET[d] Levels	0	10	0.8 ± 0.04	14.4	--	--	---
	4	8	1.2 ± 0.22	23.7	4	10	1.1 ± 0.09
	9	8	0.4 ± 0.02	10.3	9	9	0.4 ± 0.02
	18	4	0.8 ± 0.08	20.0	18	8	0.9 ± 0.11
	25	9	0.8 ± 0.07	19.7	25	10	0.5 ± 0.09
	32	3	1.6 ± 0.17	43.0	32	3	0.6 ± 0.03
	36	7	0.7 ± 0.05	16.0	33	4	0.5 ± 0.12

PTH Levels in PTH-treated Rats[a]

Treatment Day	No. Rats	Mean ± Standard Error	
4	7	4.6	0.27
9	8	5.0	0.16
17	2	5.1	0.38
21	8	5.5	0.39

[a]Means expressed as nanomoles/g wet tissue ± standard error.

[b]Significant difference with $\alpha = 0.05$ in a two-tailed t test.

[c]Significant difference with $\alpha = 0.005$ in a two-tailed t test.

[d]The total thiamin determination includes the TDP + TTP, free thiamin and TMP but does not include HET.

TABLE 15--Continued

Trmt. % Tot. Th	Trmt. day	No. Rats	Treatment OTH[a]	% Tot. Th	Trmt. day	No. Rats	Treatment PTH	% Tot. Th
---	--	--	---	---	--	--	---	---
100	4	10	4.7 + 0.20	100	4	10	2.1 + 0.12[c]	100
100	9	10	4.6 + 0.19	100	9	10	1.0 + 0.06[c]	100
100	18	7	3.8 + 0.22	100	17	6	0.4 + 0.08[c]	100
100	25	8	3.7 + 0.22	100	21	10	0.3 + 0.01[c]	100
100	--	--	---	---	--	--	---	---
100	--	--	---	---	--	--	---	---
--	--	--	---	--	--	--	---	--
84	4	10	3.6 + 0.28	76	4	10	2.0 + 0.22[c]	95
--	9	7	4.2 + 0.08	92	9	8	1.1 + 0.05[c]	--
78	18	8	3.1 + 0.07	82	17	6	0.4 + 0.03[c]	100
86	25	8	3.3 + 0.21	89	21	10	0.3 + 0.02[c]	--
93	--	--	---	--	--	--	---	--
79	--	--	---	--	--	--	---	--
--	--	--	---	--	--	--	---	--
26	4	10	1.0 + 0.08	21	4	9	0.6 + 0.07[c]	27
12	9	10	0.5 + 0.03	10	9	10	0.2 + 0.01[c]	17
29	18	7	0.7 + 0.10	19	17	6	0.2 + 0.12[c]	62
26	25	8	0.7 + 0.09	19	21	9	0.1 + 0.03[c]	36
47	--	--	---	--	--	--	---	--
30	--	--	---	--	--	--	---	--

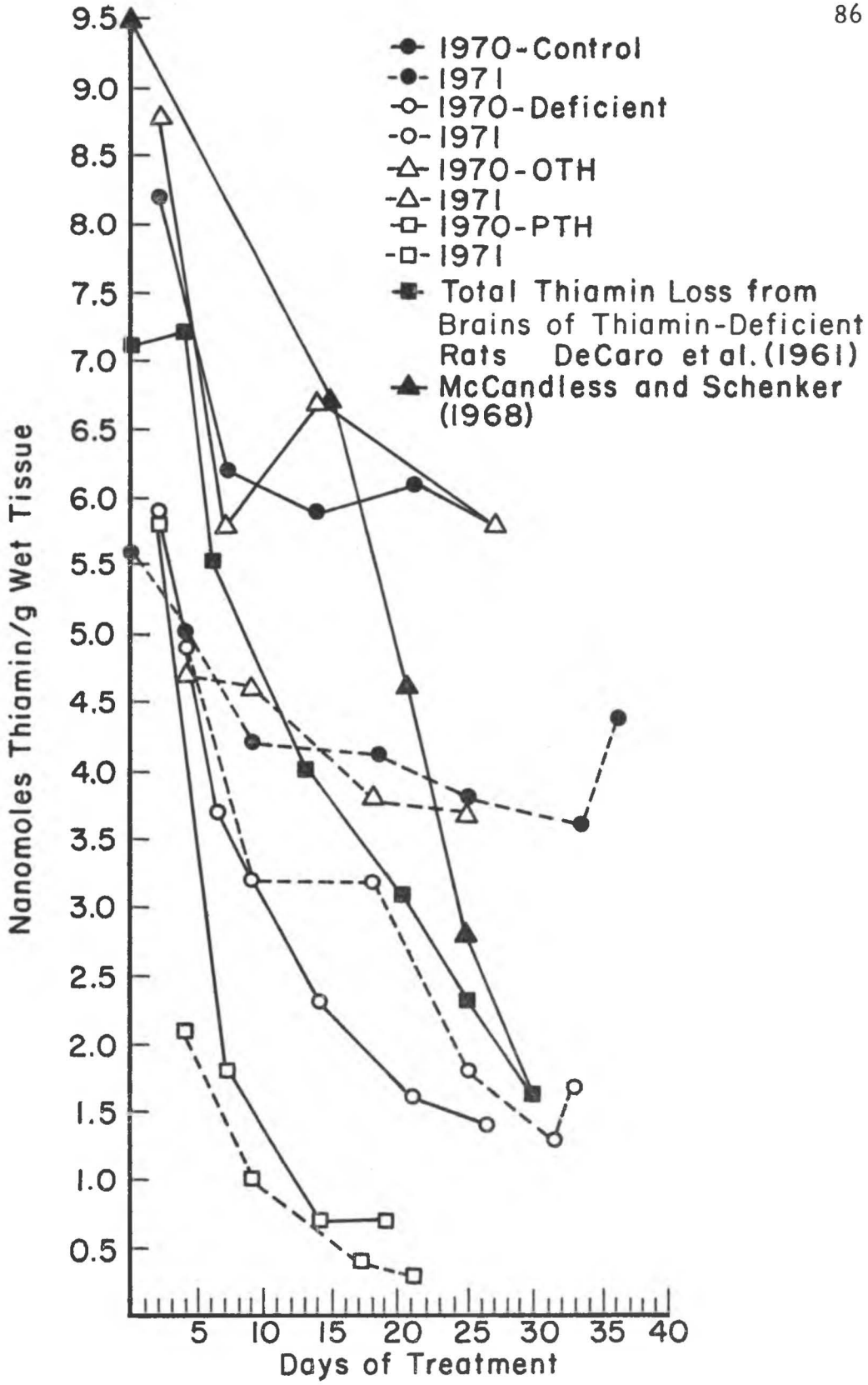


Fig. 12. --Brain levels of total thiamin in control, thiamin-deficient, OTH- and PTH-treated rats.

PTH assay due to the presence of thiamin or HET. The brain total thiamin levels in the PTH-treated rats decreased rapidly in both the 1970 and 1971 analyses and there was no meaningful difference between the total thiamin levels in the two periods of analysis. By treatment day 2 and 4 there was a significant drop of 30% in the brain total thiamin levels in the PTH-treated rats ($\alpha = 0.005$) as compared to the corresponding control levels. These levels decreased on the average to 25% of the control levels by treatment day 10, decreasing in the terminal PTH-treated rats to one-seventh of the control values.

TDP + TTP, TMP and Free Thiamin Levels

The quantity represented by TDP + TTP in all groups varied in direct proportion to the brain total thiamin levels (see Tables 14 and 15 and Figure 13) remaining about 85% of the respective total thiamin levels. The level of TDP + TTP in the OTH-treated rats as compared to the level in the control rats within the same set was not significantly different in the 1970 nor in the 1971 assays ($\alpha = 0.005$); however, the brain levels in the same groups in the 1970 assay were distinctly higher than in the 1971 assay ($\alpha = 0.005$). The quantity TDP + TTP varied in the control and OTH-treated group around 85% of the total thiamin levels. The rate of depletion of TDP + TTP as observed in the thiamin-deficient rat in the 1970 assay was slightly higher than in the 1971 assay. There was a 30% decrease in the 1970

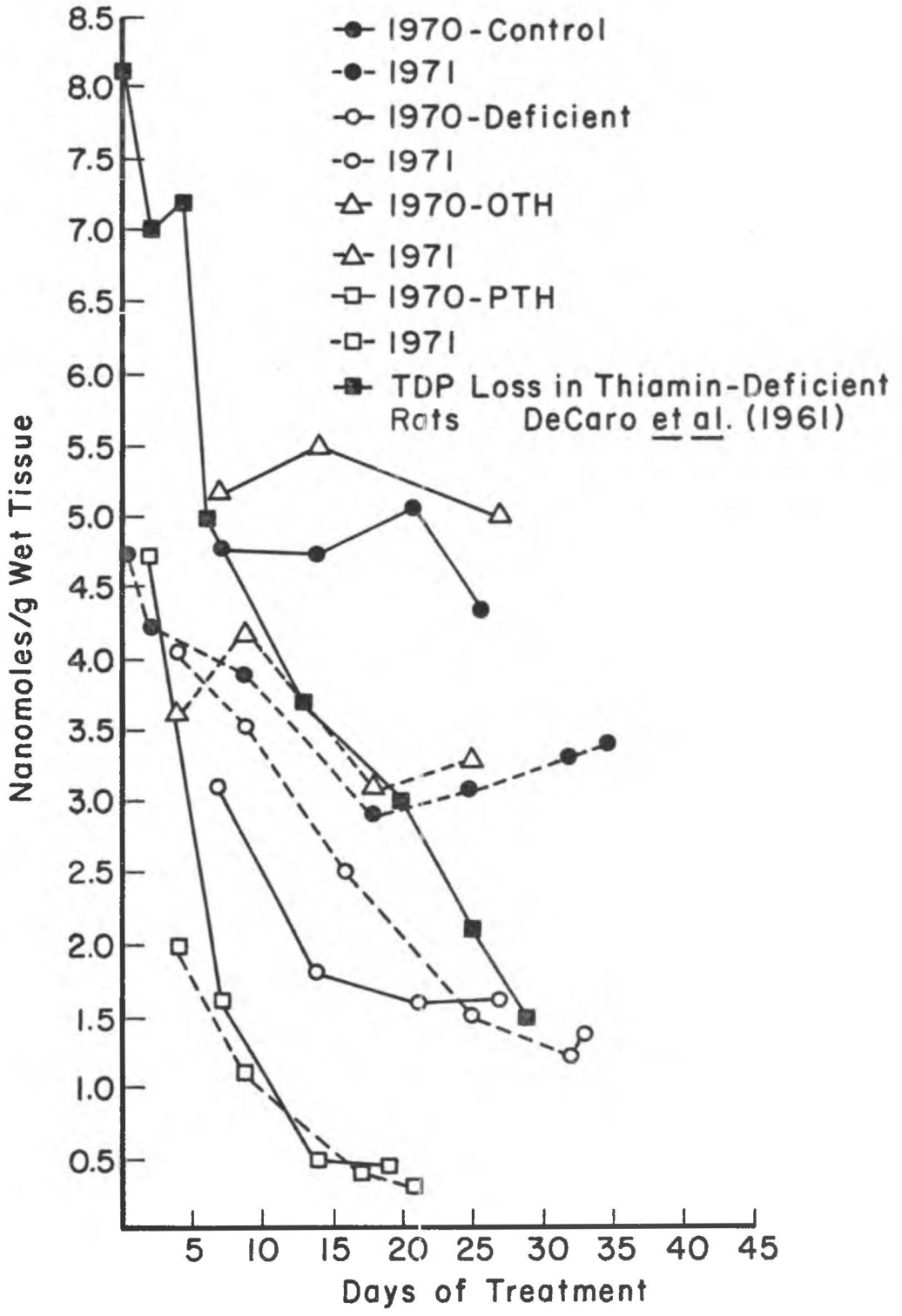


Fig. 13. --Brain levels of TDP + TTP in control, thiamin-deficient, OTH- and PTH-treated rats.

assay from the control group ($\alpha = 0.005$) on treatment day 7, decreasing to and remaining at 36% of the control values from treatment day 14 for the duration of the treatment period. Although the brain TDP + TTP levels in the terminal thiamin-deficient rats in the 1971 analysis decreased to 43% of the control rat brain levels, the TDP + TTP level was not significantly different from the control level until after treatment day 18.

The TDP + TTP levels in the brains of the PTH-treated rats decreased to 30% of the corresponding control values by the first analysis period in each set then decreased to a minimum average value of 13% of the control values after two weeks of treatment (see Tables 14 and 15, Figure 13).

The per cent of free thiamin in the brain decreased much more rapidly in the brains of thiamin-deficient and PTH-treated rats than in the control and OTH-treated rats (Figure 14). The free thiamin levels in the brain of OTH-treated rats were significantly higher than in the control rats on the second day of treatment, but by the seventh day of treatment, and until the experiment was terminated, the level of free thiamin in the brains of the controls and OTH-treated rats was not meaningfully different. The TMP levels in the brain of the control and OTH-treated rats did not vary in a regular fashion. The TMP levels decreased in the brain of the thiamin-deficient and PTH-treated rats to very low levels.

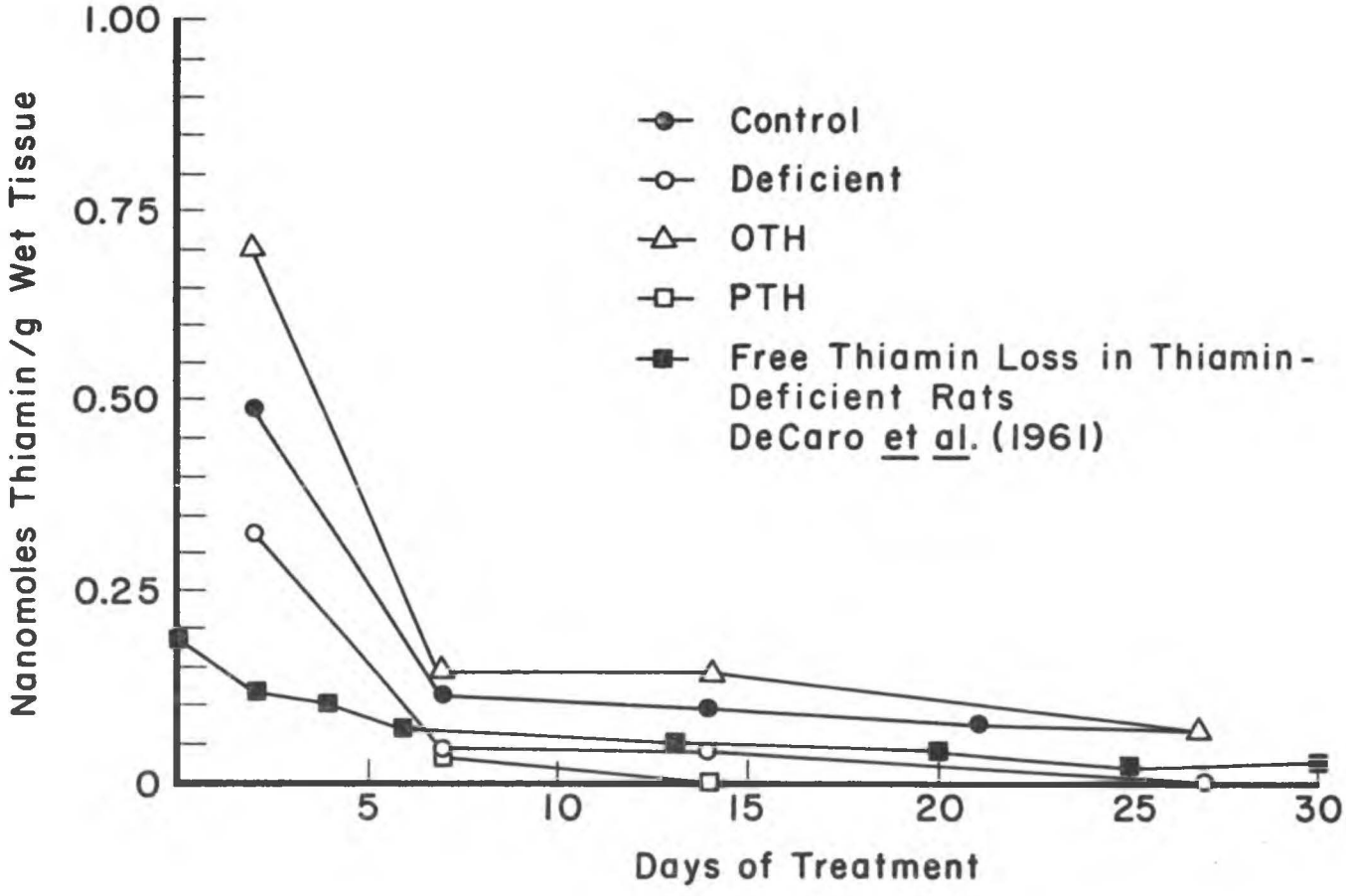


Fig. 14. --Brain free thiamin levels in control, thiamin-deficient, OTH- and PTH-treated rats.

HET and PTH Levels

The HET levels in the brains of the control, thiamin-deficient, and OTH-treated rats were not significantly different ($\alpha = 0.05$). The brain HET levels of the PTH-treated rats on treatment day 4 were 40% lower than the control rats (see Tables 14 and 15, Figure 15) and decreased rapidly to less than one-sixth of the control levels by the terminal treatment period. Note that the ug of PTH/ug of HET ratio based on the HET assay varies up to 50/1, which means that the error in the HET determination may be 50% or more. However, since the PTH levels after the first week of treatment are maintained at a constant level in the brain, the decrease of the HET levels is meaningful. The brain level of HET in the control rats varied significantly in a regular way around 0.7 nanomoles/g wet tissue. The cause of this large variation has not been identified.

The PTH accumulates in the brain as the treatment progresses. By treatment day 7 the brain PTH levels, as determined in the 1970 and 1971 analyses, approach maximum levels of 5.0 nanomoles/g wet tissue, which levels are close to the concentration of thiamin in brains of the control rats (see Figure 16).

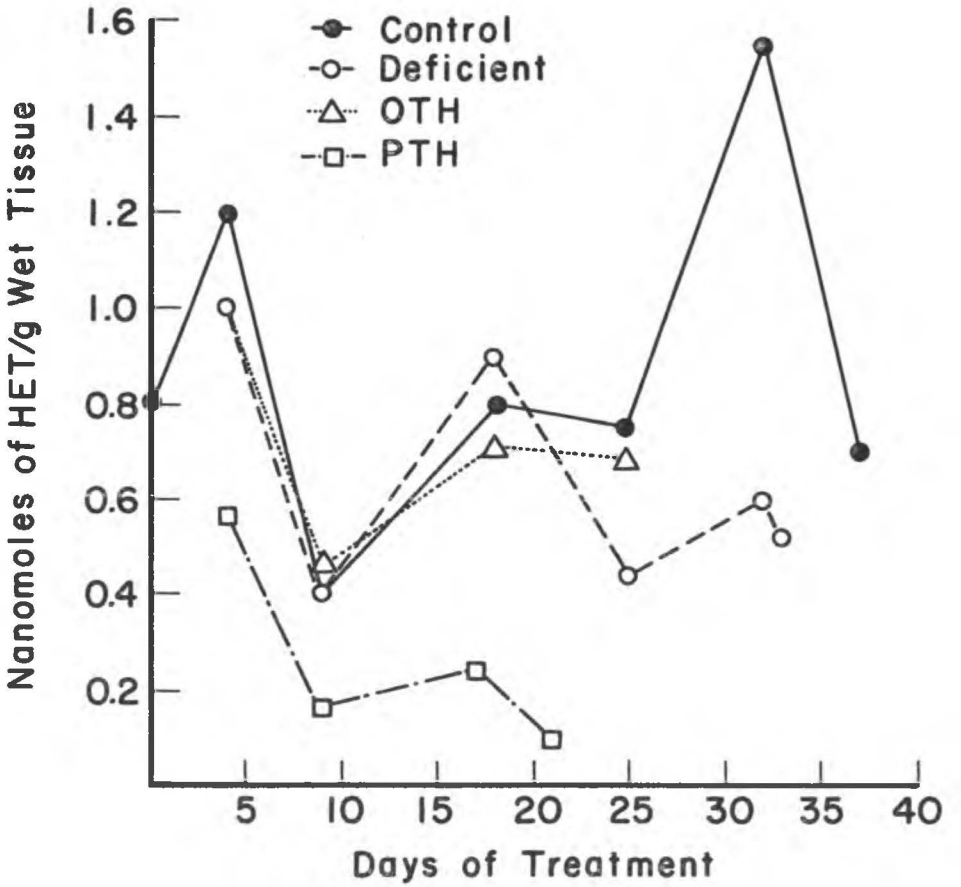


Fig. 15. --Brain HET levels in control, thiamin-deficient, OTH- and PTH-treated rats.

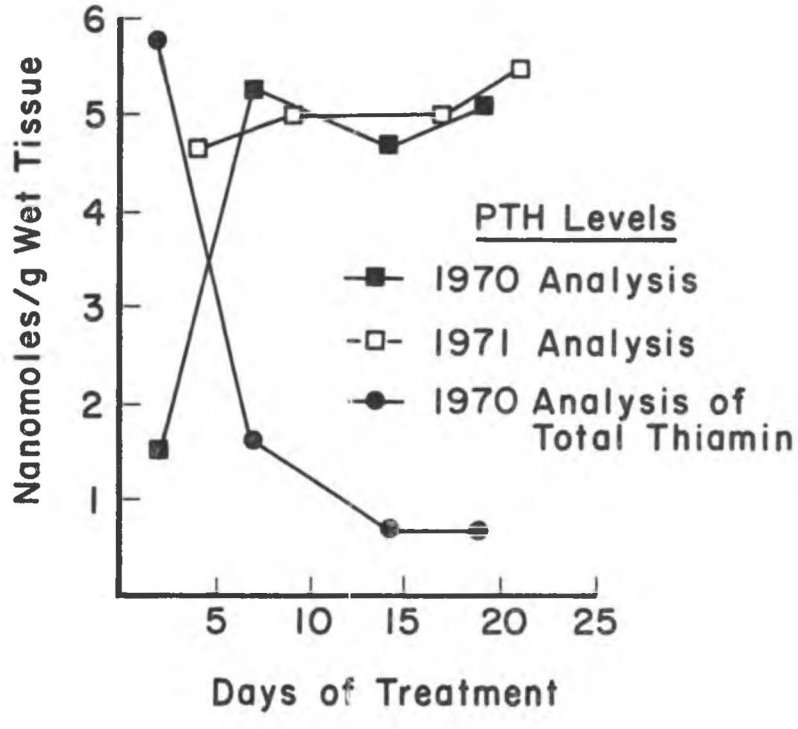


Fig. 16. --Brain PTH levels in PTH-treated rats.

DISCUSSION

The rate of weight gain of the four groups of rats in both the 1970 and 1971 analyses was similar to previous reports from Dr. Gubler's laboratory [Gubler (1961), Cheney et al. (1969), Park and Gubler (1969), Bai et al. (1971)].

The recovery of thiamin and its derivatives and PTH from rat brain tissue was good in spite of the numerous steps required to process the extremely small amount of thiamin and PTH. Many investigators have reported excellent recoveries of thiamin and PTH from rat tissues [see Rindi et al. (1961), DeCaro et al. (1961), Morita et al. (1968a, b, c, 1969)]. The standard error determined in each assay expressed as the average per cent error using standard solutions of thiamin, HET, and PTH was 1% of the mean. The standard deviation for each assay determined using up to ten individual rat brains, however, is several fold higher. The columns, techniques and procedures used throughout this dissertational study were uniform so it is felt that this high standard error reflects individual biological differences in levels rather than technological errors inherent in the respective assays.

It is important to note that there was a rapid decrease of the

brain total thiamin of the control rats in the first week of treatment. This decrease was due to the drastic change in the thiamin intake. The rats, when received, were allowed to feed on Purina Rat Chow ad libitum for four days prior to the beginning of the treatment. During this time, the rat tissues became saturated in thiamin. The chow contains 16 ug of thiamin/g, and rats consumed about 15 g of chow per day; therefore, the rats consumed about 240 ug of thiamin per day when fed the Purina Rat Chow. When treatments started on day zero, the control rats then received only 10 ug thiamin/100 g body weight daily (s.c.). This drastic decrease in thiamin intake caused the brain levels in the control rat to decrease 25% from the day zero levels. The levels of thiamin in the tissues of the control rats established by the first week of treatment represent minimal levels since 10 ug thiamin/100 g body weight is the daily minimal thiamin intake required for a normal weight gain [Scott and Griffith (1957)]. Thus this initial decrease in the brain thiamin of the thiamin-deficient, OTH- and PTH-treated rats includes this 25% decrease in the thiamin levels from the corresponding day zero levels.

The brain total thiamin, TDP + TTP, TMP, free thiamin, and HET levels in the OTH-treated rats were not meaningfully different from the levels in the control rats. OTH must exert its effect on other organs of the rat. The fact that the OTH-treated rat does not gain weight during treatment cannot be correlated with the

level of thiamin derivatives in the brain. The blood-brain barrier allows only a small amount of OTH to accumulate in the brain [Rindi et al. (1963)] so the daily thiamin plus OTH injections provide an adequate amount of thiamin to the brain tissue. The OTH-treated rats served as another "control" group. Note that the brain free thiamin levels decrease to one-tenth their initial values in four weeks of treatment in the control and OTH-treated rats. The TMP level in these two groups is somewhat erratic and does not show any particular trend during treatment. Total thiamin levels reported in this dissertational study in control rat brains was 8.2 and 5.6 nanomoles/g wet tissue. DeCaro et al. (1961), Rindi and Guiseppe (1961), and Morita et al. (1968c, 1969) report brain total thiamin levels in normal rats of 7.7, 7.1, 9.1, and 9.5 nanomoles/g wet tissue respectively.

In the thiamin-deficient rat, the rate of loss of total thiamin from the brain is close to the rate of depletion reported by DeCaro et al. (1961), and McCandless and Schenker (1968) (see Tables 14 and 15, Figure 12). The techniques used by the above authors probably did not discriminate between HET and thiamin. This may account partly for their higher values of total thiamin in the brains of the thiamin-deprived rats. In the 1970 and 1971 analyses, the thiamin-deficient rats reached a peak average weight gain on treatment day 11 and 15 respectively. The brain total thiamin level in the rats at these times of treatment are 2.8 and 3.3 nanomoles/g wet tissue

respectively (see Figures 5, 6 and 12). This total thiamin concentration seems to be a critical value, for the rats decrease in weight rapidly as the total thiamin levels decrease further. The TDP + TTP levels on treatment day 11 and 15 in the 1970 and 1971 analyses respectively is 2.3 and 2.6 nanomoles/g wet tissue and drops to much lower levels with further thiamin deprivation treatment. The PTH-treated rats reach their peak weight about the same time as the thiamin-deficient group, yet the brain total thiamin levels in the former group are one-third to one-fifth the levels of the latter group at this time of treatment (see Figures 5, 6 and 12). Perhaps the rate at which thiamin is lost from other organs of the body in the thiamin-deficient rats and the PTH-treated rats help determine the rate at which the treated rats gain weight. Another explanation might be that PTH treatment may not deplete thiamin at the same rate from all areas of the brain. Thiamin has been reported to be depleted in a uniform rate in all areas of the brain in the thiamin-deficient rat [Dreyfus (1961)].

The more rapid rate and extent of depletion of thiamin from the brain of PTH-treated rats as compared to thiamin-deficient treated rats causes the former group of rats to reach a terminal condition one to two weeks sooner than in the latter group. None of the thiamin-deficient rats showed signs of convulsions. In the terminal thiamin-deficient and PTH-treated rats, the total thiamin levels and TDP + TTP levels averaged 1.5 and 0.5 nanomoles/g wet tissue respectively.

It is significant that there is a striking difference in the total thiamin (and TDP + TTP) levels in the brains of terminal thiamin-deficient and PTH-treated rats when only the latter group experiences convulsions. It would be extremely interesting to determine the brain level of total thiamin, TDP + TTP, and HET in terminal, convulsive thiamin-deficient rats.

The reason for the depletion of thiamin in the brain of the PTH-treated rats was not due to an insufficient level of daily thiamin intake, as in the case of the thiamin-deficient rats, for 10 ug of thiamin is injected subcutaneously daily along with the 50 ug of PTH/100 g of body weight. The thiamin levels in the heart and liver of thiamin-deficient rats is much less than in PTH-treated rats, so there is a much larger supply of tissue thiamin for the brain to draw upon in the PTH-treated rats [Gurtner (1961), DeCaro et al. (1954, 1958, 1961), unpublished work in Dr. Clark J. Gubler's lab].

It is interesting that PTH accumulates in the brain rapidly with treatment and levels off at about 5.0 nanomoles/g wet tissue in the first week of treatment and remains at about this level for the next two or three weeks of treatment (see Figure 16). The daily intake of thiamin in the PTH-treated rat tends to displace PTH from the tissues [Morita et al. (1968b)], and hence acts to keep PTH from accumulating in the brain to higher levels than was observed in this dissertational study. It has been established that free PTH inhibits the following reaction in vitro:

$$\text{thiamin} \xrightarrow[\text{purified thiamin pyrophosphokinase}]{\text{ATP}} \text{TDP}$$

Johnson and Gubler (1968), Peterson (1970)

PTH also inhibits the active transport of thiamin into the animal cell [Sharma and Quastel (1965), Rindi and Ventura (1967)]. The conversion of free thiamin to the diphosphate is closely associated with the active transport process. The PTH + thiamin injections caused, in this dissertational study, a 50% depletion of total thiamin from the brain by treatment day 3 (see Figure 12). By treatment day 15, the total thiamin levels had decreased to about one-tenth of the control values. The rate at which PTH accumulates in the brain equals approximately the rate at which thiamin is displaced from the brain. One cannot tell from the data presented in this dissertational analysis how much thiamin is entering the brain after the maximum levels of PTH are reached in this tissue. Obviously since the total thiamin levels decrease in the brain tissue, the rate of entry of thiamin into the cell is much less than the rate at which the thiamin is displaced from its active sites within the cell, dephosphorylated and excreted out of the cell. The free thiamin and TMP decrease to very low levels with treatment showing that the TDP and TTP breakdown products do not accumulate in the brain tissue. The mechanisms which control the rate of entry of thiamin into the cell, the rate of dephosphorylation of

the thiamin phosphates, and the rate at which the TMP or free thiamin leave the cell has not been defined but seems to operate up to the time of death of the rat. The distribution of thiamin in the brain tissue of all treated groups is rather constant throughout the treatment period. The TDP + TTP levels remain at around 85% of the total thiamin in all the groups.

The Ki of free PTH is 0.2 uM toward thiamin pyrophosphokinase from rat brain [Johnson and Gubler (1968)] but the PTH diphosphate ester has a Ki of 78 uM for yeast pyruvate decarboxylase [Wittorf and Gubler (1971)]. Therefore, the initial effect of PTH on the brain tissue is probably due to the inhibition of the thiamin pyrophosphokinase activity. Thiamin entering the tissue that cannot be phosphorylated to the diphosphate ester would leave the cell. As the PTH/(TDP + TTP) ratio increased from about 4/1 on treatment day 7 to about 10/1 by treatment day 14, the PTH diphosphate [Morita et al. (1968b)], and unpublished work in Dr. Clark J. Gubler's lab which is probably formed in the tissue may effectively compete with TDP for the active site on the TDP requiring enzymes, for the Ki of PTH diphosphate is only three times the Km of TDP for yeast pyruvate decarboxylase [Wittorf and Gubler (1971)]. As the treatment progresses, PTH may antagonize both of these thiamin requiring enzymes in the brain tissue.

The continuously decreasing level of HET observed in the

brains of PTH-treated rats can be coordinated with the reduced pyruvate dehydrogenase activity in the brain of PTH-treated rats [Gubler (1961), Bennett et al. (1966)] and in mice [Holowach et al. (1968)] (see Table 1). No studies have been reported dealing with the preterminal effect of PTH on the pyruvate dehydrogenase activity in the brain. Note also that the pyruvate dehydrogenase activity in the brain of OTH-treated rats is normal which fact correlates well with the brain HET levels in the OTH-treated rats. It is not known why the report by Reinauer et al. (1968) of the pyruvate dehydrogenase activity in the brain in thiamin-deficient rats does not agree with the same determination by Gubler (1961) and McCandless and Schenker (1968). The pyruvate dehydrogenase activity in the brain of thiamin-deficient rats is decreased 25, 30, and 72% from the control rats according to Gubler (1961), McCandless and Schenker (1968), and Reinauer et al. (1968) respectively. The first two reports tend to support the somewhat decreased levels of HET observed in this dissertational study in the thiamin-deficient rat brains. The 24% decrease of brain HET in the terminal thiamin-deficient rats from the control group is not significant in a two-tailed t test at the 95% level of confidence. If one felt that since HET decreases in the PTH-treated rat as the thiamin level decreased, an increase in the HET level of the thiamin-deficient rat is not likely, a one-tailed test is justified. Even under these conditions, a 24% decrease in the terminal thiamin-deficient group is not significant at the 95% level of confidence.

Another interpretation of a lowering of the HET levels would be that HET is used up via the citric acid cycle at a faster rate as compared to the control rat. The AcCoA could be converted to cholesterol and/or fatty acids at an increased rate. The decrease in the HET levels might be due to a rapid breakdown of the HET molecule with no concurrent formation of AcCoA. The first hypothesis does not seem likely in the PTH-treated rats since the α-ketoglutarate dehydrogenase activity decreases in the brain of the PTH-treated rats during treatment. The decrease in the transketolase activity of the brain of thiamin-deficient rats [McCandless and Schenker (1968), and Brin (1962)] would not favor increased synthesis of fatty acids or cholesterol since both processes require NADPH. Breakdown of the HET moiety with no concurrent AcCoA formation would be a wasteful process and is, therefore, an unlikely route.

SUMMARY

The minimal level of thiamin in the rat brain that is necessary for an adequate level of metabolism has not been reported in the literature. However, the brain level of total thiamin, TDP + TTP, and HET in rats receiving a minimal yet sufficient daily amount of thiamin that is required for a normal weight gain seems to be about 3.8, 3.3, and 0.7 nanomoles/g wet tissue respectively.

The TDP + TTP levels in the brain of the thiamin-deficient rat are at least twice as high as is observed in the brains of the PTH treated rats during the entire treatment period. After fourteen days of treatment the TDP + TTP levels are from three to five times higher in the thiamin-deficient than in the PTH-treated rat brains. The minimal levels of TDP + TTP in the thiamin-deficient and in the PTH-treated rat brains were initially observed at three weeks and two weeks respectively, although the terminal levels in the thiamin-deficient rat brains are three times as high as the PTH-treated rats. The minimal level of TDP + TTP observed in the brains of the thiamin-deficient and PTH-treated rats was 36 to 43% and 10 to 15% respectively of the levels in the brain of the control rats.

OTH treatment does not alter the amount or distribution of

the thiamin derivatives in the brain of rats treated up to twenty-seven days so OTH probably exerts its effects on organs outside the central nervous system.

This report is the first comprehensive study of the effect of thiamin-deficiency, OTH and PTH treatment on the level of HET in mammalian tissues. Normal levels of HET were observed in the brain of the OTH-treated rats for the duration of treatment. The brain levels in the thiamin-deficient treated rats were not significantly different ($\alpha > 0.05$) from the control group even though the HET levels decreased 24% from the control group during the last two weeks of treatment. The brain HET levels in the PTH-treated rats decreased markedly and significantly ($\alpha = 0.005$) below the control group by treatment day 4, decreasing to around one-sixth the level of HET in the controls by the third week of treatment. The decrease in the level of HET in brains of PTH-treated rats was assumed to mean that the conversion rate of pyruvate to AcCoA was depressed significantly.

The PTH levels increased rapidly in the brain to normal thiamin levels by the end of the first week of treatment. The PTH/(TDP + TTP) ratio was high enough by treatment day 10 so that the PTH diphosphate could compete with TDP for the active site on the pyruvate dehydrogenase enzyme.

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THE DETERMINATION OF THIAMIN AND ITS DERIVATIVES IN BRAIN TISSUE OF CONTROL, THIAMIN-DEFICIENT, OXYTHIAMIN- AND PYRITHIAMIN-TREATED RATS

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ABSTRACT

The determination of total thiamin, free thiamin, thiamin diphosphate (TDP) plus thiamin triphosphate (TTP), and total α-hydroxyethylthiamin (HET) levels in rat brain in control, deficient, oxythiamin- (OTH) and pyrithiamin- (PTH) treated rats was accomplished. It was found that the TDP + TTP/thiamin ratio observed in the thiamin-deficient, OTH- and PTH-treated rats was constant and did not differ from the ratio observed in the control rat brains. The brain levels of TDP + TTP decreased to 39% and 12% of the control thiamin levels in deficient and PTH-treated rats respectively. The brain HET and TDP + TTP levels of the OTH-treated rats were not significantly different from the controls. The HET levels in the PTH rats decreased significantly ($\alpha = 0.005$) by treatment day 4 and decreased to one-seventh of the control values in the terminal stages. A significant drop in the HET levels from the control levels was interpreted to mean that the pyruvate utilization was significantly impaired in the brain.