1968-08-01

The effect of thiamine and its antagonists on plasma and tissue lactic dehydrogenase in rats

Dong Hwa Park
Brigham Young University - Provo

THE EFFECT OF THIAMINE AND ITS ANTAGONISTS ON PLASMA AND TISSUE LACTIC DEHYDROGENASE IN RATS

A Thesis

Presented to the

Department of Chemistry

Brigham Young University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Dong H. Park

August 1968

This thesis, by Dong H. Park, is accepted in its present form by the Department of Chemistry of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

ACKNOWLEDGMENTS

The writer wishes to express sincere thanks to Dr. Clark J. Gubler for his guidance and advice throughout this investigation.

Special appreciation is expressed to Dr. John H. Mangum for his suggestions in the writing of this thesis.

Support for this investigation was provided by a research grant from the National Institute of Mental Health.

Gratitude is expressed to my wife, Min Jung, for her continuing encouragement and help.

Sincere thanks is also extended to the writer's uncles and aunt for their encouragement and support throughout his academic career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

INTRODUCTION

Meyerhof (1) first observed the oxidation of lactic acid in the presence of muscle and demonstrated the participation of a coenzyme in this oxidation. Szent-Györgyi (2) then studied the properties of the lactic coenzyme prepared from heart muscle and contributed much towards elucidating its chemical nature.

Von Euler *et al.* (3) and Meyerhof and Ohlmeyer (4) independently demonstrated that the presence of the coenzyme I, i.e., nicotinamide-adenine dinucleotide (NAD^+),[a] is necessary for this oxidoreduction to take place. Von Euler *et al.* presented spectroscopic evidence that the coenzyme functions in these reactions by undergoing a cycle of reduction by α-glycerophosphate and of oxidation by pyruvate.

Von Euler *et al.* (5) and Warburg and Christian (6) found that the reduced form of coenzyme I (NADH) showed a band at 340 mμ. This characteristic absorption band of the reduced coenzyme has been utilized by Warburg and von Euler for studying reversible oxidations and reductions spectrophotometrically.

---

[a]The following abbreviations will be used in this thesis: NAD^+ for nicotinamide-adenine dinucleotide; NADH for reduced nicotinamide-adenine dinucleotide; LDH for lactic dehydrogenase; Th for thiamine; OTh for oxythiamine; PTh for pyrithiamine; Tris for tris (hydroxymethyl) aminomethane.

Wieland and Pfleiderer (7) first demonstrated that various components exhibiting lactic dehydrogenase (LDH)[a] activity can be separated by high-voltage electrophoresis from extracts of liver, heart, kidney, skeletal muscle, spleen, brain, and erythrocytes derived from a single animal. Five electrophoretically distinguishable forms of LDH arise from the combination of two distinct types of protein subunits into the tetrameric forms called isozymes[b] of active LDH. The subunits have been referred to as H and M[c] (heart and muscle) types, which appear to be controlled by two separate and independent genes. These two forms are different in amino acid composition, thermal stabilities, substrate specificities, abilities to utilize coenzyme analogues, susceptibilities to inhibitors, and other properties. The native enzymes are designated as H_4, H_3M, H_2M_2, HM_3, and M_4 in order of their decreasing mobility toward the anode (8-10).

The work of Wieland and Pfleiderer (7) and of Pfleiderer and Jeckel (11) has been especially noteworthy in demonstrating the species and tissue specificity of the isozymes of LDH. Markert and Møller (12) have been able to confirm their general results. Markert and Møller proposed the use of the term "isozyme"[b] to describe the different molecular forms in which proteins with the same enzymatic specificity may exist.

---

[b]The alternative form "isoenzyme" is also used in the literature.

[c]The terms "A" (M) and "B" (H) are also used to describe the particular subunits.

The reaction of glycolysis in animal tissues leads to the end products, pyruvate and lactate, which are reversibly convertible to each other by LDH and its co-enzyme (NADH or NAD^+). The equilibrium position of this reaction strongly favors formation of lactate rather than its oxidation (13).

Thiamine deficiency is associated with an increase in the blood pyruvate and lactate levels (14-24). Therefore, it seemed of importance to see if these elevated levels were associated with changes in the level of total LDH activity and of isozyme distribution in plasma and tissues of thiamine-deficient and antagonist-treated rats.

In diseases leading to a leakage of enzyme from a damaged tissue the isozyme pattern of the plasma and tissue may change from the normal towards that of the particular tissue involved (25-32). Since heart and brain are particularly involved in thiamine deficiency, it was felt that it would be of interest to study the isozyme patterns in these and other tissues and in the plasma of thiamine-deficient and antagonist-treated rats.

LITERATURE REVIEW

Numbering of isozymes

Since 1957 when the diagnostic importance of the LDH isozymes was first recognized, it has become an established practice to distinguish the various components by numbering them according to their electrophoretic mobilities. Unfortunately two contradictory systems have been evolved. One scheme of numbering designates the fastest-moving isozyme as LDH_1 (H_4) and the successively slower-moving isozymes as LDH_2, LDH_3, LDH_4, and LDH_5. The other scheme reverses the numbering, LDH_1 being the slowest-moving isozyme.[a]

LDH isozymes

Most animal organs contain one or more LDH isozymes as demonstrated by electrophoresis on paper, starch or agar. The electrophoretic differences are due to differences in their amino acid composition (7). Pfleiderer *et al.* (7,11) found that sulfite preferentially inhibits the fast-migrating isozymes of the rat.

Since both heart and muscle LDH can be dissociated in either guanidine or mercaptoethanol into 4 subunits of

---

[a]The former scheme of numbering will be used for LDH isozymes in this thesis.

equal molecular weight, Markert et al. (33) and Cahn et al. (8) suggest that the intermediate forms of the enzyme are in fact hybrids containing both heart and muscle type subunits. Representing the subunits of the heart and skeletal muscle enzymes by H and M respectively, five forms of the enzyme are possible: H_4 (LDH_1); H_3M (LDH_2); H_2M_2 (LDH_3); HM_3 (LDH_4); and M_4 (LDH_5). Formation of H and M subunits are probably controlled by different genes. Thus in this case the existence of five isozymes is due to the operation of two genes in a single cell.

Fine et al. (9) and other workers (10,34-39) present further evidence supporting the view that the five electrophoretically distinguishable forms of LDH found in animals represent two distinct types of enzyme subunits (H and M) with three intermediate hybrids. In the rat the embryonic form of LDH is the M form; during development and maturation in rat heart, there is a shift from M type of LDH units to H type. Fine et al. (9) also found that the rabbit and bovine embryonic forms are the M type of LDH. In contrast, the human embryonic type is the H form.

The M type is found largely in the more anaerobic tissues such as voluntary skeletal muscle. The H type is found in aerobic tissues, such as cardiac muscle (33,40-42). In the immature uterus, the level of M unit is considerably less than that found in the mature uterus. Injection of estradiol leads to a marked increase in M units in the

immature uterus, but there is no significant change in the concentration of the H form. Testosterone and progesterone, in contrast, promote a proportional increase in both LDH forms in the immature uterus. Testosterone, however, induces a selective synthesis of M units in the seminal vessels of the immature rat. Hypophysectomy leads to a decrease in M units of skeletal muscle (40).

Wieland et al. (38) digested isozymes of LDH from the pig by trypsin, after oxidation with performic acid, and found that fingerprints of the peptides, performed on thin layer plates, showed isozymes H and M to be polypeptides of different primary structure and of different amino acid compositions (42).

Amino acid analyses of all the isozymes from a number of vertebrates indicate that the LDH_1 and LDH_5 proteins from the same organism are significantly different from each other. Furthermore, there is a close relationship between the amino acid content of either the LDH_1 or LDH_5 from closely related species.

The molecular weights of LDH_1 and LDH_5 are 140,000 to 150,000 and the sedimentation constants of all the isozymes are in the same range, $S^{\circ}_{20,w} = 7.2$ to 7.7 (43). Fondy et al. (42) and Pesce et al. (43) have concluded that the molecular weights are very similar for all of the isozyme forms.

The molecular weight of LDH, derived from sedimentation velocity and diffusion as well as light scattering data was reported by Jaenicke et al. (44) to be 115,000 $\pm$ 7,000

at pH 7.0. From sedimentation velocity data Fromm (45) calculated the molecular weight of LDH from rabbit muscle to be 132,000.

The heat stabilities of the isozymes increases simultaneously with their increasing electrophoretic mobilities (46,47). The fraction stable at 65° was considered LDH_1, the fraction labile at 56°, LDH_5, and the ones stable at 56° but labile at 65°, LDH_{2-4} (22).

The difference in the degree of inhibition of LDH isozymes by excess pyruvate appears to be of considerable metabolic significance, since reduction of pyruvate to lactate by LDH_1 is strongly inhibited by quite low concentrations of pyruvate. LDH_5, on the other hand, functions more efficiently when exposed to concentrations of pyruvate inhibitory to LDH_1, and is inhibited only by much higher concentrations. The relative K_m values for both pyruvate and lactate indicate that the LDH_5 forms bind the substrates less tightly than do LDH_1 forms (8,41,42,48,49).

In spite of the marked differences between the two types of LDH subunits, important structural similarities are retained. The H and M subunits are similar in molecular weight and shape. They possess the identical sulfhydryl peptide at the active site (42,43).

Kaplan et al. (50) observed that the pyridine-3-aldehyde analogue of NAD^+ was reduced by beef heart LDH at a faster rate than the 3-acetylpyridine analogue of NAD^+ (APNAD), whereas with the same enzyme from rabbit skeletal

muscle the reaction was more favorable with APNAD.

The plasma isozyme pattern seen in the course of myocardial infarction seemed to be a more sensitive, specific, and lasting indicator of myocardial necrosis than was the total plasma enzyme activity (26). In case of myocardial infarction, chronic polyarthritis and acute polyarthritis with heart trouble, there was an above average increase in the LDH_1 activity. In cases of liver ailment the LDH_4 and LDH_5 were increased (25,27-32). In cases with malignant tumors, LDH_{2-4} showed the greatest elevation (25,31).

Reaction mechanism

Takenaka et al. (51) and Fromm (45) observed that NAD^+ was bound at approximately four sites on the LDH molecule. Approximately 4 moles of sulfhydryl groups/mole of enzyme reacted rapidly with p-chloromercuribenzoate.

Millar et al. (52) studied the effect of photo-oxidation on the LDH activity and confirmed the view that coenzyme binding depends upon a sulfhydryl group on the enzyme surface.

Recently, Fondy et al. (53) treated 19 species of crystalline LDH with p-hydroxymercuribenzoate in 8 M urea and determined the number of thiol groups bound to the mercurial. Four thiol groups per molecule were essential for the catalytic operation of the various LDH tetramers, suggesting the presence of one active site thiol group per subunit.

The variation with pH of the kinetic constants, characterizing the reversible reaction catalyzed by crystalline LDH of beef heart, was studied by Winer et al. (54). They proposed a model, based on the kinetic behavior of this system. In this model, the proton generated during the oxidation of lactate is accepted by an uncharged imidazole group on the enzyme surface and the proton, which must be supplied for the reduction of pyruvate, is provided by a charged imidazolium group (55).

In the course of photo-oxidation of crystalline beef heart LDH in the presence of methylene blue, enzyme activity decreased directly with loss of methionine, histidine and tryptophan. Enzyme activity was wholly lost when approximately half the histidine and one-fourth of the tryptophan residues were photo-oxidized (52,56).

Quite recently, Balinsky (57) suggested a mechanism of LDH action involving two histidine residues, acting as a conjugate acid-base pair ("push-pull" mechanism), as tentatively indicated in Figure 1. It is suggested that the lone pair of electrons on the basic imidazole ring is donated, as shown by arrows, to the NADH ring, the hydride ion so produced is donated to the pyruvate, and a proton is added to the carbonyl oxygen from the acidic imidazole ring, as shown by the electron shifts, thereby producing lactate.

Pyruvate and lactate are not bound by LDH to a measurable extent. The inactivation of the enzyme by mercuribenzoate is reversed by NADH but not by pyruvate.

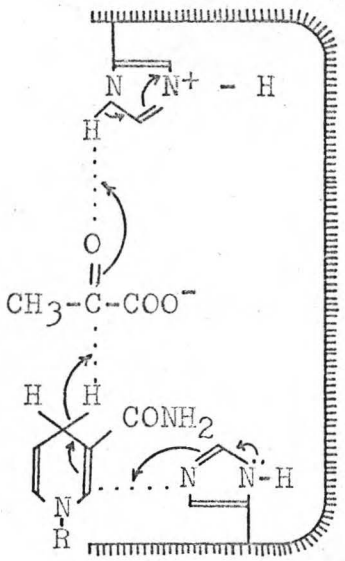

Fig. 1.--Proposed mechanism of action of LDH.

These results support the view that the enzyme forms a complex with one form of the coenzyme and that the resulting binary complex then forms a ternary complex with the substrate, mediated by the imidazole ring of a histidine residue at the active center of the enzyme (45,51,52,54, 58-60).

The inhibitory action of oxamate, the salt of the half-amide of oxalic acid, and of oxalate upon the reversible reaction catalyzed by beef heart muscle LDH has been investigated by Novoa et al. (61). Oxamate acts principally by competing with pyruvate by combining with the enzyme-reduced NAD^+ complex to form an inactive ternary complex, and oxalate acts principally by competing with lactate.

Recently, Markovich et al. (62) examined swine muscle LDH spectrophotometrically for the content of the α-helix. In forming the complex with NADH the secondary structure of the LDH changed with probable uncoiling of some of the α-helix. Oxalate ion did not cause change in the secondary structure of the apoenzyme, confirming the view that the inhibitor does not add to the apoenzyme. However, oxalate added to the LDH-NADH complex did result in a change in the secondary structure of the enzyme by increasing the concentration of α-helical regions. Evidently oxalate formed a ternary complex with the above composition.

Specificity

Meister (63,64) has found that a series of α,γ-diketo acids, ranging in chain length from five to eleven carbons, are reduced at an essentially uniform rate which is about one-tenth the rate of reduction of pyruvate. Since only one mole of NADH reacts with each mole of these acids, it is presumed that reduction occurs at the α-keto group. α,γ-diketo-δ-methyl caproic and α,γ-diketo-ϵ-methyl-heptanoic acids are also reduced by crystalline bovine heart LDH.

In contrast to the behavior of the α,γ-diketo acids, the rates fell off rapidly with increasing length of the carbon chain for the α-keto acids, pyruvate, and α-keto-butyrate being reduced most rapidly. It was also noted that the branched chain α-keto acids were considerably less

susceptible than the corresponding straight chain keto acids. Glyoxylate, hydroxypyruvate, and thiopyruvate are reduced almost at the same rate as pyruvate. Phenylpyruvate and p-hydroxyphenylpyruvate are also reduced at a low rate, but very little activity is displayed against oxalacetate and α-ketoglutarate by the bovine heart LDH. It was also observed that α-keto-γ-methiolbutyrate, α-keto-γethiol-butyrate, α-ketophenylacetate, α-keto-ε-hydroxycaproate, α-keto-ε-N-chloroacetylcaproate, α-keto-β-cyclohexyl-propionate, α-keto-δ-carbamidovalerate, α-ketoglutaric-γ-ethylester and α-keto-β-indolyl-propionate are reduced at a low rate.

It was observed by Kun (65) that LDH of heart muscle (2 µg) reversibly catalyzes reduction of β-mercaptopyruvate by NADH. The michaelis constant for pyruvate was found to be 5.4×10^{-5} at pH 7.4 (0.01 M potassium phosphate buffer) 8.2×10^{-4} for β-mercaptopyruvate. According to these measurements β-mercaptopyruvate has about one-fifteenth the affinity of pyruvate for LDH.

Markert et al. (12) studied qualitatively the substrate specificity of the LDH isozymes of beef heart with eight substrates: lactate; β-phenyllactate; α-hydroxy-butyrate; α-hydroxy-N-caproate; α-hydroxyvalerate; α-hydroxy-isovalerate; α-hydroxyisobutyrate; and α-hydroxy-β-methyl-valerate. All these substrates were oxidized in the presence of the LDH isozymes, though not at the same rate. However, all of the LDH isozymes were alike in the relative

catalytic efficiency displayed toward these different substrates.

It was reported by Frank et al. (66) that glyceric acid and a number of β-substituted halogen and amino derivatives of lactic acid are oxidized at about one-hundredth the rate at which lactate is oxidized by crystalline rabbit muscle LDH.

Plummer et al. (67,68) observed greater activity of the principal heart isozymes with α-ketobutyrate relative to that with pyruvate than isozymes from other tissues. The principal liver isozymes are shown to display low ratios for α-ketobutyrate activity/pyruvate activity.

Sawaki et al. (69) recently reported on the reduction of glyoxylate to glycolate by LDH as seen from the decrease in optical density of NADH at 340 mμ. LDH, M type (2μg), from rabbit muscle and LDH, H type (6 μg), from pig heart were 3-4 fold more active when pyruvate (0.001 M) was substrate than when glyoxylate (0.01 M) was substrate.

According to Balinsky (57), the ratio of human heart LDH to α-hydroxybutyrate dehydrogenase was constant for LDH_1 at all stages of purification. Also LDH_1 activity, measured in the presence of both pyruvate and α-ketobutyrate was the arithmetic mean of the activity measured with each substrate separately.

Banner et al. (70) found that a comparison of the effect of the LDH isozymes on glyoxylate with that on pyruvate and α-ketobutyrate showed a similarity despite the

dissimilar structures of these substrates. Both glyoxylate and ∝-ketobutyrate also showed greater activity with LDH_1 from pig heart than with LDH_5 from rabbit muscle. Differentiation between the two isozymes, using glyoxylate, was less sensitive than with ∝-ketobutyrate as substrate. This result is consistent with the findings of Plummer et al. (67,68).

The levels of pyruvate and lactate in thiamine deficiency

Rosenwald (71) reported that the lactic acid elimination through the urine was increased during avitaminosis of the B complex. The lactic acid constituted only a part of the incompletely oxidized carbon appearing in the urine which was greatly increased in avitaminosis of the B complex. He concluded that avitaminosis of the B complex affected primarily carbohydrate metabolism, and the disturbance of carbohydrate oxidation was attributed to "inner" oxygen want.

According to Collazo et al. (72), lactic acid is found in the blood of all species, and appears to be independent of the blood sugar level. Physiologically, variations of blood lactic acid depends on the state of repose or of work, or of temperature, respiration, and alimentary regimen of the animals. Vitamin B complex deficiency leads to hyperlactacidemia and lactaciduria.

No significant difference in lactate oxidation was observed by Sherman et al. (73) in minced brain tissues from normal and polyneuritic chicks. However, in avitaminous heart

tissue the oxygen uptake was lowered with lactic acid as substrate and a decreased rate of lactic acid removal was found. Lactic acid oxidation was not affected in brain tissue by the addition of vitamin B_1, but in heart tissue, a similar addition caused an increased oxygen uptake. The addition of pyruvate inhibited LDH activity to a greater extent in avitaminous heart and kidney tissue than in normal tissues.

Fornaroli et al. (14,15) found that in avitaminosis B_1 of rats there was a slight increase in the lactic acid content of blood and muscles. Results with the brain were inconclusive. There was a definite increase in pyruvic acid in all three instances.

In rats depleted of the vitamin B complex, a rapid increase in blood lactic acid, even up to 58 per cent, was reported by Krusius et al. (74). Feeding vitamin B_1 alone (instead of vitamin B complex) caused an enormous increase of up to 200 per cent on the average, of the blood lactic acid, which was attributed to the lack of flavine. The lactic acid content of the blood was not affected by fasting.

Li et al. (16) found that by the end of the first month of thiamine depletion the amount of blood pyruvate in rats increased to 3.49 mg per cent over a normal basal level of 0.96 mg per cent. During the second month the amount increased to 5.62 mg per cent and shortly before death there was usually a remarkable accumulation. The concentration of pyruvate corresponded roughly with the severity of the

clinical manifestations. At first the only signs of avitaminosis were loss of weight, weakness and anorexia, but later definite neurologic symptoms developed. Upon injections of thiamine in these polyneuritic animals, there was a prompt drop in pyruvate and the death of the animals was avoided. Li suggested that the estimation of pyruvate could be used for the diagnosis of thiamine deficiency.

The accumulation of pyruvic acid in the blood of thiamine-deficient animals was first observed in 1935 by Thompson and Johnson (17) in rats and pigeons, and by Platt and Lu (18) in human beriberi cases. The blood pyruvic acid, in mg per cent, of normal and thiamine-deficient rats was 1.09 and 3.21, respectively (19). The blood pyruvate level and bradycardia ran parallel in deficient rats. These results are in agreement with the findings of Li et al. (16).

Shimizu (20) showed that the contents of pyruvic, α-ketoglutaric, and lactic acid in the blood of rats on thiamine-deficient diets increased with development of the avitaminosis. If 150 μg of oxythiamine (OTh) was injected into rats intraperitoneally, marked elevations in blood pyruvic and lactic acid concentrations were noted after four hours. Simultaneous injections of 150 μg quantities of OTh and thiamine (Th) did not produce any changes in blood pyruvic and lactic acid concentrations during a four-hour period. The excretion of Th in the urine is greatly augmented by OTh. Frohman et al. (75) presumed that the

antimetabolite displaces Th in the body.

According to de Caro et al. (21), glucose metabolism of rats seemed to be more disturbed by administration of the antagonist, pyrithiamine (PTh) than by depriving the rats of Th. Blood pyruvate was 2.5 mg per 100 ml after PTh administration and 2.38 mg when the animals were given low Th diets. The level in control animals was 1.12 mg.

De Caro et al. (76) gave male mice on a Th-deficient diet a single oral dose of 0.5 mg PTh with or without a subsequent daily dose of 2 μg Th. Others received single oral doses of 0.5 or 2 mg OTh without subsequent Th. The mice which received Th and PTh showed no difference in blood pyruvate or liver Th when compared to controls. Although Th in muscle and brain was lower, no neurologic symptoms developed. Mice which received PTh without Th showed increases in blood pyruvate, marked decreases in brain Th, but no decreases in liver and muscle Th. All developed neurologic symptoms. OTh without Th exerted only a lowering of liver Th without affecting blood pyruvate, muscle Th, and brain Th and caused no neurologic symptoms.

Rindi et al. (22) observed that in rats on a Th-deficient diet, the lowest Th levels were first reached in the muscle. Normal concentrations are maintained in the brain for a longer time. The blood pyruvate has definitely increased on approximately the eighteenth day. Statistically it is apparently more closely correlated with adrenal hypertrophy than with decreased tissue Th.

In normal rats (23) the liver removes pyruvic acid from the circulating blood while the muscles lack this power. In Th deficiency produced by a Th-deficient diet or by Th antagonists, liver function is depressed, pyruvic acid enters the blood from the muscles, and its concentration is thus increased. Similar changes occur in normal rats treated with cortisone.

Gubler (24) found that blood levels of pyruvate were high in Th-deprived and OTh + Th-treated rats but were normal in PTh + Th-treated rats. The levels of blood pyruvate in mg per cent were 1.56 (control), 2.38, 3.05 and 1.87, respectively for Th-deprived, Oth + Th-treated and PTh + Th-treated rats.

In rats injected with 100 mg OTh a sharp increase in the level of blood pyruvate occurred (77).

Von Muralt (78) found that the pyruvate content of the blood is almost normal in the PTh-treated rats and only doubled in the group with Th deficiency. The OTh-treated rats had four times as much pyruvate in the blood.

In the findings (16,19,24,77,78) cited above the changes in blood pyruvate are quite consistent with one another except those of de Caro et al. (21), although the relative ratios of pyruvate in Th-deficient and antagonist-treated rats are different.

MATERIALS AND METHODS

Equipment

A Beckman DU spectrophotometer was used for measurement of the enzyme activity in tissues. A Beckman DB-G spectrophotometer, attached to a linear/log Varicord model 43 recorder was used for measuring the levels of the enzyme, pyruvate, and lactate in blood. Water at 25° was circulated through the thermospacers surrounding the cell compartment of the spectrophotometer.

A Servall automatic superspeed refrigerated centrifuge, model RC-2, equipped with a 4.25 inch rotor, was used for centrifugation. Temperature of the centrifugal compartment was maintained at 0°.

A Beckman model G pH meter was used for the pH adjustment of buffers and reagents.

The isozymes were separated on Sepraphore III cellulose polyacetate strips, using the Gelman Rapid Electrophoresis Chamber connected with a Duostat regulated power supply. A Scan-A-Tron attached to the Spinco Analytrol densitometer was used to quantitate the electropherograms.

Chemicals

Thiamine-HCl, Oxythiamine-HCl, Pyrithiamine-HBr, β-NAD^+, disodium salt (Grade III), β-NADH, disodium salt

(Grade III), LDH from rabbit muscle (type II), Nitro Blue Tetrazolium and Phenazine Methosulfate were obtained from Sigma Chemical Company, St. Louis, Missouri. Heparin, sodium salt, was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Spinco buffer B-1 (barbital-barbituric acid buffer, pH 8.6, ionic strength 0.05) was obtained from Spinco Division of Beckman Instruments, Inc., Palo Alto, California. Sepraphore III cellulose polyacetate strips (1" x 6 3/4") were purchased from Gelman Instrument Company, Ann Arbor, Michigan.

Deionized distilled water was prepared by using a Crystalab Deeminizer model CL-5, with Deeminite L-10 ion exchange resin, purchased from Crystal Research Laboratories, Inc., Hartford, Connecticut. Deionized distilled water was used throughout the experiments. The salt concentration did not exceed three parts per million.

Outline of animal experiments

Male white Sprague-Dawley strain rats weighing 170-200 g were used in the experiments. They were purchased from the Northwest Rodent Company, Pullman, Washington. The rats were housed at random in individual cages with wire-mesh screen bottoms. Temperature in the animal room was kept at 26 ± 4°. All rats were maintained for 5-7 days with Purina Laboratory Chow, obtained from Ralston Purina Company, St. Louis, Missouri, and with water given "ad libitum." After this period the rats were randomly divided into four groups and fasted for 24 hours. They were then placed on

a basal Th-deficient diet. The composition of this diet is shown in Table I and is the same as that used previously by Gubler (24). At the same time appropriate supplements of Th and its antagonists, PTh and OTh, were given daily by subcutaneous injection under the right foreleg, using a 26 or 27 gauge hypodermic needle and one cm^3 syringe.

Group I rats served as the control group and were treated by daily subcutaneous injections of 10 micrograms of Th per 100 g of body weight in 0.2 ml of 0.9 per cent sodium chloride solution. Group II rats served as the Th-deprived group and received no supplement. Group III were supplemented by daily injections of 10 micrograms of Th + 2 mg of OTh per 100 g of body weight in 0.2 ml of 0.9 per cent sodium chloride solution, and Group IV rats received 10 micrograms of Th + 50 micrograms of PTh per 100 g of body weight in 0.2 ml of 0.9 per cent sodium chloride solution. All groups were continued on the basal diet for the period of the experiments. The rats were weighed daily and allowed unlimited access to water and food. In order to avoid any emotional disturbance or unnecessary handling of the rats, weighing, injection, food and water supply were done at the same time each day.

When a rat developed symptoms of severe Th deficiency such as rapid loss of weight, weakness, anorexia, or polyneuritic convulsions, it was sacrificed, along with a control rat, by anesthesia with ethyl ether. The blood was collected from the abdominal aorta, using a 20 gauge

TABLE I

BASAL THIAMINE-DEFICIENT DIET

Ingredient	% of diet	G/20 kg
Sucrose	68.5	13,700
Casein (vitamin-free)	20.6	4,400
Salt mix (No. IV, University of Wisconsin)[a].	4.5	900
Corn oil	5.4	1,000
Vitamin mix[b]		44.5
Choline chloride		80.0

[a]The salt mix contained the following ingredients with weight given in grams: $CaCO_3$, 1200; K_2HPO_4, 1290; $CaHPO_4 \cdot 2H_2O$, 300; $MgSO_4 \cdot 7H_2O$, 408; NaCl, 670; $Fe(C_6H_5O_7) \cdot 6H_2O$, 110; KI, 3.2; $MnSO_4 \cdot H_2O$, 15.2; $ZnCl_2$, 1.0; $CuSO_4 \cdot 5H_2O$, 1.2.

[b]The vitamin mix contained the following ingredients with amounts given in grams for a 20-kg diet: inositol, 40.000; p-aminobenzoic acid, 2.000; calcium pantothenate, 1.200; nicotinic acid, 0.800; pyridoxine, 0.120; riboflavin, 0.240; biotin, 0.004; folic acid, 0.010; 2-methyl-1,4-naphthoquinone, 0.080.

hypodermic needle and syringe wetted with 0.2 ml of heparin solution (10 mg/ml). The tissues were quickly removed, weighed and stored at -20° until used.

Tissue LDH preparation and determination

The method of extraction of LDH from tissue was a modification of the method described by Fine et al. (9). Frozen tissues were thawed, sliced into small pieces, added to cold 0.25 M sucrose solution in a ratio of 250 mg tissue per ml of sucrose solution and homogenized in an all-glass Potter-Elvehjem motor-driven homogenizer with a teflon pestle. The vessel was kept immersed in an ice bath. These homogenates were centrifuged in a Servall automatic super-speed refrigerated centrifuge at 23,500 x G and 0° for one hour. One volume of supernatant solution was diluted with three volumes of cold 0.25 M sucrose solution. The final volume of the tissue homogenate in ml was 16 times the wet weight in grams of the tissue taken.

Two-hundredths ml of this diluted supernatant solution was added to a 3.0-ml cuvette (with one-cm light path) containing the preincubated reaction mixture shown in Table II in a water bath at 25°. This is a modified method, originally described by Amador et al. (79). The above mixture was mixed by gentle inversion. Absorbance increases at 340 mμ were read each minute for five minutes against a cuvette filled with deionized distilled water.

The absorbance of the reagent blank was deducted from the measured value for the tissue sample to obtain the

TABLE II

THE ASSAY SYSTEM FOR THE ACTIVITY OF TISSUE LDH

Reagent	Conc.	pH	Blank	Sample
Sodium pyrophosphate	0.05 M	8.8	2.40 ml	2.40 ml
Deionized distilled H_2O			0.28 ml	"
NAD^+	0.027 M	6.0	0.30 ml	0.30 ml
Sodium lactate	775 mM	8.8	"	0.28 ml
Preincubated at 25°				
Diluted tissue homogenate[a]			0.02 ml	0.02 ml

[a]The volume of this diluted tissue homogenate in ml is 16 times the wet weight in grams of the tissue taken.

change in absorbance caused by LDH. One unit of tissue LDH activity is that amount of enzyme which will catalyze the conversion of one micromole of substrate per minute at 25°. One milliunit is equal to 0.001 unit. The LDH activity in tissues was calculated from the $\Delta A_{340m\mu}$, the dilution factor, and the molar absorbance index for NADH (6.22×10^6 cm^2/mole). The formula is as follows:

$$\frac{\Delta A \frac{340\ m\mu}{1\ min}}{6.22} \times 10^3 \times \text{milliunits/ml} \times 150\ \text{(dilution factor)} = \text{milliunits/ml of tissue homogenate}$$

Determination of blood lactate and pyruvate

An aliquot of the whole blood was immediately added to an equal volume of cold 0.6 M perchloric acid and mixed thoroughly with a plastic spatula. It was centrifuged in a Servall automatic superspeed refrigerated centrifuge at 23,500 x G for twenty minutes. The resulting supernatant solution was used for the determination of lactate and pyruvate.

Blood lactate was determined by the method of Scholz et al. (80). In the assay 0.1 ml of the deproteinized supernatant solution was added to the reaction mixture, containing glycine buffer, NAD^+, and LDH, to initiate the reaction. This mixture, as shown in Table III, was mixed well by gentle inversion and allowed to stand in a water bath at 25° for one hour. Absorbance at 340 mμ was read against a cell filled with deionized distilled water. The

TABLE III

THE REACTION MIXTURE FOR THE DETERMINATION OF BLOOD LACTATE

Reagent	Conc.	pH	Blank	Sample
Glycine and hydrazine	0.5M 0.4M	9.0	2.67 ml	2.67 ml
NAD+	0.027M	6.0	0.20 ml	0.20 ml
LDH (2 mg protein/ml)			0.03 ml	0.03 ml
Diluted $HClO_4$	0.3M		0.10 ml	" "
Deproteinized blood sample			" "	0.10 ml

absorbance of the reagent blank was subtracted from the measured value for the blood sample to obtain the change in absorbance ($\Delta A_{340\ m\mu}$) caused by lactate. The amount of lactate per 100 ml blood was calculated from the $\Delta A_{340\ m\mu}$, the molar absorbance index for NADH (6.22×10^6 cm^2/mole), and the dilution factor. The formula used for this calculation is as follows:

$$\frac{\Delta A_{340\ m\mu}}{6.22 \times 10^3} \times 90.08 \text{ (molecular weight of lactic acid)} \times 60 \text{ (dilution factor)} \times 100 \text{ (volume of blood)} = \text{mg lactate/100 ml blood}$$

The method of Bücher et al. (81) was used for the measurement of pyruvate levels in the blood. One volume of 1.1 M K_2HPO_4 solution was added to three volumes of the deproteinized supernatant solution, mixed, and allowed to stand for ten minutes in an ice bath. The precipitated potassium perchlorate was removed by filtration. This solution (buffered to ca. pH 7) was equilibrated to a temperature of ca. 25°, and a 2.00 ml aliquot was used for the assay by adding to 0.09 ml of 3×10^{-3} M NADH (pH 7.5) in a 1 cm cuvette. The optical density was followed at 340 mμ. Then 0.05 ml of LDH (0.75 mg protein/ml) was added to the cuvette and mixed thoroughly to start the reaction. After two minutes three measurements at one minute intervals were taken to obtain more precise values with subsequent extrapolation to zero time.

The amount of pyruvate reduced to lactate was calculated from the change in absorbance at 340 mμ, the molar absorbance index for NADH at 340 mμ (6.22×10^6 cm^2/mole), the dilution factor and the volume of blood used. The formula used is as follows:

$$\frac{\Delta A_{340\ m\mu}}{6.22 \times 10^3} \times 88.06 \text{ (molecular weight in mg)} \times 2.85 \text{ (dilution factor)} \times 100 \text{ (volume of blood)} = \text{mg pyruvate/100 ml blood}$$

Determination of the LDH activity in plasma

The remainder of the whole blood was centrifuged at 1,085 x G for fifteen minutes to remove erythrocytes. The

supernatant plasma was used for the determination of the LDH activity and the study of the LDH isozyme pattern in plasma. Plasma with distinct hemolysis was discarded.

Plasma was kept in the cold room until used. Before use, the buffer solution and plasma were brought to 25° in a water bath. The following were successively pipetted into a 3 ml-cuvette and mixed well by gentle inversion: 2.90 ml of phosphate/pyruvate buffer pH 7.5 (0.05 M phosphate and 3.1×10^{-4} M in potassium pyruvate), 0.05 ml of 8×10^{-3} M NADH (pH 7.5) and 0.05 ml of plasma. The change in absorbance at 340 mμ was measured for a period of 3 to 5 minutes at 1-minute intervals (82-84). The mean value determined from the recorded absorbance differences per minute was used for calculation.

One unit is that amount of the enzyme which will catalyze the conversion of one micromole of substrate per minute at 25°. One milliunit is equal to 0.001 unit. Enzyme units were calculated from the change in absorbance per minute, the molar absorbance index for NADH, the dilution factor, and the volume of plasma used. The formula for calculation is as follows:

$$\frac{\Delta A_{1\ min}^{340\ m\mu}}{6.22} \times 10^3 \text{ milliunits/ml} \times 60 \text{ (dilution factor)} = \text{milliunits/ml plasma}$$

Electrophoresis

A packet of Spinco B-1 buffer (barbital-barbituric acid buffer, ionic strength 0.05, pH 8.6) was dissolved and

diluted to one liter with deionized distilled water. Gelman Sepraphore III strips (1" x 6 3/4") were first floated on top of the buffer, thoroughly wetted in order to avoid the possible trapping of air pockets and then immersed for at least 30 minutes. If the trapping of air pockets occurs, the cellulose polyacetate strip will not be activated and this will interfere with electrophoresis in these areas, thereby disturbing the electropherogram. Soaking produces a colloidal change which brings Sepraphore III strips back to its original gel structure. A Gelman Rapid Electrophoresis Chamber was filled with about 450 ml of cold Spinco B-1 buffer, tilted to obtain equal levels in the compartments, and connected to the power supply. A five lambda sample of plasma or tissue homogenate was applied by applicator 2.0 cm from the center line to the cathode side of each Sepraphore III strip. In order to prevent diffusion of the sample, the strip was lightly blotted prior to the application. After the sample was applied, the strips (eight) were immediately transferred to the chamber in the cold room and run for $1^1/2$ hour at a constant voltage of 200 volts.

On completion of the $1\frac{1}{2}$-hour electrophoresis, the strips were removed and stained by immersion in the staining solution for five minutes. The staining solution (9) was composed of 23.3 ml of 0.1 M Tris buffer (pH 8.5), 3.5 ml of 775 mM sodium lactate (pH 7.0), 0.9 ml of 0.027 M NAD^+, 1 ml of Nitro Blue Tetrazolium (10 mg/ml) and 0.12 ml of phenazine methosulfate (5 mg/ml).

NADH formed from the reaction between lactate and NAD^+ does not react directly with ditetrazolium salts, and some hydrogen-transferring agent is required as an intermediate. Phenazine methosulfate as an intermediate carrier catalyzes the reaction between NADH and the ditetrazolium salt. Thus Nitro Blue Tetrazolium (ditetrazolium salt) undergoes reduction to sparingly soluble, but intensely colored diformazan. This diformazan, therefore, remains compactly at the site of its formation and gives sharp definition of the isozyme bands separated by the electrophoresis (85).

The strips were then treated with three rinses of 5 per cent acetic acid in water until the background stain was removed. They were then dried in air.

The transparent strips were placed in a black paper (7/16" x 4") in which a "window" had been cut and were then scanned by using the Gelman Scan-A-Tron attached to the Spinco Analytrol densitometer. This attachment merely changes the strip speed of the Spinco Analytrol so as to read the narrower and sharper bands of Sepraphore III electropherograms.

Reduction of α-keto acids by LDH

The relative rates of reduction were compared with the following keto acids: potassium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate, sodium α-keto isovalerate, sodium α-ketoisocaproate, sodium α-keto-β-methylvalerate, and sodium p-hydroxyphenylpyruvate. The

keto acids were dissolved in 0.05 M phosphate buffer at pH 7.5 to prepare 3.1×10^{-4} M solutions, respectively.

Enzymatic activity was determined by following the decrease in the absorption of NADH at 340 mμ. The reaction mixture, as shown in Table IV, contained NADH, a substrate, enzyme solution, and 0.05 M phosphate buffer at pH 7.5 in a volume of 3 ml. The reaction was started by addition of 0.05 ml of LDH (0.200 mg protein/ml or 0.750 mg protein/ml) and mixed well by gentle inversion. The change in absorbance at 340 mμ was automatically recorded on the chart which was moving at a rate of one inch per minute. The activity values were obtained from the initial linear portion of the time curve. The recorded ΔA per minute values were compared with one another. Experiments were conducted at 25° (81-84).

TABLE IV

THE REACTION MIXTURE FOR REDUCTION OF ∝-KETO ACIDS BY LDH

Reagent	Conc.	pH	Volume
Phosphate buffer/ potassium pyruvate[a]	0.05 M 3.1×10^{-4} M	7.5	2.81 ml[b]
NADH in 1% $NaHCO_3$[c]	8×10^{-3} M	7.5	0.14 ml
LDH	0.200 mg protein/ml or 0.75 mg protein/ml		0.05 ml

[a]The other keto acids replace pyruvate for the respective assay.

[b]The proper amount was added to make the final volume 3 ml.

[c]0.14 ml of NADH solution was used in case of pyruvate and 0.06 ml or 0.07 ml of NADH solution was used in case of the other keto acids, depending on the concentration of LDH added, i.e., 0.200 mg protein/ml or 0.75 mg protein/ml, respectively.

EXPERIMENTAL RESULTS

Growth of the animals

The growth curves are shown in Figure 2. The growth curves from all experiments were essentially the same and only one is presented.

The curve for the control group supplemented with adequate amount of Th showed an increase at a normal rate throughout the experiments. The Th-deprived group grew normally for 10 to 14 days, then started to lose weight gradually and reached the starting weight around 26 days later. They showed symptoms of severe Th deficiency such as weakness and anorexia.

The group which was treated with Th + OTh (1:200) gained weight at a subnormal rate for four days and gradually lost weight.

The group which received Th + PTh (1:5) followed the usual pattern of normal growth for eight days and began losing weight very rapidly. Showing the preconvulsive symptoms such as weakness, anorexia, rapid loss of weight for a couple of days, the rats suddenly started the polyneuritic convulsion and died within 48 hours or so.

Stress and weight of adrenal glands

As shown in Table V, Th deprivation or administration

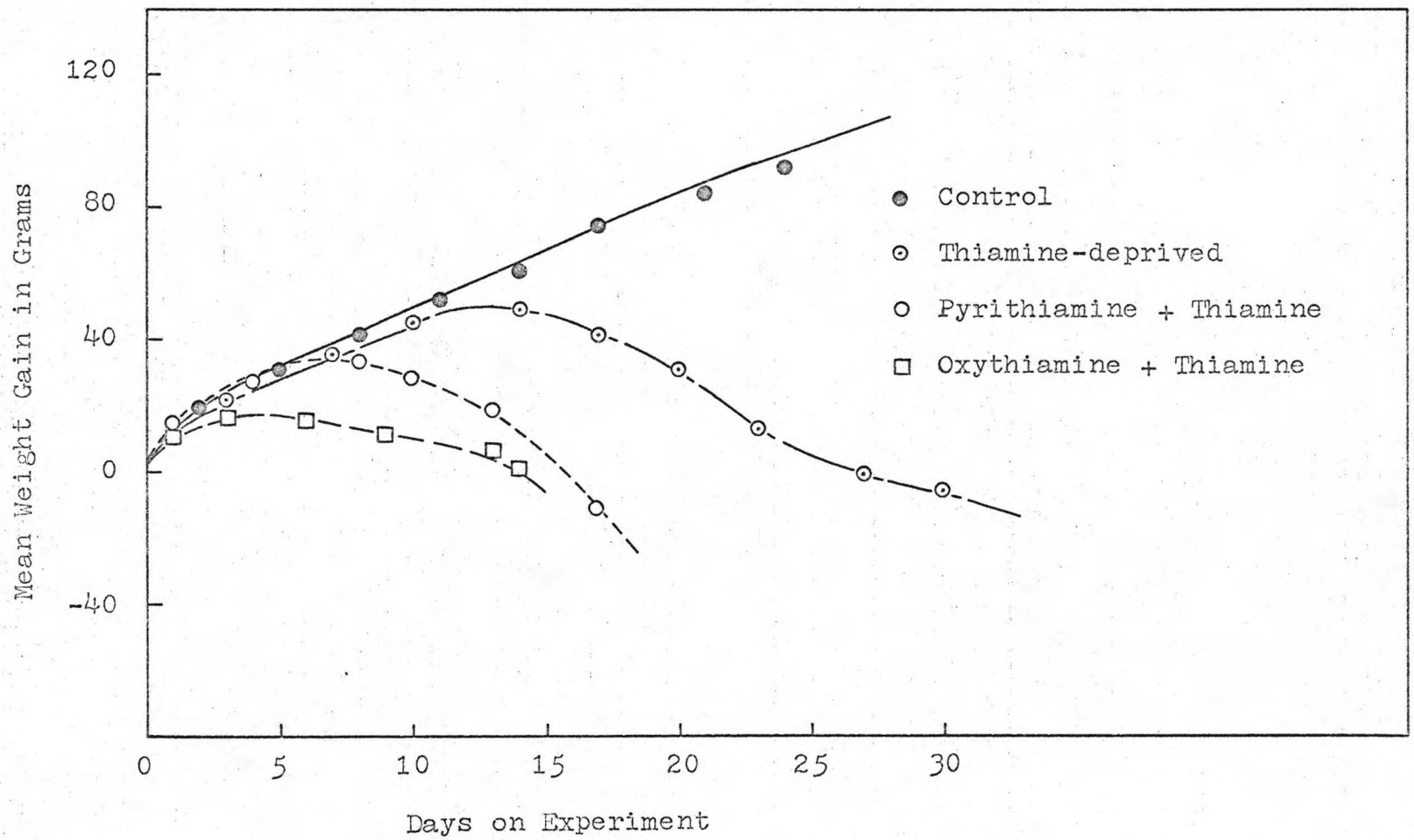

Fig. 2.--Growth curves for rats in thiamine deficiency experiment.

TABLE V

WEIGHT OF THE ADRENAL GLANDS (EXP. 1)

Group	Number	Adrenal glands wt mg/100 g body wt[a]	Significance level (p)
Control	23	13.5 ± 0.38	. .
Th-deprived	28	20.4 ± 0.50	<0.0005
OTh-treated	18	19.6 ± 0.99	<0.0005
PTh-treated	24	27.4 ± 0.64	<0.0005

TABLE VI

WEIGHT OF THE ADRENAL GLANDS (EXP. 2)

Group	Number	Adrenal glands wt mg/100 g body wt[a]	Significance level (p)
Control	6	12.0 ± 0.86	. .
PTh-treated[b] (before convulsion)	11	20.9 ± 1.24	<0.0005
PTh-treated[c] (after convulsion)	8	23.8 ± 1.24	<0.0005

[a]Mean ± standard error.

[b]The rats were sacrificed just before the convulsion started.

[c]The convulsion lasted at least for one day. The rats in convulsion were kept alive as long as possible, depending upon the body condition of the particular rat involved.

of OTh with Th resulted in a significant increase ($p<0.0005$) in the weight of the rat adrenal glands per 100 g body weight, as compared with the control group. The degree of increase in the weight of adrenal glands was similar in both groups.

A marked increase ($p<0.0005$) in the weight of adrenal glands was caused by the injection of PTh with Th. The weight of adrenal glands seems to be built up mostly before the polyneuritic convulsion in the PTh + Th treated rats. During the convulsion, a further increase of about 10 per cent of the preconvulsion weight was observed, as shown in Table VI.

Pyruvate and lactate levels in blood

The blood pyruvate level in the Th-deprived rats, as shown in Table VII, did not increase significantly, as compared with the control group, but the administration of OTh or PTh with Th resulted in the significant increase ($p<0.0005$) in the respective blood pyruvate level. The blood pyruvate in the PTh-treated group seems to be mainly built up in the convulsion period, as compared with the preconvulsion PTh-treated group, as summarized in Table VIII.

The blood lactate levels, as presented in Table IX, were correspondingly increased in the OTh-treated group ($p<0.005$) and the PTh-treated group ($p<0.0005$). Th-deprived group did not exhibit any marked increase in the blood lactate level ($p<0.10$). In the PTh-treated group the lactate level, summarized in Table X, was mainly built up during the convulsion ($p<0.05$).

TABLE VII

PYRUVATE LEVEL IN BLOOD (EXP. 1)

Group	Number	Mg pyruvate/100 ml blood[a]	Significance level (p)
Control	12	1.31 ± 0.125	• •
Th-deprived	11	1.54 ± 0.186	>0.15
OTh-treated	10	2.43 ± 0.176	<0.0005
PTh-treated	10	2.39 ± 0.136	<0.0005

TABLE VIII

PYRUVATE LEVEL IN BLOOD (EXP. 2)

Group	Number	Mg pyruvate/100 ml blood[a]	Significance level (p)
Control	6	0.77 ± 0.131	• •
PTh-treated[b] (before convulsion)	9	1.36 ± 0.220	<0.05
PTh-treated[c] (after convulsion)	6	2.26 ± 0.263	<0.005

[a]Mean ± standard error.

[b]The rats were sacrificed just before the convulsion started.

[c]The convulsion lasted at least for one day. The rats in convulsion were kept alive as long as possible, depending upon the body condition of the particular rat involved.

TABLE IX

LACTATE LEVEL IN BLOOD (EXP. 1)

Group	Number	Mg lactate/100 ml blood[a]	Significance level (p)
Control	33	30.71 ± 2.735	• •
Th-deprived	31	35.98 ± 2.857	<0.10
OTh-treated	35	42.26 ± 2.957	<0.005
PTh-treated	28	56.59 ± 5.058	<0.0005

TABLE X

LACTATE LEVEL IN BLOOD (EXP. 2)

Group	Number	Mg lactate/100 ml blood[a]	Significance level (p)
Control	6	32.20 ± 3.380	• •
PTh-treated[b] (before convulsion)	9	30.30 ± 5.427	>0.25
PTh-treated[c] (after convulsion)	6	46.68 ± 5.957	<0.05

[a]Mean ± standard error.

[b]The rats were sacrificed just before the convulsion started.

[c]The convulsion lasted at least for one day. The rats in convulsion were kept alive as long as possible, depending upon the body condition of the particular rat involved.

Reduction of α-keto acids by LDH

The activity of LDH from rabbit muscle with seven α-keto acid substrates, i.e., potassium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate, sodium α-keto-isovalerate, sodium α-ketoisocaproate, sodium α-keto-β-methylvalerate, and sodium p-hydroxyphenylpyruvate, was determined with two different enzyme concentrations, i.e., 10 μg protein and 37.5 μg protein (the enzyme concentration used for the determination of the blood pyruvate levels). The purpose of this experiment was to find a keto acid or keto acids interfering with the determination of pyruvate.

LDH acted upon only pyruvate, α-ketobutyrate, α-ketovalerate, and p-hydroxyphenylpyruvate, as shown in Tables XI and XII. The reduction rate of α-ketobutyrate is about one-twelfth of that for pyruvate. α-ketovalerate and p-hydroxyphenylpyruvate were very slowly reduced as compared with pyruvate.

The LDH activity in plasma and tissues

Table XIII shows the LDH activity in plasma of rats suffering from three types of the induced Th deficiency. The LDH activity in plasma was not affected by Th deprivation or by administration of OTh with Th when compared with the control group. However, the treatment with PTh and Th caused a marked increase of the LDH activity in plasma ($p<0.005$).

In Table XIV, the levels of LDH in various tissues,

TABLE XI

THE RELATIVE RATES OF REDUCTION AMONG
∝-KETO ACIDS BY LDH (10 μg)

∝-Keto acids	Rate in $\Delta A^{340\ m\mu}_{1\ min}$ [a]
Potassium pyruvate	1.970
Sodium ∝-ketobutyrate	0.147
Sodium ∝-ketovalerate	0.005
Sodium ∝-ketoisovalerate[b]	. .
Sodium ∝-ketoisocaproate[b]	. .
Sodium ∝-keto-β-methylvalerate[b]	. .
Sodium p-hydroxyphenylpyruvate	0.004

TABLE XII

THE RELATIVE RATES OF REDUCTION AMONG
∝-KETO ACIDS BY LDH (37.5 μg)

∝-Keto acids	Rate in $\Delta A^{340\ m\mu}_{1\ min}$ [a]
Potassium pyruvate[c]	. .
Sodium ∝-ketobutyrate	0.495
Sodium ∝-ketovalerate	0.021
Sodium ∝-ketoisovalerate[b]	. .
Sodium ∝-ketoisocaproate[b]	. .
Sodium ∝-keto-β-methylvalerate[b]	. .
Sodium p-hydroxyphenylpyruvate	0.016

[a]This value is mean of two measurements.

[b]In these particular LDH and NADH concentrations, no appreciable activity of LDH toward these keto acids was detected.

[c]The reaction goes too fast to measure.

TABLE XIII

LDH ACTIVITY IN PLASMA

Group	Number	Milliunits[a] LDH/ml plasma[b]	Significance level (p)
Control	23	109 ± 9.0	• •
Th-deprived	24	116 ± 10.5	>0.25
OTh-treated	24	115 ± 8.4	>0.25
PTh-treated	17	161 ± 17.5	<0.005

[a]One unit is that amount of the enzyme which will catalyze the conversion of one micromole of substrate per minute at 25°. One milliunit is equal to 0.001 unit.

[b]Mean ± standard error.

i.e., brain, kidney, heart, and liver, were summarized for Th deprivation, and for treatment with OTh or PTh with Th.

The LDH levels in brain and kidney were significantly increased ($p<0.05$) by OTh administration, whereas in Th-deprived or PTh-treated rats the levels were not different from the normal rats. In heart, Th deprivation resulted in about 11 per cent decrease ($p<0.005$) of the activity but no noticeable change was found in the other two types of induced Th deficiency. In liver, the activity of LDH was markedly decreased in all three Th deficiencies ($p<0.0005$). The PTh-treated, the OTh-treated and the Th-deprived groups are in the order of the decreasing activity.

TABLE XIV

LDH ACTIVITY IN TISSUES

Tissue	Group	Number	Milliunits[a] LDH/ ml homogenate[b,c]	Significance level (p)
Brain	Control	25	1610 ± 41.5	. .
	Th-deprived	25	1651 ± 74.0	>0.25
	OTh-treated	29	1734 ± 47.9	<0.05
	PTh-treated	21	1531 ± 51.6	<0.15
Kidney	Control	25	2145 ± 58.4	. .
	Th-deprived	25	2267 ± 74.2	<0.15
	OTh-treated	29	2324 ± 69.1	<0.05
	PTh-treated	21	2182 ± 80.9	>0.25
Heart	Control	23	5420 ± 140.6	. .
	Th-deprived	25	4791 ± 152.3	<0.005
	OTh-treated	28	5680 ± 167.2	<0.15
	PTh-treated	21	5050 ± 190.0	<0.10
Liver	Control	25	8217 ± 243.9	. .
	Th-deprived	25	4573 ± 282.8	<0.0005
	OTh-treated	29	5057 ± 199.6	<0.0005
	PTh-treated	21	5811 ± 248.3	<0.0005

[a]One unit of tissue LDH activity is that amount of the enzyme which will catalyze the conversion of one micromole of substrate per minute at 25°. One milliunit is equal to 0.001 unit.

[b]The final volume of the tissue homogenate in ml was 16 times the wet weight in grams of the tissue taken.

[c]Mean ± standard error.

Electropherograms of LDH isozymes

The isozyme patterns of LDH were examined in adult rat tissues and plasma, and the results for four of these are presented as photographs of electropherogram patterns in Figures 3-6. Close examination of these electropherograms indicate that plasma and four tissues examined, i.e., brain, kidney, heart, and liver, show the usual pattern for the isozymes. All tissues except liver and plasma clearly exhibit all five isozymes in pictures, even though liver and plasma also contain all five isozymes.

In brain, $LDH_1 = LDH_2$, $LDH_3 = LDH_4$ and LDH_5 are the decreasing order in the relative abundance of isozymes, as shown in Figure 3. The same patterns of isozyme distribution were observed in all the groups, although there was a significant increase ($p<0.05$) of LDH activity in the OTh-treated rats.

The electropherograms of the isozyme pattern in the rat kidney are shown in Figure 4. The relative abundance decreased in the following order of $LDH_1 = LDH_2$, LDH_5, LDH_4, and LDH_3. No significant difference was found among the groups. The significant increase ($p<0.05$) of the LDH levels resulting from OTh administration did not show any change in the isozyme distribution.

In heart, the fast-moving isozymes, such as LDH_1 and LDH_2 are predominant in the control group and all of three types of Th deficiency, as shown in Figure 5. LDH_2, LDH_1, LDH_3, and $LDH_4 = LDH_5$ are the decreasing order of the relative

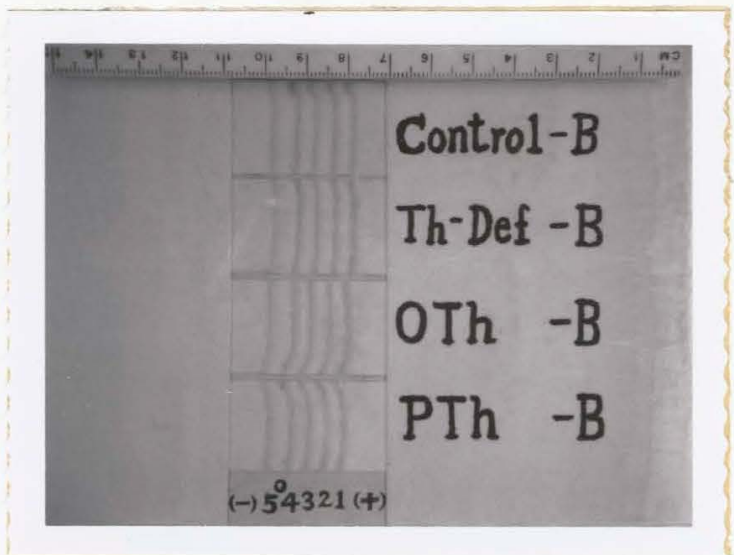

Fig. 3.--Photograph of LDH electropherograms of rat brain.

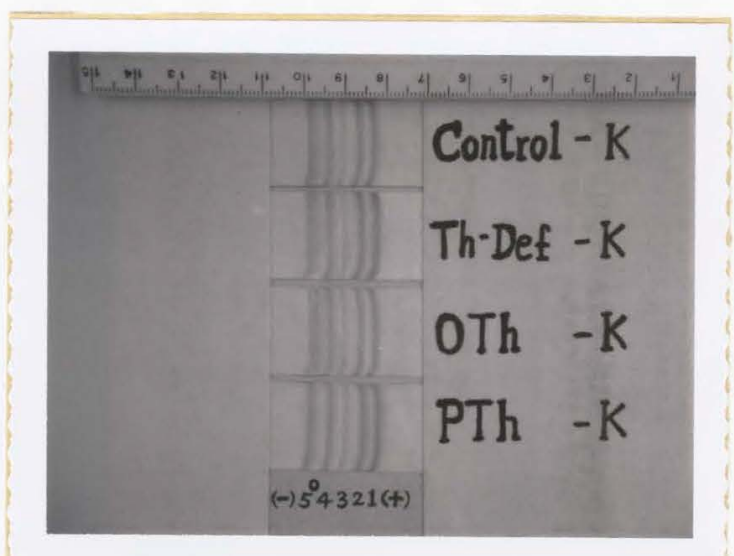

Fig. 4.--Photograph of LDH electropherograms of rat kidney.

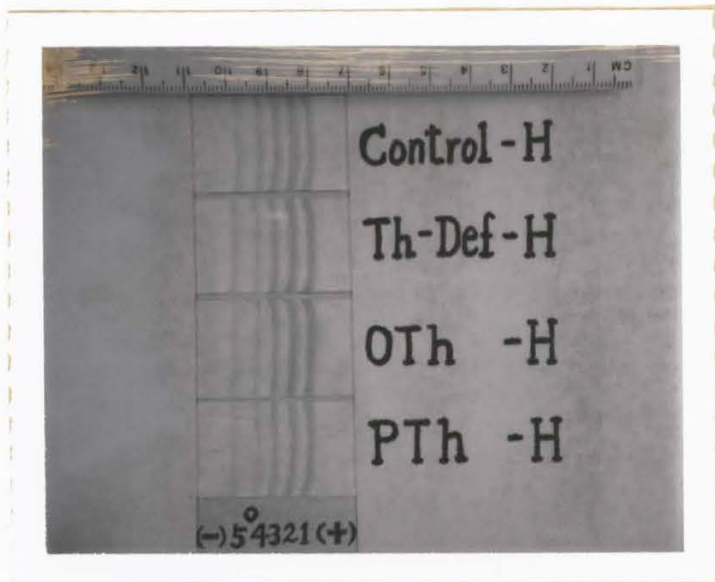

Fig. 5.--Photograph of LDH electropherograms of rat heart.

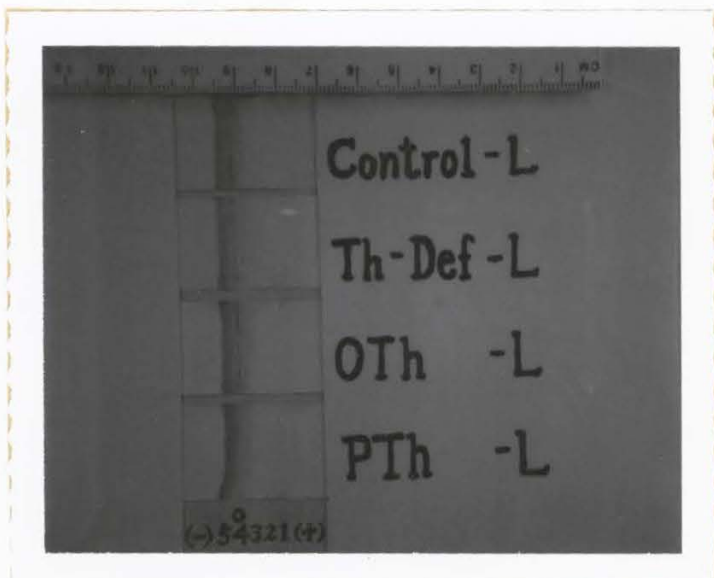

Fig. 6.--Photograph of LDH electropherograms of rat liver.

proportions of isozymes in all the groups. No noticeable difference in the isozyme patterns among the groups was seen, although Th deprivation resulted in a significant decrease of the total LDH activity in heart ($p<0.005$).

The patterns observed in liver are quite complex. Two extra bands, in addition to the five major bands, are located between LDH_2 and LDH_3, and LDH_3 and LDH_4, respectively. LDH_5 is the most predominant isozyme in rat liver. The other bands are too faint to detect in the picture, given in Figure 6. The order of the relative abundance in the isozymes of all the groups is as follows: LDH_5, LDH_4, LDH_2, LDH_1, and LDH_3. However, in some cases of the Th-deprived group, LDH_3 is noticeably more abundant than LDH_2. In the PTh-treated group one extra band between LDH_2 and LDH_3 was absent. The fast-moving isozymes and two extra bands can be detected only by illuminating the background of the electropherograms.

Direct assay of plasma LDH activity showed it, as presented in Table XIII, to contain about 100 units per ml, a very small amount compared to a couple of thousand units per ml tissue homogenate, as given in Table XIV. However, the electropherograms of plasma still show the specific isozyme patterns. The picture is not presented here because the other bands except LDH_5 can not be observed. The isozyme patterns are similar in all of the groups. The LDH_5 is predominant in plasma. LDH_4, LDH_3, LDH_2, and LDH_1, present in much smaller amounts, are in the decreasing order of the relative abundance. In spite of high LDH level in plasma by the

treatment with PTh, no noticeable difference in the isozyme pattern of this group from the control group was observed.

DISCUSSION

The usual pattern for the growth curves (24) was observed among control, Th-deprived and two Th antagonist-treated animals (Fig. 2).

Significant increases ($p<0.0005$) in weights of the adrenal glands were observed in all three types of Th deficiency (Tables V and VI). Shinozaki (86) found that this increase in weight of the adrenal glands in Th deficiency was caused by hyperfunction of adrenal cortex. Th deprivation or OTh treatment had a similar effect on weight of the adrenal glands. A more drastic increase in the weight was observed in PTh treatment and its unique poly-neuritic convulsion seemed to make some further contribution to this increase.

Among the α-keto acids examined, only α-ketobutyrate could possibly interfere with the determination of blood pyruvate in this particular assay system. α-ketobutyrate was the only α-keto acid, that was reduced at a sufficient rate (about one-twelfth that of pyruvate) to cause interference.

The results presented in Tables VII and VIII showed that blood pyruvate was increased in all three types of Th deficiency. However, there were some differences in the

degree of increase among the groups. This data is quite consistent with previous findings (14-17,19-24,73,76,87,88), even though the level of pyruvate was low in the Th-deprived group. Other investigators (21,24,74,76,88) found that blood pyruvate levels in the PTh-treated group did not differ from the control group. Unlike the previous reports, the data given here provide evidence that blood pyruvate was also increased by the treatment with PTh along with Th. This increase was almost the same as observed with the OTh group. Table VIII indicates that blood pyruvate in the PTh-treated group increases in parallel with the progression of the polyneuritic convulsion.

Rindi et al. (22,87) showed that the accumulation of pyruvate in the rat blood can be correlated statistically with the adrenal hypertrophy rather than with Th deficiency. The results shown here are not in agreement with the view of Rindi et al., since the weight of adrenal glands in the PTh-treated group was mostly built up in the preconvulsion stage of stress, without any change in blood pyruvate from the control group.

As shown in Tables IX and X, the level of blood lactate is usually around 20 times as much as the pyruvate level. The blood lactate levels generally paralleled the pyruvate levels, that is blood lactate was increased along with pyruvate in three types of induced Th deficiency. The data presented here are consistent with the findings of the other investigators (14,15,20,75). The lactate levels in the

blood of PTh-treated rats showed the same patterns as the pyruvate levels in the preconvulsion and the convulsion stages, respectively. This fact confirms the view given above that blood pyruvate mainly builds up in parallel with the progression of the convulsion in the PTh-treated group.

Rats made deficient by Th deprivation or PTh treatment showed no significant difference from the control in LDH activity of brain, whereas by OTh treatment the brain LDH levels were significantly increased (Table XIV). In kidney the LDH levels of the OTh-treated rats were also significantly higher than in the control rats. No significant change in the LDH activity of kidney resulted from Th deprivation or PTh administration, when compared with normal rats. Th deprivation caused a significant decrease of the LDH levels in heart, whereas no significant change from the LDH activity of the control group resulted from antagonist treatment. Liver LDH levels were decreased by 29-44 per cent in all three types of induced Th deficiency (Table XIV). Th deprivation caused the most significant decrease of the liver LDH activity. In OTh-treated rats the liver LDH levels were lower than in PTh-treated rats. These data on the liver LDH activity are generally in agreement with the findings of van Eys (89). The data given above suggest a different locus or mechanism of action for these two Th antagonists (91). The fact that only PTh treatment causes convulsions supports this idea and the tissue data also suggest that these two forms of Th deficiency have different effects, depending on the tissues involved.

It has been reported that the LDH levels of the liver are decreased about 30 per cent by starvation (89,90). These observations indicate that the effects of Th deficiency may be partly due to starvation.

Only in PTh-treated rats was a marked increase in plasma LDH activity observed, even though the blood pyruvate and lactate levels were significantly increased both in the OTh-treated and the PTh-treated group. Several tissues in addition to brain, kidney, heart and liver appear to contribute to the plasma LDH levels in the three types of Th deficiency, since all deficient groups showed marked decreases in the LDH levels of liver and only the PTh-treated group exhibited the buildup of LDH in plasma. The contribution of tissues to the plasma LDH levels are in all likelihood complicated and so it is difficult to attribute the buildup of the plasma LDH levels to a single tissue. Another possible explanation for this difference is that the sudden buildup of lactate in muscle, caused by a severe convulsion of the PTh-treated rats for a short time can not be removed properly by liver and therefore accumulates in the blood stream. This sudden accumulation of lactate in blood may cause an increase of the plasma LDH levels by a positive feedback mechanism.

Although tissues exhibit remarkable specificity in their patterns of isozymes, it is obvious that this specificity is based upon the relative amounts, rather than the presence or absence, of particular isozymes (33). It appeared that there was no noticeable difference in the distribution

patterns of brain and kidney LDH isozyme obtained from three types of Th deficient and control animals, even though the OTh-treated rats showed a significant increase in the LDH levels. The LDH levels of heart in Th deficiency exhibited a marked decrease (Table XIV) but the electropherograms of isozymes showed a similar distribution when compared with the control. In the above three tissues all five isozymes were always present in different relative abundance, depending upon the particular tissue involved. Evidently, these forms of Th deficiency have no effect on the isozyme distribution in the above tissues.

Quite complicated isozyme patterns were observed in liver. LDH_5 is the most predominant form and the other bands in electropherograms can hardly be detected (Fig. 6) even by densitometry. However, those weak bands can be shown by illuminating the background of the electropherograms. By careful examination, two extra bands in addition to five major bands were observed between LDH_2 and LDH_3, and LDH_3 and LDH_4, respectively. There have been several reports (34,92-94) that a greater number than five LDH isozymes may be detected, especially when starch-gel electrophoresis is employed. Wieland and Pfleiderer (95) report occasional observations of extra bands of activity in rat tissues separated by paper or cellulose acetate electrophoresis. It is quite difficult to explain the presence of two extra bands in liver. These may be isozymes similar to a sixth isozyme, which is present in testes of some species such

as human and rabbit, or dissociation products of a particular tetramer which might arise during electrophoresis. Since the other tissues examined did not show any extra bands and extra bands in liver were observed only among the faint bands their source or cause can not be explained from the data available. In some cases of Th deficiency, the LDH_3 band was more abundant than LDH_2, whereas the opposite pattern was observed in controls. In the PTh-treated rats, one extra band between LDH_2 and LDH_3 was absent. This gives at least some indications that Th deprivation or PTh treatment may have some effects on the pattern of isozyme distribution in liver.

In plasma all the groups showed the same pattern of isozyme distribution. LDH_5 was the most predominant. The other four bands except LDH_5 were difficult to observe. All three Th-deficient types seem to have no effect on the isozyme distribution of plasma.

SUMMARY

Thiamine deficiency was induced by thiamine deprivation, and treatment with oxythiamine or pyrithiamine. Thiamine deprivation and oxythiamine administration caused striking increase in weight of adrenal glands. The weight was even higher in pyrithiamine treatment.

The levels of blood pyruvate as well as lactate were markedly increased in the OTh-treated and the PTh-treated rats. The increase in these levels occurred in parallel with the progression of the polyneuritic convulsion in the PTh-treated rats, whereas there was no increase in the preconvulsion period. Only α-ketobutyrate, among the α-keto acids examined, might possibly interfere with the determination of blood pyruvate. However, since the reduction rate of α-ketobutyrate was about one-twelfth that of pyruvate, the interference would be small.

Thiamine deprivation and pyrithiamine administration caused no significant change in the LDH levels of brain and kidney. However, oxythiamine treatment caused a significant increase in the LDH activity of brain and kidney. In the heart, a different effect was observed. The LDH level was significantly decreased only by thiamine deprivation. All three types of deficiency resulted in marked decrease in the LDH levels of liver. In spite of the various changes in the

total LDH activity of tissues examined, the plasma LDH level was significantly increased only by pyrithiamine treatment.

LDH isozymes of plasma and tissues were electrophoretically separated on Sepraphore III cellulose polyacetate strips. The patterns of isozyme distribution in brain, kidney and heart were not noticeably different among the three Th-deficient groups. All five isozymes were observed in proportions that are highly specific for the tissues involved. Liver contains two extra bands, which were located between LDH_2 and LDH_3, and LDH_3 and LDH_4. LDH_5 was the predominant isozyme and the other bands are very faint, compared with LDH_5. In thiamine deprivation liver LDH_3 was noticeably more abundant than liver LDH_2 in some cases, which is opposite to that found in the control. One extra band between LDH_2 and LDH_3 was absent after pyrithiamine treatment. In plasma, LDH_5 was predominant. The other bands were also present but in relatively small proportions. No significant difference in the isozyme distribution was observed among all three types of thiamine deficiency and the control.

BIBLIOGRAPHY

1. Meyerhof, O., Z. Physiol. Chem., 102, 1 (1918).

2. Szent-Gyorgyi, A., Biochem. Z., 157, 50 (1925).

3. Von Euler, H., Adler, E., Gunther, G., and Hellstrom, H., Hoppe-Seyl. Z., 245, 218 (1936).

4. Meyerhof, O., and Ohlmeyer, P., Naturwissenschaften, 24, 741 (1936).

5. Von Euler, H., Adler, E., and Hellstrom, H., Z. Physiol. Chem., 241, 239 (1936).

6. Warburg, O., and Christian, W., Biochem. Z., 287, 291 (1936).

7. Wieland, T., and Pfleiderer, G., Biochem. Z., 329, 112 (1957).

8. Cahn, R. D., Kaplan, N. O., Levine, L., and Zwilling, E., Sci., 136, 962 (1962).

9. Fine, I. H., Kaplan, N. O., and Kuftinec, D., Biochem., 2, 116 (1963).

10. Fondy, T. P., Pesce, A., Freedberg, I., Stolzenbach, F., and Kaplan, N. O., Biochem., 3, 522 (1964).

11. Pfleiderer, G., and Jeckel, D., Biochem. Z., 329, 370 (1957).

12. Markert, C. L., and Møller, F., Proc. Nat. Acad. Sci. U. S., 45, 753 (1959).

13. White, A., Handler, P., and Smith, E. L., Principles of Biochemistry, 3rd Ed., McGraw-Hill Book Co., New York, 1964, p. 376.

14. Fornaroli, P., Quaderni Nutriz, 7, 155 (1940).

15. Fornaroli, P., and Boni, I., Boll. Soc. Ital. Biol. Sper., 15, 218 (1940).

16. Li, P. K., and Kato, K., J. Lab. Clin. Med., 26, 1314 (1941).

17. Thompson, R. H. S., and Johnson, R. E., Biochem. J., 29, 694 (1935).

18. Platt, B. S., and Lu, G. D., Chinese Physiol. Soc. 16, Proc. (1935).

19. Lu, G. D., Biochem. J., 33, 774 (1939).

20. Shimizu, T., Igaku to Seibutsugagu, 18, 195 (1951).

21. De Caro, L., and Rindi, G., Boll. Soc. Ital. Biol. Sper., 28, 1689 (1952).

22. Rindi, G., Ferrari, G., and Perri, V., Intern. z. Vitaminforsch., 25, 210 (1954).

23. Ferrari, G., and de Caro, L., Intern. Z. Vitaminforsch., 28, 386 (1958).

24. Gubler, C. J., J. Biol. Chem., 236, 3112 (1961).

25. Wuest, H., Med. Welt, 1965, 1798 and 1803 (1965).

26. Wroblewski, F., Ross, C., and Gregory, K. F., New Eng. J. Med., 263, 531 (1960).

27. Vesell, E. S., Ann. N. Y. Acad. Sci., 94, 877 (1961).

28. Hendrich, F., and Hule, V., Z. Ges. Inn. Med. Ihre Grenzgegiete, 19, 351 (1964).

29. Nutter, D. O., Trujillo, N. P., and Evans, J. M., Am. Heart J., 72, 315 (1966).

30. Warburton, F. G., Smith, D., and Laing, G. S., Nature, 198, 386 (1963).

31. Cohen, L., Djordjevich, J., and Ormiste, V., J. Lab. Clin. Med., 64, 355 (1964).

32. Wieme, R. J., and van Maercke, Y., Ann N. Y. Acad. Sci., 94, 898 (1961).

33. Markert, C. L., and Ursprung, H., Develop. Biol., 5, 363 (1962).

34. Fritz, P. J., and Jacobson, K. B., Sci., 140, 64 (1963).

35. Boyer, S. H., Fainer, D. C., and Watson-Williams, E. J., Sci., 141, 642 (1963).

36. Shaw, C. R., and Barto, E., Proc. Nat. Acad. Sci. U. S., 50, 211 (1963).

37. Dawson, D. M., Goodfriend, T. L., and Kaplan, N. O., Sci., 143, 929 (1964).

38. Wieland, T., Georgopoulos, D., Kampe, H., and Wachsmuth, E. D., Biochem. Z., 340, 483 (1964).

39. Gold, A. H., and Segal, H. L., Biochem., 4, 1506 (1965).

40. Kaplan, N. O., J. Cellular Comp. Physiol., 66, Pt. 2, 1 (1965).

41. Salthe, S. N., Comp. Biochem. Physiol., 16, 393 (1965).

42. Fondy, T. P., and Kaplan, N. O., Ann. N. Y. Acad. Sci., 119, 888 (1965).

43. Pesce, A., Fondy, T. P., Stolzenbach, F., Castillo, F., and Kaplan, N. O., J. Biol. Chem., 242, 2151 (1967).

44. Jaenicke, R., and Pfleiderer, G., Biochim. Biophys. Acta, 60, 615 (1962).

45. Fromm, H. J., J. Biol. Chem., 238, 2938 (1963).

46. Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F., Biochem. Z., 334, 37 (1961).

47. Wroblewski, F., and Gregory, K. F., Ann. N. Y. Acad. Sci., 94, 912 (1961).

48. Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F., J. Biol. Chem., 235, 2288 (1960).

49. Latner, A. L., Siddiqui, S. A., and Skillen, A. W., Sci., 154, 527 (1966).

50. Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E., J. Biol. Chem., 221, 833 (1956).

51. Takenaka, Y., and Schwert, G. W., J. Biol. Chem., 223, 157 (1956).

52. Millar, D. B. S., and Schwert, G. W., J. Biol. Chem., 238, 3249 (1963).

53. Fondy, T. P., Everse, J., Driscoll, G. A., Castillo, F., Stolzenbach, F. E., and Kaplan, N. O., J. Biol. Chem., 240, 4219 (1965).

54. Winer, A. D., and Schwert, G. W., J. Biol. Chem., 231, 1065 (1958).

55. Dobry-Duclaux, A., Biochim. Biophys. Acta, 89, 1 (1964).

56. Robinson, D. R., Stollar, D., White, S., and Kaplan, N. O., Biochem., 2, 486 (1963).

57. Balinsky, D., Biochim. Biophys. Acta, 122, 537 (1966).

58. Anderson, S. R., Florini, J. R., and Vestling, C. S., J. Biol. Chem., 239, 2991 (1964).

59. Winer, A. D., and Schwert, G. W., J. Biol. Chem., 234, 1155 (1959).

60. Winer, A. D., Acta Chem. Scand., 17, Suppl. 1, S 203 (1963).

61. Novoa, W. B., Winer, A. D., Glaid, A. J., and Schwert, G. W., J. Biol. Chem., 234, 1143 (1959).

62. Markovich, D. S., Zavodskii, P., and Volkenstein, M. V., Dokl. Akad. Nauk. S. S. S. R., 170, 204 (1966).

63. Meister, A., J. Biol. Chem., 184, 117 (1950).

64. Meister, A., J. Biol. Chem., 197, 309 (1952).

65. Kun, E., Biochim. Biophys. Acta, 25, 135 (1957).

66. Frank, W., and Holz, E., Z. Physiol. Chem., 314, 1 (1959).

67. Plummer, D. T., Elliott, B. A., Cooke, K. B., and Wilkinson, J. H., Biochem. J., 87, 416 (1963).

68. Plummer, D. T., and Wilkinson, J. H., Biochem. J., 87, 423 (1963).

69. Sawaki, S., Hattori, N., and Yamada, K., J. Vitaminol., 12, 210 (1966).

70. Banner, M. R., and Rosalki, S. B., Nature, 213, 726 (1967).

71. Rosenwald, L., Biochem. Z., 168, 324 (1926).

72. Collazo, J. A., and Morelli, E., J. Physiol. Path. Gen., 24, 508 (1926).

73. Sherman, W. C., and Elvehjem, C. A., Biochem. J., 30, 785 (1936).

74. Krusius, F. E., and Simola, P. E., Biochem. Z., 290, 428 (1937).

75. Frohman, C. E., and Day, H. G., J. Biol. Chem., 180, 93 (1949).

76. De Caro, L., Rindi, G., Perri, V., and Ferrari, G., Experientia, 12, 300 (1956).

77. Ostrovskii, Y. M., Vopr. Pitaniya, 24, 41 (1965).

78. Von Muralt, A., Ann. N. Y. Acad. Sci., 98, 499 (1962).

79. Amador, E., Dorfman, L. E., and Wacker, W. E. C., Clin. Chem., 9, 391 (1963).

80. Scholz, R., Schmitz, H., Bucher, T., and Lampen, J. O., Biochem. Z., 331, 71 (1959).

81. Bucher, T., Unpublished, quoted by Bohringer, C. F., Sohne, G. H., Biochemische Abteilung, (1963).

82. Warburg, O., and Christian, W., Biochem. Z., 286, 81 (1936).

83. Kubowitz, F., and Ott, P., Biochem. Z., 314, 94 (1943).

84. Wroblewski, F., and LaDue, J. S., Proc. Soc. Exp. Biol., 90, 210 (1955).

85. Wilkinson, J. H., Isoenzyme, J. B. Lippincott Co., Philadelphia, Penn., 1966, p. 29.

86. Shinozaki, Y., Bitamin, 18, 161 (1959).

87. De Caro, L., and Rindi, G., Experientia, 11, 196 (1955).

88. Johnson, L. R., and Gubler, C. J., Biochim. Biophys. Acta, 156, 85 (1968).

89. Van Eys, J., J. Nutrition, 73, 403 (1961).

90. Weber, G., J. Nutrition, 71, 156 (1960).

91. Woolley, D. W., and Merrifield, R. B., Federation Proc., 11, 459 (1952).

92. Allen, J. M., Ann. N. Y. Acad. Sci., 94, 937 (1961).

93. Blanco, A., and Zinkham, W. H., Sci., 139, 601 (1963).

94. Goldberg, E., Sci., 139, 602 (1963).

95. Wieland, T., and Pfleiderer, G., Ann. N. Y. Acad. Sci., 94, 691 (1961).

THE EFFECT OF THIAMINE AND ITS ANTAGONISTS ON PLASMA AND TISSUE LACTIC DEHYDROGENASE IN RATS

An Abstract of

A Thesis

Presented to the

Department of Chemistry

Brigham Young University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Dong H. Park

August 1968

ABSTRACT

The lactic dehydrogenase activity in plasma and tissues was measured in the thiamine-deprived, the oxythiamine-treated and the pyrithiamine-treated rats as well as the control rats. The lactic dehydrogenase levels of brain and kidney were significantly increased by oxythiamine treatment. The enzyme activity in heart was markedly decreased only in the thiamine-deprived rats. Unlike the above tissues, the enzyme levels in liver were decreased by 29-44 per cent in all three types of thiamine deficiency. However, the enzyme activity in plasma was significantly increased only by pyrithiamine administration.

The distribution patterns of lactic dehydrogenase isozymes were electrophoretically examined in these deficiencies. No significant difference among the three thiamine-deficient groups was observed in brain, kidney, and heart. All five isozymes were observed in proportions that are highly specific for the tissues involved. Two extra bands, in addition to five major bands, were found in liver. In thiamine deprivation liver LDH_3 was noticeably more abundant than LDH_2 in some cases, which is opposite to that found in the control. One extra band between LDH_2 and LDH_3 was absent in liver after pyrithia-

mine treatment. No noticeable difference in the isozyme distribution of plasma was found among the three thiamine-deficient groups. Blood pyruvate along with lactate was significantly increased by oxythiamine treatment. Pyrithiamine administration also caused a marked increase of blood pyruvate along with lactate only in the phase of the polyneuritic convulsion. Remarkable increases in weight of the adrenal glands were observed in all three cases.