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# Effect of Lead Poisoning on the Thiamine Status and Function in Liver and Blood of Rats

ELISABETH TOKARSKI and LEMBITU REIO

Nutrition Laboratory, National Food Administration, P.O.B. 622, S-751 26 Uppsala, Sweden

Three groups of Sprague-Dawley rats were fed a thiamine deficient diet, which was supplemented by daily subcutaneous injections of a minimum requirement of thiamine, and treated with lead(II) acetate in different molar ratios to thiamine (1:1, 2:1, 10:1) for 5 and 9 months, respectively. The prolonged administration of lead(II) acetate decreases the thiamine level in lead-treated rats and diminishes the enzymatic activity of pyruvate dehydrogenase as well as that of transketolase. The thiamine level in the liver decreased by 30 to 40 % compared with a reference group and the activity of the erythrocyte transketolase diminished by 5 to 40 %. The level of the blood pyruvate increased by about 20 % and the rate of the oxidative decarboxylatoin of pyruvate by liver mitochondria decreased.

Thiamine (vitamin $B_1$) plays three major roles at the cellular level. The first is related to energy metabolism and concerns the oxidative decarboxylation of α-keto acids. The second is concerned with biosynthetic pathways as reflected by the transketolase reaction of the pentose phosphate shunt yielding nicotinamide adenine dinucleotide phosphate (NADPH) and pentose. The third deals with the function of membranes and nerve conduction. Thiamine depletion affects neurons and their functions in selected areas of the central nervous system.[1] Neurological defects, such as peripheral neuritis and encephalopathy, have been recognized in association with thiamine deficiency.[2,3] Similar neurological defects have been observed to accompany lead intoxication. Damage to the central nervous system causing encephalopathy and neuropathy is a common feature, but the mechanism by which lead affects the nervous system is still only partially understood.[4–7]

This coincidence of neurological alterations may suggest that interaction between lead and thiamine may occur to some extent.

This study intends to investigate the possible interaction between subcutaneously administered lead(II) acetate and thiamine *in vivo* systems such as the blood and liver of rats. The possible influence of lead poisoning on the thiamine status and activity was observed by measuring the following thiamine pyrophosphate (TPP) functions: the transketolase activity (TPP effect) and the pyruvate level in the blood, the thiamine level in the liver, and oxidative decarboxylation of pyruvate by liver mitochondria.

## MATERIALS AND METHODS

*Reagents.* β-Nicotinamide adenine dinucleotide in reduced form (NADH), lactic dehydrogenase (LDH) in ammonium sulfate solution, adenosine 5′-triphosphate, thiamine HCl, thiamine pyrophosphate (TPP), D-ribose-5-phosphate, and pyruvic acid were obtained from Sigma Chemical Co., St Louis, Mo., U.S. Potassium hexacyanoferrate(III) and lead(II) acetate were delivered by Merck Co., Darmstadt, G.F.R. Cod-liver oil and Mebumal were available from ACO, Sweden. Casein (vitamin-free) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Taka-diastase was obtained from Parke-Davis, S.P.A., Lainate, Milano, Italy and activated Decalso from Fisher Scientific Co., New York, U.S.

*Animals.* Sprague-Dawley rats of both sexes weighing 110 to 130 g were used in all experiments.

*Treatment of animals.* All rats were placed on a basal thiamine deficient diet. Vitamins A and D were supplied by addition of cod-liver oil (about 3 g/100 g of food). For all animals the basal thiamine deficient diet had the following composition calculated for 1 kg of diet: sucrose

685.0 g (68.5 % of diet), casein (vitamin-free) 220.0 g (20.6 %), salt mix [11] 45.0 g (4.5 %), corn oil 50.0 g (5.4 %), vitamin mix [11] 2.23 g, choline chloride 4.0 g. Casein (vitamin-free) contained 0.14 μg of thiamine.HCl/g. All the rats were fed *ad libitum* and allowed unlimited access to tap water. They were divided into three groups, each with its respective control. Each animal was placed in an individual iron cage. After the initial 4 days on the basal thiamine deficient diet all animals received daily subcutaneous injections of 10 μg of thiamine/100 g body weight in 0.2 ml of 0.9 % solution of sodium chloride, which was the minimum amount necessary for optimal growth. At the same time they were given different amounts of lead(II) acetate.

Group I of the experimental animals was treated by daily subcutaneous injections of 9.89 μg lead(II) acetate/100 g body weight in 0.2 ml $H_2O$ (molar ratio of lead(II):thiamine was 1:1) for 5 months.

Group II was given daily subcutaneous injections of 19.78 μg lead(II) acetate/100 g body weight in 0.2 ml $H_2O$ (lead(II):thiamine = 2:1) for 9 months.

By daily subcutaneous injections, group III was given 98.90 μg of lead(II) acetate/100 g body weight (lead(II):thiamine = 10:1) for 9 months. All control animals were given daily subcutaneous injections of 0.2 ml $H_2O$ at the same time as their respective experimental groups.

*Preparation of blood samples, liver homogenate and mitochondria.* After an appropriate time had passed the animals were anaesthetized by the injection of Mebumal. Heart puncture was performed to obtain blood samples (2 – 6 ml) for determination of the pyruvate level and the TPP effect, followed by the removal of the liver.

The tissue was quickly removed and divided into two parts. One part (for mitochondria preparation) was quickly weighed and immediately placed in ice-cold 0.25 M sucrose. The sample was homogenized for 2 min in the Potter-Elvehjem glass homogenizer with a Teflon pestle. This sample was immersed in an ice bath. Cold sucrose solution (0.25 M) was added to the homogenate (homogenizing medium) so that the final volume of the homogenate in millilitres was 10 times the wet weight in grams of the tissue taken. Liver mitochondria were separated essentially according to Johnson and Lardy.[8]

The other part of the tissue was weighed for preparation of liver homogenate and homogenized in 0.1 M HCl in the Ultra-Turrax homogenizer (type TP 10 – 18) and suspended in at least 15 times its weight of 0.1 M HCl.

*Determination of erythrocyte transketolase activity and TPP effect.* Blood was collected by cardiac puncture into a heparinized syringe, and centrifuged in the graduated centrifuge tube. After the plasma and the buffy coat had been removed by suction, a volume of distilled water equal to twice the volume of the cells was added. The cells were then resuspended by stirring with a wooden applicator stick. The mixture was frozen in tipped vials to prevent breakage. The transketolase activity was estimated by measuring the amount of hexose produced in the enzymic reaction when D-ribose-5-phosphate was added to a buffered medium containing hemolyzed red blood cells.

In addition, the percentage stimulation of hexose formed by the system involving transketolase was determined after thiamine pyrophosphate had been added to hemolysate.

The resulting transketolase activity is referred to as the TPP effect and is due to the initial deficiency of thiamine pyrophosphate and is given as a percentage of the normal activity. The transketolase activity is expressed as the number of μg hexose per ml of hemolysate formed per hour during incubation at 38 °C.

*Determination of pyruvate in blood.* The level of pyruvate in blood was measured with lactic dehydrogenase (LDH) and β-nicotinamide adenine dinucleotide in reduced form (NADH) according to Sigma Technical Bulletin.[10] The amount of pyruvate determined was expressed in mg/100 ml blood.

*Determination of pyruvate oxidation by liver mitochondria.* The activity of pyruvate oxidation was determined by the reduction of hexacyanoferrate(III) to hexacyanoferrate(II) ion. This reduction was determined by following the change in absorbance ($\Delta A$) at 420 nm. The change was measured with a Beckman model DB-GT spectrophotometer according to the method of Gubler.[11]

The reaction mixtures were prepared directly in cuvettes with a 10 mm light path and contained: Potassium phosphate buffer (0.15 M pH 7.4) 75 μmol, magnesium sulfate 20 μmol, Versene (EDTA) 2 μmol, ATP 6 μmol, pyruvic acid 20 μmol, 0.2 ml of 1:5 dilution of mitochondria suspension in sucrose (0.25 M), followed by the addition of sucrose to a total volume of 3.0 ml. Furthermore, an addition of 0.7 ml of 0.0666 M potassium hexacyanoferrate(III) (4.66 μmol) was made to the cuvettes containing sample and endogenous blank. In the reference cuvette 0.25 M sucrose was added to a final volume of 3.7 ml. All ingredients were kept in an ice bath until mixing in reaction cuvettes. Measurements were carried out at room temperature under uniform conditions.

The reference blank did not contain any hexacyanoferrate(III), nor did the endogenous blank contain any substrate. At zero time and at successive 5-min intervals for 30 min after the addition of hexacyanoferrate (III) ion, the sample and the endogenous blank were read simultaneously against the reference blank in a double-beam spectrophotometer.

The absorbance obtained at each time interval was subtracted from that measured at zero time in order to give the change in absorbance.

The enzyme activity was expressed as $\Delta A_{420}/30$ min per 0.2 ml mitochondria suspension. The protein analyses were made according to the method given by Hartree.[12]

*Determination of thiamine level in liver.* The thiamine level in liver was estimated by the thiochrome method [13] and adapted to Technicon autoanalyser procedure [14] and modified by the Nutrition Laboratory (National Food Administration, Uppsala, Sweden). The principle of this assay is the oxidation of thiamine to thiochrome by potassium hexacyanoferrate(III) in the presence of a strong alkali. The oxidation product is extracted into isobutyl alcohol and its UV fluorescence is measured. The sample was treated with Taka-diastase. The hydrolyzed product (free thiamine) is then purified on a base-exchange silicate column (activated Decalso), and eluted with potassium chloride. The thiamine content is expressed as μg thiamine/g liver.

## RESULTS AND DISCUSSION

*Effect of lead poisoning on the appearance of hexose in rat red cell hemolysates.* The amount of hexose produced in the red blood cells in the enzymic reaction catalyzed by transketolase is a good indicator of the thiamine status. As seen from Table 1 all experimental groups showed a diminished level of hexose. The depression in the hexose formation increased with an increased ratio of lead to thiamine supplied to the rats.

It has also been observed that the prolonged supply of only the necessary minimum amount of thiamine also decreased the formation of hexose in the control animals of groups I, II and III.

After addition of 100 μg TPP *in vitro* to control and poisoned rat hemolysates, a stimulation of the formation of hexose in the transketolase assay was observed in both cases. However, this stimulation (expressed as TPP effect in %) was always greater in the lead-treated animals and increased with the amount of lead administered to the rats. The increase reached its maximum between groups II and III where the amount of administered lead was 5 times greater. In group III the TPP effect increased by about 100 % compared with the control.

The results of groups II and III are not completely comparable with those of group I. The animals of the first group were poisoned within 5 months, whereas the other groups were treated with lead(II) acetate for 9 months. However, the following tendencies can be clearly seen in all the groups. The production of hexose in the erythrocyte hemolysates of poisoned rats (μg hexose/ml hemolysate per hour) was lower than their respective controls in all cases; this difference is expressed as a depression from control (Table 1). The greatest depression from control occurs in group III. This observation may lead to the conclusion that, with prolonged poisoning of rats with lead(II) acetate, the thiamine is inactivated or that its function as a coenzyme to apoenzyme is inhibited. On the other hand, it is also possible that lead poisoning may reduce, at least partially, the level of the apoenzyme present in the blood.

*Effect of lead poisoning on pyruvate level in*

*Table 1.* Effect of lead poisoning on erythrocyte transketolase activity in rats, expressed as μg hexose/ml hemolysate. The TPP effect (%) represents the percentage change due to the presence of the added coenzyme. The results given are means ±S.D in parenthesis.

| Group | Months of test | No. of rats | μg Hexose/ml hemolysate per hour | | | |
|---|---|---|---|---|---|---|
| | | | | Addition of 100 μg TPP | TPP effect % | Depression from control % |
| Control rats [a] | | | | | | |
| I | 5 | 6 | 818(66) | 889(65) | 9(1) | |
| II | 9 | 5 | 526(51) | 608(56) | 15(1) | |
| III | 9 | 6 | 552(46) | 641(59) | 16(2) | |
| Lead-fed rats [a] | | | | | | |
| I | 5 | 6 | 780(115) | 876(120) | 12(1) | 5 |
| II | 9 | 6 | 398(35) | 490(54) | 23(3) | 24 |
| III | 9 | 6 | 330(40) | 450(73) | 35(6) | 40 |

[a] The amounts of thiamine and lead administered are given in Table 2 under supplements.

*Table 2.* Effect of lead poisoning on blood pyruvate and liver thiamine level of rats. Thiamine values represent total thiamine content after hydrolysis with phosphatase. Supplements were daily administered by subcutaneous injections. The results given are means ± S.D × $10^3$.

| Group | Month of test | No. of rats | Supplements μg/100 g of body weight | Molar ratio $B_1/Pb^{2+}$ | Pyruvate mg/100 ml | Increase from control % | No. of rats | Thiamine μg/g |
|---|---|---|---|---|---|---|---|---|
| Control I | 5 | 4 | Thiamine 10 | | 1.13(12) | | 6 | 3.91(37) |
| Lead-fed I | 5 | 4 | Thiamine 10<br>Lead 6.3 | 1:1 | 1.26(10) | 11.5 | 6 | 2.80(30) |
| Control II | 9 | 6 | Thiamine 10 | | 1.20(6) | | 6 | 4.00(49) |
| Lead-fed II | 9 | 7 | Thiamine 10<br>Lead 12.6 | 1:2 | 1.39(9) | 15.8 | 6 | 2.65(48) |
| Control III | 9 | 5 | Thiamine 10 | | 1.21(5) | | 7 | 4.19(30) |
| Lead-fed III | 9 | 7 | Thiamine 10<br>Lead 63 | 1:10 | 1.52(8) | 20.8 | 6 | 2.45(42) |

*blood.* Thiamine is known to be involved in the enzymatic decarboxylation of pyruvate. One of the earliest observations of a biochemical abnormality in thiamine deficiency was the accumulation of pyruvate in tissues and the increase in its concentration in circulating blood.[15] In this study the level of pyruvate in the blood was estimated by an assay utilizing the enzymic conversion of pyruvate into lactate with simultaneous oxidation of NADH to $NAD^+$, which was measured spectrophotometrically. The results obtained (Table 2) show a small increase in the pyruvate level of the blood. The greatest increase is recorded for group III and the smallest for group I. This observation supports the view that high lead(II) administration results in a higher pyruvate level in the blood.

*Effect of lead poisoning on thiamine level in rat liver.* As shown in Table 2, the liver thiamine content decreased by 30 to 40 % in all groups of lead-treated rats compared with the controls. There is, however, no significant difference between the respective groups in spite of a tenfold increase in the lead(II) dose between groups I and III and a longer time of poisoning. The observed decrease in the thiamine content of the liver may, in addition, confirm our earlier laboratory results revealing diminution in thiamine and its activity in the blood as an effect of the lead poisoning of the rats.

*Oxidation of pyruvate by rat liver mitochondria from normal and lead-poisoned rats.* There are two thiamine-dependent enzymic reactions in the Krebs cycle. One of these is oxidative decarboxylation of pyruvate, where thiamine is a cofactor of pyruvate dehydrogenase catalyzing its conversion to "active acetyl". Studies were carried out using a spectrophotometric method with hexacyanoferrate(III) ion as the electron acceptor.[11] The investigations were performed on the animals of experimental group III in which the rats had been poisoned for 9 months with lead(II) (the ratio of lead to thiamine was 10:1) and the measurements were made in duplicate. The values for the rate of oxidative decarboxylation of pyruvate by rat liver mitochondria of control and lead-poisoned rats were: 0.390 ±0.035 average for 6 rats and 0.240±0.028 average for 7 rats, respectively. These values are expressed as $\Delta A$ 420 nm/0.2 ml of mitochondria suspension for 30 min. The original intention was to present the results on the basis of mitochondria protein content. During this investigation, however, it became apparent that the protein content of mitochondria from the poisoned rats had decreased by 15 to 20 %. Therefore, it was considered better to express the obtained results on the basis of a unit volume of 0.2 ml of mitochondria suspension (see methods) which was constant for both the control and poisoned rats.

As seen from the values obtained, there is a significant decrease in the rate of oxidative decarboxylation of pyruvate by the liver mitochondria of the lead-poisoned rats compared with the oxidation rate by control group mitochondria. The amount of information ob-

tained from this experiment is not sufficient to draw a definite conclusion as to whether or not the decreased oxidation rate of pyruvate is due to the inhibition of thiamine as a cofactor of puryvate dehydrogenase or even to that of the sulfhydryl groups of lipoic acid. Lipoic acid also participates in the overall reaction cycle of the pyruvate dehydrogenase complex. The above investigation of the oxidation of pyruvate is not complete and should only be considered a preliminary observation. However, it was thought to be important to present these results as a complement to the earlier inventigations. Further investigations into this subject will be required.

This study revealed that in all groups of lead-treated rats the enzymatic activities of pyruvate dehydrogenase as well as of transketolase decreased considerably in comparison with the controls. The diminished activity of transketolase might be caused by the lack of cocarboxylase, which has been proven by an increase in the production of hexose after the addition of cocarboxylase – thiamine pyrophosphate – in the hemolysate *in vitro*. This increase, however, never reached the level of the hexose production in the control rats, which might be explained by a partial inhibition of the apoenzyme. The decrease in the thiamine level of poisoned rat liver (Table 2) indicates the diminished availability of this coenzyme for erythrocyte transketolase. On the other hand, the level of pyruvate in the blood increased in the lead-treated rats, which could indicate a decrease in the oxidative decarboxylation of pyruvate. This is in good agreement with the observed diminished activity of pyruvate dehydrogenase in liver mitochondria of poisoned animals. The addition of TPP *in vitro* gave only a partial recovery of the control activity of the mitochondria pyruvate dehydrogenase. This could again indicate the lack of TPP as well as the inhibition or deficiency of the enzymes pyruvate dehydrogenase and dihydrolipoyl transacetylase (or lipoic acid itself). The fact that the total protein content of the liver mitochondria in the lead-treated rats decreased by about 20 % could indicate that lead(II) acetate might also have in inhibitory effect on the synthesis of the protein as well as of the apoenzymes.

The interaction between thiamine compounds has been demonstrated by the described experiments. However, more data would be needed to establish the dependence of this interaction upon the existence of a redox system between thiamine-thiamine disulfide,[16] where thiamine could be present in thiol form as an intermediate. This is theoretically possible and could be a subject for further investigation. At the same time heavy metals (for instance lead) are known to exhibit a strong affinity for a common biochemical ligand such as organic phosphate.[17]

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