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BIOCHEMICAL STUDIES ON THE MECHANISM OF DRUG ACTION

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PREFACE

The present is an annual report for Grant No. DAHC 19-71-G-0002 (Army Project Number 2N061102B71D) and represents detailed results and discussion of work accomplished from August 1, 1970 to July 31, 1971.

This report deals with basic biochemical alterations caused by thiamin deficiency and the effect of aspirin on these processes.

The author wishes to thank the U. S. Army Research Office for their support of this project. The close association and contributions of Dr. Eugene M. Sporn, Chief, Special Projects Branch, Life Sciences Division, with this research is acknowledged.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animals, Resources, National Academy of Sciences, National Research Council.

ABSTRACT

The effect of acylcarnitines on pyruvate metabolism was compared in liver mitochondria from thiamin-deficient and pair-fed rats. Respiration and oxidative phosphorylation were investigated with pyruvate, succinate, α-ketoglutarate or α-glycerophosphate as substrate. The respiratory rate was significantly decreased in state 4 when α-glycerophosphate was used as substrate ($P < 0.001$). In state 3, the respiratory rates in the presence of α-ketoglutarate, pyruvate or α-glycerophosphate were significantly decreased ($P < 0.05$). The ratio of ADP to oxygen was decreased when α-ketoglutarate was used as substrate ($P < 0.025$). Pyruvate utilization by liver mitochondria from thiamin-deficient rats was markedly decreased but the percentages of pyruvate carboxylation and oxidation were not altered. Acylcarnitines stimulated pyruvate carboxylation more in deficient mitochondria than in mitochondria from pair-fed controls. Maximum stimulation in the percentage carboxylation of pyruvate was obtained in the presence of 0.83 mM L-octanoylcarnitine. Pyruvate oxidation was greatly reduced by the addition of acylcarnitines. These experiments indicate that the ability of fatty acids to alleviate the symptoms of thiamin deficiency may be related to their effect on the regulation of pyruvate carboxylase, a key enzyme in the pathway of gluconeogenesis.

Diets containing 0.2% acetylsalicylic acid were fed to control and thiamin-deficient rats. Acetylsalicylic acid caused a significant decrease in body weight and adipose tissue weight in thiamin-deficient acetylsalicylic acid-treated but not in normal acetylsalicylic acid-treated rats. A large decrease in the total blood ketone bodies in thiamin-deficient acetylsalicylic acid-treated rats was observed. A significant decrease in the blood α-hydroxybutyrate to acetoacetate

ratio in both control and thiamin-deficient rats treated with aspirin was noted.

Rat liver mitochondria from both control, thiamin-deficient, and acetylsalicylic acid fed rats were incubated in the presence of ATP, Mg^{+2}, P_i, pyruvate, and radioactive bicarbonate, and synthesis of dicarboxylic acids was examined. It was found that in acetylsalicylic acid-treated rats, the pyruvate utilization and $^{14}CO_2$ incorporation and organic acids formation were greatly decreased.

Addition of octanoylcarnitine greatly stimulated $^{14}CO_2$ incorporation into organic acids in thiamin-deficient and acetylsalicylic acid-treated rats. The percentage of pyruvate carboxylated in the presence of octanoylcarnitine was significantly increased in mitochondria from all animals.

3. Respiration and oxidative phosphorylation by liver mitochondria isolated from rats fed 0.2% dietary aspirin for thirty-one days with pyruvate, α-ketoglutarate and succinate were studied polarographically. There was an increase in the state 3 respiration with pyruvate (150%), α-ketoglutarate (138%) and succinate (128%). A significant ($P < 0.05$) increase in state 4 respiration with all three substrates was found. The ADP/O ratios were significantly decreased with pyruvate ($P < 0.025$), α-ketoglutarate ($P < 0.005$) and succinate ($P < 0.01$).

Addition of aspirin *in vitro* to isolated mitochondria decreased state 3 respiration with pyruvate ($P < 0.05$), α-ketoglutarate ($P < 0.05$) and succinate ($P < 0.01$). The state 4 respiration was significantly ($P < 0.025$ to $P < 0.001$) increased with all three substrates. The oxidative phosphorylation was decreased with all substrates. It is suggested that *in vitro*, the uncoupling by aspirin was due to an increase in the ATPase activity.

4. Glucose synthesis from pyruvate or lactate is significantly decreased in perfused liver and kidney slices from thiamin-deficient rats. The addition of octanoate or preincubation of the tissue with thiamin restored its ability to synthesize glucose. Glucose synthesis from malate or α-ketoglutarate was unaltered in kidney slices by thiamin deficiency. Oral administration or intraperitoneal injection of octanoate significantly increased glucose synthesis by kidney slices from both pair-fed control and thiamin deficient rats.

The activities of phosphoenolpyruvate carboxykinase, fructose diphosphatase, and glucose-6-phosphatase were increased in both liver and kidney from thiamin deficient rats. Pyruvate carboxylase was increased in kidney only. Malic enzyme activity was decreased in liver and unchanged in kidney.

The concentrations of L-lactate, pyruvate, and α-ketoglutarate were significantly increased in thiamin-deficient liver; whereas the concentrations of aspartate, acetoacetate, β-hydroxybutyrate, acetyl coenzyme A and orthophosphate were significantly decreased. The concentrations of L-malate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, dihydroxyacetone phosphate, α-glycerophosphate, citrate, isocitrate, L-glutamate, ammonium ion, coenzyme A, ATP, ADP, and AMP were unaltered in thiamin-deficient liver. In addition, the concentrations of serum free fatty acids, phospholipids and triglycerides were significantly decreased by thiamin deficiency.

The mitochondrial ratio of $NAD^+/NADH$, calculated from both the glutamate dehydrogenase and the β-hydroxybutyrate dehydrogenase equilibriums was significantly increased. Thus, the mitochondrial compartment of thiamin-deficient rat liver appears to be highly oxidized. Similar calculations using the lactate dehydrogenase equilibrium constant indicated that the $NAD^+/NADH$ ratio in the cytoplasm of thiamin-deficient rat liver was unchanged. The

cytoplasmic $NADP^+$/NADPH ratio, calculated using both the isocitrate dehydrogenase and malic enzyme equilibriums was significantly increased.

These data suggest a functional block in glucose synthesis via pyruvate carboxylation in vitro which is apparently related to decreased pyruvate oxidation. The metabolite concentrations of the liver support the conclusion that the activities of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase are markedly decreased in thiamin-deficient rat liver and that these changes may result in further alterations in carbohydrate and lipid metabolism.

Glucose synthesis from pyruvate, malate and fructose is inhibited by preincubation of rat kidney slices with oxythiamin. Oxythiamin ($1X10^{-1}$mg/ml) caused a 78% and a 65% decrease in glucose synthesis from pyruvate and malate respectively. The conversion of fructose to glucose was inhibited by 27% in the presence of oxythiamin ($1X10^{-2}$mg/ml); pyrithiamin ($1X10^{-5}$-$1X10^{-1}$mg/ml) did not inhibit glucose synthesis from pyruvate. Preincubation of kidney slices with both oxythiamin and pyrithiamin did not inhibit glucose synthesis from pyruvate or fructose.

Fluorocitrate (0.1 mM) inhibited glucose synthesis from pyruvate by 59%. Fluorocitrate (0.1 mM) and oxythiamin ($1X10^{-3}$mg/ml) together decreased glucose synthesis from pyruvate by 76%. Octanoate (0.8 mM) and β-hydroxybutyrate (5 mM), but not acetate (5 mM), restored the synthesis of glucose from pyruvate or malate in the presence of oxythiamin. Octanoate (0.8 mM) partially restored glucose synthesis from pyruvate in the presence of fluorocitrate (0.1 mM).

Glucose synthesis from α-ketoglutarate was not altered in the presence of oxythiamin. Octanoate (0.8 mM) significantly decreased glucose synthesis from α-ketoglutarate. Oxythiamin did not inhibit pyruvate oxidation or carboxylation when added to intact rat kidney mitochondria.

These results strongly support the hypothesis that oxythiamin must be phosphorylated in order to inhibit pyruvate oxidation and that pyrithiamin acts by inhibiting the phosphorylation of oxythiamin in vitro. The lack of effect of oxythiamin on pyruvate metabolism by intact kidney mitochondria indicates that thiamin pyrophosphorokinase (E.C.2.7.6.2 ATP: Thiamin pyrophosphotransferase) is a soluble enzyme in rat kidney. It is also concluded that inhibition of pyruvate oxidation, in the absence of alternative sources of acetyl CoA, NADH and ATP, greatly decreases pyruvate carboxylation in vitro.

6. Aspirin has been fed to rats maintained on thiamin supplemented and thiamin deficient diets. Aspirin produced no evidence of thiamin deficiency in rats receiving adequate B_1 but significantly decreased the time necessary for the development of deficiency in rats not receiving thiamin. Dietary aspirin also caused inhibitions of pyruvate and α-ketoglutarate oxidations in mitochondria from rats fed both the thiamin supplemented and thiamin deficient diets.

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INTRODUCTION

The primary objective of this research project was to study mode of action of drugs utilizing thiamin deficient animals. In order to use these deficient animals as a model system a comprehensive study was conducted on biochemical lesions of thiamin deficiency and regulation of these lesions. Up to present time most of the research with thiamin deficiency has been concerned with the biochemical lesions associated with the pyruvate and α-ketoglutarate dehydrogenase complexes as well as the transketolase reactions (1-6). Numerous studies have dealt with the "thiamin sparing" effect of fatty acids (7-18). In spite of these studies the sparing action of fatty acids still remains obscure. Westenbrink (19) was the first to suggest such an effect, reporting that high fat diets conserved the thiamin present in various tissues. Evans et al (11) later substantiated this hypothesis and demonstrated that fats were variable in their ability to alleviate symptoms associated with thiamin deficiency; the optimal effects were obtained with medium-chain-length fatty acids, principally octanoic acid.

In 1940 Krebs and Eggleston (20), while studying the biological synthesis of oxaloacetic acid from pyruvate and carbon dioxide in avian liver, suggested that thiamin may be important for the carboxylation of pyruvate. Barron et al (21) demonstrated that glucose formation in rat kidney slices was impaired by thiamin deficiency and could be restored to normal by the addition of thiamin in vitro. Contrary to these findings, Benevenga et al (22) found that more pyruvate-2-^{14}C was incorporated into glutamate via pyruvate carboxylation in thiamin-deficient calf liver; there was no decrease in the ability of thiamin-deficient calves to synthesize glucose.

It is now well established that biotin and not thiamin is the essential cofactor for pyruvate carboxylase [EC 6.4.1.1 pyruvate: carbon-dioxide ligase (ADP)] (23-25), and that this enzyme catalyzes a key reaction in the pathway of gluconeogenesis. Acetyl coenzyme A is a required allosteric activator for pyruvate carboxylase. Thus, the effects which Krebs and Eggleston (20) observed on pyruvate carboxylation during thiamin deficiency may be attributed to the production of insufficient amounts of acetyl CoA from the oxidation of pyruvate.

Salicylates are known to inhibit glycogenesis (26) and amino-transferase enzymes which may lead to decreased gluconeogenesis (cf. Reference 27). Administration of salicylates to diabetic, adrenalectomized, and hypophysectomized animals resulted in a decrease in blood glucose levels (28-30). A depletion of muscle and liver glycogen in normal animals given salicylates was also reported (31, 32). It has been suggested that these effects of salicylates on carbohydrate metabolism are associated with stimulation of oxygen consumption (33-35), presumably through the uncoupling of oxidative phosphorylation (36).

This report reports the investigation on the sites and possible mode of action of aspirin. Thiamin-deficient animals were used in these studies as a model system since thiamin deficiency is known to cause well defined biochemical lesions of carbohydrate metabolism. A detailed study was conducted on basic mitochondrial processes, oxidative phosphorylation, tissue slices technique, metabolites levels, and enzymes activities of normal, thiamin deficient and aspirin treated rats.

EXPERIMENTAL

A. Methods

Animals and Diets

Male albino rats (Sherman strain) weighing 120-150 g kept in individual cages with wire-mesh screen bottoms, were used in all experiments. Deficient animals were fed a thiamin deficient diet ad libitum. Control animals were fed the same diet and were given thiamin hydrochloride (60 μg/100 g body wt /day). This dosage was found to be adequate for maintaining normal growth when the rats were fed ad libitum. For all experiments pair-fed controls were used to eliminate the effects of starvation. All animals had free access to tap water. The degree of thiamin deficiency was determined by growth rates and rectal temperature.

Preparation of Liver Homogenate

The liver was removed rapidly and one piece was suspended in 0.25 M sucrose, pH 7.2, containing reduced glutathione at a concentration of 1 mM and the other piece in 0.1 M Tris-buffer, pH 7.2, containing reduced glutathione and EDTA, both at a concentration of 1 mM. A 10% homogenate (w /v) was prepared using a Potter-Elvehjem homogenizer for 2 minutes at a setting of 70 on the variac. The homogenate in Tris-buffer was sonicated* for 90 seconds and centrifuged at 30,000 X g for 20 minutes. The activities of pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose-1,6-diphosphatase (EC 3.1.3.11), and glucose-6-phosphatase (EC 3.1.3.9) were determined in the supernatant. The homogenate in sucrose medium was

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*Branson Sonifier Model W-185D; Branson Sonic Power Co., Plainview, Long Island, N. Y.

centrifuged at 105,000 X g for 1 hour without prior sonication and the supernatant was used for determining the activities of malic enzyme (EC 1.1.1.40), total HMP shunt dehydrogenase (EC 1.1.1.49 and EC 1.1.1.44), isocitrate dehydrogenase (EC 1.1.1.42) and citrate cleavage enzyme (EC 4.1.3.6).

Enzyme Assays

Pyruvate carboxylase. Pyruvate carboxylase assay was essentially as described by Deodhan and Mistry (38). The reaction mixture contained 25 μmoles Tris-HCl, pH 7.4, 50 μmoles (2 μCi) $NaH^{14}CO_3$, 10 μmoles Na pyruvate, 2.5 μmoles ATP, 5 μmoles $MgCl_2$, 0.43 μmole CoA, 2.5 μmoles acetylphosphate, 2.5 units phosphotransacetylase, 0.3 unit citrate synthase and 0.1 ml twice diluted homogenate in a final volume of 1 ml. The mixture was incubated at 37° for 10 minutes and the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid. An aliquot of 0.05 ml of the supernatant was spotted on a filter paper strip, and the radioactivity was determined by suspending the dried strip in 15 ml of 0.5% PPO in toluene, using a liquid scintillation spectrophotometer. The values were corrected for $NaH^{14}CO_3$, fixed in the absence of the acetyl CoA generating system.

Phosphoenolpyruvate carboxykinase. The assay system, essentially as described by Sillero et al (39) contained 10 μmoles imidazole buffer, pH 7.0; 1.5 μmoles $MnSO_4$, 50 μmoles (5 μCi) $NaH^{14}CO_3$, 1.5 μmoles PEP, 2 μmoles ADP, 15 μmoles K-glutamate, 0.3 unit glutamate oxalacetate transaminase and 0.1 ml twice diluted supernatant in a final volume of 1 ml. The mixture was incubated at 30° for 7 minutes and the reaction was stopped by adding 0.1 ml 50% trichloroacetic acid. After centrifugation an aliquot of the supernatant was used to determine the radioactivity as described above. The control had no GDP in the system.