Differential Decrease of "Shuttle" Enzymes of Extramitochondrial $NADH_2$ Oxidation of Heart Muscle during Progressive Thiamine Deficiency[1,2] (34569)

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Oxidation of extramitochondrially generated $NADH_2$ may involve: (a) direct oxidation probably by passage through the mitochondrial membrane, (b) oxidation by pyruvate catalyzed by the cytoplasmic lactic dehydrogenase, and (c) oxidation by the α-glycerophosphate (α-GP), β-hydroxybutyrate (β-HB), and malate-oxaloacetate "shuttle" systems. However, liver mitochondria are virtually impermeable to $NADH_2$ and oxygen uptake is nil; hypotonic treatment brings about respiration indicating that the treatment establishes accessibility of $NADH_2$ to the respiratory chain (1, 2). On the other hand, mitochondria from heart muscle normally show oxygen uptake with $NADH_2$ (2–4) although hypotonic treatment substantially enhances oxidation of $NADH_2$ by both the respiratory chain (2) and by cytochrome *c* or dichlorophenolindophenol (3). While direct oxidation by means of the respiratory chain appears to be one pathway whereby extramitochondrial $NADH_2$ is oxidized in heart muscle, the α-GP and β-HB cycles may represent parallel pathways of $NADH_2$ oxidation (3, 5). There is evidence that the α-GP cycle is operative in skeletal and insect flight-muscle mitochondria (*e.g.*, 6).

Since progressive thiamine deficiency brings about gradual blockage at the level of pyruvic and α-ketoglutaric decarboxylase, a reasonable assumption is that this results in increased reliance on glycolytic ATP synthesis with simultaneous increase in the rate of reduction of extramitochondrial NAD. This, in turn, requires an increase in the rate of reoxidation of extramitochondrially generated $NADH_2$. The present study explores the changes in soluble cytoplasmic and mitochondrial particle-bound α-GP dehydrogenase (α-GPDH) as well as β-HB dehydrogenase (β-HBDH) activities during the time course of progressive thiamine deficiency.

Materials and Methods. Diets and isolation of mitochondria. Male Sprague-Dawley rats, weighing 320–370 g at the beginning of the experiment were used. Three groups were fed basal, thiamine-low (0.7 mg thiamine per kg diet) and thiamine-devoid (no thiamine) diets, respectively (3). The rats were sacrificed by decapitation, and the hearts were removed below the atria, rinsed with ice-cold physiological saline, blotted, and weighed. Mitochondria were isolated as previously described (7) except that the pH of the 0.44 *M* sucrose was adjusted to 6.8.

Measurements of enzyme activities. Mitochondrial α-GPDH. A modification of the indirect method of Henley *et al.* (8) was used: approximately 5 mg protein in 0.25 ml of mitochondrial suspension was incubated at 37° with 0.75 ml of 0.05 *M* triethanolamine buffer (pH 7.5) and 0.25 ml of 0.2 *M* α-GP (*dl*-α-GP disodium salt, Sigma), for 10 min. The reaction was stopped with 0.5 ml 0.6 *M* perchloric acid, the incubation mixture was shaken vigorously, then cooled, neutralized with 0.6 *N* KOH, and centrifuged. Dihydroxyacetone phosphate (DHAP) in the supernatant fluid was assayed enzymatically as follows: 0.3 ml of supernatant fluid was mixed in the cuvette with 1.7 ml of 0.05 *M* triethanolamine buffer (pH 7.5) and 254 μmoles $NADH_2$ (Sigma) in 0.1 ml. The reaction mixture was read at 340 mμ in a Beckman

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DB spectrophotometer against a blank containing all cofactors except $NADH_2$. After the initial reading, 0.86 IU of α-GPDH (Calbiochem) in 0.1 ml was added. The cuvette was kept at room temperature for final reading after which no further change in optical density occurred (approximately 10–15 min). A control containing all cofactors except α-GP was used. Any change in optical density in the control was subtracted from the experimental.

Extramitochondrial α-GPDH. The postmitochondrial fraction was centrifuged at 105,000*g* for 1 hr to remove the microsomes. The supernatant fluid was used for the determination of the soluble α-GPHD following a modification of the procedure of Baronowski (9). The reaction mixture, before the addition of substrate, contained 1.8 ml 0.05 *M* triethanolamine buffer (pH 7.5), approximately 1 mg protein in 0.2 ml myocardial supernatant fluid and 508 μmoles $NADH_2$ in 0.2 ml. The cuvette was kept at room temperature until no decrease in optical density at 340 mμ could be observed (approximately 10 min). One tenth milliliter of 2.5×10^{-3} *M* sodium salt of DHAP (obtained by hydrolysis from the dicyclohexylamine salt of dimethylketal monohydrate; Calbiochem.), was then added. Oxidation of $NADH_2$ was followed at 340 mμ at 10-sec intervals for 2 min.

Mitochondrial β-HBDH. Activity of this enzyme was determined exactly following the method of Jurtshuk *et al.* (10), using the variation with "Asolectin" phospholipid (Associated Concentrates Inc., Long Island, N. Y.). Protein content in the mitochondrial suspension and myocardial supernatant fluid were determined following Lowry (11).

The control enzyme activities represent the overall averages of 10 groups of 3 determinations on individual animals, fed basal diet from 1 through 10 weeks. Enzyme activities of each experimental group represent averages of four to six rats on thiamine-devoid diet and three to five rats on thiamine-low diet.

Results and Discussion. The results of these studies are summarized in Table I. The enzyme activities of the controls represent the overall average of normal animals throughout the time course of the experiment. This average was used because for none of the three enzyme activities is there any significant change during the time course of the 10 weeks. The probabilities of significance of difference of the experimental animals were calculated against these averaged control values. However, results not given here indicate that comparison of the experimental animals with controls maintained on basal diet for the same lengths of time give identical probability values.

Eys (12) reported earlier that thiamine-deficient rats show a decrease of α-GPDH activity in both liver and skeletal muscle. On the basis of the present findings with heart muscle (Table I), the decrease observed by Eys is probably due to a differential decrease of only the particle-bound, mitochondrial α-GPDH. It is to be noted that mitochondrial α-GPDH activity remains unchanged during the initial stage of thiamine deficiency in both thiamine-low and thiamine-devoid rat hearts, and significant decrease of activity begins beyond 7 weeks with the thiamine-low diet and beyond 2 weeks with the thiamine-devoid diet. Also, in the thiamine-devoid rats this enzyme activity decreased to a greater extent than in thiamine-low rats.

An electron microscopic study (to be published elsewhere) indicates that progressive thiamine deficiency brings about severe alterations of the mitochondria. There is irregularity of the orientation of the cristae and decrease of their number. In the terminal phase many mitochondria are enlarged and tightly packed together; some mitochondria fuse to giant structures (cf. 13). Since the mitochondrial α-GPDH is localized in the mitochondrial membrane, the decrease of this enzyme activity during progressive thiamine deficiency appears to correlate with the alteration of mitochondrial ultrastructure. In contrast to the particle-bound α-GPDH, the extramitochondrial α-GPDH showed an increase in the initial stage of deficiency with both diets, then activity gradually returned to normal and remained at the control level up to the end of the experiment. Although the signifi-

TABLE I. Change in α-Glycerophosphate and β-Hydroxybutyrate Dehydrogenase Activities in the Rat Heart During Progressive Thiamine Deficiency.[a]

	Weeks of feeding	Mitochondrial α-GPDH (mμmoles DHAP formed/mg protein/min)		Extramitochondrial α-GPDH (mμmoles $NADH_2$ oxidized/mg supernatant protein/min)		Mitochondrial β-HBDH (mμmoles NAD reduced/mg protein/min)	
Control	0	18.2 ± 0.44		88.8 ± 2.19		48.2 ± 1.27	
Thiamine-devoid diet	2	19.1 ± 1.27	$0.60 > p > 0.50$	116.3 ± 3.82	$p < 0.001$	50.9 ± 2.98	$0.50 > p > 0.40$
	4	12.7 ± 0.88	$p < 0.001$	99.8 ± 4.77	$0.05 > p > 0.02$	51.9 ± 2.64	$0.30 > p > 0.20$
	5	11.7 ± 0.60	$p < 0.001$	88.9 ± 2.04	$p > 0.90$	57.1 ± 3.94	$0.05 > p > 0.02$
Thiamine-low diet	1	20.0 ± 1.50	$0.30 > p > 0.20$	103.9 ± 5.25	$p < 0.001$	50.7 ± 5.43	$0.70 > p > 0.60$
	2	21.0 ± 2.08	$0.20 > p > 0.10$	93.9 ± 5.18	$0.40 > p > 0.30$	45.1 ± 5.88	$0.70 > p > 0.60$
	3	21.0 ± 1.55	$0.20 > p > 0.10$	86.7 ± 7.40	$0.80 > p > 0.70$	51.5 ± 2.72	$0.30 > p > 0.20$
	4	17.9 ± 2.62	$p > 0.90$	78.7 ± 5.07	$0.10 > p > 0.05$	41.7 ± 3.53	$0.10 > p > 0.05$
	5	18.4 ± 2.10	$p > 0.90$	90.8 ± 9.69	$0.90 > p > 0.80$	52.8 ± 3.12	$0.20 > p > 0.10$
	6	16.7 ± 1.75	$0.50 > p > 0.40$	93.6 ±11.51	$0.70 > p > 0.60$	48.9 ± 3.52	$0.90 > p > 0.80$
	7	18.9 ± 0.35	$0.30 > p > 0.20$	78.9 ± 3.29	$0.05 > p > 0.02$	43.7 ± 4.50	$0.40 > p > 0.30$
	8	14.3 ± 0.59	$p < 0.001$	93.2 ± 7.73	$0.60 > p > 0.50$	40.5 ± 4.87	$0.20 > p > 0.10$
	9	13.6 ± 0.81	$p < 0.001$	84.6 ± 2.19	$0.20 > p > 0.10$	46.4 ± 3.59	$0.70 > p > 0.60$
	10	14.2 ± 0.53	$p < 0.001$	89.6 ± 5.02	$0.90 > p > 0.80$	47.8 ± 3.14	$p > 0.90$

[a] Activities were calculated from the rate curves by means of a $NADH_2$ reference curve.

cance of this result is not clear, it may represent a compensatory response affecting the rate of oxidation of extramitochondrial $NADH_2$.

Hydroxybutyrate dehydrogenase activity was found only in the mitochondria, not in the supernatant fluid. There was no significant difference in this activity between the deficient and control rat heart mitochondria during the whole time course, except for a small increase at the fifth week with the thiamine-devoid diet. This increase in activity could be a result of increase of accessibility to the substrate across the mitochondrial membrane in severe deficiency (cf. 3), since, surprisingly, the change of membrane structure even in the terminal stage of thiamine deficiency does not seem to affect β-HBDH activity itself. It is known that this membrane-bound enzyme is relatively stable in the presence of thiols even after sonication or digitonin treatment of the mitochondria (14).

Since α-GP (and also β-HB) strongly stimulate mitochondrial $NADH_2$-cytochrome *c* reductase activity between 2 and 6 weeks of thiamine deficiency (3), it is unexpected that neither of the two α-GP dehydrogenases showed an increase in this period of deficiency. It is of interest in this connection that exercising fails to enhance either the cytoplasmic or the mitochondrial α-GPDH activity in skeletal muscle (15) although the levels of respiratory chain enzymes are increased (16). It has been suggested that a possible physiological role of the outer membrane-localized $NADH_2$ oxidase(s) in the mitochondria could be to serve as intermediate(s) for transferring reducing equivalents from α-GPDH to the respiratory chain (17, 18). Accordingly, α-GPDH would represent only the initial segment of α-GP oxidase, and it is possible that α-GPDH is not the rate-determining step in overall α-GP oxidase activity in the mitochondria. For these reasons the rates of α-GP-supported mitochondrial oxygen uptake and α-GP cytochrome *c* reductase activity are now being studied at intervals of thiamine deficiency.

Preliminary results indicate that α-GP "shuttle" activity is operative in our system. In these experiments, patterned essentially on those of Boxer and Devlin (2), mitochondrial oxidation of $NADH_2$ was measured with a Clark electrode in the presence of ADP, and the effect of DHAP on the respiratory rate was measured. Respiration was found to be notably stimulated by DHAP when the experiment was carried out in the absence of EDTA, but stimulation by DHAP was blocked when EDTA (a competitive inhibitor of mitochondrial α-GPDH) was added.

Summary. In spite of the apparent functioning of the α-glycerophosphate "shuttle" in heart muscle, progressive thiamine deficiency does not bring about an expected compensatory increase in the soluble cytoplasmic and the mitochondrial particle-bound α-glycerophosphate dehydrogenases. This was suggested by the previous finding that α-glycerophosphate (and also β-hydroxybutyrate) strongly stimulate mitochondrial $NADH_2$-cytochrome *c* reductase between 2 and 6 weeks of thiamine deficiency. Although the soluble cytoplasmic α-glycerophosphate dehydrogenase shows a temporary increase at the beginning of thiamine deficiency, it returns immediately to and then remains at the control level up to death. On the other hand, the level of the particle-bound enzyme gradually decreases, the onset of decrease depending on the severity of thiamine deficiency. Except for a small increase at the terminal stage of severe thiamine deficiency, mitochondrial β-hydroxybutyrate dehydrogenase level remains constant throughout the feeding of thiamine-deficient diets.

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