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Branched chain amino acids
maple syrup urine disease
mitochondrial membranes
thiamine
vitamin responsiveness

In Vivo and *in Vitro* Response of Human Branched Chain α-Ketoacid Dehydrogenase to Thiamine and Thiamine Pyrophosphate

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Summary

In a homozygous affected patient with maple syrup urine disease, pharmacologic doses of thiamine lowered urinary excretion of branched chain α-ketoacids and stimulated branched chain α-ketoacid dehydrogenase (BCKAD) in his peripheral blood leukocytes. Supplementation of his branched chain aminoacid restricted diet with 100 mg/day of thiamine eliminated recurrent episodes of ketoacidosis. These clinical responses were studied *in vitro* using mitochondrial inner membranes prepared from his cultured skin fibroblasts and those from another thiamine-responsive patient from Canada. BCKAD in both mutant cell lines had similarities to normal enzyme including: identical apparent K_m value for thiamine pyrophosphate; similar heat inactivation profiles which were slowed by the presence of thiamine pyrophosphate; and stimulation above basal activity by thiamine pyrophosphate. Differences in the enzymes included: decreased apparent V_{max} for thiamine pyrophosphate; increased lability at 37°; and failure to respond to added NAD^+, CoASH, and Mg^{2+}.

We propose that "excess" thiamine led to increased available thiamine pyrophosphate which stabilized the branched chain α-ketoacid dehydrogenase, decreased biologic turnover, increased enzyme specific activity and produced *in vivo* tolerance to branched chain aminoacids in these patients with maple syrup urine disease.

Speculation

By studying the partially purified normal and mutant branched chain α-ketoacid dehydrogenases from cultured human fibroblasts, direct *in vitro* effects of thiamine pyrophosphate can be measured and related to *in vivo* clinical responses. This should improve and extend the treatment and management of patients with maple syrup urine disease and provide a method for study of other mutant human enzymes located in the mitochondrial membrane.

INTRODUCTION

Thiamine responsive maple syrup urine disease (MSUD) was first described in 1971. Oral loading with vitamin B_1 decreased previously elevated plasma branched chain amino acid concentrations in a presumably homozygous affected Canadian female child (22). In a preliminary study we reported a similar effect from high doses of oral thiamine on two homozygous affected brothers (6). MSUD results from a specific decrease in the activity of branched chain α-ketoacid dehydrogenase (BCKAD) which oxidatively decarboxylates α-ketoisocaproate (KIC), α-keto-ß-methylvalerate (KMV) and α-ketoisovalerate (KIV), the transaminated products of leucine, isoleucine and valine respectively (9,25). BCKAD is thought to be a multienzyme complex composed of a decarboxylase, a transacylase and lipoamide oxidoreductase in which thiamine pyrophosphate (TPP) is a cofactor for the first component (5,24). Intracellular concentration of this cofactor can most likely be increased by oral loading since thiamine kinase is active in the intestine and most other tissues of man (15, 17,18,23).

Vitamin responsive inborn errors of metabolism have been recognized for a number of years and our understanding of the mechanism by which these responses are realized continues to advance (1,12,13,16,19,20,22). Classically, holoenzyme function was augmented by mass action through increased coenzyme production and binding to apoenzyme. In a more recently postulated mechanism, the presence of "excess" coenzyme decreased the degradation of holoenzyme (12,13). This mechanism was invoked as the means by which TPP improved BCKAD activity (4,6). Supraphysiologic ingestion of thiamine increased BCKAD activity in normal adult liver by 2 fold, but required three weeks, the approximate biological half life of mitochondrial turnover (4). Because BCKAD is expressed incultured human skin fibroblasts, and a decreased activity is observed in cells from MSUD patients, this tissue was used to study in vitro the effect of TPP (2-6,9). Here we report the effect of oral loading with thiamine on urine concentrations of the branched chain α-ketoacids and on enzyme function in isolated white blood cells from a single homozygous affected patient. We also report studies on the effect of TPP on BCKAD activity in isolated mitochondrial inner membranes from fibroblasts cultured from this thiamine responsive patient, the Canadian patient and normal controls.

METHODS AND MATERIALS

Following detailed description of the research project and obtaining informed parental consent, a homozygous affected MSUD patient was admitted to the Clinical Research Facility at Emory University where continuous supervision of his diet was possible. This patient's synthetic diet was restricted in branched chain amino acids and supplemented with Recommended Dietary Allowances of all vitamins including 5 mg/day of thiamine. After 1 week of stabilization, the study was begun. After no added thiamine, the diet was then supplemented with 50, 100 and 150 mg of thiamine-HCl, respectively for each of three succeeding weeks. A 24 hour urine was collected on day 1,3, and 7, and 10 ml of heparinized blood was collected after day 3 of each of four weekly periods. White blood cells were prepared from the unclotted blood using the polyvinyl pyrollidone method (20). These cells were used fresh for assay of the BCKAD activity.

Punch skin biopsy was used to obtain a primary culture of fibroblasts from this patient (Mutant A). Fibroblasts cultured from the previously reported thiamine responsive patient were purchased from The Repository for Human Mutant Cell Strains in Canada (Mutant B). Cells were grown in 690 cm^2 Bellco roller bottles using Dulbecco-Vogt medium supplemented with 15% fetal calf serum. Cells were harvested when confluent, usually 7 days, by treatment with 0.25% trypsin for 30 minutes at 37°. Three to four roller bottles yielded between 0.75 and 1.50 g wet weight of cells. The cells were then suspended in 0.27M mannitol buffered with 10 mM Tris-HCl, at pH 7.4 and made 0.1 mM with respect to EDTA, hence MTE buffer, to a final concentration of 400 mg wet weight per 10 ml buffer. Protease VI was added at 2.5 µg/40 mg wet weight and allowed to react for 7 minutes at 0°. At the end of this incubation, the cells were homogenized with a glass-Teflon grinder and the volume doubled with MTE buffer. Centrifugation at 700 xg for 10 minutes separated undisrupted cells and nuclei from the mitochondria-containing supernatant. Mitochondria were pelleted by centrifugation of this supernatant at 10,000 xg for 10 minutes. Mitochondria prepared by this method showed coupled respiration in the presence of succinate and ADP (5). This mitochondria rich pellet was treated with 0.5 µg digitonin per mg mitochondrial protein for 20 minutes at 0°. Detergent action was stopped by the addition of MTE buffer equal to the suspending volume and inner membrane vesicles pelleted by centrifugation at 10,000 xg for 15 minutes. This inner membrane rich pellet was suspended in 30 mM K_xPO_4 buffer pH 7.2 to a concentration of 80 to 150 µg protein per ml for use as the enzyme source (5,21).

ASSAY

Urinary ketoacid concentrations were determined as their methyl derivatives by gas chromatography (7). Enzyme activity was assessed by liquid scintillation of $^{14}CO_2$ released from the 1-^{14}C labeled ketoacid prepared from the 1-^{14}C labeled precursor amino acid as previously described (8). Incubation at 37° was for the indicated times in the legends to figures and tables.

HEAT INACTIVATION

Aliquots of isolated mitochondrial inner membranes were placed in a water bath at the indicated temperature for the appropriate time, with intermittant shaking to prevent superheating. The samples were then placed on ice until assay. Cofactors were present or absent as indicated. A minimum of 100 µg of protein were used for aliquots. Protein was determined by the method of Lowry using bovine serum albumin as a standard (14).

Data analyses were aided by computer programs. All reagents were of highest purity available and made up in deionized water.

RESULTS

In order to evaluate the biochemical response of MSUD patients to oral loading with thiamine, urinary output of the α-ketoacids was monitored along with BCKAD activity in isolated white blood cells. Figure 1 summarizes the in vivo response. The upper panel demonstrates reduction in urinary α-ketoacid excretion with increased oral thiamine. On a branched chain amino acid restricted diet at 1.25 gm protein per pound body weight, the patient excreted 199 ± 80 and 194 ± 18 mg per 24 hr of α-ketoisocaproic and α-keto-ß-methylvaleric acids respectively. After 3 weeks of supplemental thiamine these values had fallen to 34 ± 19 and 42 ± 18 mg per 24 hr. The lower panel represents the effect of oral thiamine on peripheral leukocyte BCKAD. To normalize the enzyme data, a single control individual was used who was maintained on Recommended Dietary Allowance doses of thiamine and a normal diet. Before thiamine administration, valine, leucine, and isoleucine decarboxylation was 4.7, 2.3, and 5.5% of control, respectively. After thiamine these values rose to 25.7, 16.6 and 23.0% of the same control.

The patient was dismissed from the facility and instructed to continue daily oral doses of 100 mg thiamine. After three years on thiamine therapy, the patient was again admitted to the Clinical Research Facility for reevaluation. Thiamine above Recommended Dietary Allowance level was removed from his diet for three weeks and urinary branched chain α-ketoacid excretion and peripheral white blood cell BCKAD activity was assayed. Both 24 hr urinary excretion and BCKAD activity on admission was the same as day 28 of the initial study and remained at this level throughout the three week test period. The patient's monthly ketoacidosis of unknown cause disappeared and for six years the patient has remained stable on the restrictive diet supplemented with thiamine at 100 mg/day and has required no further hospitalizations for treatment of metabolic acidosis.

Since the activity of the dehydrogenase was reflected in cultured skin fibroblasts, this tissue was used to investigate the mechanisms producing the observed clinical responses to high doses of thiamine. Isolated inner mitochondrial membranes provided a partially purified enzyme preparation which eliminated complicating variables in branched chain α-ketoacid decarboxylation such as membrane transport of substrates and cofactors and transamination of aminoacid precursors. Data of Johnson and Connelly with beef liver mitochondria suggested that the branched chain α-ketoacid dehydrogenase was localized on the outside of the inner membranes, facing the intramitochondrial space (11). When the outer membrane was removed by digitonin treatment, properly oriented inner membranes were obtained (21). Thus, the enzyme was made directly accessible to the substrate and cofactors.

When these membrane preparations were incubated at 37° without exogenous cofactors followed by a 15 minute assay, $^{14}CO_2$ release from the α-ketoacids became dependent upon the addition of cofactors during the assay. The most important cofactor to eliminate was thiamine pyrophosphate since the fall to baseline activity did not occur if this cofactor alone were added to the buffer (5). The left panel in figure 2 shows that BCKAD from normal fibroblasts fell to basal activity after 90 minutes. Without exogenous cofactors during the assay, activity was only 3% of that found after 5 minutes of incubation. The 5 minute time point reflects the temperature equilibration period used in all experiments and was used as our zero time point. When all four cofactors were present during the 15 minute assay, full restoration of activity was possible. The right panel describes a similar effect with mutant enzyme. In contrast to normal enzyme, after 90 minutes it was only possible to restore 34% of the mutant enzyme activity by the addition of cofactors. Full restoration of CO_2 release was possible at the 30 minute time point with these preparations from the mutant. Thus, the mutant enzyme complex appeared more sensitive to increasing duration of preincubation reflecting an increased lability at 37°.

Cofactors were then tested singly and in combination for their ability to restore decarboxylating activity to the proteins in inner mitochondrial membranes. All possible combinations were tested but only the results of the most pertinent data are reported in Table I. Ninety minutes of incubation was used to lower CO_2 release to basal level. Again, note that the absence of thiamine pyrophosphate during this preincubation was necessary to attain basal activity (5). When NAD^+, CoASH and Mg^{2+} were present during preincubation and assay, BCKAD from normal cells was maintained at 18 fold above basal level. This was not seen for enzyme from the mutant cells. When thiamine pyrophosphate was added along with the other cofactors during the assay period, an additional 3 fold stimulation was observed with normal enzyme and a similar effect was now seen with BCKAD from the mutant cells. Identical results were seen with mutant A, data not shown.

The response to added TPP after 90 minutes incubation without cofactors made it possible to compare affinity constants for thiamine pyrophosphate in the mitochondrial inner membrane preparations from mutant and normal cell lines. Double reciprocal plots of velocity versus thiamine pyrophosphate concentrations with saturating concentrations of KIC, NAD^+, CoASH and Mg^{2+} showed a common apparent K_m value of 1.6 µM, figure 3. Apparent V_m values were 2632 and 1053 pmoles $^{14}CO_2$/mg protein/15 minutes for normal cell BCKADs and 400 and 180 pmoles $^{14}CO_2$/mg protein/15 minutes for mutants B and A respectively. These findings indicated that both mutants had a decreased apparent V_m for thiamine pyrophosphate and the two fold higher overall activity expressed by the Canadian patient (Mutant B) as compared to the Georgian (Mutant A) might reflect their clinical findings of a more benign expression for the Canadian patient when compared to the Georgian.

Heat inactivation can often distinguish a mutant enzyme from its normal counterpart. This was not true in our inner membrane preparations. When denaturation at 50° with time was measured, identical rates of inactivation were observed in the mutant and normal membrane preparations (Figure 4). Of interest, however, was the stabilizing effect of thiamine pyrophosphate on these rates of inactivation. When thiamine pyrophosphate was included during the heating process, the inactivation rate was retarded in a similar manner for normal and mutant. $T_{\frac{1}{2}}$ for normal BCKAD went from 5min without thiamine pyrophosphate to 10 min in the presence of thiamine pyrophosphate and for mutant BCKAD the respective times were 6 and 12 min.

DISCUSSION

These data explore the mechanism of vitamin response and expand an earlier report from Canada about a thiamine-responsive patient with MSUD who had decreased plasma branched chain amino acids in response to high oral doses of thiamine (22). A response to thiamine in pharmacological doses was characterized by both lowering of urinary branched chain α-ketoacids and an increase in BCKAD activity in isolated white cells. Initially, the patient's condition was controlled by dietary restriction of the branched chain amino acids which maintained urinary ketoacid excretion at approximately 200 mg/24 hour (days 0-7, figure 1). After three weeks of added thiamine, the output of these ketoacids was further reduced and has remained at these lowered levels for six years. In addition, the specific activity of his peripheral leukocytes' BCKAD increased at least 5 fold during this treatment period and suggested that improved enzyme function was the direct effect of pharmacological doses of thiamine. It was found previously that the specific activity of normal adult liver BCKAD increased after three weeks of oral loading with thiamine (4). A clinical hypothesis to correlate these findings suggests that greater intake of thiamine produced higher intracellular concentrations of thiamine pyrophosphate, which increased the biological half-life of mitochondrial branched chain α-ketoacid dehydrogenase. Acute effects of high-dose vitamin administration and removal would not be expected by this hypothetical mechanisim. This, in fact, was seen in the Georgia patient. Additionally, removal of high levels of thiamine from this patient's diet did not result in increased urinary ketoacid levels and decreased BCKAD activity in white blood cells to pretreatment levels within three subsequent weeks of observation.

Direct biochemical data to explain the in vivo effect of high thiamine was sought by analysis of normal and mutant BCKAD partially purified but

bound to inner mitochondrial membranes of cultured human skin fibroblasts. Mitochondrial inner membranes prepared by treatment with digitonin resulted in a 5 fold purification of BCKAD and eliminated the need for transmembrane transport and conversion of precursors to cofactors and substrates before catalysis (5,21). The use of these organelles as the enzyme source also minimized contamination by other catabolic reactions which might compete for the exogenous cofactors and substrate.

Despite marked reduction in overall activity, BCKAD prepared in this way from MSUD mutant cells had several similar physical characteristics when compared to enzyme prepared from cells with normal BCKAD activity. Decarboxylation of the branched chain α-ketoacids by both normal and mutant enzymes was reduced to basal level by incubation for 90 minutes without exogenous cofactors. Addition of TPP during this period prevented this fall in activity (5). Addition of TPP to both preparations at basal levels produced a three fold stimulation in CO_2 release in the presence of NAD^+, CoASH and Mg^{2+} suggesting similar cofactor requirements. Inactivation of both normal and mutant BCKAD occurred within 15 minutes at 50°. This rate was slowed and $T_{\frac{1}{2}}$ doubled by including thiamine pyrophosphate during heating in both the normal and mutant preparations indicating that thiamine pyrophosphate produced thermostability. Finally, an apparent K_m value of 1.6 μM for thiamine pyrophosphate binding in BCKAD was identical for the enzyme from mutant and normal cells indicating a similar affinity for this cofactor.

Important differences were found for the BCKAD from these mutants as compared to the enzyme from control cells. The maximal rate of CO_2 release at saturating concentrations of thiamine pyrophosphate (V_m) was greatly reduced for the two mutant enzymes studied. Since binding for thiamine pyrophosphate was normal (K_m) we suggest that the first protein in the complex, decarboxylase, functions normally in substrate and cofactor binding. Thus, decreased activity would have to be due to an abnormality in association of the component proteins, an abnormal catalytic site in one of the other two components or decreased number of functioning decarboxylase subunits. The concept of unstable protein-protein interaction is supported by the fact that the normal BCKAD could be reconstituted by the addition of NAD^+, CoASH and Mg^{2+} without thiamine pyrophosphate while both mutant complexes were unresponsive to this treatment (Table 1). Differences in V_m between the mutants were reflected in the in vivo study where it was reported that the Canadian patient required only 10 mg thiamine per day for an acute lowering of plasma branched chain aminoacids while in this study 100 mg thiamine/day was determined to be necessary and required three weeks for maximal effect. It has also been reported that the Canadian patient had 40% of normal BCKAD activity with 0.1 mM isoleucine but approached normal with 5 mM substrate (22). Enzyme activity from the Georgia patient has never exceeded 25% of control. This suggests that different mutations are responsible for these two MSUD phenotypes.

These results do not provide clear, simple answers to the mechanism by which thiamine was able to improve the phenotypic expression of MSUD in these two patients. They do suggest that the mutation does not involve the transmembrane transport of thiamine, its conversion to thiamine pyrophosphate or its binding to the decarboxylase protein. Several other possibilities for the thiamine effect exist. We know that thiamine pyrophosphate functions as the acceptor molecule for the acyl moity (24). This primary role may be limited in a mutant multienzyme complex. A secondary role for thiamine pyrophosphate might be to aid in the proper alignment of component proteins. This role was demonstrated for thiamine pyrophosphate with bacterial pyruvate decarboxylase (10). Since BCKAD from mutants A & B had a decreased activity (V_m) at saturating levels of thiamine pyrophosphate the larger amounts of this cofactor could be necessary for optimum component association. Thus, when thiamine pyrophosphate was present in quantity sufficient for total binding, then proper association might result and lead to a more stable complex better able to catalyze this reaction. This postulate is supported by the fact that mutant enzyme was more labile at 37° and thiamine pyrophosphate stabilized both normal and mutant BCKAD at both 37° and 50°. A stabilization effect of B_6 on mutant cystathionine β-synthase has also been proposed (12,13).

More direct answers concerning the specific mutant gene product may be obtained through immunologic studies. Currently, normal mammalian BCKAD is being purified and antibodies raised to its component proteins. Inner mitochondrial membranes from MSUD mutant fibroblasts will then be studied by immuno-histochemical methods in an attempt to specifically identify the abnormal protein and record more precisely the effects of thiamine pyrophosphate on the biological rates of protein degradation.

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26. Informed consent was obtained from parents of all children used in this study.

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TABLE I. REQUIREMENT FOR TPP TO RESTORE BRANCHED CHAIN α-KETOACID DEHYDROGENASE ACTIVITY TO MITOCHONDRIAL INNER MEMBRANES FROM CULTURED SKIN FIBROBLASTS

CONDITIONS OF 90 MIN. PREINCUBATION	CONDITIONS OF 15 MIN. ASSAY	CONTROL	MUTANT (B)
		P MOLES $^{14}CO_2$/MG PROTEIN/15'	
NO ADDITIONS	NO ADDITIONS	53.13±12.61 (4)[b]	99.35±69.34 (4)
NAD^+ CoASH[a] Mg^{2+}	NAD^+ CoASH[a] Mg^{2+}	971.91±64.12 (5)	78.13±17.09 (3)
NAD^+ CoASH[a] Mg^{2+}	TPP[a] NAD^+ CoASH Mg^{2+}	2564.45±74.63 (3)	266.75± 9.82 (3)

[a] Cofactors were present at a final concentration of 0.2 mM; 0.1 mM 1-^{14}C-α-ketoisocaproate as substrate.

[b] Average ± SEM for number of determination indicated in parentheses.

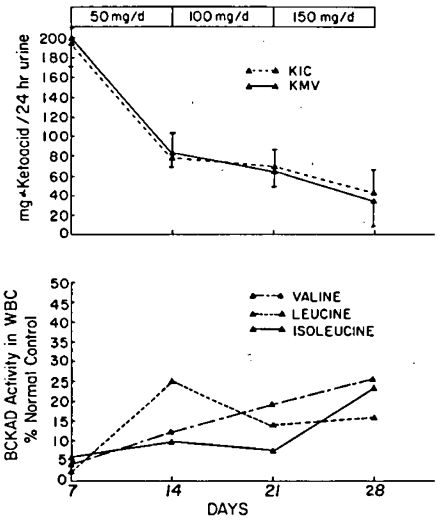

FIGURE 1 In vivo response of patient to oral loading with thiamine-HCl. Upper panel demonstrates the fall in urinary α-ketoacids during the test period. Values represent the average of 4 determinations ± SEM. KIC = α-ketoisocaproate, KMV = α-keto-β-methylvalerate. The lower panel represents the concomitant increase in branched chain α-ketoacid dehydrogenase (BCKAD) activity in isolated white blood cells during this same period. The values are an average of 4 determinations expressed as percent of activity in white blood cells from the same unaffected adult control prepared and assayed on the same day. Assay is described in Methods and Materials.

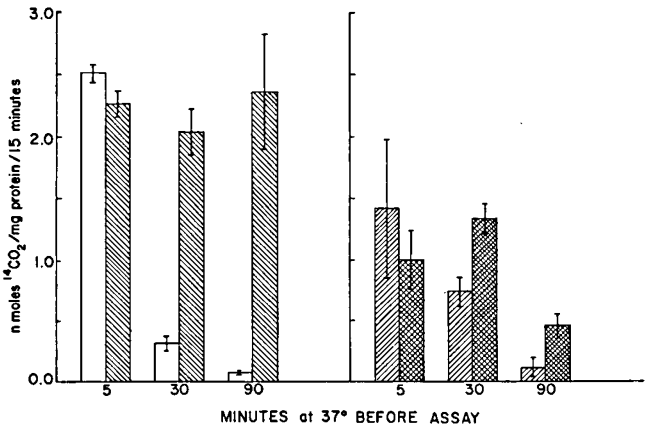

FIGURE 2 Reconstitution of branched chain α-ketoacid dehydrogenase activity with mitochondrial inner membranes from control □, and mutant B ▨ fibroblasts. ▧ represents assay in the presence of exogenous cofactors, during 15 minute assay. Data is the average of 6-8 determinations ± SEM. Assay is as described in Methods and Materials, 0.1 mM 1-^{14}C-α-ketoisocaproate served as substrate.

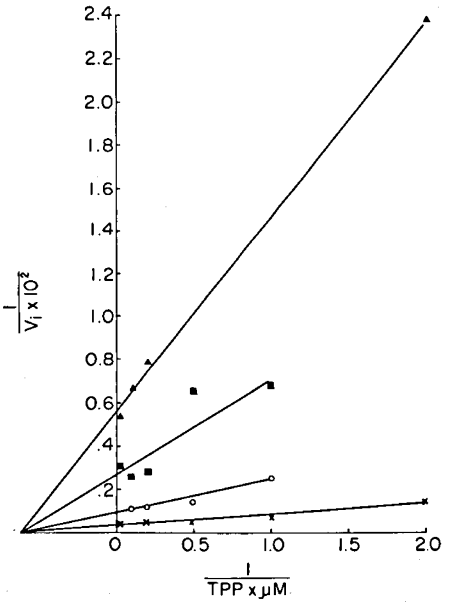

FIGURE 3 Lineweaver Burk plot of activity vs TPP concentration for branched chain α-ketoacid dehydrogenase in mitochondrial inner membranes isolated from cultured skin fibroblasts. Tissue preparations were preincubated in 30 mM K_xPO_4, pH 7.2 buffer for 90 minutes. Reactions were initiated by addition of 1-^{14}C-α-ketoisocaproate at 0.1 mM and CoASH, NAD^+, Mg^{2+} at 0.2 mM. TPP was present at the indicated concentrations and incubation was for 15 minutes. Data represent the average of 4-8 determinations for each point. ▲—▲, ■—■ = mutant A & B, respectively; O—O, X—X = control.

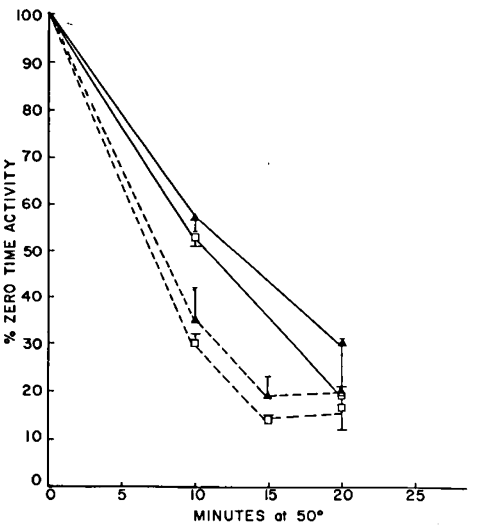

FIGURE 4 Effect of heating mitochondrial inner membranes at 50° on branched chain α-ketoacid dehydrogenase in the absence (- - -) and presence (——) of 0.2 mM thiamine pyrophosphate. Assay was for 15 minutes as described in text with all cofactors present during assay. 0.1 mM 1-^{14}C-α-keto-isocaproate served as substrate. Data is the average of 4 determinations and expressed as % of unheated sample + or - SEM. □ = Control; ◬ = Mutant A.

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