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CORRELATION OF ENZYMATIC, METABOLIC, AND BEHAVIORAL DEFICITS IN THIAMIN DEFICIENCY AND ITS REVERSAL*

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To clarify the enzymatic mechanisms of brain damage in thiamin deficiency, glucose oxidation, acetylcholine synthesis, and the activities of the three major thiamin pyrophosphate (TPP) dependent brain enzymes were compared in untreated controls, in symptomatic pyrithiamin-induced thiamin-deficient rats, and in animals in which the symptoms had been reversed by treatment with thiamin. Although brain slices from symptomatic animals produced $^{14}CO_2$ and ^{14}C-acetylcholine from [U-^{14}C]glucose at rates similar to controls under resting conditions, their K^+-induced-increase declined by 50 and 75%, respectively. In brain homogenates from these same animals, the activities of two TPP-dependent enzymes transketolase (EC 2.2.1.1) and 2-oxoglutarate dehydrogenase complex (EC 1.2.4.2, EC 2.3.1.61, EC 1.6.4.3) decreased 60–65% and 36%, respectively. The activity of the third TPP-dependent enzyme, pyruvate dehydrogenase complex (EC 1.2.4.1, EC 2.3.1.12, EC 1.6.4.3) did not change nor did the activity of its activator pyruvate dehydrogenase phosphate phosphatase (EC 3.1.3.43). Although treatment with thiamin for seven days reversed the neurological symptoms and restored glucose oxidation, acetylcholine synthesis and 2-oxoglutarate dehydrogenase activity to normal, transketolase activity remained 30–32% lower than controls. The activities of other TPP-independent enzymes (hexokinase, phosphofructokinase, and glutamate dehydrogenase) were normal in both deficient and reversed animals.

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Thus, changes in the neurological signs during pyrithiamin-induced thiamin deficiency and in recovery paralleled the reversible damage to a mitochondrial enzyme and impairment of glucose oxidation and acetylcholine synthesis. A more sustained deficit in the pentose pathway enzyme, transketolase, may relate to the anatomical abnormalities that accompany thiamin deficiency.

INTRODUCTION

The mechanisms of neurological damage in thiamin deficiency have been intensively studied since the discovery in the early 1900's that a trace constituent in rice husks cured beri-beri (1). The decreases in glucose and pyruvate oxidation in thiamin deficient brain, that were first demonstrated in 1929 (2, for review see 3, 4), have been confirmed repeatedly. Furthermore, the alterations in glucose utilization parallel the characteristic anatomical lesions that accompany thiamin deficiency (5). However, their relation to the pathophysiology of thiamin deficiency is unknown.

The deficits in oxidative metabolism may subsequently inhibit acetylcholine synthesis. Impaired acetylcholine synthesis was first demonstrated by Quastel and coworkers (6) and has been confirmed by several groups (for references, see 3). More recent studies indicate that the cholinergic deficit occurs early in thiamin deficiency and is functionally significant (7–10).

The enzymatic basis of the deficits in oxidative metabolism during thiamin deficiency is controversial. Activities of the pyruvate dehydrogenase complex (PDHC; EC 1.2.4.1, EC 2.3.1.12, EC 1.6.4.3), 2-oxoglutarate dehydrogenase complex (KGDHC; EC 1.2.4.2, EC 2.3.1.61, EC 1.6.4.3), and transketolase (EC 2.2.1.1), the three major thiamin-pyrophosphate (TPP)-dependent enzymes of mammalian brain, have been reported to decline or be unchanged at various stages of thiamin deficiency (for reference see 3, 4). However, activities have typically been measured with assays that have maximal activities 2- to 10-fold less than newer methods (11–13), and have often failed to distinguish between enzyme activities and pathways in which the enzyme takes part. For example, the earlier reports of defects of pyruvate "oxidase" (1) or in PDHC (14–16) were based on preparations in which mitochondria and synaptosomes were largely intact; pyruvate utilization under those circumstances may reflect overall mitochondrial oxidative activity rather than the activity of a particular enzyme.

The relationship between the enzymatic deficiencies and the deficits in glucose oxidation, neurotransmitter synthesis, neurological function, and anatomic lesions remains unclear. Therefore, we have examined brain acetylcholine synthesis, overall oxidative metabolism, the activities of

the three major TPP-dependent enzymes of glucose oxidation and non-TPP-dependent enzymes, both in animals with overt neurological symptoms from pyrithiamin-induced thiamin deficiency and in animals in which the symptoms had been reversed by seven-day treatment with thiamin. Total enzyme activities were measured with new spectrophotometric assays that give activities significantly higher than previous assays, in homogenates that were treated with detergent to disrupt mitochondria and synaptosomes, and in the presence of excess TPP. Transketolase was also measured in the absence of added TPP.

EXPERIMENTAL PROCEDURE

Materials. Rats (40–50 gm males) were purchased from Charles River Breeding Laboratory Inc. (Wilmington, Massachusetts). Thiamin hydrochloride and pyrithiamin hydrobromide were from Sigma Chemical Co. (St. Louis, Missouri). [U-^{14}C]Glucose (285 Ci/mol) was from New England Nuclear Corp. (Boston, Massachusetts). The polypropylene cups for $^{14}CO_2$ collection were from Kontes (Vineland, California). Reagents for extraction of acetylcholine were as described previously (17). The control diet (vitamin B complex diet complete) and the control diet minus thiamin were from ICN Nutritional Biochemical Corp. (Cleveland, Ohio). The composition of this diet is detailed elsewhere (9). The reagents for each of the enzyme assays were as described in the original "method" paper for each.

Animal Preparation. Male Wistar rats (55–85 g) were housed individually as described previously (8). Thiamin deficiency was induced with a thiamin-deficient diet and daily injections of the centrally-acting thiamin antagonist, pyrithiamin hydrobromide (0.5 mg/kg in 0.9% NaCl; 8, 19). Gross neurological symptoms such as tremors, seizures and Wooley-White reflexes developed after thirteen days. At this time, one group of treated animals (n = 5) and controls (n = 5) were sacrificed. A second group of animals (n = 5) were injected with thiamin hydrochloride (100 mg/kg) for seven days and fed a thiamin-containing diet before sacrifice ("reversed" group). Both experiments were repeated three times with a control (n = 5) and treatment (n = 5) group. A group of "chronically" thiamin-deficient animals were also examined. These rats were made thiamin deficient (i.e., pyrithiamin injections plus a thiamin-deficient diet) for thirteen days and then treated with thiamin for seven days. They were then again made thiamin deficient and after appearance of gross neurological symptoms (seizures and loss of righting reflex), they were treated with thiamin for seven days. Both steps were repeated a third time before the rats were sacrificed.

Tissue Preparation. The brain anterior to the cerebellum but without the olfactory bulbs was removed, sliced in two dimensions (0.3 mm × 0.3 mm) with a McIlwain chopper as described previously (17), and washed with ice-cold buffer (pH 7.4): 5 mM-KCl, 2.3 mM-$CaCl_2$, 1.3 mM-$MgSO_4$, 141 mM-NaCl and 10 mM-Na_2HPO_4. $^{14}CO_2$ production and ^{14}C-acetylcholine synthesis by brain slices were determined as described previously (17). The tissue (2–3 mg protein) was incubated for 60 min in siliconized 20 ml scintillation vials in one ml of the rinse buffer with the addition of 5 mM-[U-^{14}C]glucose (μCi/μmol) and 40 μM-paraoxon. The high K^+-buffer contained 31 mM-KCl. The $^{14}CO_2$ was collected with hyamine hydroxide on fluted filter paper in suspended polypropylene cups. Acetylcholine was extracted from the buffer into dichloromethane as an ion pair with dipicrylamine. The nmoles of acetylcholine were calculated from the specific activity of the radioactive glucose as described previously (17).

For enzyme measurements, slices were separated from the media by centrifugation (900 *g* for 4 min) and stored at −80°C. They were subsequently homogenized in 9-vol of ice-cold buffer with 0.2 mM-2-mercaptoethanol and 20 mM-3-(N-morpholino)propane sulfonic acid (MOPS) that was adjusted to pH 7.2 with tris-(hydroxymethyl)amino methane (Trizma). Aliquots for activity measurements of PDHC, phosphofructokinase (PFK, EC 2.7.1.11), glutamate dehydrogenase (GDH, EC 1.4.1.2) and transketolase were stored at −80°C and subsequently diluted. For pyruvate dehydrogenase phosphate phosphatase (PDH_b phosphatase; EC 3.1.3.43), hexokinase (EC 2.7.1.1) and KGDHC determinations, the homogenate also contained 5 mM-$MgCl_2$, 0.1 mM-$CaCl_2$, 1 g/l Triton X-100 and 1 g/l Lubrol-PX before storage at −80°C.

Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was measured in the direction of NADH oxidation in the presence of 1 mM-ADP at 30°C as described by Plaitakis et al. (19) except that NADH was increased to 0.15 mM. Phosphofructokinase (PFK; EC 2.7.1.11) was assayed by the method of Massey and Deal (20). PDHC was determined with the arylamine acetyltransferase-coupled assay (11) except that 2-mercaptoethanol and lactate dehydrogenase were omitted. Transketolase activity was measured as described by Blass et al. (12) except that the amount of glycerol phosphate dehydrogenase-triose phosphate isomerase was increased to 5 U/ml and the reaction was initiated with a mixture of D-xylulose-5-phosphate and ribose-5-phosphate. Transketolase measurements were done in the absence and presence of 0.3 μM-TPP. The activity of KGDHC was determined as the 2-oxoglutarate-dependent formation of NADH at 30°C. The reaction mixture (0.8 ml) contained: 50 mM-MOPS that was adjusted to pH 7.6 with Trizma base, 0.5 mM-$MgCl_2$, 0.1 mM-$CaCl_2$, 50 μM-ethylenediamine tetraacetic acid, 0.5 mM-dithiothreitol, 0.15 mM-TPP, 1 mM-2-oxoglutarate, 1 mM-NAD, 0.12 mM-CoA, 40 μM-rotenone and 1 g/l Triton X-100 (K. F. R. Sheu, unpublished method). The rate of NADH formation was linear with protein concentrations from 50–200 μg for 3–5 minutes. The hexokinase assay monitored the formation of NADPH in the presence of $NADP^+$ and glucose-6-phosphate dehydrogenase. The reaction mixture contained: 50 mM-MOPS, that was adjusted to pH 7.6 with Trizma base, 5 mM-$MgCl_2$, 5 mM-glucose, 1.5 mM-NADP, 1 mM-ATP, 1 g/l Triton X-100, and 0.5 U/ml glucose-6-phosphate dehydrogenase (21). PDH_b phosphatase activity was based on the rate of activation of the inactive, phospho-PDHC, as described previously (13). Phospho-PDHC, the substrate for PDH_b phosphatase, was prepared by phosphorylating the highly purified, bovine kidney PDHC (13).

RESULTS

The gross behavioral changes and weight changes of the animals were as described previously (8). The pyrithiamin-treated rats began to lose weight compared to the controls after ten days of treatment. By day 13, many animals were "catatonic" and some had seizures. Injections of thiamin ameliorated any overt neurological signs within one day, and the animals subsequently gained weight normally for the seven-day "reversal" period.

Pyrithiamin treatment and its reversal altered acetylcholine synthesis and the oxidation of [U-^{14}C]glucose similarly (Table I). ^{14}C-Acetylcholine formation was unaffected under resting conditions (i.e., 5 mM-KCl) by either pyrithiamin treatment or its reversal. However, brain slices from

TABLE I

THE EFFECT OF THIAMIN DEFICIENCY ON ^{14}C-ACETYLCHOLINE SYNTHESIS AND $^{14}CO_2$ PRODUCTION FROM [U-^{14}C]GLUCOSE

	Control	Pyrithiamin	Thiamin Afer Pyrithiamin
^{14}C-ACh			
5 mM-KCl	100.1 + 3.1	106.5 ± 6.0	88.5 ± 5.6
	(19)	(10)	(11)
31 mM-KCl	153.5 ± 9.6[a]	119.3 ± 7.5[b]	142.7 ± 9.9[a,c]
	(19)	(10)	(11)
High KCl/Low KCl	1.54	1.12	1.61
^{14}C-CO_2			
5 m*M*-KCl	100.0 ± 1.8	91.4 ± 2.1	98.5 ± 2.5
	(19)	(10)	(11)
31 m*M*-KCl	128.0 ± 4.4[a]	103.8 ± 2.4[b]	127.6 ± 4.2[a,c]
	(19)	(10)	(11)
High KCl/Low KCl	1.28	1.14	1.30

Values are the percentage ± SEM of the 5 mM-KCl control values. The control values (nmol/mg protein per hour) for acetylcholine were 2.12 ± 0.26 (n = 19) and 1.34 ± 0.10 (n = 19) in high and low potassium buffers, respectively. The control values (nmol/mg protein per hour) for $^{14}CO_2$ were 90.7 ± 7.2 (n = 19) and 72.6 ± 6.6 (n = 19) in high and low potassium buffers, respectively. Each experiment was done twice with five animals per group both times and the value for each animal was determined in triplicate. The total n is given in parentheses. The pyrithiamin group was treated for thirteen days with injections of pyrithiamin and a thiamin deficient diet. The reversed group was then treated with thiamin for seven days. Saline-injected controls were sacrificed with each group. See text for details. Statistical comparisons were by analysis of variance with the least significance difference test ($p < 0.05$; 22).

[a] Denotes significant K^+ effect

[b] Denotes significant effect of pyrithiamin at same K^+

[c] Denotes that pyrithiamin and thiamin-reversed groups differ.

pyrithiamin-treated animals did not significantly increase acetylcholine synthesis in response to K^+ stimulation. High K^+ stimulated acetylcholine production by 54% in controls, but only by 12% in thiamin-deficient animals. This deficit was completely reversed by in vivo thiamin treatment. $^{14}CO_2$ production responded to the treatments in an analogous manner. Thus, in low K^+ buffer no effect of pyrithiamin treatment or of the thiamin reversal was noted. However, $^{14}CO_2$ production due to K^+-depolarization was depressed by half (from 28% to 14%) with pyrithiamin treatment and the depression was reversed by thiamin treatment.

Pyrithiamin treatment did not diminish the activities of any of the non-thiamin requiring enzymes, and only changed some of the TPP-dependent

TABLE II
THE EFFECT OF THIAMIN DEFICIENCY ON KEY ENZYMES OF CARBOHYDRATE METABOLISM

	Control	Pyrithiamin	Thiamin After Pyrithiamin
Tranketolase			
no TPP in reaction mixture	100.0 ± 1.3 (30)	30.8 ± 1.9[a] (15)	69.5 ± 1.5[a,b] (15)
TPP in reaction mixture	100.0 ± 1.8 (30)	35.4 ± 2.2[a] (15)	68.3 + 1.7[a,b] (15)
Hexokinase	100.0 ± 1.7 (20)	114.4 ± 7.9[a] (10)	102.1 ± 2.7 (9)
Phosphofructokinase	100.0 ± 3.4 (30)	97.4 ± 6.6 (10)	102.9 ± 3.1 (10)
Pyruvate dehydrogenase complex	100.0 ± 1.4 (20)	96.4 ± 3.9 (10)	107.0 ± 2.8[a,b] (10)
Pyruvate dehydrogenase phosphate phosphatase	100.1 ± 4.2 (20)	105.9 ± 7.8 (9)	100.8 ± 4.3 (10)
2-Oxoglutarate dehydrogenase complex	100.1 ± 2.0 (30)	64.4 ± 4.5[a] (15)	104.2 ± 6.0[b] (15)
Glutamate dehydrogenase	100.0 ± 1.6 (20)	97.4 ± 3.0 (10)	103.1 ± 2.6 (10)

Values are % of control ± SEM for the n in parentheses. Each experiment was run three times with 5 animals per group in each experiment. The pyrithiamin group was treated for thirteen days with injections of pyrithiamin and a thiamin deficient diet. The reversed group was then treated with thiamin for seven days. Saline-injected controls were sacrificed with each group. Controls values (nmol/mg protein per min except for phosphatase where the unit is mU PDHC activated/min per mg protein) for each enzyme were as follows: transketolase without TPP (8.68 ± 0.19; n = 30), transketolase with TPP (8.64 ± 0.25; n = 30), hexokinase (91.5 ± 3.1; n = 20), PFK (178 ± 9; n = 20), PDHC (79.7 ± 1.5; n = 20), PDH_b phosphatase (156.3 ± 7.5; n = 20), KGDHC (13.9 ± 0.7; n = 30), and GDH (496 ± 8; n = 20). Since the values for the two control groups were similar, they were combined. Comparisons were made by analysis of variance and the least significant difference test ($P < 0.05$; 22).

[a] Denotes values that differ significantly from control.

[b] Denotes that the pyrithiamin-reversed animals differ from pyrithiamin treated.

enzymes (Table II). The activities of the non-TPP-dependent enzymes either increased slightly (+14.4%, hexokinase), or were unchanged (PFK and GDH) even in severely deficient rats. The activity of the TPP-dependent PDHC and its regulatory PDH_b phosphatase were unaffected under the conditions used. The activity of KGDHC declined 36% after pyrithiamin treatment. The pentose cycle enzyme transketolase was the most sensitive enzyme to pyrithiamin treatment; its activity declined 69% and 65% when assayed in the absence or presence of TPP respectively.

TABLE III
THE EFFECT OF THREE EPISODES OF THIAMIN DEFICIENCY ON BRAIN ENZYMES

	Control	Chronic Thiamin Deficient
Transketolase	(n = 3)	(n = 3)
no TPP in reaction mixture	8.48 ±0.54	6.28 ±0.42[a]
TPP in reaction mixture	8.64 ±0.36	7.16 ±0.30[a]
Hexokinase	66.6 ±3.3	60.5 ±10.0
2-Oxoglutarate dehydrogenase complex	10.3 ±0.6	11.4 ±1.0
Glutamate dehydrogenase	418 ±7	436 ±24
^{14}C-Acetylcholine		
5 mM-KCl	1.21 ±0.09	1.04 ±0.22
31 mM-KCl	2.04 ±0.07	1.85 ±0.41
^{14}C-CO_2		
5 mM-KCl	54.9 ±1.4	48.4 ±2.8
31 mM-KCl	72.0 ±6.4	60.0 ±8.0

Three cycles of a pyrithiamin-induced thiamin deficiency followed by reversal were produced. Values (nmol/mg protein per min) are mean ± SEM except for CO_2 (nmol glucose/mg protein per hour) and ACh (nmol/mg protein per hour). [a] Denotes significantly different ($P < 0.05$) from control by Student's t-test.

Thiamin treatment for seven days increased the activities of all of the depressed enzymes (Table II). KGDHC activity returned to normal within this time. Although transketolase activity increased, it was still 30% lower than normal, whether measured in the presence or absence of TPP.

In one series of experiments, a group of animals were made thiamin deficient, then injected with thiamin for seven days (initial reversal), then injected daily with pyrithiamin until they showed neurological signs, then reinjected with thiamin for seven days for a second reversal (Table III). A total of three thiamin reversals were performed. Controls were injected with saline over this same period. The time required for development of neurological symptoms and the effects of reversal varied considerably. Thus, after the initial reversal, one rat never developed neurological symptoms and two rats died during the second cycle even after administration of thiamin. Transketolase activity remained low whether it was measured with (−17%) or without (−26%) TPP in these triple-reversed animals.

DISCUSSION

Thiamin deficiency in man classically induces two types of central nervous system dysfunction. The first (Wernicke syndrome) is a motor dis-

order that is associated with clouding of consciousness and typically responds to treatment with large doses of thiamin. The second (Korsakoff psychosis) is associated with defects in new memory and with permanent anatomical lesions, especially of the periventricular gray matter. The two conditions tend to merge in the clinical setting and are referred to as the the "Wernicke-Korsakoff syndrome" (23).

The data in this paper suggest that the reversible symptoms of thiamin deficiency are related to a diminished activity of the TPP-dependent tricarboxylic acid enzyme, KGDHC. The decline in activity agrees with the results of all three groups who have previously studied this enzyme in thiamin deficient brains (25–27), although the 36% deficit in the present studies was less than the 44 to 70% decrease described previously by others. A decrease in the activity of this tricarboxylic acid cycle enzyme would be expected to impair oxidation of pyruvate by intact mitochondria, but not the activity of PDHC. Previous studies of PDHC, in which mitochondria may have been variably disrupted, have in fact given inconsistent results. Of ten earlier studies of pyruvate oxidation by thiamin deficient brain, six (14–16, 24–26) reported deficiencies and four (15, 27–29) normal values. The PDHC activities by the technique used in the present studies is not increased by increased Triton concentrations (11), and no evidence was found for a deficiency of this enzyme. The decline in KGDHC activity (36%) was comparable to the decrease in glucose oxidation (22%). The control activity of KGDHC (14 nmol/min/mg protein) was close to the calculated flux of 3-carbon units derived from glucose in vivo; a typical resting rate of glucose utilization (0.62 μmol.g^{-1} min^{-1}; 30) corresponds to a flux of 3-carbon units of 1.24 μmol.g^{-1} min^{-1} which approximates 12.4 nmol.mg protein^{-1} min^{-1}. Thus, a small inhibition of KGDHC activity might proportionally depress carbohydrate oxidation. Even small reductions in carbohydrate oxidation due to KGDHC loss appear to lead to proportional impairment of acetylcholine synthesis (Tables I and II), and these decreases appear functionally significant (9, 10). Similarly, decreases in the incorporation of [U-^{14}C]glucose into amino acids of pyrithiamin-treated animals occur in symptomatic, but not in asymptomatic animals (31). The possible roles of these two neurotransmitters in the pathophysiology of thiamin deficiency have been reviewed (32). The comparison of enzyme activity of KGDHC to carbohydrate flux may not be accurate, since measured enzyme activities often rise as assay methods improve (e.g., 11). However, treatment with thiamin for seven days simultaneously reversed the neurological symptoms, the deficits in acetylcholine synthesis, glucose oxidation and KGDHC activity, which suggests that they are related.

The decrease in transketolase activity persists despite thiamin treatment and may be related to the permanent anatomical lesions, although it does not correlate with the neurological symptoms or the deficiencies in glucose oxidation or acetylcholine synthesis. Thiamin deficiency characteristically reduces transketolase activity in brain and in peripheral tissues (33, 34). Indeed, low transketolase activity in red blood cells and an unusually large stimulation by added TPP are used to diagnose thiamin deficiency (35). The decrease in transketolase activity, however, preceeds the onset of definite neurological abnormalities, and it persists after they are reversed acutely (34) or, in the present studies, chronically (Tables II and III). The lack of correlation with the functional and metabolic changes holds even if treatment with thiamin for more than seven days would have returned transketolase activity to normal. Indeed, decreases in transketolase activity do not correlate with any significant change in metabolism of the pentose phosphate pathway or related metabolic processes (36). The observation that transketolase fell equally whether measured in the presence or absence of TPP indicates that there was loss of the apoenzyme and suggests a loss of cells rich in transketolase (Tables II and III). The distribution of transketolase between different neural cell types and/or between glia and neurones is not known. Previous studies (37) did not find regional differences in transketolase, but areas sensitive to thiamin deficiency were not examined. Constitutive abnormalities of transketolase have been reported in patients with permanent brain damage due to thiamin deficiency (38).

The stability of the PDHC and the relative low activity of KGDHC and transketolase in thiamin deficiency accord with the known properties of these enzymes. TPP easily dissociates from PDHC and activity is restored by adding TPP back. Removing TPP from transketolase and restoring activity by adding it back is so difficult (39–40) that several workers proposed that TPP was covalently bound to transketolase (41). KGDHC has never been freed of TPP without inactivating it irreversibly. It seems reasonable to suggest that transketolase or KGDHC synthesized in the absence of TPP is less stable and more difficult to restore by adding TPP than is PDHC.

Further studies of other models of thiamin deficiency will be necessary to test the hypothesis that the functional lesions consistently relate to an oxidative defect and to KGDHC and the more permanent lesions to a deficit in transketolase. Pyrithiamin thiamin deficiency differs in detail from dietary thiamin deficiency, so that it will be necessary to study these relationships in dietary thiamin deficiency as well. Recent studies indicate that cellular acidosis precedes cell necrosis in pyrithiamin-thiamin deficiency (42), raising the possibility that a local oxidative defect with lactate

acidosis is an important factor in the generation of some of the permanent, anatomic lesions (43). A persistent glutamate decarboxylase deficit in the thalamus of deficient-reversed animals correlates with the thalamic damage which has been related to the memory disturbance in human Korsakoff psychosis (44). The evidence presently available is consistent with the hypothesis that the oxidative defect in thiamin deficiency relates primarily to the acute, reversible neurological disabilities.

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