

## Erythrocyte transketolase activity in suspected cases of Leigh's disease, or subacute necrotising encephalomyelopathy

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**SUMMARY** Erythrocyte transketolase activity and the effect of adding thiamine pyrophosphate (% thiamine pyrophosphate effect) were measured in 111 subjects suspected to suffer from Leigh's disease (subacute necrotising encephalomyelopathy). From clinical evidence these subjects were divided into five groups: (1) necropsy-proved cases of subacute necrotising encephalomyelopathy, (2) cases positive for urinary thiamine pyrophosphate: adenosine triphosphate phosphotransferase inhibitor, (3) clinically likely cases of subacute necrotising encephalomyelopathy (patients still alive, or on whom no necropsy was performed), (4) cases diagnosed as diseases other than subacute necrotising encephalomyelopathy (control group), (5) cases for which no diagnosis had been made.

Comparison of erythrocyte transketolase activities with and without added thiamine pyrophosphate and of the % thiamine pyrophosphate effect for each group compared with the control group showed no statistically significant differences from normal values for any of these parameters. Similarly, there were no differences between the two sexes in transketolase activity, and no correlation between transketolase activity and age. These results indicate that erythrocyte transketolase activity is not altered in subacute necrotising encephalomyelopathy and is unlikely to be of value for the diagnosis of Leigh's disease.

The symptoms and clinical and pathological findings in subacute necrotising encephalomyelopathy (SNE) or Leigh's disease were reviewed by Monpetit *et al.*<sup>1</sup> and Pincus.<sup>2</sup> The recessive mode of inheritance and the various biochemical abnormalities that have been detected suggest that the condition is the result of an inborn error of metabolism.<sup>3</sup>

Although it generally presents before age 2 years in children who had previously been healthy, SNE can first manifest itself in adolescence or early adulthood. Clinically it is difficult to diagnose, but psychomotor retardation, the presence of cranial nerve lesions and respiratory disorders in the hypotonic child, with exacerbation and remission of the symptoms and signs may suggest the diagnosis. The difficulty in clinical diagnosis leads to uncertainty in evaluating the results of any biochemical investigations except in cases in which the diagnosis has been confirmed at necropsy, or in cases where a sibling has died with SNE proved at necropsy. However, the discovery by Cooper *et al.*<sup>4</sup> of a substance present in

the body fluids of patients with SNE which inhibits the formation of thiamine triphosphate by the enzyme thiamine pyrophosphate (TPP): adenosine triphosphate (ATP) phosphotransferase, led to an assay for this inhibitor substance in the urine of patients suspected of having SNE. The specificity of the assay was discussed by Pincus *et al.*;<sup>5</sup> their data suggested that the inhibitor was linked with the presence of the disease in sick children. However, others, with the exception of Murphy *et al.*,<sup>6</sup> have been unable to operate the assay successfully, and at present there are some doubts about it.

The inhibitor substance disappears from the urine if the patient is receiving thiamine;<sup>7</sup> referring physicians are not always aware of this fact, and a negative finding will result if the sample is not satisfactory. Nevertheless, at the present time, the presence of this inhibitor substance in samples of urine is a potentially valuable guide for the diagnosis of SNE in children presenting with symptoms and signs of this disease.

The brain lesions which occur in SNE are very

similar to those found in the Wernicke-Korsakoff syndrome, except that in SNE the mamillary bodies are not generally affected. As the Wernicke-Korsakoff syndrome has been shown to involve a defect in thiamine metabolism, it has been suggested that SNE may also reflect a disturbance in thiamine metabolism.<sup>4, 8-9</sup> To date however, the manner in which thiamine is associated with the formation of brain lesions in SNE is not understood. In a few cases, the activities of enzymes requiring TPP as cofactor have been investigated but have produced equivocal results.<sup>3, 10-15</sup>

As there have been no extensive studies of the involvement of TPP-dependent erythrocyte transketolase in cases of SNE it was decided to investigate activity of this enzyme in suspected cases of Leigh's disease.

### Patients

Samples were obtained from 111 children in whom the diagnosis of SNE had been suspected. Samples were received from hospitals in the UK and West Germany. Clinical records were studied and a follow up made, as far as was possible, into the course of the illness.

Particular attention was given to the diagnostic opinion of the referring physician, and on this basis the subjects were divided into five groups: (1) necropsy-proved cases of SNE (n=4), (2) cases positive for urinary TPP:ATP phosphotransferase inhibitor (n=17), (3) clinically possible cases of SNE (cases still alive, or on whom no necropsy was performed) (n=21), (4) cases diagnosed as diseases other than SNE (control group) (n=36), (5) cases for which no diagnosis had been made (n=33).

### Materials and methods

Dithiothreitol (DTT); nicotinamide adenine dinucleotide, reduced (NADH); thiamine pyrophosphoric acid chloride; and glycerol-3-phosphate: NAD 2-oxidoreductase (D-glyceraldehyde-3-phosphate: ketol isomerase, EC 1.1.18/EC 5.3.1.1. or GDH/TIM) were obtained from Boehringer Mannheim. D-Ribose-5-phosphate (disodium salt) and tris (hydroxymethyl) aminomethane were from Sigma London Ltd; magnesium chloride was supplied by BDH, Poole, Dorset.

Venous blood was collected in tubes containing lithium heparin, centrifuged at 0°C and 3000 rev/min for 15 min, and plasma and buffy coat removed. The erythrocytes were then either frozen at -20°C and stored, or were prepared immediately for assay as follows: 0.5 ml erythrocytes were diluted with 2 ml distilled water and haemolysed by rapid freezing and

thawing (three times) in dry ice/methanol. Stroma were removed by centrifugation at 0°C and 3000 rev/min for 15 min and the supernatant used for assay of transketolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycoaldehyde transferase, EC 2.2.11 or TK).

The method used for determination of transketolase activity was essentially that of Vo-Khactu *et al.*<sup>16</sup> The only differences were as follows: 50 µl haemolysate were assayed using 100 mmol/l tris-HCl buffer pH 7.6 containing 1 mmol/l DTT and 5 mmol/l MgCl<sub>2</sub>; reference cuvettes contained 2.7 ml buffer and 50 µl haemolysate; readings at 340 nm were taken at 30-second intervals for 15 min.

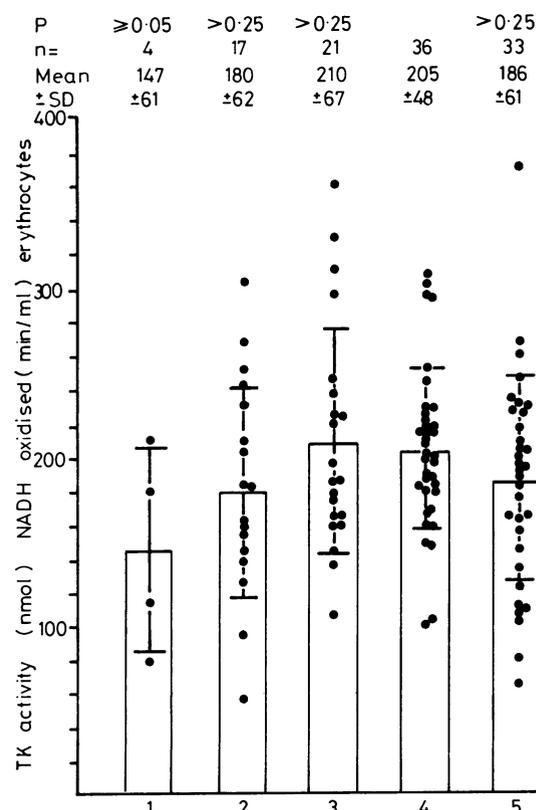


Fig. 1 *Transketolase activity in erythrocyte haemolysates from: (1) necropsy-proved cases of SNE, (2) TPP:ATP phosphotransferase inhibitor positive cases, (3) clinically possible cases of SNE, (4) cases diagnosed as other than SNE, (5) as yet undiagnosed cases. The mean ± 1 standard deviation in each group is shown in addition to the individual values. The P values denote the statistical significance, by the Student's *t* test, for differences between the control group (group 4) and the others.*

Activity was expressed as nmol NADH oxidised/min/ml erythrocytes. Transketolase activity was determined in duplicate in the presence and absence of exogenous TPP and the stimulatory effect expressed as follows:

$$\% \text{ TPP effect} = 100 \times [(\text{TK activity with TPP}) - (\text{TK activity without TPP})] / (\text{TK activity with TPP})$$

Urine, minimum volume 5 ml, was dried on filter paper (Whatman 3 MM, 10 × 8 cm) as described by Cooper<sup>5</sup> and despatched to Yale University for determination of TPP:ATP phosphotransferase inhibition by the method previously described.<sup>5</sup> Inhibition in excess of 40% was regarded as a positive result for the inhibitor substance.

**Results**

The reproducibility of the transketolase assay was determined by assaying one erythrocyte sample 10 times in the presence and absence of TPP. This

resulted in coefficients of variation from the means, of 6.5 and 6.4% respectively. For the % TPP effect, the mean value was 15.5 ± 8.2 and its coefficient of variation was 52.9%. This figure compares favourably with other reported values for small increments (see Discussion).

The results of erythrocyte transketolase determinations are shown for all groups of subjects in Fig. 1. Each group was compared with the control group (group 4) and there were no significant differences.

Transketolase activities after adding the TPP are presented in Fig. 2. All groups showed increased activity and the relationships were the same as before the TPP was added. Similarly, no significant differences in the % TPP effect were observed between the groups (Fig. 3). There was no correlation between age or sex and erythrocyte transketolase activity.

For 10 subjects blood and urine samples were

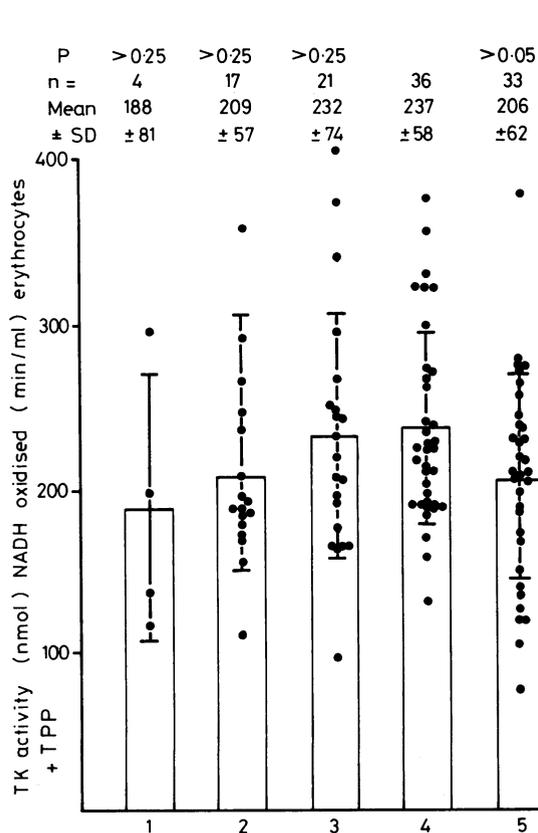


Fig. 2 Transketolase activity in erythrocyte haemolysates when thiamine pyrophosphate, final concentration 0.36 mmol/l was added before incubation. Other details are as in Fig. 1.

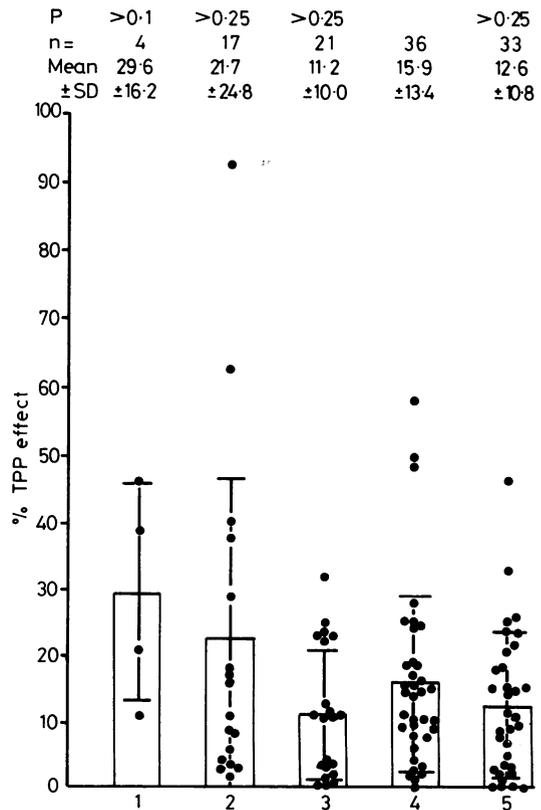


Fig. 3 The percentage increase in transketolase activity in erythrocyte haemolysates after addition of thiamine pyrophosphate (final concentration 0.36 mmol/l) to the incubation medium. Other details are as in Fig. 1.

Table *Family studies of urinary inhibitor and erythrocyte transketolase activities*

Case	Group	Inhibitor	TK activity		% TPP effect
			-TPP	+TPP	
1	1	Negative	214	298	39.6
Brother		Positive	261	310	18.8
Mother		Negative	175	267	52.6
Father		Positive	110	118	7.3
2	1	Positive	Not assayed		
Sister		Positive	233	224	0
Mother		Negative	204	235	15.2
Father		Positive	202	211	4.5
3	2	Positive	140	197	40.7
Sister		Negative	181	219	20.9
Mother		Negative	141	170	20.6
Father		Negative	139	159	14.4
4	2	Positive	268	294	9.7
Mother		Negative	157	162	3.2
Father		Negative	195	230	17.9
5	2	Positive	189	195	3.2
Mother		Negative	215	235	9.3
Father		Negative	207	217	4.8
6	2	Positive	184	192	4.3
7	2	Positive	232	247	6.5
Brother		Not assayed	229	255	11.4
Mother		Not assayed	100	121	21.0
Father		Not assayed	205	240	17.1
8	3	Negative	138	169	22.5
Sister		Negative	232	281	21.1
Mother		Negative	206	193	0
Father		Negative	Not assayed		
9	3	Negative	165	168	1.8
Mother		Negative	127	204	60.6
Father		Negative	164	180	9.8
10	3	Negative	182	187	2.7
11	3	Negative	159	181	13.8
Sister		Negative	179	233	30.2
Mother		Negative	200	216	8.0
Father		Negative	150	158	5.3
12	4	Negative	204	226	10.8
Mother		Negative	180	207	15.0
Father		Negative	164	210	28.0

TK activity expressed as nmol NADH oxidised min/ml packed erythrocytes.

obtained from siblings and parents also. The results of these studies are shown in the Table. In 2 patients with SNE confirmed at necropsy, clinically normal siblings and fathers were found to be positive for the inhibitor while the mothers were negative. In the remaining cases, the results were as expected in that clinically normal siblings and parents were negative for the inhibitor; there were no pronounced differences from the propositus in erythrocyte transketolase levels. In all subjects erythrocyte transketolase levels were within normal ranges.

### Discussion

Erythrocyte transketolase activity has been used clinically to determine thiamine status and has been

shown to be a specific and accurate reflection of thiamine sufficiency.<sup>17-18</sup> Methods of measuring the enzyme activity include colorimetric determination of sedoheptulose production, and ultraviolet spectrophotometric determination of NADH oxidation. Both methods, so far, have been shown to give comparable results for normal subjects. However, the activities reported here are higher than previously obtained, perhaps because of minor improvements in the assay procedure.

The determination of transketolase activity in the presence and absence of TPP, and the % TPP effect in particular, has been regarded as a sensitive index of thiamine deficiency and a % TPP effect greater than 25% is generally regarded as indicative of a deficiency state.<sup>17-18</sup> However, it has often been

reported that a high TPP effect does not always correlate with clinical findings<sup>19-21</sup> and this, together with the large variation usually found in TPP effect determinations (coefficient of variation from the mean 50-85%), limits the clinical significance of this parameter. The values of the TPP effect quoted for our control group agree with results reported for healthy individuals.

Apart from a role in determining thiamine deficiency the activity of erythrocyte transketolase has been investigated in a number of disease states—such as alcoholism, cancer, uraemia, neurosis, diabetes mellitus, anaemia, polyneuritis, and malnourishment secondary to vascular disease of the brain. In these conditions, enzyme levels have been shown to be significantly altered only in alcoholics, neurosis, and pernicious anaemia. In alcoholics, low enzyme levels were thought to reflect poor thiamine status as alcohol has been shown to block intestinal absorption of thiamine. Patients with neurosis were concluded to be thiamine-deficient due to poor food intake.<sup>22</sup> Patients with pernicious anaemia showed significantly increased transketolase activity and this was believed to be due to a larger population of young blood cells.<sup>23</sup>

For the few cases of Leigh's disease, in which transketolase activity has been determined, the results so far have been equivocal. In one study<sup>10</sup> no change in activity was observed; in another<sup>15</sup> significantly reduced enzyme levels accompanied by a high TPP effect were observed.

In addition to erythrocyte transketolase, there is also evidence, in SNE, to implicate deficiencies in other enzymes which require thiamine pyrophosphate as cofactor—for example pyruvate decarboxylase<sup>11 24</sup> and pyruvate dehydrogenase.<sup>25-26</sup> So far results of investigations of these enzymes have also proved equivocal.

This study has established that erythrocyte transketolase activity is not affected in necropsy-proved cases of SNE, nor is it affected in clinically likely cases, or in patients shown to be positive for an inhibitor of TPP: ATP phosphotransferase. In addition, studies of unaffected siblings and parents showed no pronounced differences from the propositus in terms of erythrocyte transketolase activities.

Results for the urinary inhibitor assay in these cases bear on the question of how this assay is used as a diagnostic criterion for SNE. Samples from 3 of the 4 necropsy-proved cases of SNE were negative for the inhibitor. It was also observed that 2 clinically normal siblings and their fathers in 2 necropsy-confirmed cases of SNE gave positive inhibitor results. These findings appear inconsistent with those of Pincus *et al.*<sup>5</sup> in which the inhibitor was positive in all 10 necropsy-proved cases of SNE. The reason for

this discrepancy is not known, but it may result from delays in assaying the urine.

At present it seems that there is no single laboratory procedure which will diagnose Leigh's disease before death and it is possible that the disease may be the result of more than one metabolic abnormality.

Current results suggest that involvement of thiamine pyrophosphate in Leigh's disease will be in a role other than that of coenzyme in erythrocyte transketolase or as precursor of thiamine triphosphate. Evidence has suggested an additional neural function of thiamine<sup>27-28</sup> and studies have been reported on a possible function of thiamine in neuromuscular transmission.<sup>29</sup> It remains to be seen whether or not this approach will be more fruitful in elucidating the role of thiamine in the pathogenesis of Leigh's disease.

We thank the physicians and staff of 24 hospitals in Britain and West Germany for their co-operation in this study, and the Research Fund, Bethlem Royal Hospital and Maudsley Hospital, for support.

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Received 5 June 1979