

Circumvention of Defective Neutral Amino Acid Transport in Hartnup Disease Using Tryptophan Ethyl Ester

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Abstract

Tryptophan ethyl ester, a lipid-soluble tryptophan derivative, was used to bypass defective gastrointestinal neutral amino acid transport in a child with Hartnup disease. The child's baseline tryptophan concentrations in serum ($20 \pm 6 \mu\text{M}$) and cerebrospinal fluid ($1.0 \pm 0.2 \mu\text{M}$) were persistently $< 50\%$ of normal values. Cerebrospinal fluid 5-hydroxyindoleacetic acid (5-HIAA), a serotonin metabolite, was also $< 50\%$ of normal ($21 \pm 2 \text{ ng/ml}$). Serum tryptophan concentrations increased only modestly and briefly after an oral challenge with 200 mg/kg of oral L-tryptophan, reflecting the absorptive defect. An oral challenge with 200 mg/kg of tryptophan ethyl ester resulted in a prompt increase in serum tryptophan to a peak of $555 \mu\text{M}$. Sustained treatment with 20 mg/kg q6h resulted in normalization of serum ($66 \pm 15 \mu\text{M}$) and cerebrospinal fluid tryptophan concentrations (mean = $2.3 \mu\text{M}$). Cerebrospinal fluid 5-HIAA increased to more normal concentrations (mean = 33 ng/ml). No toxicity was observed over an 8-mo period of treatment, chronic diarrhea resolved, and body weight, which had remained unchanged for 7 mo before ester therapy, increased by $\sim 26\%$. We concluded that tryptophan ethyl ester is effective at circumventing defective gastrointestinal neutral amino acid transport and may be useful in the treatment of Hartnup disease.

Introduction

Hartnup disorder is an autosomal recessive condition that is characterized by defective renal and gastrointestinal transport of neutral amino acids (1, 2). Multiple clinical forms of varying severity have been described (3–5). Many affected individuals appear to be totally asymptomatic (Hartnup disorder), while others suffer from an episodic pellagra-like illness with dermatitis, diarrhea, ataxia, and neuropsychiatric symptoms (Hartnup disease). Infrequently, patients are severely affected in childhood with progressive neurodegeneration leading to death by adolescence. There is little information available concerning the reasons for these different clinical presentations although general nutrition may play a role. It has been suggested that Hartnup disorder is a multifactorial problem, but these factors have not been well identified (6).

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Gastrointestinal absorption of tryptophan, an essential amino acid, is severely impaired in Hartnup disorder and may lead to systemic deficiency of tryptophan and its metabolites, nicotinic acid and NAD. Alternative carriers appear to exist for the uptake of other neutral amino acids that may be present in adequate amounts in serum. Treatment with nicotinamide supplementation to enable NAD synthesis may alleviate some symptoms (7), and a protein enriched diet may also help by providing tryptophan-containing peptide fragments that are absorbed through dipeptide transport systems (8).

Treatment of systemic tryptophan deficiency with nicotinamide may permit synthesis of NAD, but does not directly provide substrate for the synthesis of proteins or serotonin. Under normal circumstances circulating tryptophan enters the central nervous system (CNS) via a transport system for neutral amino acids (9–12). There it is hydroxylated to form 5-hydroxytryptophan and subsequently converted to serotonin by decarboxylation. CNS serotonin concentrations, which are reflected by measurement of the degradation product 5-hydroxyindoleacetic acid (5-HIAA)¹ in cerebrospinal fluid, are directly related to serum tryptophan concentrations (13–15). Marked increases in brain tryptophan and 5-HIAA concentrations occur within 1 h after the administration of intravenous tryptophan and are detectable in lumbar cerebrospinal fluid ~ 6 –8 h later (12, 13). While restoration of normal serum tryptophan concentrations would be desirable in Hartnup disorder, this has not been readily achieved. Previous studies demonstrated that amino acid esters readily cross membranes due to their lipid-soluble characteristics and are subject to intracellular enzymatic hydrolysis, thus regenerating the native amino acid (16–19). We investigated tryptophan ethyl ester as a means of bypassing defective amino acid transport in a severely affected child with Hartnup disease whose CNS tryptophan and serotonin levels remained low despite nicotinamide therapy.

Methods

Study patient. A 3-yr-old child was evaluated for growth failure, marked developmental delay, hyperactivity, chronic diarrhea, episodic ataxia, and weakness. Dietary history was unremarkable and family history was unobtainable. At 3 yr of age height was 87.5 cm and weight was 10.5 kg, both less than the third percentile for age. His extrapolated Bayley mental development index was 50 and magnetic resonance imaging revealed cortical atrophy. Muscle biopsy of the vastus lateralis showed a nonspecific pattern of type II fiber atrophy. He was noted to have an aminoaciduria that was specific for neutral amino acids with the exception of glycine and imino acids consistent with the pattern seen in Hartnup disease. A D-xylose absorption test was normal.

1. *Abbreviations used in this paper:* CSF, cerebrospinal fluid; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylethylene glycol.

Protocols. Informed consent for the following studies was obtained from the child's parents under protocols that had been approved by the local institutional review board. Informed consent for oral tryptophan loading was also obtained from an unrelated adult control.

Oral tryptophan loading. L-Tryptophan was administered orally in a dose of 200 mg/kg to both the patient and an unrelated normal control. Tryptophan was measured in serum samples using a fluorometric method (20), while other amino acids were measured by automated amino acid analysis (21). Urine indicans were measured spectrophotometrically (22).

Dietary therapy. A diet containing 3 g/kg of protein was instituted and maintained throughout the study. Oral nicotinamide supplementation was started at a daily dose of 2 mg/kg and increased monthly by 2 mg/kg until a total dose of 10 mg/kg (given in two divided doses) was achieved. Serum and cerebrospinal fluid (CSF) samples were obtained 4 wk after each increase in nicotinamide supplementation. Tryptophan-derived 5-HIAA, tyrosine-derived 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), and homovanillic acid (HVA) were measured in CSF by gas chromatography and mass spectrometry (23). NAD levels in red cells were measured spectrophotometrically (24).

Intravenous tryptophan loading. Intravenous grade, pyrogen-free L-tryptophan (Ajinomoto USA, Inc., Raleigh, NC) was prepared just before use as a 1% solution (wt/vol) in 0.5 normal saline, adjusted to pH 7.4 with 1 N sodium hydroxide, and sterilized by filtration through a 0.22- μ m filter. After an overnight fast tryptophan was administered intravenously over 30 min at a dose of 100 mg/kg. Serum samples were obtained for tryptophan measurement over a 6-h period. CSF for tryptophan and serotonin metabolite studies was obtained both before and 8 h after infusion.

Oral tryptophan ethyl ester loading. After an overnight fast, L-tryptophan ethyl ester (Sigma Chemical Co., St. Louis, MO) was administered as a 2% solution (wt/vol) in water by nasogastric tube. Serum samples were obtained over a 6-h period. Tryptophan ethyl ester levels were measured by both HPLC using a C18 bondapack column and methanol solvent with detection at 280 nm and by gas chromatography with mass spectrometry. Serum ethanol levels were measured using a NAD-generating system (25).

Tryptophan ethyl ester therapy. The patient was maintained on a protein enriched diet without nicotinamide supplementation. Tryptophan ethyl ester (1.25% solution wt/vol) was administered orally at a dose of 20 mg/kg q6h. This dose was chosen to elevate serum tryptophan levels to within the normal range.

Synthesis of tryptophan ethyl ester. L-[3 H]Tryptophan (ICN Nutritional Biochemicals, Cleveland, OH) was esterified using ethanolic HCl (20). The product was ~98% pure as determined by TLC on cellulose acetate plates using a solvent system of 96% methanol/3.7% H₂O/0.3% formic acid (26).

In vitro studies. Female Sprague-Dawley rats were anesthetized with CO₂ and killed by decapitation. Tissues were removed and homogenized in PBS, pH 7.4, at 4°C using a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Small intestine was flushed extensively with buffer before the mucosa was removed by scraping with a glass slide. Incubations were performed at 37°C with 10 mM L-[3 H]-tryptophan ethyl ester and then terminated by the addition of 4 vol cold ethanol. After centrifugation for 2 min at 10,000 g supernatants were assayed by TLC. Protein was determined spectrophotometrically (27).

Results

Oral tryptophan and dietary therapy. Challenge with 200 mg/kg of oral L-tryptophan resulted in a small increase in serum tryptophan concentration (Fig. 1) with marked indicanuria 16–24 h later, confirming the diagnosis of Hartnup disease. In contrast, serum levels rose to ~600 μ M in the normal control without marked indicanuria (data not shown). Treatment with a protein enriched diet and oral nicotinamide

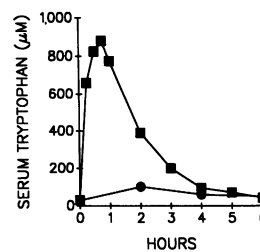


Figure 1. Serum tryptophan levels after 200 mg/kg of oral L-tryptophan (●) and 100 mg/kg of intravenous L-tryptophan (■).

resulted in little or no clinical improvement despite nicotinamide doses as great as 10 mg/kg per d. Red cell NAD levels on this therapy were 127 ± 6 nmol/ml packed red blood cells vs. 81 ± 7 nmol/ml for normal controls and literature normal values of 79 ± 9 nmol/ml (24). Tryptophan concentrations in serum were approximately one-third of normal at 20 ± 6 μ M vs. 62 ± 21 for age-matched controls. CSF tryptophan and 5-HIAA concentrations were consistently <50% of normal for age (Table I). During a 12-mo period of treatment with nicotinamide his symptoms continued and he had poor growth. His weight reached a peak of 11.6 kg at 4 yr of age and remained unchanged over the following 7 mo.

Intravenous tryptophan. Intravenous tryptophan was administered to test his ability to synthesize serotonin in the CNS. Serum tryptophan concentrations rose promptly after intravenous loading with a peak of 880 μ M at 45 min (Fig. 1). 8 h after infusion the CSF concentration of tryptophan rose sevenfold and the level of 5-HIAA in CSF increased to 50 ng/ml, within the range of normal (Table I). Transient, mild sedation was the only observed side effect during the study.

Oral tryptophan ethyl ester trial. The lipid-soluble ethyl ester derivative of tryptophan was administered in an effort to bypass the defect in tryptophan absorption. In vitro studies showed that tryptophan ethyl ester is readily hydrolyzed to tryptophan by homogenates of rat intestinal mucosa, liver, and kidney, but not brain (Fig. 2). Virtually no hydrolysis was observed using human plasma or washed human red cells (data not shown). The drug was found to be stable in acidic aqueous solution while refrigerated and protected from light. Over a 5-mo period the purity of a 2% (wt/vol) solution in 100 mM citrate buffer, pH 4.0, decreased slightly from 98 to 95%.

After a 200 mg/kg oral dose the child's serum tryptophan concentration rose to a peak of 555 μ M and remained elevated

Table I. CSF Metabolites during Tryptophan Ethyl Ester Therapy

Treatment	Tryptophan	5-HIAA	MHPG	HVA
	μ M	ng/ml	ng/ml	ng/ml
Nicotinamide	1.0 ± 0.2	21 ± 2	11 ± 1	95 ± 31
Intravenous tryptophan	7.0	50	9	115
Oral ester	3.3 ± 1.4	34 ± 5	11 ± 3	$63 \pm 24^*$
Controls	2.5 ± 1.1	52 ± 15	13 ± 3	103 ± 29

CSF was obtained by lumbar puncture 7 h after intravenous tryptophan infusion and after treatment with 20 mg/kg q6h of tryptophan ethyl ester. Control values were established using CSF from children ages 3–4 yr who had spinal taps performed to rule out infection.

* HVA value includes one extremely low value (35 ng/ml) after which the dose of ester was decreased.

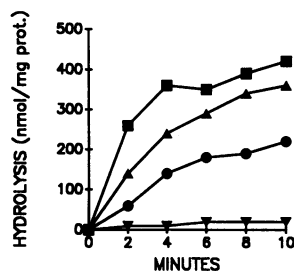


Figure 2. Hydrolysis of 10 mM L-[³H]tryptophan ethyl ester in PBS, pH 7.4, at 37°C by homogenates of rat intestinal mucosa (●), liver (▲), kidney (■), and brain (▼).

for 5 h (Fig. 3). Hydrolysis of the ester during absorption was apparently complete since only tryptophan was detected in serum samples by both HPLC and gas chromatography with mass spectrometry. The lower limit of detection for the ester in serum was 400 nM by the latter method. Ethanol generated by ester hydrolysis was below measurable limits in serum samples as well. Side effects were limited to transient nausea and sedation similar to what has been reported with intravenous tryptophan loading (28). No changes were observed in hematocrit, white count, electrolytes, calcium, phosphorus, or indices of hepatic and renal function 24 h after the study.

Tryptophan ethyl ester therapy. The dose of tryptophan ethyl ester was adjusted to 20 mg/kg and was repeated every 6 h based on kinetic data (Fig. 4). Peak serum tryptophan concentrations, measured 1 h after a dose, were reproducible at $78 \pm 17 \mu\text{M}$, mean \pm SD, $n = 5$ (normal, $62 \pm 21 \mu\text{M}$; $n = 11$) over a 1-wk period. No significant changes were observed in the serum concentrations of other amino acids after each dose. Red cell NAD levels were normal at $91 \pm 6 \text{ nmol/ml}$ packed red blood cells. The urinary excretion of tryptophan increased fourfold (from 0.28 to 1.07 mmol/g creatinine) on this treatment, while the excretion of other amino acids remained unchanged. Urine concentrations of indoleacetic acid as measured by gas chromatography and mass spectrometry remained unchanged by treatment. During therapy the CSF tryptophan concentration approximately tripled over basal levels to $3.3 \pm 1.4 \mu\text{M}$, while concentrations of other amino acids remained in the normal range. The concentration of 5-HIAA also increased from $21 \pm 2 \text{ ng/ml}$ to $34 \pm 5 \text{ ng/ml}$, while concentrations of MHPG and HVA, tyrosine-derived neurotransmitter metabolites, remained essentially unchanged and within the normal range (Table I). The dose of ethyl ester was decreased by 25% to 20 mg/kg three times daily after 5 mo because the CSF tryptophan concentration increased to $5 \mu\text{M}$ and the concentration of HVA markedly decreased to 37 ng/ml. Study 2 mo later showed normal CSF tryptophan and neurotransmitter concentrations.

His weight, which had been unchanged at 11.6 kg for the preceding 7 mo, increased by 26% during 8 mo of treatment with tryptophan ethyl ester (Fig. 5). During this same time his

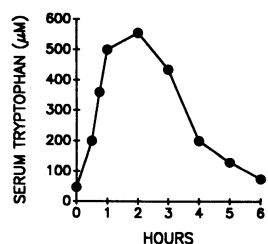


Figure 3. Serum tryptophan levels after a 200 mg/kg dose of oral L-tryptophan ethyl ester. Baseline serum tryptophan concentration was $35 \mu\text{M}$ at the start of the study.

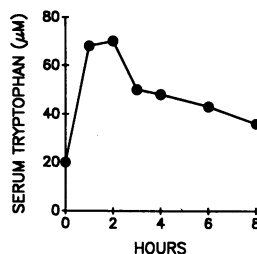


Figure 4. Serum tryptophan levels after a 20 mg/kg dose of oral L-tryptophan ethyl ester.

linear growth rate doubled as his height increased from 98 to 103 cm. His chronic diarrhea completely resolved and his gait improved. Peak tryptophan concentrations remained normal during treatment at $66 \pm 15 \mu\text{M}$, $n = 4$. Serum concentrations of many essential and nonessential amino acids which were either below or at the lower limit of the normal range before the administration of tryptophan ethyl ester increased (Table II). No side effects were observed other than a faint smell of alcohol on his breath associated with drug ingestion. No changes were observed in either blood counts, serum chemistries, renal function, or hepatic function. Aminoaciduria in spot urine specimens remained unchanged despite therapy.

Discussion

This patient, with clinical and biochemical criteria diagnostic of Hartnup disease, was unimproved after therapy with nicotinamide and a protein enriched diet. Clinical symptoms including diarrhea, neurologic dysfunction, and poor growth persisted. Serum concentrations of many amino acids, particularly tryptophan, remained low, while concentrations of tryptophan and its metabolites in CSF were also markedly decreased. It has been suggested, but not proven, that tryptophan deficiency is the basis for symptoms in this disorder. Since both transport of tryptophan across the blood-brain barrier and subsequent serotonin synthesis by the CNS appeared to be intact, we directed our efforts toward restoration of normal serum tryptophan concentrations.

We considered tryptophan ethyl ester as a means of bypassing defective gastrointestinal transport of tryptophan in Hartnup disease in light of studies in which lipid-soluble amino acid esters had been used successfully to bypass defective lysosomal transport systems (16–19). The theoretical attractiveness of this approach was strengthened by in vitro experiments that demonstrated that the ester was hydrolyzed by intestinal mucosa, liver, and kidney to provide tryptophan (Fig. 6). As suggested by these studies, tryptophan ethyl ester administration successfully corrected our patient's tryptophan deficiency state. Serum and CSF tryptophan concentrations

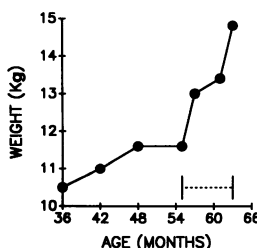


Figure 5. Weight gain over a period of 27 mo. Dashed line, interval of therapy with L-tryptophan ethyl ester.

Table II. Serum Amino Acid Measurements

Amino acid	Normal	Pretherapy	Posttherapy	Ratio
	μM	μM	μM	
Taurine	72±44	24	69	2.9
Aspartate	14±12	5	25	5.0
Threonine*	147±124	37	70	1.9
Serine	141±59	58	109	1.8
Glu/gln	561±220	359	373	1.0
Proline	226±147	143	282	2.0
Glycine	244±60	135	152	1.1
Alanine	407±175	159	157	1.0
Valine*	202±102	97	102	1.1
Cystine	44±16	13	23	1.8
Methionine*	52±35	9	67	7.4
Isoleucine*	58±34	35	71	2.0
Leucine*	99±55	55	137	2.5
Tyrosine	167±170	40	34	0.9
Phenylalanine*	79±46	21	32	1.5
Ornithine	138±133	14	105	7.5
Lysine*	173±100	53	146	2.8
Histidine*	78±24	10	31	3.1
Arginine*	83±54	10	66	6.6

Measurements were performed after an overnight fast both before and after 3 mo of therapy with tryptophan ethyl ester. Normal values are based on data from 20 healthy age-matched controls and are represented by mean±1 SD.

* Essential amino acids.

increased with the subsequent synthesis of serotonin in the CNS. Interestingly, his diarrhea resolved and he had a substantial improvement in growth.

The similarity between symptoms in some individuals with Hartnup disease and those with the niacin deficiency state, pellagra, has focused attention on tryptophan deficiency as a critical etiologic factor. While isolated tryptophan deficiency may lead to chronic neurotransmitter imbalance and theoretically could limit protein synthesis and therefore growth, deficiencies of many other amino acids may also occur in Hartnup disorder (8). In our patient some of these deficiencies may have been aggravated by chronic diarrhea since many, although not all, abnormalities in serum amino acid concentrations resolved with the cessation of diarrhea. The clinical and biochemical improvements observed in our patient in response to tryptophan replacement are suggestive that tryptophan deficiency was a crucial factor in the appearance of his symptoms. Since tryptophan ethyl ester is a specific means of providing tryptophan to these patients, its use in clinical stud-

ies should now permit the role of tryptophan deficiency in Hartnup disease to be clearly defined.

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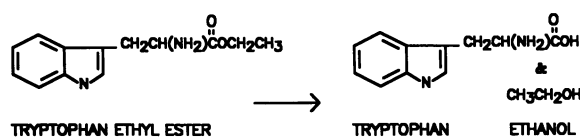


Figure 6. Hydrolysis of tryptophan ethyl ester.

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