The Effect of Tyrosine-Deficient Total Parenteral Nutrition on the Synthesis of Dihydroxyphenylalanine in Neural Tissue and the Activities of Tyrosine and Branched-Chain Aminotransferases

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The poor solubility of tyrosine (Tyr) limits the amount of this amino acid in total parenteral nutrition (TPN). In rats maintained on a standard pediatric TPN mixture, plasma and brain concentrations of Tyr are reduced to about 25% of the levels in chow-fed controls. To determine whether these low concentrations of Tyr affect the synthesis of catecholamines in neural tissue, the rate-limiting step (conversion of Tyr to dihydroxyphenylalanine [DOPA]) is studied by administering NSD-1015 to block the pyridoxal phosphate (PLP)-dependent decarboxylation of DOPA. However, in TPN rats, plasma concentrations of Tyr are increased by drug treatment. Because brain Tyr is also increased, these and other experiments using NSD-1015 clearly overestimate the rate of DOPA synthesis for drug-free rats on TPN. Nevertheless, in TPN rats, there is less DOPA in the brain in one experiment and less DOPA in the olfactory bulbs in another, versus control rats. Further examination of the metabolic effects of NSD-1015 reveals that the drug also elevates the concentration of branched-chain amino acids (BCAAs) in the plasma of TPN rats. These findings result from inhibition by NSD-1015 of the PLP-dependent aminotransferases that initiate catabolism of Tyr in the liver and BCAAs in the muscle. Despite the pronounced reduction in plasma Tyr, TPN rats showed a marked increase in the activity of hepatic Tyr aminotransferase compared with chow-fed controls. Conversely, although TPN elevates BCAA concentrations in plasma, the activity of branched-chain aminotransferase (BCAT) in the heart muscle of TPN rats is not different from control values. Different values but the same relationships are seen in drug-free rats.

TOTAL PARENTERAL NUTRITION (TPN) has contributed significantly to survival in a number of clinical situations. Although the mixtures of nutrients used for TPN have undergone many improvements since the pioneering studies by Wilmore and Dudrick,[1] TPN inevitably creates abnormal conditions in the circulation. These include constant high concentrations of glucose, a loss of meal-associated fluctuations in amino acid concentrations, and infusion of an emulsion of lipids not associated with lipoproteins. TPN also lacks or is deficient in certain amino acids, due to limited solubility (tyrosine [Tyr] and cystine) or stability (glutamine). In an attempt to compensate for the deficiency of Tyr, high concentrations of phenylalanine (Phe), the metabolic precursor of Tyr, are included in the amino acid mixture. However, plasma Phe concentrations are elevated and Tyr levels remain at about 30% of normal values in rats and in infants maintained on a pediatric TPN mixture.[2] In rats, substitution of the peptide γ-glutamyltyrosine (Glu(Tyr)) for equimolar amounts of Phe and glutamate in the TPN mixture normalizes plasma Tyr concentrations; Tyr levels in the brain are doubled but remain below control values.[2]

The initial aim of this study was to determine whether the subnormal plasma and brain concentrations of Tyr created by TPN affect the conversion of Tyr to catecholamines in neural tissue. The drug NSD-1015 (*m*-hydroxybenzylhydrazine) can be used to block this pathway after the rate-limiting enzyme, Tyr hydroxylase (EC1.14.16.2), has converted Tyr to dihydroxyphenylalanine (DOPA).[3] Normally, DOPA is not detected in neural tissue, because it is efficiently converted to dopamine by pyridoxal phosphate (PLP)-dependent aromatic amino acid decarboxylase (EC4.1.1.28); when the decarboxylase is inhibited, DOPA accumulates at a rate reflecting the activity of Tyr hydroxylase.[4] It has been suggested[5] that this hydrazine drug inhibits aromatic amino acid decarboxylase by reacting with the coenzyme, PLP, which is required for the activity of many enzymes involved in amino acid metabolism. Lightcap et al[6,7] have shown that NSD-1015 inactivates PLP-dependent γ-aminobutyric acid aminotransferase of pig brain; the drug forms a hydrazone with free PLP and with PLP bound to the enzyme.

In the present investigation, we studied the effect of low-Tyr TPN on the rate of formation of DOPA in the olfactory bulb, retina, and brain of rats treated with NSD-1015. We found that dosages of the drug that permit measurement of DOPA in neural tissues also increase plasma concentrations of Tyr and the three branched-chain amino acids (BCAAs), valine (Val), isoleucine (Ile), and leucine (Leu), over pretreatment values. Thus, we also examined the effects of the drug on the aminotransferases that initiate the catabolism of Tyr and BCAAs and thus affect the plasma concentrations.

MATERIALS AND METHODS

Animals

Upon receipt, male Sprague-Dawley rats (Charles River, Wilmington, MA) were caged individually in an animal room maintained on a daily light cycle from 6 AM to 6 PM. Rats were housed for at least 4 days on-site before surgery and gained weight normally during this period. The protocol for the use of rats in these experiments was approved by the Institutional Animal Care and Use Committee of the University of Louisville.

The surgical procedure to implant a catheter via the femoral vein into the inferior vena cava was described previously.[8] At the time of surgery, rats weighed 123 to 195 g. After surgery, the animals were allowed a 3-day recovery period. During this time, they were infused and fed as described previously[2]; all animals gained weight during the recovery

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period. At time 0, blood was collected in heparinized tubes from the tails of experimental (TPN) and control (saline [Sal]) rats. Food was removed from the cages of TPN rats, and they were infused at 21 to 22 mL/kg body weight/h with a TPN solution[2] containing a pediatric formulation of amino acids (Aminosyn-PF 10%; Abbott Laboratories, North Chicago, IL) with a 10:1 molar ratio of Phe to Tyr. Tyr was infused at a rate of 20 μmol/kg body weight/h. All Sal rats had access to rodent chow (Purina Mills, St Louis, MO) ad libitum while they were infused with heparinized saline at 21 to 22 mL/kg body weight/h. TPN solutions were sterilized by filtration through sterile 0.22-μm Millex-GS filters (Millipore, Marlboro, MA).

Rats on the TPN mixture received 1.0 μg pyridoxine (PN) (as the hydrochloride)/g body weight/d; rats on the vitamin B_6–supplemented TPN mixture received an additional 3.2 μg PN/g body weight/d. In experiment A, PN concentrations in the infused TPN mixtures were measured by high-performance liquid chromatography (HPLC) with fluorometric detection[9] both before and after the 2-day infusion period and were found to be unaffected by light in the animal room. This extends the observation on vitamin stability in TPN reported by Dahl et al.[10] By measuring the amount of chow consumed by Sal rats, we determined their intake of PN to be 0.6 to 0.8 μg/g body weight/d.

A custom amino acid mixture for preparation of the TPN in experiment C was purchased from PharmaThera (Memphis, TN). The TPN mixture was prepared by modifying this amino acid mixture by addition of pyrogen-free Phe and glutamic acid, kindly provided by Dr T. Furukawa of Nutri-Quest (Chesterfield, MO), to duplicate the amino acid composition of the Abbott Aminosyn-PF 10% used in experiments A and B. To prepare peptide-containing TPN (PTPN), the custom mixture was supplemented with Glu(Tyr) (Peptides International, Louisville, KY) as described previously.[2] The amino acid mixtures were isonitrogenous; they were adjusted to pH 5.5 to 6.0 with NaOH and sterilized by filtration as described earlier.

Rats treated with NSD-1015 (experiments A, C, and D) and drug-free rats (experiment B) were studied. Blood was sampled from the tail at day 2 in experiments A, C, and D and at day 1 in experiment B. We reported previously that in TPN rats, below-normal concentrations of Tyr and above-normal concentrations of Phe were noted at 24 hours and maintained in daily measurements made through 96 hours.[2] In experiments A, C, and D, the final sample of tail blood was taken 20 minutes before administration of the drug. Rats in experiment B received no drug before collection of trunk blood. All rats were removed from the animal room to a lighted, quiet laboratory at least 40 minutes before killing. Rats in experiments A and B were killed in the morning during their normal sleep cycle. Some of the rats closed their eyes and appeared to sleep during the period before death. To determine whether closed eyes affect the concentration of DOPA in the retina, rats in experiments C and D were killed in the evening at least 1 hour after the dark cycle (normal wakeful time) began. These rats did not sleep during the period between administration of the drug and death.

Drug treatment of animals in experiments A, C, and D consisted of an intraperitoneal injection of NSD-1015 (75 mg/mL in water) at 100 mg/kg body weight 20 minutes after tail blood was drawn. Exactly 20.0 minutes later, they were killed swiftly by a guillotine. Infusion pumps were turned off at the moment of decapitation. Trunk blood was collected immediately in heparinized tubes. Animals in group D were not catheterized and thus served as unoperated controls; in these rats, a single sample of tail blood was taken 20 minutes before the drug was injected. Animals in experiments A and B were killed on day 2 at 46 to 50 hours; animals in experiment C were killed on day 2 at 48 to 53 hours.

Table 5 includes a summary of the treatments in each experiment.

In rats of experiments A, C, and D, portions of two lobes of the liver, the heart (ventricles), olfactory bulbs, and residual brain tissue were removed, weighed, and frozen rapidly in liquid nitrogen. The eyes were removed and chilled on ice during dissection of the retinas under magnification. Retinas were weighed and frozen as before. All dissections were made by a single individual to minimize variations in technique. The time between decapitation and freezing of each tissue was standardized. Location of the catheter tip within the inferior vena cava was verified at necropsy. Tissues were stored at −80°C until analyzed. Within a single experiment, the storage time of neural tissue was constant so that the stability of DOPA was not a variable. The storage time of tissues in different experiments varied; thus, DOPA concentrations in rats from different experiments are not compared.

In rats of experiment B (no drug treatment), DOPA was undetectable in neural tissue. All tissues except for brain were removed and treated as before. From the brain tissue (after removal of olfactory bulbs), the pituitary gland, prefrontal cortex, hippocampus, and cerebellum were dissected and frozen rapidly in liquid nitrogen. The cerebellum and remaining brain tissue were weighed, frozen immediately in liquid nitrogen, and stored at −80°C. The olfactory bulb, pituitary gland, prefrontal cortex, hippocampus, and retina were subsequently analyzed for serotonin, hydroxyindoleacetic acid, catecholamines, and metabolites. These data are not included in this report.

Preparation of Samples

The preparation of the samples was performed on ice unless stated otherwise. Blood plasma, separated in a refrigerated hematocrit centrifuge, was recentrifuged in 0.4-mL microfuge tubes at 13,000 × *g* in a refrigerated Eppendorf centrifuge (Model 5415; Brinkman Instruments, Westbury, NY) to ensure removal of platelets and any remaining cells. A 100-μL aliquot of plasma was then transferred to a 1.5-mL microfuge tube and diluted with 400 μL deionized water containing 25 μmol/L norleucine, the internal standard for automated amino acid analyses. Protein was removed with $HClO_4$, and excess perchlorate was precipitated as the potassium salt using potassium bicarbonate. The supernate (pH 6, Hydrion paper) was frozen at −80°C until analyzed. Tyr, Phe, Glu(Tyr), and tryptophan (Trp) levels were measured by HPLC as described previously.[2] NSD-1015 does not affect these measurements; it elutes among several other species detected near the solvent front. In selected samples, the levels of BCAAs and other plasma amino acids were measured by automated amino acid analysis[11] with correction for recovery of norleucine.

Frozen brain tissue, olfactory bulbs, and retinas were thawed on ice in measured amounts of 0.1N $HClO_4$ (~1 mL $HClO_4$/100 mg tissue) containing dihydroxybenzylamine as internal standard. The homogenization and centrifugation of brain tissue were described previously.[2] Olfactory bulbs and retinas were homogenized in the 1.5-mL microfuge tubes in which they had been frozen, using a Teflon pestle designed to fit the microfuge tube. The final concentrations of brain tissue, olfactory bulbs, and retinas in homogenates were calculated. After centrifugation at 4°C for 15 minutes at 13,000 × *g*, DOPA levels were measured in the supernates of homogenates of olfactory bulbs and retinas. Aliquots of the supernate of brain tissue were frozen at −80°C and later analyzed for aromatic amino acids, PLP, pyridoxamine phosphate (PMP), and DOPA.

Aromatic amino acid levels in the supernates of brain homogenates were measured by the HPLC method used for plasma, except that Tyr was measured by absorbance at 230 nm during elution with 18% methanol in 0.095% trifluoroacetic acid; Phe and Trp were measured by absorbance at 212 nm during elution with 15.5% methanol in 0.095% trifluoroacetic acid. Glu(Tyr) levels could be measured in brain by the HPLC method used for elution of Phe and Trp; however, the peptide was not detectable in brain samples from PTPN rats of experiment C.

The DOPA content of supernates of homogenates of brain, olfactory bulbs, and retinas was determined by HPLC with electrochemical detection by the method of Sved and Fernstrom.[12] Separation was made on a Waters μBondapak C_{18} column (3.9 × 30 cm, 10-μm particle size; Waters Division, Millipore, Marlborough, MA). The electrochemical detector (Model LC-4B; Bioanalytical Systems, West Lafayette, IN)

was set at +700 mV versus an Ag/AgCl reference electrode. Measurements of DOPA were corrected for recovery of the internal standard.

A portion of each liver was homogenized in 4 vol 50-mmol/L potassium phosphate, pH 7.6, using an Ultra-turrax tissuemizer (Tekmar, Cincinnati, OH) at a setting of 50. An aliquot of the homogenate was immediately mixed with an equal volume of cold 11% trichloroacetic acid, and the sample was incubated at 50°C for 15 minutes to release PLP. The acid extract was centrifuged for 5 minutes at 13,000 × *g*. The remainder of the original homogenate was centrifuged at 13,000 × *g* for 10 minutes, and the supernate was used in the assay of the activity of Tyr aminotransferase (TAT).

Each heart was homogenized in 4 vol 50-mmol/L potassium phosphate, pH 7.8, using the tissuemizer at a setting of 70. An aliquot was immediately treated with trichloroacetic acid as described for the liver. The heart homogenate was centrifuged at 400 × *g* for 10 minutes, and the supernate was assayed for activity of branched-chain aminotransferase (BCAT).

Enzyme Assays

TAT activity in liver cytosol was determined in both the absence and presence of supplementary PLP by the method of Granner and Tomkins.[13] Enzyme activity in liver cytosol was measured. The activity of BCAT in heart was determined using [1-^{14}C]-L-Leu as substrate in a modification of the procedure of Hutson et al.[14] The reaction mixture (1 mL) contained 50 mmol/L potassium phosphate, pH 7.8, 3 mmol/L [1-^{14}C]-L-Leu (specific activity, 20 μCi/mol), 1 mmol/L α-ketoisovalerate, 2.5 mmol/L dithiothreitol, 0.4% CHAPS, and 10 μL heart homogenate, with and without supplementary PLP. Each assay mixture was incubated for 30 minutes at 37°C with shaking in a 10-mL Erlenmeyer flask closed with a stopper fitted with a plastic center well (Kontes, Vineland, NJ). The center well contained 40 μL phenethylamine (Research Products International, Mount Prospect, IL). The reaction was stopped by addition of 0.5 mL 2-mol/L sodium acetate, pH 3.4, followed by 0.5 mL 30% hydrogen peroxide to release ^{14}C-labeled CO_2. The samples were shaken at 37°C for 60 minutes. The well was placed in a minivial containing 4 mL Econo-safe scintillation cocktail (Research Products International). Radioactivity was measured with a Beckman LS7500 scintillation counter (Beckman Instruments, Fullerton, CA). Background samples in which the heart homogenate was added after the acetate were included with each experiment. Protein was determined using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

Analysis of Vitamin B_6 Compounds

Vitamin B_6 compounds were separated by HPLC and measured fluorometrically.[15] Aliquots of acid extracts of the tissues were injected, and PLP and PMP were separated at a flow rate of 1.5 mL/min using 100% A (20 mmol/L HCl) from 0 to 4 minutes; a linear gradient from 100% A at 4 minutes to 50% A and 50% B (0.1 mol/L sodium phosphate, pH 3.3) at 9 minutes; a linear gradient from 50% A and 50% B at 9 minutes to 90% B and 10% C (0.5 mol/L sodium phosphate, pH 5.9) at 14 minutes; and a linear gradient from 90% B and 10% C at 14 minutes to 100% C at 21 minutes. The concentration of PLP in the deproteinized extracts of tissues and plasma was also determined using the L-Tyr apodecarboxylase assay with L-[1-^{14}C] Tyr (specific activity, 44 μCi/mmol) as substrate.[16]

Chemicals

Reagents used for measurement of DOPA levels in neural tissue included Dowex 50 W-X4 resin (200-400 mesh; Bio-rad Laboratories, Richmond, CA) and EDTA (International Biotechnologies, New Haven, CT). Citric acid, NaH_2PO_4 monohydrate, 1-octanesulfonic acid, *m*-hydroxybenzylhydrazine, dihydroxybenzylamine, DOPA, CHAPS, dithiothreitol, and α-ketoisovalerate were purchased from Sigma Chemical (St Louis, MO). L-[1-^{14}C]-Leu (specific activity, 58 mCi/mmol) and L-[1-^{14}C]-Tyr (specific activity 55 mCi/mmol) were obtained from Dupont New England Nuclear (Boston, MA). Other chemicals were supplied as noted previously.[2,8,9]

Statistical Analysis

Data were examined statistically by one-way ANOVA or Student's *t* test using the Epistat Statistical Package (Tracy L. Gustafson, Richardson, TX). The α value was set at 0.05. The statistical significance of differences in aromatic amino acid concentrations in plasma and brain was confirmed by Levine's test for equality of variances.[17]

RESULTS

Aromatic amino acid concentrations in the blood plasma of the rats are shown in Table 1. No differences were found between initial (time 0) blood samples collected in the morning (experiments A and B) and those collected in the afternoon (experiments C and D). In experiment A, we also found no differences between rats on TPN versus TPN supplemented with additional vitamin B_6. Thus, these data are not reported separately. In a comparison of plasma from the final samples of tail blood from Sal (n = 10) and TPN (n = 12) rats, Tyr was decreased 75% and Phe was increased 72% with TPN, in general agreement with previous studies.[2] Similar changes were apparent when the final samples of tail blood in TPN rats were compared with those at time 0. Inclusion of Glu(Tyr) in PTPN in experiment C maintained plasma Tyr and Phe at values similar to those at time 0.

A comparison of Phe and Tyr concentrations in trunk blood and in final samples of tail blood in Table 1 indicates that values in trunk blood are affected by factors other than the drug. For instance, in drug-treated, chow-fed rats (n = 9), the mean concentrations of Phe and Trp were lower in trunk than in tail blood. This suggests dilution of the trunk blood either by the pressure of saline in the line when the pumps are turned off or by the body fluids (experiment D, no pumps) or both. Also, in TPN rats of experiment B (no drug), the mean concentrations of Phe and Trp either remained the same or increased in trunk blood compared with tail blood. This may be due to the opposing influences of dilution by body fluids and increases caused by contamination of the plasma with amino acids in TPN in the line when the pumps are turned off.

Despite the difficulty in measuring these influences, the data in Table 1 support the conclusion that NSD-1015 increases plasma Tyr concentrations. This increase is apparent when Tyr concentrations are compared in drug-treated TPN rats (n = 8) and TPN rats of experiment B (no drug). However, the drug-induced increase may be blunted by the sources of dilution already noted. The following observations provide additional support for the stated conclusion: (1) When trunk and tail blood were compared in drug-treated Sal rats (n = 6), mean plasma concentrations of Tyr were not significantly different, whereas mean concentrations of Phe and Trp were decreased to the same degree (36% to 37%) in trunk blood. (2) In drug-treated TPN rats (n = 8), the 49% increase in Phe can be attributed to contamination of the plasma with Phe-rich TPN; however, since there is very little Tyr in TPN, the 150% increase in Tyr in trunk blood must be ascribed primarily to the drug treatment. (3) When no drug was administered (experiment B), the mean

Table 1. Concentrations of Aromatic Amino Acids (μmol/L) in Rat Plasma

Experiment(s)	Treatment	No. of Rats	Time 0 Sample of Tail Blood			Final Sample of Tail Blood			Trunk Blood		
			Tyr	Phe	Trp	Tyr	Phe	Trp	Tyr	Phe	Trp
A, B, C	Sal	10	93 ± 9	64 ± 5	99 ± 9	106 ± 12	68 ± 6	113 ± 10			
A, B, C	TPN	12	81 ± 4	61 ± 3	100 ± 5	26 ± 2*†	117 ± 4*†	125 ± 6†			
C	PTPN	4	77 ± 12	59 ± 5	89 ± 7	64 ± 6*	68 ± 4	126 ± 9†	96 ± 8‡	91 ± 5‡	139 ± 16
A, C	Sal	6				114 ± 21	70 ± 9	121 ± 15	128 ± 19	45 ± 6‡	76 ± 12‡
A, C	TPN	8				26 ± 2*	116 ± 6*	132 ± 7	65 ± 3*‡§	173 ± 19*‡	144 ± 10*
A, C, D	Chow-fed	9				107 ± 14	75 ± 6	128 ± 13	116 ± 14§	47 ± 4‡§	79 ± 9‡
B	Sal	4				95 ± 4	65 ± 6	100 ± 2	81 ± 5	57 ± 3	77 ± 8‡
	TPN	4				27 ± 4*	121 ± 7*	111 ± 9	36 ± 6*	188 ± 22*‡	119 ± 8*
D	Unoperated	3				93 ± 11	85 ± 1	141 ± 29	94 ± 13	51 ± 2‡	85 ± 18‡

NOTE. Results are the mean ± SEM. Rats in experiment B received no drug. All others received NSD-1015 as described.
*Different from value in comparable group of Sal rats.
†Different from time 0 in same experiment or group of experiments.
‡Different from final sample of tail blood in same experiment or group of experiments.
§Different from value in comparable group of rats in experiment B.

concentrations of Tyr, Phe, and Trp in Sal rats were decreased to a similar extent (12% to 23%) in trunk compared with tail blood. (4) In TPN rats of experiment B (no drug), there was no significant increase in Tyr or Trp in trunk compared with tail blood; the 55% increase in Phe in trunk blood versus tail blood can be ascribed to contamination of the plasma with high-Phe TPN.

Aromatic amino acid concentrations in the rat brains are shown in Table 2. In rats of experiment B, we found no difference in the concentration of aromatic amino acids in the cerebellum and residual brain tissue. Thus, we believe the values for rats of experiment B in Table 2 are representative of brain tissue (less the olfactory bulbs). In rats of experiment C, drug treatment elevated brain Tyr in both Sal and TPN rats compared with the rats of experiment B (no drug). An increase in brain Tyr (*v* rats in experiment B) was also noted in rats of experiment A, although the small number of Sal rats (n = 2) affects the significance of this difference.

The brain to plasma ratios were calculated using the concentrations of amino acids in trunk blood. Thus, the factors already noted that affect the measured concentrations of amino acids in trunk blood also affect these ratios. However, the trend is clear: brain to plasma ratios were markedly lower in TPN rats than in chow-fed controls. This suggests that TPN alters the transport of aromatic amino acids into the brain. In experiment C, although PTPN (containing Glu(Tyr)) maintained the normal concentration of plasma Tyr (Table 1), brain Tyr, albeit elevated, was not normalized by the peptide. To examine the possible reasons for these differences, complete amino acid analyses on plasma in selected rats were performed. It was found that TPN produces significantly higher plasma concentrations not only for Phe but also for the BCAAs, arginine, glutamate, proline, and threonine; of 20 amino acids analyzed, only the Tyr concentration is consistently less than normal in rats maintained on TPN for 24 hours or longer (Table 1).

Pertinent to the altered brain to plasma ratios of aromatic amino acids in TPN rats are the concentrations of BCAAs shown in Table 3. The marked elevation of BCAAs in the final samples of tail blood from TPN rats, compared with values at time 0 and also final samples of tail blood from Sal rats, explains the reduced brain to plasma ratios of aromatic amino acids in TPN rats in Table 2, since BCAAs compete with aromatic amino acids for transport into the brain.

In Sal rats of experiment A, the normal concentrations of BCAAs in final samples of tail blood were not increased by treatment with NSD-1015 (trunk blood). However, when the

Table 2. Concentrations of Aromatic Amino Acids in the Brain and DOPA in Neural Tissue

Experiment	Treatment	No. of Rats	Brain (nmol/g)			Brain to Plasma Ratio (nmol/g/μmol/L)			DOPA (pmol/g)		
			Tyr	Phe	Trp	Tyr	Phe	Trp	Brain	Olfactory Bulb	Retina
A	Sal	2	108 ± 21	43 ± 8	18 ± 6	1.10 ± 0.01	1.16 ± 0.03†	0.27 ± 0.05	1,004 ± 60	1,346 ± 287	1,032 ± 54
	TPN	4	28 ± 2*†	43 ± 1	17 ± 1	0.44 ± 0.03*	0.25 ± 0.05*	0.13 ± 0.03*	687 ± 34*	911 ± 17	795 ± 133
B	Sal	4	78 ± 11	45 ± 4	16 ± 1	0.96 ± 0.11	0.80 ± 0.05	0.22 ± 0.02			
	TPN	4	16 ± 3*	53 ± 5	18 ± 1	0.44 ± 0.05*	0.30 ± 0.04*	0.15 ± 0.01*			
C	Sal	4	109 ± 10†	39 ± 2	17 ± 1	0.80 ± 0.07	0.83 ± 0.10	0.23 ± 0.04	879 ± 88	923 ± 21	634 ± 134
	TPN	4	28 ± 2*†	45 ± 1*	19 ± 1	0.42 ± 0.02*	0.32 ± 0.06*	0.14 ± 0.01*	790 ± 9	676 ± 83*	340 ± 9
	PTPN	4	45 ± 3‡	29 ± 1‡	18 ± 0	0.48 ± 0.02*	0.32 ± 0.01*	0.14 ± 0.01*	837 ± 29	686 ± 18*	449 ± 68
D	Unoperated	2	101 ± 5	57 ± 8	21 ± 3	0.84 ± 0.12	1.16 ± 0.18	0.31 ± 0.07	1,111 ± 6	1,002 ± 68‖	798 ± 152‖

NOTE. Results are the mean ± SEM. Rats in experiment B received no drug. All others received NSD-1015 as described.
*Different from corresponding value in Sal rats.
†Different from corresponding value in experiment B (no drug).
‡Comparison between PTPN rats and TPN rats in experiment C shows significant differences.
§Concentrations in plasma from trunk blood used to calculate ratio.
‖Brain sample from 1 rat lost; 3 pairs of olfactory bulbs and retinas were analyzed.

Table 3. Concentrations of BCAAs (μmol/L) in Rat Plasma

Experiment	Treatment	No. of Rats	Time 0 Sample of Tail Blood			Final Sample of Tail Blood			Trunk		
			Val	Ile	Leu	Val	Ile	Leu	Val	Ile	Leu
A	Sal	2	403 ± 14§	177 ± 10	263 ± 15	353 ± 37	158 ± 5§	216 ± 9	280 ± 59	118 ± 19	166 ± 17
	TPN	4	390 ± 22	171 ± 11	260 ± 16	732 ± 56*†	364 ± 23*†	671 ± 130†	1,190 ± 136*†‡§	775 ± 101*†‡§	1,158 ± 153*†
B	Sal	2‖	325 ± 3	148 ± 0	215 ± 4	288 ± 10	135 ± 2†	179 ± 12	267 ± 67	129 ± 33	173 ± 59
	TPN	2‖	291 ± 30	136 ± 18	198 ± 24	607 ± 21*†	364 ± 4*†	502 ± 17*†	672 ± 53*†	493 ± 86*†	698 ± 157*

NOTE. Results are the mean ± SEM. Rats in experiment B received no drug; all others received NSD-1015 as described.

*Different from Sal rats in same experiment.

†Different from time 0 value in same experiment.

‡Different from final sample from tail in same experiment.

§Different from corresponding value in experiment B.

‖Two samples were lost due to malfunctioning of the autosampler of the amino acid analyzer.

mean concentrations of BCAAs were doubled by TPN, NSD-1015 caused further elevations in plasma concentrations of Val and Ile (experiment A). In experiment B (no drug), there was no significant elevation of BCAAs in the trunk blood of TPN rats compared with the final samples of tail blood.

The combined effect of the drug in elevating plasma and brain Tyr and the high plasma levels of BCAAs in attenuating this elevation in the brain produced a difference between Sal and TPN rats in the rate of synthesis of DOPA in the brain in experiment A and in the olfactory bulb in experiment C (Table 2). In experiment A, mean DOPA concentrations in the retina of combined TPN rats (n = 4) were not different from those in Sal rats (n = 2). However, the mean DOPA content of the retina in rats on TPN (n = 2) was 576 ± 79, and in rats on TPN plus vitamin B_6 (n = 2), 1,015 ± 63. When all TPN rats (n = 4) were combined, the mean values for retinal DOPA in TPN and Sal rats were not different. Further experiments would be required to determine whether vitamin B_6 affects DOPA synthesis in the retina. We prefer to draw no conclusion from these data, because of the difficulty in controlling the time required to dissect each retina and also because of the small number of animals in each group.

Additional experiments might produce more consistent data but would be difficult to justify, since the drug artificially elevates Tyr concentrations in neural tissue. From these experiments with NSD-1015, it is unwise to conclude that low-Tyr TPN does not consistently reduce the rate of synthesis of catecholamines in neural tissue.

Because NSD-1015 has been widely used to study the conversion of Tyr to DOPA, further investigation of the metabolic effects of the drug was undertaken. The elevation of both Tyr and BCAAs in the plasma of drug-treated TPN rats suggests that NSD-1015 inhibits not only the target enzyme, aromatic amino acid decarboxylase, but also the vitamin B_6–dependent enzymes, TAT and BCAT, which normally regulate plasma levels of Tyr and BCAAs, respectively. Plasma and tissue concentrations of the vitamin B_6 derivatives PLP and PMP, which are interconverted during transamination, are shown in Table 4. In experiment A, a fourfold increase in the concentration of PN (TPN plus vitamin B_6) did not increase the plasma concentration of PLP over the level in rats on unsupplemented TPN; thus, these data are not reported separately. The data in Table 4 indicate the following: (1) TPN (and PTPN) rats (n = 16) showed a significant increase in the plasma concentrations of PLP in final samples of tail blood compared with values at time 0 and with final samples of tail blood in chow-fed rats (n = 13). This elevation was observed in tail blood sampled at day 1 (experiment B) and did not increase further when tail blood was sampled on day 2 (experiments A and C). (2) In either Sal or TPN rats of experiment B (no drug), PLP in trunk blood was not different from that in the final samples of tail blood. (3) When rats of experiment B were compared with drug-treated rats, NSD-1015 decreased PLP in the plasma from trunk blood, PLP and PMP in the liver, and PMP but not PLP in the heart of chow-fed (n = 9) and TPN (n = 12) rats; in drug-treated TPN rats (n = 12), brain PLP was lower than in TPN rats of experiment B. (4) In drug-treated TPN rats (n = 12), the concentrations of PLP and PMP in the liver and PMP in the heart were lower than in chow-fed drug-treated rats (n = 9).

PLP concentrations reported in Table 4 were measured by the Tyr apodecarboxylase assay,[16] in which liver extracts are diluted 1,500-fold. HPLC assays[15] for PLP in the heart and brain of all rats and in the liver of rats not treated with NSD-1015 yielded results similar to those in Table 4. However, HPLC analyses for PLP in undiluted extracts of liver from drug-treated rats yielded much lower values than the decarboxylase assay. This may be due to increased dissociation of the hydrazone formed between NSD-1015 and PLP[6] in the diluted samples of liver extract used in the enzymatic assay. We investigated the reaction of PLP and NSD-1015 and estimated the dissociation constant of the hydrazone to be approximately 6 μmol/L at pH 7.6.

In drug-free rats, the concentration of PMP in the heart was severalfold higher than for PLP, unlike the relationship of PMP to PLP in the liver and brain (Table 4). Since NSD-1015 decreases the concentration of PMP in the heart, the effect of the drug on PMP in vitro was examined. PMP and NSD-1015, both at 0.1 mmol/L, were incubated in phosphate buffer, pH 7.6, for 60 minutes. There was no evidence of a reaction; the incubation did not change the concentration of PMP, as determined spectrophotometrically or by HPLC.

Measurements of the activity of aminotransferases are shown in Table 5. Assays were conducted both with and without added PLP. In assays of TAT activity, it should be noted that the homogenates (and thus both NSD-1015 and TAT) are diluted 2,000-fold. When assays of TAT activity in drug-free Sal rats were supplemented with PLP, the observed increase indicates that in these highly diluted samples only 40% of hepatic TAT was present as holoenzyme. From the data in Table 5, we conclude the following: (1) In both drug-treated and drug-free rats, TPN causes a marked increase in the activity of TAT over

Table 4. Concentrations of PLP and PMP in Plasma, Liver, Heart, and Brain

Experiment(s)	Treatment	No. of Rats	Plasma PLP (nmol/L) Time 0	Plasma PLP (nmol/L) Final Sample (tail)	Plasma PLP (nmol/L) Trunk	Liver (nmol/g) PLP	Liver (nmol/g) PMP	Heart (nmol/g) PLP	Heart (nmol/g) PMP	Brain (nmol/g) PLP	Brain (nmol/g) PMP
A, B, C, D	Chow-fed	13	738 ± 55‖	824 ± 64							
A, B, C	TPN, PTPN	16	728 ± 48¶	1,280 ± 90*†							
A, C, D	Chow-fed	9	740 ± 142#	860 ± 84	434 ± 60†‡§	22.6 ± 2.1§	15.6 ± 0.8§	9.2 ± 0.3	18.9 ± 0.7§	7.6 ± 0.4	9.6 ± 0.2
A, C	TPN	12	723 ± 66**	1,320 ± 120*†	270 ± 22*†‡§	14.6 ± 0.8*§	9.5 ± 0.3*§	9.1 ± 0.3	16.6 ± 0.6*§	7.4 ± 0.2§	9.3 ± 0.1
B	Sal	4	745 ± 35	743 ± 84	812 ± 90	38.3 ± 1.1	26.6 ± 1.8	9.5 ± 0.2	33.8 ± 1.4	7.8 ± 0.4	10.0 ± 0.3
	TPN	4	738 ± 70	1,140 ± 10*†	1,130 ± 70*†	32.1 ± 0.9	22.1 ± 1.0	9.6 ± 0.2	34.2 ± 0.9	8.3 ± 0.1	9.5 ± 0.4

NOTE. Results are the mean ± SEM. Rats in experiment B received no drug; all others received NSD-1015 as described.
*Different from corresponding value for chow-fed or Sal rats in same experiment or group of experiments.
†Different from time 0 value for same animals.
‡Different from value in final sample of tail blood in same group.
§Different from comparable group of rats in experiment B.
‖No time 0 values in experiments A and D; n = 8.
¶No time 0 values in experiment A; n = 12.
#n = 4.
**n = 8.

the level in chow-fed rats in the same experiment. (2) NSD-1015 decreases the activity of TAT in both TPN and chow-fed rats killed in the morning (experiment A compared with experiment B). Yanagi et al[18] have shown that protein feeding rapidly increases the activity of hepatic TAT in rats. This can explain why the TAT activity in drug-treated chow-fed rats in experiments C and D (killed in the evening) was increased over that of drug-free rats in experiment B (killed in the morning); however, feeding is not a factor in TPN rats, and in these animals, the activity of TAT was consistently lower in drug-treated versus drug-free rats. (3) TAT activity is generally increased when the assay mixture is supplemented with PLP. In drug-treated animals, addition of PLP causes a greater numerical increase in TAT activity in TPN versus Sal rats.

Measurements of BCAT activity in the heart indicate the following: (1) Within an experiment, the activity of BCAT in TPN and Sal rats does not differ. This is true for both drug-treated and drug-free rats. Thus, BCAT activity in the heart is not affected by TPN. (2) NSD-1015 decreases the activity of BCAT in both chow-fed and TPN rats compared with drug-free rats. (3) Stimulation of BCAT activity by addition of PLP to the assay mixture is seen in all drug-treated rats, but not in drug-free TPN rats. (4) Supplementation of the assay mixture with PLP restores normal BCAT activity in drug-treated rats of experiments C and D; in experiment A, normal BCAT activity is not fully achieved by supplementation with PLP. (5) In experiment C, the presence of Glu(Tyr) in PTPN does not change the activity of either TAT or BCAT versus the values in TPN rats.

Dyck[5] reported that NSD-1015 inhibits rat liver TAT activity in vitro with an IC_{50} of 30 μmol/L. We also found that addition of NSD-1015 to approximately 30 μmol/L in assay mixtures produces 50% inhibition of TAT and BCAT activities in homogenates of liver and heart, respectively, from a drug-free Sal rat.

DISCUSSION

The initial aim of this study was to examine the effect of low-Tyr TPN on the synthesis of catecholamines in neural tissue. The hydrazine drug, NSD-1015, was used to inhibit aromatic amino acid decarboxylase, blocking the further metabolism of DOPA and thus permitting measurement of the activity of Tyr hydroxylase via the rate of accumulation of DOPA. Tyr

Table 5. Activities of TAT in Liver and BCAT in Heart

Experiment	Time Tissue Obtained	Drug Dosage (mg/kg body weight)	Treatment	No. of Rats	Liver TAT (nmol/mg protein/30 min) 0 PLP	Liver TAT (nmol/mg protein/30 min) +PLP§	Heart BCAT (nmol/mg protein/30 min) 0 PLP	Heart BCAT (nmol/mg protein/30 min) +PLP§
A	AM	100	Sal	2	186 ± 16‡	477 ± 105	185 ± 36‡	615 ± 19†‡
			TPN	2	576 ± 65*‡	2,060 ± 370*†	150 ± 17‡	563 ± 20†‡
			TPN + B_6‖	2	743 ± 158	2,410 ± 330*†	144 ± 10‡	504 ± 31‡
B	AM	0	Sal	4	310 ± 19	840 ± 91†	684 ± 32	796 ± 31†
			TPN	4	1,250 ± 150*	2,610 ± 240*†	767 ± 37	889 ± 51
C	PM	100	Sal	4	293 ± 16	817 ± 85†	290 ± 56‡	843 ± 65†
			TPN	4	515 ± 56*‡	1,600 ± 140*†‡	210 ± 29‡	766 ± 78†
			PTPN	4	467 ± 34*‡	1,430 ± 160*†‡	217 ± 21‡	781 ± 67†
D	PM	100	Unoperated	3	314 ± 47	739 ± 77†	340 ± 47‡	814 ± 58†

NOTE. Results are the mean ± SEM.
*Different from corresponding value for Sal rats in same experiment.
†Different from 0 PLP value in same experiment.
‡Different from corresponding value for rats in experiment B.
§Assay mixture supplemented with 70 μmol/L PLP.
‖Contains a fourfold increase in the pyridoxine HCl content of TPN.

hydroxylase, which occurs as a single isozyme in rat neurons,[19] catalyzes the initial and rate-limiting reaction in the biosynthesis of the neurotransmitters dopamine, norepinephrine, and epinephrine.

Unfortunately, administration of the drug that permits DOPA to accumulate also elevates the concentration of Tyr in the brain, altering one of the parameters to be studied. However, the increase in brain Tyr (Table 2) was not commensurate with the large increase in plasma Tyr (Table 1) in drug-treated rats. We attribute this to the high circulating levels of BCAAs produced by the use of pediatric TPN. These "large neutral amino acids" compete with Tyr, Trp, and Phe for transport into the brain.[20] This is clearly illustrated by the significantly higher brain to plasma ratios of Tyr, Phe, and Trp in chow-fed compared with TPN and PTPN rats (Table 2). Thus, the combined influences of the drug and the high circulating concentrations of BCAAs keep brain Tyr concentrations at 26% of the levels in Sal rats, although, in TPN rats, brain Tyr concentrations are 75% higher than in drug-free TPN rats (Table 2).

It is thus understood that measurements of DOPA in neural tissue in these studies underestimate the influence on the activity of Tyr hydroxylase of the more severe deficiency of brain Tyr that exists before drug treatment. Nevertheless, in experiments A and C, the brain and olfactory bulb, respectively, of TPN rats contain significantly lower concentrations of DOPA than those of Sal rats.

The conclusion that NSD-1015 elevates plasma Tyr in both Sal and TPN rats (Table 1) supplements Dyck's report[5] that NSD-1015 increases brain Tyr. The relationship between the activity of Tyr hydroxylase and the ambient concentration of Tyr has been difficult to establish because Tyr hydroxylase activity is enhanced by increased neuronal firing, whether mediated by nerve stimulation[21] or by stresses such as cold[22] and immobilization,[23] and may be influenced by ambient concentrations of catecholamines,[24] as well as Tyr,[25] Phe,[26] and the cofactor tetrahydrobiopterin.[27] In the retina, Tyr hydroxylase is also activated by light.[28] Rapid increases in the activity of Tyr hydroxylase occur through covalent modification of the enzyme by phosphorylation.[29] Because of the many factors that influence the activity of Tyr hydroxylase and the elevation of plasma Tyr by NSD-1015 in these experiments, the finding of any reduction in the rate of DOPA synthesis is surprising. It is possible that the lower concentrations of brain Tyr that would occur in the absence of drug treatment would more significantly decrease the rate of synthesis of DOPA and thus of catecholamines. These findings arouse concern about the very low concentrations of plasma Tyr that result from the use of TPN in premature infants.[2]

It should be possible to produce normal concentrations of Tyr in the brain by limiting BCAAs to amounts that maintain normal plasma concentrations and by adding peptides such as Glu(Tyr) to produce normal plasma concentrations of Tyr. This and a prior report[2] showing that Glu(Tyr) provides normal concentrations of Tyr in the plasma but not in the brain of rats can now be explained by the concomitant high plasma concentrations of BCAAs that compete with Tyr for transport into the brain.

Because NSD-1015 elevates Tyr in the plasma and brain, we realized that our original question of the effect of low-Tyr TPN on catecholamine synthesis could not be definitively answered by these experiments. However, we were able to investigate further the effects of the drug on the metabolism of both Tyr and BCAAs. We found that the observed elevation of Tyr by NSD-1015 may be attributed to inhibition of the activity of liver TAT. Lightcap et al[6,7] have reported that NSD-1015 forms a hydrazone with both free and enzyme-bound PLP and that a PLP-enzyme is inhibited best by hydrazines that are isosteres of the substrate of the enzyme. We found that the drug decreases plasma and tissue concentrations of PLP, the coenzyme of TAT. Thus, it is not surprising that we observed inhibition of TAT by NSD-1015 in vivo.

It is apparent that TAT activity is strongly induced in the liver of rats on TPN compared with chow-fed rats (Table 5). A high-protein diet is known to induce TAT[18]; the continuous administration of amino acids in TPN simulates the effect of a high-protein diet. The infusion of TPN in this study was set at a rate that prevented weight loss in TPN rats.[2] The macronutrients in the TPN mixture consist (by weight) of 76.6% carbohydrate (glucose), 6.5% lipid (chiefly triacylglycerols), and 16.9% protein (amino acids). This compares with the 63.2% carbohydrate, 5.9% fat, and 30.8% protein that comprise the digestible nutrients of the rodent chow provided to all chow-fed rats. Plasma concentrations of BCAAs, as well as Phe, are significantly elevated by infusion of TPN containing an amino acid mixture designed for pediatric use (Tables 1 and 2). Leu has been shown to induce TAT in rat hepatoma cells.[30]

Because the requirement for vitamin B_6 increases with a high-protein diet,[31] the PN content of the TPN was quadrupled in one group of rats (experiment A). This supplementary PN had no significant effect on plasma concentrations of aromatic amino acids or BCAAs, concentrations of PLP or PMP in the liver, heart, or brain, PLP in plasma (data not shown), or the activities of TAT or BCAT (Table 5) compared with the values in rats on unsupplemented TPN. A difference in retinal DOPA in supplemented versus unsupplemented rats was noted.

Data on the effect of NSD-1015 on BCAA concentrations in the plasma (Table 3) suggest that the drug also affects the activity of BCAT in muscle, the enzyme that initiates catabolism of BCAAs. We investigated the activity of BCAT in heart muscle, which expresses the same isozyme of BCAT as found in skeletal muscle.[32] The data in Table 3 indicate that the drug affects the ability of BCAT to influence plasma concentrations of BCAAs only when these concentrations are significantly above normal (TPN rats); the drug does not affect plasma concentrations of BCAAs in Sal rats. Since in Sal rats the activity of BCAT in the heart is reduced about 60% by drug treatment (Table 5), it appears that under normal conditions less than half of the available BCAT activity in muscle suffices to maintain normal plasma concentrations of BCAAs.

In these experiments, TPN does not induce BCAT activity in the heart. The drug-induced decrease in BCAT activity in the heart muscle of Sal rats is overcome by supplementation of the assay mixture with PLP. NSD-1015 may form a hydrazone with TAT and BCAT, as Lightcap et al[7] have reported for γ-aminobutyric acid aminotransferase. Such a complex might increase the rate of turnover of TAT, which in rats normally has a half-life of approximately 2 hours.[33] It is possible that the hydrazone remains bound to the TAT and is only slowly displaced by PLP during the assay.

Aminotransferases are unique among the vitamin B_6–

requiring enzymes in that enzyme-bound PLP and PMP are interconverted during catalysis. In both drug-treated and drug-free animals, less PLP and PMP are found in the liver of TPN versus chow-fed rats (Table 4). This may be related to increased lipid in the liver of TPN rats. In previous studies in this laboratory,[34] we found that the liver lipid content of Sal and TPN rats was 4% and 7%, respectively.

A number of conclusions can be drawn from the results of these studies: (1) The use of NSD-1015 increases brain concentrations of Tyr above those in drug-free rats, and thus cannot adequately address the question of the effect of chronically low Tyr levels, created by the use of low-Tyr TPN, on the synthesis of catecholamines in neural tissue. Any observed decrease in the rate of synthesis of DOPA in drug-treated TPN rats must be presumed to underestimate that which would obtain in drug-free animals on TPN. (2) Use of low-Tyr TPN in rats induces TAT, the major enzyme responsible for the catabolism of Tyr. This has the potential to exacerbate the already marked deficiency of Tyr in TPN mixtures, which is not compensated by the presence of high concentrations of Phe. This could be of special significance in the response of premature infants to TPN, since no current commercial mixture of amino acids provides adequate Tyr to maintain plasma Tyr levels similar to those in breast-fed infants. (3) It is of interest that chronically low levels of plasma Tyr do not reverse the induction of TAT, well documented to be under the control of glucocorticoids.[35] (4) NSD-1015 inhibits other PLP-dependent enzymes (eg, TAT and BCAT) in addition to aromatic amino acid decarboxylase. This results in changes in the concentration of other amino acids (eg, Tyr and BCAAs) and presumably their metabolites. (5) Above-normal concentrations of BCAAs in TPN limit the drug-induced elevation of Tyr in the brain. (6) Although rats on TPN containing Glu(Tyr) (PTPN) have normal concentrations of Tyr in the plasma (Table 1), brain Tyr remains low (Table 2) because transport into the brain of Tyr provided by Glu(Tyr) is impeded by the high circulating concentrations of BCAAs in these rats. The use of Glu(Tyr) in conjunction with normalization of plasma concentrations of BCAAs should provide the brain with normal concentrations of Tyr.

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