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Research Article

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ABSTRACT In vitro studies have suggested that catabolism of branched chain amino acids is linked with alanine and glutamine formed in, and released from, muscle. To explore this possibility in vivo, static and kinetic studies were performed in three patients with classical, and one patient with partial, branched chain α-ketoacid decarboxylase deficiency (maple syrup urine disease, MSUD) and compared to similar studies in eight age-matched controls. The subjects underwent a 24–30-h fast, and a glucose-alanine flux study using stable isotopes. Basal plasma leucine concentrations were elevated ($P < 0.001$) in patients with MSUD ($1,140\pm125$ μM vs. 155 ± 18 μM in controls); and in contrast to the controls, branched chain amino acid concentrations in plasma increased during the fast in the MSUD patients. Basal plasma alanine concentrations were lower ($P < 0.01$) in patients with classical MSUD (153 ± 8 μM vs. 495 ± 27 μM in controls). This discrepancy was maintained throughout the fast despite a decrease in alanine concentrations in both groups. Plasma alanine and leucine concentrations in the patient with partial MSUD were intermediate between those of the controls and the subjects with the classical form of the disease. Circulating ketone bodies and glucoregulatory hormones concentrations were similar in the MSUD and normal subjects during the fast.

Alanine flux rates in two patients with classical MSUD (3.76 and 4.00 μmol/Kg per min) and the patient with partial MSUD (5.76 μmol/Kg per min) were clearly lower than those of the controls (11.72 ± 2.53 [SD] μmol/Kg per min). After short-term starvation, glucose flux and fasting concentrations were similar in the MSUD patients and normal subjects.

These data indicate that branched chain amino acid catabolism is an important rate limiting event for alanine production in vivo.

INTRODUCTION

During periods of caloric deprivation, muscle serves as a source of potential gluconeogenic amino acids. Two amino acids, alanine and glutamine, are released from muscle in excess of their content in tissue protein and are presumed to be formed from other substrates within the myocyte (1). Recent in vitro studies have implicated branched chain amino acid catabolism in the production of alanine from both rat diaphragm (2) and epitrochlaris muscle (3).

In normal human subjects, it is difficult to document the relative contribution of branched chain amino acid catabolism to alanine production. Therefore, we have studied the hormone and substrate responses to fasting and the dynamic flux of glucose and alanine in four children with congenital enzymatic defects in the oxidative decarboxylation of branched chain amino acids (maple syrup urine disease, MSUD[1]). These studies demonstrate that alanine flux in these children inversely correlates with the severity of the enzymatic defect, when compared to the flux in normal children.

Patient population. Three related Caucasian children with classical MSUD (C-MSUD) and one Negro child with partial MSUD (P-MSUD) were studied (Table I). During this study they were consuming an artificial diet containing 50–60 mg/kg body wt of leucine, 1.5–2 g/kg body wt of crystalline amino acids (MED-71004, General Biochemicals Div., The Mogul Corp., Chagrin Falls, Ohio) and 70–90 cal/kg body wt per day. The patient with P-MSUD was 30-mo-old and was controlled on a 1-g/kg body wt protein diet. Her branched chain α-keto acid oxidative decarboxylase activity was 15% of normal in peripheral leukocytes.

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[1] *Abbreviations used in this paper*: C-MSUD, classical MSUD; MSUD, maple syrup urine disease; P-MSUD, partial MSUD.

TABLE I
Patient Population Studied

Patients	Sex	At time of diagnosis			At time of study			
		Age	Plasma leucine	Plasma alanine	Age	Height	Weight	Head circumference
			μM	μM	mo	cm (percentile)	kg (percentile)	cm (percentile)
C-MSUD								
1	M	6 wk	3,175	46	37	94.7 (50)	15.8 (60)	51.5 (50)
2	F	1.5 days	286	79	77	113.7 (40)	22.1 (70)	51.2 (50)
		3.0 days	782	90				
3	F	20 days	3,308	40	106	114.5 (<3)	20.9 (3)	51 (50)
P-MSUD								
4	F	14 mo	1,824	177	30	90.0 (50)	14.5 (75)	50 (50)
Normals								
1	F				102	124.7 (40)	27.7 (50)	54.5 (60)
2	M				69	106.3 (5)	18.5 (25)	51.0 (50)
3	F				52	100.8 (25)	15.3 (25)	50.5 (50)
4	M				88	124.1 (50)	23.0 (50)	51 (40)
5	F				86	122.4 (50)	20.3 (25)	51 (50)
6	M				122	137.1 (50)	31.2 (50)	54.4 (60)
7	M				57	106.2 (10)	15.8 (25)	52.5 (60)
8	F				107	136.3 (75)	24.9 (25)	49 (3)
Mean					85	119.7	22.1	51.7
SE					9	4.9	2.0	0.7

The fasting and flux data from the MSUD patients were compared to those of eight normal children (85±9-mo-old, mean±SE), who underwent a 30-h fast and deuterium-labeled alanine and glucose flux studies. The normal children were studied as part of an evaluation of symptoms which were suggestive of hypoglycemia. None had documented hypoglycemia before, during, or after these studies (Table I).

Study protocol. After written parental consent, all children were admitted to the Washington University Clinical Research Center at St. Louis Children's Hospital. All subjects were fasted after an evening meal (1700 h). 2–3 ml of blood were drawn at 1800 (zero time), 2400, 0800 h (14 h fasting), and every 4 h for 30 h in the normal children and 26 h in the patients with MSUD. The patients were monitored carefully throughout the fast and blood samples handled as described (4). Two of the C-MSUD patients (77- and 105-mo-old), the patient with P-MSUD, and the eight normal subjects underwent a deuterium-labeled alanine and glucose flux study.

After an overnight fast (14 h), a scalp vein needle was inserted in one arm for infusion of isotope. Another needle was placed in the contralateral anticubital fossa for blood sampling and maintained patent with a slow infusion of saline. Isotope was infused at a constant rate to enrich the circulating glucose and alanine pools between 1 and 1.5%. Samples were drawn at 0, 60, 120, 150, 180, 210, 240, 270, and 300 min during

the study. 2–3 ml of whole blood were drawn at each sampling. 0.7 ml was precipitated immediately with an equal volume of cold 3 M perchloric acid and with the remaining blood, centrifuged, separated, and stored at −80°C until assayed.

METHODS

Substrate and hormone determinations. Glucose, alanine, glutamine, glutamate, lactate, pyruvate, β-hydroxybutyrate, and acetoacetate (4, 5) were determined microfluorometrically. Insulin, glucagon, and growth hormone were measured by radioimmunoassay and cortisol by protein binding assay as described (4). Quantitative plasma amino acids were performed on a Spinco-Beckman model 119 amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (6).

Measurement of stable isotope enrichment. Flux rate determinations were carried out according to the methods of Bier et al. (7). 6, 6-di-deuteroglucose (D_2-glucose) and $2d_1$-3,3,3d_3 L-alanine (D_4-alanine) were purchased from Merck, Sharp & Dohme Canada Ltd., Montreal, Quebec, Canada. Each isotopic preparation was pyrogen-free as determined by a Food and Drug Administration-licensed commercial laboratory. The isotopes were dissolved in sterile saline, millipore filtered (0.2 μm), and autoclaved.

Glucose and amino acids from the perchloric acid extracts were separated by liquid chromatography according to the procedure of Kriesberg et al. (8) with appropriate reduction in all reagents to accommodate smaller sample size. Glucose-and amino acid-containing fractions were lyophilized to dryness. 6-0 Acetyl 1,2:3,5-di-0 (n-butane boronyl)-αD-glucofuranose was prepared from the dried glucose-containing fraction by the method of Sherman and Wiecko (9). N,O Tri-

methylsilylalanine was prepared from the dried amino acid-containing fraction by the procedure of Gehrke and Leimer (10) with the exception that the reaction was carried out at room temperature for 24 h (7).

Deuterium enrichment of glucose and alanine was measured on a PDP-12 computer-controlled LKB-9000 gas chromatograph-mass spectrometer (GC-MS) (LKB Instruments, Inc., Rockville, Md.) or a computer controlled Finnigan 3200 GC-MS (Finnigan Corp., Sunnyvale, Calif.) with an ionizing voltage of 70 eV. Because of day-to-day variation in accelerating voltage and mass spectrometer settings, a glucose or alanine standard curve of known enrichment was run before the samples. N,O Trimethylsilylalanine was separated from other amino acid derivatives on a 2 m × 2 mm 10% OV-11 column programmed from 90° to 200°C at 4°C/min. αD-glucofuranose was separated on a 2 m × 2 mm 3% OV-17 column at 210°C. Calibration curves, sensitivity, and reproducibility have been published (11).

Calculation of flux rates and statistics. Substrate inflow and outflow rates were calculated according to standard formula (12, 13). Data comparisons between the normal and MSUD subjects were performed by nonpaired two tailed Student's t test. Comparisons within groups were carried out by a paired Student's t test. All data are expressed as mean ± 1 SE unless specifically stated otherwise.

RESULTS

During the fast, plasma glucose and insulin concentrations decreased and blood β-hydroxybutyrate and acetoacetate concentrations increased in the MSUD patients and were indistinguishable from those of the normal subjects (Fig. 1). Lactate concentrations were elevated postprandially in the MSUD patients (2,737 ±292 μM, C-MSUD; 4319 μM, P-MSUD) when compared with those of the normal subjects (1,862 ±137 μM, $P < 0.02$ from C-MSUD patients). However, subsequent blood lactate and all the pyruvate concentrations were similar between the MSUD patients and the normal subjects (Table II). Plasma concentrations of glucagon, cortisol, and growth hormone obtained in both groups were similar throughout the fast (Fig. 2).

Postprandial, plasma leucine concentrations were significantly elevated in the C-MSUD patients when compared to those of normal subjects (Fig. 3) (1,137 ±25 μM vs. 155±18 μM respectively, $P < .001$). An intermediate concentration of 505 μM was observed in the P-MSUD patient. During the first 22 h of fasting the leucine concentrations of the normal subjects decreased to 105±9 μM and subsequently increased by 30 h fasting to 192±16 μM. In the C-MSUD patients plasma leucine concentrations rose throughout the fast (1,352±101 μM at 26 h fasting, $P < 0.05$ by paired t test). No change in plasma leucine was observed in the P-MSUD subject (Fig. 3).

During the first 22 h of fasting plasma isoleucine and valine concentrations decreased ($P < 0.02$) in the normal subjects and returned to basal values by 30 h of the fast. In the C-MSUD subjects, isoleucine ($P < 0.05$) and valine ($P < 0.025$) concentrations in-
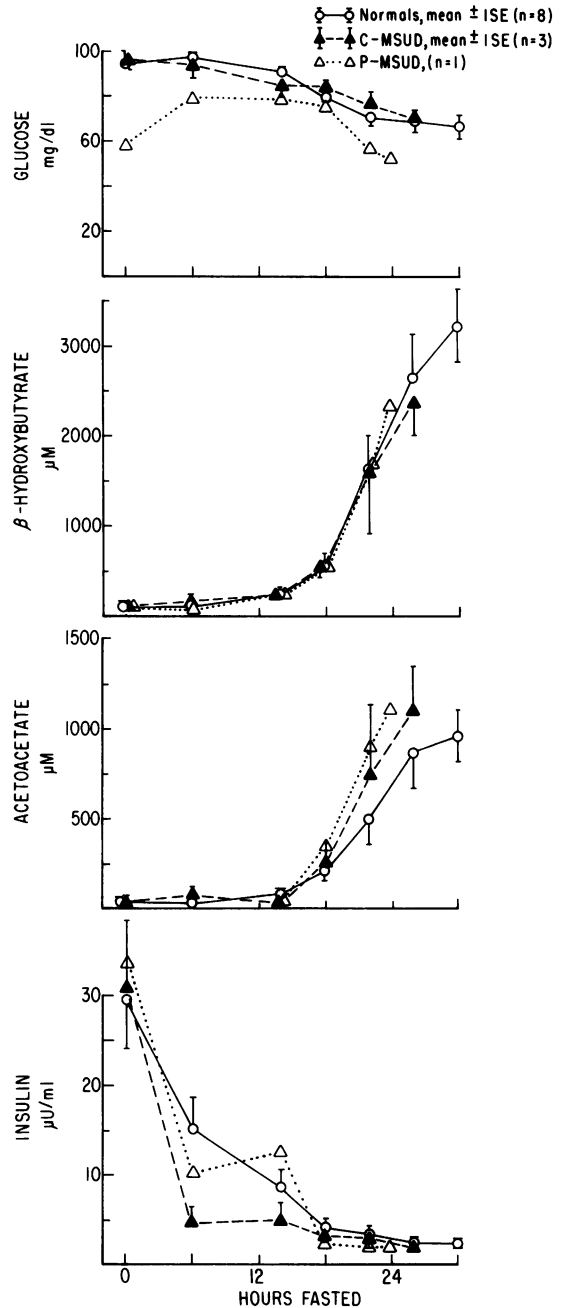

FIGURE 1 Plasma glucose, blood β-hydroxybutyrate and acetoacetate, and plasma insulin concentrations during a 24–30 h fast in three children with (C-MSUD ▲), one with (P-MSUD △), and eight normal children. Eight normal children (○): zero time of the fast is 1800 h, 1 h after their last meal. Data are expressed as mean$\pm$1 SE.

creased throughout the period of fasting. Plasma isoleucine and valine concentrations changed little in the P-MSUD subject during the 26 h of fasting (Fig. 3, Table II).

Postprandial plasma alanine concentrations were

Blood Lactate and Pyruvate and Plasma Amino Acid Concentrations during Fasting in Three C-MSUD
and One P-MSUD Patients and Eight Normal Children

										End of the fast		
Hours fasted......	0			14			22			26	24	30
Subjects..........	C-MSUD	P-MSUD	Normal	C-MSUD	P-MSUD	Normal	C-MSUD	P-MSUD	Normal	C-MSUD	P-MSUD	Normal
Substrate*							μM					
Lactate	2,740±290‡	4,320	1,860±140	1,550±260	2,180	1,420±240	1,940±520	2,980	1,360±140	1,970±520	2,950	1,390±170
Pyruvate	59±3	97	63±11	54±14	80	61±12	40±16	108	53±14	30±11	59	41±8
Aspartate	—	20	7±2	7±1‡	10	12±1	10±3	—	10±3	10±4	19	9±2
Threonine	156±12	108	151±21	81±2	84	118±11	58±13	50	78±7	51±9	40	51±3
Serine	146±13	150	162±17	121±4	98	138±12	114±31	105	107±8	97±25	98	74±7
Proline	167±17	188	309±48	101±24‡	98	175±11	71±10	99	135±21	69±7§	82	113±5
Glycine	257±33	297	219±21	253±32	229	199±17	223±59	173	116±21	196±43	155	101±13
Alanine	153±8‡	268	495±27	81±9"	96	264±34	66±1"	131	229±21	65±7§	98	185±15
Glutamine	422±52	757	587±69	468±55	454	566±50	409±56	492	528±49	409±59	308	413±32
Glutamate	77±2	60	83±15	70±9	51	63±11	65±12	66	49±9	71±12	73	40±7
Methionine¶	157±19‡	92	65±15	163±15"	87	31±6	145±12"	85	27±5	140±19"	81	14±6
Valine	282±20	469	300±37	319±111‡	389	227±22	421±18"	399	184±13	441±20§	421	291±20
Isoleucine	184±37	252	103±12	230±29"	211	69±4	260±26"	207	69±7	278±21"	209	129±11
Leucine	1,137±125"	505	155±18	1,200±75"	451	103±9	1,292±75"	468	105±9	1,352±101"	485	192±16
Tyrosine	94±36	55	110±22	27±2‡	33	56±7	32±5‡	38	49±3	35±6	42	31±6
Phenylalanine	84±15	42	68±8	37±5	34	48±4	38±6	40	48±3	38±5	46	42±2

* Data expressed as mean±SE.
‡ $P < 0.05$ from normals.
§ $P < 0.01$ from normals.
" $P < 0.001$ from normals.
¶ Methionine plus alloisoleucine (14).

lower in the C-MSUD patients when compared to those of the normal subjects (153±8 μM vs. 495±27 μM, $P < 0.001$) with an intermediate concentration observed in the P-MSUD patient (268 μM) (Fig. 4). Plasma alanine decreased in all subjects during the fast (end of fast data; C-MSUD, 69±7 μM; P-MSUD, 82 μM; normal subjects, 185±15 μM at 26 h fasting and 113±5 μM at 30 h fasting) (Fig. 4). In the normal subjects, plasma glutamine concentrations decreased from 567±34 μM to 409±59 μM by 30 h fasting. In contrast, glutamine concentrations were lower than controls ($P < 0.05$) and changed little in the C-MSUD patients during the fast (420±52 μM). The postprandial glutamine concentration was elevated (759 μM) in the P-MSUD patient and decreased throughout the fast to a concentration (308 μM) below the mean of the normal subjects (Fig. 4).

Plasma proline concentrations were lower in the C-MSUD patients when compared to those of the normal subjects but were only significantly different at the end of the fast ($P < 0.05$, Table II). Plasma proline concentrations in the P-MSUD subject were similar to those of the C-MSUD subjects during the fast (Table II). No differences were observed in the plasma concentrations of aspartate, threonine, serine, glycine, glutamate, tyrosine, or phenylalanine throughout the fast between the groups studied (Table II).

Deuterated alanine-glucose turnover studies. Dur-

ing the 5 h of the stable isotope infusion, plasma glucose concentrations and isotopic enrichment in the C-MSUD patients and P-MSUD patient were similar to those obtained in the normal subjects. Glucose flux was calculated over the last hour of the study during which time both glucose concentration and glucose enrichment were nearly constant in all subjects studied. Glucose inflow and outflow in the MSUD patients were within 2 SD of the mean of the normal subjects (Table III).

Plasma alanine concentrations were lower in all MSUD patients when compared to the normal subjects and fell during the period of study. Good isotopic equilibrium was accomplished in all subjects by 210 min of the infusion. Alanine inflow and outflow in the C-MSUD patients were >3 SD below those of the control subjects (inflow in the C-MSUD patients was 3.76, and 4.00 $\mu mol/kg$ per min vs. control of 11.72 ±2.53 $\mu mol/kg$ per min, mean±1 SD; outflow in the C-MSUD was 3.78 and 4.0 $\mu mol/kg$ per min vs. controls of 11.81±2.53 μmol, kg/min, mean±1 SD). The P-MSUD patient had analanine flux >2 SD below those of the normal subjects (inflow of 5.76 $\mu mol/kg$ per min; outflow was 5.88 $\mu mol/kg$ per min) (Table III).

DISCUSSION

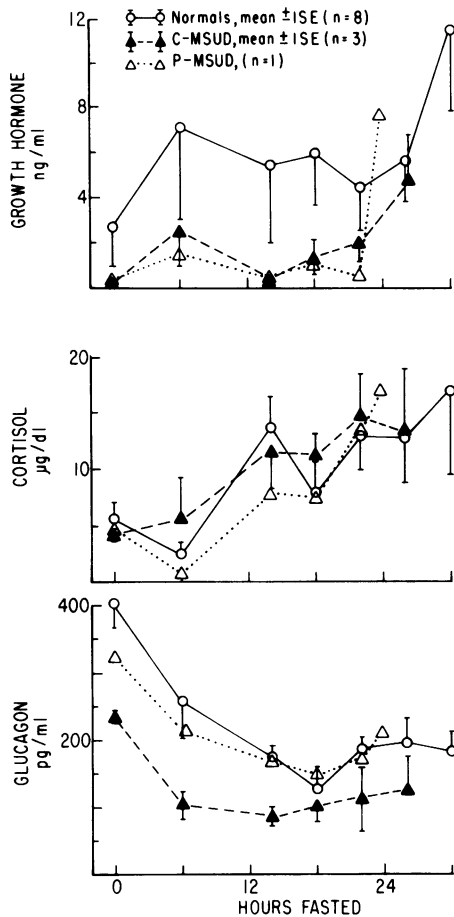MSUD is an autosomal recessive deficiency in the branched chain α-keto acid oxidative decarboxylase.

FIGURE 2 Plasma growth hormone, cortisol, and glucagon during a 24–30-h fast in three children with C-MSUD (▲), one patient with P-MSUD (△), and eight normal children (○). Zero time of the fast is 1800 h, 1 h after their last meal. Data expressed as mean±1 SE.

FIGURE 3 Plasma leucine, isoleucine, and valine concentrations during a 24–30-h fast in three children with C-MSUD (▲), one patient with P-MSUD (△), and eight normal children (○). Zero time of the fast is 1800 h, 1 h after their last meal. Data are expressed as mean±1 SE.

Several varieties of MSUD are now recognized: classical (14) and partial (intermediate and intermittent) (15–17). The severity of the clinical expression of the disease is most likely the result of the amount of residual oxidative decarboxylase activity. Less than 2% of normal activity of the enzyme has been observed in classical MSUD whereas 2–15% of normal activity have been found in patients with the partial forms of the disease (16). Because there is no endogenous de novo synthesis of branched chain amino acids, the plasma concentrations must reflect the net result of dietary intake and endogenous protein turnover. The increasing concentrations of leucine in the C-MSUD subjects during the fast are likely the result of net endogenous protein catabolism and leucine flux into the vascular space. Therefore, the decrease in plasma leucine concentrations in the normal subjects during the first 22 h of fasting may reflect accelerated leucine catabolism during this period of time; however, de-
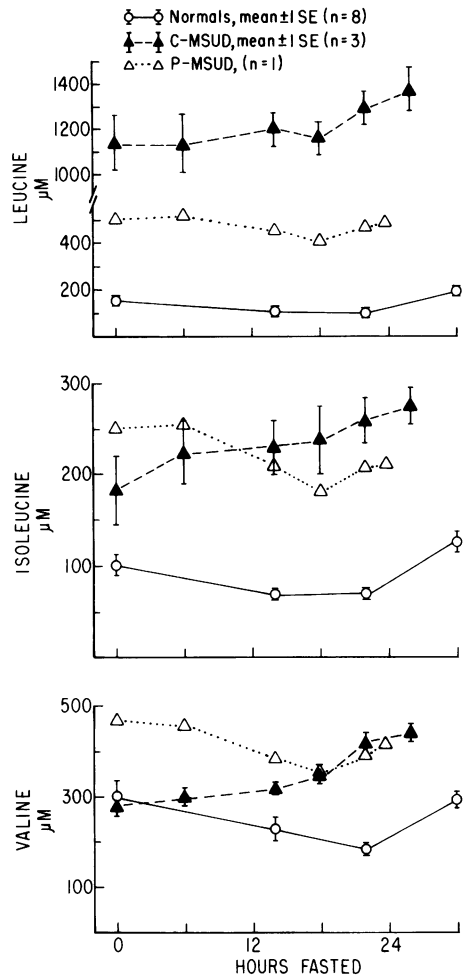
creased inflow of branched chain amino acids cannot be directly ruled out. Although the mechanisms involved are not understood, it is of interest that the subsequent rise in branched chain amino acid concentrations in both the MSUD and normal subjects paralleled the increase in blood ketone body concentrations.

The branched chain amino acids (leucine, isoleucine, and valine) are essential amino acids which are ingested in large excess to the amount necessary to sustain protein synthesis and normal growth (18). Therefore, the catabolism of branched chain amino acids may make a significant contribution as energy and nitrogen sources (19). Availability of leucine has been linked to the rates of alanine and glutamine production by muscle in vitro (2, 3). The source of the carbon skeleton of alanine is pyruvate, but whether the pyruvate is derived from glycolysis (20), catabolism of
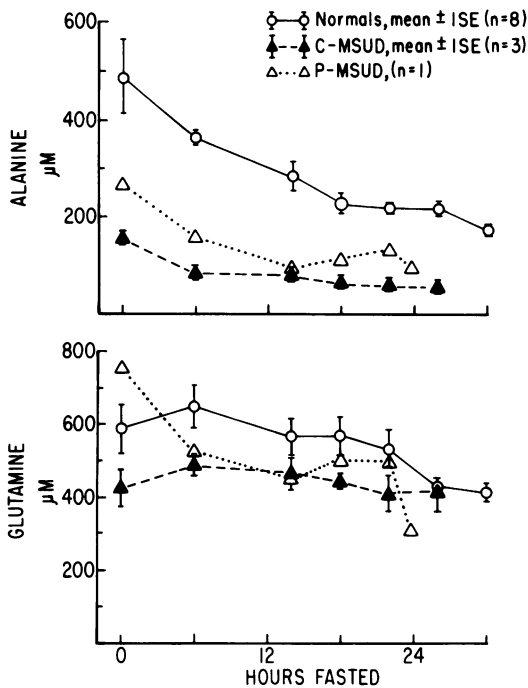
FIGURE 4 Plasma alanine and glutamine concentrations during a 24–30-hour fast in three children with C-MSUD (▲), one with P-MSUD (△), and eight normal children (O). Zero time of the fast is 1800 h, 1 h after their last meal. Data are expressed as mean±SE.

TABLE III
Glucose and Alanine Flux in MSUD and Normal Subjects

Patient	Glucose		Alanine	
	Inflow	Outflow	Inflow	Outflow
	μmol/kg/min		μmol/kg/min	
C-MSUD				
2	23.2	24.4	4.00	4.00
3	23.2	23.8	3.76	3.78
P-MSUD				
4	36.7	35.4	5.76	4.88
Normals				
1	31.3	31.6	10.62	10.43
2	41.8	41.5	12.39	12.44
3	40.3	40.8	15.09	15.29
4	31.1	31.1	15.11	15.17
5	35.2	34.8	7.78	7.96
6	21.6	18.2	11.77	11.84
7	42.4	42.3	11.65	11.55
8	25.2	25.6	9.41	9.78
Mean	33.6	33.2	11.72	11.81
SD	7.8	8.4	2.53	2.53

other amino acids (via malic enzyme [3]) or some combination of the two remains to be clarified. In contrast, the carbon skeleton of glutamine is derived from glutamate via glutamate synthetase. Because alanine is formed by the transamination of glutamate and pyruvate, glutamate is a common intermediate for both alanine and glutamine synthesis. Branched chain amino acid transaminase, the first enzymatic step in the catabolism of branched chain amino acids, converts α-ketoglutarate and branched chain amino acid to glutamate and the respective branched chain α-keto acid (21). Therefore, it is not surprising that addition of branched chain amino acids to muscle preparations in vitro whether in physiologic (2) or pharmacologic (3) concentrations, results in accelerated production of alanine and glutamine (Fig. 5A).

At the time of initial diagnosis, plasma concentrations of alanine in MSUD patients are extremely low and with dietary therapy increase but never return to normal (22). A defect in the oxidative decarboxylation of branched chain amino acids and decreased transamination of branched chain amino acids would result in decreased branched chain amino acid dependent glutamate generation (Fig. 5B). Therefore, it is of note that the alanine flux inversely correlated with the presumed relative activities of the oxidative decarboxylase in the children studied ($r = 0.841$, $P < 0.01$).

Infusion of ketone bodies has resulted in decreased alanine concentrations in adults (23), and we have observed low alanine concentrations in fasted ketotic normal children (24). Neither ketosis per se nor any other circulating hormone measured can be causally related to the decrease in alanine concentrations or flux in the MSUD subjects because the ketone body and glucoregulatory hormone concentrations were similar in all subjects studied. Although secondary metabolic effects on alanine synthesis by intracellular accumulation of the branched chain α-keto acids cannot be excluded, we believe that the primary effect is the rate of
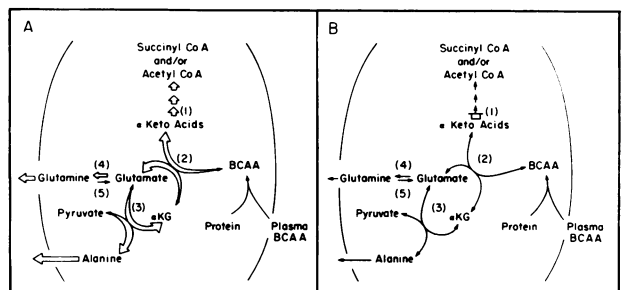

FIGURE 5 Schematic representation of the proposed metabolic event in normal subjects (A) and in MSUD patients (B). α-KG, α-keto acid, and BCAA represents α-ketoglutarate, branched chain α-keto acids, and branched chain amino acids, respectively. Key numbers in the above diagram represent the following enzymes: (1) branched chain α-keto acid oxidative decarboxylase, (2) branched chain amino transaminase, (3) glutamate-pyruvate transaminase, (4) glutamine synthetase, (5) glutaminase.

branched chain amino acid-dependent glutamate generation. If this assumption is correct, as much as 70% of alanine production in normal children may be dependent on branched chain amino acid catabolism.

Although specific flux studies of glutamine were not carried out, the observed decreased plasma concentrations of glutamine in the C-MSUD subjects throughout the fast and the P-MSUD patient at the end of the fast also could be secondary to decreased availability of glutamate. Further indirect evidence of decreased intracellular glutamate pool is the reduction in proline, because proline can be metabolized to glutamate.

During fasting in adults it has been estimated that as much as 50% of hepatic glucose production is derived from amino acids, and alanine alone may contribute up to half of this (25). Hypoalaninemia has been associated with hypoglycemia in individuals with hypopituitarism (24), ketotic hypoglycemia (4), renal failure (26) and MSUD (22). Pharmacologic testing of hepatic gluconeogenesis by alanine infusion in these disorders has demonstrated normal glucose production with the exception of MSUD. In a single patient studied in this laboratory before and after dietary therapy, alanine infusion resulted in normal alanine disappearance, no rise in plasma glucose, lactate, or pyruvate, but a sustained accumulation of glutamine. We concluded that there was a preferential shunting of three carbon units into glutamine leading to decreased net oxaloacetate production and a secondary impairment in hepatic glucose production from alanine (22). The normal glucose flux rates in the postabsorptive state and the absence of hypoglycemia in our patients were unexpected. Two explanations might be proposed: to avoid severe metabolic acidosis the MSUD subjects were fasted for only 26 h and this may not have been a sufficient challenge to elicit hypoglycemia; or, another substrate(s) (e.g., lactate, other amino acids) may have a greater fractional contribution to hepatic glucose production. Until carbon labeled compounds (^{13}C or ^{14}C) are used, the specific contribution of gluconeogenic precursors to hepatic glucose production remains to be clarified.

Although these studies were performed in the complex in vivo setting, it is only through the study of individuals with selective enzymatic deficiencies that we are able to appreciate the role of selected substrates or metabolic pathways as they relate to overall fuel homeostasis in man. Our results are consistent with in vitro studies which demonstrate an important role for branched chain amino acids in the formation of alanine in vivo and indicate that a significant percentage of alanine production in children may be dependent on branched chain amino acid catabolism.

ACKNOWLEDGMENTS

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