



## LABORATORY FINDINGS

Serum electrolyte determinations indicated the presence of a large anion gap (32–44 mEq/liter) during four of five ketotic attacks. Carbon dioxide content ranged from 13.0 to 20.8 mEq/liter, and the pH was 7.18 and 7.29. Urines were generally strongly positive for acetone. Six of the ketotic episodes were associated with leukopenia, which ranged from 1,400 to 3,600/mm<sup>3</sup>. During these episodes nonsegmented polymorphonuclear cells (mean: 33%) predominated over segmented cells (mean: 22%). Platelet counts were normal. Plasma amino acids were normal, except for glycine, which was slightly elevated on one of five tests as discussed below. Urine amino acids were also normal. Urinary amino acid values after acid hydrolysis were similar to two normal control subjects, including a 10-fold increase of glutamate over values without acid hydrolysis. This increase is apparently due to hydrolysis of phenylacetylglutamine (20). Urinary excretion of methylmalonic acid was normal. Propionic acidemia was ruled out through determination of leukocyte propionyl-CoA carboxylase (11). Because intermittent ketoacidotic episodes accompanied with the characteristic offensive odor were suggestive of isovaleric acidemia, a leucine loading test was performed as described in the following section. The results from this test led to the diagnosis of isovaleric acidemia as shown in Table 1. The basal metabolic rate (BMR) was 87.6 kcal/24 hr/kg at 6 months of age. An elevation of this degree can be attributed to a change in body composition due to malnutrition, mainly the loss of fat which has relatively low metabolic activity. However, underweight infants have a normal BMR per unit of body weight predicted from height, and when the BMR of this patient was expressed on this basis it was still elevated (60.5 kcal/24 hr/kg predicted weight vs. normal  $53.09 \pm 2.62$  SD) (13). Plasma thyroxine was normal. Blood-urea-nitrogen and serum creatinine were repeatedly normal. Since 14 months of age urinalyses consistently showed heavy proteinuria both during and between attacks. Quantitative determinations showed 518 mg protein and 135 and 250 mg  $\alpha$ -amino nitrogen in 24-hr urine specimens collected at 25 months of age. Fasting blood sugar at 5.5 years was 62 mg/100 ml and cholesterol, 81 mg/100 ml.

Oral loading tests were done with l-isoleucine (150 mg/kg), l-threonine (125 mg/kg), l-valine (250 mg/kg), and l-leucine (125 mg/kg). The details of the leucine loading test are described under Results. All other amino acids were well tolerated.

## METHODS AND MATERIALS

Serum and urine samples were stored frozen until analysis. Serum isovaleric acid was measured by the method described pre-

Table 1. Effect of glycine on acute leucine loading test<sup>1</sup>

Subjects	Serum isovaleric acid, mg/100 ml	Urine, mg/mg creatinine	
		Isovaleric acid	Isovaleryl-glycine
Control	0.05 (24)	<0.002 (22) <sup>2</sup>	<0.02 (23) <sup>2</sup>
Patient			
Baseline	0.77	0.013	6.30
Leucine (125 mg/kg)	5.60 <sup>3</sup>	0.023	9.90
Leucine (125 mg/kg) + glycine (250 mg/kg)	0.93 <sup>3</sup>	<sup>4</sup>	26.20 <sup>5</sup>

<sup>1</sup> Leucine and glycine were given as aqueous solutions; glycine was given 15 min after leucine administration.

<sup>2</sup> Calculated from the referenced data on the assumption that 3- to 5-year-old normal children excrete 100–300 mg creatinine/24 hr (3).

<sup>3</sup> Blood samples 1 3/4 hr after leucine administration. Urine samples were collected for 24 hr after leucine loading except for that described in footnote 5.

<sup>4</sup> Not measured.

<sup>5</sup> This urine sample was collected for only 12 hr.

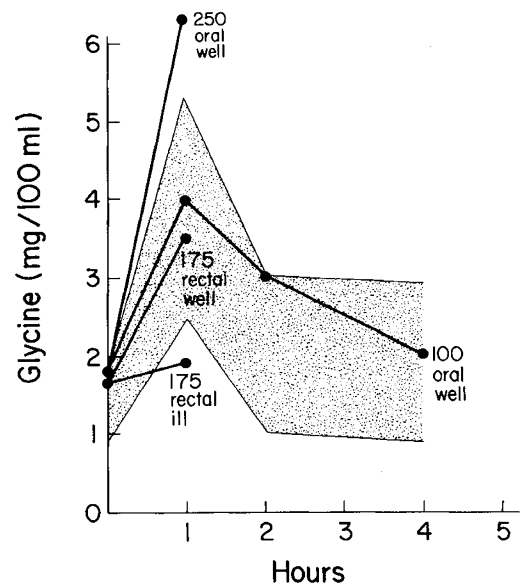


Fig. 1. Plasma glycine after oral or rectal administration of glycine in the specified amounts: 100, 175, or 250 mg/kg. Shaded area represents range of values observed in three normal infants (19) given 200 mg/kg orally.

viously (24). Urinary isovalerylglycine was determined according to the method of Tanaka and Isselbacher (23) with slight modifications. Plasma amino acids were analyzed by column chromatography with the use of a Technicon amino acid analyzer after resin deproteinization. For oral as well as rectal loading tests, amino acids were dissolved in 60 ml water.

## RESULTS

## EFFECT OF GLYCINE ADMINISTRATION ON LEUCINE LOADING TEST

In an attempt to find the proper dosage and the patient's response, glycine was given first alone in an amount of 250 mg/kg. The plasma glycine level at 1 hr was 6.12 mg/100 ml (Fig. 1). Following a dose of 100 mg glycine/kg with a meal, plasma glycine was 3.95 mg/100 ml. This value and the values obtained after 2 and 4 hr were similar to those of normal control subjects receiving a roughly comparable amount of glycine (19). A glycine dose of 175 mg/kg was then given rectally, once when the patient was well, and once during ketoacidosis. When the patient was well, plasma glycine rose to 3.54 mg/100 ml within 1 hr. In contrast, during ketoacidosis, no increase was observed in the plasma glycine level (Fig. 1). Since the amount of isovaleryl-CoA is assumed to be higher during ketoacidosis, one explanation for the low plasma glycine level would be the utilization of exogenous glycine for conjugation.

Three types of leucine loading tests were done. Each set of loading tests was performed with and without glycine administration. The first set of loading tests was done by oral administration of leucine solution in the amount of 125 mg/kg body weight. The patient excreted 6.30 mg isovalerylglycine and 0.013 mg isovaleric acid/mg creatinine, respectively, during the 24 hr preceding the loading tests. When leucine was given, vomiting started 1 3/4 hours after the loading and continued for 8 hr. Serum isovaleric acid rose from 0.77 before loading to 5.60 mg/100 ml in 1 3/4 hr. After the leucine loading, urinary excretion of isovalerylglycine and isovaleric acid during the 24 hr increased to 9.90 and 0.023 mg/mg creatinine, respectively (Table 1). When the same amount of leucine was given with 250 mg glycine/kg, vomiting did not occur. The serum sample which was taken before the leucine-glycine load was lost during analysis. However, serum isovaleric acid was elevated only to 0.93 mg/100 ml at 1 3/4 hr. It is much lower than the

value observed after the leucine loading alone and not significantly different from the baseline value in the previous loading test. Unfortunately, urine was collected for only 12 hr after the leucine-glycine loading. The amount of urinary isovalerylglycine was 26.20 mg/mg creatinine in this period (Table 1). This value is significantly higher than the values observed with leucine loading alone even when we assume that only the basal amount of isovalerylglycine was excreted in the next 12 hr (such a mean value for 24 hr would be 16.3 mg/mg creatinine).

To confirm the beneficial effects of glycine on leucine loading, two more sets of experiments were performed. In the second set of experiments, the patient was given three meals a day for 5 days, each containing about 400 mg leucine (total: 100 mg/kg/24 hr). Each meal was followed by 1 g glycine within 15 min. During this 5-day period the urine was pooled. Total urinary isovalerylglycine excretion in the 5-day period was 9,210 mg. In the following 5-day period, she was given the same diet without glycine. In this period she excreted 6,910 mg isovalerylglycine (Table 2). However, when urinary isovalerylglycine excretion in these two 5-day periods was expressed in milligrams per mg creatinine, there was no significant difference. Had the leucine load been large enough to cause ketoacidosis, we might have seen a greater difference in the excretion of isovalerylglycine during the two periods.

In the third experiment a protein meal which contained 80 mg leucine/kg body weight was given with or without glycine administration. The serum isovaleric acid levels after the meal without glycine were 1.14 and 1.01 mg/100 ml at 3 hr and at 6 hr, respectively. However, when 2 g glycine were given as an aqueous solution 15 min after an identical meal, serum isovaleric acid levels were 0.53 and 0.79 mg/100 ml at 3 and 6 hr, respectively. These values are significantly lower than the values observed when the same meal was given without glycine (Table 3).

#### CLINICAL OBSERVATIONS ON EFFECT OF GLYCINE ADMINISTRATION DURING KETOTIC EPISODES AND IN REMISSION

After demonstrating the beneficial effect of glycine through leucine loading tests the patient had another severe attack in 1974, again associated with loss of hair. Informed consent was obtained from the mother, and the child was placed on 250 mg glycine/kg/24 hr for 6 months. The glycine was administered in three doses after meals. The daily protein allowance of 1.5 g/kg was also divided into three meals with equal leucine content (total: 125 mg/kg/24 hr). Thus, the daily intake of leucine and glycine was planned to be similar to the amounts given in the loading experiments. However, repeated 24-hr dietary recall did not indicate a persistent change in food intake. Reliable assessment of the average food intake over a long period of time during chronic glycine administration was not possible because the patient was not in the hospital during this period.

In the 6 months of glycine treatment, linear growth of 5.8 cm exceeded that of the preceding 6 months by 50%. Weight gain during the same period was 2.05 kg in contrast to 0.80 kg gained in 6 months without glycine. It was noted during chronic glycine

Table 2. Effect of glycine on urinary excretion of isovalerylglycine in a 5-day period<sup>1</sup> on a diet with fixed leucine content (100 mg/kg/day)

	Urinary isovalerylglycine <sup>2</sup>	
	mg/5 days	mg/mg creatinine
No glycine	6,910	8.46
With glycine (3 g/24 hr)	9,210	8.76

<sup>1</sup> These experiments were done while the patient was in good health.

<sup>2</sup> In pooled 5-day collection.

Table 3. Effect of glycine on serum isovaleric acid after a meal containing 80 mg leucine/kg body weight

Time after meal, hr	Serum isovaleric acid, mg/100 ml	
	With glycine (2 g) <sup>1</sup>	No glycine
3	0.53	1.14
6	0.79	1.01

<sup>1</sup> Glycine was given in aqueous solution 15 min after the meal.

therapy that the child occasionally craved meat. In March 1974, glycine was discontinued for 2 days when the patient refused foods because of a sore throat. This infection induced a ketoacidotic attack. Instructions were then changed to provide for three glycine doses per day, to be given even if meals were refused. Six months later she developed a mild cold followed by diarrhea. Although she appeared well at bedtime, she awoke at 7 o'clock the next morning, after a 14-hr fast, and vomited 14 times until noon. Extra doses of oral glycine, given at the onset of vomiting, were not retained. She was therefore admitted and given 200 mg/kg glycine rectally every 6 hr as well as intravenous glucose (1 g/kg) in normal saline with insulin (1 U/5 g glucose). Whereas previous attacks lasted for at least 6 days despite intravenous fluid therapy for ketoacidosis, on this occasion vomiting stopped in the afternoon of the day of admission. She retained fluids during the night and ate a full breakfast the next morning. An anion gap of 32–44 mEq/liter was observed in the beginning but improved to 13 mEq/liter within 36 hr of treatment with glycine. In the last attack, which occurred 1.5 months later, vomiting subsided promptly with rectal glycine and intravenous fluid therapy but without insulin. An anion gap of 30 mEq/liter was observed initially in this episode but was reduced to 11 mEq/liter within 24 hr.

Plasma amino acid levels before and during glycine therapy are summarized in Table 4. Only amino acids which showed changes are listed. Before glycine therapy, plasma glycine levels observed during attacks tended to be lower than the values during remission. In one of two determinations which were conducted during remission, plasma glycine was slightly elevated. Glycine loading induced a rise of alanine and glutamate. Consequently, glycine-alanine ratios were near unity, except for two attacks of ketoacidosis, when the level of glycine was lower than that of alanine.

#### DISCUSSION

In 1967 Tanaka and Isselbacher (23) identified a large amount of isovalerylglycine in urine of patients with isovaleric acidemia. They have shown that conjugation of isovaleryl-CoA with glycine plays an important role in its detoxification in these patients. Since then treatment of patients with isovaleric acidemia by glycine administration appeared to be promising. But such an attempt has never been reported until now. In other organic acidemias such as propionic (10) and methylmalonic acidemia (17) and  $\alpha$ -methylacetoacetyl-CoA  $\beta$ -keto-thiolase deficiency (8), severe hyperglycinemia is often observed. In contrast, the plasma glycine level is usually not elevated in patients with isovaleric acidemia, with the exception of two patients (1, 6) who had a slight to moderate elevation of plasma glycine. This difference in plasma glycine concentration in these organic acidemias appears to be due to at least two factors. First, in propionic acidemia (2) and  $\alpha$ -methylacetoacetyl-CoA  $\beta$ -keto-thiolase deficiency (8), cleavage of [1-<sup>14</sup>C]glycine to <sup>14</sup>CO<sub>2</sub> was inhibited but was normal in patients with isovaleric acidemia (1). The second factor is the difference of affinity of glycine *N*-acylase for various acyl-CoA's. Propionyl-CoA and methylmalonyl-CoA are poor substrates for this enzyme (4). Therefore, no glycine conjugates of methylmalonate or propionate, or very few (16), are detected in these diseases. Isovaleryl-CoA, on the other hand, is a very good substrate for this

Table 4. Levels of plasma amino acids<sup>1</sup> before and after glycine administration

Amino acids	Plasma amino acid levels, mg/100 ml											
	Control subjects <sup>2</sup>		Patient									
	Before 1974	After 1974	Well		Ketoacidosis			Well (1974)		Ketoacidosis (1974)		
			1970	1974	1970	1971	1974	Oral glycine load <sup>3</sup>		Rectal glycine load <sup>3</sup>		
							250 mg/kg	100 mg/kg <sup>4</sup>	175 mg/kg <sup>4</sup>	175 mg/kg <sup>4</sup>		
Threonine <sup>5</sup>	1.54	2.14	1.03	1.10 <sup>5</sup>	1.18	0.31	0.58 <sup>5</sup>	0.51 <sup>5</sup>	2.57 <sup>5</sup>	tr. <sup>5</sup>	4.89 <sup>5</sup>	
Serine	1.96	1.79	1.36	1.41	1.28	0.36	0.53	2.09	2.42	2.14	1.07	
Glutamate	3.67	3.63	1.48	3.49	1.42	1.37	0.73	3.94	2.94	3.68	4.98	
Glycine	1.98	2.33	1.98	2.10	1.55	0.46	1.73	6.12	4.44	3.51	1.57	
Alanine	3.17	3.65	2.15	2.14	1.53	0.84	1.47	5.63	5.92	3.55	2.02	
Valine	2.56	3.28	1.13	1.12	1.66	1.03	3.66	2.41	2.64	2.58	3.05	
Isoleucine	0.83	1.71	0.42	0.63	0.57	0.29	1.14	1.09	1.12	0.90	1.93	
Leucine	1.68	2.89	1.00	1.28	1.01	0.51	1.95	1.98	1.90	1.87	2.88	

<sup>1</sup> Only amino acids which showed changes are listed. Levels of other amino acids remained within normal range.

<sup>2</sup> Upper limit of normal. Two different control values for each amino acid is due to a change of instrumentation.

<sup>3</sup> Blood samples were taken one hour after a single load of specified amounts. Loading tests conducted while the patient was well were started after a 12-hr fast.

<sup>4</sup> These tests were done while the patient was on glycine therapy. The patient was receiving glycine (250 mg/kg/24 hr) during the 6-month period.

<sup>5</sup> Includes glutamine and asparagine.

enzyme (4), and glycine is very well utilized for the conjugation of isovaleryl-CoA in isovaleric acidemia (1). Thus, a large amount of isovalerylglycine is always excreted in urine of patients with isovaleric acidemia (22, 23). In the present case and also in the patient reported previously (24), plasma glycine levels seem to be lower during ketoacidotic attacks than in remission. These findings are indicative of an inadequate supply of glycine to handle increased amounts of isovaleryl-CoA produced in ketoacidotic attacks. We therefore considered it possible to enhance glycine conjugation of isovaleryl-CoA by raising the glycine concentration in tissues by exogenous administration.

The effect of exogenous glycine administration on acute leucine loading tests were therefore investigated. A marked elevation of serum isovaleric acid has been observed after leucine loading in the two patients originally reported by Tanaka *et al.* (24). In the present case a similar elevation of serum isovaleric acid was observed when leucine alone was given. However, when leucine was given with glycine the elevation of serum isovaleric acid was minimal. Vomiting occurred when leucine alone was given but it did not occur when leucine was given with glycine. More significantly, the amount of urinary isovalerylglycine was much larger after joint administration of leucine and glycine than after administration of leucine alone. A similar effect was observed when leucine was administered in the form of protein diet. Thus we are able to show that an exogenous supply of glycine is effective in enhancing glycine conjugation of an excessive amount of isovaleryl-CoA produced by leucine loading. The conjugation prevented the accumulation of free isovaleric acid, a toxic substance.

Our observations *in vivo* on this patient seem to be in accordance with the enzymologic data on bovine glycine *N*-acylase by Bartlett and Gompertz (4). They observed a very high  $V_{max}$  (12.3  $\mu\text{mol}/\text{min}/\text{mg}$  protein) and a relatively high  $K_m$  (0.19 mM) for isovaleryl-CoA. The  $V_{max}$  and  $K_m$  values are 4.4  $\mu\text{mol}/\text{min}/\text{mg}$  protein and 0.009 mM for benzoyl-CoA. If we assume that human glycine *N*-acylase has similar characteristics, then isovaleryl-CoA has a very high reactivity with glycine when concentration of isovaleryl-CoA is elevated in tissues, as in the case of patients with isovaleric acidemia. Furthermore, with the bovine enzyme, the  $K_m$  value for glycine is high (3.3 mM). Since glycine concentration in human liver tissue (74.4  $\pm$  32.2  $\mu\text{g}/\text{g}$ ) is low (21), acylation of glycine with isovaleryl-CoA may be greatly enhanced if glycine concentration in liver tissue is raised. Although they must

be confirmed by investigation of human glycine *N*-acylase *in vitro*, our assumptions seem to be supported by the observation of Ando *et al.* (1) who injected [<sup>14</sup>C]glycine into a patient with isovaleric acidemia. They observed a very high incorporation of [<sup>14</sup>C]glycine into urinary isovalerylglycine, whereas the labeling of hippurate was almost not detectable in their study. Hippurate is the glycine conjugate of benzoic acid which arises from diet as well as from metabolism of phenylalanine. It is a normal constituent in human urine and is highly labeled with [<sup>14</sup>C]glycine when [<sup>14</sup>C]glycine is injected to a control. These data indicated that exogenous glycine is utilized much more extensively for the synthesis of isovalerylglycine than for that of hippurate in the patient with isovaleric acidemia.

After we confirmed the effectiveness of glycine administration in acute leucine loading tests, the patient was placed on long term glycine administration in the hope of preventing a recurrence of ketoacidotic attacks. Long term treatment with glycine was considered safe because glycine was widely used in large amounts (7.5–25 g/24 hr) in the past in the treatment of patients with muscular dystrophy apparently without any significant side effects (7). It has also been shown that the metabolism of glycine to CO<sub>2</sub> was normal in a patient with isovaleric acidemia (1). Our patient ate more meat and grew more in weight and height in the 6 months of glycine administration than the preceding 6 months. However, it must be pointed out that during the time the patient was receiving chronic glycine therapy it was difficult to control her diet. Glycine administration did not prevent two ketotic attacks which occurred when she developed upper respiratory infections. However, the duration of these attacks was much shorter when ordinary intravenous therapy was supplemented with rectal glycine administration. Thus, although the therapeutic effect of glycine administration in acute ketotic attacks appears to be ascertained, chronic glycine therapy must be tested carefully and evaluated in the future, especially in view of the moderate elevation of alanine and glutamate observed in the course of the treatment.

Since glycine conjugation with isovaleryl-CoA is instrumental in preventing isovaleric acid accumulation during remission, it is advisable to restrict substances which compete for glycine conjugation, such as benzoic and salicylic acids. Benzoic acid is derived from fruits and increased intake of fruits seemed to have played a role in precipitating attacks during infancy in this patient. Salicylate ingestion may have been a contributing factor in the

case described by Ando *et al.* (1). In ketoacidotic episodes, correction of acidosis is important. Schachter *et al.* (18) demonstrated decreased reaction velocities for glycine conjugation at low pH levels with bovine glycine *N*-acylase. Thus, acidosis which develops from other causes may set in motion a vicious cycle in which glycine conjugation decreases because of the low pH causing accumulation of isovaleric acid and further lowering of the pH. Consequently, prevention and prompt correction of acidosis is crucial in the management of acute ketoacidotic episodes.

Two peculiar findings were observed in this patient, although they are unrelated to glycine therapy. One is the elevation of BMR. Since there was no clinical sign of hyperthyroidism and serum thyroxin was normal, the elevation of BMR is not due to hyperthyroidism. It is interesting to note that short chain fatty acids including isovaleric acid are uncouplers of oxidative phosphorylation (9). Therefore, it is possible that the observed elevation of BMR is due to the effect of elevated isovaleric acid in tissues. The other observation is the loss of hair which occurred twice during ketoacidotic episodes. Its mechanisms are currently unknown.

#### SUMMARY

Leucine, which is not normally metabolized in isovaleric acidemia, was given to a patient with this disorder as a single amino acid load and in the form of a protein meal, first alone and then in conjunction with oral glycine. It was shown that glycine has a detoxifying effect because it decreased the level of serum isovaleric acid and caused an increased urinary excretion of the nontoxic conjugation product, isovalerylglycine. Long term glycine administration was well tolerated but did not prevent ketotic episodes induced by infections. During several attacks rectal glycine was effective in shortening the duration of the ketosis.

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