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Research Article

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Inhibition by Propionyl-Coenzyme A of *N*-Acetylglutamate Synthetase in Rat Liver Mitochondria

A POSSIBLE EXPLANATION FOR HYPERAMMONEMIA IN PROPIONIC AND METHYLMALONIC ACIDEMIA

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ABSTRACT In the search for the mechanism by which hyperammonemia complicates propionic and methylmalonic acidemia the effects of a series of acyl-coenzyme A (CoA) derivatives were studied on the activity of *N*-acetylglutamate synthetase in rat liver mitochondria using acetyl-CoA as substrate. Propionyl-CoA was found to be a competitive inhibitor. The inhibition constant of 0.71 mM is in the range of concentrations of propionate found in the serum of patients with propionic and methylmalonic acidemia. Propionyl-CoA was also found to be a substrate for *N*-acetylglutamate synthetase, forming *N*-propionylglutamate. This compound was a weak activator of rat liver carbamoylphosphate synthetase; the activation constant was 1.1 mM as compared with 0.12 mM for *N*-acetylglutamate. A decreased level of *N*-acetylglutamate in liver mitochondria that would follow inhibition of *N*-acetylglutamate synthetase by propionyl-CoA would be expected to lead to hyperammonemia.

Methylmalonyl-CoA, tiglyl-CoA, and isovaleryl-CoA at a concentration of 3 mM caused 30–70% inhibition of *N*-acetylglutamate synthetase. The latter two compounds are readily detoxified by the formation of *N*-acylglycine conjugates in liver, which may prevent large accumulations and could explain why hyperammonemia is not characteristic of patients with β-ketothiolase deficiency or isovaleric acidemia in whom these compounds would be expected to be elevated.

INTRODUCTION

Propionic and methylmalonic acidemia are inherited disorders of branched-chain amino acid metabolism in which there are specific deficiencies of the activity of propionyl-coenzyme A (CoA)[1] carboxylase and methylmalonyl-CoA mutase, respectively. These enzymes are in the pathway by which four amino acids, L-valine, L-isoleucine, L-methionine, and L-threonine are catabolized. Patients with these abnormalities present clinically with the ketotic hyperglycinemia syndrome with elevated concentrations of propionate in the blood (1–7). Two other disorders, isovaleric acidemia (8) and α-methyl-β-hydroxybutyric aciduria (9) may also present with the ketotic hyperglycinemia syndrome. Involved patients have recurrent ketoacidosis, hyperglycinemia, neutropenia, thrombopenia, osteoporosis, and protein intolerance. This syndrome may be distinguished from a nonketotic hyperglycinemia (10).

Severe hyperammonemia has often been encountered in infants with propionic acidemia (11, 12) and methylmalonic acidemia (13, 14). A high degree of correlation has been obtained between the blood concentrations of ammonia and of propionate (15). Hyperammonemia has also been reported in one patient with α-methyl-β-hydroxybutyric aciduria (9). Enzymes of the urea cycle have been studied in patients with propionic and methylmalonic acidemia and have generally been found to be normal (11, 14) although carbamoylphosphate synthetase activity has been observed to be low (15).

The pathway of mammalian urea biosynthesis, which serves for the removal of ammonia, is shown in Fig. 1. The synthesis of *N*-acetylglutamate provides the natural allosteric activator of the ammonia-dependent mitochondrial carbamoylphosphate synthetase (16–18). *N*-Acetylglutamate synthetase is a mitochondrial enzyme activated by L-arginine (19, 20). Our

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[1] *Abbreviations used in this paper*: CoA, coenzyme A; V_{max}, maximum velocity.

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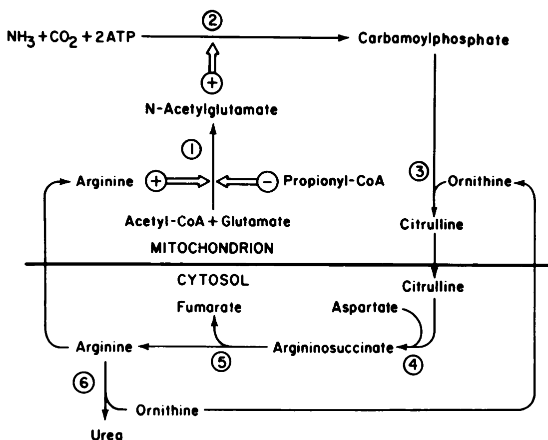

FIGURE 1 Enzymatic steps in the biosynthesis of urea: (1) N-acetylglutamate synthetase; (2) carbamoylphosphate synthetase; (3) ornithine carbamoyl transferase; (4) argininosuccinate synthetase; (5) argininosuccinase; (6) arginase. (+) indicates activation of N-acetylglutamate synthetase (1) by L-arginine and carbamoylphosphate synthetase (2) by N-acetylglutamate, and (−) indicates inhibition of acetylglutamate synthetase (1) by propionyl-CoA.

hypothesis has been that the accumulation of propionyl-CoA in the liver mitochondria of patients with propionic and methylmalonic acidemia might lead to hyperammonemia through an inhibition of the biosynthesis of N-acetylglutamate (21). It was the purpose of this study to assess the effects of propionyl-CoA and methylmalonyl-CoA on N-acetylglutamate synthetase in rat liver mitochondria. It was found that propionyl-CoA was a significant inhibitor.

METHODS

Acetyl-CoA and propionyl-CoA were prepared by the method of Simon and Shemin (22). Tiglyl-CoA, isovaleryl-CoA, and methylmalonyl-CoA were purchased from the Sigma Chemical Co. (St. Louis, Mo.). N-Propionylglutamate was synthesized according to a modification of the method of Marshall et al. (23); 60 mmol of glutamic acid suspended in 0.15 ml of H_2O were shaken at room temperature with 2 ml of propionic anhydride and then with 2 ml of water until only one phase was present; the solution was dried under nitrogen to remove propionic acid and anhydride; and then the residue was dissolved in water and adjusted to pH 6 with NaOH. A portion was analyzed by liquid-partition chromatography (24). [1-^{14}C]L-glutamic acid was purchased from Amersham Corp. (Arlington Heights, Ill.) and purified by anion-exchange chromatography on Aminex-A 25 with 0.1 N acetic acid (25).

Wistar rats weighing 200–300 g were kept on a laboratory diet that contained 24% protein. They were killed by decapitation, and the livers were rapidly removed and kept at 4°C during the preparation of mitochondria according to the method of Myers and Slater (26). Mitochondria was suspended in 0.25 M sucrose and subjected to sonic disintegration at 30 kcycles for 30 s.

Measurement of N-acetylglutamate synthetase. N-Acetylglutamate synthetase was assayed by measuring the incorporation of radioactivity from [1-^{14}C]L-glutamic acid into N-acetylglutamate. The standard assay system of 0.1 ml contained these final concentrations: mitochondrial extract (0.25 mg of protein), 50 mM Tris HCl, pH 8.5, 1 mM [1-^{14}C]L-glutamic acid (2.25 mCi/mM), 4 mM L-arginine, and acetyl-CoA and propionyl-CoA at different concentrations. After incubation for 15 min at 37°C the reaction was stopped by the addition of 0.025 ml of 0.5 N H_2SO_4. Acetyl-CoA was omitted in the blank. The reaction was linear for 20 min and gave a concave upward curve with increasing protein as reported by Tatibana and Shigesada (27). For the study of N-propionylglutamate synthesis, the blank contained propionyl-CoA but not the mitochondrial extract, because N-propionylglutamate was formed nonenzymatically at high concentrations of propionyl-CoA.

Isolation of N-acetyl-[1-^{14}C] or N-propionyl-[1-^{14}C]L-glutamate. The labeled product was separated from free [1-^{14}C]glutamic acid with 0.9 × 4-cm cation exchange columns of BIORAD AG 50 W-X4, H^+ form, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.). The acyl-[^{14}C]glutamates were eluted with 6 ml of water and were measured in routine assays by counting a portion in a liquid scintillation spectrometer. The unreacted [1-^{14}C]L-glutamate was retained on the columns. A small amount of [1-^{14}C]α-ketoglutarate formed and eluted with the acylglutamates and was corrected for by the blanks. N-Acetylglutamate and N-propionylglutamate were separated from each other and from α-ketoglutarate by liquid-partition chromatography of a sample of the water eluate on silicic acid columns eluted with 30% t-amyl alcohol in chloroform. With gradient elution in this system (24) N-acetyl-[1-^{14}C]L-glutamate coeluted with authentic standard at R_f 79.3 relative to citrate at 100, and N-propionyl-[1-^{14}C]L-glutamate coeluted with authentic N-propionylglutamate at R_f 61.5.

Identity of the product of the reaction of acetyl-CoA and [1-^{14}C]L-glutamate isolated by liquid-partition chromatography with N-acetylglutamate was confirmed by measuring its ability to activate rat liver carbamoylphosphate synthetase in an enzymatic assay (28), modified to increase the sensitivity to 2.5–40 nmol of N-acetylglutamate. The system contained the following in a final vol of 1 ml: acetylglutamate extract, sonicated rat liver homogenate (2 mg of protein), 5 mM ATP, 20 mM NH_4Cl, 15 M MgCl, 10 mM KCl, 20 mM L-ornithine, 50 mM Tris HCl (pH 7.2), and 0.2 mM $NaH^{14}CO_3$ (10 mCi/mM). Incubation was carried out at 37°C for 15 min and stopped by the addition of TCA to a final concentration of 5%. The sample was dried overnight under a heat lamp to remove free $^{14}CO_2$, and its radioactivity was determined. The labeled product cochromatographed on ion-exchange column chromatography with authentic nonlabeled citrulline. The sensitivity of the assay was in the range of 2,500 dpm/nmol of N-acetylglutamate.

Measurement of carbamoylphosphate synthetase activity and its activation by N-acetyl- and N-propionylglutamate. The system contained the following in a final vol of 1 ml: sonicated rat liver mitochondria (5 mg of protein), 5 mM ATP, 20 mM NH_4Cl, 15 mM $MgCl_2$, 10 mM KCl, 20 mM L-ornithine, 50 mM Tris HCl (pH 7.2), 10 mM $NaH^{14}CO_3$ (0.2 mCi/mM), and N-acetyl- or N-propionylglutamate at varying concentrations. Incubation was carried out at 37°C for 15 min and stopped by the addition of TCA to a final concentration of 5%. The sample was dried overnight under a heat lamp to remove free $^{14}CO_2$, and its radioactivity was determined by liquid scintillation counting.

Protein was determined by a modification of the procedure of Lowry et al. (29). All kinetic data were calculated from double reciprocal plots weighted by V^4 according to Wilkinson (30). Apparent K_m values for each sub-

strate of N-acetylglutamate synthetase were calculated as the mean of the K_m values obtained at the various concentrations of the alternate substrate. The patterns of the kinetic plots and the apparent K_m and K_i values are not precise because of problems inherent in kinetic studies with crude enzyme preparations.

RESULTS

N-Acetylglutamate synthetase in rat liver mitochondria was found to have an absolute requirement for L-arginine with an apparent activation constant (K_a) of 1 mM and, therefore, 4 mM of L-arginine was employed in all incubations. With acetyl-CoA and [1-^{14}C]L-glutamate as substrates the radioactive product was shown to be N-acetylglutamate by elution from a cation-exchange column with water, cochromatography with authentic N-acetylglutamate on liquid-partition chromatography, and the ability of the product isolated by liquid-partition chromatography to activate rat liver carbamoylphosphate synthetase. The kinetic properties of N-acetylglutamate synthetase are shown in Figs. 2A and B. The apparent mean K_m for L-glutamate was 5.0 mM and that for acetyl-CoA was 0.6 mM. The maximum velocity (V_{max}) was 5.7 nmnol/min per mg protein.

Propionyl-CoA was also a substrate for N-acetylglutamate synthetase. The radioactive product formed when propionyl-CoA and [1-^{14}C]L-glutamate were used as substrates cochromatographed with authentic N-propionylglutamate on liquid-partition chromatography. The kinetics of the synthetase in the presence of propionyl-CoA and L-glutamate are shown in Figs. 3A and B. The K_m for propionyl-CoA was 2.1 mM. This value was somewhat higher than the K_m for acetyl-CoA. The K_m for L-glutamate was 1.5 mM. The V_{max} was 0.23 nmol/min per mg protein when propionyl-CoA was used as substrate, which was only one-twenty-fifth of the V_{max} obtained with acetyl-CoA as substrate.

The synthesis of N-acetylglutamate from acetyl-CoA was competitively inhibited by propionyl-CoA (Fig. 4A).

The replot of the slopes from the double reciprocal plot against the concentration of propionyl-CoA showed that the inhibition was hyperbolic (Fig. 4B). The replot of the reciprocal of the slopes in the presence of propionyl-CoA minus the slope in the absence of propionyl-CoA vs. the reciprocal of the concentration of propionyl-CoA is shown in Fig. 4B (31). This gives a straight line with a K_i of 0.71 mM calculated from the negative reciprocal of the intercept on the abcissa (Fig. 4B). The K_i was somewhat lower than the K_m for propionyl-CoA as a substrate. Other acyl CoA esters were tested as possible substrates or in-

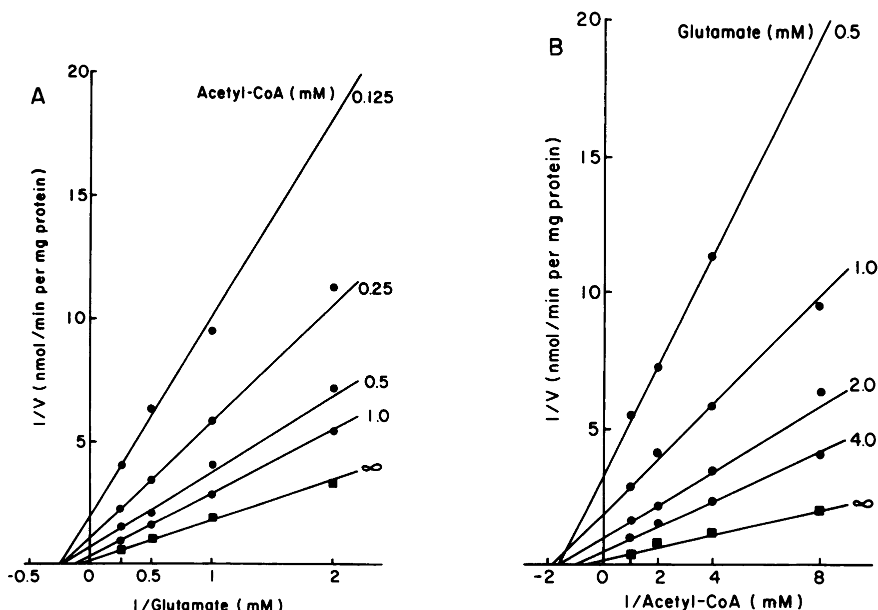

FIGURE 2 Kinetic properties of N-acetylglutamate synthetase. The substrates were acetyl-CoA and [1-^{14}C]L-glutamate. (A) Kinetics for L-glutamate: N-acetyl-[1-^{14}C]glutamate formation was measured in the presence of varying concentrations of L-glutamate and fixed concentrations of acetyl-CoA. For acetyl-CoA at infinite concentration, $1/V_{max}$-(V) from Fig. 2B was plotted. Other conditions were as specified in Methods. (B) Kinetics for acetyl-CoA: N-acetyl-[1-^{14}C]N-glutamate formation was measured in the presence of varying concentrations of acetyl-CoA and fixed concentrations of L-glutamate. For glutamate at infinite concentrations $1/V_{max}$ (V) from Fig. 2A was plotted.

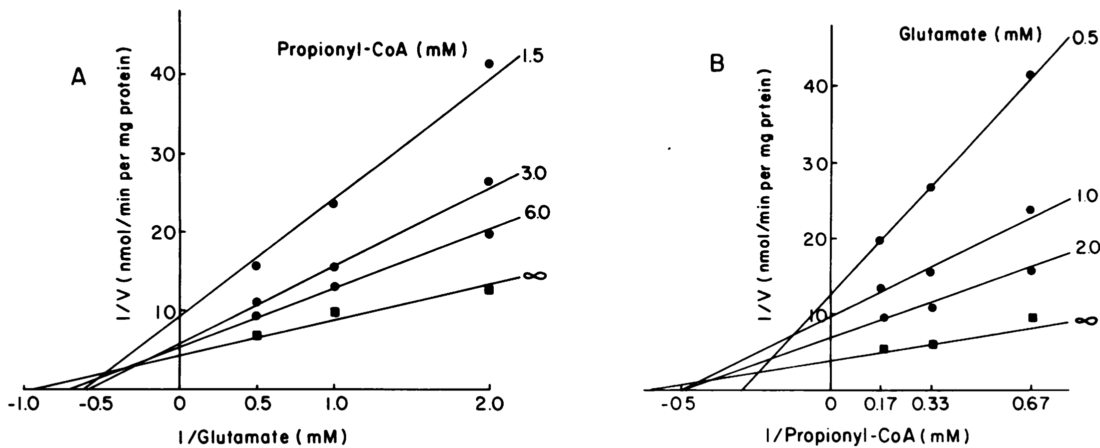

FIGURE 3 Kinetic properties of N-acetylglutamate synthetase in the presence of the substrates propionyl-CoA and [1-^{14}C]L-glutamate. (A) Kinetics for L-glutamate: N-propionyl-[1-^{14}C]glutamate formation was measured in the presence of varying concentrations of L-glutamate and fixed concentrations of L-glutamate and fixed concentrations of propionyl-CoA. For propionyl-CoA at infinite concentration, $1/V_{max}$ (V) from Fig. 3B was plotted. (B) Kinetics for propionyl-CoA: N-propionyl-[1-^{14}C]glutamate formation was measured in the presence of varying concentrations of propionyl-CoA and fixed concentrations of L-glutamate. For glutamate at infinite concentrations, $1/V_{max}$ from Fig. 3A was plotted.

hibitors of an N-acetylglutamate synthetase (Table I). Methylmalonyl-CoA, isovaleryl-CoA, and tiglyl-CoA at concentrations of 3 mM were not substrates. However, isovaleryl-CoA and tiglyl-CoA were inhibitors of the synthesis of N-acetylglutamate by 70 and 60%, respectively, at concentrations of 3 mM (Table II). Methylmalonyl-CoA was slightly inhibitory.

Because N-propionylglutamate was synthesized by N-acetylglutamate synthetase, the ability of N-propionylglutamate to serve as an activator of rat liver carbamoylphosphate synthetase was compared with that of N-acetylglutamate. N-Propionylglutamate was found to be an activator; the K_a was 1.1 mM (Fig. 5B), which was ten times the K_a of 0.12 mM for N-

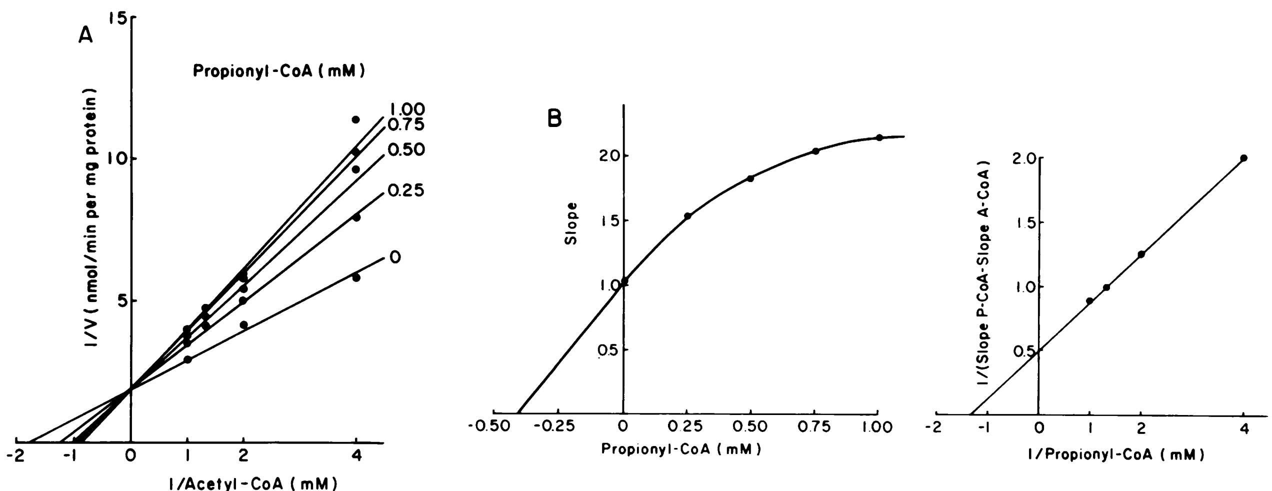

FIGURE 4 Inhibition of N-acetylglutamate synthetase by propionyl-CoA. (A) N-acetyl-[1-^{14}C]-glutamate synthesis was measured in the presence of varying concentrations of acetyl-CoA and fixed concentrations of propionyl-CoA. (B) The graph on the left shows the hyperbolic curve for the replot of the slopes from Fig. 4A vs. propionyl-CoA concentration. The graph on the right shows the straight line obtained when the reciprocal of the slope in the presence of propionyl-CoA minus the slope in the absence of propionyl-CoA was plotted against the reciprocal of the concentration of propionyl-CoA.

TABLE I
Substrate Specificity of N-Acetylglutamate Synthetase

Labeled substrate	Acyl CoA thioester	[^{14}C]*N*-acylamino acids formed
		nmol
[1-^{14}C]L-glutamate	1 mM Acetyl-CoA	0.770*
	3 mM Propionyl-CoA	0.143*
	3 mM Isovaleryl-CoA	0.008
	3 mM Methylmalonyl-CoA	0.000
	3 mM Tiglyl-CoA	0.000

Incubation conditions and separation of the products have been specified in Methods.
* The labeled acylamino acids formed cochromatographed respectively with authentic *N*-acetyl- and propionylglutamate on liquid-partition chromatography.

acetylglutamate (Fig. 5). The average V_{max} with *N*-propionylglutamate was 68% of that with *N*-acetylglutamate, and significantly lower by Student's *t* test ($P < 0.005$). Activation of carbamoylphosphate synthetase by *N*-acetylglutamate and *N*-propionylglutamate was approximately additive when both were present simultaneously (Figs. 5A and B).

DISCUSSION

N-Acetylglutamate synthetase in rat liver mitochondria preparations was found to have a K_m of 0.6 mM for acetyl-CoA and a K_m of 5.0 mM for L-glutamate, which were similar to the K_m values of 0.7 mM for acetyl-CoA and 3.0 mM for L-glutamate reported for the purified enzyme from rat liver (20, 32). Propionyl-CoA was shown to be a substrate with an affinity approximately three times less than that for acetyl-CoA. The V_{max} when propionyl-CoA was the substrate was one-twenty-fifth of the V_{max} for acetyl-CoA. Shigesada and Tatibana (19) reported that propionyl-CoA was a substrate for *N*-acetylglutamate synthetase with an activity 6% of that with acetyl-CoA at 0.5 mM. We have shown that propionyl-CoA is a competitive inhibitor of *N*-acetylglutamate synthetase with a k_i value similar to its K_m value.

These results suggest that elevated concentrations of propionyl-CoA in mitochondria of the liver of patients with propionic acidemia and methylmalonic acidemia may significantly inhibit the synthesis of *N*-acetylglutamate. Evidence for elevated propionyl-CoA is provided by the fact that these patients have plasma propionate levels as high as 5–8 mM (1, 7) and excrete propionylglycine (33). The synthesis of propionylglycine in the liver is catalyzed by glycine-*N*-acylase with a K_m for propionyl-CoA of 0.18 mM for the bovine enzyme (34). In vitamin B_{12}-deficient rats, which provide a model for methylmalonic acidemia, levels of propionyl-CoA were elevated 15-fold to 81.5 nmol/g liver. This was similar to the levels of acetyl-CoA of 79.6 nmol/g and higher than those of methylmalonyl-CoA of 57 nmol/g (35). In isolated rat livers perfused with 10 mM propionate, the concentrations of propionyl-CoA rose 10-fold to 62.15 nmol/g (36).

These data suggest that concentrations of propionyl-CoA may be elevated in the liver of patients with propionic acidemia and methylmalonic acidemia to levels higher than acetyl-CoA. Because the K_i for propionyl-CoA is similar to the K_m for acetyl-CoA, significant inhibition of *N*-acetylglutamate synthetase could result. A decreased level of *N*-acetylglutamate would decrease the activation of carbamoylphosphate synthetase and, hence, clearance of ammonia by the urea cycle (27). Some *N*-propionylglutamate may be synthesized in the liver of the patients but with an K_a for carbamoylphosphate synthetase ten times higher than *N*-acetylglutamate would not be expected to stimulate ureagenesis.

In vitro studies have suggested that a metabolite of propionate inhibits ureagenesis. Propionate did not directly inhibit carbamoylphosphate synthetase or ornithine transcarbamylase, but 5 mM of propionate

TABLE II
Inhibition of N-Acetylglutamate Synthetase by CoA Esters of Organic Acid Catabolites of the Branched-Chain Amino Acids

Labeled substrate	Acyl CoA thioester	*N*-acetyl-[1-^{14}C]glutamate formed	Percent inhibition
		nmol	*%*
[1-^{14}C]L-glutamate	0	0.770	0
+ 1 mM acetyl-CoA	3 mM Propionyl-CoA	0.204	73
	3 mM Isovaleryl-CoA	0.229	70
	3 mM Tiglyl-CoA	0.310	60
	3 mM Methylmalonyl-CoA	0.552	28

The methodology was as in Table I. The radioactive product was cochromatographed with authentic *N*-acetylglutamate on liquid-partition chromatography.

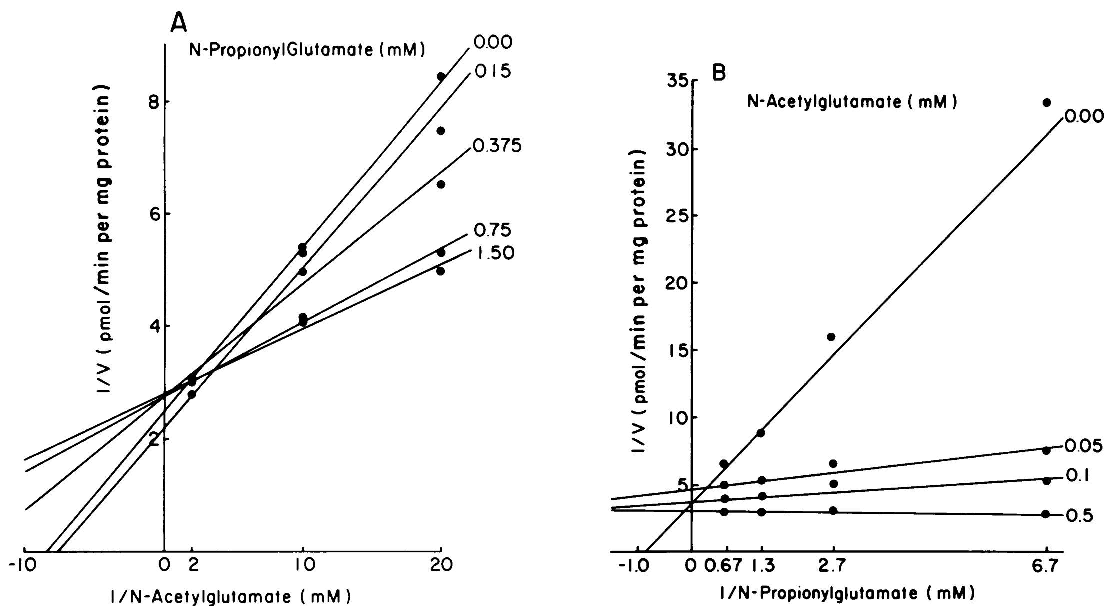

FIGURE 5 Activation of carbamoylphosphate synthetase by *N*-acetylglutamate and *N*-propionylglutamate. The formation of [^{14}C]citrulline from $NaH^{14}CO_3$ was determined at various concentrations of *N*-acetylglutamate and *N*-propionylglutamate as described in Methods.

inhibited ureagenesis from ammonia and ornithine by 40% in rat liver slices (37). Propionate also inhibited the synthesis of citrulline in isolated rat liver mitochondria (38). It was reported in a recent abstract that propionyl-CoA, and to a lesser extent tiglyl-CoA and methylmalonyl-CoA, produced time- and concentration-dependent inhibition of carbamoylphosphate synthetase activity in homogenates of human and rat liver (39). This was apparently a result of a direct inhibition of carbamoylphosphate synthetase and not of inhibition of *N*-acetylglutamate synthetase because 10 mM of *N*-acetylglutamate was added as an activator. Thus, there may be more than one site for inhibition of ureagenesis by propionyl-CoA.

In our study, methylmalonyl-CoA was only slightly inhibitory of *N*-acetylglutamate synthetase activity, and, therefore, the hyperammonemia in methylmalonic acidemia is probably caused by increased levels of propionyl-CoA. Isovaleryl-CoA and tiglyl-CoA at high concentrations were shown to inhibit *N*-acetylglutamate synthetase almost as much as did propionyl-CoA. The fact that hyperammonemia has not been described in patients with isovaleric acidemia and in only one patient with β-ketothiolase deficiency (9) may be explained by the finding that isovaleryl-CoA and tiglyl-CoA are more rapidly metabolized by glycine *N*-acylase than propionyl-CoA (34). Large amounts of isovalerylglycine are excreted by patients with isovaleric acidemia (40) and tiglylglycine by patients with β-ketothiolase deficiency (9), whereas only small amounts of propionylglycine and tiglylglycine are excreted by patients with propionic acidemia (31, 41).

It may be possible to prevent acute episodes of hyperammonemia in patients with propionic and methylmalonic acidemia by administering an activator of carbamoylphosphate synthetase to compensate for decreased levels of *N*-acetylglutamate. Analogues of *N*-acetylglutamate such as *N*-carbamoyl-L-glutamate have been shown to activate carbamoylphosphate synthetase (42). Kim et al. (43) demonstrated that *N*-acetylglutamate given intraperitoneally was ineffective in protecting against lethal doses of ammonium acetate in rats but that *N*-carbamoylglutamate along with L-arginine was effective. *N*-carbamoylglutamate might provide effective therapy in patients with propionic and methylmalonic acidemia when acute episodes of hyperammonemia first occur.

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