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N-carbamylglutamate Markedly Enhances Ureagenesis in *N*-acetylglutamate Deficiency and Propionic Acidemia as Measured by Isotopic Incorporation and Blood Biomarkers

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ABSTRACT: *N*-acetylglutamate (NAG) is an endogenous essential cofactor for conversion of ammonia to urea in the liver. Deficiency of NAG causes hyperammonemia and occurs because of inherited deficiency of its producing enzyme, NAG synthase (NAGS), or interference with its function by short fatty acid derivatives. *N*-carbamylglutamate (NCG) can ameliorate hyperammonemia from NAGS deficiency and propionic and methylmalonic acidemia. We developed a stable isotope ^{13}C tracer method to measure ureagenesis and to evaluate the effect of NCG in humans. Seventeen healthy adults were investigated for the incorporation of ^{13}C label into urea. [^{13}C]urea appeared in the blood within minutes, reaching maximum by 100 min, whereas breath $^{13}CO_2$ reached a maximum by 60 min. A patient with NAGS deficiency showed very little urea labeling before treatment with NCG and normal labeling thereafter. Correspondingly, plasma levels of ammonia and glutamine decreased markedly and urea tripled after NCG treatment. Similarly, in a patient with propionic acidemia, NCG treatment resulted in a marked increase in urea labeling and decrease in glutamine, alanine, and glycine. These results provide a reliable method for measuring the effect of NCG on nitrogen metabolism and strongly suggest that NCG could be an effective treatment for inherited and secondary NAGS deficiency. **(*Pediatr Res* 64: 213–217, 2008)**

N-acetylglutamate (NAG) is an obligatory activator of the carbamyl phosphate synthetase 1 (CPS1) reaction, the first step of the urea cycle (1). Regulation of ureagenesis, particularly in response to the ingestion of ammonia or a protein load, depends in large measure on the NAG concentration in hepatic mitochondria (2–4). A congenital absence of NAG, usually caused by a deficiency of NAG synthase (NAGS), results in hyperammonemia and developmental delay, and can result in death (5–8).

Previous studies indicate that *N*-carbamylglutamate (NCG), a stable synthetic derivative of glutamic acid, effectively activates CPS1 (1,9). Indeed, the administration of NCG to patients with a deficiency of NAGS can normalize the blood ammonia concentration (10–12). Treatment with NCG also benefits some patients with inherited diseases like propionic and methylmalonic acidemia (13,14) indicating that these patients may have diminished ammonia flux through CPS1. Thus, NCG therapy may be able to promote carbamyl phosphate synthesis in a variety of clinical settings, in which this reaction is compromised, including some forms of CPS1 deficiency, organic acidemias, disorders of fatty acid oxidation, and hepatic encephalopathy. Similarly, administration of NCG also might benefit patients who have hyperammonemia consequent to treatment with valproic acid (15).

The wide-scale application of NCG to manage patients with hyperammonemia would be enabled by the availability of a sensitive and reproducible method to assess the efficacy of this agent in promoting ureagenesis. To realize this goal, we investigated using mass spectrometry to monitor the *in vivo* synthesis of [^{13}C]urea following administration of [1-^{13}C]acetate. Our rationale was that hepatic mitochondria swiftly converted labeled acetate to $^{13}CO_2$, which is first incorporated into ^{13}C-carbamyl phosphate and, ultimately, into [^{13}C]urea. This noninvasive, stable isotope approach entails no exposure to radioactivity and is essentially free of any major risk to human subjects.

In this report, we demonstrate the feasibility of the method in healthy human volunteers. We also document that the method quantifies the normalization of ureagenesis by NCG in a patient with NAGS deficiency and in a patient with propionic acidemia.

METHODS

Controls. The healthy volunteers who were studied were young adults (age 20–40 y). A negative pregnancy test was required of all female subjects before study. The study was approved by the Institutional Review Boards at the Children's Hospital of Philadelphia and Children's National Medical Center. Informed consent was obtained from each individual before enrollment.

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Abbreviations: CPS1, Carbamyl phosphate synthetase 1; **NAG,** *N*-acetylglutamate; **NAGS,** *N*-acetylglutamate synthase; **NCG,** *N*-carbamylglutamate

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After an overnight fast, an indwelling catheter was placed in a vein for blood drawing. A baseline sample (5 mL) of heparinized blood was obtained and a sample of expired air was injected into a 20 mL glass tube under vacuum.

At time 0, each subject ingested 0.33 mmol/kg, (27 mg/kg) [1-^{13}C]sodium-acetate (98 atom % excess) dissolved in 60 mL of water. Blood samples (5 mL) were subsequently obtained at 15, 30, 45, 60, 75, 90, 120, 180, and 240 min. Each blood specimen was placed in a precooled, heparinized tube and immediately centrifuged to separate the plasma, which was kept frozen (−70°C) until analysis. The study was concluded at 240 min. Total plasma urea was measured in each sample.

Breath samples were taken concomitantly with the blood by having the subject exhale through a one-way valve into a plastic collection device.

Patient 1 (NAGS deficiency). The patient, now a 57-y-old woman, was in good health until age 40 when she developed frequent migraine headaches, intermittent staring spells, nausea, recurrent vomiting, lethargy, and ataxia followed by coma. The plasma ammonia level was as high as 500 μM. In addition, she manifested an increased blood glutamine level and diminished blood urea nitrogen. The hyperammonemia was attributed to a metabolic disorder rather than liver disease, but a specific enzyme deficiency was not identified. For 17 y, she had multiple attacks of hyperammonemia that required repeated hospitalizations. She was tentatively diagnosed with partial ornithine transcarbamylase deficiency and treated with a low protein diet and lactulose. The latter was discontinued and she was treated with Na-phenylbutyrate (Buphenyl) and L-citrulline as well as ondansetron for chronic nausea. These interventions failed to prevent persistent hyperammonemia. A high carbohydrate and low protein diet resulted in obesity and type II diabetes requiring insulin. A subsequent study in our laboratory revealed NAGS deficiency, based on mutation analysis that showed compound heterozygosity for V350I (GTC to ATC) and L442V (CTG to GTG) mutations (8).

Patient 2 (propionic acidemia). The patient was a 6-y-old boy with propionic acidemia who presented at 4 d of age with severe hyperammonemia and metabolic acidosis requiring hemodialysis. Peak ammonia levels at that time exceeded 1000 μM. The diagnosis of propionic acidemia was established by urine organic acid analysis and therapy was instituted with a special formula, L-carnitine and biotin. The addition of sodium-phenylbutyrate was required to maintain a plasma ammonia level within an acceptable range. The infant was discharged at 1 mo of age without sodium-phenylbutyrate but readmitted 2 wk later with an acute exacerbation of his illness and a plasma ammonia level of 1100 μM. He was discharged on sodium-phenylbutyrate and this was continued for a period of 7 mo. Following discontinuation of the phenylbutyrate, plasma ammonia levels ranged from slightly above normal to twice normal when the patient was well. He was admitted to the hospital on multiple occasions during the first 3 y of life, usually for acute exacerbations of his disorder but once for acute pancreatitis. During these episodes, more significant elevations of plasma ammonia were observed, ranging from 3 to 6 times normal. His development was significantly delayed. He is now ambulatory but has no speech. He is fed entirely by gastrostomy tube. Mutation analysis revealed that he is homozygous for the G216fs mutation in the PCCA gene.

The two patients described above underwent an identical stable isotope procedure described above for the healthy controls, before, and then following, treatment for 3 d with NCG (Carbaglu®, Orphan Europe, Paris, 2.2 g/m^2/d). In addition, their blood samples from each time point were also analyzed for plasma ammonia (RXL Dade Behring, Siemens Healthcare Diagnostics, Deerfield, IL) and quantitative amino acids (Biochrom, Cambridge, UK).

Measurement of [^{13}C]urea. In a plastic tube, 40 μL of 60% perchloric acid and 0.5 mL of deionized water were added to 0.5 mL of plasma. The precipitated protein was removed by centrifugation. The plastic tube was uncapped for 30 min to facilitate evaporation of CO_2. The supernatant was then transferred to a new tube and the pH was adjusted to 6–7 with KOH solution. After removal of precipitated potassium perchlorate, the sample was applied to an AG-1 column (1 mL; Cl^- form; 100–200 mesh) to remove traces of bicarbonate. The eluate was combined with a 2 mL wash of 10 mM HCl. The sample was taken to dryness in a glass test tube that was heated to 80°C. The dried sample was then left overnight in an airtight container that contained gauze soaked with 1N NaOH to remove any residual trace of CO_2. The glass tube was then flushed with helium and capped with a rubber stopper. Four hundred microliters of 0.5 M potassium phosphate, pH 6.0, containing 3 mg of urease/0.4 mL was injected through the stopper. The phosphate buffer was boiled and allowed to cool before addition of urease to avoid exogenous contamination with any CO_2. After 60 min incubation, 100 μL of 20% phosphoric acid was injected through the rubber stopper. The final phosphoric acid concentration was 0.4 mol/L. After 60 min, an aliquot of air was removed with a gas-tight syringe and was analyzed for $^{13}CO_2$ by a Finnigan Delta Plus isotope ratio-mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A commercial CO_2 source (Airgas, Radnor, PA) was used as the standard.

RESULTS

Controls. Figure 1*A* illustrates the appearance of $^{13}CO_2$ in breath for 33 studies in 17 control subjects (9 women and 8 men, 16 subjects were studied twice) who consumed a dose of [1-^{13}C]acetate. Oxidation of labeled acetate to CO_2 was rapid, with a peak label in $^{13}CO_2$ of ~0.6 atom % excess being observed by 45–60 min. Thereafter, labeling declined gradually, declining by 240 min to a level (~0.01 atom % excess) that was just above baseline.

Figure 1*B* shows the label in plasma [^{13}C]urea. This parameter increased in a sigmoid fashion during the initial hour of the study, attaining a plateau of ~0.08–0.09 atom % excess by 60 min. Enrichment in urea declined slowly thereafter, especially in comparison with $^{13}CO_2$, reflecting the slower turnover of urea compared with that of bicarbonate.

The absolute concentration of plasma [^{13}C]urea, corresponding to the product of urea concentration (mmol/L) and isotopic abundance, is shown in Figure 1*C*. By 60 min, the average concentration of this parameter was 4.01 ± 0.59 μM. Assuming a body urea distribution space approximately equal to body water (~55% of body weight), we can estimate urea synthesis after normalizing this value to the label in precursor $^{13}CO_2$ at 60 min (0.609 atom % excess; Fig. 1*A*). This gives a value of 362.2 μmol/kg/h, similar to values reported in the literature (16–18).

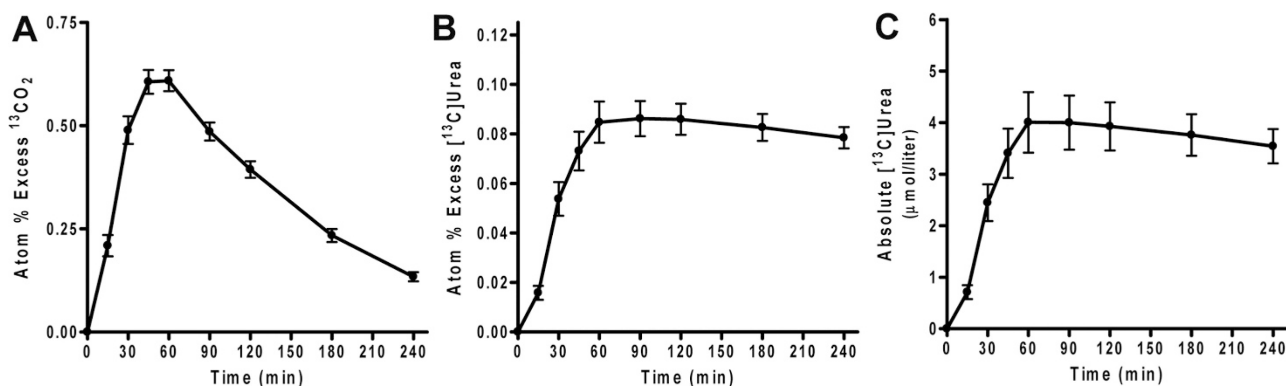

Figure 1. Increase over time of isotopic enrichment in breath $^{13}CO_2$ (*A*) plasma [^{13}C]urea (*B*) and the concentration of plasma [^{13}C]urea (*C*) in healthy adult volunteers who were administered orally 0.33 mmol/kg (27 mg/kg) of [1-^{13}C]sodium-acetate. Results are mean ± SEM of 33 individual studies in 17 subjects.

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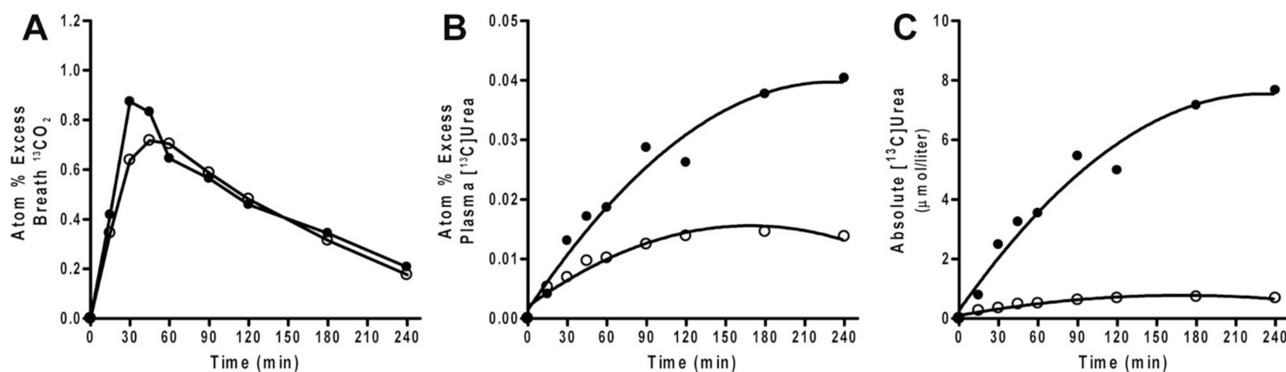

Figure 2. Increase over time of isotopic enrichment in breath $^{13}CO_2$ (*A*) plasma [^{13}C]urea (*B*) and the concentration of plasma [^{13}C]urea (*C*) in a patient with NAGS deficiency before (○) and after (●) 3-d treatment with *N*-carbamylglutamate.

Patient 1 (NAGS deficiency). The above method proved useful for measuring *in vivo* ureagenesis in a patient with NAGS deficiency that was studied before and after treatment with NCG for 3 d. As shown in Figure 2*A*, the oxidation of [1-^{13}C]acetate to $^{13}CO_2$ attained a peak of about 0.7 atom % excess before NCG therapy and about 0.8 atom % excess after, suggesting that the drug did not significantly affect flow of acetate carbon through the tricarboxylic acid cycle. In contrast, the production of [^{13}C]urea from $^{13}CO_2$ was profoundly altered. Pretreatment label in plasma [^{13}C]urea attained a peak of only 0.01 atom % excess (Fig. 2*B*), or about 10% of the control value (Fig. 1*B*). Posttreatment, this parameter increased nearly fourfold. Absolute blood [^{13}C]urea before administration of NCG scarcely increased above baseline and remained less than 1 μM (Fig. 2*C*). Therapy with NCG for 3 d augmented this value around eightfold to a peak of about 8 μM, a value that was almost twofold greater than the control value (Fig. 1*C*).

As illustrated in Figure 3, these isotopic results were congruent with the response of selected blood metabolites to NCG treatment. Thus, the plasma ammonia level declined almost to normal (Fig. 3*A*) and plasma urea increased (approximately three- to fourfold, Fig. 3*B*). The response to NCG therapy of the plasma ammonia level is noteworthy, because the patient never manifested a single normal concentration during the 17 y following her initial clinical presentation. The concentration of plasma glutamine decreased by about 200 μM posttreatment (Fig. 3*C*).

Patient 1 continued treatment with NCG under the aegis of a separate, single-patient treatment IND. During the ensuing 2 y, she enjoyed a great improvement of overall health. Treatment with ondansetron and Na-phenylbutyrate (Buphenyl) was discontinued with no recurrence of hyperammonemia. Dietary protein intake has been normalized and diabetes resolved on a diet that no longer entailed high carbohydrate intake.

Patient 2 (propionic acidemia). The positive effect of NCG treatment in the patient with NAGS deficiency prompted an examination of the use of this agent in a patient with propionic acidemia. Figure 4 illustrates these results. An interesting finding in this subject is that the peak label in expired $^{13}CO_2$ (Fig. 4*A*) before NCG therapy was lower than the peak control value (Fig. 1*A*) (0.5 *vs* >0.6 atom % excess), perhaps denoting an impairment of mitochondrial respiration, a probable factor in the pathophysiology of this disorder (19,20). However, following the administration of NCG, the peak of $^{13}CO_2$ in breath increased markedly (0.7 atom % excess).

The baseline label in plasma [^{13}C]urea attained a peak of only ~0.02 atom % excess, or about one-fourth of the control value (Figs. 4*B* and 1*B*). After therapy with NCG for 3 d, this value doubled, but only to about half the control value. Similarly, NCG therapy augmented the production of absolute [^{13}C]urea by almost threefold (Fig. 4*C*), but this was still considerably lower than the normal maximal value (Fig. 1*C*). As shown in Table 1, the plasma concentrations of glutamine, alanine, and glycine all were significantly diminished after

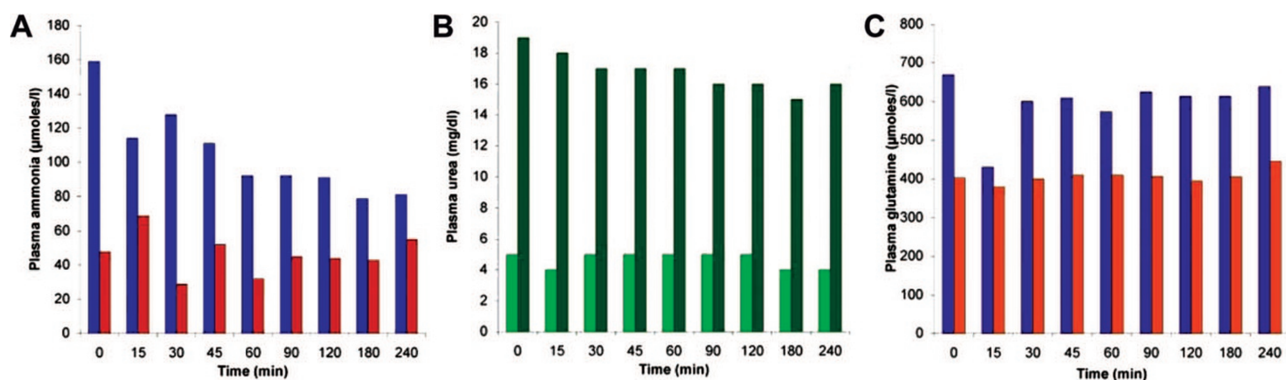

Figure 3. Plasma levels of ammonia (*A*), blood urea nitrogen (*B*) and glutamine (*C*) in a patient with NAGS deficiency before (*A*-blue, *B*-light green, *C*-magenta purple) and after (*A*-red, *B*-dark green, *C*-orange) 3-d treatment with *N*-carbamylglutamate.

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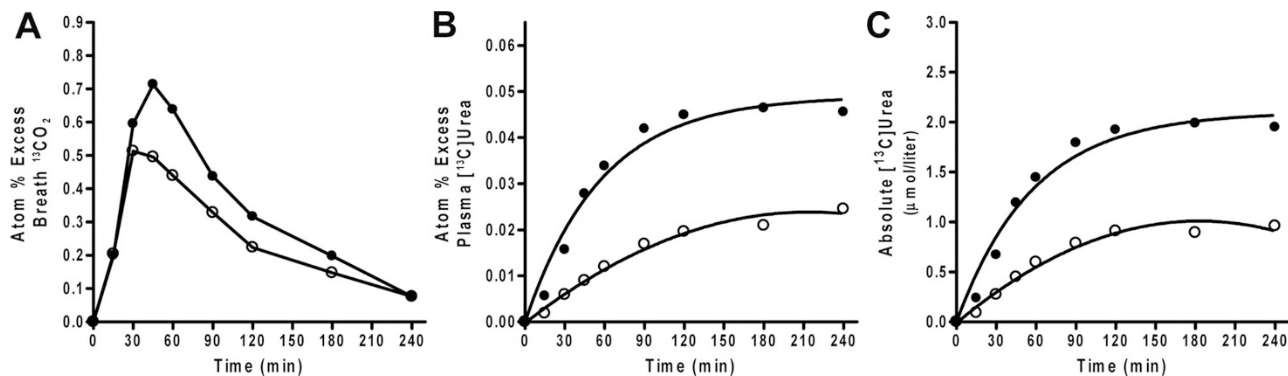

Figure 4. Increase over time of isotopic enrichment in breath $^{13}CO_2$ (*A*) plasma [^{13}C]urea (*B*) and the concentration of plasma [^{13}C]urea (*C*) in a patient with propionic acidemia before (○) and after (●) 3-d treatment with *N*-carbamylglutamate.

Table 1. *Plasma ammonia, urea, and amino acids (mean ± SD, n = 9 in μmol/L except urea mg/dL) in a patient with propionic acidemia before and after 3 days treatment with NCG*

Marker	Pre-NCG	Post-NCG	*p* (two-tailed paired *t* test)
Ammonia	39.7 ± 4.7	34.4 ± 7.1	0.1
Urea	13.2 ± 1.2	12.1 ± 0.3	0.01
Glutamine	621 ± 34	363 ± 28	<0.0001
Glycine	1313 ± 53	633 ± 33	<0.0001
Alanine	522 ± 44	306 ± 37	<0.0001

treatment with NCG, although there was little change in ammonia and urea levels which were normal or close to normal before NCG treatment. Thus, in this subject, NCG seemed to improve overall nitrogen metabolism, but not as dramatically as in the patient with NAGS deficiency.

DISCUSSION

Stable isotope methodology has enabled a large number of clinical investigations. These isotopes emit no radioactivity and are safe for administration to human subjects, including children. The mass spectrometric analysis of isotopic enrichment in body fluids is very sensitive and reproducible, thereby rendering this approach useful for kinetic studies of metabolite turnover and the delineation of pertinent precursor-product relationships (21,22).

We previously measured *in vivo* ureagenesis by tracing the rate of [^{15}N]urea production after an oral load of $^{15}NH_4Cl$ in patients with ornithine transcarbamylase deficiency (23). More recently, we used this technique to document normalization of urea production in a patient with NAGS deficiency (24). This method proved useful, but it presented three shortcomings. First, it obliged the administration of ammonia (albeit in relatively small amounts) to patients who have a compromised urea cycle. Second, the administration of $^{15}NH_4Cl$ to an individual with hyperammonemia results in substantial dilution of the tracer within the active "metabolic pool," thereby confounding comparisons with control data. Finally, the measurement of label in [^{15}N]urea entails the use of gas chromatography-mass spectrometry, a method that is not very sensitive for detecting isotopic abundance.

The use of [1-^{13}C]acetate circumvents these difficulties. Acetate administration poses no hazard to human subjects, including individuals with hyperammonemia. Indeed, sodium acetate is commonly added to i.v. solutions. Furthermore, isotope ratio-mass spectrometry measurement of label released from [^{13}C]urea by urease is 1000-fold more sensitive than gas chromatography-mass spectrometry detection of label in [^{15}N]urea.

Most of the administered ^{13}C label (>99%) is eliminated as $^{13}CO_2$ in breath, reflecting the efficiency of acetate oxidation in humans. Some of this $^{13}CO_2$ appears in plasma [^{13}C]urea, and with isotope ratio-mass spectrometry, we were able to detect and quantify this conversion as soon as 15 min following isotope administration (Fig. 1*B*). Indeed, the rate of [^{13}C]urea enrichment paralleled the rise of breath $^{13}CO_2$ during the initial 60 min of study (Figs. 1*A* and *B*). Interindividual and intraindividual differences in the rate of formation of both $^{13}CO_2$ and [^{13}C]urea were relatively small, as indicated by the relatively modest values for the standard errors of the mean for each time point.

This method also reliably documented the severe compromise (~one-tenth of control) of ureagenesis in patient 1, a woman with NAGS deficiency. Furthermore, the tracer study reliably documented the robust response of this individual to a relatively brief (3 d) period of treatment with NCG. Indeed, the method revealed the unexpected finding that NCG therapy increased the rate of ureagenesis to a level that exceeded (by about two times) the control value (comparing Figs. 1 and 2). The method should allow identification of the minimal effective dose of NCG that rectifies otherwise deficient ureagenesis in this individual. Furthermore, results of the isotopic technique were consonant with those of conventional biochemical measurements, including quantification of ammonia, urea, and glutamine.

The patient with propionic acidemia manifested a rate of ureagenesis that was compromised far less than in patient 1. However, even in this individual the ^{13}C-based approach disclosed a therapeutic effect of NCG, as documented by a rise of [^{13}C]urea synthesis posttreatment (Figs. 4*B* and *C*). These data suggest a functional NAG deficiency in patients with propionic acidemia and, very likely, in patients with methylmalonic acidemia. The posttreatment decline in the concentrations of amino acids (glutamine, alanine, and glycine), each of which is a major nitrogen carrier, is consistent with the conjecture that NCG therapy favored the flow of nitrogen away from amino acids and toward urea. Of note is the finding

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that, unlike the patient with NAGS deficiency, the subject with propionic acidemia did manifest a small, but statistically nonsignificant decline of blood ammonia, which was very close to normal to begin with. The average concentration of plasma urea that was normal before NCG showed a small decrease of 1 mg/dL, which may be physiologically insignificant because the increased urea that was produced in response to NCG could have been eliminated in the urine. Thus, the current method documented a therapeutic response that would have probably remained hidden had the indicators been conventional biochemical measurements.

The use of carbon-13 as a tracer of ureagenesis should prove useful to assess the efficacy of NCG in diverse contexts. Similar studies in healthy individuals seem warranted, because an enhancement by NCG of ureagenesis in control subjects would confirm *in vivo* the regulatory role of NAG in controlling urea synthesis and would afford a rationale for clinical trials of NCG in individuals with hepatic encephalopathy, as previously suggested (25). In addition, this methodology should facilitate evaluation of the efficacy of NCG in CPS1 deficiency, in which the drug could provide additional cofactor to individuals whose mutation results in diminished binding of NAG to the enzyme. It also is conceivable that NCG would increase residual CPS1 activity or improve the stability of selected CPS1 mutants in a manner similar to the effect of vitamins and cofactors in other inborn errors of metabolism (26,27).

We demonstrated that NCG improves nitrogen disposal in propionic acidemia. A similar salutary effect might be elicited in patients with methylmalonic acidemia, fatty acid oxidation defects, or patients with hyperammonemia secondary to valproate therapy. In each instance, it has been proposed that there is a relative deficiency of NAG because of the accumulation of excessive amounts of CoA esters, which may compete with acetyl-CoA, and a concomitant diminution in the level of acetyl-CoA, which becomes rate-limiting for the synthesis of NAG. Studies *in vitro* seem to support this formulation (28–30).

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