Human hepatic *N*-acetylglutamate content and *N*-acetylglutamate synthase activity

Determination by stable isotope dilution

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N-Acetyl-L-glutamate (*N*-acetylglutamate) content and *N*-acetylglutamate synthase activity ranges were established in human liver tissue homogenates by stable isotope dilution. The methods employ *N*-[methyl-²H₃]acetyl[¹⁵N]glutamate as internal standard, extraction of *N*-acetylglutamate by anion-exchange technique and its determination by g.l.c.–mass spectrometry by using selected ion monitoring. Hepatic *N*-acetylglutamate content in 16 different human livers, normal in structure and function, ranged from 6.8 to 59.7 nmol/g wet wt. (25.0 ± 13.4 mean $\pm$ S.D.) or from 64.6 to 497.6 nmol/g of protein (223.2 ± 104.2 mean $\pm$ S.D.). *In vitro*, *N*-acetylglutamate synthase activity in liver tissue homogenate ranged from 44.5 to 374.5 (132.0 ± 90.6 mean $\pm$ S.D.) nmol/min per g wet wt. or from 491.7 to 3416.9 (1159.6 ± 751.1 mean $\pm$ S.D.) nmol/min per g of protein. No correlation was found between hepatic *N*-acetylglutamate concentrations and the respective maximal enzymic activities *in vitro* of *N*-acetylglutamate synthase. The marked variability in this system among individual livers may reflect its regulatory role in ureagenesis.

INTRODUCTION

N-Acetyl-L-glutamic acid is an obligatory cofactor of mitochondrial carbamoyl-phosphate synthase I (EC 6.3.4.16), the first enzyme of the urea cycle in ureotelic animals (Grisolia & Cohen, 1953; Hall *et al.*, 1958). It is an allosteric activator of this enzyme (Marshall *et al.*, 1958), altering its conformation (Guthohrlein & Knappe, 1968). Without *N*-acetylglutamate, the enzymic activity of carbamoyl-phosphate synthase I *in vitro* is very low or undetectable. *N*-Acetylglutamate is synthesized in the mitochondria from acetyl-CoA and L-glutamate by an enzymic reaction catalysed by *N*-acetylglutamate synthase (EC 2.3.1.1) (Shigesada & Tatibana, 1971).

The enzymic activity of rat and human *N*-acetylglutamate synthase in the presence of saturating concentrations of substrates can be markedly increased in the presence of L-arginine (Shigesada & Tatibana, 1978; Bachmann *et al.*, 1982b; Kawamoto *et al.*, 1985). The dependence of carbamoyl-phosphate synthase I activity on availability of *N*-acetylglutamate, and the correlations between hepatic *N*-acetylglutamate content and amounts of ingested protein or ammonia in animals (Shigesada & Tatibana, 1971; Felipo *et al.*, 1988), prompted investigations on the possible role of hepatic *N*-acetylglutamate content and *N*-acetylglutamate synthase activity in the regulation of urea synthesis. Research on the biology of this system has been hampered by the lack of direct and specific chemical methodologies for measurement of *N*-acetylglutamate. The biological assay for the measurement of *N*-acetylglutamate, exploiting its effect on the activity of carbamoyl-phosphate synthase I, may be highly inaccurate, and it has been a major point of disagreement with respect of interpretation of measured hepatic *N*-acetylglutamate levels (Lund & Wiggins, 1984). Moreover, even the chemical methods employed did not estimate *N*-acetylglutamate directly, but rather used quantification of glutamate after enzymic hydrolysis of *N*-acetylglutamate (Lund & Wiggins, 1984; Alonso & Rubio, 1985), which may affect the specificity of the assay (Sonoda & Tatibana, 1990).

Very little is known about the *N*-acetylglutamate content or *N*-acetylglutamate synthase activity in normal human liver, because no detailed studies of human tissue have been reported.

In this study, new specific and sensitive assays were developed to measure tissue *N*-acetylglutamate content and *N*-acetylglutamate synthase enzymic activity in liver tissue homogenates. These methods involve stable isotope dilution and employ anion-exchange extraction and determination by g.l.c.–mass spectrometry. These methods were utilized to study normal human liver tissue and establish apparent normal ranges for *N*-acetylglutamate content and *N*-acetylglutamate synthase activity. The accuracy, sensitivity and specificity of these methods should be helpful for the study of small variations in this system and for learning more about its physiology and pathology.

EXPERIMENTAL

Materials

N-Acetyl-L-glutamic acid, [²H₃]acetic anhydride and DEAE-Sephadex, 40–120 µm particle size, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[¹⁵N]Glutamic acid was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. NO-Bis(trimethylsilyl)trifluoroacetamide + 10% trimethylchlorosilane (Regisil RC-3) was purchased from Regis Chemical Company, Morton Grove, IL, U.S.A.

Preparation of a stable isotope derivative of *N*-acetylglutamic acid

Equimolar amounts of L-[¹⁵N]glutamic acid and [²H₃]acetic anhydride were allowed to react in an aqueous solution for 2 h. The *N*-[methyl-²H₃]acetyl[¹⁵N]glutamate formed was isolated as follows. The solution's pH was adjusted to 7.5–8.0 with 0.05 M-Tris/HCl buffer, pH 8.5, and 1% (w/v) KOH solution, and it was applied on small anion-exchange gravity columns each containing 5 ml of DEAE-Sephadex charged with 0.5 M-pyridinium formate. The columns were washed with 10 ml of

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deionized water and then with 10 ml of 0.1 M-pyridinium formate to eliminate excess L-[^{15}N]glutamic acid (Tuchman & Holzknecht, 1990). *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate was then eluted with 10 ml of 0.3 M-pyridinium formate solution. After freeze-drying of this eluate, the dry residue was dissolved in methanol and was used as the stock solution of the internal standard. The purity of *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate was tested by g.l.c.-mass spectrometry and was found to be greater than 99%. The concentration of its solution was determined after drying a small fraction of the solution under N_2 and derivative formation with 100 μl of Regisil RC-3 solution at 60 °C for 20 min to form the bistrimethylsilyl derivative. Quantification was then performed by g.l.c. using a flame ionization detector, comparing the unknown amounts of the isotope with known amounts of *N*-acetylglutamate within the linear range of the detector. When high concentrations (> 2 $\mu\text{g}/100 \mu\text{l}$) of *N*-acetylglutamate are present in the derivative-formation solution and the solution is left for several hours before injection, an additional chromatographic peak of *N*-acetylglutamate tristrimethylsilyl derivative may be formed. This could be avoided by using a higher volume of derivative-formation solution and/or injecting the solution shortly after derivative formation, resulting in a single *N*-acetylglutamate bistrimethylsilyl peak.

Human tissue

Frozen normal human liver tissue used in this study was obtained through the University of Minnesota 'Liver tissue procurement and distribution system' by contract from the National Institutes of Health. The age of the individuals ranged from 5 to 67 years (38.4 ± 17.8 , mean $\pm$ s.d.). All 16 livers studied were structurally normal with no dysfunction before harvest. The livers were frozen immediately after harvesting in liquid N_2 and were stored at -80 °C until analysis. The studies on these liver tissues have been reviewed by the University of Minnesota Review Board on Human Subjects in Research and were approved on the basis of secondary use of data obtained from another project.

Preparation of liver homogenates

Sections of frozen liver were weighed and were homogenized in 8 vol. of deionized water on ice by a Polytron apparatus for 20 s. Protein concentration of the liver homogenate was measured by the bicinchoninic method (Smith *et al.*, 1985) with bovine serum albumin used as standard. The homogenate of each liver sample was assayed in duplicates both for *N*-acetylglutamate content and for *N*-acetylglutamate synthase activity. Five separate sections of a single liver were also studied in order to assess the variability of this system within different areas of a liver.

Assays of *N*-acetylglutamate content and *N*-acetylglutamate synthase activity in frozen liver tissue

Immediately following homogenization, two 200 μl fractions of the homogenate were removed, 5.3 nmol of *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate was added as internal standard to each fraction and the protein was rapidly precipitated with 1 ml of ice-cold 30% (w/v) trichloroacetic acid. These two portions of the homogenate were used to measure *N*-acetylglutamate content in duplicate and also served as controls for the *N*-acetylglutamate synthase assay performed on the other two 200 μl portions of the homogenate. The enzyme assay mixture contained 200 μl of tissue homogenate, 10 mM-L-glutamate, 2.5 mM-acetyl-CoA and 1 mM-L-arginine dissolved in a total volume of 1 ml containing 0.05 M-Tris/HCl buffer, pH 8.5. Each assay mixture was incubated for 5 min at 37 °C, after which it was precipitated with 1 ml of 30% trichloroacetic acid. An additional control sample for the assay contained all the assay components except for the

tissue, and was used as the blank reflecting the chemical formation of *N*-acetylglutamate during acid precipitation. After treatment with trichloroacetic acid, the assay mixtures and the control samples were handled by an identical procedure. The mixture supernatants were separated from the precipitate by centrifugation, and the acid was then removed by extraction with diethyl ether three times. The sample pH was adjusted to 7.5–8.0 with 1% KOH, and *N*-acetylglutamate and *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate were then extracted and derivatives prepared as described above.

G.l.c.-m.s. determination of *N*-acetylglutamate

A 2 μl portion of the derivative of the extract was injected into the gas chromatograph (5890 gas chromatograph; Hewlett Packard, Avondale, PA, U.S.A.) and separated on a cross-linked 5% phenylmethylsilicone capillary column (0.2 mm outer diam., 25 m long) with a film thickness of 0.52 μm (Ultra 2; Hewlett Packard). The oven's temperature program started at 100 °C, increasing by 20 °C/min up to 270 °C. Injection-port and transfer-line temperatures were 250 °C and 280 °C respectively. Split injection mode was used, with a split ratio of 1:50. Flow rate of the carrier gas, helium, was 1 ml/min and its linear velocity was 35 cm/s. Detection was by mass-spectrum detector (5971 quadrupole MSD; Hewlett Packard) equipped with 70 eV electron-impact ion source. Selected ion-monitoring mode was employed, by monitoring m/z 216 for *N*-acetylglutamate and m/z 220 for *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate. Dwell time was 20 ms and resolution was 0.5 atomic mass unit. Quantification was done by the internal-standard calibration based on the ion ratios between m/z 216 (*N*-acetylglutamate) and m/z 220 (*N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate). Results of *N*-acetylglutamate content were expressed as nmol per g tissue wet wt. and per g of protein. Results of *N*-acetylglutamate synthase activity were expressed as nmol of *N*-acetylglutamate generated/min per g tissue wet wt. or per g of protein after subtracting the sum of liver and 'blank' content of *N*-acetylglutamate. Chemical formation of *N*-acetylglutamate in the assay mixture was acid-catalysed, and ranged from 0.69 to 1.75 nmol (1.3 ± 0.3 mean $\pm$ s.d., $n = 27$). The length of incubation of the reaction mixture of neutral pH did not contribute to chemical formation of *N*-acetylglutamate.

RESULTS AND DISCUSSION

The synthesis of labelled *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate from L-[^{15}N]glutamic acid and [$^2\text{H}_3$]acetic anhydride provided an internal standard with a relative molecular mass of 193, higher by 4 compared with natural *N*-acetylglutamate ($M_r = 189$) (Fig. 1). Fig. 2 shows the mass spectra of bistrimethylsilyl derivatives of both compounds, with several ions different by m/z 4 between the two compounds. No detectable m/z -220 ion was found in the mass spectrum of *N*-acetylglutamate, and no detectable m/z -216

Fig. 1. Structure of *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamic acid, the stable isotope derivative used as the internal standard for the quantification of *N*-acetylglutamate

Fig. 2. Mass spectra of bistrimethylsilyl derivatives of *N*-acetylglutamic acid (a) and *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamic acid (b)

ion was found in the mass spectrum of *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate. Therefore, m/z -216 and m/z -220 ions were chosen for selected ion monitoring of both compounds respectively. This internal standard should markedly improve the accuracy of *N*-acetylglutamate determination, compensating for variations in extraction efficiency as well as in g.l.c.-m.s. determination.

Owing to the relatively low liver concentrations of *N*-acetylglutamate, sensitive and reliable methods for its measurement are crucial, especially for studies designed to determine small changes in its content. The use of the above stable isotopic-dilution technique easily enabled detection and quantification of hepatic *N*-acetylglutamate content and measurement of *N*-acetylglutamate synthase activity in 25 mg of tissue. These two assays can also be performed on a smaller amount of tissue, and thus are applicable for needle liver-biopsy material. Fig. 3 shows the ion chromatograms obtained from an enzymic assay. The labelled internal standard (m/z 220 and 322) has a retention time 0.01 min shorter than natural *N*-acetylglutamate (m/z 216 and 318). So far we have not encountered interfering chromatographic peaks, probably owing to the selective extraction and the high efficiency of separation of the capillary column. Depending on the tuning of the mass-spectrum detector, the ratio between the currents generated by m/z -216 and m/z -220 species when equimolar amounts of *N*-acetylglutamate and *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate were injected ranged between 1.1 and 2.4. This ratio was stable over time and changed only after a new tune of the detector. Calibration of the ion ratios by using known amounts of *N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate and *N*-acetylglutamate was performed daily before analysing the liver extracts. We think that *N*-acetylglutamate content and *N*-acetylglutamate synthase activity are very stable in frozen liver samples. Studies on the stability may be hampered by the variability of this system in different sections of the liver. However, repeated studies of the same liver (in a patient with carbamoyl-phosphate synthase deficiency and elevated *N*-acetylglutamate levels; Tuchman & Holzkecht, 1990) over several months gave very similar results (results not shown).

The results obtained from *N*-acetylglutamate and *N*-acetylglutamate synthase assays are summarized in Table 1. Liver

Fig. 3. Selected ion-monitoring chromatograms of m/z 216, m/z 318, m/z 220 and m/z 322 ion currents obtained after extraction of *N*-acetylglutamate following an assay of *N*-acetylglutamate synthase in 25 mg of human liver tissue

The labelled internal-standard peak was eluted from the column 0.01 min before *N*-acetylglutamate. The ratio between m/z 216 (*N*-acetylglutamate) and m/z 220 (*N*-[methyl- $^2\text{H}_3$]acetyl[^{15}N]glutamate) peak areas was used to quantify the amounts of *N*-acetylglutamate present in the sample.

content of *N*-acetylglutamate in human liver tissue ranged from 6.8 to 59.7 (25.0 ± 13.4 mean $\pm$ S.D.) nmol/g wet wt. or from 64.6 to 497.6 (223.2 ± 104.2 mean $\pm$ S.D.) nmol/g of protein. In the 16 livers studied, no consistent correlation was found between *N*-acetylglutamate levels and the age of the individual. In five different 25 mg sections of a single liver, *N*-acetylglutamate content ranged from 14.0 to 22.1 nmol/g wet wt. (17.5 ± 3.3 mean $\pm$ S.D.) or from 144.6 to 250.8 nmol/g of protein (191.4 ± 38.0 mean $\pm$ S.D.). These results indicate that *N*-acetylglutamate may not be evenly distributed within the liver. No previously reported *N*-acetylglutamate levels in human liver are available; rat liver has been reported to contain 20–40 nmol/g wet wt. measured by a biological assay. This biological assay determined *N*-acetylglutamate indirectly as the capacity of liver tissue extract to induce citrulline synthesis in a carbamoyl-phosphate synthase I assay (Shigesada & Tatibana, 1971; McGivan *et al.*, 1976; Staddon *et al.*, 1984; Zollner, 1981). The biological method has inherent problems, such as non-specific inhibitors in the tissue extract, which will cause underestimation of *N*-acetylglutamate concentrations (Cohen, 1984). In addition, the enzyme extract may also contain *N*-acetylglutamate, making it difficult to estimate low concentrations. Discrepancies in results of *N*-acetylglutamate determinations owing to diverse methodologies were the major argument in the controversy about the role of *N*-acetylglutamate in the control of urea synthesis (Meijer & Hensgens, 1982; Lund & Wiggins, 1984). More specific methods for the measurement of *N*-acetylglutamate were developed which utilize radiochemical techniques (Shigesada & Tatibana, 1971). Endogenous *N*-acetylglutamate was first labelled *in vivo* by administration of radiolabelled glutamate, and it was then isolated by a cation-exchange technique. Alternatively, exogenous radioactively labelled *N*-acetylglutamate was used as the internal standard, and the natural *N*-acetylglutamate was estimated indirectly from the amounts of labelled and natural L-glutamate generated after incubation of the sample with an aminocyclase that hydrolyses *N*-acetylglutamate to glutamic acid (Lund & Wiggins, 1984). A sensitive h.p.l.c. method has been developed for the quantification of glutamate formed by the enzymic hydrolysis of liver *N*-acetylglutamate (Alonso & Rubio, 1985). However, also this method did not quantify *N*-acetylglutamate directly.

Most previously reported methods for assaying *N*-acetyl-

Table 1. *N*-Acetylglutamate content and *N*-acetylglutamate synthase activity in human liver

Assay 1 and Assay 2 represent the results of two separate assays on separate fractions of one homogenate.

Liver no.	<i>N</i> -Acetylglutamate*		<i>N</i> -Acetylglutamate synthase†	
	Assay 1	Assay 2	Assay 1	Assay 2
1	15.8 (149.8)	16.2 (153.2)	78.6 (744.2)	83.4 (789.7)
2	40.9 (384.9)	42.4 (398.8)	52.3 (491.7)	54.2 (509.3)
3	19.8 (180.0)	17.3 (156.8)	93.3 (848.3)	94.0 (854.3)
4	32.2 (240.4)	30.2 (225.9)	187.5 (1400.6)	182.1 (1360.6)
5	20.1 (226.0)	25.3 (285.2)	46.7 (525.4)	44.5 (500.7)
6	28.4 (259.3)	36.1 (329.1)	374.5 (3416.9)	356.3 (3251.1)
7	16.1 (191.7)	14.3 (169.8)	96.9 (1153.3)	95.4 (1135.2)
8	10.9 (84.8)	11.6 (89.9)	89.0 (690.9)	94.2 (731.2)
9	7.0 (66.3)	6.8 (64.6)	55.1 (522.1)	54.8 (519.2)
10	14.8 (188.4)	14.0 (187.3)	85.5 (809.4)	85.9 (800.6)
11	29.8 (237.3)	26.7 (212.8)	182.1 (1450.0)	207.0 (1648.0)
12	23.8 (219.9)	18.7 (172.8)	56.9 (526.9)	61.3 (567.4)
13	43.1 (294.3)	43.1 (294.4)	288.4 (1969.7)	284.7 (1944.8)
14	59.7 (497.6)	55.3 (461.1)	191.5 (1596.0)	201.5 (1678.7)
15	14.5 (173.0)	14.3 (169.7)	153.8 (1831.3)	142.7 (1698.3)
16	26.5 (203.3)	22.8 (174.7)	76.2 (584.0)	72.7 (557.5)
Mean $\pm$ s.d.	25.0 $\pm$ 13.4 (223.2 $\pm$ 104.2)		132.0 $\pm$ 90.6 (1159.6 $\pm$ 751.1)	

* Expressed as nmol/g wet wt., and as nmol/g of protein in parentheses.

† Expressed as nmol/min per g wet wt., and as nmol/min per g of protein in parentheses.

glutamate synthase activity employ radioactive glutamate as substrate incubated with acetyl-CoA in the presence of liver mitochondrial extract as the source of the enzyme and arginine as its activator. The reaction was stopped with a strong acid such as HClO₄ or trichloroacetic acid. The labelled *N*-acetylglutamate formed during the reaction was separated by paper or ion-exchange chromatography, and its radioactivity quantified (Bachmann *et al.*, 1982b; Shigesada & Tatibana, 1971). Our results show that there is a substantial amount of chemical formation of *N*-acetylglutamate (1.3 ± 0.3 nmol, $n = 27$) when acid is added to a solution containing millimolar concentrations of glutamate and acetyl-CoA. This formation of *N*-acetylglutamate in the 'blank' could cause inaccurate estimation of low enzymic activity. One previous report only mentioned assaying *N*-acetylglutamate synthase in liver tissue homogenate without prior isolation of mitochondria (Bachmann *et al.*, 1982a), but no details were given on the method. One could assume that, owing to the presence of aminoacylases in liver cell cytosol (Reglero *et al.*, 1977), *N*-acetylglutamate produced in the reaction will be converted by those enzymes into glutamate during incubation. However, our results suggest that it is possible to measure substantial *N*-acetylglutamate synthase activity in liver tissue homogenate. The amount of *N*-acetylglutamate generated in the reaction was linear with respect to incubation time and amount of tissue in the assay mixture (Fig. 4). When five different 5 mg sections of the same liver were assayed for *N*-acetylglutamate synthase activity, very similar results were obtained in all sections. The enzymic activity in the five sections ranged from 75.6 to 85.9 nmol/min per g wet wt. (80.9 ± 4.6 , mean $\pm$ s.d.) or from 694.7 to 809.4 nmol/min per g of protein (746.7 ± 53.9 , mean $\pm$ s.d.). This indicates that the assay is reproducible and that the enzyme was equally distributed within the liver section that was studied.

It is unknown, however, whether the activity of enzyme measured in this study is mitochondrial or cytosolic, although it seems to be membrane-bound, because *in vitro* *N*-acetylglutamate synthase activity was found to be manyfold higher in whole tissue homogenate than in ultracentrifuged supernatants (Table

Fig. 4. Effects of incubation time (a) and of liver tissue amount (b) on the generation of *N*-acetylglutamate in *N*-acetylglutamate synthase assay mixtures containing 10 mM-L-glutamic acid, 2.5 mM-acetyl-CoA, 1 mM-L-arginine and liver tissue homogenates

N-Acetylglutamate formed in the reaction mixtures was extracted by anion exchange and quantified by g.l.c.-m.s. by using stable isotope dilution.

Table 2. Hepatic N-acetylglutamate synthase activity in tissue homogenates and ultracentrifuged supernatants

Results are expressed as nmol/min per g of tissue, and as nmol/min per g of protein in parentheses. Two normal livers were studied in duplicate.

Liver no.	Homogenates*	Supernatants†
1	78.6 (744.2)	4.0 (71.2)
	83.4 (789.7)	3.9 (68.4)
2	187.5 (1400.6)	3.2 (62.2)
	182.1 (1360.6)	3.8 (73.2)

* Whole tissue homogenates obtained with a Polytron apparatus.

† Supernatants of tissue homogenates obtained by ultracentrifugation at 105000 g for 30 min.

2). N-Acetylglutamate synthase activity was previously found to reside mainly in the mitochondria (Uchiyama *et al.*, 1981).

The enzymic activity in tissue homogenates ranged widely among individual livers, from 44.5 to 374.5 (132.0 ± 90.6 , mean $\pm$ S.D.) nmol/min per g wet wt. or from 491.7 to 3416.9 (1159.6 ± 751.1 , mean $\pm$ S.D.) nmol/min per g of protein. No correlation was found between hepatic N-acetylglutamate concentrations and the respective maximal enzymic activities of N-acetylglutamate synthase *in vitro*. This finding suggests that the regulation of N-acetylglutamate synthase activity may be post-translational, involving activators and/or inhibitors.

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