Is *N*-acetylglutamate a short-term regulator of urea synthesis?

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A method is described for determining *N*-acetylglutamate as glutamate. *N*-Acetylglutamate content of hepatocytes from 48 h-starved rats is high. It shows no parallelism with rates of urea synthesis from glutamine. We question its accepted function as a short-term regulator of urea synthesis.

N-Acetylglutamate, the obligatory cofactor of carbamoyl phosphate synthetase (EC 6.3.4.16), is considered an important short-term regulator of urea synthesis (see Meijer & Hensgens, 1982; Rabier *et al.*, 1982). It has been suggested that its rate of synthesis is regulated by the supply of mitochondrial glutamate (Tatibana & Shigesada, 1976; Stewart & Walser, 1980; Zöllner, 1981). This led us to investigate whether the allosteric phosphate-dependent mitochondrial glutaminase that is present exclusively in liver could control synthesis of acetylglutamate and, therefore, the synthesis of urea. Glutaminase is linked to urea synthesis by its products (NH_4^+ and glutamate) and by its effector, NH_3 . Glucagon, which activates glutaminase (Lacey *et al.*, 1981; Corvera & Garcia-Sainz, 1983), is also reported to increase the *N*-acetylglutamate content of liver (Cathelineau *et al.*, 1980; Hensgens *et al.*, 1980; Rabier *et al.*, 1982). These links appear to be more than coincidental.

N-Acetylglutamate is generally measured as the capacity of a tissue extract, sometimes crudely fractionated, to increase citrulline synthesis by comparison with known amounts of *N*-acetylglutamate. We considered that this was inherently unsatisfactory and so devised a specific assay for *N*-acetylglutamate, which is described in this paper. Measured by this assay, the amounts of *N*-acetylglutamate in freshly isolated hepatocytes are much higher than any published values, which leads us to question the *N*-acetylglutamate theory of regulation of urea synthesis.

Experimental

Tissue preparations

Hepatocytes were isolated from livers of 48 h-starved female Wistar rats (about 200 g) as described by Farrell & Lund (1983), a procedure

taking a total of approx. 45 min. Incubations were in 50 ml Erlenmeyer flasks, each containing 200–250 mg wet wt. of tissue in 10 ml of physiological saline (Krebs & Henseleit, 1932) with additions as shown. Flasks were gassed with O_2/CO_2 (19:1), sealed with rubber stoppers, and incubated in a shaker bath. At the time stated, the suspension was poured off, rapidly centrifuged (2000 *g* for 30 s), and the pellet deproteinized with 2 ml of 10% (v/v) HClO_4 . A portion of the incubation medium was also deproteinized and centrifuged. Supernatants from both tissue and medium were neutralized with KOH.

To obtain sufficient liver extract for comparison of different types of assay of *N*-acetylglutamate, livers from fed male rats were freeze-clamped (Wollenberger *et al.*, 1960), ground in a mortar under liquid N_2 , and 1 part of tissue was deproteinized with 2 parts of 10% HClO_4 . The acid supernatant was neutralized with KOH.

Metabolite assays

Ammonia and urea were determined in the samples of medium by using the method, enzymes and coenzymes as described previously (Farrell & Lund, 1983). Glutamate, after deacylation of *N*-acetylglutamate, was determined essentially as described by Cornell *et al.* (1974) in a double-beam spectrophotometer (Aminco DW-2).

Preparation of N -acetyl[1- ^{14}C]glutamate

L-Glutamate (10 μmol), L-[1- ^{14}C]glutamate (5 μCi) (Amersham International, Amersham, Bucks., U.K.) and acetic anhydride (20 μl) in a final vol. of 0.22 ml were left together at room temperature for 2 h. *N*-Acetyl[1- ^{14}C]glutamate was separated by chromatography on a Dowex 1-X8 column (24 cm $\times$ 1.2 cm) by continuous gradient elution (water $\rightarrow$ 3 M-formic acid) in a system with a chart recorder and a radioactivity detector inserted

in the line to the fraction collector. Two peaks appeared in addition to residual [$1\text{-}^{14}\text{C}$]glutamate. The fractions corresponding to each peak were pooled, dried by rotary evaporation, re-dissolved in 6ml of water and re-dried to complete the removal of formic acid. The compound that was quantitatively converted into [^{14}C]glutamate on incubation with aminoacylase was identified as *N*-acetylglutamate: the other formed [^{14}C]glutamate after boiling in 0.5M-NaOH, and was presumed to be pyrrolidone-5-carboxylic acid.

In this gradient elution system, with 3M-formic acid drawn into water in a 125ml mixing vessel, glutamate and *N*-acetylglutamate were well separated: [^{14}C]glutamate appeared as a sharp peak in the 30–40ml fraction, and *N*-acetyl[^{14}C]glutamate as a broader peak after approx. 70ml. Thus there was no possibility of overlap, even in liver extracts, where the glutamate content may be over 100-fold higher than that of *N*-acetylglutamate.

Measurement of N-acetylglutamate

Tissue extract (1ml) plus *N*-acetyl[$1\text{-}^{14}\text{C}$]glutamate marker (20 μl ; approx. 1.2×10^4 d.p.m.) was run in the column-chromatography system, and the fraction containing ^{14}C was treated as described above. The dried material was dissolved in 1.2ml of 40mM-potassium phosphate buffer, pH7, treated with 0.1ml of aminoacylase (Boehringer) (after dialysis to remove NH_4^+) for 2h at 37°C, further deproteinized and neutralized before determination of glutamate. All values were corrected for the amount of glutamate contributed by the marker [^{14}C]acetylglutamate (4nmol).

To shorten the procedure, we later did a two-step elution on small (3cm $\times$ 0.5cm) Dowex 1-X8 columns: first with 0.15M-formic acid (3ml) to elute glutamate, then with 2M-formic acid to elute *N*-acetylglutamate. These conditions were established by recovery of radioactivity from labelled glutamate or *N*-acetylglutamate. Rotary evaporation and further treatment were as described above. Comparable results were obtained with the two procedures.

Quantitative aspects of measurement of N-acetylglutamate as glutamate

To satisfy ourselves that the method was quantitative, we showed (i) by ion-exchange chromatography that *N*-acetyl[$1\text{-}^{14}\text{C}$]glutamate, in the small amount used as marker, was quantitatively converted into [^{14}C]glutamate, (ii) that recovery of ^{14}C in the sample after column chromatography was essentially 100% of the added ^{14}C marker, (iii) that the enzymic assay of glutamate at low concentration was quantitative in the double-beam spectrophotometer, and (iv) that

before deacylation nothing in the sample reacted in the glutamate assay.

Validation of method: comparison with kinetic assays for N-acetylglutamate

N-Acetylglutamate is generally measured as the capacity of a tissue extract to increase citrulline synthesis, which is then either measured colorimetrically or as the incorporation of $\text{H}^{14}\text{CO}_3^-$ into acid-stable products. The enzyme system may be liver mitochondrial preparations or purified carbamoyl phosphate synthetase and ornithine carbamoyltransferase (EC 2.1.3.3). The unknowns are compared with a range of standards of the activator. We compared the colorimetric and ^{14}C -incorporation methods in freeze-clamped liver extracts with a view to explaining the discrepancy between our values and the lower published values.

The standard assay system contained, in 1ml, 50mM-Tris/HCl buffer, pH7.2, 20mM-ornithine, 20mM- NH_4Cl , 5mM-ATP, 15mM- MgCl_2 , 10mM- KHCO_3 (see McGivan *et al.*, 1976), with or without addition of 0.5 μCi of $\text{NaH}^{14}\text{CO}_3/\text{ml}$, 0.1–0.6ml of tissue extract and 0.1ml of sonicated mitochondria (final concn. approx. 3mg of protein/ml). Liver mitochondria were prepared by the rapid method of Kun *et al.* (1979). In the context of the assay, 'tissue extract' refers to: (A) neutralized liver extract in which *N*-acetylglutamate was separated by continuous gradient elution, with or without added ^{14}C marker (see 'Measurement of *N*-acetylglutamate'); (B) extract fractionated by the two-step procedure described by Hensgens *et al.* (1980); (C) untreated extract. In each case, the fractionated sample was re-constituted to 0.5–1.0 times the original volume and, in the following text, percentage recovery refers to the value obtained on the same extract when *N*-acetylglutamate was assayed as glutamate (86.9 ± 6.5 nmol/g wet wt. for liver from fed male rats; mean $\pm$ S.E.M. for seven animals).

Colorimetric assay

Incubation was for 15 min at 37°C. A range of *N*-acetylglutamate concentrations (0–100nmol) was included in each assay. The reaction was stopped with 0.2ml of 8% (w/v) trichloroacetic acid, and citrulline was measured as described by Nuzum & Snodgrass (1976). In this assay, treatment (A) gave $101 \pm 8.7\%$ recovery for the equivalent of 0.25ml of original extract, and $78 \pm 5.5\%$ for 0.5ml (means $\pm$ S.E.M. for four liver extracts). Treatment (B) gave extremely low values, no stoichiometry with sample size, and standard *N*-acetylglutamate (20nmol) incubated in the presence of tissue extract was not recovered. Unfractionated extracts could not be assayed, because urea interferes in the colour reaction. In our opinion this is not a satisfactory assay: all unknowns fell below 10nmol

on the standard curve; even treatment (A) gave poor stoichiometry, and extracts after treatment (B) appear to contain an inhibitor of the reaction.

Incorporation of $H^{14}CO_3^-$ into acid-stable products

Samples (containing no ^{14}C marker) and standards (0–32 nmol of *N*-acetylglutamate) were preincubated with the sonicated mitochondrial suspension for 10 min at room temperature before addition of substrates (see Meijer & Van Woerkom, 1978). Further incubation was for 15 min at 37°C. Reactions were stopped with 0.1 ml of 60% $HClO_4$. Supernatant (0.5 ml) in a scintillation vial was heated at 70°C for 2 h before addition of 10 ml of scintillation fluid [5.5 g of Permablend III (Packard), 60 g of naphthalene, 600 ml of toluene, 400 ml of methoxyethanol]. In this method, standards always gave an excellent correlation coefficient ($r = >0.98$ in eight experiments). Treatment B gave low values, which were not reproducible from day to day, and added standard *N*-acetylglutamate (20 nmol) was not recovered. Low volumes (<0.3 ml) of untreated extract (C) gave values of the same order as when *N*-acetylglutamate was measured as glutamate (approx. $120 \pm 10\%$). Again, lower values were found as sample size was increased. This was not the result of inhibition by perchlorate (see McGivan *et al.*, 1976), because livers deproteinized with 5 M-HCl showed the same phenomenon. In this, as in the colorimetric assay, there is the problem that all unknowns fall below the 10 nmol standard and stoichiometry is poor because tissue extracts appear to contain an inhibitor.

Results and discussion

No parallelism between N-acetylglutamate content of hepatocytes and rate of urea synthesis from glutamine

The *N*-acetylglutamate content of freshly isolated hepatocytes is considerably higher, when

measured as glutamate after deacylation, than when measured as the capacity of tissue extracts to increase citrulline synthesis: 116 ± 5.4 nmol/g wet wt. after 48 h starvation in our experiments (Table 1), compared with, in male rats, 40 nmol/g wet wt. (Zöllner, 1981) or, after 18–24 h starvation, approx. 50 nmol/g dry wt. (Hensgens *et al.*, 1980) (i.e. 14 nmol/g wet wt., taking the wet-wt./dry-wt. ratio as 3.7; Krebs *et al.*, 1974). Our high initial values raise questions concerning the proposed function of *N*-acetylglutamate as an important short-term regulator of urea synthesis. The concentration in the mitochondrial matrix must far exceed the K_a of 0.1 mM for activation of carbamoyl phosphate synthetase (Lof *et al.*, 1983) when the following factors are taken into account: 28% of the 3H_2O -permeable space of packed hepatocytes from 48 h-starved rats is extracellular (P. Lund, unpublished work); intracellular *N*-acetylglutamate is reported to be at least 65% mitochondrial (Shigesada & Tatibana, 1971; Hensgens *et al.*, 1980); the matrix volume of mitochondria of hepatocytes from 24 h-starved rats is 7–10% of the intracellular volume (Quinlan *et al.*, 1983); glutaminase and the urea-cycle enzymes are located in the periportal cells (Haüssinger, 1983). If *N*-acetylglutamate is intimately linked to the urea cycle, it too would presumably be restricted to the same cells. Altogether these corrections would make the matrix concentration over 1 mM before incubation.

On incubation of the hepatocytes without substrate, *N*-acetylglutamate content decreases by about 30%. The effect of 5 mM-glutamine is to maintain, but not to increase significantly, the amount of *N*-acetylglutamate present initially, in spite of the fact that the rate of urea synthesis increases about 3-fold between 20 and 40 min compared with the 0–20 min rate. This may be partly explained by the necessity for self-activation of glutaminase by ammonia (see Verhoeven *et al.*, 1983). When 0.05 mM-dibutyl cyclic AMP is

Table 1. *N*-Acetylglutamate content of hepatocytes from 48 h-starved rats during urea synthesis from glutamine. For experimental details see the Experimental section. Additions were: glutamine (5 mM); dibutyl cyclic AMP (0.05 mM); phenylephrine (0.1 mM). Values are means $\pm$ S.E.M. for the numbers of experiments in parentheses. Significances of differences in amounts of *N*-acetylglutamate from the corresponding 5 mM-glutamine control are: * $P < 0.05$; ** $P < 0.01$. † 0–20 min rate; †† 20–40 min rate.

Additions	Incubation (min)	<i>N</i> -Acetylglutamate (nmol/g wet wt.)	Urea synthesis (μ mol/min per g wet wt.)
None	0	116 ± 5.4 (6)	
None	40	77 ± 9.2 (4)**	0.13 ± 0.01 (4)
Glutamine	20	109 ± 12 (6)	0.27 ± 0.02 (6)†
Glutamine	40	126 ± 7.8 (6)	0.79 ± 0.08 (6)††
Glutamine + dibutyl cyclic AMP	20	140 ± 8.6 (6)*	0.46 ± 0.03 (6)†
Glutamine + dibutyl cyclic AMP	40	206 ± 17 (5)**	1.74 ± 0.10 (5)††
Glutamine + phenylephrine	20	108 ± 5.0 (6)	0.50 ± 0.04 (6)†
Glutamine + phenylephrine	40	145 ± 11 (5)	1.52 ± 0.11 (5)††

present, glutaminase is further activated (see Lacey *et al.*, 1981; Corvera & Garcia-Sainz, 1983). Both *N*-acetylglutamate and the rate of urea synthesis are increased, and the two appear to be linked (see the introduction). However, the link does not hold with phenylephrine, which, as an α -agonist, also increases assayable activity of glutaminase (Corvera & Garcia-Sainz, 1983) and urea synthesis from glutamine (Titheradge & Haynes, 1980; see Corvera & Garcia-Sainz, 1983) without a significant increase in *N*-acetylglutamate (Table 1; see also Titheradge & Haynes, 1980).

Objections to the N-acetylglutamate theory of regulation of urea synthesis

Apart from the doubts introduced by conflicting results obtained with the different methods of assay for *N*-acetylglutamate, there are other unsatisfactory aspects of the *N*-acetylglutamate theory. Firstly, *N*-acetylglutamate is reported to have a half-life of 20–30 min (Meijer & Van Woerkom, 1982; see also Meijer & Hensgens, 1982). How is it so well maintained, when the liver is depleted of endogenous substrates during the 45–60 min procedure for isolation of hepatocytes? Secondly, *N*-acetylglutamate content and citrulline synthesis from NH_4Cl are reported to be increased in mitochondria isolated from glucagon-treated livers (Cathelineau *et al.*, 1980; Hensgens *et al.*, 1980; Rabier *et al.*, 1982; see also Meijer & Hensgens, 1982). How is citrulline synthesis from NH_4^+ stimulated by glucagon, whereas the synthesis of urea is not (P. Lund, unpublished work)? Thirdly, phenylephrine, which is as effective as glucagon or dibutyryl cyclic AMP in accelerating urea synthesis from glutamine, differs in that it does not increase *N*-acetylglutamate above the amount found when glutamine alone is substrate (see Table 1). Taken together, these observations suggest that either glutaminase activity or some step beyond carbamoyl phosphate synthetase regulates urea synthesis from glutamine, at least in hepatocytes from 48 h-starved rats. Our preliminary measurements indicate that neither dibutyryl cyclic AMP nor phenylephrine causes the accumulation of ammonia, glutamate or citrulline, implying limitation of glutaminase. It is not known exactly how hormonally mediated activation of the enzyme is achieved; it seems most probable that changes in the conformation or composition of the mitochondrial inner membrane alter its kinetic properties (see McGivan & Bradford, 1983). In any event, if the assumptions concerning distribution of *N*-acetylglutamate are correct, the increases that we observe in the presence of dibutyryl cyclic AMP appear to be irrelevant to activation of carbamoyl phosphate synthetase.

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