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Analysis and physiological implications of renal 2-oxoglutaramate metabolism

Itzhak NISSIM,* Suzanne WEHRLI, Beatrice STATES, Ilana NISSIM and Marc YUDKOFF
Division of Biochemical Development and Molecular Diseases, The Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

The relative significance of the flux through the glutamine aminotransferase (glutaminase II) pathway to renal ammoniagenesis is poorly understood. A basic and unresolved question is whether 2-oxoglutaramate (2-OGM), a product of the glutaminase II reaction, is deamidated to yield 2-oxoglutarate and NH_3, or whether 2-OGM accumulates as an unreactive lactam, depending on the environmental pH. In the current studies we utilized ^{13}C n.m.r. as well as ^{15}N n.m.r. to demonstrate that 2-OGM occurs as a lactam, i.e. 5-hydroxypyroglutamate, regardless of the environmental pH. Our additional aims were to determine whether human kidney cells (HK cells) in culture can produce 2-OGM and to ascertain a pH-dependent relationship between NH_3 and 2-OGM production from glutamine. We therefore developed an isotope dilution assay for 2-OGM utilizing 5-hydroxy[4-^{13}C,1-^{15}N]pyroglutamate as the labelled species. Incubations of HK cells in minimal essential medium supplemented with 1 mM-[2-^{15}N]glutamine demonstrated significantly higher production of 2-OGM at pH 6.8 and lower production at pH 7.6 compared with pH 7.4. Similarly both $^{15}NH_3$ and [^{15}N]alanine formation were significantly higher in acute acidosis (pH 6.8) and lower in acute alkalosis (pH 7.6) compared with that at physiological pH. Addition of 1 mM-amino-oxyacetate to the incubation medium at pH 7.4 significantly diminished [^{15}N]alanine and 2-OGM production, but the production of $^{15}NH_3$ via the glutamate dehydrogenase pathway was significantly stimulated. The current observations indicate that the glutaminase II pathway plays a minor role and that flux through glutamate dehydrogenase is the predominant site for regulation of ammoniagenesis in human kidney.

INTRODUCTION

Augmented urinary ammonium excretion is the principal mechanism whereby an excess of H^+ is eliminated. Van Slyke *et al.* [1] originally suggested that the renal extraction of glutamine could account for most of the ammonia formed. Three distinct pathways are known to abet glutamine metabolism in the kidney: (1) phosphate-dependent glutaminase; (2) γ-glutamyl transpeptidase; (3) glutamine aminotransferase or the glutaminase II pathway [2–6].

The cytosolic glutaminase II reaction catalyses the transamination of glutamine with a 2-oxoacid, forming 2-oxoglutaramate (2-OGM), which is deamidated by ω-amidase to yield 2-oxoglutarate and NH_3. Cooper [7] suggested that this pathway is capable of producing significant amounts of ammonia during acidosis. However, other studies indicate that 2-OGM exists predominantly as the cyclic lactam and therefore is enzymically unreactive [8,9]. Our previous studies of cultured human kidney (HK) cells [6] suggest a relatively minor role for the glutaminase II pathway during acidosis, since we found that the augmentation of NH_3 formation in acidosis is derived primarily from N-2 of glutamine rather than N-5. These data imply augmented hydrolysis of glutamine via phosphate-dependent glutaminase and the deamination via glutamate dehydrogenase of the glutamate so formed. Hence, the role of the glutaminase II reaction in glutamine metabolism remains poorly understood and the subject of conflicting observations.

Furthermore, similarly to our studies with cultured HK cells [6], recent studies in LLC-PK_1 cells [10] also indicated that the enhanced ammonia production in response to acute change in media pH is accounted for entirely by increased flux through glutamate dehydrogenase. In addition, acute acidosis also increases alanine production by LLC-PK_1 cells. The latter observation appeared to result from stimulated flux through the glutaminase II pathway and was accompanied by increased accumulation of 5-hydroxypyroglutamate [10]. Since ammonia generation from N-5 of glutamine was not increased, the low pH stimulated glutamine transamination and resulted in the production of 5-hydroxypyroglutamate, but did not result in increased metabolism through ω-amidase [10].

In the current investigation we have applied the stable isotope and gas-chromatography–mass-spectrometry (g.c.–m.s.) technique [11,12] to explore the role of the glutaminase II pathway in abetting ammonia formation. We have synthesized ^{15}N,^{13}C-labelled 2-OGM to measure the concentration of 2-OGM by an isotope-dilution method. Our purposes were: (*a*) to determine whether cultured HK cells can produce 2-OGM and (*b*) to delineate the potential relationship between ammonia and 2-OGM formation from glutamine, depending on the H^+ concentration of the incubation medium. In addition, we used ^{13}C and ^{15}N n.m.r. to delineate the effect of environmental pH on the structure of 2-OGM. The current data add further support to the hypothesis that the glutaminase II pathway plays a minor role in renal ammoniagenesis [6].

MATERIALS AND METHODS

L-[2-^{15}N]Glutamine, L-[5-^{15}N,3-^{13}C]glutamine (99 atom % excess) and 2-OGM (barium salt) were purchased from MSD (Montreal, Canada). Minimal essential medium (MEM) was purchased from Flow Laboratories (McLean, VA, U.S.A.). L-Amino acid oxidase (no. 9253), catalase (no. C-10) and 3,4-dihydroproline were purchased from Sigma. All other reagents were of the highest available grade.

Synthesis of isotopically labelled 2-OGM

Preparation of labelled 2-OGM was carried out by a modification of the procedure described by Ratner [13]. Briefly, 15 mg

Abbreviations used: 2-OGM, 2-oxoglutaramate; HK cells, human kidney cells; t-BDMS, t-butyldimethylsilyl.

* To whom correspondence and requests for reprints should be addressed.

of L-[3-^{13}C,5-^{15}N]glutamine was incubated with O_2 aeration for approx. 10 h at 37 °C in a reaction mixture containing 2 mg of L-amino acid oxidase and 2000 units of catalase in a final volume of 5 ml of Tris buffer, pH 7.7. The resulting solution (after 10 h incubation) was passed over an AG-1 column (Cl^-; 200–400 mesh; X^{-8}; 0.5 cm × 6 cm). The column was washed with 10 ml of distilled water, and the 5-hydroxy[4-^{13}C,1-^{15}N]pyroglutamate was eluted with 5 ml of 1 M-HCl. The pH of the eluate was adjusted to approx. 5 by addition of crystalline $Ba(OH)_2$. Determination of glutamine before and after the incubation with L-amino acid oxidase demonstrated that all of the L-[3-^{13}C, 5-^{15}N]glutamine was converted into 2-OGM.

Preparation of standard dilution curves

A standard dilution curve of ^{13}C- and ^{15}N-labelled 2-OGM was prepared by adding 20.2 nmol of the labelled compound to tubes containing 100, 75, 40, 25 or 12.5 nmol of unlabelled 2-OGM plus 100 nmol of 3,4-dihydroproline as internal standard. These samples (approx. 200 μl) were passed through an AG-1 column (200–400 mesh), after the pH had been adjusted to 7–8 with NaOH, then washed with 5 ml of water and eluted with 2 ml of 1 M-HCl. The samples were then evaporated to dryness under N_2, and the t-butyldimethylsilyl (t-BDMS) derivative was prepared by adding 50 μl of acetonitrile plus 50 μl of t-BDMS reagent and incubated for 10 min at 60 °C in reaction vials.

Experiments with cultured human renal epithelial cells

To determine whether HK cells are able to produce 2-OGM and to delineate a possible relationship between ammonia and 2-OGM formation from glutamine, incubations were carried out with HK cells prepared as previously described [6]. The steady-state medium was removed and replaced with 2 ml of fresh MEM supplemented with 25 mM-bicarbonate plus 5 mM-Hepes. This medium was adjusted to pH 6.8, 7.4 or 7.6. Incubations were initiated by addition of 1 mM-[2-^{15}N]glutamine in the presence or absence of 1 mM-amino-oxyacetate, an inhibitor of the aminotransferase reaction [14]. After 3 h incubation at 37 °C under compressed air/CO_2 (19:1), the medium was removed and the cell monolayer was washed three times with cold (4 °C) phosphate-buffered saline. Then 2 ml of 0.1 M-HCl was added to each dish. The bottom of the plate was scraped with a rubber spatula and the cells were then frozen and thawed twice to ensure complete cell lysis.

Amino acids and ammonia as well as their ^{15}N enrichment were determined in both the medium and intracellular compartment as previously described [6,11,12]. For determination of the 2-OGM concentration, to each 500 μl of the medium (extracellular compartment) or cell lysate (intracellular compartment) 5 nmol of labelled 2-OGM was added. After the pH had been adjusted to 7 with Tris buffer (pH 8), samples were passed over an AG-1 column (200–400 mesh), washed with 5 ml of water and eluted with 2 ml of 1 M-HCl. t-BDMS derivatives were prepared as described above.

The concentration of 2-OGM was calculated according to the equation:

$$C_2 = C_1(E_1/E_2 - 1)$$

where C_2 is the unknown concentration in the sample, and E_2 is the isotopic enrichment, i.e. atom% excess after addition of known amount (C_1) with a known isotopic enrichment (E_1) of 2-OGM.

G.c.–m.s. analysis

G.c.–m.s. analysis of the t-BDMS derivative of 2-OGM was performed with a Hewlett–Packard 5990A system. Gas-chromatographic separation was performed with a 25 m capillary column (SPB-1). The carrier gas was helium at a flow rate of approx. 2.5 ml/min. Injector temperature was 250 °C and the temperature programme was 80 °C isothermal for 1 min and then 10 °C/min for 4 min, followed by 15 °C/min for 8 min and 25 °C/min up to 300 °C. The spectrum of the t-BDMS derivative of 2-OGM was obtained by electron impact. The concentration of 2-OGM was determined with selected monitoring of ions obtained by electron impact, as discussed below.

N.m.r. experiments

To determine the chemical structure of 2-OGM, depending on the environmental pH, a 100 mM solution of unlabelled 2-OGM was prepared in 20 mM-Tris buffer in the pH range 2–10. In addition, ^{13}C and ^{15}N n.m.r. analyses were performed by using ^{15}N- and ^{13}C-doubly-labelled 2-OGM prepared as follows: a mixture of 40 mg of [3-^{13}C,5-^{15}N]glutamine, 2 mg of catalase (approx. 5000 units) and 5 mg of L-amino acid oxidase (approx. 3.5 units), in 10 ml of 20 mM-Tris buffer, pH 8.0, was incubated at 37 °C with O_2 aeration. ^{15}N and ^{13}C n.m.r. spectra were obtained before complete metabolism of glutamine, i.e. after 2 h incubation, in order to demonstrate the difference between ^{15}N and ^{13}C resonances of the precursor (glutamine), and that of the product (2-OGM), in the intact crude reaction mixture.

N.m.r. spectra were obtained on a Bruker AM-400 wide-bore spectrometer equipped with an Aspect 3000 data system. Unless otherwise indicated in the Figure legends, most of the one-dimensional ^{13}C spectra were obtained at room temperature under routine conditions as follows: ^{13}C frequency 100.62 MHz at 9.4 T, pulse flip angle 25°, 230 p.p.m. sweep width, 32 k data points, roughly 0.5 s repetition time with no additional relaxation delay. The spectra were acquired by using composite pulse decoupling (MLEV) of the protons to minimize temperature changes of the solution [15]. The processing was performed on 32 k data points by using a 1 Hz line broadening. ^{13}C shifts were measured relative to an external reference (pure 1,4-dioxan in a co-axial capillary $\delta = 67.4$ p.p.m.) and are given relative to tetramethylsilane at 0 p.p.m.

^{15}N spectra were obtained on the same spectrometer at 40.55 MHz with 20° pulse flip angle, 250 p.p.m. sweep width, 64 k data points, 3.3 s repetition time without additional relaxation delay. The acquisition was performed under MLEV decoupling of the protons at all times. Zero filling to 256 k and no line broadening were used to obtain the Fourier-transformed spectrum with sufficient digital resolution. The ^{15}N resonances exhibit partial or full nuclear Overhauser enhancement, but were nevertheless phased positive. Chemical shifts are given relative to an external reference (1 M-NH_4Cl in a co-axial capillary).

RESULTS AND DISCUSSION

The chromatogram of the total ion current obtained from the t-BDMS derivatives of 3,4-dihydroproline and 2-OGM is shown in Fig. 1. The retention time and temperature for the 3,4-dihydroproline were approx. 7.8 min and 147 °C respectively. For 2-OGM these values were 10.7 min and 220 °C.

The mass spectra and structure of the t-BDMS derivative of 2-OGM are depicted in Fig. 2. The molecular ion at m/z 487 is not seen. The peak at m/z 472 (1.2%) results from loss of $-CH_3$. The peaks at m/z 430, 402 and 328 correspond to $M^+ - 57$, $M^+ - 85$ and $M^+ - 159$ respectively (Fig. 2).

Ions at m/z 328, 329 and 330 were best suited for selected ion monitoring and quantitative studies because of their high relative intensity and minimum interference by other constituents in biological samples. The ratio of m/z 330/328 was 11.9 ± 0.21 (S.D.), representing more than 50 analyses carried out over a period of 2 months. Fig. 3(*a*) illustrates the m/z 330/328 ratio versus varying isotopic enrichment in 2-OGM when variable

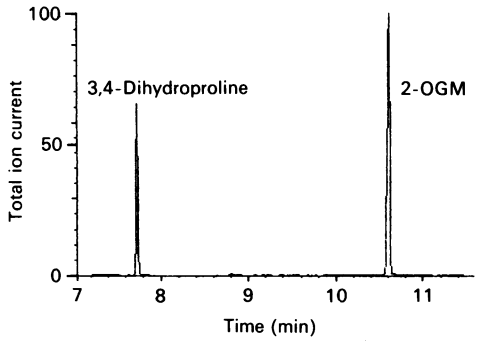

Fig. 1. Chromatogram of the total ion current obtained by electron-impact monitoring of t-BDMS derivative of 2-OGM and 3,4-dihydroproline (internal standard)

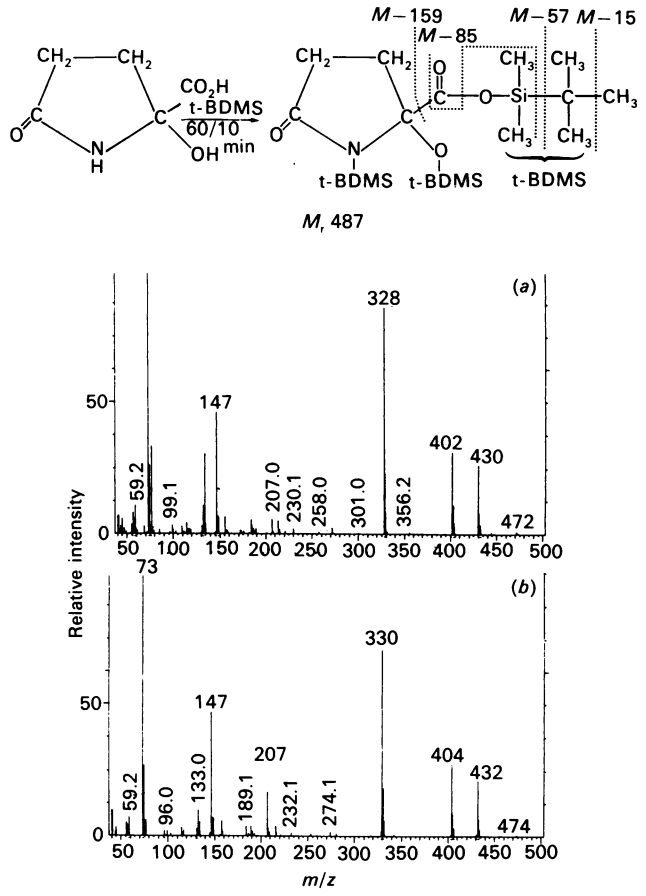

Fig. 2. Electron-impact mass spectra of (*a*) unlabelled and (*b*) $^{15}N,^{13}C$-labelled 2-OGM t-BDMS derivatives

Also is shown a schematic representation of the mass spectra produced by the tri-t-BDMS derivatives of 2-OGM.

amounts of unlabelled 2-OGM were added to a constant amount of labelled (99 atom % excess) compound. The corresponding relationship between enrichment in 2-OGM and the concentration of this compound is shown in Fig. 3(*b*). Calculations of the concentration based on the data of Fig. 3(*b*) have demonstrated an excellent agreement between observed and theoretical values: 21.79 ± 1.10 versus 20.25 mM. This agreement between expected and observed concentrations demonstrates that the isotope-dilution technique is accurate and sensitive enough to detect nmol amounts of 2-OGM in a biological sample.

Fig. 4(*a*) illustrates the ^{13}C n.m.r. spectrum of a 100 mM solution of 2-OGM in 20 mM-Tris buffer, pH 8.1. Besides the

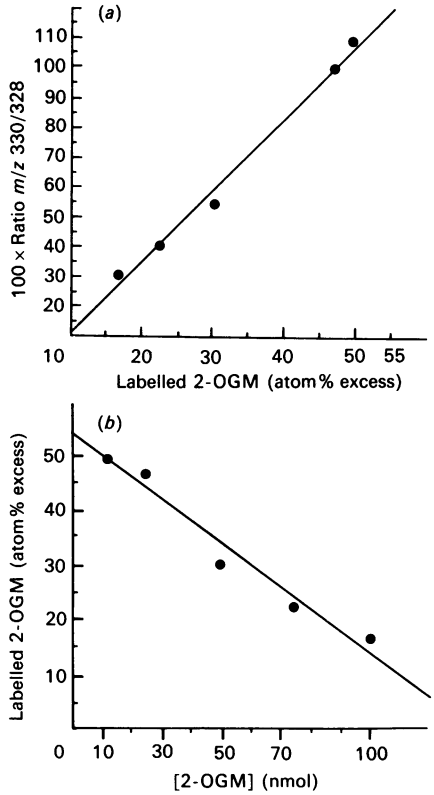

Fig. 3. Standard isotope-dilution curves for the quantification of 2-OGM

(*a*) *m/z* 330/328 ratio obtained by adding various amounts of unlabelled 2-OGM to a constant amount of labelled 2-OGM. (*b*) Calculated isotopic enrichment based on the *m/z* 330/328 (× 100) ratio (*a*) versus the concentration of unlabelled 2-OGM.

resonances due to the Tris buffer ($\delta = 60.20$, $\delta = 61.00$ p.p.m.) and a small impurity (labelled Y), probably recrystallization-solvent ethanol, the spectrum shows only one set of lines corresponding to the cyclized form of 2-OGM, i.e. 5 hydoxy-pyroglutamate. Peaks at $\delta = 29.58$ and 33.72 p.p.m. are triplets in the off-resonance spectrum and correspond to C-3 and C-4. The peak at $\delta = 89.16$ p.p.m. remains a singlet in the off-resonance spectrum and therefore is assigned to the quaternary carbon, C-5. The two peaks at $\delta = 177.48$ and $\delta = 181.57$ p.p.m. are assigned to the carbonyls. To identify each resonance further we performed a ^1H–^{13}C inverse-correlated experiment via long-range coupling [16]. As illustrated in Fig. 5, only the lactam carbonyl C-2 ($\delta = 181.57$ p.p.m.) can couple to the protons of both CH_2-3 and CH_2-4 via $^2J_{CH}$ and $^3J_{CH}$. The acid carbonyl C-6 couples only to the protons of CH_2-4. Similar observation leads to the assignments of CH_2-3 at $\delta = 29.58$ p.p.m. and CH_2-4 at 33.72 p.p.m.

Fig. 4(*b*) shows the ^{13}C n.m.r. spectrum of the crude reaction mixture used to prepare the $^{13}C,^{15}N$-labelled 2-OGM, after 2 h incubation. The peak at $\delta = 26.68$ p.p.m. (labelled X) corresponds to the remaining unchanged 3-^{13}C of glutamine. The enriched carbon of the synthesized 2-OGM coincides with the position of the C-4 carbon in the unlabelled compound and exhibits a small long-range coupling $^2J_{C\text{-}4\text{–}N\text{-}1} = 1.5$ Hz (see the inset). ^{13}C–^{13}C satellites are also visible, but coupling is more clearly observed on the resonance of the remaining natural-abundance carbons. C-3 ($\delta = 29.6$ p.p.m.), for example, is a doublet of doublets $^1J_{C\text{-}3\text{–}C\text{-}4} = 32.4$ Hz, $^2J_{C\text{-}3\text{–}N\text{-}1} = 8.0$ Hz, and C-5 ($\delta = 89.2$ p.p.m.) is also a double doublet $^1J_{C\text{-}5\text{–}C\text{-}4} = 35.9$, $^1J_{C\text{-}5\text{–}N\text{-}1} = 11.4$ Hz. The carbonyl C-6 ($\delta = 177.48$ p.p.m.) appears as an unresolved broad singlet, and the carbonyl C-2 ($\delta = 181.6$ p.p.m.) is a doublet

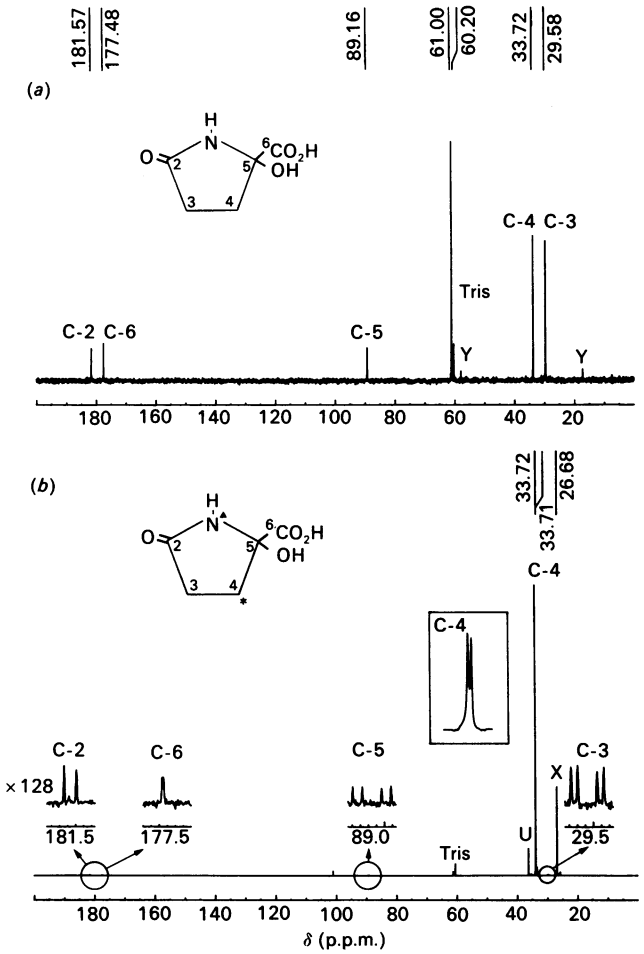

Fig. 4. (*a*) ^{13}C n.m.r. spectrum of a 100 mM solution of 2-OGM in 20 mM-Tris buffer, pH 8.1, and (*b*) ^{13}C n.m.r. spectrum of the crude reaction mixture used to prepare 4-^{13}C,1-^{15}N-labelled 2-OGM after 2 h incubation (for details see the Materials and methods section)

In (*a*), the Tris buffer gives rise to two signals, CH_2–OH at 61.00 p.p.m. and the quaternary carbon at 60.20 p.p.m. Y is a trace of recrystallization solvent ethanol. In (*b*) X is the remaining [3-^{13}C,5-^{15}N]glutamine, and U is an unidentified minor compound. The acquisition was performed with 64 k data points zero-filled to 256 k for the processing. No line broadening was used. Insets show the expansion of the natural-abundance carbon signals.

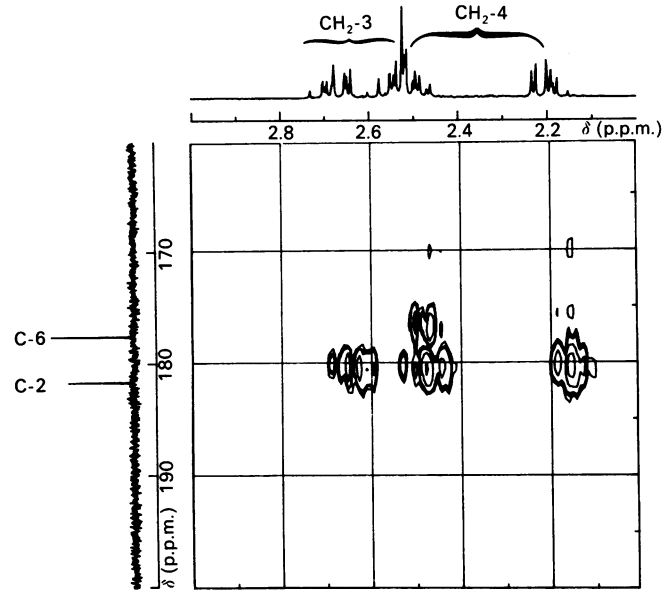

Fig. 5. Two-dimensional ^1H–^{13}C correlation via heteronuclear zero and double-quantum coherence using inverse mode optimized for long-range coupling

The spectrum results from a 1024 × 512 data matrix; acquisition times in the t1 and t2 dimensions were 11.3 and 300 ms respectively. The repetition rate was 1 s, the evolution time for the long-range coupling was 60 ms and 464 scans were recorded per t1 value. The window used in both dimensions was sinebell-shifted by $\pi/2$, and the spectrum is plotted in the absolute-value mode.

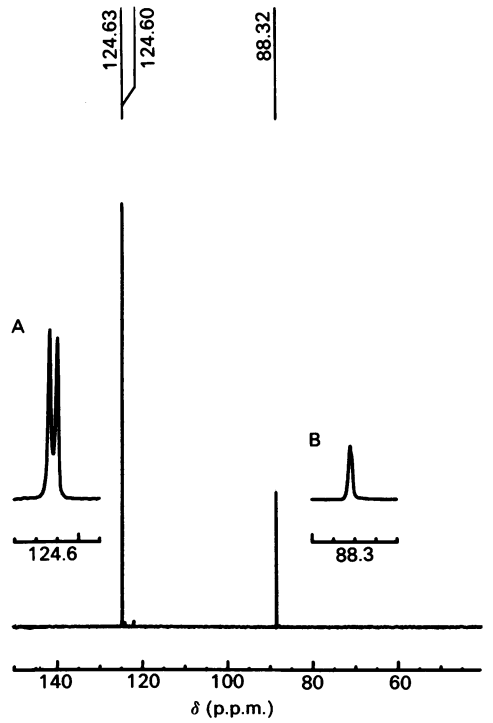

Fig. 6. ^{15}N spectrum of the crude reaction mixture used to synthesize ^{15}N- and ^{13}C-labelled 2-OGM

The spectrum was obtained from the reaction mixture after 2 h incubation as detailed in the Materials and methods section. The remaining 5-^{15}N of glutamine is at 88.32 p.p.m. and 1-^{15}N of the synthesized 5-hydroxypyroglutamate is at 124.63 p.p.m.

$^1J_{C\text{-}2\text{-}N\text{-}1}$ = 15.3 Hz. The peak at δ = 36.14 p.p.m. (labelled U) has not been identified. The ^{15}N n.m.r. spectrum of the same crude reaction mixture (Fig. 6) demonstrates three signals. Besides the Tris resonance (δ = 3.6 p.p.m., not shown on the Figure), the signal at δ = 88.3 p.p.m. is the remaining [5-^{15}N]glutamine. The most intense signal (δ = 124.6 p.p.m.) is split by the small coupling $^2J_{N\text{-}1\text{-}C\text{-}4}$ = 1.5 Hz (inset A) and is therefore assigned to the imino nitrogen of the cyclic 2-OGM. Thus the present n.m.r. data demonstrate that 2-OGM exists only in cyclic form, i.e. 5-hydroxypyroglutamate.

To delineate a possible equilibrium between the open-chain and cyclic structure of 2-OGM we studied at room temperature the ^{13}C n.m.r. spectra of the commercial 2-OGM in Tris buffer. Spectra identical with that illustrated in Fig. 4(*a*) were obtained at pH values in the range 2–10 (results not shown). We were unable to detect any changes in the linewidth or position of the peaks other than those expected when reaching the p*K* value of the acid function. It is important to note that *C*-5 has an extremely large chemical shift difference between the two forms: in the open-chain configuration this carbon, being a ketone carbonyl, would probably be found above 200 p.p.m., whereas in the cyclic structure C-5 is an sp^3 carbon substituted by oxygen and nitrogen atoms and is found at 89.16 p.p.m. Therefore, the

Table 1. Effect of pH and amino-oxyacetate on production of 5-hydroxypyroglutamate, ammonia and alanine from [2-^{15}N]glutamine

Cultured human kidney cells were incubated with minimal essential medium supplemented with 1 mM-[2-^{15}N]glutamine. Incubations were carried out for up to 4 h at the indicated pH. 5-Hydroxypyroglutamate was determined by an isotope-dilution technique (see the Materials and methods section). ^{15}N-labelled ammonia and alanine are the products of isotopic enrichment (atom % excess/100) and total [^{14}N + ^{15}N]metabolite concentration (nmol/mg of protein). Values are means ± S.D. of 5–15 cultured dishes from four separate experiments: P values (Student's t test) indicate the significance of the difference compared with experiments at pH 7.4; *$P < 0.05$; †$P < 0.002$; ‡$P < 0.001$; §$P < 0.01$.

Experiment (pH)	Metabolite production (nmol/h per mg protein)				
	Total ammonia	Total alanine	5-Hydroxypyroglutamate	[^{15}N]Ammonia	[^{15}N]Alanine
pH 7.4	307.8 ± 32.7	209.2 ± 41.9	30.6 ± 6.2	41.7 ± 10.1	81.5 ± 21.3
pH 6.8	451.0 ± 42.2†	310.6 ± 30.3‡	58.5 ± 6.4†	117.4 ± 32.6‡	133.5 ± 21.7*
pH 7.6	204.1 ± 40.8§	172.2 ± 16.7*	30.2 ± 3.7*	13.3 ± 5.3‡	48.6 ± 9.7§
pH 7.4 + 1 mM-amino-oxyacetate	415.6 ± 39.7§	67.3 ± 13.9‡	9.5 ± 2.1‡	117.6 ± 20.1‡	8.6 ± 2.4‡

broadening of C-5 in the case of intermediate exchange rate should be important and measurable over a wide range of pH values, even if the minor form has a low abundance. The observation of a unique form in which C-5 is sharp and appears at 89.16 p.p.m. could result from a slow chemical exchange on the n.m.r. time-scale with the open-chain form too low to be detected, or a fast chemical exchange, with again the open-chain form being negligible.

Formation of 5-hydroxypyroglutamate from glutamine by HK cells is shown in Table 1. We could not detect the lactam in the intracellular compartment. The non-metabolized 5-hydroxypyroglutamate formed in the cytosol probably was released into the medium. The accumulation of 5-hydroxypyroglutamate was significantly elevated at pH 6.8 and depleted at pH 7.6 compared with pH 7.4 (Table 1). As noted above, the increased production of 2-OGM at acid pH does not reflect pH-dependent cyclization of the parent compound. Furthermore, when incubations were carried out at pH 7.4 in the presence of amino-oxyacetate production of 5-hydroxypyroglutamate was significantly ($P < 0.01$) decreased (Table 1). Thus inhibition of the flux through the glutamine aminotransferase blocked the formation of 2-OGM.

In the glutamine aminotransferase reaction the amino N of glutamine would be transferred to an appropriate oxoacid receptor, e.g. pyruvate, thereby giving rise to increased [^{15}N]-alanine synthesis. This is shown in Table 1, representing the pH effect on the production of total and ^{15}N-labelled ammonia and alanine during incubations of HK cells with 1 mM-[2-^{15}N]glutamine. $^{15}NH_3$ production (nmol/mg or protein) was significantly higher at pH 6.8 and lower at pH 7.6 compared with that at pH 7.4. Similarly, production of [^{15}N]alanine was higher ($P < 0.01$) in acute acidosis and lower ($P < 0.005$) in acute alkalosis. These observations are in good agreement with our previous studies involving incubations of [2-^{15}N]glutamine as a sole nitrogen source in phosphate buffer [6].

Production of $^{15}NH_3$ from [2-^{15}N]glutamine most probably occurred via the sequential action of phosphate-dependent glutaminase and glutamate dehydrogenase [1,3,6,11,12]. Hence the current results further support the conclusion that flux through glutamate dehydrogenase is primarily responsible for augmented renal ammoniagenesis in response to acidosis [6].

Formation of [^{15}N]alanine at various pH values of the incubation medium is correlated with the formation of $^{15}NH_3$ (Table 1). In the presence of amino-oxyacetate, at pH 7.4 the production of [^{15}N]alanine was significantly diminished ($P < 0.001$), but $^{15}NH_3$ formation increased ($P < 0.05$) to a level obtained at pH 6.8. These observations are in accord with previous studies demonstrating an enhanced flux through glutamate dehydrogenase in the presence of amino-oxyacetate [17,18]. Production of [^{15}N]alanine could have been derived via either glutamate–pyruvate aminotransferase or the glutaminase II pathway [6]. The decreased formation of [^{15}N]alanine and 2-OGM in the presence of amino-oxyacetate (Table 1) suggests that either the glutamate–pyruvate aminotransferase and/or glutaminase II pathway is inhibited. The fact that $^{15}NH_3$ production from [2-^{15}N]glutamine is greater in the presence of amino-oxyacetate at pH 6.8 indicates that the glutaminase II pathway has a minor role in enhanced ammoniagenesis by cultured HK cells. Furthermore, to the extent that the glutaminase II pathway is more active, less NH_3 would be derived from the amide N of glutamine, this nitrogen now being trapped as non-metabolizable 5-hydroxypyroglutamate. Although we cannot differentiate between [^{15}N]-alanine formed via glutamate–pyruvate aminotransferase and/or glutaminase II, the results with amino-oxyacetate (Table 1) suggest that the augmented $^{15}NH_3$ is mediated via glutamate dehydrogenase from [^{15}N]glutamate derived through the phosphate-dependent glutaminase pathway. In this regard, the stimulation of both total ammonia and $^{15}NH_3$ formation in the presence of amino-oxyacetate indicates that the latter enhanced not only flux through glutamate dehydrogenase but also flux through the phosphate-dependent glutaminase pathway. Similarly, Schoolwerth & LaNoue [17] have suggested that amino-oxyacetate stimulates flux through both glutamate dehydrogenase and glutaminase pathways.

In summary, contrary to the previous view that significant amounts of ammonia can be produced via the sequential action of glutamine aminotransferase and ω-amidase pathways [7,19,20], the present data indicate that the glutaminase II pathway has a minor role in renal ammoniagenesis, regardless of H^+ homoeostasis. Further studies to elucidate the mechanism(s) abetting and regulating 2-OGM formation are of special importance because of the possibility that 2-OGM may have a nephrotoxic effect, causing tubular-cell necrosis and renal injury in a manner analogous to that of other imino compounds [21] and β-lactam antibiotics [22].

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