A QUANTITATIVE STUDY OF RECYCLING OF ISOTOPE FROM
GLYCINE-1-C¹⁴, α -N¹⁵ INTO VARIOUS SUBUNITS OF THE
URIC ACID MOLECULE IN A NORMAL SUBJECT *

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Glycine is the precursor of three atoms of the purine ring, carbons 4 and 5 and nitrogen 7 (1, 2). When glycine-N¹⁵ is administered orally in man N¹⁵ is incorporated predominantly into N-7, but N¹⁵ is also found in N-1, N-3, and N-9 which are donated not by glycine but by aspartic acid (N-1) and the amide-N of glutamine (N-3 and N-9) (3). The N¹⁵ found in N-atoms other than N-7 of uric acid increases with time and may reach 35 per cent of the total N¹⁵ after several days (1). In contrast, when glycine-1-C¹⁴ is administered orally in man the isotope labels C-4 of uric acid almost exclusively, and very little C¹⁴ is incorporated into other carbon atoms by secondary pathways (4). As a consequence of these diverse fates of the α -N¹⁵ and 1-C¹⁴ atoms of glycine, the N¹⁵/C¹⁴ ratio in uric acid exceeds 1.0 after administration of doubly labeled glycine (5). Values of 1.7 and 1.8 were reported in control subjects, and of 2.0 to 2.8 in gouty subjects by Gutman and co-workers (5). Their results further suggested that the ratio of (N¹⁵ in N-7)/(C¹⁴ in uric acid) was also greater than 1.0. In one "normal excretor" gouty subject the ratios were generally in excess of 1.2. Additionally, ratios of 1.08 to 1.92 (mean, 1.34) and of 0.86 to 1.37 (mean, 1.16) were calculated for two nongouty control subjects, the same (N¹⁵ in N-7)/(total N¹⁵ in uric acid) assumed as in one normal subject studied by Shemin and Rittenberg (1).

An incorporation of glycine-N¹⁵ into N-7 greater than of glycine-1-C¹⁴ into C-4 would have important implications that might signify either an accessory pathway of purine synthesis, or a disparity in the rates of "isotopic turnover" of the α -N and 1-C of glycine. There are known mecha-

nisms whereby such a disparity of isotopic turnover might arise. A major fate of glycine involves interconversion with serine. The glycine-serine "shuttle" would not of itself alter the C¹⁴/N¹⁵ ratio of reconstituted glycine, since it is the α -C of glycine that becomes the β -C of serine, and in the case of glycine-1-C¹⁴, α -N¹⁵, the α -C is unlabeled. However, serine serves as a precursor of glyoxylic acid, a compound readily converted to glycine by glyoxylic-glutamic transaminase (6-8). The glyoxylic acid arising from glycine-1-C¹⁴, α -N¹⁵ via serine (9-12) would be unlabeled, since prior decarboxylation and deamination steps would have removed both C-1 and α -N of serine, and both carbons of glyoxylic acid would represent carbons originally in the α -position of glycine. Glycine can also be deaminated directly by glycine oxidase to form glyoxylic acid and ammonia (13). If this labeled glyoxylic acid was extensively diluted by unlabeled molecules, and if ammonia-N¹⁵ from glycine found its way rather directly into glutamic acid, it is theoretically possible that active resynthesis of glycine by the glyoxylic-glutamic transaminase route could result in reincorporation of N¹⁵ in excess of C¹⁴. In this case the glycine pools should show more rapid isotopic turnover of C¹⁴ than of N¹⁵, and such differential rates of turnover might be reflected in unequal labeling of various products synthesized from glycine. Such mechanisms might offer an explanation for the (N¹⁵ in N-7)/(C¹⁴ in uric acid) ratios more than 1.0 found by Gutman and colleagues in control subjects (5), and perturbations of certain reactions of these cycles might be present in gout to explain the higher ratios found in subjects with this disorder.

Since the calculation cited above, suggesting that relatively more N¹⁵ than C¹⁴ enters the glycine subunit of uric acid after injection of glycine N¹⁵, 1-C¹⁴ in control subjects, also involved

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an assumed N^{15} partition in urate, it seemed desirable to explore this problem more fully. This study was performed to obtain direct evidence on incorporation of N^{15} into N-7 and of C^{14} into C-4 of uric acid after administration of glycine α - N^{15} , 1- C^{14} to a normal subject, and to determine how faithfully the glycine subunit of uric acid reflects the pattern of labeling of the glycine pool, as sampled during formation of hippuric acid. In this subject there was no evidence for disparate labeling of either the glycine subunit of uric acid or of glycine in hippuric acid. These results do not, of course, bear on the possibility of such disparity in subjects with derangements of purine or amino acid metabolism.

MATERIALS AND METHODS

Glycine-1- C^{14} , specific activity 4.0 mc per mmole, was purchased from Isotopes Specialties Co. Glycine- N^{15} was synthesized according to Schoenheimer and Ratner (14) from potassium phthalimide- N^{15} , containing approximately 95 atom per cent excess N^{15} , obtained from Isomet Corp.

Uric acid was isolated from urine by precipitation with copper (15) and repeatedly recrystallized from Li_2CO_3 solution (16), after decoloration with Darco-S51. All uric acid analyses were performed by the differential spectrophotometric method of Praetorius (17) employing purified uricase purchased from Worthington Biochemical Corp.

Hippuric acid was isolated according to Quick (18) and recrystallized repeatedly from hot water after decoloration with Darco until each sample gave an uncorrected melting point of 186° to 187° C.

Each uric acid sample was degraded by several procedures to permit analysis of individual atoms. C-(4+5) was obtained as glyoxylic acid semicarbazone according to Buchanan, Sonne and Delluva (19). N-7 was obtained as glycine by hydrolysis of uric acid in 12 N HCl at 160° for 18 hours (1). After alkalization and aeration to collect NH_3 (20) derived from N-(1+3+9), the residual solution was neutralized and the glycine chromatographed on a Dowex 50- H^+ column, 1.0×7.5 cm. Any undegraded uric acid was washed off the column with water. Glycine was eluted with 0.1 M citrate, pH 3.4 (21), and was located with ninhydrin in the 30 to 70 ml fractions. Residual traces of NH_3 were retained by the column. N-(1+7) was obtained as NH_3 according to Brandenberger (22). Uric acid, and glycine derived from it, hippuric acid, and the glycine standard were all degraded by the micro-Kjeldahl procedure, and steam distilled to collect NH_3 for N^{15} analyses. Samples were prepared for N^{15} analysis according to Rittenberg (23). N^{15} analyses were performed in duplicate on a 180° Consolidated Electrodynamics mass spectrometer.¹

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Samples checked on both instruments were in excellent agreement. All C^{14} determinations were performed on solid samples of uric acid, glyoxylic acid semicarbazone, glycine or hippuric acid in a Harvard-Robinson gas-flow counter (24). Most of these were done by plating samples in triplicate. All samples were corrected for self-absorption to a standard mass of 3.3 mg per 1.54 cm^2 planchet area. The efficiency of counting at this sample thickness is 24.6 per cent.

Design of experiment. The subject of the study was a 27 year old normal adult male weighing 84.0 kg. He was placed on a standard low purine diet containing 2,600 calories, 70 g protein and 350 g carbohydrates, which was prepared by the diet kitchen of Duke Hospital and which he ingested throughout the study. After 5 days of pretreatment with this diet, the subject's serum uric acid was 5.4 mg per 100 ml and the 24-hour urinary uric acid was 523 mg. These values varied only minimally throughout the next 24 days, the urinary uric acids ranging from 452 to 529 mg per day, averaging 495 ± 18 mg per day. Urinary uric acid and hippuric acid isolated before labeled glycine was given showed no biological enrichment with N^{15} . The subject received 5.01 g C^{14} , N^{15} glycine orally. This glycine showed by analysis 95.1 atom per cent excess N^{15} , and 886,340 cpm per mmole (mol wt of glycine, corrected for N^{15} content, 76.02). The total N^{15} administered was 293.0 mg; total C^{14} was 57,533,000 cpm.

Urine samples were collected in sequential aliquots of 10, 2, 10, and 2 hours, respectively, during the first 2 days. Thereafter they were collected in sequential 22- and 2-hour aliquots for 22 additional days. Uric acid was isolated from the 10- and 22-hour urine samples. Hippuric acid was isolated from urine samples collected for 2 hours after intravenous administration of 400 mg sodium benzoate. All samples were analyzed in triplicate for uric acid content. The concentrations of N^{15} and C^{14} in uric acid of these 2-hour samples were obtained by interpolation from the plots of data obtained from analysis of uric acid isolated from 10- or 22-hour samples.

RESULTS

Gross incorporations of N^{15} and C^{14} into uric acid. The incorporations of N^{15} and C^{14} into individual samples of uric acid are shown in Figure 1. Both incorporation curves show secondary maxima on Days 10 to 12 in addition to the early maxima on Days 2 and 3. As anticipated, the incorporation of N^{15} exceeded that of C^{14} , and the disparity increased gradually with time. By the end of the 24-day period, an N^{15}/C^{14} ratio of 1.5 had been reached. The cumulative incorporation of N^{15} into

for the majority of N^{15} analyses, and to Dr. Henry Kamin and Mr. Bernard Bulos of the Department of Biochemistry of Duke University Medical Center for some of the total uric acid- N^{15} and hippurate- N^{15} analyses.

FIG. 1. INCORPORATION OF N^{15} AND C^{14} INTO URIC ACID AFTER ORAL ADMINISTRATION OF GLYCINE- α - N^{15} , 1- C^{14} .

uric acid was 0.360 per cent in 24 days, whereas that of 1- C^{14} was 0.264 per cent during the same period.

Incorporation of N^{15} into individual nitrogen atoms of uric acid. Values for incorporation of N^{15} into N-1 and N-(3 + 9), in atom per cent excess, may be calculated from analytical data as follows.

$$N^{15-1} = 2 \left[\frac{N^{15}-(1+7)}{2} \right] - (N^{15-7})$$

$$N^{15}-(3+9) = 3 \left[\frac{N^{15}-(1+3+9)}{3} \right] - (N^{15-1})$$

Since both N-3 and N-9 are derived from the amide-N of glutamine (3), the enrichment of each may be taken as approximately $\frac{1}{2} [N^{15}-(3+9)]$, assumed that the same pool of intracellular glutamine contributes both N-3 and N-9, and that the turnover of this pool is slow with respect to the time lapse between these contributions. Thus, individual enrichment values may be obtained for N-1, N-3, and N-9 in addition to N-7. The cumulative incorporations of N^{15} into individual N-atoms are shown in Figure 2, in which the sum of N^{15} in N-1, N-3, N-7, and N-9 is also shown. The sum of the individual N^{15} values is in excellent agreement with values of uric acid itself. In no case did the values on any given day differ by more than 10 per cent. The cumulative incorporation of N^{15} into uric acid, as determined by addition of the cumulative incorporation values into the individual N-atoms (Figure 2) was 0.260 per cent. This value is virtually identical with that obtained by analysis of intact uric acid samples.

N^{15} in N-7 of uric acid accounted for 88 per

FIG. 2. INCORPORATION OF N^{15} INTO INDIVIDUAL N-ATOMS OF URIC ACID. The triangles represent N^{15} in N-3 and N-9 individually; they are assumed to be equally labeled (see text). The squares represent the sums of the individual values, and also the N^{15} in total uric acid, since these are virtually identical (see text).

cent of the N^{15} in uric acid collected over the first 10 hours. By the end of the third week, however, it accounted for only about 60 to 65 per cent of the total N^{15} (Figure 3). These data show a general conformity with those of Shemin and Rittenberg (1).

Incorporation of C^{14} into C-4 of uric acid. When uric acid was degraded to glyoxylic acid semicarbazone, the enrichment of the latter in all cases approximated that of uric acid itself. The data are given in Table I. The average ratio of enrichment of glyoxylic acid semicarbazone to uric acid was 0.981, signifying that virtually all of the C^{14} of the uric acid molecule was present in

FIG. 3. PERCENTAGE OF N^{15} FOUND IN THE N-7 ATOM OF URIC ACID VERSUS TIME.

TABLE I

Comparison of total enrichment of uric acid and of the C-(4 + 5) fragment of uric acid, isolated as glyoxylic acid semicarbazone

Day	C-(4+5) cpm/mmole	Uric acid cpm/mmole	C-(4+5)/ uric acid
3	2,738	2,859	0.96
4	2,659	2,758	0.97
5	2,646	2,804	0.94
6	2,702	2,506	1.08
7	2,568	2,503	1.03
8	2,528	2,412	1.05
9	2,371	2,489	0.95
10	2,621	2,608	1.00
11	2,337	2,535	0.92
12	2,332	2,510	0.93
14	2,333	2,302	1.01
15	1,977	1,962	1.01
16	1,978	1,993	0.99
17	1,821	1,853	0.98
18	1,716	1,784	0.98
19	1,702	1,844	0.92
20	1,637	1,717	0.95
21	1,565	1,704	0.92
22	1,454	1,401	1.04
Average			0.981

the C-(4 + 5) fragment. Although in this study the C-6 and C-(2 + 8) fragments of uric acid were not isolated, in an earlier study of eight samples from four subjects any isotope that may have been in these fragments after administration of glycine-1-C¹⁴ was too low in concentration to be detected when counted as BaCO₃ (4).

In these earlier studies of the distribution of C¹⁴ in the various carbon atoms of urinary uric acid, from 95 to 100 per cent of the isotope was found in the C-(4 + 5) fragment. Two samples of glyoxylic acid semicarbazone derived from C-(4 + 5) were further degraded. Only a very small amount of activity (< 10 per cent) was detected in CO₂ derived chiefly from C-5, and since the degradation method employed (4, 19) is known to separate C-4 and C-5 imperfectly, this was considered contamination from C-4. This explanation may be applied also to the small degree of apparent labeling of C-5 found by Buchanan and colleagues (19) and by Karlsson and Barker (2) after experiments with glycine-1-C¹⁴ in pigeon systems. Significant labeling of C-5 occurs only under circumstances in which glycine 2-C¹⁴ is employed (2) or in which the "formyl pool" itself becomes highly labeled and contributes isotope to C-(2 + 8) and by indirect pathways to C-5 also (25). Secondary enrichment of C-5, from gly-

cine-1-C¹⁴, is most unlikely, since even administration of C¹⁴O₂ or formate-C¹⁴ does not result in enrichment at this position beyond 1 to 3 per cent of the total label in uric acid (2, 26). In the present study there is no significant fraction of C¹⁴ unaccounted for that could be in C-(2 + 8) or C-6. Therefore it appears reasonable to conclude that virtually all C¹⁴ is in C-4 of uric acid after administration of glycine-1-C¹⁴ although, admittedly, rigorous proof is lacking.

Comparison of N¹⁵ in N-7 and of C¹⁴ in C-4. The isotopic enrichments of N-7 and C-4 are plotted in Figure 4, where it can be seen that there is no significant difference in the concentrations of N¹⁵ and C¹⁴ recovered in the glycine subunit of uric acid. Comparison of 19 individual samples on which both analyses were available gave an N¹⁵/C¹⁴ incorporation ratio of 1.009. Individual values ranged from 0.865 (Day 2) to 1.118 (Day 9). The secondary maxima previously observed in the gross incorporation data (Figure 1) are again in evidence in N-7 and C-4. These maxima in uric acid on Days 10 to 12 may reflect a contribution of granulocyte nucleic acid purines, since granulocytes have a life span of about 10 days (27).

The cumulative incorporations of N¹⁵ into N-7 and of 1-C¹⁴ into C-4 are shown in Figure 5. Again, good agreement is seen. The values differ by only 0.005 per cent after 24 days.

Analysis of urinary hippuric acid. Small doses of sodium benzoate (400 mg) were given periodically to promote synthesis of hippuric acid. Quantities of 1.5 to 3.0 g of benzoate have been used by others (28, 29), but under these circumstances

FIG. 4. INCORPORATION OF N¹⁵ INTO N-7 AND OF C¹⁴ INTO C-4 OF URIC ACID, AFTER ORAL ADMINISTRATION OF GLYCINE-α-N¹⁵, 1-C¹⁴.

FIG. 5. CUMULATIVE INCORPORATION OF N^{15} INTO N-7 AND OF C^{14} INTO C-4.

benzoate stimulates mobilization of glycine from protein or synthesis of new glycine (28, 30), giving lower enrichment values of hippurate. The smaller dose was chosen to minimize these phenomena. The hepatic glycine pool concerned with synthesis of hippurate (31) was sampled to permit comparison of its N^{15}/C^{14} concentrations with those of administered glycine as functions of time. The values are plotted on semilogarithmic coordinates in Figure 6, where it is apparent that there is no significant differential in turnover of one isotope in glycine with respect to the other in the pool participating in hippuric acid synthesis. Although the analytical data on individual samples of hippuric acid are somewhat more erratic than on the glycine subunit [C-(4 + 5) and N-7] of uric

FIG. 6. SEMILOGARITHMIC PLOT OF N^{15} AND C^{14} CONTENT OF HIPPURIC ACID VERSUS TIME. The squares represent the differences between the values of N^{15} and C^{14} observed and those anticipated from the regression line, $T_{1/2} = 197$ hours, and these data ($\blacksquare = N^{15}$, $\square = C^{14}$) serve as the basis for placement of the regression line, $T_{1/2} = 18.2$ hours. The difference points on Day 1 fall well above the second line and signify the existence of a fractional turnover of the glycine pool too rapid to be analyzed from available data (see text).

acid, the N^{15}/C^{14} ratio on 19 samples of hippuric acid on which both analyses were obtained averaged 1.002.

The semilogarithmic plot of isotope decay in hippuric acid indicates at least three fractional turnover rates of the glycine pool. The data fit the equation

$$C_t = C_0 e^{-\lambda_1 t} + 1.84 e^{-0.0380 t} + 0.35 e^{-0.00352 t}$$

The value $\lambda_2 = 0.0380$ is in good agreement with the second rate constant calculated by Watts and Crawhall, $\lambda_2 = 0.0377$, after administration of glycine-1- C^{13} to a normal subject (32). They also found a more rapid component of glycine turnover with a rate constant of $\lambda_1 = 0.552$; in the present study no samples were obtained earlier than 10 hours after glycine administration, and data are therefore insufficient to plot the most rapid component of glycine turnover. The rate constants calculated are somewhat greater than those found by Berlin, Hewitt and Lotz (29) in three subjects given glycine-2- C^{14} , but in their subjects some recycling of C^{14} from the 2-position back into resynthesized glycine was evident.

DISCUSSION

This study demonstrates that in this normal subject all of the incorporation of N^{15} into N-7 of uric acid is accounted for on the basis of incorporation of the intact glycine molecule. The processes that result in transfer of N^{15} from glycine to aspartic acid and the amide-N of glutamine, and secondarily into N-1, and N-3 and N-9 of uric acid, respectively, do not result in detectable re-labeling of glycine. Had this occurred, N^{15}/C^{14} values significantly in excess of 1.0 would have been expected in N-7/C-4 of uric acid, and in hippuric acid.

Recycling of N^{15} from glycine into the "nitrogen pool" and back into endogenously produced glycine remains a possible explanation for $[(N^{15} \text{ in N-7})/(C^{14} \text{ in uric acid})] > 1.0$ found in gouty subjects by Gutman and colleagues (5). The pathways by which $N^{15}H_4^+$ is incorporated excessively in such patients into uric acid might well include labeling of N-7 via glycine. A detailed analysis of these possibilities in gouty patients is warranted.

The present study shows, however, that in this normal subject there is no disparity of isotopic

turnover of α -N¹⁵ and 1-C¹⁴ in body glycine, and no evidence for other than direct incorporation of intact glycine into the glycine subunit of uric acid.

SUMMARY

1. Glycine-1-C¹⁴, α -N¹⁵ was administered orally to a control subject, and incorporations of C¹⁴ and N¹⁵ into individual atoms of uric acid, and into hippuric acid, were followed for 24 days.

2. Glycine-1-C¹⁴ labeled C-4 of uric acid almost exclusively. Glycine N¹⁵ labeled N-1, N-3, and N-9 appreciably, in addition to its direct incorporation into N-7. The percentage of N¹⁵ in atoms other than N-7 reached 35 to 40 per cent in 3 weeks.

3. A parallel incorporation of N¹⁵ and C¹⁴ into N-7 and C-4 of uric acid was observed. Analyses of hippuric acid similarly gave no evidence for differential rates of isotopic turnover of α -N and carboxy-C of glycine. The data indicate that all of the N¹⁵ in N-7 of uric acid in this normal subject was accounted for by direct incorporation of the intact glycine molecule; there was no detectable recycling of N¹⁵ into N-7 by secondary pathways.

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