The First Glycine Metabolic Pool in Man

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The number of reported investigations which have been designed to determine directly with the help of isotopically labelled intermediates whether a given precursor–product relationship operates in man *in vivo* are still relatively few. In connexion with our studies on the conversion of glycine into oxalate in subjects with primary hyperoxaluria (Scowen, Crawhall & Watts, 1958), we required to know the extent of the dilution which an oral dose of [^{13}C]glycine would undergo in the human subject. We thus calculated the size and turnover rate of the pool of glycine with which such a dose of glycine mixes immediately after absorption and distribution, and have called this pool the 'first glycine metabolic pool', a term previously used by Arnstein & Neuberger (1951). The location of this metabolic pool cannot be defined in anatomical terms, but the uncombined visceral glycine together with the glycine which is dissolved in the plasma and interstitial fluid presumably constitute a large part of it. The free urinary glycine is separated directly and continuously from the plasma by glomerular filtration. After the administration of isotopically labelled glycine, the isotope content of glycine isolated from urine would be expected to be the same as the isotope content of the plasma glycine at the moment of filtration, and we suggest that it may therefore be used as a measure of the isotope content of the glycine in the first glycine metabolic pool. The glycine moiety of the urinary hippuric acid that is excreted after oral doses of sodium benzoate which are sufficiently large to increase the urinary excretion of hippurate has been used as a sample of the glycine metabolic pool (Gray & Neuberger, 1950; Berlin, Hewitt & Lotz, 1954). This can be criticized on the grounds that the administration of these loading doses of sodium benzoate may cause the deviation of glycine from other metabolic pathways to participate in hippurate synthesis (Arnstein & Neuberger, 1951), or promote the synthesis of glycine from non-nitrogenous precursors (Gray & Neuberger, 1950). Moreover, since the liver is the main site of hippurate synthesis, dietary glycine reaching the liver via the portal vein might take part in this reaction before it had mixed uniformly in the glycine metabolic pool. We have studied this problem in man by comparing the isotope contents of the urinary hippuric acid and the first glycine metabolic pool during a period of repetitive feeding with, and after a single dose of, isotopically labelled glycine.

Stable isotopes were used in the present work as in the subsequent clinical investigations to eliminate any slight risk of radiation injury. Some of the results presented here have been the subject of preliminary communications (Crawhall & Watts, 1958*a*, *b*).

METHODS

Analyses were by Weiler and Strauss, Oxford. Melting points are uncorrected.

Synthesis of [1-^{13}C]*glycine and* [^{15}N]*glycine*

[1-^{13}C]Glycine had to be synthesized on a much larger scale than in previously described isotopic syntheses (cf. Arnstein & Bentley, 1950), and it was therefore necessary to design a new apparatus for the preparation of the first intermediate, [1-^{13}C]acetic acid. The direct amination of bromoacetic acid with NH_3 and $(NH_4)_2CO_3$ proved unsatisfactory and was abandoned, as we were unable to separate glycine from iminodiacetic acid on our scale of work (cf. Chase & Downes, 1953), the latter impurity being present to the extent of about 10% of the glycine yield in our preliminary experiments.

[1-^{13}C]*Acetic acid from* $Ba^{13}CO_3$. The reaction was performed in the carbonation apparatus (Fig. 1), which was not assembled until the Grignard reagent had been prepared in the triple-necked 3 l. flask (*A*). Ether-washed Mg-turnings (16 g., 0·6 mole) were covered with diethyl ether (1 l. previously dried over Na wire) and methyl iodide (41·4 ml., 0·6 mole) was added dropwise at such a speed as to keep the ether boiling gently under reflux. When the reaction appeared complete, the reagent was heated under reflux for 1 hr. The flask was then cooled and incorporated into the carbonation apparatus. The gas-inlet tube (Fig. 1) was of wide bore (8 mm. internal diam. proved satisfactory), as the Grignard complex which separates out as the carbonation proceeds tends to occlude it. The Hg-sealed paddle stirrer was operated by a powerful low-speed motor. The Dewar-type reflux condenser (*B*) was filled with a solid CO_2–ethanol cooling mixture. Spiral trap (*C*) was cooled in liquid O_2 to trap any unreacted CO_2; trap (*D*) was cooled in solid CO_2–ethanol and served to prevent the diffusion of water vapour into the apparatus. The bubbler (*E*) contained phosphoric acid (84%, w/v) and indicated the rate of flow of gas through the apparatus. Flask (*F*) contained saturated $Ba(OH)_2$ to collect any unreacted $^{13}CO_2$ not trapped in (*C*). This $Ba(OH)_2$ was only faintly opalescent at the end of the reaction. $Ba^{13}CO_3$ (66·1 g., 0·3 mole; 60·4 atoms % excess

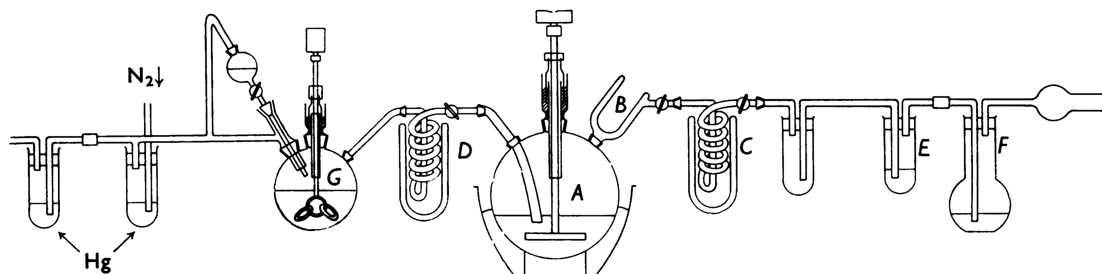

Fig. 1. Carbonation apparatus. For explanation see Methods section.

of ^{13}C) was placed in the triple-necked flask (*G*). This was fitted with a Hg-sealed paddle stirrer, an N_2-inlet tube and a dropping funnel, from which phosphoric acid (100 ml.; 84 %, w/v) was added dropwise. Flask (*G*) was connected with flask (*A*) through the spiral trap (*D*), which was cooled in solid CO_2–ethanol to retain any water liberated from the phosphoric acid. Before commencing the liberation of CO_2, flask (*A*) was cooled to $-10°$, in solid CO_2–ethanol and maintained at this temperature throughout the course of the reaction. Nitrogen was passed through the apparatus at about 30 ml./min. The phosphoric acid was added to the $Ba^{13}CO_3$ over the course of about $1\frac{1}{2}$ hr.; initially the stirrer in flask (*G*) had to be turned by hand but as the reaction proceeded the contents of the flask became more fluid and mechanical stirring was possible. When the addition of phosphoric acid was complete flask (*G*) was heated with a naked flame to ensure that all the $^{13}CO_2$ had been evolved. The Grignard reaction mixture was then stirred for a further 0·5 hr. The liquid O_2 trap (*C*) was quickly placed in the position of the solid CO_2–ethanol trap (*D*) and the system of traps beyond flask (*A*) was brought together again. Nitrogen was passed through to sweep any unreacted $^{13}CO_2$ back into the reaction mixture, and stirring was continued for a further $\frac{1}{2}$ hr., after which the inlet tube of flask (*A*) was removed and replaced by a dropping funnel. Sulphuric acid (350 ml.; 16 %, w/v) was added to the Grignard complex, the temperature of the reaction flask being maintained below 35°. Much gas was liberated (probably mainly methane) but no CO_2 appeared to be collected in the $Ba(OH)_2$ trap. When this reaction appeared to be complete Ag_2SO_4 (150 g.) was added to remove I^- ions and the reaction flask (*A*) was transferred to a steam bath and warmed gently under reflux with mechanical stirring until no more I_2 was visible in the ether layer. Insoluble material (AgI) was removed by centrifuging and filtration, and the residue was washed with 0·1 N-H_2SO_4 (2 × 100 ml.) and water (100 ml.). The combined aqueous phase and washings were distilled to dryness, water (300 ml.) was added to the non-volatile residue ($MgSO_4$) and steam-distillation was performed until the distillate showed a neutral reaction. The combined distillates were brought to pH 10·0 by the addition of approx. 5 N-NaOH and the water was removed by distillation. Sulphuric acid (50 %, v/v) was added to the concentrated residue to give pH 1·0 and the solution was extracted for 24 hr. with ether (200 ml.) in a continuous-extraction apparatus. The ether was removed by slow distillation from a water bath (45°). The residue from this procedure was transferred to a Vigreaux flask, the conventional fractionating column of which had been replaced by a column (15 × 1 cm.) packed with Fenske helices, and the distillation was continued from an oil bath. The fractions collected between 100° and 118° had a combined weight of 17·34 g. and contained 16·34 g. (0·27 mole) of acetic acid (determined by titrating weighed 10 μl. portions of each fraction) with 1 g. of water (by difference). Yield, 90 % based on $Ba^{13}CO_3$.

[1-^{13}C]*Bromoacetic acid.* [1-^{13}C]Acetic acid (15·8 g., 0·26 mole; 60·4 atoms % excess of ^{13}C) in conjunction with water (1 g.; 0·056 mole) as impurity was mixed with acetyl chloride (4·8 g.; 0·061 mole) in order to remove the water. Red phosphorus (0·3 g.) was then added and the mixture heated in a 100 ml. round-bottomed flask fitted with a dropping funnel and reflux condenser in an oil bath at 80°. Bromine (62·2 g., 0·78 mole; dried under H_2SO_4) was added dropwise during 2 hr. so that a gentle stream of HBr was evolved and the temperature retained at 80–100°. This temperature was maintained for a further 4 hr. The reaction mixture solidified on cooling. It was then distilled from an oil bath at reduced pressure (14 mm. Hg). A fraction (2 g.) was obtained at 60°. The main crop distilled at 110° (40·3 g., 0·29 mole; 90 % based on total 'acetyl'). Most of this crystallized on cooling though some remained liquid, which may have been bromoacetyl bromide.

Ethyl bromo-[1-^{13}C]*acetate.* Bromo-[1-^{13}C]acetic acid (40·3 g.) was melted and transferred to a 250 ml. round-bottomed flask. A mixture of benzene and ethanol (80 ml.; 5:3) was added and the mixture heated under reflux on a steam bath with a Dean and Stark azeotropic distillation head. Water was separated by this apparatus and removed. After 2 hr. no more water was being formed, so extra benzene–ethanol mixture (50 ml.) was added and refluxing continued for a further 3 hr. The mixture was then poured into saturated $NaHCO_3$ soln. (100 ml.) and ether (100 ml.). The ether phase was separated and the aqueous phase again extracted with ether (2 × 100 ml.). Ether extracts were combined and dried over anhydrous Na_2SO_4. Sodium sulphate was removed by filtration and the ether solution distilled through a Vigreaux-type lagged fractionating column (1 in. × 11 in.) until the temperature rose to 56°. The residue was transferred to a 100 ml. Vigreaux flask and heated by an oil bath. The main fraction distilling between 150 and 153° was collected (42·5 g., 0·26 mole; 80 % based on [^{13}C]acetic acid).

[1-^{13}C]*Glycine.* Ethyl bromo-[1-^{13}C]acetate (42·6 g.; 0·255 mole) was added to potassium phthalimide (49·3 g.; 0·266 mole) and heated by an oil bath at 115–120° under reflux for 4 hr. with occasional stirring. The mixture was never more than semi-fluid but the use of dimethylform-

amide as a solvent did not improve the yield. The residue, whilst still warm, was added to conc. HCl–acetic acid–water (1 l.; 1:1:1) and heated under reflux for 3 hr. Some of the solvent (800 ml.) was then removed by distillation. Conc. HCl–acetic acid (1 l.; 1:1) was then added and heating under reflux continued for 24 hr. The acid was again removed by distillation until phthalic acid began to crystallize. The mixture was cooled and the phthalic acid removed by filtration. The solid residue was carefully washed with water (500 ml.). The filtrate and washings were combined and the rest of the solvent was removed by distillation at reduced pressure from a water bath. The residue was dried *in vacuo* over P_2O_5 and NaOH flakes. The residue was then suspended with stirring in ethanol (300 ml.) for 0·5 hr. The insoluble residue was removed by filtration and washed carefully with ethanol (2 × 150 ml.). Water (100 ml.) was added to the combined filtrates to prevent esterification of the amino acid and the solvent removed by distillation *in vacuo*. The residue was dried and then dissolved in water (2 × 25 ml.). There was an insoluble brown residue which was removed by filtration. The amino acid hydrochloride solution was then passed through a column of Amberlite IR-45 (2 × 60 cm.) in the OH form and eluted with water. The first five fractions (50 ml.) were combined and the water was removed by distillation. The residue was dried *in vacuo*. Yield 16·2 g. (0·216 mole; 85 % yield based on ethyl bromoacetate; 59 % yield based on $Ba^{13}CO_3$). It gave a single spot corresponding to glycine when tested chromatographically (Found: C, 32; H, 6·5; N, 18·1; $C_2H_5O_2N$ requires C, 32; H, 6·7; N, 18·7 %). Attempts had been made in preliminary experiments to isolate glycine from the ethanol solution of its hydrochloride by precipitation with pyridine. This gave rise to a compound, m.p. 182°, which was probably diglycine hydrochloride (Bentley, 1951) (Found: C, 25·6; H, 5·7; N, 13·8. Calc. for $C_4H_{11}O_4NCl$: C, 25·7; H, 5·9; N, 14·9 %).

[^{15}N]*Glycine*. $^{15}NH_4NO_3$ (4·2 m-moles) was converted into [^{15}N]glycine (yield 58 %) by the method of Schoenheimer & Ratner (1939).

Isolation of urinary hippuric acid and free urinary glycine (as 2:4-dinitrophenylglycine)

The urine samples were collected in plastic bottles and stored in a deep-freeze cabinet (−20°). Each specimen was concentrated to a volume of about 150 ml. by distillation under reduced pressure at 37° (this avoids hydrolysis of the urinary peptides) in an atmosphere of N_2. The pH of the concentrate was adjusted to about 1 by the addition of conc. HCl and extracted twice (in a continuous-extraction apparatus) with ethyl acetate. The first extraction lasted 45 min.; hippuric acid was isolated from this extract and recrystallized repeatedly from water until its m.p. and mixed m.p. agreed with those of an authentic sample of the acid. Sufficient material for duplicate mass-spectrometric analyses (at least 20 mg.) was obtained in almost all cases; where the amount of the final product was insufficient for this purpose, it was diluted with a known amount of unenriched hippuric acid and recrystallized. The second ethyl acetate extraction was continued for 4 hr. to remove the last traces of hippuric acid and some of the urinary pigments, and this extract was discarded. The pH of the aqueous phase was then adjusted to approx. 9·0 with 5N-NaOH; the precipitate of phosphates which formed was removed by centrifuging, washed twice with water and discarded. The combined aqueous phase and washings were transferred to a three-necked flask and the NH_3 and amino compounds present were treated with excess of benzoyl chloride under the Schotten–Baumann conditions. A twofold excess of benzoyl chloride, calculated on the basis of the upper limit of the normal range of the published values for the urinary excretion of NH_3 and amino acids (Hawk, Oser & Summerson, 1949; King & Wootton, 1956), was employed to allow for possible diurnal variations in the amounts of these substances excreted in the urine. Special care was taken to provide effective stirring, cooling (0°) and pH control during the reaction. When the reaction was complete 5N-NaOH was added until the pH was approx. 10·0, and the benzamide which had been formed by the reaction of the urinary NH_3 with the benzoyl chloride was removed by continuous extraction with ethyl acetate. The aqueous phase was acidified (pH 1·0) by the addition of conc. HCl and the mixed *N*-benzoylamino acids were removed by continuous extraction with ethyl acetate. This extract was distilled to dryness and the residue hydrolysed with 6N-HCl for 24 hr. The hydrolyzate was distilled to dryness, the brown residue was dissolved in 150 ml. of water and benzoic acid removed by continuous ether extraction. The aqueous solution, which contained the free urinary amino acids, some urinary pigments and a little NH_3, was evaporated to dryness on a steam bath. The residue was dissolved in water and $NaHCO_3$ was added until the evolution of NH_3 ceased; the solution was again evaporated to dryness; the residue was redissolved in water and the addition of $NaHCO_3$ and evaporation was repeated to remove the last traces of NH_3. The final residue was transferred to a light-tight stoppered bottle with the minimum volume of water; a twofold excess (calculated on the basis of published data for the normal urinary free amino acid excretion) of 1-fluoro-2:4-dinitrobenzene dissolved in redistilled methanol (2 ml.) was added and the bottle shaken mechanically for 24 hr.

The subsequent chromatographic isolation of 2:4-dinitrophenyl (DNP)-glycine was based on the separation procedure described by Krol (1952) in connexion with the determination of glycine in small samples of proteins. The reagents used were prepared as described by this author, except where the contrary is specifically stated, and all manipulations were performed with the minimum exposure to light. The reaction mixture was transferred to a separating funnel and shaken with peroxide-free ether (50 ml.), and the ether extract was washed with portions of water (5 ml.) until the washings were colourless. The washings were added to the aqueous phase, the pH of which was brought to 1·0 by the addition of conc. HCl, and a second extraction with peroxide-free ether was carried out in a continuous-extraction apparatus and continued until no more yellow colour passed into the ethereal phase. The ethereal extract was dried by the addition of anhydrous Na_2SO_4, filtered off and the solvent removed by distillation. The mixed DNP-amino acids were dissolved in acetone and the solution was made up to 50 ml. A sample (0·1 ml.) of this solution was evaporated to dryness, the residue was dissolved in 0·5 % $NaHCO_3$ soln. (100 ml.), the light absorption at 360 mμ was measured and the mixed DNP-amino acid content obtained from a calibration curve constructed with solutions of synthetic DNP-glycine. It is appreciated that this value may not be strictly accurate because the DNP-derivatives of the different amino acids and other yellow substances

present will have slightly different light-absorption maxima, but the value obtained provided a satisfactory guide to the number of chromatographic columns of the size described below which would be needed for a given separation.

Columns of buffered Celite 545 (Johns-Manville Co. Ltd., London, S.W. 1) (2 cm. × 20 cm., pH 6·05) were prepared essentially as described by Krol (1952). It was found that satisfactory separation of DNP-glycine was obtained provided that not more than 250 mg. of 'mixed DNP-derivatives' (above) were loaded on to any one column. The DNP-glycine formed an intense band (R_f 0·12–0·14), which was preceded by three fast-running bands; a fourth faint band was also sometimes apparent and just ahead of the DNP-glycine band. No difficulty was experienced in eluting the DNP-glycine band alone, provided that the initial development of the chromatogram was allowed to take place slowly (i.e. the eluate being collected at a rate of 0·5 ml./min.). The DNP-glycine-containing eluate was distilled to dryness *in vacuo* and the DNP-glycine crystallized from ethyl acetate–*cyclo*hexane (1:9). The yields of DNP-glycine in the present work varied between 19 and 65 mg./3 hr. urine sample. [Evered (1956) quotes values for the daily excretion of free urinary glycine which are equivalent to 37–64 mg. of DNP-glycine/3 hr.] The identity and purity of each sample isolated was confirmed by chromatography on paper with the *tert.*-amyl alcohol and propanol–light petroleum (100–120°) solvent systems described by Blackburn & Lowther (1951). DNP-glycine isolated in this way melted at 200–203°, unchanged by admixture with a synthetic sample of the derivative.

Preparation of nitrogen samples for mass-spectrometric analysis

Glycine and hippuric acid were digested by the normal Kjeldahl procedure and the NH_3 so formed was converted into N_2 by treatment with sodium hypobromite (Francis, Mulligan & Wormall, 1954). DNP-glycine is not completely digested by this procedure and the modification described by Elek & Sobotka (1926) and by Baker (1955) was employed. DNP-glycine (approx. 6 mg.) and pure glucose (approx. 150 mg.) were weighed in the digestion flask. Kjeldahl reagent (3 ml.) (Francis *et al.* 1954) was added and the mixture heated gently for 6 hr. At the end of this period the mixture was still black. The rate of heating was then considerably increased and continued for 17 hr. Titration of the NH_3 formed by this method indicated that all the N_2 in the sample had been converted into NH_3, but comparison of isotopic enrichments between [^{15}N]glycine, [^{15}N]hippuric acid and DNP-[^{15}N]glycine gave a value for DNP-glycine 3% higher than for the corresponding glycine or hippuric acid (Table 1).

Preparation of carbon dioxide samples for mass-spectrometric analysis

[1-^{13}C]Glycine was subjected to combustion to form CO_2 in a Pregl type of combustion tube, and the CO_2 was collected in an apparatus similar to that described by Bradley, Holloway & McFarlane (1954) for the preparation of $^{14}CO_2$ samples. Any oxides of nitrogen present were decomposed by the hot-wire treatment described by those authors. Carbon dioxide from the carboxyl group only was obtained from DNP-[1-^{13}C]glycine and from [1-^{13}C]hippuric acid by the acylative decarboxylation procedure described previously (Crawhall & Watts, 1959).

EXPERIMENTAL

The experiments were all performed on the same healthy male subject (weight 72 kg.; height 187 cm.). Each experiment was preceded by an overnight fast and complete emptying of the bladder. Frequent draughts of water were taken to promote diuresis.

Experiment 1. The subject took [^{15}N]glycine (339 mg., 36·2 atoms % excess of ^{15}N), dissolved in water, by mouth. Urine specimen 1 comprised all urine voided during the subsequent 6 hr. and urine specimen 2 comprised all urine voided between 6 and 24 hr. after the ingestion of [^{15}N]-glycine. No food was taken during the collection of specimen 1; the subject's usual diet was taken during the collection of specimen 2. The free glycine was isolated from each of these specimens and its ^{15}N content was measured.

Experiment 2. The subject took [^{15}N]glycine (100 mg., 36·2 atoms % excess of ^{15}N) by mouth. This was followed by the collection of six 6 hr. urine collections, and a further dose of [^{15}N]glycine (100 mg.) was taken at the beginning of each urine collection period. After 36 hr., 3-hr. urine collections were made, no further [^{15}N]glycine being taken during this period. The total protein intake was restricted to 40 g./day during the experimental period and the subject's meals were arranged so that the dietary protein intake was spread approximately uniformly over each 24-hr. period. The hippuric acid and free glycine were isolated from each urine specimen and their ^{15}N contents determined.

Experiment 3. A single dose of [1-^{13}C]glycine (344 mg., 49·3 atoms % excess of ^{13}C) and eight 3 hr. urine samples were collected during the subsequent 24 hr. Hippuric acid and the free glycine were isolated and their ^{13}C contents determined. The subject's diet was arranged as in Expt. 2.

RESULTS

Experiment 1. The results of this experiment (Table 2) demonstrated that sufficient enrichment could be obtained in the first glycine metabolic pool for mass-spectrometric analysis of the free urinary

Table 1. *Content of* ^{15}N *in nitrogen obtained from* [^{15}N]*hippuric acid and* 2:4-*dinitrophenyl*-[^{15}N]*glycine synthesized from the same batch of* [^{15}N]*glycine, the isotope content of which was determined simultaneously*

Compound	^{15}N (atoms % excess) Mean	Extreme range of values	No. of observations
[^{15}N]Glycine	0·688	0·685–0·693	3
2:4-Dinitrophenyl-[^{15}N]glycine	0·709	0·705–0·714	3
[^{15}N]Hippuric acid	0·680	0·674–0·688	3

Table 2. *Content of* ^{15}N *in the free urinary glycine after a single oral dose of* [^{15}N]*glycine* (*Expt.* 1)

The subject took [^{15}N]glycine (339 mg., 32·1 atoms % excess) orally.

Urine collection No.	Time after administration of [^{15}N]glycine (hr.)	^{15}N Content of the isolated 2:4-dinitrophenyl-glycine (atoms % excess)
1	0–6	0·308
2	6–24	0·036

glycine isolated as the 2:4-dinitrophenyl derivative from a 6-hr. urine collection. However, the isotope content of the isolated glycine decreased so rapidly that the isotope enrichment of the sample isolated from the urine collected between 6 and 24 hr. after the administration of the [^{15}N]glycine could only be measured with an error of $\pm 10\%$, and it was apparent that either a single very large (and therefore unphysiological) dose, or multiple smaller doses, of the labelled amino acid would be needed for an investigation in which it was desired to keep the glycine in the first glycine metabolic pool measurably enriched with the isotope for a longer period.

The observed isotope enrichments represented the mean value for the ^{15}N content of the free urinary glycine during the time intervals concerned. As only two urine collections were made, the first glycine metabolic-pool size and the turnover rate of glycine in the pool could only be deduced if a simple exponential decay of the isotope content of the pool was assumed, and evidence bearing on the validity of such an assumption was obtained from the results of Expts. 2 and 3. We are indebted to Professor J. Rotblat for the following calculation.

If it is assumed that the glycine pool is a single-compartment system in equilibrium and containing some [^{15}N]glycine, then

$$C_t = C_0 e^{-\lambda t}, \quad (1)$$

where C_t is the concentration of [^{15}N]glycine in the pool at time t and C_0 is the concentration of [^{15}N]-glycine immediately after the administration of the isotope. The length of time required for orally administered [^{15}N]glycine to come into equilibrium with the endogenous glycine is not known but can be assumed to be short compared with the time of collection of the first sample (6 hr.).

If urinary glycine is being excreted at a constant rate then

$$C_{\text{mean }0-t_1} = \frac{C_0\int_0^{t_1} e^{-\lambda t}\,dt}{t_1} = C_0\frac{1-e^{-\lambda t_1}}{\lambda t_1} \quad (2a)$$

and

$$C_{\text{mean }t_1-t_2} = \frac{C_0\int_{t_1}^{t_2} e^{-\lambda t}\,dt}{t_2-t_1} = C_0\frac{e^{-\lambda t_1}-e^{-\lambda t_2}}{\lambda(t_2-t_1)}, \quad (2b)$$

where $C_{\text{mean }0-t_1}$ is the mean value of concentration of [^{15}N]glycine in the pool and in the urine over the period 0–t_1 and $C_{\text{mean }t_1-t_2}$ is a similar value found over the period t_1-t_2. Therefore

$$\frac{C_{\text{mean }0-t_1}}{C_{\text{mean }t_1-t_2}} = \frac{1-e^{-\lambda t_1}}{e^{-\lambda t_1}-e^{-\lambda t_2}}\cdot\frac{t_2-t_1}{t_1}. \quad (3)$$

In this experiment $t_1 = 6$ hr. and $t_2 = 24$ hr., so that $e^{-\lambda t_2}$ may be neglected by comparison with $e^{-\lambda t_1}$. Equation (3) can most easily be solved by employing this simplification. The values of $C_{\text{mean }0-t_1}$ and $C_{\text{mean }t_1-t_2}$ have been found (Table 2). Therefore

$$\frac{0{\cdot}308}{0{\cdot}036} = \frac{t_2-t_1}{t_1}(e^{\lambda t_1}-1).$$

By substituting values of $t_1 = 6$ hr. and $t_2 = 24$ hr. it is found that

$$\lambda t_1 = 1{\cdot}36,$$

therefore

$$\lambda = 0{\cdot}23 \text{ hr.}^{-1},$$

which is equivalent to a half-life time of 3 hr. Substituting these values in equation (2a), $C_0 = 0{\cdot}566$. Therefore

$$\text{dilution of original [}^{15}\text{N]glycine fed} = \frac{10{\cdot}7}{0{\cdot}566} = 18{\cdot}9 \text{ times.}$$

This value of 10·7 atoms % excess of ^{15}N is the enrichment of fed glycine calculated as DNP-glycine, as that is the form in which it was isolated.

First glycine metabolic-pool size (0·339 × 18·9 g.) = 6·4 g. Turnover rate of glycine in pool ($\lambda \times$ pool size) = 1·73 g./hr. As the subject weighed 72 kg. this is a turnover rate of 0·32 m-mole/kg./hr

Experiment 2. This experiment was designed to determine whether a constant level of isotope enrichment in the first glycine metabolic pool could be achieved by the administration of multiple doses of isotopically labelled glycine and to compare the isotope contents of the urinary hippuric acid and the first glycine metabolic pool in this type of experiment. We also attempted to study the rate of disappearance of the isotope from these situations after the cessation of feeding with [^{15}N]glycine to test the validity of the assumption made in the calculation of pool size and the glycine-turnover rate from the data obtained in Expt. 1.

The average values for the ^{15}N contents of the urinary hippuric acid and the free urinary glycine were 0·546 (S.D. 0·124) atoms % excess and 0·261 (S.D. 0·040) atoms % excess respectively during the period of feeding with [^{15}N]glycine. It also appeared that the plateau value for the isotope enrichment of both substances was reached during the first 6 hr. of the experiment (Fig. 2). The isotope content of the DNP-glycine isolated from the urine specimens

collected after the cessation of feeding with [^{15}N]-glycine was too small for accurate mass-spectrometric analysis, so that no information could be obtained about the time course of the disappearance of the labelled amino acid from the free urinary glycine; this was partly due to the presence of two unenriched nitrogen atoms in the DNP-glycine molecule. The isotope content of the hippuric acid, which contains only the isotopically enriched amido group nitrogen atom, could be followed satisfactorily up to 69 hr. after the cessation of feeding with [^{15}N]glycine. These results, which have been plotted on a semilogarithmic scale in Fig. 3, indicate that the isotope enrichment is not decreasing in a simple exponential manner. Although the later points lie on a straight line, the gradient of which corresponds to a half-life time of 18·5 hr., the early points are too scanty to permit the resolution of the apparently composite portion of the curve.

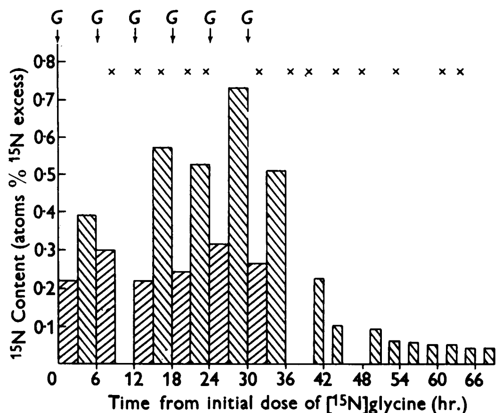

Fig. 2. Isotope content of the free urinary glycine (▨) and the urinary hippuric acid (▧) which resulted from repeated doses of [^{15}N]glycine (100 mg., 32·1 atoms % excess) given at the times indicated by *G*. Food was taken at the times indicated by ×. Further experimental details are described under 'Expt. 2' (Experimental section).

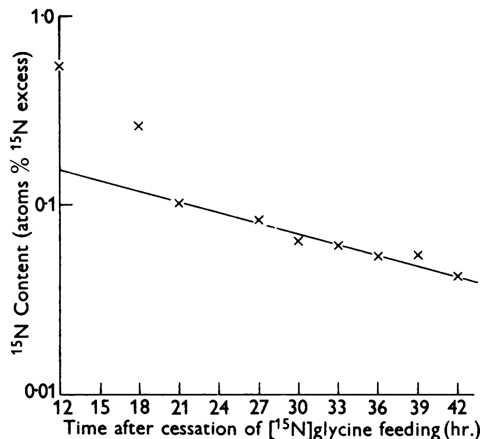

Fig. 3. Change with time of the isotope content of the urinary hippuric acid after the cessation of repetitive [^{15}N]glycine feeding (Expt. 2), plotted on semilogarithmic co-ordinates. Each point represents the average isotope content of the free urinary glycine for the 3 hr. period preceding the time opposite which it is plotted.

Experiment 3. This experiment was designed to permit the calculation of the first glycine metabolic-pool size and turnover rate from the time course of the disappearance of an isotopic label from the free urinary glycine after a single oral dose of isotopically labelled glycine, without having to assume that we were dealing with a single-compartment system (cf. Expt. 1). We also compared the isotope contents of the free urinary glycine and the urinary hippurate. The isotope content of the free urinary glycine did not decrease as a single exponential function with time after a single dose of [1-^{13}C]glycine (Fig. 4); the first two hippuric acid values were considerably greater than the corresponding free urinary glycine values, but the subsequent results were closely similar (Table 3). In order to calculate the first glycine metabolic-pool size from these results it

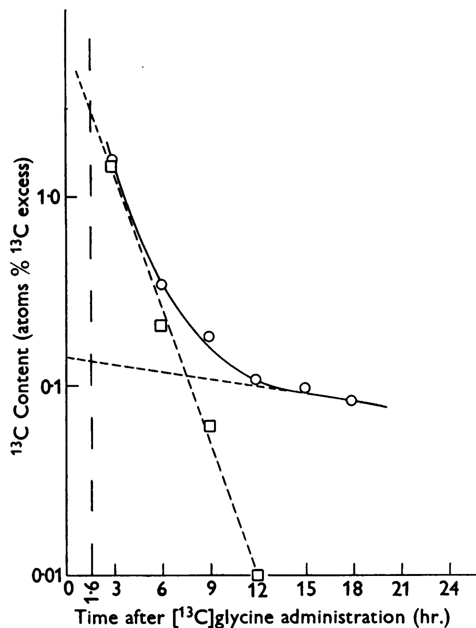

Fig. 4. Change with time of the isotope content of the free urinary glycine after a single oral dose of [1-^{13}C]glycine (344 mg., 49·3 atoms % excess of ^{13}C, Expt. 3) plotted on semilogarithmic co-ordinates. Each point represents the average isotope content of the free urinary glycine for the 3 hr. period preceding the time opposite which it is plotted. The resolution of the composite curve into two exponential components and the effective zero time (1·6 hr. after the ingestion of [1-^{13}C]glycine) are shown.

Table 3. *Content of* ^{13}C *in the free urinary glycine and the urinary hippuric acid after the administration of* [1-^{13}C]*glycine* (*Expt.* 3)

The subject took [1-^{13}C]glycine (344 mg., 49·3 atoms % excess) orally. Hippuric acid and 2:4-dinitrophenyl-glycine were isolated and degraded to yield their carboxyl group C atoms in the form of CO_2 as described in the Methods section.

	Urine collection	Isotope content (atoms % excess) derived from carboxyl group only	
No.	Time after administration of [1-^{13}C]glycine (hr.)	2:4-Dinitrophenyl derivative	Hippuric acid
1	0–3	1·59	3·16
2	3–6	0·321	0·412
3	6–9	0·181	—
4	9–12	0·107	0·111
5	12–15	0·098	0·094
6	15–18	0·085	0·083
7	18–21	—	0·066
8	21–24	—	0·062

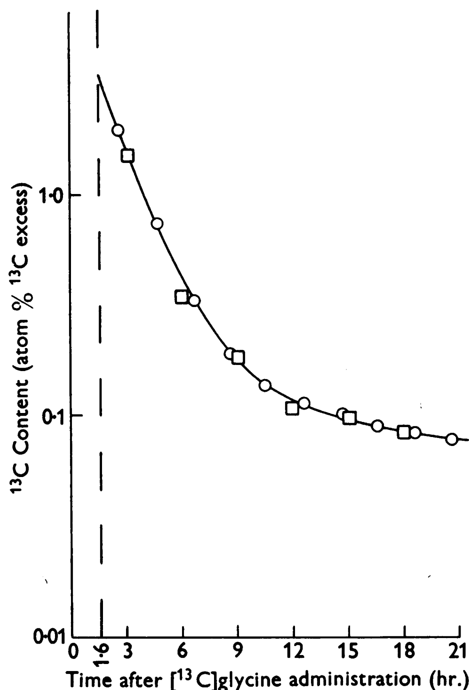

Fig. 5. Comparison of the observed ^{13}C content of the free urinary glycine (□) after a single oral dose of [1-^{13}C]-glycine (Expt. 3) with the corresponding value (○) calculated from the equation

$$C_t = 2{\cdot}95\ e^{-0{\cdot}552t} + 0{\cdot}16\ e^{-0{\cdot}0377t},$$

where C_t is the ^{13}C content of the free urinary glycine at time t. This time is 1·6 hr. greater than the experimental time, which was measured from the moment of [1-^{13}C]glycine ingestion.

would be desirable to know the isotope enrichment in the free urinary glycine at zero time. However, no such value is available because of the delayed mixing which occurs with administration by mouth, and because it is necessary to collect urine for 3 hr. to obtain sufficient DNP-glycine for the replicate mass-spectrometric analyses. More rapid mixing would have been obtained if the [^{13}C]glycine had been given intravenously, but this would have involved raising the plasma glycine to unphysiological levels. The values for the ^{13}C content of the free urinary glycine have been plotted in Fig. 4 at times corresponding to the end of each urine collection period, although they are in fact average values for each of the 3 hr. collection periods. It is possible to calculate a theoretical value for the initial isotope content (C_0) of the first glycine metabolic pool and hence to determine the effective zero time for the experiment in the following manner:

$$C_{\text{mean}} = \frac{C_0}{\lambda t}[1 - e^{-\lambda t}], \qquad (4)$$

where C_{mean} is the observed ^{13}C enrichment between 0 and 3 hr., from which the small contribution of the second exponential component has been deducted; C_0 is the theoretical initial ^{13}C enrichment which would have given rise to this mean value; λ is the exponential decay constant (0·552); t is the length of time covered by the collection period (i.e. 3 hr.).

Solution of this equation shows that the value of C_0 is 2·95 atoms % excess of ^{13}C. The administered glycine weighed 343 mg. and contained 49·8 atoms % excess of ^{13}C, so that it was diluted 16·9-fold between ingestion and its excretion as free urinary glycine, and the first glycine metabolic pool size is 5·8 g. By definition, the turnover rate of the pool [$\lambda \times$ pool size] is therefore 3·2 g./hr. (0·59 m-mole/kg. body wt./hr.) in this experiment. With the value of C_0 calculated from equation (4), it is found (Fig. 4) that the effective zero time of the experiment is 1·6 hr. after the experimental zero time (i.e. the time at which [^{13}C]glycine was administered). The C_0 value for the second exponential component of the curve based on the experimentally determined values (Fig. 4) is therefore 0·16 atom % ^{13}C

Table 4. *Comparison of experimentally determined values of the content of ^{15}N in free urinary glycine in Expt. 2 with corresponding calculated values*

Calculated values were obtained by application of equation (5), modified to allow for the different amounts of isotope administered in the two experiments.

Urine collection no.	^{15}N Content of free urinary glycine (atoms % excess)	
	Observed	Calculated
1	0·219	0·187
2	0·300	0·216
3	0·223	0·231
4	0·246	0·245
5	0·316	0·256
6	0·264	0·265

excess. By employing these two C_0 values and the observed slopes of the lines representing the two exponential rate processes, equation (5), which expresses the experimentally derived curve, can be calculated:

$$C_t = 2{\cdot}95\ e^{-0{\cdot}522t} + 0{\cdot}16\ e^{-0{\cdot}0377t}, \quad (5)$$

where C_t is the ^{13}C content of the free urinary glycine at a time t, where $t = 0$ corresponds to 1·6 hr. on the experimental curve.

In Fig. 5, the curve derived from this equation has been plotted on semilogarithmic co-ordinates and the experimentally determined values have been superimposed.

The validity of equation (5) and hence of the deductions drawn from Expt. 3 is demonstrated by using this equation to calculate theoretical values for the isotope enrichment of the free urinary glycine during each of the 6 hr. urine-collection periods in Expt. 2. The agreement between the calculated and the experimentally determined values (Table 4) is considered to be satisfactory from this point of view.

The second exponential component of the curve shown in Fig. 4 has a half-life time of 16 hr. and corresponds to a second pool containing 107 g. of glycine with a turnover rate of 4 g./hr. The isotope disappeared from the urinary hippuric acid at a similar rate to this during the later parts of Expts. 2 and 3 (Fig. 3 and Table 3).

DISCUSSION

Size and turnover rate of the first glycine metabolic pool

The metabolic-pool concept is an abstract one and the term requires careful definition in the light of the context in which it is being used. In the present case, we wished to determine the amount of dilution which a dose of isotopically labelled glycine would undergo immediately after absorption and distribution. The amount of glycine with which the dose of labelled glycine would mix under these circumstances we term the 'first glycine metabolic pool'. This term was previously used by Arnstein & Neuberger (1951) to describe the rapidly turned-over glycine in the liver, kidney and intestine with which an injected dose of glycine would mix very quickly and which is in relatively slow equilibrium with the intracellular glycine of other tissues, with serine, glutathione, peptides and proteins. Henriques, Henriques & Neuberger (1955) obtained quantitative data for the turnover rate of glycine in a number of situations and concluded that, for the distribution of a labelled amino acid, a model consisting of a 'system of semi-isolated compartments communicating with a central compartment–the blood and extracellular fluid–at widely differing rates' approximates to the truth in mammals. Although there is no rigid proof that our first glycine metabolic pool corresponds exactly with the pool studied by Arnstein & Neuberger (1951) or with the 'central compartment' of Henriques *et al.* (1955), we feel that the correspondence is sufficiently close to make the introduction of yet another name unjustified. The most satisfactory source of a sample of the pool would probably be the free plasma glycine, but the published values for the glycine content of human plasma (or serum) vary between 11·6 and 39·0 μg./ml. (Arnstein, 1954), so that it was clearly impracticable to obtain sufficient amounts of glycine (at least 30 mg. of the DNP-derivative) for duplicate mass-spectrometric analysis from this source. The rationale underlying the use of the free urinary glycine as a sample of the first glycine metabolic pool has been explained in the introduction to this paper.

The results of Expt. 1 served to demonstrate that measurable amounts of the isotope would appear in the pool after a dose of labelled glycine which was small (339 mg.) relative to the approximate pool size (5·8 g.). It is clear from the results of Expt. 3 that, as was originally suspected, the assumption made in calculating the pool size and turnover rate from the data obtained in Expt. 1 was an over-simplification, the overall effect being to over-estimate the pool size and to underestimate the turnover rate. The rate of disappearance of the isotope from the free urinary glycine in Expt. 3 corresponded well to an equation containing two exponential functions (Fig. 5), and the size and turnover rate of a 'second' as well as a 'first' glycine metabolic pool could in fact be calculated (see end of Results section). In view of the increasing multiplicity of factors (e.g. differential diffusion and reaction rates) which influence the distribution of the labelled amino acid, we do not think that speculation about the physiological significance or

the anatomical location of the second pool is justified. Henriques *et al.* (1955) found that after the intravenous injection of [^{14}C]glycine into a rabbit, the rate of loss of isotope from the plasma during the first 18 hr. after injection could be represented as the sum of three exponential processes. Although the method of graphical analysis which we have employed is a standard one in work of this type (see, for example, Robertson, 1957), it is open to the general criticism that it can be satisfactorily employed with many curves which do not in fact represent exponential functions. Also the number of components into which a given composite curve can be resolved may be influenced by the number of experimentally determined values which can be obtained for the parameter under discussion, and this may be limited in biological work. Thus it may be that the apparent difference between our results and that of Henriques *et al.* (1955) may result only from the smaller number of experimentally determined values upon which our curve is based.

In general the calculation of a pool size and hence of a turnover rate by an isotope-dilution technique presupposes that the labelled material mixes instantaneously with the pool at zero time. In our experiments, labelled glycine was given orally so that mixing could not be regarded as occurring instantaneously. This difficulty was overcome in Expt. 3 by calculating a value (C_0) for the isotope content of the glycine in the first glycine metabolic pool at a hypothetical zero time such that had the same dose of [^{13}C]glycine been administered at this hypothetical time and had it mixed instantly with the first glycine metabolic pool it would have given rise to the excretion pattern which we observed. Although errors of approximation are unavoidable in the mathematical treatment of these types of data, our values in man for the first glycine metabolic-pool size (1·07 m-moles/kg.) and turnover rate (0·59 m-mole/kg./hr.), which we found in Expt. 3, can be compared with some published values for small animals obtained in different types of experiment. Arnstein & Neuberger (1951) report a 'first glycine metabolic pool' size of 1·7 m-moles/kg. for the rat; in their experiments [^{14}C]glycine and sodium benzoate were injected into the peritoneal cavity. Turnover rates of 1·05 m-moles/kg./hr. (Arnstein & Neuberger, 1953) and 1·13 m-moles/kg./hr. Arnstein & Stanković (1956) have been reported for the rat, and a value of 1·66 m-moles/kg./hr. was found for the guinea pig (Arnstein & Stanković, 1956); these values were obtained by comparing the ^{14}C content of the dietary and visceral-protein glycine after prolonged feeding with [^{14}C]glycine.

When the isotope contents of the free urinary glycine and the urinary hippuric acid were compared during a period of repetitive [^{15}N]glycine feeding, values for the latter were consistently higher and more variable than for the former (Fig. 2). This suggests that the glycine which is used for hippurate synthesis is drawn preferentially from the portal blood stream as it enters the liver and before it has equilibrated with the first glycine metabolic pool. Simkin & White (1957) suggested that a large proportion of the glycine used for hippurate synthesis is provided by the free liver glycine; our findings lend further support to this idea.

Previous workers who have attempted to equate the isotope content of the glycine moiety of the urinary hippurate with that in the glycine metabolic pool have employed loading doses of benzoate to increase the urinary hippurate excretion. Stein, Paladini, Hirs & Moore (1954) isolated the urinary hippuric acid by ion-exchange chromatography and report that the normal adult male excretes 1·0–2·5 g. of this acid/24 hr. Armstrong, Chao, Parker & Wall (1955), who separated hippuric acid from urine by ethyl acetate extraction, likewise report excretion rates of 1–3 mg./kg. body wt./24 hr. for human subjects. These reports suggested that it might be possible to isolate sufficient amounts of hippurate for mass-spectrometric analysis without the use of loading doses of benzoate. This was possible in the majority of cases, although there were occasional failures. (Similar failures have not been encountered in the isolation of DNP-glycine.) We had hoped that when no loading dose of benzoate was given, and the possible disturbance of glycine metabolism which abnormal amounts of this ion itself might introduce (see, for example, Gray & Neuberger, 1950) was thereby avoided, the isotope content of the urinary hippurate would prove to be the same as that of the sample of the free glycine metabolic pool represented by the free urinary glycine. This was not so with urine samples obtained during or shortly after the administration of [^{13}C]-glycine (Fig. 2 and Table 3), although at time intervals more remote from the administration of the labelled amino acid the two sets of values agree (specimens 4–6 in Table 3). It is concluded that the isotope content of the glycine moiety of the urinary hippurate is not representative of that in the first glycine metabolic pool during or shortly after the administration of isotopically labelled glycine, and therefore that it could not be used as a means of sampling the pool under these conditions. It appears that at periods more than 9 hr. after the administration of isotopically labelled glycine (Table 3) the urinary hippuric acid could be used for this purpose, although further experimentation would be needed to determine the critical time interval exactly. This is comparable with the result of Arnstein & Neuberger's (1951) study, which

showed that the isotope content of the hippurate excreted by rats which were given benzoate after a dose of labelled glycine was only independent of the dose of benzoate when the interval between their administration was 6 hr. or longer.

SUMMARY

1. The amount of dilution which orally administered isotopically labelled glycine undergoes immediately after absorption and distribution has been investigated in man, and the 'pool' of glycine with which the labelled amino acid becomes diluted under these circumstances is termed the 'first glycine metabolic pool'.

2. The free urinary glycine (isolated as the 2:4-dinitrophenyl derivative) has been used as a sample of the first glycine metabolic pool in man. In experiments with [1-^{13}C]glycine and [^{15}N]glycine 3 hr. urine collections yield sufficient material for mass-spectrometric analysis.

3. The rate of change in the isotope content of the first glycine metabolic pool after a single oral dose of isotopically labelled glycine can be represented as the sum of two exponential functions. The first glycine metabolic-pool size and turnover rate of a 72 kg. adult were of the order of 5·8 g. (1·07 m-moles/kg. body wt.) and 3·2 g./hr. (0·59 m-mole/kg. body wt./hr.), respectively.

4. The isotope content of urinary hippurate and of the first glycine metabolic pool have been compared during a period when repeated doses of isotopically labelled glycine were being given and after a single oral dose of the labelled amino acid. It is concluded that the isotope content of the glycine moiety of the urinary hippuric acid even in the absence of loading doses of benzoate does not indicate the isotope content of the first glycine metabolic pool during or shortly after the administration of labelled amino acid although it may do so later.

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