THE ROLE OF THE SUCCINATE-GLYCINE CYCLE IN GLUTAMATE SYNTHESIS

by

John Power Kane, B.S.

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INTRODUCTION

In the course of studying porphyrin biosynthesis, Shemin et al (1-7) identified as a porphyrin precursor, δ-aminolevulinic acid (ALA). They were able to demonstrate that this compound arose from the decarboxylation of α-amino-β-ketoadipic acid (8) which in turn is formed from the condensation of one molecule each of glycine and succinate. It was found (3) that two molecules of ALA are then condensed to yield one molecule of the monopyrrole, porphobilinogen. The porphyrin ring is then formed by the condensation of four molecules of porphobilinogen.

It was also found (7) that the δ-carbon atom of ALA contributes "C_1" units for the synthesis of the ureido groups of purines, the β-carbon of serine and the methyl carbon of methionine in precisely the same fashion as does the α-carbon of glycine, from which it is derived.

Upon the addition of C^{14}-labeled ALA to a cell-free extract of duck erythrocytes, Shemin (7) was able to isolate labeled α-ketoglutaric acid, thus establishing that the succinate-glycine condensation and subsequent reactions could function as a cycle, originating with one tricarboxylic acid cycle intermediate, succinate, and passing through another, α-ketoglutaric acid. Oxidative deamination of the δ-carbon of ALA is the only alteration of that molecule necessary to produce α-ketoglutaric acid. A similar type of reaction is known to occur in the conversion of ornithine to glutamic acid (9). Fig. 1 is a graphic representation of this cycle.

As well as being a source of porphyrin precursor and C_1 units, this cycle represents another mechanism whereby the carbons of glycine may be oxidized. The carboxyl carbon of glycine becomes the C-1 carbon of α-amino-β-ketoadipic acid which is decarboxylated in the formation of ALA. This is substantiated by the fact that porphyrins isolated after the administration of C^{14} carboxyl labeled glycine contain no radioisotope[7]. The α-carbon of glycine would then become the C-1 carbon of α-ketoglutaric acid which is decarboxylated in the formation of succinate.

In the course of studying the effects of deficiencies of vitamin B_{12} and folic acid on the synthesis of the constituent amino acids of glutathione in the rat, Anderson and Stekol[10] observed that glutamic acid isolated from liver after the administration of α-C^{14} labeled glycine, contained the radioisotope. They did not degrade the molecule to localize the activity, however. The metabolic pathways which they postulated for the transfer of the α-carbon activity from the glycine to glutamic acid involved proven *in vivo* reactions beginning with the conversion of glycine to serine by the addition of a carbon atom in the β-position, which may come, itself, from the α-position of glycine. The serine thus formed, then may be converted to pyruvic acid which can enter the tricarboxylic acid cycle either as oxaloacetic acid by the C_3-C_1 type condensation of the Wood-Werkman reaction, or as acetate, by decarboxylation. After entering the tricarboxylic acid cycle the label originating from glycine could then appear in any of the five carbon atoms of α-ketoglutaric acid.

This compound could then be transaminated to yield labeled glutamic acid. That label which entered the tricarboxylic acid cycle as acetate would appear in both the carboxyl and the α-carbons of the acetate molecule and would label only the 4 and 5 positions of glutamic acid with one passage through the cycle, but would be evenly distributed among the 1, 2, and 3 positions with subsequent cycling. That label which entered the cycle by way of the Wood-Werkman reaction would appear in the 1 and 2 positions of glutamic acid, upon the first passage through the cycle and would be distributed among the 1, 2, and 3 positions on further circuits. Thus, if the principal pathway involved is decarboxylation to acetate, the greatest activity should appear in the C-5 position of the glutamic acid formed. Wang et al[11] isolated and degraded glutamic acid from yeast subsequent to the administration of α-C^{14} labeled pyruvate. They found that under aerobic conditions thirty-nine per cent, and under anaerobic conditions fifty per cent, of the total activity for the five carbon atoms resided in the C-5 carbon atom, indicating the principal pathway to be that of decarboxylation to acetate, and thence to the tricarboxylic acid cycle.

Since oxidative deamination of the δ-amino group and transamination of the γ-carbonyl group are the only alterations necessary to convert ALA to glutamate, and since labeled α-ketoglutaric acid has been isolated subsequent to the administration to rats of 5-C^{14} ALA, the succinate-glycine cycle presented itself as an additional possible pathway for the transfer of the α-carbon from glycine to glutamate, as observed by Anderson and Stekol. It can be seen that

all the activity contributed to glutamic acid from α-C^{14} glycine by this pathway would reside in the C-1 position of the glutamate produced.

Experiments were planned to confirm the transfer of α-C^{14} glycine activity to glutamate and to evaluate the role of the succinate-glycine cycle in glutamate synthesis. Since isotopically labeled ALA was unavailable, dilution experiments were undertaken in which two groups of animals received α-C^{14} glycine, one group receiving in addition an excess of non-labeled ALA, with subsequent isolation of glutamate and determination of its radioactivity. If this pathway were contributing to glutamate synthesis, the activity reaching the glutamate from the α-carbon of the glycine would be decreased because of the dilution of the labeled ALA pool by the non-labeled ALA, providing that the administered ALA were able to be metabolized. Shemin[7] demonstrated that exogenous non-labeled ALA did depress the incorporation of activity from α-C^{14} glycine into hemin in duck erythrocyte hemolysates. He also showed that the depression in isotope incorporation was a result of dilution rather than inhibition of the reaction by demonstrating N^{15} incorporation into the hemin when the experiment was repeated with N^{15}-containing ALA.

Degradation of the glutamate molecule to determine the proportion of activity residing in the C-1 position and to quantitate any dilution effect in the activity of that carbon atom alone would provide further evidence as to the extent of the contribution of the succinate-glycine cycle to glutamate synthesis.

Determination of the activity of aspartic acid isolated from the same animals would give an index of the relative rates of utilization of the isotopic glycine in the two experimental groups since no activity reaching α-ketoglutaric acid via the succinate-glycine cycle can reach aspartic acid. Such activity would reside in the C-1 position of α-ketoglutaric acid which is lost in the formation of succinic acid prior to the reactions leading to oxaloacetic acid, the cycle precursor of aspartic acid. Thus, the exogenous ALA would be expected to have no dilutional effect on the activity reaching aspartic acid.

EXPERIMENTAL

Two essentially equivalent experiments were carried out. In the first experiment, aspartic acid, in addition to glutamic acid, was isolated and counted as an index of the relative rates of absorption of the α-C^{14} glycine in the two experimental groups of animals. Only in the second experiment was sufficient glutamic acid recovered to permit degradation studies to be carried out.

A. Animals:

All animals used were female rats of the Sprague-Dawley strain. In the first experiment two groups of three animals each, weighing 95 to 104 grams, were used. In the second experiment the two experimental groups consisted of four animals each, weighing from 145 to 155 grams.

In each experiment the animals were subjected to a 24 hour fasting period prior to the administration of the metabolites in

order to minimize the effect of differences in their states of alimentation.

B. Administration of isotopic glycine and δ-aminolevulinic acid (ALA):

In both experiments the α-C^{14} labeled glycine, dissolved in one milliliter of physiologic saline, was injected intraperitoneally. Where used, crystalline ALA* was dissolved in the glycine solution just prior to its administration.

In the first experiment three animals received 20 uc. (2.37 uM) of C^{14} activity as α-labeled glycine only, and three animals received an identical dose of isotopic glycine with the addition of 4 mg. (308 uM) of non-labeled ALA per animal.

In the second experiment eight animals received 16.6 uc. (1.97 uM) of α-C^{14} glycine each, four receiving in addition 4 mg. of non-labeled ALA.

All the animals were allowed to metabolize the compounds administered for exactly one hour, at which time they were sacrificed by decapitation.

C. Initial treatment of livers:

Immediately upon decapitation of the animals, the livers were dissected out and chilled in cracked ice to arrest further metabolic activity. The livers of each group were then pooled, pureed, and desiccated in a drying oven, after which time they were reduced to a fine powder by means of a mortar and pestle.

Each sample was then extracted by 24 hours of continuous flushing with ether in a Soxhlet type extractor in order to remove the

*Supplied by the Parke-Davis Company, Detroit, Michigan

ether soluble lipid fraction.

D. Hydrolysis:

Hydrolysis of the liver proteins was accomplished by refluxing with 6.0 N HCl for 48 hours. The humin fraction was then removed by filtration through Whatman No. 2 paper filters. The residual free hydrochloric acid was removed by repeated vacuum distillation.

E. Isolation of Amino Acids:

Glutamic acid and aspartic acid were isolated by column chromatography, using the method of Hirs, Moore and Stein[12]. An anion exchange resin, Dowex 1-X8 was employed as the acetate form, in columns 18 mm. in diameter and 45 cm. in length. The hydrolysate of liver proteins was put on the columns in 0.5 N acetic acid. Glutamic and aspartic acids were eluted with the same solution, the concentrations of amino acids in the eluate fractions being determined by the ninhydrin method of Moore and Stein[13,14]. A complete separation of these amino acids from each other and from all the other amino acids in the hydrolysate was effected.

The eluate fractions containing aspartic and glutamic acids were concentrated by evaporation over a steam bath and the amino acids were crystallized by the addition of 10 volumes of 95 per cent ethanol with storage at -20°C., overnight. The crystals were washed with ethanol and dried.

The identity and purity of the crystalline amino acids obtained were determined by comparison with known pure samples of glutamic and aspartic acids on paper chromatograms using both a n-butanol/

acetic acid/water system and a pyridine/water system.

E. Combustion of Amino Acids:

Samples of the crystalline amino acids were combusted with hot chromic acid under vacuum, the evolved CO_2 being trapped in 0.25 N NaOH. After combustion was completed, the carbonate formed from the evolved CO_2 was precipitated as $BaCO_3$ by the addition of 0.15 M BaOH and 0.25 M NH_4 Cl. The precipitate was allowed to flocculate while held under vacuum and then rapidly filtered on a sintered glass suction filter. The residue was washed repeatedly with water and then with 95 per cent ethanol, and dried at 110°C.

F. Decarboxylation of Glutamic Acid:

Decarboxylation of glutamic acid to isolate carbon from the C-1 position was accomplished by a modification of the method of Van Slyke[15]. The reaction was conducted in the same vacuum combustion apparatus as was the combustion of the whole molecule. The amino acid sample, dissolved in several milliliters of water, was placed in the reaction vessel with several boiling chips, and the vessel evacuated. Four mols of ninhydrin per mol of amino acid, and an equal weight of solid citric acid/sodium citrate buffer of pH 2.5 were dissolved in 15 ml. of water and added through a sidearm tube. The reaction mixture was heated for 30 minutes at 100°C. The evolved CO_2 was trapped in NaOH, filtered, washed and dried as in the combustion of the whole molecule. It was found that flaming of the glassware with a microburner during the reaction, to prevent condensation of water droplets in the communicating arm of the apparatus,

was essential to obtain maximum yields. Using known pure samples of glutamic acid, yields averaging 98.5 per cent of theory were obtained.

G. Measurement of the radioactivity of $BaCO_3$ samples:

The samples of $BaCO_3$ were finely ground in an agate mortar and suspended in 95 per cent ethanol. They were evenly plated on 25 mm. steel planchets and dried under an infrared lamp.

The activity was counted in a lead shield, using a Tracerlab end-window Geiger-Müller tube with a window density of less than 1.8 mg./cm.^{2} and an efficiency of 2.2 per cent with samples of infinite thickness. The counts were recorded by means of a Nuclear Model 165 Scaler and were then corrected to infinite thickness.

RESULTS

The relative C^{14} activities derived from combustion of the whole molecules of glutamic and aspartic acids in the two groups of animals of Experiment I are presented in Table I. It can be seen that the activity reaching the glutamic acid in the group of animals which received ALA was reduced by a ratio of 2.98:1. The activity of the aspartic acid isolated from the animals receiving ALA was also reduced, but to a much lesser extent, the ratio being 1.55:1.

In Table II are presented both the activities of the carbon derived from the whole molecule of glutamic acid and the activity residing in the C-1 position of glutamate in the two groups of animals of the second experiment. It can be seen that the activity of the

Table I

Experiment I

Total C^{14} Activity of Aspartic and Glutamic Acids

Isolated from Rat Liver

	α-C^{14} glycine administered	α-C^{14} glycine + ALA administered
Glutamic acid	367 c.p.m.	123 c.p.m.
Aspartic acid	113 c.p.m.	73 c.p.m.

Counts corrected to infinite thickness.

Table II

Experiment II

C^{14} Activity Incorporated into Glutamic Acid in Rat Liver

	α-C^{14} glycine administered	α-C^{14} glycine+ALA administered
Whole Molecule	26.3 c.p.m.	27.9 c.p.m.
Carbon from C-1 position	17.5 c.p.m.	8.7 c.p.m.
Per cent of total activity in C-1 position	13.3	6.2

Counts corrected to infinite thickness.

glutamic acid in Experiment II was of the order of one-tenth as great as in Experiment I. It can also be seen that no dilution occurred in the activity of the whole molecule of glutamic acid in those animals receiving ALA. However, a depression of greater than 2:1 proportion occurred in the activity of the C-1 carboxyl carbon alone. In the animals receiving α-C^{14} glycine alone, the carboxyl carbon of glutamate contained 13.3 per cent of the total activity in the molecule, and in those receiving ALA also, this was reduced to 6.2 per cent.

DISCUSSION

It can be seen from the data that the α-carbon of glycine is incorporated into the glutamic acid of rat liver. If the succinate-glycine cycle makes an important contribution to glutamate synthesis, a substantial reduction in the activity of carbon obtained from the whole molecule should occur when a non-labeled intermediate in that cycle is administered, providing that the intermediate is absorbed from the site of injection and is able to enter the metabolic pool of that particular compound. It has been pointed out that non-labeled ALA was capable of diluting the incorporation of C^{14} from glycine into hemin in duck erythrocyte hemolysates and that the decreased incorporation was not due to inhibition of the system. An even more specific measure of dilution by this intermediate than dilution of the total molecule activity would be a depression in the activity of the C-1 position of glutamic acid, since all the activity reaching glutamate via the succinate-glycine cycle should

reside in this position. In the first experiment a reduction of about two-thirds in the activity of the whole molecule occurred with the administration of ALA. In the second experiment, no reduction of total activity was demonstrated; however, a decrease of greater than one-half occurred in the activity of the C-1 position alone. In this experiment, however, the total activity of the glutamate isolated from animals receiving isotopic glycine alone was only about one-tenth of that in the first experiment.

The decrease in glutamate activity observed in the group receiving glycine plus ALA in the first experiment cannot be attributed in large part to differences in the absorption of the labeled glycine since the difference in the activity of the aspartic acid isolated from the two experimental groups was of a much lesser order than that of the glutamate. It will be remembered that C^{14} reaching α-ketoglutaric acid from the α-carbon of glycine is lost in the decarboxylation of α-ketoglutaric acid to succinate and hence would not be transferred to aspartic acid via the tricarboxylic acid cycle. Hence, the administration of ALA should not be expected to dilute the incorporation of label into aspartic acid, and a similarity of activity of aspartic acid from both groups should indicate similar rates of absorption of the isotopic glycine.

The only factors in the experimental plan which would tend to reduce the activity of the glutamate in the second experiment were: the dosage of glycine, which was about one-fifth less, and the weight of the animals, which was about one-third more, than in the first experiment. Since the decrease in total activity of the

glutamate isolated in the second experiment was unaccountable for entirely on these bases, it must be assumed that one or more of the pathways between glycine and glutamic acid were functioning to a much lesser degree or that the preexisting glutamate pool was larger than in the first experiment.

It can be seen from the small percentage of the total activity residing in the C-1 carbon of glutamic acid isolated from animals receiving the isotopic glycine alone that the contribution which could have come from the succinate-glycine cycle in the second experiment was very small, amounting to not over fourteen per cent of the total activity. It also may be assumed that if the two-thirds reduction observed with ALA administration in the first experiment was due to dilution, the succinate-glycine cycle made a major contribution to glutamate synthesis in that group of animals.

If it may be assumed that both the reduction in total activity of glutamate in the first experiment and the reduction in the C-1 carbon activity in the second are due to dilution by unlabeled ALA, then the tentative conclusion may be reached that in both experiments the succinate-glycine cycle contributed to glutamate synthesis, but that its quantitative importance varied considerably between experiments, its contribution in the second experiment being so slight that the small change in total activity effected by ALA dilution could easily have been nullified by small fluctuations in some of the more important contributing pathways. The tentative conclusion, then, may be made that the succinate-glycine cycle is variable in its contribution to glutamate synthesis.

One factor which is capable of sharply altering the amount of glycine entering the succinate-glycine cycle is the size of the acetyl CoA pool. Shemin[7] has demonstrated that the administration of acetate and pyruvate depresses the condensation of succinate with glycine and that the inhibition can be overcome by the addition of succinate. He postulates that the inhibition results from the condensation of acetate and glycine in a fashion analogous to that of succinate and glycine, forming α-amino acetoacetic acid which is decarboxylated to aminoacetone. Thus, if an animal were degrading a large amount of fatty acids, as in the starving state, the excess of acetyl CoA would competitively sweep the glycine into the acetate-glycine condensation.

Shemin[7] also postulates the participation of the original product of succinate-glycine condensation, α-amino-β-ketoadipic acid as an active form of glycine in the synthesis of serine and other glycine derivatives. If this pathway is functional, it provides another alternate pathway which could compete for injected glycine. Still another variable factor in the competition for glycine is tissue protein synthesis.

That glycine can be oxidized by pathways other than the succinate-glycine cycle, and by conversion to serine with the subsequent formation of pyruvate has been demonstrated by Ratner *et al*[16] who isolated an enzyme, glycine oxidase, which is capable of oxidizing glycine to glyoxylic acid which may yield formate and CO_2 or be oxidized to oxalate.

Since ALA is a precursor of both porphyrins and purines, variations in the rates of synthesis of these compounds could greatly alter the pool of ALA available for glutamate synthesis.

Since glutamic acid is easily deaminated to form α-keto-glutaric acid, and in fact α-ketoglutaric acid may be an intermediate in the formation of glutamic acid from ALA, a rapid rate of oxidation of α-ketoglutamate would depress the activity reaching the glutamate pool in the liver.

Considerable labeling of liver aspartic acid occurred upon administration of α-C^{14} glycine. Possible pathways for this isotope transfer include the formation of serine and then pyruvate, which may be labeled in both the C-2 and C-3 positions, from glycine, with subsequent condensation in the Wood-Werkman reaction with CO_2, which may itself be labeled, to form oxalacetic acid, which can be transaminated to form aspartic acid. Another possible pathway involves the decarboxylation of the pyruvate formed, to yield acetate which would condense with oxaloacetate to enter the tricarboxylic acid cycle, emerging subsequent to one complete cycle as oxaloacetate bearing the label of the acetate which was derived from the labeled pyruvic acid. It is to be noted that this is the same combination of pathways proposed by Anderson and Stekol to explain the transfer of label from α-C^{14} glycine to glutamate.

SUMMARY

The role of the succinate-glycine cycle in the transfer of the α-carbon of glycine to glutamic acid has been studied by means of

two experiments in which α-C^{14} glycine with, and without non-labeled ALA, an intermediate in that cycle, was administered to rats with subsequent isolation of liver glutamate and determination of its radioactivity. Dilution of the incorporation of the label by ALA into glutamate would evidence the function of that pathway in glutamate synthesis. Since all the labeled carbon from α-C^{14} glycine which enters the glutamate molecule via the succinate-glycine cycle should reside in the C-1 position of the glutamate formed, isolation of carbon from that position was carried out in the second experiment as a more specific measure of dilution by ALA of the incorporation of α-carbon activity from glycine into glutamate than measurement of total activity of the glutamate molecule.

A marked dilution of the activity of the whole molecule of glutamate in those animals receiving the intermediate, ALA, occurred in the first experiment, but was not demonstrable in the second, where the conversion of glycine to glutamic acid was markedly depressed. However, a dilution of greater than one-half did occur in the activity of the C-1 position of the glutamate isolated in the second experiment. Since the percentage of the total activity found in the C-1 carbon was small, variation in the labeling of other carbon atoms apparently overshadowed the effect on total activity of the molecule by the dilution in the C-1 position.

Because of the dilution due to ALA of the total activity reaching glutamic acid in the first experiment, and of that reaching the C-1 position of the glutamate in the second experiment, it was

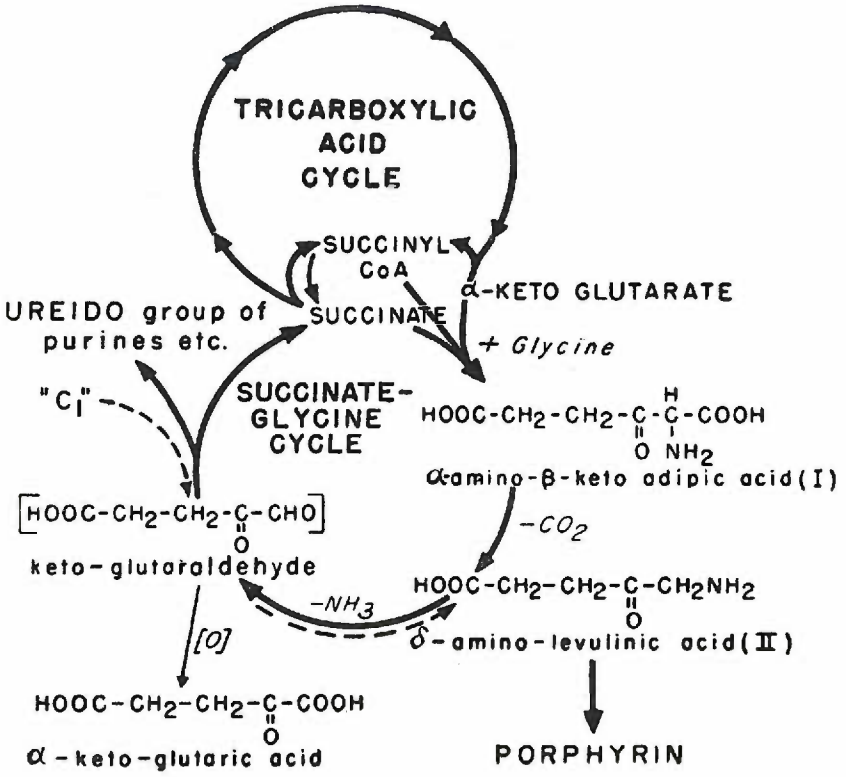
concluded that the succinate-glycine cycle does contribute to the synthesis of glutamate, but quantitatively is highly variable. It is to be concluded that further studies are needed to determine the quantitative contribution of this cycle to glutamate synthesis.

Possible explanations of the markedly diminished incorporation of isotope into glutamate in the second experiment have been discussed.

The incorporation of isotope from α-C^{14} glycine into the aspartic acid molecule has been noted and possible pathways discussed.

Figure 1

The Succinate-Glycine Cycle


TRICARBOXYLIC ACID CYCLE
SUCCINYL CoA
α-KETO GLUTARATE
UREIDO group of purines etc.
SUCCINATE
+ Glycine
"C1"
SUCCINATE-GLYCINE CYCLE
HOOC-CH2-CH2-C(=O)-C(H)(NH2)-COOH
α-amino-β-keto adipic acid (I)
-CO2
[HOOC-CH2-CH2-C(=O)-CHO]
keto-glutaraldehyde
HOOC-CH2-CH2-C(=O)-CH2NH2
-NH3
δ-amino-levulinic acid (II)
[O]
HOOC-CH2-CH2-C(=O)-COOH
α-keto-glutaric acid
PORPHYRIN

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