BIOLOGICAL PRECURSORS OF GLYCINE*

by

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The fact that the mammal, apparently, does not require dietary glycine for growth raises the question as to the nature of the compounds that can serve as precursors of glycine. Serine[1-3] and threonine[4,5] are the only natural amino acids found to be effective as glycine precursors, and of these only threonine is nutritionally indispensible. In feeding experiments, KRUEGER[6] observed an increase of free glycine in the liver of rats fed on a high carbohydrate diet, without a corresponding increase of serine; whereas an increase of serine, but not of glycine, was found on a high fat diet. The same author reported that large doses of pyruvate caused an increase of glycine and alanine, but not of serine[7]. Acetate was reported to yield glycine to the extent of about 0.1% of the administered dose[8]. It has recently been shown that glyoxylic and glycolic acids are converted extensively to glycine[9], but no extensive metabolic processes are known in vertebrates which lead to the production of these acids.

The purpose of the present work was to conduct a systematic investigation of the precursors of glycine in the rat by the use of certain compounds labeled with ^{14}C and of amino acids labeled with ^{14}C and ^{15}N. This was carried out by determining the formation of hippuric acid containing labeled glycine, following the administration of the selected compounds and sodium benzoate to rats.

The ^{14}C-labeled compounds employed were DL-serine-3-^{14}C[10], DL-threonine-1, 2-^{14}C, DL-allothreonine-1, 2-^{14}C[11], glycolate-2-^{14}C, lactate-1-^{14}C, lactate-3-^{14}C and fructose-^{14}C. The ^{15}N-labeled compounds were glycine (32 atom per cent. excess) and DL-threonine[11] (11.5 atom per cent. excess).

EXPERIMENTAL

Long-Evans strain rats, weighing 240 to 250 g were fasted for 24 hours prior to the injection of sodium benzoate and the ^{14}C- and ^{15}N-labeled compounds. The rats were placed in individual cages without food but with water, and two 24-hour urine samples were collected in graduated cylinders, to which a few drops of toluene were added as preservative. Urine samples less than 10 ml were made up to that volume by adding distilled water. To determine total radioactivity excreted, two 0.1 ml samples were placed on aluminum disks of 4.5 cm diameter, absolute alcohol was added dropwise on top of the samples in order to secure a thin layer of the solid material on the disk and the ^{14}C activity was counted with a thin mica end-window Geiger-Müller counter.

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The hippuric acid was isolated according to the procedure of HAMPTON[12] by acidifying the remaining rat urine, with 10 *M* acetic acid to pH 3.8 with a Beckman pH meter and extracting successively with 50, 25 and 25 ml portions of ethyl acetate. The extracts were combined, washed with 10 ml of acidified water at pH 3.5 and then allowed to stand overnight in a separatory funnel for careful separation of the two layers of liquid. The washed ethyl acetate, containing the hippuric acid, was then distilled off in a water bath at 90° and the last trace of it removed in a vacuum desiccator. To establish purity of the hippuric acid, when required, it was recrystallized and the melting point checked against an authentic sample.

For isotopic analysis, the hippuric acid was dissolved in 10 ml of hot water and two 0.1 ml samples were taken as before for counting the ^{14}C activity. The remaining hippuric acid was used to determine the nitrogen by microKjeldahl, using borate buffer to absorb the ammonia[13], and the ammonium salt was further used for ^{15}N assay, which was performed on a Consolidated-Nier mass spectrometer.

The reliability of the above procedure was checked by analysis of total N and ^{14}C activity in rat urine to which inert hippuric acid and ^{14}C-glycine were added. Recovery of nitrogen was above 95% (90 to 100% by Hampton), and there was no contamination by ^{14}C glycine.

Collection of hippuric acid was carried out for two 24 h periods. Since about 95% of the radioactivity was in the first day's collection, only the data for the first period are reported in the tables.

To compare the data obtained, the results of the ^{14}C and ^{15}N determinations are expressed in terms of the specific activity (S.A.) ratios of the hippuric acid (HA) to the administered labeled compound. For ^{14}C this is given by $\frac{\text{S.A. of HA}}{\text{S.A. of compound}}$ and for ^{15}N by $\frac{\text{atom per cent. excess } ^{15}N \text{ in HA}}{\text{atom per cent. excess } ^{15}N \text{ in compound}}$.

These ratios multiplied by the isolated hippuric acid nitrogen represent glycine (in m.eq.) formed from the administered labeled compound. The percentage conversion is 100 times the fraction of labeled compound converted to glycine in the isolated hippuric acid. It is to be noted that the reciprocal of the above ratios (see Table IV) is equivalent to the dilution factor of SHEMIN[1].

RESULTS AND DISCUSSION

The first experiments were run to determine to what extent the carbon and nitrogen were utilized intact in the glycine in the formation of hippuric acid and how resynthesis of glycine from the degraded isotopic products might affect the results. Glycine, doubly labeled with ^{14}C and ^{15}N, was injected together with an equimolar quantity of sodium benzoate. The results (Table I) show that the ratios of $^{14}C/^{15}N$ for both the α-carbon and carboxyl-labeled glycine were approximately unity. This agrees with the independent observations of ARNSTEIN AND NEUBERGER[14]. Hence, it is apparent that, within the limits of the present method, the glycine was used intact in the animal to combine with benzoate to form hippuric acid. The ratio of $^{14}C/^{15}N$ probably would not be unity if deamination of the glycine with re-utilization of the carbon skeleton occurred to any great extent.

TABLE I

RECOVERY OF HIPPURIC ACID FROM RAT URINE AFTER INJECTION OF EQUIVALENT QUANTITIES OF SODIUM BENZOATE AND LABELED GLYCINE

Glycine	*Benzoate m.eq*	*Nitrogen m.eq*	*Hippuric Acid*				$^{14}C/^{15}N$
			^{14}C *Recovered*		^{15}N *Recovered*		
			m.eq	*per cent.*	*m.eq*	*per cent.*	
1 ^{14}C–^{15}N	0.20	0.16	0.017	10.3	0.016	10	1.04
1 ^{14}C–^{15}N	0.40	0.31	0.043	14.2	0.045	14.9	0.95
1 ^{14}C–^{15}N*	0.20	0.145	0.027	18.8	0.026	18.1	1.04
2 ^{14}C–^{15}N	0.20	0.20	0.02	10.0	0.018	9.0	1.08
2 ^{14}C–^{15}N	0.40	0.27	0.036	13.1	0.044	16.1	0.82

* Hippuric acid twice recrystallized (MP = 187°)

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To secure further evidence on the extent of the resynthesis of glycine from the labeled products, experiments were performed with $^{15}NH_4Cl$. In work done by FOSTER, SHOENHEIMER AND RITTENBERG[15] in which $^{15}NH_4Cl$ was fed, it was reported that approximately 4% of the label appeared in glycine. This figure, obtained in an experiment where isotopic ammonium ion and sodium benzoate were administered orally, is considerably higher than any conversion observed in the present work. In the present experiments (Table II) equivalent amounts of labeled ^{14}C precursor (glycine-2-^{14}C or glycolate-2-^{14}C), $^{15}NH_4Cl$ and sodium benzoate were injected intraperitoneally in a single dose. The isolated hippuric acid was recrystallized to assure its purity. The results show (Table II) that less than a fraction of 1% of the ^{15}N was reintroduced into the glycine of the hippuric acid following either glycine or sodium glycolate administration. Resynthesis of glycine, once degraded, therefore, can exert no appreciable influence on the interpretation of the present results. It is apparent that the labeled products of glycine degradation are greatly diluted and so do not appear to any noteworthy extent in the reformed glycine.

TABLE II

EXTENT OF RESYNTHESIS OF GLYCINE FROM LABELED PRODUCTS

*Compound**	*Nitrogen m.eq*	*Hippuric Acid*			
		^{14}C Recovered		*^{15}N Recovered*	
		m.eq · 10^3	*per cent.*	*m.eq · 10^5*	*per cent.*
Glycine-2-^{14}C	0.051	9.3	19.8	7.7	0.15
Glycine-2-^{14}C	0.037	5.3	14.2	4.1	0.11
Na glycolate-2-^{14}C	0.051	4.9	9.1	26	0.50
Na glycolate-2-^{14}C	0.051	7.0	13.6	31	0.60

* 0.20 m.eq. of ^{14}C-labeled compound and ^{15}N H_4Cl injected in each instance except the last, in which the quantities were 0.40 m.eq.

The data on the recovery of labeled hippuric acid upon injection of DL-serine-3-^{14}C and of labeled DL-threonine and allothreonine are recorded in Table III. The results show that the β-carbon of serine appears in the hippuric acid glycine only to a slight extent, of the order of about 0.1% as compared to the 10 to 15% from 1 or 2 ^{14}C-labeled glycine. From the fact that the extent of utilization of the β-carbon of serine was similar to that of acetate, together with the work of ELWYN AND SPRINSON[16], who showed that this carbon could go to the methyl carbon of acetate, it appears possible that, in the degradations of serine via pyruvate, acetate was formed which, in turn, was converted to a slight extent to glycine.

With DL-threonine, the amount of hippuric acid derived from the 1,2 carbon fraction was increased from as little as 1 up to 6% as the amount of threonine injected was raised from 0.20 to 0.80 m.eq. per rat. In all but one instance twice the equivalence of threonine to benzoate was given on the assumption that D-threonine would follow a different metabolic pathway. The ratio of $^{14}C/^{15}N$ converted is of the order of 1.5. This indicates that the utilization of nitrogen for glycine formation is less extensive than that of the 1,2 carbons and that some of the D-threonine was deaminated and the 1,2 carbons reconverted to glycine, a process that would entail a considerable

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TABLE III

RECOVERY OF HIPPURIC ACID FROM RAT URINE AFTER ADMINISTRATION OF SODIUM BENZOATE AND CERTAIN LABELED AMINO ACIDS

Amino Acid	*m.eq*	*Benzoate m.eq*	*Nitrogen m.eq*	*Hippuric Acid*				$^{14}C,^{15}N$
				^{14}C *Recovered*		^{15}N *Recovered*		
				m.eq · 10^3	*per cent*	*m.eq* · 10^3	*per cent*	
			Injected					
DL-Serine-3-^{14}C	0.20	0.20	0.20	0.25	0.12			
DL-Serine-3-^{14}C	0.20	0.20	0.20	0.30	0.15			
			Injected					
DL-Threonine* 1, 2-^{14}C–^{15}N**	0.20	0.10	0.021	0.89	4.3	0.36	1.7	2.5
DL-Threonine* 1, 2-^{14}C–^{15}N**	0.40	0.20	0.055	2.5	4.5	1.7	3.0	1.5
DL-Threonine* 1, 2-^{14}C–^{15}N**	0.20	0.20	0.16	1.7	1.1	1.2	0.8	1.4
DL-Threonine* 1, 2-^{14}C–^{15}N**	0.80	0.40	0.23	12.0	5.2	6.7	3.0	1.8
DL-Threonine* 1, 2-^{14}C–^{15}N**	0.80	0.40	0.23	15.0	6.3	10.0	4.1	1.5
			Fed					
DL-Threonine 1, 2-^{14}C	0.80	0.40	0.29	8.1	2.8			
DL-Threonine 1, 2-^{14}C	0.80	0.40	0.28	17.0	6.0			
			Injected					
DL-Allothreonine* – 1, 2-^{14}C	0.40	0.20	0.15	23.0	15.3			
DL-Allothreonine* – 1, 2-^{14}C	0.80	0.40	0.30	47.0	15.7			

* Twice the equivalence of amino acid to sodium benzoate employed because of DL-forms of amino acids.

** Hippuric acid recrystallized twice.

dilution of the ^{15}N. Comparison of the ^{15}N of hippuric acid and of the simultaneously excreted urea from the first two threonine experiments given in Table III gave values in hippuric acid of 0.190 and 0.334 and in the urea of 0.052 and 0.110 atom per cent. excess respectively. Consequently, the dilution of the ^{15}N in the urea was about 3 fold greater than in the hippuric acid. As the nitrogen in the hippuric acid was diluted by glycine nitrogen alone, while the nitrogen in the urea was diluted by all nitrogen sources, the above results suggests a rather extensive deamination of the DL-threonine.

The results for allothreonine were striking in that, although not normally present in the rat, it was quite extensively converted to glycine. The conversion was about three times that from DL-threonine and the labeled carbons of the allothreonine suffered very little greater dilution in the hippuric acid than was found for glycine itself. This result confirms the observation of BRAUNSHTEIN AND VILENKINA[5] with liver slices.

Knowledge of the metabolism of L-threonine is rather limited. ELLIOTT AND NEUBERGER[17] found that when glycine-^{15}N was fed to animals the amino nitrogen of threonine isolated from the protein of muscle and viscera, like lysine, showed no exchange with the ^{15}N-containing nitrogen. The work of MELTZER AND SPRINSON[4] suggested that there is a direct cleavage of L-threonine into acetate and glycine. Hence, it would be expected that the glycine should be used intact in the hippuric acid and that the ratio of the 1,2 ^{14}C/^{15}N should be unity. That this is not the case, indicates that the D-isomer may partially be used by the rat. This is supported by the observation that 25 to 30% of the total ^{14}C instead of the expected 50% was recovered in the urine,

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and it was possible that deamination took place prior to splitting of the carbon skeleton, in which case the two carbon fragments become reaminated to give glycine. The difference in the metabolism of D- and L-threonine, of course, remains to be solved.

DL-allothreonine also gave about a 30% recovery of the total ^{14}C activity in the urine.

The ability of the rat to grow on a diet lacking both glycine and serine suggests that carbohydrate provides the precursors of glycine. This was tested with the compounds given in Table IV. The results clearly show that lactate-1-^{14}C and -3-^{14}C did not form glycine at all in the rat. This excluded any mode of cleavage of lactate to form the carbon skeleton of glycine. Furthermore, following injection of either form of labeled lactate, the non-protein hot water extract of the tissues obtained 20 minutes after administration exhibited no ^{14}C activity in the glycine fraction when chromatographed by the method of LIEN AND GREENBERG[18]. This rules out the possibility that carbohydrate forms glycine via a three carbon metabolic product. The results of KRUEGER[7] that pyruvate may serve as a source of glycine, although interesting, cannot be the result of a direct conversion.

Another test of the capability of carbohydrate to form glycine is provided by the experiments with labeled fructose. The extent of conversion found was slight. These

TABLE IV

RECOVERY OF HIPPURIC ACID FROM RAT URINE AFTER ADMINISTRATION OF SODIUM BENZOATE AND CERTAIN LABELED CARBON COMPOUNDS

Compounds *	*Benzoate m.eq*	*Nitrogen m.eq*	*Hippuric Acid*	
			^{14}C Recovered	
			m.eq	*per cent.*
		Injected		
Zn lactate-1-^{14}C	0.20	0.10	$7.0 \cdot 10^{-6}$	
Zn lactate-1-^{14}C	0.20	0.26	$1.6 \cdot 10^{-5}$	
Zn lactate-3-^{14}C	0.20	0.10	$3.8 \cdot 10^{-4}$	0.38
		Fed		
Zn lactate-1-^{14}C	0.20	0.17	$7.8 \cdot 10^{-5}$	
Zn lactate-3-^{14}C	0.20	0.19	$1.7 \cdot 10^{-5}$	
		Injected		
Ca glycolate-2-^{14}C	0.20	0.10	0.013	13.3
Na glycolate-2-^{14}C	0.20	0.16	0.040	25.0
Na glycolate-2-^{14}C	0.20	0.15	0.029	28.9
		Fed		
Na glycolate-2-^{14}C	0.20	0.24	0.012	5.0
Na glycolate-2-^{14}C	0.20	0.17	0.013	7.7
		Injected		
Fructose-^{14}C **	0.20	0.20	$3.8 \cdot 10^{-4}$	0.19
Fructose-^{14}C **	0.20	0.20	$2.2 \cdot 10^{-4}$	0.11

* Dose of each compound in amount equivalent to benzoate except for fed Na glycolate-2-^{14}C which was 0.02 m.eq.

** Labeled in all carbons.

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results appear to rule out the possibility that the hexoses provide the precursors of glycine.

Glycolate, on the other hand, was very extensively converted to glycine, a result also observed by WEINHOUSE AND FRIEDMAN[9]. At the same dose level it formed hippuric acid to an even greater extent than did glycine. The explanation for this may be that the liver can absorb glycolate much faster than glycine and that, once absorbed, the conversion to glycine also occurs rapidly. Twenty minutes after injection of 0.10 m.eq. of glycolate, about 20% of the radioactivity was present in the hot water extract of liver, compared to about 4% in the case of glycine itself.

Although glycolate could serve as a very good source of the carbon skeleton of glycine, it is not considered a normal biological constituent and no reaction is known which gives rise to glycolate in the animal, although some of the hypothetical pathways proposed for the metabolism of pentoses suggest the formation of this compound.

The excretion of hippuric acid was not altered when the DL-threonine and sodium benzoate were given orally (by stomach tube). This has also been shown to be true for glycine[19] and for acetate[8]. The results with glycolate are not strictly comparable since the oral dose was much smaller than the dose injected.

From the foregoing results it appears that, in the main, the glycine precursors must furnish glycine as a whole and only the amino acids serine and threonine, in addition to exogenous glycine, can fulfil this requirement. Certain compounds, such as allothreonine and glycolate, are excellent sources of glycine.

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SUMMARY

Compounds that could be considered to be precursors of glycine were tested by determining the excretion of labeled hippuric acid after administration of the isotopically-labeled compound together with sodium benzoate. Compounds that yielded glycine in considerable amounts were DL-threonine, DL-allothreonine and glycolic acid. Only traces of glycine were derived from lactic acid, fructose and the β-carbon of serine.

The labeling of the glycine in hippuric acid following injection of glycine-^{14}C–^{15}N showed that the amino acid was used intact in the animal. A precursor of glycine for the animal must, therefore, supply the molecule of glycine as a whole and the amino acids serine, threonine, and dietary glycine, appear to be the only known natural metabolites that can fulfil this requirement.

RÉSUMÉ

Des composés qui pouvaient être considérés comme des précurseurs de la glycine ont été étudiés de la façon suivante: le composé en question, marqué isotopiquement, était administré en même temps que du benzoate de sodium et la quantité excrétée d'acide hippurique marqué était déterminée. La DL-thréonine, la DL-allothréonine et l'acide glycolique donnèrent des quantités considérables de

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glycine, tandisque l'acide lactique, le fructose et la sérine (carbone-β) ne donnèrent que des traces de glycine.

La façon dont la glycine de l'acide hippurique était marquée après injection de glycine ^{14}C–^{15}N montra que l'acide aminé était utilisé tel quel dans l'animal. Un précurseur de la glycine doit donc fournir à l'organisme animal la molécule de glycine entière; les acides animés sérine et thréonine et la glycine de diète sont les seuls métabolites naturels connus qui puissent satisfaire à cette condition.

ZUSAMMENFASSUNG

Verbindungen, welche als Vorläufer von Glycin betrachtet werden könnten, wurden geprüft, indem sie isotopisch markiert, zusammen mit Natriumbenzoat verabreicht wurden und darauf die ausgeschiedene markierte Hippursäure bestimmt wurde. Verbindungen, welche bedeutende Mengen von Glycin lieferten waren DL-Threonin, DL-Allothreonin und Glykolsäure. Spuren von Glycin lieferten Milchsäure, Fructose und Serin (β-Kohlenstoff).

Die Markierung des Glycins in der Hippursäure nach Injektion von Glycin-^{14}C–^{15}N zeigte, dass die Aminosäure im Tierkörper intakt verwendet wird. Ein Vorläufer des Glycins muss also dem Tierkörper das Glycinmolekül als Ganzes liefern. Serin und Threonin und Diätglycin scheinen die einzigen bekannten natürlichen Metaboliten zu sein, welche diese Bedingung erfüllen.

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