Hippuric Acid Synthesis in Man after the Administration of [α - ^{14}C]Glycine

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Previous studies from this laboratory have reported the excretion of ^{14}C in the breath (Berlin, Tolbert & Lawrence, 1951) and urine (Berlin, Tolbert & Lee, 1953) following the intravenous injection of α -labelled glycine; autopsy data on the ^{14}C content of the tissues at various intervals up to 525 days after administration have also been given (Berlin, Tolbert & Lotz, 1952). These studies have demonstrated that approximately 98 % of the administered ^{14}C can be accounted for in the breath and urine. These studies did not give any information regarding the specific activity of the various glycine fractions in the body.

In studying the incorporation in man of glycine into haemoglobin either with ^{15}N (Shemin & Rittenberg, 1946) or ^{14}C (Berlin, Lawrence & Lee, 1951) it has been found that in the latter part of the curve, i.e. after approximately 150 days, there is an isotope content corresponding to about 10–15 % of the plateau level and that the specific activity in this tail portion of the curve decreases slowly with time. In the interpretation of this tail portion of the curve it is important to know if there is sufficient label (^{15}N or ^{14}C) present in haemoglobin precursors to account for a significant fraction of the observed haemoglobin specific activity, or if this persistence of label in the haemoglobin is due to re-utilization of the catabolic products of haemoglobin. The present study was designed to test the first hypothesis.

Arnstein & Neuberger (1951) have reviewed the literature on the *in vivo* synthesis of hippuric acid, and as a result of their experiments concluded that in the rat about 10 mg. of free glycine/100 g. body weight is immediately available for conjugation with benzoate. They also showed that when the time interval between glycine and benzoate administration was at least 6 hr., the isotope content of the hippuric acid was independent of the dose of sodium benzoate used. Though similar data are not available for man, it would seem likely that the isotope content of the hippuric acid after the administration of a small dose of sodium benzoate would give an approximate measure of the isotope content of the free glycine at that time.

EXPERIMENTAL

Three patients (two with lymphoblastoma and one with aplastic anaemia) were given [α - ^{14}C]glycine (100 μC , approx. 8 mg.). Later they were given orally 1.5 g. of sodium benzoate and the urine was collected during the ensuing 4 hr. This dose and the time after the administration of the glycine were such that, based on the data of Arnstein & Neuberger (1951), it was unlikely that the size of the dose of sodium benzoate was a significant factor affecting the isotope content of the hippuric acid.

For the isolation of the hippuric acid, urine (150 ml.) was made acid to litmus, then NaCl (45 g.) was added and the mixture heated until the salt was dissolved. After cooling, 6N- H_2SO_4 was added until the solution was acid to congo red. After standing overnight the solution was filtered. The precipitate was dissolved in 100 ml. of boiling water and made alkaline to litmus. Charcoal was added, the solution boiled and filtered while hot. Then the solution was acidified with 6N- H_2SO_4 and concentrated to 30 ml. The precipitate which formed was dried at 40–50° and then recrystallized twice. The m.p. of the product, was unchanged by admixture with authentic hippuric acid. (Found: C, 60.38; H, 5.18. Calc. for $\text{C}_9\text{H}_9\text{O}_3\text{N}$: C, 60.28; H, 5.06 %.) For separation of the glycine moiety the hippuric acid was hydrolysed by refluxing with 2N-KOH for 4 hr. The benzoic acid was precipitated with 2N- H_2SO_4 and removed by continuous extraction with ether for 6–8 hr. The aqueous solution was considered to contain no other organic material besides glycine.

For determination of the relative labelling of the carboxyl and methylene carbon atoms of the glycine moiety the hippuric acid was hydrolysed with 2N-KOH and the glycine decarboxylated with ninhydrin according to the method of Van Slyke, Dillon, MacFayden & Hamilton (1941) and the evolved CO_2 collected in NaOH .

Although the ratio of specific activities of haemin carbon/haemoglobin carbon was not measured in these three patients, thirty-four measurements in four individuals with polycythemia vera have been carried out giving an average ratio of 6.28 ± 0.29 (S.E.M.). Individual averages were 5.77, 6.23, 6.29 and 6.74. In thirty measurements on three pigs the ratio averaged 6.25 (individual pig averages 6.25, 6.25 and 6.54) (Cartwright & Berlin, to be published). In five measurements done on two groups of rats (four normal and three anaemic rats) Altman, Casarett, Masters, Noonan & Salomon (1948) found after administration of [α - ^{14}C]glycine that the haemin carbon/haemoglobin carbon specific activity ratio averaged 6.78. It is then not unreasonable to

Fig. 1a-c. The specific activities of the hippuric acid excreted by patients *W*, *F* and *G*. The two straight lines in each figure represent the two rate processes (for method of analysis see text).

suppose that the ratio observed in patients with polycythemia vera will also hold for the present study since it largely reflects the relative incorporation of glycine into haemin and globin and the conversion of glycine into serine and incorporation of the latter into globin.

For measurement of the specific activity the compounds were converted into BaCO_3 by the method of Van Slyke & Folch (1940). The BaCO_3 was then converted into CO_2 and introduced into a 100 ml. ionization chamber operating at atmospheric pressure. A vibrating reed electrometer and a recording potentiometer were used to measure the rate of voltage change in the ionization chamber. In an ionization chamber of this size the sensitivity is 0.02 disintegrations/min./mg. BaCO_3 (Tolbert & Baker, to be published).

RESULTS

Fig. 1a-c shows the specific activity of the excreted hippuric acid in the three patients. The specific activity decreases sharply during the first 30 days and then slowly during the remaining period of observation. A convenient method of analysis and mathematical description of these curves is to assume that they represent two first-order processes. The graphical method of analysis is carried out by fitting the data after approximately 50 days to a straight line, which represents the rate for the slowest observable process. This line is then extrapolated to zero time, and the line representing the rate of the fast process is obtained by subtracting from the experimental data the contribution due to the slow process. (This is the same method as is used in the graphical analysis of two radioactive isotopes). Because 4 hr. are required for the collection of hippuric acid and because small additional quantities of hippuric acid will be excreted after

this time, several discrete repeat determinations during the first and second days are not possible, and the curve for the first 2 days for the ^{14}C content of the hippuric acid cannot be compared with that of the respiratory $^{14}\text{CO}_2$. In such an analysis it is found that the two components have half-times averaging 6.8 and 75 days respectively. Table 1 shows, in addition to the calculated half-times for the components of the hippuric acid ^{14}C content, the half-times obtained by a similar method of analysis for the two slowest breath components of respiratory $^{14}\text{CO}_2$ (Berlin, Tolbert & Lawrence, 1951; Berlin & Tolbert, to be published). It is readily seen that in each individual there is close agreement between the observed half-times for the slowest two components of the breath $^{14}\text{CO}_2$ and the corresponding hippuric acid ^{14}C component. This indicates that the breath $^{14}\text{CO}_2$ was in large measure derived from free glycine present at any given time.

The relative labelling of the carboxyl and methylene carbon atoms is shown in Table 2, from which

Table 1. *The half-times for the components of hippuric acid ^{14}C content and for components III and IV of the breath $^{14}\text{CO}_2$ excretion*

For nature of the components see text. Half-times are expressed in days.

	Hippuric acid component		Breath component	
	I	II	III	IV
Patient F	4.5	58	4.5	52
Patient G	8.0	105	7.5	117
Patient W	8.0	63	7.0	57
Average	6.8	75	6.3	75

Table 2. *The relative labelling of the carboxyl and methylene carbon atoms of the glycine moiety of hippuric acid in patient W*

Specific activity ($\pm$ probable error of the instrument) is expressed as disintegrations/min./mg. BaCO_3 . There was no significant percentage of ^{14}C in the carboxyl group of hippuric acid between days 22 and 71.

Day after administration	Specific activity of		Percentage of ^{14}C in carboxyl group of hippuric acid
	Hippuric acid	Carboxyl group	
0	217*	55.35 $\pm$ 0.01	2.8
1	11.3 $\pm$ 0.02	2.64 $\pm$ 0.074	2.6
5	4.47 $\pm$ 0.02	0.675 $\pm$ 0.112	1.7
15	1.744 $\pm$ 0.02	0.231 $\pm$ 0.09	1.5
22	1.04 $\pm$ 0.02	0.127 $\pm$ 0.084	—
29	0.803 $\pm$ 0.03	0.119 $\pm$ 0.14	—
43	0.579 $\pm$ 0.03	0.173 $\pm$ 0.08	—
50	0.484 $\pm$ 0.02	0.059 $\pm$ 0.07	—
62	0.306 $\pm$ 0.03	0.185 $\pm$ 0.16	—
64	0.391 $\pm$ 0.03	0.087 $\pm$ 0.12	—
71	0.227 $\pm$ 0.024	0.144 $\pm$ 0.116	—
85	0.201 $\pm$ 0.04	0.280 $\pm$ 0.104	14.7
92	0.213 $\pm$ 0.026	0.203 $\pm$ 0.07	10.5
106	0.175 $\pm$ 0.027	0.555 $\pm$ 0.08	35.2
113	0.212 $\pm$ 0.04	0.134 $\pm$ 0.15	—
120	0.156 $\pm$ 0.066	0.014 $\pm$ 0.18	—
127	0.091 $\pm$ 0.026	0.07 $\pm$ 0.06	—
141	0.130 $\pm$ 0.036	None	—

* Counted on an end-window Geiger-Müller tube.

it is seen that in the first four samples there is a significant labelling of the carboxyl carbon atom to the extent of 1.5–2.8% of the total hippuric acid activity. It is not until the samples taken on the 85th, 92nd and 106th days after administration that the specific activity of the carboxyl carbon atom is again sufficiently above the limits of sensitivity to be significant, at which time 10.5–35.2% of the ^{14}C is in the carboxyl position. It is also at this time that the red cells which have a relatively large ^{14}C content are being destroyed (see Fig. 2). This labelling of the carboxyl carbon atom probably arises from the initial labelling of the carboxyl carbon atom by $^{14}\text{CO}_2$ fixation during the first few days after the administration of the labelled glycine and subsequent incorporation of the carboxyl-labelled glycine into globin. Then, upon catabolism of haemoglobin, free glycine which is labelled in the carboxyl position becomes available for hippuric acid synthesis. As was anticipated the benzoic acid did not contain ^{14}C .

Fig. 2 shows the specific activity of the haemoglobin of these three patients. In patients *F* and *G* the red-cell life may be estimated by the method of Shemin & Rittenberg (1946) as 105 and 112 days respectively. In patient *W* an accurate estimate cannot be made because of numerous transfusions; however, the decrease in the ^{14}C content of the haemoglobin between the 80th and 100th days suggests a life span of the red cell in this patient that is somewhat shorter than normal.

DISCUSSION

All previous interpretations of the curve depicting the isotope content of the haemin were based on the assumption that after 20–30 days the isotope content of the haemoglobin precursors was negligible. The present study shows clearly that this assumption is not valid.

If the observations on rats obtained by Arnstein & Neuberger (1951) can be quantitatively applied to

man, the dose of sodium benzoate and the time after the administration of the glycine should not appreciably affect the isotope content of the hippuric acid. However, other data on a patient with porphyria suggest that a higher dose of sodium benzoate (3 g.) than the one used in the present work (1.5 g.) will lower the isotope content of the hippuric acid, presumably by stimulating the formation of glycine (Gray & Neuberger, 1950). If this also occurs with the dose of sodium benzoate used in the present work, the isotope content of the free glycine may be higher than that of the glycine moiety of the hippuric acid measured.

Eight of the thirty-four carbon atoms of haemin are derived from the α -carbon atom of glycine (Muir & Neuberger, 1950; Radin, Rittenberg & Shemin, 1950); thus the specific activity of the haemin should be $\frac{8}{34}$ ths that of the α -carbon atom of the glycine available for haemin synthesis, if no other haemin precursor contained a significant amount of ^{14}C . The other simple direct precursor of haemin is succinate (Shemin & Kumin, 1952) of which both the carboxyl and methylene groups are used. It is unlikely that a large amount of ^{14}C will enter the haemin through succinate in the present study owing to the large pool size of the tricarboxylic acid cycle intermediates.

The degree to which the observed haemoglobin specific activity curve may be affected by this persistence of labelled glycine can be calculated by making certain assumptions. At any time more than 10 days after the administration of the labelled glycine the isotope content of the haemoglobin may be divided into two fractions, (1) that which was incorporated into haemoglobin within the first few days after administration, and (2) that which is present as a result of the continual synthesis of labelled haemoglobin from labelled glycine. If it is assumed that the entire activity of the hippuric acid is in the methylene carbon atom of the glycine moiety, that the rate of production of red cells is constant and that only the eight carbon atoms of haemin which are specifically derived from the α -carbon atom of glycine contain ^{14}C , then the contribution to the observed isotope content of haemoglobin may be evaluated by determining the average specific activity of the persisting labelled glycine between 10 days after administration and the date of observation. Since the rate of decrease of the specific activity of the hippuric acid may be described in terms of two exponential components, the contribution of each to the observed specific activity may be evaluated by integrating between the date of measurement and 10 days after the administration of the glycine up to 140 days and after 140 days between the date of measurement minus the observed red-cell life span. This relatively simple treatment neglects the distribution of the

Fig. 2. The specific activity of the haemoglobin in patient *F* (Δ), patient *W* ($\circ$) and patient *G* ($\square$).

cell lives about the mean, but is sufficient for the present purpose. The mean specific activity of the hippuric acid will be

$$\frac{\int_{t_1}^{t_2} N_{O_1} e^{-k_1 t} dt + \int_{t_1}^{t_2} N_{O_2} e^{-k_2 t} dt}{t_2 - t_1} = \frac{N_{O_1}(e^{-k_1 t_1} - e^{-k_1 t_2})}{k_1(t_2 - t_1)} + \frac{N_{O_2}(e^{-k_2 t_1} - e^{-k_2 t_2})}{k_2(t_2 - t_1)},$$

where N_{O_1} and N_{O_2} represent the values of components I and II extrapolated to zero time, and k_1 and k_2 the decay constants for components I and II of the decrease in hippuric acid ^{14}C content.

Fig. 3 shows a comparison between the observed specific activity of the haemoglobin and a curve depicting the specific activity of the haemoglobin synthesized up to 10 days after administration of the labelled glycine. This latter curve was obtained by subtracting from the observed specific activity of the haemoglobin the value calculated, as above, for the specific activity of the haemoglobin present at any time and synthesized more than 10 days after administration of the labelled glycine. This shows that the true mean red-cell life span is probably at least a few days shorter than when calculated from the curve of the observed specific activity, and that to a large degree the tail portion of the curve is due to continual synthesis of labelled haemoglobin. This figure also shows the specific activity of the newly synthesized haemoglobin at any given time as calculated from the observed hippuric acid specific activity. These show that by 250 days the observed haemoglobin specific activity is almost, if not entirely, due to synthesis of haemoglobin from persisting precursors. In this patient (*G*) 96 % of the observed ^{14}C in the haemoglobin can be shown to be due to this process. At 200 days in patient *F* 55 %

Fig. 3. The specific activity of the haemoglobin in patient *G* (Δ), together with the calculated value for specific activity of the haemoglobin after correction for the haemoglobin synthesized after 10 days ($\square$) and the specific activity of the newly synthesized haemoglobin ($\circ$) as calculated from the observed hippuric acid isotope content.

of the observed ^{14}C in the haemoglobin can also be accounted for in this way. This figure also indicates that the prolonged uptake of ^{14}C by haemoglobin is due to synthesis of labelled haemoglobin more than 10 days after administration of the glycine. In patient *W*, who had an aplastic anaemia, an accurate determination of the isotope content of the haemoglobin synthesized is not possible because the circulating haemoglobin contains large amounts of transfused haemoglobin. Even if the isotope content of this patient's own haemoglobin could be determined, the variation in the frequency of transfusions makes it difficult to assume a constant rate of haemoglobin synthesis.

In these calculations it is assumed that the isotope content of the hippuric acid is a precise measure of that of the free glycine available for haemoglobin synthesis. Since the actual figure for the isotope content of the free glycine may be higher than indicated by hippuric acid (Gray & Neuberger, 1950), this correction is a minimum and it is possible that when more accurate data on the free glycine specific activity become available the tail portion of the curve will be shown to be entirely due to synthesis of haemoglobin long after administration of the glycine. Further investigations along this line would necessitate a systematic degradation of haemin to determine the specific activity of the carbon atoms not specifically derived from glycine. It is not likely that this will be significant but it nevertheless should be investigated.

From the present study it appears that there is an appreciable concentration of ^{14}C in the α -carbon atom of glycine up to 260 days after administration. Studies of the ^{15}N content of stercobilin after feeding [^{15}N]glycine show that there is a large incorporation of ^{15}N in the first few days after feeding and that the ^{15}N level in the stercobilin then falls to a low but measurable level to rise again at the time of the breakdown of the red cells formed at the time of the glycine feeding (Gray, Neuberger & Sneath, 1950; London, West, Shemin & Rittenberg, 1950). In the intervening period, i.e. between the two peaks in the ^{15}N content of the stercobilin this small but measurable continuous excretion of [^{15}N]stercobilin could arise from (a) the catabolism of haemoglobin implying a small but definite destruction of red cells containing [^{15}N]haemoglobin or (b) the catabolism of haemoglobin in intact red cells, or (c) the incorporation of the ^{15}N into stercobilin by the same process that gives rise to the first peak in the ^{15}N content of the stercobilin, the ^{15}N being derived from the same pool of free glycine as is available for hippuric acid synthesis. The data from this study lend support to the hypothesis that the incorporation between 20 and 80 days arises at least in part from persisting [^{15}N]glycine, but we cannot exclude either of the other two possibilities.

Because the haemin carbon/haemoglobin carbon specific activity ratio was not measured in these patients and because the hippuric acid ^{14}C content may be lower than the ^{14}C content of the free plasma glycine, only general deductions can be made, and the calculations as made above on the effect of the persistence of labelled glycine on the isotopic content of the haemoglobin are only approximate. This does not, however, negate the general conclusion that there is a continual synthesis of labelled haemoglobin from labelled glycine, long after administration of the labelled glycine and that this continual synthesis can account for a large part and possibly all of the tail portion of the curve depicting the isotope content of haemoglobin.

SUMMARY

1. The specific activity of the glycine available for conjugation with benzoate has been determined in three persons after the administration of labelled glycine.

2. The rate of decrease of the specific activity of the hippuric acid can be described in terms of two exponential components having average half-times of 6.8 and 75 days.

3. The rates for these two components agree well with the rates of the two slowest components of the breath $^{14}\text{CO}_2$ excretion.

4. The observed specific activity of the hippuric acid was almost entirely confined to the α -carbon atom of the glycine moiety except during the period 85–106 days after administration.

5. It is concluded that the tail portion of the curve of the specific activity of haemin or haemoglobin could be due, in large measure if not entirely,

to the continual formation of red cells containing ^{14}C derived from persisting labelled glycine and is probably not due to a population of red cells having life spans far in excess of about 120 days.

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Preparation of ^{32}P -labelled Adenosine 5'-phosphate, Inosine 5'-phosphate and Ribose 5-phosphate

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In studies on the metabolism of adenosine and inosine phosphates it was desirable to use compounds labelled with ^{32}P in the α -phosphate group. Ostern & Terszakowec (1937), Ostern, Baranowski & Terszakowec (1938), Caputto (1951) and Kornberg & Pricer (1951) have synthesized adenosine 5'-phosphate (AMP) by incubating adenosine with adenosine triphosphate (ATP) in a yeast extract

(Lebedev juice), according to the reaction: adenosine + ATP $\rightarrow$ AMP + ADP (adenosine diphosphate). Experiments in this laboratory showed that the labile phosphate of ATP readily exchanges with inorganic [^{32}P]phosphate in Lebedev juice without added substrate. This has been utilized for the preparation of radioactive AMP (AM^{32}P). The resultant AM^{32}P , chromatographically homo-