HEMOGLOBIN SYNTHESIS FROM GLYCINE LABELED WITH RADIOACTIVE CARBON IN ITS \( \alpha \)-CARBON ATOM*

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Important progress in our knowledge concerning the biological precursors of hemin was made when Bloch and Rittenberg (1) presented evidence that significant amounts of deuterium are incorporated into the hemin of rats fed deuterioacetic acid. Further advances in this field were made by Shemin and Rittenberg (2–4), who showed that glycine labeled with \( \text{N}^{15} \) is utilized as a nitrogenous precursor of hemin, whereas other possible amino acids, such as glutamic acid, proline, and leucine, do not act in a similar fashion and contribute only in an indirect way to hemin N by enriching the \( \text{N}^{15} \) concentration of the body. It has been reported recently that hemin synthesis from glycine occurs \textit{in vitro} in the presence of avian blood (5) and with blood from patients with sickle cell anemia (6). It is of considerable interest for an understanding of the mechanism of hemin synthesis to determine whether the \( \alpha \)-carbon atom of glycine is also incorporated in hemin.

Experiments demonstrating the participation of the \( \alpha \)-carbon atom of glycine in the synthesis of hemin are reported in this paper. A preliminary report has been published elsewhere (7).

EXPERIMENTAL

In order to show that the methylene carbon atom of glycine is incorporated in hemin, two groups of animals were studied, one group consisting of four normal adult rats, and one group consisting of three adult rats which had been rendered anemic by previous phenylhydrazine feeding and which were responding to this anemia by increased hemoglobin production, as evidenced by an increased reticulocyte count.

These rats were fed methylene-labeled glycine \((\text{C}^{14}\text{H}_2\text{NH}_2\text{COOH})\)\(^1\) of a specific activity of 1.83 microcuries per mg. in a single dose in aqueous solution by stomach tube. The total dose administered in this way was 2 microcuries, \(i.e\). approximately 1 microcurie per 100 gm. of body weight.

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\(^1\) This preparation of glycine was synthesized by Dr. R. Ostwald and kindly made available to us through the courtesy of Dr. B. M. Tolbert of the Radiation Laboratory of the University of California.
At varying intervals after the glycine feeding, these animals were anesthetized with sodium pentobarbital and bled as completely as possible from the carotid artery by means of the technique of Buchwald and Hudson (8). This technique was modified first by the injection of a solution of sodium heparin into the femoral vein prior to bleeding from the carotid artery, and second by perfusion with physiological saline solution through the inferior vena cava, this perfusion being continued until the animal died. These modifications were adopted in order to secure maximum yields of hemoglobin and in order to free the organs of contained blood.

Hemoglobin was isolated by crystallization according to the method of Warburg and Reid (9). Crystalline hemin was prepared as described by Nencki and Zaleski (10). Globin was isolated according to Anson and Mirsky (11). Such globin preparations were carefully washed with acetone and ether and were found by spectroscopic analysis to contain no bound hemin.

Method of Analysis—A method developed by Bale and Masters was employed for the determination of C\textsuperscript{14} activity. This method involved the conversion of organic material to carbon dioxide by the wet digestion method of Van Slyke and Folch (12). The CO\textsubscript{2} thus produced was then introduced into a 1200 ml. ionization chamber, together with inert CO\textsubscript{2}, to produce a total pressure of 1 atmosphere. The ionization current was read through the use of a ballistic vane electrometer, a version of the dynamic condenser electrometer developed at The University of Rochester. The background ionization current is equivalent to approximately 200 C\textsuperscript{14} disintegrations per minute. Activities of this order are read to ±0.10 per cent accuracy and net activities above twice the background to an accuracy of approximately ±0.3 per cent.

Results

Glycine Feeding of Normal Rats—Data obtained from the feeding of methylene-labeled glycine to normal, untreated rats are presented in Table I, which shows the activities of hemin, globin, and hemoglobin, each of which was analyzed separately for C\textsuperscript{14} activity.

A comparison of the C\textsuperscript{14} content of globin with that of hemin is made for the following reasons: (1) a comparison of the respective C\textsuperscript{14} activity of hemin and globin would reveal whether the incorporation of C\textsuperscript{14} in hemin was due to the specific precursor activity of the α-carbon atom of glycine, or whether C\textsuperscript{14} incorporation in hemin was due to a non-specific enrichment of the C\textsuperscript{14} concentration of the body available for the synthesis of proteins and other large molecular components of the tissues; (2) data available on the C\textsuperscript{14} content of globin would permit the establishment of hemin-globin

\textsuperscript{2} Bale, W. F., and Masters, R. E., unpublished method.
ratios (shown in the last column of Table I). This ratio may be considered an index of the C\textsuperscript{14} partition between hemin and globin and might possibly show measurable variations under pathological conditions, such as anemia, etc.

The C\textsuperscript{14} content of the hemoglobin found checked fairly well with the theoretical value calculated from the C\textsuperscript{14} content of hemin and globin determined independently. In making this calculation a molecular weight of 68,000 was assumed for hemoglobin (13), and it was also assumed that 1 molecule of globin is capable of combining with 4 molecules of hemin (14). These theoretical values for hemoglobin are included in Tables I and II. The exact reason for the discrepancies is not apparent, although the most likely cause for this disagreement is contamination of hemoglobin or globin samples. In both groups of animals, one of the calculated hemoglobin values deviates markedly from the value found, in each case the measured value being lower than the calculated value. It is conceivable that a low activity contaminant, e.g. stroma, precipitated with hemoglobin, thus bringing about the observed disagreement between the calculated and measured values.

In Table I are also shown calculations of the percentage of the total dose of C\textsuperscript{14} incorporated in hemin, globin, and hemoglobin. These percentages were calculated from independent colorimetric hemoglobin determinations\textsuperscript{3} before the animals were sacrificed or on the assumption that the hemoglobin of the rat represents 13.8 per cent of the total rat blood, which in turn

\textsuperscript{3} Acid hematin was determined with the photoelectric colorimeter of Klett and Summerson.

\begin{table}
\centering
\caption{C\textsuperscript{14} Activity of Hemin, Globin, and Hemoglobin in Normal Rats after Feeding C\textsuperscript{14}H\textsubscript{2}NH\textsubscript{2}COOH}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Time after} & \multicolumn{2}{|c|}{\textbf{C\textsuperscript{14} activity of hemin}} & \multicolumn{2}{|c|}{\textbf{C\textsuperscript{14} activity of globin}} & \multicolumn{2}{|c|}{\textbf{C\textsuperscript{14} activity of Hb}} & \textbf{Activity} \\
\textbf{glycine} & \textbf{Units,} 10\textsuperscript{4} & \textbf{Per cent} & \textbf{Units,} 10\textsuperscript{4} & \textbf{Per cent} & \textbf{Units,} 10\textsuperscript{4} & \textbf{Per cent} & \textbf{ratio,} \\
\textbf{feeding} & \textbf{disintegrations} & \textbf{dose in} & \textbf{disintegrations} & \textbf{dose in} & \textbf{disintegrations} & \textbf{dose in} & \textbf{hemin-} \\
& \textbf{per gm.} & \textbf{hemin}\textsuperscript{*} & \textbf{per gm.} & \textbf{globin}\textsuperscript{t} & \textbf{per gm. Hb} & \textbf{Hb}\textsuperscript{*} & \textbf{globin,} \\
\textbf{days} & \textbf{per min.} & \textbf{hemin} & \textbf{per min.} & \textbf{globin} & \textbf{per min.} & \textbf{Hb} & \textbf{gm. basis}\textsuperscript{*} & \textbf{measured} & \textbf{calculated} & \textbf{measured} & \textbf{calculated} & \textbf{measured} & \textbf{calculated} & \textbf{measured} & \textbf{calculated} & \textbf{measured} & \textbf{calculated} \\
\hline
1 & 25.2 & 0.49 & 2.84 & 1.42 & 3.54 & 3.69 & 1.83 & 1.91 & 8.9 \\
6 & 31.0 & 0.66 & 4.61 & 2.57 & 2.93 & 5.65 & 1.69 & 3.23 & 6.7 \\
8 & 18.3 & 0.29 & 2.20 & 0.86 & 2.69 & 2.82 & 1.10 & 1.15 & 8.3 \\
8 & 17.4 & 0.46 & 2.31 & 1.56 & 2.04 & 2.87 & 1.43 & 2.02 & 7.5 \\
\hline
\end{tabular}
\end{table}

\begin{itemize}
\item \textsuperscript{*} On the basis of total circulating blood.
\item \textsuperscript{t} On the basis of hemin and globin measurements.
\end{itemize}
amounts to 7 per cent of the total body weight. The value assumed for hemoglobin is based upon that published by Thewlis and Meyer (15) and checks with a small series of rats used in our laboratory. In general, 0.5 per cent of the total dose of C\(^{14}\) administered was incorporated in hemin.

**Glycine Feeding of Phenylhydrazine-Treated Rats**—Three rats were each given 24 mg. of phenylhydrazine hydrochloride by stomach tube on 2 alternate days, resulting in a depression of the erythrocyte count. These animals were then allowed to enter the recovery period, in the course of which the reticulocyte count reached a level of 15 to 20 per cent. When this level was reached, methylene-labeled glycine was administered.

Since these animals were synthesizing hemoglobin at a more rapid rate than normal rats, it is not surprising to find that the hemoglobin contained considerably higher concentrations of C\(^{14}\). This is shown in Table II. It

**Table II**

<table>
<thead>
<tr>
<th>Time after glycine feeding (days)</th>
<th>C(^{14}) activity of hemin, units, (10^4) disintegrations per min. per gm. hemin</th>
<th>C(^{14}) activity of globin, units, (10^4) disintegrations per min. per gm. globin</th>
<th>C(^{14}) activity of Hb, units, (10^4) disintegrations per min. per gm. Hb</th>
<th>Activity ratio, hemin-globin, gm. basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.2</td>
<td>7.18</td>
<td>3.42</td>
<td>7.3</td>
</tr>
<tr>
<td>4</td>
<td>58.5</td>
<td>8.24</td>
<td>10.9</td>
<td>7.1</td>
</tr>
<tr>
<td>5</td>
<td>160.0</td>
<td>16.6</td>
<td>23.5</td>
<td>9.6</td>
</tr>
</tbody>
</table>

* Calculated on the basis of hemin and globin measurements.

is interesting to note that the ratio of hemin to globin, expressed on a gm. to gm. basis, remains fairly constant and agrees quite well with that of the normal animals. This suggests that the partition of C\(^{14}\) in normal animals and in animals recovering from phenylhydrazine anemia varied within similar, narrow limits and was of the same order of magnitude. It cannot be said with certainty at this time whether the ratio of newly formed hemin to newly formed globin is constant under all conditions. There are indications, however, that under certain nutritional conditions this hemin to globin ratio is altered extensively.

The significance of the hemin-globin ratios cannot be completely assessed at this time. However, the hemin-globin ratio is considered to reflect the rates at which hemin and globin are synthesized and the differences between

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4 For the blood volume, the assumed value is based on that cited by Griffith and Farris (16).

5 Unpublished experiments of the authors.
these respective rates. The rates of incorporation of glycine in hemin and globin could be assessed by comparing the C\textsuperscript{14} activity per glycine residue incorporated in hemin and in globin. Such a comparison reveals that the C\textsuperscript{14} activity per glycine residue is approximately 1\frac{1}{2} times as high for hemin as for globin, suggesting that the rate of hemin synthesis is slightly faster than the rate of globin synthesis.

For the purpose of making an approximate preliminary calculation, it is assumed that 75 per cent of the total C\textsuperscript{14} activity of globin is present as glycine residues,\textsuperscript{6} and that there are 50 glycine residues per mole of globin.\textsuperscript{7} It is furthermore assumed that 4 glycine residues are incorporated in every molecule of newly formed hemin.

**DISCUSSION**

The experiments reported in this paper indicate that the methylene carbon atom of glycine is incorporated into the hemin molecule, and that glycine labeled with C\textsuperscript{14} in its \(\alpha\)-carbon atom acts as a precursor of the tetrapyrrrole structure of hemin. Because of the finding that the amount of C\textsuperscript{14} activity of hemin is significantly greater than that of globin, it may be concluded that the incorporation of glycine into hemin is a process which does not depend upon the enrichment of the C\textsuperscript{14} concentration of the body. Thus, it appears probable that the \(\alpha\)-carbon atom of glycine is incorporated directly into the hemin molecule. The mechanism of this reaction is at present unknown.

Shemin and Rittenberg (4) have pointed out that glycine may participate in hemin synthesis in a manner analogous to a reaction recently described by Fischer and Fink (18) in which a pyrrole-like substance is formed as a result of the condensation of glycine and a \(\beta\)-ketoaldehyde. Such a concept is tenable even in view of the report that the carboxyl carbon atom of glycine is not incorporated in hemin.\textsuperscript{8} If the Fischer and Fink reaction is operative in hemin synthesis, it must be assumed that the carboxyl group is removed at some point during the condensation reaction or after the formation of the pyrrole ring.

It appears more likely that the carboxyl group is removed after the pyrrole ring has been formed, since no enzyme system capable of decarboxylating glycine has so far been discovered, the possible exception being the fermentation of glycine by *Diplococcus glycinophilus* (20, 21). This point

\textsuperscript{6} It is quite conceivable that the entire C\textsuperscript{14} activity of globin does not reside in the glycine residues, but that the C\textsuperscript{14} activity of other amino acids also contributes to the C\textsuperscript{14} activity of globin.

\textsuperscript{7} The glycine content of rat hemoglobin is assumed to be analogous to that of horse hemoglobin as determined by Shemin and Foster (17).

\textsuperscript{8} Radin, N., Rittenberg, D., and Shemin, D., personal communication.
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of view is also supported by the experiment of Lorber and Olsen (22) who reported that no appreciable decarboxylation of glycine, labeled with C\textsubscript{13} in the carboxyl group, takes place in isolated mammalian heart preparations. Furthermore, the hemin of rats to which CaC\textsubscript{14}O\textsubscript{2} has been administered contains significant amounts of C\textsubscript{14} (23). Thus, one would expect that, if carboxyl-labeled glycine were decarboxylated, the CO\textsubscript{2} thus formed could contribute to hemin synthesis, provided that its concentration was high enough.

Indirect evidence that the carbon-nitrogen bond in glycine remains intact is derived from the observation of Shemin and Rittenberg (3) that N\textsuperscript{15}-containing ammonium citrate fed to rats is not incorporated into hemin. These experiments also suggest that the oxidative deamination described by Ratner, Nocito, and Green (24) for a variety of tissues does not play a significant rôle as far as glycine utilization for hemin synthesis is concerned.

Suitable methods for the degradation of the hemin molecule are as yet not available. For this reason it has not been possible to determine the distribution of C\textsubscript{14} in the tetrapyrrole structure of hemin. Attempts to develop such methods are now in progress in this laboratory.

SUMMARY

1. It has been shown that α-carbon-labeled glycine is incorporated into hemin and globin of normal and phenylhydrazine-treated animals, and that the hemoglobin of phenylhydrazine-treated animals contains higher concentrations of C\textsubscript{14} activity than that of normal animals.

2. The data presented suggest that the partition of C\textsubscript{14} activity between hemin and globin is essentially the same in normal animals and in animals recovering from an anemia produced by phenylhydrazine feeding.

The authors wish to express their thanks to Dr. William F. Bale for his continuing interest in this investigation.

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