THE MECHANISM OF PORPHYRIN FORMATION*

THE FORMATION OF A SUCCINYL INTERMEDIATE FROM SUCCINATE

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(Received for publication, May 12, 1952)

In previous publications the location of the carbon atoms of protoporphyrin derived from acetate was determined (3, 4). It was found that 26 carbon atoms of protoporphyrin biologically synthesized from C\textsuperscript{14}-methyl-labeled and C\textsuperscript{14}-carboxyl-labeled acetate contained C\textsuperscript{14} (4). It was concluded from the C\textsuperscript{14} activity pattern in the porphyrin that the acetate was utilized by being converted to a 4-carbon atom unsymmetrical compound via the tricarboxylic acid cycle. It was suggested that this precursor was a succinyl intermediate, possibly a succinyl-coenzyme complex, and an intermediate of the α-ketoglutarate-succinate reaction (4). The existence of an intermediate of this reaction was independently ascertained by Sandri and Littlefield (5, 6) and Kaufman (7). Kaufman found that the oxidation of α-ketoglutarate is coenzyme A-dependent, and the former authors presented evidence for the formation of an “active” succinate from α-ketoglutarate in the presence of coenzyme A, capable of succinylating sulfanilamide.

Since it also appeared from the C\textsuperscript{14} activity pattern in protoporphyrin that this “active” succinate arises not only from α-ketoglutarate but also from succinate (4), the relationship of the citric acid cycle to porphyrin formation can be diagrammatically represented as in Diagram 1. This formulation suggested the existence of a reaction that hitherto had not been described, namely Reaction C of Diagram 1, and indicated that aspects of the tricarboxylic acid cycle could be studied by investigating the mechanism of porphyrin formation. In this communication direct evidence is presented for the above scheme: that a 4-carbon atom compound is the precursor of 26 carbon atoms of protoporphyrin and that this succinyl intermediate can arise from succinate via both Reactions C and F (Diagram 1) in the tricarboxylic acid cycle.

* This work was supported by grants from the National Institutes of Health, United States Public Health Service, from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and from the Rockefeller Foundation. A preliminary report was presented at a Ciba Foundation Conference (1), and the results reported at the meeting of the American Society of Biological Chemists at New York, April 16, 1952 (2).
It may be well to point out that, although succinic acid should give rise to the succinyl intermediate by the two pathways (Diagram 1), the labeling of protoporphyrin from radioactive succinate will depend on the position of C^{14} in the succinate. It was possible to demonstrate both Reactions C and F and to differentiate between them by investigating the synthesis of protoporphyrin from C^{14} carboxyl labeled and C^{14} methylene labeled succinate in the presence and absence of malonate. By determining the C^{14} distribution in protoporphyrin synthesized from C^{14}-carboxyl-labeled succinate with and without malonate more evidence for Reaction C was obtained.

Washed duck red blood cells (8) or hemolyzed duck red blood cells (2, 9) were incubated with equal concentrations of C^{14}-carboxyl-labeled and C^{14}-methylene-labeled succinate in the presence and absence of malonate. Theoretically C^{14}-carboxyl-labeled succinate cannot give rise to C^{14}-labeled protoporphyrin via Reaction F, but only via Reaction C. On entering the tricarboxylic acid cycle, i.e. in the oxidative direction of the cycle (Reaction F, Diagram 1), carboxyl-labeled succinate would give rise to a-carboxyl-labeled α-ketoglutarate. The resulting succinyl intermediate arising by oxidative decarboxylation and utilized for porphyrin formation would therefore contain no C^{14}. However, if Reaction C occurs, carboxyl-labeled succinate would produce labeled protoporphyrin. If this postulation is correct, then carboxyl-labeled succinate should produce labeled protoporphyrin only by Reaction C, and therefore malonate, which blocks Reaction F, should have little or no influence on the C^{14} activity of protoporphyrin.

Methylene-labeled succinate, in contrast to carboxyl-labeled succinate, should produce labeled protoporphyrin via two pathways: (1) Reaction C and (2) the oxidative direction of the tricarboxylic acid cycle (Reaction F). Methylene-labeled succinate should produce α-ketoglutaric acid labeled in its α- and β-carbon atoms by Reaction F. As a result the succinyl intermediate formed by this pathway, and utilized for porphyrin formation,
should have 2 of its carbon atoms labeled with C$^{14}$. Malonate should, in this case, have a marked inhibitory effect on the utilization of methylene-labeled succinate for porphyrin formation, and any C$^{14}$ activity in protoporphyrin made from methylene-labeled succinate in the presence of malonate will be the result of Reaction C.

**Table I**

**Comparison of C$^{14}$ Activities of Hemin Made from C$^{14}$-Carboxyl-Labeled Succinic Acid and from C$^{14}$-Methylene-Labeled Succinic Acid in Presence and Absence of Malonate**

<table>
<thead>
<tr>
<th>Experiment No.*</th>
<th>Hemin synthesized from C$^{14}$-carboxyl-labeled succinic acid</th>
<th>Hemin synthesized from C$^{14}$-methylene-labeled succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without malonate</td>
<td>With malonate, 0.02 M</td>
</tr>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>1</td>
<td>353</td>
<td>307</td>
</tr>
<tr>
<td>2</td>
<td>247</td>
<td>208</td>
</tr>
<tr>
<td>3</td>
<td>128</td>
<td>211</td>
</tr>
<tr>
<td>4</td>
<td>132</td>
<td>114</td>
</tr>
<tr>
<td>5</td>
<td>540</td>
<td>697</td>
</tr>
<tr>
<td>6</td>
<td>139</td>
<td>105</td>
</tr>
<tr>
<td>7‡</td>
<td>94§</td>
<td>104</td>
</tr>
</tbody>
</table>

* Experiments 1 to 5 were carried out with intact red cells; Experiments 6 and 7, with hemolyzed preparations.
† The observed activities were multiplied by factors, since the activities of the carboxyl-labeled succinate samples were different from those of methylene-labeled succinate samples; see the experimental section.
‡ The malonate concentration of this experiment was 0.01 M.
§ The C$^{14}$ activity of hemin made from carboxyl-labeled succinate with intact red cells of this preparation was 120 c.p.m.

In Table I the C$^{14}$ activity of the hemin samples obtained by incubating equal amounts of C$^{14}$-carboxyl-labeled and C$^{14}$-methylene-labeled succinate, with and without malonate, is given. It can be seen from Table I that the C$^{14}$ activity of the hemin samples made from methylene-labeled succinate is much higher than that made from carboxyl-labeled succinate. Whereas malonate had a large inhibitory effect (compare Columns c and e, Table I) on the utilization of methylene-labeled succinate, it had either only a very slight inhibitory effect or even an enhancing effect on the uti-
The enhanced utilization of carboxyl-labeled succinate in the presence of malonate may be due to the greater availability of the succinate for Reaction C, since the succinate was not drawn off by oxidation. These results are in complete agreement with the postulation that carboxyl-labeled succinate produces labeled protoporphyrin by Reaction C only and that methylene-labeled succinate forms labeled protoporphyrin by Reactions C and F.

Support of the labeling of protoporphyrin from methylene-labeled succinate by the two pathways can be gathered by comparing the C14 activity values of Columns a and d of Table I. If the methylene-labeled succinate were only utilized via Reaction C, the C14 activity of the hemin samples would be only 16/10 as high as that found for carboxyl-labeled succinate, since methylene-labeled succinate would label equally 16 carbon atoms of the porphyrin, while carboxyl-labeled succinate would label 10 carbon atoms (see Fig. 1). The C14 activities of the hemin samples made from methylene-labeled succinate (Column c, Table I) were multiplied by 10/16 and are given in Column d. It can be seen that the activities calculated in Column d are still much higher than those of Column a, demonstrating the labeling of the porphyrin from methylene-labeled succinate by the two pathways.

However, 10/16 of the C14 activities of the hemin samples made from methylene-labeled succinate with malonate (Column f) are quite similar to those made from carboxyl-labeled succinate (Column b). These results demonstrate the utilization of the methylene-labeled succinate under these experimental conditions through Reaction C. The slightly higher values in Column f in Experiments 1 to 4 with whole cells are, in all likelihood, due to the lack of complete inhibition of the oxidation of succinate in the presence of malonate. On the other hand, in the experiments in which hemolyzed preparations were used (Experiments 6 and 7, Table I) the malonate inhibition of Reaction F was more complete; the C14 activities of Columns b and f are equal.

Further evidence for the existence of Reaction C and proof for the utilization of a 4-carbon atom compound for porphyrin formation were obtained by investigating the C14 distribution in protoporphyrin synthesized from carboxyl-labeled succinate. If Reaction C occurs and if the succinate is utilized as a unit, carboxyl-labeled succinate would not only give rise to labeled protoporphyrin, but the resulting porphyrin should have the C14 activity pattern shown in Fig. 1. This predicted C14 activity pattern is based on our previous evidence that each side of all the pyrrole units is made from the same 4-carbon atom compound (4). If the succinate were converted into the "active" derivative and 2 moles of the latter compound condensed with glycine, the common precursor pyrrole formed would con-
tain C\textsuperscript{14} in positions shown in Fig. 1. On utilizing 4 moles of this pyrrole to form protoporphyrin 4 radioactive carbon atoms from position 7 and 2 from position 10 would be lost and the protoporphyrin would contain 10 carbon atoms equally labeled in positions shown in Fig. 1: carbon atoms 3 and 5 of pyrrole rings A and B and carbon atoms 3, 5, and 10 of pyrrole rings C and D. Therefore, 40 per cent of the C\textsuperscript{14} activity should reside in rings A and B and 60 per cent should be found in rings C and D. Further, if the succinate were utilized as a unit, the carboxyl groups of the protoporphyrin should contain 20 per cent of the C\textsuperscript{14} activity of the porphyrin and its activity should be one-half and one-third of rings A and B and of rings C and D respectively.

The labeled hemin obtained from carboxyl-labeled succinate was degraded in order to determine the C\textsuperscript{14} distribution pattern. Methylethylmaleimide, representing pyrrole rings A and B, and hematinic acid,
representing rings C and D, were isolated. The hematinic acid was decarboxylated to yield methylethylmaleimide and CO$_2$, representing the carboxyl groups of the protoporphyrin. This latter sample of methylethylmaleimide from rings C and D represents comparable carbon atoms to those found in rings A and B (Fig. 2). It can be seen in Experiments 1 and 2 of Table II that the activities found in these fractions agree, within experimental limits, with the theoretical values calculated for the formation of protoporphyrin from carboxyl-labeled succinate through Reaction C (Diagram 1), as outlined in Fig. 1; rings A and B and rings C and D contained respectively 40 and 60 per cent of the activity. The finding that the carboxyl groups contained respectively one-half and one-third of the activities of those of rings A and B and of rings C and D is further evidence for the utilization of all 4 carbon atoms as a unit. It should be noted that the succinyl intermediate utilized for porphyrin formation will be unsymmetrically labeled if it arises from α-ketoglutarate, as previously demonstrated (4), and symmetrically labeled when formed directly from succinate (Reaction C).

In support of the above evidence, hemin was synthesized from carboxyl-labeled succinate in the presence of malonate. The labeled hemin was degraded and it was found ( Experiment 3, Table II) that the C$^{14}$ distribution

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1 In unpublished experiments of J. C. Wriston, Jr., and D. Shemin, it was found that the C$^{14}$ activity pattern among the carbon atoms of the protoporphyrin biosynthesized from C$^{14}$-γ-carboxyl-labeled α-ketoglutaric acid was in agreement with the above results.
tion pattern in the protoporphyrin made from carboxyl-labeled succinate in the presence of malonate was the same as that made in the absence of malonate.

The above results furnish more evidence that a succinyl intermediate is utilized, as previously postulated, for 26 of the carbon atoms of protoporphyrin. The mechanism of formation of the "active" succinate for porphyrin formation appears to be similar to the formation of "active" acetate or acetyl coenzyme A from pyruvate and acetate (10, 11), since this succinyl intermediate is formed from both α-ketoglutarate and succinate.

### Table II

**Distribution of C14 Activity in Protoporphyrin Made from C14-Carboxyl-Labeled Succinic Acid**

<table>
<thead>
<tr>
<th>Compound analyzed for C14 activity</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3 with malonate (0.02 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found* c.p.m.</td>
<td>Theory † c.p.m.</td>
<td>Found* c.p.m.</td>
</tr>
<tr>
<td>Hemin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole porphyrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylethylmaleimide</td>
<td>3520</td>
<td>3600</td>
<td>3820</td>
</tr>
<tr>
<td>Pyrrole rings A and B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematinic acid</td>
<td>5250</td>
<td>5400</td>
<td>5880</td>
</tr>
<tr>
<td>Pyrrole rings C and D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylethylmaleimide</td>
<td>3670</td>
<td>3600</td>
<td>3920</td>
</tr>
<tr>
<td>Pyrrole rings C and D minus carboxy groups</td>
<td>1900</td>
<td>1800</td>
<td>1930</td>
</tr>
<tr>
<td>Barium carbonate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxyl groups</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The observed activities are given in the experimental section; the values reported in this table are the calculated molar activities.
† Based on the activity of porphyrin.
‡ Determined for mesoporphyrin.

Whether the succinyl intermediate utilized for porphyrin formation is succinyl coenzyme A is in the process of being determined. Although Sanadi and Littlefield (5), in their enzyme system, were unable to demonstrate the formation of succinylsulfanilamide from succinate, the experiments of Kaufman (7), however, indicate the formation of succinyl coenzyme A from succinate.

While the demonstration of Reaction C has been interpreted above as the formation of a succinyl intermediate, it may also be interpreted as a reversal of the ketoglutarate-succinate reaction. If α-ketoglutarate were

The addition of a boiled liver extract or Armour's coenzyme concentrate to a hemolyzed preparation increased 2- to 3-fold the utilization of succinate for porphyrin formation (Shemin, D., and Kumin, S., unpublished experiments).
formed from carboxyl-labeled succinate by Reaction C and subsequent carboxylation and if the ketoglutarate were directly utilized for porphyrin formation with the concomitant loss of its α-carboxyl group, the same results would have been obtained. However, the ketoglutarate-succinate reaction has not as yet been shown to be reversible, and thus far in duck red blood cells, which can utilize succinate for porphyrin formation, it has as yet not been possible to demonstrate the reversibility of this reaction. In view of this and the experiments of Kaufman (7) and Sanadi and Littlefield (5, 6), it is most reasonable to interpret Reaction C (Diagram 1) as the formation of a succinyl intermediate.

**EXPERIMENTAL**

*Measurement of Radioactivity*—All the organic and barium carbonate samples were assayed for their radioactivity with an end-window counter as "infinitely thick" samples and the results are reported as counts per minute above background and as molar activity. The term molar activity is introduced in order to be able to relate the C\(^{14}\) activity of parts of a molecule to the C\(^{14}\) activity of the molecule from which it is derived. Molar activity for the compound analyzed is defined as follows:

\[
\text{Molar activity} = \frac{\text{activity (found)}}{\% \text{ C in compound}} \times \frac{\text{No. of carbon atoms in compound}}{\text{No. of moles of compound in parent molecule}} \times \frac{100}{12 \text{ gm.}} \times \frac{\text{No. of moles of compound in parent molecule}}{\text{in parent molecule}}
\]

*C\(^{14}\)-Carboxyl- and C\(^{14}\)-Methylene-Labeled Succinate*—The carboxyl-labeled succinate was obtained from the Oak Ridge National Laboratory and the methylene-labeled succinate from Tracerlab. The succinic acid samples were neutralized with sodium hydroxide before use and diluted in the experiments with non-radioactive succinate. The activities of the samples used are given in each of the experiments. Succinate having an activity of 0.1 mc. per mm counted, under our conditions, at the rate of 1 \(\times\) \(10^6\) c.p.m.

*Preparation of Duck Red Blood Cells*—Ducks were exsanguinated and the heparinized blood centrifuged and the plasma removed. The red blood cells were washed five times with isotonic saline and finally suspended in a volume of saline approximately equal to the original volume of plasma. Each incubation sample represented 25 to 30 ml. of the original blood volume. In experiments in which many blood samples were needed the washed red blood cells of several ducks were pooled and then divided (8).

*Lyzed Preparation of Duck Red Blood Cells*—The washed duck red blood

\(^a\) Wriston, J. C., Jr., and Shemin, D., unpublished experiments.
cells were hemolyed by addition of 1.5 volumes of water. After 1 hour in the refrigerator, solid potassium chloride was added in order to make the mixture isotonic and magnesium chloride in an amount so that its final concentration was 0.002 M. This suspension, quite capable of synthesizing protoporphyrin, was then utilized (2, 9). In many experiments the C\textsuperscript{14} activities of the hemin samples made from succinate in intact and hemolyzed preparations were quite similar (e.g. Table I, Experiment 7).

Comparison of Utilization of C\textsuperscript{14}-Carboxyl-Labeled and C\textsuperscript{14}-Methylene-Labeled Succinate—In the experiments of Table I samples of pooled washed red blood cells of the duck and hemolyzed preparations were incubated with equal concentrations of both labeled types of succinate (1 mg. per ml.). Each flask also contained 1 to 2 mg. per ml. of glycine. After shaking at room temperature for 20 hours, the hemin was isolated and purified by recrystallization, and its activity determined. In samples in which malonate was used its concentration was 0.02 M.

The radioactivity of the carboxyl-labeled succinate used in Experiments 1 to 7 of Table I was 0.060, 0.053, 0.055, 0.046, 0.14, 0.060, and 0.060 mc. per mm, respectively, while that of the methylene-labeled succinate in Experiments 1 to 4 and 6 and 7 was 0.02, 0.02, 0.05, 0.05, 0.05, and 0.05 mc. per mm, respectively. In order to compare the C\textsuperscript{14} values, the observed activities of the hemin made from methylene-labeled succinate were multiplied by 3, 2.65, 1.1, 0.92, 1.2, and 1.2 in Experiments 1 to 4 and 6 and 7 of Table I, respectively.

Preparation of Labeled Hemin from C\textsuperscript{14}-Carboxyl-Labeled Succinate—In the experiments of Table II, pooled washed red blood cells were shaken for 24 hours at room temperature with carboxyl-labeled succinate (activity 0.1 to 0.14 mc. per mm) and glycine. The concentration of succinate was 0.5 mg. per ml. and that of the glycine 1.5 mg. per ml. In Experiment 3 of Table II, the incubation mixture contained 0.02 M malonate. Sufficient blood was utilized so that about 1 gm. of labeled hemin could be isolated in the experiments. For degradation purposes the labeled hemin was diluted 3-fold with non-radioactive hemin.

The observed activities of the porphyrin samples of Experiments 1, 2, and 3 of Table II were 191, 178, and 183 c.p.m. above background, respectively. In Experiment 1 mesoporphyrin was counted, while the activities of the hemin samples were determined in Experiments 2 and 3.

**Degradation of Labeled Hemin**

The methods of degradation were similar to those previously published (4, 12).

**Preparation of Mesoporphyrin**—The hemin was converted to mesoporphyrin by methods previously published (12–14). However, in these ex-
periments the protoporphyrin in the formic acid was not isolated but directly reduced to mesoporphyrin after removal of the iron by filtration.

**Oxidation of Mesoporphyrin to Methylethylmaleimide and Hematinic Acid** —Mesoporphyrin was oxidized to methylethylmaleimide and hematinic acid as previously described (12, 15, 16). The hematinic acid isolated was further purified by sublimation and recrystallization from water. The melting points of the samples of methylethylmaleimide and hematinic acid were 65–66° and 114–115°, respectively.

The observed activities of the methylethylmaleimide samples (rings A and B) of Experiments 1, 2, and 3 of Table II were 152, 165, and 160 c.p.m. above background, respectively, while those of the hematinic acid samples were 172, 193, and 204 c.p.m. above background, respectively.

**Conversion of Hematinic Acid to CO₂ and Methylethylmaleimide** —The hematinic acid was decarboxylated by heating the material at 300° for 30 minutes under a stream of nitrogen. This method is similar to that of Küster (17) who reported, however, that the decarboxylation occurred at 187–191°. We have not observed any significant evolution of carbon dioxide before 290–300°. The carbon dioxide was collected in barium hydroxide; yield 90 to 95 per cent. The black tarry residue was extracted with chloroform and the solution taken to dryness after filtration. The residue was sublimed and the methylethylmaleimide purified by several subsequent sublimations and recrystallizations from 0.2 per cent ammoniacal water. The yield of methylethylmaleimide was 40 to 50 per cent, m.p. 65–66°.

The observed activities of these methylethylmaleimide samples (rings C and D) in Experiments 1, 2, and 3 of Table II were 158, 169, and 175 c.p.m., above background, respectively. The observed activities of the barium carbonate samples (corrected for back-scattering) representing the carboxyl groups of the porphyrin, in Experiments 1, 2, and 3, Table II, were 58, 59, and 62 c.p.m., respectively.

**SUMMARY**

An investigation of the mechanism of protoporphyrin synthesis was carried out with both intact and hemolyzed duck erythrocytes. The C¹⁴ distribution pattern in protoporphyrin, synthesized from carboxyl-labeled succinate, demonstrated the utilization of succinate, as a unit, for 26 of the carbon atoms of protoporphyrin. The C¹⁴ activities of hemin samples synthesized from C¹⁴-carboxyl-labeled and C¹⁴-methylene-labeled succinate, in the presence and absence of malonate, were compared. It was found that, although both types of labeled succinate produced labeled hemin, malonate inhibited only the labeling of the hemin synthesized from methylene-labeled succinate. It is concluded from the data that a succinyl intermedi-
ate or an "active" succinate which condenses with glycine to form each 
pyrrole unit of the porphyrin is a product of the tricarboxylic acid cycle 
and arises from both succinate and α-ketoglutarate.

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