The Enzymatic Synthesis of Uroporphyrins from Porphobilinogen

IV. INVESTIGATIONS ON THE PARTICIPATION OF FORMALDEHYDE*

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It has been reported (1) and confirmed (2) that the production of uroporphyrin III from porphobilinogen, either by heating in acid solution or enzymatically by hemolysed avian erythrocytes, is accompanied by the formation of formaldehyde from the aminomethyl group of the pyrrole. These observations have been cited as evidence in support of two hypotheses on the mechanism of uroporphyrin III formation (1, 2). The hypothesis of Shemin et al. (1) requires the accumulation of 1 mole of formaldehyde per mole of uroporphyrin III formed. The availability of purified enzyme preparations which catalyze the formation of uroporphyrinogen I or III from porphobilinogen (3, 4) has permitted a quantitative investigation of formaldehyde accumulation during the enzymatic synthesis of these porphyrinogens; under these conditions interpretation of the results is least complicated by possible side reactions. Furthermore, a comparison of formaldehyde accumulation during the enzymatic formation of the I and III isomers of uroporphyrin should provide evidence regarding the involvement of formaldehyde specifically in synthesis of the III isomer.

In addition, it has been reported (3) that formaldehyde accelerates the nonenzymatic synthesis of porphyrins from porphobilinogen although it appears to inhibit the enzymatic reactions. Some of the present investigations were designed to determine whether formaldehyde can be a reactant in the formation of porphyrins from porphobilinogen. This information is also pertinent to an evaluation of a proposal by Treibs and Ott (5) and the hypothesis of Cookson and Rimington (6); both of these suggestions require the liberation of formaldehyde from the aminomethyl group of porphyrinogen and the subsequent utilization of this formaldehyde for the formation of uroporphyrinogen III.

The data presented here indicate that formaldehyde is neither a stoichiometric by-product of the enzymatic formation of uroporphyrinogen III from porphobilinogen nor a reactant in this process.

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METHODS

The procedures used for the estimation of porphyrins and PBG* have been described (3). Labeled PBG was prepared from 5-aminolevulinic acid-5-C14 by incubation with a purified fraction from chicken blood essentially according to the method of Schmid and Shemin (7).

Radioactivity incorporated into uroporphyrin was determined by counting the uroporphyrin octamethyl esters at infinite thinness in a gas flow counter. Porphyrins were recovered from reaction mixtures and converted to the methyl esters as described previously (3).

Some of the experiments described here were performed with the use of wheat germ uroporphyrinogen III cosynthetase (uroporphyrinogen isomerase) Fraction C (4) or PBG deaminase Fraction H 30-50, or both (3); in other experiments more highly purified preparations of these enzymes were used.

An example of the procedure, carried out at 0–4°, for obtaining PBG deaminase Fraction L from Fraction H 30-50 follows: 7 ml containing 3.96 g of Fraction C protein in 73 ml of 0.2 M phosphate buffer was mixed with 22 g of charcoal (the charcoal is weighed dry and then moistened with water and centrifuged before use). After 30 minutes the suspension was centrifuged and the supernatant fluid recovered. Ninety milligrams of uroporphyrinogen III cosynthetase Fraction C-2 from wheat germ was added. The following is an example of the procedure, also carried out at 0–4°, for the preparation of Fraction C-2 from wheat germ uroporphyrinogen III cosynthetase Fraction C: A solution containing 3.96 g of Fraction C protein in 73 ml of 0.2 M phosphate buffer was mixed with 22 g of charcoal. After 30 minutes the suspension was centrifuged and the supernatant fluid obtained after centrifuging this suspension was dialyzed against distilled water for 5 hours and stored at -20° until used. The best preparations of this fraction, Fraction L, catalyzed the consumption of 1.73 μmoles of PBG per ml per hour per mg of protein.

The following is an example of the procedure, also carried out at 0–4°, for the preparation of Fraction C-2 from wheat germ uroporphyrinogen III cosynthetase Fraction C: A solution containing 3.96 g of Fraction C protein in 73 ml of 0.2 M phosphate buffer was mixed with 22 g of charcoal. After 30 minutes the suspension was centrifuged and the supernatant

1 The abbreviations used are: PBG, porphobilinogen; EDTA, ethylenediaminetetraacetic acid.

2 Darco G-60, Mathison, Coleman, and Bell, Inc., East Rutherford, New Jersey.
Enzymatic Synthesis of Uroporphyrins from Porphobilinogen. IV

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Incubation period</th>
<th>Carrier CH$_2$O added</th>
<th>Recovered methylene bis-methone</th>
<th>PBG-C$^4$ giving rise to CH$_2$O, calculated</th>
<th>Uroporphyrin isomer produced$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m./m mole</td>
<td>hrs</td>
<td>$\mu$moles</td>
<td>c.p.m./m mole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3$^b$</td>
<td>147.3</td>
<td>7</td>
<td>5.0</td>
<td>1.247</td>
<td>1.84%</td>
<td>I</td>
</tr>
<tr>
<td>11$^c$</td>
<td>126.8</td>
<td>2</td>
<td>3.5</td>
<td>0.881</td>
<td>1.50%</td>
<td>I</td>
</tr>
<tr>
<td>4$^d$</td>
<td>147.3</td>
<td>7</td>
<td>5.0</td>
<td>2.630</td>
<td>4.00%</td>
<td>III</td>
</tr>
<tr>
<td>10$^e$</td>
<td>128.8</td>
<td>2</td>
<td>3.5</td>
<td>0.655</td>
<td>0.75%</td>
<td>III</td>
</tr>
</tbody>
</table>

$^a$ Based on paper chromatography of the octamethyl esters (9).
$^b$ The reaction mixture contained in 10 ml: PBG deaminase (2.41 mg of protein, Fraction L); 1 mmole of Tris buffer, pH 8.2; 0.035 mmole of EDTA; and 3.7 $\mu$moles of PBG-C$^4$. Incubated anaerobically at 37°.

* The reaction mixture contained in 7 ml: PBG deaminase (4.05 mg of protein, Fraction L); 0.53 mmole of Tris buffer, pH 8.2; 0.035 mmole of EDTA; and 3.7 $\mu$moles of PBG-C$^4$. Incubated anaerobically at 37°.

* The reaction mixture and incubation conditions were the same as those in Experiment 3 except that uroporphyrinogen III cosynthetase (84.4 mg of protein, Fraction C-2) was included.

* The reaction mixture and incubation conditions were the same as those in Experiment 1 except that PBG deaminase; PBG deaminase preparation or with both this enzyme and uroporphyrinogen III cosynthetase; PBG was not included in these reaction mixtures. Recovery of formaldehyde was 920/, in the first case and 89% in the second case.

* The hypothesis of Shemin et al. (1) for the biosynthesis of uroporphyrinogen III requires that 20% of the PBG used in the reaction should give rise to formaldehyde. In our experiments, the formaldehyde produced from PBG in the above experiments arose non-enzymatically is suggested by the following experiments.

**TABLE II**

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>PBG</th>
<th>CH$_2$O</th>
<th>Uroporphyrin produced</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmole/ml</td>
<td>mmole/ml</td>
<td>c.p.m./m mole</td>
<td>min</td>
</tr>
<tr>
<td>Nonenzymatic: Incubated at 90°: 0.1 M, pH 8.2 Tris buffer</td>
<td>1.</td>
<td>0.435</td>
<td>1.04</td>
<td>204 256 10</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td>0.435</td>
<td>0.65</td>
<td>204 160 10</td>
</tr>
<tr>
<td>Enzymatic Enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. PBG deaminase</td>
<td>0.501</td>
<td>0.975</td>
<td>979</td>
<td>3 120</td>
</tr>
<tr>
<td>4. PBG deaminase</td>
<td>0.505</td>
<td>0.350</td>
<td>979</td>
<td>0 120</td>
</tr>
<tr>
<td>5. PBG deaminase</td>
<td>0.495</td>
<td>0.975</td>
<td>979</td>
<td>1 120</td>
</tr>
<tr>
<td>U III-co$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. PBG deaminase</td>
<td>0.430</td>
<td>1.00</td>
<td>979</td>
<td>8 150</td>
</tr>
<tr>
<td>U III-co$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. PBG deaminase</td>
<td>0.425</td>
<td>1.00</td>
<td>204</td>
<td>11 150</td>
</tr>
</tbody>
</table>

$^a$ Reaction mixture: PBG deaminase (2.1 mg of protein, Fraction H 30-50); 0.41 mmole of Tris, pH 8.2; 0.02 mmole of EDTA; 0.24 mmole of cysteine; C$^4$-formaldehyde; and PBG. Total volume 6 ml. Incubated anaerobically at 37°.

$^b$ Reaction mixture: As in 3 and 4 but contained, in addition, uroporphyrinogen III cosynthetase (U III-co$^c$) (45 mg of protein, Fraction C-2).

$^c$ Reaction mixture: As in 3 and 4 but contained, in addition, uroporphyrinogen III cosynthetase (U III-co$^c$) (45 mg of protein, Fraction C-2).

$^d$ Reaction mixture: As in 6 but tetrahydrofolic acid omitted. The amount predicted to be recovered from 20% to 18%; much less than this was found (Table I). The possibility that the formaldehyde produced from PBG in the above experiments arose non-enzymatically is suggested by the following experiments.
128,800 c.p.m. per μmole, it appears that the formaldehyde arose from the aminomethyl group of 8% of the PBG used.

In our hands the yield of porphyrin in experiments of this kind is approximately 30 to 37%.

Studies on Incorporation of Formaldehyde into Uroporphyrins—The incorporation of C\textsuperscript{14} from C\textsuperscript{14}-formaldehyde into uroporphyrin during its enzymatic and nonenzymatic formation from PBG was investigated. Formaldehyde has been shown to inhibit the enzymatic synthesis of uroporphyrin from PBG and to accelerate the nonenzymatic synthesis (3).

Data on the incorporation of C\textsuperscript{14} from C\textsuperscript{14}-formaldehyde into uroporphyrin formed upon heating PBG solutions at 80°C are presented in Table II; these findings are in general agreement with the earlier report of Mauzerall and Granick (10). The maximum yield of porphyrin obtained in nonenzymatic experiments of this type was approximately 14%. In contrast to the marked incorporation of formaldehyde carbon during nonenzymatic synthesis of uroporphyrin III, insignificant amounts, probably attributable to nonenzymatic synthesis alone, were detected in uroporphyrins formed enzymatically. Examples of results obtained in some of these experiments are also given in Table II. The addition of up to 200 μg per ml of tetrahydrofolic acid, prepared from folic acid by the method of Rabinowitz and Pricer (11), to enzymatic reaction mixtures had no effect on the incorporation of formaldehyde. Also, tetrahydrofolic acid, with or without added formaldehyde, could not substitute for uroporphyrinogen III cosynthetase preparation in catalyzing the formation of uroporphyrinogen III from PBG in the presence of PBG deaminase.

These data indicate that (a) during the enzymatic synthesis of either uroporphyrinogen I or III, carbon at the level of formaldehyde is not formed from the aminomethyl group of PBG, liberated into the reaction mixture, and then taken up and incorporated into porphyrin, and (b) that essentially no exchange of carbon occurs between formaldehyde in the medium and any intermediates.

Numerous hypotheses have been proposed regarding the mechanism of the enzymatic formation of uroporphyrin III from PBG (e.g. 1, 6, 12–15). At present the only hypotheses which are still not wholly excluded are those which have not yet been tested experimentally. The data in the present paper appear to exclude formaldehyde as a product or reactant in the enzymatic synthesis; however, its participation in catalytic amounts is not excluded.

**SUMMARY**

1. The production of formaldehyde from the aminomethyl group of porphobilinogen during the nonenzymatic synthesis of uroporphyrins has been confirmed.

2. Formaldehyde carbon is readily incorporated into uroporphyrin during the nonenzymatic synthesis from porphobilinogen, as has been reported earlier, but it is not incorporated during the enzymatic synthesis from the pyrrole.

3. With the use of partially purified enzyme preparations, it has been found that only small amounts of formaldehyde are produced from the aminomethyl group of porphobilinogen during the synthesis of either uroporphyrinogen I or III; these amounts might be accounted for by nonenzymatic reactions.

**Acknowledgment**—We are indebted to Mr. Charles Kung for the conscientious application of his technical skill in many of these experiments.

**REFERENCES**

The Enzymatic Synthesis of Uroporphyrins from Porphobilinogen: IV.
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