The partial purification of porphobilinogen deaminase from spinach leaf tissue and some details of the action of this enzyme in the synthesis of uroporphyrinogen I, have been reported in earlier communications (2-5). Uroporphyrin I has been found in nature in the excreta of humans and other animals under certain pathological conditions (6, 7).

The present report describes the preparation, from wheat germ, of an enzyme, designated here as uroporphyrinogen isomerase, which catalyzes certain steps in the enzymatic synthesis of uroporphyrinogen III or a mixture of the I and III isomers of this porphyrin. The following procedure leads to the preparation, from wheat germ, of fractions which differ from one another with respect to types of enzymatic activities in the biosynthesis of porphyrins from PBG. All preparative operations were conducted at about 4°C.

Wheat germ (250 gm.) is extracted with 1 liter of cold distilled water for 30 minutes with continuous mechanical stirring. This is followed by centrifugation of the slurry at about 15,000 \( \times \) g for 20 minutes; the sediment is discarded, and the supernatant fluid is collected after filtration through muslin to remove most of the fatty layer which separates out on centrifugation. Glacial acetic acid is then added, drop by drop, to adjust to pH 5. A Beckman model G pH meter is used to monitor the pH, and the fluid is stirred mechanically during the addition of the acetic acid. After 30 minutes in the cold, the material is centrifuged for 20 minutes at about 15,000 \( \times \) g and again the sediment is discarded and the supernatant fluid collected.

Next, solid ammonium sulfate (21.18 gm. per 100 ml. of fluid) is added to the supernatant fluid slowly while it is stirred mechanically. After 30 minutes, the suspension is centrifuged, and supernatant fluid is collected. The sediment (Fraction A) is discarded after one washing with ammonium sulfate solution (21.18 gm. per 100 ml.).

To the combined supernatant fluid and wash of Fraction A is added solid ammonium sulfate (7.06 gm. per 100 ml. of fluid), and the procedure described above is repeated with the exception that an ammonium sulfate solution containing 28.24 gm. of ammonium sulfate per 100 ml. is used for washing. The supernatant fluid and wash of this fraction (B) are combined and washed with a solution of ammonium sulfate (35.3 gm. per 100 ml.). The supernatant fluid and wash of Fraction C are discarded.

Each precipitate is then suspended in a minimal amount of cold distilled water and dialyzed against 200 volumes of distilled water at 0°C to 5°C for 4 hours. The dialyzed material is then frozen and stored at \(-20^\circ\).
The activity of the uroporphyrinogen isomerase is best conserved by carrying out the preparation without interruption. The interposition of a nucleoprotein-precipitating step (by the addition of protamine or MnCl₂) between the isoelectric precipitation and the ammonium sulfate fractionation, provided no benefit with respect to ease of subsequent manipulations or sharpness of separation of the desired components.

Fraction B—Fraction B catalyzes the consumption of PBG and the appearance of uroporphyrins; however, different preparations of this fraction vary with respect to the proportions of the I and III isomer which appear during incubation with the substrate. Fig. 1 illustrates tracings of chromatograms (11) of methyl esters of uroporphyrins produced from PBG through the catalysis of this fraction.

No measurable alteration in enzymatic activity of preparations of Fraction B has been observed even after storage for 12 months at -20°, but the capacity of similar preparations to catalyze the synthesis of uroporphyrin III appears to be completely lost if they are heated at 55° for 15 minutes or aged in the refrigerator (about 4°) for 2 days. The ability of heated or aged preparations to catalyze the consumption of PBG appears to be unaltered by either of these treatments; however, only uroporphyrin I is formed. Similarly, frozen and thawed preparations of Chlorella which have been heated at 55°, catalyze the synthesis of only uroporphyrin I from PBG (12). These observations suggest that at least two enzymes are required for the biosynthesis of uroporphyrinogen III from PBG.

Fraction C—The PBG-consuming activity of preparations of Fraction C made to date ranged downward from 0.01 µmole of PBG consumed per ml. per hour per mg. of protein, to a level at which no activity was measurable. Uroporphyrin III is produced by preparations which are capable of catalyzing the consumption of PBG.

Experiments in which PBG, PBG-D prepared from spinach leaf tissue (5), and Fraction C from wheat germ are incubated together, reveal that wheat germ Fraction C contains an enzyme (uroporphyrinogen isomerase) which catalyzes some step(s) in the synthesis of uroporphyrinogen III. In these experiments, PBG is consumed at a rate commensurate with the concentration of the deaminase; however, unlike experiments in which Fraction C is omitted, the bulk of the porphyrin recovered at the termination of the incubation period is uroporphyrin III rather than the I isomer. Traces of the latter isomer are sometimes also present. Below certain limits, the proportions of the two isomers produced is a function of the relative concentrations of PBG-D and uroporphyrinogen isomerase in the reaction mixtures (Fig. 2). These estimates are based on the relative fluorescence intensity and the area of the spots in the Falk and Benson method (11) of paper chromatography.

With most preparations of Fraction C, under standard assay conditions (0.2 to 0.4 µmole of PBG per ml.), it has not been possible to detect a difference in the rate of PBG consumption (a) in the presence of PBG-D alone, as compared with (b) the rate in the presence of PBG-D plus uroporphyrinogen isomerase at the minimum concentration of the isomerase required for the production of uroporphyrin III without chromatographically detectable uroporphyrin I. However, kinetic studies at low substrate concentrations reveal clear differences.

The apparent Michaelis-Menten constant in the presence of both enzymes is estimated to be 10 × 10⁻⁵ M per liter, and the concentration at which the maximum velocity of the reaction was achieved was 0.125 µmole per ml. In the absence of the isomerase, maximum reaction velocity was achieved at 0.076 µmole per ml. Fig. 3 illustrates Lineweaver-Burk plots (13) of the kinetic data. At the enzyme concentration used in these experiments, the preparation of uroporphyrinogen isomerase employed catalyzed no consumption of PBG when incubated alone with this pyrrole.

Rate of Consumption of PBG and Porphyrin Yield—The rate of consumption of PBG by the isomerase (Fraction C)-deaminase
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FIG. 2. Tracing of a paper chromatogram (Falk and Benson method (11)) of methyl esters of porphyrins recovered from assay of a preparation of wheat germ Fraction C in which different proportions of Fraction C and PBG-D (Stage H 30-50) were incubated with PBG. The Fraction C preparation contained 136 mg. of protein per ml.; the H 30-50 preparation of 28 mg. of protein per ml. Each 3 ml. of reaction mixture contained 0.05 ml. of H 30-50 and the following amounts of Fraction C: 1, 1.3 ml.; 2, 1.0 ml.; 3, 0.6 ml.; and 4, 0.3 ml. The initial concentration of PBG ranged from 0.42 to 0.44 μmole per ml. Dotted lines show position of pigments at the beginning of the second development; solid lines show final positions. U I = uroporphyrin I marker, U III = uroporphyrin III marker.

TABLE I
Yield of uroporphyrin from PBG and effect of aerobic or anaerobic incubation

<table>
<thead>
<tr>
<th></th>
<th>PBG consumed</th>
<th>Uroporphyrin accumulated</th>
<th>Yield</th>
<th>Conditions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.446</td>
<td>0.068</td>
<td>61.2</td>
<td>Aerobic</td>
</tr>
<tr>
<td>b</td>
<td>0.373</td>
<td>0.085</td>
<td>91.3</td>
<td>Anaerobic; cysteine</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.319</td>
<td>0.060</td>
<td>75.4</td>
<td>Aerobic</td>
</tr>
<tr>
<td>b</td>
<td>0.337</td>
<td>0.083</td>
<td>98.5</td>
<td>Anaerobic; cysteine</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.380</td>
<td>0.089</td>
<td>93</td>
<td>Anaerobic; cysteine</td>
</tr>
<tr>
<td>b</td>
<td>0.374</td>
<td>0.084</td>
<td>90</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>c</td>
<td>0.374</td>
<td>0.062</td>
<td>66</td>
<td>Acridine</td>
</tr>
<tr>
<td>d</td>
<td>0.400</td>
<td>0.066</td>
<td>66</td>
<td>Aerobic</td>
</tr>
</tbody>
</table>

* Each reaction mixture contained 0.03-0.05 ml. of porphobilinogen deaminase (PBG-D). Stage H 30-50 (0.65 to 1.08 mg. of protein), 0.5 ml. of wheat germ Fraction C (49 mg. of protein), 3 mmoles of pH 8.2 Tris buffer, and 0.01 mmole of ethylenediaminetetraacetic acid. Total volume 3 ml. Incubated at 37°. Cysteine, where included, 0.12 mmole. The uroporphyrinogen accumulated in each experiment was oxidized to uroporphyrin by I2, which was added to each reaction mixture after the PBG had been completely consumed.

Thus, uroporphyrinogens can be oxidized enzymatically to a compound(s) spectroscopically different from uroporphyrin by an enzyme present in the preparations used here, and this may be the principal cause of reduced yields of uroporphyrin from PBG under aerobic conditions. This oxidizing enzyme is inhibited by hydroxylamine and appears to have some properties in common with an oxidizing enzyme described in extracts of system is the same whether the reaction mixture is incubated in air or anaerobically. Under the latter conditions, uroporphyrinogen, but little or no porphyrin, is present at the time of exhaustion of the substrate. The porphyrin formed upon oxidation of the accumulated uroporphyrinogen was found to be uroporphyrin III.

On the other hand, the yield of porphyrin is adversely affected by the presence of air. The yield approaches 100 per cent when incubation is under anaerobic conditions but is considerably lower when the reaction mixture is incubated aerobically (Table I). There is no similar effect of aeration upon yield when the isomerase is omitted from the reaction mixture, that is, when uroporphyrinogen I is produced enzymatically from PBG by the action of PBG-D prepared from spinach leaf tissue (Stage H 30-50 (5)).

The occurrence of low yields of uroporphyrin is positively correlated with the appearance in the reaction mixtures of an absorption band with a maximum at 638 mp; this is in addition to the uroporphyrin maxima at 502, 538, 561, and 612 mp in aqueous solution. The peak at 638 mp does not occur in preparations which have been incubated under anaerobic conditions but is prominent in reaction mixtures which have been incubated aerobically. However, under aerobic conditions, the development of this band is reduced by about two-thirds if hydroxylamine (final concentration 0.01 M) is included in the reaction mixture. The 638 mp absorption band also appears when either uroporphyrinogen I or III (prepared as described in (9)) is incubated with Fraction C and PBG-D under aerobic conditions. However, incubation of either uroporphyrin I or III under these conditions does not result in the development of this band. It also fails to appear when uroporphyrinogens are oxidized by air or with iodine (5), (Fig. 3).

FIG. 3. Paper chromatogram (Falk and Benson Method (11)) of methyl esters from preincubation experiment. See text and Table II for treatments. Dotted lines show position of pigments at the beginning of the second development; solid lines show final positions. U I = uroporphyrin I marker. U III = uroporphyrin III marker.
spinach leaf tissue (5). The nature of the substance which absorbs at 638 mK has not been determined.

Inhibition of Uroporphyrinogen Isomerase—Fig. 4 is a tracing of a chromatogram (11) of the methyl esters of uroporphyrins recovered from experiments in which PBG-D and uroporphyrinogen isomerase were incubated with hydroxylamine or some other compounds prior and subsequent to the introduction of PBG into the reaction mixtures. Hydroxylamine, at a final concentration of 1 or 2 X 10⁻⁴ M, inhibits uroporphyrinogen isomerase; uroporphyrin I as well as III accumulated in hydroxylamine-treated mixtures.

Dimedon, 3-amino-1,2,4-triazole, and semicarbazide, at concentrations ranging from 0.001 to 0.02 M, have no effect on the enzymatic production of uroporphyrin III in this system.

Preincubation Experiments—As described elsewhere (5), the incubation of PBG-D preparations with PBG under anaerobic conditions leads to the consumption of substrate without the appearance of porphyrin, and uroporphyrinogen I accumulates. The addition, at this point, of a uroporphyrinogen isomerase solution was found to have no effect upon the nature of the product, i.e. uroporphyrin I was recovered after oxidation of the porphyrinogen. Thus, the isomerase participates in the synthesis of uroporphyrin III prior to the formation of a cyclized tetrapyrrole; that is, it does not act to rearrange the acetic and propionic acid side chains.

In another type of experiment, very small amounts of uroporphyrinogen isomerase were incubated with PBG-D for 30 minutes at room temperature prior to the addition of substrate and subsequent incubation. The preincubation appeared to have no effect upon the relative amounts of the two uroporphyrin isomers recovered.

**Table II**

<table>
<thead>
<tr>
<th>Initial contents of reaction mixtures</th>
</tr>
</thead>
</table>

| Numbers 13 to 17: 0.2 ml. of wheat germ Fraction C (24 mg. of protein); 0.7 ml. of 0.5 M phosphate buffer, pH 8.2; 1 ml. of porphobilinogen (PBG) (600 µg.); 1.1 ml. of water.  |
| Number 18: as above but 0.2 ml. of water substituted for wheat germ Fraction C. |

**TABLE II**

<table>
<thead>
<tr>
<th>Subsequent additions and manipulations</th>
<th>Products*</th>
</tr>
</thead>
<tbody>
<tr>
<td>b. At 180 minutes, 0.2 ml. of 117 HIIB added†</td>
<td>U III &gt;&gt; U I</td>
</tr>
<tr>
<td>a. Incubated 180 minutes at 29°C</td>
<td>U III</td>
</tr>
<tr>
<td>b. After incubation (a), heated 30 minutes at 55°C, cooled, and 0.2 ml. of 117 HIIB added†</td>
<td>U I</td>
</tr>
<tr>
<td>a. Incubated at 29°C for 30 hours</td>
<td>U I</td>
</tr>
<tr>
<td>a. Heated 30 minutes at 55°C prior to addition of PBG; cooled, 1 ml. of PBG solution added; incubated 180 minutes at 29°C</td>
<td>U III &gt;&gt; U I</td>
</tr>
<tr>
<td>b. At 180 minutes, 0.2 ml. of 117 HIIB added†</td>
<td>U I</td>
</tr>
<tr>
<td>b. In ice bath for 180 minutes</td>
<td>U I</td>
</tr>
<tr>
<td>b. At 180 minutes, 0.2 ml. of 117 HIIB added†</td>
<td>U III &gt;&gt; U I</td>
</tr>
<tr>
<td>a. Incubated 180 minutes at 29°C</td>
<td>U I</td>
</tr>
<tr>
<td>b. After incubation (a), heated 30 minutes at 55°C, cooled, 0.2 ml. of 117 HIIB added†</td>
<td>U I</td>
</tr>
</tbody>
</table>

* U I = uroporphyrin I; U III = uroporphyrin III.
† Subsequently incubated at 29°C for 17 hours. 117 HIIB is a preparation from spinach leaf acetone powder which contains both porphobilinogen deaminase (PBG-D) and the enzyme which oxidizes the colorless precursor (uroporphyrinogen) to uroporphyrin. This preparation contains 12.3 mg. of protein per ml.

In a third type of experiment, an attempt was made to determine whether preincubation of the isomerase with PBG might have some effect on the nature of the product; that is, although no detectable consumption of PBG (as measured by the Ehrlich reaction) occurs in the presence of the isomerase alone, perhaps other alterations in the molecule, not reflected as a decrease in the total number of free α-positions, might be effected. The procedures followed in these experiments and the nature of the products (based on Falk and Benson (11) chromatography) are outlined in Table II. A tracing of a chromatogram of the porphyrin methyl esters from such an experiment is shown in Figure 3. The 55°C heat treatment serves to inactivate the uroporphyrinogen isomerase.

As is illustrated, incubation of PBG with the isomerase, followed by the inactivation of this enzyme, and then the addition of PBG-D, leads to the production of the same isomer as if the PBG had never been exposed to the isomerase, i.e. uroporphyrin I was recovered. Thus, it appears that under the conditions described here, PBG, PBG-D, and uroporphyrinogen isomerase must be present simultaneously for the synthesis of uroporphyrinogen III from PBG.

Identification of the Product as Uroporphyrin III—The porphyrin product(s) of the PBG-uroporphyrinogen isomerase-PBG-D reaction move as a single spot corresponding to an octacarboxylic porphyrin (uroporphyrin) in the chromatographic system of Nicholas and Rimington (14). The behavior of the
methyl esters in the Falk and Benson (11) chromatographic system is shown in Figs. 1 to 4.

Porphyrin pooled from five different experiments was collected by precipitation at pH 4. The precipitate was dried, esterified with methanol containing 30 per cent (w/w) dry HCl, and crystallized from chloroform-methanol after chromatography on magnesium oxide (Grade III) (15). The crystals of the methyl ester were crushed between cover glasses and observed on the micromelting point apparatus between crossed Nicol prisms. Softening of the crystals commenced at 257°, and all birefringence was gone at 262°. This would correspond to 85 to 90 per cent uroporphyrin III in mixture with uroporphyrin I, according to published, mixed melting point curves (16).

An aliquot of the uroporphyrin was partially decarboxylated to coproporphyrin (17), esterified, and chromatographed on magnesium oxide (15), and the melting behavior was observed after recrystallization from benzene-petroleum ether followed by recrystallization from chloroform-methanol. The crystals formed as small curved needles or crescents in rosettes. These coproporphyrin methyl ester crystals softened considerably at 158°, at 175° small birefringent pieces lay in pools of melted material, by 195° the residual birefringence was disappearing, and by 199° to 200° all birefringence had disappeared. Upon cooling, resolidification began at 155°. In the apparatus used here a sample of coproporphyrin III tetramethyl ester from Corynebacterium diptheriae began to lose birefringence at 155°, and in the range 178° to 179° all birefringence disappeared quickly; some resolidification occurred at 169°, and by 162°, large pieces of crystalline material were evident throughout the field. These data on the melting behavior of the coproporphyrin methyl ester prepared from the uroporphyrin produced in these experiments are in accord with the conclusion that the isomeric composition is about 85 to 90 per cent III and 10 to 15 per cent I as based on the mixed melting point curves of Jope and O’Brien (18). Consideration of the lability of the isomerase and of the stability of the deaminase (5) suggests that the uroporphyrin I present may be made from PBG after all the isomerase has deteriorated.

The coproporphyrin methyl ester, prepared from the uroporphyrin synthesized enzymatically in these experiments, moved as a single spot corresponding to the III isomer in the paper chromatographic method of Graick and Mauzerall.3

**DISCUSSION**

The present data describe what appears to be a system of at least two enzymes which catalyze the synthesis of uroporphyrinogen III from PBG.

The data provided by the kinetic studies (Fig. 5) suggest very strongly that there is, at some point in the synthesis of uroporphyrinogen III, a direct interaction between PBG and the isomerase. On the other hand, at isomerase concentrations

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which catalyze the synthesis of uroporphyrin III when PBG-D is present, the isomerase cannot act on the PBG when present alone with it. This is indicated by the production of uroporphyrin I when the isomerase is incubated with PBG and then inactivated prior to the addition of PBG-D to these solutions.

In view of the above observations, it seems reasonable to assume that the isomerase requires two substrates for its action. One of these is PBG, and the other would probably be some product of the action of PBG-D on PBG, short of a cyclized tetrapyrrole, i.e. a di- or tripyrrole. The nature of this second substrate is, as yet, unknown. The detailed mechanisms of the action of the enzyme, involving the two substrates, is unclear at present. Unfortunately, the data available do not serve as a guide to favor strongly any one of the current hypotheses (12, 19-21) regarding the formation of uroporphyrinogen III.

Of the two hypotheses of the biosynthesis of uroporphyrinogen III which have been advanced which involve a "T" pyrromethane intermediate (12, 20), data are available now to evaluate only the proposal of Shemin and associates (20). In the case of this scheme the isomerase might catalyze the synthesis of a tripyrromethane from a dipyrrole plus PBG, although in this system the participation of a third enzyme, to remove an aminomethyl or formyl group from the a-position of one of the dipyrrolic intermediates, might be expected. (It is entirely possible that the wheat germ preparations used here do contain more than one enzyme which is active in uroporphyrinogen III synthesis.) One discrepancy between the present findings and the hypothesis of Shemin and associates is that, although the product of the reactions is principally or entirely uroporphyrinogen III, no detectable pyrrole remains in solution at the termination of the reaction. This is the case even when sufficient enzyme is introduced to have the reaction go to completion in 1 hour. According to the hypothesis of Shemin and associates, 1 mole of opsopyrrole dicarboxylic acid should be accumulated for each mole of uroporphyrin III formed; that is, the level of pyrrole, as measured by the Ehrlich reaction, should not fall below 20 per cent of the initial concentration. Polymerization of the residual pyrroles, or their failure to react with p-dimethylaminobenzaldehyde, could account for the failure to detect them, but opsopyrrole dicarboxylic acid is relatively stable under the incubation conditions employed in the present experiments, and it reacts with p-dimethylaminobenzaldehyde to give a complex with a higher molar extinction value than that of the PBG complex. In addition, Shemin's hypothesis is incompatible with the report of Dresel and Falk (22), that about 90 per cent of the PBG incubated with avian red cell preparations can be accounted for as porphyrin and with the present data, where the yield of uroporphyrinogen III from PBG exceeded 80 per cent in anaerobic experiments.

Cookson and Rimington (19) have proposed that, at least nonenzymatically, a transfer of an aminomethyl or a methyl radical from one a-position of PBG to the unsubstituted a-position of the same or another molecule of PBG might be involved in the synthesis of uroporphyrin III. If this were the case in the enzymatic reactions under study here, the isomerase could be acting to catalyze such transfers. The evidence presented indicates, however, that the isomerases preparations are incapable of acting on PBG alone. Thus, if the transfer of, e.g. an aminomethyl (or equivalent) group is involved, it would appear most likely that this transfer occurs while both substrates of the isomerase are on the surface of the enzyme.

The present data suggest a possible site of the metabolic lesion in congenital porphyria. If it is assumed that enzymes with the same mode of action as PBG-D and uroporphyrinogen isomerase occur in mammals, it would appear that impaired uroporphyrinogen isomerase activity, or increased PBG-D activity, or a combination of both, in at least some tissues, might be associated with this disease.

**SUMMARY**

The preparation of uroporphyrinogen isomerase from wheat germ and some properties of this enzyme are described. Evidence is presented which is compatible with the conclusion that this enzyme catalyzes the synthesis of uroporphyrinogen III from porphobilinogen and some product of the action of porphobilinogen deaminase on porphobilinogen. Uroporphyrinogen isomerase is inhibited by hydroxylamine.

Current hypotheses of porphyrin biosynthesis and the possible sites of metabolic lesions in congenital porphyria are discussed in the light of the present data.

**Acknowledgment**—It is a pleasure to acknowledge the technical assistance of Mrs. D. Jacobsohn and Mr. Charles Kung during various phases of this work.

4 Bogorad, L., unpublished results. At pH 8.2 and an initial concentration of 86.6 μg per ml. of opsopyrrole dicarboxylic acid, no detectable change in concentration was observed after incubation for 60 minutes at 37°.

The opsopyrrole dicarboxylic acid used in these experiments was kindly provided by Dr. S. F. MacDonald, Division of Pure Chemistry, National Research Council, Ottawa, Canada.

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