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# Studies on the Porphobilinogen Deaminase–Uroporphyrinogen Cosynthetase System of Cultured Soya-bean Cells

By ELENA B. C. LLAMBÍAS and ALCIRA M. DEL C. BATLLE
*Cátedra de Química Biológica I, Departamento de Química Biológica, Facultad de Ciencias Exactas, Perú 272, Buenos Aires, Argentina*

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1. Porphobilinogenase was isolated and purified from soya-bean callus tissue; its components, porphobilinogen deaminase and uroporphyrinogen isomerase, were separated and purified. 2. The purified porphobilinogenase was resolved into two bands on starch-gel electrophoresis. The molecular weights of porphobilinogenase, deaminase and isomerase fractions were determined by the gel-filtration method. Porphobilinogenase activity was affected by the presence of air; uroporphyrinogens were only formed under anaerobic conditions, although substrate consumption was the same in the absence of oxygen as in its presence. 3. pH-dependence of both porphobilinogenase and deaminase was the same and a sharp optimum at pH 7.2 was obtained. Isomerase was heat-labile, but the presence of ammonium ions or porphobilinogen afforded some protection against inactivation. The action of several compounds added to the system was studied. Cysteine, thioglycollate, ammonium ions and hydroxylamine inhibited porphobilinogenase; certain concentrations of sodium and magnesium salts enhanced activity; some dicarboxylic acids and 2-methoxy-5-nitrotropone inhibited the deaminase. 4. δ-Aminolaevulate and ethionine in the culture media stimulated porphyrin synthesis and increased porphobilinogenase activity, whereas iron deficiency resulted in porphyrin accumulation. 5. The development of chlorophyll and porphobilinogenase on illumination of dark-grown callus was followed. 6. A hypothetical scheme is suggested for the enzymic synthesis of uroporphyrinogens from porphobilinogen.

In this paper the trival name porphobilinogenase, suggested by Lockwood & Rimington (1957), is used to designate the porphobilinogen deaminase–uroporphyrinogen III cosynthetase system.

The mechanism of synthesis of porphyrins is of interest because of their involvement in a variety of biological reactions. A part of the pathway that has stimulated much speculation is the step involving the condensation of porphobilinogen to uroporphyrinogens. It has been known since 1953 that porphobilinogen is an intermediate in the biosynthesis of porphyrins (Falk, Dresel & Rimington, 1953; Bogorad & Granick, 1953) and a good deal of information is available on the enzyme system concerned, which is widely distributed (Lockwood & Rimington, 1957; Bogorad, 1958*a*,*b*; Granick & Mauzerall, 1958; Heath & Hoare, 1959*a*,*b*; Lockwood & Benson, 1960; Levin & Coleman, 1967; Stevens, Frydman & Frydman, 1968; Levin, 1968; Sancovich, Batlle & Grinstein, 1969*a*,*b*).

In plant tissue extracts (Bogorad, 1962) the formation of uroporphyrinogen I from porphobilinogen is enzymically catalysed by the relatively heat-stable deaminase;* in the presence of both deaminase and isomerase, uroporphyrinogen III, the physiological intermediate in haem biosynthesis is formed. The mode of action of the isomerase is unknown since it does not catalyse the disappearance of porphobilinogen or the conversion of one uroporphyrinogen isomer into another (Bogorad & Marks, 1960). The mechanism whereby uroporphyrinogens are formed from porphobilinogen is still obscure. Although no intermediate steps have been detected so far, numerous hypotheses have been proposed for this enzymic reaction (for review see Margoliash, 1961). Cornford (1964) and Bullock (1965) have since proposed other possible mechanisms.

Soya-bean callus-tissue cultures, which undergo

* Abbreviations: deaminase, porphobilinogen deaminase; isomerase, uroporphyrinogen III cosynthetase.

active cell division, fail to synthesize chlorophyll in amounts equivalent to that found in mature leaves; this inability could be due either to the absence or to the low activity of the enzymes involved in chlorophyll synthesis. Therefore investigations have been carried out on the presence and activity of two of the enzymes involved, namely δ-aminolaevulate dehydratase (Tigier, Batlle & Locascio, 1968, 1970) and succinyl-CoA synthetase (Wider & Tigier, 1970). Granick (1954*a*,*b*) and Bogorad (1958*a*,*b*) have reported the presence of enzymes involved in the steps leading from δ-aminolaevulate to chlorophyll in higher plants and investigations on the activities of these enzymes and those related to prior steps in callus-tissue cultures have been made by Tigier *et al.* (1968, 1970) and Stobart & Thomas (1968*a*). During the extraction of plant tissues, accumulation of products of the phenol oxidase reaction may powerfully inhibit plant enzymes and subcellular organelles (Loomis & Battaile, 1966; Anderson, 1968); it appears that these problems are less in tissue-culture cells and that the efficiency of enzyme extraction from such cells is very good.

The present paper reports the isolation and purification from light-grown soya-bean callus of porphobilinogenase and the separation of its components, deaminase and isomerase. Some properties of these enzymes are also described.

A mechanism for the enzymic conversion of porphobilinogen into uroporphyrinogens is proposed.

## MATERIALS AND METHODS

Porphobilinogen was obtained by the method of Sancovich, Ferramola, Batlle & Grinstein (1970), with δ-aminolaevulate and purified δ-aminolaevulate dehydratase (Batlle, Ferramola & Grinstein, 1967), and it was determined as described by Moore & Labbe (1964). Solutions of porphobilinogen were prepared either in 0.1M-tris–HCl buffer or 0.1M-phosphate buffer, pH7.2.

Sephadex or Sepharose gels were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and Bio-Gel P from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Starch gel for electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto, Canada. Calcium phosphate gel was prepared as described by Keilin & Hartree (1951). Protamine sulphate was kindly supplied by Dr J. Mendive and gibberellic acid was a generous gift from Dr M. Grinstein. Tris–HCl and $Na_2HPO_4$–$NaH_2PO_4$ buffers were used throughout the work. All other reagents were of A.R. grade obtained from several commercial sources. All solutions were made up in ion-free glass-distilled water.

*Source material of enzyme.* Undifferentiated callus cultures from soya-bean seeds were obtained by the procedure of Miller (1963). The growth medium and culture conditions for these tissue cultures have been reported by Tigier *et al.* (1970); any additional ingredient is indicated in the text or in the legends to the figures and tables. 'Dark-grown callus' refers to callus cultures that were grown in the dark with regular 14-day subculturing. 'Light-grown callus' refers to callus cultures that were subcultured from dark-grown callus tissues and then grown under 100ft-candles of white light. Illumination was supplied by 60W bulbs at an appropriate distance from the flasks. Unless otherwise stated, experiments were carried out with cultures grown semi-anaerobically as it was not found necessary to make the conditions strictly anaerobic.

Acetone-dried powders of these cultures were prepared as described by Tigier *et al.* (1970) and stored in a vacuum desiccator over $CaCl_2$ at 0°C.

Protein concentrations were determined by using the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951) and chlorophyll was determined spectrophotometrically in 80% (v/v) acetone (Arnon, 1949).

*Determination of enzyme activity.* The standard incubation system contained, unless otherwise stated: enzyme preparation (usually 8ml); 0.05M buffer, pH7.2; 250μg of porphobilinogen; 0.5ml of 0.6M-NaCl and 0.5ml of 0.12M-$MgCl_2$ in a final volume of 10ml at pH7.2; the pH was controlled before and after incubation with a combination-electrode pH-meter and the average value was always within the optimum pH range. Incubations were carried out anaerobically in Thunberg tubes, in the dark, with mechanical shaking, at 38°C for 4–6h. When the effect of additives was studied, the incubation system contained only enzyme, substrate and buffer, with or without the addition of other reagents, in a standard final volume and at pH7.2. Blanks were always run with porphobilinogen and without enzyme. After incubation, trichloroacetic acid was added (to a final concentration of 5%, w/v) to precipitate the protein, the mixture was then illuminated with white light (50ft-candles) for 20–25min to oxidize the porphyrinogens formed, the precipitated protein was filtered off and total porphyrins were determined in acid solution (Rimington, 1960).

*Fractionation and determination of porphyrins.* Porphyrins were fractionated and esterified by the usual methods (Dresel & Falk, 1954; Rimington & Benson, 1967), but it was later found that the procedure of evaporating the acid solution to dryness *in vacuo* and dissolving the residue in the esterification mixture 5% (v/v) $H_2SO_4$ in methanol (Batlle & Grinstein, 1964) was simpler and it was therefore adopted; nevertheless results obtained with both procedures were much the same, although losses were smaller with the latter. Identification and quantitative determination of porphyrins formed were made by the methods of Batlle & Grinstein (1962). Isomeric composition of uroporphyrin fractions was determined by the methods of Cornford & Benson (1963) and Batlle & Benson (1966). Spectrophotometric measurements were carried out in a Beckman DB spectrophotometer.

*Enzyme unit.* This is defined as the amount of enzyme that catalyses the formation of one μmol of uroporphyrinogen/min from porphobilinogen, under the standard conditions described above; specific activity is defined as units of enzyme/mg of protein.

For the determination of isomerase activity, the reaction mixture was as described above, except that the 36-fold purified deaminase was also added together with

various amounts of the isomerase (5–6mg of isomerase/mg of deaminase).

Ammonium ions are known to inhibit porphobilinogenase (Bogorad, 1958*b*, 1962, 1963; Sancovich *et al.* 1969*a*,*b*). They were therefore removed from enzyme preparations before measurement of enzymic activity, by the method of molecular sieving with columns (2cm × 30cm) of Sephadex G-25 or Bio-Gel P-20, 0.05M-tris–HCl or phosphate buffer, pH7.2, being used as eluent. When Sephadex G-100, Sepharose 6B or other gel columns were used, protein was eluted with the same buffers. Columns were run at 4°C. Protein content and enzyme activity were determined in each fraction and those containing activity were pooled. Gel columns were prepared as described by Batlle, Benson & Rimington (1965).

Starch-gel electrophoresis was based on the methods described by Bodman (1960).

Ultrafiltration experiments were performed by using LKB ultrafilter equipment or viscose tubes as described by Wildy, Nizet & Benson (1961).

*Extraction of porphyrins from callus cultures.* The callus cells were harvested and the fluorescent pink material was extracted from them with either ethyl acetate–acetic acid (3:1, v/v) or acetic acid–HCl (9:1, v/v) and then processed by a procedure similar to that described by Dresel & Falk (1954).

*Determination of molecular weights.* This was carried out by the methods of Andrews (1964, 1965), Batlle (1967) and Locascio, Tigier & Batlle (1969).

*Radioactivity determinations.* The radioactivity of labelled compounds was measured in a thin-window gas-flow counter (Nuclear–Chicago model D47); samples prepared were infinitely thin.

## RESULTS

### *Preparation of enzymes*

All operations were carried out in the cold-room at 4°C unless otherwise stated and centrifugations were performed in an International Refrigerated Centrifuge or in a Spinco model L Centrifuge.

(*a*) Preparation of porphobilinogenase (Table 1*a*).

(1) Homogenate. Homogenate (50% wet wt./vol.) of light-grown wound callus (50–100g) was prepared in a Potter–Elvehjem-type homogenizer (glass tube and glass pestle); the homogenizing medium was 0.1M-tris–HCl buffer, pH7.4.

(2) Centrifugation at 600*g*. The homogenate was centrifuged at 600*g* for 10min. The sediment was washed three times with a total volume of buffer equal to that used to prepare the homogenate. All supernatants were collected together, constituting

Table 1. *Purification of porphobilinogenase and separation of isomerase from deaminase in soya-bean callus*

Incubation conditions were as described in the Materials and Methods section. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fractions were carried out by the methods described in the text. For the determination of isomerase activity, purified soya-bean deaminase was used (1mg of deaminase/5mg of isomerase). As the exact isomerase/deaminase ratio in the homogenate and supernatant is not known, results cannot be expressed in terms of specific activity of isomerase.

| Fraction | $10^6 \times$ Sp. activity | Purification | Porphyrins formed: UI (%) | Porphyrins formed: UIII (%) |
|---|---|---|---|---|
| (*a*) Porphobilinogenase | | | | |
| (1) Homogenate | 0.26 | 1 | 10 | 90 |
| (2) 600*g* supernatant | 0.46 | 1.8 | 10 | 90 |
| (3) 24000*g* supernatant | 0.76 | 2.9 | 10 | 90 |
| (4) Sephadex G-25 column | 1.35 | 5.1 | 10 | 90 |
| (5) Protamine sulphate treatment | 1.55 | 5.8 | 20 | 80 |
| (6) 0–70% $(NH_4)_2SO_4$ fraction | 2.06 | 8.0 | 20 | 80 |
| (7) Sephadex G-100 column (peak) | 19.66 | 73.0 | 20 | 80 |
| (*b*) Deaminase | | | | |
| (1)–(3) as in (*a*) | 0.76 | 2.9 | 10 | 90 |
| (4*b*) Calcium phosphate-gel treatment | 2.13 | 8.0 | 50 | 50 |
| (5*b*) Heat treatment | 4.88 | 18.3 | 70 | 30 |
| (6*b*) 0–70% $(NH_4)_2SO_4$ fraction | 9.58 | 36.0 | 75 | 25 |
| (7*b*) Sephadex G-100 column (peak) | 50 | 150.0 | 85 | 15 |
| (*c*) Isomerase | | | | |
| (1)–(6) as in (*a*) | 2.06 | — | 20 | 80 |
| (7*c*) 70–90% $(NH_4)_2SO_4$ fraction | — | — | — | — |
| 70–90%+soya-bean deaminase (from stage (6*b*) of purification) | — | — | 0 | 100 |
| (8*c*) Sephadex G-100 column (peak) | — | — | — | — |
| Sephadex G-100 + deaminase | — | — | 0 | 100 |

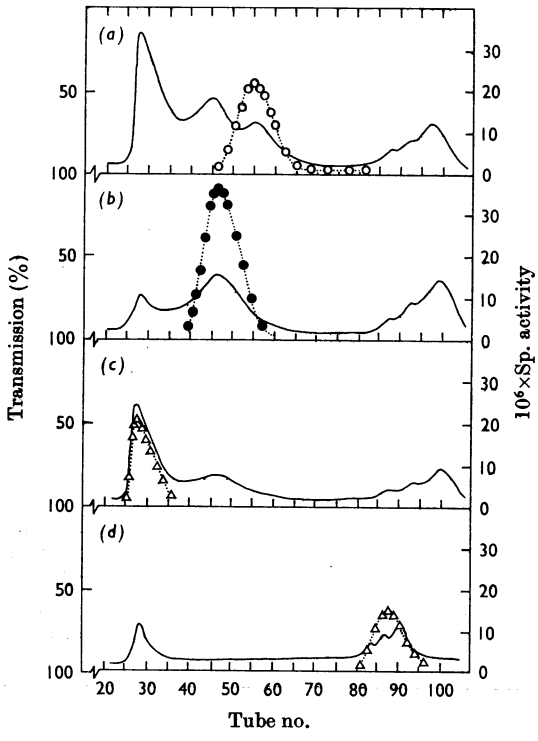



Fig. 1. Elution diagram on Sephadex G-100 of (*a*) eight-fold purified porphobilinogenase, (*b*) 36-fold purified deaminase, (*c*) isomerase preparation obtained after step 7*c* of purification, and (*d*) first peak of the Sephadex G-100 column shown in (*c*) rechromatographed on a Sepharose 6B column. ——, U.v. absorption as recorded with a Uvicord I; ○, porphobilinogenase activity; ●, deaminase activity; △, isomerase activity. Fractions of 4.3ml were collected. Activities were determined as described in the Materials and Methods section.

the crude extract, and the final sediment was discarded.

(3) Centrifugation at 24000*g*. The crude extract was centrifuged at 24000*g* for 20min; the sediment had only some deaminase activity and was discarded.

(4) Gel filtration on Sephadex G-25. The 24000*g* supernatant was passed through a Sephadex G-25 column (2.5cm × 80cm).

(5) Protamine sulphate treatment. The pH of the eluate from Sephadex G-25 columns was adjusted to pH 5.0 with acetic acid; after 10min, the material was centrifuged at 11000*g* for 10min and the sediment was discarded. The supernatant solution was then treated with 0.075ml of 2% (w/v) protamine sulphate (pH 5.0)/ml of enzyme solution (5mg of protein/ml). After stirring for 20min the precipitate was centrifuged and discarded.

(6) First ammonium sulphate fractionation. To the supernatant from step (5) solid ammonium sulphate was added up to 70% saturation, the pH being kept close to 7.2 by adding conc. ammonia. After 1h, the protein precipitate was collected by centrifuging at 12000*g* for 10min, and dissolved in a small volume of 0.05M-tris–HCl buffer, pH 7.2.

(7) Gel filtration on Sephadex G-100. The enzyme preparation from stage (6) was applied to a Sephadex G-100 column (2.5cm × 80cm). Fig. 1(*a*) shows a typical elution diagram, where porphobilinogenase activity was associated with the third protein peak and the specific activity of the active peak was nine times higher than that of the preparation from stage (6). It must be noted however, that the main protein peak had isomerase activity. The third peak when rechromatographed on the same Sephadex G-100 column again gave three peaks, the main one of which had porphobilinogenase activity and the other two were eluted in volumes corresponding to isomerase and deaminase.

(*b*) Preparation of deaminase (Table 1*b*). Steps 1–3 were the same as those described for porphobilinogenase.

(4*b*) Calcium phosphate-gel treatment. The 24000*g* supernatant was treated with calcium phosphate gel (1mg of protein/2–3mg of gel). After being stirred for 10min the mixture was centrifuged at 1000*g* for 5min and the sediment discarded.

(5*b*) Heat treatment. The supernatant was heated with constant stirring at 70°C for 30min, and then cooled in an ice bath, centrifuged at 10000*g* for 10min and the sedimented protein precipitate was discarded.

(6*b*) Ammonium sulphate fractionation. The supernatant solution was made to 70% saturation with solid ammonium sulphate and the precipitate, collected by centrifugation, was dissolved in a small volume of 0.05M-tris–HCl buffer, pH 7.2.

(7*b*) Gel filtration on Sephadex G-100. The protein solution was applied to a Sephadex G-100 column (2.5cm × 80cm). Fig. 1(*b*) shows a typical elution diagram; deaminase was associated with the second protein peak.

(*c*) Preparation of isomerase (Table 1*c*). Steps 1–6 were the same as those described for porphobilinogenase.

(7*c*) Ammonium sulphate fractionation. The supernatant from stage (6) was fractionated with ammonium sulphate to 70–90% saturation; this precipitate was dissolved in a small volume of 0.05M-tris–HCl buffer, pH 7.2.

(8*c*) Gel filtration on Sephadex G-100. The protein solution was applied to a Sephadex G-100 column (2.5cm × 80cm). Fig. 1(*c*) shows a typical elution diagram, where isomerase activity was associated with the first protein peak; evidence was obtained suggesting the presence of a nucleoprotein in this fraction.

(9c) Gel filtration on Sepharose 6B. The pooled isomerase-containing fractions from the Sephadex column were concentrated by ammonium sulphate precipitation and applied to a Sepharose 6B column (2.5cm × 80cm). Fig. 1(*d*) shows a typical elution diagram where isomerase activity was associated with the second, third and fourth peaks.

No substantial further purification of any one enzyme fraction was attained by chromatography on DEAE-cellulose (or DEAE-Sephadex) nor was the separation of deaminase from isomerase achieved by using DEAE-cellulose.

Gel filtration on Sephadex G-200 or Bio-Gel P-200, instead of or after Sephadex G-100 did not increase the degree of purification of the enzyme preparations.

*Extracts from acetone-dried powder of wound callus.* Extracts from acetone-powder stocks were prepared by adding 1g of acetone-dried powder to 30ml of 0.1M-tris–HCl buffer, pH7.4. After being stirred for 30min the slurry was centrifuged at 24000*g* as in step (3) of purification and the supernatant thus obtained was processed as described above. Specific activities of crude extracts of acetone-dried powders were usually twice or three times higher than those obtained from fresh wound callus.

*Isomer analysis of reaction products.* Preparations of different stages of purification of porphobilinogenase formed uroporphyrinogen III predominantly. The main product of deaminase preparations obtained after Sephadex G-100 columns was uroporphyrinogen I. Isomerase obtained either after 70–90% ammonium sulphate saturation or Sephadex G-100 chromatography consumed no porphobilinogen and formed only uroporphyrinogen III when deaminase from soya-bean callus, cow liver (Sancovich *et al.* 1969*b*) or avian erythrocyte was added together with the soya-bean isomerase to the system.

## *Properties*

*Electrophoresis.* The enzyme preparations obtained in each step of the above procedures were subjected to electrophoresis on starch gel at different pH values. At alkaline pH, the most pure fraction exhibiting porphobilinogenase activity was resolved into two bands (Fig. 2), whereas deaminase and isomerase preparations migrated as a single band each; isomerase migrated to the same position as the faster running band of porphobilinogenase and deaminase behaved as the slower band. The electrophoretic migration rate of these preparations was compared with that of the same enzymes obtained from other sources and differences were observed. Liver porphobilinogenase migrated as a single band, running as the slower one of soybean porphobilinogenase, and avian erythrocyte porphobilinogenase produced three bands, the fastest one running slower than the one corresponding to soybean porphobilinogenase.

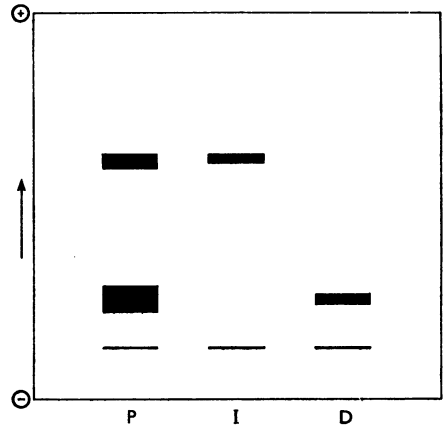



Fig. 2. Electrophoretic behaviour of 70-fold-purified porphobilinogenase (P), most highly purified isomerase (I) and 140-fold purified deaminase (D) on starch gel. Borate buffer, pH8.2, was used with a current of 30mA for 4h at room temperature.

Purified enzyme fractions were run on calibrated gel columns to determine their molecular weights. The molecular weight of the porphobilinogenase was 22000±2200; for deaminase a value of 40000± 4000 was obtained, and a value of 210000±21000 was calculated for the isomerase preparations obtained after step 5 of purification; values of 6000, 12000 and 24000 resulted when determinations were performed with Sepharose columns (see the Discussion section).

*Changes of enzyme specific activity on alteration of growth conditions.* The influence of the environment, including gas phase and light conditions, was studied. It was found that the activity of the porphobilinogenase was modified by the conditions of growth (Table 2). The highest activity was measured in callus grown semi-aerobically and exposed to light. Other enzymic activities have also been followed, to see whether the observed increases in the porphobilinogenase reflect a general increase in all the enzymic systems associated with pyrrol formation, but both δ-aminolaevulate dehydratase activity (Tigier *et al.* 1970) and succinyl-CoA synthetase activity were almost the same in callus grown semi-aerobically or anaerobically in the light or in the dark; however, light caused an increase in the activity of δ-aminolaevulate synthetase from 0.526 to 0.858nmol of δ-aminolaevulate formed/ min per mg of protein (E. W. Xifra, A. M. del C. Batlle & H. A. Tigier, unpublished work).

*Porphobilinogenase assay conditions.* A preliminary survey was made to find the most suitable

conditions for measuring porphobilinogenase activity.

*Extraction medium and incubation atmosphere.* The best medium for extracting the enzyme was found to be 0.1M-tris–HCl buffer, pH7.4 (Table 3). Phosphate buffer extracted proteins and porphobilinogenase very badly; on the other hand glycine buffer is a good medium for extracting proteins, but it is less specific than tris in the present case. Other buffer concentrations were also tried, but 0.1M proved to be the best. Glycine buffer at pH values lower than 8.5 showed lower efficiency in the extraction.

The yield of porphyrin was affected by the presence of air in the incubation system, the highest formation of uroporphyrinogen III being observed under anaerobic conditions. No porphyrins were obtained when the reaction mixture was incubated aerobically; however, consumption of porphobilinogen was the same under aerobic and anaerobic conditions.

*Dependence on* pH. When porphyrinogen formation was studied as a function of pH, a sharp maximum at pH7.2 was found either in tris or phosphate buffer with porphobilinogenase activity decreasing rapidly at higher or lower pH values. Enzyme activity was slightly better in phosphate buffer than in tris buffer and very low in glycine buffer. The pH-dependence of deaminase was identical, with a sharp optimum pH at 7.2.

The shape of the pH curves for both porphobilinogenase and deaminase was the same when porphobilinogen consumption was measured. Two maxima at pH6.7 and 7.2 were observed; the relative activities for porphobilinogenase were 4.4 and 6.5nmol of porphobilinogen consumed/h per mg respectively, whereas deaminase activity was approximately the same at the two pH values.

*Formation of a new intermediate and its binding to the enzyme.* The formation of a pyrrolic intermediate formed by the action of porphobilinogenase on porphobilinogen has been reported by Llambías & Batlle (1970*a*). It was found that, under the standard conditions described, uroporphyrinogen formation or porphobilinogen consumption were linear with increasing enzyme concentrations over a wide range, but as can be seen in Fig. 3, both in phosphate and tris buffer with either porphobilinogenase or deaminase, uroporphyrinogen formation occurred after a 3–4h lag, after which uroporphyrinogen increased linearly with time. There was no evidence of a lag when porphobilinogen consumption was measured. Further, yields of uroporphyrin based on porphobilinogen consumption were unusually low, differing greatly from stoicheiometric values.

These findings strongly suggested the formation of some compound, probably a polypyrrolic one, that could be the substrate partner of porphobilinogen for the isomerase. In addition, the lag was less pronounced when this new intermediate was present in the reaction mixture since the beginning of incubation (see also Fig. 3), suggesting again that its absence was responsible for the delay in the formation of uroporphyrinogen.

It has been shown (Llambías & Batlle, 1970*a*)

Table 2. *Effect of growth conditions on porphobilinogenase*

Callus cultures were grown as indicated. Cell-free extracts were prepared and porphobilinogenase was assayed in the 24000*g* supernatant as described in the Materials and Methods section.

| Growth conditions | $10^6 \times$ Sp. activity |
|---|---|
| Semi-aerobic, light-grown | 1.61 |
| Anaerobic, light-grown | 1.08 |
| Semi-aerobic, dark-grown | 0.83 |
| Anaerobic, dark-grown | 0.60 |

Table 3. *Effect of extracting medium and incubation conditions on porphobilinogenase*

Callus tissues were extracted as indicated in the text. Cell-free extracts were prepared and porphobilinogenase was assayed in the 0–70% $(NH_4)_2SO_4$ fraction after being passed through a Sephadex G-25 column to eliminate $NH_4^+$ and eluted with 0.05M-phosphate buffer, pH7.2, in which activity was determined as described in the Materials and Methods section. Incubations were carried out for 6h. Semi-anaerobic incubation was carried out by incubating the system anaerobically for half the time and then allowing air to enter the Thunberg tube for the rest of the time.

| Incubation conditions | Extracting buffer | Protein extracted (mg/ml) | $10^6 \times$ Sp. activity |
|---|---|---|---|
| Anaerobic | 0.1M-tris–HCl, pH7.4 | 3.8 | 2.31 |
| Semi-anaerobic | 0.1M-tris–HCl, pH7.4 | 3.8 | 0.76 |
| Aerobic | 0.1M-tris–HCl, pH7.4 | 3.8 | 0 |
| Anaerobic | 0.1M-phosphate, pH7.4 | 1.5 | 0.43 |
| Anaerobic | 0.1M-glycine–NaOH, pH8.5 | 6.3 | 1.31 |

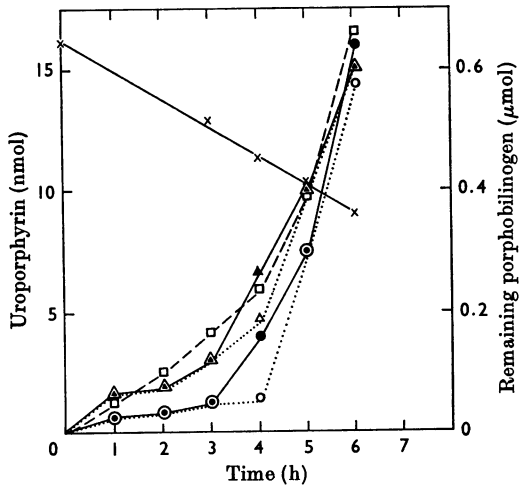



Fig. 3. Porphobilinogen consumption and uroporphyrinogen formation. Uroporphyrinogen III was formed when porphobilinogen was incubated with 30-fold-purified porphobilinogenase in 0.05M-phosphate buffer, pH7.2 (○), or 0.05M-tris–HCl buffer, pH7.2 (●). Uroporphyrinogen I was formed when porphobilinogen was incubated with 36-fold purified deaminase in 0.05M-phosphate buffer, pH7.2 (△), or 0.05M-tris–HCl buffer, pH7.2 (▲). × Porphobilinogen consumed when it was incubated with either porphobilinogenase or deaminase in either buffer. □, Uroporphyrinogen III formed when porphobilinogen and the tripyrrol intermediate (4ml of the supernatant solution obtained as described in Table 4) were incubated together with porphobilinogenase in 0.05M-phosphate buffer, pH7.2.

that the intermediate product of the action of the porphobilinogenase on porphobilinogen acts as the second substrate in the formation of uroporphyrinogen III by purified isomerase preparations from various sources. When it is incubated with porphobilinogen and purified deaminase, a mixture of uroporphyrinogens I and III is formed. Soya-bean callus deaminase, acting on porphobilinogen, produces a different pyrrolic intermediate which also behaves as a second substrate for deaminase but with the formation mainly of uroporphyrinogen. I

When 5.8mg of porphobilinogenase (sp. activity × $10^6 = 1.02$) was incubated anaerobically with [$^{14}$C]porphobilinogen (250μg, 12000c.p.m.) in 0.05M-phosphate buffer, pH7.2 (final volume 10ml), at 38°C for 3h, and the reaction mixture was passed through a Sephadex G-25 column (1cm × 100cm), equilibrated and eluted with the same buffer, non-coincident peaks of $^{14}$C radioactivity and enzyme activity emerged from the column. This indicated that at least under these conditions the intermediate was not tightly bound to the enzyme and that the binding was a physical one rather than through the formation of a stable covalent linkage.

*Stability and effect of heat.* Wound callus porphobilinogenase was found to be relatively stable at all stages of purification. Ammonium sulphate (70% saturation) precipitates the enzyme in a form that is very stable at −15°C; even in solution in 0.1M-tris–HCl or phosphate buffer at 4°C it was fairly stable for a month. Repeated freezing and thawing rapidly inactivated the enzyme, which lost 50% of its activity when stored at room temperature for 17h. The stability of the deaminase was found to be the same as that of the whole system. As has been shown in other tissues (Booij & Rimington, 1957; Lockwood & Rimington, 1957; Bogorad, 1958*a*,*b*; Granick & Mauzerall, 1958; Lockwood & Benson, 1960; Cornford, 1964; Sancovich *et al.* 1969*a*,*b*), soya-bean callus isomerase is also a heat-labile enzyme. Preheating of the porphobilinogenase at 70°C for 30min destroyed isomerase activity almost completely, resulting in an increase of total uroporphyrin formation (Table 4). These results agree with the findings of Booij & Rimington (1957), Lockwood & Benson (1960), Cornford (1964) and Llambías & Batlle (1970*d*). Heat inactivation is slightly greater, occurring after shorter time-intervals, when isomerase is heated alone. The addition of 0.01M-ammonium sulphate or porphobilinogen (25μg/ml) afforded some protection against inactivation. At higher temperatures or longer heating intervals there was significant loss of enzyme activity.

*Effect of dialysis and ultrafiltration.* The possibility of diffusible cofactors was investigated. Dialysis of the 24000*g* supernatant of the 0–70% ammonium sulphate fraction against glass-distilled water at 4°C for 4, 18, 24 and 40h produced a precipitate, which was separated by centrifugation. Enzyme activity was determined in both the supernatant and the precipitate. Whereas the supernatant had 30% of the original porphobilinogenase activity (Table 5) neither the precipitate nor the diffusate had any activity.

When the 24000*g* supernatant was ultrafiltered at 4°C for different time-intervals (4–18h) (Table 5) the ultrafiltrate residue had 55–65% less porphobilinogenase activity, whereas the ultrafiltrate had no activity. Addition of the ultrafiltrate to the ultrafiltrate residue did not restore the porphobilinogenase activity. These results indicate that some component weakly bound to the enzyme was lost on dialysis or ultrafiltration and that it could not bind the enzyme in the same way when ultrafiltrate was added back to the ultrafiltrate residue.

*Effect of various substances on enzymic activity.* Table 6 shows that both cysteine and thioglycollate

Table 4. *Effect of heat on uroporphyrinogen isomerase activity*

Incubation conditions were as described in the Materials and Methods section. Heated supernatant: 24000*g* supernatant was heated at 70°C for 30min, and after cooling in an ice bath the mixture was centrifuged at 10000*g* for 10min and the protein precipitate was discarded. In Expts. 3 and 4 heating was performed in the presence of porphobilinogen or $NH_4^+$ at the concentrations stated, and before determination of enzyme activity these compounds were removed from the supernatant by the gel-filtration method.

| Expt. | System | Addition | $10^6 \times$ Porphobilinogenase sp. activity | Uroporphyrin formed Isomer type (%) I | III |
|---|---|---|---|---|---|
| 1 | 24000*g* supernatant | None | 0.58 | 10 | 90 |
| 2 | Heated supernatant | None | 1.20 | 72 | 28 |
| 3 | Heated supernatant | 0.1mM-Porphobilinogen | 1.83 | 60 | 40 |
| 4 | Heated supernatant | 10.0mM-$NH_4^+$ | 1.0 | 42 | 58 |

Table 5. *Effect of dialysis and ultrafiltration on porphobilinogenase activity*

Cell-free extracts were prepared and dialysis or ultrafiltration experiments were carried out on the 24000*g* supernatants. Enzyme activity was assayed as described in the Materials and Methods section.

| Experimental | Time (h) | $10^6 \times$ Sp. activity Supernatant | Residue | Ultrafiltrate | Ultrafiltrate+ residue |
|---|---|---|---|---|---|
| Dialysis | 0 | 1.66 | | | |
| | 4 | 0.58 | | | |
| | 18 | 0.43 | | | |
| | 40 | 0.41 | | | |
| Ultrafiltration | 0 | 1.66 | | | |
| | 3 | | 0.76 | 0 | 0.66 |
| | 4 | | 0.55 | 0 | 0.50 |
| | 18 | | 0.53 | 0 | 0.43 |

at 1 and 0.1mM inhibited the enzyme. It was also found that the addition of 3mM-sodium chloride and 0.6mM-magnesium chloride to the reaction mixture produced a great increase in activity; at the same time they inhibited uroporphyrinogen-decarboxylase activity. When the chlorides were replaced by acetate some activation was found, but the effect was less marked than with the chloride. The isomer composition of the reaction products was practically unchanged.

The effect of some other compounds was studied (Table 7). Ammonia and hydroxylamine inhibited isomerase and deaminase. Deaminase activity was also diminished by the presence of some dicarboxylic acids. 2-Methoxy-5-nitrotropone (1mM), a new agent for chemical modification of free amino groups of proteins (Tamaoki, 1967), inhibited deaminase by 30%.

*Development of chlorophyll and porphobilinogenase activity on illumination of dark-brown callus.* It is a common finding that when etiolated plants are exposed to light a lag phase in the biosynthesis of chlorophyll is found (Virgin, 1955, 1960; Wolf & Price, 1957) and then a rapid synthesis of chlorophyll occurs (Sisler & Klein, 1963). However, some plant tissue cultures form chlorophyll in the light at a low rate (Sunderland, 1966; Stobart, McLaren & Thomas, 1967). The early effects of illumination on chlorophyll development in such chlorophyllous tissues in culture have not been reported. We have measured the chlorophyll content and porphobilinogenase activity of colourless callus grown either in the dark or in the light, and in various stages of greening. As is shown in Fig. 4, three steps were observed during the synthesis of chlorophyll by colourless callus on exposure to light: (1) a rapid conversion of previously accumulated protochlorophyllide into chlorophyllide, (2) a lag phase of 90–100min, during which no additional pigment was formed, and (3) a period of rapid synthesis, which continued until chlorophyll content reached a maximum.

Table 6. *Effect of thiols and salts on porphobilinogenase activity*

Porphobilinogenase, eightfold purified, was used as enzyme preparation. Incubation conditions were as described in the Materials and Methods section. Incubation was in 0.1M-phosphate buffer, pH7.2, for 6h. The activity of the system in the presence of other reagents is expressed on the basis of the amount of uroporphyrinogens formed by the control as 100% activity.

| Additives | Activity (%) |
|---|---|
| Control | 100 |
| Cysteine (1mM) | 42 |
| Cysteine (0.1mM) | 60 |
| Thioglycollate (1mM) | 46 |
| Thioglycollate (0.1mM) | 70 |
| Control | 100 |
| NaCl (3mM)+$MgCl_2$ (0.6mM) | 173 |
| Sodium acetate (3mM)+magnesium acetate (0.6mM) | 120 |

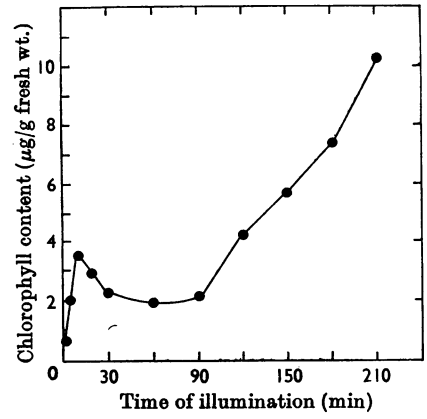



Fig. 4. Early effects of light on chlorophyll synthesis in callus transferred from the dark to the light.

Table 7. *Effect of some reagents on enzymic activity*

Incubation conditions were as described in the Materials and Methods section. The activity of the system in the presence of other reagents is expressed on the basis of the amount of uroporphyrinogen formed by the control as 100% activity. Porphobilinogenase, 18-fold purified, and deaminase, 40-fold purified, were used. Identification and quantitative determination of uroporphyrinogens formed and isomer analysis were carried out by procedures described in the Materials and Methods section.

| Enzyme addition (mM) | Activity (%) | Uroporphyrin formed Isomer type (%) I | III |
|---|---|---|---|
| Porphobilinogenase | | | |
| None | 100 | 20 | 80 |
| $NH_4^+$ (100) | 70 | 60 | 40 |
| Hydroxylamine (10) | 17 | 50 | 50 |
| Adenine (1) | 81 | 37 | 63 |
| Adenine (10) | 80 | 65 | 35 |
| Deaminase | | | |
| None | 100 | | |
| $NH_4^+$ (100) | 85 | | |
| Hydroxylamine (10) | 40 | | |
| Succinic acid (1) | 77 | | |
| Succinic acid (10) | 65 | | |
| Adipic acid (1) | 70 | | |
| Phthalic acid (1) | 70 | | |
| 2-Methoxy-5-nitrotropone (1) | 70 | | |

Porphobilinogenase activity increased very slowly when colourless callus was transferred from the dark to the light and up to 90min later it did not differ much from that of dark-grown callus. The results in Table 8 suggest a correlation between the appearance of porphobilinogenase activity and the synthesis of chlorophyll.

The intracellular distribution in these cultures was investigated by using conventional fractionation procedures; some differences in activity were detected in comparable fractions from these four types of callus.

*Effect of compounds added to the culture media on the synthesis of porphyrins and porphobilinogenase activity* (Table 9). The effect of the addition of various compounds to the culture media was studied to see whether they produce any significant change in porphobilinogenase activity or porphyrin synthesis.

The content of porphyrins in colourless callus, dark- or light-grown, or in the presence of gibberellic acid (100–250μg/ml), ATP (2–10mM) and biotin (20–50μg) was very low. Iron deficiency or the addition of δ-aminolaevulate (0.5–2.5mM) or ethionine (0.5–2mM) stimulated porphyrin accumulation; within a few hours after subculturing, the callus had the characteristic red fluorescence of porphyrins and a greenish-brown colour. Porphyrins, and presumably the porphyrinogens also, were bound very tightly to the tissue, as no porphyrin could be detected in the culture media. A preliminary analysis of the porphyrin content showed coproporphyrin to be the main component, although uroporphyrin, phyriaporphyrin and protoporphyrin were also detected.

Growth was prevented by δ-aminolaevulate and iron deficiency. Biotin had no effect on porphobilinogenase, and no activity was detected when 2mM-ATP was present in the media. Gibberellic acid, δ-aminolaevulate and ethionine produced a measured increase in enzyme activity.

Table 8. *Chlorophyll content and porphobilinogenase activity in soya-bean callus cultures*

Porphobilinogenase activity was assayed in the 24000*g* supernatant obtained as described in the Materials and Methods section. The cultures used in these experiments were as follows: dark-grown callus, callus cultures that were grown in the dark; first-generation callus, callus that was subcultured from dark-grown and then grown in the light; subcultures from this, after 14 days growth in the same conditions of illumination provided second-generation callus, from which third-generation callus was derived by subculturing after 14 days. N.D. = not determined.

| Material assayed | Chlorophyll content ($\mu$g/g fresh wt.) | $10^6 \times$ Sp. activity | $10^8 \times$ Sp. activity / chlorophyll content ratio |
|---|---|---|---|
| Dark-grown bean leaves | 31 | N.D. | |
| Light-grown bean leaves | 237 | N.D. | |
| Dark-grown callus | 1.7 | 0.34 | 20 |
| First-generation callus | 10.5 | 1.83 | 17 |
| Second-generation callus | 7.5 | 1.38 | 18 |
| Third-generation callus | 7.3 | 0.52 | 7 |

Table 9. *Effect of various compounds added to the basal medium on the synthesis of porphyrins and porphobilinogenase activity*

Cell-free extracts were prepared from callus grown semi-aerobically in the light or dark under standard conditions, in the presence of different additives or in the absence of iron; porphobilinogenase activity was determined in the 24000*g* supernatant as described in the Materials and Methods section. Concentrations of the additives shown are final concentrations in the media.

| Light conditions | Additives | $10^6 \times$ Sp. activity | Porphyrin formed by callus (nmol/g fresh wt.) |
|---|---|---|---|
| Light | None | 1.60 | 0.060 |
| Dark | None | 0.88 | 0.065 |
| Light | Gibberellic acid (3.3 $\mu$g/ml) | 3.71 | 0.100 |
| Dark | Gibberellic acid (3.3 $\mu$g/ml) | 3.38 | 0.030 |
| Dark | δ-Aminolaevulate (0.5 mM) | 3.16 | 0.800 |
| Dark | δ-Aminolaevulate (0.3 mM) | 2.71 | 0.500 |
| Dark | δ-Aminolaevulate (0.1 mM) | 2.63 | 0.115 |
| Dark | Minus iron | 0.76 | 0.538 |
| Light | Minus iron | 1.83 | 0.250 |
| Dark | ATP (2 mM) | 0.07 | 0.050 |
| Light | Ethionine (2 mM) | 3.70 | 0.200 |
| Light | Biotin (0.5 mM) | 1.40 | 0.030 |

## DISCUSSION

Procedures described for the isolation and purification of porphobilinogenase and the separation of isomerase and deaminase from each other are reproducible, although initial activities varied with different batches of callus culture. It has been found that the physical properties of the soybean enzymes are very different from those of enzymes with the same activities obtained from other sources.

Porphobilinogenase has been resolved into two components on starch-gel electrophoresis, indicating that at least in callus tissue two distinct enzymes contribute to the overall activity.

Molecular-weight determinations suggest that there should exist some association–dissociation of the individual components of this system. Although it seems strange that the molecular weight of the porphobilinogenase is less than that of the deaminase, determination of molecular weights under some conditions, particularly at low concentrations of protein, provided evidence of some dissociation of the deaminase into fractions of molecular weight $10000 \pm 1000$, although complete dissociation has not been observed.

When a preparation of isomerase obtained after step 7*c* of purification was chromatographed on dextran or polyacrylamide gel, a high molecular weight resulted. Evidence was obtained suggesting that a nucleoprotein might still be bound to the enzyme. The complex was dissociated on Sepharose 4B or 6B (Loeb, 1968; Locascio *et al.* 1969) and

it was found that the molecular weight of the isomerase is low. It has also been found that δ-aminolaevulate dehydratase, isolated from the same tissue, was firmly bound to a nucleoprotein (Tigier *et al.* 1970).

Bogorad (1958*a*,*b*), Lockwood & Benson (1960) and Sancovich *et al.* (1969*b*) have shown that porphobilinogenase and deaminase were as active in the absence of oxygen as in its presence; we have observed that the activity of porphobilinogenase was affected by the presence of air. The yield of uroporphyrinogens was highest under anaerobic conditions, although consumption of substrate was not modified by air, suggesting that some intermediate in the reaction was still being formed, but that this compound, probably some polypyrrolic intermediate (Llambías & Batlle, 1970*a*), must be in the reduced form to be further enzymically converted into uroporphyrinogens; the presence of air would prevent this reduction (A. M. Stella, V. E. Parera, E. B. C. Llambías & A. M. del C. Batlle, unpublished work).

So far callus tissue is the only system tested where a pronounced lag in uroporphyrinogen formation has been observed, which has allowed us to detect the new pyrrole intermediate.

A sharp optimum pH at 7.2 has been obtained for both porphobilinogenase and deaminase when the activity was measured in terms of uroporphyrinogens formed. A second broad optimum pH at about 6.7 was observed when porphobilinogen consumption was measured, suggesting perhaps that consumption of porphobilinogen occurs in two steps with two somewhat different pH optima.

Although isomerase was inactivated by preheating porphobilinogenase at 70°C, uroporphyrin synthesis was increased. The addition of $NH_4^+$ or porphobilinogen afforded some protection against heat inactivation. These results might suggest that the isomerase associates with the positively charged aminomethyl group of porphobilinogen or the intermediate polypyrrol by virtue of a negatively charged group, so when any amino derivative is present, it could bind the enzyme at the same active site and become stabilized.

The inhibitory action of cysteine and thioglycollate may be due either to competition with thiol groups in the enzyme for the substrate, an intermediate or to direct binding with any of them.

The activating effect of certain concentrations of sodium salts and magnesium salts is still under study, but they could probably act by producing some association–dissociation phenomena, giving the enzyme a definite structural arrangement necessary for maximal activity.

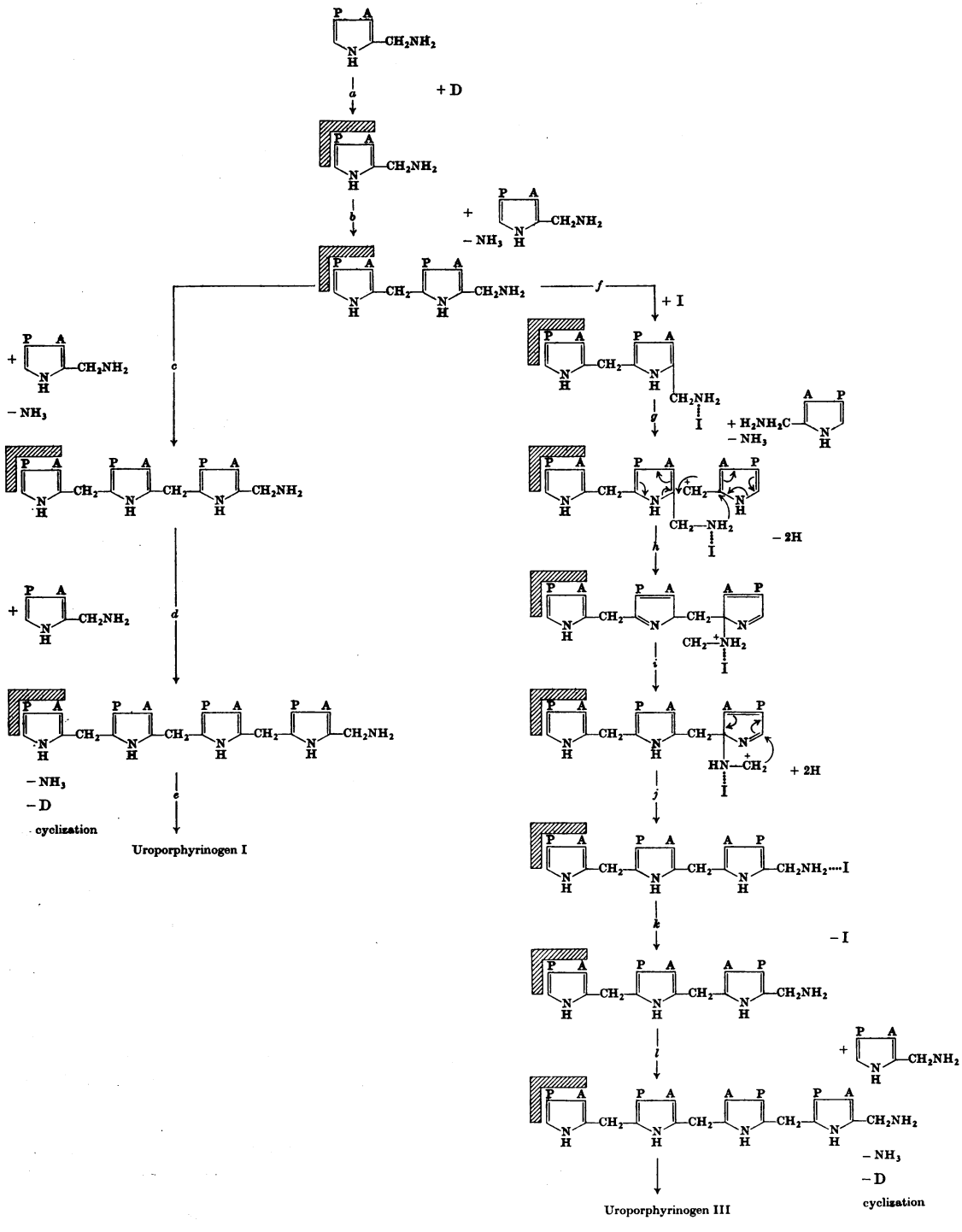
The inhibitory effect of nitrogen bases on the isomerase could be due to a competition between isomerase and the base for its substrates. On the other hand, dicarboxylic acids might also inhibit deaminase by competition with porphobilinogen for some positively charged groups on the enzyme, perhaps the amino groups; this hypothesis was in some way supported by the fact that 2-methoxy-5-nitrotropone also inhibited deaminase.

When dark-grown callus was exposed to light, porphobilinogenase activity increased, being paralleled by an expected increase in chlorophyll content, but after 1 generation in the light the enzyme and chlorophyll contents adapted to the new light conditions and did not increase further. The enzyme activity, on a protein basis, increased with time in greening callus, probably owing to synthesis of a new protein rather than the activation of existing protein. Porphobilinogenase, expressed on a chlorophyll basis, was the same in dark-grown callus and first and second generation of light-grown callus, indicating that porphobilinogenase plays a direct part in chlorophyll synthesis. It has in fact been proposed that light induces the synthesis of enzymes necessary for chlorophyll production (Stobart & Thomas, 1968*b*). The qualitative picture that has emerged from the study of chlorophyll development when dark-grown callus was placed in the light was similar to the picture obtained when etiolated leaves were illuminated (Gassman & Bogorad, 1967).

The addition of ethionine or δ-aminolaevulate to the culture media or iron deficiency stimulated porphyrin synthesis, as has been observed with cultures of *Rhodopseudomonas spheroides* (Lascelles, 1959, 1969; Gibson, Neuberger, & Tait, 1962*a*,*b*; Gajdos, Gajdos-Török, Gorchein, Neuberger & Tait, 1967). δ-Aminolaevulate produced an increase of porphobilinogenase activity, consistent with the accumulation of porphyrins. δ-Aminolaevulate dehydratase activity was also enhanced under these conditions, so it is possible that δ-aminolaevulate has induced or activated δ-aminolaevulate dehydratase, which then formed greater amounts of porphobilinogen, and this in turn resulted in an increased activity of porphobilinogenase.

In the presence of ATP, porphobilinogenase activity was not detected. Gajdos *et al.* (1967) showed that all nucleoside triphosphates diminish porphyrin excretion in *Rh. spheroides* and they suggested that ATP could control porphyrin excretion by modifying the activities of the enzymes involved in its pathway; however, in callus cultures, since ATP was present in the growth medium, it could probably function by repressing enzyme formation rather than by inhibiting its activity.

*Mechanism for the conversion of porphobilinogen into uroporphyrinogens* (Scheme 1). The way in which the cyclic uroporphyrinogens are formed from porphobilinogen is unknown and many




Scheme 1. Scheme for the enzymic formation of uroporphyrinogens I and III from porphobilinogen. A, Acetate side chain; P, propionate side chain; D or ⌐, porphobilinogen deaminase; I, uroporphyrinogen isomerase; see the text for explanation.

hypotheses have been put forward, although some of them have been rejected on the basis of experimental evidence. The scheme postulated by Cornford (1964) seems to be one of the most likely, but must be modified to take account of the latest experimental work (Sancovich *et al.* 1969*a*,*b*; Llambías & Batlle, 1970*a*,*b*,*c*,*d*, and the present paper).

It is suggested that the first porphobilinogen molecule is bound to deaminase through its two carboxyl groups and its α-free position (reaction *a*); the binding groups on the enzyme might be positively charged amino acid side chains. Then, deaminase catalyses the formation of a dipyrrol with the second porphobilinogen molecule (reaction *b*); from this dipyrrol a linear tripyrrol, with already the structure of series III, is formed by the action of the isomerase, which catalyses a reaction between the substituted α-positions of the dipyrrol and a third porphobilinogen molecule (reactions *f*, *g*, *h*) and a subsequent migration of the side chain (reactions *i*, *j*) (Cookson & Rimington, 1954; Robinson, 1955; Bullock, Johnson, Markham & Shaw, 1958). As stated above, isomerase could bind either the dipyrrol or the new porphobilinogen unit through the positively charged aminomethyl groups by means of a negative residue. In reaction (*k*), the tripyrrol is liberated and then a fourth porphobilinogen unit is added, forming uroporphyrinogen III. As suggested by Cornford (1964) and Sancovich *et al.* (1969*b*), a competition between isomerase and porphobilinogen is proposed to occur at specific points (reactions *c* and *f*) so that in the absence of isomerase or in the presence of an excess of substrate an abnormal tripyrrol would be formed, which would finally produce a greater proportion of uroporphyrinogen I. Considering the accumulation of the polypyrrolic intermediate in these tissues, it is also postulated that reactions (*a*)–(*k*) or (*a*)–(*c*), occur very rapidly and that the fourth porphobilinogen molecule is added less easily (as was also suggested by Cornford, 1964).

The mechanism proposed is in many respects similar to that of Cornford (1964). It is speculative but takes into account experimental data already considered in that scheme and further evidence obtained later.

We have also found that hydroxylamine and $NH_4^+$ are poor inhibitors of deaminase but greatly inhibited isomerase. This could be due to the interference by hydroxylamine and $NH_4^+$ with the attachment of the charged α-aminomethyl groups of the substrate to isomerase. H. A. Sancovich, A. K. Rosenberg, A. M. del C. Batlle & M. Grinstein (unpublished work) have observed that many nitrogen bases exhibit a similar effect.

Some aliphatic and aromatic compounds with two carboxyl groups inhibited deaminase activity (Llambías & Batlle, 1970*d*, and the present paper), supporting the idea that two of the sites of attachment on the deaminase are specific for the propionate and acetate side chains of porphobilinogen; in addition, 2-methoxy-5-nitrotropone, which specifically reacts with free amino groups of proteins, inhibited deaminase.

The formation of a new intermediate resulting from the action of porphobilinogenase on porphobilinogen and an abnormal intermediate that is formed by the action of deaminase on porphobilinogen (Llambías & Batlle, 1970*a*) suggested that isomerase would function at a step after the formation of a dipyrrol but before the insertion of the last molecule of porphobilinogen, although the possibility that isomerase could act at an earlier stage can not be excluded. However, it has not been possible to identify which enzyme adds the fourth porphobilinogen molecule.

It has also been found that the soya-bean callus enzymes have kinetic behaviour and parameters very different from those of the same enzymes obtained from other sources (Lockwood & Benson, 1960; Sancovich *et al.* 1969*b*; Llambías & Batlle, 1970*b*,*c*).

Finally, the possibility that isomerase could participate stoicheiometrically rather than catalytically in the formation of uroporphyrinogen III, or that it could be inactivated during the reaction, cannot be discarded (Levin, 1968).

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