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Polypyrroles Formed from Porphobilinogen and Amines by Uroporphyrinogen Synthetase of *Rhodopseudomonas spheroides*

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1. Uroporphyrinogen I synthetase of *Rhodopseudomonas spheroides* was purified more than 200-fold from the soluble protein of broken bacterial cells. The enzyme had molecular weight 36000, an isoelectric point of 4.46 and migrated as a single active protein band on disc-gel electrophoresis at pH 7.5 and 8.9. 2. The enzyme consumed porphobilinogen and formed uroporphyrinogen at pH 8.2 without the accumulation of intermediates. In the presence of hydroxylamine, ammonia or methoxyamine the production of porphyrinogen was inhibited and the enzyme formed open-chain polypyrroles instead. 3. These polypyrroles behaved like uroporphyrinogen on Sephadex G-25; they were colourless and had unsubstituted α-pyrrolic positions. The inhibitory amines were incorporated into the molecules. 4. The polypyrroles formed porphyrins non-enzymically and the cyclization reaction was accompanied by the release of the inhibitory amine. Exchange of the amino function of the original porphobilinogen in the polypyrrole was complete with hydroxylamine and almost complete with methoxyamine, both ammonia and methoxyamine being present in the polypyrrolic material. 5. The behaviour, properties and composition of the radioactive hydroxylamine derivative were consistent with a tetrapyrrolic structure, probably a pyrrylmethane, that was not cyclized, rather than with di-, tri- or penta-pyrrolic structures. No monopyrrolic or dipyrrolic Ehrlich-positive material was released on cyclization. The ammonia and methoxyamine derivatives had properties similar to the hydroxylamine derivative. 6. Another modified pyrrole was detected only in experiments with hydroxylamine. It differed from both porphobilinogen and known dipyrroles and appeared to be a monopyrrole. 7. The participation of positively charged reaction centres in the enzymic mechanism, particularly in the cyclization step, is discussed.

Uroporphyrinogen synthetase catalyses the condensation of 4 molecules of porphobilinogen to uroporphyrinogen isomer I with the elimination of 4 molecules of ammonia. A second enzyme, uroporphyrinogen III cosynthetase, which is more labile to heat than the synthetase, is inactive towards porphobilinogen alone or preformed uroporphyrinogen I, but used in conjunction with the synthetase alters its activity so as to form uroporphyrinogen III, the precursor of haem and chlorophyll.

In spite of much speculation on the type and nature of possible intermediates (Margoliash, 1961; Mathewson & Corwin, 1961; Cornford, 1964; Dalton & Dougherty, 1969; Llambias & Batlle, 1971*a*; Frydman, B. *et al.*, 1971) the mechanism of the action of the enzyme is not understood. With enzymes from most sources, e.g. wheat germ, spinach leaves, cow liver and erythrocytes, intermediates do not accumulate, which suggests that the complete series of reactions occurs on the enzyme surface, although intermediate polypyrroles accumulate if hydroxylamine or ammonia is present (Bogorad, 1963). Llambias & Batlle (1971*a*) showed that enzymes from soya-bean callus tissue give intermediates.

In the present work we prepared uroporphyrinogen synthetase from the photosynthetic bacterium *Rhodopseudomonas spheroides* and looked for the occurrence of intermediates. The separation of the enzymic activities of synthetase and co-synthetase in this organism was first demonstrated by Hoare & Heath (1959).

Bogorad (1963) found that hydroxylamine and ammonia prevented the formation of porphyrins, but did not alter the rate of consumption of porphobilinogen by enzymes isolated from wheat germ and spinach leaves. Recently he and his co-workers reported the isolation of a dipyrrole (Pluscec & Bogorad, 1970) and of a tetrapyrrole (Radmer & Bogorad, 1972) from systems containing hydroxylamine and ammonia respectively.

Batlle and co-workers (Llambias & Batlle, 1970; Stella *et al.*, 1971) found that enzymes from soya-bean callus consumed porphobilinogen initially at a

much faster rate than expected for the amount of porphyrin formed, suggesting the presence of intermediates. Two apparent tripyrroles have been isolated.

We have investigated the reaction of uroporphyrinogen synthetase and porphobilinogen with hydroxylamine and ammonia in more detail and have, in addition, used another compound methoxyamine, whose incorporation into polypyrroles has been demonstrated.

Materials and Methods

Organisms

R. spheroides (N.C.I.B. 8253) was grown in medium S of Lascelles (1956) semi-anaerobically in the light at 32–34°C for 40–48h in 3 litre conical flasks. Cells were harvested, washed with 0.05M-potassium phosphate buffer, pH7.5, resuspended in phosphate buffer and stored at −20°C until needed (Tait, 1970).

Chemicals

5-Amino[4-^{14}C]laevulinic acid hydrochloride, [^{3}H]dimethyl sulphate, [^{3}H]acetic anhydride and sodium [^{125}I]iodide were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Methoxyamine hydrochloride was purchased from Eastman Kodak Co., Rochester, N. Y., U.S.A. AnalaR hydroxylamine hydrochloride, calcium phosphate gel and protamine sulphate were from BDH Chemicals Ltd., Poole, Dorset, U.K. *p*-Dimethylaminobenzaldehyde for Ehrlich's reagent was obtained from Fisons Scientific Co. Ltd., Loughborough, Leics., U.K., and from May and Baker Ltd., Dagenham, Essex, U.K. DEAE-cellulose (DE-23) was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; Sephadex G-15, G-25 and G-100 from Pharmacia, Uppsala, Sweden. Opsopyrroledicarboxylic acid, porphobilinogen monohydrate, porphobilinogen lactam and aminolaevulinic acid hydrochloride were recrystallized samples prepared in this laboratory. 4,3′-Di(carboxymethyl)-3,4′-di-(2-carboxyethyl)-dipyrrylmethane (dipyrrole 1), 3,3′-di(carboxymethyl)-4,4′-di-(2-carboxyethyl)dipyrrylmethane (dipyrrole 2) and 5,5′-di(aminomethyl)-4,4′-di(carboxymethyl)-3,3′-di-(2-carboxyethyl)dipyrrylmethane (dipyrrole 3) were obtained from Dr. S. F. Macdonald, National Research Council, Ottawa, Ont., Canada.

Substrates

Porphobilinogen was produced enzymically from 5-aminolaevulinic acid and partially purified aminolaevulinate dehydratase from *R. spheroides*. The partial purification is described below.

Enzyme (activity 330000nmol of porphobilinogen/h at 37°C) in 210ml of solution was added to 50mmol of potassium phosphate, 40mmol of sodium bicarbonate, 10mmol of cysteine and 2mmol of $MgCl_2$ at pH8.0 in 700ml of a solution that had been bubbled vigorously with N_2 for 20min. After 10min, 2g of aminolaevulinic acid hydrochloride in 100ml of water was adjusted to pH7.6 and added to the incubation mixture. The flask was flushed with N_2 and stoppered. After 16h at 37°C the formation of porphobilinogen had ceased and was 39.5% complete. Protein and salts were precipitated with 40ml of 25% (w/v) $CuSO_4$, the solution was centrifuged and the supernatant adjusted to pH6 with acetic acid. Porphobilinogen was then adsorbed on a column of Dowex 2 (acetate form; 720ml). The column was washed well with de-aerated water and the porphobilinogen was eluted with successive volumes of 500ml of 1M-acetic acid and 500ml of 0.2M-acetic acid.

Porphobilinogen (445mg) was freeze-dried and purified according to Cookson & Rimington (1954) and crystallized as the monohydrate (Westall, 1952). Very pale-pink crystals (128mg) were obtained, which were 98% pure by weight based on the value of $\epsilon = 57700$ litre·mol^{-1}·cm^{-1} for the reaction with Ehrlich's reagent (Bogorad, 1958*a*, 1962). Mother liquor from crystallizations and column washings were freeze-dried in fractions containing 50mg of porphobilinogen and freed of coloured impurities and polymeric material by gel filtration on Sephadex G-25 in 0.05M-potassium phosphate buffer, pH7.5. Colourless solutions of porphobilinogen containing 3mg/ml were stored at −20°C.

[^{14}C]Porphobilinogen was prepared on a smaller scale by incubation of 50μCi of 5-amino[4-^{14}C]-laevulinic acid with 250mg of aminolaevulinic acid hydrochloride and enzyme (activity 218000nmol of porphobilinogen/h) in a total volume of 150ml at 37°C for 5h, when 42% conversion into porphobilinogen had occurred. Porphobilinogen was separated from aminolaevulinate, freeze-dried, purified and crystallized from water, pH4.0, as above. After gel filtration on Sephadex G-25, the specific radioactivity of porphobilinogen in each fraction was 121c.p.m./nmol.

Acetylporphobilinogen

N-Acetylporphobilinogen was prepared by acetylation of porphobilinogen with acetic anhydride at pH8. The mixture was adsorbed on Dowex 2 (acetate form) and the resin was washed with 1M-acetic acid. *N*-Acetylporphobilinogen was eluted with 6M-acetic acid and the solution was freeze-dried.

Methoxyamine

A radioactive sample of methoxyamine hydrochloride was prepared on a small scale from [^{3}H]-

dimethyl sulphate, $NaNO_2$, $Na_2S_2O_5$, acetic acid and ice according to the method developed by Hjeds (1965) but scaled down 100-fold. The product was recrystallized from ethanol; it did not reduce Fehling's solution, and hence was free of hydroxylamine. The specific radioactivity was 124d.p.m./nmol as determined after 100-fold isotope dilution and recrystallization. The purity was 89% as radioactive methoxyamine.

Purification of enzymes

Uroporphyrinogen I synthetase (porphobilinogen deaminase) from R. spheroides. Concentrated suspensions of cells were disrupted at 0°C by ultrasonication or by passage through a French pressure cell. Suspensions were centrifuged at 100000*g* for 1.5h. The clear supernatant was the 'crude extract'. Uroporphyrinogen synthetase was precipitated by adding $(NH_4)_2SO_4$ to the crude extract (7–10mg of protein/ml) in 0.05M-potassium phosphate buffer, pH7.5, at 0°C. Protein precipitating between 40% and 60% saturation with $(NH_4)_2SO_4$ was centrifuged, dissolved in small volumes of the phosphate buffer and dialysed against 4 litres of buffer. The solution containing approx. 1g of protein was applied to a column [2.5cm×50cm; packed under N_2 pressure at 35kPa (5lb/in^2)] of DEAE-cellulose, equilibrated with either 0.025M-potassium phosphate buffer, pH7.5, or 0.02M-Tris–HCl buffer, pH7.5, at 5°C. The enzyme was eluted with a linear gradient formed from either 0.025M-potassium phosphate, pH7.5 (500ml), and 0.25M-potassium phosphate buffer, pH7.5 (500ml), or alternatively from 0.02M-Tris–HCl, pH7.5 (750ml), and 0.02M-Tris–HCl buffer, pH7.5, containing 0.3M-NaCl (750ml). The flow rate was 18ml/h and 25ml fractions were collected. The enzyme was eluted at conductivity of 0.012–0.015mho, equivalent to 0.09M-potassium phosphate buffer, pH7.5, or 0.11M-NaCl. Samples containing enzyme were combined and dialysed against 2 litres of potassium phosphate buffer (5mM). At this stage of purification a large amount of nucleoprotein material was usually present ($\lambda_{max.}$ 259nm) so the sample in the dilute phosphate buffer (125ml; 1.6mg of protein/ml) was treated with washed calcium phosphate gel (67ml; 2g), stirred gently at 5°C for 40min and centrifuged. The supernatant solution was applied to a second column (16.5cm×1.6cm) of DEAE-cellulose in 0.05M-Tris buffer, pH9.5, and enzyme was eluted with a linear gradient formed from 0.05M-Tris, pH9.5 (75ml), and 0.05M-Tris buffer, pH9.5, containing 0.3M-NaCl (75ml). Fractions (2.5ml) were collected at a flow rate of 10ml/h.

The enzyme was eluted together with a yellow protein at a conductivity of 0.009–0.0115mho, i.e. 0.09M-NaCl. Enzyme fractions were combined, concentrated to 5ml with Carbowax (G.T. Gurr Ltd., London S.W.6, U.K.) and applied to a column (2.25cm×50cm) of Sephadex G-100 in 0.025M-potassium phosphate buffer, pH7.5. The flow rate was 18.5ml/h and 4.5ml fractions were collected. The enzyme was eluted close behind a yellow protein band that constituted the major protein material detected by disc-gel electrophoresis after the DEAE-cellulose step. The solution of the enzyme was concentrated 10-fold either with an Amicon ultrafiltration cell and a UM-10 membrane or by treatment with Carbowax. Enzyme samples at this stage of purification (activity 6000nmol/h per mg) were used for the preparation and investigation of the polypyrrole intermediates.

Smaller samples of enzyme were purified further. The enzyme was largely separated from the yellow protein by a second filtration on Sephadex G-100. The enzyme sample (0.8mg) was analysed with the LKB isoelectric-focusing apparatus model 8101 and with ampholytes covering the pH ranges 3–10 and 3–6. Two main protein components with isoelectric points of 4.05 and 4.46 were present. These were separated by chromatography on DEAE-cellulose at pH4.5. Enzyme fractions from Sephadex containing 1.0–1.5mg of protein were absorbed on a small column (1.5ml vol.) of DEAE-cellulose in 0.05M-sodium acetate buffer, pH4.5, and enzyme was eluted with a linear gradient formed from 0.05M-sodium acetate (40ml) and 0.05M-sodium acetate buffer containing 0.1M-NaCl (40ml); the flow rate was 10ml/h and fractions (2.5ml) were collected. The enzyme was eluted at the start of the gradient (conductivity 0.004mho; 0.015M-NaCl) and another protein fraction was eluted at conductivity 0.005mho. Fractions were combined, dialysed against 0.05M-potassium phosphate buffer, pH7.5, concentrated to 1ml with Carbowax, dialysed against 0.05M-potassium phosphate buffer, pH7.5, and the purest samples of enzyme were investigated.

All purification steps were monitored for enzymic activity by both the consumption of porphobilinogen and by formation of uroporphyrin, for protein by absorbance at 280nm and by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard protein (Armour Pharmaceutical Co., Eastbourne, U.K.) and for the number of protein bands detected by disc-gel electrophoresis at both pH8.9 and pH7.5.

Variation of the order of the purification steps and the treatment of the crude extract with calcium phosphate and protamine sulphate at pH7.5 did not alter the overall degree of purification. Treatments with alumina C_γ gel and hydroxyapatite in 1.5mM-phosphate buffer, or with CM-cellulose and phosphocellulose at pH7.5 were not satisfactory. Chromatography of the crude extract on DEAE-cellulose at pH10.0 and subsequent filtration on Sephadex G-100 and chromatography on DEAE-cellulose at pH6.5

and 8.5 or gel filtration with Sephadex G-200 did not improve the purification significantly.

Aminolaevulinate dehydratase. Preparation of this enzyme from *R. spheroides* has been described by Nandi *et al.* (1968). Dehydratase for preparation of porphobilinogen was partially purified; disrupted cells were centrifuged at 100000*g* and the enzyme was precipitated from the supernatant solution between 20 and 40% saturation with $(NH_4)_2SO_4$, dissolved in buffer, dialysed and chromatographed on DEAE-cellulose at pH 8.0 in 0.01 M-Tris buffer with a linear salt gradient of KCl (0.07–0.33 M). The enzyme eluted at 0.18 M-KCl and was assayed by the method of Burnham & Lascelles (1963).

Determinations

Uroporphyrinogen synthetase assay. The enzyme has two measurable activities, namely the consumption of porphobilinogen and the formation of uroporphyrin. A dissociation of the two activities was sought. Assays were based on those used by Bogorad (1962) and by Levin & Coleman (1967). The bacterial enzyme was 20–1000 times more active than mammalian enzymes and it proved unnecessary to remove protein from the incubation mixture before assay.

(i) Porphobilinogen. Substrate (0.4 mM; 100–250 μl) in 0.1 M-Tris–0.5 mM-EDTA buffer, pH 8.2, was incubated in small tubes with and without enzyme (5–10 μl) at 37°C for up to 1 h. The reaction was stopped and the remaining porphobilinogen was measured by the addition of 100 μl of solution to 1.4 ml of water and 1.5 ml of modified Ehrlich's reagent (Mauzerall & Granick, 1956). The full magenta colour at 553 nm was reached and measured after 15 min. The difference in absorbance for test and control samples measured the consumption of porphobilinogen. A value of $\epsilon = 57700$ litre·mol^{-1}·cm^{-1} at 553 nm was used in all experiments (Bogorad, 1958*a*, 1962). The concentration of porphobilinogen was always kept above 0.1 mM as changes in absorbance were then linear over 2 h and the rate was independent of substrate concentration. The non-enzymic loss of porphobilinogen in 1 h at 37°C was negligible.

(ii) Porphyrin. Samples (100 μl) of the incubation mixture were added to 100 μl of either 0.05 M-potassium phosphate, pH 7.5, or 0.1 M-Tris buffer, pH 8.2. The excess of porphobilinogen was iodinated and the uroporphyrinogen was oxidized to uroporphyrin in the same step by addition of 2 μl amounts of 0.1 M-I_2 in KI until the solution retained a pale-yellow colour. The volume was brought to 3.0 ml with either phosphate or Tris buffer and a crystal of $Na_2S_2O_3$ was added to remove excess of I_2. The Soret band at 399 nm was measured with a Unicam SP. 700. A value of $\epsilon = 224 \times 10^3$ litre·mol^{-1}·cm^{-1} at 399 nm was used for uroporphyrin I in phosphate or Tris buffer, pH 7.5–8.2, and a value of 495×10^3 litre·mol^{-1}·cm^{-1} used at 406 nm in 5% HCl.

These values were consistent with published values for the extinction coefficients (Bogorad, 1958*a*, 1962; Mauzerall & Granick, 1958; Rimington, 1962; Falk, 1964) of the absorption bands in the Soret and visible regions of the spectrum. The specific radioactivity of uroporphyrin I and of uroporphyrin octamethyl ester was exactly four times that of porphobilinogen if the values reported here were used (see below). The value in 5% HCl is lower than that used by Rimington (1962) and slightly higher than that used by Batlle & Grinstein (1964). Because of the variable value reported in acid, spectra were nearly always recorded in phosphate or Tris buffer. Oxidation of uroporphyrinogen with I_2 was more convenient than long autoxidation or photo-oxidation of uroporphyrinogen in HCl. Since orange uroporphomethenes were formed in air and were not rapidly oxidized to uroporphyrin by I_2 in acid solution, all oxidations were done at pH 7.5 or 8.2. It was essential to avoid contamination of porphyrin solutions by metal salts, especially Zn^{2+}, by including EDTA in the buffers. In phosphate or Tris buffers Zn^{2+}–uroporphyrin was formed very readily and was irreversibly oxidized by traces of I_2, resulting in loss of the characteristic porphyrin spectrum and destruction of the fully conjugated porphyrin ring system.

(iii) Isomer percentage. Relative amounts of uroporphyrins I and III were estimated after paper chromatography. Porphyrins were absorbed on to talc at pH 4.5, eluted with dilute ammonia, dried and esterified with methanol–H_2SO_4. The esters were extracted into chloroform and spectra examined from 380 to 700 nm to eliminate possible contamination by metals or other porphyrins. A value of $\epsilon = 213 \times 10^3$ litre·mol^{-1}·cm^{-1} at 406 nm was used. The ester (1 μg) was chromatographed on paper according to the method of Cornford & Benson (1963). A solvent mixture of paraffin (b.p. 190–215°C)–dioxan (4:1.5, v/v) was best for separation of the isomers at room temperature. Comparisons were made visibly with standard mixtures of uroporphyrin I and III containing 0, 25, 50, 75 and 100% of uroporphyrin I. Uroporphyrin III was prepared enzymically from porphobilinogen and a crude extract of *R. spheroides*. Solvents were purified by procedures given by Falk (1964).

Ehrlich reaction. All pyrroles were detected and determined with modified Ehrlich's reagent in acetic acid–perchloric acid (Mauzerall & Granick, 1956). The rates of colour development, wavelength of maximum absorption and the shape of the absorption curve were monitored for all pyrroles and intermediates by using 1 cm cuvettes in a Unicam SP. 600 and a Unicam SP. 700 recording spectrophotometer.

Initial spectra were determined within 15s of mixing samples (50–100 μl) with reagent (2.9ml).

Radioactivity. [^{14}C]Porphobilinogen, polypyrroles, porphyrins and [^{3}H]methoxyamine were counted with a Nuclear–Chicago scintillation counter model 6860 in scintillation fluid containing a toluene solution of 2,5-diphenyloxazole (4g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (1g/l)–Triton X-100 (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) (2:1, v/v). Samples (50μl) in aqueous buffer solution from the Sephadex column were added to 10ml of scintillation solution to give clear solutions at 5°C and counted two to four times for 10min periods. Quenching was corrected with the external-standard channels-ratio quench correction method; the counting efficiency was usually about 22% for ^{3}H and 82% for ^{14}C for all samples. ^{125}I was counted in solution with a Packard Tri-Carb scintillation γ counter. By using the specific radioactivities all subsequent amounts of radioactivity were converted into nmol of radioactive material. [^{14}C]Uroporphyrin I was isolated from several experiments by gel filtration and precipitation. The specific radioactivity of the uroporphyrin I and of the octamethyl ester calculated with the aid of the extinction coefficients given above was 480c.p.m./nmol, i.e. 120±2c.p.m./nmol of monopyrrole, in agreement with the value determined for porphobilinogen.

Separation of polypyrroles from proteins and porphobilinogen on Sephadex columns. In many experiments described below, polypyrroles can be made to accumulate. The general procedure described here is adopted for their preparation and separation from protein and substrate.

[^{12}C]Porphobilinogen or [^{14}C]porphobilinogen (5–7μmol; 1.3–1.5mg) in 0.1M-Tris–5mM-EDTA buffer, pH8.2, was incubated in small tubes under N_2 with portions (0.09ml) of 2M-methoxyamine hydrochloride or 4M-NH_4Cl or 2M-methoxyamine hydrochloride adjusted to pH8.2 and 0.1–0.2ml of solution of enzyme (activity 46000nmol/h per ml). The higher concentration of enzyme was used for experiments with ammonia and methoxyamine, since the rate of consumption of porphobilinogen was slightly decreased. The mixture (0.85–0.95ml) was incubated at 37°C under N_2 in the dark for 1.5h. Samples (5μl) were withdrawn at zero time and 1.5h and analysed for porphobilinogen, porphyrin and radioactivity. For experiments with [^{3}H]methoxyamine hydrochloride, buffer solutions were bubbled with N_2, added to solid methoxyamine hydrochloride and then 0.65ml of this solution was added directly to 1.3mg of porphobilinogen monohydrate.

After incubation for 1.5h the almost colourless clear solution of uroporphyrinogen plus polypyrrole was applied under N_2 to the standard column (0.95cm×170cm) of Sephadex G-25 that had been equilibrated at 5°C with 0.05M-potassium phosphate buffer, pH7.5, or 0.05M-phosphate buffer containing 10mM-EDTA and saturated with N_2. In a few experiments, 0.025M-phosphate or ammonium acetate was used. The column was wrapped in aluminium foil and N_2 was bubbled continuously through the eluting buffer to minimize oxidation of polypyrrole and porphyrinogen. The flow rate was 25ml/h at 5°C. After the first 25ml, fractions (3ml) were collected and kept cold away from the light. Porphyrinogen was eluted after 2h (51ml) and porphobilinogen was eluted after 3.25h (79ml). Free uroporphyrin was retarded and eluted free of other pyrroles near fraction 26 (103ml) and a heptacarboxylic acid porphyrin was eluted near fraction 44 (156ml).

Solutions were monitored for protein by absorbance at 280nm, and for porphyrin in the Soret region after oxidation. Samples (0.2ml) were oxidized with 3μl of 0.1M-I_2 in KI and diluted to 3.0ml with Tris or phosphate buffer. Polypyrroles and porphobilinogen were detected by reaction with Ehrlich's reagent. The amount of polypyrrole intermediate, X, was determined by heating samples (0.2ml) at 55°C for 1.25–1.5h and determining the increase in porphyrin formed non-enzymically. Heating for 1h, however, produced the maximum amount of porphyrin. Absorption spectra of porphyrins were then recorded from 380 to 700nm both before and after oxidation with I_2 to ensure that porphyrinogen was not present and that Zn^{2+}–uroporphyrin had not been formed. The amount of polypyrrole X was expressed as nmol of porphyrin. Individual 3ml fractions were investigated immediately after their isolation. When large batches were examined samples in phosphate or ammonium acetate buffer were combined under N_2, freeze-dried and filtered on Sephadex G-25 the next day. This column was used for all the experiments reported in this work.

Determination of free hydroxylamine. Free hydroxylamine and simple hydroxamates in solutions were measured by the method of Seifter *et al.* (1960) scaled down 10-fold. This involved oxidation of hydroxylamine to nitrite by I_2 in acetic acid, diazotization of sulphanilic acid and coupling to α-naphthol. Samples (0.5ml) containing 0–100nmol of hydroxylamine were analysed in a total volume of 3.0ml. Absorbance at 520nm after 10min was linear to 0.80 with 100nmol of hydroxylamine. No interference was noted with 40μmol of potassium phosphate. Test solutions, test solutions+40nmol of hydroxylamine as internal standard, blanks and controls were used with and without α-naphthol and sulphanilic acid.

Determination of free ammonia. Free ammonia was determined by the method of Chaney & Marbach (1962), involving the nitroprusside-catalysed conversion of ammonia, alkaline hypochlorite and phenol into the stable blue colour of indophenol. The method was scaled down to measure 0–100nmol of

ammonia with 300μl of both reagents and 2.4ml of sample. E_{625} increased linearly with concentration of ammonia.

No interference was found with 50μmol of phosphate, 2.5μmol of EDTA, enzyme alone, hydroxylamine or methoxyamine (100nmol). Tris (40μmol) prevented the colour development completely; porphobilinogen, uroporphyrinogen and uroporphyrin all interfered strongly. Thus for determining stoicheiometries ammonia was removed from the solution by microdiffusion in small Conway vessels. Ammonia from samples (0.4 or 1.2ml) was collected in 0.1ml of 0.1M-HCl for 20h at 5°C and adjusted to 3ml in a volumetric flask. The recovery of ammonia was quantitative within 2%. Standard NH_4Cl (100nmol) gave E_{625} 0.77 above a control blank after 30min at room temperature.

Ammonia released when polypyrrole is heated. Sample fractions (3ml) containing the intermediate polypyrrole in phosphate buffer were obtained from Sephadex G-25. Portions (0.2ml) of column fractions were analysed for porphyrinogen and portions (1.2ml) were analysed for ammonia in small Conway units at 5°C. The remainder was heated at 55°C for 1.25h, cooled and again analysed for porphyrin and ammonia. The unheated fractions contained only traces of ammonia, probably owing to a very slow release from the intermediate at 5°C. Finally, the porphyrin in the alkaline solution was determined to check that unheated polypyrrole had not formed much porphyrin and hence released more ammonia.

Thin-layer electrophoresis. This was carried out in 0.05M-veronal buffer, pH9.2 at 5°C, by the method of Pluscec & Bogorad (1970). Several samples were applied on small pieces of filter paper to cellulose plates (0.5mm thick) sandwiched between top layers of Parafilm and glass and held with clips. A potential gradient of only 19–20V/cm was applied over a 15cm plate, and may thus afford less resolution than systems used by others. Pyrroles were detected by spraying the plate with Ehrlich's reagent. On a preparative scale the sample was applied as a band and electrophoresis was continued for 1.5h. The centre portion was covered up and the outer edges were sprayed with Ehrlich's reagent; the centre portion was scraped off and free pyrroles were eluted with water. Mobilities of pyrroles (m_p) are expressed relative to the mobility of porphobilinogen ($m_p = 1$).

Disc-gel electrophoresis was carried out with the Shandon apparatus (model SAE 2731) and method (Shandon Scientific Co. Ltd., London N.W.10, U.K.), but omitting the spacer gel. Samples were applied in 100μl of 0.75% sucrose or glycerol solution to the top of 7.5% (w/v) polyacrylamide gels, followed by layers of water and running buffer. Electrophoresis at pH8.9 showed only one enzyme and protein band for fractions after the first chromatographic step, so conditions were altered to improve the resolution. The gel was prepared in 1.2M-glycine–0.06M-KOH buffer as described in the Shandon instruction manual for pH6.6; the running buffer contained 7.6ml of lutidine and 2.75g of glycine/l. But after prior electrophoresis at 3mA/tube for 1.5h at 5°C the pH value in the gel was constant at 7.44. Samples were then applied to the gel in sucrose and water and electrophoresis was continued for 1.5h at a very low current (1mA/tube at 200V). Protein bands were stained with Amido Schwarz and were extremely sharp. The enzyme was located by immersing the whole gel in substrate solution (0.3ml) at 40–50°C for 5–10min and looking in u.v. light for the red fluorescence.

Molecular weight of enzyme. This was determined by gel filtration on a column (2.25cm × 50cm) of Sephadex G-100 at pH7.5 according to the method of Andrews (1964). Comparison was made with standards of bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.), ovalbumin (R. D. Marshall, of this Department) chymotrypsinogen (L. Light and Co., Colnbrook, Bucks., U.K.) and cytochrome *c* (BDH Chemicals Ltd.). Determinations of molecular weight for samples of purified enzyme were made in the presence and in the absence of 2-mercaptoethanol by the method of Weber & Osborn (1969) with 10% polyacrylamide gels in 0.1% sodium dodecyl sulphate. Comparisons were made with reduced samples of bovine serum albumin, catalase (C.F. Boehringer und Soehne, Mannheim, Germany), ovalbumin, pepsin and trypsin (Armour Pharmaceutical Co.) and lysozyme [Sigma (London) Chemical Co., London S.W.6, U.K.].

Results and Discussion

Uroporphyrinogen I synthetase from R. spheroides

Uroporphyrinogen I synthetase was purified more than 200-fold from the crude extract (Table 1). Our most purified samples had a specific activity of over 27000nmol of porphobilinogen/h per mg of protein at 37°C. They showed a single active protein band on disc-gel electrophoresis at pH8.9 and only one active major protein band at pH7.5. Traces of very sharp minor bands of protein were detected at pH7.5. Only one active band was detected by disc-gel electrophoresis of the crude extract and of enzyme at each stage of purification.

Uroporphyrinogen I synthetase had an isoelectric point of 4.46 at 5°C. The isoelectrically focused enzyme gave only a single active protein band in disc-gel electrophoresis at pH7.5, whereas the other inactive proteins appeared as three faster-moving bands when stained either with Amido Schwarz or with Bromophenol Blue in ethanol. The molecular weight of the synthetase determined by gel filtration was

Table 1. *Isolation and purification of uroporphyrinogen synthetase of R. spheroides*

The preparation of crude extract from 14g dry wt. of cells and the determination of specific activity (nmol/h per mg of protein) towards porphobilinogen at pH8.2 are described in the text. Before filtration on Sephadex the protein was concentrated to approx. 5ml with Carbowax. Protein from the DEAE-cellulose step at pH9.5 had one major and ten minor protein bands detected by disc-gel electrophoresis at pH7.5 after pre-running the gels for 1.5h (see the text for details).

Fraction	Total activity (μmol/h)	Specific activity (nmol/h per mg)	Purification (x-fold)	Number of major protein bands on electrophoresis at pH7.5
Crude extract	800	130	1	
$(NH_4)_2SO_4$ and dialysis	392	330	2.5	
DEAE-cellulose, pH7.5	263	1305	10	
Calcium phosphate gel	195	1990	15.3	
DEAE-cellulose, pH9.5	144	2930	22.5	
First Sephadex G-100 (combined)	109	10000	77	3
Second Sephadex G-100				
(Combined)	73			3
(Peak)	25.8	18050	139	2
DEAE-cellulose, pH4.5 (peak), dialysed, concentrated, dialysed	8	27000	208	1

35000 and the molecular weight of purified enzyme determined in sodium dodecyl sulphate–polyacrylamide gels in the presence and in the absence of 2-mercaptoethanol was 36000. Agreement of the molecular weights determined by the two methods suggests that the enzyme consists of a single polypeptide chain.

The purified enzyme at 0°C was stable in solution at pH7.5 and 9.5 for 3 weeks but was more unstable at pH4.5. At pH7.5 and 22°C 50% of the activity disappeared over 24h. Thiols (0.1mM and 1mM) did not stabilize the enzyme significantly. The pH optimum for purified enzyme for both consumption of porphobilinogen and formation of uroporphyrinogen was 7.8–8.0 with 0.1–0.4mM-substrate, with activity falling to 50% of maximum values at pH6.5 and 9.2. The K_m for porphobilinogen was 40μM, a value similar to that obtained for enzyme from wheat germ (Frydman & Frydman, 1970). Enzyme used in the preparation of intermediate polypyrroles had specific activity of 6000nmol/h per mg of protein and remained fully active when stored at −20°C for over 1 year.

The partially purified enzyme from *R. spheroides* was inhibited almost completely at pH8.2 by $HgCl_2$ or *p*-chloromercuribenzoate (0.1mM) and completely by sodium dodecyl sulphate (1mM) or guanidine (0.5M) as found for the enzyme from other sources (Frydman & Frydman, 1970; Bogorad, 1958*a*). This enzyme from *R. spheroides* was not inhibited by EDTA, *o*-phenanthroline (2mM) or iodoacetate (9mM). Ca^{2+} or Mg^{2+} (4mM) were only slightly inhibitory (12% and 8% respectively) in contrast to enzyme isolated from wheat germ (Frydman & Frydman, 1970). Hydrazine and aniline (45mM) inhibited the formation of uroporphyrinogen by 15% and 8% respectively. Methylamine (up to 0.2M) at pH8 inhibited both the consumption of porphobilinogen and formation of uroporphyrinogen to the same extent (40%), yet was more inhibitory to both at pH9.2 than at pH8. *N*-Acetylporphobilinogen (0.8mM) did not inhibit the formation of porphyrinogen, whereas opsopyrroledicarboxylic acid (1mM) inhibited by 40%. A brief report of the preparation and properties of uroporphyrinogen I synthetase from *R. spheroides* has recently been given by Jordan & Shemin (1971).

Formation of uroporphyrinogen in the absence of inhibitors

When porphobilinogen (0.2–10mM) was incubated with either crude, partially purified, or our most purified enzyme the consumption of porphobilinogen and the formation of uroporphyrinogen were both linear with time and concentration of enzyme. No differentiation between the two activities was noticed over all times examined whether the consumption of porphobilinogen was partial or complete.

By using the extinction coefficients for porphobilinogen and porphyrins given in the Materials and Methods section, between 4.5 and 4.7 molecules of porphobilinogen were apparently consumed for 1 molecule of uroporphyrin determined. The lowest ratio of 4.40, corresponding to a 90% yield, was obtained when air was completely excluded or when agents like dimedone (0.05M) were present at pH7.0. With 0.05M-cysteine or in Thunberg tubes the ratio was still in the range 4.60–4.50. The ratio 4.5–4.70

was obtained for uroporphyrinogen synthetase (a) in the initial stages of purification, (b) for even the most purified samples of enzyme, (c) with purified enzyme when assayed at 0°C or 37°C and (d) even when a large excess of enzyme was present so that the whole reaction was complete in 5min. The rate of the enzymic reaction and the ratio of about 4.5 were not altered markedly by the presence of air. Assays at pH values of 6.5, 7.0, 8.0 and 8.5 for 1h did not alter the ratio appreciably, although the presence of high concentrations of salts, phosphate or Tris buffer decreased the yield of porphyrin, e.g. 0.2M-Tris–HCl gave a ratio of 6.0, which at 0.01M fell to 4.7.

The stoicheiometry with respect to ammonia of the complete enzymic reaction was determined after incubation at pH8.2 at 37°C for 4h when all the porphobilinogen had been consumed. Test samples (0.1, 0.2 and 0.4ml) each contained 463nmol of ammonia/ml from a loss of 470nmol of porphobilinogen/ml and formation of 104.4nmol of porphyrin/ml. Correction for the non-enzymic release of 18nmol of ammonia/ml from porphobilinogen gave an overall stoicheiometry of 0.98 molecule of ammonia/molecule of porphobilinogen and 4.25 molecules of ammonia/molecule of porphyrin in approximate agreement with expectation.

Several possibilities may account for the ratio being higher than the theoretical value of 4.0. (*a*) Extinction coefficients used may be too low for porphobilinogen and too high for uroporphyrin. However, the values given in the literature and used in this work, which are given in the Materials and Methods section, were consistent with the weight of porphobilinogen monohydrate, the release of ammonia and the radioactivity of both porphobilinogen and porphyrin. (*b*) Oxidation of uroporphyrinogen to uroporphyrin with I_2 may either be incomplete or form products other than uroporphyrin. Bogorad (1958*a*,*b*), Mauzerall & Granick (1958) and Mauzerall (1962) have found that small amounts of an unknown species absorbing at 630–660nm are often present in oxidation experiments. Also traces of species absorbing at 445nm are detected on oxidation with I_2. We have noticed these absorptions (640nm, 465nm) in several experiments with uroporphyrinogen in air or on oxidation with small amounts of I_2, but could not prevent their formation. Treatment with I_2 was otherwise an efficient way of stopping the enzymic reaction in the presence of porphobilinogen. The results of experiments with [^{14}C]porphobilinogen suggest that the method of assay may lead to ratios higher than the theoretical stoicheiometric values (Table 2, Expt. D). (*c*) Other enzymic side reactions that consume porphobilinogen may occur, but this seems unlikely since all enzyme preparations assayed in Tris–EDTA buffer showed the same ratio. Frydman *et al.* (1972) have recently found a porphobilinogen oxygenase in wheat germ and rat liver which can consume porphobilinogen and not form porphyrin; it is strongly inhibited by EDTA. However, all assays discussed in this work contained EDTA (5mM), which should be expected to inhibit an oxygenase, if present. (*d*) Oxidation of intermediate species may occur during the incubation. Although the ratio was the same both during and at the end of the reaction with porphobilinogen, which suggests that an intermediate is not being built up, heating enzymically formed [^{14}C]uroporphyrinogen gave appreciable amounts of non-porphyrin material. Subsequent treatment with I_2 only removed about 1% free porphyrin, though greater losses were found when Zn^{2+}–uroporphyrin was present. Oxidation reactions became more prominent when large amounts of polypyrroles were present and this is discussed below. Possibility (*b*) seems to account for the high values generally observed but point (*d*) may become more important when polypyrrole intermediates are present.

Differentiation between the two enzymic activities

In the absence of inhibitors the consumption of porphobilinogen and formation of uroporphyrinogen occur together. In the presence of hydroxylamine, ammonia and the less basic methoxyamine, however, the consumption of porphobilinogen and formation of porphyrinogen are affected to different extents. These three bases did not appreciably alter the rate of consumption of porphobilinogen by either purified uroporphyrinogen I synthetase or the combined crude synthetase containing cosynthetase from *R. spheroides*, although both the yield of porphyrin and the nature of the isomer formed were drastically altered. Whenever these bases were present at concentrations 0.01–0.1M uroporphyrin I was formed almost

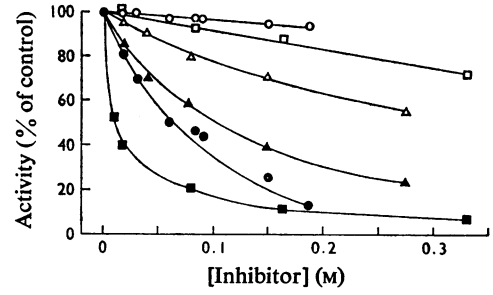


Fig. 1. *Effect of amines on the activity of uroporphyrinogen synthetase*

Inhibition of porphyrin formation (▲, ■, ●) and consumption of porphobilinogen (△, □, ○) by ammonia (▲, △), hydroxylamine (■, □) and methoxyamine (●, ○). Activity of control enzyme assayed in the absence of inhibitor is 100% with 0.4mM-porphobilinogen, 0.1M-Tris buffer, pH8.2, at 37°C for 1h.

exclusively with enzyme preparations of synthetase or synthetase containing the cosynthetase, which shows that the cosynthetase was inhibited much more effectively than the synthetase. The further effects on the two activities of uroporphyrinogen I synthetase are shown in Fig. 1. Only the formation of porphyrin was appreciably decreased. The rate of formation was decreased to one-half with either 0.015M-hydroxylamine, 0.1M-ammonia or 0.07M-methoxyamine. The formation of porphyrin, however, continued to increase even after all the monomeric porphobilinogen had been consumed and the incubation was continued beyond 1h. With 0.2M-hydroxylamine the final recovery of porphyrin from porphobilinogen did not reach 100%, but increased from 10% after 1h to a final value of 60% after 6–24h. An intermediate, X, that can proceed to form porphyrin (Bogorad, 1963) must have been present in solution. The rate of reaction was such that if longer assay times were used (e.g. 3–6h at 37°C) then the intermediate species would not have been observed.

Incubation mixtures containing either methoxyamine, ammonia or hydroxylamine, which had consumed all the porphobilinogen, reacted with modified Ehrlich's reagent in the same way; all produced a very transient purple colour ascribed to reaction of a polypyrrole. The absorption spectra were very similar to the spectra obtained with Ehrlich's reagent and synthetic dipyrrylmethanes (see below) and with incubation mixtures containing di- and tetra-pyrroles reported by Bogorad and his colleagues (Pluscec & Bogorad, 1970; Radmer & Bogorad, 1972).

Separation of polypyrroles by gel filtration

The polypyrroles were separated from both unused porphobilinogen and protein by gel filtration on columns of either Sephadex G-15 or G-25 in phosphate buffer at 5°C. A typical elution pattern from the standard column (0.95cm × 170cm) of Sephadex G-25 is shown in Fig. 2 for an incubation mixture that still contained residual porphobilinogen after 1h. The enzyme (mol.wt. 36000) was eluted in two fractions (5 and 6) and remained fully active. Very similar elution patterns were obtained with each of the three amines examined, i.e. 0.2M-hydroxylamine, 0.4M-ammonia and 0.2–1.0M-methoxyamine. More polypyrrole was found the higher the concentration of amine.

Control experiments with [^{14}C]porphobilinogen or enzymically formed [^{14}C]uroporphyrinogen, or uroporphyrin reduced chemically with borohydride, established that pyrrolic material was confined to fractions that were of the same molecular size as tetrapyrroles and monopyrroles respectively. Uroporphyrinogen and polypyrrole were eluted close together. The polypyrrole reacted with Ehrlich's reagent and produced an intense purple colour ($\lambda_{max.}$ 565–570nm) at once, which faded very quickly with a half-life of approx. 50s; a second absorption maximum ($\lambda_{max.}$ 490–485nm) remained (Fig. 3). After treatment with I_2 solution the polypyrrole solution no longer reacted with Ehrlich's reagent, did not have extra porphyrins present and porphyrin was not formed on heating.

The colourless samples of intermediate X were

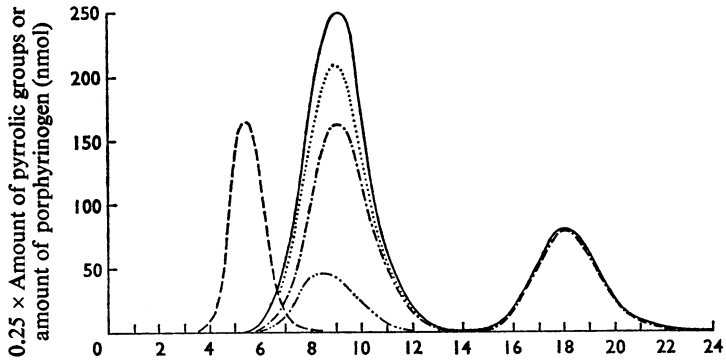


Fig. 2. *Gel filtration on Sephadex G-25 of an incubation mixture of enzyme,* [^{14}C]*porphobilinogen and amine (ammonia, hydroxylamine or methoxyamine)*

Fractions (3ml) were collected in 0.025M-potassium phosphate buffer, pH7.6 at 5°C. Protein was determined by absorbance at 280nm (----); total [^{14}C]pyrrolic groups by radioactivity (——); free porphobilinogen by reaction with Ehrlich's reagent (·––·); porphyrinogen measured spectrophotometrically as porphyrin after oxidation with I_2 (–···–); total porphyrin after heating samples at 55°C for 1.25h (· · · · ·); intermediate polypyrrole X, measured by difference between total porphyrin after heating and uroporphyrinogen (–·–·–).

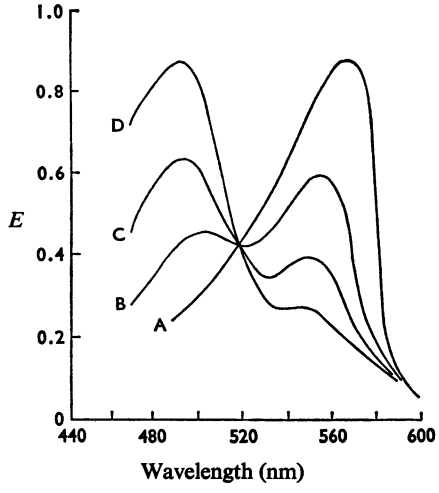


Fig. 3. *Spectra at various times in the reaction of intermediate X_{NH_2OH} with Ehrlich's reagent*

The solution contained 35nmol of polypyrrole X in 3 ml. Spectra were recorded in 1cm cuvettes and are shown for times: A, 20s; B, 1min; C, 2.5min; D, 5min. For further details see the text.

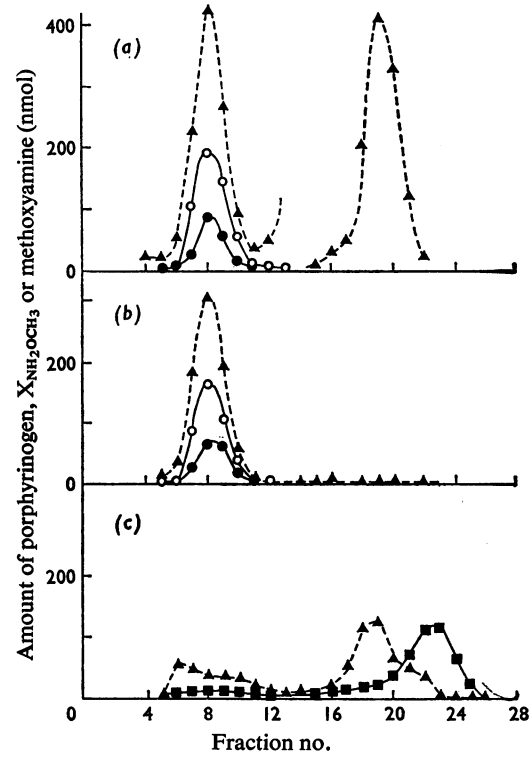


Fig. 4. *Gel filtration on Sephadex G-25 of incubation mixtures containing $[^3H]X_{NH_2OCH_3}$*

Fractions (3ml) were collected in 0.025M-potassium phosphate buffer, pH7.6. Porphyrinogen was measured spectrophotometrically as uroporphyrin at 400nm (●), intermediate $X_{NH_2OCH_3}$ (○), bound [^{3}H]methoxyamine (▲) and free amine diluted 600-fold in fractions 15–22; uroporphyrin was measured as E_{400} (■). (*a*) Initial incubation mixture described in the text with 1.04M-methoxyamine; (*b*) combined fractions 5–11 from (*a*) freeze-dried, dissolved in 1ml of water and re-filtered on Sephadex; (*c*) combined fractions 5–11 from (*b*) freeze-dried, dissolved in 1ml of water, heated at 55°C for 1.25h and refiltered on Sephadex.

stable at 0°C in the absence of air and light. They could be freeze-dried, re-filtered on Sephadex G-25 and recovered unchanged. Fig. 4 shows the pattern obtained for the methoxyamine intermediate ($X_{NH_2OCH_3}$). After being left to stand, fractions containing polypyrrole spontaneously formed uroporphyrin I via colourless porphyrinogens, very quickly at 55°C, at a moderate rate at 37°C, but only very slowly at 0°C. The rate of conversion into porphyrin of polypyrrole in both the isolated fractions and the initial incubation mixture was not increased by the addition of fresh enzyme.

Intermediate $X_{NH_2OCH_3}$ emerged with, or slightly ahead of, uroporphyrinogen and intermediates X_{NH_3} and X_{NH_2OH} (the ammonia and hydroxylamine intermediates respectively) emerged with, or slightly behind, uroporphyrinogen. Thus whereas these compounds may all have molecular weights similar to tetrapyrroles, they are eluted in slightly different positions. The compounds do not behave as dipyrroles or monopyrroles and their elution behaviour is compatible with their being open-chain tetrapyrroles, although the possibility of their being tri- or pentapyrroles cannot be excluded by this experiment alone.

The synthetic dipyrrylmethane (dipyrrole 1, named in the Materials and Methods section) with two free α positions was used as a marker and was eluted near fraction 16. As incubation mixtures of enzyme and [^{14}C]porphobilinogen with ammonia, methoxyamine or even hydroxylamine did not show pyrrolic material in this region, dipyrroles were probably not major intermediates. However, the αα′-disubstituted compound, dipyrrole 2, was retarded a little and emerged near fraction 20 adjacent to porphobilinogen. Evidence presented below shows that pyrrolic material that is not unchanged porphobilinogen can be found in experiments with hydroxylamine. Thus the complete absence of dipyrroles in the porphobilinogen fractions cannot be ruled out. Free methoxyamine

was eluted in the same position as porphobilinogen, whereas hydroxylamine and salts eluted near fractions 20–22. Free uroporphyrin was retarded appreciably on Sephadex G-25 in phosphate–EDTA buffer, pH7.6, and was eluted in fractions 23–29 with a maximum in fraction 26. It was clearly separated from other pyrrolic material. Traces (5%) of a heptacarboxylic acid porphyrin were detected only in incubation mixtures of enzyme and porphobilinogen alone, and this porphyrin was eluted in pure form in fractions 37–51. Porphyrins containing a smaller number of carboxyl groups would be more firmly held to Sephadex G-25, but were not detected.

Porphomethenes

The formation of orange compounds by ready oxidation of both porphyrinogens and pyrromethanes was often encountered in this work. The orange compounds ($\lambda_{max.}$ 490–500nm) are all considered to contain a conjugated dipyrromethene unit within a larger chain (Bogorad, 1958*a*; Mauzerall, 1962).

Colourless solutions of porphyrinogens in air slowly turn orange–pink due to oxidation of the hexahydroporphyrins to tetra- and di-hydroporphyrins (porphomethenes) and to porphyrins. Uroporphyrinogen and the uroporphomethene formed in phosphate buffer can be rapidly oxidized to uroporphyrin by I_2, but when the porphomethene is formed in acid solution it could not be oxidized by I_2 at that pH. It formed porphyrin, however, on illumination.

Samples of polypyrroles could be both oxidized and iodinated by I_2 at pH7.5. The products have no sharp absorption bands in the visible region. In acetic acid–perchloric acid an orange species was readily formed from intermediate X_{NH_2OH} by oxidation with air, which was not affected further by I_2. This sample in acid gave porphyrin when left for 24h, even after being treated with I_2, which suggests that a free α position of the polypyrrole had not been iodinated, resembling in this respect the behaviour of porphobilinogen in acid.

The component of heated intermediate $X_{NH_2OCH_3}$ or X_{NH_2OH} that was not porphyrin was also orange ($\lambda_{max.}$ 490–500nm), but this absorption band disappeared on oxidation of a bridge methane group with I_2 at pH7.5 without the production of porphyrin. The non-porphyrin material was probably formed from intermediate X either by oxidation of the α position of the pyrrole or by intermolecular condensation of the polypyrrole instead of intramolecular cyclization.

Experiments with hydroxylamine

Hydroxylamine is ten times more effective than ammonia and twice as effective as methoxyamine in forming polypyrroles. Porphyrin synthesis was inhibited 50% with 0.015M-hydroxylamine and 0.4mM-porphobilinogen. The same degree of inhibition was obtained with 0.01M-hydroxylamine at 37°C for 1h at five concentrations of porphobilinogen between 0.04 and 0.4mM, which suggests a non-competitive inhibition of porphyrinogen synthesis. The rate of consumption of porphobilinogen was not apparently altered by 0.01M-hydroxylamine, which suggests that it is the final cyclization step to porphyrinogen that is inhibited by hydroxylamine. Intermediate X_{NH_2OH} gave a very intense colour with Ehrlich's reagent, but after its ready conversion into porphyrin by heating no colour was obtained. As heating did not result in the release of Ehrlich-positive material, it can be deduced that intermediate X is a tetrapyrrole. The conversion of intermediate X_{NH_2OH} into uroporphyrin was not catalysed by uroporphyrinogen synthetase and both the rate and extent of the enzymic formation of uroporphyrin I from porphobilinogen (60μM) was not altered by the presence of intermediate X, which suggests that free polypyrrole cannot re-enter the enzymic reaction.

The number of pyrrole rings/mol of polypyrrole was examined with radioactive porphobilinogen. [^{14}C]Porphobilinogen (1.37mg), 0.16ml of enzyme and 0.2M-hydroxylamine were allowed to react for 1.5h at 37°C and the products were separated to yield a pattern shown in Fig. 2. Fractions that contained intermediate X were combined, heated at 55°C for 1.25h, freeze-dried and dissolved in 0.9ml of water. The solution which now contained porphyrin was again applied to the Sephadex column. A large amount of non-porphyrin pyrrolic material, named 'Y', was eluted in fractions 6–14 and free uroporphyrin I was retarded.

For all subsequent calculations of stoicheiometry we assume that solutions of the polypyrrole contain a mixture of uroporphyrinogen which can be oxidized to porphyrin and another compound, X, which is converted into porphyrin on heating. In addition, another compound Y was detected after heating which was not porphyrin. Compound Y may be a side product of the conversion of compound X into porphyrin or a third component of the original unheated mixture. We were unable to distinguish between these two possibilities.

It can be seen from Table 2, Expt. A, that the fractions contain more than the stoicheiometric ratio of four [^{14}C]pyrrole units/mol of porphyrin finally present after heating, clearly indicating the presence of a significant amount of the non-porphyrin material Y. The amount of this material in each fraction was determined from gel filtration of the heated sample as described above, and is shown in line 4, Table 2, Expt. A. The non-porphyrin material has an apparent molecular weight similar to compound X and uroporphyrinogen. If both this amount of non-porphyrin

Table 2. *Composition of individual fractions from gel filtration of incubation mixtures containing synthetase, porphobilinogen and inhibitory amines*

Mixtures incubated for 1.5h were separated and analysed by methods described in the text. Amounts of polypyrrole X, porphyrinogen and [^{14}C]pyrroles in the total 3ml of solution are given in nmol, in terms of either porphyrin measured spectrophotometrically or in units of monopyrrole measured by radioactivity. Incubations were: Expt. A, with 0.2M-hydroxylamine, and Expt. B, without hydroxylamine, both gel-filtered in 0.025M-potassium phosphate buffer, pH7.5; Expt. C, with 0.86M-methoxyamine, and Expt. D, without methoxyamine, both incubated with additional 0.02M-cysteine and gel filtered in 0.05M-potassium phosphate–0.01M-EDTA buffer, pH7.5. The amount of non-porphyrin material Y is given in units of monopyrrole and was the polypyrrolic material of heated samples that was obtained from a second filtration on Sephadex G-25.

Expt. A. +Hydroxylamine

Fraction no. ...	5	6	7	8	9	10	11	12	13	Total
Polypyrrole X	1	1	22	100	164	118	50	14	2	472
Total porphyrinogen (P+X)	1	2	35	141	207	142	57	16	3	605
Total [^{14}C]pyrrole		18	193	702	1025	716	272	76	19	3021
Non-porphyrin [^{14}C]pyrrole (Y)		48	69	88	87	85	77	57	32	553
Ratio of total [^{14}C]pyrrole/ total porphyrinogen		9.0	5.5	5.0	4.9	5.0	4.8	4.9	6.3	5.0
Ratio of (total [^{14}C]pyrrole–Y–4P)/X			3.2	4.5	4.7	4.6	3.3			4.1

Expt. B. Control, no inhibitor

Fraction no. ...	5	6	7	8	9	10	11	12	13	Total
Total porphyrinogen			62	246	275	140	43	10	3	779
Total [^{14}C]pyrrole		27	293	1147	1295	687	226	61	27	3763
Non-porphyrin [^{14}C]pyrrole (Y)	6	22	42	64	88	92	86	78	72	550
Ratio of total [^{14}C]pyrrole/ total porphyrinogen			4.7	4.7	4.7	4.9	5.2	6.1		4.8
Ratio of (total [^{14}C]pyrrole–Y)/ total porphyrinogen			4.0	4.4	4.4	4.2	3.2			4.1

Expt. C. +Methoxyamine

Fraction no. ...	5	6	7	8	9	10	11	12	13	Total
Polypyrrole X			21	90	137	96	44	14	4	406
Total porphyrinogen (P+X)			27	123	203	161	75	22	6	617
Total [^{14}C]pyrrole		30	230	766	1170	875	383	122	39	3615
Non-porphyrin [^{14}C]pyrrole (Y)	6	19	41	68	89	89	85	74	46	517
Ratio of total [^{14}C]pyrrole/ total porphyrinogen			8.5	6.2	5.8	5.4	5.1	5.5		5.85
Ratio of (total [^{14}C]pyrrole–Y)/ total porphyrinogen			7.0	4.9	5.3	4.9	4.0	2.2		5.0
Ratio of (total [^{14}C]pyrrole–Y–4P)/X			7.9	6.3	6.0	5.3	4.0			5.5

Expt. D. Control, no inhibitor

Fraction no. ...	5	6	7	8	9	10	11	12	13	Total
Total porphyrinogen		2	62	215	279	187	82	27		854
Total [^{14}C]pyrrole		31	297	926	1185	809	359	133	52	3792
Non-porphyrin [^{14}C]pyrrole (Y)		8	16	23	35	40	40	30	23	215
Ratio of total [^{14}C]pyrrole/ total porphyrinogen			4.8	4.3	4.2	4.3	4.4	4.9		4.4
Ratio of (total [^{14}C]pyrrole–Y)/ total porphyrinogen			4.5	4.2	4.1	4.1	3.8	3.6		4.2

pyrrole and the amount of pyrrole present as uroporphyrinogen (i.e. 4×P) is subtracted from the total number of [^{14}C]pyrrole molecules present, the number of pyrrole groups in 1 molecule of polypyrrole can be calculated for each fraction (Table 2, line 6). Thus each intermediate X_{NH_2OH} apparently contains between 4.1 and 4.5mol of pyrrole. The results of control experiments for the formation of

Table 3. *Composition of combined fractions from gel filtration of incubation mixtures containing, synthetase, porphobilinogen and inhibitory amines*

Incubation mixtures were separated on Sephadex G-25 and analysed by methods described in the text. Each 3 ml fraction was analysed and the summed values for fractions 6–12 are reported. Values are given as nmol of each species.

	Expt. A. +Hydroxylamine			Expt. B. +Ammonia		
Inhibitor concn. (M) ...	0	0.20	0.21	0*	0.40	0.40
Total [^{14}C]pyrrole	3763		3002	14	3956	
Porphyrinogen (P)	779	138	132		367	297
Total porphyrinogen (P+X)	779	569	602		645	585
∴ Polypyrrole X		431	470		278	288
Non-porphyrin [^{14}C]pyrrole (Y)	550		521		773	
Polypyrrole X/total porphyrinogen (%)		76	77		43	44
Ratio of total [^{14}C]pyrrole/ total porphyrinogen	4.83		5.0		6.13	
Ammonia released		0				372
Ratio of ammonia/polypyrrole X		0				1.29

	Expt. C. +Methoxyamine								
Inhibitor concn. (M) ...	0†	0.18†	0.21	0.86†	0.94	1.0	1.0	1.04	1.04‡
Total [^{14}C]pyrrole	3759			3574		4183	5244		
Porphyrinogen (P)	853	446	440	208	192	229	273	182	170
Total porphyrinogen (P+X)	853	698	718	610	679	716	756	688	564
∴ Polypyrrole X		252	278	402	487	487	483	506	394
Non-porphyrin [^{14}C]pyrrole (Y)	250			524			1300		
Polypyrrole X/total porphyrinogen (%)		36	39	66	71	68	64	73	
Ratio of total [^{14}C]pyrrole/ total porphyrinogen	4.40			5.85		5.83	6.92		
[^{3}H]Methoxyamine		406	504					995	783
Ammonia released					135				
Ratio of methoxyamine/ polypyrrole X		1.62	1.81					1.96	1.98

* Run for porphobilinogen without enzyme; only 0.3% pyrrole units were not present as monomeric porphobilinogen.

† Incubation mixture contained added 0.02M-cysteine; separation was achieved in 0.05M-potassium phosphate–0.01M-EDTA buffer, pH 7.6.

‡ Second gel filtration of freeze-dried sample; a third gel filtration showed that free methoxyamine was released on heating.

uroporphyrinogen from enzyme and porphobilinogen alone give a value near 4.1 (Table 2, Expts. B and D).

Release of ammonia and hydroxylamine from intermediate X_{NH_2OH}. No ammonia was released when intermediate X_{NH_2OH} was heated (Fig. 6 and Table 3, Expt. A). This indicates that the aminomethyl group of porphobilinogen was no longer present in this polypyrrole, unlike the ammonia and methoxyamine compounds discussed below. Attempts were made to measure the amount of hydroxylamine released on heating. Eight determinations were made on samples that had been freeze-dried from solutions of either ammonium acetate or potassium phosphate buffer. Values varied between 0.24 and 0.67 nmol of hydroxylamine/nmol of intermediate X. As eight test and control tubes were needed for each determination only small amounts of intermediate X could be used (30–40 nmol). The recovery of hydroxylamine (40–50 nmol) added as internal standard in the presence of intermediate X, uroporphyrinogen or uroporphyrin was between 72 and 82%, which indicates interference by these compounds. As hydroxylamine could not be removed from these samples and in view of the above interferences no definite statement on the exact stoicheiometry can be made, but the presence of

a -NHOH group in intermediate X_{NH_2OH} can be inferred.

Further reactions of the polypyrrole formed in the presence of hydroxylamine. (i) Iodination. I_2 reacts rapidly with pyrroles and preferentially attacks the free α position. With porphobilinogen and polypyrroles in alkaline solution I_2 stops both the enzymic reaction and reaction with Ehrlich's reagent. ^{125}I should thus be incorporated into any polypyrrole with a free α position.

A solution of polypyrrole (1.0ml) containing 340nmol of compound X and 170nmol of porphyrinogen was treated with an eightfold molar excess of $^{125}I_2$ in KI for 5min ($3\times20\mu l$) and excess of I_2 was removed by $Na_2S_2O_3$. The solution was analysed on the standard Sephadex G-25 column in 0.025M-ammonium acetate buffer and samples (1ml) were counted for radioactivity. Visible spectra were also recorded. Approx. 260ng-atoms of ^{125}I were incorporated in the orange–brown fractions 5–12. Since uroporphomethene was not present, this product absorbing at 500nm was an open-chain pyrromethene that could be further oxidized by I_2. No radioactivity was associated with porphyrin recovered in fractions 23–29, whereas the oxidized polypyrroles had approx. 0.75g-atom of ^{125}I/mol of original polypyrrole, which confirms that compound X possessed a free α position and thus was an open-chain compound. The radioactive polypyrrole was freeze-dried and filtered a second time on Sephadex G-25. It was eluted in the same position but was now free of all residual I^- ion. The fractions were brown and had absorption bands at 305nm and at 279, 371, 405 and 490nm; the last was removed by further reaction of a remaining bridge methane group with I_2. E_{305} was equivalent to $\epsilon = 25000$ litre·mol^{-1}·cm^{-1} per atom of ^{125}I.

(ii) Effects of acid and alkali. Treatment of intermediate X_{NH_2OH} with methanol–H_2SO_4 caused ready cyclization to porphyrin and no esterified polypyrrole was obtained. The polypyrrole in solution of ammonium acetate was freeze-dried and samples containing 19nmol of compound X and 7.5nmol of uroporphyrinogen were left in the dark at 22°C for 24h with solutions of various acidity. Clearly, cyclization to porphyrin is strongly favoured in acidic solution (Table 4). Although the original solution and the heated samples only contained isomer I, treatment at pH values 4.2 or less produced increased amounts of isomer III or isomer IV. Treatment with 1% or 5% HCl produced up to 50% uroporphyrin III. Thus a non-enzymic rearrangement of the pyrrole rings had occurred very readily and this is consistent with compound X being a labile linear bilane (Jackson *et al.*, 1967). It is known that isomers of uroporphyrinogen rearrange readily in hot acid, e.g. 10min in 1M-HCl at 98°C (Mauzerall, 1960), but with the open-chain tetrapyrrole this rearrangement occurred at low temperatures.

(iii) Acetylation. Attempts to detect a free amino group in either intermediate X_{NH_2OH} or X_{NH_3} by reaction with acetic anhydride or with dansyl chloride have not proved successful. An orange porphomethene-type species ($\lambda_{max.}$ 493 and 377nm) was produced rapidly on reaction of a mixture of intermediate X_{NH_2OH} and uroporphyrinogen with acetic anhydride at pH8.

Experiments with ammonia

With 0.4M-ammonia up to 44% of the total tetrapyrrolic material was present in the open-chain form. Intermediate X_{NH_3} was much more labile than the hydroxylamine derivative and could not be freeze-

Table 4. *Effect of pH on the formation of porphyrin from intermediate* X_{NH_2OH}

A sample of intermediate X_{NH_2OH} in ammonium acetate buffer was freeze-dried and dissolved in 5mM-potassium phosphate buffer (1ml). Samples (50μl) were: Expt. A, oxidized with I_2; Expt. B, dried under a stream of N_2 and esterified; Expt. C, heated at 55°C for 1h; Expt. D, treated with solution (0.5ml) at various pH values for 24h at 22°C. Total porphyrin was measured spectrophotometrically for 100μl samples diluted into 2ml of 0.3M-Tris buffer, pH8.2. The remaining porphyrin was absorbed on talc, eluted with ammonia, dried, esterified and the isomer composition determined after paper chromatography.

	Solution	Porphyrin (nmol)	Isomer (% of isomer III)
Expt. A	Start	7.5	0–25
Expt. B	Dried and esterified	15.1	25–50
Expt. C	Heated	26.2	0–25
Expt. D	5% HCl	26.9	50
	1% HCl	26.1	50
	0.01M-Sodium acetate, pH4.2	Precipitate	25
	0.025M-Potassium phosphate, pH7.5	21.4	0
	0.10M-Tris, pH8.2	13.2	0
	0.01M-NaOH	9.5	0

dried and stored intact; consequently, all investigations with it were made with freshly prepared solutions in phosphate buffer. Intermediate X_{NH_3} gave an intense transient purple colour with Ehrlich's reagent and on heating produced isomer I almost exclusively. It differed from the hydroxylamine derivative in one important respect in that it released ammonia on cyclization (Fig. 6). Individual fractions from Sephadex released up to 1.8mol of ammonia for every extra mol of porphyrin formed, although the value for the combined fractions was 1.29 (Table 3, Expt. B).

With [^{14}C]porphobilinogen radioactivity was detected only in fractions that contained compound X or porphobilinogen. From the results of Expt. B, Table 3, 1 porphyrin molecule is apparently formed from 6.1 pyrrole rings. The samples of intermediate X_{NH_3} are relatively unstable and over 19% of pyrrolic material was present as non-porphyrin pyrrole Y in the heated samples. When the amount of this material was taken into account, as described above for hydroxylamine, a value of 6.2 was obtained for the stoicheiometry. A value of 6 would correspond to 2 tripyrrole molecules condensing to 1 porphyrin and 2 monopyrrole molecules, a reaction not in agreement with experiments with hydroxylamine and other properties of the polypyrrole. Thus owing to the instability of intermediate X_{NH_3} and the relatively small amounts of polypyrrole a reliable determination of the stoicheiometry was not possible.

However, should compound Y or its precursor, as well as compound X, release ammonia on heating, the calculated ratio would fall from 1.29 ammonia molecules/porphyrin molecule to 0.8 ammonia molecule/

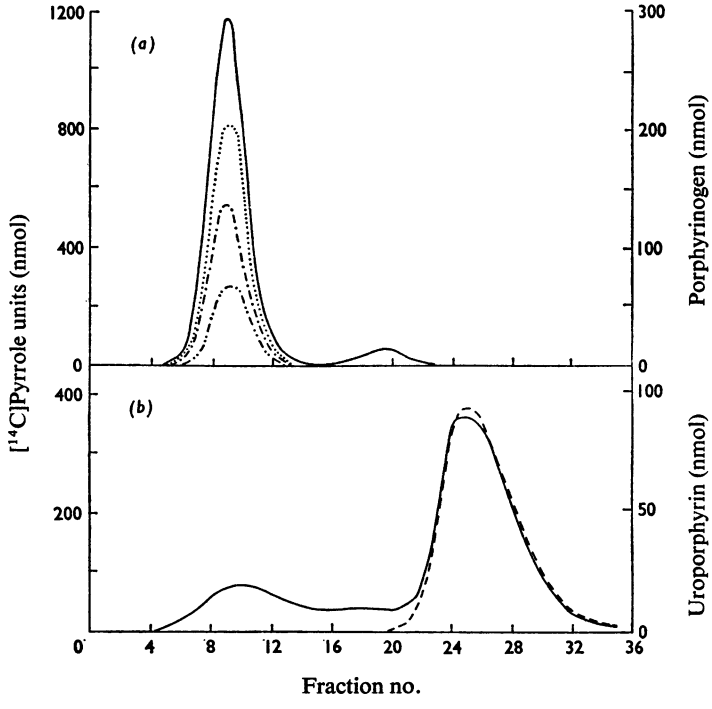


Fig. 5. *Gel filtration of incubation mixtures containing* [^{14}C]$X_{NH_2OCH_3}$

Enzyme, [^{14}C]porphobilinogen (7mM) and methoxyamine (0.84M) in 0.9ml of solution were incubated at pH8.2 at 37°C for 1.5h, then applied to Sephadex G-25. Fractions (3ml) were collected in 0.05M-potassium phosphate–0.01M-EDTA buffer, pH7.6, at 5°C and analysed as described in the text. Total [^{14}C]pyrrole units (——); porphyrinogen (– · ·–); total porphyrin after heating (· · · ·); intermediate polypyrrole measured by difference between total porphyrin after heating and porphyrinogen (–·–·–); uroporphyrin measured by E_{503} and E_{400} (– – – –). (*a*) Initial incubation mixture; (*b*) combined fractions 6–12 heated at 55°C for 1.5h, left to oxidize in air for 2h, freeze-dried, dissolved in 0.9ml of water, left for 1h and then re-filtered on Sephadex.

4 pyrrole rings for the combined fractions and to a range of 1.31–0.5 for the individual fractions. Thus although the terminal groups of the polypyrrole can be considered as $—CH_2NH_2$, in the absence of more data on the composition of compound Y it cannot be decided whether more than 1 ammonia molecule is associated with the polypyrrole.

Experiments with methoxyamine

Methoxyamine, with pK4.60, is less basic than ammonia and hydroxylamine. It is, however, a good nucleophile (Dixon & Bruice, 1971; Jencks & Carriuolo, 1960). Polypyrroles were prepared with concentrations of methoxyamine between 0.18 and 1.0M. The incorporation of [^{3}H]methoxyamine and [^{14}C]porphobilinogen and the release of ammonia by heat were determined for the polypyrroles in separate experiments (Table 3, Expt. C). Methoxyamine was incorporated into the polypyrrole X (Fig. 5) to 2.1–1.3nmol/nmol of compound X for fractions 8–12 in each experiment (Table 5). Samples that contained compound X were combined under N_2, freeze-dried and re-filtered on the Sephadex column. The solutions were now free of excess of methoxyamine and the same stoicheiometry of about 2.0 groups/mol of compound X was obtained. Gel filtration of samples that had been freeze-dried, dissolved in water and heated for 1.5h showed that small amounts of radioactive methoxyamine were still associated with the non-porphyrin material Y. By far the major portion of [^{3}H]methoxyamine had been released on cylization of compound X and was detected as free methoxyamine (Fig. 4*c*). Samples that had already been heated lost all their methoxyamine on freeze-drying.

In addition to containing methoxyamine, intermediate $X_{NH_2OCH_3}$ also contained ammonia (Fig. 6 and Table 5). The relative proportion of methoxyamine to ammonia varied from fraction to fraction, but the sum came to about 2.2nmol/nmol of intermediate X. Thus the polypyrrole $X_{NH_2OCH_3}$ is not a single compound but is a mixture of species which contain either $—NH_2$ or $—NHOCH_3$ end groups. Experiments with lower concentrations of methoxyamine gave ratios of methoxyamine/compound X that decreased from 2.0 to 1.0 for fractions 8–10, which probably indicates a greater proportion of the amino species. The material Y that is present in all preparations of the polypyrrole X also contains methoxyamine groups and thus the value of 2 groups/

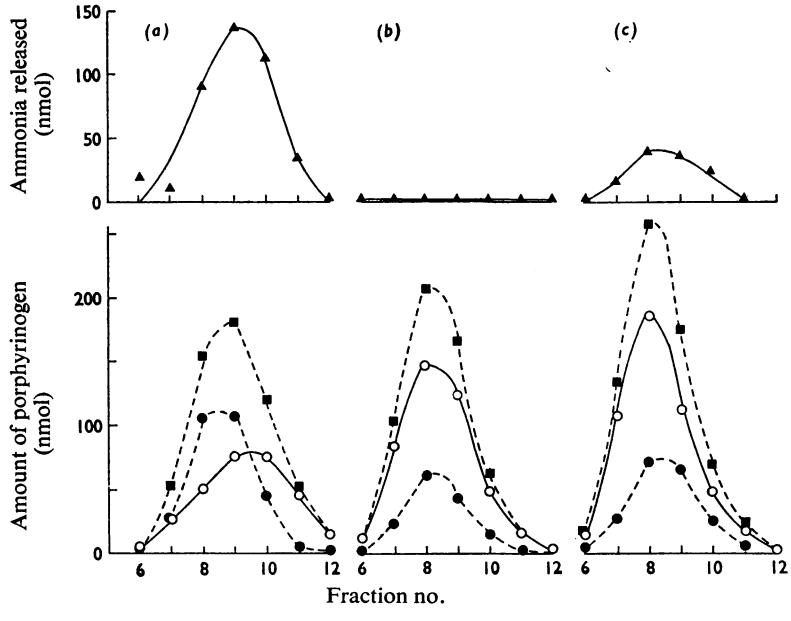


Fig. 6. *Ammonia released by heating the polypyrroles*

Polypyrrole was formed in the presence of (*a*) 0.4M-ammonia, (*b*) 0.2M-hydroxylamine and (*c*) 0.94M-methoxyamine and separated by gel filtration on Sephadex G-25. Fractions were analysed by methods described in the text; the amounts of ammonia and porphyrin in the total fraction (3ml) are shown. Ammonia released by heating each fraction at 55°C for 1.25h (▲); porphyrinogen (●); total porphyrin after heating (■); intermediate polypyrrole measured by difference between total porphyrin and porphyrinogen (○).

Table 5. *Methoxyamine and ammonia content of intermediate* $X_{NH_2OCH_3}$

Results are given for two samples of intermediate X prepared under almost identical conditions (see Table 3, Expt. C; methoxyamine, 1.04 and 0.94M) and expressed as ratios of the amount of amine accompanying 1 molecule of compound X in solution. Methoxyamine was measured by radioactivity and ammonia was the amount released when samples of compound X were heated (see the text for details).

Fraction no. ...	7	8	9	10
Methoxyamine/compound X				
First gel filtration	1.98	2.14	1.74	1.35
Second gel filtration	2.16	1.88	1.88	1.54
Ammonia released/compound X	0.18	0.23	0.36	0.63
Sum of (methoxyamine+ammonia)/compound X	2.16	2.37	2.10	1.98

compound X is an over-estimate. However, the amount of ^3H associated with compound Y was too low to be measured accurately and was not used in the calculations.

Experiments with [^{14}C]porphobilinogen listed in Tables 2 and 3 show that the stoicheiometry is still greater than 4.0. Even when intermediate X was the major component of the solution the ratio pyrrole units/total porphyrin varied from 5.8 to 6.9, which shows that polypyrroles incapable of forming porphyrin on heating were present. Thus a reliable estimation of the stoicheiometry was prevented. Although it has been calculated that about 2mol of methoxyamine and ammonia are present in fractions containing 1mol of compound X and hence form 1mol of porphyrin on heating, it cannot be concluded that compound X is the only compound that contains methoxyamine and ammonia. Thus calculation shows that 1.1 mol of methoxyamine and ammonia is found for every 4.0 pyrrolic groups not present as uroporphyrinogen in the unheated samples. This value of 1.1 mol must be regarded as the lower limit for the stoicheiometric value for a modified tetrapyrrole.

Reaction of pyrroles with modified Ehrlich's reagent

Fractions 8–10 from Sephadex that contained the hydroxylamine, ammonia and methoxyamine polypyrroles (X) all gave an intense purple colour with Ehrlich's reagent ($\lambda_{max.}$ 565–570nm). The spectra were very similar in shape to those reported for a synthetic dipyrrylmethane (Pluscec & Bogorad, 1970) and the tetrapyrrole isolated from experiments with ammonia (Radmer & Bogorad, 1972). This purple colour faded very rapidly with a half-life of 55s at room temperature to leave a yellow-coloured solution. Extrapolation of the E_{565} to zero time for each derivative gave ϵ values in the range 85000–95000 litre·mol^{-1}·cm^{-1} per mol of compound X, which decreased to 46000 after 1min. This suggests that all intermediates had the same general structure of an open-chain polypyrrole.

With modified Ehrlich's reagent porphobilinogen gave a magenta colour that developed over 10–15min and two absorption maxima were seen at 553 and 525nm ($\epsilon = 57700$ litre·mol^{-1}·cm^{-1} at 553nm). Opsopyrroledicarboxylic acid lacks α substituents and also gave a stable magenta colour within 1min with one absorption maximum at 550nm ($\epsilon = 30000$ litre·mol^{-1}·cm^{-1}). *N*-Acetylporphobilinogen gave one absorption maximum at 556nm within 1min that slowly faded, as did porphobilinogen lactam ($\epsilon = 57700$ litre·mol^{-1}·cm^{-1} at 556nm). Solutions of synthetic dipyrroles with both α positions free, i.e. dipyrroles 1 and 2, gave blue colours almost at once ($\lambda_{max.}$ 600–595nm, $\epsilon = 65000$ litre·mol^{-1}·cm^{-1}) that faded very quickly through a blue–grey colour to yellow ($\lambda_{max.}$ 485–488nm, $\epsilon = 73000$ litre·mol^{-1}·cm^{-1}) after 1h. The substituted $\alpha\alpha'$-diaminomethyldipyrrole, dipyrrole 3, did not react with Ehrlich's reagent, but very slowly under the influence of acid developed a yellow colour over 3–20h ($\lambda_{max.}$ 475nm). Synthetic 5-aminomethyl-4,4′-di(carboxymethyl)-3,3′-di-(2-carboxyethyl)dipyrrylmethane (dipyrrole 4) apparently reacts like the unsubstituted dipyrroles. The absorption maximum at 566nm faded after 30s to another at 483nm (Pluscec & Bogorad, 1970). Uroporphyrinogen was Ehrlich-negative, but under the oxidizing conditions in acid formed an orange–yellow porphomethene ($\lambda_{max.}$ 490–500nm) that appeared similar to the yellow compounds produced by reaction of the polypyrrole with Ehrlich's reagent. Reactions of other substituted 2-aminomethylpyrroles have been described by Frydman, R. B. *et al.* (1971); electron-donating groups in position 4 increased the rate of reaction with Ehrlich's reagent, whereas a hydrogen atom on C-4 slowed down the rate of reaction so that a maximum development time of 60min was needed.

Thus both the shape of the absorption curve and the rate of colour development with Ehrlich's reagent enable the pyrrolic compounds to be distinguished.

Modified pyrrole found in incubation mixtures of synthetase, porphobilinogen and amines

The presence of a dipyrrole in incubation mixtures of spinach leaf uroporphyrinogen I synthetase, porphobilinogen and hydroxylamine was reported by Pluscec & Bogorad (1970). Incubation mixtures containing uroporphyrinogen and intermediate X_{NH_2OH} or $X_{NH_2OCH_3}$ were prepared from solutions of 0.2M-hydroxylamine or 0.8M-methoxyamine and examined by electrophoresis at pH9.2. Mixtures containing intermediate $X_{NH_2OCH_3}$ and uroporphyrinogen showed only the presence of unused porphobilinogen and polypyrrole X that moved just behind the now fluorescent uroporphyrin. Mixtures containing intermediate X_{NH_2OH} showed a third compound as well as traces of porphobilinogen and large amounts of compound X. This new compound migrated behind the polypyrrole to a region corresponding to the dipyrrole described by Pluscec & Bogorad (1970) and to the dianions of opsopyrroledicarboxylic acid and *N*-acetylporphobilinogen; it migrated more slowly than the tetra-anion of dipyrrole 1. This compound was examined more fully in the following experiments.

No significant amounts (<2%) of radioactive pyrrolic material other than the tetrapyrrole X and porphobilinogen were detected by gel filtration on Sephadex G-25 or G-15, whether residual porphobilinogen was present or not. However, in experiments with hydroxylamine and in which all the porphobilinogen had not apparently been consumed porphobilinogen and the unknown pyrrole (described above) were eluted in the same position. The mixture gave a different type of colour with Ehrlich's reagent with a lower absorbance/mol of [^{14}C]pyrrole. By contrast, remaining porphobilinogen ([^{14}C]porphobilinogen) separated from incubation mixtures of enzyme and either methoxyamine or ammonia gave the normal colour with Ehrlich's reagent with the correct $\lambda_{max.}$ and ϵ values. The unknown pyrrolic compounds produced red colours with Ehrlich's reagent. Absorption reached a maximum after 4min ($\lambda_{max.}$ 555nm) with only a slight shoulder at 525nm unlike porphobilinogen (cf. Fig. 7*a* and 7*d*). A second maximum was present at 486nm which reached its maximum value after 10min. The red colour subsequently faded and left the peak at 486nm. In two large-scale experiments the relative heights of the 555nm and 485nm absorption maxima differed, but the general findings were similar.

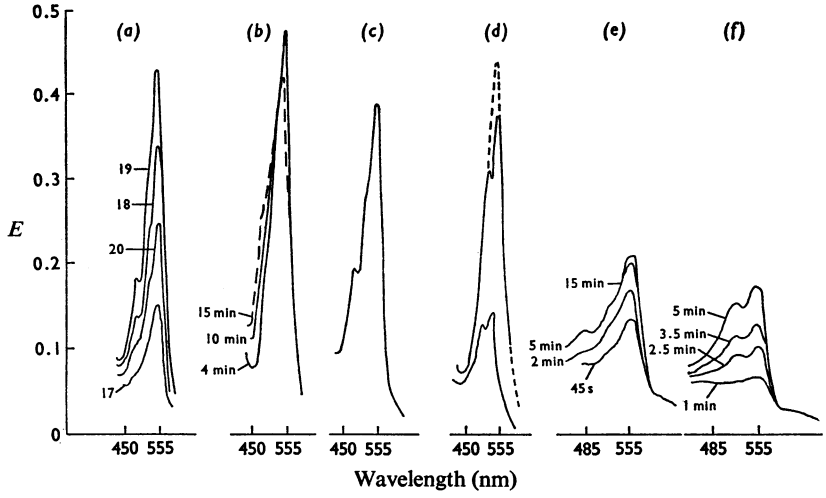


Fig. 7. *Absorption spectra taken at various times after reaction of samples of 'modified pyrrole' with Ehrlich's reagent*

For details see the text. (*a*) Fractions 17–20 from Sephadex chromatography after reaction for 15min with Ehrlich's reagent. The numbers show the fraction used. (*b*) Pyrrole fractions from Dowex 2 (acetate form) after 4, 10 and 15min reaction. The numbers show the reaction times. (*c*) Pyrrole fraction from Dowex 2 after 15min reaction. (*d*) Opsopyrroledicarboxylic acid, (----) and porphobilinogen at two concentrations (——) after 10min reaction. (*e*) 'Modified pyrrole' after electrophoresis after 45s, 2min, 5min and 15min reaction with reagent. The numbers show the reaction times. (*f*) Porphobilinogen after electrophoresis after 1, 2.5, 3.5 and 5min reaction with reagent. The numbers show the reaction times.

A mixture of 1.4mg of porphobilinogen, 0.15ml of enzyme in 0.9ml of 0.1M-Tris–0.006M-EDTA buffer with 0.2M-hydroxylamine, pH8.2, was incubated at 37°C under N_2 for 1h. Samples (5μl) were tested for porphobilinogen at zero time and after 1h; in these experiments 65%, 73% and 80% porphobilinogen had been consumed. The mixture was separated on the standard column of Sephadex G-25 in phosphate buffer. The remaining 'porphobilinogen' was collected and combined (10ml) and the fractions were monitored for reaction with Ehrlich's reagent (Fig. 7*a*).

The pyrroles were adsorbed on a column (4ml vol.) of Dowex 2 (acetate form), washed with water and eluted with 5ml of 1M-acetic acid followed by 8ml of 0.2M-acetic acid, as in the isolation of porphobilinogen (Mauzerall & Granick, 1956). This fraction gave the same colour with Ehrlich's reagent as the original solution (Fig. 7*a* and 7*b*) and was clearly not due to unchanged porphobilinogen. The sample was frozen under N_2 and freeze-dried. In contrast, opsopyrroledicarboxylic acid, *N*-acetylporphobilinogen and tetra-anionic, dipyrrole 1 were firmly held by the resin and were not eluted with 0.2M- or 1.0M-acetic acid. The monopyrroles could, however, be eluted with 6M-acetic acid. Dipyrrole 1 could be eluted rapidly with 3M-HCl, but it decomposed on attempted elution with 1–6M-acetic acid.

The sample of modified pyrrole was subjected to electrophoresis at pH9.2 on cellulose plates and was a mixture of normal porphobilinogen (m_p 1.0) and a faster-moving pyrrole (m_p 1.9). This pyrrole migrated more slowly than the di-anions of opsopyrroledicarboxylic acid and *N*-acetylporphobilinogen (m_p 1.9–2.2), the tetra-anion of dipyrrole 1 (m_p 2.5–2.8), the octa-anion of uroporphyrinogen (m_p 2–2.5) and the tetrapyrrole X, which migrated just behind uroporphyrinogen. The pyrroles from Sephadex gave a similar electrophoretic pattern. The faster-moving pyrrole (m_p 1.9) had the same mobility as the third compound detected by electrophoresis of the original incubation mixture and was neither porphobilinogen nor the tetrapyrrole.

The compound was eluted from the cellulose; it reacted with modified Ehrlich's reagent and gave a red solution which developed maximum absorbance at 555nm after 5min and then faded slowly and was similar to the original sample of pyrrole. A second absorption peak was present at 486nm which reached a maximum value after 10min (Fig. 7*e*). The recovered porphobilinogen had a normal peak at 555nm after 15min with a smaller peak at 525nm (Fig. 7*f*). The modified pyrrole was not very stable; oxidation and polymerization occurred with acetic acid and with HCl or on freeze-drying. Both 'red porphobilin' type compounds and porphyrins were formed after 20h. Chromatographed on cellulose plates in butanol–acetic acid–water (63:11:25, by vol.), the pyrrole moved with, or very slightly ahead of, porphobilinogen (R_F 0.50–0.47), but behind opsopyrroledicarboxylic acid and *N*-acetylporphobilinogen (R_F 0.80–0.84). The synthetic dipyrrole 1 decomposed very readily in the acidic solvent and gave red material. Solutions of the tetrapyrrole X and uroporphyrinogen were converted into uroporphyrin too easily in acid and could not be compared with the unknown pyrrole.

Porphobilinogen was incubated without enzyme with 0.2M- or 0.4M-hydroxylamine for 1.5h at 37°C. The mixture did not show an altered Ehrlich colour, and after incubation for 2.5h and 4h, the electrophoretic pattern was not different from incubations of porphobilinogen alone. Thus the modification of the porphobilinogen arose from an enzymic reaction.

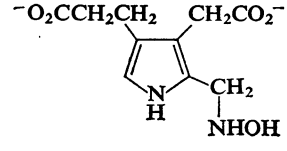
Nature of the modified pyrrole

The elution from Sephadex indicates that the pyrrole is a small molecule like porphobilinogen with an unsubstituted pyrrolic α position. The electrophoresis results at pH9.2 suggest the pyrrole is a di-anion at that pH, whereas porphobilinogen is a mono-anion. The replacement of an aminomethyl group of porphobilinogen by a hydroxylaminomethyl group would be expected to lower the p*K* value from about 10.1 to about 6.0 and so make the molecule a di-anion at pH9.2. However, the possibility of the pyrrole being a tri- or tetra-anion cannot be completely excluded from the electrophoresis results.

On the other hand, the adsorption to and ease of elution from Dowex 2 before electrophoresis suggest that the pyrrole is a di-anion rather than a tri- or tetra-anion and hence a monopyrrole rather than a dipyrrole, and also suggest that in dilute acetic acid the pyrrole carried charges more like porphobilinogen than like opsopyrroledicarboxylic acid or *N*-acetylporphobilinogen. Ion-exchange chromatography at pH7.2 (not reported here) also indicates a di-anionic structure at pH7.2.

The pyrrole differed from both porphobilinogen and the synthetic dipyrrole described by Pluscec & Bogorad (1970) in that the absorption maximum with Ehrlich's reagent and the rate of colour development were slightly different. The variable, but definite, absorption at 486nm in the reaction of the non-purified fractions with Ehrlich's reagent may be caused by the presence of some additional dipyrrolic material, but the amount was appreciably decreased after treatment with Dowex 2.

The pyrrole probably has the structure:



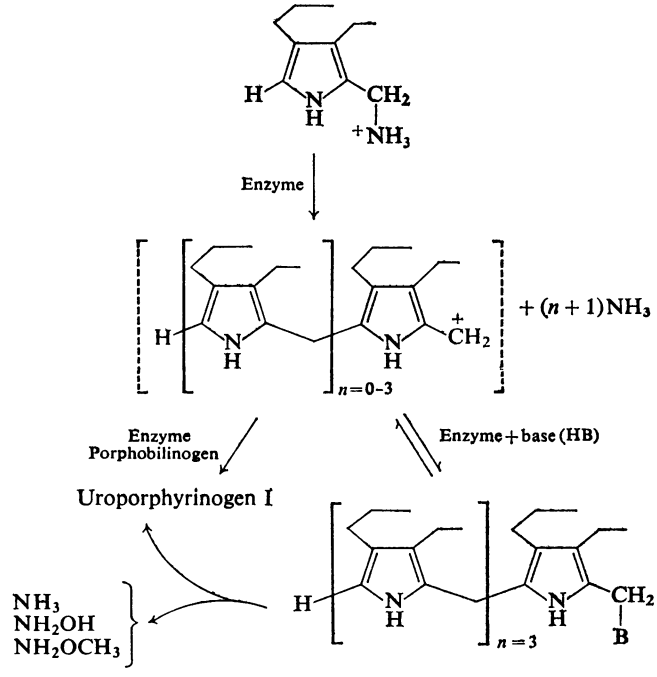
The dipyrrole isolated by Pluscec & Bogorad (1970) from spinach leaf uroporphyrinogen synthetase, porphobilinogen and hydroxylamine may also be a modified derivative rather than an unmodified 5-aminomethyldipyrrylmethane. Such a compound might be expected to differ only slightly from the synthetic 5-aminomethyldipyrrylmethane in its n.m.r. spectrum, spectral shape and rate of formation of the Ehrlich colour as described above. The reported results of Pluscec & Bogorad (1970) suggest that such modified compounds are present.

The overall conclusion is that with enzyme from *R. spheroides* a modified porphobilinogen has been detected in which the amino group has been replaced by a hydroxylamino group with a consequent lowering of the p*K*.

General Discussion

It is generally accepted that substitutions on pyrrolic rings are of the electrophilic type. We may therefore assume that the formation of uroporphyrinogen from porphobilinogen must consist, whether it is catalysed by enzyme or not, of a series of reactions in which an electrophilic group attacks the electron-rich α position in pyrrole rings. In the case of uroporphyrinogen I synthetase the attacking group could be the protonated aminomethyl group, or possibly an enzyme-bound carbonium ion formed by loss of ammonia, or a compound containing an unsaturated methylene group. It also appears that the three condensations leading to the linear tetrapyrrolic compound and the final cyclization occur sequentially on the enzyme and that normally no intermediates appear free in solution (Frydman, R. B. *et al.*, 1971). It is still uncertain whether all the four condensations proceed at one active site with the substrate sliding along the active surface of the enzyme, or whether there are two sites, one for the first three steps and another for the cyclization. Information about the number of binding sites for pyrroles is also scanty.

The results obtained when the enzymic reaction proceeded in the presence of the three inhibitors, ammonia, hydroxylamine and methoxyamine, indicate that the rates of condensation leading to an open-chain tetrapyrrole are not apparently affected, but that the rate of cyclization is greatly decreased. These findings could be explained by a hypothesis postulating two active sites on the enzyme with only one being sensitive to the inhibitors. However, it is also possible that a rate factor is important. It may be that with enzymes from *R. spheroides* the rate of



Scheme 1. *Possible mechanism of the inhibition of the enzymic formation of uroporphyrinogen from porphobilinogen by ammonia, hydroxylamine and methoxyamine*

For details see the text. HB represents ammonia, hydroxylamine or methoxyamine.

the linear condensations is very fast compared with that of the final cyclization and release of uroporphyrinogen, which together may be rate-limiting. If this is the case inhibition of the earlier steps might also occur but the intermediate would remain associated with the enzyme and would not be observed. A third possible explanation is that the stereochemical constraint on the enzyme–substrate complex for the cyclization is much greater than for the linear condensation steps and is thus more susceptible to inhibition.

The results reported here indicate that with the three inhibitors tetrapyrrolic intermediates which differ from one another in structure are formed enzymically from porphobilinogen (Scheme 1). With ammonia as inhibitor it is likely that the compound, i.e. X_{NH_3}, differs only in one respect from the normal intermediate in being released into the solution instead of remaining bound to the enzyme. Thus in the presence of ammonia the postulated open-chain tetrapyrrolic intermediate is prevented from cyclizing and in addition is made to dissociate from the enzyme. A rational explanation of both these findings might be as follows. At the pH of about 8, and less so at 7 or 6, ammonia acts in its non-protonated form as a nucleophile and occupies a position normally taken up by the α position of the terminal pyrrolic group before cyclization. Thus the linear tetrapyrrole with the protonated aminomethyl group is continuously regenerated by exchange with ammonia instead of participating in the final cyclization, possibly through the intermediate formation of a carbonium ion or methylene compound. It is possible that the normal intermediate can dissociate but that in the absence of an inhibitor its rate of dissociation is negligibly small. However, when the life of the complex is greatly extended by continuous regeneration the rate of dissociation becomes sufficiently large to lead to a liberation of the tetrapyrrole, which cannot readily recombine with the enzyme. Once released from the enzyme the tetrapyrrole can only slowly be converted into uroporphyrin non-enzymically and is not a substrate for any of the enzyme systems (Radmer & Bogorad, 1972, and the present work with hydroxylamine).

The action of the enzyme in the presence of the other two inhibitors yields open-chain tetrapyrrolic intermediates in which we may assume, from the evidence given in the present paper, that the aminomethyl group is replaced by the hydroxylaminomethyl group or a methoxyaminomethyl group. Uroporphyrinogen I synthetase appears to catalyse the exchange of a suitable base with the ammonia group of the aminomethyl side chain of the linear tetrapyrrole and also possibly of porphobilinogen, but this substitution does not occur in the absence of enzyme. In such a reaction hydroxylamine and methoxyamine would react as the nucleophilic partners in a substitution reaction. In addition, this exchange reaction may lead to an inversion of the configuration at the aminomethyl group and a subsequent release from the enzyme of the new tetrapyrrole, if it has a stereochemistry at the —CH_2— group that resembles that in the product uroporphyrinogen,which is normally released. Hydroxylamine and methoxyamine act as nucleophiles in the substitution reactions with the exocyclic amino group of cytidine and in the addition reactions with the pyrimidine rings of cytosine and uridine (Budowsky *et al.*, 1971, 1972) and also with the positive centre of NAD^+ in the presence of alcohol dehydrogenase (Burton & Kaplan, 1954), but such reactions with pyrrole rings have not been recorded. Consequently, attention is focused on reaction with the aminomethyl side chain rather than on additions to the pyrrole ring.

The question then arises as to whether this substitution of the aminomethyl group also occurs at the first, second or third condensations leading to modified porphobilinogen, modified di- and tri-pyrroles and modified tetrapyrroles. If this were the case we might expect to find such intermediates in experiments in which such inhibitors were used. With the exception of one hydroxylamine derivative, which we shall consider presently, no such modified intermediates other than the tetrapyrroles have been formed and released, which implies again that the first three condensations differ in an important respect from the cyclization. Alternatively, we assume the modified mono-, di- and tri-pyrroles react like their normal counterparts in the linear condensation, although inactive in the final cyclization. In experiments with hydroxylamine, by far the most effective of the three inhibitors, a modified porphobilinogen has been isolated which appears to have a hydroxylaminomethyl group instead of the aminomethyl group. This compound was not further acted on by the enzyme and it is possible, therefore, that the formation of linear modified tetrapyrroles does not involve the formation of intermediate modified lower pyrroles. Thus with enzyme from *R. spheroides* substitution appears to occur at the final cyclization step and clarification of the differences between the two types of condensation step must be obtained from further experiments involving ammonia.

With enzymes from other sources interference with the condensation may also occur at some of the earlier linear condensation steps, so that, for example, a dipyrrole is found with enzyme from spinach leaves and hydroxylamine (Pluscec & Bogorad, 1970) and a presumed tripyrrole is found with enzyme from soya-bean callus tissue treated with porphobilinogen in the absence of added inhibitors (Llambias & Batlle, 1970), and both pyrroles could be incorporated into porphyrin when re-incubated with porphobilinogen and their respective enzymes. In addition, although the consumption of porphobilinogen is not affected

by hydroxylamine, inhibition of either uroporphyrinogen I synthetase of avian erythrocytes (Llambias & Batlle, 1971*b*) or uroporphyrinogen III co-synthetase of *R. spheroides* or spinach leaves may precede or accompany the type of inhibition considered in the present paper. Apparent competition between the amines and a pyrrole for a binding site as described here for enzyme from *R. spheroides* may be responsible for these effects.

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