Jones, R. G. W. (1967), *Biochem. J. 103*, 714.
King, F. E., King, T. J., and Manning, L. C. (1957), *J. Chem. Soc.*, 563.
Law, A., Threlfall, D. R., and Whistance, G. R. (1971), *Biochem. J. 123*, 331.
Lester, R. L., Crane, F. L., and Hatefi, Y. (1958), *J. Amer. Chem. Soc. 80*, 4751.
Nilsson, J. L. G., Farley, T. M., and Folkers, K. (1968), *Anal. Biochem. 23*, 422.
Nowicki, H. G., Dialameh, G. H., Trumpower, B. L., and Olson, R. E. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 884.
Olsen, R. K., Smith, J. L., Daves, G. D., Moore, H. W., Folkers, K., Parson, W. W., and Rudney, H. (1965), *J. Amer. Chem. Soc. 87*, 2298.
Olson, R. E. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 85.
Olson, R. E. (1966), *Vitamins Hormones 24*, 551.
Olson, R. E., and Aiyar, A. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 217.
Parson, W. W., and Rudney, H. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 599.
Raman, T. S., Rudney, H., and Buzzelli, N. K. (1969), *Arch. Biochem. Biophys. 130*, 164.
Rudney, H., and Raman, T. S. (1966), *Vitamins Hormones 24*, 531.
Rudney, H., and Sugimura, T. (1961), *in* Quinones in Electron Transport, Wolstenholme, G. E. W., and O'Connor, C. M., Ed., London, J. E. Churchill, Ltd., p 211.
Spiller, G. H., Threlfall, D. R., and Whistance, G. R. (1968), *Arch. Biochem. Biophys. 125*, 786.
Stoffel, W., and Martius, C. (1960), *Biochem. Z. 333*, 440.
Trumpower, B. L., Aiyar, A. S., Opliger, C. E., and Olson, R. E. (1972), *J. Biol. Chem.* (in press).
Whistance, G. R., Brown, B. S., and Threlfall, D. R. (1970), *Biochem. J. 117*, 119.
Whistance, G. R., Dillon, J. F., and Threlfall, D. R. (1969), *Biochem. J. 111*, 461.
Whistance, G. R., Field, F. E., and Threlfall, D. R. (1971), *Eur. J. Biochem. 18*, 46.
Whistance, G. R., and Threlfall, D. R. (1967), *Biochem. Biophys. Res. Commun. 28*, 295.
Winrow, M. J., and Rudney, H. (1969), *Biochem. Biophys. Res. Commun. 37*, 833.

A Tetrapyrrylmethane Intermediate in the Enzymatic Synthesis of Uroporphyrinogen†

Richard Radmer‡ and Lawrence Bogorad*

ABSTRACT: An uncyclized tetrapyrrylmethane has been identified and recovered from reaction mixtures of the enzyme uroporphyrinogen I synthetase and the pyrrole porphobilinogen incubated with ammonium ions. The polypyrrole has been judged to be a tetrapyrrylmethane on the basis of its electrophoretic behavior and the observation that it forms uroporphyrinogen I *via* a nonenzymatic first-order reaction. The effect of pH on the rate of formation of uroporphyrinogen can be taken to suggest that the tetrapyrrylmethane bears an aminomethyl group on an α position. The tetrapyrrylmethane is not converted to uroporphyrinogen III in an enzymatic system capable of forming this isomer from porphobilinogen. Some hypotheses for the formation of uroporphyrinogen III are reevaluated in the light of these observations.

The enzymatic formation of uroporphyrinogen III (urogen III)[1] from the monopyrrole porphobilinogen (PBG) requires the action of two enzymes—urogen I synthetase and urogen III cosynthetase (Bogorad, 1958b). Urogen III is a precursor of hemes and chlorophylls.

Urogen I is formed when PBG is incubated with urogen I synthetase (Bogorad, 1958a). Although urogen I is not a substrate for the synthesis of urogen III by urogen III cosynthetase, it seems likely that some earlier product of urogen I synthetase is utilized for the enzymatic formation of urogen III.

It has been observed that polypyrrolic precursors of urogen I accumulate in reaction mixtures of PBG and urogen I synthetase in the presence of hydroxylamine or ammonium ions (Bogorad, 1963; Pluscec and Bogorad, 1970). These products can be separated by paper or thin-layer electrophoresis. One of the intermediates which accumulates in hydroxylamine-inhibited reactions has been identified as a dipyrrylmethane by Pluscec and Bogorad (1970). It was also observed in these experiments that hydroxylamine and ammonium ions inhibited the formation of urogen I in different ways;

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[1] Abbreviations used: urogen I, uroporphyrinogen I; urogen III, uroporphyrinogen III; PBG, porphobilinogen; tle, thin-layer electrophoresis; uro, uroporphyrin; Copro, coproporphyrin.

i.e., these inhibitors seemed to bring about the accumulation of different groups of intermediates. For example, at comparatively low concentrations of hydroxylamine the yields of urogen I were found to be substantially less than 100% even after prolonged incubation, while at 10-fold higher concentrations of ammonium ions PBG was converted to urogen I virtually quantitatively *via* intermediate polypyrroles which accumulated and could be detected. This suggested that an open-chain tetrapyrrole which could subsequently cyclize to form urogen I might be accumulating in reaction mixtures inhibited by ammonium ions.

The present communication describes the isolation and identification of a tetrapyrrylmethane from reaction mixtures of PBG and urogen I synthetase inhibited by ammonium ions. A compound of this type has been suggested as a precursor of urogen III by Wittenberg (1959) and by Mathewson and Corwin (1961). This possibility has been investigated in some of the experiments described here.

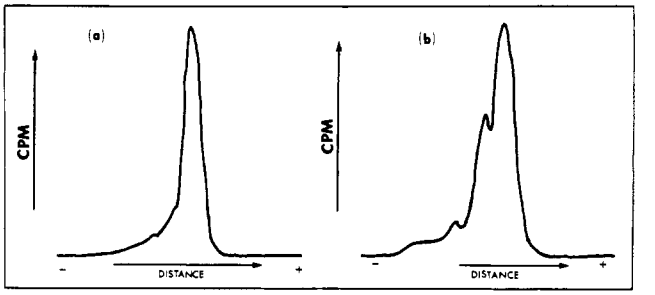

FIGURE 1: Distribution of radioactivity after thin-layer electrophoresis of an enzymatic reaction mixture inhibited by (A) NH_4^+ and (B) NH_2OH. PBG-^{14}C was supplied as the substrate. The distribution was determined using a Packard radiochromatogram scanner with the following settings: time constant = 10; range = 1000; scan speed = 1 cm/min; slit = 2 mm × 1 in. Other experiments revealed that the major peak consists of a mixture of tetrapyrrylmethane and -methene trailing just behind a red-fluorescing mixture of urogen and Uro.

Methods and Results

Thin-Layer Electrophoresis of Pyrrolic Products Which Accumulate in Reaction Mixtures Containing PBG, Urogen I Synthetase, and NH_2OH or NH_4^+. The following incubation mixtures were used for these experiments. EXPERIMENT 1. PBG (0.255 μmoles), Tris (pH 8.5, 15 μmoles), ammonium acetate adjusted to pH 8.5 (10 μmoles), cysteine adjusted to pH 8.5 (1.25 μmoles), sufficient urogen I synthetase ammonium sulfate fraction B (Bogorad, 1962) to consume the PBG in 60–90 min, and distilled water to bring the total volume to 50 μl. The PBG-^{14}C was prepared from δ-aminolevulinic acid-^{14}C as outlined by Pluscec and Bogorad (1970) and had a specific activity of about 10,000 dpm/μg. The enzyme was prepared from spinach leaf tissue at the New England Enzyme Center, Tufts University School of Medicine. The PBG was a generous gift from Dr. S. F. MacDonald of the NRC of Canada; it was synthesized by the method of Arsenault and MacDonald (1961).

EXPERIMENT 2. The incubation mixture for this experiment was the same as was used in expt 1 except that 0.2 M NH_2OH was used as the inhibitor instead of 0.2 M NH_4^+.

After incubation, 1 μl of each incubation mixture was tested for the presence of polypyrroles with 1 μl of modified Ehrlich reagent (Pluscec and Bogorad, 1970).

Thin-layer electrophoresis was performed on 5 × 20 cm glass plates coated with MN-cellulose 300 (Brinkmann Instruments, Inc.) at a thickness of 0.5 mm (wet). Before use each plate was sprayed with buffer (0.05 M barbital sodium, pH 9.3) and equilibrated 20 min in a potential gradient of 50 V/cm using the same buffer. Samples of 5–10 μl each were applied near the negative end of the plate and electrophoresis was carried out at 4° for 75 min in the same potential gradient. After electrophoresis the plates were dried under a heat lamp and scanned using a Packard Model 7201 radiochromatogram scanner.

The results of this experiment (Figure 1) show that several products accumulate if the enzyme reaction is inhibited with either ammonium ions or hydroxylamine, although the spectrum of intermediates produced is different in the two cases. Inhibition by hydroxylamine (Figure 1b) results in the accumulation of fairly large amounts of di- and tripyrroles in addition to the tetrapyrrole and urogen I (Pluscec and Bogorad, 1970). Inhibition by ammonium ions, on the other hand (Figure 1a), seems to cause tetrapyrrole and urogen I to accumulate almost to the exclusion of di- and tripyrroles. These observations corroborate the data of Bogorad (1963) in which it was shown that yields of urogen I from PBG approaching 100% were obtained upon extended incubation of NH_4^+-inhibited reaction mixtures, while a maximum of 70% of the PBG was recovered as urogen when the reaction was inhibited with 0.2 M hydroxylamine. It was because of the relative homogeneity of product in the NH_4^+-inhibited reaction mixture that this system was selected as the source of the presumed tetrapyrrole for these experiments.

The subsequent experiments were performed to isolate the presumptive linear tetrapyrrole in order to establish some of its properties and to determine whether this compound is an intermediate in the enzymatic synthesis of urogen III.

Preparation and Purification of the Tetrapyrrole. The preparative incubation mixture contained the following compounds in a final volume of 2.0 ml: PBG, 9 μmoles; Tris, pH 8.5, 600 μmoles; ammonium acetate adjusted to pH 8.5, 400 μmoles; cysteine adjusted to pH 8.5, 50 μmoles; distilled water; and sufficient urogen I synthetase ammonium sulfate fraction B to consume the PBG in 60–90 min.

The reaction was carried out in Thunberg tubes which were evacuated and then filled with oxygen-free N_2 before incubation at 37°. The PBG present was estimated in 10-μl aliquots removed from the reaction vessel before evacuation and after 30-min incubation, using the modified Ehrlich reagent described by Mauzerall and Granick (1956). From these values the time of PBG exhaustion plus 30 min was calculated assuming a linear rate of PBG consumption.

After incubation for 30 min longer than the time calculated to be required for complete consumption of PBG, the components of this preparation were separated by column chromatography at 4° using a Whatman DE-52 column with stepwise elution. The column was made from 1 g of Whatman DE-52 equilibrated with a solution of 0.15 M Tris (pH 8.5) and 25 mM cysteine (pH 8.5), supported in a Pasteur pipet. After the reaction mixture had been applied, the column was washed with 8 ml of 0.25 M Tris–0.025 M cysteine (pH 8.3) which removed the NH_4^+ ions, urogen I synthetase, and some smaller pyrrolic components. The desired pyrrolic product was then eluted with 8 ml of 0.5 M Tris–0.025 M cysteine (pH 8.3). Solid $BaCl_2$ was then added (0.25 g/ml), after which the pyrrole was precipitated by the addition of four volumes of methanol according to a method described for dipyrrylmethanes (S. F. MacDonald, personal communication). After

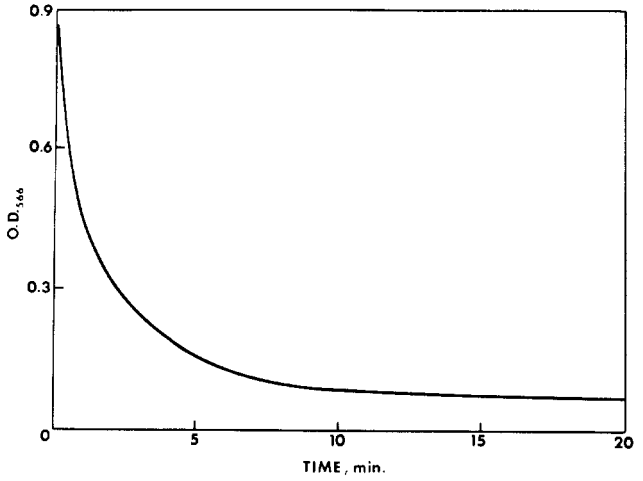

FIGURE 2: Spectra from 480 to 580 nm showing the change in the absorption spectrum of the Ehrlich complex of the tetrapyrrole with time (see text). Scans started A, 30 sec; B, 1 min; C, 3 min; D, 10 min after mixing Ehrlich solution and sample. Each scan was completed in 20 sec.

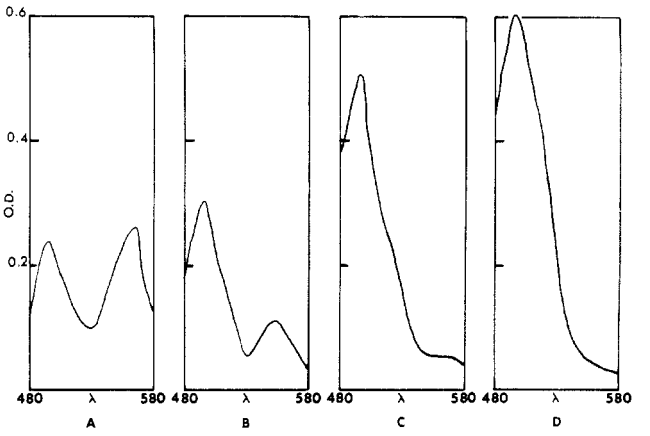

FIGURE 3: Change in absorbance at 566 nm with time of tetrapyrrole–Ehrlich reaction (see text).

centrifugation the barium salt was dissolved in 0.15 M Tris–0.025 M cysteine (pH 8.5) and, when required, the Ba^{2+} was precipitated by the addition of solid K_2SO_4. (The amounts used here depended on the experiment to be performed.)

As is shown below, this compound is extraordinarily labile and is subject to several types of alteration, including oxidation and spontaneous cyclization. Accordingly, a new preparation, isolated immediately before use, was employed in each experiment.

In order to determine the homogeneity of this type of preparation the pyrrolic components were labeled using PBG-^{14}C as the substrate (400,000 dpm of PBG-^{14}C in a total volume of 1 ml). After the components of the reaction mixture had been separated on DE-52, as described above, the tetrapyrrole was dissolved in a minimal amount of buffer and analyzed electrophoretically as described in the previous section. The radioactivity after electrophoresis of the purified tetrapyrrole was in one symmetrical peak. However, the spot on the plate corresponding to this peak could be resolved into two components, a red fluorescent leading edge containing Uro (formed easily by oxidation of urogen) and a trailing edge containing partially oxidized linear tetrapyrrole. Apparently, some of the tetrapyrrole cyclized during electrophoresis, yielding a ratio of uncyclized to cyclized tetrapyrrole of approximately 1:1, and the peak appeared symmetrical. The fact that the Uro migrates ahead of the uncyclized tetrapyrrole suggests that the latter has a protonated amino group. The Uro has a net charge of -8 and the tetrapyrrole thus has a net charge of -7 or less.

This electrophoretic evidence also demonstrates that the tetrapyrrole preparation has no pyrrolic contaminants other than urogen. In addition, there was no NH_4^+ ion detectable by Nesslerization and no detectable urogen I synthetase activity in the material obtained from DE-52. Material purified in this way was used to study the reaction of this compound with *p*-dimethylaminobenzaldehyde in the Ehrlich reaction and to establish the absorption spectrum and extinction coefficient of the oxidized form of the polypyrrole.

Reaction of the Tetrapyrrole with Ehrlich Reagent. Material for the study of the Ehrlich reaction was prepared by taking up the barium salt of the purified tetrapyrrole preparation in 8 ml of 0.15 M Tris–0.025 M cysteine (pH 8.5). An aliquot of this solution was then diluted with three volumes of 0.15 M Tris–0.025 M cysteine–0.1 M EDTA. Immediately before the solution was to be used the cysteine was removed by the addition of one-fourth volume of 0.25 M $HgCl_2$. Equal volumes of this preparation and of modified Ehrlich reagent were mixed and the spectra recorded from 580 to 480 nm at a scanning speed of 5 nm/sec using a Cary recording spectrophotometer Model 14 to record the time course of the reaction (Figure 2).

A similar experiment was also performed to determine the change in absorbance with time at 566 nm, the absorption maximum of the complex between the tetrapyrrole and *p*-dimethylaminobenzaldehyde. Within 5 sec after mixing, the sample was introduced into the spectrophotometer and recording was begun (Figure 3). Recording was continued until the optical density remained constant (about 20 min).

The Ehrlich reaction of this compound is extremely intense and rapid. The time course of this reaction indicates that the absorption at 566 nm declines rapidly (Figure 3) at the same time that an absorption peak at 495 nm increases in intensity (Figure 2). After 30 sec, the Ehrlich peak at 566 nm is roughly equal to that at 495 nm. At the end of 1 min, however, the 566-nm peak is only about one-half as intense as the 495-nm peak, and at the end of 10 min, there is no discernible peak in the 566-nm region. The residual absorption at 566 nm is probably not due to the Ehrlich reaction but rather to the base of the strong 495-nm peak. This series of changes is probably due to the extremely rapid formation of a complex between the pyrrolic compound and the *p*-dimethylaminobenzaldehyde followed by the rapid oxidation by the perchloric acid in the Ehrlich reagent of the bilane to the bilidiene with the concomitant loss of absorbance at 566 nm.

Spectral changes which in some respects resemble those observed in the tetrapyrrole–Ehrlich reaction have been reported for the reaction between a dipyrrylmethane and the Ehrlich reagent (Pluscec and Bogorad, 1970). However, these two reactions differ markedly at least in their kinetics (compare Figure 2 with Figure 1, Pluscec and Bogorad, 1970).

The isolated tetrapyrrole has no absorption in the visible region and, therefore, it is a bilane. Photooxidation in HCl yields a product with an absorption maximum at 495 nm, although some cyclization probably occurs in the time required for photooxidation.

Extinction Coefficient of the Derived Bilidiene. For this experiment a tetrapyrrylmethane preparation purified by chro-

TABLE I: Data Used to Determine the ϵ_{mM} of the Derived Tetrapyrrolic Bilidiene in 1 N HCl.

Expt	A (495 nm)	Concn of Tetrapyrrole (μmoles/ml $\times 10^3$)	Uro A 495 nm	Uro A ϵ_{mM}
1	1.336	25	0.150	48
2	0.667	13.8	0.036	46

matography on DE-52 and precipitated by $BaCl_2$ was taken up in 4 ml of Tris-cysteine–EDTA and the urogen concentration was determined by I_2 oxidation of three 0.3-ml aliquots at time zero. Three other 0.3-ml aliquots were made 1 N with HCl and illuminated (350 ft-candles General Electric cool-white fluorescent lights) for 30 min. After appropriate dilution with 1 N HCl the spectra were recorded on the Cary spectrophotometer. A set of 0.3-ml aliquots was incubated 3 hr at 50° under N_2 before oxidation with I_2 to determine their content of urogen I. From the difference in porphyrin concentrations of these two Uro determinations the amount of tetrapyrrole present initially was estimated. This value along with the photooxidation data allows the molar extinction of the bilidiene to be calculated. The ϵ_{mM} of the bilidiene was estimated from the values in Table I. It was assumed that the precursor was converted to urogen I with a yield of 100%. This assumption is probably valid because (a) the absorption spectrum of the solution after oxidation was that of urogen without detectable abnormalities and (b) earlier data (Table I in Pluscec and Bogorad, 1970) showed that final yields of almost 90% were obtained in unfractionated reaction mixtures of spinach leaf urogen I synthetase, PBG, and 0.2 M ammonium acetate.

The correction for absorbance by Uro present initially was made by measuring the absorption of the oxidized solution at 406 nm and using this figure to calculate the amount of absorbance due to Uro at 495 nm, the maximum for the bilidiene.

Photooxidation in acid as well as oxidation by I_2, O_2, HNO_3, H_2O_2, or $FeCl_3$ yielded a compound with a bilidiene-type spectrum. Attempts to oxidize this compound further to the state of a bilitriene (which would be expected to absorb at about 600–650 nm) by the method of Fisher *et al.* (1952) were unsuccessful, even though bilirubin was oxidized to biliverdin in a parallel experiment. (This behavior is not unusual; many bilidienes exist which cannot be oxidized to the corresponding bilitrienes (D. Dolphin, personal communication).)

Nonenzymatic Production of Urogen I. Determination of the Reaction Order. Material for the kinetic analysis was prepared by diluting the purified tetrapyrrole preparation to a final volume of 14 ml in 10 ml of 0.15 M Tris–0.025 M cysteine and 4 ml of 1 M EDTA at a final pH of 8.5. This solution was then divided into 0.3-ml aliquots and incubated at 50° under N_2. At time zero and at 30-min intervals thereafter the urogen concentration of three aliquots was determined using the I_2 oxidation technique described by Bogorad (1962). This assay was continued until the concentration of porphyrinogen remained constant (about 3 hr).

The results of this experiment are presented in Figure 4. These data indicate that the nonenzymatic formation of urogen from its polypyrrolic precursor is first order with respect

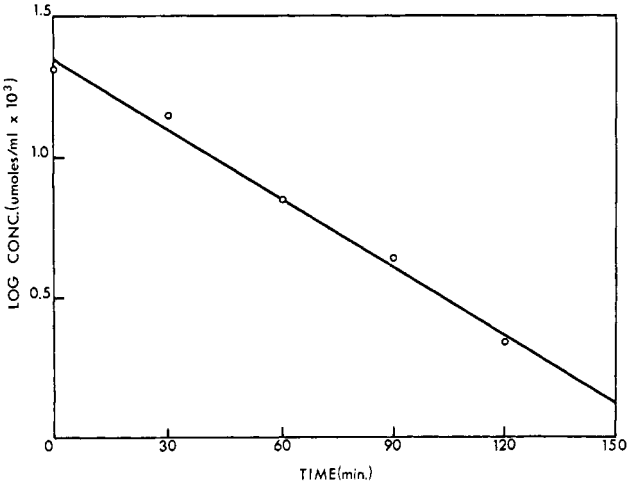

FIGURE 4: First-order plot of kinetic data for nonenzymatic formation of urogen I from the polypyrrylmethane. k_1 = slope × 2.303; slope = 8×10^{-3}; k_1 = 0.018 min^{-1}. The tetrapyrrole concentration was taken as the difference between the concentration of urogen at the commencement and at the end of the reaction.

to this precursor which has a half-life of about 30 min at 50°.

As a further demonstration that urogen I formation is first order, *i.e.*, that it results from the cyclization of a single molecule of a tetrapyrrole rather than a condensation of mono- or polypyrroles, the rate of the reaction was measured starting with different concentrations of substrate. If the formation of urogen is indeed first order, there should be no difference in the apparent rate constant regardless of the initial substrate concentration.

The rate equation for a first-order reaction is $\ln([A_0]/[A]) = k_1t$; for a second-order reaction: $(1/[A]) - (1/[A_0]) = k_2t$, where $[A_0]$ = concentration at time zero and $[A]$ the concentration at time t. If t is constant k_1 and k_2 can be compared directly—set $x_1 = k_1t$ and $x_2 = k_2t$. If the reaction is first order and is run at different initial concentrations, the calculated x_1 will remain constant while the calculated x_2 will vary widely.

Material for this experiment was prepared by diluting the purified tetrapyrrylmethane preparation to a volume of 8 ml with 0.5 M Tris–0.025 M cysteine–0.1 M EDTA at a final pH of 8.5. Four different groups of solutions were then prepared in triplicate: (1) solutions oxidized immediately with I_2; (2) solutions containing the same concentrations of tetrapyrrole as in group 1; (3) solutions diluted four times; and (4) solutions diluted 16 times. The reaction mixtures in groups 2–4 were then incubated 30 min at 50° under N_2, after which the porphyrin concentrations were determined spectrophotometrically after oxidation with I_2. Table II shows the results of this experiment. The data are in agreement with the kinetic experiment and support the conclusion that the formation of urogen is first order. The precursor of urogen in this reaction thus appears to be an uncyclized tetrapyrrole.

Effect of pH on the Rate of Nonenzymatic Urogen I Formation. The rates of formation of urogen I in the absence of enzyme at pH 5.9, 8.5, and 12.1 were studied to determine whether there might be an ionizable amino group on the tetrapyrrole which would affect the cyclization reaction.

Purified tetrapyrrole preparation was diluted up to 6 ml with 0.15 M Tris–0.025 M cysteine–0.1 M EDTA at a final pH of 8.5. Four groups of three sets of tubes were prepared. Each tube contained 0.3 ml of this solution plus: sets 1 and 2 0.1

TABLE II: Experimental and Derived Data Obtained to Determine the Effect of Substrate Concentration on the Rate of Nonenzymatic Urogen I Formation.[a]

Expt	Tetrapyrrole (mM × 10^2) At T_0	Tetrapyrrole (mM × 10^2) At T_{30}	Calcd X_1	Calcd X_2
1	4.48	2.05	3.08	0.265
2	1.12	0.578	2.96	0.840
3	0.28	0.128	3.14	4.77

[a] These data show that X_1 is essentially constant while X_2 varies. It therefore appears that the nonenzymatic formation of urogen is first order with respect to substrate and that the compound isolated is a tetrapyrrole.

TABLE III: The Effect of pH on the Nonenzymatic Formation of Urogen I.

pH	Tetrapyrryl-methane at T_0[a] (mM × 10^2)	Amt of Urogen Formed in 30 min (mM × 10^2)	Amt Consumed at pH X: Amt Consumed at pH 8.5[b]
		Expt 1	
5.9	5.48	2.32	1.07
12.1	5.48	1.05	0.49
8.5	5.48	2.16	1.00
		Expt 2	
5.9	2.80	1.33	0.96
12.1	2.80	0.56	0.40
8.5	2.80	1.39	1.00

[a] The initial concentration of tetrapyrrylmethane is based on estimates in which it is assumed that urogen I is formed (from it) in 100% yield during incubation at 50° for 3 hr under N_2. [b] Rates were calculated assuming first-order kinetics. The composition of each incubation mixture is given in the text.

ml of H_2O; set 3 0.1 ml of 1 M acetate (pH 4.6); set 4 0.1 ml of 1 N NaOH. Set 1 was analyzed at time zero to measure initial tetrapyrrylmethane and urogen; sets 2–4 were incubated 30 min at 50° under N_2. After incubation, 0.1 ml of 4 M Tris (pH 8.0) was added to each tube, the urogen was oxidized with I_2, and the porphyrin was estimated spectrophotometrically. In addition, 1 ml of the diluted tetrapyrrole preparation was incubated at 50° under N_2 for 3 hr and then analyzed in triplicate for comparison with set 1 to estimate the initial concentration of presumptive tetrapyrrole in each reaction mixture.

The results of this experiment (Table III) suggest that an amino group is present and that the rate of cyclization is higher when this group is protonated. The pK_a of the amino group of PBG is 10.1 (Granick and Bogorad, 1953). Assuming that the pK_a of the amino group of the tetrapyrrole has roughly the same value, it can be inferred that at pH 8.5 the amino group is almost fully protonated, while at pH 12.1 only about 1% of the groups are protonated. This is reflected in the lower rate of cyclization although the effect is smaller than would be expected if protonation were the factor-limiting cyclization. These data furnish at least presumptive evidence that the tetrapyrrole bears an aminomethyl group from one of the molecules of PBG from which it was formed.

Determination of Biological Significance. The hypotheses of Wittenberg (1959) and Mathewson and Corwin (1961) postulate that a pyrrolic compound of the type isolated here is the key intermediate in the formation of urogen III. Both of these hypotheses are of the form PBG → open-chain tetrapyrrole (I-isomer type) → urogen III. In the following experiments these hypotheses as well as the possibility that both enzymes plus PBG might be required to utilize the tetrapyrrylmethane for the synthesis of urogen III were tested.

Tetrapyrrole purified by DE-52 chromatography yielded only urogen I after incubation with urogen III cosynthetase. Thus, contrary to the prediction of the hypotheses of this type, this intermediate in urogen I biosynthesis is not converted to urogen III by urogen III cosynthetase alone.

Another type of experiment was designed to test the possibility that both enzymes or both enzymes *plus* PBG might somehow be required to utilize the tetrapyrrylmethane for the synthesis of urogen III. ^{14}C-Labeled tetrapyrrylmethane was isolated and incubated with PBG-^{12}C and both urogen I synthetase and urogen III cosynthetase. The formation of urogen III would show that the entire enzyme system was working; the presence of any radioactivity in urogen III would demonstrate that the tetrapyrrylmethane precursor of urogen I can also serve as a precursor of urogen III.

^{14}C-Labeled tetrapyrrole was prepared from 10^6 dpm of PBG in a 1-ml preparative incubation mixture. The tetrapyrrylmethane was purified on DE-52 in the usual manner and taken up in 4 ml of 0.15 M Tris–0.025 M cysteine (pH 8.5). To 1 ml of the solution of purified tetrapyrrylmethane was added 4.5 μmoles of PBG and sufficient urogen I synthetase and urogen III cosynthetase to catalyze the formation of 0.3 μmole of urogen III in 2 hr.

After incubation the porphyrin was isolated by the method of Bogorad (1958a). The uroporphyrin was decarboxylated to coproporphyrin (Copro) by the procedure of Edmondson and Schwartz (1953). The porphyrin was then spotted on Whatman No. 1 filter paper and the descending chromatogram was developed for 24 hr in darkness using a mixture of 2,6-lutidine–ammonia–water–0.1 M EDTA (25:10.5:7:0.05, v/v). After development, the Copro isomers were located by their fluorescence and their position was marked. A 2-in. wide strip of the chromatogram was then analyzed for ^{14}C with a Packard radiochromatogram scanner. As a further check the chromatogram was cut into squares and analyzed using a Packard scintillation counter. In such experiments, all of the radioactivity appeared only in the I isomer; Copro III was produced entirely from PBG-^{12}C.

In summary, the following reactions occurred during incubation with the enzymes: (a) PBG-^{12}C → urogen-^{12}C III and (b) tetrapyrrole-^{14}C → urogen-^{14}C I. The tetrapyrrole we isolated did *not* serve as a substrate for the urogen III synthesizing system. The urogen I which formed arose at least in part by the spontaneous cyclization of the tetrapyrrole which has its side chains arranged in the symmetrical order of urogen I. These observations will be evaluated more fully in the next section.

An experiment was also performed to determine whether the cyclization of the linear tetrapyrrole can be accelerated by urogen I synthetase. After the tetrapyrrole had been iso-

lated in the usual manner, aliquots were incubated at 37° under N_2 with or without urogen I synthetase for 90 min. After oxidation with I_2 the protein was precipitated by bringing the reaction mixture to a final trichloroacetic acid concentration of 20%. After centrifugation the solution was made 1 N with respect to HCl and the amount of Uro was determined spectrophotometrically.

Those experiments showed that the rate of cyclization of the isolated tetrapyrrole is not increased by urogen I synthetase. Furthermore, since the rate of cyclization of the free tetrapyrrylmethane is comparatively slow—its half-life is about 30 min—it probably does not exist free in solution as a precursor of urogen I in a normal, noninhibited enzymatic reaction. However, since tetrapyrrylmethane can exist in many configurations, it is conceivable that only one or a few forms can become associated with the enzyme and that these very configurations are highly favorable for nonenzymatic cyclization. Thus the enzymatic reaction might not proceed more rapidly than the nonenzymatic one even though the enzyme can catalyze cyclization of this tetrapyrrylmethane.

Discussion

The following observations indicate that the compound studied in these experiments is a linear tetrapyrrole with the structure shown in Figure 5a. (1) The compound is produced from PBG and can be converted into urogen I; it is therefore pyrrolic. (2) It reacts with the Ehrlich reagent very rapidly at room temperature and therefore has a free α position. (3) This compound is not a dipyrrole because, first, it is electrophoretically different from the dipyrrole formed from PBG and, second, its nonenzymatic conversion to urogen follows first-order kinetics. (4) Since PBG does not have to be present to form urogen from the polypyrrole, the precursor is not a tripyrrole. (5) Since it migrates slightly behind urogen on electrophoresis at pH 9.4, it contains no more than eight negative charges and therefore has not more than four pyrroles. This also indicates that the compound is a tetrapyrrole. (6) The isolated compound does not absorb visible light. However, upon oxidation it is converted to a compound which has an absorption maximum at 495 nm. This indicates that the isolated compound is a bilane which is converted to a bilidiene or bilidiene-like compound upon oxidation (Figure 5b). (7) Because it cyclizes to form urogen I, its β substituents are known to be alternating acetic and propionic acid residues. (8) From the effect of high pH on the nonenzymatic rate of urogen formation it may be inferred than an amino group is present and participates in the cyclization of this compound. The presence of an amino group may also be inferred from the slightly slower electrophoretic mobility of this tetrapyrrole than urogen.

The tetrapyrrole described in these experiments has the same structure as the tetrapyrrole proposed as an intermediate in the enzymatic formation of urogen III by Wittenberg (1959). The fact that this compound is not utilized in the formation of urogen III in the present experiments tends to cast doubt on the validity of the Wittenberg hypothesis. However, the observation that urogen I synthetase does not accelerate the conversion of the tetrapyrrole to urogen I points up the possibility that such a tetrapyrrylmethane may be an intermediate in urogen III formation but that it does not ordinarily occur free in solution. The nascent bound tetrapyrrylmethane could, for example, be converted as part of a complex with the two enzymes or be transferred from urogen I synthetase to urogen III cosynthetase during the existence of a transitory complex.

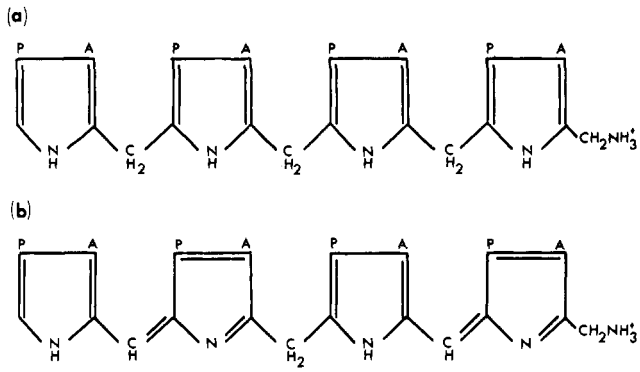

FIGURE 5: Proposed structures of (A) the isolated tetrapyrrylmethane P = $CH_2CH_2COO^-$; and (B) the tetrapyrryle after photooxidation. A = CH_2COO^-.

A similar compound was proposed as an intermediate in the biosynthesis of urogen III by Mathewson and Corwin (1961) on the basis of studies using Stuart models. However, the intermediate that they proposed retains its α-hydrogens and has the pyrrole rings in the pyrrolenine form, thereby forming a more flexible tetramer. This hypothesis cannot be tested directly using the isolated tetrapyrrole since its α-hydrogens are presumably lost and the imine hydrogens retained. S. F. MacDonald *et al.* (in preparation; also compare Kim, 1969) have synthesized a dipyrrylmethane which is an intermediate in the enzymatic synthesis of urogen (Pluscec and Bogorad, 1970). This compound has both rings in the pyrrole form and retains both imine hydrogens at the expense of its α-hydrogens. Although no data are available to resolve this question, it seems quite likely that the tetrapyrrole being studied has this same arrangement of hydrogen atoms.

However, the requirement for the pyrrolenine forms in the Mathewson–Corwin hypothesis can be questioned on theoretical grounds. Using Corey–Pauling models (Ealing Corp., Cambridge, Mass.) it is possible to demonstrate the formation of urogen III according to the proposed scheme without the necessity of retaining the α-hydrogens on the pyrroles. These contradictory results may be due to the improved molecular models available since this hypothesis was advanced in 1960. Thus, although this hypothesis cannot be tested directly, at least one aspect of it can be questioned on the same sorts of grounds on which it was proposed.

Bogorad (1958b) has demonstrated that although urogen III cosynthetase consumes no PBG when incubated in the absence of urogen I synthetase, the V_{max} of the enzymatic reaction is higher when both enzymes are incubated with PBG than when urogen I synthetase alone is incubated with PBG. This suggests that urogen III cosynthetase might catalyze the consumption of PBG in the presence of a product formed by urogen I synthetase; the present work shows that this product is not a free tetrapyrrylmethane. The critical juncture in the enzymatic synthesis of urogen III is probably not the rearrangement of a preformed tetrapyrrole; the addition of PBG to a tripyrrole with concomitant rearrangement seems more likely although it has not yet been tested directly.

It has been shown that urogen III cosynthetase does not isomerize PBG to produce an intermediate which is later used by urogen I synthetase—*i.e.*, the sequence of the enzymatic synthesis of urogen III is not: (a) urogen III cosynthetase–PBG → isoPBG; (b) urogen I synthetase–1isoPBG + 3PBG → urogen III (Bogorad, 1958b). It has also been demonstrated by Carpenter and Scott (1961) that isoPBG is not incorporated

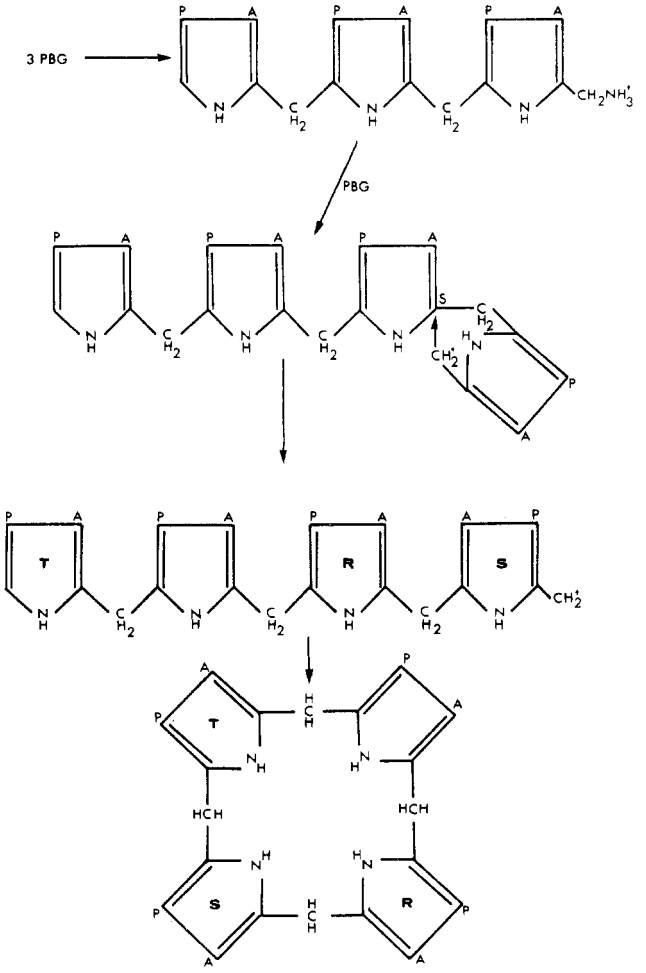

FIGURE 6: Hypothetical scheme for the enzymatic formation of urogen III from PBG (see text). S = substituted α-carbon which is attacked by carbonium ion. P = $CH_2CH_2COO^-$; A = CH_2COO^-.

into urogen III. An alternative which has been considered frequently (*e.g.*, Bogorad and Granick, 1953; Bogorad, 1960) is that, first, a tripyrrylmethane is formed from PBG by urogen I synthetase and then urogen III cosynthetase (or this enzyme in concert with urogen I synthetase) catalyzes the addition of a fourth molecule of PBG and the rearrangement and cyclization of the resulting tetrapyrrylmethane.

Figure 6 illustrates a possible way in which urogen III might be formed by the two enzymes in association with one another. This hypothesis postulates the existence of two different types of catalytic sites on the urogen I synthetase molecule. The first of these would catalyze the formation of a tripyrrole by the head-to-tail condensation of three molecules of PBG. (This process could take place at two different enzymatic sites or at one site which acts as a polymerase.) The second type would be an "isomerase" site at which urogen III cosynthetase would associate. At this site deaminations would be performed by the urogen I synthetase and critical bond directing done by urogen III cosynthetase in the following manner. (1) The tripyrrole is deaminated and the carbonium ion thus formed attacks the free α position of the PBG molecule (this would be mechanistically equivalent to the formation of a dipyrrole from two molecules of PBG). (2) Urogen I synthetase deaminates the nascent tetrapyrrole aided by urogen III cosynthetase. The urogen III cosynthetase, in this instance, would aid in positioning the substrate properly to facilitate the deamination. The urogen III cosynthetase then would aid the attack of the carbonium ion on the substituted α position of the nascent tetrapyrrole (S in Figure 6) which would result in the displacement of the methylene group at this position. (3) The carbonium ion which is formed by this displacement would then attack the free α position at the opposite end of the tetrapyrrole to form urogen III.

If this hypothesis is correct, inhibition by hydroxylamine and ammonia in this system might be explained as follows. (1) Hydroxylamine acts at both the polymerase site and the isomerase site of urogen I synthetase. This causes the accumulation of di- and tripyrroles from reactions at the polymerase site and tetrapyrroles at the isomerase site. (2) Ammonium ions act only or mainly at the isomerase site of urogen I synthetase. This results in the accumulation of tetrapyrroles almost to the total exclusion of di- and tripyrroles. (3) Because of their disruptive influence on the isomerase site, both of these inhibitors block the formation of urogen III by the two enzyme system.

According to this hypothesis, urogen III cosynthetase would not directly catalyze the consumption of the fourth molecule of PBG used in the biosynthesis of urogen III but would facilitate utilization of the pyrrole by urogen I synthetase. This hypothesis implies the existence of a complex of the two enzymes and some substrate or substrates. Attempts to detect such complexes on sucrose density gradients in the presence of PBG have been unsuccessful thus far (M. Higuchi and L. Bogorad, unpublished). The complex, if it exists, might be short-lived or very labile. Additional experiments are in progress to explore possible associations of urogen I synthetase and urogen III cosynthetase during urogen III synthesis.

References

Arsenault, G. P., and MacDonald, S. F. (1961), *Can. J. Chem. 39*, 2043.

Bogorad, L. (1958a), *J. Biol. Chem. 233*, 501.

Bogorad, L. (1958b), *J. Biol. Chem. 233*, 510.

Bogorad, L. (1960), *in* Comparative Biochemistry of Photoreactive Systems, Allen, M. B., Ed., New York, N. Y., Academic Press, p 277.

Bogorad, L. (1962), *Methods Enzymol. 5*, 885.

Bogorad, L. (1963), *Ann. N. Y. Acad. Sci. 104*, 676.

Bogorad, L., and Granick, S. (1953), *Proc. Nat. Acad. Sci. U. S. 39*, 1176.

Carpenter, A. T., and Scott, J. J. (1961), *Biochim. Biophys. Acta 52*, 195.

Edmondson, P. R., and Schwartz, S. (1953), *J. Biol. Chem. 205*, 605.

Fisher, H., Baumgartner, H., and Hess, R. (1952), *Hoppe-Seyler's Z. Physiol. Chem. 206*, 201.

Granick, S., and Bogorad, L. (1953), *J. Amer. Chem. Soc. 75*, 3610.

Kim, Y. C. (1969), *Can. J. Chem. 47*, 3259.

Mathewson, J. H., and Corwin, A. H. (1961), *J. Amer. Chem. Soc. 83*, 135.

Mauzerall, D., and Granick, S. (1956), *J. Biol. Chem. 219*, 435.

Pluscec, J., and Bogorad, L. (1970), *Biochemistry 9*, 4736.

Wittenberg, J. B. (1959), *Nature (London) 184*, 876.