A Dipyrrylmethane Intermediate in the Enzymatic Synthesis of Uroporphyrinogen*

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ABSTRACT: Porphobilinogen is normally converted virtually quantitatively into uroporphyrinogen I by uroporphyrinogen I synthetase. However, intermediates which can be separated electrophoretically accumulate in the presence of ammonia or hydroxylamine.

One of these intermediates has been identified as 5-aminomethyl-4,3′-di(carboxymethyl)-3,4′-di(2-carboxylethyl)dipyrrylmethane (DPM) by comparison with synthetic material. It is shown here that the enzyme uroporphyrinogen I synthetase does not catalyze the condensation of two molecules of DPM to form uroporphyrinogen I. However, in the presence of porphobilinogen (PBG) DPM is incorporated into uroporphyrinogen I.

It is concluded that the cyclic tetrapyrrole is formed from DPM by the successive addition of porphobilinogen residues but not by the fusion of two 5-aminomethyl-4,3′-di(carboxymethyl)-3,4′-di(2-carboxyethyl)dipyrrylmethane units.

The cyclic tetrapyrrole Urogen[1] I is synthesized from the monopyrrole PBG by Urogen I synthetase (Bogorad, 1958a). Urogen III is produced from PBG by the combined action of the enzymes Urogen I synthetase and Urogen III cosynthetase (Bogorad, 1958b). Urogen III is the precursor of hemes and chlorophylls (Neve *et al.*, 1956; Bogorad, 1958c).

Urogen I can be visualized as forming by the head-to-tail condensation of four molecules of PBG with the elimination of four molecules of ammonia. The intermediate pyrrylmethanes are easily predicted but have not heretofore been isolated or demonstrated. The identification and preparation of such intermediates should permit the elucidation of at least some aspects of the mechanism of action of Urogen I synthetase and the mode of synthesis of Urogen III.

Various competitive and noncompetitive inhibitors of Urogen production have been described by Bogorad (1958a, 1960) and by Carpenter and Scott (1961) but most of these compounds have roughly equivalent effects on the rate of consumption of PBG and formation of cyclic tetrapyrroles. The experiments described here show that hydroxylamine and ammonia, on the other hand, can interfere markedly and preferentially with the appearance of Urogen.[2]

These experiments also demonstrate that intermediates in Urogen I synthesis accumulate when appropriate concentrations of ammonium ions or NH_2OH are incubated with PBG and Urogen I synthetase. One of the intermediates from NH_2OH-containing reaction mixtures is shown to be DPM. Synthetic DPM, as well as the enzymatically produced dipyrrylmethane, is utilized for Urogen I synthesis only in the presence of PBG.

Materials and Methods

Urogen I synthetase ammonium sulfate fraction B (Bogorad, 1962) prepared from spinach leaves was used in these experiments. Cysteine, hydroxylamine, and EDTA, when included in reaction mixtures, were added from solutions adjusted to the pH of the buffer used. All enzymatic reactions were carried out in Thunburg tubes evacuated and filled with oxygen-free N_2.

DPM and PBG were the synthetic products of J. M. Osgerby, J. Pluscec, Y. C. Kim, F. Boyer, N. Stojanac, and S. F. MacDonald (1970, in preparation), and Arsenault and MacDonald (1961), respectively.

PBG-^{14}C was produced enzymatically from ALA-5-^{14}C. The latter was a gift from Drs. D. Mauzerall and F. Sparatore of The Rockefeller University who synthesized the compound from glycine-2-^{14}C. The ALA dehydrase used to form PBG from the ALA-5-^{14}C was a dialyzed ammonium sulfate fraction (33–50% of saturation) of a chicken red cell hemolysate prepared essentially according to the method of Schmid and Shemin (1955). After incubation of glutathione, enzyme, pH 7 phosphate buffer and ALA-^{14}C, until the PBG concentration stopped increasing, the solution was deproteinized by the addition of trichloroacetic acid. The PBG-^{14}C was adsorbed on Dowex-2 acetate. The pyrrole was eluted from the resin with 1 M acetic acid, was further purified by chromatography on alumina, and was finally crystallized following the procedure of Westall (1952). Yields were about 75%. The PBG-^{14}C was prepared with Dr. G. S. Marks (now at Queen's University, Kingston, Ontario, Canada).

PBG was estimated quantitatively using the modified Ehrlich reagent described by Mauzerall and Granick (1956). DPM was estimated colorimetrically using the same reagent. The dipyrrylmethane reacts with the Ehrlich reagent very

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[1] Abbreviations used are: Urogen, uroporphyrinogen; PBG, porphobilinogen; DPM, 5-aminomethyl-4,3′-di(carboxymethyl)-3,4′-di(2-carboxyethyl)dipyrrylmethane; ALA, δ-aminolevulinic acid.

[2] Brief preliminary reports of some of these experiments have been made (Bogorad, 1963).

rapidly. Figure 1 shows absorption spectra recorded starting 30 and 60 sec and at 15 min after a solution of DPM was mixed with the modified Ehrlich reagent. The rapid decline in absorbance at 566 nm, the absorption maximum of the complex, is apparent in Figure 1. Continuous monitoring at 566 nm revealed that the optical density at this wavelength was declining within 30 sec after addition of the reagent to DPM and that a much slower rate of decline began after about 40–60 sec. The millimolar extinction coefficient of DPM 30 sec after the addition of the reagent is 1.84×10^3, within 60–120 sec it is 1.51×10^3 at 566 nm. The Ehrlich reaction with PBG is slower—maximum absorption is reached within approximately 10 min after addition of *p*-dimethylaminobenzaldehyde in acetic–perchloric acid to a solution of PBG. The major absorption maximum for the latter reaction is at 553 nm; a smaller maximum occurs at about 525 nm.

Urogens were oxidized by iodine and measured spectrophotometrically as uroporphyrins (Bogorad, 1962). In some experiments the uroporphyrins were esterified and chromatographed by the method of Falk and Benson (1953).

For purification of porphyrinogens produced in radioisotope experiments, enzymatically formed Urogen was oxidized, esterified in H_2SO_4–methanol (Bogorad, 1962), and decarboxylated to coproporphyrin (Edmonson and Schwartz, 1953). Separation and further purification of coproporphyrin isomers was accomplished by thin-layer chromatography. Glass plates (5 × 20 cm) were coated with MN-cellulose Powder 300 (Brinkman Instruments, Inc.) to a thickness of 0.5 mm and dried for 10 min at 105°. After 0.5 mμmole of porphyrin had been applied per spot, plates were developed in a mixture of 2,6-lutidine–ammonia–water–0.1 M EDTA (10:4.2:2.8:0.02) for 3–4 hr in darkness. Coproporphyrin I moved with an R_F of 0.44 and coproporphyrin III with an R_F of 0.49.

PBG, DPM, and other pyrrolic components of enzymatic incubation mixtures were separated from one another by paper or thin-layer electrophoresis. For the latter, glass plates were coated with MN-cellulose powder 300 or 300 HR. To prepare, 0.5-mm thick coating on 12 plates, each 5 × 20 cm, 15 g of cellulose powder was suspended in a blender with 85–90 ml of water. After spreading, the cellulose plates were dried at 105° for 10 min and stored until needed. Before use, each plate was moistened with buffer (0.05 M Veronal sodium, pH 9.2) by spraying or permitting the solvent to rise by capillarity. Plates were then equilibrated for 20–30 min in a potential gradient of 25 V per centimeter using the same buffer. Samples of 10–25 μl each were applied near the negative end of the plate and electrophoresis was carried out at 4°. After electrophoresis the plates were examined with a 3600-Å lamp to detect red fluorescing regions and then sprayed with the modified Ehrlich reagent to locate PBG and polypyrrylmethanes. (The progress of the electrophoresis can be checked during the run by inspection with the 3600-Å lamp; uroporphyrin migrates more rapidly than any of the other components of the reaction mixture. Generally some of the Urogen produced is spontaneously oxidized to uroporphyrin.)

Results

Effect of NH_3 and NH_2OH on the Enzymatic Synthesis of Urogen I from PBG. ACCUMULATION OF INTERMEDIATES. During the enzymatic formation of Urogen I from PBG *in vitro*, the rate of disappearance of substrate is normally proportional to the rate of production of the cyclic tetrapyrrole. However, when hydroxylamine was included in reaction mixtures the rate of PBG consumption exceeded that of Urogen formation. The effect of hydroxylamine was initially attributed to a possible inhibitory action on some component of crude Urogen I synthetase preparations which promotes the oxidation of the porphyrinogen (Bogorad, 1958a). Upon reexamination it has been found that hydroxylamine as well as NH_4^+ alter the rate of PBG consumption only slightly but severely limit Urogen I formation; intermediates between PBG and Urogen I accumulate under these circumstances.

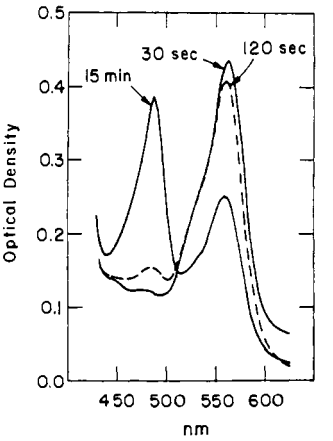

FIGURE 1: Absorption spectra taken at various times after combining 2 ml of a solution containing 0.53 μmole of DPM with 2 ml of modified Ehrlich reagent. Scans were run at 5 nm/sec from 625 nm toward shorter wavelengths beginning 30 and 60 sec and 15 min after the two solutions were mixed.

In the present experiments PBG was incubated with spinach leaf Urogen I synthetase and one of various concentrations of hydroxylamine or ammonium acetate. The concentration of PBG was estimated colorimetrically by the Ehrlich reaction using 0.05-ml aliquots of the incubation mixture taken before it was raised to the incubation temperature of 37°. After 30 min other 0.05-ml samples were removed and the concentration of PBG was estimated again. From the two values of PBG concentration the time required for exhaustion of substrate was estimated, assuming the rate of consumption to be linear and interference with the Ehrlich reaction by accumulated intermediates to be negligible. Enough enzyme was included to permit all of the PBG to be consumed within 90–180-min incubation. The reaction mixture was maintained at 37° long enough for the PBG to become exhausted, making the assumptions noted with regard to the linearity of the reaction and interference with the colorimetric measurements of PBG. After this time, plus an additional 30 min at 37° (t_1), 0.3-ml aliquots were removed for analysis by the Ehrlich reaction and 0.8-ml aliquots were taken for estimation of the Urogen which had been formed.

As shown in Table I, the effects of hydroxylamine and ammonium ions on the rate of PBG consumption are relatively small, however, the yield of porphyrin at t_1, based on the amount of porphobilinogen consumed, can be strikingly reduced. After continued incubation overnight (t_2) the yield of Urogen in some cases approached that observed in controls. At t_1, in the presence of 0.10–0.60 M NH_4^+ or 0.005–0.20 M

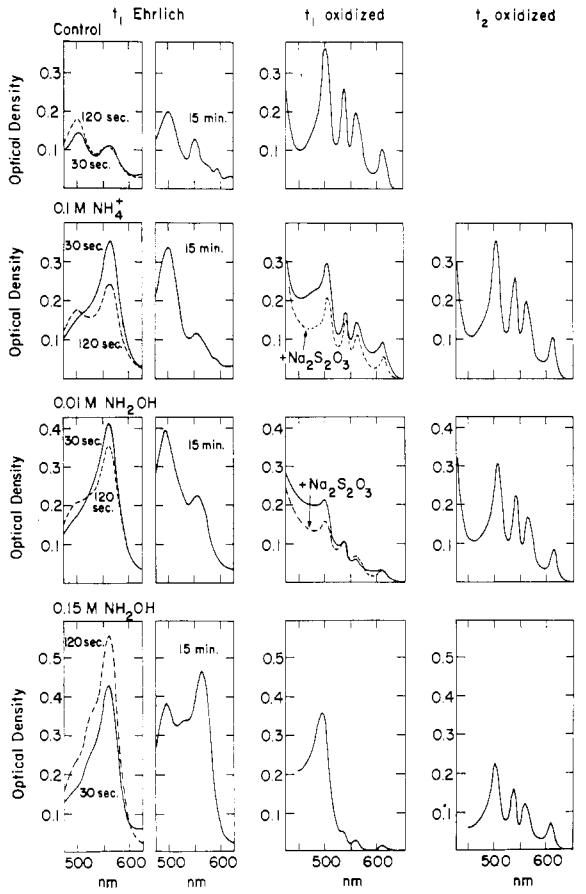

FIGURE 2: Some effects of NH_4^+ and NH_2OH on the action of Urogen I synthetase. See text for description of procedures and notes to Table I for components of incubation mixtures, etc.

NH_4OH about 45–60% of the PBG consumed was in an intermediate which could be converted into Urogen I in the absence of free PBG. (However, the possible presence of trace amounts of PBG could not be excluded.) Within the same range of concentration of inhibitors, up to about 30% of the PBG used was in compounds which were not converted into Urogen I under the same circumstances. At equivalent concentrations larger amounts of Urogen intermediates accumulate in NH_2OH- than in NH_4^+-containing mixtures.

Hydroxylamine or ammonium acetate in the concentrations used in the experiments for which data are given in Table I do not affect the Ehrlich reaction with PBG or the oxidation or Urogen with iodine.

Aliquots of incubation mixtures containing NH_2OH or ammonium acetate but from which PBG has been removed enzymatically react with the Ehrlich reagent. Absorption spectra of 0.3-ml aliquots of a control and of incubation mixtures containing 0.10 M NH_4^+, 0.01 M NH_2OH, or 0.15 M NH_2OH taken at t_1 made to 2 ml with water, and mixed with 2 ml of modified Ehrlich's reagent are shown in Figure 2 (first two columns). Absorption spectra were determined, using a Cary recording spectrophotometer, beginning 30 sec, 120 sec, and 15 min after the addition of the Ehrlich's reagent. Scanning at a rate of 5 nm/sec toward shorter wavelengths was begun at 625 nm. Oxidized solutions do not exhibit a positive Ehrlich reaction.

TABLE I: Effects of Ammonium Ions and Hydroxylamine on the Enzymatic Formation of Urogen I from PBG.[a]

Concentration of NH_4^+ or NH_2OH	Rate of PBG Consumption[b]	Urogen I: Yield at t_1[c]	Urogen I: Yield after Incubation Overnight (t_2)
0[d]	100	100	100
0.005 M NH_4^+	105	85	98
0.01	100	85	94
0.02	106	73	91
0.05	87	71	89
0.10[d]	85	49	94
0.20	78	40	89
0.40	52	27	71
0.60	59	22	70
0.005 M NH_2OH	97	20	85
0.01[d]	101	30	85
0.03	107	25	82
0.05	91	25	82
0.10	94	12	72
0.15[d]	82	15	70
0.20	77	15	70

[a] Incubation at 37°. Components incubated: NH_4^+ (as ammonium acetate pH 8) or NH_2OH (NH_2OH–HCl, pH adjusted to 8) as shown plus 0.3 mmole of Tris, pH 8.2; 90 μmoles of cysteine; 10 μmoles of EDTA; 1.43 μmoles of PBG; and 0.9 mg of protein Urogen I synthetase preparation all in 3.0 ml. [b] Calculated on the basis of the first 30 min of incubation. [c] Calculated time of PBG exhaustion plus 30 min. In these experiments t_1 ranged from 90 to 180 min. [d] See Figure 2 for absorption spectra of Ehrlich reaction at t_1 and of oxidized samples at t and t_2.

The Ehrlich reaction at t_1 appears to differ quantitatively in incubation mixtures containing from 0.005 to 0.6 M ammonium acetate. The curves shown for the preparation with 0.1 M NH_4^+ is representative. The absorption at about 566 nm falls very rapidly. On the other hand, the Ehrlich reaction at t_1 differs qualitatively depending upon the concentration of NH_2OH. At low concentrations, rapidly declining absorption roughly resembling the pattern of NH_4^+-containing reaction mixtures is characteristic but at concentrations of 0.1 M and above (the crossover is probably in the range between 0.05 and 0.1 M) a slower rising but subsequently slightly declining absorption is seen (for example, in the curves shown for "0.15 M NH_2OH" in Figure 2). The absorption spectra after 15 min also differ with NH_2OH concentration. These observations suggest that different Ehrlich reacting species accumulate depending on whether NH_4^+ or NH_2OH is present and also depending on the concentration of NH_2OH used.

Absorption spectra of 0.8-ml aliquots taken at t_1 and t_2, diluted to 3.4 ml and oxidized with iodine are also shown in Figure 2 (t_1 oxidized). The absorption spectrum of the oxidized control at t_1 is that of uroporphyrin at pH 8.2. The absorption

spectra of the other preparations shown in this column differ from the control in displaying relatively greater absorption in the 500-nm region; all or most of this absorption is eliminated by the addition of sodium hydrosulfite. The fourth column of Figure 2 shows that additional Urogen is formed upon prolonged incubation of reaction mixtures containing NH_4^+ or NH_2OH (compare Table I).

Analyses by paper or thin-layer electrophoresis after incubation of reaction mixtures containing NH_2OH confirm the disappearance of PBG and reveal the presence of Ehrlich-reacting compounds which move slower than Urogen I but more rapidly than PBG (Figure 3). Generally three Ehrlich-reacting spots can be observed. The pattern of Ehrlich-reacting compounds which accumulate in NH_4^+-inhibited incubation mixtures is different and, as will be described in a subsequent report, consists mainly of a linear tetrapyrrylmethane (R. Radmer and L. Bogorad, in preparation).

Substrates for Urogen I Synthetase. Urogen I could be formed by the condensation of two molecules of PBG followed either by (a) condensation of pairs of dipyrrylmethanes or (b) the stepwise addition of PBG first to a dipyrrylmethane and then to a tripyrrylmethane. The following experiments indicate that b is the mode of enzymatic synthesis of Urogen I.

When synthetic DPM was incubated alone with Urogen I synthetase, neither a change in concentration of DPM nor the production of Urogen I could be detected. On the other hand, incubation mixtures of PBG and DPM yielded larger amounts of Urogen I than could be accounted for by utilization of PBG alone. Results of experiments of this kind are shown in Table II—all the PBG plus up to about 50% of the DPM present in the incubation mixture could be accounted for by the Urogen formed. These are minimal values for the utilization of the synthetic DPM because it is assumed that PBG is utilized equally in incubation mixtures with and without DPM. No Urogen could be detected after incubating together at 37° for 170 min 1.5 μmoles each of DPM and PBG in the reaction mixture shown for expt 2, Table II, but from which the enzyme preparation was omitted.

The conclusion that DPM is incorporated into Urogen I in the presence of PBG was confirmed by isotope dilution experiments. As shown in Table III, the specific radioactivity of Urogen I produced enzymatically when synthetic DPM-^{12}C was incubated with Urogen I synthetase and PBG-^{14}C was lower than that expected if the PBG-^{14}C alone had been utilized for tetrapyrrole synthesis.

PBG could be participating catalytically in the consumption of DPM by Urogen I synthetase. However, when DPM was incubated with the enzyme and trace amounts of PBG (DPM:PBG molar ratios of 10 or 100) Urogen production was not detectable.

Enzymatically Formed DPM. The following experiments were performed to investigate the nature of a material formed enzymatically from PBG which moves like synthetic DPM in thin-layer electrophoresis.

PBG (12 μmoles) was incubated at 37° with Urogen I synthetase and hydroxylamine for 150 min (see Table IV for composition of the reaction mixture). After incubation, each 0.3-ml portion of reaction mixture was applied, 25 μl at a time, as a series of spots on a 20 × 20 cm glass plate coated with a 0.75-mm thick layer of cellulose. After electrophoresis for 1 hr in a potential gradient of 25 V per cm, the location of Ehrlich-reacting materials was determined by spraying two

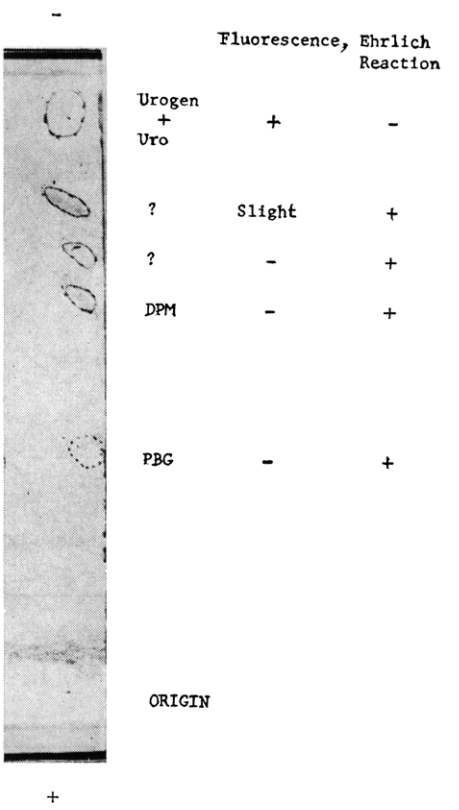

FIGURE 3: Electrophoretic separation of substrate and of products in a reaction mixture containing the following in a total volume of 0.36 ml: 1 μmole of PBG; 10 μmoles of EDTA, pH 8; 30 μmoles of Tris, pH 8.2; 8 μmoles of cysteine; 100 μmoles of NH_2OH; and 0.88 mg of protein of Urogen I synthetase preparation. After incubation at 37° for 150 min 10 μl of the solution was applied to a 0.25-mm thick cellulose layer for electrophoresis in 0.05 M barbital, pH 9.2, for 120 min at a potential gradient of 25 V/cm. The plate was examined with a 3660-Å lamp for fluorescence and then sprayed with the modified Ehrlich reagent.

opposite edges of the plate (the center of the plate was shielded during spraying). The cellulose powder containing the material which migrated as does DPM (about 6.5 cm from the origin toward the positive end under these conditions) was scraped from the unsprayed center portion of the plates, suspended in water, and the liquid was filtered off. The eluted material behaved like synthetic DPM in the Ehrlich reaction with regard to kinetics of color development and the position of the absorption maximum of the complex produced with *p*-dimethylaminobenzaldehyde. Using the colorimetric Ehrlich assay, approximately 4.2 μmoles of DPM was calculated to have accumulated during the reaction; 0.83 μmole of PBG remained unconsumed.

The presumptive DPM produced enzymatically and separated electrophoretically was concentrated by freeze-drying. This material dissolved in D_2O yielded an nuclear magnetic resonance spectrum essentially identical with that of synthetic DPM (Figure 4). When incubated with Urogen I synthetase the enzymatically produced compound, like synthetic DPM, was used for Urogen formation only when PBG was present (Table IV, compare Table II).

The two Ehrlich-positive compounds besides DPM which can be detected on thin-layer electrophoretograms of solutions

TABLE II: Enzymatic Incorporation of DPM into Urogen I.[a]

Expt	Substrate (μmoles)		PBG Consumed (μmoles)	Urogen Formed (μmole)		Yield Based on PBG (%)	DPM Used (%)
	PBG	DPM		Calcd	Found		
1	1.50		1.40	0.349	0.342	98	
		1.50			0		0
	1.50	1.50			0.699	204	47
2	1.50		1.40	0.349	0.307	88	
		1.50			0		0
	1.50	1.50			0.431	140	16
3	1.33			0.286	0.284	99	
	1.33	0.67	1.14		0.397	139	34

[a] Incubation 37°: expt 1, 135 min; expt 2, 170 min; expt 3, 150 min. Incubation mixtures: PBG and/or DPM as shown; 8 μmoles of EDTA; 240 μmoles of pH 8.2 Tris; 60 μmoles of cysteine; and, Urogen I synthetase. Experiment 1: final volume 3.55 ml; 10.5 mg of protein of Urogen I synthetase preparation. Experiments 2 and 3: final volume 3.00 ml; 3.5 mg of protein of Urogen I synthetase preparation.

TABLE III: Enzymatic Incorporation of DPM into Urogen I. Isotope Dilution Data.

Expt	Substrates[a]			Urogen[b] (cpm/mμmole)		
				Calcd		
		μmoles	cpm/mμmole	PBG + DPM	PBG only	Found
1	PBG-^{14}C	1.30	37			
				49	148	56
	DPM	1.30				
2	PBG-^{14}C	1.33	74			
				148	296	212
	DPM	0.67				

[a] PBG-^{14}C prepared from ALA-5-^{14}C. Incubation mixtures as described for expt 2 and 3 of Table II. [b] Measured as coproporphyrin I. See text.

TABLE IV: Utilization of Enzymatically Produced DPM by Urogen I Synthetase.[a]

Substrates (μmoles)		PBG Consumed (μmoles)	Urogen Formed (μmoles)		Yield Based on PBG	DPM Used (%)
PBG	DPM[b]		Calcd	Found		
2.30		1.78	0.419	0.418	100	
	1.15			0		0
2.30	1.15			1.862	206	76.5

[a] Incubation mixture: PBG and/or enzymatically formed DPM as shown; 75 μmoles of cysteine; 10 μmoles of EDTA; 300 μmoles of Tris, pH 8.5; and 8.75 mg of protein Urogen I synthetase preparation. Total volume 2.07 ml. Incubation at 37° for 180 min. [b] Isolated electrophoretically after incubation for 150 min at 37° of 12 μmoles of PBG; 25 μmoles of cysteine; 40 μmoles of EDTA; 120 μmoles of Tris, pH 8.5; 200 μmoles of NH_2OH; and 5.25 mg of protein Urogen I synthetase preparation. Total volume 1.05 ml.

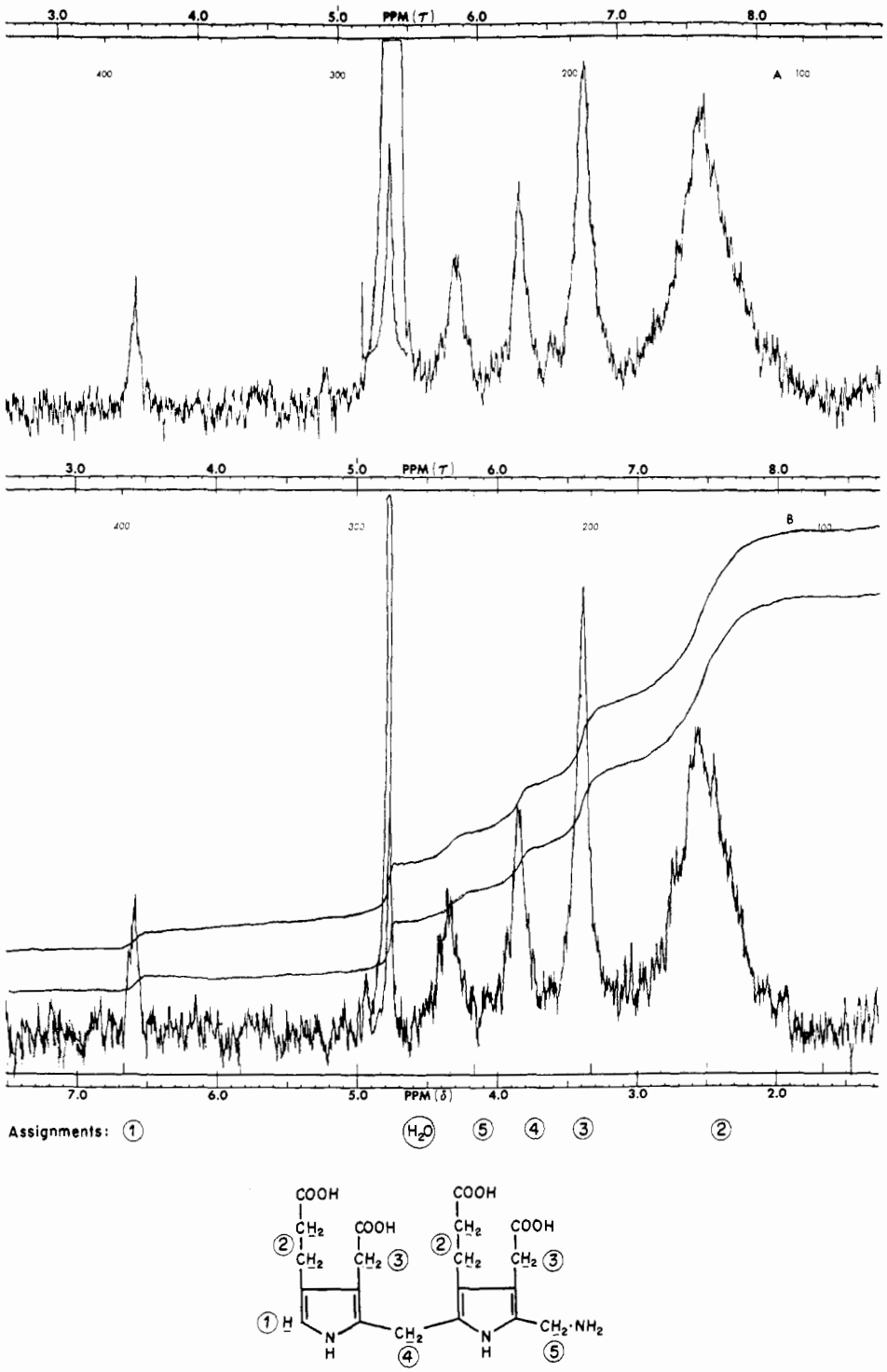

FIGURE 4: Nuclear magnetic resonance spectra of synthetic DPM (upper) and of DPM produced from PBG (lower). See text for procedural details. Assignments are noted at the bottom of the figure.

of PBG, Urogen I synthetase and NH_2OH after exhaustion of the monopyrrolic substrate have not been identified unequivocally but seem likely to be tri- and tetrapyrrylmethanes. [The material which moves just slower than Urogen appears to be a linear tetrapyrrylmethane (R. Radmer and L. Bogorad, in preparation).]

Effects of Ammonia and Hydroxylamine on Urogen III Formation. Hydroxylamine at concentrations of 1 or 2×10^{-2} M inhibits the formation of Urogen III from PBG (Bogorad, 1958b). This phenomenon was reinvestigated and the effect of NH_4^+ on Urogen III production was also examined to try to determine whether NH_4^+ or NH_2OH interfere directly or by blocking the production of a substrate for Urogen III cosynthetase.

TABLE V: Effect of Ammonium Ions and Hydroxylamine on the Enzymatic Formation of Urogen III from PBG.[a]

Concentration of NH_4^+ or NH_2OH	Urogens: Yield at t_1	Urogens: Yield at t_2	Urogen Isomers Formed[b]
0	100	100	III
0.005 M NH_4^+	95	97	I < III
0.01	92	97	I < III
0.02	93	97	I > III (tr)
0.05	86	97	I > III (tr)
0.10	83	90	I > III (tr)
0.001 M NH_2OH	79	97	I > III
0.005	57	87	I > III
0.01	44	87	I > III (tr)
0.02	35	83	I > III (tr)

[a] Composition of reaction mixtures: as shown in Table I except that each 3-ml incubation mixture also included 18 mg of wheat germ Urogen III cosynthetase preparation purified through step 6 (Bogorad, 1962). [b] The presence of the two isomeric constituents were determined by paper chromatography (Falk and Benson, 1953) of uroporphyrin octamethyl esters prepared by oxidation and esterification of Urogens present at t_2; (tr) = trace of uroporphyrin III octamethyl ester; presence questionable.

Table V shows the consequences of including various concentrations of NH_4^+ or NH_2OH in reaction mixtures of Urogen I synthetase, Urogen III cosynthetase, and PBG in which Urogen III is normally the sole tetrapyrrolic product. In general, porphyrin yields at t_1 (time of PBG exhaustion + 30 min) were not as severely affected as in the experiments for which data are given in Table I. This difference may result simply from the much larger amounts of protein present when the Urogen III cosynthetase preparation is included, however, this possibility was not investigated further.

The data of Table V show that concentrations of NH_4^+ which only slightly depress the overall yield of Urogens arrest Urogen III formation severely. Urogen III formation is largely blocked by NH_2OH at concentrations as low as 10^{-3} M.

It is not possible, in as complex a situation as this, to conclude from these data whether NH_4^+ and NH_2OH are interfering with Urogen III formation by (a) blocking the production by Urogen I synthetase of substrates required for Urogen III synthesis, or by (b) directly affecting the action of Urogen III cosynthetase, or by (c) somehow otherwise preventing a normal interaction between the two enzymes which might be required for Urogen III synthesis. However, the fact that some concentrations of NH_4^+ which interfere with Urogen III synthesis have little effect on Urogen yield at t_1 make possibility a less attractive and thus favor consideration of the two latter suggestions; a similar tentative conclusion could be reached with regard to the action of NH_2OH in this system.

The following compounds had no affect on yield at t_1 or t_2 and had no influence on Urogen III synthesis: 0.25 M sodium chloride, sodium acetate, sodium sulfate, or sodium propionate; 0.2 M glycine or urea; 0.10 M glucosamine, histamine, methylamine, β-phenylethylamine, Sedormid, or 3-amino-1,2,4-triazole; 0.05 M glutamine; 0.02 M tryptamine, 5-hydroxytryptamine, creatine, or creatinine.

Discussion

Urogen I synthetase does not catalyze the condensation of two molecules of DPM but apparently acts as a polymerase. When PBG and DPM are both included in the reaction mixture, PBG is condensed with DPM and a fourth PBG residue is presumably added to the linear tripyrrylmethane formed from PBG and DPM. After the tetrapyrrole is formed the probability of spontaneous ring closure to form Urogen I seems very high. Mauzerall (1960) has estimated the mean distance apart of the ends of the open-chain tetrapyrrole to be about 9 Å; *i.e.*, roughly 4 M.

Various types of inhibitors of the enzymatic synthesis of Urogen I are known. Among these are *p*-mercuribenzoate, Hg^{2+}, and formaldehyde (Bogorad, 1958a). Opsopyrrole dicarboxylic acid is a competitive inhibitor of PBG consumption (Bogorad, 1960) and isoPBG acts similarly (Carpenter and Scott, 1961). Inhibition of the Urogen I synthetase reaction by NH_2OH and NH_4^+ is different from that exhibited by either of the other two classes of inhibitors; the initial rate of PBG consumption is little reduced but completion of tetrapyrroles and cyclization of completed tetrapyrroles seems sharply arrested. Hydroxylamine and NH_4^+ also seem to act somewhat differently from one another both qualitatively and quantitatively.

It is shown here that Urogen I synthetase does not catalyze the condensation of pairs of DPM molecules. Thus the enzyme cannot normally first condense all the PBG present to make DPMs and still produce Urogen I. Although the data on utilization of DPM provided here do not necessarily support such a conclusion, it would seem to be required that the enzyme has a greater affinity for DPM than for PBG. (The probability that the enzyme has sites for four PBG molecules and condenses them simultaneously seems low in view of the failure to condense pairs of DPMs.)

It seems possible that NH_2OH and NH_4^+ alter the affinity of the enzyme for polypyrroles. No PBG is detectable after incubation of Urogen I and Urogen I synthetase with high concentrations of NH_4^+ thus ruling out a simple—and unlikely—equilibrium effect of NH_4^+. The mechanism by which Urogen I production is altered by NH_4^+ or NH_2OH is unclear. However, these agents are useful for forcing the accumulation of open-chain polypyrroles. This may help in studies on nature of the substrate or substrates of Urogen III cosynthetase. It is not unlikely that one substrate for Urogen III formation is a polypyrrolic product of the activity of Urogen I synthetase on PBG—*e.g.*, a tripyrrylmethane (Bogorad, 1960).

The new Ehrlich-reacting materials formed enzymatically from PBG when either NH_4^+ or NH_2OH is present could conceivably be addition products of these compounds with PBG or with enzymatically formed polypyrroles and thus not normal intermediates in Urogen I synthesis. The principal arguments against *all* the non-PBG Ehrlich-reacting materials detected on electrophoresis being of this class are: (a) the electrophoretic behavior of PBG is not altered by incubation with NH_2OH, (b) one of them has now been identified as

DPM, and (c) materials similar in electrophoretic mobility to those formed in the presence of NH_2OH can be found after short incubation of high concentrations of PBG with Urogen I synthetase in the *absence* of NH_4^+ or NH_2OH.

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Biochemical Similarity of Papain-Solubilized *H-2*^{d} Alloantigens from Tumor Cells and from Normal Cells*

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ABSTRACT: Papain-solubilized *H-2*^{d} alloantigens were purified from crude cell membranes of Meth-A tumor cells by $(NH_4)_2SO_4$ fractionation, Sephadex G-150, DEAE-Sephadex A-25 column chromatography, and 7.5% polyacrylamide disc gel electrophoresis. The purified preparations (class I and II) showed slightly broad single bands on disc gel electrophoresis at pH 9.3 and 4.3. The preparations were glycoprotein in nature having 85–90% protein, 3–4.5% neutral carbohydrate, 1.5–4% glucosamine, and 1–1.5% sialic acid. A comparison of peptide maps obtained by cellulose thin-layer chromatography for CNBr-treated and trypsin-digested class I and II glycoproteins showed that about half of the peptides of class II were identical with those found in the class I preparation.

The purified Meth-A materials and similarly prepared purified *H-2*^{d} alloantigens from DBA/2 normal mouse spleen cells, both of which carry the same *H-2* specificities, were found to have not only similar serological profiles by several criteria but also similar amino acid analyses, overall chemical analyses, molecular weight, chromatographic, and electrophoretic properties. Furthermore, a peptide analysis of CNBr-treated *H-2* antigens by column chromatography on PA-35 resin showed that the elution profiles of the two materials were very similar. Thus, *H-2* alloantigen preparations from normal and tumor cells sources which share the *H-2*^{d} genetic make-up but differ in several non-*H-2* properties as well as in cell type, were found to be exceptionally similar as far as could be discerned by our techniques.

Mouse *H-2* alloantigens are membrane-located products of the *H-2* genetic locus and carry the immunological determinants involved in tissue graft rejection. Of more than 15 histocompatibility loci, *H-1*, *H-2*, etc., the *H-2* locus and its products form the major system, and differences between individuals at this locus cause the most rapid graft rejection. Over 20 different alleles or "haplotypes" have been found for the *H-2* genetic region, and each allele determines a different mosaic of *H-2* specificities many of which are cross-reactive between the different alleles (*cf.* Snell and Stimpfling, 1966; Shreffler, 1966).

At the biological level, the *H-2* alloantigen is an unusually stable genetic product. Its expression in numerous cultured cell lines has shown complete resistance to loss even under conditions arranged to select against its presence (Klein,

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