Relation between Structure and Reactivity in Porphobilinogen and Related Pyrroles*

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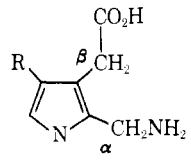
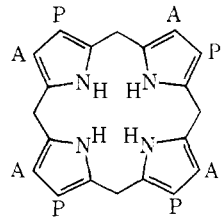
ABSTRACT: Porphobilinogen, 2-aminomethyl-3-carboxymethyl-4-carboxyethylpyrrole, is the only monopyrrole involved in a major metabolism. To study the structural features which make porphobilinogen self-condense chemically and enzymatically to uroporphyrinogens, its reactivity was compared to a series of synthetic 2-aminomethylpyrroles. The propionic acid side chain of porphobilinogen at C-4 was replaced by a methyl, a carboxymethyl, and an hydrogen group in order to examine the influence of the inductive effect of the substituent at C-4 on the reactivity of the pyrroles. The existence of a pyrrolenine intermediate in an electrophilic attack on the α-occupied position of the pyrroles can be inferred by measuring the decarboxylation rates of the 5-carboxypyrrolelactams of the corresponding 2-aminomethylpyrroles. The condensation of the four 2-aminomethylpyrroles to porphyrins was then measured under anaerobic conditions at pH 3.5 and 7.4. From the determinations of the pyrrole consumption rates, the rates of porphyrin formation, the porphyrin yields, the effect of temperature, the inhibition of porphyrin formation by dimedon, and the incorporation of labeled formaldehyde from the reaction mixture into the porphyrins, the following conclusions were reached. (a) The self-condensation of 2-aminomethyl-3-carboxymethylpyrroles was governed by the inductive effect of the substituent at C-4. When the propionic acid residue of porphobilinogen was replaced by hydrogen the resulting Mannich base was nonreactive. When it was replaced by a methyl group, the resulting pyrrole was extremely reactive. (b) The same inductive effect at C-4 steered the attack of one 2-aminomethylpyrrole unit to the free C-5 position of a second unit or to the occupied C-2 position. While porphobilinogen reacted preferentially by the latter mechanism, the C-4 methyl analog reacted by the former mechanism. (c) Porphobilinogen was the only one among the examined pyrroles that formed stable intermediates during the condensation process. The obtained results were compared with the enzymatic results of porphobilinogen condensation and the important differences were discussed.

All the natural porphyrins are originatated biosynthetically in a single pyrrole–porphobilinogen (PBG),[1] **1**. The enzymatic polymerization of this Mannich base affords the first natural cyclic tetrapyrrole derivative, uroporphyrinogen III, **2**, which is then transformed into the great variety of natural porphyrins through secondary transformation of the β substituents of the porphyrin nucleus. The structure



1, R = $CH_2CH_2CO_2H$
3, R = CH_3
4, R = CH_2CO_2H
5, R = H



2, A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

of porphobilinogen is unique and no other single monopyrrolic metabolites are known. The acetic acid and propionic acid side chains of porphobilinogen are the metabolic precursors of all the known β substituents (methyl, ethyl, vinyl, and formyl groups) of the porphyrins and chlorins. It is thus apparent that the unique structure of porphobilinogen possesses the best array of β substituents to allow the efficient (100% yield) enzymatic polymerization to uroporphyrinogen III, a metabolic step that occurs without formation of intermediates, and whose mechanism is still not elucidated (Lascelles, 1964). Porphobilinogen was also chemically polymerized to uroporphyrinogens, but the reaction yields varied with the pH of the reaction (Mauzerall, 1960) and were lower than the enzymatic ones. It was recently claimed (Whitlock and Buchanan, 1969) that the β substituents of porphobilinogen play a secondary role in its condensation mechanism to uroporphyrinogen III. Since there are no natural analogs of porphobilinogen with which to compare its reactivity, several synthetic 2-aminomethylpyrroles were prepared for comparison purposes. The propionic acid side chain at C-4 was replaced by methyl, carboxymethyl, and hydrogen groups. The synthetic substituents at C-4 were chosen according to their relative inductive effects. It was expected that the susceptibility to electrophilic attack at C-5 will decrease along the series: 2-aminomethyl-4-methyl-3-pyrroleacetic acid (**3**), porphobilinogen, 2-aminomethyl-3,4-pyrrolediacetic acid (**4**), and 2-aminomethyl-3-pyrroleacetic acid (**5**), which should be the least reactive. The self-condensation of the 2-aminomethylpyrroles was then considered to occur according to the two possible reactions by which porphobilinogen can polymerize to uroporphyrinogens (Cookson and Rimington, 1954; Mauzerall, 1960). We will formulate them by introducing an intermediate pyrrolenine step according to the results presented in Scheme I. It was assumed that reaction (A) will predominate at low pH (elimination of ammonia will be

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[1] Abbreviations used are: PBG, porphobilinogen; PDA, 2-aminomethyl-3,4-pyrrolediacetic acid; PMA, 2-aminomethyl-3-pyrroleacetic acid; MPMA, 2-aminomethyl-4-methyl-3-pyrroleacetic acid.

favored) and will not exchange formaldehyde with the reaction medium. Reaction B instead will be favored by a neutral medium, since the enamine-type elimination of the aminomethyl residue at the pyrrolenine intermediate step can be excluded in acidic media. Reaction B will also be followed by formaldehyde release, which will then recondense with the formed dipyrromethanes and propagate the polymerization (Mauzerall, 1960). It was also assumed that formaldehyde will recondense with the dipyrromethanes at a very fast rate as compared to pyrroles, according to our results on electrophilic substitution of dipyrromethanes (B. Frydman and S. J. Reil, 1970, unpublished data). The relative rates of condensation of the 2-aminomethylpyrroles to form porphyrinogens was then examined with the purpose of establishing how the structural features of porphobilinogen influence its reactivity.

Experimental Section

Materials

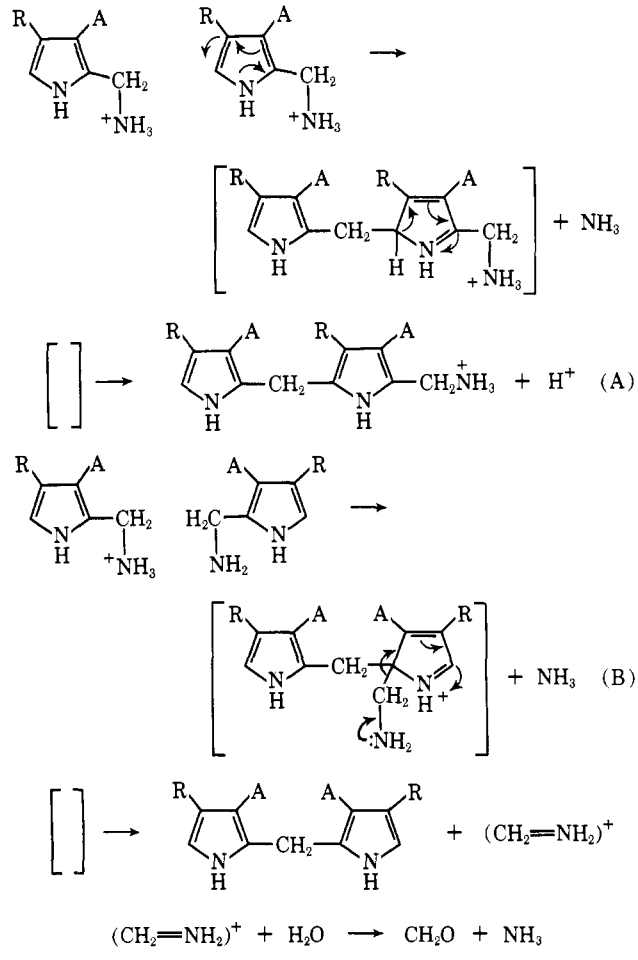
Porphobilinogen, 2-aminomethyl-3-pyrroleacetic acid, 2-aminomethyl-3,4-pyrrolediacetic acid, 2-aminomethyl-4-methyl-3-pyrroleacetic acid, and the corresponding 5-carboxypyrrolelactams, **6**, and pyrrolelactams, **7**, were obtained from 6-azaindoles by synthesis (Frydman *et al.*, 1969). Formaldehyde-^{14}C was obtained from New England Nuclear Corp. Other chemicals were Reagent Grade.

Methods

Decarboxylations were carried out at 71° in water (pH 5.5). 5-Carboxypyrrolelactams (1.5 mg) were dissolved in 10 ml of water (approximately 3×10^{-4} M) and aliquots (approximately 200 μl) were withdrawn at the indicated times. The precentage of decarboxylation was determined by measuring the amount of pyrrolelactam formed with Ehrlich's modified reagent (Falk, 1964), and comparing with standard curves. The amount of free pyrroles was measured colorimetrically with the same reagent. Optimum reading time was 5 min after reagent addition for MPMA, 15 min for PBG, 30 min for PDA, and 60 min for PMA. Porphyrins were determined, after oxidation of the porphyrinogens to porphyrins with a 1% iodine solution and elimination of excess of iodine with a sodium thiosulfate solution, by reading the Soret band in 2% hydrogen chloride for uroporphyrins and the porphyrins formed by PDA and PMA, and in 1% hydrogen chloride for the porphyrin formed from MPMA. A Beckman DU spectrophotometer was used. For the former porphyrins the amount of porphyrin was calculated according to the method of Rimington and Sveinsson (1950). For the MPMA porphyrin, which had the same λ_{max} as coproporphyrin, the ϵ value of the latter was used for calculations. Nuclear magnetic resonance spectra were determined in a Varian A-60 apparatus with Me_4Si or sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standards, and infrared spectra were obtained in potassium bromide wafers.

Analysis of the Reaction Mixture. The amount of pyrrole used was 10^{-4} M. The exact amount will be given in each case. The reactions were carried out at pH 3.5 (buffer acetate 0.1 M) and pH 7.4 (buffer phosphate 0.1 M). The temperature used, except when otherwise specified, was 70°. The total volume of the reaction was 100 μl. The reagents were mixed in seal-off tubes, freezed, evacuated at 0.1 mm, flushed with pure nitrogen, thawed, freezed again, and the operation was repeated four times before sealing the tubes *in vacuo*. The tubes were heated during the specified times, cooled to 0°, opened, 100 μl of water was added to the mixture, aliquots were removed, and determinations of pyrrole and porphyrin were made as described. Blanks were run under the same conditions, but keeping the tubes at room temperature. When the reaction mixture contained formaldehyde-^{14}C, three aliquots were separated for the determination of: (a) pyrrole consumption, (b) porphyrin formation, and (c) formaldehyde incorporated into the porphyrins. The last determination was carried out after the porphyrins were separated from pyrrole and unreacted formaldehyde by partition chromathography on polyethylene (Richards and Rapoport, 1966). The porphyrin-containing tubes were pooled and counted in a gas-flow counter.

SCHEME I



Results

Physical Properties of the Pyrroles. The 2-aminomethylpyrroles, **1–5**, and their corresponding lactams, **7**, were devoid of any ultraviolet or visible absorption spectra. The substituted 5-carboxypyrrolelactams, **6**, had ultraviolet maximum (ethanol) at 276 mμ (ϵ 12,600) (**6**, R = CH_3, $CH_2CH_2CO_2H$, and CH_2CO_2H), while the unsubstituted 5-carboxypyrrole lactam (**6**, R = H) had an ultraviolet maximum (ethanol) at 270 mμ (ϵ 16,000) (Frydman *et al.*, 1969). The infrared spectra of the lactams had ν_{max} at 3200 cm^{-1} (NH) and at 1690 and 1630 cm^{-1} (CO). The nuclear magnetic magnetic resonance spectra are detailed in Table I. Several features of the spectra are noteworthy. As could be expected,

TABLE I: Nuclear Magnetic Resonance Spectra of Pyrroles.[a]

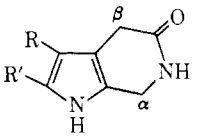
Compounds R	CF_3CO_2H				Py-d_5				0.1 N NaOD				Me_2SO-d_6			
	α-CH_2	β-CH_2	CH_2CH_2	H_5	α-CH_2	β-CH_2	CH_2CH_2	H_5	α-CH_2	β-CH_2	CH_2CH_2	H_5	α-CH_2	β-CH_2	CH_2CH_2	H_5
1					4.2 (s)	3.6 (s)	2.7 (m)	6.7 (s)	4.1 (s)	3.3 (s)	2.6 (m)	6.6 (s)				
3[b]					4.1 (s)	3.6 (s)		6.4 (s)	4.1 (s)	3.2 (s)		6.2 (s)				
4[c]					4.2 (s)	3.5 (s)		7.0 (s)	4.0 (s)	3.3 (s)		6.7 (s)				
5[d]									4.2 (s)	3.4 (s)		6.9 (d)				
6 $CH_2CH_2CO_2H$	5.15 (b)	4.0 (s)	3.1 (m)	4.9 (b)					4.15 (b)	3.15 (t)	2.4 (m)					
6[e] CH_3	5.2 (b)	3.9 (s)		4.8 (b)									4.3 (b)	3.3 (b)		6.4 (s)
6[f] CH_2CO_2H	5.2 (b)	4.0 (s)		5.0 (b)	4.9 (b)	3.6 (b)			4.2 (b)	3.2 (t)						
6[g] H	5.1 (b)	4.0 (s)		4.9 (b)					4.2 (b)	3.1 (t)						
7 $CH_2CH_2CO_2H$	5.15 (b)	4.0 (s)	3.1 (m)	5.3 (2, m)					4.15 (b)	3.15 (t)	2.4 (m)	6.55 (s)	4.4 (b)	3.3 (b)	2.7 (m)	6.5 (s)
7[h] CH_3	5.15 (b)	3.9 (s)		5.25 (2, m)									4.3 (b)	3.3 (b)		6.4 (s)
7 CH_2CO_2H	5.1 (b)	3.95 (s)		5.35 (2, m)					4.15 (b)	3.15 (t)		6.7 (s)				
7[i] H	5.2 (b)	4.0 (s)		5.4 (2, m)									4.3 (b)	3.35 (b)		6.7 (d)

[a] Values in δ. [b] CH_3, 2.4 (s) (Py-d_5); 2.3 (s) (0.1 N NaOD). [c] CH_2CO_2H, 4.2 (s) (Py-d_5); 3.3 (s) (0.1 N NaOD). [d] H_4, 6.2 (d), 4.2 (s) (Py-d_5); 3.4 (0.1 N NaOD). [e] CH_3, 2.5 (s). [f] CH_2CO_2H, 3.9 (s) (trifluoroacetic acid); 6.5 (s) (0.1 N NaOD). [g] CH_3, 2.45 (s) (trifluoroacetic acid): 2.3 (s) (Me_2SO-d_6). [h] H_4, 7.0 (t) (trifluoroacetic acid); 6.1 (d) (Me_2SO-d_6).

the inductive effect of the substituent at C-4 influenced the chemical shift of the proton at C-5 in **1**–**5** and **7** in the expected sense. In trifluoroacetic acid solution the lactams, **7**, and the 5-carboxyporphobilinogen lactams, **6**, exist entirely as the conjugate acids in the α-pyrrolenine forms **9** and **8**, which revert to the pyrrole forms **7** and **6** in alkaline or neutral solution. This is in line with the observations of Chiang and Whipple (1963) that methylpyrroles formed α-protonated salts in aqueous sulfuric acid. The methylene signal at C-5 was a nonresolved multiplet.

Homoallylic coupling was also observed in several of the lactams **6** and **7** (in 0.1 N NaOD) between the ring α-methylene and β-methylene ($J\alpha,\beta$ = 3Hz). This type of coupling was already described by Kim (1969) for two of the lactams of Table I, but at 150°. We observed several well-resolved triplets for β-methylenes at 25°.

Decarboxylation of Lactams. The existence of a pyrrolenine intermediate step in the electrophilic attack on 2-aminomethyl-3-pyrroleacetic acids (reactions A and B) can be deduced from the examination of the decarboxylation rates of the corresponding 5-carboxylactams **6**. The decarboxylation of 5-carboxypyrrole lactams to the corresponding lactams **7**

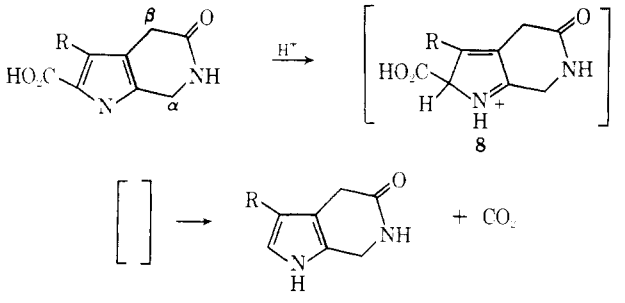


R = CH_3, $CH_2CH_2CO_2H$, CH_2CO_2H, H

6, R′ = CO_2H

7, R′ = H

was achieved by heating in water at low pH (4–5). At low acidity (pH 7) the decarboxylation rate was very slow and it stopped entirely at higher pH (8–9). If the decarboxylation reaction was visualized as an electrophilic substitution at C-5 with formation of a pyrrolenine intermediate **8**; it had to be



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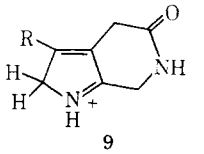
expected that the decarboxylation rates will increase with the increasing inductive effects of R. Figure 1 shows that the difference in the decarboxylation rates is in agreement with the expected electron-releasing properties of R.

The same effect can be established independently by nuclear magnetic resonance determinations. The H-5 proton of the lactams **7** appeared in neutral solutions (dimethyl sulfoxide) at approximately 6.5 ppm and was transformed in acid solution (trifluoroacetic acid) into a methylene band at



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approximately 5.3 ppm due to α-pyrrolenine formation (Table I). It was then possible to measure the rate of exchange of H-5 by adding a catalytic amount of DCl to the dimethyl sulfoxide solution followed by an adjustment to pH 7–8 with alkali at various times periods. On addition of DCl the chemical shift at 6.5 ppm disappeared and a nonresolved band appeared at 5.2 ppm. On adjustment to pH 7–8 with 5 M NaOD the signal at 6.5 ppm was again detectable. It was thus found that H-5 exchanged in MPMA lactam in 5 min (at room temperature), in porphobilinogen lactam (benzyl ester) in 20 min, and in PMA lactam the exchange was only 50% after 60 min. Thus the rate of electrophilic substitution at C-5 by the way of a pyrrolenine intermediate was again dependent on the electron releasing properties of R.

Pyrrole Condensation. The comparative rates of pyrrole consumption were then examined. The 2-aminomethylpyrroles were polymerized at pH 3.5 and at pH 7.4 as described in Methods. At pH 3.5 (Figure 2a) there was good difference among the disappearance rates of the four pyrroles and the difference was in agreement with the respective inductive effects of the substituent at C-4. At pH 7.4 (Figure 2b) the same difference existed but while PBG and PDA were more reactive than at pH 3.5, MPMA was less reactive and the three reactions rates became more similar. The results are in agreement with the reactions A and B. At low pH, reaction A (head to tail attack) will predominate and the formation of the pyrromethanes and pyrrole consumption will be a function of the electron-releasing properties of R. At neutral pH, reaction B will be the predominant one and hence the inductive effect of R will be less noticeable. However, since the rate of recombination of formaldehyde with the formed dipyrromethanes will also depend on the inductive effect of R, the difference in the rates will remain: MPMA > PBG > PDA ≫ PMA.

Porphyrin Formation. When the rates of porphyrin formation were examined under the same conditions it was found that they paralleled the rate of pyrrole consumption. Figure 3a shows that at pH 3.5 porphyrin formation was closely related to pyrrole consumption. It must be noted that the porphyrins formed by MPMA were acid labile after heating at 70° during 30 min, and the data indicated in Figure 3a represent corrected values. When the porphyrin yields were examined under the same conditions (Figure 3a, inserted), it was found that only in the case of PBG were the porphyrin yields increased with time, thus indicating that only in this case were stable intermediates accumulated. The same conclusions were reached by examining the rates of porphyrin formation at pH 7.4 (Figure 3b). The initial rate of porphyrin formation was faster for MPMA than for PBG, although this was not the case for pyrrole consumption (Figure 2b). When the porphyrin yields were measured (Figure 3b, inserted), it was found again that only uroporphyrin yield increased with time. This indicated that in the polymerization of PBG stable intermediates were formed at pH 7.4, which were then transformed with time into porphyrins. If the polymerization was done in the presence of air uroporphyrin yields never exceeded 25% even at very long times, since the intermediates were undoubtedly oxidized in the process. PMA did not form porphyrins at acid pH and only traces at neutral pH.

Influence of Temperature. When porphyrin formation was examined with temperature at pH 3.5 and 7.4 (Figure 4a,b), MPMA was again the most reactive pyrrole followed by PBG and PDA. PMA formed only traces of porphyrins. As could be expected from a temperature-directed polymerization, porphyrin formation gave exponential curves. The only exception was the formation of uroporphyrins at pH 7.4 (Figure 4b), confirming again that discrete and stable intermediates were formed in the chemical condensation of porphobilinogen.

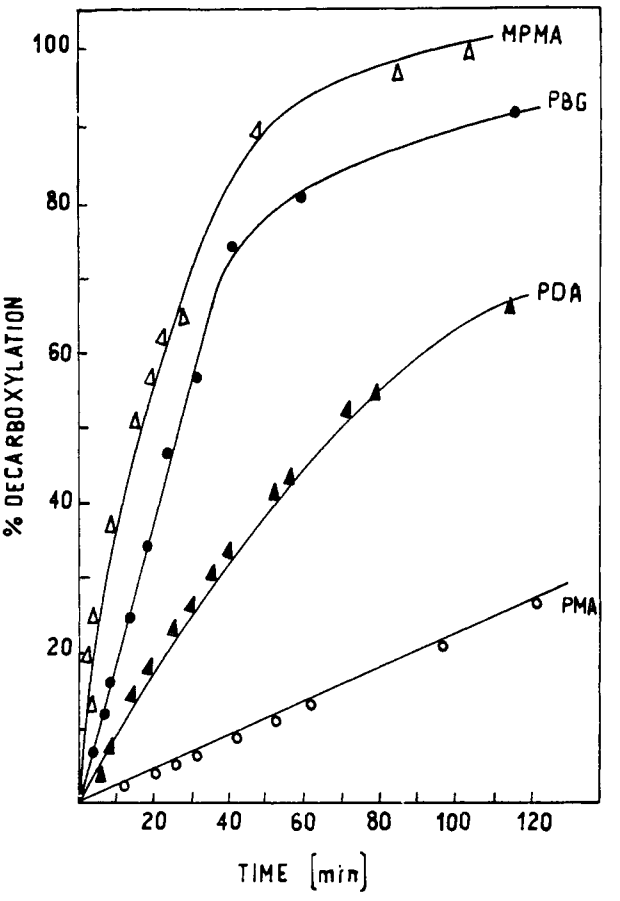

FIGURE 1: Decarboxylation rates of 5-carboxypyrrolelactams. The concentrations of 5-carboxypyrrolelactams and the assay procedure were given in Methods.

Effect of Dimedon and Formaldehyde. According to reaction mechanism B, formaldehyde is liberated when the condensation of two 2-aminomethylpyrroles occur, and can then recondense at the next step with the formed dipyrrylmethanes to yield porphyrins. This was demonstrated for porphobilinogen (Mauzerall, 1960). Dimedon, which is a good trapping agent for formaldehyde, should then have an inhibitory effect on porphyrin formation when pathway B predominated in the pyrrole condensation reaction. As Table II shows this was the case with the porphyrins formed at the expense of PBG and PDA at pH 7.4, while it had no effect on porphyrin formation at expense of MPMA. The same results were obtained at pH 3.5, but the effect of dimedon on the amount of porphyrin formed by PBG was less noticeable.

The addition of low concentrations of formaldehyde to the reaction mixture should have the opposite effect on porphyrin formation as dimedon addition. It must also be expected that the condensation of formaldehyde with the dipyrrylmethanes accumulating through reaction mechanism B should be faster at low pH than at neutral pH. Table II shows that this was the case for PBG and PDA, but that the porphyrins originated in MPMA remained essentially unaffected by addition of formaldehyde to the reaction mixture.

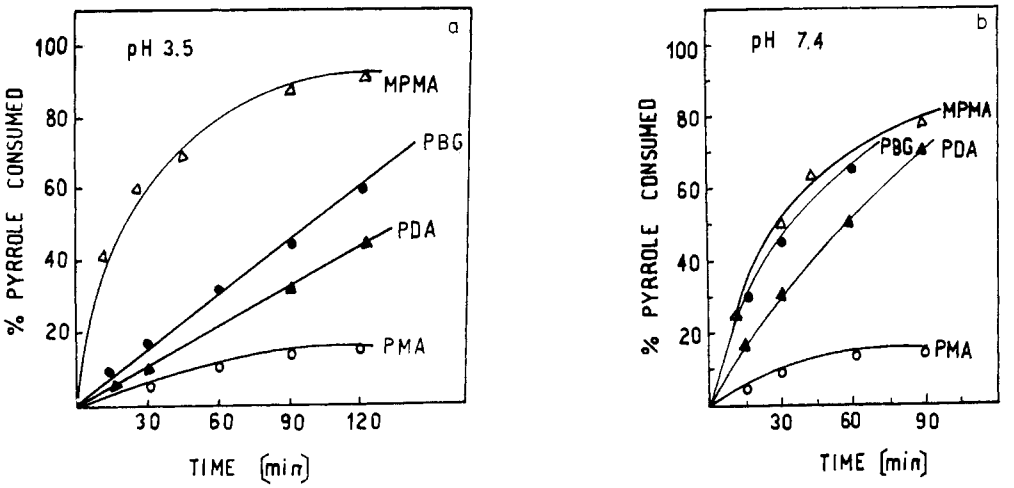

FIGURE 2: Pyrrole consumption rate. The reaction mixture was heated for the given times at 70° and pH 7.4 (b) and pH 3.5 (a). Pyrrole concentrations were: PBG, 0.26 mM; MPMA, 0.27 mM; PDA, 0.22 mM; and PMA, 0.34 mM. Per cent of pyrrole consumption was expressed as the percentage of pyrrole consumed of the total amount of pyrrole present at the start of the reaction.

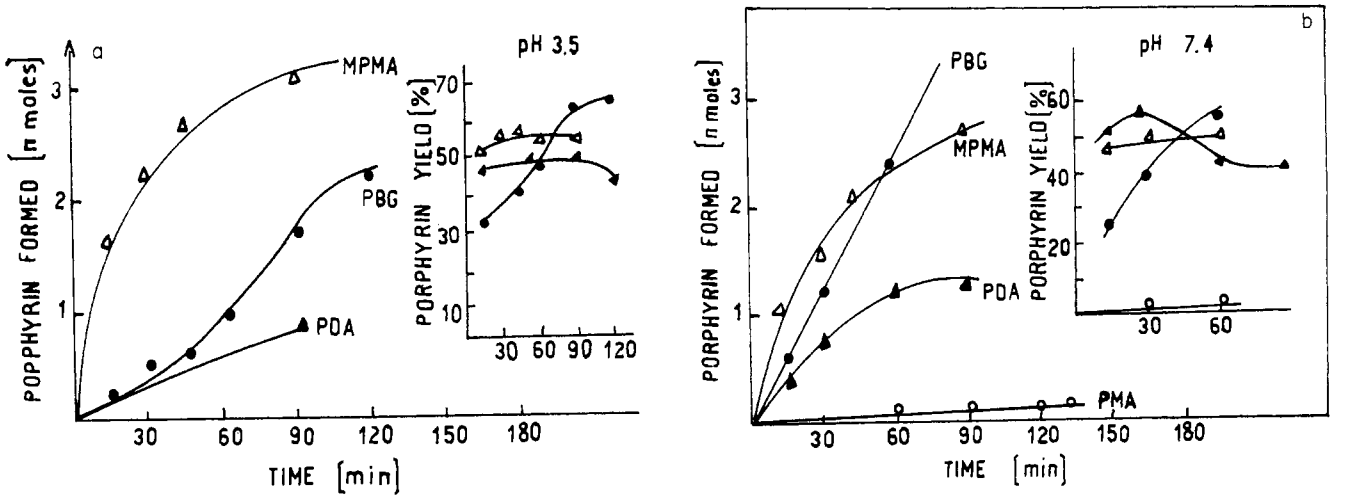

FIGURE 3: Time course of porphyrin formation and porphyrin yields. The reaction mixture and the conditions were the same as indicated in Figure 2. As 100% porphyrin yield was considered the theoretical amount of porphyrin formed if all the pyrrole consumed could be transformed into porphyrins.

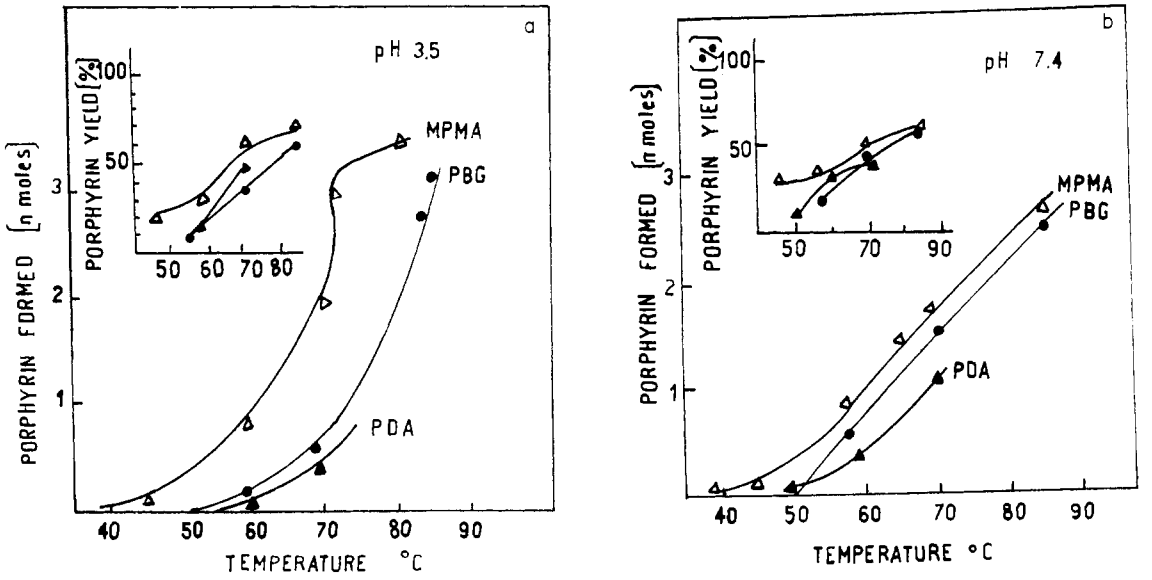

FIGURE 4: Effect of temperature on porphyrin formation and porphyrin yield. The pyrroles were heated for 60 min at the indicated temperatures and pH. The concentrations were the same as in Figure 2. Pyrrole consumption and porphyrin formation were measured as described.

TABLE II: Effect of Dimedon and Formaldehyde Addition on Porphyrin Yields.[a]

Pyrrole	pH	Addition	Porphyrin Formed (mμmoles)	Addition		Porphyrin Formed (mμmoles)
PBG	7.4		1.07			1.05
		Dimedon	0.26	Formaldehyde	0.25	1.6
	3.5		0.6			0.53
		Dimedon	0.3	Formaldehyde	0.25	1.00
PDA	7.4		0.7			0.7
		Dimedon	0.37	Formaldehyde	0.25	0.88
	3.5		0.32			0.32
		Dimedon	0.16	Formaldehyde	0.25	0.54
MPMA	7.4		2.08			2.2
		Dimedon	2.10	Formaldehyde	0.25	2.2
	3.5		2.25			2.8
		Dimedon	2.24	Formaldehyde	0.25	2.8

[a] The incubation mixture was prepared and the porphyrins estimated as described in Methods. Pyrrole concentrations were those given in Figure 2a. Dimedon concentration was 0.5 mM and formaldehyde concentration was one-fourth (0.25) of the pyrrole concentration. The reaction was carried out at 70° for 30 min as indicated. The addition to the reaction mixture of ammonium hydroxide, in an equimolecular amount to the added formaldehyde, did not alter the results.

These conclusions were supported by the data obtained when the reaction was run in the presence of formaldehyde-^{14}C. Table III shows that while formaldehyde-^{14}C was appreciably incorporated into the porphyrins formed by PBG and PDA, it was incorporated to a lesser extent into the porphyrins formed at expense of MPMA, indicating again that the condensation of MPMA to porphyrins went predominantly through reaction mechanism *A*. The amount of formaldehyde-^{14}C incorporated into the porphyrins originating in PBG and PDA was higher at pH 3.5 than at pH 7.4. This was due mainly to the well known fact, from Fischer's and Corwin's work, that formaldehyde condenses at low pH with pyrroles to give α-pyrrylcarbinoles which then polymerize very fast to porphyrins with elimination of water, while at neutral or alkaline pH the α-pyrrylcarbinols are more stable and are only slowly transformed into porphyrins. It is also likely that the imonium cation $(CH_2{=}NH_2)^+$ liberated through reaction mechanism B is not totally hydrolyzed to formaldehyde, but recombines as such with the pyrrylmethanes. Since the imonium cation is undoubtedly a stronger electrophile than formaldehyde it will prevent the latter's incorporation into the porphyrins.

Formaldehyde-^{14}C was also found to be incorporated to some extent into the porphyrins formed from MPMA, due undoubtedly to the higher sensitivity of the ^{14}C measuring method as compared to the spectrophotometric method of porphyrins determination; but when each set of results was compared separately it was clear that the incorporation of label into the porphyrins formed from MPMA indicated less exchange of formaldehyde with the reaction medium than in the case of PBG and PDA.

Discussion

The structure of porphobilinogen has several features which could explain its unique reactivity. It is a α-Mannich base of a pyrrole, and as such it reacts by releasing ammonia and giving a reactive positive carbon (presumably a carbonium ion or the equivalent diene) which initiates a polymerization by an electrophilic attack on the C-2 or C-5 of a second porphobilinogen unit. It has been suggested that the C-3 acetic acid side chain stabilizes the carbonium ion by way of a lactone formation (Mauzerall, 1960). We examined in this paper the influence of the propionic acid side chain on the reactivity of porphobilinogen.

Of the two mechanisms by which porphobilinogen con-

TABLE III: Incorporation of Formaldehyde-^{14}C into Porphyrins.[a]

Pyrrole	pH	Formaldehyde-^{14}C	Sp Act. (cpm/mμmole of Porphyrin)
PBG	7.4	0.15	2080
		0.25	4280
	3.5	0.15	4140
		0.25	5000
PDA	7.4	0.15	3160
		0.25	3240
	3.5	0.15	5700
MPMA	7.4	0.15	1000
		0.25	1820
	3.5	0.15	608
		0.25	1050

[a] Pyrrole concentrations and reaction conditions were as described in Table I. Formaldehyde-^{14}C was used at concentrations of one-eight (0.15) and one-fourth (0.25) of the pyrrole concentrations (16,500 and 33,000 cpm, respectively). Specific activity was determined as indicated in Methods.

densed, reactions A and B, it appeared that reaction B (electrophilic attack at C-2) was the preferred one. This was shown in Figure 2 and 3 which indicate that pyrrole consumption and porphyrin formation were considerably higher at neutral pH than at pH 3.5. Since reaction mechanism B will be strongly inhibited at pH 3.5, we conclude that this mechanism is the preferred for porphobilinogen polymerization. Reaction mechanism A (electrophilic attack at C-5) is preferred only when the electron-releasing properties of the substituent at C-4 increase. By replacing the propionic acid residue by a methyl group, as in MPMA, pyrrole consumption rates and porphyrin formation rates became higher at pH 3.5 and were independent of activation by formaldehyde or inhibition by dimedon (Table I). Thus MPMA reacted almost exclusively by mechanism A. When the propionic acid residue was replaced by hydrogen, the resulting pyrrole (PMA) was very slow to self-condense and it almost did not yield porphyrins, thus indicating that the Mannich base is in this sense a nonreactive compound.

Porphobilinogen appears to be the "best choice" among the examined 2-aminomethylpyrroles. MPMA, which is more reactive, is very unstable and it starts to form porphyrins already at 37° (Figure 4). The uroporphyrins are the most stable among the homologous porphyrins. The porphyrins originated in MPMA are acid labile and the porphyrins originated PDA are unstable in the air. Porphobilinogen was the only pyrrole, among the examined, to form stable intermediates during the condensation process, which were transformed with time into porphyrinogens.

When the chemical condensation of porphobilinogen was compared with the enzymatic one, there was a striking difference. In the enzymatic reaction the yields were approximately 100%, even when measured at the possible shortest times (minutes). When the yields were lower than 100% (Frydman and Frydman, 1970), they did not increase with time. This was also the case when the enzymatic reaction was carried out at very low concentrations (1×10^{-5} M) of porphobilinogen. The enzymatic condensation is certainly an anaerobic process, but occurs also in the air giving almost the same porphyrin yields (Frydman and Frydman, 1970). We already mentioned in Results that this was not case when the uroporphyrins were formed chemically. It is thus apparent that the enzymatic polymerization of porphobilinogen takes place on the enzymatic surface without accumulation of free intermediates, at least under normal conditions.

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On the Structure of Ovotransferrin. Isolation and Characterization of the Cyanogen Bromide Fragments and Evidence for a Duplicate Structure*

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ABSTRACT: The reaction of cyanogen bromide with ovotransferrin produced three polypeptide fragments, which were recovered in a mole ratio of 1:1:1. For every mole of ovotransferrin cleaved and fractionated, 2 moles of each fragment was recovered. Molecular weights of the fragments were estimated as 21,000, 9400, and 7000, the sum of which is approximately one-half that of the native protein (76,600). The amino acid and carbohydrate composition of the fragments was one-half that of the native protein. These results are consistent with a protein structure in which duplicate polypeptide fragments are linked to form a single polypeptide chain.

The transferrins are a group of homologous glycoproteins which bind two atoms of ferric ions in two separate but equivalent sites on the protein molecule (Warner and Weber, 1953; Warner, 1959; Aasa *et al.*, 1963; Windle *et al.*, 1963; Aisen *et al.*, 1966; Feeney and Komatsu, 1966). Because of their high molecular weights (*ca.* 80,000), it has been sug-

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