THE CHEMICAL POLYMERIZATION OF PYRRYL MANNICH BASE METHIODIDE SALT TO PORPHYRINS

SONG-SWEE ER

The Chemical Polymerization of Pyrryl Mannich Base Methiodide Salt to Porphyrins

A Thesis

by

Song-Swee Er, B.Sc.

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Abstract

Pyrryl Mannich base methiodides were polymerized in both anhydrous hydroxylic and nonhydroxylic solvents with the hope of clarifying some aspects of the mechanism of porphyrin formation. Although a mixture of type isomers were obtained, the product composition was found to consist of ~ 95% type (III and IV) and ~ 5% type (I and II) isomers from various solvents. The results suggested that the isomer composition is not dependent on the presence of "active" hydrogen in the solvent, and polymerization is not due to separation of formaldehyde (or equivalent) from the pyrrole and then a "random" polymerization; formation of type III and IV porphyrins is an inherent property of trimethylaminopyrrole salts and rearrangement in some form is a normal part of polymerization. On the basis of these results and published data , an intermediate with ring D porphobilinogen unit in the pyrrolenine form at right angles to the macrocycle formed from the other three pyrrole units and methane bridges is suggested and a scheme for the natural type III porphyrin formation is also proposed.

A new compound diethyl 2,5-dimethyl-3,6-pyrazine-dithiolcarboxylate is reported as a by-product of a pyrryl thiolester synthesis.

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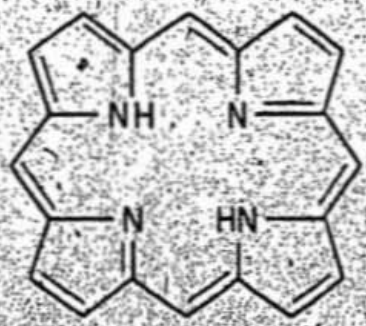
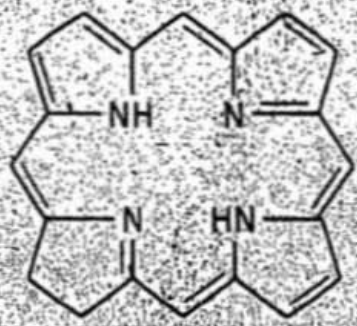
Introduction

Tetrapyrrolic macrocyclic compounds have been subjected to investigation chemically and biologically for over a century, but the work concerning their biosynthesis has only been done in the past thirty years. These macrocycles can be classified into two main series (1) : (i) the porphins and dihydroporphins; (ii) the corroles and their octahydro-derivatives, i.e. corrins. The typical examples for the dihydroporphins and corrins are the chlorophylls and vitamin B_{12} coenzyme respectively. The basic skeleton of these pyrrole pigments is formed from four pyrrole nuclei, joined together by four methene bridge carbon atoms; but in the corrole and corrins series, one of the methene bridge carbon atoms is missing and instead, two pyrrole units are joined directly in their α -position. The basic ring system for each are porphin (I) and chlorin (II); corrole (III) and corrin (IV) as shown.

It has been found that the porphins can occur either in the free state or as metal complexes. When each of the four pyrrole rings of a porphin bears two different substituents A and B in the β -position, then four isomers of the porphyrin (V) exist. This is also true for the porphyrinogen where the pyrrole units are joined by the methane bridge linkages instead of the methene bridge as in the porphins. The only porphyrins known to exist naturally in the free state are protoporphyrin IX (VI) and the I and III type isomers of uroporphyrins and coproporphyrins (Va, Vb) (2).



(I)



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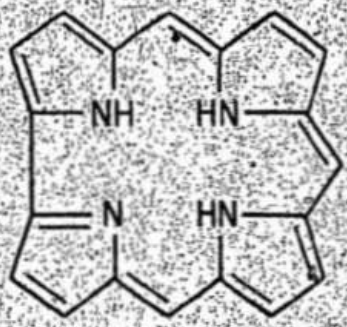

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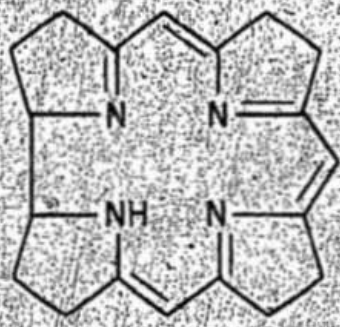

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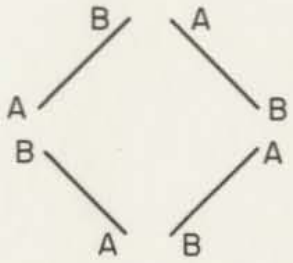
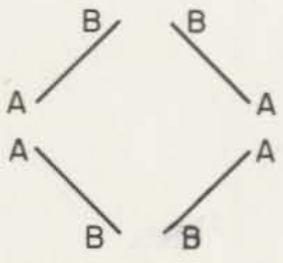


Type I



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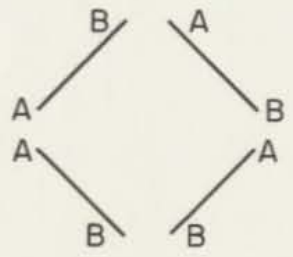
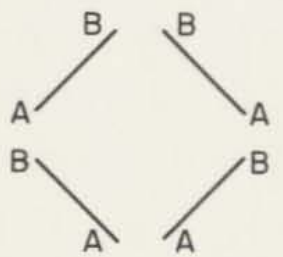


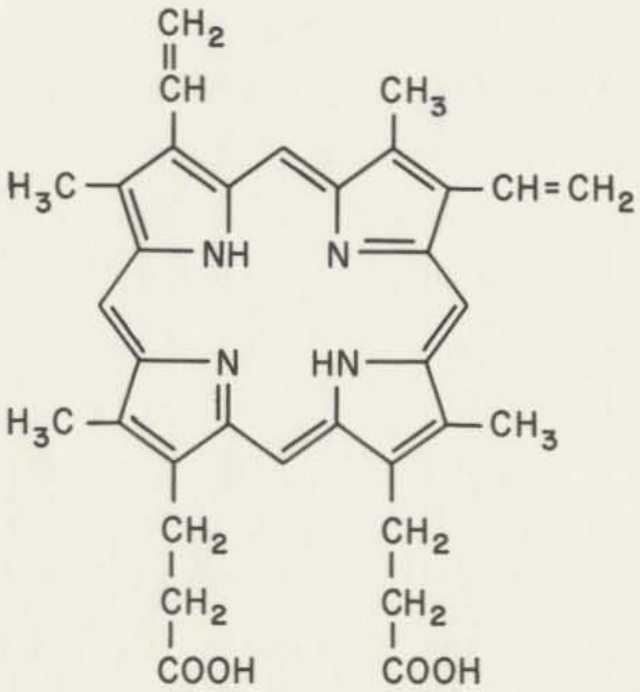
Type III



Type IV

(Va): For Uroporphyrins : A = CH_2COOH , B = CH_2CH_2COOH

(Vb): For Coproporphyrins : A = CH_3 , B = CH_2CH_2COOH



(VI)

The first δ pathway of porphyrin biosynthesis was demonstrated by Shemin and Rittenberg (3, 4). Nothing was known about the chemical precursors of haem in the body until 1946. They found that after feeding the ^{15}N-glycine to humans, the haem of haemoglobin was enriched with ^{15}N. Later experiments with ^{14}C labelling showed that the methylene but not the carboxy carbon atom of glycine is incorporated into haem (5, 6, 7). Degradation also showed that all four methene bridge carbon atoms were derived from the glycine (8, 9). Later it was found that δ-aminolaevulic acid, in vitro, was a more reactive precursor for haem, porphobilinogen and porphyrins than glycine (10, 11 - 13). It has been proposed that δ-aminolaevulic acid was formed from the glycine and succinyl-CoA with δ-aminolaevulic acid synthetase; pyridoxal phosphate is the only co-factor required (14 - 17).

The enzymic condensation of two molecules of δ-aminolaevulic acid to porphobilinogen is thought to involve, firstly, an aldol type condensation and secondly, a Schiff base linkage, which are catalysed by the enzyme δ-aminolaevulic acid dehydrase (10 - 12, 18).

The polymerization of porphobilinogen to uroporphyrinogen both in vitro and in vivo has been the most interesting subject in the biosynthesis of porphyrins. It was known for some time that both I and III type isomers occur in nature with most of them being the type III; the type II and IV isomers apparently do not occur naturally (19). It has been shown that two enzymes are responsible for the formation of

uroporphyrinogen. In the presence of the first enzyme, uroporphyrinogen I-synthetase, porphobilinogen is converted into uroporphyrinogen I. However,when the second enzyme, uroporphyrinogen III-cosynthetase was incubated together with uroporphyrinogen I-synthetase and porphobilinogen, it brought about the production of uroporphyrinogen III instead of the I isomer. Uroporphyrinogen I is not a substrate for the cosynthetase (13, 21, 22). Several mechanisms for the biosynthesis of uroporphyrinogen from porphobilinogen have been suggested (23-31). Bullock (32) has suggested that uroporphyrinogen I-synthetase is a reversible deaminase enzyme which holds an equilibrium between porphobilinogen and a uroporphyrinogen mixture (plus ammonia in some form) containing mostly uroporphyrinogen I; the cosynthetase is a surface which picks uroporphyrinogen III by its peculiar stereochemistry, thus forcing the equilibrium to give entirely the III isomer. This is an agreement with Bogorad's work (20, 21, 33), where kinetic studies suggested that the action of the synthetase on porphobilinogen is the rate determining process and the cosynthetase step, which also involves porphobilinogen as a substrate, is much faster.

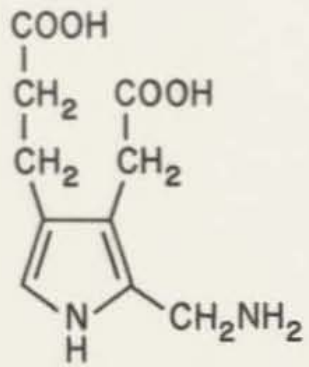
Much of the earlier work concerning the chemical polymerization of monopyrroles to porphyrins involved the formation of uroporphyrin from porphobilinogen, which can also be isolated from the patients with acute porphyria. Later, the readily accessible synthetic pyrroles were used. In recent years, various methods were used and results reported, with the hope of elucidating the pathway by which Nature converts

porphobilinogen to uroporphyrin III almost exclusively.

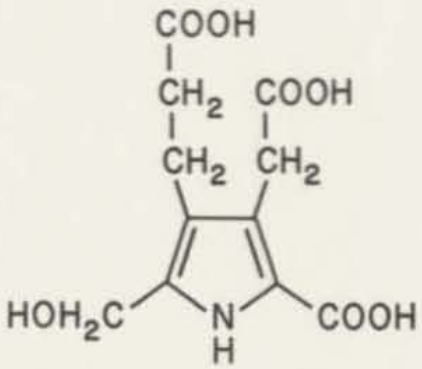
The synthesis of porphyrin directly from the self-condensation of a pyrrole was first done by Fischer and Treibs (34) in 1926. They found that aetioporphyrin was formed when opsopyrrole was treated with formic acid. In 1935, Rothemund (35) found that when pyrrole was treated with aldehydes in the presence of pyridine under pressure and elevated temperature, it gave rise to small yields of meso-substitued porphyrins. As early as 1939, Waldenstrom and Vahlquist (36) claimed that uroporphyrin III was obtained by warming weakly acid solutions containing porphobilinogen. This was later confirmed by Westall (37). However, Cookson and Rimington (38) found that other isomers were also formed in small quantities in this reaction.

In 1943, Siedel and Winkler (39) reported that when 4-ethyl-5-hydroxymethyl-3-methyl-pyrrole-2-carboxylic acid (later shown to be 5-acetoxymethyl compound (24)) was heated with dilute hydrochloric acid, the reaction gave a mixture of aetioporphyrins I and II.

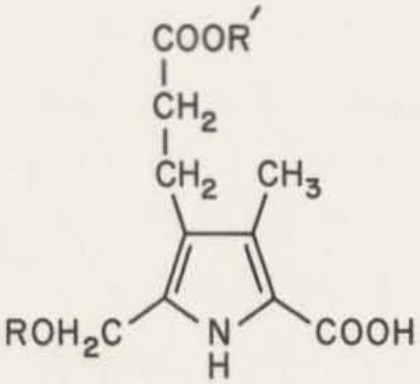
Having established the structure of porphobilinogen (VII) in 1953, Cookson and Rimington (38) performed the polymerization of porphobilinogen <u>in vitro</u>, at 100 °C in 0.5N hydrochloric acid and at 20 °C under different pH conditions. They found that at 100 °C (in acid) only a single product, uroporphyrin III was formed rapidly and in high yield with no trace of other isomers;but at 20 °C the polymerization took place over several days and was dependent on pH; at pH 6.5,



(VII)



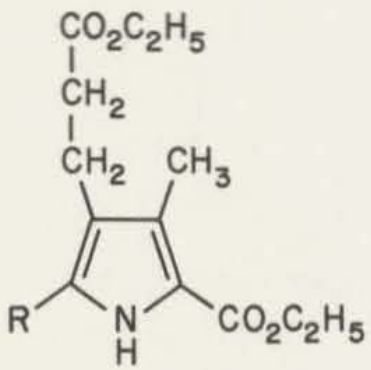
(IX)



(VIII)

(VIIIa): R = CH_3CO- , R' = C_2H_5

(VIIIb): R = R' = CH_3



(X)

(Xa): R = -COOH

(Xb): R = CH_3

only the type III isomer was formed; but at pH >10, about equal amounts of the type I and III isomers were obtained (Table 1.). Based on these results, and theoretical considerations, they proposed a mechanism for the acid catalyzed polymerization of porphobilinogen to give a random mixture of the four uroporphyrin isomers.

In 1958, Bullock et al (24) claimed that copro-porphyrin-III tetraethyl ester (m.p. 147 - 149°C) was obtained almost exclusively by the action of glacial acetic acid on 5-acetoxy-4,2'-ethoxycarbonylethyl-3-methyl-pyrrole-2-carboxylic acid (VIIIa) followed by aerial oxidation. A mechanism based on the Hayashi rearrangement of substituted o-benzoylbenzoic acids (40, 41) was proposed for the polymerization of 2-(α-substituted methyl)pyrroles to the type III porphyrins, which includes the biological polymerization of porphobilinogen: In the same year, Treibs and Ott (28) found that uroporphyrin III was the main product when the pyrrole (IX) was heated in acid solution. In 1960, Falk and Dresel (42) also reported the exclusive formation of uroporphyrin III under acidic conditions. Later work by Mauzerall (43, 44) indicated that a random mixture of uroporphyrins (1/8 isomer I, 1/8 II, 1/2 III and 1/4 IV) was obtained when porphobilinogen was heated in vacuo, in 1N hydrochloric acid. He also found that in neutral and alkaline solution, uroporphyrinogen neither isomerises nor incorporates formaldehyde. When porphobilinogen was heated under neutral conditions the isomer ratio found

Table 1. Conversion of PBG* into uroporphyrins in various solution at 20°C (38).

* PBG = porphobilinogen

	Composition	pH	Time of appearance of fluorescence (days)	Uroporphyrin yield in (µg/mg PBG)	Unchanged PBG (mg.)	Apparent composition of uroporphyrin mixture (%)
A.	2.4 mg. PBG, 50 mg. Na_2CO_3, 0.5 ml. water, normal urine	>10	7	65	0	'I' 60 'III' 40
B.	2.3 mg. PBG, approx. 8.1 ml. HOAc, 4.75 ml. normal urine, approx. 1.2 ml. 2N NaOH	6.5	2	160	0	'I' 0 'III' 100
C.	2.0 mg. PBG, 5.0 ml. 0.2N NH_3	>10	4	80	0	'I' 50 'III' 50
D.	2.2 mg. PBG, 5.0 ml. 0.5N HCl	<1	no fluorescence purple pigment formed	0.35	1.0	not enough material

was : 1/2 I, 1/2 (III and IV) and in alkaline conditions 3/4 I, 1/4 (III and IV); but in the presence of formaldehyde, incorporation of formaldehyde into uroporphyrinogen occurred and an approximately random mixture of isomers was obtained (Table 2, & 3).

The apparent contradiction between Mauzerall's work and that of the earlier workers may be due to the fact that the earlier polymerizations were carried out in the presence of air, where, if the porphyrinogen of type III was kinetically preferred product, it might undergo rapid oxidation to the porphyrin before randomization. To test this hypothesis, Kay (45) tried some experiments similar to those of Mauzerall but under the conditions used by Bullock et al. He also polymerized the pyrrole ether (VIIIb) under various conditions. He claimed that a random mixture of coproporphyrins was formed by the polymerization of monopyrroles in acidic condition. He also reported that isomerization of coproporphyrinogen I took place in acid solutions. His results are given in Table 4. It is interesting to note that when the pyrrole ether (VIIIb) was polymerized in the presence of cupric salts in acid, the ratios are altered in favor of the isomer I.

Interesting differences appear to exist between the chemical and enzymic reactions as regards the participation of free formaldehyde. Shemin et al (27) reported that when porphobilinogen labelled with ^{14}C in the aminomethyl group was heated in dilute acid solution or converted enzymically

Table 2. Uroporphyrin formed by condensing porphobilinogen under various conditions. (44).

Expt.	Conditions	Uroporphyrin (% yield)	Uroporphyrin (% preoxidized)	Isomer composition I.	II	III and IV
1.	Acid	78	0.1	1/8	1/8	3/4
2.	Neutral	55	0.6	1/2	0	1/2
3.	pH 10.0	70	0.45	5/8	0	3/8
4.	Alkaline	40	1.1	3/4	0	1/4

Table 3. Uroporphyrin formed by condensing porphobilinogen under various conditions in the presence of ^{14}C-formaldehyde. (44)

Expt.	Conditions	Molar Ratio HCHO/PBG	Uroporphyrin (% yield)	Isomer I	Isomer II	Ratio of Molar Activities URO/HCHO Found	Ratio of Molar Activities URO/HCHO Calcd.
1.	Acid	0.27	33	1/8	1/8	0.51	0.85
2.	Neutral	0.98	48	1/8	1/8	1.7	1.98
3.	Alkaline	0.28	34	1/8	1/8	0.8	0.88

Table 4. Paper chromatographic resolution of mixture of coproporphyrin isomers formed under various conditions. (45)

Expt.	Compound Used	Conditions	yield (%)	% isomers I	II	III & IV
1.	Coproporphyrinogen I	pH 4, 20 C for 24 hr.	67	80		20
2.	Coproporphyrinogen I	pH 4, 100 C for 30 min.	75	75		25
3.	Coprpponphyrinogen I	pH 4, Cu^{++}, 20 C for 48 hours.	92	92		8
4.	Pyrrole ether (VIIIb)	pH 4, 20 C for 24 hr.	34	19	14	67
5.	Pyrrole ether (VIIIb)	pH 4, 100 C for 30 min.	52	16	16	68
6.	Pyrrole ether (VIIIb)	pH 4, Cu^{++}, 20 C for 48 hours.	28	57	6	37
7.	Pyrrole ether (VIIIb)	pH 4, Zn^{++}, 20 C for 48 hours.	38	18	17	65

into porphyrin in cell-extracts, radioactive formaldehyde could be isolated as the dimedon derivative. This indicated the indirect participation of formaldehyde in porphyrin formation in either process. In later work Lockwood (46) found that with unlabelled porphobilinogen and addition of ^{14}C-formaldehyde to the medium, the uroporphyrin III isolated after heating in acid solution was highly radioactive, while that formed enzymically was completely devoid of activity. Thus, formaldehyde was neither a product nor a reactant in the enzymic synthesis.

Bogorad et al (30, 56) reported the isolation and identification of intermediates (a dipyrrylmethane and an uncyclized linear tetrapyrrylmethane with an aminomethyl group on an α -position) from the reaction mixtures of the enzyme uroporphyrinogen I synthetase and porphobilinogen incubated with ammonium ions. The tetrapyrrylmethane was not converted to uroporphyrinogen III in an enzymatic system capable of forming this isomer from porphobilinogen. Recent work by Battersby et al (53, 55) using double labelled ^{13}C-porphobilinogen and some related dipyrrylmethanes provides certain limitations on the possibilities for the type III porphyrin formation during porphyrin biosynthesis. They found that only the porphobilinogen unit forming ring D and no other porphobilinogen unit undergoes intramolecular rearrangement. Their results also indicate that tetrapyrrane formation is (2 + 2) reaction and not a (1 + 1 + 1 + 1) process as

has commonly been assumed previously.

A decision about the mechanism of the type III porphyrin formation, both *in vitro* and *in vivo* will clearly soon be possible. The object of present work was to examine the porphyrin formation in mild conditions (especially in the absence of acid) from pyrryl Mannich base methiodides, with a view to finding conditions where formation of a single type isomer might occur.

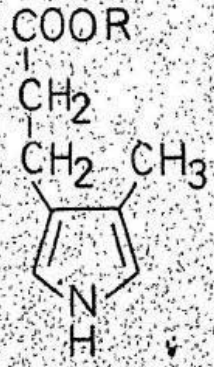
Discussion

(A). The chemical polymerization of monopyrroles to porphyrins

It has been known for some time that many pyrroles of the general type (XVI) when refluxed in either acid or base solution and followed by aerial oxidation produce porphyrins. The composition of the mixture of isomers formed may vary with pH (36, 37, 44). In general, if both R and R' are alkyl groups and Z is a good leaving group, then greater than 50% conversion to the porphyrins is often obtained. Among the monopyrroles, porphobilinogen (VII) is the one which has been studied most extensively over a few decades. A variety of mechanisms has been offered to rationalize its polymerization reactions, reflecting the large number of pathways that are possible. Some of these (36, 27, 44) may be ruled out because of the later experimental evidence against them (21, 29, 48, 49) while others have not been tested (24 - 26). The basic assumption in these mechanisms is that the Z group is dissociated from the pyrrole leaving a "benzyl type" carbonium ion, which can then undergo electrophilic attack on another pyrrole unit. Since substituents of the type ($-CH_2Z$) are also known to be lost from the α-position of pyrroles during the course of a reaction, therefore, in porphyrin synthesis in vitro

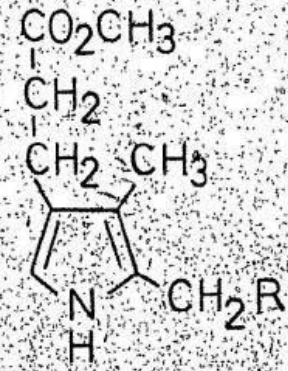
there are at least two possible ways of attack by one pyrrole unit on another (Figure 1.). If these reactions are in competition with each other, then polymerization of a pyrrole with different R and R' will give a mixture of different isomers. However, there are some other complications after the initial reaction; different routes of attack are possible. It was known that in hot acid (but not neutral) solution, considerable isomerization of the uroporphyrinogens occurs (42). Therefore, if one assumes that in neutral solution, there is no isomerization of the porphyrinogens formed, then the production of a mixture of different type of isomers must be due to the competition of the two reactions given in Figure 1.

It was found that in the preparation of the Mannich base, only a single product was formed from opsopyrrole monocarboxylic acid methyl ester. Although there are two possible structures, it is most likely (XIIa), since according to Fischer (57), treatment of opsopyrrole monocarboxylic acid with hydrocyanic acid and subsequent hydrolysis gave 3-methyl-4-propionic acid-2-pyrrolecarboxaldehyde only. In a similar reaction with opsopyrrole dicarboxylic acid dimethyl ester, a mixture of two products (XIII) and XIV) was obtained. The N.M.R. spectrum showed two aromatic protons as well as two peaks corresponding to the dimethylamino groups. The ratio of these peaks was approximately 2:1. Attempts to separate this mixture failed, thus, it was impossible to identify which isomer was present in larger amount.



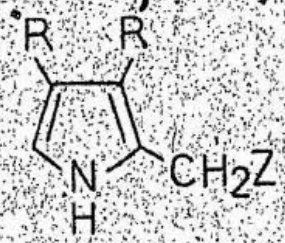
(XIa) : R = H

(XIb) : R = CH_3



(XIIa) : R = $-N(CH_3)_2$

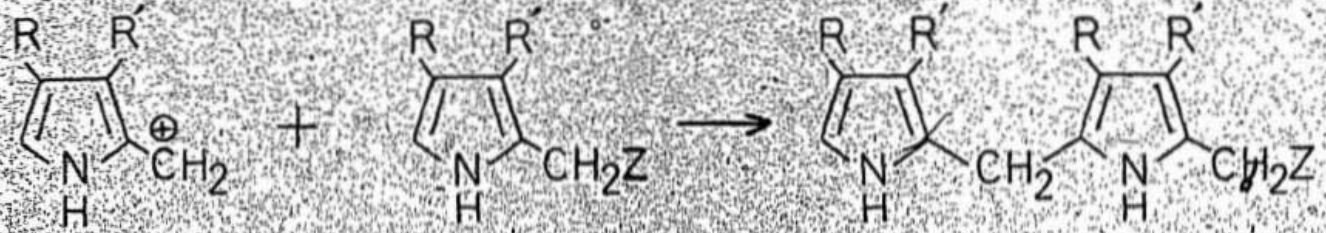
(XIIb) : R = $-\overset{+}{N}(CH_3)_3\ I^-$



(XV) Z = $\overset{+}{N}(CH_3)_3\ I^-$, $-OCH_3$ etc.

Fig. 1.

SCHEME A :



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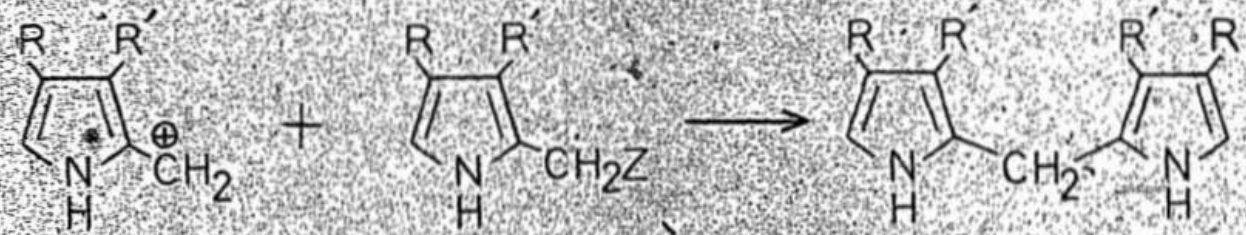


In the system studied here, 2-N,N-dimethylaminomethyl-4-carbomethoxyethyl-3-methylpyrrole methiodide salt (XIIb) was refluxed in both anhydrous hydroxylic and nonhydroxylic solvents, followed by aerial oxidation with the hope that displacement of the substituent would be minimized and a unique type of isomer produced. When this methiodide salt was polymerized by refluxing in methanol, a good yield of coproporphyrin tetramethyl ester (XXa) was obtained. Paper chromatographic analysis (2, 51) showed that it was mainly type III and IV isomers. Elution of the pigments from the paper strip and use of the intensity of the Soret band as the analytical parameter (2) showed that the porphyrin was ~ 95% type (III and IV) and ~ 5% type (I and II) isomers. Variation of the polymerization solvent (including anhydrous dioxane, dry dimethyl sulfoxide, anhydrous tetrahydrofuran, and glacial acetic acid) produced different yields of coproporphyrin, but the product composition scarcely varied from solvent to solvent (Table 5.). The hydroxylic solvents gave higher yields but all were > 35%. Porphyrin formation from 4-carbomethoxyethyl-3-methylpyrrole in the presence of excess formaldehyde with pyridine as catalyst in methanol gave a very low yield of coproporphyrin. Thus, it seems likely that porphyrin formation from the trimethylaminomethyl-pyrrole salt does not involve dissociation of a one carbon α-substituent, and the resulting mixture of type isomers is not a "random" mixture, but is controlled by the polymerization reaction and

Table 5. Coproporphyrin tetramethyl ester formed by condensing 2-N,N-dimethylaminomethyl-4-carbomethoxyethyl-3-methylpyrrole methiodide salt (XIIb) in various solvents.

Expt.	Conditions	Coproporphyrin (% yield)	% isomer	
			I and II	III and IV
1.	Refluxed in anhydrous dioxane for 18 hr., followed by aerial oxidation for 3 hours.	41	3.4	96.6
2.	Refluxed in anhydrous THF for 18 hr., followed by aerial oxidation for 3 hr.	38	4.5	95.5
3.	Heated in anhydrous DMSO at 90° C for 18 hr., followed by aerial oxidation for 3 hours.	40	5.1	94.9
4.	Refluxed in methanol for 4 hours, followed by aerial oxidation for 3 hr.	71	5.8	94.2
5.	Refluxed in glacial acetic acid for 4 hr., followed by aerial oxidation for 3 hr.	59	4.4	95.6
6.	Stirred with Amberlite IR-120 resin (acid form) in distilled water at room temperature for 24 hours.	23	9.0	91.0

the relative reactivities of two α-positions.

Further analysis of the mixture using N.M.R. spectra recorded in both deuterio δ chloroform and deuterated trifluoroacetic acid (Figures 2, 3, 4) also suggested that it is mainly type III and IV isomers. The N.M.R. spectra recorded for the products from the reactions in different solvents are found to be similar to one another. The spectra recorded in deuterated chloroform showed a drastic change with concentration (Figures 2 & 3). Because of this dependance on concentration, the proton chemical shifts of the mixture of different isomers recorded in chloroform solution are of little use. However, in the more concentrated solution (Figure 3), there were seven peaks for the meso- protons as well as a well-defined triplet for the β-methyl groups at τ 6.47, 6.54 and 6.62 which according to Abraham et al (50), is the indication of type III isomer. Interpretation of the spectra recorded in deuterated trifluoroacetic acid also suggested that the product consisted mostly type III with some type IV isomer. According to Abraham et al (50), the pattern of the meso- proton resonances for coproporphyrin I, II III and IV tetramethyl esters in deuterated trifluoroacetic acid was singlet, doublet (2:2), triplet (1:2:1) and triplet (1:2:1) respectively. The spectrum of the product obtained, shown in deuterated trifluoroacetic acid (Figure 4), has a well defined triplet for the meso- proton at τ values around -1.18, -1.09 and -1.01, this is close to the reported result for

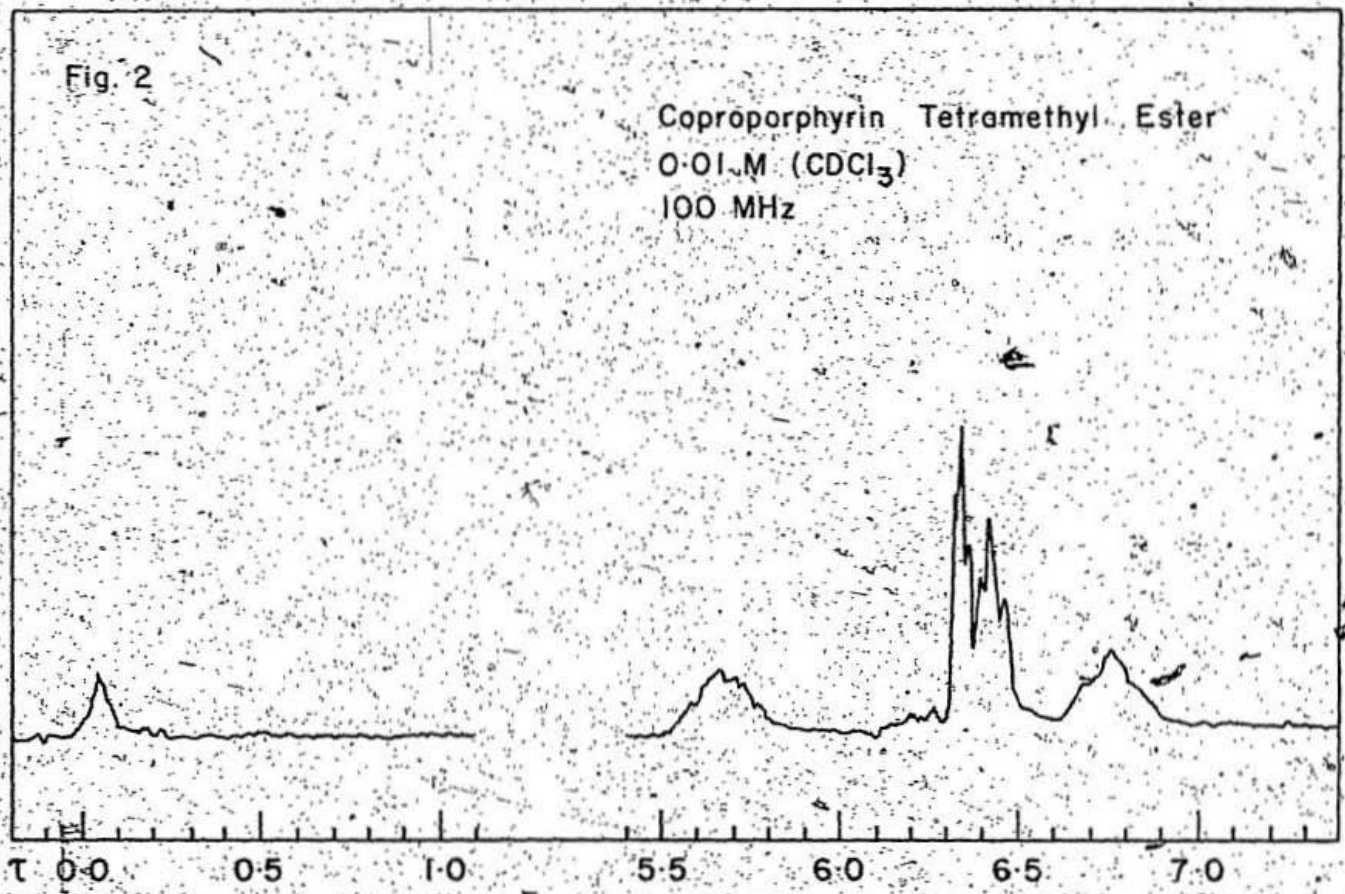


Fig. 2
Coproporphyrin Tetramethyl Ester
0·01 M ($CDCl_3$)
100 MHz
τ 0·0
0·5
1·0
5·5
6·0
6·5
7·0

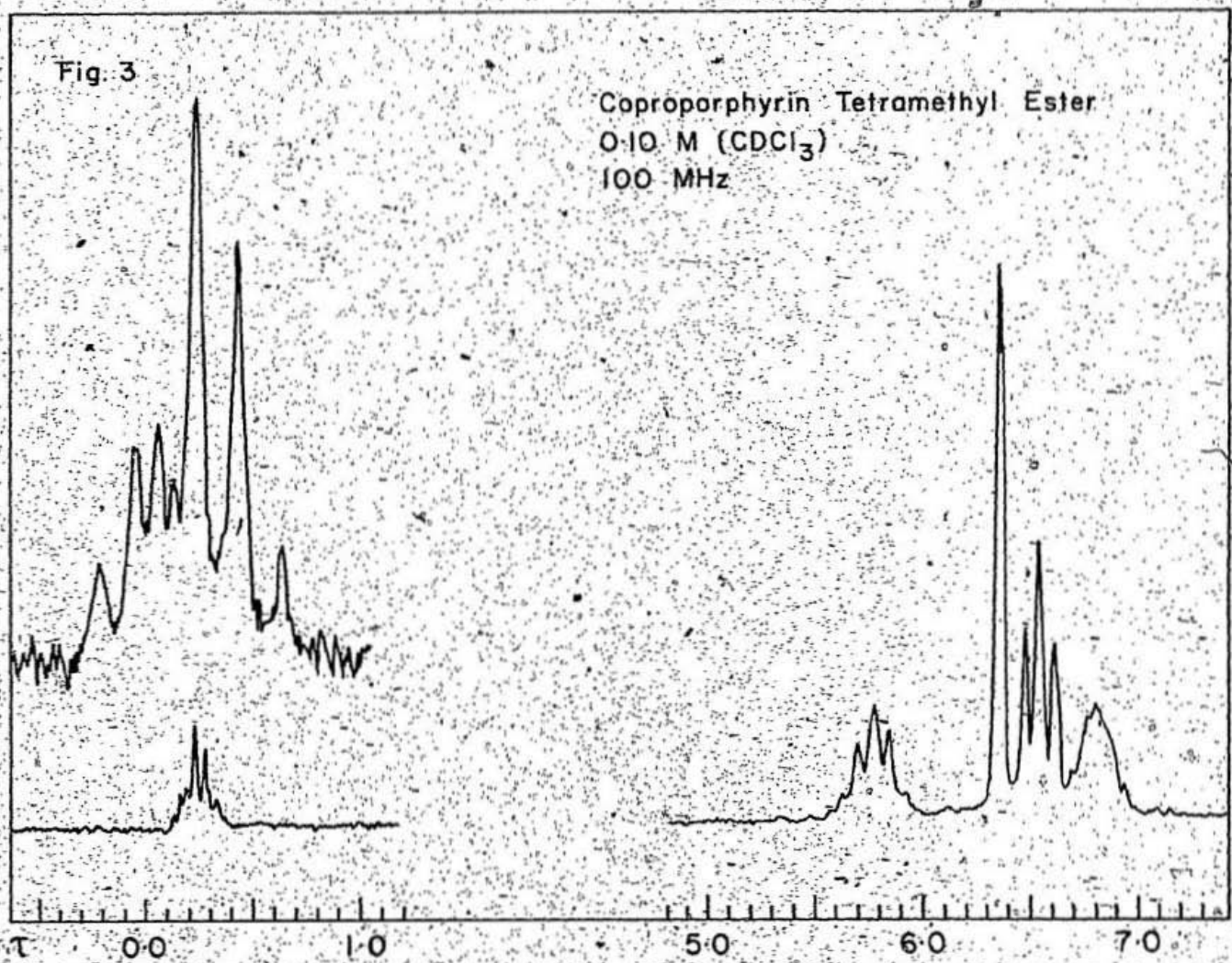
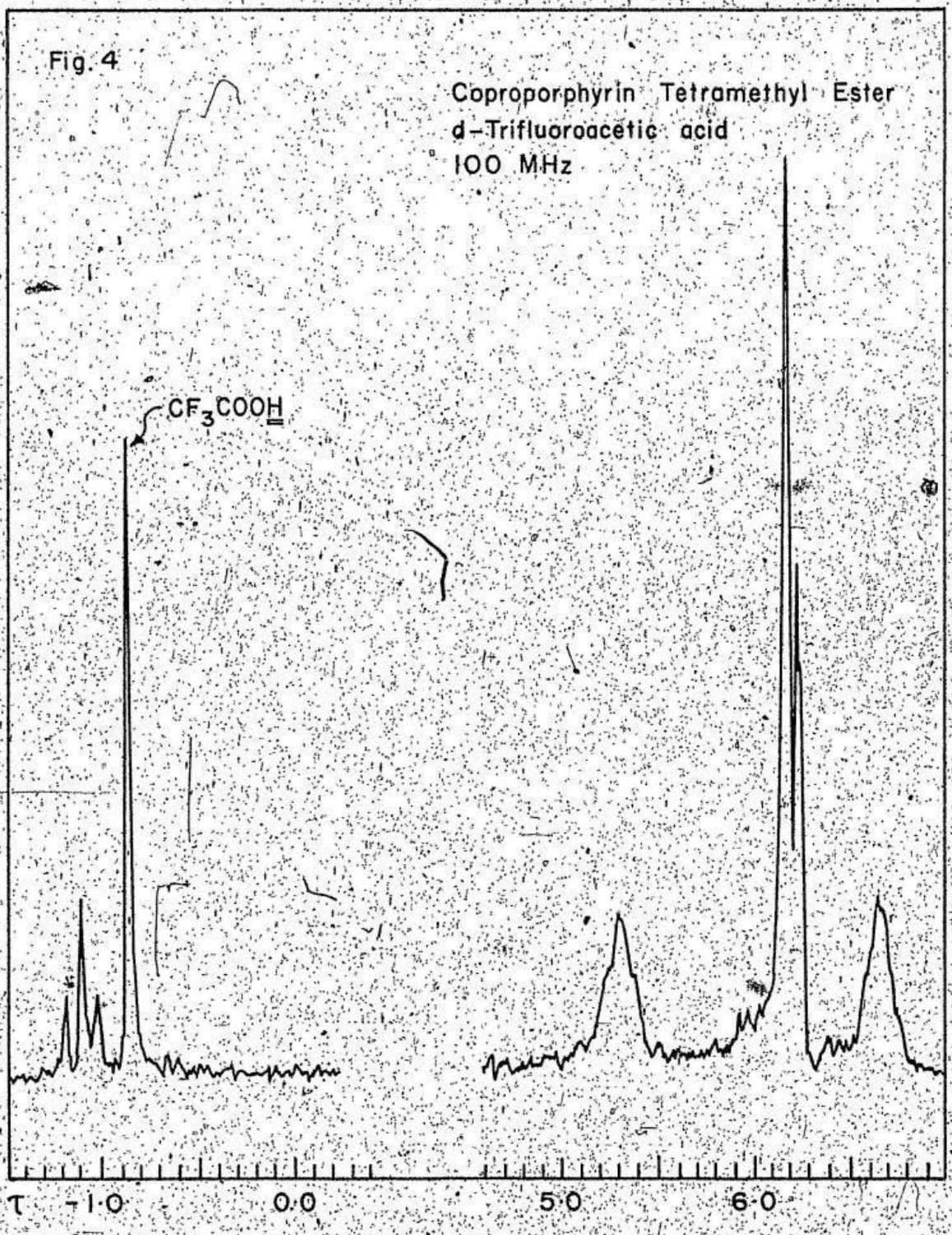


Fig. 3
Coproporphyrin Tetramethyl Ester
0·10 M ($CDCl_3$)
100 MHz
τ
0·0
1·0
5·0
6·0
7·0

Fig. 4


Coproporphyrin Tetramethyl Ester
d-Trifluoroacetic acid
100 MHz
CF_3COOH
τ -1·0
0·0
5·0
6·0

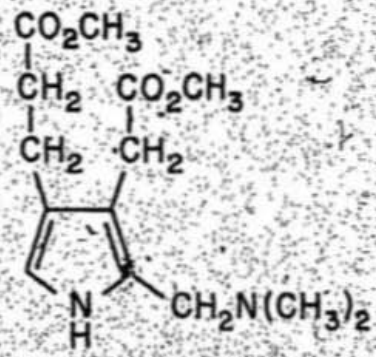
the positions of the triplets for the coproporphyrin III and IV tetramethyl esters. Thus the product is evidently very largely type III isomer i.e. the naturally occuring isomer type. The evidence presented here suggested that the " reversal " of one pyrrole unit in type III porphyrin formation is an inherent property of pyrroles with the (XVa) system, and hence rearrangement in some form is a normal part of the polymerization.

The Mannich base methiodides (XVa) present a particularly interesting case of porphyrin formation, since a dimerization reaction which requires the loss of $-CH_2Z$ from the α -position (Figure 1), in this case appears to demand the attack of a " benzylic " carbonium ion on an already positively charged species, with the loss of a group which already bears a positive charge. This seems fundamentally unlikely on general chemical grounds, but a displacement of some type must occur if type III and IV isomers are to be generated by other than the " random " process. It seems likely, therefore, that any " reversal " takes place after dipyrryl methane formation, where an electrically neutral pyrrole ring is the group Z in $-CH_2Z$, and not the $-\overset{+}{N}(CH_3)_3$ as in the original Mannich base salt. The observations reported here appear quite inconsistent with Johnson's mechanism (24), which requires rearrangement of the α -substituent at each step of the polymerization. Porphobilinogen (VII) must be present in physiological systems as a Zwitterion form (XVI).

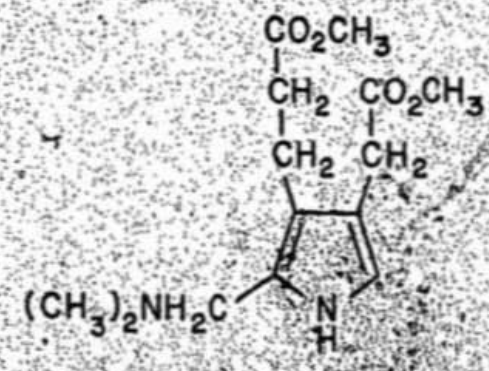
and thus it formally resembles the Mannich base methiodide salts. Unlike the salts, of course, it has the ability to dissociate proton (perhaps under specific conditions at the enzyme site), and thus has a group $-CH_2Z$ which need not be charged and presumably, therefore, would be more readily rearranged.

The "benzylic" carbonium ion (XVII) has been used by many authors in hypothetical schemes of porphyrin formation, though as Kenner et al have pointed out (52) a form (XVIII) seems more plausible for this species. Since the proton is now dissociable, one could visualise a process in which protonated and unprotonated species (XIX) react together to form a dipyrromethane.

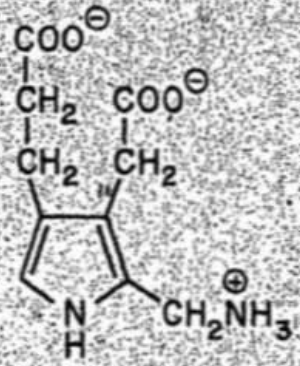
Johnson et al (24) postulated a Hayashi type rearrangement in a reversal step, but their porphyrin generating scheme led exclusively to type III isomer (XXb). Bogorad (30), Corwin (25) and others prefer a mechanism which involves a reversal at the tripyrrane $\longrightarrow$ tetrapyrrane step which is mediated by uroporphyrinogen III synthetase, using a rearrangement reaction which chemically, at least, seems less plausible than Johnson's. This scheme however has the advantage that it can also lead to uroporphyrin I in a simple way, whereas, Johnson's scheme cannot. Recently, Battersby et al (53) based on the ^{13}C-N.M.R. studies, reported that during the biosynthesis of the macrocycle of natural porphyrins (type-III isomer), the porphobilinogen (PBG, VII) unit forming



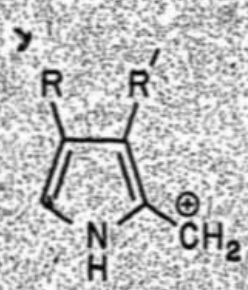
(XIII)



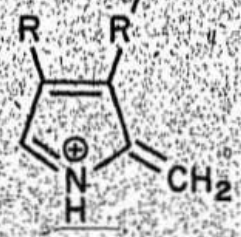
(XIV)



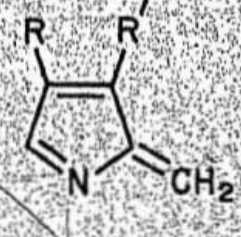
(XVI)



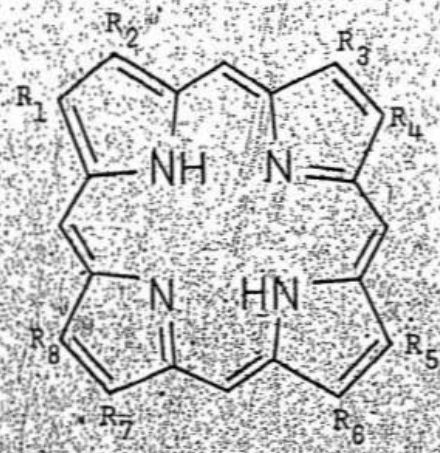
(XVII)



(XVIII)



(XIX)



(XX)

(XXa) For Coproporphyrin III Tetramethyl Ester :

$R_1 = R_3 = R_5 = R_8 = CH_3$

$R_2 = R_4 = R_6 = R_7 = -CH_2CH_2COOCH_3$

(XXb) For Uroporphyrin III Octamethyl Ester :

$R_1 = R_3 = R_5 = R_8 = -CH_2COOCH_3$

$R_2 = R_4 = R_6 = R_7 = -CH_2CH_2COOCH_3$

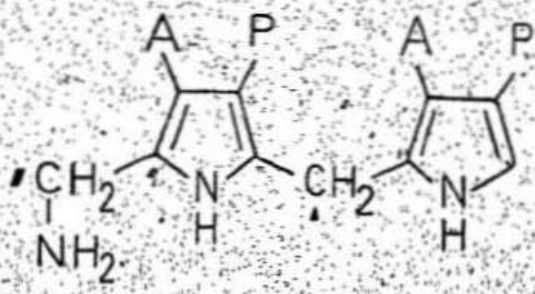
ring D, and no other PBG unit, was found to undergo intramolecular rearrangement. Thus the rearrangement schemes proposed by Bogorad and Corwin etc. can then be eliminated.

The specific reactivity of the α -position adjacent to the methyl group in opsopyrrole monocarboxylic acid seems consistent with the results that polymerization of Mannich base methiodide salt (XIIb) gives mainly coproporphyrin III. In the uroporphyrin series, there is evidently far less difference between the reactivities of the two α -positions in opsopyrrole dicarboxylic acid (54) and hence this system appears to have the ability to yield both rearranged (Type III) and unrearranged (Type I) porphyrins quite readily in " non-random " process.

Nature of the " Type III " rearrangement

The results cited here indicated that the " type III " rearrangement is a normal reaction of porphobilinogen-type pyrroles. The in vitro reaction does not involve predissociation of formaldehyde or an equivalent one carbon substance, because use of the methiodide salt appears to prevent reaction of one pyrrole unit at the substituted α -position of a second unit. If the in vivo and in vitro reactions are the same then recent work of Battersby et al (55) places severe limitations on the possibilities.

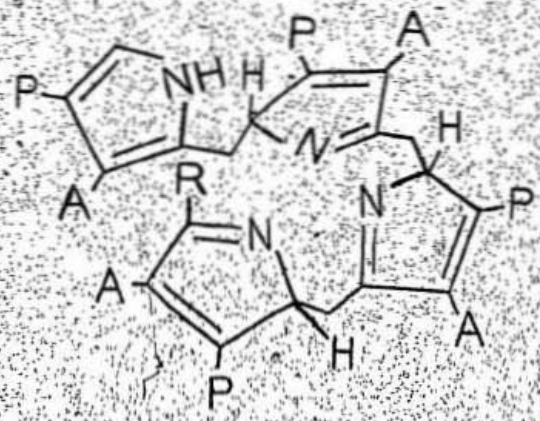
Thus, his observation that (XXI) can form



(XXI)

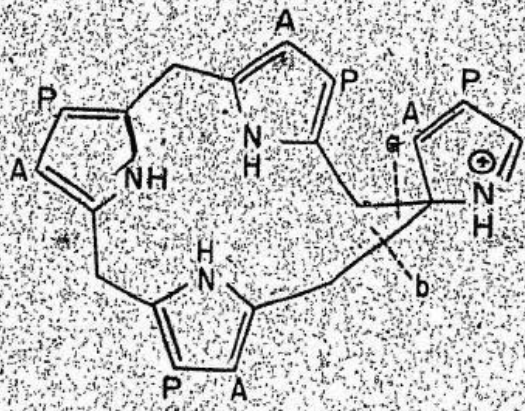
• ^{13}C at α, γ in protoporphyrin IX

• ^{13}C at β, δ in protoporphyrin IX



(XXII) R = $-CH_2\overset{+}{N}H_3$

protoporphyrin IX with enzyme systems from Euglena gracilis in the absence of porphobilinogen suggested that uroporphyrinogen formation is a (2 + 2) reaction and not (1 + 1 + 1 + 1) process as was commonly assumed previously. Secondly, the observations of specific meso - carbon ^{13}C labelling mean that the rearrangement does not involve an intramolecular 1-carbon shift but must require the specific reversal of a ring, presumably ring A or D. This makes a mechanism resembling that of Corwin and Mathewson (25) most likely. Corwin's original proposal required that in the tetrapyrrane intermediate (XXII) at least three of the heterocyclic rings should be in the α - pyrrolenine form. The use of Stuart models imposed this restriction because of " strain " in the unprotonated form. This protonation is the major drawback with Corwin's mechanism since the pKa of the conjugate acid to a tetrasubstituted pyrrole is about 3.5 (56) and such acid conditions are hardly normal in cells. The use of Dreiding models suggested that this limitation is not real. An aminomethyl-tetrapyrrane (XXIII) can readily take up a conformation which brings the aminomethyl group close to the methane bridge at the other end of the tetrapyrrane. Furthermore, a model of the intermediate (XXIV) with one ring in the α -pyrrolenine form is remarkably strainless with three pyrrole rings and methane bridges essentially coplanar, but with the fourth ring at right angles to this plane. This model is an excellent potential intermediate to account for the formation of type I or type III porphyrins



(XXIV)

A = CH_2COOH , P = CH_2CH_2COOH

since the result merely depends on which methane bridge migrates from the α -position of the pyrrolenine ring. Intramolecular migration may occur via the nitrogen atom or directly across the ring, but in any case it provides an excellent control point for uroporphyrinogen III synthetase but allows uroporphyrinogen I formation from the common intermediate, the type I aminomethyl-tetrapyrrane. Thus a mechanism based on the following sequence of events appears to meet the available experimental data (Scheme C.).

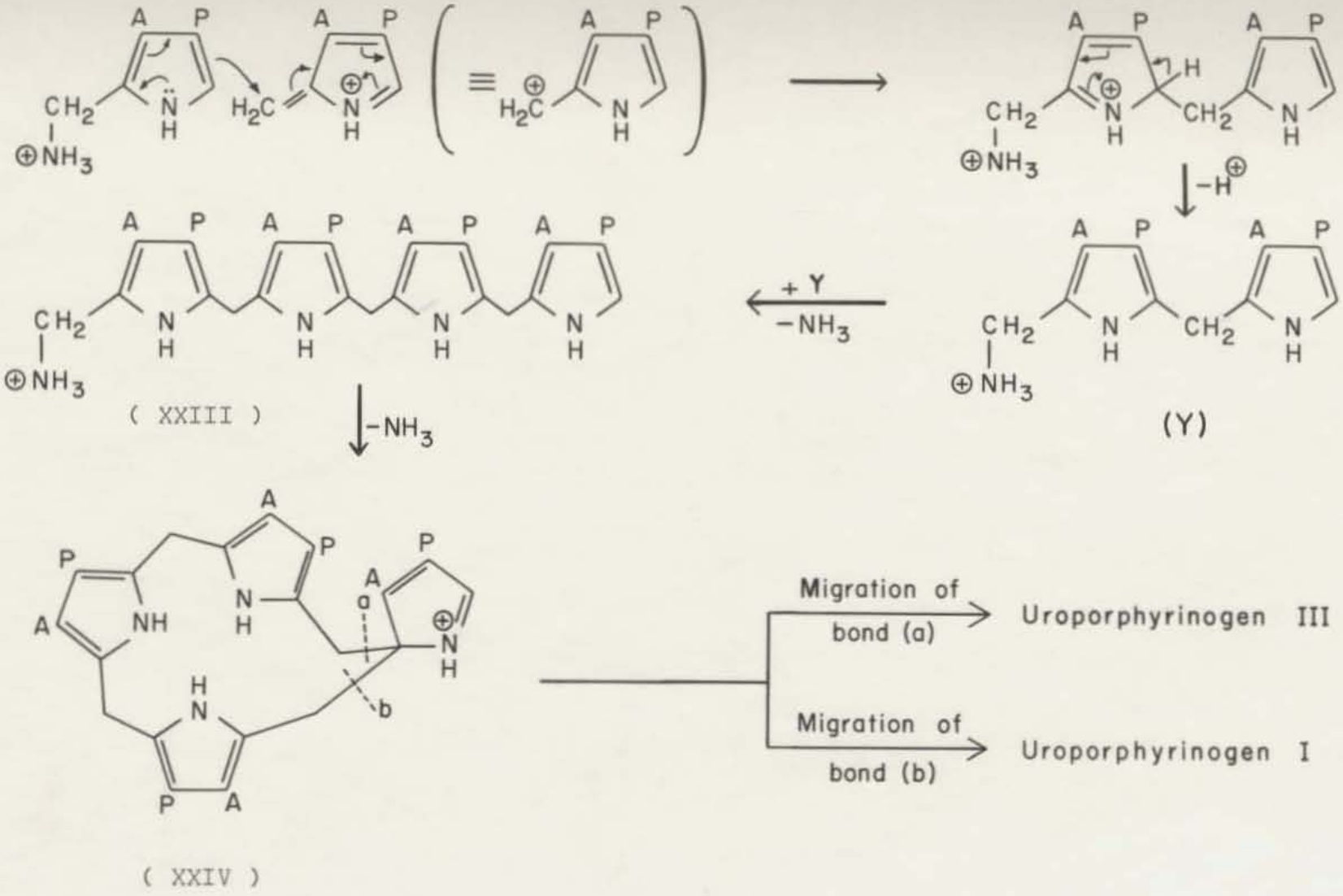
(a). Polymerization of two porphobilinogen units to an aminomethyl-dipyrrylmethane, via the unsubstituted α -position.

(b). Formation of a tetrapyrrane by (2 + 2) mechanism giving a product with the " type I " substituent arrangement.

(c). Either simultaneous or subsequent attack of the aminomethyl substituent (through the " benzylic " carbonium ion) on the most remote methane bridge-pyrrole linkage to form an intermediate with the ring D PBG unit in the pyrrolenine form at right angles to the macrocycle formed from the other three pyrrole units and methane bridges (XXIV).

(d). Migration of a methane bridge from the pyrrolenine form, possibly via nitrogen, to give either type I or type III porphyrinogen, depending on which methylene migrates. If bond (a) migrates, the product is type III with ring D reversed; if bond (b) migrates, the product is type I.

SCHEME C

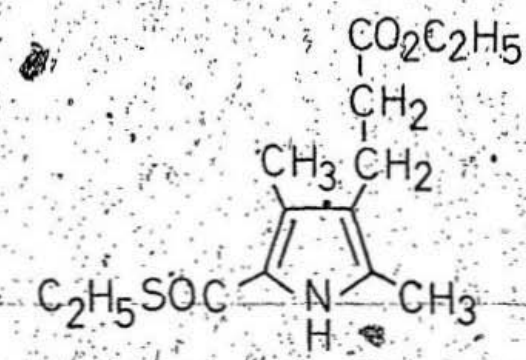

A P
CH2
⊕NH3
N H
H2C
≡
-H⊕
+ Y
-NH3
(XXIII)
(Y)
-NH3
a
b
(XXIV)
Migration of bond (a)
Uroporphyrinogen III
Migration of bond (b)
Uroporphyrinogen I

B. Bromination of the pyrrole thiolester (XXV)

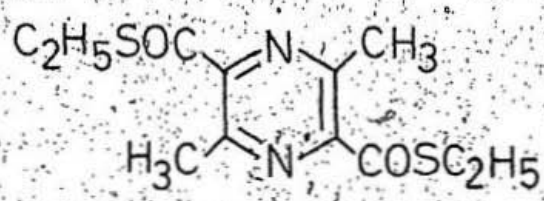
In the preparation of pyrrole thiolester (XXV) by a modified Knorr synthesis, a non-pyrrole by-product (yellow needles) was also obtained. It was confirmed that this by-product is diethyl 2,5-dimethyl-3,6-pyrazine-dithiolcarboxylate (XXVI). (Attempts to recrystallize this yellow by-product from hot methanol yielded colorless needles which were found to be 2,5-dimethyl-3,6-dicarbomethoxypyrazine (XXVII). This presumably comes from ester exchange.

Wells (54) has demonstrated that when treated with bromine, the thiolester group of the pyrrole thiolester (XXV), rather than the methyl group was undergoing bromination to give most likely the corresponding acyl bromide. He also observed that when this brominated product was treated with a pyrrole which bears a free α-position, a dipyrryl ketone was obtained.

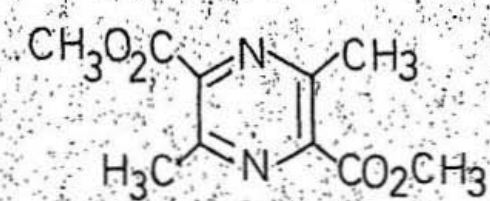
Attempts were therefore made to isolate the intermediate for dipyrryl ketone formation by treating the bromination product of pyrrole thiolester (XXV) with (a) dry pyridine in anhydrous ether and (b) anhydrous sodium acetate under absolute ether. Attempts to separate and identify the components of the mixture ended in failure.



(XXV)



(XXVI)



(XXVII)

Experimental

Melting points (uncorrected) were determined on a Thomas Hoover capillary melting point apparatus, unless otherwise stated. Infrared spectra were recorded on a Perkin-Elmer 237B grating spectrophotometer. Ultraviolet spectra were recorded on a Perkin-Elmer 202 Ultraviolet spectrophotometer. Nuclear magnetic resonance spectra were recorded on a Varian HA-100 spectrometer and a Varian A-60 analytical spectrometer, the resonance positions are reported on the τ scale, using tetramethylsiliane as an internal reference. Mass spectra were recorded on a Hitachi-Perkin-Elmer RMU-6E mass spectrometer.

The following compounds were prepared in bulk as starting materials for the experimental work. Given with the name of each compound is the number of the literature reference.

Compound	Literature
Ethyl 4-acetyl-5-oxohexanoate	24
2,4-Dimethyl-5-carboxy-pyrrole-3-propionic acid diethyl ester	24

Method for the determination of relative isomer composition of the coproporphyrin mixture in the product.

Separation of the coproporphyrin isomer was carried out according to Chu's method (51). After separation, each fraction was cut from the paper chromatogram and extracted with chloroform. The chloroform solution was then

concentrated to 1 ml. and the visible spectrum for each fraction was recorded. Comparison of the absorbance of the major band (Soret band) of each fraction (on the assumption that its extinction coefficient for each isomer is identical) permits determination of the relative isomer composition in the product.

2-Carboxy-3-(2-carbethoxyethyl)-4-methyl-5-carbethoxy-pyrrole (Xa)

Bromine (8.0 g.) was added to a stirred solution of 2,4-dimethyl-5-carboxy-pyrrole-3-propionic acid diethyl ester (Xb, 13.3 g.) in dry CCl_4 (150 ml.) at room temperature under UV light. After the reaction was completed, the solvent was removed under reduced pressure. Absolute ether (200 ml.) was then added to the dry residue, followed by dropwise addition of sulfuryl chloride (13.5 g.) with stirring at ≤ 4 °C and protected from moisture. Stirring was continued for 30 min. then overnight with the cooling bath removed. The next day, the clear solution was refluxed for 30 min., after which the solvent was removed under reduced pressure. Three portions of anhydrous ether (100 ml. each) were succesively added and removed in the same way. The oily residue was then stirred on the steam-bath for 15 min. with a hot solution of sodium acetate (50 g.) in water (500 ml.). On cooling, the crystals

separated and sodium bicarbonate was then added to dissolve the precipitate. The solution was next extracted with ether (four times), which was in turn washed with 5% sodium bicarbonate solution (twice with 100 ml.). The combined aqueous layers were filtered and finally acidified with sulfur dioxide. After cooling the crystalline product was filtered and washed three times with water, then recrystallized from aqueous ethanol to give colorless needles. (10.2 g. 69%). m.p. 150 - 151 °C. N.M.R. spectrum ($CDCl_3$): τ -1.91 (-COOH), 0.1 (broad, N-H), 5.60, 5.83 (overlapping quartets, $-OCH_2$), 6.9 & 7.35 (A_2B_2 multiplets, $-CH_2CH_2CO$), 7.68 (singlet, $-CH_3$), 8.61, 8.73 (overlapping triplets, ester CH_3).

3-Methylpyrrole-4-propionic acid (Opsopyrrole monocarboxylic acid) (XIa)

2-carboxy-3-(2-carbethoxyethyl)-4-methyl-5-carboethoxy-pyrrole (4.5 g., Xa) in 10% sodium hydroxide solution (24 ml.) was heated under nitrogen in a sealed tube for 6 hr. at 175 °C. After filtration, the solution was stirred with Amberlite IR-120 resin (acid form, 5 g.), which was then passed through the same resin (60 g.) with water as eluent until the Ehrlich's reaction was weak. The eluate was concentrated at room temperature under vacuum, and finally freeze-dried. The solid residue on freeze-drying was then purified by sublimation to give colorless crystals. (1.07 g. , 46%).

m.p. 115 - 116°C. N.M.R. spectrum ($CDCl_3$) : τ -1.9 (-COOH), 1.82 (broad, N-H), 3.45 (doublet, two α -H), ~ 7.33 (A_2B_2 multiplets, - CH_2CH_2CO), 7.97 (singlet, $-CH_3$). Mass spectrum: M^+ 153.

2-N,N-dimethylaminomethyl-4-carbomethoxyethyl-3-methyl pyrrole (XIIa)

Opsopyrrole monocarboxylic acid (XIa, 306 mg.) was treated with diazomethane (generated from Diazald (1.72 g.) in ether (17 ml.) and a solution of potassium hydroxide (0.5 g.) in water (0.7 ml.) and ethanol (2 ml.) at 60 - 65°C). After one hour, the solvent was removed, and the oily residue dried under vacuum. (Thin layer chromatography studies and N.M.R. spectrum showed that the ester was a pure compound). The dry residue was then dissolved in methanol (2 ml.) and treated with a solution of anhydrous dimethylamine hydrochloride (172 mg.), potassium acetate (196 mg.) and 40% formaldehyde solution (150 mg.) in water (1 ml.) at -15°C under nitrogen. The temperature was maintained between -15 and -10°C during addition. The solution was stirred for another 2 hours at -10°C under nitrogen, then extracted with ether (four times). The ether1 extracts were washed with water (twice), and dried over anhydrous potassium carbonate for 2 hours. Removal of the ether under vacuum gave an oil (253 mg. 57%), which

was shown by the N.M.R. spectrum and thin layer chromatogram to be substantially a single product. N.M.R. spectrum ($CDCl_3$): τ 0.81 (broad, N-H), 3.6 (doublet, and singlet in the presence of D_2O), 6.4 (singlet, $-OCH_3$), 6.7 (singlet, $(-CH_2N)$, ~ 7.4 (A_2B_2 multiplets, $-CH_2CH_2CO$), 7.81 (singlet, $N(CH_3)_2$), 8.05 (singlet, $-CH_3$).

2-N,N-dimethylaminomethyl-4-carbomethoxyethyl-3-methylpyrrole methiodide salt (XIIb)

Pyrryl Mannich base (XIIa, 250 mg.) was dissolved in anhydrous ether (15 ml.), then a two-fold excess of methyl iodide was added. After 3 hours, the precipitated methiodide salt was collected and dried in a vacuum desiccator for 4 hours to give the product (XIIb, 298 mg. 73%). Anal. Calc'd. for $C_{13}H_{23}N_2O_2I$: C, 42.62; H, 6.28; N, 7.65; I, 34.68. Found : C, 42.69; H, 6.20; N, 7.56; I, 34.89.

Polymerization of 2-N,N-dimethylaminomethyl-4-carbomethoxyethyl-3-methylpyrrole methiodide salt (XIIb) to coproporphyrin tetramethyl esters

(A). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was treated with anhydrous dioxane (15 ml.) under reflux

for 18 hours, and the mixture was protected from moisture. After cooling, the mixture was aerated for a further 3 hours. Removal of the dioxane under reduced pressure gave a residue which was passed through an Alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C) with chloroform as eluent. The porphyrin fractions (determined by visible spectra) were collected then concentrated, finally all the chloroform was boiled off while maintaining the volume with hot methanol. On cooling, the coproporphyrin tetramethyl ester separated as reddish-brown crystals (14 mg. 41%). m.p. 168 - 185°C. Visible spectrum ($CHCl_3$) : λ max 402 (Soret), 500, 533, 569 and 620 mμ (log ε max 5.19, 4.13, 3.98, 3.82 and 3.71 respectively). The N.M.R. spectra, as determined in $CDCl_3$ and trifluoroacetic acid-d, are given in Figures 2, 3 & 4. It was found by the paper chromatographic study that the product probably consists of all the four isomers (I - IV) with 3.4% of isomers I and II; 96.6% of isomers III and IV.

(B). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was heated with dry THF (15 ml.) under reflux for 18 hours, and the mixture was protected from moisture. After cooling, the reaction mixture was aerated for 3 hours. The solvent was removed under reduced pressure

to give a residue which was then dissolved in small amount of chloroform and finally chromatographed on an Alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C) with chloroform as eluent. Removal of chloroform from the porphyrin fractions (determined by visible spectra) and crystallization from chloroform-methanol gave reddish-brown crystals. (12.9 mg. 38%). m.p. 168 - 182 °C. Visible spectrum ($CHCl_3$) : λ max 401 (Soret), 501, 534, 570 and 621 mu (log ε max 5.20, 4.14, 4.00, 3.82 and 3.70 respectively). The N.M.R. spectra, as determined in $CDCl_3$ and d-trifluoroacetic acid, are similar to those shown on Figures 2, 3 & 4. The product was found chromatographically to be a mixture of all the four isomers (I-IV) with 4.5% of isomers I and II; 95.5% of isomers III and IV.

(C). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was heated with anhydrous DMSO (15 ml.) at 90 °C for 18 hours, and the mixture was protected from moisture. After cooling, it was aerated for a further 3 hours. The solution was poured into cold water and immediately extracted with chloroform (five times). The chloroform solution was then washed with water (twice) and dried over anhydrous sodium sulfate. The chloroform solution was finally concentrated and chromatographed on an

Alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C) with chloroform as eluent. Removal of the chloroform from the porphyrin fractions (determined by visible spectra) and crystallization from chloroform-methanol gave reddish-brown product. (13.6 mg. 40%). m.p. 164 - 186 °C. Visible spectrum ($CHCl_3$) : λ max 402 (Soret), 501, 534, 569 and 620 mμ (log ε max 5.20, 4.15, 3.99, 3.83 and 3.71 respectively). The N.M.R. spectra as determined in $CDCl_3$ and trifluoroacetic acid-d, are similar to those shown on Figures 2, 3 & 4. The product was found to consist of 5.1% of isomers I and II ; 94.9% isomers III and IV.

(D). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was heated with methanol (15 ml.) under reflux for 4 hours and then aerated for 3 hours at room temperature. After removal of the methanol under reduced pressure, the residue was dissolved in small amount of chloroform which was then chromatographed on an alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C) with chloroform as eluent. Removal of the chloroform from the porphyrin fractions (determined by visible spectra) gave copro-porphyrin tetramethyl esters. The product was recrystallized from chloroform-methanol to give reddish-brown crystals. (24.2 mg. 71%). m.p. 168 - 188 °C. From the paper

chromatographic study, it was found that the product was a mixture of all four isomers with 5.8% of isomers I and II; 94.2% of isomers III and IV. Visible spectrum ($CHCl_3$) : λ max 402 (Soret), 501, 533, 568 and 621 mμ (log ε max 5.19, 4.15, 3.98, 3.83 and 3.69 respectively). The N.M.R. spectra, as determined in $CDCl_3$ and d-trifluoro-acetic acid, are similar to those shown on Figures 2, 3 and 4.

(E). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was dissolved in glacial acetic acid (15 ml.), which was then heated under reflux for 4 hours. After cooling and aerating for 3 hours at room temperature, the solution was poured into cold water and extracted five times with chloroform which was in turn washed with water (four times) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure. The dry residue was then kept overnight with 30 ml. of saturated methanolic hydrogen chloride with stirring. The next day, the solution was poured into ice-water which was then extracted with chloroform (several times). The combined chloroform solution was washed with aqueous resorcinol which was in turn washed with a little chloroform. The chloroform solutions were then combined and washed with water (three times) and finally dried over anhydrous

sodium sulfate. After filtration, the filtrate was concentrated and chromatographed on an alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C), chloroform as eluent. Removal of the chloroform from the porphyrin fractions (determined by visible spectra) and recrystallization from chloroform-methanol gave reddish-brown crystals (20.2 mg. 59%), m.p. 154 - 163°C. The product was found to be a mixture of all four isomers (I - IV), with 4.4% of isomers I and II; 95.6% of isomers III and IV. Visible spectrum ($CHCl_3$) : λ max 401 (Soret), 500, 534, 570 and 620 mμ (log ε max 5.20, 4.13, 3.99, 3.82 and 3.69 respectively). The N.M.R. spectra , as determined in $CDCl_3$ and d-trifluoroacetic acid, are similar to those shown on Figures 2, 3 & 4.

(f). Pyrryl Mannich base methiodide salt (XIIb, 70 mg.) was stirred with distilled water (25 ml.) in the presence of Amberlite IR-120 resin (acid form, 5 g.) at room temperature for 24 hours. The solution was filtered and the resin washed with dry methanol several times to remove as much water as possible. The choloroform was next added to extract the porphyrin out from the filtrate and methanol washings. The chloroform solution was then dried over anhydrous sodium sulfate. After filtration, the chloroform was removed under reduced

pressure till dryness. The residue was then combined with the dry resin (from first filtration) and kept overnight in 30 ml. of saturated methanolic hydrogen chloride with stirring. The next day, the solution was filtered and the resin washed three times with dry methanol. The filtrate was poured into ice-water which was then extracted with chloroform (several times); meanwhile, the resin was stirred with chloroform (20 ml.) for 30 min., then filtered. The chloroform solutions were then combined and washed with aqueous resorcinol which was in turn washed with small amount of chloroform. The chloroform solution was finally washed with water (four times) and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated and chromatographed on an alumina column (Neutral, Aluminiumoxid Fluka Typ 507 C) with chloroform as eluent. Removal of the chloroform from the porphyrin fractions and recrystallization from chloroform-methanol gave reddish-brown product. (7.8 mg. 23%). m.p. 128 - 149°C. From the chromatographic study, it was found that the product is a mixture of all four isomers (I-IV) with 9 % of isomers I and II; 91% of isomers III and IV. Visible spectrum ($CHCl_3$) : λ max 400 (Soret), 501, 533, 569 and 620 mμ (log ε max 5.18, 4.14, 4.00, 3.83 and 3.71 , respectively).

Condensation of 4-carbomethoxyethyl-3-methylpyrrole (XIb) with formaldehyde

(A). Opsopyrrole monocarboxylic acid (XIa, 15.3 mg.) was treated with freshly prepared diazomethane. After 30 min., the solvent was removed, and the oily residue dried in vacuo. The methyl ester so formed was then treated with a molar equivalent of formaldehyde in methanol (10 ml.) under reflux for 24 hours and then further aerated for 3 hours. The solvent was removed and the residue was dissolved in small amount of chloroform. The amount of porphyrin formed was very small, it could only be detected under the UV lamp.

(B). In this experiment, the amount of chemicals used were same as in reaction (A), except the ratio of pyrrole (XIb) to formaldehyde is 1:2. The amount of coproporphyrin tetramethyl ester formed was found to be about 1 mg. (5.6%), determined spectrophotometrically.

(C). In this experiment, the reaction conditions were same as reaction (B), but this was refluxed in the presence of 2 drops of pyridine as catalyst. The yield of coproporphyrin tetramethyl ester was found to be about 1.4 mg. (7.9%) as determined spectrophotometrically.

Preparation of ethyl 3-carboxyethyl-2,4-dimethylpyrrole-5-thiolcarboxylate (XXV)

A solution of sodium nitrite (12.7 g.) in water (45 ml.) was added to an ice-cooled, well-stirred solution of ethyl acetothiolacetate (25 g.) in glacial acetic acid (70 ml.) at such a rate that the temperature remained less than 14 °C. The mixture was stirred at this temperature for 3 hours and then stirred overnight at room temperature. After adding ethyl 4-acetyl-5-oxohexanoate (34.4 g.) to the solution, zinc dust (26 g.) was added at such a rate that the temperature remained almost constant at 65 °C. Stirring was continued for another 30 minutes. Following this, the mixture was heated on a steam bath for 1 hour then cooled and finally poured onto crushed ice. After 4 hours, the product was collected and recrystallized from ethanol to give pale yellow crystals. After passing the pale yellow crystals through a silica gel column with chloroform as eluent, two compounds were obtained. The major product was the desired pyrrole derivative (XXV) (identical with an authentic sample), which forms colorless needles (17 g. 35%), m.p. 71.5 - 72.5 °C.

The side-product obtained after recrystallizing from ethanol was found to be diethyl 2,5-dimethylpyrazine-3,6-dithiolcarboxylate (XXVI), which gave yellow needles (610 mg.). m.p. 144 - 145 °C. Ultraviolet spectrum ($CHCl_3$) : λmax 245 and 319 mμ (broad), (log ε max 4.05 and 4.06).

N.M.R. spectrum ($CDCl_3$) : τ 6.92 (quartet, $-SCH_2$), 7.1 (singlet, $-CH_3$), and 8.63 (triplet, thioester CH_3). Mass spectrum : M^+ 284 (6), 256 (23), 224 (44), 196 (48), 195 (100), 108 (30). Anal. Calc'd. for $C_{12}H_{16}N_2O_2S_2$: C, 50.73; H, 5.5; N, 9.77; S, 22.66. Found : C, 50.70; H, 5.63; N, 9.85; S, 22.54.

An attempt to recrystallize the yellow crystals of (XXVI) from hot methanol δ to colorless needles, which were found to be 2,5-dimethyl-3,6-dicarbomethoxypyrazine (XXVII). m.p. 132 - 134°C. Ultraviolet spectrum (CH_3OH) : λ max 221 and 290 mμ (log ε max 4.05 and 4.07 respectively). N.M.R. spectrum ($CDCl_3$) : τ 5.93 (singlet, ester CH_3), 7.14 (singlet, $-CH_3$). Mass spectrum : M^+ 224 (37), 194 (30), 192 (27), 166 (100), 165 (33.5), 164 (45).

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