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Biosynthesis of uroporphyrinogens from porphobilinogen: mechanism and the nature of the process

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The enzymic self-polymerization of porphobilinogen gives rise to the cyclic tetrapyrroles uroporphyrinogen III and uroporphyrinogen I. The former is the precursor of all the natural porphyrins and chlorins. The formation of uroporphyrinogen III is catalysed by a dual enzymic system, porphobilinogen deaminase and uroporphyrinogen III cosynthase. Deaminase polymerizes four porphobilinogen units on the enzymic surface, without liberation of free intermediates into the reaction medium, and forms uroporphyrinogen I. Cosynthase enters into association with the deaminase, and acts as a 'specifier protein' of the latter, changing the mode of porphobilinogen condensation on the enzymic surface. The association is independent of the presence of substrate. While deaminase catalyses the head-to-tail condensation of the porphobilinogen units, the association deaminase-cosynthase catalyses the head-to-head condensation of the same units. As a result different enzyme-bound dipyrrylmethanes are formed from the beginning of the process, and this can be demonstrated by using synthetic dipyrrylmethanes and tripyrranes.

Introduction

Porphyrin derivatives play an important part in the biochemistry of all the living systems. The mere mention of haem, the chlorophylls, the cytochromes, vitamin B_{12}, the prosthetic groups of many haemoproteins, is sufficient to show the deep involvement of porphyrins in all types of metabolic phenomena. It also suggests that porphyrin biosynthesis must be one of the oldest metabolic processes that developed on Earth. As is the case with other large and diversified molecules of living systems (e.g. steroids, alkaloids), porphyrins are built up from very simple molecules. The final structural complexity is produced by the sequential and irreversible self-polymerization of every new intermediate formed, thus giving rise to structures which increase in size and complexity. Finally when the successive polymerization reactions produce a large enough molecule with many potential reactive sites, a series of minor secondary reactions introduce a great variety of structural changes in this intermediate producing the great number of chemical structures suited for the different metabolic needs. These secondary reactions occur without or with little expense of energy. Porphyrin metabolism is schematically depicted in figure 1. The polymerization reactions proceed until uroporphyrinogens are formed. Glycine and succinyl-CoA condense to form δ-aminolaevulinic acid. The condensation of two units of δ-aminolaevulinic acid form porphobilinogen, and the polymerization of four units of porphobilinogen form uroporphyrinogen III and uroporphyrinogen I. Uroporphyrinogen III undergoes a series of structural modifications to afford all the natural porphyrin derivatives (Lascelles 1964) and very likely also cobyrinic acid (Ian Scott, Iagen & Lee 1973). In higher plants δ-aminolaevulinic acid might be originated in a different pathway.

Uroporphyrinogen III is the precursor of all the natural porphyrins, while uroporphyrinogen I was only found in its oxidized form (uropophyrin I) in the excreta of humans and animals under pathological conditions or is an exotic natural product (Bogorad 1960). Uroporphyrinogen I is not an intermediate of the porphyrin biosynthetic pathway.

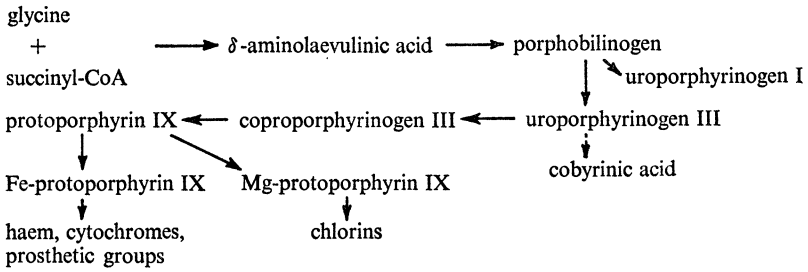
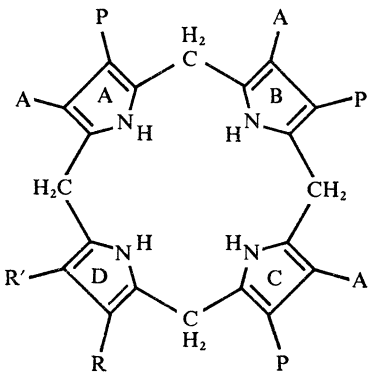


FIGURE 1



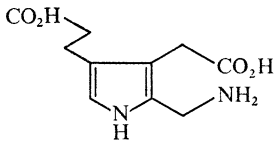
A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

uroporphyrinogen III, R = P; R′ = A

uroporphyrinogen I, R = A; R′ = P

FIGURE 2

Since porphobilinogen **1** is an α-Mannich base it was easy to visualize a sequential head-to-tail condensation of four units of the pyrrole giving rise to a cyclic tetramer where the order of the side chains is that found in uroporphyrinogen I. During the process 4 mol of ammonia have to be released and this was experimentally found to be the case. In uroporphyrinogen III,



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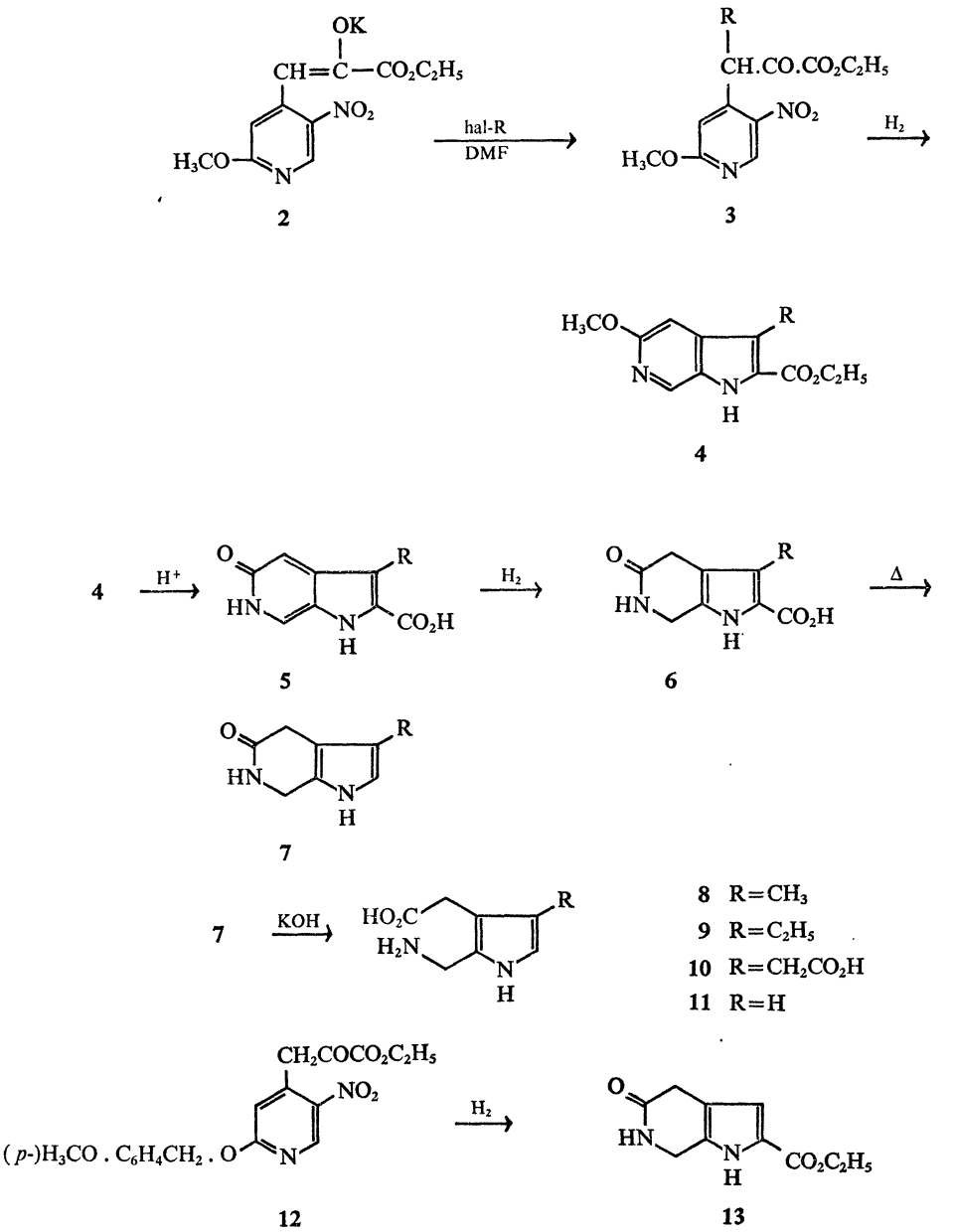
however, there is an inversion in the order of the β-substituents of ring D, and hence the skeleton of a type III porphyrin is present in all the natural porphyrin derivatives. Few biochemical transformations have produced so many hypotheses on the nature of its mechanism, as the inversion process which takes place during the biosynthetic formation of uroporphyrinogen III from porphobilinogen. More than 20 hypotheses have been advanced to explain this reaction

and new ones are constantly being proposed (Margoliash 1961; Mathewson & Corwin 1961; Cornford 1964; Bullock 1965; Dalton & Dougherty 1969; Battersby 1971; Llambias & Battle 1971; Bogorad & Radmer 1972; Whitlock, Whitlock & Alles 1974).

Since porphobilinogen also forms uroporphyrinogens by its chemical polymerization, the problem could be approached by postulating that the enzymic polymerization makes use of this chemical property driving it either to the formation of uroporphyrinogen III or of uroporphyinogen I. If the chemical self-condensation of porphobilinogen takes place by two possible reaction schemes – one potentially leading to the formation of uroporphyrinogen III, and the other to the formation of uroporphyrinogen I – then the enzymic formation of both uroporphyrinogen isomers could be explained in terms of the two reaction schemes.



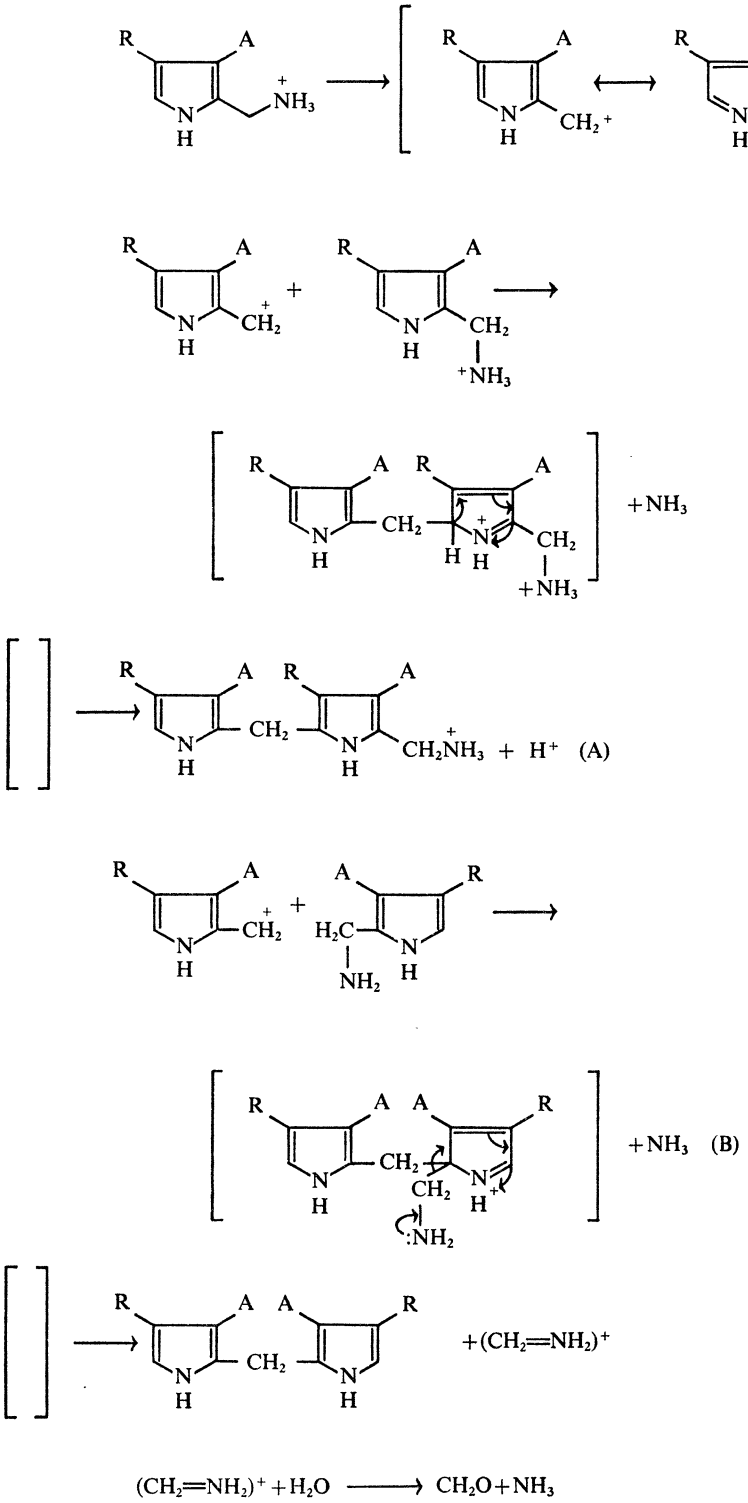
SCHEME 1

The chemical polymerization of porphobilinogen

To examine the structural features which make porphobilinogen **1** self-condense to form uroporphyrinogens, its reactivity had to be compared with a number of synthetic 2-aminomethylpyrroles since there are no natural analogs of **1**. The synthetic pyrroles were obtained by introducing suitable modifications in our synthesis of porphobilinogen from 6-azaindoles (Frydman, Reil, Despuy & Rapoport 1969). Alkylation of the potassium enolates of conveniently protected ethyl 3-nitro-4-pyridinepyruvates **2** with alkyl halides afforded substituted pyridinepyruvates **3** (scheme 1). By reductive cyclization of the latter the 2-carbethoxy-5-methoxy (or 5-benzyloxy)-6-azaindoles **4** were obtained (Frydman, Buldain & Repetto 1973). Cleavage of the ether groups afforded the 6-azaindanones **5**, which were hydrogenated to the 5-carboxypyrrole lactams **6**. The latter were decarboxylated to the pyrrole lactams **7**, which were saponified to the 2-aminomethyl-3-carboxymethylpyrroles **8**–**10** (Frydman, Buldain & Repetto 1973). 2-Aminomethyl-3-carboxymethylpyrrole **11** itself, could be obtained by the catalytic hydrogenation of ethyl 2-anisyl-3-nitro-4-pyridinepyruvate **12**, which was thus transformed in a one-step reaction into the pyrrole lactam **13**. The latter was transesterified to its benzyl ester, and after hydrogenolysis, decarboxylation, and saponification, **11** was obtained in very good overall yields.

The structure of porphobilinogen **1** has several features which could explain its unique reactivity. It is an α-Mannich base of a pyrrole, and as such it reacts by releasing ammonia and giving rise to a reactive positive carbon (a carbonium ion or the equivalent diene) which initiates the polymerization by an electrophilic attack on the C2 or the C5 of a second porphobilinogen unit (scheme 2). The reactions take place by formation of an intermediate pyrrolenine derivative, since electrophilic substitutions on 2-aminomethyl pyrroles and its derivatives produce α-pyrrolenines (Frydman, Reil & Frydman 1971; Chiang & Whipple 1963). Reaction A – attack at the α-free position – will predominate at low pH, since at that pH the 2-aminomethyl groups will be protonated and the elimination of ammonia will be favoured. Reaction B – attack at the α-substituted position – will be favoured at a neutral pH where the enamine-type elimination of the aminomethyl residue at the α-pyrrolenine intermediate step will be possible (scheme 2). The liberated imonium ion $(CH_2{=}NH_2)^+$ will either recondense with the α,α′-free dipyrrylmethane formed in the reaction giving rise to a 2-aminomethyl dipyrrylmethane, or will be hydrolysed to give formaldehyde and ammonia. The formed formaldehyde will also recondense with the α,α′-free dipyrrylmethane and propagate the polymerization reaction. The formed 2-aminomethylpyrromethanes will self-condense to form uroporphyrinogens.

It was found that formaldehyde was released during the chemical polymerization of porphobilinogen, and that the former was incorporated from the reaction medium into the formed uroporphyrinogens (Mauzerall 1960; Frydman, Reil & Frydman 1971). Dimedon, a trapping agent of formaldehyde, inhibited the formation of uroporphyrinogens when added to the reaction mixture. When porphobilinogen was heated under anaerobic conditions at pH 3.5, the pyrrole disappeared and uroporphyrinogens were formed at a slower rate than when the reaction was carried out at pH 7.4 (figures 3, 4). When the reaction was carried out with the 2-aminomethylpyrrole **8**, which is analogous to porphobilinogen **1** but has a stronger electron releasing substituent at C4; it was found that the pyrrole consumption and porphyrinogen formation rates were higher at pH 3.5 than at pH 7.4 (figures 3, 4).



Scheme 2

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When the reaction was carried out with the 2-aminomethyl-3,4-dicarboxymethylpyrrole **10** the effect was reversed, pyrrole consumption and porphyrinogen formation took place at higher rates at neutral pH than at acid pH (figures 3, 4). It is interesting that 2-aminomethyl-3-carboxymethylpyrrole (**11**) itself was very unreactive and formed only traces of porphyrinogens (Frydman *et al.* 1971). These results are in agreement with the reactions outlined in scheme 2.

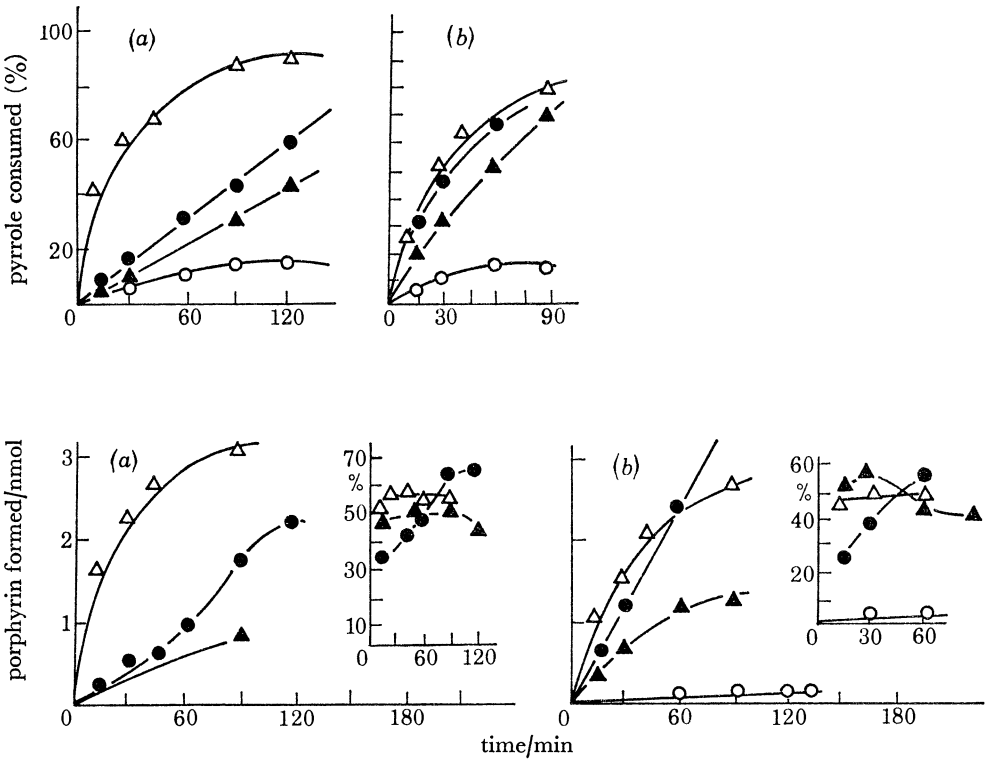


FIGURE 3 (top). Pyrrole consumption rate at 70 °C and (*a*) pH 3.5; (*b*) pH 7.4. Pyrrole concentrations were: ●—●, porphobilinogen, 0.26 mM; △—△, 2-aminomethylpyrrole **8**, 0.27 mM; ▲—▲, 2-aminomethylpyrrole **10**, 0.22 mM; and ○—○, 2-aminomethylpyrrole **11**, 0.34 mM. Percentage of pyrrole consumption was expressed as the percentage of pyrrole consumed of the total amount of pyrrole present at the start of the reaction.

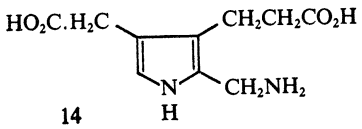
FIGURE 4 (bottom). Rate of porphyrin formation and porphyrin yields. (*a*) pH 3.5; (*b*) pH 7.4. The reaction mixtures and conditions were the same as indicated in figure 3. As 100% porphyrin yield was considered the theoretical amount of porphyrin formed if all the pyrrole consumed would be transformed into porphyrins. ●—●, Porphobilinogen; △—△, 2-aminomethylpyrrole **8**; ▲—▲, 2-aminomethylpyrrole **10**; ○—○, 2-aminomethylpyrrole **11**.

At pH 3.5 reaction A (head-to-tail attack) will predominate and the 2-aminomethylpyrroles having the stronger electron-releasing substituent R at C4 will self-condense at the highest rates (CH_3 > $CH_2CH_2CO_2H$ > CH_2CO_2H ⩾ H). At pH 7.4 reaction B (head-to-head attack) will be the predominant one, and the inductive effect of R will be less important. The Mannich base itself **11** was almost a non-reactive compound. A very strong electron-releasing residue at C4 (R = CH_3) drove the polymerization almost entirely through reaction pathway A. As a consequence, the self-condensation of (**8**) was not inhibited by the addition of dimedon, or activated by the addition of formaldehyde (Frydman *et al.* 1971). The fact that porphobilinogen **1** (and also the 2-aminomethylpyrrole **10**) self-condensed at higher rates at pH 7.4 than at pH 3.5, indicates that reaction B is the preferred one for porphobilinogen polymerization.

Uroporphyrinogen yields increased with the reaction time during the chemical polymerization of porphobilinogen (figure 4, insert), indicating that stable and discrete intermediates were formed during the process. These results were at variance with what was found during the enzymatic polymerization of porphobilinogen, and are of help in understanding this latter process.

The enzymic polymerization of porphobilinogen

Bogorad (1958) isolated from spinach leaves an enzyme – porphobilinogen deaminase – which polymerized 4 mol of porphobilinogen giving rise to the formation of 1 mol of uroporphyrinogen I and of 4 mol of ammonia. From wheat-germ he isolated a second enzyme which he called uroporphyrinogen III isomerase or cosynthetase† and which did not consume porphobilinogen.



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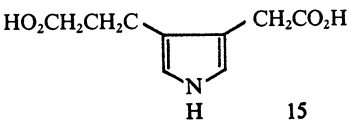

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When added to porphobilinogen deaminase the system formed uroporphyrinogen III when porphobilinogen was consumed (Bogorad 1958). The distribution of both enzymes was found to be ubiquitous in all living systems (Lascelles 1964). Uroporphyrinogen III was not produced when uroporphyrinogen I was incubated with uroporphyrinogen III cosynthase or with a system which catalysed the formation of uroporphyrinogen III from porphobilinogen. Consequently it was clear that uroporphyrinogen I is not a substrate for the cosynthase, which does not open the macrocycle to invert ring D. The cosynthase did not consume porphobilinogen nor transformed it either into isoporphobilinogen (Bogorad 1960) **14**, and the latter was not only not incorporated into urporphyrinogen III (Carpenter & Scott 1959), but exerted an inhibitory effect on the enzymatic system (Frydman & Feinstein 1974). It was impossible to detect an enzymic release of formaldehyde from the 2-aminomethyl group of porphobilinogen (Bogorad & Marks 1960), or an enzymic incorporation of [^{14}C]formaldehyde (Bogorad & Marks 1960) or [^{14}C]opsopyrroledicarboxylic acid **15** (Bogorad 1960) from the reaction medium into uroporphyrinogen III. Hence, the action of cosynthase did not involve the release or recombination of free formaldehyde with any intermediate formed by the deaminase.

The enzymic conversion of porphobilinogen into uroporphyrinogen III must then take place through an intramolecular rearrangement reaction, since no other pyrrole apart from porphobilinogen **1** takes part in the process and no exchange of C_1 fragments with the medium could be detected.

A number of questions had to be solved simultaneously with the answer sought for the side-chain inversion problem in the biosynthesis of uroporphyrinogen III. They are those concerned with the nature of porphobilinogen's polymerization by the deaminase. Is this a non-stop reaction which takes place entirely on the enzymic surface? Is it possible to separate the deaminase in more than one protein, each catalysing a partial polymerization reaction, and thus obtain pyrrylmethane intermediates formed under normal conditions? What can be learned about the interaction of deaminase and cosynthase that could be helpful in understanding the nature of

† Porphobilinogen deaminase is also called uroporphyrinogen I synthetase, while uroporphyrinogen isomerase is also called uroporphyrinogen III cosynthase or cosynthetase. We prefer to use the names porphobilinogen deaminase and uroporphyrinogen III cosynthase which are more appropriate for their mode of action.

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uroporphyrinogen III formation? Since uroporphyrinogen III cosynthase was a heat-labile enzyme while porphobilinogen deaminase was heat-stable, the usual procedure for obtaining deaminase was to heat the enzymatic system at the temperatures needed to destroy the cosynthase (Bogorad 1958), while the cosynthase was obtained from a different source (Bogorad 1958; Levin 1968). A non-destructive separation of the activities from a system containing both enzymes was achieved with extracts from wheat germ (Stevens & Frydman 1968) and from human erythrocytes (Stevens, Frydman & Frydman 1968). This separation allowed an estimation of the relative amounts of both enzymes in the same system, and it was found that the cosynthase was present in a great excess over the deaminase, thus ensuring that uroporphyrinogen III will always be formed under normal physiological conditions. The porphobilinogen deaminase obtained from wheat germ and from human erythrocytes was purified and its properties were studied. The enzyme from both sources had similar properties. It behaved as a single protein of low molecular mass (25000 ± 5000) (Frydman & Frydman 1970; Frydman & Feinstein 1974).

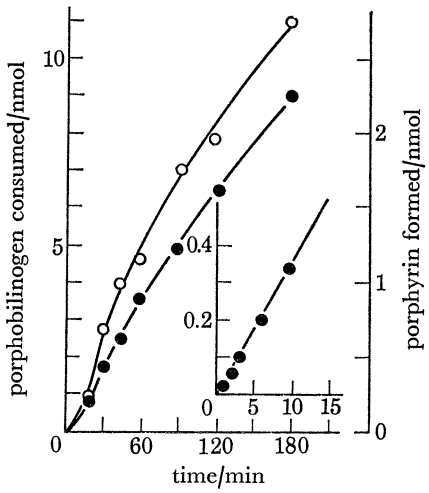


FIGURE 5. Rates of substrate consumption (○—○) and products formation (●—●). Wheat-germ deaminase (DEAE-cellulose purified fraction) was used.

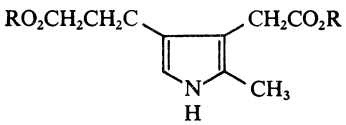
A molecular mass of 36000 was obtained for the deaminase of *Rhodopseudomonas spheroides* (Jordan & Shemin 1973; Davies & Neuberger 1973). Porphobilinogen consuming activity and uroporphyrinogen forming capacity could not be separated by the action of different dissociating agents. In all cases the enzyme behaved as a sole functional unit and was a single active band when examined by polyacrylamide gel electrophoresis (Frydman & Frydman 1970; Frydman & Feinstein 1974). Through all the purification steps the porphobilinogen consuming activity could not be separated from the uroporphyrinogen forming activity. During the enzymatic polymerization no lag in uroporphyrinogen formation was observed, even at the shortest possible times (figure 5).

When these results were compared with the reaction rates obtained during the chemical polymerization of porphobilinogen (figures 3, 4), it was clear that while in the latter case discrete intermediates accumulated, the enzymic reaction took place in a non-stop process on the enzyme's surface.

Some deaminase preparations formed less uroporphyrinogen than expected from the stoichiometric values (Frydman & Frydman 1970; Frydman & Feinstein 1974). This effect was attributed to the presence of two active sites in the deaminase: one to which porphobilinogen

units became bound and where polymerization starts; and a second one more labile, where the final cyclization step takes place. Studies with inhibitors lent support to this proposal (Frydman & Frydman 1970; Stevens *et al.* 1968).

Since porphobilinogen has no natural analogues, the problem of the substrate specificity of deaminase was examined with the help of the synthetic analogues **8**–**11** of porphobilinogen. None of them was found to act as a substrate even at the lowest rate (Frydman & Frydman 1970; Frydman & Feinstein 1974). Opsopyrroledicarboxylic acid **15** was not a substrate of deaminase and inhibited competitively porphobilinogen consumption (Bogorad 1960). The structural requirements of the deaminase substrate seems thus limited to the array of substituents present in the porphobilinogen molecule, since even such a close analogue as 2-aminomethyl-3,4-pyrrolediacetic acid (**10**) was not a substrate of the enzyme. The 2-aminomethylpyrroles exerted, however, an inhibitory effect on the enzyme. Isoporphobilinogen **14** and 2-aminomethyl-3,4-pyrrolediacetic acid **10** inhibited the activity by addition to the incubation mixture, while 2-aminomethyl-3-pyrroleacetic acid **11** and 2-aminomethyl-4-ethyl-3-pyrroleacetic acid **9** exerted their effect only by preincubation. When the deaminase was preincubated with 2-aminomethyl-3-pyrroleacetic acid and the mixture was dialysed to eliminate the pyrrole, the enzyme remained partially inhibited (40 % inhibition), both for porphobilinogen consumption and for porphyrin formation. Thus, the inhibition produced by this pyrrole is irreversible and of a non-competitive nature. The partially inhibited deaminase was more resistant to further inhibition by *p*-hydroxymercuribenzoate and photo-oxidation in the presence of methylene blue, at concentrations and conditions which inhibited the non-treated enzyme (Frydman & Feinstein 1974). It was also more resistent to further inhibition by oxidation with tryptophan pyrrolooxygenase (Frydman & Frydman 1973). 2-Methylopsopyrroledicarboxylic acid



16 R=H

17 R=CH_3

also inhibited the enzymic activity when added to the incubation mixture while its dimethyl ester **17** was devoid of any inhibitory effect (Frydman & Feinstein 1974). It can be concluded that the pyrroles which inhibited the deaminase directly and hence competed with porphobilinogen for the substrate's binding site have all in common two β-acetic acid or propionic acid side chains and are α- or α,α′-free pyrroles. When the propionic acid residue of the porphobilinogen structure was missing or was replaced by an alkyl residue, the inhibitory effect could only be obtained by preincubation in the absence of substrate. Hence only the presence of both acidic side chains conferred to the analogues a sufficient binding rate as to compete with the substrate for the locus on the enzyme.

The second enzyme of the system is uroporphyrinogen III cosynthase. Its study was made difficult by the fact that the cosynthase had no measurable substrate consumption and had to be measured by the amount of uroporphyrinogen III formed. Since deaminase must always be present in the system, uroporphyrinogen I is usually formed in variable amounts and the estimation of uroporphyrinogen III depends on a reliable separation method of both isomers.

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This can only be achieved by oxidizing the uroporphyrinogens to uroporphyrins and by decarboxylating the latter to coproporphyrins in dilute hydrochloric acid at 180 °C (Edmonson & Schwartz 1953). Coproporphyrins I and III had then to be separated either by paper chromatography or by t.l.c. on cellulose (Frydman & Feinstein 1974).

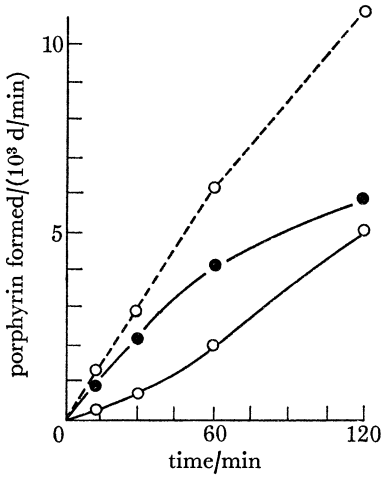


FIGURE 6. Rate of uroporphyrinogen III formation. The enzymes used were a Sephadex G-100 purified human erythrocyte deaminase and a DEAE-cellulose purified cosynthase. [^{14}C]porphobilinogen (9 nmol, 2500 d/min) was used. The uroporphyrinogen formed and the isomer composition were estimated as coproporphyrins. ○—○, Total porphyrin; ●—●, isomer III; ○—○, isomer I.

Cosynthase interacts with deaminase irrespective of the source of both enzymes. Although minor variations could be detected in the properties of the enzymes according to their origin, no fundamental differences were found among them. Kinetic determinations of the cosynthase can only reflect the way in which cosynthase affects the kinetic constants of porphobilinogen deaminase, the substrate consuming enzyme. Deaminase consumed porphobilinogen and formed uroporphyrinogen I following a classical Michaelis–Menten kinetics. The addition of cosynthase did not change this reaction pattern, apart from changing the isomer type formed during the reaction (Frydman & Feinstein 1974). However, in the case of human erythrocyte enzymes the affinity for the substrate of the dual enzymic system increased while the maximum velocity decreased (Frydman & Feinstein 1974). The opposite effects were obtained when wheat germ cosynthase was added to preheated spinach leaf deaminase (Bogorad 1958). These changes in the affinity for the substrate of the dual system reflect either a direct interaction of the cosynthase with porphobilinogen, or a conformational modification of the deaminase produced by its association with the cosynthase. Since it was impossible to demonstrate any direct interaction between cosynthase and porphobilinogen by dialysis equilibrium, the second possibility must be the cause of the aforementioned changes. The existence of this association could also be inferred from an examination of the rates of uroporphyrinogen III and uroporphyrinogen I formation (figure 6). Both isomers seem to share a common intermediate and since this intermediate is not a pyrrylmethane compound (see below), it must be the deaminase-cosynthase association.

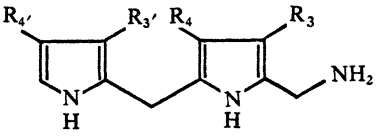
The association is a loose one. It efficiently forms uroporphyrinogen III in short reaction times, but as the reaction time goes by the cosynthase starts to inactivate while deaminase recovers its original activity and uroporphyrinogen I is predominantly formed. An independent proof of the formation of the two-enzyme complex in the absence of any added substrate or

pyrrylmethane derivative can be found working with immobilized enzymes. Deaminase bound to Sepharose was associated with cosynthase when the latter was filtered through the system. The immobilized complex formed uroporphyrinogen III when incubated with porphobilinogen (Frydman & Feinstein 1974). Since deaminase catalyses the polymerization of porphobilinogen in a non-stop reaction without liberation of free intermediates, the association deaminase-cosynthase must carry out the reaction in a similar way. This led us to propose (Frydman *et al.* 1971) for this enzymic complex a model similar to that found in lactose synthetase (Ebner 1970), where cosynthase plays the role of the 'specifier protein' of the system.

Synthesis and chemical polymerization of 2-aminomethylpyrrylmethanes

Since it is not possible to obtain pyrrylmethane intermediates of the enzymic polymerization, the latter had to be prepared by synthesis.

There are four isomeric 2-aminomethyldipyrrylmethanes which can be derived from a formal dimerization of two units of porphobilinogen **1** and isoporphobilinogen **14** by the loss of one mole of ammonia.



18 $R_3=R_{3'}=CH_2CO_2H$; $R_4=R_{4'}=CH_2CH_2CO_2H$

19 $R_3=R_{4'}=CH_2CO_2H$; $R_4=R_{3'}=CH_2CH_2CO_2H$

20 $R_4=R_{3'}=CH_2CO_2H$; $R_3=R_{4'}=CH_2CH_2CO_2H$

21 $R_4=R_{4'}=CH_2CO_2H$; $R_3=R_{3'}=CH_2CH_2CO_2H$

Figure 7

Dipyrrylmethane **18** is the result of the formal head-to-tail condensation of two units of porphobilinogen, while dipyrrylmethane **20** is the result of the formal head-to-head condensation of two units of porphobilinogen followed by a 2-aminomethyl migration. Dipyrrylmethanes **19** and **21** are 'nonsense dipyrrylmethanes' from the biosynthetic standpoint.

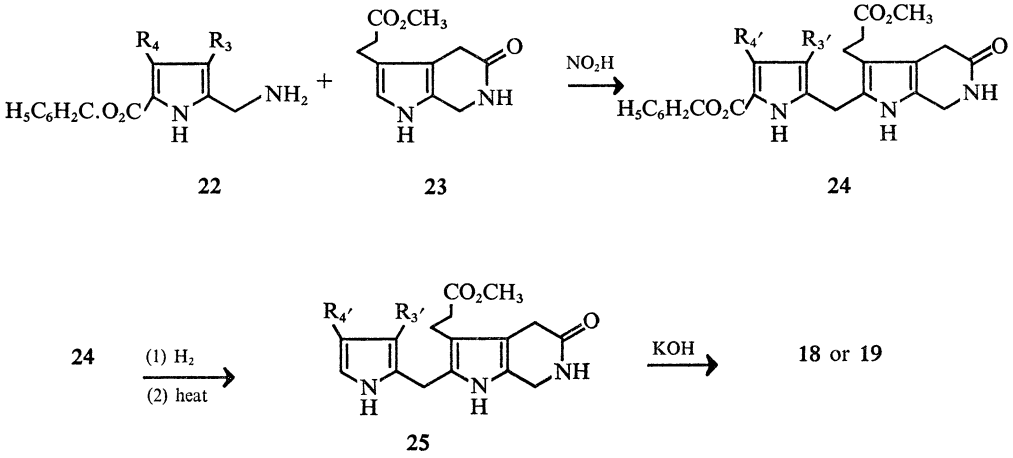
Their synthesis had to be planned with the prior assumption that they would be extremely reactive and would condense rapidly to porphyrins if submitted to extensive manipulation. Thus, it was decided to keep the reactive aminomethyl group blocked until the last step, and then to free it by a mild reaction.

Dipyrrylmethanes **18** and **19** were obtained by condensation of 5-benzyloxycarbonyl-2-aminomethylpyrroles **22** with porphobilinogen lactam methyl ester (**23**) (scheme 3).

The condensation was achieved using nitrous acid. The 5'-benzyloxycarbonyl-dipyrrylmethanes **24** were submitted to hydrogenolysis and decarboxylation, and the resulting dipyrrylmethane lactams **25** were saponified to obtain the dipyrrylmethanes **18** and **19** (Frydman *et al.* 1971). This synthetic outline was also adopted by other research groups (Osgerby *et al.* 1972; Battersby *et al.* 1973) to obtain **18** and **19**

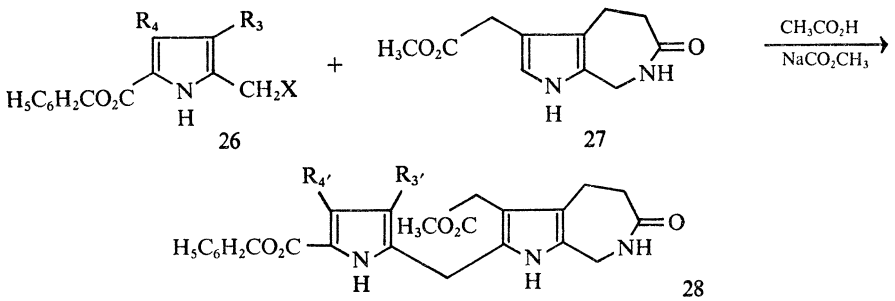
The synthesis of the dipyrrylmethanes **20** and **21** was obtained by a prior condensation of properly protected 2-halomethylpyrroles **26** with the seven-membered pyrrole lactam **27** (scheme 4).

The obtained 5′-benzyloxycarbonyldipyrrylmethane lactams **28** were transformed into the 2-aminomethyldipyrrylmethanes **20** and **21** as described in scheme 3 (Valasinas, Levy & Frydman 1974; Battersby, Beck & McDonald 1974).



R = $CH_2CO_2CH_3$ or $CH_2CH_2CO_2CH_3$

SCHEME 3



X = Cl or Br

R = $CH_2CO_2CH_3$ or $CH_2CH_2CO_2CH_3$

SCHEME 4

The dipyrrylmethanes **18–21** were very similar in their properties. They were amorphous unstable solids, which precipitated from their solutions at pH 3.5, and were readily transformed into porphyrins by handling. They were very stable in aqueous solutions at alkaline pH, but decomposed readily at pH below 6–7. They were colourless substances (when pure), devoid of any absortion in the ultraviolet or visible spectra, and could not be subjected to t.l.c. or paper chromatography in solvents containing traces of acids. They were, however, stable to t.l.c. on cellulose in alkaline medium, and to electrophoretic procedures at pH above 9. The C5′ hydrogen exchanged very rapidly with deuterium at pH 7–8 (Frydman *et al.* 1971), in contrast to the stability of analogous protons in 2-aminomethylpyrroles and to the slow exchange of the

equivalent C5″ hydrogens in the homologous 2-aminomethyltripyrranes. This susceptibility to electrophilic attack could explain the very rapid polymerization of the 2-aminomethyldipyrrylmethanes to uroporphyrinogens (see below).

In figure 8 are depicted the time course of the shift in the absortion maxima of the compounds formed by Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) with the dipyrrylmethane lactam **25** ($R_{3'} = CH_2CO_2CH_3$; $R_{4'} = CH_2CH_2CO_2CH_3$) and the 2-aminomethyldipyrrylmethane **18**. The peak shifted rapidly from 557 nm (λ_{max} of the *p*-dimethylaminobenzyldipyrrylmethane) to 484 nm (λ_{max} of the *p*-dimethylaminobenzyldipyrrylmethene) in the case of the dipyrrylmethane lactam, and more slowly in the case of the open ring derivative. This slow shift is a useful analytical tool to identify the presence of 2-aminomethylpyrrylmethanes in solution.

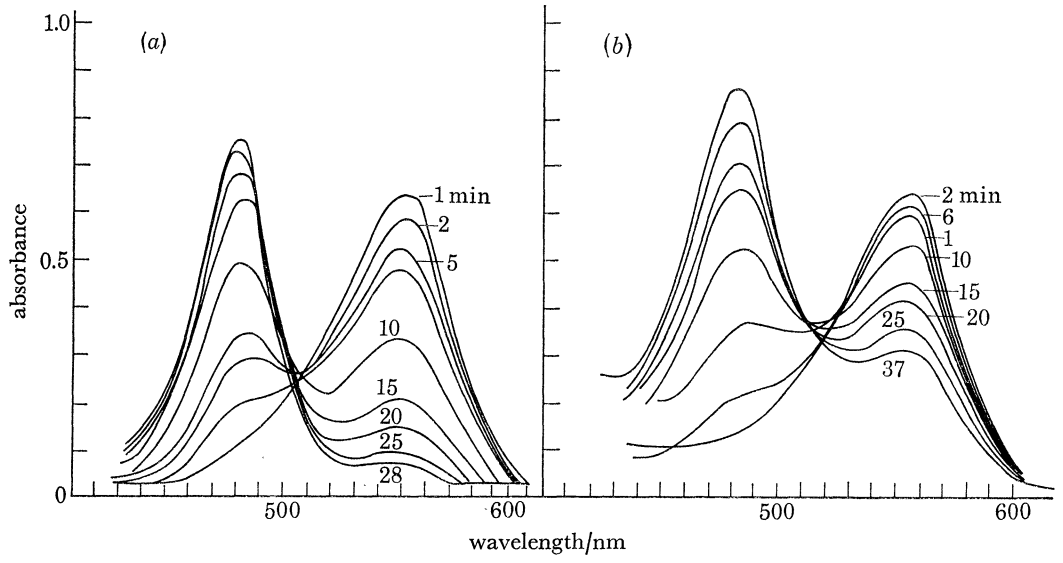


FIGURE 8. Absorption spectra taken at various times after reaction of (*a*) dipyrrylmethane lactam **25** (2×10^{-5} M) and (*b*) 2-aminomethyldipyrrylmethane **18** (1×10^{-4} M) with Ehrlich's reagent.

The synthesis of tripyrranes (pyrrylmethyldipyrrylmethanes) followed a similar pattern to that used for the synthesis of the dipyrrylmethanes. By condensation of 5-benzyloxycarbonyl-2-chloromethylpyrroles **26** with the dipyrrylmethane lactams **29** or **30** in neutral solutions, the corresponding 5″-benzyloxycarbonylpyrrylmethyldipyrrylmethane lactams **31** and **32** were obtained (scheme 5). Cleavage of the benzyloxy groups, thermal decarboxylation of the resulting 5″-carboxytripyrranes, and saponification of the tripyrrane lactams **33** and **34** afforded the 2-aminomethyltripyrranes **35**–**37**.

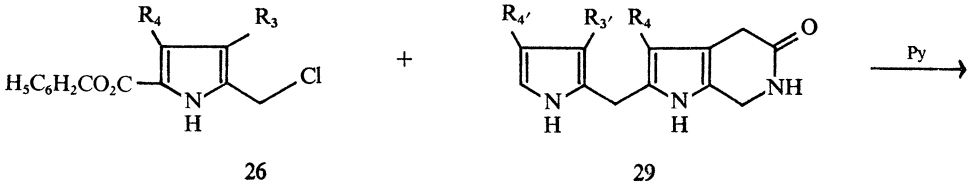
This synthetic method is useful for the synthesis of a large number of isomeric tripyrranes.

Tripyrrane **35** (figure 9) is formally derived from the condensation head-to-tail of three units of porphobilinogen by the loss of two moles of ammonia; tripyrrane **36** is formally derived from the condensation head-to-tail to dipyrrylmethane **20** with a third porphobilinogen unit and tripyrrane **37** has been proposed as an intermediate in the biosynthesis of uroporphyrinogen III (Llambias & Battle 1971).

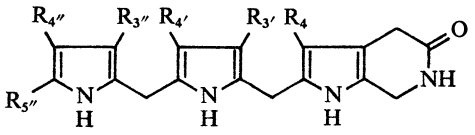
The 2-aminomethyltripyrranes were amorphous and unstable solids, but were relatively stable in alkaline solutions when kept in the cold. They readily decomposed by t.l.c. on paper chromatographic methods, even with neutral or alkaline solvents. They were unstable to thin

layer electrophoresis on cellulose except at high pH. They were devoid of any ultraviolet or visible absorption spectra, but could be identified by their n.m.r. spectra.

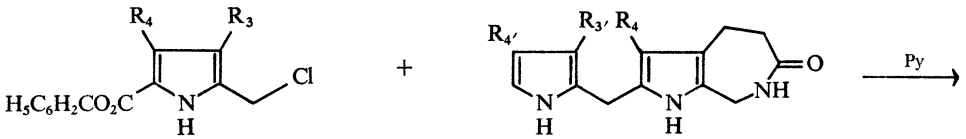
2-Aminomethyldipyrrylmethanes were more unstable than porphobilinogen and dimerized already at 37 °C under conditions similar to those used in the enzymic studies. An analogous sequence to that proposed for porphobilinogen condensation (scheme 2) could be advanced for dipyrrylmethane condensation (scheme 6). The dimerization of dipyrrylmethane **18** could proceed by either a head-to-tail condensation (reaction A), or by a head-to-head condensa-



26 29



31, $R_{5''}=CO_2CH_2C_6H_5$; 33, $R_{5''}=H$



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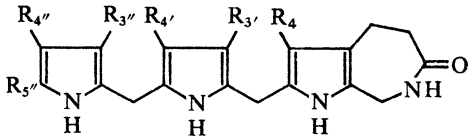
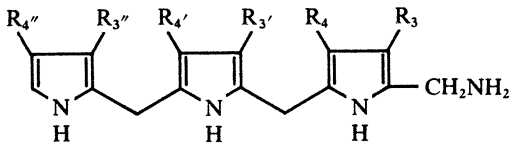


32, $R_5''=CO_2CH_2C_6H_5$; 34, $R_{5''}=H$
$R=CH_2CO_2CH_3$ or $CH_2CH_2CO_2CH_3$

SCHEME 5



35 $R_3=R_{3'}=R_{3''}=CH_2CO_2H$; $R_4=R_{4'}=R_{4''}=CH_2CH_2CO_2H$

36 $R_3=R_{4'}=R_{3''}=CH_2CO_2H$; $R_4=R_{3'}=R_{4''}=CH_2CH_2CO_2H$

37 $R_4=R_{3'}=R_{3''}=CH_2CO_2H$; $R_3=R_{4'}=R_{4''}=CH_2CH_2CO_2H$

FIGURE 9

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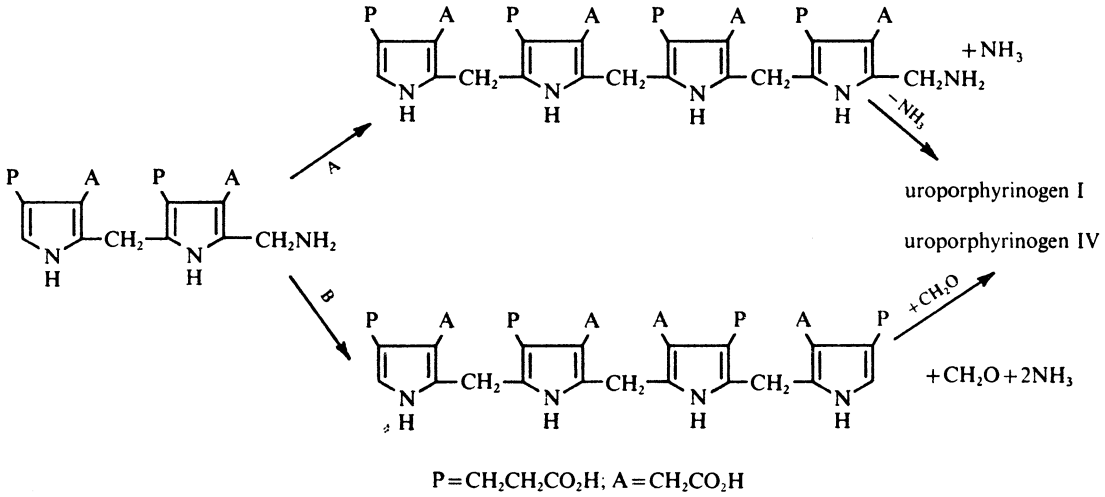

SCHEME 6

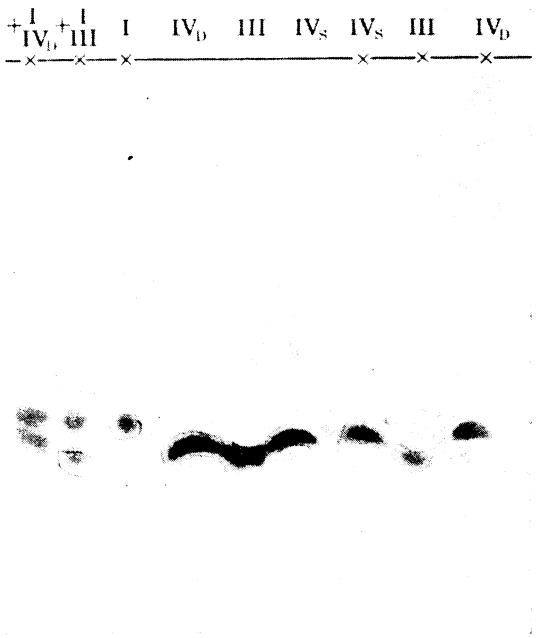

FIGURE 10. Separation by t.l.c.-cellulose of coproporphyrins IV, III and I: III, coproporphyrin III; IV_S, synthetic coproporphyrin IV; IV_D, coproporphyrin IV prepared from dipyrrylmethane **18**; I, coproporphyrin I, I + IV_D, coproporphyrin mixture obtained by chemical dimerization of **18**.

tion (reaction B) followed by a release of formaldehyde and a recombination of the same with the α,α′-free bilane to give the corresponding uroporphyrinogen. The dimerization of dipyrrylmethane **18** will thus afford uroporphyrinogens I and IV, while the dimerization of dipyrrylmethane **19** and **20** will afford only uroporphyrinogen II, and the dimerization of dipyrrylmethane **21** will give rise to uroporphyrinogens I and IV.

It was experimentally found that the chemical dimerization of dipyrrylmethane **18** at 37 °C and pH 7.4 afforded two isomeric uroporphyrinogens which were identified as of types I and IV. This was achieved by decarboxylating both isomers to coproporphyrins and by separation of the isomers on cellulose coated plates at 15 °C (figure 10).

The proportion of both isomers in the reaction mixture (table 1) oscillated with the concentration of the dipyrrylmethane and the temperature of the reaction. The dimerization of the dipyrrylmethanes **19** and **20** at 37 °C and pH 7.4 formed only uroporphyrinogen II (Frydman, Valasinas & Frydman 1973).

TABLE 1. CHEMICAL DIMERIZATION OF DIPYRRLMETHANES

dipyrrylmethane		temperature	uroporphyrinogen formed		isomer composition (%)		
	nmol	°C	nmol	%	I	II	IV
18	50	37	1.48	6	64	—	36
	50	46	2.5	10	63	—	37
	70	37	1.4	4	60	—	40
	100	37	3.5	7	54	—	46
19	20	37	1.06	10.6	—	100	—
20	22·5	37	0.95	8.6	—	100	—
	70	37	1.8	5	—	100	—
	140	37	2.9	4	—	100	—
21	25	37	0.73	6	73	—	27
	25	46	1.43	11.5	74	—	26
	75	37	1.86	5	70	—	30
	75	46	3.3	9	71	—	29
	125	37	3.5	5.5	75	—	25
	125	46	6.3	10	70	—	30

When the 2-aminomethyltripyrranes were heated at 37 °C and pH 7.4 uroporphyrinogens were also formed but with lower yields than obtained with 2-aminomethyldipyrrylmethanes. A similar reaction scheme to the one proposed for the dipyrrylmethane dimerization can be proposed for the tripyrranes, in which the possible hexapyrrylmethanes will be formed instead of the bilanes. They will then cyclize at the thermodynamically favoured tetrapyrrole position to give uroporphyrinogens after cleavage of a dipyrrylmethane segment. Thus, it could be predicted that tripyrrane **35** will form uroporphyrinogens I and III and tripyrrane **36** will form uroporphyrinogens III and II. It was found that tripyrrane **35** formed 50–65 % of uroporphyrinogen I and 35–50 % of uroporphyrinogen III, and that tripyrrane **36** formed 70 % of uroporphyrinogen III and 30 % of uroporphyrinogen II.

2-AMINOMETHYLPYRRYLMETHANES AS INTERMEDIATES IN THE ENZYMIC POLYMERIZATION

The possibility of using synthetic 2-aminomethylpyrrylmethanes as intermediates to study this biosynthetic process deserves a prior comment. When the biosynthesis of natural products which are formed by unknown enzymic reactions are studied, it is customary to use whole systems and to examine the preferential incorporation of the possible precursors into the final product. In the biosynthesis of porphyrins however, the metabolic pathway has already been firmly established, the enzymes involved are known and can be isolated and their properties studied. In this case if synthetic intermediates are used with whole systems and their incorporation into the final product is measured, the obtained results must fall in line with what is known or can be learned about the properties of the enzyme involved at that stage to have any biological

significance. Hence, we preferred to examine the interaction of any synthetic possible intermediates directly with the enzymes involved in the reaction under study instead of looking for their incorporation into the final products of the pathway.

The use of pyrrylmethanes in the study of this reaction mechanism is based on the prior assumption that they are equal or similar to the enzyme-bound intermediates formed in the process. The possibility then exists that if the natural enzyme-pyrrylmethane association is very stable, no exchange with the reaction medium will take place and no incorporation into the porphyrinogens of an external added pyrrylmethane will be observed. An alternative possibility is that the added pyrrylmethane will bind to the enzyme competing with porphobilinogen for its binding site. In this case it must be expected that the enzyme will have a much greater affinity for the natural substrate than for the added external polymers and the incorporation of the latter will necessarily be low. However, if the enzymic polymerization takes place in various steps and the product of the first step is the substrate of the second one, then added free dimer or trimer identical with the natural ones will be incorporated in a higher proportion than the starting monomer into the final tetramer (uroporphyrinogen).

Enzymes from different sources were used during the studies with the pyrrylmethanes, and no fundamental differences were found in their behaviour. The dipyrrylmethanes **18**–**21** were not substrates of either porphobilinogen deaminase, uroporphyrinogen III cosynthase or the combined enzymic system (Frydman *et al.* 1971; Frydman *et al.* 1973; Frydman, Valasinas, Rapoport & Frydman 1972). The only uroporphyrinogens formed during the incubations were those originated by the chemical dimerizations of the dipyrrylmethanes described above. Hence, neither deaminase nor cosynthase are dimerizing enzymes and do not form uroporphyrinogens by polymerizing two dipyrrylmethane units. When the dipyrrylmethanes were incubated together with porphobilinogen in the presence of cosynthase, no enzymatic uroporphyrinogen formation could be detected (Frydman *et al.* 1971; Frydman *et al.* 1973; Frydman *et al.* 1972). This discarded the proposals (see Margoliash 1961; Cornford 1964; Llambias & Battle 1971) that the deaminase formed dipyrrylmethanes which could serve as further substrates of the cosynthase. When dipyrrylmethane **18** was added to an enzymic system which formed uroporphyrinogens it inhibited porphobilinogen consumption and increased the porphyrin yields, after the corrections were made for the chemical blanks (Frydman *et al.* 1971). This indicated that the dipyrrylmethane was incorporated into the uroporphyrinogens. A detailed study was then performed in the presence of [^{14}C]porphobilinogen and it was found that dipyrrylmethane **18** was incorporated in low yields into uroporphyrinogen I (about 10 % of the total uroporphyrinogen I formed), when incubated either with deaminase or with deaminase-cosynthase (Frydman *et al.* 1971). No incorporation into uroporphyrinogen III was detected. Those results were confirmed by using [^{14}C]dipyrrylmethane **18** and [^{12}C]porphobilinogen (Frydman *et al.* 1973). The dipyrrylmethane also exerted a strong inhibitory effect on the enzymic formation of uroporphyrinogen III, which was simultaneous with an increase in the enzymic formation of uroporphyrinogen I (Frydman *et al.* 1973). This effect was not enhanced by a previous incubation of the cosynthase and the dipyrrylmethane, suggesting that the inhibition in the formation of uroporphyrinogen III is due to an interference of the dipyrrylmethane with the deaminase-cosynthase association. These results indicated that the polymerization catalysed by the deaminase proceeds by a sequential addition of porphobilinogen units, since the latter must be added to the dipyrrylmethane to complete the reaction toward uroporphyrinogen formation. Pluscec & Bogorad (1970) also found that dipyrrylmethane **18** was incorporated into uroporphyrinogen I in the presence

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of porphobilinogen and deaminase, but the high incorporation values reported are at variance with our findings.

When the dipyrrylmethane **18** was incubated with haemolysed duck erythrocytes which formed protoporphyrin IX from porphobilinogen it was found that a protoporphyrin was formed at expense of the dipyrrylmethane. This was due to the action of the decarboxylating enzymes present in the system on the uroporphyrinogen IV formed during the chemical dimerization of the dipyrrylmethane (table 2).

TABLE 2. TRANSFORMATION OF DIPYRRLMETHANE **18** INTO A PROTOPORPHYRIN BY DUCK'S BLOOD

system	addition	uroporphyrin formed nmol	coproporphyrin formed nmol	protoporphyrin formed nmol
porphobilinogen	—	—	—	—
porphobilinogen + duck's blood enzyme	—	—	0.1	0.72
porphobilinogen + duck's blood enzyme	$HONH_2$ (50 mM)	0.16	0.8	—
porphobilinogen + duck's blood enzyme†	—	0.25	0.8	0.47
dipyrrylmethane (25 nmol)	—	1.3	—	—
dipyrrylmethane + duck's blood enzyme	—	0.16	0.37	0.48
dipyrrylmethane	$HONH_2$ (50 mM)	0.67	—	—
dipyrrylmethane + duck's blood enzyme	$HONH_2$ (50 mM)	0.16	0.24	0.2
dipyrrylmethane†	—	2.1	—	—
dipyrrylmethane + duck's blood enzyme†	—	0.48	0.48	0.69

† The incubations were carried out at 49 °C.

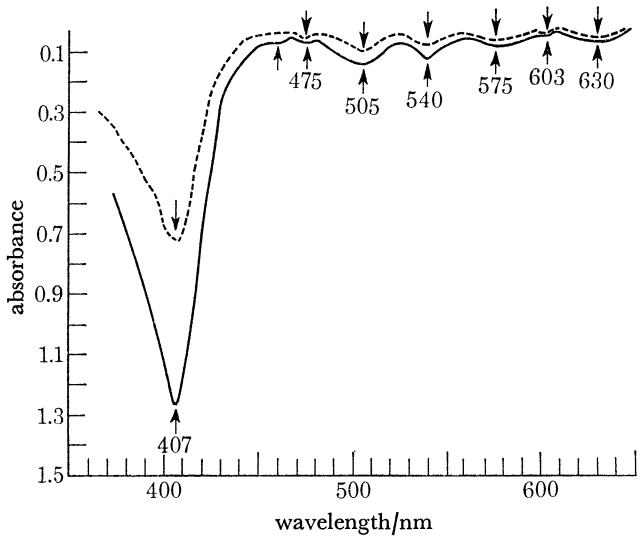


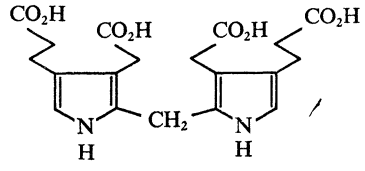
FIGURE 11. Absorption spectra (Cl_3CH) (——) of protoporphyrin IX and (- - -) protoporphyrin obtained from coproporphyrinogen IV.

Under this condition a protoporphyrin is obtained which is identical in its chromatographic and spectral properties (figure 11) with protoporphyrin IX. The same protoporphyrin was obtained when the enzymic system was incubated with coproporphyrinogen IV, where 52 % of the latter was consumed as compared with a 32 % consumption of coproporphyrinogen III.

The interaction of dipyrrylmethane **18** and the enzymic system afforded a number of clues to understand its mechanism. The obtained incorporation into uroporphyrinogen I indicates that the dipyrrylmethane is an intermediate of the process. The low values obtained fall in line with the properties of the deaminase described above, and with our early proposal (Frydman & Frydman 1970; Frydman *et al.* 1971) that the condensation of porphobilinogen must take place on the enzyme's surface. No free pyrrylmethanes are liberated at any stage into the solution. An externally added dipyrrylmethane competed very unfavourably with the intermediate bound to the enzyme. It was clear that if dipyrrylmethane **18** would be liberated into the reaction medium under normal conditions and then be reused again by the enzyme in the following stage, its incorporation into uroporphyrinogen I would be more favoured than the incorporation of porphobilinogen itself.

The properties of the enzymes and the lack of incorporation of dipyrrylmethane **18** into uroporphyrinogen III lead us to suggest (Frydman *et al.* 1971) that the cosynthase forms an association with the deaminase which changes the mechanism of porphobilinogen condensation on the deaminase surface. The obvious conclusion was that a different dipyrrylmethane must be the first intermediate when the polymerization was catalysed by the deaminase-cosynthase system. When dipyrrylmethane **20** – formally derived from a head-to-head condensation of two porphobilinogen units – was added to the enzymatic system in the presence of porphobilinogen, it was found to be incorporated only into uroporphyrinogen III and not into uroporphyrinogen I (Frydman *et al.* 1972). The incorporation values were low and of the same order of magnitude as for dipyrrylmethane **18**. The incorporation of dipyrrylmethane **20** into uroporphyrinogen III agree with results obtained by studying the incorporation of double-labelled [2,11-^{13}C]porphobilinogen into protoporphyrin IX (Battersby, Hunt & McDonald 1973).

Dipyrrylmethane **19** was not incorporated into either isomer and it did not affect the distribution of isomers produced by the enzymes (Frydman *et al.* 1971; Frydman *et al.* 1973). Dipyrrylmethane **21** was not incorporated into any isomer either. It exerted an inhibitory effect on the formation of uroporphyrinogen III which was not reflected in an increase in the formation of uroporphyrinogen I. When dipyrrylmethane **38** was incubated with a uroporphyrinogen III forming system in the presence of porphobilinogen it exerted a strong inhibitory effect on the formation of isomer III, and was not incorporated into either isomer. This discards the possibility that during the enzymatic formation of uroporphyrinogen III the 2-aminomethyl group is entirely detached from the pyrrylmethane unit.



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Tripyrranes always played an important part in all the mechanistic speculations about uroporphyrinogen III biosynthesis. This was also favoured by the fact that they were rather exotic compounds from the synthetic standpoint and not readily available for testing. We

examined first the interaction between tripyrrane **35** and the enzymatic system, since this tripyrrane was repeatedly proposed as an intermediate in uroporphyrinogen III biosynthesis. Tripyrrane **35** was not a substrate for cosynthase either alone or in the presence of porphobilinogen (Frydman, Valasinas, Levy & Frydman 1974). When a small amount of deaminase was added to the reaction mixture to trigger the action of cosynthase, the tripyrrane **35** was not only not incorporated into uroporphyrinogen III, but exerted an inhibitory effect on the formation of that isomer. Addition of tripyrrane **35** to an enzymic system forming both isomers inhibited the formation of total uroporphyrinogen. A stronger inhibition was exerted on the formation of uroporphyrinogen III than on the total formation of uroporphyrinogens (figure 12*a*). As a result a net increase in the proportion of uroporphyrinogen I present in the reaction products was observed.

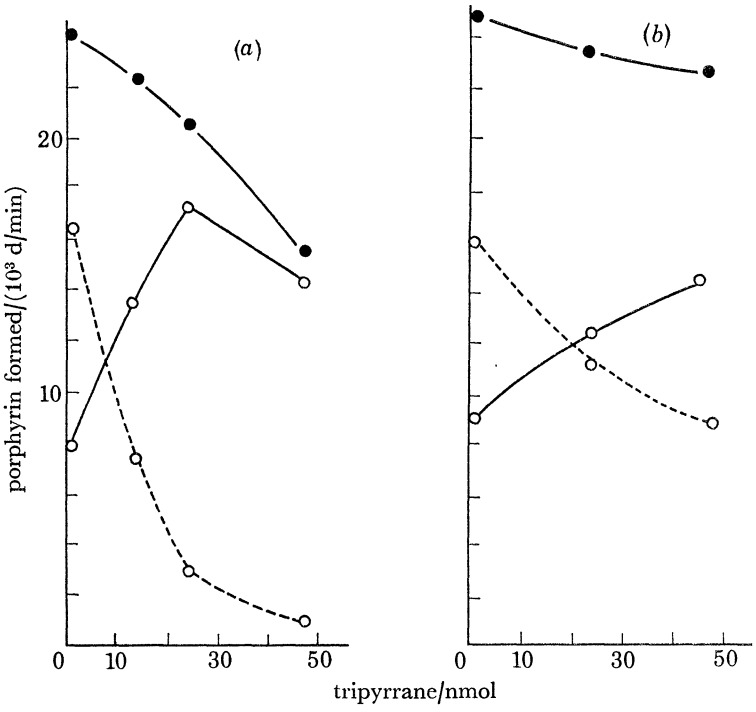

FIGURE 12. Effect of (*a*) tripyrrane **35** and (*b*) tripyrrane **36** on the enzymic formation of uroporphyrinogen I and uroporphyrinogen III. ●—●, Total porphyrin; ○—○, uroporphyrinogen I; ○- - -○, uroporphyrinogen III.

By using a system which formed only uroporphyrinogen I from porphobilinogen and by adding to it tripyrrane **35** in a great excess over the stoichiometric values, a small (2–8 %) but significant enzymic incorporation of tripyrrane **35** was obtained (Frydman *et al.* 1974). When the enzymic system formed both isomers, tripyrrane **35** was incorporated to a small extent only into isomer I (table 3). The concentrations of tripyrrane **35** needed to obtain a significant incorporation into uroporphyrinogen I inhibited the formation of uroporphyrinogen III to such an extent that it was impossible to measure its incorporation into this last isomer. It was clear that any incorporation of this sort has to be discarded.

The results obtained with tripyrrane **35** are consistent with the general mechanism outlined for the deaminase-cosynthase system as well as with the results obtained with dipyrrylmethane **18**. The larger the intermediate, the less is it incorporated into a normally non-stop sequence. Its

strong inhibitory effect on isomer III is similar to the effect of dipyrrylmethane **18** and no evidence was found of its incorporation into this isomer. It was not a substrate for cosynthase either and all the hypotheses in this sense can be discarded.

The biosynthetic pathway toward uroporphyrinogen III can proceed onwards from the formation of dipyrrylmethane **20** (or its enzyme-bound analogue), by two different sequences.

TABLE 3. EFFECT OF TRIPYRRANE **35** ON THE ENZYMIC FORMATION OF UROPORPHYRINOGENS III AND I

		isomers formed						
		I				III		
system†	tripyrrane added nmol	nmol	count/min	molar act. (count/min)/nmol	Δ	nmol	count/min	molar act. (count/min)/nmol
incubated		0.235	10400	44500		0.305	13350	43700
incubated‡	21.5	0.43	1700	39500	3000	0.072	3200	45000
control§	21.5	0.26	11000	42500		—	—	—
incubated	43	0.475	14305	30000	5000	0.014	650	45500
control	43	0.315	10600	35000		—	—	—

† Wheat germ deaminase–cosynthase. Porphobilinogen (9 nmol, molar act. 11000 (count/min)/nmol) was used.
‡ Controls corrected for isomer III inhibition were deducted for estimation of that isomer.
§ The incubated system was mixed with a blank to account for the chemical polymerization of **34**.

They will differ depending on if the third porphobilinogen unit became attached by its α-free position affording an enzyme-bound tripyrrane **36**, or if the new porphobilinogen unit attaches through its occupied α-position affording a different tripyrrane (figure 13). When the interaction of tripyrrane **36** with the enzymes was examined, it was found that it did not behave as a substrate of the deaminase, of the cosynthase, or of the deaminase-cosynthase complex, either alone or in the presence of porphobilinogen. In the presence of tripyrrane **36** no more uroporphyrinogen was formed by the enzymic system than that formed in its absence, after the amount of porphobilinogen contributed by the chemical polymerization of **36** was deducted. On the contrary, the addition of tripyrrane **36** had an inhibitory effect on the formation of total uroporphyrinogens (figure 12*b*) and specially on the formation of uroporphyrinogen III. This last inhibition was less pronounced that the one exerted by tripyrrane **35**. Tripyrrane **36** was not incorporated into uroporphyrinogen I either.

CONCLUSIONS

From the studies of the properties of the enzymes involved, as well as from the results obtained by examining the interactions among the synthetic 2-aminopyrrylmethanes and the enzymes, a mechanism can be outlined which should define the nature of the biosynthetic process leading to the formation of uroporphyrinogens from porphobilinogen. The mechanism must be based first on the evidence that under normal conditions the reaction takes place through a 'zipping up' process on the enzyme's surface. The next basic tenet must be that cosynthase acts as a 'specifier protein' of deaminase changing the mode of porphobilinogen condensation from the beginning of the reaction and steering it toward the formation of uroporphyrinogen III. The obvious conclusion is, then, that there are no pyrrylmethane intermediates common to both uroporphyrinogens I and III. The formation of both isomers by the two different enzymatic

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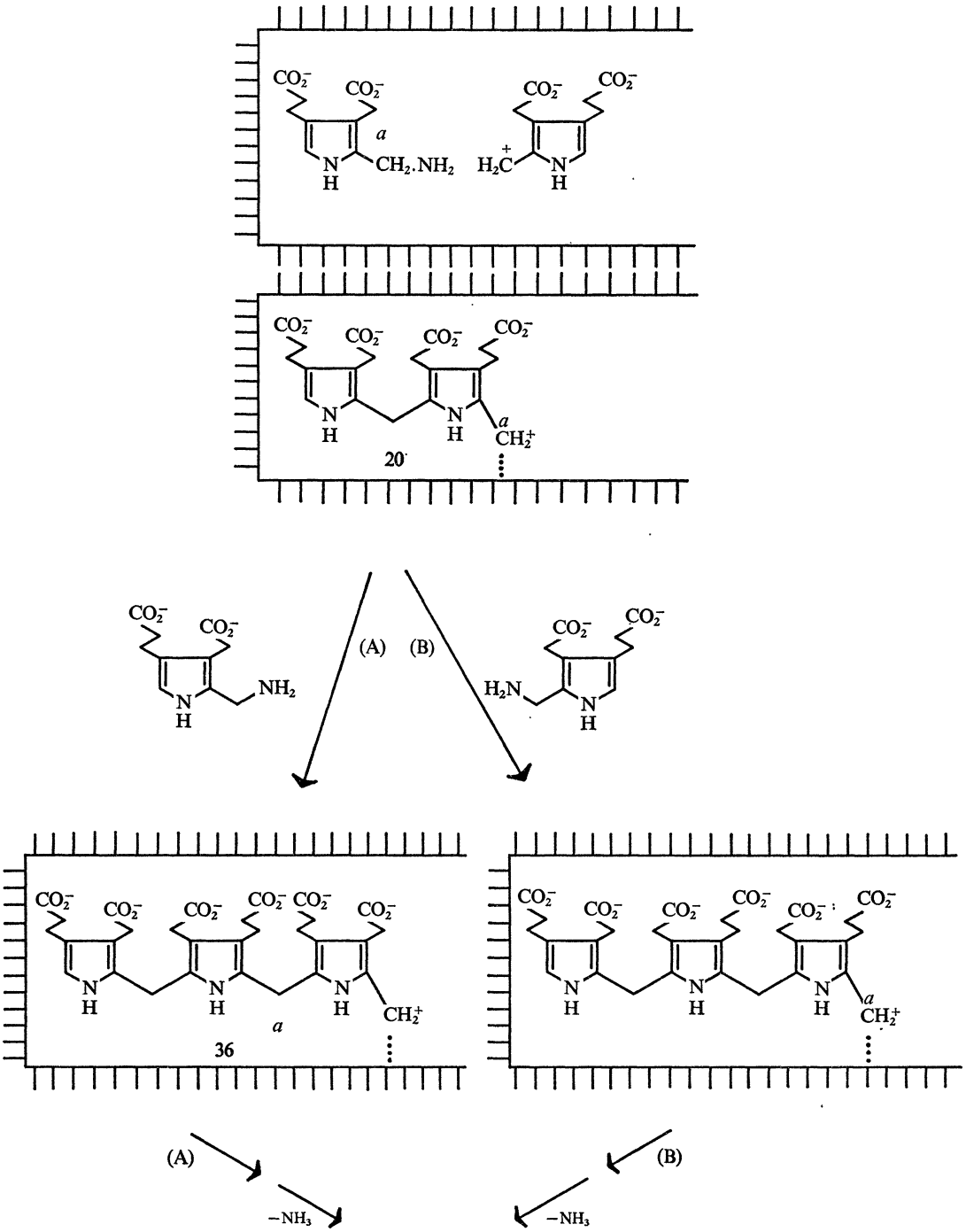

FIGURE 13. The schematic biosynthetic pathway to uroporphyrinogen III. The head-to-head condensation of two porphobilinogen units by the deaminas–ecosynthase association gives rise to an enzyme-bound dipyrrylmethane **20** residue. The 'active methyl' group *a* of the first unit is transferred and remains either covalently to the dipyrrylmethane, or in a partial equilibrium with the protein. In the next steps the third and fourth units may attack (A) either through the α-free position, or (B) through the α-occupied position, producing a constant transfer of the initial 'active methyl' group *a*.

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systems (deaminase and deaminase-cosynthase) represent both ways by which porphobilinogen starts its chemical self-condensation (scheme 2); a head-to-tail condensation by elimination of ammonia, or a head-to-head condensation by displacement of an activated methyl group (figure 13). This 'active methyl' group which in the chemical polymerization of porphobilinogen is liberated as formaldehyde must be constantly transferred through the growing pyrrylmethane chain until the ultimate formation of the macrocycle. The chemical driving force for this transfer might be the presence of the propionic acid residue in the β-position above the methyl or aminomethyl group. The stronger electron-releasing inductive effect of the propionic acid residue – as compared to an acetic acid residue – will favour the electrophilic attack at the occupied α-position by the incoming porphobilinogen units and generate a continuous displacement of an 'active' or positively charged methyl group. Hence, while porphobilinogen deaminase function only as a deaminating enzyme, the deaminase-cosynthase complex must also be a methyl-transferase type of enzyme. The intimate and detailed mechanism of the reaction must still await further studies with new synthetic pyrrylmethane intermediates as well as with the enzymes itself.

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