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APPLICATION OF CMR SPECTROSCOPY TO THE STUDY OF PORPHYRIN AND CORRIN BIOSYNTHESIS IN VITRO AND IN VIVO

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Abstract — The pathway of porphyrin and corrin biosynthesis has been studied by application of CMR spectroscopy using whole cells, cell-free extracts and purified enzymes (porphobilinogen deaminase, uro'gen III cosynthetase). Methods for following the course of these biochemical processes in the NMR tube have been developed in conjunction with isolation, structural and stereochemical studies. Several intermediates in corrin biosynthesis which belong to the isobacteriochlorin family have been discovered and their structures proved by spectroscopic analysis and biotransformation to corrins. Proposals for the unknown segment of the porphyrinogen-corrin conversion have been made.

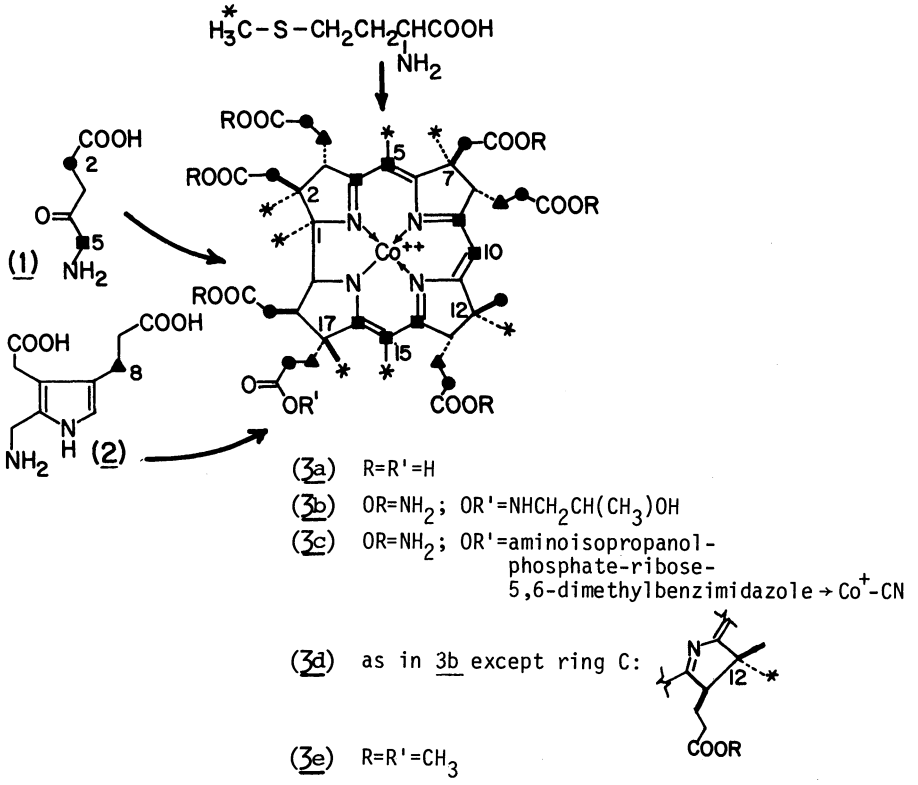
INTRODUCTION

The discovery (Ref. 1) that corrins, like heme and the chlorophylls, are formed in Nature from δ-aminolevulinic acid (ALA, 1) and, by implication, porphobilinogen (PBG, 2) was made over twenty years ago (Ref. 2). Cobyrinic acid (3a), the "simplest" natural corrin, is the precursor of the vitamin (as cyanocobalamin 3c) and its coenzyme (Ref. 3a). The biosynthesis of vitamin B_{12} which has evolved as a unique, dynamic function of the cell, presents a challenge which finds analogy in the discovery of an ancient monument built from familiar materials but by unknown techniques of construction. We shall see that a careful reconstruction of the design and execution of each stage leads to the conclusion that certain structural features of the corrin nucleus have been removed and later restored in a different style.

With the knowledge that the corrin nucleus is constituted from the building blocks of ALA, methionine and cobalt ion (Ref. 3b), we began, in 1970, the experiments necessary for the observation of regiospecifically enriched carbons in the CMR spectra of corrins derived from Propionibacterium shermanii. We first review experiments designed to establish both the number and the mode of insertion of the methionine-derived methyl groups and of the ALA molecule into corrin as a background for the development of the enzymology of cobyrinic acid synthesis, including a brief glimpse of some novel probes for the enzymes at work on porphyrin and corrin construction *in vitro* and *in vivo*. Finally, the isolation and structures of new intermediates and their implication for the mechanism of B_{12} formation will be described.

THE CARBON BALANCE AND THE ORIGIN OF THE METHYL GROUPS IN VITAMIN B_{12}

Administration of [2-^{13}C]-ALA to *P. shermanii* (ATCC 9614) afforded a sample of cyanocobalamin (3c) in which eight high-field signals in the CH_2 and CH_3 region were enriched (Ref. 4a-c) in the proton noise-decoupled ^{13}C FT-NMR spectrum. Assignments of the eight ^{13}C resonances were made to the seven CH_2CONH_2 methylenes and one of the gem-dimethyl groups of ring C in full accord with earlier ^{14}C studies (see Fig. 1 and Table 1). A sample of B_{12} enriched by feeding [5-^{13}C]-ALA provided the surprising result that, of the eight anticipated enriched carbons, only seven signals appeared in the low field region associated with sp^2 (C = C and C = N) functions. The distribution of label is illustrated in Fig. 1. Such an array is in harmony with current ideas on the mechanism of type III uro'gen formation (*vide infra*) and this result (Ref. 4) was simultaneously and independently confirmed in Shemin's laboratory (Ref. 5). However, there was no ^{13}C-enhanced signal above 95 ppm downfield from HMDS showing that no enrichment of the C-1 methyl occurred. This indicates that one of the $^{13}CH_2NH_2$ termini of ALA (and hence of PBG or uro'gen III) has been extruded in the formation of the vitamin. The origin of the "missing" C-1 methyl group turned out to be methionine (Ref. 4a). The ^{13}C FT spectrum of dicyanocobalamin obtained by feeding [$^{13}CH_3$]-methionine revealed seven signals highly enriched above natural abundance (C* in Fig. 1) between 15 and 23 ppm (Table 1), a result which was to receive confirmation from the work of the Cambridge group (Ref. 6) in which the use of cobester (3e) simplified the CMR analysis.



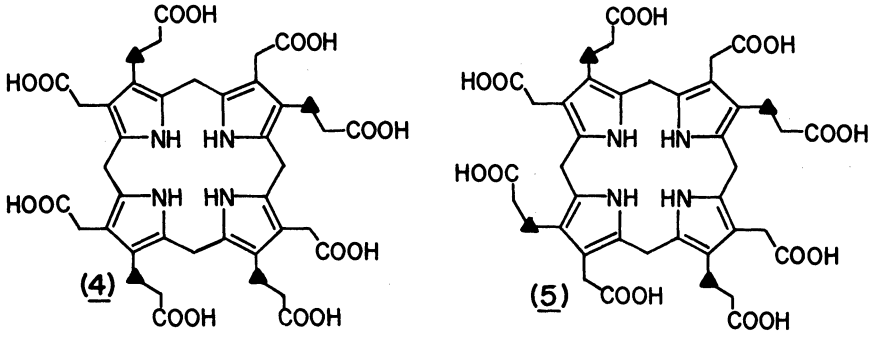


Fig. 1. Labeling of corrins from ^{13}C-enriched ALA (● ■), PBG (▲) and methionine (*).

TABLE 1. ^{13}C Chemical shifts[a] for cyanocobalamin and dicyanocobalamin with proton decoupling (δ from HMDS) based on enrichment data for 26 carbon atoms

Enriched position[†]		Cyanocobalamin	Dicyanocobalamin
C Methyl at C_{12} (pro-S)	●	32.4	31.6
C Methyl at C_{12} (pro-R)	*	20.1	19.6
C Methyl at C_5 and C_{15}	*	16.2, 16.5	15.8, 16.3
C Methyl at C_1, C_2, C_7, C_{17}	*	16.9, 17.9 20.5, 20.4	17.5, 18.4 19.7, 22.7
Propionamide α-methylenes ($\underline{CH_2}CONH_2$) at C_3, C_8, C_{13}, C_{17}	●	32.4, 32.8, 33.6 35.7 or 36.0	32.9, 33.0 33.2, 33.5
Acetamide α-methylenes ($\underline{CH_2}CONH_2$) at C_{18}, C_2, C_7	●	35.7 or 36.0 43.9, 44.1	36.1 43.3, 44.9
Propionamide β-methylenes ($\underline{CH_2}CH_2CONH_2$) at C_3, C_8, C_{13}	▲	27.0(2C), 29.1	25.9, 26.8, 27.8
Propionamide β-methylene at C_{17}	▲	33.3	34.1
C_5	■	108.6	106.3
C_{10}	■	96.0	92.2
C_{15}	■	105.2	104.2
C_4, C_{16}	■	180.1, 181.1	179.6, 179.7
C_9	■	174.6	173.1
C_{14}	■	166.4	164.1

[a] In ppm (± 0.2 ppm). These values correct earlier data (Ref. 4) which contain a computer-derived error. The assignments are unchanged.

[†] See Fig. 1 for a summary of the enrichment scheme and the numbering system.

It was next established (Ref. 7-11) that the [$^{13}CH_3$]-methionine methyl (*) is inserted into the corrin template at C-12 from the α-face and that the absolute configuration at C-12 is (R). Furthermore, the ^{13}C results rationalize the apparent anomaly (observed previously) that the β-methyl group (Ref. 4) of the gem-dimethyl grouping at C-12, derived from C-2 of ALA (1), resonates at substantially lower field (31.6 ppm) than the methyl region tentatively assigned by Doddrell and Allerhand (10). At this stage of the investigation (1972-1973) the technique of cell-free corrin biosynthesis with P. shermanii was developed at Yale (Ref. 12) and at Cambridge (Ref. 13). The supernatant fraction (100,000 g) with appropriate additives (Ref. 12 & 13) is capable of transforming ALA, methionine, PBG and, as described below, uro'gen III (Ref. 14) to cobyrinic acid (3a). A similar system from Clostridium tetanomorphum which makes cobyrinic acid, but not heme, was developed simultaneously by Müller (15).

FORMATION OF URO'GEN III AND ITS ROLE IN CORRIN BIOSYNTHESIS

With the establishment of proper pH control and feeding conditions, we found reproducible conversion of [8-^{13}C]-PBG and of the chemically synthesized type I-IV uro'gen mixture labeled in the propionate side chains to specimens of dicyanocobalamin whose ^{13}C-NMR enrichments [four carbons in each case at 25.9, 26.8, 27.8 and 34.1 ppm] occurred with identical chemical shift (Fig. 1, Table 1). Since uro'gen I (5) had been shown to be quite ineffective in labeling B_{12}, the conclusion seemed inescapable — uro'gen III must be the precursor of vitamin B_{12} (Ref. 4). This finding restored considerable confidence and led to confirmation by a further set of ^{13}C labels inserted into uro'gen III by total regiospecific synthesis at C-5 and C-15 (Yale) and of ^{14}C at CH_2CO_2H in ring C (Cambridge), and the appearance of enrichments at C-5, C-15 (108.4 and 105.2 ppm) in the cyanocobalamin derived from whole-cell feeding of these precursors and of radioactivity at C-12β (●) in cobester as shown in Fig. 2 (Ref. 16), results which were also confirmed at Stuttgart (Ref. 15).

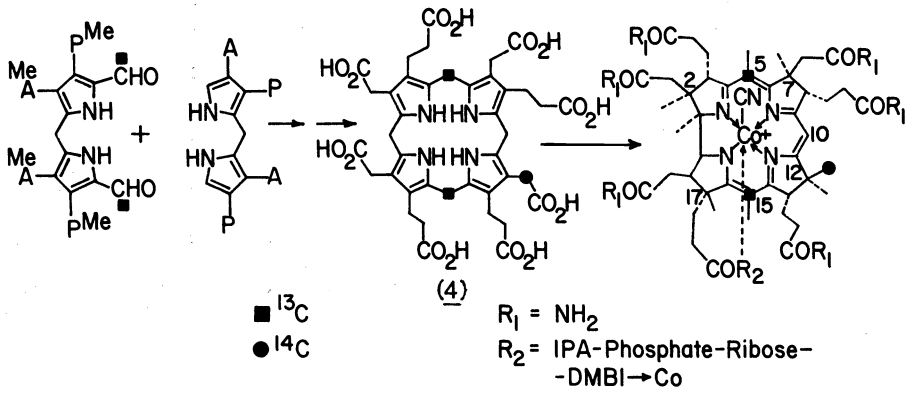


Fig. 2. Synthesis and incorporation of [5,15-$^{13}C_2$]- and of [^{14}C]-uro'gen III.

A parallel series of experiments with [5,15-$^{14}C_2$]- and [5,15,20-$^{14}C_3$]-uro'gen disclosed that, after proper care was taken to solve the severe problems associated with the chemical production of formaldehyde from the meso positions of uro'gen, a stoichiometric amount of formaldehyde from C-20 of uro'gen could be trapped (as the dimedone adduct) during the conversion of uro'gen III to cobyrinic acid in the cell-free system described above (Ref. 17). The logical but unlikely possibility exists in this experiment that, by an amazing coincidence, the radioactive yield (~3%) of cobyrinate from ^{14}C at C-5 and C-15 of uro'gen (analyzed as cobester) fortuitously matches that of formaldehyde from ^{14}C-20! We recall at this juncture Shemin's classic experiment (Ref. 14) with [5-^{14}C]-ALA in which ca. 10% of the radioactivity from the labeled position must have been returned to the "C-1" pool in order to explain the appearance of a small amount of radioactivity in the methyl groups of B_{12}, including C-1. We shall return to the logic of this fascinating point when we later describe the structures and biointermediacy of the methylated isobacteriochlorins.

We have initiated a study of the application of low temperature, high resolution CMR spectroscopy to the enzymes of the B_{12} pathway in vitro and in vivo. As a first example we chose the deaminase/cosynthetase-mediated conversion of PBG to uro'gens I and III. This work sets the stage for subsequent experiments designed to trace the complete metabolism of glycine to corrin via cryo-enzymological NMR techniques.

The formation of uro'gen III requires the participation of two enzymes, PBG deaminase (uro'gen I synthetase) and uro'gen III cosynthetase (Ref. 18). In the presence of deaminase alone, PBG is converted into uro'gen I (5) which is not further involved in the pathway of tetrapyrrole or corrin biosynthesis. We have followed the events of enzymic conversion of PBG (2), enriched with ^{13}C, into uro'gens in the NMR tube, thus avoiding the isolation of potentially labile compounds. In the first experiment highly purified deaminase (80 units per mg) was incubated with 300 µg of [11-^{13}C]-PBG (Fig. 3) in tris buffer at 37°C under anaerobic conditions. NMR spectra were recorded on Varian SC-300 and XL-200 NMR spectrometers.

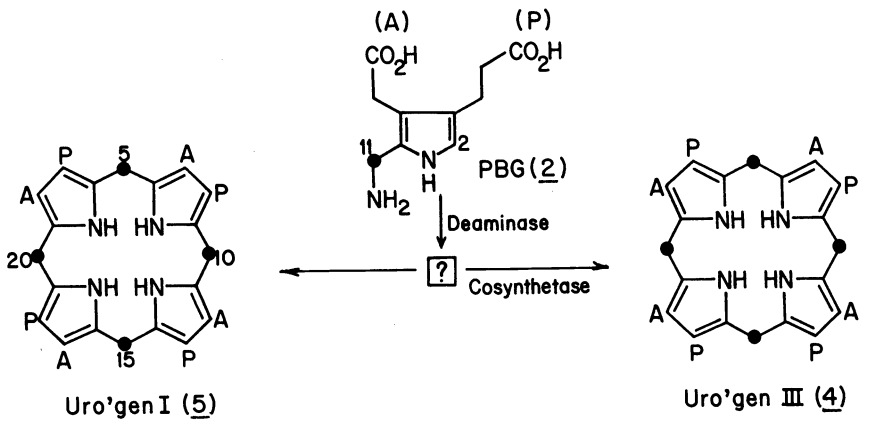


Fig. 3. Conversion of [11-^{13}C]-PBG to uro'gens I and III.

The spectrum of uro'gen I, obtained after the enzyme reaction had been allowed to proceed to completion, is shown in Fig. 4a. The triplet centered at 21.63 ppm (reduced $^{1}J^{13}C\text{-}^{1}H$ = 100 Hz) results from the four equivalent meso carbon atoms (5, 10, 15 and 20 in uro'gen I). When the enzyme reaction is allowed to proceed until 47% of the PBG has been consumed (11 min), in addition to the remaining PBG signal (triplet centered at 34.95 ppm, reduced $^{1}J^{13}C\text{-}^{1}H$ = 125 Hz) and the uro'gen signal (21.63 ppm) at 0°C, a complex signal is also present at 21.85-22.15 ppm which integrated as 17% of the ^{13}C (Fig. 4b). In addition, a further signal (integral: 5% of ^{13}C) appears as a triplet at 54.78 ppm (reduced $^{1}J^{13}C\text{-}^{1}H$ = 130 Hz). These latter signals disappear as the enzymic conversion proceeds to completion. Treatment of the incubation medium with either acid or base under conditions where the deaminase is inactive affords only uro'gen I which is also the sole product of the reaction with deaminase in buffer. When the NMR experiment is performed under identical conditions in the presence of sufficient cosynthetase (Ref. 19) to produce 100% of uro'gen III, after 47% of the PBG has been consumed (11 min), none of the complex signals at 21.85-22.15 ppm or at 54.78 ppm is observed, whilst the uro'gen (III) peak is proportionally increased (Fig. 4c).

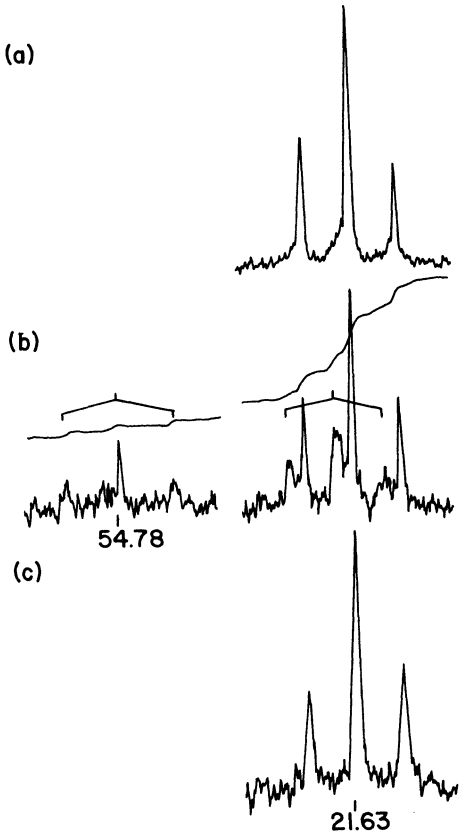


Fig. 4. 75.5 MHz ^{13}C spectra of enriched uro'gen I (a), uro'gen I and pre-uro'gen (b), and uro'gen III (c) at 0°C under single frequency off-resonance proton decoupling conditions. Typically, 35,000 90° pulses were accumulated over a spectral width of 9,000 Hz while locked to internal D_2O (10%) with a repetition rate of 0.6 sec. The lines were broadened approximately 2 Hz by exponential multiplication of the F.I.D. (Ref. 20).

From these data we conclude that during the enzymic conversion of PBG into uro'gen a hitherto undetected intermediate is accumulated in solution which ultimately yields uro'gen I. The absence of ^{13}C signals at 21.85-22.15 and 54.78 ppm under conditions where the enzymic system is forming only uro'gen III suggested that the intermediate, which we have named pre-uro'gen, responsible for these signals was involved in the normal pathway of uro'gen III biosynthesis, thus favoring the hypothesis of rearrangement at the tetrapyrrole level and the formation of pre-uro'gen from an enzyme-bound version of the bilane (6). Before discussing the most recent evidence for the structure of pre-uro'gen, we turn to the biochemical experiments which define this species as the substrate for cosynthetase. Evidence that pre-uro'gen is indeed the

long-sought substrate for cosynthetase was obtained as follows. The deaminase-free pre-uro'gen filtrates (stage 1) were incubated with uro'gen III cosynthetase (Ref. 21) (16 u/ml, 37 u/mg) together with the appropriate controls (stage 2) which showed the complete absence of deaminase, since during stage 2 there was no further consumption of PBG. The amounts of uro'gen III formed during these experiments are shown in Table 2.

TABLE 2. Formation of pre-uro'gen assayed by conversion to uro'gen III and by CMR spectroscopy

Incubation time (mins)		% Isomer III formed[a]		% of pre-uro'gen present	% Conversion of pre-uro'gen into uro'gen III
Stage 1 deaminase	Stage 2 cosynthetase	cosynthetase	buffer		
11	10	37[b]	0[b]	43[d]	86
23	10	21[b]	0[b]	24[d]	87
37	10	7[b]	0[b]	-	-
60	10	0[b]	0[b]	0[d]	-
30	10	13[c]	0[c]	-	-

[a]Remaining % is isomer I[b,c]

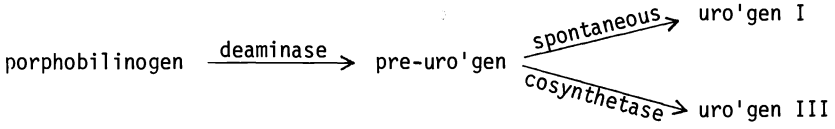
[b]Determined by hplc of uroporphyrin methyl esters

[c]Determined by hplc of coproporphyrin methyl esters

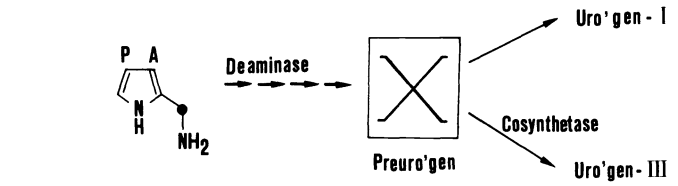
[d]Determined by ^{13}C NMR in a separate experiment

The data clearly demonstrate several important features of the uro'gen III synthesizing system. a) Pre-uro'gen is formed transiently by the action of PBG deaminase and, in the absence of uro'gen III cosynthetase, rearranges to form uro'gen I in acidic, basic or neutral media. b) The formation of uro'gen I from pre-uro'gen occurs in the absence of deaminase. c) Pre-uro'gen is converted into uro'gen III in high yield by uro'gen III cosynthetase. d) PBG deaminase is not required for the conversion of pre-uro'gen into uro'gen III. e) Pre-uro'gen is tetrapyrrolic since PBG is not consumed during its conversion to uro'gen III. f) Uro'gen I does not act as a substrate for uro'gen III cosynthetase.

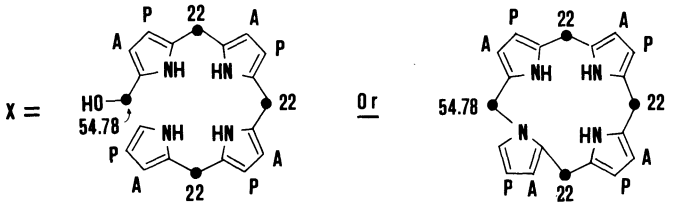
All of the evidence suggests that a hitherto unsuspected pathway for the biosynthesis of uro'gen III is operative in which the enzymes act sequentially and independently as shown below, with pre-uro'gen as the key intermediate (Ref. 21).



With the importance of pre-uro'gen as the long-sought substrate for cosynthetase firmly established, it now remains to offer structural proof for this reactive intermediate. Figure 5 summarizes the NMR data which led to two possible structures promulgated in late 1978 (Ref. 20). The presence of a signal at 54.78 ppm in the ^{13}C-spectrum of pre-uro'gen was attributed to a C-X bond and on this basis the hydroxymethylbilane (6) and the unusual N-alkyl tetrapyrrole (7) were chosen. Multiply labeled versions of PBG were synthesized and from a series of experiments in which [11-^{13}C; 1-^{15}N]-PBG was incubated with deaminase it was concluded (Ref. 22) that at 0°C, pH 7-8.2, the most abundant species present showed one bond ^{13}C-^{15}N coupling (J = 6 Hz) at 54.78 ppm, a result compatible with structure (7). However, at 37°C, using large amounts of substrate and purified deaminase, the spectrum shown in Fig. 6 was obtained from PBG labeled with ^{13}C at C-11 and with ^{15}N at the pyrrole nitrogen (Ref. 23). The coupling constant and chemical shift are in excellent agreement with two-bond ^{13}C-^{15}N coupling (^{2}J = 1.8 Hz), i.e. the hydroxybilane 6 is observable as a short-lived species at pH 8, 37°C (Ref. 22 & 23). The hydroxybilane structure has been confirmed by independent work at Cambridge (Ref. 24) where the NMR experiment was repeated, and the structural elucidation completed by total synthesis of the bilane (6). The interpretation of these experiments is shown in Fig. 7.



"X" has δ 54.78ppm (1Ċ) 22 ppm (3Ċ)



G.Burton, P.E.Fagerness, S.Hosozawa, P.M.Jordan, and A.I.Scott, J.C.S. Chem. Comm., 1979, 202.

Fig. 5. Earlier proposed structures for pre-uro'gen.

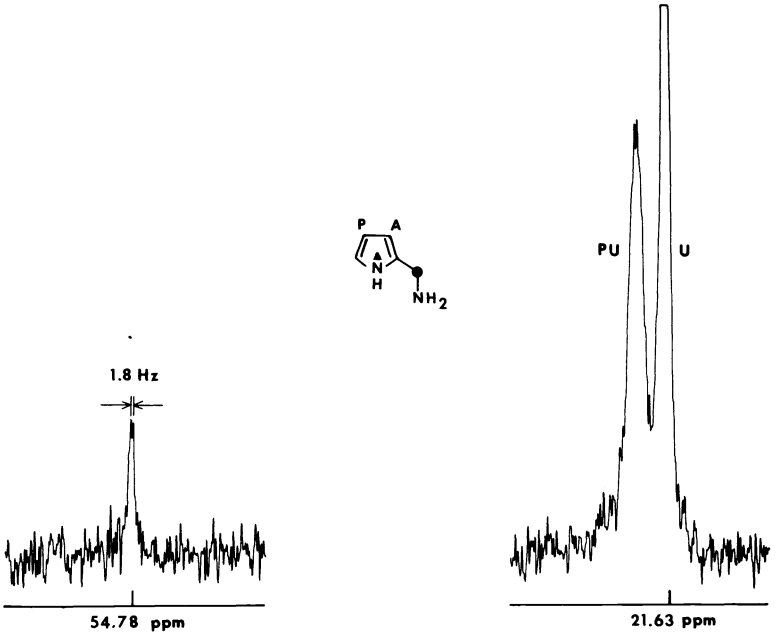


Fig. 6. C-13 NMR spectrum of pre-uro'gen from PBG at 37°C and pH 8.3 (4 mg labeled PBG/ml).

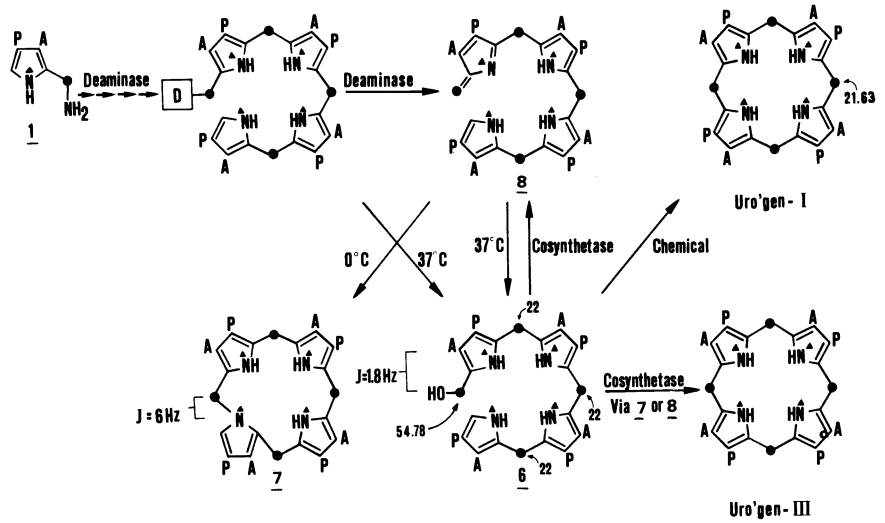
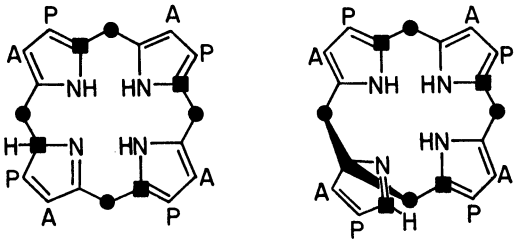


Fig. 7. Proposed mechanisms for deaminase/cosynthetase.

Even before the excitement of the chase for the elusive intermediate subsided, the availability of high-field, wide-bore NMR instrumentation allowed the rapid accumulation and observation of yet another set of ^{13}C-spectra derived from 30-90 sec incubations at high substrate concentration. In these experiments only the 22 ppm signals can be observed in the first 30-60 secs of incubation. Subsequently, the addition of base (→ pH 12) again reveals the hydroxybilane signal at 54 ppm. These very recent results (Ref. 25) now suggest that although it has not yet been possible to detect the obvious reactive intermediate (8), the first observable macrocycle appears to correspond to either the 2H-pyrrolenine tautomer (9) or possibly a spiro compound (10) since all of the observed carbons are at 22 ppm, i.e. are C-C rather than



(9) (10)

■ From C−2 of PBG ● From C-11 of PBG

C-O or C-N bonded. The function of deaminase is therefore even more complex than either we or our colleagues at Cambridge had suspected in that the enzyme can form an enzyme-free macrocycle under kinetically observable conditions which may be reversibly changed by hydroxide ion to 6, or carried forward via the second enzyme, cosynthetase, to uro'gen III. It remains to be seen whether, using double-labeled PBG, structures such as 8 or the 2H tautomer of uro'gen I can be detected. Whatever the outcome, our latest results show that neither the recently favored hydroxybilane and certainly not the previously suggested aminobilane (6; OH = NH_2) are the true physiological substrates of deaminase, but rather represent nucleophilic, reversible traps for the exomethylene pyrrolenine (8).

NMR STUDIES OF PORPHYRINOGEN SYNTHESIS IN VIVO

The technique of CMR spectroscopy for direct, non-invasive observation of metabolic events has now been developed to study the metabolism of ALA and PBG in P. shermanii and R.spheroides. The study of the dynamics of formation of tetrapyrroles in live cells of R. spheroides and of P. shermanii is admirably suited to the technique of whole-cell NMR, since the intermediate porphyrinogens are highly oxygen-sensitive and the presence of large amounts of pigments makes the estimation of porphyrins and/or porphyrinogens difficult. Direct observation of porphyrinogen biosynthesis in intact R. spheroides and P. shermanii (Ref. 26) set the stage

for a detailed evaluation of the metabolic flux of ALA into tetrapyrroles involving time course experiments during adaptation of these organisms to different growth conditions.

In R. spheroides the tetrapyrrole pathway is of interest with respect to metabolic control since three major groups of pigments are produced, namely haem, bacteriochlorophyll and corrins. In addition, the Athiorhodacae, of which R. spheroides is a member, have the ability to grow in the dark or light either aerobically or anaerobically. Levels of bacteriochlorophyll may change 100-fold during changes in growth conditions, thus providing scope for the investigation of adaptive mechanisms associated with the change from photosynthetic to non-photosynthetic metabolism.

The ^{13}C-NMR spectrum of anaerobically grown R. spheroides incubated with [5-^{13}C]-ALA (Fig. 8)

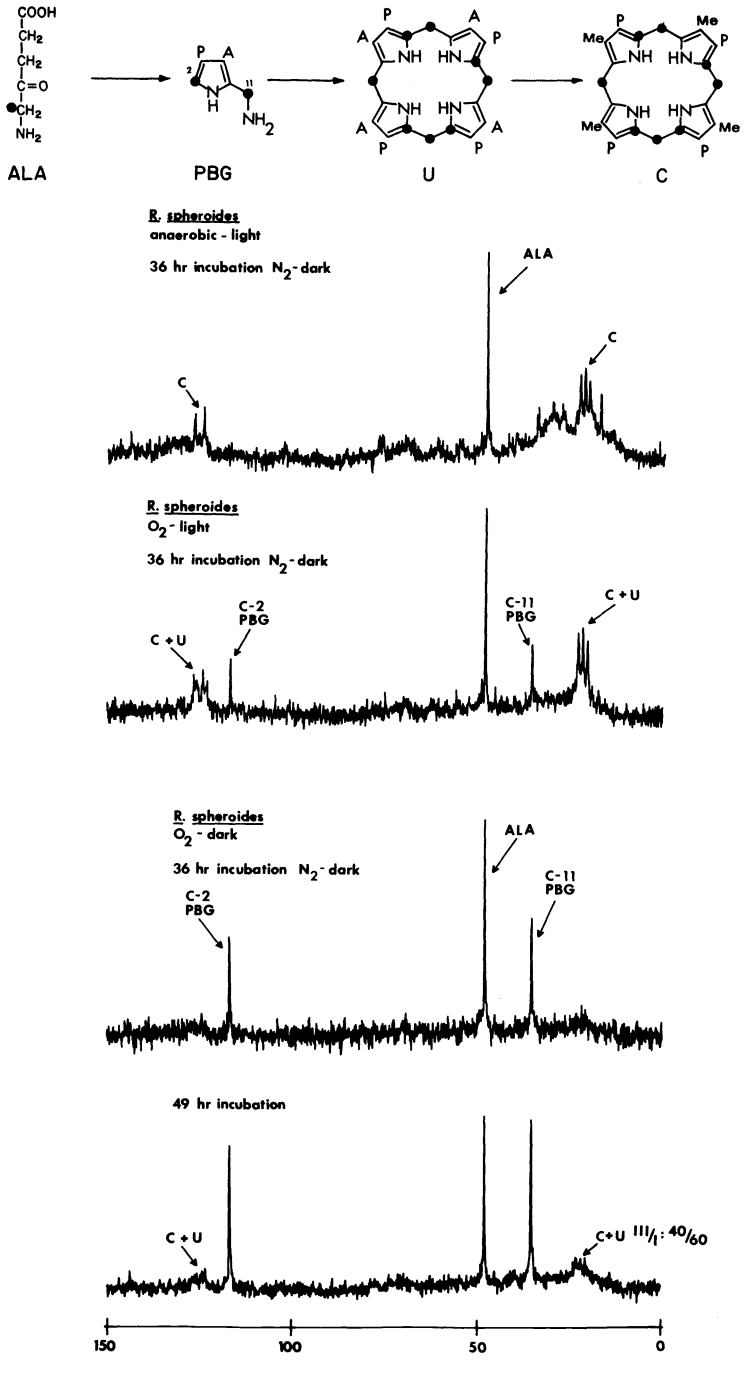


Fig. 8. Time course of anaerobically and aerobically grown R. spheroides during metabolism of ^{13}C-ALA under light and dark conditions.

indicates that most of the substrate has been incorporated into porphyrinogens with no accumulation of the intermediate porphobilinogen (PBG, 2). Examination of the signals at 20-24 ppm and 123-125 ppm indicate the presence of type III porphyrinogens as judged by the expected couplings and signal heights from labeling in carbons 4, 5, 9, 10, 14, 15, 16 and 20 (see Fig. 8 for labeling patterns) (Ref. 22). Subsequent isolation and tlc of the cell extract showed that the major porphyrinogen formed was coproporphyrinogen III (80%) together with some uroporphyrinogen III and protoporphyrinogen IX (5%), some of which had been excreted extracellularly. A time course experiment (24 hr) failed to show the appearance of PBG, suggesting that the enzymes PBG deaminase and uroporphyrinogen III cosynthetase were not present in rate limiting quantities. Parallel studies with crude cell-free extracts of R. spheroides using PBG as substrate gave similar results, indicating that in light-adapted, anaerobically grown cells the rate determining reactions of porphyrinogen biosynthesis are those catalyzed by ALA synthetase and coproporphyrinogen III oxidase.

R. spheroides when grown aerobically in the dark produces essentially no carotenoid pigments and is cream colored, in contrast to the dark reddish-brown coloration of anaerobic cultures. Under the former conditions no bacteriochlorophyll can be detected. When the organism grown in this way was incubated with [5-^{13}C]-ALA in the NMR tube as before (anaerobically in the dark) for 36 hr, 15% of the starting ALA remained and the only intermediate formed in significant quantities was PBG, amounting to approximately 80% of the ^{13}C signal with only trace quantities of porphyrinogens visible (Fig. 8). Further incubation (49 hr total) at 28°C resulted in partial consumption of the remaining [5-^{13}C]-ALA, the porphobilinogen signals increased slightly, while porphyrinogen synthesis was minimal compared with the anaerobic light-grown cultures (Fig. 8). Furthermore, the isomer ratio of porphyrinogens formed was estimated (from the peak heights) to be 60:40, I:III. Under these conditions uroporphyrin and coproporphyrin were also formed.

When aerobic-dark grown cells of R. spheroides were subjected to anaerobic-light conditions and the cells harvested between 12 and 48 hr to monitor any changes in the flux of [5-^{13}C]-ALA into tetrapyrroles during the adaptation from non-photosynthetic to photosynthetic metabolism, no accumulation of porphobilinogen or porphyrinogens was observed, suggesting that any intermediates were being carried through to bacteriochlorophyll and were not accumulating either intra- or extracellularly. ALA was consumed very slowly indicating that the adaptation was not immediate and that adaptation probably involved de novo enzyme synthesis. When cells were grown aerobically in the light and incubated with [5-^{13}C]-ALA (for 36 hr in the dark under nitrogen), accumulation of type III porphyrinogen was evident after 36 hr (Fig. 8). Notice that some PBG remains and that decarboxylase activity is incomplete as judged by the presence of uro'gen III and copro'gen III signals at 123-125 ppm.

The cumulative results of this work suggest that in addition to ALA synthetase, coproporphyrinogen III oxidase is also a key regulating enzyme in tetrapyrrole biosynthesis in R. spheroides since coproporphyrinogen III accumulates in large quantities under anaerobic light conditions.

On changing from anaerobic to aerobic respiratory metabolism in the dark, the enzymes of R. spheroides most dramatically affected appear to be porphobilinogen deaminase and to an even greater extent, uroporphyrinogen III cosynthetase, resulting in accumulation of porphobilinogen, an observation not recorded in earlier studies. The enzymes ALA-dehydratase and ALA-synthetase have to date been assumed to be the major control points in the tetrapyrrole biosynthetic pathway as both enzymes are four to five times less active in cell-free extracts from aerobic dark grown cells compared with those from semi-anaerobic light grown cells. However, the build-up of large amounts of PBG not only in R. spheroides but in P. shermanii tends to suggest that greater control lies with PBG deaminase and uroporphyrinogen III cosynthetase in whole cells. This point is significant in that ALA dehydratase, traditionally accepted as being the most oxygen sensitive of the enzymes of the tetrapyrrole pathway, still remains substantially active under aerobic growth conditions. In the whole cell, the effect of oxygen is clearly diminished, pointing to the danger of correlating results with whole cells and cell-free extracts and highlighting the uniqueness of the non-invasive NMR approach as a powerful experimental tool for studying the normal cellular machinery at work. The stage is now set for a detailed investigation of corrin synthesis via ^{13}C-Met and ^{13}C-ALA metabolism studied by NMR in vivo.

THE FINAL STAGES. CHARACTERIZATION AND INTERMEDIACY OF THE ISOBACTERIOCHLORINS OF P. SHERMANII

As soon as uro'gen III was defined as the precursor of the corrin nucleus (Fig. 2) the search for partially methylated intermediates on the way to B_{12} began in earnest and it was noted as early as 1973 (Ref. 27) that the preferred structure for sirohydrochlorin, the iron-free prosthetic group of the enzyme siroheme, could be modified to accommodate its possible role as a biosynthetic intermediate. At first sight this would have seemed an extraordinary coincidence but as the study of sirohydrochlorin developed in parallel with the isolation of orange-fluorescent substances from cobalt-deficient, ALA-supplemented incubations of

P. shermanii, complete identity of the dimethyl isobacteriochlorin from the six-electron reducing enzyme and from the B_{12}-producing organism was established. Thus, inspection of the UV, CD, mass and PMR spectral data for the methyl ester of the P. shermanii metabolite and of sirohydrochlorin from E. coli sulfite reductase leaves no doubt that the substances are identical in every respect. The molecular constitution was also confirmed by high resolution mass determination of the molecular ion.

The UV spectrum of sirohydrochlorin is diagnostic of the isobacteriochlorin class requiring the two reduced rings to be adjacent. Since sirohydrochlorin is incorporated intact into cobyrinic acid (see later), structures 11 - 14 represent the possible alternatives.

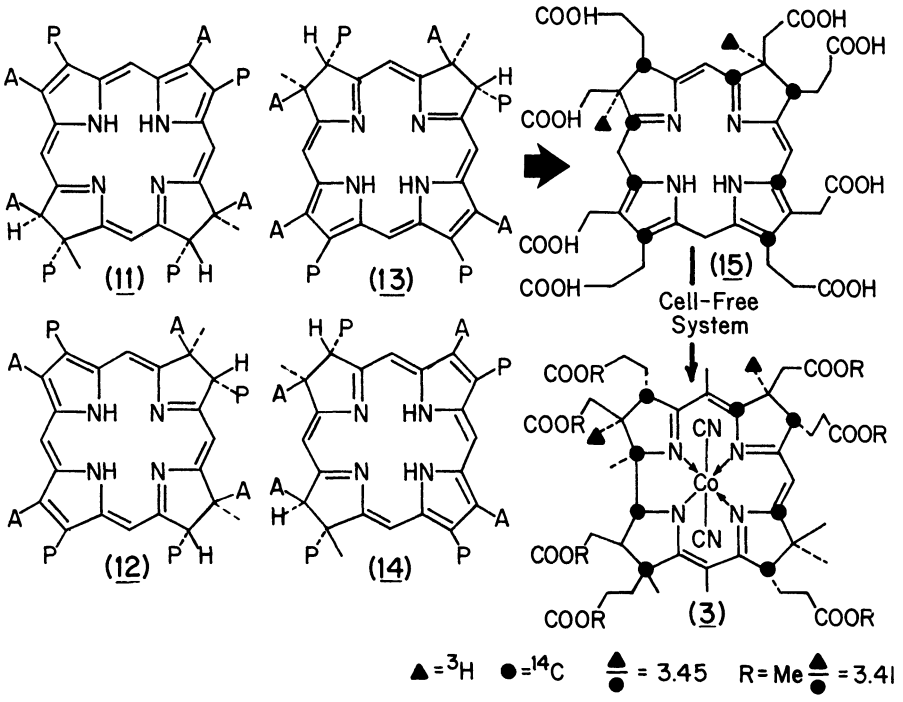


Fig. 9. Incorporation of Factor II (sirohydrochlorin) into cobyrinic acid.

At this stage in the investigation the structural proposals for sirohydrochlorin were considerably reduced by biosynthetic experiments. To cite one of several examples, a doubly labeled sample to reduced sirohydrochlorin methyl ester (15; OH = OCH_3) was prepared by separate incubations with [4-^{14}C]-ALA and [$^3H_3$C]-S-adenosylmethionine. When the samples were combined ($^3H/^{14}C$ = 3.45), purified and re-fed after hydrolysis and sodium amalgam reduction (15), the resultant cobester (3; R = Me) was obtained in 2.4% radiochemical yield with retention of the ratio ($^3H/^{14}C$ = 3.41) (see Fig. 9).

In view of the correspondence of the oxidation level of 15 and of its biosynthetic product, cobyrinic acid (3; R = H), it was surprising to find that incubation of the unreduced species 13 led to equally good (0.3-2.8%) incorporation. We discuss below the implication of this finding. At this stage in the investigation Müller (28) described the isolation and cell-free conversion of a P. shermanii metabolite, Factor II, to cobyrinic acid. Comparison of the reported physical data for Factor II and sirohydrochlorin leaves little doubt that they are identical.

Two structures, (12) and (13), are still consistent with the spectroscopic and bioincorporation data mentioned above. However, since it is unlikely that methylation of ring C occurs before decarboxylation of the acetate side chain during B_{12} biosynthesis, structure (13) becomes attractive for sirohydrochlorin. This complete stereostructure 13 was confirmed by a series of biosynthetic experiments in which ^{13}C-labeling again proved to be of diagnostic value in arriving at a unique solution. It was first shown that a natural abundance CMR spectrum could be obtained on a 500 µg sample in which 10^6 transients were collected over 252 hr. Next, incubation of [4-^{13}C]-ALA (90% enriched) with the P. shermanii cell-free homogenate yielded a purified sample of sirohydrochlorin (400 µg) whose proton-decoupled and proton-coupled spectra showed that only structures 12 and 13 are compatible with the observation of a doublet (J = 135 Hz) for each sp^3 carbon resonance (C-3 and C-8), since structures 11 and 14 would exhibit only one such enriched sp^3 carbon-bearing hydrogen.

Finally, incubation with [5-^{13}C]-ALA enabled us to show that sirohydrochlorin has structure 13. The expected labeling pattern is as shown in Fig. 10. In the proton decoupled spectrum (Fig. 10, spectrum A), the C-15 resonance is a triplet (J = 71.1 Hz) due to 1,2 coupling with

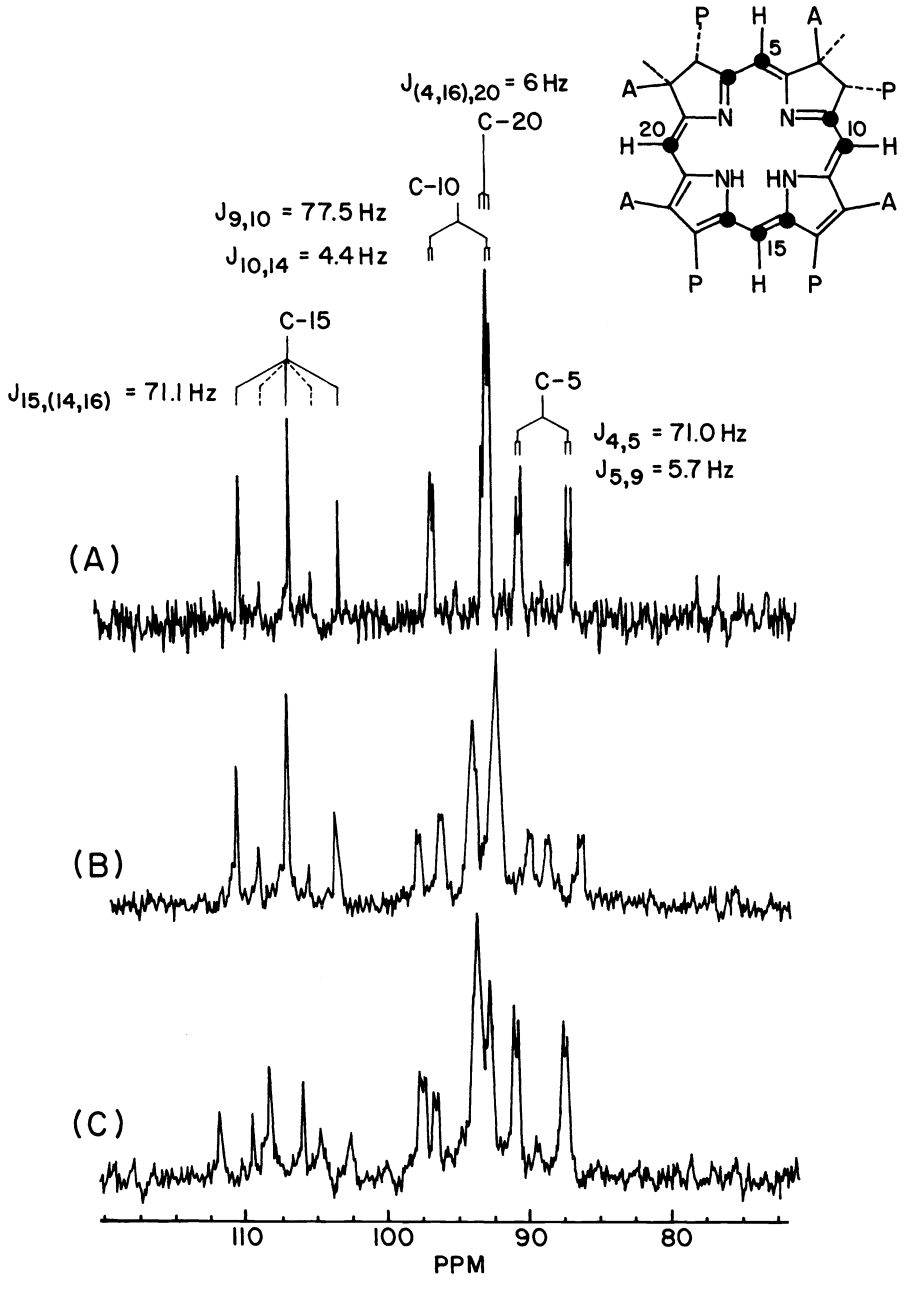


Fig. 10. Selective irradiation of the CMR spectrum of sirohydrochlorin (A), at C-15 (B) and C-5 (C). For explanation see text.

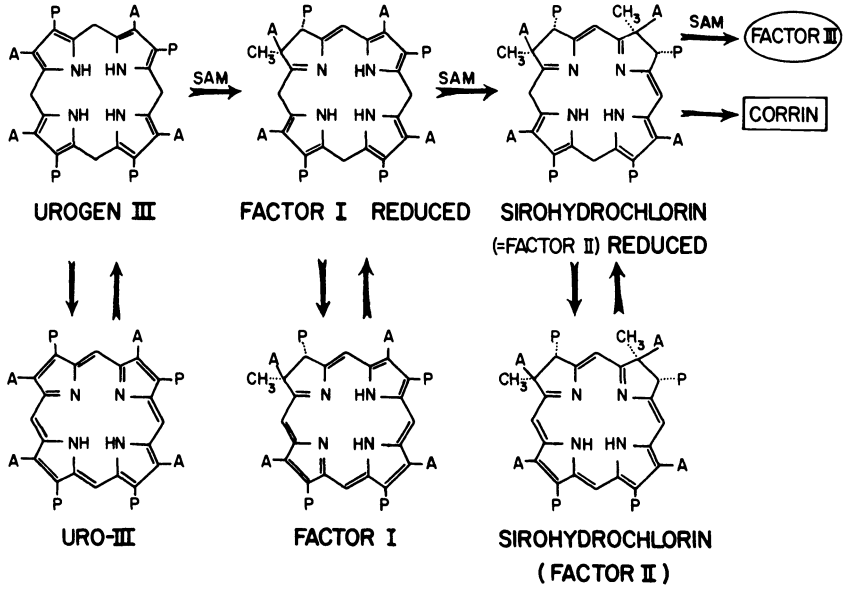
two adjacent enriched sites. (A lower intensity doublet is also present due to those molecules having only one adjacent enriched site.) The C-5 and C-10 resonances both occur as doublet of doublets due to 1,2 and 1,4 couplings whilst C-20 shows only 1,4 interactions. The four meso-hydrogen PMR resonances occurred as doublets at δ 8.54, 7.46, 7.36 and 6.78 ppm. As discussed by Bonnett et al. (29), the upfield meso-hydrogen resonance of an isobacteriochlorin may be assigned to that between the reduced (methylated) rings and the downfield resonance to that between the non-reduced rings. Thus, the δ 8.54 hydrogen could be coupled to C-15 in structure 13 and to C-20 in structure 12. The former case was confirmed by selective irradiation of the δ 8.54 hydrogen resonance. As can be seen from spectrum B in Fig. 10,

the C-15 resonance at δ 107.4 remained a triplet whilst the other meso-carbon resonances showed coupling to meso-hydrogen resonances. Similarly, spectrum C shows the effect of decoupling C-5 by irradiation at δ 6.78.

Our colleagues in Stuttgart, Cambridge and Moscow independently isolated metabolites from P. shermanii with similar UV and mass spectral characteristics to sirohydrochlorin. The Anglo-Russian group postulated structure 13 for their metabolite after assuming that it was on the B_{12} pathway and further that ring C was not methylated. Further work with the lactones and free esters of this series (Ref. 30) independently confirmed the above conclusions and showed that sirohydrochlorin is indeed identical with a P. shermanii metabolite, that it has structure 13 and that it is an intermediate on the corrin pathway. The possibility that siroheme represents a prebiotic sulfate-reducing agent (Ref. 27) and further, that both sirohydrochlorin and vitamin B_{12}-producing anaerobic organisms predate the evolution of heme-synthesizing aerobes (Ref. 23), suggests that the reductive methylation of reduced porphyrins may be a phenomenon of considerable antiquity (three billion years). We also note that the same dimethylisobacteriochlorin prosthetic group is active in the enzymes of nitrite → ammonia reduction (Ref. 31). Thus, it appears that the biosynthetic architecture of corrins evolved at a very primitive stage of life on this planet and that the problem we are investigating has existed for even longer than we had realized at the outset. The intermediacy of the dimethylisobacteriochlorin (13) (Ref. 32) in corrin biosynthesis requires not only reappraisal of the specific incorporation of uro'gen III heptacarboxylic acid into cobyrinic acid, which could be explained by non-specific enzymatic conversion of a substrate closely related to, but not identical with, the normal metabolic intermediate, but also a significant modification of our present working hypothesis (Ref. 33) for the post-uro'gen III segment of corrin biosynthesis. With the knowledge that 13 is established as an intermediate by isolation in cobalt-deficient incubations and by intact specific incorporations into cobyrinic acid (thereby defining the absolute stereochemistry of sirohydrochlorin), the remaining stages of the sirohydrochlorin-cobyrinic acid pathway appear, not necessarily in this order, to involve: a) introduction of five additional methyl groups from S-adenosylmethionine with retention of the methyl protons; b) loss of C-20; c) reclosure of the seco-corrin system; d) two-electron reduction to corrin; e) insertion of cobalt with valency change $Co^{2+} \rightarrow Co^{3+}$.

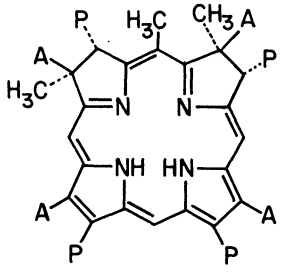
As a guide to future experimentation we recently offered (Ref. 32) a speculative mechanistic rationale of these processes based on the known or presumed chemistry of the reduced porphyrins and of corrins. Many of the ideas embodied in the Schemes are presently being tested experimentally and include certain key modifications of a proposal for corrin biosynthesis published several years ago (Ref. 33), some of which stem from the recent elegant chemical analogies uncovered in Eschenmoser's (34) several synthetic approaches to corrins. These Schemes also embody the observation that two new metabolites (Factors I and III) had been isolated at Stuttgart (Ref. 28 & 35) and that Factor III appeared to be related to the bislactone corriphyrin-3 (Ref. 36).

Scheme 1 shows the incorporation of sirohydrochlorin (= Factor II) and the presumably intact incorporations of Factors I and III all taking place at the reduced level. The experimental

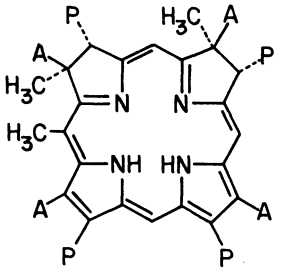


SCHEME 1

observation of incorporation of Factors II and III (Ref. 35), but only the reduced form of Factor I (Ref. 28) and uro'gen III, indicates the presence of a non-specific oxido-reductase system which, however, is not capable of reducing either uro III or Factor I. At this juncture, the evidence for the structures suggested for Factors I and III were still permissive rather than diagnostic. Thus, Factor I could be methylated in ring B rather than in ring A. We felt that Factor III, while undoubtedly a trimethylisobacteriochlorin to which the structure (16) had been assigned (Ref. 36) on PMR evidence, could, in fact, be another isomer and in the absence of location of ^{14}C label, did not occupy the position of proven intermediacy in cobyrinate biosynthesis at this stage.



(16)



(17) A = CH_2CO_2H
P = $CH_2CH_2CO_2H$

(18) A = $CH_2CO_2CH_3$
P = $CH_2CH_2CO_2CH_3$

Fig. 11. Proposed (16) and revised (17) structures for Factor III.

In a recent collaborative study with Professor G. Müller (Ref. 37) we have provided spectroscopic and biochemical evidence that the "extra" methyl group is added to sirohydrochlorin at C-20 rather than at C-5 leading to the revised structure (17) for Factor III, i.e. 20-methylsirohydrochlorin, which suffers loss of both C-20 and its attached methyl group during biotransformation to cobyrinic acid, probably as acetic acid.

Factor III was isolated from δ-aminolevulinic acid (ALA)-supplemented cobalt-free incubations of P. shermanii (ATCC 9614) and from a B_{12}-deficient mutant of this organism. High resolution FD mass spectrometry of the octamethyl ester (18) established the formula $C_{51}H_{64}N_4O_{16}$ (988.4342) and analysis of the PMR spectrum (300 MHz) revealed only three signals at δ 6.43, 7.21 and 8.33 ppm in contrast to the four signals in this region in the spectrum of (13) which have been assigned to the four meso protons at C-5, C-10/C-20 and C-15 (δ 6.78, 7.36/7.46 and 8.54, respectively). Factor III is therefore 10- or 20-methylsirohydrochlorin. In order to decide between these alternatives a specimen of Factor III (400 μg) was prepared from a suspended-cell incubation from P. shermanii containing $^{13}CH_3$-methionine and [5-^{13}C]-ALA. When this ^{13}C-enriched species (as the octamethyl ester) was examined by microprobe CMR spectroscopy (Fig. 12) it became possible to deduce the complete structure (18). First, the downfield position of the C-15 meso-carbon triplet at δ 108.98 (J = 72 Hz) confirms that rings A and B are methylated (Ref. 32), and since the meso-carbon signals at δ 89.5 and 95.4 each show ^{13}C-^{13}C coupling to an enriched neighbor (J = 70 Hz) these are assigned to C-5 and C-10, respectively, by analogy with the corresponding resonances in sirohydrochlorin derived by biochemical enrichment with [5-^{13}C]-ALA (Ref. 32). Thus, the remaining meso-carbon resonance at δ 104.8 which consists of a doublet (J = 44.5 Hz) must correspond to C-20, the additional fine structure being due to long-range coupling with C-4 and C-16. That the ^{13}C-^{13}C coupling constant of 44.5 Hz for C-20 is due to substitution by a methionine-derived methyl group is confirmed by inspection of the methyl region of the CMR spectrum which displays three enriched species consisting of singlets at δ 20.17 and 19.62 and a doublet at δ 18.79 (J = 44.5 Hz). It can be seen that, due to different efficiencies of incorporation of ^{13}C-SAM and of [5-^{13}C]-ALA, the enrichments in the methyl groups and in the ALA-derived sp^2 carbons are not identical. Hence the satellite intensities reflect a greater enrichment in C-20 than in its pendant methyl group and allow unambiguous assignment of structure 18 to the octamethyl ester. Thus Factor III is 17, i.e. 20-methylsirohydrochlorin, rather than the C-5 methylated isobacteriochlorin (Ref. 36). The C-20 methyl structure for Factor III has been deduced independently at Cambridge (Ref. 38). The absolute stereochemistry of Factor III and its relationship to cobyrinic acid was obtained by the following biochemical experiment. Doubly labeled (^3H/^{14}C) 20-methylsirohydrochlorin is converted to cobyrinic acid with loss of the "C_2" unit consisting of C-20 (derived from C-5 of ALA) and methyl group originating from methionine. An alternative explanation, that the C-20 methyl group migrates to C-1 (or →

C-19 → C-17) with complete loss of ^{3}H can be ruled out, for earlier experiments have shown that all seven methionine-derived methyl groups of B_{12} are inserted without exchange of the CH_3 protons (Ref. 39, 40 & 41).

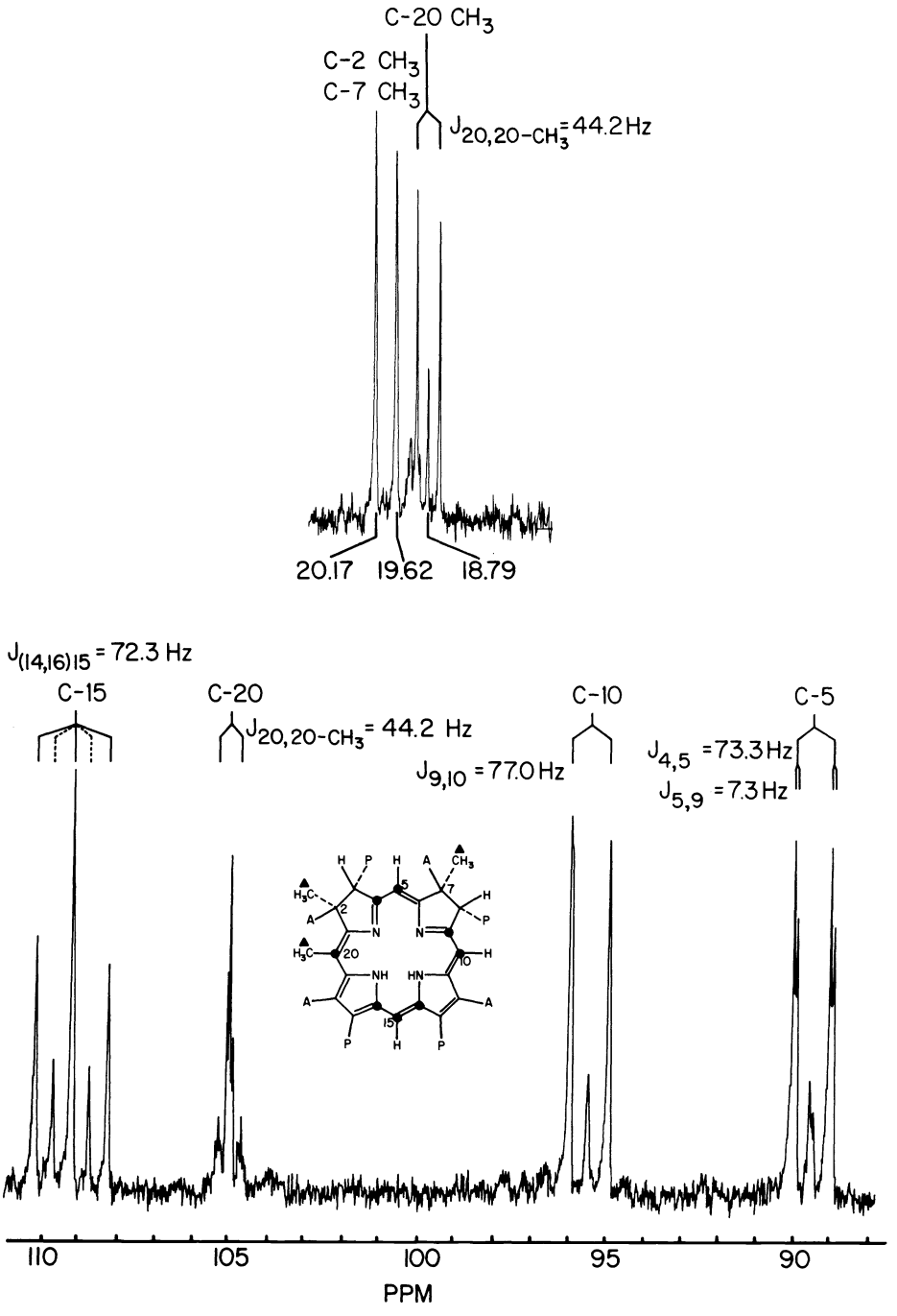
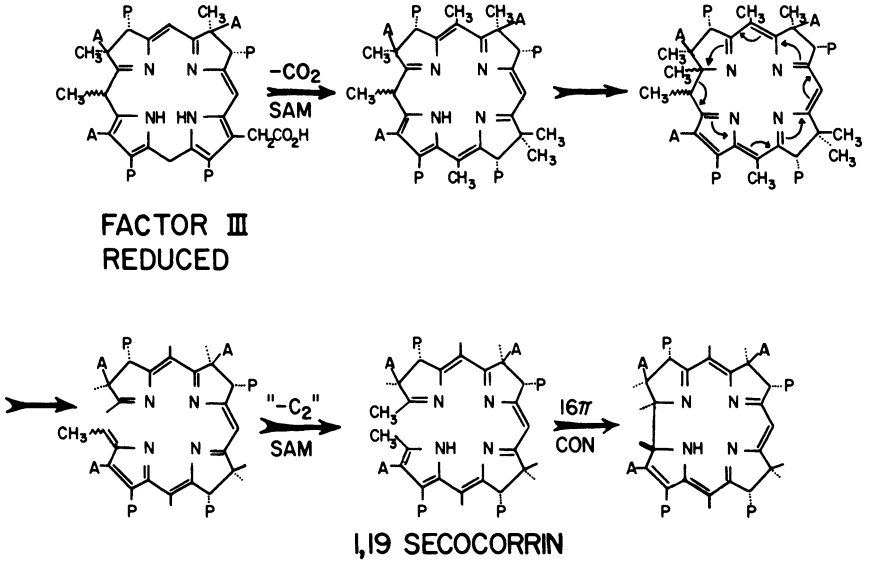


Fig. 12. CMR spectrum of Factor III. For explanation see text.

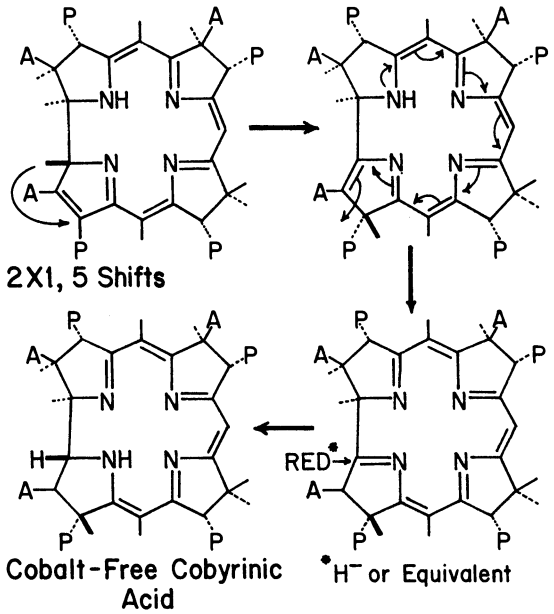
The nature of the loss of C-20 (from C-5 of ALA) in corrin biosynthesis must now be discussed. In earlier experiments it was clearly shown (Ref. 16 & 17) that, under carefully controlled conditions, ^{14}C formaldehyde could be trapped from the C-20 position of uro'gen III. The data presented above can be interpreted in several ways: a) methylation at C-20 is followed by loss of a "C_2" unit which is further cleaved to "C_1" units, one of which is trapped as

formaldehyde; b) the formaldehyde is released [under enzymic control (Ref. 17)] only from the uro'gen III molecule and not from Factor III which could release a "C_2" unit; c) more than one pathway exists for the biotransformation of uro'gen III to cobyrinic acid. It thus appears that in order to achieve the inter-corrin A → D ring junction the biosynthetic route requires not only the specific formation and subsequent disruption of the type III uro'gen macrocycle but the sacrifice of at least one methionine-derived methyl group and the carbon to which it is attached (C-20), since both of these must be excised from the species undergoing (or having undergone) seco-corrin → corrin closure (Ref. 33 & 42). This apparently prodigal series of events is portrayed in Scheme 2 which represents only one of several working hypotheses consonant with published data. Further work is in progress to clarify post-Factor III metabolism in vitamin B_{12} biosynthesis. Meanwhile, we note that Factor I indeed has the proposed structure and stereochemistry shown in Scheme 1 (Ref. 43). In fact, the volatile fragment released during conversion of Factor III to corrin has recently been found by Dr A S Irwin to be acetic acid, whose methyl and carboxyl groups are derived from methionine and C-20 respectively.



SCHEME 2

As shown in Scheme 2, decarboxylation at ring C, methylation (SAM) and ring opening can lead via loss of C-20 either directly to dehydrocorrin (Ref. 33 & 42) or, in an interesting variant, by methylation at C-1 to the 1,19 dimethyl seco-corrin whose electrocyclic closure reveals a 1,19 trans-methyl dehydrocorrin. The last part of the sequence (Scheme 3) portrays



SCHEME 3

the 1,5 shifts of the β-oriented C-19 methyl in ring D to C-17, its final resting place on the upper face of the molecule. A splendid analogy for this step has been discovered by A. W. Johnson (44). The pathway could then terminate by two-electron reduction to cobalt-free cobyrinic acid followed by, or synchronized with, insertion of cobalt (Ref. 33). It is hoped that, by examining intact cells and enzymes by NMR (wide bore-high resolution), corrin biosynthesis can be "viewed" by the non-invasive technique described earlier (Ref. 26).

Obviously, many formidable problems (and even more surprises) remain in the elucidation of the final details of corrin biosynthesis. In conclusion, it is a pleasure to pay tribute to the outstanding energy and devotion of my younger colleagues who have contributed so much to the progress of the work described in this lecture and whose names are mentioned in the references.

Acknowledgements — The microbiological and analytical techniques necessary for this exacting work with sub-milligram quantities of enriched, purified isolates were superbly handled by A. Brown, D. Brownstein, D. Stem, J. Petrillo, P. Copsey and M. Smith. Our work has been made possible by grants from the National Institutes of Health (AM 20528), the National Science Foundation (CHE 77-04877) and The Robert A. Welch Foundation (A-713).

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