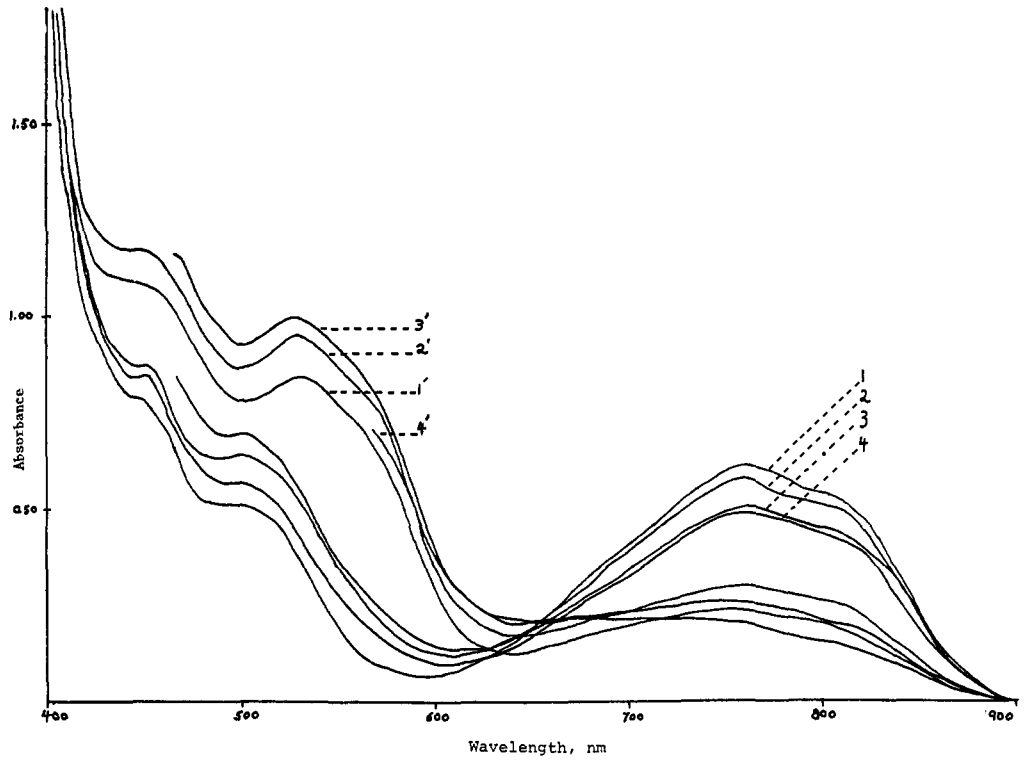


Figure 1. Reversible oxygenation of **1**. Curves 1–4 represent the reduced complex; curves 1′–4′ represent the oxygenated complex.

Pyridine *N*-oxide is not formed (glpc analysis) during this process.

In contrast the less hindered species **3** reacted with oxygen (toluene, pyridine (4%)) at −78° to give a red brown solution, $\lambda_{max}$ 565 nm, with absorption of 0.5 mol of $O_2$ (±0.02). This uptake is apparently irreversible.

These experiments prove that the stoichiometry and reversibility of combination of these iron(II) complexes with oxygen are dependent upon the steric hindrance provided by the ligand. In the hindered case (**1**), a reversible 1:1 complex (**5**) has been formed, whereas in case **3** a complex of stoichiometry 2Fe(II) per $O_2$, **6**, was obtained.[15] Complex **1** combined smoothly with carbon monoxide to give a 1:1 complex (**7**), $\lambda_{max}$ (DMF) 505 mμ.

The formation of 1:1 and 2:1 complexes of various Co(II) species with dioxygen is well known,[16] but these differ greatly from the iron species in stability and consequently do not require steric encumbrance in the ligand. However, an X-ray analysis of a 2:1 cobalt–dioxygen complex indicates a Co–Co distance of 4.5 Å.[17] A similar Fe–Fe distance would be available at the closest approach of two molecules of complex **3**, as in structure **6**. This fact offers an explanation for the sterically dependent phenomena described here.

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(15) We suggest these formulations, **5**–**7**, without implication of the nature of binding of oxygen, as the most likely ones on the basis of present evidence.

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(17) M. Calligaris, G. Nardin, and L. Randaccio, *Chem. Commun.*, 763 (1969).

## Stereochemistry of Methyl Group Insertion in Corrinoid Biosynthesis.[1] Determination of Carbon Isotope Chirality by $^{13}C$ Nuclear Magnetic Resonance[2]

*Sir:*

Recent experiments with whole cells of *Propionibacterium shermanii*[3] implicating uroporphyrinogen III (**2**, urogen III) as a precursor for vitamin $B_{12}$ (**3a**, cyanocobalamin) have placed restrictions on the timing of the methylation process in corrin biosynthesis. Before developing further mechanistic proposals for the latter sequence, which appears to be controlled by both steric and electronic consequences of methyl group insertion *via* *S*-adenosylmethionine (leading to α orientation in rings A and B and β orientation in ring D), resolution of the problem of the stereochemistry of methylation at C-12 in ring C became necessary. Thus, although it has been rigorously demonstrated[4] that one

(1) Presented in part at the Peter A. Leermakers Symposium, Wesleyan University, Nov 29, 1972, and available on a "Science Symposia on Tape" from the American Chemical Society.

(2) Carbon-13 Fourier Transform Nmr, Part VII. Part VI: H. H. Wasserman, R. J. Sykes, P. Peverada, C. K Shaw, R. J. Cushley, and S. R. Lipsky, *J. Amer. Chem. Soc.*, in press.

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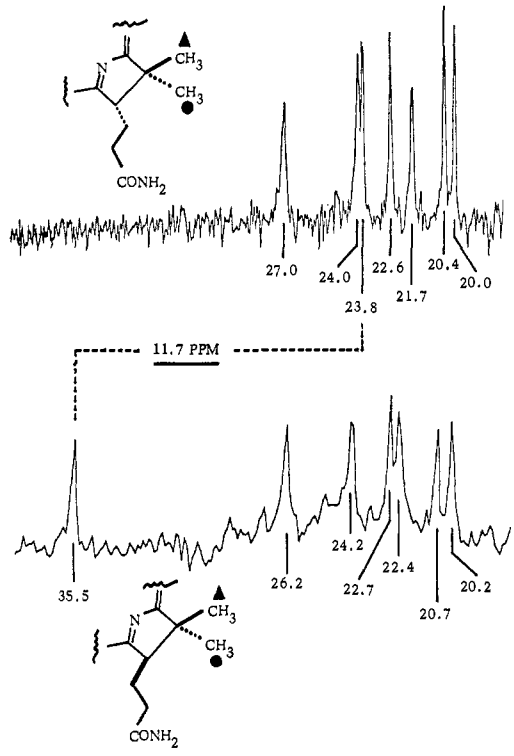
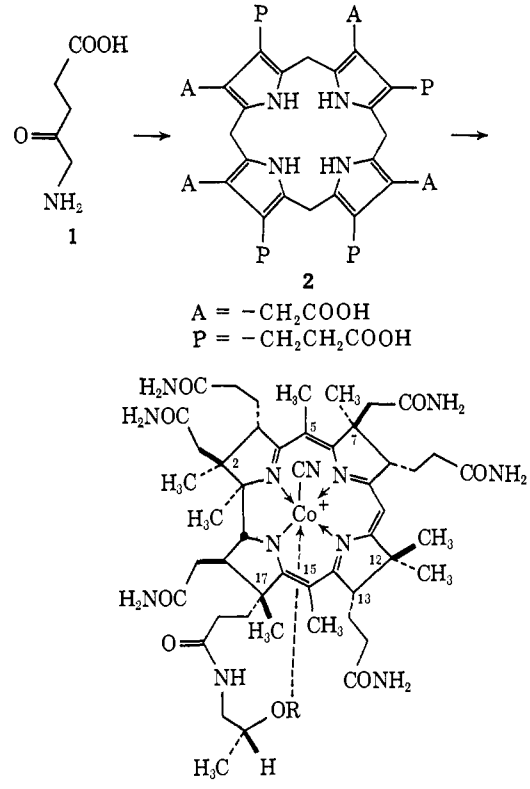
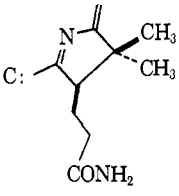



Figure 1. (Top) Proton noise-decoupled $^{13}C$ FT spectrum of [$^{13}CH_3$]methionine (●) enriched dicyanocobinamide (9 mg) in 0.1 *M* KCN. (The external $^{19}F$ lock ($C_6F_6$) and $^{13}C$ reference (hexamethyldisilane, HMDS) were contained in a 5-mm tube mounted coaxially in a 10-mm sample tube using a computer controlled FT system described previously (R. J. Cushley, D. R. Anderson, and S. R. Lipsky, *Anal. Chem.*, **43**, 1281 (1971)). Only the range 38.1–15.8 ppm is shown: data set = 8K points; digitizing rate = 10 kHz; pulse width = 50 μsec; receiver skip = 100 μsec. (Bottom) Proton noise-decoupled $^{13}C$ FT spectrum of [$^{13}CH_3$]-methionine (●) enriched dicyanoneocobinamide (2 mg) in 0.1 *M* KCN. Conditions and frequency range same as above except data set = 4K points.




3a, R = phosphate-ribose-DMBI ⟶ Co
b, R = H




of the methyl groups at C-12 is derived from methionine and the other from C-2 of δ-aminolevulinic acid (**1**, ALA), the *stereospecificity* of this process has not been established. Described in this communication are experiments that provide a ready solution to this problem with its attendant mechanistic implications and which demonstrate the particular usefulness of $^{13}C$ chemical shifts for the determination of carbon isotope chirality.

A labeled specimen of dicyanocobinamide (**3b**) was obtained where one of the C-12 methyl groups was specifically enriched by feeding [$^{13}CH_3$]methionine to a vitamin $B_{12}$ producing culture.[5] The conformation[6,7] of the C ring of cobinamide (**3b**) places the α-methyl synperiplanar to the adjacent axially oriented propionamide side chain at C-13. Such a juxtaposition would be predicted to produce a γ effect[8] on the $^{13}C$ chemical shift of the α-methyl group (analogously this effect should be reflected in the chemical shifts of the methyls at C-1, C-2, C-7, and C-17). The $^{13}C$ FT nmr spectrum of [$^{13}CH_3$]methionine enriched **3b** derived from **3a** by treatment with $CF_3COOH$[9] is shown in Figure 1 (top). The spectrum consists of seven methyl resonances 20–27 ppm downfield from HMDS. In addition to hydrolysis of the nucleotide, epimerization at C-13 also occurs in the same treatment of **3a** with $CF_3COOH$ to yield neocobinamide (**4**) which is easily separated from cobinamide (**3b**) above.[9] Neocobinamide differs from cobinamide solely by virtue of a configurational inversion of the propionamide group at C-13 accompanied by a conformational change in the skew of the C-12–C-13 bond. Thus, if the methionine-derived methyl at C-12 is α oriented, in the neo series it will bear an anticlinal relationship to the propionamide side chain and the concomitant removal of the γ effect should result in a downfield shift of the methyl resonance signal. That this is indeed the case is shown by the downfield shift of 11.7 ppm in the $^{13}C$ FT nmr spectrum for one of the methyl resonance lines in neocobinamide (Figure 1, bottom).

These results prove that the [$^{13}CH_3$]methionine methyl (●) is inserted into the corrin nucleus 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[5] that the β-methyl group (▲) of the *gem*-dimethyl grouping at C-12, derived from C-2 of ALA (**1**), resonates at substantially lower field than the methyl region tentatively assigned by Doddrell

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and Allerhand.[10] It should be noted that all the remaining methyl groups at $sp^3$ carbons appear at higher field, *i.e.*, within that region proposed by Doddrell and Allerhand, because of $\gamma$ interaction with adjacent syn groups.

Based on the foregoing argument, together with the X-ray diffraction data for the normal and neo series, four of the seven methionine-derived methyl groups are introduced on the $\alpha$ face of the corrin nucleus, the C-17 methyl is delivered from the opposite ($\beta$) side, and the two remaining alkylations occur at $sp^2$ meso positions (C-5 and C-15). We believe that these findings have important implications for the sequence of events in the biosynthesis of corrins from urogen III (2) that have been outlined earlier[1] and which will be presented in full shortly.[11]

**Acknowledgment.** We thank NSF and the National Institutes of Health (Grant RR-00356) for support of this work and Mr. M. Kajiwara and Ms. D. Brownstein for the culture of *P. shermanii*.

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## Biosynthesis of Corrins. A Cell-Free System from *Propionibacterium shermanii*

*Sir:*

Earlier studies from these laboratories have demonstrated the incorporation of porphobilinogen and uroporphyrinogen III (**1**, urogen III) into cyanocobalamin (**2**) using whole cell preparations of *P. shermanii*.[1,2] Recognizing the difficulties experienced by ourselves and other workers[3,4] in achieving uniformly substantial, reproducible incorporations of an advanced intermediate such as urogen III, especially where microgram quantities of this sensitive substrate are incubated in whole cell suspensions for long periods, we have developed a stable, cell-free preparation from *P. shermanii* which carries out reproducible biosynthesis of corrins from appropriate precursors at the submicromolar level.

The crude mixture of "corrin synthetase" was prepared from wet cells of *P. shermanii* (ATCC 9614) grown as described previously.[5] Disruption by a French press[6] in phosphate buffer (pH 7.6, 0.01 *M*) and centrifugation at 37,000*g* afforded an active supernatant fraction which was used in all of the following experiments and which can be stored at −30° without measurable loss of activity for up to 4 months. Incubations of [5-$^{14}$C]δ-aminolevulinic acid (ALA), [*methyl*-$^{14}$C]- and [*methyl*-$^{3}$H]*S*-adenosylmethionine (SAM) and of [α,β,γ,δ-*meso*-$^{14}$C]urogen I–IV mixtures were carried out with the cofactors and additives as shown in Table I. Post-incubation mixtures were freeze dried and subjected to exhaustive methanolysis after dilution with various combinations of corrins. Cobyrinic acid heptamethyl ester (**3a**, cobester) was the sole corrinoid product of methanolysis and was purified by three different tlc separations, coupled with autoradiography, and finally recrystallized to constant activity after dilution with authentic, nonradioactive sample.

**Table I.** Incorporation of ALA, Urogen, and SAM into Cobyrinic Acid by a Cell-Free System

| Expt no. | Substrate | Weight per incubation (μg) | Incubation conditions[a] (mg of protein[c]) | % radiochemical yield[b] |
|---|---|---|---|---|
| 1 | 5-[$^{14}$C]ALA[d] | 65 | 400 | 22.8[k] |
| 2 | 5-[$^{14}$C]ALA[d] | 18 | 65 (boiled enzyme) | <0.001[k] |
| 3 | [$^{14}$C]Urogen[e] | 400 | 65 | 3.4[h,k] |
| 4 | [$^{14}$C]Urogen[e] + | 106 | 65 | 3.1 ($^{14}$C)[h,i] |
| | [$^{3}H_3$C]SAM[f] | 0.44 | | 23.6 ($^{3}$H) |
| 5 | [$^{14}CH_3$]SAM[g] | 7.7 | 65 | 36[j] |
| 6 | [$^{3}H_3$C]SAM[f] | 0.44 | 65 (boiled enzyme) | <0.001[j] |

[a] Incubation mixture contains amount of protein indicated and the following components in a final volume of 10 ml of phosphate buffer pH 7.6, 0.02 *M*: GSH, 4 mg; ATP, 3 mg; DPN, 3 mg; DPNH, 2 mg; TPNH, 6 mg; $CoCl_2$, 1 mg; 5,6-dimethylbenzimidazole, 1 mg; cysteine, 1 mg; mercaptoethanol, 5 μl; 16 hr, 37°. [b] Total radioactivity in isolated heptamethyl ester of cobyrinic acid divided by total radioactivity in the substrate multiplied by 100. [c] The protein concentration of crude enzyme solution was estimated by the procedure of O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951). [d] Specific activity: 26.2 mCi/mmol. [e] Specific activity: 73.2 mCi/mmol. [f] Specific activity: 4550 mCi/mmol. [g] Specific activity: 52 mCi/mmol. [h] The real yield should be based on urogen III. [i] $^{3}$H/$^{14}$C ratio of the substrates 0.53, $^{3}$H/$^{14}$C ratio of the product 5,3. [j] Incubation conditions as in footnote *a* plus 1.25 mg of ALA. [k] Incubation conditions as in footnote *a* plus 10 mg of SAM.

The presence of ALA dehydratase,[7] urogen I synthetase,[8] urogen III cosynthetase,[9] as well as the complete methylative, reductive, and cobalt inserting enzymes in the crude enzyme mixture can be inferred from the remarkably efficient conversion (*ca.* 23%) of [5-$^{14}$C]ALA to corrins isolated as cobester (experiment 1). Confirmation of the role of urogen III is evident from experiment 3 where the observed radiochemical yield corresponds to at least 6–7% incorporation of urogen III, since the synthetic mixture contains at most 50% of the type III isomer. Previous work has demonstrated that type I, II, and IV urogens are not incorporated into the corrin system. Isolation and identification of the methyl esters of penta-, hexa-, and heptacarboxylporphyrins as well as the copro- and uroporphyrins were carried out by established procedures and these were

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