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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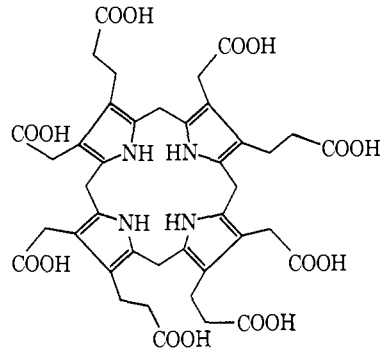
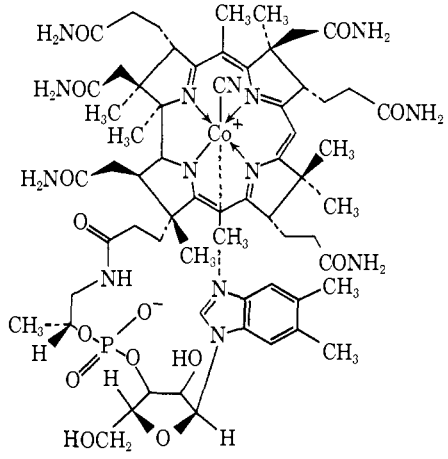
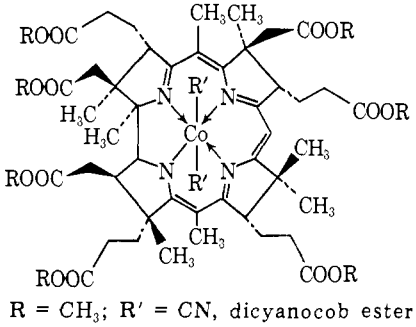


1, uroporphyrinogen III



2, cyanocobalamin



3a, R = $CH_3$; R′ = CN, dicyanocob ester
b, R = H; R′ = OH, diaquocobyrinic acid

found to be radioactive indicating that the decarboxylative enzymes of urogen metabolism are also present in the cell-free system, in common with similar preparations from bacteria,[10a] avian red cells,[10b] mammalian reticulocytes,[10c] and mouse spleen.[10d] Experiment 4 was carried out with [*methyl*-$^3$H]SAM using [$^{14}$C]urogen as internal standard. The by-products of the incubation, *viz.* uro, copro, and the partially decarboxylated porphyrins (as their methyl esters), contained $^{14}$C but no tritium isotope. On the other hand, repeated crystallization of cobester from experiment 4 gave a constant $^3$H/$^{14}$C ratio (in agreement with the separate feeding experiments 3 and 5) providing an internal check that both decarboxylating and methylating systems were operative. Since all of the cell-free assays depend on the purification of cobyrinic acid as the crystalline heptamethyl ester, experiment 4 removes any ambiguity of *in vitro* methyl transfer in the esterification process and also shows that no secondary incorporation of $^3$H from [*methyl*-$^3$H]SAM occurs during the incubation.

Experiment 5 demonstrates that, in the presence of added ALA, the incorporation of [*methyl*-$^{14}$C]SAM reaches 36% while the methylating enzyme system is inactivated by boiling (experiment 6).

Using an entirely different assay procedure, the actual biosynthesized corrin was found to be cobyrinic acid (**3b**) (experiment 3). The post-incubation mixture was subjected to phenol extraction after treatment with corrin mixture (as carrier) and the purified solution was separated by electrophoresis (Whatman 3MM and ET 81) and ion exchange paper chromatography (Whatman ET 81). Autoradiographs showed cobyrinic acid to be the only detectable radioactive corrin in all of these separations.

In summary, the stable crude enzyme mixture described in this communication contains the requisite system for converting both ALA and urogen III to cobyrinic acid (**3b**), the prototype of the more complex corrins, in the presence of SAM.

With the establishment of the comparatively rapid assay technique described herein, separation of the component synthetase(s) responsible for the intriguing steps between urogen III and cobyrinic acid is now in progress.

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## Determination of Relative Glycosyl Bond Strengths in Nucleosides by Chemical Ionization Mass Spectrometry. A Comparative Study of 7- and 9-β-D-Ribofuranosylpurines

*Sir:*

Several in-depth studies have been recently published of the acid-catalyzed hydrolysis of purine nucleosides.[1-4] It has been suggested[1-4] that these nucleosides hydrolyze by an A-1 mechanism which involves a preequilibrium protonation of the purine followed by a rate-limiting cleavage of the ribosyl–purine bond.

A similar reaction of protonation followed by cleavage of the glycosyl bond has been shown to occur in the vapor phase.[5] The ion–molecule reaction has been studied by chemical ionization mass spectrometry (CIMS)[6,7] and provides a highly effective means of following the reactions of protonated species in the absence of solvent effects, under carefully controlled conditions.

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