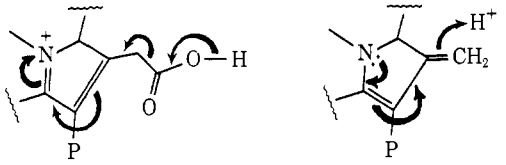
The implications of these results[14] in terms of mechanistic rationale are discussed in the following communication.

**Acknowledgment.** We thank the National Science Foundation and the National Institutes of Health (Grant RR-00356) for support of this work.

wise block a process such as, *e.g.*



(13) J. Lascelles, "Tetrapyrrole Biosynthesis and Its Regulation," W. A. Benjamin, New York, N. Y., 1964.

(14) Essentially identical results using [5-$^{13}$C]-ALA have been obtained by Professor D. Shemin (personal communication). See C. E. Brown, J. J. Katz, and D. Shemin, *Proc. Nat. Acad. Sci. U. S.*, **69**, 2585 (1972).

(15) Carbon-13 Fourier Transform Nuclear Magnetic Resonance. IV.

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*Received August 28, 1972*

## Biosynthesis of Corrinoids. Uroporphyrinogen III as a Precursor of Vitamin $B_{12}$

*Sir:*

In considering the plethora of mechanistic proposals[1] for the conversion[2] of porphobilinogen (**1**, PBG) to vitamin $B_{12}$ (**2**) we have been guided by the essential simplicity, symmetry, and energetic economy of a scheme[1,3] wherein the cobalt (corrin), iron (heme), and magnesium (chlorophyll) pathways diverge *after* the formation of uroporphyrinogen III (**3**, urogen III). Regardless of the details of urogen III formation, an experimental distinction can be made between the intermediacy of **3** and the ingenious corrin synthetase mechanism of Corwin[4] (Scheme I, path A) which bypasses the urogens to form the ring A → D corrin linkage directly. At the same time many of the other interesting hypotheses[1] which involve the A → D linkage at an earlier assembly stage could be discarded if proof for the intervention of urogen III were forthcoming. Recent feeding experiments, however, with whole cells of *Propionibacterium shermanii* have indicated that

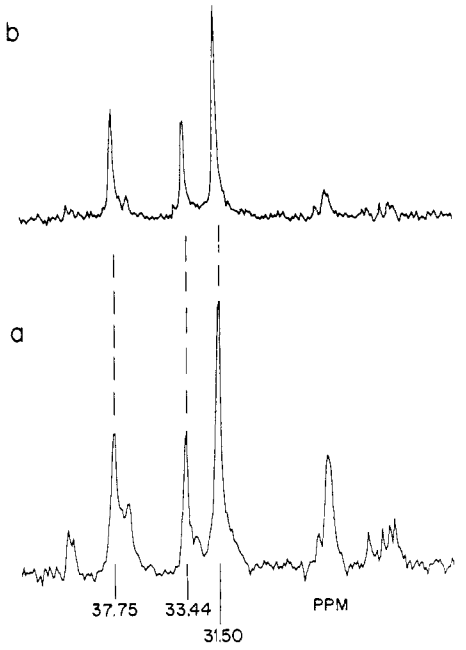
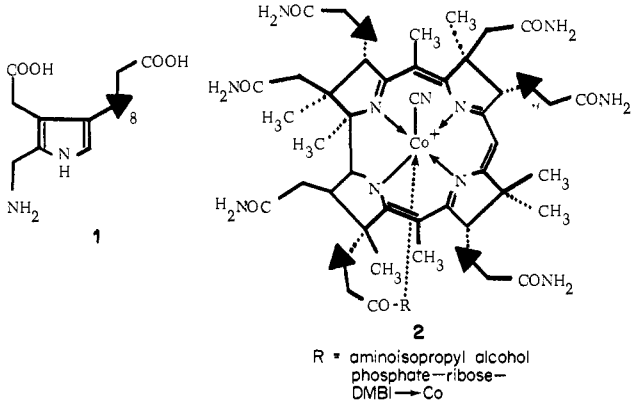



Figure 1. (a) Portion, 44.73–17.22 ppm downfield from external HMDS [the external $^{19}$F lock ($C_6F_6$) and $^{13}$C reference (hexamethyldisilane, HMDS) were contained in a 5-mm sample tube mounted coaxially in a 10-mm sample tube containing the aqueous cyanocobalamin solutions; the $^{13}$C FT spectra were determined at ambient probe temperature (40–45°) using a computer-controlled FT system previously described (R. J. Cushley, D. R. Anderson, and S. R. Lipsky, *Anal. Chem.*, **43**, 1281 (1971))] of the proton noise-decoupled $^{13}$C FT spectrum of 41 mg of [8-$^{13}$C]-PBG enriched cyanocobalamin (vitamin $B_{12}$, **2**) in $H_2O$; data set = 4K points; digitizing rate = 10 kHz; pulse width = 50 μsec; receiver skip = 100 μsec (Cushley, *et al.*). (b) Portion, 44.73–17.22 ppm downfield from external HMDS, of the proton noise-decoupled $^{13}$C FT spectrum of 40 mg of [$^{13}$C]urogen enriched cyanocobalamin (**2**) in $H_2O$; same conditions as for spectrum 1a.

virtually no specific incorporation of enzymically[5] or chemically[6] synthesized [$^{14}$C]urogen III could be observed. We believe that these negative results may be attributed to the conditions of the feeding experiment and although valid for the concentrations and/or pH, aeration, heat treatment, and cellular ages specified, may be contrasted with the successful incorporations described in this communication.




(1) B. F. Burnham in "Metabolic Pathways," 3rd ed, Vol. III, D. M. Greenburg, Ed., Academic Press, New York, N. Y., 1969, Chapter 18.

(2) S. Schwartz, K. Ikeda, I. M. Miller, and C. J. Watson, *Science*, **129**, 40 (1959). The bioconversion of PBG to vitamin $B_{12}$ has been assumed from the isolation of radioactive $B_{12}$ from a feeding experiment. No degradations to locate the label were performed but the assumption seems reasonable on the basis of the specific, nonrandomized incorporation of various $^{14}$C radiomers of δ-aminolevulinic acid into the corrin molecule, since the latter amino acid is a well proven precursor of PBG [D. Nandi and D. Shemin, *J. Biol. Chem.*, **243**, 1224, 1231, 1236 (1968)].

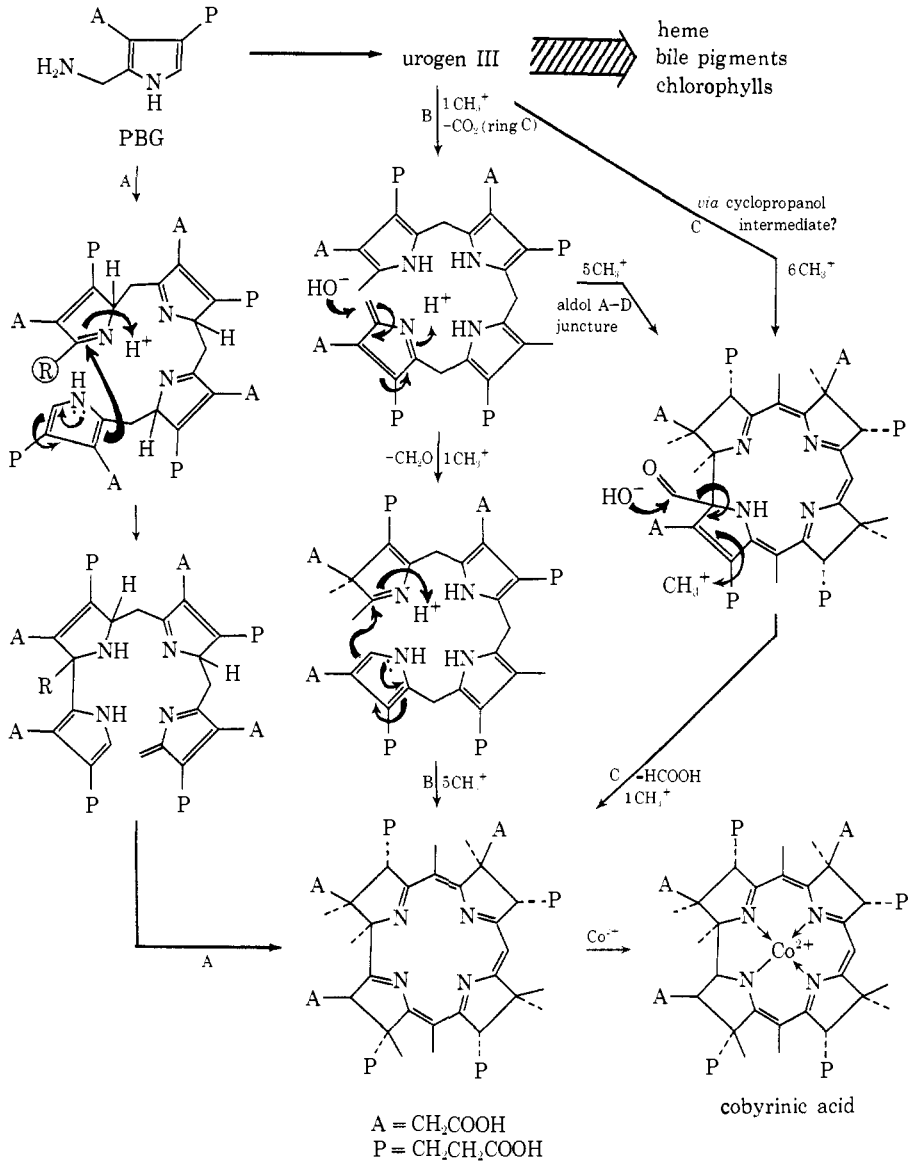
(3) B. F. Burnham and R. A. Plane, *Biochem. J.*, **98**, 13c (1966). The isolation of urogen III from vitamin $B_{12}$ producing organisms[1] lends further support to this concept.

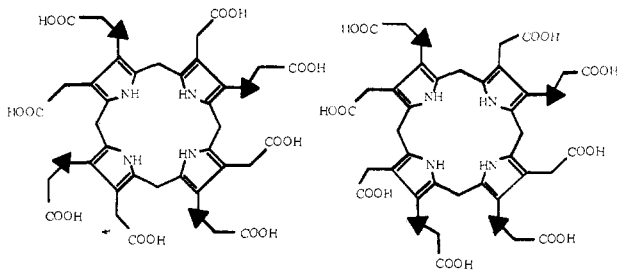
(4) J. H. Mathewson and A. H. Corwin, *J. Amer. Chem. Soc.*, **83**, 135 (1961).

(5) G. Müller and W. Dieterle, *Hoppe-Seyler's Z. Physiol. Chem.*, **352**, 143 (1971).

(6) B. Franck, D. Gantz, and F. Hüper, *Angew. Chem., Int. Ed. Engl.*, 11, 421 (1972).

**Scheme I**



4 3

Urogen II: AP-PA-AP-PA
Urogen IV: AP-AP-PA-PA

A = $-CH_2COOH$
P = $-CH_2CH_2COOH$

Using freshly harvested cells of *Propionibacterium shermanii* ATCC 9614 (25–30 g) suspended in degassed phosphate buffer (100 ml) containing $CoCl_2$ (1 mg), methionine (20 mg), 5,6-dimethylbenzimidazole (20 mg), and glucose (1.6 g), specific incorporations of chemically (expt 2)[7] and enzymically (expts 5 and 6)[8] synthesized [$^{14}$C]urogen III (**3**) were obtained as recorded in Table I. Experiment 5 was run with 100 $\mu$g of a urogen III–I mixture which approximates to the previously reported conditions[5] in which very low (but not zero) incorporation occurs. The resultant cyanocobalamin (**2**), after extensive purification and recrystallization to constant radioactivity, was degraded by hydrolysis and Kuhn–Roth oxidation and showed that none of the original $^{14}$C label (from [8-$^{14}$C]-PBG) had been randomized into the nucleotide segment, or into those carbons ($CH_3C$–) which afford acetic acid on oxidation. Although the $^{14}$C label was not directly located by this experiment it could be inferred that the positions shown in **2** are labeled and

(7) (a) [8-$^{14}$C]-PBG was prepared by a modification of Rapoport's method[7b] using [$^{14}$C]formaldehyde. The purified PBG was converted to the statistical mixture of urogens I (12.5%), II (12.5%), III (50%), and IV (25%) in 80% yield by Mauzerall's technique[7c] and the isomer ratio estimated by the standard assay.[7d] The absence of any remaining PBG was demonstrated by preparative electrophoresis and the purity of the preparation checked by paper chromatography and autoradiography. (b) B. Frydman, S. Reil, M. Despuy, and H. Rapoport, *J. Amer. Chem. Soc.*, **91**, 2338 (1969). (c) D. Mauzerall, *ibid.*, **82**, 2601 (1960). (d) L. Eriksen, *Scand. J. Clin. Lab. Invest.*, **10**, 319 (1958).

(8) L. Bogorad, *Methods Enzymol.*, **5**, 891 (1962). It is essential to use a wheat germ source which has not been subjected to heat treatment; otherwise the main product of the incubation is urogen I. We thank Professor L. Bogorad for valuable information on this and related techniques with the spinach enzyme.

the evidence is clearly in favor of specific and intact incorporation of urogen III.

Table I. $^{14}C$ Feeding Experiments Using Suspended Cells of *P. shermanii* ATCC No. 9614

| Expt | Substrate fed | mg fed | Hr | Spec incorp/C |
|---|---|---|---|---|
| 1 | PBG | 21 | 70 | 5.1 |
| 2 | Urogens I–IV | 25 | 70 | 0.91 |
| 3 | Urogen I | 7 | 40 | 0.000 |
| 4 | Urogen I[a] | 25 | 70 | 0.017 |
| 5 | Urogens III + I | 0.1 | 70 | 0.0052 |
| 6 | Urogens III + I | 24 | 70 | 0.40 |
| 7 | Uroporphyrins I–IV | 34 | 60 | 0.000 |

[a] See ref 10.

On the other hand urogen I (**4**) (prepared by the spinach synthetase procedure[9]) gave either zero or very low[10] incorporation when administered in carefully monitored parallel feeding experiments (Table I; expts 3 and 4).

With proof for the inertness of urogen I in hand, any concern over the use of urogen III–I mixture (70:30 to 50:50) obtained from the wheat germ preparation can be discounted as can the use (expt 2) of the chemically synthesized statistical mixture of the types I–IV isomers which contain 50% of the type III isomer together with 12.5% of type I, the remainder being biologically inert types II and IV isomers. In conformity with heme and chlorophyll biosynthesis,[1] no incorporation of the same statistical mixture of the [$^{14}C$]uroporphyrins I–IV (from which urogens I–IV were prepared) was observed (expt 7).

In order to confirm these results without recourse to the tedium of carbon-by-carbon degradation of the vitamin, the experiment was repeated using [8-$^{13}C$]-PBG and the urogen I–IV isomers labeled with $^{13}C$ (90% enrichment per carbon) as shown above. A preliminary report[2] that PBG was specifically incorporated could be confirmed by using first [8-$^{14}C$]-PBG (Table I, expt 1; ~5% specific incorporation) and then [8-$^{13}C$]-PBG. The $^{13}C$ FT nmr spectrum of [8-$^{13}C$]-PBG enriched vitamin $B_{12}$ is shown in Figure 1a. The spectrum contains three resonances at 37.75, 33.44, and 31.50 ppm arising from four enriched centers. That four enriched carbons were actually present was demonstrated by conversion of the vitamin to the dicyano form. The sharp signal at 31.50 ppm was cleanly resolved and the $^{13}C$ spectrum showed four enhanced peaks of equal intensity. When the [$^{13}C$]-urogen isomers were administered to *P. shermanii* (~12 g of cells/100 ml of medium per flask) and the resultant vitamin $B_{12}$ was subjected to similar $^{13}C$ nmr analysis, the enriched spectrum showed enhancement[11] (Figure 1b) (~6%) of the *same set of four methylene* carbons as were labeled in the [$^{13}C$]-PBG experiment.

These results together with the enrichment data for [$^{13}C$]-ALA[12] not only corroborate positive incorporation with carbon-14 but also provide unequivocal evidence for the location of the label, and strongly support the sequence PBG → urogen III → vitamin $B_{12}$ in *P. shermanii*. Thus, with the important *proviso* that sufficient substrate must be present to permeate the cell wall and to survive *in vitro* oxidative destruction it is our view that vitamin $B_{12}$ is produced by a reductive contraction of urogen III.

A summary of several hypothetical routes from urogen III to the corrinoids is given in Scheme I, paths B and C, both of which imply a hydrolytic ring contraction. Further delineation of these and other alternatives must await the results of cell free experiments which are now in progress.

**Acknowledgment.** We thank the National Institutes of Health (Grant RR-00356) and the National Science Foundation and Merck & Co. for support of this work.

(9) L. Bogorad, *Methods Enzymol.*, **5**, 855 (1962).

(10) In fact the apparent, small incorporations of the type I isomer (*ca.* 5% of urogen III incorporation) could be traced to minor amounts of the type III isomer present in the spinach enzyme preparation when run for more than 6–8 hr. This effect has been observed during prolonged incubations with this enzyme (Professor L. Bogorad, private communication).

(11) Reference to Table I indicates that the specific incorporation of the [$^{14}C$]urogen mixture is 0.91%. This experiment utilized *ca.* 25 g of cells/100 ml of medium. In several runs in which the cell weight was reduced to 10–12 g, incorporations of 2–3% were observed. The [$^{13}C$]-urogen isomers (780 mg; 90% $^{13}C$) were therefore administered in high concentration (3.6 l.) to a relatively small cell mass (400 g) and incubated under very strict anerobic conditions in stoppered vessels for 70 hr with pH adjustment (to 7.0) each day.

(12) A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, P. J. Whitman, and R. J. Cushley, *J. Amer. Chem. Soc.*, **94**, 8267 (1972).

(13) Carbon-13 Fourier Transform Nuclear Magnetic Resonance. V.

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## Nucleophilic Reactions of Cationic Transition Metal *Dihapto*allene Complexes

*Sir:*

There has been considerable current interest in nucleophilic reactions of hydrocarbon ligands which are polyhapto bonded to transition metals.[1] We now have extended investigations on such reactions to the previously unexplored *dihapto*allene complexes.[2] Communicated herein are the remarkable reactivity of $[h^5\text{-}C_5H_5Fe(CO)_2(h^2\text{-}CH_2{=}C{=}CHR)]^+$ toward several nucleophiles and the relevance of these processes to the mechanism of metal-promoted cycloaddition reactions of 2-alkynyl complexes.[3]

The cations **1a** and **1b**[3,4] have been obtained as the crystalline $BF_4^-$ salts by treatment of the corresponding $h^5\text{-}C_5H_5Fe(CO)_2CH_2C{\equiv}CR$ with $HBF_4$ in $(CH_3CO)_2O$.

(1) See, for example, (a) L. Busetto, A. Palazzi, R. Ros, and U. Belluco, *J. Organometal. Chem.*, **25**, 207 (1970); (b) P. J. C. Walker and R. J. Mawby, *Inorg. Chem.*, 10, 404 (1971); (c) F. Haque, J. Miller, P. L. Pauson, and J. B. Pd. Tripathi, *J. Chem. Soc. C*, 743 (1971); (d) M. H. Chisholm, H. C. Clark, and L. E. Manzer, *Inorg. Chem.*, **11**, 1269 (1972).

(2) Although nucleophilic additions to a coordinated *dihapto*allene have not been investigated for isolable metal complexes, such reactions have been reported for some transient intermediates. Accordingly, products of reactions of several palladium(II) complexes ($PdX_2$) with allene most certainly arise *via* the formation of a metal-$h^2$-allene intermediate followed by addition of X to the coordinated $CH_2{=}C{=}CH_2$. For a summary of these reactions, see P. M. Maitlis, "The Organic Chemistry of Palladium," Vol. I, Academic Press, New York, N. Y., 1971, pp 188–191, 248–249; Vol. II, pp 41–42.

(3) D. W. Lichtenberg and A. Wojcicki, *J. Organometal Chem.*, **33**, C77 (1971).

(4) J. Benaim, J.-Y. Merour, and J.-L. Roustan, *C. R. Acad. Sci., Ser. C*, **272**, 789 (1971).