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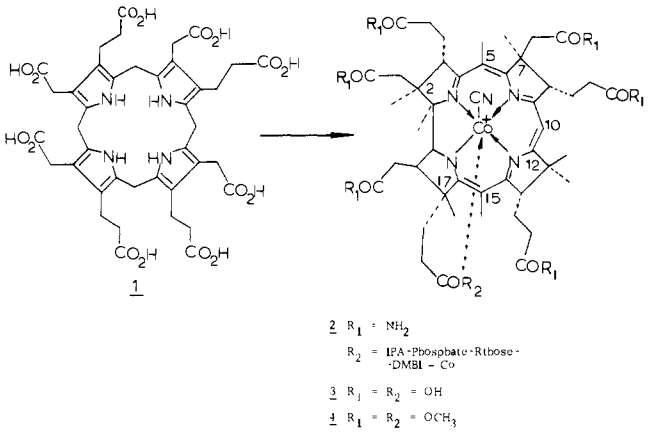
Concerning the Intermediacy of Uro'gen III and of a Heptacarboxylic Uro'gen in Corrinoid Biosynthesis

Sir:

Previous work from this laboratory has provided evidence that in whole-cell[1,2] and cell-free[3,4] systems, the enzymes of *Propionibacterium shermanii* utilize uro'gen III (**1**) for the production of cyanocobalamin[1,2] (**2**) via cobyrinic acid[3,4] (**3**). These experiments employed side-chain labeled samples of uro'gen III obtained both by chemical synthesis (in admixture with types I, II, and IV uro'gens) and enzymatic preparation (together with uro'gen I). It was clearly demonstrated in experiments with ^{13}C-enriched substrates that the label in the side-chain propionate groups was carried to the corresponding positions in the appropriate corrin. However,



the intrinsic symmetry of these labeling patterns together with the problems of employing an isomer mixture left open the logical (if unlikely) possibility that in vitro dissociation of the uro'gen into labeled fragments capable of assimilation by the enzyme system could give rise to the observed regiospecific enrichments *without mediation of the intact uro'gen III molecule.* In order to resolve this question of vital importance for the mechanism of corrin biosynthesis,[2,5] we have undertaken the regiospecific synthesis of a set of uro'gens whose patterns of enrichment with both stable and radioisotopes are designed to provide unambiguous probes for intact biotransformation and for the nature of the overall mechanism connecting the uro'gen and corrin structures.

The regiospecific syntheses of [α,γ-$^{14}C_2$]- and of ring B propionic acid [$^3H_2$]uro'gen III were carried out by the procedures of MacDonald[6] and Franck,[7] modified where appropriate for the introduction of radioisotope.[8] Incubation of the doubly labeled uro'gen ($^3H/^{14}C$, 4.10; Scheme I) in the cell-free system from *P. shermanii*[9] gave, after dilution with carrier, conversion to cobester (**4**), and crystallization to constant activity, a sample of cobester (**4**) with $^3H/^{14}C$

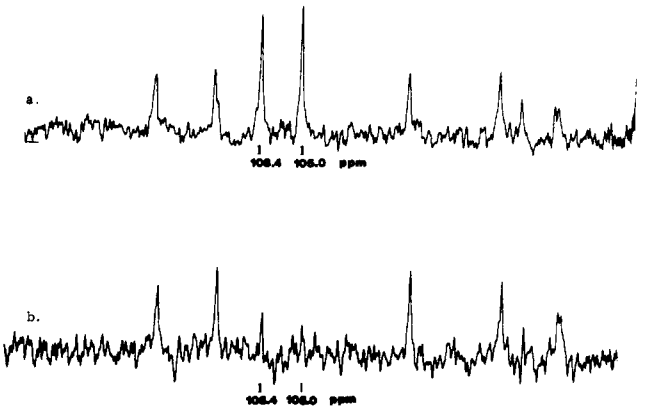
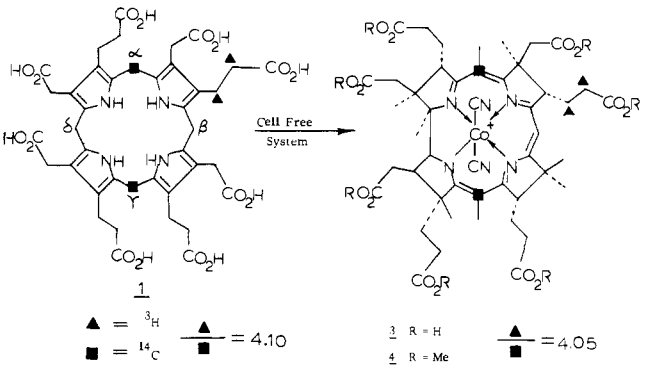
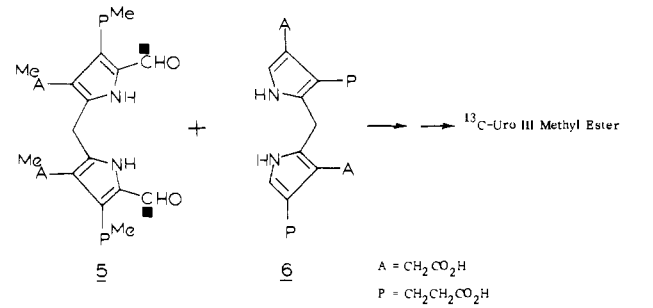


Figure 1. (a) Proton noise-decoupled ^{13}C-FT NMR spectrum of [^{13}C]uro'gen III enriched cyanocobalamin (D_2O; 4K points) and assigned labeling patterns. (b) Proton noise-decoupled ^{13}C-FT NMR spectrum of natural abundance cyanocobalamin (D_2O; 4K points).

Scheme I

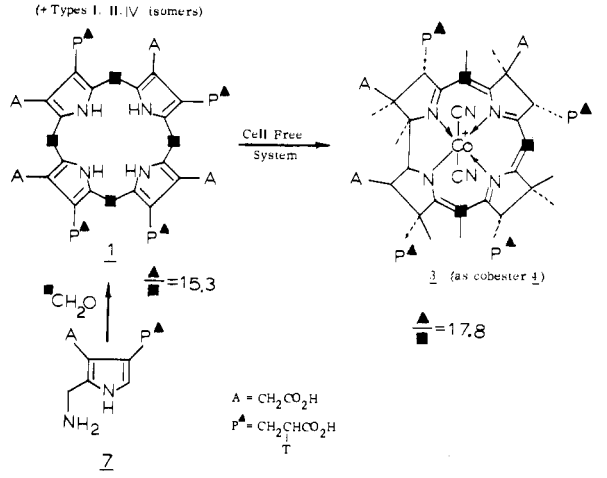


4.05. Any randomization via fragmentation-recombination would have led, in the case of this unsymmetrically labeled substrate, to a profound change in the tritium-carbon ratio. To confirm this result and at the same time locate the site of label in the corrin, a specimen of [α,γ-$^{13}C_2$]uro'gen III was prepared via condensation of the dipyrromethane dial-



dehyde (**5**) and dipyrromethane (**6**), with introduction of ^{13}C from dimethylformamide (90% ^{13}C) by a procedure established above for the synthesis of the ^{14}C radiomer to give finally a sample of the α,γ-^{13}C-enriched uro'gen (90% ^{13}C). Administration of 365 mg of this "north-south" labeled substrate to resting whole cells of 340 g of *P. shermanii*[10] *gave (after the usual work-up)*[2,11] *pure cyanocobalamin (11 mg)* (**2**) whose FT ^{13}C NMR spectrum (Figure 1a) on comparison with the natural abundance spectrum taken under identical conditions (Figure 1b) revealed enhancement (4.5% specific incorporation) at only two resonances in the sp^2 region, viz., at 105.0 and 108.4 ppm downfield from Me_4Si. These signals had previously been assigned to C_{15} and C_5, respectively, both by the correlations of Allerhand[12] and by biosynthetic labeling.[1,2] This experiment

Scheme II



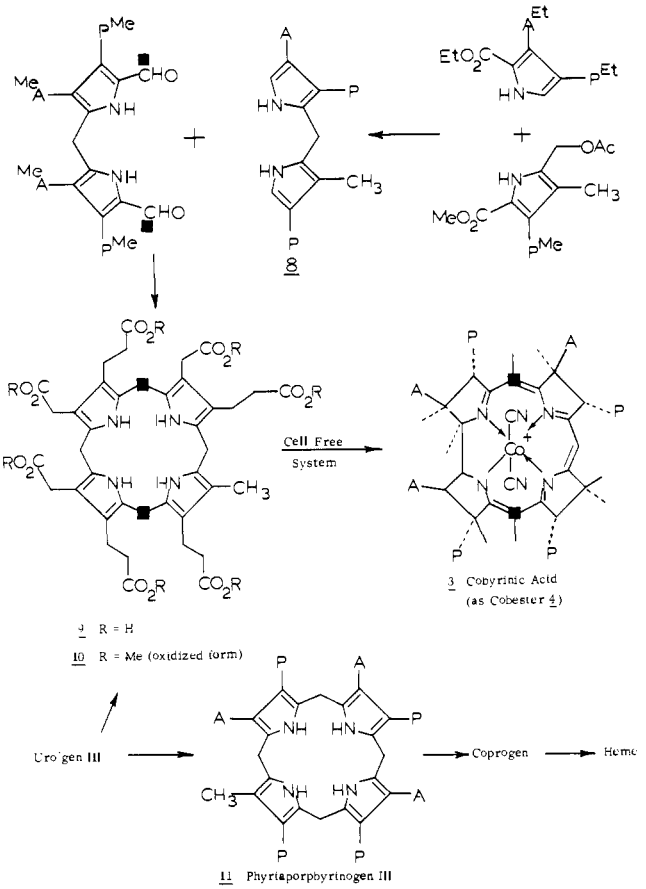
confirms the intact incorporation of doubly labeled **1** and unambiguously locates the labeled sites according to Scheme I where ■ now denotes both ^{14}C and ^{13}C.

A complementary set of experiments was then conducted with a different, multiply labeled version of uro'gen III obtained (in admixture with the types I, II, IV isomers) by in vitro condensation at pH 7.6 of [9-^{3}H]porphobilinogen (**7**) and [^{14}C]formaldehyde followed by acidic equilibration[13] of the meso ^{14}C label (see Scheme II). Incubation of this substrate ($^3H/^{14}C$ 15.3 in recovered uroporphyrin)[14] with the cell-free preparation, recovery, and work-up in the usual way gave cobester (**4**) with $^3H/^{14}C$ = 17.8, a value which is 10% below that expected (20.4) on the basis of complete loss of the "western" (δ) meso carbon from the system as formaldehyde, formate,[2,15,16] or possibly CO_2. Thus although strict stoichiometry was not realized in this experiment, there is a clear trend toward a ratio representing the loss of the δ-meso carbon from uro'gen III.[16-18]

Finally, in order to test the earlier hypothesis[3] that decarboxylation of the acetic acid side chain in ring C takes place at the uro'gen level, a regiospecific total synthesis of the type III heptacarboxylic acid (**9**) was carried out as summarized in Scheme III. The melting point (238–240°) of the heptamethyl ester (**10**) was in excellent agreement with that reported by Battersby et al.[19] for this isomer (prepared by an analogous route), and spectroscopic and mixture melting point comparison confirmed their identity.[19] The synthesis was repeated (using ^{14}C-dimethylformamide) and the resultant [α,γ-$^{14}C_2$]heptacarboxylic uro'gen III incubated with the cell-free system[3] to afford (after crystallization to constant activity) cobester (**4**) (0.1% incorporation).[20] This experiment provides the first clear indication that the hepta acid (**9**) is an intermediate in corrin biosynthesis (Scheme III) and that uro'gen III suffers decarboxylation *prior to* the reductive methylation sequence necessary to generate the rearranged corrin structure. It is of considerable interest to note that, since phyriaporphyrinogen III[19] (**11**) is considered to be the obligatory biosynthetic precursor for coprogen and heme, the new isomer (**9**) represents the branchpoint at which the heme and corrin pathways, having shared a common route from glycine and succinate to uro'gen III, diverge.[21]

Acknowledgment. We thank Ms. J. Petrillo and Mr. A. Brown for skilled technical assistance, Drs. A. Brossi and W. Leimgruber (Hoffmann-LaRoche) for kindly supplying starting materials for uro'gen synthesis, and NIH (Grant AM 17014) for generous support.

Scheme III



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(8) Full details of the methodology of the isotopic labeling of the synthetic intermediates, which followed in large measure the published procedures for the regiospecific synthesis of uro'gen III with introduction of carbon label via [^{14}C]- or [^{13}C]dimethylformamide, will be given elsewhere.
(9) Incubation mixtures contain 1.2 ml of 100,000 g of supernatant (10 mg of protein; prepared as described in ref 3 and 4), 100 μM K_3PO_4 buffer (pH 7.6), 10 μM $CoCl_2$, 1.4 μM *S*-adenosylmethionine, 8 μM dithiothreitol, 2.4 μM glutathione, and 0.3 μM uro'gen III (4.10 μCi in ^{3}H, 1.0 μCi in ^{14}C; prepared from uro III by sodium amalgam reduction) in a total volume of 2.0 ml. Tubes were incubated in vacuo and in the dark at 34° in a shaking water bath. After 12 hr of incubation, air was admitted into the tubes, and the incubation mixtures were worked up as described earlier to afford, after 3 TLC's and recrystallization to constant specific activity and ratio, cobester (8.20×10^{-3} μCi in ^{3}H; $^3H/^{14}C$ = 4.05).
(10) The conditions of the whole cell feeding experiments are critical and are available both as a summary[2] and in full detail.[11]
(11) A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, R. J. Cushley, and P. J. Whitman, *J. Am. Chem. Soc.*, **96**, 8069 (1974).
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(14) Determined after incubation and recovery by established procedures.[11]
(15) R. C. Bray and D. Shemin, *J. Biol. Chem.*, **238**, 1501 (1963). These authors noted that in a secondary process some radioactivity (ca. 10%) from carbon-5 of δ-aminolevulinic acid is returned to the methyl groups of corrin via "C_1" metabolism.
(16) Careful control experiments on the nature of the extruded, δ-meso carbon of uro'gen III indicate that there is a high "blank" for the in vitro escape of formaldehyde[17] from all four meso positions, but only in the case of the ^{14}C-labeled δ position is there evidence for some return of radioactivity to cobyrinic acid as revealed by (a) a $^3H/^{14}C$ ratio in the derived cobester some 10% lower than required by theory, (b) an unchanged $^3H/^{14}C$ ratio in the [α,γ-$^{14}C_2$]-labeled experiment, and (c) recovery of ca. 50% of the expected [^{14}C]formaldehyde (as the dime-

done derivative) in the experiment where the [$\alpha,\beta,\gamma,\delta$-$^{14}C_4$]uro'gen mixture was used.

(17) Prolonged incubation or heat treatment of any of the ^{14}C-meso labeled uro'gen species described in this communication led to extensive decomposition to $^{14}CH_2O$ as determined by dimedone trapping and crystallization of the adduct to constant activity.

(18) In one experiment, the uro'gen mixture was isolated from the [$^{14}CH_2O$-^{3}H]porphobilinogen condensation without acid-catalyzed equilibration. When this sample ($^{3}H/^{14}C$, 15.2) was incubated with the complete enzyme system as before, the resultant cobester showed little change in $^{3}H/^{14}C$ ratio (16.0) indicating that the δ position has been exchanged with $^{14}CH_2O$ to a minor extent compared with the other (α,β,γ) positions, and also that the mechanism of in vitro synthesis of uro'gens[13] is worthy of reexamination.

(19) A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine, III, F. Satch, and J. Saunders, *J. Chem. Soc., Chem. Commun.*, 994 (1974). We thank Professor Battersby (Cambridge) for exchanging samples of synthetic **10** and for confirming their identity.

(20) Administration of [α,γ-$^{14}C_2$]-(**9**) to whole cells of *P. shermanii* afforded cyanocobalamin (**2**) with a specific incorporation of 1.0% after crystallization to constant activity.

(21) The role of intact, unsymmetrically labeled uro'gen III as a specific precursor for the corrin nucleus has recently been confirmed at Cambridge by Professor A. R. Battersby and coworkers (private communication, Mar 10, 1975).

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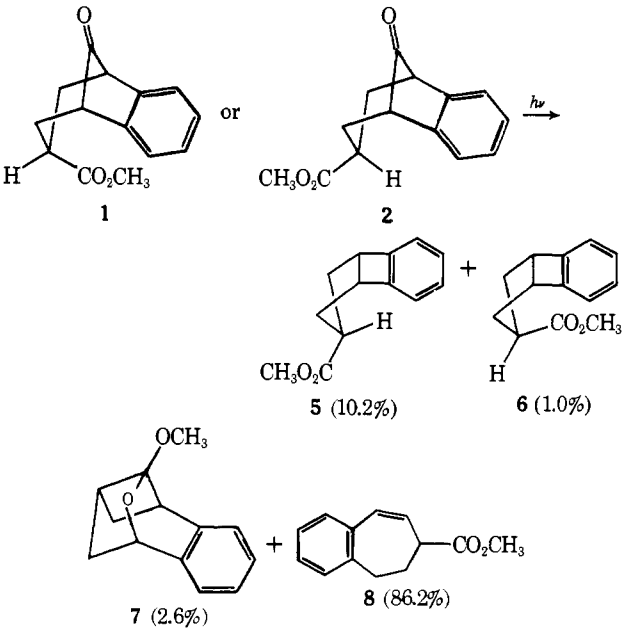
Photodecarbonylation of Methyl Benzobicyclo[3.2.1]octen-8-one-3-*exo*- and -*endo*-carboxylate. Orbital Symmetry Control in the Chemistry of a Twisted *o*-Quinodimethane

Sir:

Photochemical elimination of carbon monoxide or carbon dioxide from appropriate precursors has been successfully used to produce *o*-quinodimethanes.[1] Photodecarbonylations of 2-indanones have been studied by Quinkert and coworkers.[1a] Their work focused mainly on phenyl substituted 2-indanones where for example it was elegantly shown that *cis*- and *trans*-1,3-diphenyl-2-indanone afforded diphenyl substituted *o*-quinodimethanes in a two step nonstereospecific decarbonylation from the singlet state. These underwent subsequent thermal and photochemical reactions but could be observed by low temperature ultraviolet spectroscopy and trapped with dienophiles. Jones and coworkers have examined the photochemical production of relatively strain-free *o*-quinodimethanes related to 2,3-dihydronaphthalene.[1b] These molecules were found to be detectable by ultraviolet spectroscopy if the facile 1,5-suprafacial hydrogen shift was prevented by appropriate substitution and isolable if in addition 1,4-diphenyl substitution was present.

We have examined the photochemistry of methyl benzobicyclo[3.2.1]octen-8-one-3-*endo*- and -*exo*-carboxylate, **1** and **2**.[2] Our principal goal was to determine the effects of phenyl substitution absence coupled with structural constraints on the propensity for formation and subsequent reactions of the expected *o*-quinodimethane, **4**. Due to the constraining influence of the three-carbon bridging group **4** must exist in the *Z,Z* configuration and must be substantially twisted. We anticipated that this effect coupled with the absence of phenyl substitution would destabilize **4** relative to the strain-free biradical **3** and a one- or two-step photodecarbonylation would perhaps lead to products derived from the biradical. These reactions might show stereospecificity since inversion of the benzylic radical requires transient formation of **4** and the pendant carbomethoxy group would serve as a nonparticipating stereochemical probe.



Irradiation[3] of **1** or **2** gave rise to several products:[4] methyl benzobicyclo[3.2.0]hepten-3-*exo*- and -*endo*-carboxylate, **5** and **6**, 7-methoxy-2,3-benzo-8-oxatricyclo-[4.2.1.$0^{4,7}$]nonene, **7**, and methyl 1,2-benzo-1,3-cycloheptadiene-5-carboxylate, **8**, in a nonstereospecific reaction; see equation.[5] The reaction is moderately efficient,[6] not sensitized by acetone, and only slightly quenched by *trans*-1,3-pentadiene.[7] These facts indicate that the reaction may occur from the excited singlet state.

Product formation can be rationalized in terms of an intermediate biradical, **3**, or *o*-quinodimethane, **4**, undergoing closure to epimeric bicyclic benzocyclobutenes, **5** and **6**;[1] 1,5-hydrogen shift to **8**,[1,8] or an intramolecular Diels-Alder reaction to **7**.[9]

Experiments in which temperature and excitation wavelength were varied (Tables I and II) showed that **5**, **6**, and **7** are produced in a common reaction independent of formation of **8**. Thus the ratio of **5:6:7** remains essentially constant and irradiation at low temperatures (Table I) or simultaneous irradiation with 366 and 313 nm light (Table II) results in striking decreases in the relative amount of **8**.

Table I. Effect of Temperature on Photochemistry of 1 and 2[a]

Compound	Temp, °C	Solvent	% 8 in product mixture	5:6:7
1 or 2	35	Cyclohexane	86.2	1.00:0.07:0.19
1	0	Cyclohexane–Benzene (1:1)	48[b]	1.00:0.05:0.26
2	0	Cyclohexane–Benzene (1:1)	47[b]	1.00:0.05:0.23
1	−40	Dichloromethane	0	1.00:0.06:0.13
2	−40	Dichloromethene	0	1.00:0.08:0.15

[a] Rayonet reactor 3000 Å lamps. [b] Cyclohexane–benzene (2:1) is the solvent in these experiments.