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Biosynthesis of Vitamin B_{12}. Concerning the Mechanism of the Uro'gen III → Cobyrinic Acid Transformation[1]

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Incubation of [5,15,20-$^{14}C_3$]uro'gen III (*1*) with a cell-free system from *P. shermanii* has revealed that during the formation of cobyrinic acid (*3*) from this substrate C-20 is lost to the medium and can be trapped as the dimedone adduct of formaldehyde. Similarly, [5,15,20-$^{14}C_3$]uro'gen heptacarboxylic acid (**5**) is converted with loss of C-20 to cobyrinic acid although much less efficiently. The significance of these results for the mechanism of corrin biosynthesis is discussed.

Although several of the key intermediates on the biosynthetic pathway to corrins have been identified (*1*, *2*), nothing is yet known about the fascinating but mysterious mechanisms underlying the steps whereby uro'gen III (**1**) is decarboxylated in ring C (*3*), alkylated with seven methionine-derived methyl groups (*2*), and, in a sequence which ruptures the macrocycle by removing the original C-20 unit from (**1**) (*2*), produces a version of secocorrin (*3b*) (*eg.*, **2**) which is poised for recyclization and conversion to cobyrinic acid (**3**). Regardless of the structural details of a species such as **2**, knowledge of the oxidation level of the termini (C-1, C-19) involved in the recyclization step becomes crucial in the design of the mechanistic rationale and hence synthetic and trapping approaches toward the delineation of the true biosynthetic secocorrins. With respect to C-1, three research groups have recently commented (*4*) on the non-exchangeable nature of the C-1 methyl-group protons during the overall operation of the biosynthetic machinery. In this communication we wish to report on the oxidation level of the departing "C_1 unit" as it pertains to current biogenetic theory involving C-19 and also to comment further on the role of heptacarboxylic uro'gen (**5**) in corrin biosynthesis.

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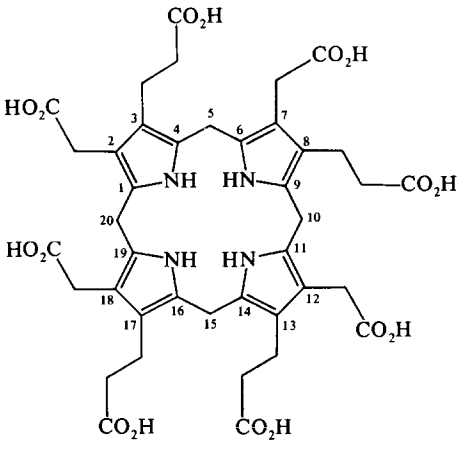
Previous experiments (*2*, *5*) have provided rigorous evidence that C-20 is indeed lost to the medium in *Propionibacterium shermanii* during the process whose carbon balance is summarized in Eq. (1):

$$\text{Uro'gen III (40C)} + \text{methionine (7C)} \xrightarrow[-\text{"C-20"}]{-CO_2} \text{cobyrinic acid (45C)} \qquad (1)$$

Indeed the C-20 unit could depart from the system at any oxidation level between methanol and carbon dioxide. However, many attempts in this laboratory (*5*) to obtain a stoichiometric relationship between capture of such a C_1 unit and cobyrinic acid synthesis in a cell-free system were frustrated by the well-known (*6*) *in vitro* release and recapture of formaldehyde during the equilibration of the types I to IV isomers of the reduced (uro'gen) series at pH 4. Thus the demonstration that "formaldehyde" or its biochemical equivalent is formed enzymically becomes dependent on the development of an incubation/assay system which is virtually devoid of a chemical blank. Using totally synthetic [5,15,20-$^{14}C_3$]uro'gen III (*7*) (Scheme 1) we have devised a



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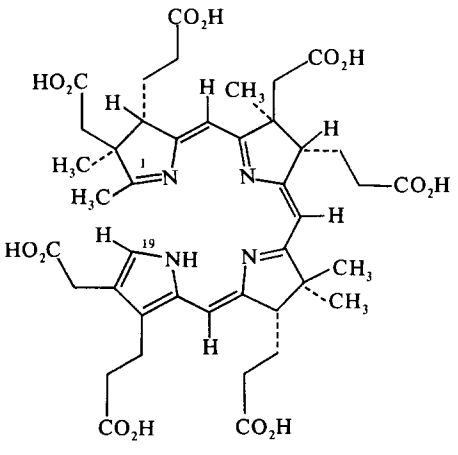

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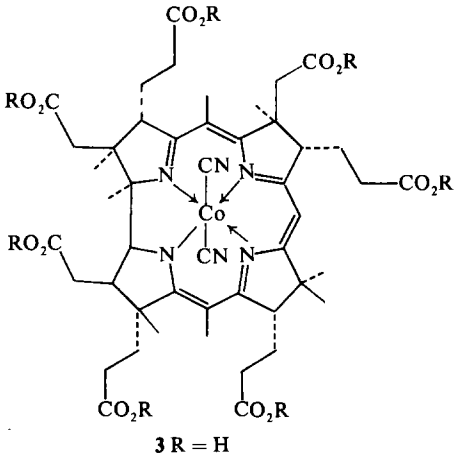


3 R = H
4 R = CH_3

SCHEME 1

TABLE 1
CONVERSION OF URO'GEN SUBSTRATES TO CORRIN, FORMALDEHYDE AND CO_2

Experiment	Substrate	CO_2–Hyamine[a,b] dpm	CH_2O–Dimedone adduct[a,c] dpm	CH_2O–Dimedone adduct[a,c] % Incorporation	Cobester dpm	Cobester % Incorporation
1	[5,15,20-$^{14}C_3$]Uro'gen III[d]	470	2.29×10^5	3.81	2.67×10^5	3.98
2	[5,15,20-$^{14}C_3$]Uro'gen III[e]	667	732	0.012	246	0.0037
3	[5,15,20-$^{14}C_3$]Heptacarboxylic uro'gen[f]	485	3476	0.17	19 250	0.21
4	[5,15,20-$^{14}C_3$]Heptacarboxylic uro'gen[e]	700	135	0.006	574	0.0062

[a] After incubation, the mixture was oxidized by I_2 solution and the pH was adjusted to 4.5 with 0.6 ml of 3*N* HCl before injecting 1.5 ml of saturated dimedone solution (MeOH : H_2O, 1 : 1). After 3 hr, 1 ml of 37% formaldehyde was added and the mixture was set aside at room temperature overnight.

[b] A flask containing a dipper of 2.0 ml of hyamine was evacuated, attached to the incubation flask, and the pressure was equilibrated. Nitrogen was then bubbled for 10 min through the incubation mixture. The mechanistic origin of this small (but constant) $^{14}CO_2$ value is being studied.

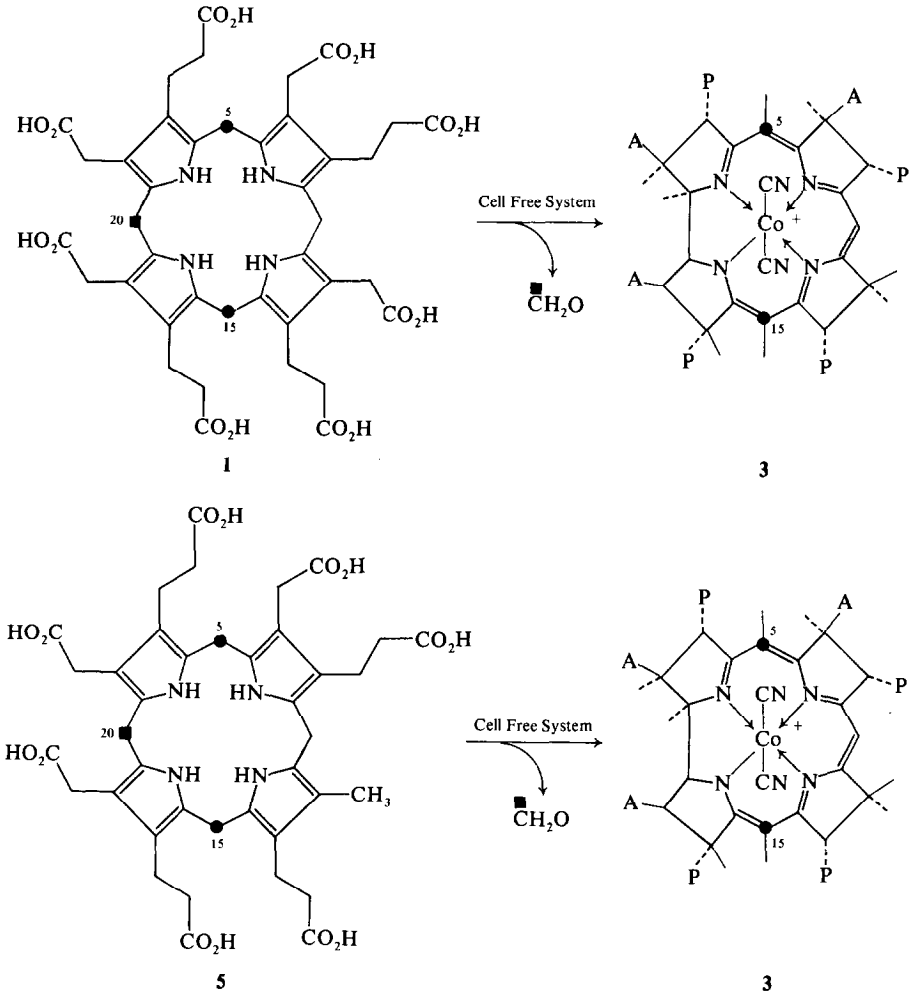
[c] After CO_2 trapping, the formaldehyde–dimedone adduct was extracted with chloroform, purified by tlc, and recrystallized to constant activity (mp 189–191°).

[d] Each incubation contained 16 ml of protein in a 37 000 *g* supernatant of *P. shermanii* homogenate prepared by sonication. Additives were dissolved in 3.5 ml of 0.1 *M* degassed potassium phosphate buffer (pH 7.6): GSH, 39.0 μmol; NADH, 28.1 μmol; $COCl_2 6H_2O$, 12.6 μmol; DTT, 51.8 μmol; $MgCl_2 6H_2O$, 49.1 μmol; and SAM, 34.4 μmol. A solution of radioactive uro'gen III (2 ml; prepared by reducing radioactive uro'gen III solution with 3% sodium amalgam) was added to the incubation. The final mixture (21.5 ml, pH 7.6) was incubated anaerobically at 37°C for 16 hr in the dark. The incubation mixture contained a total of 0.79 μmol of [5,15, and 20-$^{14}C_3$]uro ([5,15-$^{14}C_2$], 6.7×10^6 dpm; [20-$^{14}C_1$], 6.00×10^6 dpm, respectively).

[e] In Experiments 2 and 4, boiled enzyme controls using identical conditions and the same amounts of additives and substrates were run.

[f] Conditions as in Experiment 1 using 2.5 ml of a solution of heptacarboxylic uro'gen containing 1.68 μmol of [5,15, and 20-$^{14}C_3$]hepta ([5,15-$^{14}C_2$], 9.2×10^6 dpm; [20-$^{14}C_1$], 2.1×10^6 dpm, respectively).

reproducible protocol in which the unconsumed uro'gen III in an aliquot of the post-incubation mixture is oxidized to uroporphyrin III and the formaldehyde–dimedone adduct is isolated subsequently from this preparation at pH 4. A second aliquot is analyzed in the usual way (*10*) for cobyrinic acid synthesis (conversion to cobester **4**, crystallization to constant activity). The results of these experiments are shown in Table 1, where it can be seen (Experiment 1) that not only do the formaldehyde–dimedone and cobester conversions correlate well, but, most importantly, the removal of enzyme (Experiment 2) generates a negligible amount of "C_1 unit." These results are to be contrasted with typical runs where dimedone is added to the incubation mixture and the pH is adjusted to 4, without prior oxidation. In these experiments no correlation of the formaldehyde number with cobester yield was observed. It is now safe to conclude that the extruded C-20 carbon of uro'gen III is trapped at the oxidation level of formaldehyde. Since the evolution of free formaldehyde is a rare biochemical event, the



1 3 5 3

SCHEME 2

intervention of a tetrahydrofolate-mediated reaction (*11*) becomes an attractive possibility which can now be tested, albeit by indirect biochemical methodology (*12*) requiring the preparation of chiral versions of **1** and a coupled enzyme assay to distinguish the stereochemical fate of the prochiral center at C-20.

Turning to the timing of the loss of the second carbon from uro'gen III, as CO_2 from the ring C acetate side chain of **1** [see Eq. (1)], previous work had shown (without location of the final ^{14}C label) that the heptacarboxylic acid (**5**) labeled at C-5 and C-15 was consistently but rather inefficiently incorporated into cobyrinic acid (*3a*, *c*, *d*). Faced with the difficulty of interpreting these results we carried out the total synthesis (*14*) of triply labeled **5** (^{14}C at C-5, C-15, and C-20) and incubated this substrate with the cell-free system (Scheme 2). If incorporation of **5** is controlled by the same enzymic mechanism utilized by **1** then the radiochemical yield of methylene–dimedone adduct and cobester (**4**) should match, irrespective of the efficiency of the process. Reference to Table 1 indicates that although the bioconversion of uro'gen hepta acid (**5**) to cobyrinic acid (**3**) is, in accord with previous work (*3*), an order of magnitude (0.2%) less than that of uro'gen III (~3–4%), the enzymic release of formaldehyde (0.17%) from this substrate (^{14}C-20) and its otherwise intact conversion (^{14}C-5) are no longer in doubt. We conclude that the acid (**5**) is either a true, but sparingly incorporated, intermediate in corrin biosynthesis, or that the enzymes of *P. shermanii* are capable of utilizing (**5**) as an intact species for the subsequent steps of C-20 extrusion and reductive methylation (*16*).

ACKNOWLEDGMENTS

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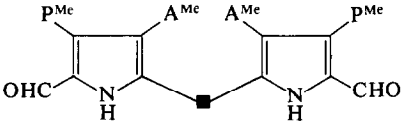
REFERENCES

1. δ-Aminolevulinic acid (ALA): D. Shemin, J. W. Corcoran, C. Rosenblum, and I. M. Miller, *Science* **124**, 272 (1956); J. W. Corcoran and D. Shemin, *Biochem. Biophys. Acta* **25**, 661 (1957). Porphobilinogen (PBG): S. Schwartz, K. Ikeda, I. M. Miller, and C. J. Watson, *Science* **129**, 40 (1959); A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, and R. J. Cushley, *J. Amer. Chem. Soc.* **94**, 8269 (1972); A. R. Battersby, M. Ihara, E. McDonald, F. Satoh, and D. C. Williams, *J. Chem. Soc. Chem. Commun.*, **436** (1975); H. O. Dauner and G. Müller, *Hoppe–Seyler's Z. Phys. Chem.* **356**, 1353 (1975).
2. A. I. Scott, *Tetrahedron* **31**, 2639 (1975); A. I. Scott, *Phil. Trans. Roy. Soc. Lond. B* **273**, 303 (1976).
3. (a) A. I. Scott, N. Georgopapadakou, K. S. Ho, S. Klioze, E. Lee, S. L. Lee, G. H. Temme, III, C. A. Townsend, and I. M. Armitage, *J. Amer. Chem. Soc.* **97**, 2548 (1975); (b) A. I. Scott, E. Lee, and C. A. Townsend, *Bioorg. Chem.* **3**, 229 (1974); (c) A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh, and D. C. Williams, *J. Chem. Soc. Perkin I*, 166, (1977); (d) R. Deeg, H. Kriemler, K. Bergmann, and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, **358**, 339 (1977).
4. M. Imfeld, C. A. Townsend, and D. Arigoni, *J. Chem. Soc. Chem. Commun.*, 541 (1976); A. R. Battersby, R. Hollenstein, E. McDonald, and D. C. Williams, *J. Chem. Soc. Chem. Commun.*, 543 (1976); A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demou, and D. Petrocine, *J. Chem. Soc. Chem. Commun.*, 544 (1976).

5. E. LEE, Ph.D. thesis, Yale University, 1974; N. GEORGOPAPADAKOU, Ph.D. thesis, Yale University, 1975.
6. D. MAUZERALL, *J. Amer. Chem. Soc.* **82**, 2601, 2605 (1960).
7. The regiospecific synthesis of [20-^{14}C]uro'gen III was carried out according to the procedure of Tarlton et al. (*8*) using the methylene ^{14}C-labeled 5,5'-diformylpyrromethane (**i**) and the 5,5'-unsubstituted pyrromethane (**ii**). The corresponding [5,15-$^{14}C_2$]uro'gen III was synthesized according to the procedure of Franck et al. (*9*). The specimens were then combined as indicated in Table 1.



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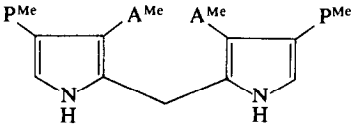

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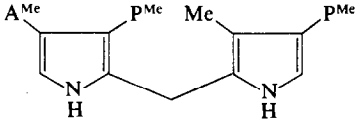
8. E. J. TARLTON, S. F. MACDONALD, AND E. BALTAZZI, *J. Amer. Chem. Soc.* **82**, 4389 (1960).
9. B. FRANCK, D. GANTZ, AND F. HÜPER, *Angew. Chem. Int. Ed. Engl.* **11**, 420 (1972).
10. A. I. SCOTT, B. YAGEN, AND E. LEE, *J. Amer. Chem. Soc.* **95**, 5761 (1973).
11. P. R. FARINA, L. J. FARMA, AND S. J. BENKOVIC, *J. Amer. Chem. Soc.* **95**, 5409 (1973), and references cited. Direct proof for the intervention of tetrahydrofolate (THF) as a *mediator* of the extrusion process rather than a trap for free formaldehyde is, at the present stage of a crude enzyme mixture, difficult to obtain. A mechanism involving free *formate* as the expelled species followed by THF trapping and reduction of methenyl- to methylene-THF is rendered unlikely by our failure to trap formate (*13*) in the incubation. A. series of experiments with purified enzyme(s) and chiral substrates is in hand to settle this point.
13. M. KAJIWARA, unpublished data.
14. The specimen of [20-^{14}C]heptacarboxylic acid (**5**) was prepared from the dipyrromethane (**i**) and the ^{14}C-labeled pyrrolaldehyde (**ii**) according to the procedure of Engel and Gossauer (*15*). The corresponding [5,15-$^{14}C_2$]heptacarboxylic acid (**5**) was prepared analogously using the ^{14}C-labeled pyrrolaldehyde (**iii**), and the specimens were combined as indicated in Table 1.



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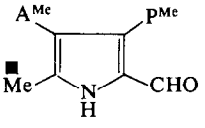

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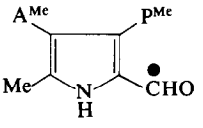

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15. J. ENGEL AND A. GOSSAUER, *Liebigs Ann. Chem.*, 1637 (1976).
16. The usual criteria for intermediacy include biotransformation to product, direct isolation, or indirect (hot or cold) trapping. In the case of the uro'gen hepta carboxylic acid (**5**) the problem of trapping is complicated by the presence of substantial, isolable quantities of the isomeric ring D-hepta carboxylic acid, the precursor of coprogen III which cocrystallizes with and is difficult to separate from the ring C acid (**5**). It has so far proved impossible to resolve this mixture for radiochemical assay.