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(35) Significant geranyl chloride remained in experiment 5 (*ca.* 80%) and about 20% geranyl bromide remained in experiment 6.

(36) Other coupling reactions on substrates bearing a nerol geometry have been even less successful; *cf.* ref 18b and 34a.

(37) A generous gift of the Reynolds Tobacco Co; Δ^2 >99% trans.

(38) R. Ruegg, U. Gloor, R. N. Goel, G. Ryser, O. Wiss, and O. Isler, *Helv. Chim. Acta*, **42**, 2616 (1959).

(39) Limiting use of AgO (2.5 equiv) led to MK-9 in 70% yield or 89% conversion based upon recoved **26**; see Experimental Section.

(40) All reactions were performed at room temperature and under nitrogen atmosphere unless otherwise noted. Melting points were determined on a hot-stage microscope and are uncorrected. Column chromatographies and tlc plates both employed Camag kieselgel as absorbent. Unless otherwise noted, nmr spectra were determined in CDCl$_3$ solution with a Varian T-60 instrument and are reported as δ values relative to internal TMS. Ultraviolet absorption measurements were made in isooctane using a Cary 14 recording spectrophotometer. Gc comparisons were accomplished with a 10 ft $\times$ 0.25 in. column containing 5% QF-1 liquid phase on 100–120 mesh AW-DMCS treated Chromosorb W. A CEC-103 mass spectrometer was used for determining mass spectra. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif. All solvent evaporations were performed *in vacuo* using a Berkeley rotary evaporator.

(41) P. I. Gaultier and C. Hauw, *Acta Crystallogr., Sect. B*, **25**, 51 (1969).

(42) Nerol–geraniol ratios were determined by derivatization as trimethylsilane ethers followed by gc analysis ($T = 105°$): *cis*-**16**, retention time 13 min; *trans*-**16**, retention time 15 min.

(43) O. Isler, R. Ruegg, L. Chopard-dit-Jean, H. Wagner, and K. Bernhard, *Helv. Chim. Acta*, **39**, 897 (1956).

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(45) *cis-/trans*-Menaquinone-2 and -9 ratios were determined by medium pressure liquid chromatography (Chromatronix) using a 254-nm absorption monitor. A 2 mm $\times$ 30 cm column was packed with Spherisorb S20W and eluted with 3% ether in isooctane at a flow rate of 10 μl/min: *cis*-**1a**, retention time 4.0 hr; *trans*-**1a**, 4.6 hr, Δ^2-*cis*-**1b**, 3.3 hr; Δ^2-*trans*-**1b**, 4.3 hr.

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Biosynthesis of Corrins. I. Experiments with [14C]Porphobilinogen and [14C]Uroporphyrinogens

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Abstract: Previous work in the area of corrin biosynthesis is summarized, and the results of administering regiospecifically synthesized versions of [8-14C]porphobilinogen (PBG) and [14C]uroporphyrinogen (uro'gens) of types I–IV to resting cells of *Propionibacterium shermanii* are discussed in terms of the distribution of radioactivity in vitamin B$_{12}$ (cyanocobalamin). The development of satisfactory feeding conditions, isolation procedures, and some improvement for the synthesis of intermediates are described.

Vitamin B$_{12}$ (cyanocobalamin, **1**), one of nature's most complex nonprotein structures (C$_{63}$H$_{88}$N$_{14}$O$_{14}$PCo), has presented a formidable challenge at every stage of its investigation. The isolation of the crystalline "antipernicious anemia factor" from liver by Folkers[1] and Smith[2] in 1948 marks the beginning of chemical studies[3-5] which culminated in 1955 with Hodgkin's X-ray diffraction analysis.[6] In 1958, the coenzyme **2** was characterized by Barker[7] and its structure again deduced by X-ray diffraction (Hodgkin).[8] The discovery of the cobalt–carbon bond in turn opened up a whole new area of research on the remarkable rearrangements catalyzed by the coenzyme. The recent achievement[9]

of the total synthesis of vitamin B$_{12}$ represents the solution of yet another outstanding problem posed by the complex functional and stereochemical array contained in the corrin nucleus. The same structural and stereochemical features of **1** also constitute a major problem in considering the possible mode of biosynthesis of corrins (as **3**), for although it has been known for almost 20 years that vitamin B$_{12}$ shares the "early" part of heme, chlorophyll and tetrapyrrole biosynthesis in that it is built up *via* the succinate–glycine/ δ-aminolevulinate sequence,[10] the point at which the "cobalt" route divides from the "iron" and "magnesium" pathways was unknown at the outset of our investigation. From
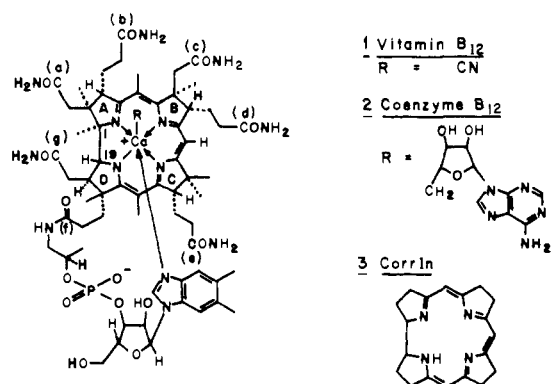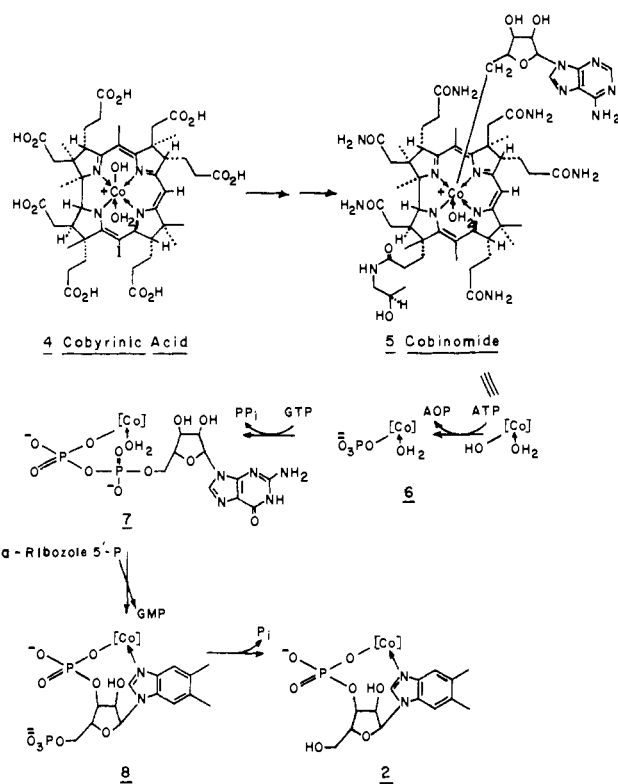
Figure 1. Structures of the corrin family.

Figure 3. Presumed labeling pattern of [2,3-^{14}C]ALA in vitamin B$_{12}$.

4 Cobyrinic Acid   5 Cobinomide

Figure 2. Metabolic pathway from cobyrinic acid (4) to cobalamin 5'-phosphate (8).
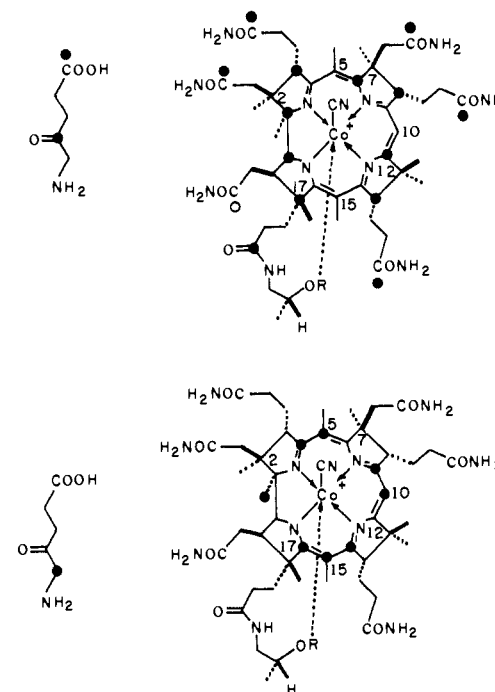
Figure 4. Presumed labeling pattern of [1,4-^{14}C]ALA and [5-^{14}C]ALA in vitamin B$_{12}$.

the corrin ring system there extends a nucleotide loop which consists of (R)-1-amino-2-propanol bound by an amide linkage to the "f" propionic acid side chain and esterified to the phosphate moiety of a unique 3'-mononucleotide in which dimethylbenzimidazole (DMBI) is attached by means of an N-α-glycosidic bond to D-ribofuranose. It has been shown by Sprinson[11] that L-threonine is the precursor of the amino-2-propanol segment. The biosynthesis of 5,6-dimethylbenzimidazole shares certain features in common with that of riboflavine since Alworth[12] has provided good evidence for the derivation of the unusual nucleotide base from 6,7-dimethyl-8-ribityllumazine.

Most importantly, the meticulous work of Bernhauer[13] has revealed that the simplest of the natural corrins is co-byrinic acid (4), and that a series of successive amidations leads from 4 first to cobinamide (5), thence to the phosphate 6, guanosine diphosphate–cobinamide (7), and co-balamin 5'-phosphate (8) (all as 5'-deoxyadenosyl deriva-tives with the exception of diaquocobyrinic acid) (see Fig-ure 2). Crude enzyme extracts capable of carrying out these transformations have been reported.[14]
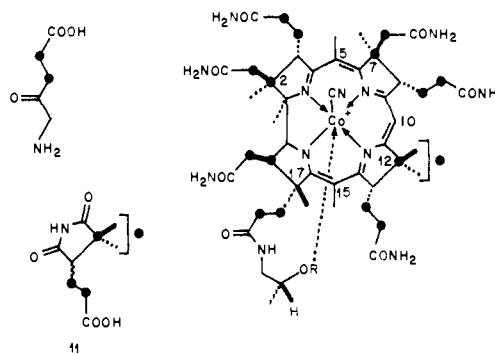
With the elucidation of the steps from cobyrinic acid to the cobalamins, the remaining gap in our knowledge con-cerning the biosynthesis of corrins resides in the uncharted route between δ-aminolevulinic acid (ALA) (9) and cobyri-nic acid (4). Thus although the pioneering experiments of Shemin[15] using variously labeled ^{14}C versions of ALA clearly indicated that the tetrapyrrole pathway was in-volved, the severe experimental problems associated with the degradative chemistry required to locate each label did not allow a completely rigorous solution. Using [2,3-^{14}C]ALA, we found all of the degradative work on the de-rived vitamin was compatible with the labeling pattern shown in Figure 3 which showed that the corrin nucleus was derived from eight molecules of ALA with loss of one of the carboxyl groups of an acetic acid side chain of the presumed intermediate porphobilinogen (10), PBG. The involvement of 8 equiv of ALA was also confirmed in the case of admin-istration of [1,4-^{14}C]ALA and the [5-^{14}C] radiomer (Fig-ure 4). In the case of the [5-^{14}C]ALA feeding experiments, some radioactivity was found in the C$_1$-methyl group by Kuhn–Roth oxidation of the derived cobinamide.[16] In spite of the low activity (8–9% theoretical) associated with the C$_1$-methyl group, the result was tentatively interpreted as a transfer of the amino methyl terminus of ALA (C$_5$) to the angular methyl group at C$_1$ in ring A of the corrin system.
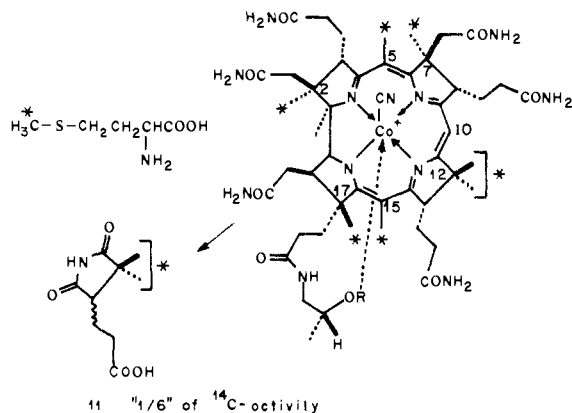
Figure 5. Presumed labeling pattern of $[^{14}CH_3]$-L-methionine in vitamin B_{12}.

As discussed later, the interpretation of this experiment had profound implications for all of the proposals concerning corrin biogenesis. The remaining carbons of the complete system were shown to be derived from methionine by feeding this substrate with $^{14}CH_3$ label.[15-17] The difficulties associated with the degradative chemistry of the corrins allowed only a reasonable estimate for the amount of radioactivity in ring C, *i.e.*, one-sixth of the total activity (in **11**) (Figure 5). Kuhn–Roth oxidation studies were in accord with the introduction of six "extra" methyl groups from methionine (Figure 5), that at C_1 being derived from C_5 of ALA and that at C_{12} from C_2 of ALA *via* decarboxylation. Thus it was recognized from the outset that the biosynthetic mechanism for corrin formation is, in fact, a study of a methylation sequence and stereochemistry imposed upon a tetrapyrrolic template of unknown structure and oxidation level. The overall carbon balance of these experiments is summarized in eq 1. One further piece of experimental evidence

$$8(ALA) + 6(methionine) - CO_2 \longrightarrow cobyrinic\ acid \quad (1)$$
$$(C_5) \hspace{8cm} (C_{45})$$
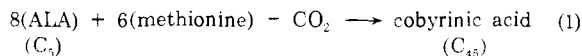
dence concerned the report[18] that a ^{14}C-labeled specimen of PBG (**10**) was incorporated into vitamin B_{12}, although the location of the label in the vitamin was not determined.

From the beginning of our studies, we were intrigued. as others have been by the structural similarity of uroporphyrinogen III (uro'gen III) (**12**) and cobyrinic acid (**4**) and we were attracted by the economy of a biosynthetic scheme wherein the corrin and porphyrin pathways diverge *after* the formation of uro'gen III.[10] The fact that the latter reduced porphyrin system is an authenticated metabolite of *P. shermanii* taken together with work[19,20] on *Clostridium tetanomorphum* (a B_{12}-producing organism) where relatively large amounts of uro'gen III were formed at the expense of copro'gen III (**13**) or proto'gen IX (**14**) (Figure 6) constitutes some indirect evidence for the intermediacy of the type III uro'gen (**12**). Our early simplistic notion[21] for the

Scheme I
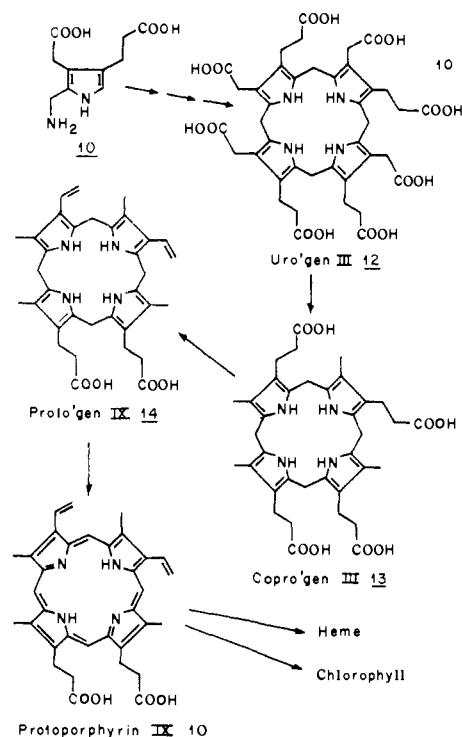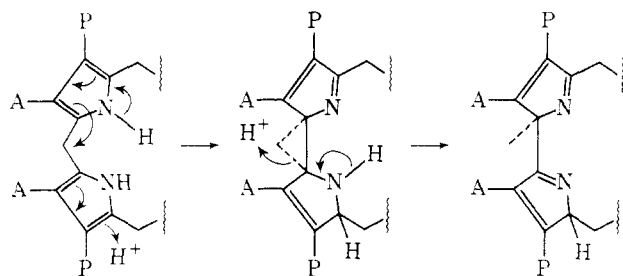
Figure 6. Intermediate stages of porphyrin biosynthesis.

uro'gen $\longrightarrow$ corrin conversion involving cyclopropane chemistry is illustrated in Scheme I. Such an idea has been independently conceived[22] by Eschenmoser *via* reductive ring contraction of *corphins* (as **15**). Another independent

15
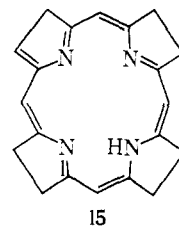
scheme involving the cobalt *uroporphyrin* III system has been suggested by Dolphin[23] (Scheme II). All of these early suggestions assumed the "carbon balance" of eq 1, *viz.* that

Scheme II
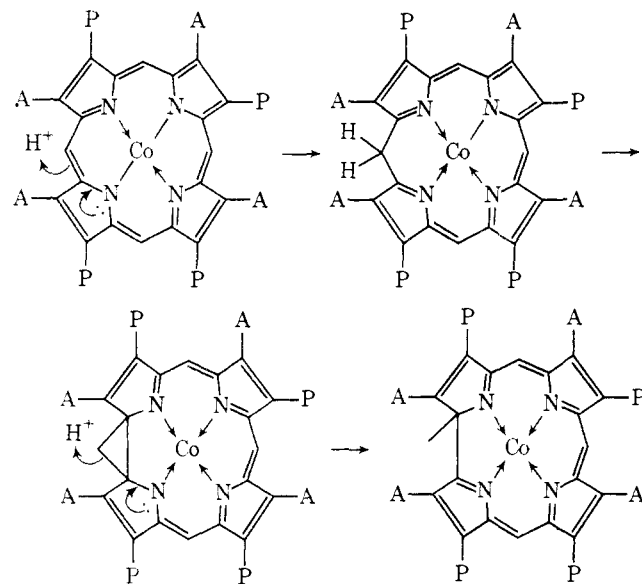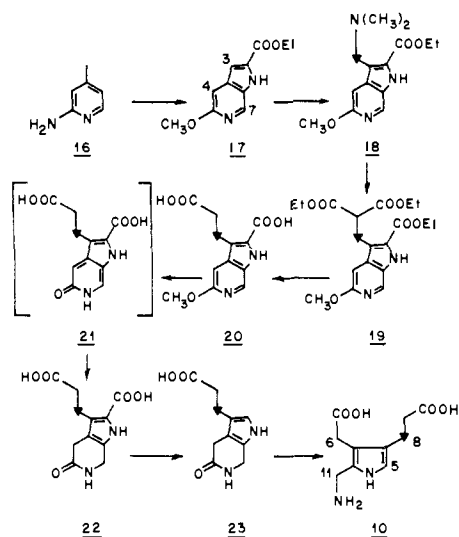
Figure 7. Synthesis of [8-^{14}C]PBG (10) by Rapoport's[25] procedure.

Figure 8. Formation of types I–IV uroporphyrins and uro'gens from PBG (10).

the C_1-methyl group in ring A is formed from the original aminomethyl function (C_5) of ALA. In view of subsequent experimentation described in the succeeding papers of this series, it has become necessary to devise a completely new concept for corrin biosynthesis.

The formation of uro'gen III (12) from PBG (10) and the further transformation of the former to corrins involve perhaps the most exciting unsolved challenges in porphyrin–corrin biochemistry, but before we reexamined the many experiments and explanations that have been made for these processes, the key experiment of testing the possible intermediacy of uro'gen III for vitamin B_{12} in *P. shermanii* became our first objective. Thus, regardless of the controversial and at times puzzling aspects of type III uroporphyrin biosynthesis in various organisms (a topic which forms the basis of a later paper of this series), clear and unambiguous proof for the intermediacy of uro'gen III constituted the first requirement in establishing a recognizable intermediate between PBG (10) and cobyrinic acid (4).

As the first test of this hypothesis, a sample of ^{14}C-labeled PBG (10) was synthesized, which could be converted by Mauzerall's procedure[24] to the statistical mixture of uro'gens I–IV (containing 50% uro'gen III) and fed to a vitamin B_{12} producing organism. [8-^{14}C]PBG was obtained by a slight modification of Rapoport's excellent procedure.[25] 2-Amino-4-methylpyridine (16) was converted[25-27] in six unexceptional steps to azaindole 17 as detailed in the Experimental Section (see Figure 7). Label was introduced into 17 *via* [^{14}C]paraformaldehyde by reaction with dimethylamine hydrochloride to give Mannich base 18. The base was displaced with sodiomalonate to give triester 19, which was saponified and decarboxylated in refluxing hydrochloric acid to yield diacid 20. Azaindole diacid (20) was demethylated to pyridone 21, which was hydrogenated to carboxyporphobilinogen lactam (22). After purification by repeated crystallization and alumina chromatography, 22 was decarboxylated in water at reflux to give [8-^{14}C]PBG lactam (23) (about 30% yield from introduction of label). As PBG (10) itself is readily oxidized, it was generated immediately from the lactam 23 prior to use by alkaline hydrolysis at room temperature for 3 days. PBG lactam was found to be stable at −20° in the dark for several months, and the labeled pyrrole was stored in this form. To obtain substances of lower specific activity, the lactam was diluted with radioinactive material before hydrolysis. As a matter of course, the diluted sample was recrystallized once before conversion to PBG, and a specimen was retained for
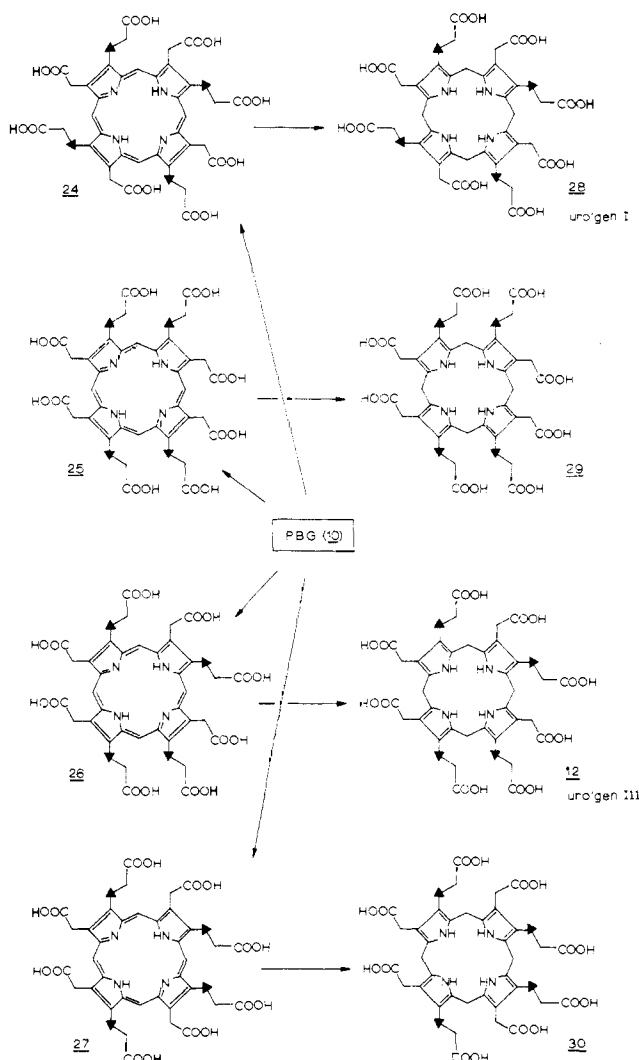
counting. In every case, the specific activities of the [8-^{14}C]PBG lactam (23) and of the [8-^{14}C]PBG (10) derived from it differed by less than 1% from their mean value. The details of these reactions are reported in the Experimental Section.

[8-^{14}C]PBG (10) was condensed in hydrochloric acid, essentially by Mauzerall's procedure,[24] and oxidized to yield [^{14}C]uroporphyrins I–IV (24, 25, 26, 27) (Figure 8). The statistical mixture of uroporphyrin isomers ($\frac{1}{8}$ I, $\frac{1}{8}$ II, $\frac{1}{2}$ III, $\frac{1}{4}$ IV) was isolated and purified by precipitation at the isoelectric point and chromatography. Immediately before use, the uroporphyrins were reduced over freshly ground 3% sodium amalgam in dim light to give [^{14}C]uro'gens I–IV, 28, 29, 12, and 30, as shown in Figure 8.

All of Shemin's classic biosynthetic studies had been performed with growing cultures of an actinomycete (ATCC 11072). During the 1960's, however, *Propionibacteria* came to be generally favored, in part because of their extensive use by Bernhauer,[13] but mainly because significantly higher yields of vitamin B_{12} were attainable. Two readily available strains of *Propionibacterium shermanii* (ATCC 9614 and 13673) were selected as the vitamin B_{12}-producing organisms to be used in this research. After some initial difficulties, the bacteria were satisfactorily cultured in a simplified version of Bernhauer's medium.[28] It was found that the best cell growth was obtained when periodic adjustments of pH and additions of glucose were made. Moreover,

Table I. Summary of ^{14}C-Feeding Experiments Using Suspended Cells of *P. shermanii*[a]

Expt	Substrate	Total substrate fed, mg	Time, hr	Spec incorp/C, %
1	[8-14C]PBG	21	70	6.1
2	[8-14C]PBG	21	70	5.1
3	[8-14C]PBG	15	50	5.1
4	[14C]Uro'gens I–IV	34	60[b]	0.15
5	[14C]Uroporphyrins I–IV	34	60[b]	0.00
6	[14C]Uro'gens I–IV	27	70[b]	0.08
7	[14C]Uro'gens I–IV	25	70[c]	2.6
8	[14C]Uro'gens I–IV	25	70[c]	0.91
9	[14C]Uro'gens I–IV	23	70[c,d]	1.88
10	[14C]Uro'gens I–IV	23	70[c]	0.71
11	[14C]Uro'gen I[e]	25	70	0.017
12	[14C]Uro'gens III + I	23	70[f]	0.008
13	[14C]Uro'gens III + I	24	70[c]	0.40
14	[14C]Uro'gens III + I	0.1[g]	70	0.007[c]
15	[14CH3]-L-Methionine	15[c]	48	15
16	[14CH3]-L-Methionine	15[c]	48	17
17	[5-14C]ALA	15	50	27
18	[5-14C]ALA	15	50	25

[a] Complete details of each experiment are given in the text (Experimental Section). [b] No pH adjustment. [c] As in expt 4 and 6 but with pH adjustment (to 7.0) at 19 and 45 hr. [d] As footnote c plus L-methionine (10 mg). [e] Contained 5-10% uro'gen III. [f] Constant pH adjustment (to 7.0–7.5). [g] Note small sample size.

P. shermanii is usually grown anaerobically, but under these conditions only "incomplete" corrinoids are obtained. For the complete nucleotide to be formed, either the last phase of growth must be aerobic, or 5,6-dimethylbenzimidazole (DMBI, **31**) must be added to the medium. A great deal of experimentation was required before standardized growth conditions were established that gave both sufficient incorporation of precursors and good yields of the vitamin. These generalized conditions are described in full in the Experimental Section.

Vitamin B_{12} remains almost entirely bound within the cell during growth owing to extensive hydrogen bonding by the side-chain amides. The isolation of corrins, therefore, only requires collection and fairly vigorous extraction of the cells. In practice, liberation of vitamin B_{12} and its congeners was achieved by heating centrifuged and washed cells at reflux in 80% methanol containing a small amount of potassium cyanide. This crude extract, after concentration and precipitation of protein, was submitted to phenol–chloroform extraction. Cyanocobalamin was separated and purified by electrophoresis or by paper chromatography and crystallized to constant specific radioactivity from acetone–water.

In establishing a uniform procedure for the conduct of feeding experiments, minimization of the oxidation of milligram quantities of uro'gen was of primary importance. Administration of such labile material to large volumes of growing cultures in the traditional fashion seemed self-defeating. Consequently, it was decided to harvest fermentation of *P. shermanii* and suspend the cells in a relatively small volume of medium (100–300 ml) whose composition could be uniformly controlled. Small-scale incubations of this sort increased the effective concentration of labeled substrates, often only available in limited quantity, and, most importantly, permitted the maintenance of strictly anaerobic conditions. To induce maximum incorporation of precursor during the suspended cell feeding experiments, the *P. shermanii* was grown in the absence of cobalt and 5,6-dimethylbenzimidazole (DMBI, **31**). Early media used for the suspended cell incubations were indirectly based on

those employed by Burnham[20] and Lascelles[29] for porphyrin studies with other organisms and consisted of 0.1 *M* phosphate buffer (pH 7.0), cobalt chloride, DMBI, L-methionine, glucose and, variously, ascorbic acid, magnesium chloride, as well as ammonium sulfate and manganese sulfate on a very few occasions.

The first feeding experiments conducted in the early spring of 1971 with [8-14C]PBG (**10**) gave radioactive vitamin B_{12}, confirming the result of Schwartz[18] from a decade before. A number of control experiments were run with radioactive PBG, and ammonium and manganese sulfate could subsequently be eliminated from the incubation medium. Of the two strains of *P. shermanii* used, No. 9614 gave consistently higher incorporations and better yields of the vitamin and was used in all further work. Finally, the addition of glucose to the suspension medium was found to result in higher incorporations, as expected. By late spring, the incubation techniques were sufficiently refined to allow the feeding of [14C]uro'gens I–IV **28, 29, 30**, and **12**, derived from [8-14C]PBG (**10**). The first trial gave 0.5% specific incorporation of the uro'gen mixture into cyanocobalamin. This was followed by two important experiments (expt 4 and 5) (see Table I) wherein a sample of [14C]uroporphyrins I–IV was divided into two equal parts, one of which was reduced to the corresponding [14C]uro'gen mixture and fed to suspended cells of *P. shermanii*. The remaining portion was fed directly without reduction and incubated under the same conditions. In accord with the previous experiment, the [14C]uro'gen mixture gave a positive incorporation into vitamin B_{12} (0.153%), whereas the cyanocobalamin derived from the [14C]uroporphyrin experiment was absolutely without activity (0.00%). The implications of these experiments were twofold. First, the incorporations observed in the [14C]uro'gen experiments were not due to radioactive impurities present in the starting [14C]uroporphyrin, e.g., [8-14C]PBG. It was assumed by analogy with the common practice of feeding DL mixtures of chiral substrates that only uro'gen III was incorporated from the uro'gen mixture and that uro'gen I and the nonnaturally occurring ("nonsense") uro'gens II and IV were merely spectators of the biochemical events involving the type III isomer. Second, the uro'gen oxidation state (hexahydroporphyrin) was biologically active, in conformity with heme and chlorophyll biosynthesis. This finding is in discord with Dolphin's mechanistic proposals,[23] centering on cobalt uroporphyrin III, that were outlined above.

The simple incorporation of radioactivity, however, does not constitute proof of biointermediacy. Classical degradations to determine position and verify nonrandomization of label would be a formidable task in the case of a molecule with the complexities and symmetries of vitamin B_{12}. The burden of such a proof for uro'gen III was compounded by the report of Müller[30] that appeared at about the same time as our first success with the chemically synthesized [14C]-uro'gen mixture. The German group synthesized a small but highly radioactive sample of [14C]uro'gens III + I enzymatically from freeze-dried cells of *P. shermanii* by a method published earlier.[31] This material was purified with meticulous care and fed to suspended cells of *P. shermanii*. Compared with a parallel experiment with ALA, the uro'gens gave an extremely low incorporation (but not zero). They considered this to demonstrate that uro'gen III was not a precursor of vitamin B_{12}.

Faced with this negative precedent, the validity of our preliminary results demanded a means to establish the location of labeled centers in the vitamin. In principle, the problem of tedious degradation of the corrin structure could be circumvented in two ways: either by judicious use of doubly

labeled precursors, difficult due to synthetic constraints; or by the technique of ^{13}C-FT nmr. This second alternative became especially attractive in view of the publication in mid-1971 by Doddrell and Allerhand[32] of the *partially* assigned ^{13}C-FT nmr spectra of vitamin B_{12} and some of its simple derivatives. The large, rigid corrin structure has the fortuitous consequence that dipole-dipole interactions dominate spin-lattice relaxation processes,[33] and most resonances (at least for those carbons with one or more protons) in the proton noise-decoupled spectra appear at roughly the same intensity (close to maximum nuclear Overhauser enhancements).[33] This behavior implied that enrichments as low as 2-3% in cyanocobalamin from ^{13}C-labeled precursors could be meaningfully detected above natural abundance. To this end considerable time and effort were expended to modify the fermentation and suspended-cell conditions to obtain higher incorporations of PBG and uro'gen. Some of Bernhauer's[28] growth procedures and Müller's[30] feeding and isolation techniques were adapted to our existing methods, and by the end of 1971, generalized procedures had been established (see Experimental Section). It was found that the use of young cells in the propagation and final anaerobic growth of the *P. shermanii* had a positive effect on incorporations of precursor and yields of the vitamin under the suspended-cell conditions. Daily pH adjustment and additions of glucose during the anaerobic culturing phase had a favorable effect on the total cell mass and hence on the amount of cyanocobalamin produced. Similarly, occasional adjustments of pH and additions of glucose during the course of suspended-cell feeding experiments dramatically increased the incorporations observed. This factor was particularly well illustrated in [14C]uro'gens I-IV, expt 6 and 7. Experiment 6 employed relatively old cells fed without adjustment of pH or addition of glucose and gave a specific incorporation of 0.081%, whereas expt 7, carried out with somewhat younger cells and only two adjustments of pH, gave a 2.56% incorporation into cyanocobalamin. Using the standardized conditions, experiments with [8-14C]PBG gave incorporations of 5-6% per labeled center (expt 1 and 2) (see Table I).

At this juncture, an attempt was made to separate the four uroporphyrin isomer types by high-pressure liquid chromatography, a separation that has never been accomplished by conventional chromatographic techniques. After an extended and unsuccessful effort, the project was abandoned, and [14C]uro'gens III and I were synthesized enzymatically from [8-14C]PBG (Figure 9) by published procedures. A preparation of uro'gen I synthetase-uro'gen III cosynthetase was obtained from wheat germ by the method of Bogorad.[34,35] For high cosynthetase activity, it is essential to use non-heat-treated wheat germ. Enzyme preparations from this material had the capacity to produce [14C]-uro'gens III + I, 12 and 28, in a ratio of III:I = 8:2-6:4, analyzed as the corresponding coproporphyrins by standard procedures.[36,37] Initial experiments with this substrate were conducted with the standardized medium (however, see below) after three cycles of freeze-pump-thaw degassing and *very* frequent adjustments of pH during the feeding period. Under these conditions, discouragingly low incorporations were observed, *e.g.*, expt 12, 0.008%. The third trial, however, was carried out, as were all succeeding [14C]uro-'gen suspended-cell feeding experiments, with pH adjustments only daily or twice daily. [14C]Uro'gens III + I (expt 13) gave a specific incorporation of 0.40% and [14C]uro-'gens I-IV (expt 8) run simultaneously and under identical conditions gave 0.91% incorporation.

It would appear, then, that while occasional pH adjustments result in better incorporations of precursor than
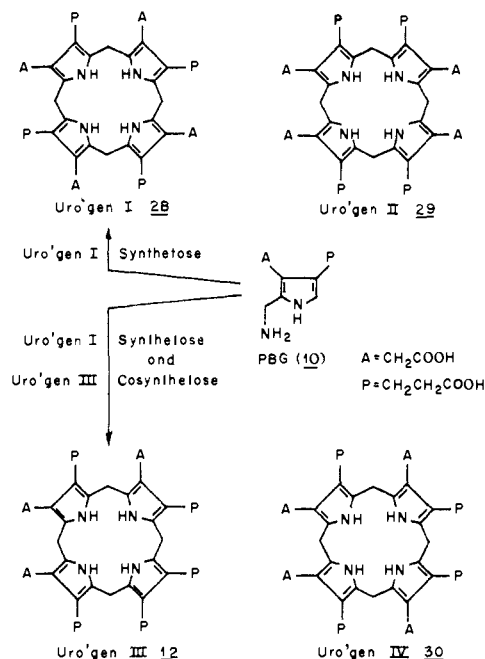

Figure 9. Enzymatic conversion of PBG (10) to uro'gens I and III.

none, overly zealous maintenance of pH at neutrality leads to poor incorporations, at least in the case of uro'gen III. At pH 4-5, uro'gen may be sufficiently less ionized so that it can pass more freely through lipophilic cell walls to the sites of corrin biosynthesis.

To show that the positive incorporation of the [14C]uro-'gen III + I mixture was due only to the type III isomer, and to demonstrate that type I does not convert to type III under the suspended-cell feeding conditions, a sample of [14C]uro'gen I was prepared enzymatically by known procedures.[38] In [14C]uro'gen I, expt 11 (Table I), it was fed under conditions virtually identical with those above and gave a slight positive incorporation into cyanocobalamin, 0.017% (5% that of uro'gen III). Paper chromatographic analysis of the [14C]uro'gen I fed (as its coproporphyrin[36,37]) clearly revealed the presence of a small amount of the type III isomer. The uro'gen I synthetase incubation, unfortunately, had been run for 12 hr, and some *in vitro* conversion of [8-14C]PBG to the statistical mixture of uro-gen isomers presumably had taken place.

All the uro'gen experiments described above were conducted without L-methionine in the suspended-cell medium. After their completion, a control experiment was conducted in which the chemically synthesized mixture of [14C]uro-'gens I-IV was fed with and without added methionine (expt 9 and 10, respectively). In the presence of methionine, the uro'gen incorporation was more than twice that of the control (1.88 *vs.* 0.71%). The incorporation figures in the experiments above presumably would have been doubled had they been conducted with added methionine (methionine was added in [8-14C]PBG experiments).

The positive incorporation of an enzymatically produced [14C]uro'gens III + I mixture into vitamin B_{12} must be reconciled with Müller's unsuccessful attempt[30] discussed earlier. The Stuttgart group, however, fed an extremely small amount of substrate (0.017 mg), whereas we used about 25 mg (in enzymatic studies, 1-2 mg of uro'gens III + I or uro'gen I of high specific activity was diluted with radioinactive uro'gens I-IV). As an approximation of Müller's conditions, 0.1 mg of highly radioactive [14C]uro'gens III + I (expt 14) was fed and run parallel to [14C]uro'gen I, expt 11 above. A 7.65×10^{-3}% incorporation resulted,

which was consistent with Müller's data, $2.4 \times 10^{-5}\%$, using the equivalent of about one-tenth as much uro'gen III. Therefore, in addition to the pH behavior noted above, the successful incorporation of uro'gen III into vitamin B_{12} depends upon sufficient substrate being present to survive *in vitro* oxidative destruction during the course of the feeding experiment. This important proviso was absent in Müller's experiment and appears to have been absent again in a more recent study by Franck and coworkers,[39] where a total of about 3.5 mg of uro'gen III, obtained by chemical synthesis, was fed to 1 l. of growing *P. shermanii*. As in Müller's experiment, a very low but nonzero incorporation was observed, $2.1 \times 10^{-3}\%$.

In conclusion, these negative results with enzymically[30] and chemically[39] synthesized uro'gen III must be contrasted with the successful incorporations presented in this paper. Uro'gen I does not convert to uro'gen III under the experimental conditions, and presumably the unnatural isomers uro'gens II and IV do not either. Furthermore, simple degradation of cyanocobalamin[34] derived from [^{14}C]uro-'gens I-IV and [8-^{14}C]PBG by hydrolysis and Kuhn-Roth oxidation (essentially by Shemin's procedures)[16] showed that *none of the original ^{14}C label had randomized into the nucleotide segment or into those carbons which yield acetic acid on oxidation.* Taken together, these observations provided preliminary evidence to support the sequence PBG → uro'gen III → vitamin B_{12} in *P. shermanii* as summarized in Figure 10. With the first publication of these early experiments,[21] a mechanistic scheme was advanced to account for the transformation of uro'gen III to cobyrinic acid that extended the tentative ideas briefly noted earlier. The use of ^{13}C-FT nmr to establish the intact incorporation of PBG and uro'gen III, however, led to some surprises that undermined these notions. These developments are described in the succeeding paper.

Experimental Section

General. Solvents throughout were reagent grade and used as supplied, unless otherwise noted. Extractions were performed in the usual manner with the indicated solvents and wash solutions; drying of solutions in organic solvents prior to rotary evaporation was accomplished by shaking with and filtration through anhydrous sodium or magnesium sulfate. Reactions performed under nitrogen were carried out in flasks thoroughly purged by alternate evacuation and admission of nitrogen through a threeway stopcock; the flask was maintained at a slight positive pressure by a bubbler connected at the nitrogen source by a t tube. Full physical and spectral data are presented for all new compounds as well as for known compounds from established procedures, where such data have not been published.

Analytical thin-layer chromatographic (tlc) separations were carried out on plastic-backed plates: silica gel with fluorescent indicator (Eastman Chromatogram 6060 or Baker-flex IB-F), polyamide (MN-Polygram 11, Machery-Nagel, Düren; Brinkmann distributor), and cellulose (Eastman Chromatogram 6065). During later synthetic work, separations were routinely run on small glass plates prepared from chloroform suspensions of silica gel (GP 254, E. Merck, Darmstadt) or alumina (GF 254 [E], E. Merck). Once developed with the solvents indicated, plates were visualized by viewing under ultraviolet light or by spraying with a suitable indicator solution.

Analytical and preparative paper chromatograms were obtained, using Whatman No. 1 and 3MM, respectively. The conditions of development (ascending or descending) and the solvents used are as indicated; visualization procedures were as above.

Paper electrophoreses were run on a Savant Model FP-22A water-cooled flat-plate apparatus, using the buffers and voltages indicated. Whatman No. 1 or 3MM paper was used, depending on the scale of the separation.

Melting points (mp) were taken on a Kofler-type hot-stage microscope apparatus (Reichert, Austria) and are uncorrected.
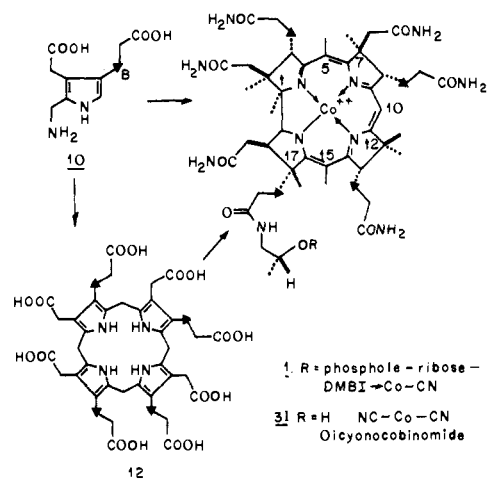

Figure 10. Summary of labeling pattern in cyanocobalamin 1 from [8-^{14}C]PBG and [^{14}C]uro'gen III.

Infrared absorption spectra (ir) of dry samples in KBr disks were recorded, using a Perkin-Elmer 237B grating spectrometer set at the normal (N) slit width. Absorption maxima are reported in reciprocal centimeters (cm^{-1}) and are accurate to ±5 cm^{-1} for the range 625–2000 cm^{-1} and ±10 cm^{-1} for the range 2000–4000 cm^{-1}. Where used, the abbreviations to indicate relative peak intensities are s = strong, and w = weak (unmarked peaks were of intermediate intensity).

Ultraviolet-visible absorption spectra (uv) were obtained with a Beckman DB-G double-beam grating instrument, using 1-cm cells and the indicated solvent, typically ethanol. Absorption maxima (λ_{max}) are reported in nanometers (nm, accurate to ±1 nm), together with the corresponding extinction coefficients (ϵ value in l. (mol^{-1} cm^{-1})); the wavelengths (approximate) of shoulders are denoted (sh).

Proton magnetic resonance spectra (nmr) were taken in the designated solvents on Varian A-60A and HA-100 instruments. The spectra were reported in the following manner: chemical shift in δ relative to tetramethylsilane (TMS), multiplicity (coupling constants in hertz), integrated intensity (assignment). Abbreviations are those commonly employed: s = singlet, d = doublet, t = triplet, q = quartet, br = broad, sym = symmetrical, m = multiplet, c = complex. For spectra recorded in deuterium oxide, dioxane was chosen as the internal reference, and the resulting chemical shifts were reported relative to TMS, using the correction 3.70 ppm for dioxane in CDCl$_3$ relative to TMS.

Determinations of radioactivity were made, using a Packard Model 3320 Tri-Carb Scintillation spectrometer for samples weighed accurately on a Cahn ratio electrobalance and counted in a liquid scintillation cocktail consisting of 15 ml of Aquasol (New England Nuclear) and 2.00 ml of either water or 0.1 N sodium hydroxide. Acidic compounds such as porphobilinogen (PBG), its lactam, and uroporphyrins were counted in the latter, alkaline combination; cyanocobalamin (vitamin B_{12}), dicyanocobinamide, methionine, and δ-aminolevulinic acid hydrochloride (ALA · HCl) were counted in the former neutral mixture. To correct the counts (cpm) obtained from the Packard Tri-Carb to the actual number of disintegrations per minute (dpm), efficiency curves were constructed in the usual fashion, employing both the external standard (AES) and channel ratio methods. In addition, as uroporphyrins and corrinoids quench especially strongly, separate curves were computed for each, using added radioinactive sample as quencher to ensure maximum accuracy. Accurate background readings over long periods were obtained prior to counting samples of low specific activity.

Synthetic Procedures. Synthesis of ^{14}C Precursors. [$^{14}CH_3$]-L-Methionine. [$^{14}CH_3$]-L-Methionine (10–15 μCi/mmol) was used as received (New England Nuclear, crystalline solid) or after dilution as necessary with cold material (Sigma, sigma grade).

δ-Aminolevulinic Acid (ALA). [4-^{14}C]ALA (10–30 μCi/mmol in 0.1 N HCl) and [5-^{14}C]ALA (20–30 μCi/mmol in 0.01 N HCl) were purchased from New England Nuclear and used at the activity received or after dilution with cold material (Sigma).

Ethyl 5-Methoxy-6-azaindole-2-carboxylate (17). Precursor of [8-14C]PBG (10). 2-Amino-4-methylpyridine (16; 502 g, 4.64 mol; 2-amino-4-picoline, Aldrich, 98%) was nitrated according to the method of Seide.[27] After neutralization, the undesired isomer **32** was removed by steam distillation, and the remaining **33** was converted to **34** by decomposition of its diazonium salt.[26] Treatment of **34** with phosphorus oxychloride and phosphorus pentachloride and subsequent steam distillation[25] yielded the 2-chloro compound **35** as a highly crystalline solid. Nucleophilic displacement of chloride by methoxide in methanol proceeded readily under mild conditions[26] to give 2-methoxy-4-methyl-5-nitropyridine (**36**; 219 g, 28% yield overall).

Ethyl 2-Methoxy-5-nitro-4-pyridinepyruvate (37). 2-Methoxy-4-methyl-5-nitropyridine (**36**) was condensed with diethyl oxalate by Rapoport's procedure[25] as modified by Dr. Philip Bays of this laboratory. Sodium hydride (31.2 g, 1.29 mol in oil dispersion) and diethyl oxalate (190 g, 1.30 mol) were added in succession with stirring under anhydrous conditions to a solution of **36** (217 g, 1.29 mol) in 2 l. of dry tetrahydrofuran. Stirring was continued at room temperature, and the progress of the reaction was monitored by analytical tlc (polyamide, chloroform). After 6 hr, starting material could no longer be detected, and the viscous, dark-red solution (sodium enolate) was evaporated to dryness. The enolate residue was suspended in water and decomposed by the addition of glacial acetic acid to pH 3.5, and, after standing overnight, the precipitated crude pyruvate **37** was filtered and dried. Continuous extraction (soxhlet, petroleum ether) of the crude pyruvate afforded **37** as a light-tan crystalline solid (195 g, 56%; lit.[25] 93% after accounting for recovered starting material), whose melting point and uv absorption spectrum were consistent with that reported.
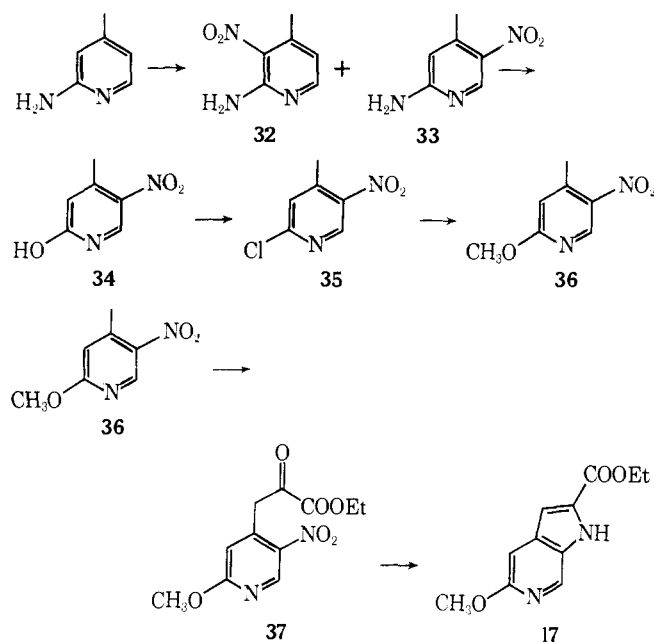
Ethyl 5-Methoxy-6-azaindole-2-carboxylate (17). The pyridinepyruvate **37** (195 g, 0.73 mol) was hydrogenated at atmospheric pressure in 38-g lots over 10% palladium on carbon (8 g) in ethanol (500 ml). When the theoretical amount of hydrogen had been absorbed (12.5 l.), the catalyst was filtered on Celite and washed with ethanol. The combined runs were worked up according to the method of Rapoport,[25] and the azaindole **17** was obtained in quantitative yield (160 g, 100%). The purification of **17** for use in the synthesis of [8-14C]- and [9-3H]porphobilinogen was achieved in the following manner. Recrystallization from ethanol-water gave azaindole, mp 97-100° (after six recrystallizations, mp 103-105°). Column chromatography (silica, chloroform:ethyl acetate, 20:1 initially to 3:1 finally) of 2.5 g of material (mp 97-100°) gave 1.6 g of **17** as very pale-yellow needles (mp 104-105.5°, lit.[25] 103-106°) after one recrystallization. The uv absorption spectrum (ethanol) was in accord with that published.[25]

Nmr (CDCl3): 1.41 (t, $J = 7$, 3 H, ester methyl), 3.97 (s, 3 H, methoxyl), 4.46 (q, $J = 7$, 2 H, ester methylene), 6.97 (s, 1 H, H-

4), 7.10 (d, $J = 2$, 1 H, H-3, split by pyrrole N-H and H-4), 8.48 (s, 1 H, H-7), 10.13 (v br s, 1 H, pyrrole N-H).

Ir (KBr): 3410, 3415 (s), 3000 (w), 2980 (w), 2935 (w), 1780 (s), 1630, 1520, 1485, 1460, 1415, 1340 (w), 1260 (s), 1240 (s), 1105 (w), 1040 (w), 1020 (w), 870 (w), 780 (w), 770 (w), 745 (w).

Ethyl 3-Dimethylamino[14C]methyl-5-methoxy-6-azaindole-2-carboxylate (18).[25] [14C]Paraformaldehyde (2.1 mg, 0.0438 mmol; New England Nuclear, 1.0 mCi, specific activity 2.3 mCi/mmol) was carefully rinsed into a 25-ml round-bottom flask containing azaindole **17** (550 mg, 2.50 mmol; mp 104-105.5°) and dimethylamine hydrochloride (700 mg, 8.65 mmol) with 5 ml of 1-butanol. Paraformaldehyde (73.68 mg, 2.4562 mmol; i.e., total formaldehyde 2.50 mmol) was added, followed by another 5 ml of 1-butanol. After heating at reflux for 30 min under nitrogen, the solvent was removed in vacuo, and the yellow, oily residue was dissolved in 25 ml of 3 N hydrochloric acid. The aqueous solution was extracted twice with 10 ml of ether and carefully neutralized with potassium carbonate. The precipitated solid was filtered and washed with 5 ml of ether, and the filtrate was extracted four times with 10-ml portions of ether. The ether fractions were dried and evaporated under reduced pressure, and the resulting residue was combined with the precipitated solid above and dissolved in 10-15 ml of acetone. Dry hydrogen chloride was passed through the solution until no more precipitate formed. After cooling, the solution was filtered, yielding the Mannich base **18** (805 mg, 92%) as the dihydrochloride, whose electronic absorption spectrum was in agreement with Rapoport's data.[25]

Ir (KBr): 3480, 3420, 2970 (w), 1725 (s), 1625, 1590 (w), 1460, 1250 (s), 1220 (s), 1015, 935, 785 (w).

Ethyl 2-Carboethoxy-5-methoxy-6-azaindole-3-(α-carboethoxy)-[β-14C]propionate (19).[25] The dry Mannich base dihydrochloride **18** (805 mg, 2.30 mmol) was added to a solution of sodium (210 mg, 9.13 mmol) in 8 ml of diethyl malonate. The mixture was heated under anhydrous conditions at 115-118° for 6 hr. When the reaction mixture had cooled to room temperature, it was vigorously stirred with 12 ml of ether and 12 ml of 10% hydrochloric acid, and after cooling overnight, the hydrochloride of **19** was filtered as very pale-yellow rhomboidal prisms and plates (correct uv spectrum).[25] After drying in air for 1 hr, the triester was carried on directly to the next step.

Nmr (CDCl3): 1.17 (t, $J = 7$, 6 H, propionate ester methyl), 1.52 (t, $J = 7$, 3 H, ring ester methyl), 3.5-4.2 (c m, 3 H, methylene and methine obscured by other resonances), 4.12 (q, $J = 7$, 4 H, propionate ester methylene), 4.18 (s, 3 H, methoxyl), 4.57 (q, $J = 7$, 2 H, ring ester methylene), 7.40 (s, 1 H, H-4), 8.44 (v br s, 1 H, disappears upon addition of D2O, pyrrole N-H), 9.44 (br s, 1 H, H-7), 13.0 (v br s, 1 H, disappears upon addition of D2O, protonated pyridine N).

Ir (KBr): 3390 (w), 2760 (br), 1750 (s), 1670, 1620, 1445, 1270, 1240, 1060 (w), 1040 (w), 880 (w).

2-Carboxy-5-methoxy-6-azaindole-3-[β-14C]propionic Acid (20). The triester **19** obtained in the previous step was heated at reflux for 6 hr in 9 ml of concentrated hydrochloric acid. The diacid **20** was isolated by Rapoport's procedure,[25] evaporating to dryness in vacuo, dissolving in approximately 10 ml of water and sodium carbonate and precipitating with glacial acetic acid (pH 4). The solid was filtered after several hours of cooling, washed with water and acetone, and recrystallized by dissolution in dilute ammonium hydroxide and precipitation with glacial acetic acid (290 mg, 48% for two steps, uv spectrum in accord with the literature).[25]

Nmr (DMSO-d_6): 2.35-2.75 (2 H obscured by DMSO; α-methylene), 3.26 (br t, $J_{app} = 7.5$, 2 H, β-methylene), 3.87 (s, 3 H, methoxyl, 7.00 (s, 1 H, H-4), 8.44 (s, 1 H, H-7), 11.6 (v br s, 1 H, pyrrole N-H).

Nmr (D2O-K2CO3): 2.25-2.7 (c m, 2 H, α-methylene), 3.1-3.5 (c m, 2 H, β-methylene), 3.98 (s, 3 H, methoxyl), 6.80 (s, 1 H, H-4), 8.17 (s, 1 H, H-7).

Ir (KBr): 3400 (w), 2680 (br), 1630 (s), 1540 (w), 1455, 1425, 1330, 1250, 1105 (w), 1020, 810, 760.

2-Carboxy-5-oxo-4,5,6,7-tetrahydro-1H-pyrrolo[2,3-c]pyridine-3-[β-14C]propionic Acid ([8-14C]Carboxyporphobilinogen Lactam (22)). Demethylation of **20** (290 mg, 1.10 mmol) to the pyridone **21** was accomplished by reaction with 48% hydrobromic acid (5 ml) under refluxing conditions for 2.5 hr. The dark-red solution was evaporated completely to dryness in vacuo, and the residue

was dissolved in approximately 5 ml of water by addition of sodium carbonate to pH 7.0-7.5 (the solution becoming green colored and finally brown). The pyridone 21 so obtained was immediately hydrogenated (Parr shaker, 50 psi) without isolation over 10% palladium on carbon (290 mg) for 2 hr. The catalyst was removed and the red-brown solution acidified to pH 4 with glacial acetic acid, the carboxyporphobilinogen lactam 22 appearing immediately as fine white crystals. After several hours at 5°, the product was collected either by centrifugation or filtration and recrystallized twice by dissolution in dilute ammonium hydroxide and precipitation with glacial acetic acid. Final purification was carried out by slow filtration of a concentrated solution (ca. 1 ml) of 22 in dilute ammonium hydroxide through a small column (1.5 × 3 cm) of neutral alumina prepared and eluted with the same 1 N ammonium hydroxide solution. The nearly colorless effluent (ca. 30 ml) was acidified to pH 4 with glacial acetic acid, and after cooling for several hours, the precipitated [8-^{14}C]carboxyporphobilinogen lactam (22) was filtered and washed with water and acetone (160 mg, 58% uv spectrum consistent with that published).[25]

Ir (KBr): 3460 (w), 3170 (w), 2570 (br), 1710, 1640 (s), 1445, 1330, 1300, 1275. 1210, 1105 (w), 835 (w).

5-Oxo-4,5,6,7-tetrahydro-1H-pyrrolo[2,3-c]pyridine-3-[β-^{14}C]propionic Acid ([8-^{14}C]Porphobilinogen Lactam (23)). The carboxyporphobilinogen lactam 22 (160 mg, 0.635 mmol) was heated at reflux under nitrogen for 4 hr in water (22 ml). The water was evaporated under reduced pressure and the residue recrystallized three times by dissolution in dilute ammonium hydroxide and precipitation with glacial acetic acid at pH 4 to yield porphobilinogen lactam 23 as colorless rhomboidal plates (110 mg, 84%). Reaction with Ehrlich's reagent (2% [w/v] p-dimethylaminobenzaldehyde in 5 N hydrochloric acid)[41] was strongly positive at room temperature.

Nmr (DMSO-d_6): 2.35-2.65 (4 H obscured by DMSO; propionate α- and β-methylenes); 3.18 (t, $J = 3$, 2 H, C-4 methylene), 4.28 (c m, 2 H, C-7 methylene), 6.50 (d, $J = 2$, 1 H, pyrrole α-H), 6.84 (v br s, 1 H, lactam N-H). 10.3 (v br s, 1 H, pyrrole N-H).

Nmr (D$_2$O-K$_2$CO$_3$): 2.25-2.8 (br sym sextet, A$_2$B$_2$, $J_{app} = 4$-5, 4 H, propionate methylenes), 3.77 (t, $J = 3$, 2 H, C-4 methylene), 4.29 (t, $J = 3$, 2 H, C-7 methylene), 6.62 (s, 1 H, pyrrole α H).

Ir (KBr): 3200 (s), 2915 (w), 2550 (br), 1690 (s), 1640, 1610, 1430. 1415, 1375, 1320, 1260 (w), 1245 (w), 1205, 1175 (w), 1070 (w), 960 (w), 815 (w), 750 (w), 735.

Paper chromatography: R_f 0.64 [Whatman No. 1, descending, 1-butanol:acetic acid:water 63:11:26 (single phase)].

Specific activity: 603,000 dpm/μmol (0.272 mCi/mmol).

Although porphobilinogen lactam rapidly turns yellow and eventually dark brown on exposure to air and light, it was found to be quite stable for several months at −20° in the dark; and, more importantly, it was more stable than porphobilinogen (PBG) under similar conditions. Therefore, PBG was only generated (as described below) immediately prior to use. It was usually the case for whole-cell feeding experiments to dilute the lactam to lower specific activity with radioinactive material of like purity before hydrolysis to PBG. After dilution, the lactam was recrystallized once by dissolution in dilute ammonium hydroxide and precipitation with glacial acetic acid. A sample of this material was saved for counting to compare its specific activity with that of PBG after hydrolysis. In no case did these activities differ by more than 1% from their mean value.

2-Aminomethyl-3-carboxymethyl-4-pyrrole-[β-^{14}C]propionic Acid ([8-^{14}C]Porphobilinogen, (10)). Porphobilinogen was generated just before use (see above) according to the corrected method of Rapoport.[25] Porphobilinogen lactam 23, (41.4 mg, 0.20 mmol) was dissolved in 0.6 ml of 2 N potassium hydroxide in a small beaker and sealed with parafilm. After standing undisturbed for 70-75 hr in the dark. glacial acetic acid was added to precipitate the product (pH 6-7). It should be noted, that *porphobilinogen crystallizes as the monohydrate*.[40] The solution was cooled at 0-5° for 1-2 hr and filtered, any yellow color (from oxidation of PBG lactam) passing completely into the filtrates, to give [8-^{14}C]porphobilinogen (10), after washing with cold water and acetone, as colorless blocks (33.5 mg, 69%; PBG is quite soluble in water. Yields of 80-90% were attainable for hydrolyses of 100 mg or more of PBG lactam). Reaction with Ehrlich's reagent was strongly positive at room temperature and gave the proper uv absorption spectrum.[41]

Attempted recrystallizations by dissolution in dilute ammonium hydroxide and precipitation with glacial acetic acid generally led to darkening of the product. If desired, PBG may be recrystallized with some loss of material as the hydrochloride from 2 N hydrochloric acid, according to the method of Cookson and Rimington.[40]

Nmr (DMSO-d_6, 100 MHz): 2.3-2.7 (4 H obscured by DMSO; propionate α- and β-methylenes), 3.25 (s, 2 H, acetate methylene), 3.96 (s, 2 H, aminomethylene), 6.60 (s, 1 H, ring H), 10.86 (br s, 1 H, pyrrole N-H).

Nmr (D$_2$O-K$_2$CO$_3$): 2.2-2.8 (br sym sextet, A$_2$B$_2$, $J_{app} = 5$-7, 4 H, propionate methylenes), 3.36 (s, 2 H, acetate methylene), 4.08 (s, 2 H, aminomethylene), 6.67 (s, 1 H, ring H).

Ir (KBr): 3600-2500 (v br), 1700 (br), 1495 (w), 1405, 1375, 1260 (w), 1230 (w), 1090 (w), 945 (w), 815 (w).

Paper chromatography: R_f 0.49 [Whatman No. 1, descending, 1-butanol:acetic acid:water 63:11:26 (single phase)].

Specific activity: 602,000 dpm/μmol (0.271 mCi/mmol).

[^{14}C]Uroporphyrins I-IV ([^{14}C]Uro I-IV), 24, 25, 26, and 27, and Reduction to [^{14}C]Uroporphyrinogens I-IV, 28, 29, 12, and 30. (i) [^{14}C]Uroporphyrins I-IV. The statistical mixture of uroporphyrin isomers ($\frac{1}{8}$ I, $\frac{1}{8}$ II, $\frac{1}{2}$ III, and $\frac{1}{4}$ IV) was synthesized by a slight modification of Mauzerall's procedure.[24] Freshly prepared [8-^{14}C]porphobilinogen (10) (114 mg, 0.467 mmol; specific activity 73,800 dpm/μmol, from porphobilinogen lactam 51, specific activity 72,900 dpm/μmol, average specific activity 73,850 dpm/μmol) was dissolved in 1 N hydrochloric acid (30 ml), and after the resulting solution had been thoroughly degassed by nitrogen ebullition for 1 hr, it was heated on a steam bath for 1.5 hr under nitrogen. The pale-orange solution was concentrated *in vacuo* to about 10 ml, and the pH was adjusted to 7-8 with ammonium hydroxide solution. The tetrapyrrolic product was completely oxidized to uroporphyrin by addition of iodine solution, and the excess was backtitrated with thiosulfate solution to the starch-iodine end point. With the addition of glacial acetic acid, the dark-red solution was adjusted to pH 3.5 (the approximate isoelectric pH of uroporphyrin) and cooled at 0-5° overnight. The precipitated uroporphyrin was collected by centrifugation as an amorphous, dark solid and washed three times with cold water (adjusted to pH 3.5 with glacial acetic acid). The precipitate was dissolved in dilute ammonium hydroxide and evaporated thoroughly to dryness *in vacuo*, and the residue was dissolved in water and reevaporated completely to dryness several times. The UV spectrum of the purified [^{14}C]uroporphyrin in 1.00 N hydrochloric acid was in precise agreement with published data[24] (79.6 mg, 82%). Conversion of a small specimen of the uroporphyrin to its octamethyl ester (5% concentrated sulfuric acid in dry methanol, 24 hr) likewise gave the correct-visible absorption parameters,[41] and analysis by tlc (silica gel, chloroform and methanol 100:1) showed it to have an R_f indistinguishable from that of authentic octamethyl ester (kindly supplied by Dr. S. F. MacDonald).

Paper chromatography:[42] R_f 0.07 (Whatman No. 1, descending, ammonia vapor, 2,4-lutidine:water 10:7; intense red fluorescence under uv light).

Thin-layer chromatography:[43] R_f 0.67 (talc, 0.5 N hydrochloric acid:acetone 3:7).

Electrophoresis: (1) Whatman No. 1, 0.5 M acetic acid pH 2.7, 50 v/cm. 1 hr. Uroporphyrin remains at origin, Ehrlich test negative for porphobilinogen 10 cm toward cathode. (2) Whatman No. 1, 0.05 M glycine-sodium hydroxide pH 9.2, 50 v/cm, 1 hr. Uroporphyrin as one spot (broad) 7-12 cm from origin toward anode.

Specific activity: 296,700 dpm/μmol (0.133 mCi/mmol). Theoretical (4 × porphobilinogen specific activity): 293,400 dpm/μmol (0.132 mCi/mmol).

Uroporphyrin is subject to photooxidation and should be prepared only a short time before it is to be used and stored after purification in foil-wrapped vessels, preferably in the cold.

(ii) [^{14}C]Uroporphyrinogens I-IV. Uroporphyrin was dissolved in a small amount of dilute ammonium hydroxide or phosphate buffer (0.1-5 mg/ml) and reduced in a dim light to the corresponding uroporphyrinogen (hexahydroporphyrin) by shaking over freshly ground 3% sodium amalgam in a stoppered flask under nitrogen atmosphere until the solution was colorless and no longer fluorescent red.[44] It is essential to use recently prepared amalgam (same day if possible and no more than 1 week old) for the reduction to proceed quickly and to avoid brown- or green-colored by-

products. When the reduction was complete, the amalgam was removed by filtration through glass wool, and the resulting uroporphyrinogen solution was adjusted to pH 7 with thoroughly degassed 2-3 N hydrochloric acid and used without delay.

Enzymatic [^{14}C]Uroporphyrinogen I ([^{14}C]Uro'gen I (28)). (i) Isolation of Crude Uroporphyrinogen I Synthetase. Acetone-dried powder of fresh spinach leaves was prepared at −20° according to the method of Axelrod, et al.[45] Performing all operations in the cold room at 0-4°, a crude water extract of the acetone powder was prepared and subjected to ammonium sulfate fractionation as detailed by Bogorad[38] (centrifugations 20,000 g, 20 min). The pellet precipitated between 30 and 50% of saturation with ammonium sulfate was dissolved in the minimum amount of water, dialyzed, diluted,[38] and submitted to heat treatment (75 ± 2°, 12-15 min) to completely inactivate any uroporphyrinogen III cosynthetase present. After cooling in an ice bath, the solution was centrifuged (20.000 g, 15 min) and the supernatant fractionated[38] with ammonium sulfate as above. The precipitated protein collected between 30 and 50% of saturation was dissolved in distilled water (1 ml for each 3-4 g of original acetone powder) and dialyzed.[38] This enzyme solution was used without further purification in the incubation outlined below.

(ii) Incubation of Uroporphyrinogen I Synthetase with [8-^{14}C]Porphobilinogen. Incubations were customarily conducted with 12 screw-cap test tubes, each containing 0.5 ml of 0.1 M Tris buffer (pH 8.2), 0.1 ml of 0.1 M ethylenediamine tetraacetate (EDTA, pH 8.2), glutathione (reduced form, 1 mg), 1.1 ml of distilled water, 1.0 ml of [8-^{14}C]porphobilinogen (10) solution (ca. 0.5 mg/ml; e.g., preparation 4: 0.512 mg/ml, specific activity 602,500 dpm/μmol), and 0.3 ml of the partially purified and dialyzed uroporphyrinogen I synthetase solution (see above). The contents of each tube (total volume. 3.0 ml) was purged by nitrogen ebullition for 5 min and incubated at 29° hr 6-8 hr. (Note: As discussed in the text, the incubation is best carried out for 2-3 hr instead of 8 hr to minimize the in vitro conversion of porphobilinogen to the statistical mixture of uro'gen isomers; see analysis of derived coproporphyrin (iv) below.)

(iii) Isolation and Purification of [^{14}C]Uroporphyrin I. When the incubation was complete, the tubes were cooled briefly, and sufficient iodine solution was added to completely oxidize the uroporphyrinogen I generated therein to uroporphyrin I. The contents of the tubes were pooled, and excess iodine was back-titrated with thiosulfate solution (starch-iodide end point). The pH of the mixture was adjusted to 3.5-4 with glacial acetic acid, and the uroporphyrin was adsorbed onto talc (Fisher).[24,41] The talc was filtered and washed thoroughly with water (pH 3.5 by addition of acetic acid). and the uroporphyrin I was eluted with 1-2 M ammonium hydroxide solution containing a small amount of EDTA to prevent the formation of metalloporphyrins. This alkaline extract was evaporated to dryness in vacuo, and the residue was dissolved in water, filtered, concentrated under vacuum, and subjected to electrophoresis for 1 hr (Whatman No. 1, 0.5 M acetic acid pH 2.7, 50 v/cm). No PBG was detected 10-15 cm toward the cathode (Ehrlich's reagent), and the red fluorescent uroporphyrin band was extracted with dilute ammonium hydroxide containing EDTA, concentrated under reduced pressure, and chromatographed on paper (Whatman No. 1, descending. ammonia vapor, 2,4-lutidine:water 10:7,[42] 36 hr). The uroporphyrin band was eluted with dilute ammonium hydroxide solution (again containing a small amount of EDTA) and evaporated completely to dryness, and the [^{14}C]uroporphyrin I was dissolved in a small volume of distilled water (2.33 mg, 45% uv estimated).[24]

Specific activity: 2,855,000 dpm/μmol (1.28 mCi/mmol). Theoretical (4 × porphobilinogen specific activity): 2,410,000 dpm/Kmol (1.09 mCi/mmol).

In contrast to the chemically synthesized [^{14}C]uroporphyrins I-IV (section F), where the agreement between the observed and theoretical specific activities was excellent. the same comparison for uroporphyrin I showed a difference of about 15%. This apparently large error derives from the inaccuracies inherent on a small scale in computing a yield of uroporphyrin based on measurement of the Soret band (406 nm), whose absorbance is notoriously sensitive to pH and the presence of inorganic ions.[52]

(iv) Analysis of Isomer Purity. The isomeric purity of enzymatically synthesized uroporphyrin I was accurately and reproducibly

determined by decarboxylation to and analysis of the corresponding coproporphyrin (no satisfactory method exists for separating the uroporphyrin isomers directly). A small sample of uroporphyrin in in 1 N hydrochloric acid (50-500 μg/ml, sealed tube) was rigorously degassed under high vacuum (three cycles freeze-pump-thaw) and sealed. The tube was heated at 175 ± 2° for 4 hr according to the procedure of Edmondson and Schwartz,[37] and the resulting coproporphyrin was analyzed by the method of Eriksen,[36] where the four coproporphyrin isomers are separated into three spots: I, III + IV, and II, in increasing order of R_f (Whatman No. 1, ascending, ammonia vapor, 2,6-lutidine:water 10:7). As the R_f values were variable, positive identification of isomer type was made by comparison with the mobilities of authentic samples (S. F. MacDonald) run simultaneously.[42] Analysis of, e.g., the [^{14}C]uroporphyrin I derived from the incubation discussed above contained about 5% of the type III isomer (visual estimation of uv fluorescence).

Enzymatic [^{14}C]Uroporphyrins III + I ([^{14}C]Uro'gens III + I (12, 28)). (i) Isolation of Crude Uroporphyrinogen III Cosynthetase-Uroporphyrinogen I Synthetase. As the cosynthetase is particularly heat sensitive, all operations were carried out in solutions maintained at 0-4° in the cold room or in an ice bath. Further, to obtain maximal activity, the preparation was carried out through the dialysis without interruption and either immediately incubated with substrate or stored at −20°. Wheat germ (non-heat-treated,[53] 1200 g) was slurried with 4 l. of cold water and ground either in a large mortar for 30 min or in a Sorval Omni mixer for 5 min. The crude extract was then prepared according to the directions of Bogorad[34,35] by centrifugation (15,000 g, 20 min) and removal of the fats by filtration. The supernatant from isoelectric precipitation[34] (pH 5, glacial acetic acid) was submitted to ammonium sulfate fractionation. The precipitated protein collected between 30 and 50% saturation (Bogorad fractions B and C) was suspended in approximately 150 ml of water and dialyzed against cold distilled water for 4 hr. The enzyme solution was then diluted to 240 ml for incubation with [8-^{14}C]porphobilinogen (10) (see below). This isolation procedure was employed several times, and in every case bioassay of the extract demonstrated uroporphyrinogen I synthetase and uroporphyrin III cosynthetase activity (III:1 isomer ratio. see section iv below).

(ii) Incubation of the Synthetase-Cosynthetase Mixture with [8-^{14}C]Porphobilinogen (10). To each of 120 screw-cap test tubes the following reagents were added 0.5 ml of Tris buffer (0.6 M, pH 8.2), 0.1 ml of ethylenediaminetetraacetate (EDTA, 0.1 M, pH 8.2), 0.1 ml of PBG solution (ca. 1 mg/ml; e.g., preparation 2: 1.167 mg/ml, specific activity 602,500 dpm/μmol), and 2.0 ml of dialyzed enzyme solution (see above). Each tube (total volume, 2.7 ml) was purged briefly with nitrogen, and the incubation was conducted at 36-37° for 1.25 hr.

(iii) Isolation and Purification of [^{14}C]Uroporphyrins III + I. The tubes were cooled to 0-5° in an ice bath, iodine solution was added to fully oxidize the uroporphyrinogens formed during the incubation to uroporphyrin, the excess iodine was back-titrated with sodium thiosulfate solution (starch-iodide end point), and the tubes were heated in a steam bath for 5 min to precipitate protein. The contents of the tubes were pooled and diluted to twice the original volume with 0.1 M Tris buffer (pH 8.2). After centrifugation (20,000 g, 20 min) and washing of the precipitated protein with the same buffer, the pH of the combined supernatants was adjusted to 3.5 with glacial acetic acid and refrigerated overnight. The following day the dark precipitate was centrifuged and extracted into a mixture of acetone and concentrated hydrochloric acid (19: 1). The dark-purple solution was filtered to remove undissolved material, and the filtrate was evaporated to dryness (rotary evaporator, bath temperature below 40°). The residue was dissolved in water and evaporated several times to remove excess hydrogen chloride. The crude uroporphyrin was dissolved in a small amount of dilute ammonium hydroxide and precipitated at its isoelectric point by adjusting the solution to pH 3.5 (glacial acetic acid) and cooling overnight. The precipitate was centrifuged, and the small amount of uroporphyrin in the supernatant was adsorbed on talc; the talc was washed thoroughly with water (pH 3.5 by addition of acetic acid) and the uroporphyrin eluted with acetone-concentrated hydrochloric acid solution (19:1). The porphyrin solution was evaporated to dryness in vacuo (bath temperature below 40°). the

residue combined with the precipitate obtained on centrifugation above, and the whole procedure of isoelectric pH precipitation, centrifugation, and talc treatment was repeated. The uroporphyrin so obtained was dissolved in a small amount of dilute ammonium hydroxide and evaporated to dryness under vacuum, and excess ammonia was removed by repeated dissolution in water and evaporation to dryness. The [^{14}C]uroporphyrins III + I were purified by electrophoresis (Whatman No. 1, 0.05 M glycine–sodium hydroxide pH 9.2, 50 v/cm, 50–60 min) and paper chromatography[42] (Whatman No. 1, descending, ammonia vapor, 2,6-lutidine:water 10:7; see sections F and G). A small sample of the purified [^{14}C]-uroporphyrin III + I mixture was chromatographed on a two-dimensional system (Whatman No. 1, ascending, (1) ammonia vapor, 2,6-lutidine:water 10:7; (2) 1-butanol:acetic acid:water 4:1:1). Analysis of the chromatogram by autoradiography revealed one and only one radioactive spot, whose R_f corresponded to that of the red fluorescence of uroporphyrin. After elution from the papers, the electronic absorption spectrum was recorded and the total yield of uroporphyrin estimated (4.28 mg, 36%).

Specific activity:[54] 2,610,000 dpm/μmol (1.18 mCi/mmol). Theoretical (4 × porphobilinogen specific activity): 2,410,000 dpm/μmol (1.09 mCi/mmol).

(iv) **Analysis of Isomer Purity.** The uroporphyrin III–I ratio was estimated visually from paper chromatographic separation of the derived coproporphyrins according to the procedure described previously.

Feeding Experiments. The procedures described below for the growth of the vitamin B$_{12}$ producing organism and for the conduct of suspended-cell feeding experiments were developed to optimize both the incorporation of tracer and the yield of labeled vitamin. Although mutually exclusive to a certain degree, maximization of these two factors was sought to enable efficient utilization of limited quantities of precursor under standardized conditions, with particular concern directed toward obtaining vitamin B$_{12}$ in sufficient amount and enrichment to allow eventual ^{13}C-FT nmr analysis. Some of the ^{14}C tracer experiments, however, were carried out before the conditions outlined below were established; where differences from the standardized procedure occur, the details of these individual experiments have been provided. It should be noted that only the feeding experiments of major importance have been recorded herein.

A. Growth of *Propionibacterium shermanii* **ATCC 9614.** A simplified version of Bernhauer's medium[28] was employed for slants and inocula containing per liter: casein hydrolysate (acid hydrolyzed, 12.5 g; Sigma), casein hydrolysate (enzymatic, 12.5 g; Sigma), yeast extract (5.0 g; Difco), potassium phosphate, monobasic (KH$_2$PO$_4$, 1.76 g), potassium phosphate (K$_3$PO$_4$, 1.76 g), magnesium chloride (MgCl$_2 \cdot$ 6H$_2$O, 400 mg), ferrous sulfate (FeSO$_4 \cdot$ 7H$_2$O, 10 mg), and deionized water (950 ml), and the solution was adjusted to pH 7. Glucose solution (6 g in 50 ml of deionized water) and the above medium were sterilized separately (120°, 40 min) and combined at the time of inoculation to complete the medium.

The final culture medium, again containing no cobalt or 5,6-dimethylbenzimidazole (DMBI), was prepared in an analogous fashion: casein hydrolysate (acid hydrolyzed, 37 g), casein hydrolysate (enzymatic, 37 g), yeast extract (15 g), potassium phosphate, monobasic (KH$_2$PO$_4$, 5.28 g), potassium phosphate (K$_3$PO$_4$, 5.28 g), magnesium chloride (MgCl$_2 \cdot$ 6H$_2$O, 1.20 g), ferrous sulfate (FeSO$_4 \cdot$ 7H$_2$O, 30 mg), and deionized water (2500 ml), and after dissolution the pH was adjusted to 7. Glucose solution (18 g in 100 ml of deionized water) was prepared, autoclaved separately as above, and combined with the sterilized medium at the time of inoculation to give a total volume of 2600 ml.

(i) **Slants.** The glucose and 2% agar (20 g/l.; Difco) were added to the medium prior to sterilization for the preparation of slants and stabs. Stabs of the source bacteria were grown for 1 week, stored in the freezer, and renewed every 4–6 months. During periods when feeding experiments were to be conducted, *P. shermanii* was maintained on slants at 29°, and fresh transfers were made weekly. At least three such transfers were made before the first inoculation (below).

(ii) **Inocula.** Approximately 380 ml of medium were autoclaved in a 2-l. culture bottle. One-week-old bacteria from four slants were suspended under sterile conditions in small amounts of medi-

um and transferred to the culture bottle, and sterilized glucose solution was added (2.4 g in 20 ml of deionized water). When the inoculation was complete, the culture bottles were maintained without agitation or pH adjustment at 29° for 7 days.

(iii) **Anaerobic Cultures.** Working under sterile conditions, each 400-ml still culture was used to inoculate 2500 ml of final growth medium containgd in a 6-l. erlenmeyer flask. Glucose (18 g in 100 ml of deionized water) was added to every flask, and each was sealed with a two-hole stopper fitted with glass tubes and cotton plugs to allow sterile nitrogen ebullition of the fermentation (total 3000 ml). The cultures were maintained at room temperature during the growth period and kept agitated and anaerobic by the steady passage of nitrogen. On the day after inoculation and on each of the next 4 days, 6 g of glucose (in 30 ml of deionized water) was added to each flask, and the pH was adjusted (indicator paper) to about 7.0 with 10% sodium carbonate solution (all operations with sterile solutions under sterile conditions). Adjustments of pH were typically as follows: day 1 (6.0–7.0), day 2 (5.5–7.0), day 3 (5.5–7.0), day 4 (6.5–7.0), and day 5 (6.5–7.0). On the seventh day of growth, the cells were harvested by centrifugation (20,000 g. 20 min, 1–4°), washed with either 0.9% sodium chloride or $^1/_{15}$ M phosphate buffer (pH 7.6), and used as soon as possible (*ca.* 15–20 g cells/l., wet weight).

B. Suspended-Cell Feeding Conditions. The generalized feeding conditions presented here evolved from published procedures of Müller[30] and others.[20] Experiments conducted with radioactive precursors each employed cells freshly collected from 1.5–3.0 l. of 7-day-old cultures of *P. shermanii*. Although the amounts of buffer solution, labeled substrate, and cofactors varied from experiment to experiment, the procedures themselves for feeding a stable (*i.e.,* methionine, ALA, and PBG) or a readily oxidizable (*i.e.,* uro'gen) intermediate were standardized as follows.

(i) **L-Methionine, ALA, and PBG.** Phosphate buffer ($^1/_{15}$ M, pH 7.6) and a small amount of cobalt chloride (CoCl$_2 \cdot$ 6H$_2$O) were autoclaved (120°, 40 min) together in a 500-ml erlenmeyer flask, to which the following were added under sterile conditions: freshly harvested cells, glucose in sterile aqueous solution, 5,6-dimethylbenzimidazole (DMBI, Aldrich, 99+%) in the minimum amount of 70% ethanol, L-methionine, and the labeled precursor. The methionine and precursor were dissolved in a small amount of water or $^1/_{15}$ M phosphate buffer and added after Seitz filtration (Nalge filter unit, 0.20 μ). When labeled methionine was fed, unlabeled ALA was administered as well to complete the incubation medium. When the additions were complete, the flasks were sealed with two-hole stoppers fitted with glass tubes and cotton plugs to allow sterile nitrogen ebullition of the cell suspensions in the same manner as the growing cultures of *P. shermanii* (*vide supra*). Nitrogen was passed through the fermentations for about 4 hr, and the flasks were transferred to a glove bag thoroughly purged with nitrogen. The rubber stopper and tubing were removed from each flask, the medium was adjusted to pH 7.0 (indicator paper) with 10% sodium carbonate solution (sterile), and the flask was quickly resealed with a solid rubber stopper (sterile) and parafilm. The suspensions were stored in the dark at 29°. At approximately 12, 24. and 40 hr after the feeding experiment was begun, the flasks were returned to the glove bag, and 50% aqueous glucose solution (1 ml, sterile) was added to each, and the pH was adjusted to 7.0, as in section A above. After 50 hr, the cells were harvested by centrifugation (20,000 g, 20 min), washed two to three times with 0.9% sodium chloride solution or $^1/_{15}$ M phosphate buffer (pH 7.6), and stored at −20° for subsequent extraction.

(ii) **Uro'gen.** The phosphate buffer suspension medium containing cobalt chloride was prepared in 500-ml erlenmeyer flasks as in i. and glucose, DMBI, and methionine were added under the same sterile conditions as above. The flasks were sealed with the same stopper and glass-tube arrangement, and the solutions were thoroughly purged fro 2–4 hr with nitrogen that had been first passed through an alkaline solution pyrogallol. The flasks containing the degassed medium were transferred to a glove bag well flushed with nitrogen. In semidarkness, the freshly harvested cells were added to each flask followed by the neutralized uro'gen solution. The flasks were immediately sealed with sterile rubber stoppers and parafilm, wrapped in foil, and maintained in the dark (to prevent photooxidation of the uro'gen) at 29°. Addition of glucose and adjustment of pH were carried out under nitrogen, as in section i,

under dim lights. After 50 hr, the cells were collected by centrifugation (20,000 g, 20 min, 0–4°), washed two to three times, and frozen.

C. Extraction of Corrins. The washed cells obtained from the suspended-cell feeding experiment were heated at reflux on a steam bath for 15–20 min with a mixture of 80% methanol containing 0.1% potassium cyanide, adjusted to pH 6.0 (50–150 ml/l. of *P. shermanii* used, depending on scale of feeding experiment). The mixture was centrifuged (20,000 g, 20 min) in tared bottles, the red-brown supernatant was removed, and the weight of the rubbery cell residue was determined. The cell mass so obtained was recorded for every experiment (see below), for comparative purposes, and accurately reflects the amount of *P. shermanii* used in each case. The methanolic extracts were concentrated under reduced pressure in semidarkness (rotary evaporator, bath temperature below 50°) to 10–160 ml, depending on the scale of the extraction. A small amount of silicone antifoam agent was found to speed this operation considerably. The concentrated extracts were transferred to centrifuge tubes, adjusted to pH 3.5–4.0 with concentrated hydrochloric acid, and stored overnight at 0–5° to precipitate protein. The dark suspensions were centrifuged (30,000 g, 40 min), and the precipitated protein was washed once with small portions of water and centrifuged. The combined supernatants were purified as below.

D. Purification of Vitamin B_{12} (Cyanocobalamin).[16,30] The dark red-brown supernatant was extracted once with 20–60 ml of phenol-chloroform (1:1) and a second time with about half the amount. The combined phenol extracts were washed three to five times with water or until little yellow color (riboflavine) appeared in the aqueous layer. Centrifugation was required during each extraction to break emulsions. The red-purple phenol layer was diluted with 10–15 volumes of ether and extracted several times with small portions of water until no red color remained in the organic phase. The resulting aqueous corrin-containing solution was washed three or more times with an equal volume of chloroform to remove any remaining phenol or antifoam agent. The red solution was evaporated to dryness (rotary evaporator, bath temperature below 50°) under dim lights, the residue was suspended in absolute ethanol and filtered, and the dark-red filtrate was evaporated to dryness under the conditions above. The viscous residue was dissolved in a small amount of water and submitted to electrophoresis (Whatman 3MM, 50 v/cm, 0.05 M glycine–sodium hydroxide buffer pH 9.2) for 1.5–2.5 hr as necessary for good separation. Under these conditions, remaining protein and acidic compounds (*i.e.*, acidic corrins and any residual precursor) move toward the anode, whereas cyanocobalamin alone migrates a short distance toward the cathode. [Note: In some early ^{14}C-tracer work, this separation was accomplished by paper chromatography: Whatman No. 1 or 3MM, descending, 2-butanol (saturated with water):glacial acetic acid 99:1.] The vitamin B_{12} containing band was cut from the partially dried papers and thoroughly eluted with deionized water. The aqueous cyanocobalamin solution was concentrated and desalted by extraction into phenol–chloroform (1:1), washing thoroughly with water, diluting with ether, and extracting into water as above. The water solution was washed several times with equal volumes of chloroform to remove any trace of phenol and was concentrated to 1 ml or less under reduced pressure. Approximately 10 volumes of acetone were added, and the mixture was cooked to induce crystallization. Radioactive samples were recrystallized in like fashion to constant specific activity.

Administration of [14]C Substrates. A. [8-^{14}C]Porphobilinogen ([8-^{14}C]PBG (10)). Experiment 1. Six liters (2 × 3 l.) of *P. shermanii* were grown as described above for 6 days from inocula that were 49 days old (the cells from these still cultures were collected by centrifugation, washed once with 0.9% sodium chloride, and used directly to inoculate the anaerobic cultures). The cells from the 6 l. were collected, washed, and suspended under sterile conditions in 300 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) containing cobalt chloride ($CoCl_2 \cdot 6H_2O$, 3 mg). L-Methionine (20 mg), 5,6-dimethylbenzimidazole (DMBI, 20 mg), [8-^{14}C]PBG (21.0 mg, 64,500 dpm/μmol), and 9.6 ml of 50% glucose solution were added. Nitrogen was passed through the suspension for 3 hr, and the incubation was stored under nitrogen (glove bag) at room temperature for the duration of the feeding experiment. After the first and second day, the pH of the medium was noted (6.0 and 6.4, re-

Table II

Time, hr	Adjust pH	50% glucose, ml
2	6.0–7.0	
4	6.8–7.0	
13.5	6.4–7.1	1
15.5	6.4–7.0	2
19.5	(6.8)	2
42	(6.7)	2
43.5	6.4–7.1	

Table-III

Time, hr	Adjust pH	50% glucose, ml
3	5.0–7.0	
15	6.0–7.0	1
25.5	6.2–7.0	1
40	6.4–7.0	1

spectively) and adjusted to 7.0 with 10% sodium carbonate, and 3 ml of 50% glucose solution was added. On the third day, the cells were harvested, washed and extracted (cell wt, 11.2 g), and the vitamin B_{12} was purified by paper chromatography (see section D above).

	Cyanocobalamin
Spec act., dpm/μmol	15,760
Spec act./C, dpm/μmol	3,940
Spec incorp/C, %	6.1

Experiment 2. *P. shermanii* (12 l.) was grown essentially by the standardized procedure of Section A (slants 6 days, inocula 8 days, anaerobic cultures 6 days). Cells from 8 l. were collected, washed, and suspended in the same medium as in expt 1, containing PBG (20.0 mg, 64,500 dmp/μmol) and the same cofactors. Nitrogen was bubbled through the fermentation for 2 hr, and pH adjustments and additions of glucose were carried out in a nitrogen atmosphere as shown in Table II. Approximately 50% of the incubation was harvested after 2 days (No. 2A; cell wt, 22.5 g) and the remainder after 3 days (No. 2B; cell wt, 31.7 g). Extraction in the usual manner and paper chromatographic purification gave the following results.

	Cyanocobalamin	
	2 days (2A)	3 days (2B)
Spec act., dpm/μmol	14,470	13,120
Spec act./C, dpm/μmol	3,690	3,280
Spec incorp/C, %	5.72	5.08

Experiment 3. Cells from 18 l. of anaerobic culture were harvested, washed with 0.9% sodium chloride, and divided equally among nine 500-ml erlenmeyer flasks, each containing a sterile solution of 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) and cobalt chloride ($CoCl_2 \cdot 6H_2O$, 1 mg). Eight of these flasks were used for [8-^{13}C]PBG studies (see following paper); to the ninth was added, by the procedures outlined in feeding description above (section B), 50% glucose solution (3.2 ml), DMBI (20 mg), L-methionine (20 mg), and [8-^{14}C]PBG (15.5 mg, 36,650 dpm/μmol). The incubation was degassed for 3 hr and stored at 29° in the dark, except for occasional adjustment of pH and addition of glucose, as indicated in Table III. After 49.5 hr the fermentation was harvested; the cells were washed once with $\frac{1}{15}$ M phosphate buffer, and extracted (cell wt, 30.7 g). The vitamin B_{12} was purified by electrophoresis and crystallized from acetone–water.

	Cyanocobalamin
Spec act., dpm/μmol	7460
Spec act./C, dpm/μmol	1870
Spec incorp/C, %	5.09

B. [^{14}C]Uroporphyrinogens I–IV ([^{14}C]Uro'gens I–IV (28, 29, 12, and 30)). Experiments 4 and 5. In this pair of early feeding experiments two 1-l. round-bottom flasks were prepared, each containing 250 ml of 0.1 M phosphate buffer (pH 7.0), cobalt chloride ($CoCl_2 \cdot 6H_2O$, 3 mg), magnesium chloride ($MgCl_2 \cdot 6H_2O$, 60

Table IV

Time, hr	Adjust pH Expt 12	Adjust pH Expt 13	50% glucose, ml Expt 12	50% glucose, ml Expt 13
13.5	5.0–7.2		1	
19.5		5.0–7.0		1
25.5	6.8–7.2		1	
45	6.2–7.0	6.0–7.0	1	1

mg), L-methionine (44.7 mg), 5,6-dimethylbenzimidazole (DMBI, 60 mg), and glucose (3 g in 50 ml of deionized water). The buffer and inorganic salts were autoclaved together (120°, 40 min), and the other cofactors were added by the procedure outlined in section B (ii). The completed medium (total volume 300 ml) was rigorously degassed under high vacuum (10^{-4} mm) by the freeze–pump–thaw method (three cycles). *P. shermanii* (12 l.) was grown anaerobically for 15–17 days without pH adjustment or addition of glucose. Cells from 10 l. were harvested, washed with 0.9% sodium chloride, divided equally, and suspended under a nitrogen atmosphere (glove bag) in the flasks of degassed medium. Ascorbic acid (20 mg) was then added to each flask. A solution of [^{14}C]uroporphyrins I–IV was prepared as described above (69.4 mg, 269,000 dpm/μmol, based on specific activity of precursor PBG lactam), 50% of which was administered, after reduction over sodium amalgam to the corresponding uro'gen mixture. to one of the flasks (expt 4). The remaining uroporphyrin was added without reduction to the other flask (expt 5). The incubations were sealed, and the feeding experiments were carried out at room temperature for 60 hr, without further addition of glucose or adjustment of pH (final pH 5–5.5). The cells were harvested, washed with 0.9% sodium chloride, and extracted (cell wt. 26.9 and 17.7 g, respectively), and the resulting vitamin B_{12} was purified by paper chromatography and crystallized to constant radioactivity (note: accurate background counts were obtained for both experiments).

	Cyanocobalamin Uro'gen I–IV (expt 4)	Cyanocobalamin Urop I–IV (expt 5)
Spec act., dpm/μmol	911	Slightly below background
Spec incorp, %	0.153	0.00

Experiment 6. Relatively old still cultures (24 days) were used to inoculate anaerobic cultures (6 l.) that were grown for 8 days without addition of glucose or adjustment of pH. [^{14}C]Uro'gens I–IV (27.0 mg, 296,700 dpm/μmol), synthesized from [8-^{14}C]PBG, specific activity 73,800 dpm/μmol, were fed in the same manner as in expt 4. The incubation was stored in the dark at 29° for 70 hr, harvested, washed, and extracted (cell wt, 13.0 g) as before. Purification of the vitamin was achieved again by paper chromatography and recrystallization to constant radioactivity.

	Cyanocobalamin
Spec act., dpm/μmol	241
Spec incorp, %	0.0812

Experiment 7. The bacteria (12 l.) employed were grown under conditions similar to those in expt 6 (slants 9 days, inocula 9 days, anaerobic cultures 9 days without addition of glucose or adjustment of pH). A 500-ml round-bottom flask was autoclaved containing 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) and 1 mg of cobalt chloride (CoCl$_2$ · 6H$_2$O), to which were added under sterile conditions DMBI (20 mg) and 50% glucose solution (3.2 ml), but no L-methionine. The medium was deaerated for 3.5 hr and [^{14}C]uro'gen fed (25.4 mg, 296,700 dpm/μmol) by the method outlined in section B. As in expt 6, the incubation was stored at 29° in the dark for 70 hr. However, after 19 and 45 hr, the pH was adjusted with sodium carbonate (pH 4.5–7.0 and 5.0–7.0, respectively), and 1 ml of 50% glucose was added (under nitrogen). The cells were harvested, washed, and extracted (cell wt, 7 g) in the

	Cyanocobalamin
Spec act., dpm/μmol	7541
2nd cryst (3rd)	7795 (7600)
Spec incorp, %	2.56

usual fashion, and the derived vitamin B_{12} was purified by electrophoresis and recrystallized to constant radioactivity.

Experiment 8 (Run Parallel to [^{14}C]Uro'gens III + I, Expt 13). *P. shermanii* (6 l.) was grown according to the standardized procedure. The cells were collected, washed with $\frac{1}{15}$ M phosphate buffer (pH 7.6), and divided equally between the present experiment and enzymatic [^{14}C]uro'gens III + I, expt 13. [^{14}C]Uro'gens I–IV (25.6 mg, 225,000 dpm/μmol) were incubated under the same conditions as in expt 9, with the addition of glucose and pH adjustment at 19 and 45 hr (pH 5.0–7.0 and 5.5–7.2, respectively). After 70 hr at 29°, the cells were harvested, washed twice with $\frac{1}{15}$ M phosphate buffer, and extracted (cell wt, 16.3 g), and the labeled vitamin B_{12} was obtained by electrophoresis and recrystallized to constant activity.

	Cyanocobalamin
Spec act., dpm/μmol	2071
2nd cryst (3rd)	1845 (2025)
Spec incorp, %	0.91

Experiments 9 and 10 (Run Parallel to [^{14}C]Uro'gen I, Expt 11). The bacteria (6 l.) were grown essentially by the standard procedure (slants 11 days old) described above. The cells were collected by centrifugation, washed with 0.9% sodium chloride, and divided equally among the four parallel feeding experiments. The medium for each experiment was prepared in the same manner as in expt 7 and 8, except that expt 9 contained 10 mg of L-methionine. The solutions were deaerated (3.5 hr) and the various uro'gen substrates fed according to the procedure described above (expt 9 and 10: 22.8 mg each, 225,000 dpm/μmol). The incubations were stored in the dark at 29°, except for periodic additions of glucose and adjustments of pH under nitrogen (glove bag) (see Table IV). After 70 hr, both fermentations were harvested (cell wt, 8.7 g and 9.3 g, respectively), and the vitamin B_{12} was extracted and purified as outlined in sections C and D.

	Cyanocobalamin Expt 12	Cyanocobalamin Expt 13
Spec act., dpm/μmol	4230[9]	1590[10]
Spec incorp, %	1.88	0.707

C. Enzymatic [^{14}C]Uroporphyrinogen I (Uro'gen I, 57). Experiment 11 (Run Parallel to [^{14}C]Uro'gens I–IV, Expt 11, 12, and [^{14}C]Uro'gens III + I, Expt 14). [^{14}C]Uro'gen I was generated enzymatically from [8-^{14}C]PBG (10) (4.5 mg, 603,000 dpm/μmol). The spinach synthetase incubation was conducted for 12 hr, and the resulting uro'gen was oxidized and diluted with radioinactive, chemically synthesized uroporphyrins I–IV (total 25.3 mg, 142,500 dpm/μmol). The feeding experiment was conducted as previously described with the addition of 50% glucose solution (1 ml) and the adjustment of pH at 19.5 and 45 hr (pH 5.5–7.0 and 6.0–7.0, respectively). After 70 hr in the dark at 29°, the cells were harvested and washed, and the vitamin B_{12} was isolated in the usual fashion (cell wt, 8.5 g). Note: The apparent small positive incorporation was, in fact, traced (by analysis of the corresponding coproporphyrin) to small amounts of the type III isomer generated in the spinach synthetase incubation, presumably from *in vitro* conversion of radioactive PBG to the statistical mixture of uro'gen isomers. Repetition with pure uro'gen I (7 mg) gave zero incorporation.

	Cyanocobalamin
Spec act., dpm/μmol	23.8
Spec incorp, %	0.017

D. Enzymatic [^{14}C]Uroporphyrinogens III + I (Uro'gens III + I (12 and 28)). Experiment 12. *P. shermanii* (6 l.) was grown by the standardized procedure outlined previously. In a 500-ml three-neck flask, 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) and cobalt chloride (CoCl$_2$ · 6H$_2$O, 1 mg) were autoclaved (120°, 40 min). Observing the usual precautions for sterility, DMBI (20 mg), L-methionine (20 mg), and 50% glucose solution (3.2 ml) were added. The medium was rigorously degassed under high vacuum (10^{-4} mm) by the freeze–pump–thaw method (three cycles). [^{14}C]Uro'gens III + I (4.28 mg, isomer ratio III:I, 7:3 or 8:2) were generated from [8-^{14}C]PBG (10) (603,000 dpm/μmol), using the wheat germ enzyme system as described previously. A sample of

Table V

Time, hr	Adjust pH	50% glucose, ml
1:20	6.3–7.4	
2:20	6.6–7.2	
3:00	6.9–7.2	
4:50	6.8–7.4	2
5:20	6.9–7.4	
9:10	(7.1)	
13:55	(6.9)	1
16:10	6.6–7.2	
24:50	6.9–7.4	1
25:25	6.9–7.5	
34:10	(7.0)	
35:50	(7.2)	1
37:40	(6.8)	
41:10	6.7–7.4	

Table VI

Time, hr	Adjust pH Expt 15	Adjust pH Expt 16	50% glucose, ml Expt 15	50% glucose, ml Expt 16
2:30	6.0–7.0	6.0–7.2		
9:30	6.5–7.4	6.5–7.4	1	1
24:40	6.8–7.0	6.8–7.0		
28:50	6.8–7.0	6.8–7.0		
36:40	6.6–7.0	6.6–7.0	1	1
42:30	6.5–7.0	6.6–7.0	1	1
45:00	6.5–7.0	6.6–7.0		

Table VII

Time, hr	Adjust pH Expt 17	Adjust pH Expt 18	50% glucose, ml Expt 17	50% glucose, ml Expt 18
2	6.0–7.0	6.0–7.1		
12		6.0–7.0		1
15	5.5–7.0		1	
24	6.2–7.1		1	
25		6.4–7.2		1
37	6.4–7.0		1	
38.5		6.8–7.0		1
Total 10% Na₂CO₃	26 ml	21 ml		

the enzymatic [¹⁴C]uro'gen III + I mixture (2.15 mg) was oxidized and diluted with radioinactive uroporphyrins I–IV (total 22.7 mg, 248,000 dpm/μmol). The labeled uro'gen mixture was reduced over sodium amalgam and fed together with ascorbic acid (20 mg) to the suspended cells, as detailed in section B. The flask was fitted with two pressure-equalizing dropping funnels, one containing 10% sodium carbonate and the other 50% glucose solution. Inlet and outlet tubes were connected to allow the entire system to be purged continuously with oxygen-free nitrogen while maintaining sterile conditions. Finally, electrodes were inserted to permit monitoring of the pH conveniently with a pH meter. The fermentation was wrapped in aluminum foil and maintained at room temperature with frequent pH adjustment and addition of glucose (see Table V). After 45 hr, the cells were harvested, washed once each with 0.9% sodium chloride and $\frac{1}{15}$ M phosphate buffer, μ and extracted (cell wt, 24.0 g), and the resulting vitamin B₁₂ was purified by electrophoresis and crystallized.

	Cyanocobalamin
Spec act., dpm/μmol	20.2
Spec incorp, %	0.0082

Experiment 13 (Run Parallel to [¹⁴C]Uro'gens I–IV, Expt 10). A portion (1.1 mg, isomer ratio III:I, 7:3 or 8:2) of the enzymatic [¹⁴C]uro'gen III + I mixture that had been prepared for expt 12 was combined with radioactive uroporphyrin (8.8 mg) recovered from expt 12 and more radioinactive, chemically synthesized uroporphyrins I–IV (total 24.5 mg, 214,000 dpm/μmol). The feeding experiment was conducted as described earlier (see expt 8), with addition of 50% glucose solution (1 ml) and pH adjustment carried out in the usual manner at 19 and 45 hr (pH 5.0 to 7.0 and 5.5 to 7.5, respectively). After 70 hr in the dark at 29°, the cells were harvested, washed twice with $\frac{1}{15}$ M phosphate buffer, and extracted (cell wt, 16.6 g), and the resulting vitamin B₁₂ was purified by electrophoresis and recrystallized to constant radioactivity.

	Cyanocobalamin
Spec act., dpm/μmol	877
Cryst	1041 (2nd), 1032 (2nd), 848 (3rd) 872 (3rd), 848 (4th),
Spec incorp, %	0.40

Experiment 14 (Run Parallel to [¹⁴C]Uro'gens I–IV, Expt 9 and 10). [¹⁴C]Uro'gens III + I were synthesized enzymatically from [8-¹⁴C]PBG (603,000 dpm/μmol) and purified according to the procedure described earlier. The uroporphyrin so obtained was further purified by conversion to the octamethyl ester (5% concentrated sulfuric acid in dry methanol, 20 hr at room temperature) and paper chromatography (Whatman No. 1, descending, chloroform:methanol 19:1). The intensely red fluorescent uroporphyrin octaester was eluted, hydrolyzed (7 N hydrochloric acid, 40 hr at room temperature), reduced, and fed (about 0.1 mg, isomer ratio III:I, 6:4 or 7:3), according to the procedure described for uro'gens I–IV in expt 10. Glucose was added, and the pH was adjusted in the usual manner at 19 and 45 hr (pH 5.0 to 7.0 and 6.0 to 7.0, respectively). After 70 hr in the dark at 29°, the cells were harvested, washed, and extracted (cell wt, 7.3 g). The labeled vitamin B₁₂ was purified by electrophoresis and crystallized.

	Cyanocobalamin
Spec act., dpm/μmol	184
Spec incorp, (based on spec act of PBG used X4)	0.00765

E. [¹⁴CH₃]-L-Methionine. Experiments 15 and 16. *P. shermanii* (6 l.), grown under standardized conditions, was apportioned equally between two 500-ml erlenmeyer flasks containing the following: 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6), cobalt chloride (CoCl₂ · 6H₂O, 1 mg), DMBI (20 mg), 50% glucose solution (3.2 ml). [¹⁴CH₃]-L-methionine (15.0 mg, 110,200 dpm/μmol), and ALA hydrochloride (ALA · HCl; expt 15, 10.0 mg; expt 16, 50.0 mg). Maintaining sterile conditions, nitrogen was passed through the fermentations for 2.5 hr, whereupon the flasks were transferred to nitrogen-purged glove bag for periodic adjustment of pH and addition of glucose (Table VI). After 48 hr at room temperature, the cells were harvested, washed with $\frac{1}{15}$ M phosphate buffer, extracted (cell wt, 29.6 and 20.3 g, respectively), and the vitamin B₁₂ were analyzed after electrophoresis and crystallization (note: the difference in methionine incorporations may be due more to variation in the cell weights used than to the ALA amounts fed).

	Cyanocobalamin Expt 15	Cyanocobalamin Expt 16
Spec act., dpm/μmol	116,700	133,500
Spec incorp/methyl group, %		
(If 6 labeled methyl groups)	17.6	20.2
(If 7 labeled methyl groups)	15.1	17.3

F. [5-¹⁴C]-δ-Aminolevulinic Acid ([5-¹⁴C]ALA). Experiments 17 and 18. Anaerobic cultures (6 l.) were grown according to the standardized procedures outlined earlier. Cells from 4 l. were divided equally between two 500-ml erlenmeyer flasks containing 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) and cobalt chloride (CoCl₂ · 6H₂O, 1 mg). In expt 17, the following were added, observing the usual precautions for sterility: 50% glucose (3.2 ml), DMBI (20 mg), L-methionine (20 mg), and [5-¹⁴C]ALA · HCl (15.0 mg, specific activity 19,800 dpm/μmol). After 2 hr of nitrogen ebullition, the fermentation was stored at 29° in the dark with occasional pH adjustments and glucose additions made as indicated in Table VII. Experiment 18, after suspension of the cells in the buffer, was maintained at 29° in the dark for 12 hr, after which time the labeled ALA and cofactors were administered as above. Nitrogen was passed through the incubation for 2 hr, and the addition of glucose and adjustment of pH are noted in the table (the times shown indicate the elapsed time from the feeding of labeled precursor). The cells were harvested 50.5 and 50.0 hr, respectively, after administration of substrate, washed with $\frac{1}{15}$ M phosphate buffer, and extracted (cell wt, 18.4 and 14.0 g, respectively), and

the vitamin B_{12} was purified by electrophoresis. The B_{12} bands were eluted, desalted, and counted as aqueous solutions without crystallization.

	——Cyanocobalamin——	
	Expt 17	Expt 18
Spec act dpm/μmol	37,400	34,200
Spec incorp/C, %		
(If 8 labeled centers)	23.6	21.6
(If 7 labeled centers)	27.0	24.7

Degradations of ^{14}C-Labeled Corrinoids. A. Degradation of Vitamin B_{12} (Cyanocobalamin, Labeled by Feeding [8-^{14}C]Porphobilinogen ([8-^{14}C]PBG (10)). (i) Acidic Hydrolysis of Cyanocobalamin 1 to Dicyanocobinamide 31. Labeled cyanocobalamin 1 was crystallized to constant specific activity (1.846 mg, 1.36 μmol; specific activity 963 dpm/μmol), rapidly dissolved in 0.15 ml of concentrated hydrochloric acid, and heated at 75° for 8 min, essentially by the procedure of Armitage, et al.,[46] as modified by Shemin.[16] The reaction was immediately cooled in ice, diluted with about 0.5 ml of water, and evaporated to dryness as quickly as possible under reduced pressure (rotary evaporator, bath temperature below 40°). The residue was dissolved in a small amount of water and reevaporated. The hydrolyzed corrin mixture was chromatographed[16] on a small column of Dowex 2X-8 (acetate form, 1 × 20 cm), eluting first with water. After the cobinamide-containing fractions (neutral) had been collected, elution was continued with increasing concentrations of acetic acid: a mixture of acidic corrinoids eluted with 0.2–0.4 M acetic acid and the nucleotide segment with 0.6–0.9 M acetic acid. Electronic absorption spectra was recorded of the separately pooled cobinamide and nucleotide fractions, and their concentrations were computed in aqueous solution, using extinction coefficients for dicyanocobalamin[55] and 5,6-dimethylbenzimidazole (λ methanol): 288 nm (6.66 × 10³), respectively. Despite small errors inherent in these estimations, the specific activities of the hydrolysis products were determined as presented below. (Note: There is in all likelihood some very slight contamination of the nucleotide by acidic corrinoids, accounting for the low activity of this fraction.)

	Yield, mg	Spec act., dpm/μmol
Cyanocobalamin		963
Dicyanocobinamide	0.967 (68%)	946
Nucleotide	0.484 (98%)	1–5

(ii) Kuhn–Roth Oxidation of Cobinamide 31.[16,49–51] Cobinamide obtained in Section i above (0.808 mg, 0.77 μmol; total activity 731 dpm) was combined with 0.5 ml of Kuhn–Roth reagent (16.8 g of chromium trioxide–100 ml of water and 25 ml of concentrated sulfuric acid) and heated at reflux 1.5 hr followed by distillation for 3 hr. Titration of the distillate with 0.01 N sodium hydroxide indicated more than the theoretical amount of acetic acid[16] (excess acetate presumably was derived from the Dowex 2X-8 chromatography). When the titration was complete (phenolphthalein end point), the solution was evaporated to dryness, and the amount of label in the sodium acetate was determined (total activity: 5 dpm, 7% of cobinamide total activity).

B. Degradation of Vitamin B_{12} (Cyanocobalamin, 1) Labeled by Feeding [^{14}C]Uroporphyrinogens I–IV ([^{14}C]Uro'gens I–IV). (i) Acidic Hydrolysis of Cyanocobalamin to Dicyanocobinamide 31. Vitamin B_{12} of constant specific activity (1.902 mg, 1.40 μmol; 1358 dpm/μmol) was subjected to acidic hydrolysis and Dowex 2X-8 chromatography as described above.

	Yield, mg	Spec act., dpm/μmol
Cyanocobalamin		1358
Dicyanocobinamide	1.137 (78%)	1619
Nucleotide	0.485 (97%)	~0

(ii) Kuhn–Roth Oxidation of Cobinamide 31. Cobinamide (0.860 mg, 0.82 μmol; total activity 1330 dpm) obtained in i above was oxidized according to the procedure outlined above and the extent of label in the derived sodium acetate determined (total activity: 11 dpm, 8% of cobinamide total activity).

C. Kuhn–Roth Oxidation[16,49,50,51] **of Vitamin B_{12} (Cyanocobalamin (1)) Labeled by Feeding [^{14}C]Uroporphyrinogens I–IV ([^{14}C]U-**

ro'gens I–IV (28, 29, 12, and 30)). Cyanocobalamin (1.390 mg, 1.025 μmol; specific activity 7250 dpm/μmol; total activity 7425 dpm) was heated at reflux for 2 hr with 5 ml of Kuhn–Roth reagent (16.8 g of chromium trioxide–100 ml of water and 25 ml concentrated sulfuric acid). Water (10 ml) was added to the mixture and distillation was carried out for 6 hr. Titration of the distillate with 0.001 N sodium hydroxide indicated 6.42 μmol of acetic acid. The solution was evaporated to dryness and the sodium acetate counted (specific activity ca. 1.5 dpm/μmol; total activity ca. 10 dpm, 0.135% of the cyanocobalamin total activity). If the ^{14}C label from uro'gen were entirely randomized throughout the vitamin, the specific activity of the derived acetic acid would have been approximately 230 dpm/μmol. To obtain a measure of the efficiency of the Kuhn–Roth degradation of the complex corrin structure, cyanocobalamin labeled at the "extra" methyl groups (by feeding [^{14}CH$_3$]-L-methionine) was submitted to the identical oxidation conditions; 23% of the vitamin's total activity was found to reside in the derived acetate.

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(52) Calculation of uroporphyrin yields for any isomer or combination of isomers employed Mauzerall's value[24] for the extinction coefficient of the Soret band in 1.0 N hydrochloric acid (5.05×10^5). Compare this, however, with other values that have been quoted: (1) 0.5 N hydrochloric acid (C. Rimington, *Biochem. J.*, **75**, 622 (1960)), uroporphyrin I (5.41×10^5), uroporphyrin III (5.28×10^5); (2) pH 7 phosphate buffer (L. Bogorad, *J. Biol. Chem.*, **233**, 501 (1958)), uroporphyrin (1.06×10^5).
(53) Wheat germ obtained through a commercial source (Sigma, nonheat treated) gave (in several trials) extracts with *no* uroporphyrin III cosynthetase activity. We acknowledge with great appreciation, therefore, a substantial gift of fresh non-heat-treated wheat germ with high cosynthetase activity provided by General Mills, Kansas City, Mo., through the courtesy of Dr. M. Zimmerman.
(54) See ref 52.
(55) As dicyanocobinamide is an amorphous noncrystalline compound,[47] its extinction coefficients have traditionally been taken to be those of dicyanocobalamin, a reasonable assumption, undoubtedly, as cyanide has displaced the nucleotide base from cobalt, and the effect of the nucleotide on the corrin chromophore can be no more than that of a remote side-chain substituent. However, it was not known in 1953[46] that two other products[13] are likely to appear in the neutral cobinamide fraction as a result of the strongly acidic hydrolysis conditions ring B lactone (i) (or lactam) and neocobinamide (48) whose extinction coefficients differ from those of cobinamide itself, particularly in the latter case, and hence would be expected to introduce some error into the calculation of the specific activity.

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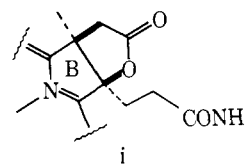
The hydrolysis conditions employed were essentially those reported by Todd's group,[46] who isolated and crystallized a mixture of 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole 2'- (and 3'-) phosphate that gave the following uv absorption data: λ (water, pH 2): 285 nm (6.61×10^3). Four years later, however, in 1957 the Cambridge group[48] conducted the hydrolysis under more prolonged, though milder, conditions (6 N hydrochloric acid, room temperature, 18 hr) and succeeded in separating and crystallizing the 2'- and 3'-nucleotides, whose absorption characteristics were cited as λ (water, pH 2): 285 (6.76×10^3) and 285 nm (7.59×10^3), respectively. Nonetheless, whatever extinction coefficient is chosen from those reported in the literature, the ϵ value employed by us (6.66×10^3) can be in error by no more than 10%.

Biosynthesis of Corrins. II. Incorporation of ^{13}C-Labeled Substrates into Vitamin B$_{12}$[1]

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Abstract: Analysis of the ^{13}C-FT nmr spectra of specimens of vitamin B$_{12}$ (1) (cyanocobalamin) enriched with ^{13}C by feeding [2-^{13}C]ALA,[3] [5-^{13}C]ALA, [8-^{13}C]PBG,[3] [^{13}CH$_3$]methionine, and [^{13}C]uro'gens[3] I–IV to resting cells of *Propionibacterium shermanii* has demonstrated excellent specific incorporation. Reinterpretation of the ^{13}C chemical shift assignments of the natural abundance spectra of corrins and of earlier ^{14}C experiments has led to several revisions of earlier assignments and of the number of "extra" methyl groups derived from methionine in the vitamin. Detailed analysis of the ^{13}C-FT[3] spectra of neocobinamide permits the allocation of the absolute stereochemistry of methylation process at C$_{12}$. The incorporation of [^{13}C]uro'gen III has been confirmed by ^{13}C nmr spectroscopy. A summary of these results is presented as a prelude to the formulation of new theories for the biosynthesis of corrins.

As indicated in the previous paper, carbon-by-carbon degradation of vitamin B$_{12}$ to establish the intact incorporation of PBG and uro'gen III promised to be an exceptionally tedious process since the repeated functional elements and stereochemical subtleties of the corrin chromophore make the exact location of labeled centers extremely troublesome to ascertain by classical methodology. With the application

of ^{13}C-FT nmr spectroscopy, however, it is precisely this structural complexity which permits the burden of the experimental difficulties to be lifted.

The natural abundance ^{13}C-FT nmr spectra of cyanocobalamin (1) and dicyanocobalamin (2), as published by Doddrell and Allerhand,[4] are reproduced in Figure 1. Two significant observations can be made on visual inspection of