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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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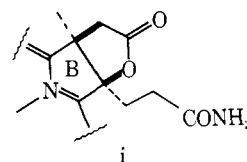
The hydrolysis conditions employed were essentially those reported by Todd's group,[46] who isolated and crystallized a mixture of 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole 2'- (and 3'-) phosphate that gave the following uv absorption data: λ (water, pH 2): 285 nm (6.61×10^3). Four years later, however, 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
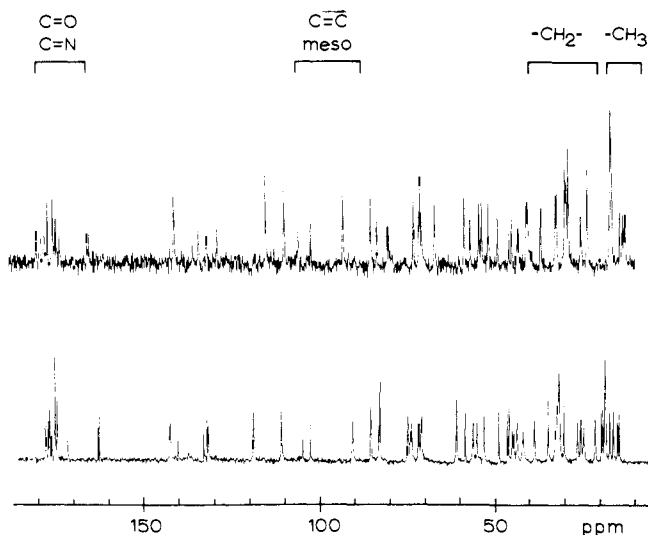
Figure 1. Proton noise-decoupled natural abundance ^{13}C-FT nmr spectra of aqueous cyanocobalamin (top) and dicyanocobalamin (bottom) (Doddrell and Allerhand, ref 4); chemical shifts are downfield from HMDS redrawn from *Proc. Nat. Acad. Sci. U.S.*, **68**, 1033 (1971).

these spectra. Firstly, there is a marked consistency in the peak intensities, except, of course, where resonances overlap or are superimposed. Allerhand and others have shown[5] that for large, rigid molecules the spin–lattice relaxation
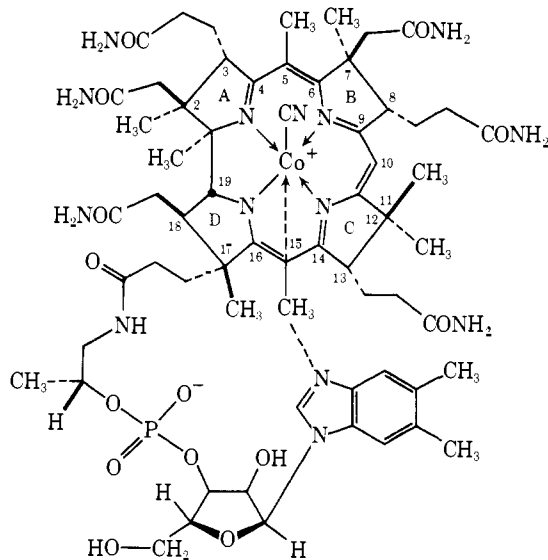
Vitamin B$_{12}$, cyanocobalamin (1)

[dicyanocobalamin (2) has CN replacing the nucleotide as the axial ligand]

processes for most ^{13}C nuclei are determined by dipole–dipole interactions. The signals in proton-decoupled spectra for carbon atoms relaxed in this manner appear with almost maximal nuclear Overhauser enhancement. Such is the case with corrins. The important implication of this behavior for biosynthetic studies is that enrichments as low as 2–3% in the vitamin from the incorporation of ^{13}C-labeled substrates in principle could be meaningfully detected above natural abundance (1.1%). Microbiological procedures, therefore, were developed as described in the previous paper[6] to achieve enrichments of this magnitude or better and to provide good total yields of cyanocobalamin for spectroscopic analysis. Secondly, the easily generated dicyano complex of vitamin B$_{12}$ gives a slightly altered spectrum owing to small conformational changes in the corrin nucleus
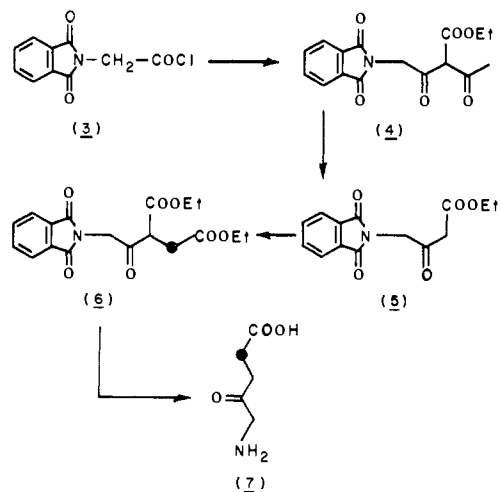

Figure 2. Synthesis of [2-^{13}C]ALA (7) by the method by Pichat.

and side chains (compare the methyl regions of the two spectra). It will be seen that the ready interconvertibility of cyano- and dicyanocobalamin becomes extremely useful in the biosynthetic studies for the separation of overlapped resonances and the assignment of spectral lines.

Doddrell and Allerhand's assignments[5] of the spectra were most detailed for the nucleotide portion of the vitamin; somewhat less so for the corrin ring system itself, with assignments being made to groups of similar carbons; and, at best, tentative for a few side-chain and methyl carbons. Unfortunately, it is these side-chain methylenes that are the most accessible in a correspondingly labeled PBG or uro-'gen precursor. The large number of closely spaced lines in the upfield portion of the spectra precludes their assignment by comparison with model compounds—even if suitable structures could have been obtained. Therefore, in a rever-. sal of the usual approach, appropriately labeled known precursors of the vitamin were employed to assign the methylene and methyl regions of the spectra prior to the feeding of side-chain-labeled PBG and uro'gen.

An adaptation of Pichat's method[7] was used to prepare [2-^{13}C]ALA (7). Phthalimide was converted by the method of Mitta, *et al.*, to phthalylglycine chloride (3), which was condensed with the magnesium enolate of ethyl acetoacetate to give diketo ester 4. Deacylation of 4 yielded ethyl phthalimidoacetoacetate (5), whose sodium enolate was generated in glyme and condensed with ethyl [2-^{13}C]bromoacetate according to the method of Pichat[7] (see Figure 2). The labeled bromoacetate was synthesized by literature procedures[8] from sodium [2-^{13}C]acetate. The diketo ester (6) so obtained was hydrolyzed under vigorous conditions to yield [2-^{13}C]ALA (7) (45–46 atom %).

Administration of [2-^{13}C]ALA (7) to a series of three large-scale incubations with suspended cells of *P. shermanii* afforded a sample of labeled vitamin B$_{12}$ (8), whose ^{13}C-FT nmr spectra as the mono- and dicyano complexes are shown in Figures 3 and 4. Comparison of the intensities of the enhanced peaks with those at natural abundance indicated an enrichment of about 6%, corresponding to a 12% specific incorporation of precursor. In accord with Shemin's earlier ^{14}C studies,[9,10,11] the enhanced signals appearing at high field were assigned to 7 of the 11 side-chain methylenes (-ĊH$_2$-CONH$_2$) and to one (•) of the geminal dimethyl groups in ring C. The methyl group was assigned to one of the two enhanced signals at highest field in cyanocobalamin and to the resonance at 35.2 ppm in dicyanocobalamin.

The reasons for these spectral assignments and the rationale for the indicated configuration of the ring C methyl
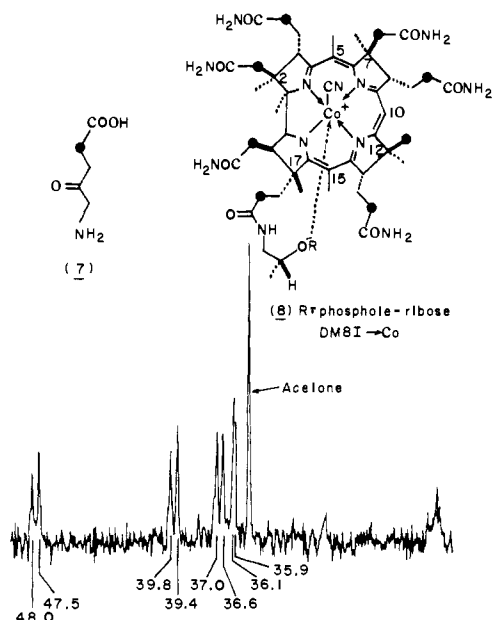
Figure 3. Proton noise-decoupled ^{13}C-FT nmr spectrum of [2-^{13}C]ALA enriched cyanocobalamin (H$_2$O; 8K points) and assigned labeling pattern.

Figure 4: Proton noise-decoupled ^{13}C-FT nmr spectrum of [2-^{13}C]ALA enriched dicyanocobalamin (0.1 M KCN; 8K points) and assigned labeling pattern.

group (Figures 3 and 4) will be discussed in the sequel. On the basis of the labeling pattern alone, however, the signal at 35.2 ppm cannot be C-18 as tentatively suggested by Allerhand;[5] and furthermore, it is to be noted that the chemical shift of the methyl resonance occurs at substantially lower field than the methyl region provisionally assigned by Allerhand[5] (Figure 1). The methylene carbons were assigned as follows. The acetamide methylenes at C-2 and C-7 were assigned to the lowest field pair of peaks at about 48 ppm in confirmation of Allerhand's assignments. The third acetamide methylene at C-18 in ring D was assigned to the resonance at 40.0 ppm in the dicyano complex and one of the two signals appearing at about 39.5 ppm in the monocyano complex. This latter methylene appears at higher field (8 ppm) because of steric interaction (γ effect)[12] with the adjacent syn methyl group at C-17. The propionamide methylenes were assigned to the cluster of peaks around 37 ppm in dicyanocobalamin and to the four remaining signals in cyanocobalamin not assigned above.

To assign the upfield resonances arising from the methyl group of methionine, [^{13}CH$_3$]-L-methionine (90 atom %) was fed to *P. shermanii* by standardized microbiological procedures. The ^{13}C-FT nmr spectrum of the resultant vitamin B$_{12}$ was run as the monocyano form and revealed six signals highly enriched (greater than 20%; compare the [^{14}CH$_3$]methionine incorporations discussed in the previous paper) above natural abundance (see Figure 5). This result appeared to be consistent with Shemin's radioactive tracer work[11] from a decade before, and on that basis the resonances were assigned to the six "extra" (*) methyl groups subtended by the corrin periphery. All these signals fell within the methyl region assigned by Allerhand; however, the resonances at 21.5 and 22.3 ppm cannot arise from 5,6-dimethylbenzimidazole, as was suggested by Allerhand[5] on the basis of model studies. The methionine-derived methyl group in ring C was assigned the α configuration (Figure 5) by analogy with the alkylation pattern in rings A and B. Further supportive evidence will be presented below.

At this juncture the stage was set to feed PBG and uro-'gen. [8-^{13}C]PBG (9) was synthesized essentially by the same procedures used previously for [8-^{14}C]PBG, but on a much larger scale. Although the first group of feeding ex-

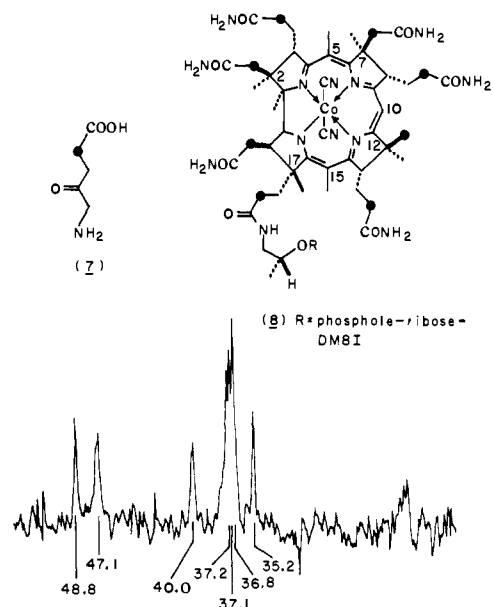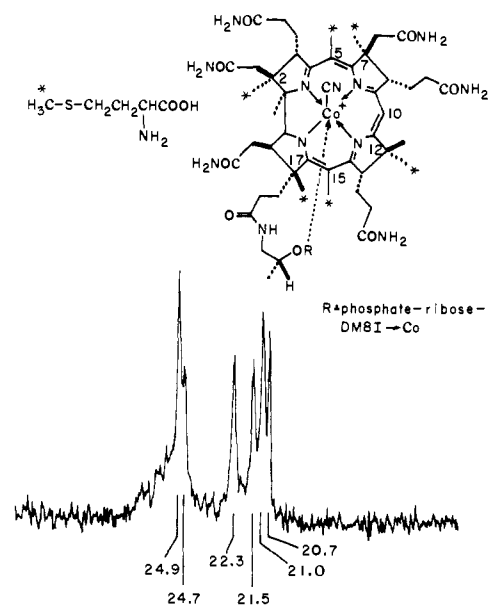Figure 5. Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}CH$_3$]-L-methionine enriched cyanocobalamin (H$_2$O; 8K points) and assigned labeling pattern by analogy with tracer studies of Shemin (also, see Figure 12).

periments gave a low incorporation of PBG (about 2%), a second series gave the expected incorporation (*ca.* 5%), as estimated from the integrated intensities of the enriched peaks. The accuracy of this method was confirmed to a remarkable degree by the 5.09%/C specific incorporation obtained for a parallel [8-^{14}C]PBG experiment. If incorporated intact into vitamin B$_{12}$, [8-^{13}C]PBG (▲) would have been expected to label the four remaining unassigned resonances in the methylene region of the cmr spectrum. That this was indeed the case is shown in Figures 6 and 7. The spectrum of the labeled cyanocobalamin (10) (Figure 6) revealed three signals which corresponded to four enriched centers (▲). Demonstration that four enriched carbons were actually present was accomplished by conversion of the vitamin to its dicyano form. The sharp signal of double inten-
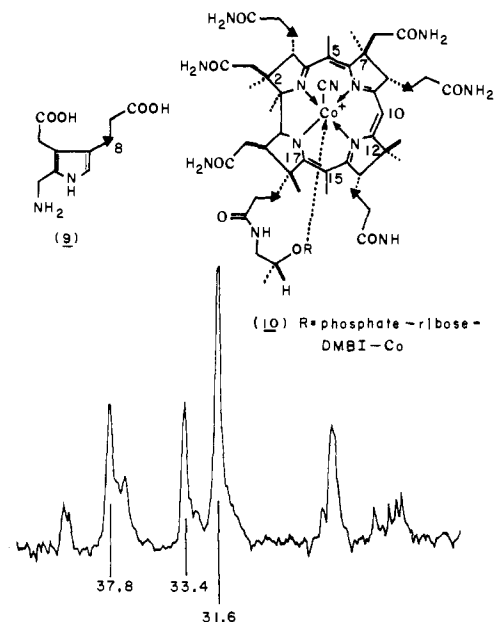
Figure 6. Proton noise-decoupled ^{13}C-FT nmr spectrum of [8-^{13}C]PBG enriched cyanocobalamin (H$_2$O; 4K points) and assigned labeling pattern.

Figure 7. Proton noise-decoupled ^{13}C-FT nmr spectrum of [8-^{13}C]PBG enriched dicyanocobalamin (0.1 M KCN; 4K points) and assigned labeling pattern.

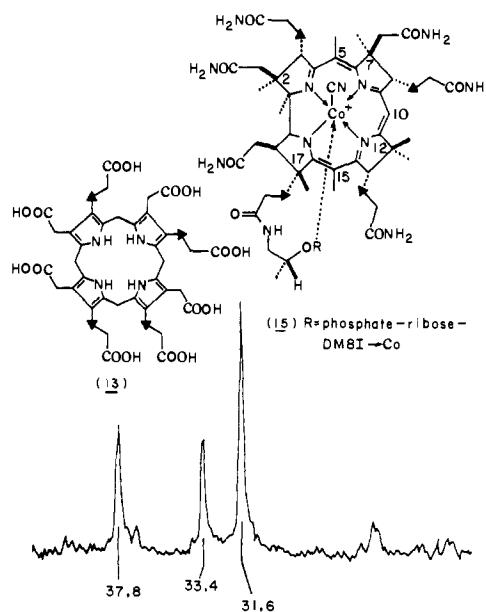**Figure 8.** Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}C]uro-'gen 111 enriched cyanocobalamin (H$_2$O; 4K points) and assigned labeling pattern.
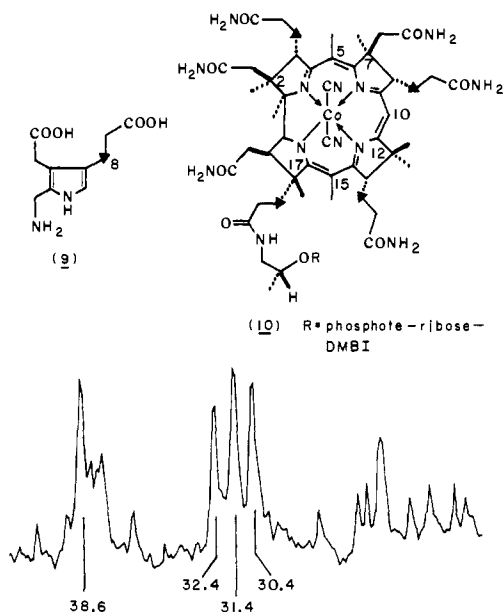
sity at 31.6 ppm in cyanocobalamin was cleanly resolved, and the ^{13}C-FT nmr spectrum showed four enhanced peaks of equal intensity (Figure 7). The other resonances visible in these spectra are methyl (right) and methylene (left) carbons at natural abundance (compare Figures 3, 4, and 5). The group of three enhanced signals upfield in dicyanocobalamin (Figure 7) was readily assigned to the structurally similar propionamide β-methylenes at C-3, C-8, and C-13 in agreement with Allerhand's tentative assignments.[5] The remaining enriched peak 6–8 ppm further downfield was assigned to the corresponding labeled center of the ring D propionamide.

At this point, the chemical shift behavior of the methyl and methylene carbons became obvious. The propionamide β-methylenes in rings A, B, and C appear at high field because of steric interaction (gamma effect)[12] with adjacent

syn methyl groups at C-2, C-7, and C-12. The ring D propionamide, however, has no alkyl groups syn at the adjacent carbon, and hence its β-methylene resonates at a "normal" unshifted position. Similarly, the acetamide methylenes at C-2 and C-7 have no group syn and appear at relatively lower field (about 8 ppm) than the acetamide methylene at C-18, as shown in the ^{13}C-FT nmr spectrum of [2-^{13}C]ALA enriched dicyanocobalamin (Figure 4). The gamma effect is reciprocal, and hence the methyl groups at C-2, C-7, C-12, and C-17 which shift the resonances of the adjacent syn side-chain methylenes to higher field must themselves be shifted upfield. Consistent with this prediction, the six "extra" methyl groups (*) derived from methionine (Figure 5) all appear in the high-field region originally proposed by Doddrell and Allerhand[5] (Figure 1). A further implication of these observations is that the problem of the stereochemistry of methylation in ring C could be resolved by simple comparison of the chemical shift behavior of the two methyl groups. As all the methionine-derived methyl groups resonate at high field, the one at C-12 must be syn to the adjacent propionamide; *i.e.*, in the biosynthesis of the corrin nucleus, it is inserted from the α face (*pro-R* configuration) as shown in Figure 5. An unequivocal proof of this assignment will be described below.

Thus, with the proof of the intact incorporation of PBG in hand, a second sample of [8-^{13}C]PBG (9) was prepared and condensed in acid to give the statistical mixture of [^{13}C]uroporphyrin isomers ($\frac{1}{8}$ I, $\frac{1}{8}$ II, $\frac{1}{2}$ III, and $\frac{1}{4}$ IV). This material, after extensive purification, was reduced to the corresponding [^{13}C]uro'gens I–IV (11–14) and fed by a modification of the procedures used in the experiments above. In an attempt to maximize the incorporation of uro-'gen, *P. shermanii* was divided and suspended in twice the usual number of flasks to which about 1.5 times the customary amount of substrate was fed. Moreover, the incubations were carried out for 70 hr instead of 45–50 hr, as was done for the feeding of the other ^{13}C-labeled substrates. The resultant vitamin B$_{12}$ (15) was subjected to ^{13}C-FT nmr analysis as before, and the enriched spectra (see Figures 8 and 9) showed enhancement of the *same set of four methylene carbons* as was labeled in the [8-^{13}C]PBG (9) experiment (▲). The precautions taken in the conduct of the feeding ex-

periment resulted in enrichments of about 10% at each labeled center, corresponding to an 11% specific incorporation of uro'gen III (13). That only uro'gen III was incorporated from the chemically synthesized mixture was based on the proven nonincorporation[6] of [^{14}C]uro'gen I (11) and by analogy with the presumed inertness of the unnatural isomers, uro'gens II and IV (12 and 14). Taken together, the radioactive tracer experiments discussed in the previous paper[6] and the mutually self-consistent spectral assignments for the ^{13}C enrichment data presented above provide unequivocal evidence for the precise location of the label and strongly support the specific incorporation of uro'gen III into corrinoids.

Doddrell and Allerhand[5] tentatively assigned the resonance at about 45 ppm in cyanocobalamin (Figure 4) to the methyl group at C-1. To check the validity of Shemin's earlier report[11,13] that this methyl is derived from C-5 of ALA (see Figures 1–7) and to complete the assignment of the biochemical origin of all the atoms of the corrin nucleus, a sample of [5-^{13}C]ALA (16) (90 atom %) was prepared[14] according to the method of Shemin[15] and fed to suspended cells of *P. shermanii* in the customary manner. As expected, ^{13}C-FT nmr analysis of the derived vitamin B$_{12}$ (17) revealed seven signals at low field associated with sp^2 carbon functions (four C=N resonances, Figure 10, left; and three C=C resonances, Figure 10, right), but it was wholly unexpected that no resonance in the range 0–95 ppm downfield from hexamethyldisilane (HMDS) was enhanced; *i.e.*, no enrichment of the C-1 methyl occurred. This observation was particularly surprising in view of our previous result with [^{13}CH$_3$]-L-methionine (Figure 5). Essentially similar results were obtained simultaneously and independently by Brown and Shemin.[16]
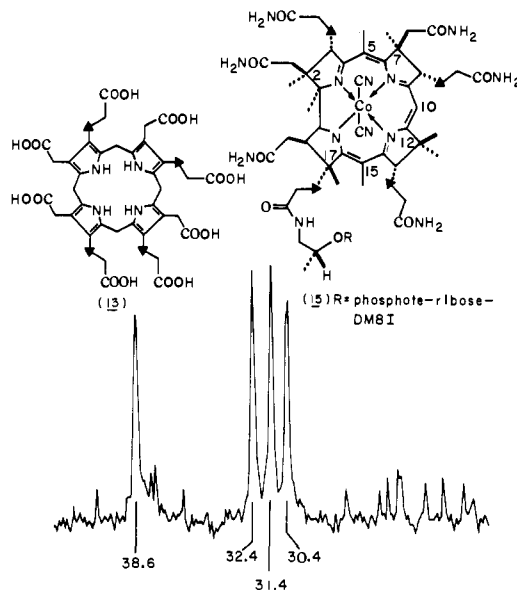
Figure 9. Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}C]uro-'gen III enriched dicyanocobalamin (0.1 M KCN; 4K points) and assigned labeling pattern.

The splitting pattern observed in the low-field region of the spectrum was consistent with the distribution of label (■) shown in (17) (Figure 10). Comparison of the chemical shifts and coupling constants allowed spectral assignments to be made as follows. Nuclear Overhauser enhancement of the resonance at 100.4 ppm permitted its assignment to the protonated C-10 meso position, in agreement with Doddrell and Allerhand.[5] This signal was split into a doublet of dou-

11, uro'gen I

9, PBG

12, uro'gen II

13, uro'gen III

14, uro'gen IV
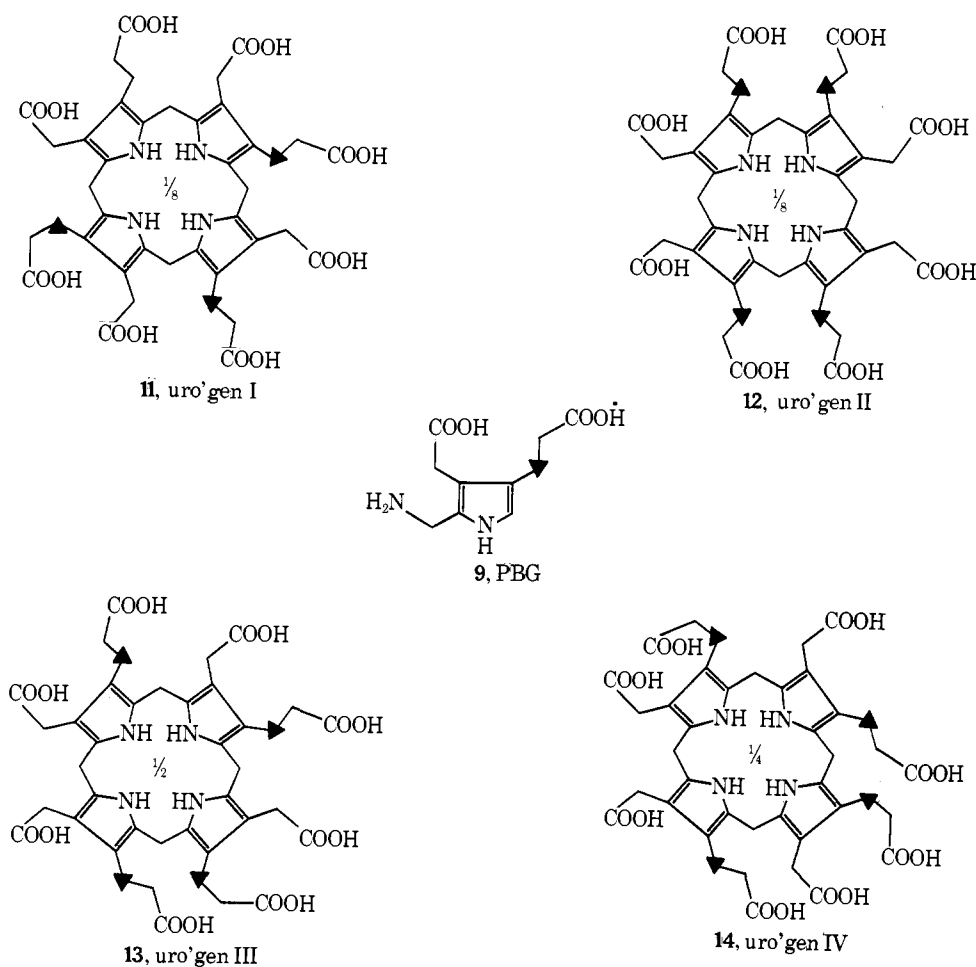
Figure 10. Proton noise-decoupled ^{13}C-FT nmr spectrum of [5-^{13}C]ALA enriched cyanocobalamin (H$_2$O; 4K points) and assigned labeling pattern.

Figure 11. Expansion of the C-5 and C-15 meso carbon region of the proton noise-decoupled ^{13}C-FT nmr spectrum of [5-^{13}C]ALA enriched cyanocobalamin (H$_2$O; 4K points).

blets owing to spin–spin coupling with the adjacent C-9 carbon ($J = 111$ Hz) and 1,4 coupling to C-14 ($J = 7$ Hz). Inspection of the region to lower field revealed that the signal at 183.6 ppm was split into a doublet with the complementary coupling constant ($J = 111$ Hz) and hence was assigned to C-9. This assignment was further supported by secondary splitting ($J = 8$ Hz) resulting from 1,4 coupling to C-5. Alkyl substitution was found to shift the resonances of the C-5 and C-15 meso carbons 10–15 ppm downfield from C-10 as predicted by Allerhand.[5] Centered on the barely visible singlet at 109.3 ppm (see Figure 11) are two doublets ($J = 100$, $J = 123$ Hz) and a doublet of doublets ($J = 100$, $J = 123$ Hz) which allowed this manifold to be easily assigned to C-15. Matching the larger of these coupling constants, the signal at 180.7 ppm was assigned to C-
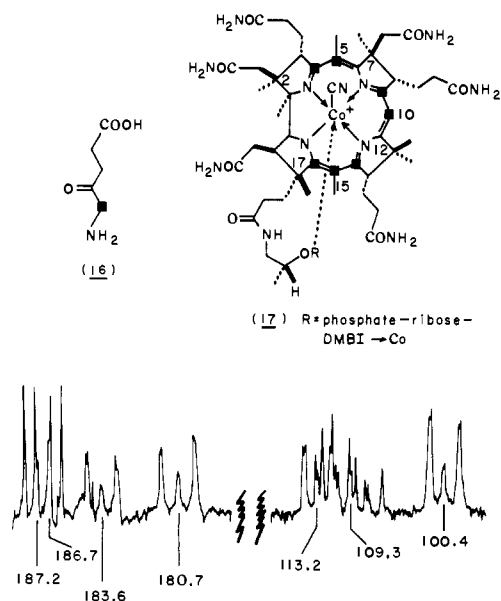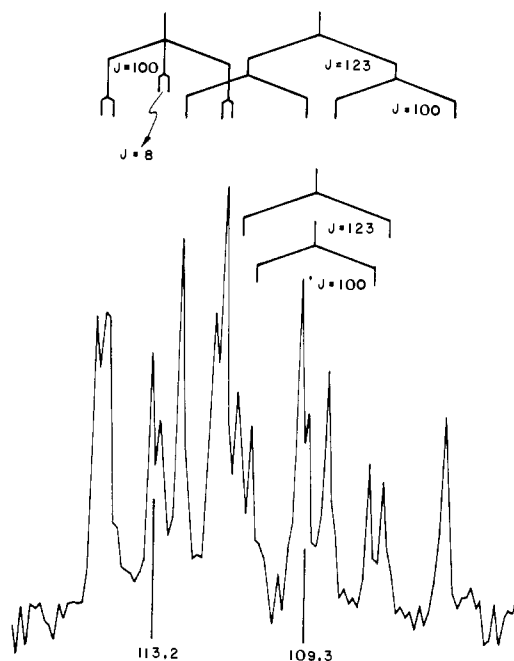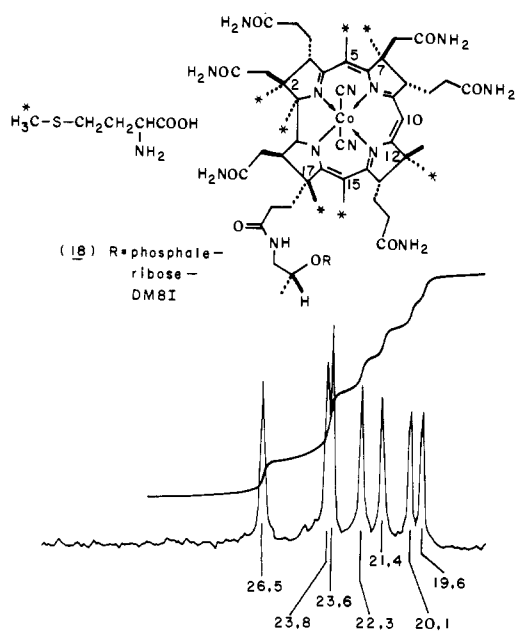

Figure 12. Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}C]-L-methionine enriched dicyanocobalamin (0.1 M KCN; 4K points) and assigned labeling pattern.

14, broadened by 1,4 coupling to C-10 noted above (not resolved at 4K). Proceeding in like manner, the finely split singlet and doublet centered at 113.2 ppm (Figure 11) were assigned to C-5, and the sharp signals (no 1,4 coupling) at 186.7 and 187.2 ppm were assigned to C-4 and C-16, but could not be further distinguished at 4K (resolution 2.5 Hz). It is noteworthy that comparison of the integrated intensities of the singlet and doublet of these resonances (their ratio is independent of nuclear Overhauser enhancements) revealed a 75–80% enrichment at each center, which corresponded to an approximately 85% incorporation of ALA *during* the course of the suspended-cell feeding experiment. Overall, however, this concentration is greatly reduced by the endogenous vitamin already present in the cells at the start of the incubation.

The distribution of label depicted in (**17**) from [5-^{13}C]ALA is in accord with current ideas[17-24] about the mechanism of uro'gen III formation, except that one of the $-^{13}$CH$_2$-NH$_2$ termini of ALA has been lost in the formation of the corrin ring, whereas in the biosynthesis of uro'gen III all the carbons of ALA are retained.[17,20,25] As the force of the evidence presented above dictates that uro'gen III serves as a good precursor of vitamin B$_{12}$, the δ-meso carbon (that between rings A and D) must be lost in the subsequent mechanistic steps to the vitamin and the methyl group at C-1 must arise from another source. *The origin of this methyl group was found to be methionine!* Conversion of the [^{13}CH$_3$]-L-methionine labeled sample of vitamin B$_{12}$ obtained earlier to its dicyano form and analysis by ^{13}C-FT nmr revealed *seven* well-defined resonances. Integration of the peak areas left no doubt (see Figure 12) that seven methionine methyl groups (*) had been incorporated. The signal at 24.9 ppm in cyanocobalamin (Figure 5) therefore corresponds to two superimposed resonances. Steric interaction between the C-1 methyl group and the adjacent syn methyl at C-2 exerts a γ effect[12] on both their chemical shifts as discussed above. While these carbons do appear at high field, the C-1 methyl would be expected to be deshielded somewhat by its proximity to nitrogen and hence is tentatively assigned to the most downfield of these seven resonances, 26.5 ppm. This carbon cannot give rise to the signal at about 45 ppm as suggested by Allerhand,[5] and we pro-
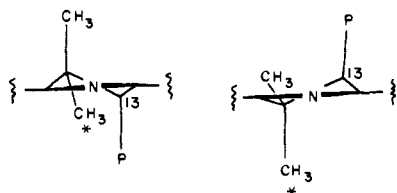
Figure 13. Conformation of ring C in cobinamide (19) (left) and neo-cobinamide (20) (right) as viewed from cobalt, based on X-ray and CD/ORD data.

pose that this latter resonance be provisionally assigned to C-18.

By analogy with the apparent γ effect[12] on the chemical shifts of the methyls at C-2, C-7, and C-17, the methionine-derived methyl in ring C was assigned the *pro-R* configuration at C-12, as discussed earlier (Figures 5 and 12). Conversely, the absence of a γ effect on the chemical shift of the *pro-S* methyl (no syn alkyl group) would rationalize the anomalously low-field position observed for the methyl at C-12 enriched by [2-^{13}C]ALA (13) (Figures 3 and 4). The validity of these assignments was proved unequivocally in the following way. The sample of [^{13}CH$_3$]-L-methionine-enriched vitamin B$_{12}$ was treated with dry trifluoroacetic acid essentially according to Bonnett's procedure[26] to gen-

18, R = phosphate-ribose-DMBI → Co-CN
19, R = H, NC-Co-CN
20, as in 19 except ring C:
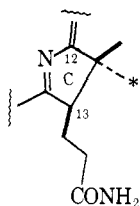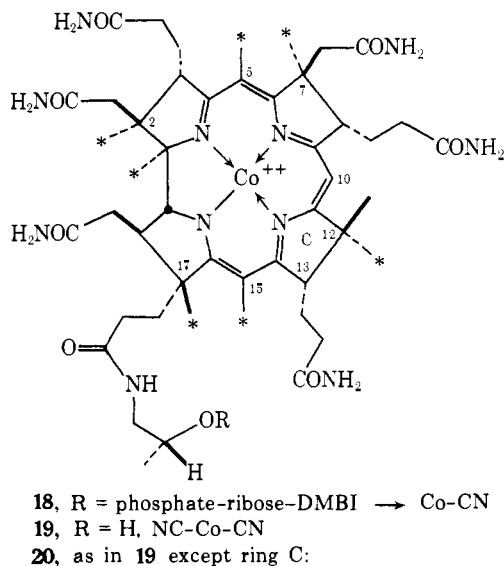
erate a mixture of labeled cobinamide (19) and neocobinamide (20). The conformation[27,28] of the C ring in cobinamide (19), as in the vitamin (18) itself, places the α methyl (*pro-R*) *syn-periplanar* to the adjacent axially oriented propionamide side chain at C-13 (Figure 13, left). Such a juxtaposition would be predicted to produce a γ effect[12] on the chemical shift of the methyl group. In addition to hydrolysis of the nucleotide, epimerization at C-13 also occurs on treatment of 18 with trifluoroacetic acid to yield neocobinamide (20), which is easily separated from 19 by paper chromatography.[26] The configurational inversion of the propionamide at C-13 in neocobinamide is accompanied by a conformational change[27,28] in the skew of ring C, as
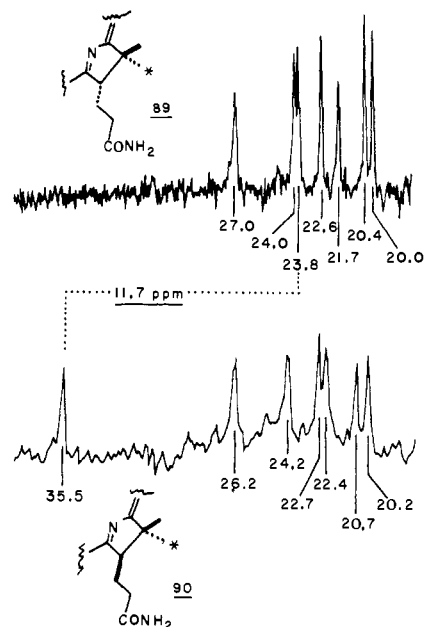

Figure 14. (Top) Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}CH$_3$]-L-methionine enriched dicyanocobalaminamide (0.1 M KCN; 8K points). (Bottom) Proton noise-decoupled ^{13}C-FT nmr spectrum of [^{13}CH$_3$]-L-methionine enriched dicyanoneocobinamide (0.1 M KCN; 4K points).

shown in Figure 13 (right). Thus, if the methionine-derived methyl (*) at C-12 is α-oriented, in the neo compound it will bear an *anticlinal* relationship to the adjacent propionamide, and concomitant removal of the γ effect should result in a downfield shift of the methyl resonance. Such was indeed observed to be the case, as shown by the downfield shift of one of the methyl resonances in the ^{13}C-FT nmr spectrum of dicyanoneocobinamide (Figure 14, bottom). A second series of [^{13}CH$_3$]-L-methionine feeding experiments was conducted, and the resulting labeled vitamin B$_{12}$ (18) was selectively hydrolyzed to cobinamide (19) by the cerium hydroxide method.[26,29,30] (The mechanism of this useful reaction has been studied by Westheimer.[31]) The ^{13}C-FT nmr spectrum of this sample is shown in Figure 14 (top). Comparison of the chemical shifts of the seven resonances in each spectrum reveals that the signal at either 23.8 or 24.0 ppm in dicyanocobinamide is shifted more than 11 ppm downfield in dicyanoneocobinamide. Furthermore, the appearance of the C-12 methionine methyl (*) in neocobinamide at 35.5 ppm correlates extremely well with the assigned chemical shift of the C-12 methyl (35.2 ppm) in [2-^{13}C]ALA enriched dicyanocobalamin (14) (Figure 4). Thus, the stereochemical assignments of the geminal methyl groups in ring C were firmly established. These conclusions were later confirmed by Battersby[32] from a completely different experimental approach. However, contradictory findings have recently been reported by Brown and Shemin.[33] The experimental evidence of these latter workers is used to formulate the methionine-derived methyl at C-12 as *pro-S*, based on proton nmr assignments of vitamin B$_{12}$ published by Brodie and Poe.[34] In considering the strength of the evidence from our own experiments and those of Battersby, we are compelled to regard Brown and Shemin's *interpretation* of their results to be in error.

The results of the ^{13}C-labeling studies presented in this paper are summarized in Figure 15. The enrichment pattern shown for [2-^{13}C]ALA and [^{13}CH$_3$]-L-methionine in vitamin B$_{12}$ was assigned on the basis of Shemin's earlier radioactive tracer experiments and allowed the positions of subsequent labeling by [8-^{13}C]PBG and [^{13}C]uro'gen III to
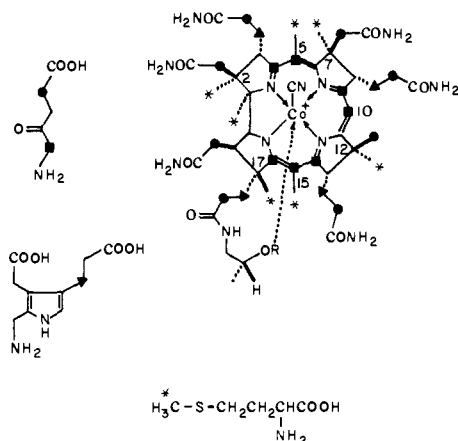
Figure 15. Summary of the assigned labeling pattern of various labeled precursors for vitamin B_{12}.

be allocated unequivocally. Together with the results obtained for [^{14}C]uro'gens III and I, these data jointly provide the first demonstration of the intact incorporation of PBG and uro'gen III into vitamin B_{12}. The results with [5-^{13}C]ALA dictate that one *of the* $-CH_2-NH_2$ *termini of ALA* (and hence of PBG) *must be lost in the formation of the vitamin and its place at C-1 taken by a methionine-derived methyl group.* In total, therefore, seven rather than six "extra" methyl groups are incorporated from methionine.

The power and versatility of cmr spectroscopy have been demonstrated throughout this study in providing evidence for the precise location of labeled centers in the vitamin. The sensitivity of ^{13}C chemical shifts to steric interactions and configurational effects has been exploited to make spectral assignments and, in particular, to give stereochemical information, *e.g.,* to establish the stereochemistry of the methylation process in ring C. The latter kind of information is often extremely difficult to obtain by classical degradative techniques. The substrate labeling patterns in the ^{13}C-FT nmr spectra of cyanocobalamin and dicyanocobalamin are summarized in Table I which at the same time provides a list of assignments for the various sets of carbon atoms in B_{12}.

One question that cannot be answered from the experiments presented in this and the previous paper[6] is at what stage in the biosynthesis of corrins and in what form the C-5 carbon of ALA is lost. If uro'gen III is in fact a true intermediate, the δ-meso carbon between rings A and D must be lost in the subsequent mechanism leading to the corrin structure. To test this requirement, to identify the nature of the carbon species lost, and to provide final unambiguous proof of the intermediacy of uro'gen III, a regiospecific total synthesis of ^{3}H/^{14}C doubly labeled uro'gen III has been completed. The details of this synthesis and of the appropriate feeding experiments will form the subject of a future publication[35] in this series.

Experimental Section

(^{13}C-FT nmr) spectra were obtained with a computer-controlled system consisting of a Brucker HFX-3 nuclear induction spectrometer operating at 21.5 kG interfaced with an IBM 1800 computer. A full description of this instrument can be found elsewhere.[36] Solutions of corrins were prepared in distilled water or 0.1 *M* potassium cyanide, depending on the ligand binding desired for cobalt. Samples were run in 10-mm tubes (total volume, 1.2–1.5 ml) at ambient probe temperature (40–45°), with a 5-mm tube mounted coaxially containing the external ^{19}F lock (hexafluorobenzene, C_6F_6) and ^{13}C reference (hexamethyldisilane, HMDS). Relative to HMDS, TMS appears at δ 2.3, CHCl$_3$ at 80.3, and CS$_2$ at

Table I. ^{13}C Chemical Shifts[a] for Cyanocobalamin and Dicyanocobalamin with Proton Decoupling (δ from HMDS) Based on Enrichment Data for 26 Carbon Atoms

Enriched position[b]	Cyano-cobalamin	Dicyano-cobalamin
C methyl at C_{12} (*pro-S*) (●)	35.9 or 36.1	35.2
C methyl at C_{12} (*pro-R*) (*)	24.7 or 24.9	23.6
C methyl at C_5 and C_{15} (*)	20.7, 21.0	19.6, 20.1
C methyl at C_1, C_2, C_7, C_{17} (*)	21.5, 22.3, 24.9 (2 C)	21.4, 22.3, 23.8, 26.5
Propionamide α-methylenes (CH_2CONH$_2$) at C_3, C_8, C_{13}, C_{17} (●)	35.9 or 36.1, 36.6, 37.0, 39.4 or 39.8	36.8 (2 C), 37.1, 37.2
Acetamide α-methylenes (CH_2CONH$_2$) at C_{18}, C_2, C_7 (●)	39.4 or 39.8, 47.5, 48.0	40.0, 47.1, 48.8
Propionamide β-methylenes (CH_2CH$_2$CONH$_2$) at C_3, C_8, C_{13} (▲)	31.6 (2 C), 33.4	30.4, 31.4, 32.4
Propionamide β-methylene at C_{17} (▲)	37.8	38.6
C_5 (■)	113.2	
C_{10} (■)	110.4	
C_{15} (■)	109.3	
C_4, C_{16} (■)	186.7, 187.2	
C_9 (■)	183.6	
C_{14} (■)	180.7	

[a] In ppm (± 0.1). [b] See Figure 15 for a summary of the enrichment scheme and the numbering system.

195.7. Proton noise-decoupled spectra were obtained in 12–48 hr, using the following uniform conditions: digitizing rate 10 kHz, pulse width 50 μsec, receiver skip 100 μsec, and sampling either 4K or 8K data points as indicated. At 8K points, the system provides a frequency resolution of 1.2 Hz or 0.05 ppm; at 4K, the resolution is 2.5 Hz or 0.1 ppm. When possible, an attempt was made to estimate the extent of enrichment in corrinoids derived from ^{13}C-feeding experiments by comparing the integrated intensity of labeled centers with that for carbons at natural abundance. By comparing carbons of the same hybridization and the same or similar substitution, differences in the nuclear Overhauser enhancements were minimized; and for large, rigid systems, such differences are comparatively small in any case.[5]

Nonetheless, as the molar concentrations of the labeled corrins were low, the noise levels were significant, and such calculations can only be regarded as approximate. Two values were calculated. Computing the ratio of the integrated intensities and subtracting the contribution of natural abundance to the enhancement at an enriched center, the *per cent enrichment, i.e.,* the per cent excess heavy isotope at a labeled center, was calculated.

$$\frac{\text{integrated intensity labeled center}}{\text{integrated intensity natural abundance}} = \frac{X\%}{1.1\%}.$$

then

$$X - 1.1 = \text{per cent enrichment}$$

By dividing the *per cent enrichment* by the extent of ^{13}C enrichment of the precursor fed, *e.g.,* 90%, an approximate measure of the *specific incorporation* during the feeding experiment was determined.

$$\frac{\text{per cent enrichment}}{\text{atom \% } ^{13}\text{C in precursor}} = \text{specific incorporation}$$

Chemical shift data are given in ppm downfield (δ) from HMDS. The abbreviations used are s = singlet, d = doublet, m = multiplet, and dxd = doublet of doublets. *J* is given in Hz.

A. Synthesis of ^{13}C-Labeled Precursors. 1. [^{13}CH$_3$]-L-Methionine. [^{13}CH$_3$]-L-Methionine was obtained from Merck of Canada and used as received (90 atom %).

2. [2-^{13}C]-δ-Aminolevulinic Acid ([2-^{13}C]ALA (7)). Phthalylglycine Chloride (3). Phthalimide (20.0 g, 136 m*M*) was converted to the acid chloride (3) (*ca.* 16 g, 56% for five steps) by the procedure

O=... NH → N—R →

3, R = CH₂OH
R = CH₂Cl
R = CH₂CN
R = CH₂COOH
R = CH₂COCl

(structure) COOEt ... N ... O ... O **4** → (structure) COOEt N O **5** →

(structure) COOEt / COOEt N O **6** → COOH / O= / NH₂ **7**
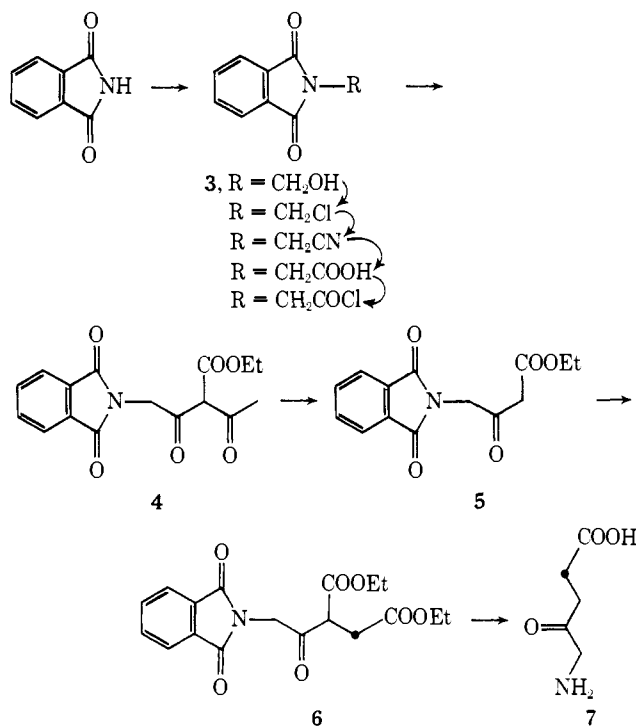
of Mitta, *et al.*[37], each intermediate being recrystallized to the literature melting point.[37]

Ethyl 2-Phthalimidoacetylacetoacetate (4). The ethoxy magnesium enolate of ethyl acetoacetate was generated in ether–benzene under anhydrous conditions[7] and condensed with phthalylglycine chloride (3) according to the procedure of Pichat, *et al.*,[7] to yield (4) (150 mM scale). Nmr (CDCl₃): 1.39 t ($J = 7$), 3 H (ester methyl); 2.46 s, 3 H (acetyl methyl); 4.36 q ($J = 7$), 2 H (ester methylene); 4.97 s, 2 H (N-methylene); 7.79 sym m, 4 H (phenyl).

Ethyl 4-Phthalimidoacetoacetate (5). Diketo ester 4 was deacylated in a mixture of ethanol and concentrated ammonium hydroxide to ethyl phthalimidoacetoacetate (5) by the method of Pichat.[7] Recrystallization from ethanol yielded purified material whose melting point was in accord with the literature (111–112°).[7] Nmr (CDCl₃): 1.29 t ($J = 7$), 3 H (ester methyl); 3.56 s, 2 H (methylene); 4.22 q ($J = 7$), 2 H (ester methylene); 4.62 s, 2 H (N-methylene); 7.78 sym m, 4 H (phenyl).

Ethyl [2-¹³C]Bromoacetate.[7,8] Sodium [2-¹³C]acetate (500 mg, 6.05 mmol; Prochem-B.O.C., 61 atom %) was treated with phosphoric acid saturated with phosphoric anhydride (3.3 g of 85% orthophosphoric acid and 2.5 g of phosphorus pentoxide). [2-¹³C]Acetic acid was distilled over about 3 hr on a vacuum line into a flask cooled in a Dry Ice-acetone bath (see ref 7). Acetic anhydride (0.2 ml, 216.5 mg, 2.12 mmol) and pyridine (2 drops) were added to the [2-¹³C]acetic acid, and the mixture was heated at reflux with an efficient condenser for 1 hr brominated by the dropwise addition of purified bromine [0.3 ml, 875 mg, 5.5 mmol (more should have been used; however, *cf.* ref 7), dried over concentrated sulfuric acid, and freshly distilled]. After 2 hr, a stream of nitrogen was passed through the cool reaction mixture to remove hydrogen bromide (and excess bromine). Distilled water (5 ml) was added to quench any acetyl bromide or acetic anhydride remaining, and the mixture was quickly evaporated to dryness *in vacuo* and stored overnight in a desiccator over phosphorus pentoxide. The thoroughly dried [2-¹³C]bromoacetic acid was slurried with about 4 ml of absolute ethanol, saturated with dry hydrogen bromide gas, and heated at reflux for 2 hr.[7] The solution was neutralized with aqueous sodium carbonate, and the ethyl [2-¹³C]bromoacetate was extracted into ether. The dried ether layer was carefully concentrated under water aspirator vacuum and chromatographed on a short column of silica gel eluting with chloroform (methylene chloride was later found preferable). Fractions containing the volatile bromo ester were cautiously evaporated under vacuum to yield the product as an oil (655 mg, 48% based on 6.05 mmol of sodium [2-¹³C]acetate and 2.12 mmol of acetic anhydride; theoretical enrichment of ¹³C, 45–46 atom %).

Ethyl 2-Carboethoxy[¹³C]methyl-4-phthalimidoacetoacetate (6).

To a suspension of sodium hydride (108 mg, 4.51 mmol; 60% in oil suspension) in 2 ml of dry 1,2-dimethoxyethane was added a solution of phthalimidoacetoacetate (5) (1.145 g, 3.92 mmol) in 8 ml of the same solvent. After stirring for 1 hr at room temperature under nitrogen, ethyl [2-¹³C]bromoacetate (655 mg, 3.92 mmol) was added in 2 ml of 1,2-dimethoxyethane, and the mixture was stirred for 2 days, as described by Pichat.[7] An equal volume of water was added to the reaction mixture, and it was neutralized with 1 N hydrochloric acid and extracted with ether. The ether layers were dried and concentrated under reduced pressure to yield the product as a very pale-yellow oil (1.367 g, about 30% starting material from nmr). Product from an earlier trial without heavy isotope gave the following. Nmr (CDCl₃) 1.25 t ($J = 7$), 3 H (ester methyl); 1.33 t ($J = 7$), 3 H (ester methyl); 2.98 d ($J = 7$), 2 H (methylene); 4.18 q ($J = 7$), 2 H (ester methylene); 4.19 t ($J = 7$), 1H (methine); 4.29 q ($J = 7$), 2 H (ester methylene); 4.83 q (AB, $J_{app} = 18$), 2 H (N-methylene); 7.83 sym m, 4 H (phenyl).

[2-¹³C]-δ-Aminolevulinic Acid ([2-¹³C]ALA) (7). The oily mixture of 5 and 6 obtained in the previous step was heated at reflux for 20 hr with 15 ml of glacial acetic acid–concentrated hydrochloric acid (1:1).[7] The solution was evaporated to dryness *in vacuo*, and the residue was successively dissolved in water and evaporated three times. Finally, the residue was dissolved in about 30 ml of water and extracted with ethyl acetate to remove phthalic acid and any other organic-soluble impurities (*e.g.*, those derived from hydrolysis and decarboxylation of keto ester (6) present in the mixture). The aqueous layer was evaporated to dryness under vacuum to give crude 7 as its hydrochloride (419 mg). Purification was accomplished by ion-exchange chromatography on Dowex 2X-8 (100 g), eluting over 1 week with 1–4 M hydrochloric acid. The ALA-containing fractions were collected and evaporated to dryness to yield purified [2-¹³C]ALA (7) as the hydrochloride (351 ml, 53% from keto ester 6 which on paper chromatography gave one spot whose R_f was identical with authentic material (Sigma). Ir (KBr): 1730 (s), 1560, 1490, 1430, 1390, 1355 (w), 1250 (w), 1210, 1185, 1140, 1100 (w), 1060 (w), 1000, 970 (w), 955, 865. Paper chromatography: R_f 0.35 [Whatman No. 1, descending, 1-butanol:acetic acid:water 63:11:26 (single phase)].

3. [5-¹³C]-δ-Aminolevulinic Acid ([5-¹³C]ALA) (16). [5-¹³C]ALA was synthesized according to the method of Shemin[15] for [5-¹⁴C]ALA using diethyl [2-¹³C]malonate (1.0 g, 6.2 mmol; Merck of Canada, 90 atom %) as the source of label.

4. [8-¹³C]Porphobilinogen ([8-¹³C]PBG) (9). [8-¹³C]PBG was synthesized according to the procedure described for [8-¹⁴C]PBG using a sixfold scale-up. Label was introduced *via* condensation with [¹³C]paraformaldehyde (1.0 g, 33.3 mmol; Merck of Canada, 90 atom %) and gave PBG in good yield (1.64 g total, 20% over seven steps based on [¹³C]paraformaldehyde), a portion of which was converted to the synthetic mixture of [¹³C]uroporphyrinogens I–IV (see below).

5. [¹³C]Uroporphyrins I–IV ([¹³C]uro I–IV) and Reduction to [¹³C]Uroporphyrinogens I–IV ([¹³C]Uroporphyrinogens I–IV, (11–14)). [8-¹³C]Porphobilinogen (9) (1.108 g, 4.52 mmol) was dissolved in 200 mol of 1 N hydrochloric acid, and the solution was degassed by nitrogen ebullition for 0.5 hr before heating on a steam bath for 1 hr under nitrogen. The pale-orange uro'gen solution was concentrated under reduced pressure, oxidized, and neutralized, and the uroporphyrin was precipitated at its isoelectric point. After cooling overnight, the precipitate was collected by centrifugation and washed five times with cold water (pH 3.5 by addition of acetic acid). The amorphous [¹³C]uroporphyrin was dissolved in a small amount of dilute ammonium hydroxide and reprecipitated at pH 3.5. As above, the precipitate after cooling several hours was washed three times and finally dried (774 mg, 82%). Paper chromatography (Whatman No. 1, ascending—ammonia vapor, 2,6-lutidine:water 10:7) of a small sample of this material had an R_f corresponding to that of an authentic sample. Immediately prior to feeding, the statistical mixture of uroporphyrin isomers was reduced over freshly prepared and ground 3% sodium amalgam to [¹³C]uroporphyrinogens I–IV (11–14), as detailed elsewhere.[6]

B. Preparation of Cobinamide and Neocobinamide. Trifluoroacetic Acid Conversion of [¹³CH₃]-L-Methionine Enriched Cyanocobalamin (18) to Cobinamide (19) and Neocobinamide (20). (i) Preliminary Trials. Unlabeled vitamin B₁₂ (40.8 mg, 0.03 mmol; Glaxo Laboratories) was dissolved in a solution of 8 ml of dry tri-

Table II. Apparent Per Cent Composition

	5 hr	25 hr	49 hr	75 hr	100 hr	150 hr
Neo B_{12}	30	5	2	1+	1	
B_{12}	25	5	2	1+	1	
Neocobinamide	20	50	53	53	54	55
Cobinamide	25	40	43	43	44	45

fluoroacetic acid and 8 drops of hydrogen cyanide and maintained at room temperature (21 ± 0.5°) in a tightly sealed, foil-wrapped flask. Samples were withdrawn at intervals shown in Table II and evaporated to dryness with a rotary evaporator (bath temperature below 20°). The residue was slurried in a small amount of benzene and evaporated to dryness as above, and this procedure was repeated several times more with ether. The thoroughly dried hydrolysis samples were sealed with parafilm and stored in the freezer for subsequent paper chromatographic analysis. Concentrated solutions of the samples in ethanol were spotted on Whatman 3MM paper and developed by a modification of Bonnett's procedure[26,38] [descending, 2-butanol:water:4% aqueous hydrogen cyanide 100: 40:1 (single phase)]. After 50 hr, the papers were dried, and the compositions of the sample mixtures were estimated visually under ultraviolet light.

(ii) **Conversion of [^{13}CH$_3$]-L-Methionine Enriched Cyanocobalamin (18).** Labeled cyanocobalamin (35–36 mg, 0.026 mmol) obtained from [^{13}CH$_3$]-L-methionine feeding experiments 1 and 2 in aqueous solution was thoroughly desalted by extraction into phenol–chloroform (1:1) and reextraction into deionized water. The aqueous solution was evaporated to dryness under reduced pressure and stored *in vacuo* over phosphorus pentoxide for 1 hr. Hydrolysis and epimerization to cobinamide and neocobinamide were carried out as in (i) above for 100 hr (temperature 22.5 ± 1°). The trifluoroacetic acid was removed under reduced pressure (rotary evaporator, bath temperature below 20°), and the residue was slurried with a small amount of benzene and evaporated. This procedure was repeated once more with benzene and four times with ether. The dry residue was dissolved in the minimum amount of ethanol and chromatographed on half sheets (3) of Whatman 3MM as in (i) above. After about 65 hr, the neocobinamide and cobinamide bands were well separated. (Note: Approximately 30% of the material had hydrolyzed to monoacidic corrinoids of greater R_f. This was not observed in the preliminary trials with authentic material and must be due either to traces of water in the trifluoroacetic acid used or to insufficient drying of the cyanocobalamin.) The papers were dried and the bands cut out and extracted as completely as possible with water and dilute hydrogen cyanide solution. Working in dim light, the solutions were concentrated nearly to dryness under reduced pressure (rotary evaporator, bath temperature below 30°) and samples prepared in 0.1 M potassium cyanide (correct uv, ord, and cd spectra)[26,28] for ^{13}C-FT nmr analysis as described below.

Cleavage of [^{13}CH$_3$]-L-Methionine Enriched Cyanocobalamin (18) to Cobinamide (19) in the Presence of Cerous Hydroxide. A solution of labeled vitamin B_{12} (18) from [^{13}CH$_3$]-L-methionine feeding experiments 3 and 4 was carefully desalted and concentrated to 10 ml under vacuum. To this solution the following were added in order: 4 ml of 4% aqueous hydrogen cyanide, 6 ml of 0.333 M cerium(III) nitrate, and 5 ml of 1.00 N sodium hydroxide. (Note: The last two solutions had been previously degassed by

nitrogen ebullition.) The resulting viscous, purple gel was heated with occasional swirling on a steam bath under nitrogen for 45 min. When the mixture had cooled to room temperature, the pH was adjusted to 7.5–8.0 with about 5 drops of concentrated ammonium hydroxide, and the mixture was cooled overnight to precipitate salts.[29,31] The salts were removed by filtration and washed with cold water, and the resulting clear, dark-purple filtrate was thoroughly desalted by the phenol–chloroform method. The final aqueous extract was concentrated under reduced pressure almost to dryness, and the viscous residue was dissolved in 0.1 M potassium cyanide for analysis by ^{13}C-FT nmr spectroscopy. Paper chromatography of a small sample of the product indicated that the hydrolysis was complete and revealed only one spot, whose R_f was coincident with authentic cobinamide (uv, ORD, and CD spectra were in accord with published data).[25,31]

C. **Feeding Experiments.** The generalized conditions described previously[6] were closely adhered to for both the growth of *P. shermanii* and the conduct of the ^{13}C feeding experiments. As large quantities of bacteria were required to produce sufficient labeled vitamin B_{12} for ^{13}C-FT nmr analysis (24–36 l.), the complete feeding of a particular precursor was broken up into a series of smaller experiments, generally 12 l./day, spread over 2 or 3 days in succession. The incubations were terminated at the times noted, and the cells were harvested, washed, and stored at −20°. When the series was complete, the cells were extracted together, and the resulting labeled vitamin B_{12} was purified by electrophoresis, desalted, and crystallized from acetone–water. The ^{13}C-FT nmr spectra were run under the conditions noted, and, where possible, the per cent enrichment of labeled centers was estimated and the specific incorporation of precursor approximated.

[2-^{13}C]-δ-Aminolevulinic Acid ([2-^{13}C]ALA (7)). Experiments 1, 2, and 3. Three experiments were conducted, each using cells from 12 l. of anaerobic culture divided equally and suspended in six 500-ml erlenmeyer flasks containing 200 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6), cobalt chloride (CoCl$_2$ · 6H$_2$O, 3 mg), DMBI (20 mg), L-methionine (20 mg), 50% glucose solution (3.2 ml), and [2-^{13}C]ALA · HCl (13.3 mg, 45–46 atom %). Nitrogen was passed through the incubations for 1 hr, and adjustments of pH and additions of glucose were made as shown in Table III.

^{13}C-FT nmr (cyanocobalamin 26.6 mg/1.5 ml of water, 13 mM; 8K points); 35.9 or 36.1 s, 1 C (β methyl (*pro-S*) at C-12); 35.9 or 36.1, 36.6, 37.0 s, 3 C (propionamide α methylenes at C-3, C-8, C-13); 39.4 or 39.8 s, 1 C (propionamide α methylene at C-17?); 39.4 or 39.8 s, 1 C (acetamide methylene at C-18); 47.5, 48.0 s, 2 C (acetamide methylenes at C-2, C-7) (see Figure 3).

^{13}C-FT nmr (dicyanocobalamin 26.6 mg/1.6 ml of 0.1 M KCN, 12 mM; 8K points); 35.2 s, 1 C (β methyl (*pro-S*) at C-12); 36.8 (2 C), 37.1, 37.2 s, 4 C (propionamide α methylenes); 40.0 s, 1 C (acetamide methylene at C-18); 47.1, 48.8 s, 2 C (acetamide methylenes at C-2, C-7) (see Figure 4).

[8-^{13}C]Porphobilinogen ([8-^{13}C]PBG (9)). Experiments 4, 5, and 6. On the first day, cells from 6 l. of *P. shermanii* were collected and distributed equally among three 500-ml erlenmeyer flasks (expt 4) containing 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6) and cobalt chloride (CoCl$_2$ · 6H$_2$O, 1 mg). On the second day, 12 l. was divided similarly among six flasks of buffer medium (expt 5), and on the third day, 18 l. was distributed among nine flasks, one of which was used for a [8-^{14}C]PBG experiment and the remaining eight for expt 6. DMBI (20 mg), 50% glucose (3.2 ml), L-methionine (20 mg), and [8-^{13}C]PBG (15 mg, 90 atom %) were added to

Table III[a]

	Experiment 1			Experiment 2			Experiment 3	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
1	6.8–7.0		1	6.8–7.0		1	6.7–7.2	
4	6.0–7.2	1	3	6.0–7.1		3.5	6.0–7.2	1
7	6.6–7.2		5.5	6.7–7.1	1	4.5	6.6–7.2	
16.5	7.0–7.2		15	6.6–7.0	1	15.5	6.4–7.1	1
19	6.4–7.1	1	19	(6.9)		23.5	6.4–7.0	1
28	6.4–7.2	1	23.5	(6.8)	1	28.5	6.6–7.2	
40	6.6–7.2	1	25.5	6.4–7.2				
			37.5	(7.0)	1	39.5	(7.0)	1

[a] The average total amount of 10% sodium carbonate added per flask in each experiment was 42, 34, and 45 ml, respectively. Incubation time, 44.5–45 hr; cell wt (after first extraction), 385.5 g; yield vitamin B_{12}, 26.6 mg; enrichment, 5.7%; specific incorporation, 12%.

Table IV[a]

	Experiment 4			Experiment 5			Experiment 6	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
4	4.5-7.0		4	4.5-7.0		3	5.0-7.0	
12.5	6.7-7.0	1	20.5	6.1-7.0	1	14.5	6.0-7.0	1
25.5	6.4-7.0	1	31.5	6.4-7.0	1	25.5	6.2-7.0	1
41.5	6.4-7.0	1	43.5	6.4-7.0	1	40	6.4-7.0	1

[a] Total 10% Na_2CO_3 added/flask, 32–39 ml; incubation time, 49.5–51 hr; cell wt (after first extraction), 481.2 g; yield vitamin B_{12}, 41–42 mg; enrichment, 4.6%; specific incorporation, 5% (cf. [8-^{14}C]PBG experiment 8, 5.09%/C).

Table V[a]

	Experiment 1			Experiment 2			Experiment 3	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
12	4.5-7.0	1	18	4.5-7.0	1	17	4.5-7.0	1
25	5.0-7.1	1	31	5.5-7.0	1	27	5.5-7.0	1
48	6.4-7.0	1	48	6.4-7.0	1	47	6.4-7.0	1

[a] Incubation time, 70 hr; cell wt (after first extraction), 443.6 g; yield vitamin B_{12}, 42.5 mg; enrichment, 10.2%; specific incorporation, 11%.

Table VI[a]

	Experiment 1			Experiment 2	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
3.5	5.5-7.2		2.5	5.5-7.2	
11	6.8-7.2	1	11	6.6-7.0	1
23.5	6.4-7.2	1	21.5	6.2-7.0	1
35	6.5-7.0	1	33.5	6.4-7.0	1
46	6.4-7.0		44.5	6.8-7.0	

[a] Total 10% Na_2CO_3 added/flask, 26–27 ml; incubation time, 51–52 hr; cell wt (after first extraction), 311.7 g; yield vitamin B_{12}, 36 mg; specific incorporation, >20%.

Table VII[a]

	Experiment 3			Experiment 4	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
4	4.5-7.2		5	4.5-7.2	
17	6.2-7.2	1	17.5	6.7-7.2	1
29	6.4-7.2	1	27.5	6.4-7.2	1
42	6.8-7.2	1	41	6.8-7.2	1

[a] Total 10% Na_2CO_3 added/flask, 28–30 ml; incubation time, 50.5–51.5 hr; cell wt (after second extraction), 280 g.

each flask in the customary manner. The suspensions were purged with nitrogen for 4 hr and stored at 29° in the dark; the pH was adjusted and glucose added as recorded in Table IV.

[13]C-FT nmr (cyanocobalamin 41 mg/1.3 ml of water, 23 mM; 4K points): 31.6 (2 C), 33.4 s, 3 C (propionamide β methylenes at C-3, C-8, C-13); 37.8 s, 1 C (propionamide β methylene at C-17) (Figure 6).

[13]C-FT nmr (dicyanocobalamin 41 mg/1.3 ml of 0.1 M KCN, 23 mM; 4K points): 30.4, 31.4, 32.4 s, 3 C (propionamide β methylenes at C-3, C-8, C-13); 38.6 s, 1 C (propionamide β methylene at C-17) (Figure 7).

[^{13}C]Uroporphyrinogens I–IV ([^{13}C]Uro'gens I–IV, 11, 12, 13, 14). Experiments 1, 2, and 3. On each of 3 succeeding days, cells from 12 l. of the total were apportioned equally among 12 500-ml erlenmeyer flasks containing the following deaerated (4 hr) medium: 100 ml of ¹⁄15 M phosphate buffer (pH 7.6), cobalt chloride (CoCl₂ · 6H₂O, 1 mg), DMBI (20 mg), 50% glucose solution (3.2 ml), and L-methionine (20 mg). [^{13}C]Uro'gens I–IV (21.4 mg, 90 atom %) were administered to each flask. Adjustments of pH were made with solid sodium carbonate, degassed under high vacuum (10⁻⁴ mm), and additions of glucose were made according to the schedule shown in Table V.

C-FT nmr (cyanocobalamin 41 mg/1.3 ml of water, 23 mM; 4K

points): 31.6 (2 C), 33.4 s, 3 C (propionamide β methylenes at C-3, C-8, C-13); 37.8 s, 1 C (propionamide β methylene at C-17) (Figure 8).

C-FT nmr (dicyanocobalamin 38 mg/1.4 ml of 0.1 M KCN, 20 mM; 4K points): 30.4, 31.4, 32.4 s, 3 C (propionamide β methylenes at C-3, C-8, C-13); 38.6 s, 1 C (propionamide β methylene at C-17) (Figure 9).

[[13]CH₃]-L-Methionine. Experiments 1 and 2. (i) Incorporation into Vitamin B_{12} (Cyanocobalamin). Over 2 days, washed cells from 24 l./flask were distributed in a total of 12 500-ml erlenmeyer flasks containing the following medium: 100 ml of ¹⁄15 M phosphate buffer (pH 7.6), cobalt chloride (CoCl₂ · 6H₂O, 1 mg), DMBI (20 mg), 50% glucose solution (3.2 ml), ALA · HCl chloride (50 mg), and [[13]CH₃]-L-methionine (15.0 mg, 90 atom %). Nitrogen ebullition of the fermentations was carried out for about 3 hr. Adjustments of pH were made with 10% sodium carbonate, and additions of glucose were made as reported in Table VI.

[13]C-FT nmr (cyanocobalamin 36 mg/1.3 ml of water, 20 mM; 8K points): 20.7, 21.0, 21.5, 22.3, 24.7, 24.9 (2 C) s, 7 C (methyls at C-1, C-2, C-5, C-7, C-12 (α) (pro-R), C-15, C-17) (Figure 5).

[13]C-FT nmr (dicyanocobalamin 36 mg/1.3 ml of 0.1 M KCN, 20 mM; 4K points): 19.6, 20.1, 21.4, 22.3, 23.6, 23.8, 26.5 s, 7 C (methyls at C-1, C-2, C-5, C-7, C-12 (α) (pro-R), C-15, C-17) (Figure 12).

(ii) Conversion of ^{13}C-Labeled Cyanocobalamin (18) to Cobinamide (19) and Neocobinamide (20). Vitamin B_{12} labeled at the methionine-derived methyl groups (species 18) was hydrolyzed and epimerized (at C-13) by the trifluoroacetic acid method[26] to a mixture of cobinamide (19) and neocobinamide (20). The paper chromatographic separation, purification, and preparation of these samples for ^{13}C-FT nmr analysis was carried out as described above.

Neocobinamide: ^{13}C-FT nmr (dicyanoneocobinamide 2 mg/1.4 ml of 0.1 M KCN, 1.4 mM; 4K points): 20.2, 20.7, 22.4, 22.7, 24.2, 26.2 s, 6 C (methyls at C-1, C-2, C-5, C-7, C-15, C-17); 35.5 s, 1 C (α-methyl (pro-R) at C-12) (Figure 14).

Experiments 3 and 4. (i) Incorporation into Vitamin B_{12} (Cyanocobalamin (18)). Cells from 24 l. of anaerobic culture were suspended and fed under the same conditions as in expt 1 and 2, except that nitrogen was passed through the incubation for 4 hr prior to the first pH adjustment (Table VII).

(ii) Conversion of Labeled Cyanocobalamin (18) to Cobinamide (19). The nucleotide was selectively hydrolyzed from the labeled vitamin B_{12} obtained above with cerous hydroxide, as previously described.

Cobinamide: ^{13}C-FT nmr (dicyanocobinamide 9 mg/1.3 ml of 0.1 M KCN, 6.6 mM; 8K points): 20.0, 20.4, 21.7, 22.6, 23.8, 24.0, 27.0 s, 7 C (methyls at C-1, C-2, C-5, C-7, C-12 (α), C-15, C-17; α-methyl at C-12 (pro-R) resonates at either 23.8 or 24.0) (Figure 14).

[5-^{13}C]-δ-Aminolevulinic Acid ([5-^{13}C]ALA (16)). Experiments 1 and 2. Washed cells from 30 l. of P. shermanii were suspended and

Table VIII[a]

	Experiment 1			Experiment 2	
Time, hr	pH	50% glucose, ml	Time, hr	pH	50% glucose, ml
2.5	5.0–7.0		2	5.0–7.0	
13.5	6.0–7.0	1	18.5	6.0–7.0	1
24	6.4–7.0	1	24	6.4–7.0	1
39		1	41.5	6.0–7.0	1

[a] Total 10% Na_2CO_3 added/flask, 24–26 ml; incubation time, 54–50 hr; cell wt (after first extraction), 385.0 g; yield vitamin B_{12}, 38 mg; specific incorporation, >20%.

fed over 2 days (18 l. the first day, 12 l. the second) in the established manner (2 l./flask), using a total of 15 500-ml erlenmeyer flasks each containing 100 ml of $\frac{1}{15}$ M phosphate buffer (pH 7.6), cobalt chloride ($CoCl_2 \cdot 6H_2O$, 1 mg), DMBI (20 mg), 50% glucose solution (3.2 ml), L-methionine (20 mg), and [5-^{13}C]ALA hydrochloride (20–30 mg, 90 atom %). The incubations were deaerated for 2 hr in the usual fashion. (Note: In expt 1 (18 l.), there was an 8-hr delay between the suspension of the cells and the actual feeding of the labeled ALA and cofactors. A subsequent pair of experiments with [5-^{14}C]ALA demonstrated that such delays have only a small effect on the incorporation of precursor (compare with [5-^{14}C]-ALA experiments, previous paper) (Table VIII).

^{13}C-FT nmr (cyanocobalamin 38 mg/1.2 ml of water, 23 mM; 4K points): 100.4 d ($J = 7$) and dxd ($J = 111, J = 7$), 1 C (C-10); 109.3 s and d ($J = 123$) and d ($J = 100$) and dxd ($J = 123, J = 100$), 1 C (C-15); 113.2 d ($J = 8$) and dxd ($J = 100, J = 8$), 1 C (C-5); 180.7 d ($J = ?(7)$) and dxd ($J = 123, J = ?(7)$), 1 C (C-14); 183.6 d ($J = 8$) and dxd ($J = 111, J = 8$), 1 C (C-9); 186.7 s and d ($J = 100$), 1 C (C-4 or C-16); 187.2 s and d ($J = 100$), 1 C (C-4 or C-16) (Figure 10).

References and Notes

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(2) (a) NIH Predoctoral Fellow, 1970–1973; (b) NIH Postdoctoral Fellow 1971–1972.

(3) Abbreviations: ALA (δ-aminolevulinic acid); PBG (porphobilinogen); uro-'gen (uroporphyrinogen); FT (Fourier transform).

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