Enzymic Synthesis of Guanosine Diphosphate Cobinamide by Extracts of Propionic Acid Bacteria*

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ABSTRACT: Several hypothetical intermediates in the conversion of cobinamide to cyanocobalamin have been synthesized. Monocyanocobinamide phosphate was synthesized by a condensation of cobinamide with β-cyanoethyl phosphate followed by hydrolysis of the cyanoethyl group. Monocyanoguanosine diphosphate cobinamide was prepared by the condensation of cobinamide phosphate with guanosine monophosphate (GMP). The Co-5′-deoxyadenosyl analog of each cobinamide derivative was prepared by chemical synthesis. Crude extracts of *Propionibacterium shermanii* were shown to convert GTP-8-^{14}C and Co-5′-deoxyadenosylcobinamide phosphate to a guanosine diphosphate cobinamide derivative. The product was characterized by its ionophoretic mobility and by its hydrolysis to GMP-8-^{14}C by potato nucleotide pyrophosphatase. 2′,3′-Isopropylidene-Co-5′-deoxyadenosylcobinamide phosphate was also active as a substrate; diaquocobinamide phosphate was inactive. An anaerobic assay for the enzyme was developed utilizing 2′,3′-isopropylidene-5′-deoxyadenosylcobinamide phosphate-^{32}P. Conversion of this substrate to guanosine diphosphate cobinamide required reduced glutathione and guanosine triphosphate (GTP). The enzymic synthesis of cobinamide phosphate could not be detected, and only tentative evidence was obtained for the conversion of Co-5′-deoxyadenosylguanosine diphosphate cobinamide to deoxyadenosylcobalamin.

In previous investigations of the enzymes involved in the formation of complete corrinoid compounds, the origin of the 5′-deoxyadenosyl moiety of cobamide coenzymes was determined (Peterkofsky and Weissbach, 1963; Brady *et al.*, 1962). An equally interesting aspect of this problem is the formation of the nucleotide side chain of cyanocobalamin and related coenzymes. Cobinamide, the nucleotide-free derivative of cyanocobalamin, has been implicated as an intermediate in cyanocobalamin biosynthesis by whole cell experiments. Bernhauer and Wagner (1962) reported that cobinamide and its ethanolamine analog, cobyrinyl a,b,c,d,e,g-hexamide f-*N*-ethanolamine, are converted to complete corrinoid compounds by *Propionibacterium shermanii* grown anaerobically in the presence of 5,6-dimethylbenzimidazole. In preliminary communications, Sanders *et al.* (1959) reported that cobinamide is converted to complete corrinoid compounds by sonic extracts of *Escherichia coli* and Pawelkiewicz and Bartosinski (1960) observed the conversion of cobinamide-^{60}Co to cyanocobalamin-^{60}Co by acetone powders of *P. shermanii*. No further details of this transformation in cell-free systems have been published. However, studies with intact organisms have implicated cobinamide phosphate, GDP[1]-cobinamide, and α-ribazole as biosynthetic intermediates. Abbreviated structural formulas are given in Figure 1. Barchielli *et al.* (1960) suggested that GDP-cobinamide is an intermediate in the formation of cyanocobalamin, and proposed the following pathway:

$$\text{cobinamide} + \text{ATP} \longrightarrow \text{cobinamide phosphate} + \text{ADP} \quad (1)$$

$$\text{cobinamide phosphate} + \text{GTP} \longrightarrow \text{GDP-cobinamide} + \text{PP}_i \quad (2)$$

$$\text{GDP-cobinamide} + \alpha\text{-ribazole} \longrightarrow \text{cyanocobalamin} + \text{GMP} \quad (3)$$

These investigators did not specify the ligands bound to the top and bottom positions of the corrinoid compounds though the monocyano forms are implied. Indirect evidence supports this proposal. Using a mutant strain of *Nocardia rugosa*, Boretti *et al.* (1960) observed that the addition of cobinamide phosphate and GDP-cobinamide to the growth medium decreased the amount of orthophosphate-^{32}P incorporated into cyanocobalamin. Under similar conditions the addi-

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[1] Abbreviations used: GMP, GDP, GTP, guanosine mono-, di-, and triphosphates; GSH, glutathione; NADH, reduced nitcotinamide–adenine dinucleotide phosphate; ADP and ATP, adenosine di- and triphosphates; UTP, uridine triphosphate; FAD, flavin–adenine dinucleotide; DA, 5′-deoxyadenosyl; DIA, 2,3-isopropylidene-5′-deoxyadenosyl; PEP, phosphoenolpyruvate; α-ribazole, 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole; DCC, dicyclohexylcarbodiimide.

tion of α-ribazole decreased the amount of radioactivity incorporated into cyanocobalamin formed by cells supplemented with ribose-^{14}C and 5,6-dimethylbenzimidazole (Barbieri *et al.*, 1962). Bartosinski (1966) found that cobinamide, cobinamide phosphate, and particularly GDP-cobinamide accumulated in larger amounts in cells of *P. shermanii* deprived of 5,6-dimethylbenzimidazole than in cells supplied with this compound. When dimethylbenzimidazole was added, there was a large and rapid increase in cobalamin while the other compounds decreased. Friedmann and Harris (1965) described a *trans-N*-glycosidase from *P. shermanii*, which, together with a phosphatase, forms α-ribazole from 5,6-dimethylbenzimidazole and nicotinic acid ribonucleotide.

The present communication describes the chemical synthesis of DIA-cobinamide phosphate, DA-cobinamide phosphate, and DIA-GDP-cobinamide, and presents evidence that DA-GDP-cobinamide is formed enzymatically from DA-cobinamide phosphate and GTP by crude extracts of *P. shermanii*.

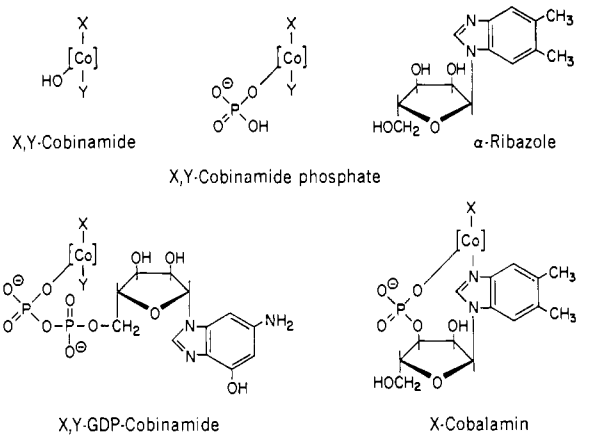

FIGURE 1: Structures of possible intermediates in the conversion of cobinamide to cobalamin. X and Y denote ligands bound to the cobalt of the corrinoid, indicated by [Co], by either a covalent or a coordinate bond.

Experimental Procedure

Materials. Commercially available compounds were obtained from the following sources: chromous chloride and barium β-cyanoethyl phosphate (K & K Laboratories); 2′,3′-isopropylidene adenosine, GTP, and DCC (California Corp. for Biochemical Research); cerous nitrate (Baker and Adamson); GSH (Mann Research Laboratory); cyanocobalamin, ATP, NADH, and PEP (Sigma Chemical Co.); sodium phosphate-^{32}P (Nuclear Consultants Corp.); GTP-8-^{14}C (Schwarz BioResearch); 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)benzene (Packard Instrument Co.). An authentic sample of cobinamide was the gift of Professor K. Bernhauer.

Pyruvate kinase (40 units/mg of protein) was obtained as a contaminant of muscle lactic dehydrogenase from Sigma Chemical Co.; deoxyribonuclease, from Worthington Biochemical Corp. Purified potato nucleotide pyrophosphatase (sp act. 4.5 units/mg protein) was a gift from Dr. A. Kornberg.

Glass capillaries (1.2–1.5-mm diameter) were obtained from the Kimble Glass Co.; analytical grade Dowex 1-Cl and Dowex 50-H$^+$ (2X, 200-400 mesh) from Bio-Rad Laboratories; phosphocellulose and DEAE-cellulose, 0.88 mequiv/g from Schleicher & Schuell.

Physical Methods. Absorption spectra were recorded at 25–28° with a Cary Model 14 spectrophotometer using matched quartz cells with a 1-cm light path. Individual absorption measurements were made with a Zeiss Model PMQ II spectrophotometer. Concentrations of 5′-deoxyadenosyl derivatives of cobinamide were determined by measuring the absorbance at 458 mμ in 25 mM NH_4HCO_3, pH 7–8, and assuming an extinction coefficient of 8.75 M^{-1} cm^{-1} (Weissbach *et al.*, 1960).

Paper ionophoresis was performed with an apparatus similar to that of Crestfield and Allen (1955) using the following buffer systems: 0.5 N acetic acid, pH 2.8; 0.1 M ammonium formate, pH 3.0; 0.1 M ammonium formate, pH 3.5; 50 mM ammonium acetate, pH 5.2; 50 mM potassium phosphate, pH 7.2; and 50 mM ethanolamine chloride, pH 10.4. Whatman No. 1 paper, previously washed with 5% oxalic acid and distilled water, was used when compounds were to be eluted from ionophoretograms. Otherwise, untreated Whatman No. 1 paper was used. The effective paper length was 52 cm and the potential gradient was 34–40 v/cm. Ionophoresis with monocyanocorrinoid compounds was conducted in subdued light. With 5′-deoxyadenosyl derivatives the operation was done in darkness.

Two reference compounds were included in each ionophoresis: picric acid (saturated aqueous solution) with a charge of −1 over the pH range used, and cyanocobalamin (5 mM) which is uncharged. The former was used to estimate the degree of uncontrolled variation in rates of migration; the latter, to correct for electroosmosis.

Paper chromatography was conducted with the following solvent systems: solvent I, isobutyric acid–water–concentrated NH_4OH (66:33:1, v/v); solvent II, *sec*-butyl alcohol–2 mM NaCN (aqueous)–3.75 M NH_4OH (100:40:14); solvent III, *sec*-butyl alcohol–water–concentrated formic acid (30:10:10). The Co-alkyl compounds were chromatographed in darkness. The position of each compound on paper was determined from its quenching of ultraviolet light.

Radioactivity of compounds on Whatman No. 1 paper was measured in a dioxane solution (Bray, 1960) with a Packard Tri-Carb Model 3324 liquid scintillation spectrophotometer. All radioactive measurements were made with a precision of ±5%. The reported counts have been corrected for background.

Growth of Microorganisms and Preparation of Cell Extracts. P. shermanii (ATCC 9614) and *Propioni-*

bacterium arabinosum (ATCC 4695) were grown at 30° in a glucose–yeast extract medium described by Brady and Barker (1961) with the exception that no $CaCO_3$ was added. The pH was maintained at 6–7 by the frequent addition of sterile 7 N NH_4OH.

Clostridium tetanomorphum (strain H1) was grown anaerobically at 37° in the medium described by Barker *et al.* (1960). Clostridial cells were suspended in 50 mM potassium phosphate buffer, pH 7.0, and treated in a Raytheon 10-kcycle sonic oscillator for 18 min at 0°. The homogenate was then centrifuged at 0° for 10 min at 12,000*g*.

For initial experiments the propionibacteria were disrupted by sonication for 45 min with an equal volume of washed glass beads. The homogenate was then neutralized with 1 N NH_4OH to pH 7 and centrifuged. Some cells were disrupted by a Hughes press; the homogenate was suspended in 50 mM Tris-Cl buffer (pH 8.2) and was neutralized as indicated above. DNase (20 μl of 0.1%) in 50 mM potassium phosphate buffer (pH 7.0) was added to degrade the nucleic acid. The extracts were centrifuged as above.

Acetone powders were prepared from freshly harvested propionic acid bacteria as described by Gunsalus (1955). Protein concentration was measured by the method of Lowry *et al.* (1951); crystalline human serum albumin was used as a protein standard.

Assay for the Synthesis of GDP-cobinamide Derivatives. The assay for synthesis of the DIA derivative of GDP-cobinamide was performed in darkness. When added, GSH and PEP were neutralized with KOH to pH 6. Ascorbic acid was neutralized with NH_4OH. The reducing agents were prepared immediately before use. Components of the reaction mixtures were placed as droplets on parafilm at 0° in dim light. To initiate the reaction, the assay mixture was mixed and drawn into a glass capillary, sealed, and placed in a constant-temperature bath at 30°. The entire operation required 30–40 sec. At the end of the assay the capillary was heated 20 sec in a boiling water bath, then emptied onto Whatman No. 1 paper with 25 mμmoles of monocyano-GDP-cobinamide for paper ionophoresis in 50 mM ammonium acetate (pH 5.2) for 60 min. At pH 5.2, monocyano-GDP-cobinamide has approximately the same mobility as DA- or DIA-GDP-cobinamide, hence it is a suitable carrier (see Table II). After ionophoresis, the paper was partially dried *in situ* and then completely dried at 80°. The GDP-cobinamide spots were cut out and radioactivity was measured.

Preparation of Substrates. Terminally labeled ATP-^{32}P, UTP-^{32}P, and GTP-^{32}P were prepared using spinach chloroplasts prepared by the method of Whatley and Arnon (1963). The reaction mixtures were similar to those employed by Krall and Purvis (1961). DA-adenosylcobamide was prepared as described earlier (Barker *et al.*, 1960) except that the final purification of the product by Dowex 50 chromatography was not done. In this preparation approximately 80% of the total corrinoid compounds was DA-adenosylcobamide. A number of unidentified incomplete cationic corrinoid compounds were also present, but the preparation was devoid of neutral or anionic compounds. Corrinoid compounds were stored in neutral solution at −10°.

Monocyanocobinamide was prepared by cerous hydroxide hydrolysis of 126 μmoles of cyanocobalamin according to the method of Friedrich and Bernhauer (1956). The hydrolysate was acidified to pH 3.0 with 1 N HCl and the corrinoid compounds were desalted by phenol extraction (Barker *et al.*, 1960). The product was purified on a phosphocellulose column (3.5 (diameter) × 9 cm). After eluting minor components with 400 ml of 5 mM sodium acetate (pH 4.0) monocyanocobinamide was eluted with 30 mM sodium acetate (pH 4.4) between 850 and 950 ml. The yield was 102 μmoles. The nucleoside, α-ribazole, was obtained in a yield of 72 μmoles by making the column basic with 100 ml of 0.1 M NH_4OH and eluting with 100 ml of 0.1 M NH_4HCO_3 (pH 8.5).

Cobinamide phosphate was prepared by the condensation of cobinamide with excess β-cyanoethyl phosphate, followed by hydrolysis of the cyanoethyl group as outlined in a brief communication by Wagner (1962). Barium β-cyanoethyl phosphate (120 μmoles) was suspended in 2 ml of water, dissolved by shaking with excess Dowex 50 H^+, and passed through a short column of the resin. The effluent was diluted to 10 ml with anhydrous pyridine, concentrated *in vacuo* to 0.5 ml, and added to 27.3 μmoles of dicyanocobinamide previously concentrated to dryness. The mixture was concentrated to dryness *in vacuo* and dissolved in 1 ml of dimethylformamide. The solution was then dehydrated by twice adding 5 ml of anhydrous pyridine and distilling it at reduced pressure. DCC (320 μmoles) was dissolved in the anhydrous solution and the reaction vessel was sealed and stored in a desiccator at 27° for 2 days. An equal volume of water was then added and, after standing at 27° for 30 min, the solution was concentrated to a small volume. The residue was taken up on 10 ml of water and the mixture was centrifuged at low speed to sediment the precipitated dicyclohexylurea. NaCN (20 mM) was added to the supernatant solution, which was then chromatographed on a DEAE-cellulose column (2 (diameter) × 11 cm). Dicyanocobinamide was not retained on the column. After washing with 200 ml of water, cyanoethyl cobinamide phosphate (21.6 μmoles) was eluted with 0.1 M NH_4HCO_3 and 2 mM NaCN (pH 8.5). The product was desalted by phenol extraction, concentrated to dryness, and hydrolyzed in 1 ml of 9 M NH_4OH containing 20 mM NaCN by heating for 15 min at 52°. The reaction mixture was concentrated to dryness *in vacuo*, then fractionated on a DEAE-cellulose column as above. Pass-through fractions contained 2.3 μmoles of dicyanocobinamide. Unhydrolyzed cyanoethyl cobinamide phosphate (2.4 μmoles) was eluted with bicarbonate between 60 and 110 ml, followed by dicyanocobinamide phosphate, between 125 and 190 ml. The product was desalted by phenol extraction. The yield was 8 μmoles. ^{32}P-labeled cobinamide phosphate (initial sp act. 9 × 10^6 cpm/μmole) was prepared in the same way using β-cyanoethyl

TABLE I: R_F Values of Cobinamide and Synthetic Derivatives.[a]

Cobalt Ligand	Corrinoid Compd	Solvent I	Solvent II	Solvent III
Monocyano	Cobinamide	0.95	0.37	0.47
Monocyano	Cobinamide phosphate	0.84	0.09	0.38
Monocyano	Cyanoethyl cobinamide phosphate	0.83	0.24	0.38
Monocyano	GDP-cobinamide	0.66	0.05	0.05
DIA	Cobinamide	0.97		0.49
DIA	Cobinamide phosphate	0.65		0.38
DIA	GDP-cobinamide	0.28		
DA	Cobinamide, peak A			0.39
DA	Cobinamide, peak B	0.91		0.26
DA	Cobinamide phosphate, peak A	0.57		0.29
DA	Cobinamide phosphate, peak B	0.57		0.21
DA	GDP-cobinamide, peak A	0.22		
DA	GDP-cobinamide, peak B	0.21		

[a] Values for the monocyano compounds were determined by ascending chromatography. Descending chromatography was used for all other determinations.

TABLE II: Ionophoretic Mobilities of Cobinamide and Synthetic Derivatives.

Cobalt Ligand	Corrinoid Compd	Mobility ($cm^2/v \times sec \times 10^5$)[a] at pH 2.8	5.2	7.2	10.4
Monocyano	Cobinamide	+5.3	+5.1	+4.6	+4.5
Monocyano	Cobinamide phosphate	−0.4	0	−3.5	−4.7
Monocyano	Cyanoethyl cobinamide phosphate	−0.7	0	−0.4	0
Monocyano	GDP-cobinamide	−3.0	−4.1	−5.3	−6.8
DIA	Cobinamide	+6.5			
DIA	Cobinamide phosphate	+4.7	0		
DIA	GDP-cobinamide	+0.3	−4.1		
DA	Cobinamide, peak B	+6.5			
DA	Cobinamide phosphate, peak A	+4.7	0		
DA	GDP-cobinamide, peak A	−0.3	−4.1		

[a] Precision is ±10%.

phosphate-^{32}P synthesized by the method of Tener (1961).

GDP-cobinamide was prepared by a condensation of cobinamide phosphate with GMP, similar to the method of Kennedy (1956). Monocyanocobinamide phosphate (20 μmoles) was passed through a Dowex 50 pyridinium column, and the effluent was concentrated to dryness. To this was added 36 μmoles of GMP in water. The solution was concentrated to dryness. A mixture of 2.5 ml of pyridine, 2.5 ml of dimethylformamide, and 0.8 ml of water was added. The corrinoid compound was dissolved by bubbling air containing HCN through the solution; 360 mg of DCC was added and the mixture was stirred at 37°. An additional 360 mg of DCC and 0.2 ml of water were added 3 days later. After 5 days 25 μmoles of GMP, 0.5 ml of water, and 360 mg of DCC were added. After 6 days, 50 ml of water was added and the mixture was filtered. The filtrate was concentrated *in vacuo;* the residue was taken up in water, adjusted to pH 8.0 with 0.1 M NH_4OH, and chromatographed on a DEAE-cellulose column (2 (diameter) × 9 cm). Two orange compounds were eluted with 25 mM NH_4HCO_3 (pH 8.5). The first, eluted between 140 and 175 ml, was unreacted monocyanocobinamide phosphate; the second, eluted between 180 and 250 ml, was monocyano-GDP-cobinamide. The yield of the latter was 47%.

R_F values of the monocyanocobinamide and its derivatives are given in Table I; the ionophoretic mobilities in Table II. In water at neutral pH, the spectrum of monocyano-GDP-cobinamide has absorb-

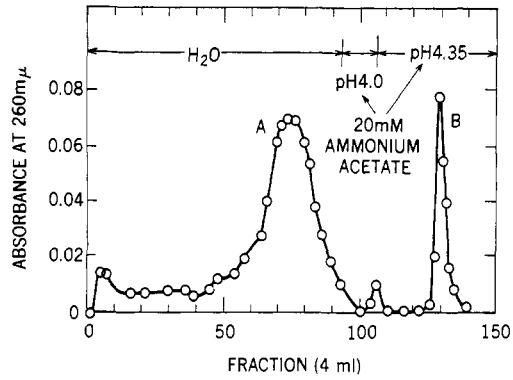

FIGURE 2: Separation of the hydrolysis products of DIA-cobinamide phosphate on phosphocellulose. DIA-cobinamide phosphate (0.62 μmole) was hydrolyzed in 0.5 ml of 0.1 N HCl for 5 min at 90°. The solution was diluted to 10 ml, the pH was adjusted to 3.0 with NH_4OH, and the solution was run into a 2 (diameter) × 7 cm column of phosphocellulose, previously equilibrated with pH 3.6 buffer. The flow rate during elution was 2.5 ml/min.

ance maxima at 352, 403, 494, and 525 mμ and minima at 290 and 418 mμ. The value for $A_{271\,m\mu}:A_{353\,m\mu}$ is 0.82; for $A_{353\,m\mu}:A_{492\,m\mu}$, the value is 3.0. In 20 mM NaCN (pH 10.2) the spectrum of the dicyano form has absorbance maxima at 273, 308, 367, 535, and 580 mμ. The ratio $A_{273\,m\mu}:A_{367\,m\mu}$ is 0.68, and the ratio $A_{367\,m\mu}:A_{580\,m\mu}$ is 2.88. These data agree with those calculated from an equimolar mixture of GMP and dicyanocobinamide, and are similar to those calculated from the data of Barchielli *et al.* (1960). Monocyano-GDP-cobinamide was converted to GMP and cobinamide phosphate by means of potato nucleotide pyrophosphatase (Kornberg, 1957). A 25-μl reaction mixture contained 50 mμmoles of monocyano-GDP-cobinamide, 25 μmoles of potassium phosphate buffer (pH 8.2), and 0.19 unit of enzyme. A control contained boiled enzyme. The reaction mixtures were incubated in sealed glass capillaries at 34°. After 3 hr they were subjected to paper ionophoresis at pH 5.2. GMP and cobinamide phosphate were identified by their ionophoretic mobilities and absorption spectra; these compounds were not formed in the control.

Cobinamide and its derivatives were converted to their DA derivatives by a modification of the methods described by Johnson *et al.* (1963). Chromous chloride was used as the reducing agent in 0.1 M Na-EDTA (pH 9.5). The reduced corrinoid compound was allowed to react with a tenfold excess of 2′,3′-isopropylidene-5′-*O*-tosyladenosine, prepared according to Sakami (1961). The reaction mixtures were acidified to pH 5, then desalted by phenol extraction. The DIA-corrinoid compounds were isolated by chromatography on small columns of phosphocellulose or DEAE-cellulose. The isopropylidene group was removed by heating for 5 min at 90° in 0.1 N HCl. The hydrolytic step, when applied to DIA-cobalamin, is known to yield two products that can be separated by chromatography on a cation exchange column, DA-cobalamin and a closedly related compound having the same absorption spectrum but lacking coenzyme activity (Hogenkamp and Pailes, 1967). The DIA derivatives of cobinamide, cobinamide phosphate, and possibly GDP-cobinamide also give two yellow products (λ_{max} 458 mμ) after hydrolysis, which are separated by column chromatography. The first product eluted is called peak A; the second, peak B.

DIA-cobinamide was prepared from 1.52 μmoles of cobinamide. The product was isolated from the reaction mixture by adsorption at pH 3 on a 2 (diameter) × 8 cm phosphocellulose column and elution with 25 mM ammonium acetate buffer (pH 4.3). The products of acid hydrolysis of DIA-cobinamide were separated by adsorption on a similar phosphocellulose column at pH 3.5 and eluted first with water and then with 50 mM ammonium acetate buffer (pH 4.2). Both the minor peak A and the major peak B yellow compounds eluted rapidly with the ammonium acetate buffer. The peak B compound, obtained in 37% yield based upon monocyanocobinamide, was indistinguishable from enzymatically prepared DA-cobinamide, whereas the peak A compound had a somewhat larger R_F value on paper chromatography in solvent III (Table I).

DIA-cobinamide phosphate was prepared from 1.0 μmole of monocyanocobinamide phosphate. The product was isolated by adsorption at pH 3.0 on a phosphocellulose column and elution with water. The pass-through fractions contained a large amount of colorless material absorbing at 260 mμ. The yellow product was eluted with water between 180 and 360 ml. The yield of DIA-cobinamide phosphate was 63%. Following acid hydrolysis, the products were separated on a phosphocellulose column (Figure 2). The over-all yield of DA-cobinamide phosphate (peak A) was 40%. This compound was indistinguishable by absorption spectrum (Figure 3), R_F values in solvents I and III (Table I), and ionophoretic mobilities (Table II) from enzymatically synthesized DA-cobinamide phosphate. The spectrum is identical with that of DA-cobinamide phosphate isolated from *P. shermanii* (Pawelkiewicz *et al.*, 1960). The second yellow compound (peak B) that eluted with 20 mM ammonium acetate (pH 4.35) had an absorption spectrum identical with that of the peak A compound, but had a lower R_F value in solvent III. After removal of the DA moiety of synthetic DA-cobinamide phosphate by treatment with NaCN, this monocyanocobinamide phosphate had the same ionophoretic mobility as synthetic cobinamide phosphate used as the starting material. As the dicyano form, it cochromatographed with the starting material on DEAE-cellulose.

DIA-GDP-cobinamide was synthesized from 1.75 μmoles of monocyano-GDP-cobinamide. The product was purified by absorption at pH 9.0 on a 2 (diameter) × 8.5 cm column of DEAE-cellulose and elution with 10 mM ammonium bicarbonate buffer (pH 8.3); it emerged from the column between 110 and 200 ml.

The yield was 84%. The compound was acid hydrolyzed as described above, and the products were separated on a DEAE-cellulose column. Two apparently distinct but overlapping yellow compounds (peaks A and B) were eluted in nearly the same position as the starting material. The spectra of both compounds resembled that of DIA-GDP-cobinamide (Figure 2). Since enzymatically prepared DA-GDP-cobinamide was not available for comparison, it is not certain which compound is the desired product. However, since in all previous syntheses the more abundant product was the expected deoxyadenosyl derivative, and since peak A contained about 70% of the two products, this compound is tentatively considered to be DA-GDP-cobinamide. The over-all yield was 17%. Both compounds yielded material closely resembling DA-cobinamide phosphate when treated with potato pyrophosphatase.

Deoxyadenosyl derivatives were prepared enzymatically for comparison with the synthetic products. An incubation mixture contained 0.1 mM monocyanocobinamide phosphate, 0.1 M potassium phosphate buffer (pH 8.2), 15 mM ATP, 5 mM $MgCl_2$, 0.05 mM FAD, 1.0 mM NADH, 4 mM sodium ascorbate, and 10 mg/ml of *P. shermanii* extract in a volume of 4 ml. After incubating anaerobically at 37° for 90 min, acetic acid was added to a concentration of 0.1 N and denatured protein was removed by centrifugation. The supernatant solution was desalted by phenol extraction, concentrated to a small volume, and subjected to paper ionophoresis at pH 2.8. The yellow cationic spot was eluted from the ionophoretogram. The yield of DA-cobinamide phosphate was 59 mμ-moles. In a similar experiment, a 21% conversion of monocyanocobinamide to DA-cobinamide was obtained. Under similar conditions no conversion of GDP-cobinamide to DA-GDP-cobinamide was detected. Diaquocobinamide phosphate was prepared by exposing DIA-cobinamide phosphate to direct sunlight for 20 min at 25°.

Results

Enzymic Formation of GDP-cobinamide. Preliminary experiments showed that when GTP and monocyanocobinamide phosphate-^{32}P and other cofactors were incubated anaerobically with a sonic extract of *P. shermanii*, significant amounts of radioactivity were observed in material with the ionophoretic mobility of GDP-cobinamide. The identity of the corrinoid substrate and the product was further investigated by an experiment (Table III) with GTP-8-^{14}C, a GTP-regenerating system, and an unlabeled corrinoid substrate, either DA-cobinamide phosphate (A) or diaquocobinamide phosphate (B). At the end of the incubation, the reaction mixtures were treated with an excess of cyanide ion to convert both the diaquo- and the deoxyadenosylcorrinoids to their dicyano forms, which were then separated on DEAE-cellulose columns after adding carrier GDP-cobinamide. The recovery of the carrier was 87% for each reaction mixture, based on

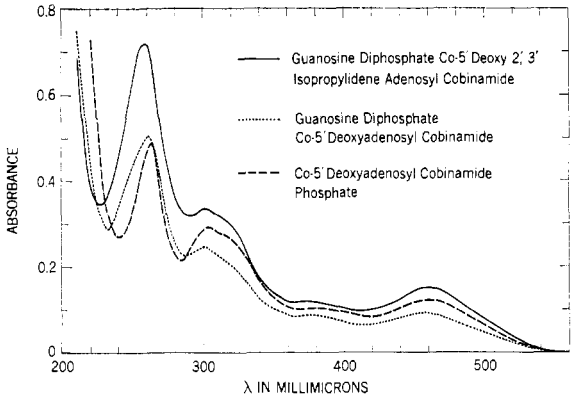

FIGURE 3: Absorption spectra of DA-cobinamide phosphate, DIA-GDP-cobinamide, and DA-GDP-cobinamide. The concentrations were: DA-cobinamide phosphate, 0.0143 mM in water, pH 6; DIA-GDP-cobinamide, 0.0171 mM in 10 mM NH_4HCO_3, pH 7.0; DA-GDP-cobinamide, 0.0109 mM in 10 mM NH_4HCO_3, pH 8.3.

the absorbance at 367 mμ. The column fractions containing the corrinoid compounds were acidified to form the monocyano derivatives and were desalted by phenol extraction. A portion of the GDP-cobinamide isolated from expt A was treated with purified nucleotide pyrophosphatase to convert it to GMP and cobinamide phosphate. The remainder of the GDP-cobinamide from expt A and that from expt B were not treated with pyrophosphatase. The three samples were then subjected to paper ionophoresis at pH 5.2. At this pH, GMP has a mobility of -9.0×10^{-5} cm^2 v^{-1} sec^{-1}. Consequently, GMP, cobinamide phosphate, and GDP-cobinamide (see Table II) were readily separated by ionophoresis. The radioactivity in these fractions was then determined. Some additional experimental details and the results are given in Table III.

The data show that the DA-cobinamide phosphate is a much better substrate than diaquocobinamide phosphate for GDP-cobinamide formation. Indeed, the slight apparent conversion with diaquocobinamide phosphate is probably not significant. When the GDP-cobinamide fraction formed from DA-cobinamide phosphate was treated with nucleotide pyrophosphatase, all the radioactivity in GDP-cobinamide was released as GMP; this supports the conclusion that radioactive GDP-cobinamide was in fact a product of the initial reaction. GMP-^{14}C could also be formed from GTP or GDP by the reaction of nucleotide phosphorylase in the crude extract. However, it is unlikely that these compounds could be present in the GDP-cobinamide fraction, since both are retained on DEAE-cellulose under the conditions used to elute dicyano-GDP-cobinamide (Staehelin, 1961), and both move much faster than monocyano-GDP-cobinamide and GMP on paper ionophoresis at pH 5.2. It is possible that guanosine and GMP contaminated the dicyanocobinamide phos-

TABLE III: Conversion of GTP-8-^{14}C and DA-cobinamide Phosphate to GDP-cobinamide.[a]

Expt	Substrate	Enzymic Hydrolysis of Products[b]	GMP (cpm)	GDP-cobinamide (cpm)	Cobinamide Phosphate (cpm)	Total (cpm)
A	DA-cobinamide phosphate	−	4.4	52.9	24.8	82.1
	DA-cobinamide phosphate	+	65.2	0	32.7	97.4
B	Diaquocobinamide phosphate	−	12.2	6.5	29.1	47.8

[a] Reaction mixtures (50 μl) contained 50 mM potassium phosphate (pH 7.0), 6 mM $MgCl_2$, 4 mM sodium ascorbate, 10 mM PEP, 2 mM GTP-8-^{14}C, 2 mM GDP-8-^{14}C (2.4×10^6 cpm/μmole), 10 mg/ml of protein (sonic extract) from frozen *P. shermanii* cells, 2 μg of pyruvate kinase, and 0.5 mM DA-cobinamide phosphate (expt A) or 0.25 mM diaquocobinamide phosphate (expt B). Reaction mixtures were prepared in 0.5-ml test tubes, flushed with purified nitrogen, then sealed and incubated for 45 min in darkness at 37°. Each reaction mixture was then diluted to 2 ml and 75 mμmoles of GDP-cobinamide and 4 μmoles of NaCN were added. After 30 min, corrinoid compounds were separated on a 1.3 (diameter) × 7 cm DEAE-cellulose column. The column was washed with 6 ml of water, then with 50 mM NH_4HCO_3 plus 2 mM NaCN (pH 8.3). Dicyanocobinamide phosphate was eluted between 12 and 24 ml, and dicyano-GDP-cobinamide between 52 and 84 ml. GMP remained on the column. The fractions containing GDP-cobinamide were combined, acidified to pH 3.5 with 1 N HCl, desalted by phenol extraction, and concentrated to 30 μl. Approximately one-half of the GDP-cobinamide from expt A was hydrolyzed with potato nucleotide pyrophosphatase (Materials). The hydrolysate was concentrated to 15 μl and 25 mμmoles of carrier monocyano-GDP-cobinamide was added. This fraction, as well as the unhydrolyzed portion, and 15 μl of unhydrolyzed GDP-cobinamide from expt B, together with 25 mμmoles of GMP and monocyanocobinamide phosphate, were subjected to paper ionophoresis at pH 5.2. The amount of radioactivity in the GMP, cobinamide phosphate, and GDP-cobinamide spots was determined. [b] By nucleotide pyrophosphatase.

TABLE IV: Corrinoid Substrate Specificity for GDP-cobinamide Formation.[a]

Corrinoid Substrate	GDP-cobinamide Formed (% of total radioactivity) Extract Number				
	1	2	3	4	5
DA-adenosylcobamide	0.108	0.057	0.118	0.052	0.057
DIA-cobinamide phosphate hydrolysis product, peak B	0.121	0.040	0.104	0.052	0.057
Diaquocobinamide phosphate	0.082	0.077	0.105	0.059	0.079
DA-cobinamide phosphate, peak A	0.310	0.130	0.264	0.145	0.114
DIA-cobinamide phosphate	0.290	0.081	0.327	0.283	0.157
Basal level (average of first three values)	0.104	0.058	0.109	0.054	0.062

[a] Each 50-μl reaction mixture, prepared at 0° in a 0.5-ml test tube, contained 50 mM Tris-Cl buffer (pH 8.2), 3 mM ammonium ascorbate, 10 mM GTP-8-^{14}C (1.86×10^5 cpm/μmole), 20 mM $MgCl_2$, 20 mM PEP, 1 mM 2-mercaptoethanol, 2 μg of pyruvate kinase, and one of the following: 0.44 mM DA-adenosylcobamide, 0.5 mM diaquocobinamide phosphate, 0.5 mM DA-cobinamide phosphate, 0.5 mM DIA-cobinamide phosphate, 0.1 mM peak B hydrolysis product of DIA-cobinamide phosphate (see Preparation of Substrates). The concentrations of the different cell extracts were the following: (1) *P. shermanii*, Hughes press extract, 11.4 mg of protein/ml; (2) *P. shermanii*, acetone powder, 20 mg/ml; (3) *P. arabinosum*, Hughes press extract, 5.4 mg of protein/ml; (4) *P. arabinosum*, acetone powder, 20 mg of powder/ml; (5) *C. tetanomorphum*, sonic extract, 10.2 mg of protein/ml. Incubations of 2 hr were performed as described in Table III. At the end of the incubation, the reaction mixtures were chilled to 0° and diluted to 100 μl with 11 mμmoles of GDP-cobinamide and 0.2 μmole of NaCN. Aliquots were removed for determination of total radioactivity. The reaction mixtures were exposed to bright light at 0° for 45 min. Denatured protein was sedimented by brief centrifugation, and the supernatant solutions were diluted to 300 μl, acidified to pH 3 with 6 μmoles acetic acid, desalted by phenol extraction, and concentrated to dryness *in vacuo*. The residues were dissolved in 20 μl of H_2O and subjected to ionophoresis at pH 5.2 with 25 mμmoles of monocyano-GDP-cobinamide. The GDP-cobinamide spots were cut out and counted.

phate and dicyano-GDP-cobinamide fractions, respectively, from the DEAE-cellulose column. This could account for the radioactivity in the neutral (cobinamide phosphate) region and in GMP in the final ionophoretogram.

The corrinoid substrate specificity for GDP-cobinamide synthesis was examined further in a second experiment summarized in Table IV. DA-cobinamide phosphate, diaquocobinamide phosphate, peak B of DIA-cobinamide phosphate hydrolysis, and DA-adenosylcobamide were incubated in the presence of GTP-8-^{14}C with cell-free preparations of *P. shermanii*, *P. arabinosum*, and *C. tetanomorphum*. In each type of extract, diaquocobinamide phosphate, DA-adenosylcobamide, and peak B hydrolysis product of DIA-cobinamide phosphate gave approximately the same basal level of radioactivity migrating with GDP-cobinamide. Both DA-cobinamide phosphate and DIA-cobinamide phosphate increased the radioactivity in GDP-cobinamide threefold over the basal level. DIA-cobinamide phosphate was generally the better substrate. Its synthesis is not complicated by hydrolysis. For these reasons it was used in subsequent experiments.

Extracts of all three organisms catalyze the synthesis of GDP-cobinamide. The propionic acid bacteria appear to give somewhat more active extracts than *C. tetanomorphum*, although a strict comparison is not possible because the extracts were prepared by different methods. With the propionic acid bacteria, the extracts obtained with the Hughes press are clearly more active than those obtained from acetone powders. All subsequent experiments were done with Hughes press extracts of the same batch of *P. shermanii* cells stored at $-10°$. A fresh extract was prepared shortly before each experiment.

Effect of Extract Concentration on the Synthesis of GDP-cobinamide. To obtain evidence that the formation of GDP-cobinamide is the result of an enzymatic reaction, the influence of extract concentration on product formation from GTP-8-^{14}C and DIA-cobinamide phosphate was determined. The assays employed 25-μl reaction mixtures and conditions similar to those described in Figure 3. Protein concentrations were varied over a range of 2–25 mg of protein/ml. In this experiment the assay was simplified by subjecting the previously heated reaction mixture to paper ionophoresis without preliminary treatment with cyanide followed by separation of the cyanocorrinoid compounds by means of a DEAE-column. The incorporation of radioactivity into DIA-GDP-cobinamide, the presumed product, was roughly proportional to the protein concentration over a limited range, from about 5 to 20 mg/ml. No reaction was observed at protein concentrations below 2 mg/ml. These results did not measure the relative rates of reaction, since subsequent experiments indicated that the reaction proceeded during only a small part of the incubation period.

Time Course of the Reaction. An experiment carried out under the conditions employed in the preceding experiment showed that the incorporation of radioactivity from GTP-8-^{14}C into DIA-GDP-cobinamide was essentially complete in 20 min, the shortest time used. Further incubation, up to 150 min, did not decrease the amount of the radioactivity in the product, which therefore appears to be stable under the assay conditions.

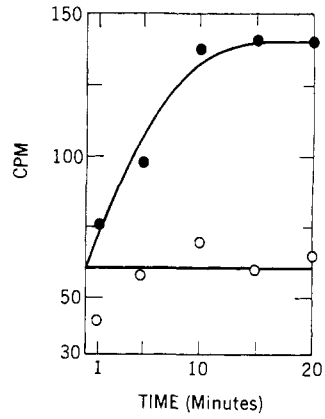

FIGURE 4: Time course of DIA-GDP-cobinamide synthesis. The complete reaction mixture (—●—) contained 0.41 mg of protein from *P. shermanii* Hughes press extract, 2 mM GSH, 10 mM KCl, 10 mM $MgCl_2$, 7.2 mM GTP-8-^{14}C (1.1×10^5 cpm/μmole) containing 0.72 mM GDP, 3 mM NADH, 0.6 mM DIA-cobinamide phosphate, 10 mM PEP, 6 μg of pyruvate kinase, and 50 mM Tris Cl (pH 8.2), in a volume of 25 μl. DIA-cobinamide phosphate was omitted from the controls (—○—). Reaction mixtures in capillaries were incubated in darkness at 30°. The assays were carried out as described in Assay for the Synthesis of GDP-cobinamide Derivatives. The ordinate gives the total radioactivity in GDP-cobinamide in each reaction mixture. Each point was obtained with a separate reaction mixture.

The course of the reaction during the first 20 min of incubation was next investigated. The experimental conditions and results are given in Figure 4. It should be noted that 3 mM NADH was added to the reaction mixture because a separate experiment showed that this significantly accelerated the conversion of GDP to GTP by pyruvate kinase, possibly due to the conversion of accumulated pyruvate to lactate by lactic dehydrogenase. Figure 4 shows that the incorporation of radioactivity into DIA-GDP-cobinamide was complete in about 10 min. The initial reaction rate estimated from the slope of the curve was 10.0 mμmoles/hr per mg of protein. No significant reaction occurred in the controls lacking DIA-cobinamide phosphate.

Requirements for the Incorporation of DIA-cobinamide Phosphate-^{32}P into DIA-GDP-cobinamide. A second assay was based upon the conversion of DIA-cobinamide phosphate-^{32}P and GTP to DIA-GDP-cobinamide-^{32}P. The approximate equivalence between the ^{32}P and the ^{14}C assays is shown in Table V. Under conditions in which 0.12 mμmole of product was formed from GTP-8-^{14}C, 0.17 mμmole of product was

TABLE V: Equivalence of ^{14}C and ^{32}P Assays for DIA-GDP-cobinamide Synthesis.[a]

Expt	Component Omitted	Fraction of Total ^{32}P or ^{14}C in Product %	Δ%	DIA-GDP-cobinamide Synthesized (mμmole)
1	None	0.191	0.040	0.12 ± 0.03
	−DIA-cobinamide phosphate	0.151		
2	None	7.64	1.68	0.17 ± 0.03
	−GTP	5.96		

[a] Each reaction mixture contained 10 mM $MgCl_2$, 16 mM PEP, 2 4 mM NADH, 10 mM KCl, 20 mM GSH, 50 mM Tris-Cl buffer (pH 8 2), 8 mM ammonium ascorbate, 6 μg of pyruvate kinase, and 0.53 mg of protein from *P. shermanii* extract in 25 μl. Experiment 1 contained in addition 12 mM GTP-8-^{14}C (1.13×10^5 cpm/μmole) and 0.4 mM DIA-cobinamide phosphate (omitted from control). Experiment 2 contained 0.4 mM DIA-cobinamide phosphate-^{32}P (1.65×10^6 cpm/μmole) and 12 mM GTP (omitted from control). The reaction mixtures were incubated at 37° foi 15 min and analyzed as described under Assay for the Synthesis of GDP-cobinamide Derivatives.

formed from DIA-cobinamide phosphate-^{32}P. The uncertainty is ±20%. The high blank in the ^{32}P assay was not dependent upon the presence of active extract or a sulfhydryl reagent (Table VI). Since the blank varied from one ionophoretogram, it is possible that a portion of the substrate was converted to the anionic sulfito derivative by impurities in the paper (Buchanan *et al.*, 1964). As shown by Table VI, omission of GTP or reducing agents decreased the incorporation to the control level. Glutathione appears to be a much more effective reducing agent than ascorbate. Omission of GSH decreased the synthesis to 27% of the complete system. In other experiments it was observed that the GTP-regenerating system stimulated incorporation when the GTP concentration was 3–7 mM, but not when it was 12 mM. The omission of $MgCl_2$ sometimes decreased the activity; it was routinely added to reaction mixtures.

TABLE VI: Requirements for the ^{32}P Assay for DIA-GDP-cobinamide Synthesis.[a]

Expt	Modification of Reaction Mixture	Product Formed (% of total ^{32}P) Uncor	Cor for Control	mμmoles
1	None	5.10	1.90	0.190
	−GSH, −ascorbate	3.20	0	0
	−GSH	3.76	0.56	0.056
	−Ascorbate	5.36	2.16	0.216
2	None	7.92	1.96	0.196
	Extract replaced by boiled extract	6.19	0.23	0.023
	−GTP	5.96	0	0
	−PEP, NADH, kinase	8.13	2.17	0.217

[a] The assays were conducted as described in Table V, with the following modifications. The complete reaction mixtures contained 0.4 mM DIA-cobinamide phosphate-^{32}P (1.3×10^6 cpm/μmole) and 0.39 mg of protein from a different *P. shermanii* extract. A control contained boiled extract (100°, 5 min) in place of unheated extract. The samples from each experiment were subjected to paper ionophoresis separately.

The Formation of Cobinamide Phosphate and DA-cobalamin. Monocyanocobinamide, DIA-cobinamide, or DA-cobinamide at a concentration of 0.5 mM was not converted to the corresponding cobinamide phosphate derivative by crude extracts of *P. shermanii* when incubated with terminally labeled ATP-^{32}P, UTP-^{32}P, or GTP-^{32}P at a concentration of 10 mM under a variety of assay conditions.

An attempt was made to detect the conversion of DA-GDP-cobinamide-^{32}P to DA-cobalamin. The ^{32}P-labeled substrate was not fractionated after removal of the isopropylidene group, and it probably contained some impurities (see Preparation of Substrates). Reaction mixtures contained 0.5 mM labeled substrate (3.2×10^5 cpm/μmole), 30 mM Tris-Cl (pH 8.2), 2 mM $MgCl_2$, 4 mM ammonium ascorbate, 1 mM GSH, 1 mM α-ribazole (omitted from one control), and 10 mg/ml of protein from a fresh Hughes press extract of *P. shermanii*. A second control contained heated extract (10 min at 100°). Reaction mixtures were incubated in sealed glass capillaries at 37° for 1 hr in darkness. DA-cobalamin (50 mμmoles) was then added and the reaction mixtures were subjected to paper ionophoresis at pH 3.0 (2 hr at 40 v/cm). Afterward the paper was dried and the radioactivity in DA-cobalamin and the GDP-cobinamide derivative was determined. The maximum α-ribazole-dependent conversion to cobalamin was 0.57% of the total ^{32}P. This value is twice the experimental error, as judged by the precision of duplicate experiments, and is equivalent to a synthesis of

0.071 mμmole of DA-cobalamin. The specific activity of the extract was 0.3 mμmole/hr per mg of protein. The product was not characterized further.

The Stability of Corrinoid Compounds in Crude Extracts. The degradation of 0.5 mM DA-benzimidazolylcobamide, as judged by the glutamate mutase assay (Barker *et al.*, 1960), was less than 5% during a 1-hr incubation under conditions similar to those described in Figure 4. When 0.1 mM monocyanocobinamide phosphate-^{32}P was incubated for 45 min under similar conditions, less than 2% of the isotope was converted to orthophosphate. Under conditions used for the enzymatic synthesis of the DA-cobinamide derivatives (see Preparation of Substrates), monocyano-GDP-cobinamide was not degraded to monocyanocobinamide phosphate. Evidently, the deoxyadenosyl group of DA-benzimidazolylcobamide, the phosphoester bond of monocyanocobinamide phosphate, and the pyrophosphate bond of monocyano-GDP-cobinamide are relatively stable under these conditions.

Discussion

The hypothesis investigated in this report proposed that the nucleotide side chain of cyanocobalamin is formed by adding α-ribazole to the cobinamide phosphate moiety of GDP-cobinamide with the release of GMP and that the intermediates are the cyano forms. It is now known that cyanocorrinoid compounds do not occur in significant quantities in bacteria that synthesize corrinoid compounds. The predominant naturally occurring corrinoid compounds are the DA derivatives, together with small amounts of the corresponding aquo compounds (Volcani *et al.*, 1961; Zagalak and Pawelkiewicz, 1962). Consequently, this investigation of biosynthetic intermediates was limited to these two forms.

The chemical synthesis of deoxyadenosyl corrinoids results in the formation of appreciable amounts of closely related products upon acid hydrolysis of the 2′,-3′-isopropylidene derivatives. These have nearly identical spectra, but can be distinguished by chromatography and by their activity as substrates or coenzymes. Thus, DA-cobinamide phosphate, but not the closely related product of hydrolysis of DIA-cobinamide phosphate, was converted enzymatically to DA-GDP-cobinamide by crude extracts. The nature of the structural difference between the two compounds is not known. It is, therefore, difficult to exclude the possibility that DA-cobinamide phosphate was partially altered by acid hydrolysis. The synthetic compound was indistinguishable from the enzymatically synthesized reference compound by several criteria. It should be noted that the monocyano compound derived from synthetic DA-cobinamide phosphate had the same mobility as synthetic monocyanocobinamide phosphate by ionophoresis at pH 7.2. It is, therefore, unlikely that synthetic DA-cobinamide phosphate was altered by the removal of an amide group.

Of the three enzymatic reactions investigated in extracts of *P. shermanii*, the phosphorylation of cobinamide, the conversion of cobinamide phosphate to GDP-cobinamide, and the conversion of the latter to cobalamin, only the formation of GDP-cobinamide derivatives was observed with some certainty. The product, after conversion to the monocyano form, was characterized by its behavior in DEAE-cellulose column chromatography and in paper ionophoresis and by the liberation of an approximately equivalent amount of GMP by the action of nucleotide pyrophosphatase. The incorporation of roughly equivalent quantities of ^{32}P from DA-cobinamide phosphate-^{32}P and ^{14}C from GTP-8-^{14}C into the product is consistent with its identification as a GDP-cobinamide derivative.

It is noteworthy that DA-cobinamide phosphate was converted to GDP-cobinamide, while diaquocobinamide phosphate and DA-adenosylcobamide were not. This indicates that DA-cobinamide phosphate is the natural substrate of the reaction and suggests that the product is DA-GDP-cobinamide. This compound was not directly identified; it was characterized only after removing the deoxyadenosyl group by treatment with cyanide. A further implication of the above observation is that the deoxyadenosyl moiety is added to the corrin ring before the formation of GDP-cobinamide. This agrees with the observations of Di Marco *et al.* (1962), who concluded that the deoxyadenosyl moiety is added to the cobalt before the attachment of the aminopropanol moiety by *N. rugosa*.

In the extracts examined, DIA-cobinamide phosphate was generally a better substrate than DA-cobinamide phosphate. Müller and Müller (1963) reported that Co-butylcobinamide and Co-5′-deoxyinosylcobinamide are partially converted by intact cells of *P. shermanii* to Co-butylcobalamin and Co-deoxyinosylcobalamin. Therefore, the enzymes involved in the conversion of cobinamide to complete corrinoid compounds require an alkyl group on the cobalt, but lack specificity for the type of alkyl group.

The specific activities observed for the formation of GDP-cobinamide in crude extracts under favorable conditions were 1.3 and 10.0 mμmoles/hr per mg of protein. This is the same order of magnitude reported for the system that adds the deoxyadenosyl moiety to corrinoid compounds in crude *P. shermanii* extracts (Brady *et al.*, 1962). The *N*-transglycosidase involved in α-ribazole formation is about 5000-fold more active in crude extracts of *P. shermanii* grown in a low cobalt medium (Friedmann and Harris, 1965). The activities of the other two systems in such cells is not known.

The present data indirectly support the hypothesis that deoxyadenosyl-GDP-cobinamide is an intermediate in DA-cobalamin biosynthesis. However, the failure to observe the formation of cobinamide phosphate and the low rate of conversion of DA-GDP-cobinamide to DA-cobalamin make this assignment less certain. The possibility that GDP-cobinamide synthesis is a side reaction unrelated to DA-cobalamin biosynthesis is considered unlikely in view of the occurrence of GDP-cobinamide in several different microbial species and the fact that activation of monophosphate esters by the

formation of nucleotide pyrophosphates is frequently encountered in biosynthetic pathways. It is possible that cobinamide is not phosphorylated directly, but instead a phosphorylated aminopropanol moiety is attached to the corrin ring. Friedrich and Sandeck (1964) reported that in whole cells of *P. shermanii* the label from cobinamide-^{60}Co appeared in cobyric acid, as well as cyanocobalamin. On the other hand, the observations by Bernhauer and Wagner (1962) and Bernhauer *et al.* (1964) that several substituted aminopropanol analogs of cobinamide are converted to the corresponding complete corrinoid compounds by intact *P. shermanii* imply that these substrates are not degraded but are transformed directly. Clarification of this problem must await further investigation.

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