Form of Vitamin B₁₂ and Its Role in a Methanol-Utilizing Bacterium, *Protaminobacter ruber*

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A methanol-utilizing bacterium, *Protaminobacter ruber*, produced a large amount of vitamin B₁₂. The compounds were isolated from the cells and identified as methylcobalamin (methyl-B₁₂) and adenosylcobalamin (adenosyl-B₁₂) by various tests. The variation in the form of B₁₂ during cultivation was examined by bioautography with cellulose acetate membrane electrophoresis. Methyl-B₁₂ and adenosyl-B₁₂ were the two main B₁₂ compounds produced in the various phases of bacterial growth. The ratio of the amount of methyl-B₁₂ to total B₁₂ compounds was higher during the earlier phases of growth. After the logarithmic phase, adenosyl-B₁₂ was the predominant form. The existence of N⁵-methyl-tetrahydrofolate:homocysteine transmethylase and methyl-B₁₂:homocysteine transmethylase was demonstrated in cell-free extracts of *Protaminobacter ruber*. Methyl-B₁₂ in *P. ruber* seems to function mainly in the B₁₂-dependent methionine synthetase system.

Much research has been conducted to produce single-cell protein or useful metabolites by methanol-utilizing microorganisms and to elucidate the pathway of methanol metabolism in microorganisms (3, 7, 12, 16; Proc. Int. Symp. on Microbial Growth on C₁-compounds, 5 Sept. 1974, Tokyo). We have searched for bacteria capable of producing large amounts of vitamins (especially vitamin B₁₂) as well as protein. Several facultative methylotrophs were isolated from industrial waste sludge in the petrochemical area of Yokkaichi, Japan, by an enrichment culture technique. A pink-pigmented strain was selected because of its production of vitamin B₁₂ and ability to grow on methanol. According to the 7th edition of *Bergey's Manual of Determinative Bacteriology*, it was classified as *Protaminobacter ruber*. The details of classification and its characteristics will be reported elsewhere.

The formation of vitamin B₁₂ by methanol-utilizing bacteria has been reported recently (9, 10, 18, 22). There is an interest in vitamin B₁₂ formation from two points of view: (i) from the practical viewpoint of commercially producing this complicated vitamin and (ii) from the basic viewpoint of elucidating its physiological role(s). An understanding of the metabolic role(s) of vitamin B₁₂ might contribute to its industrial production. This study was performed to elucidate the physiological significance of vitamin B₁₂ in cells. In this paper we report on the formation and identification of B₁₂ compounds in *P. ruber* cells and on the existence of a B₁₂-dependent transmethylase.

MATERIALS AND METHODS

Microorganism and its growth. *P. ruber* was grown in the following basal medium: NH₄H₂PO₄, 4.0 g; KH₂PO₄, 2.0 g; Na₂HPO₄·12H₂O, 3.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 10 mg; FeSO₄·7H₂O, 5 mg; MnSO₄·nH₂O, 5 mg; CoSO₄·7H₂O, 1 mg; and methanol, 20 ml; in 1 liter of tap water. The pH was adjusted to 7.2 with 40% KOH. Methanol was added aseptically after sterilization of other components. The basal medium was used as a seed medium as well as a flask production medium.

The organism was maintained on agar slants containing the basal medium at 4°C. Growth in liquid production medium was in 500-ml Sakaguchi flasks containing 100 ml of medium. The flasks were inoculated with 1% (vol/vol) seed culture cultivated aerobically for 40 h. The seed culture had been inoculated with one loopful of the slant culture and incubated on a shaker at 30°C. Growth of the bacteria was followed by measuring the dry weight of the cells.

For the isolation of B₁₂ compounds, the bacteria were grown in 10 liters of basal medium supplemented with 1 g of methionine. At the middle of the logarithmic growth phase, 2 ml of methanol was added to each flask and the pH was adjusted to 6.8 with 2 ml of mixed alkali solution (0.25 N NaOH, 0.25 N KOH, and 0.5 N NH₄OH). The cells were harvested after 92 h by centrifugation at 6,000 × g for 20 min.

Extraction and purification of vitamin B₁₂ compounds. All subsequent work was carried out in the dark because of the light sensitivity of the coenzyme form of B₁₂. B₁₂ compounds were extracted from the cells (wet weight, 250 g) with 1 liter of ethanol by boiling for 20 min. The extract was centrifuged at 6,000 × g for 20 min. The precipitate from this extract was suspended in 500 ml of ethanol and

centrifuged again. These supernatant fluids were combined and concentrated to dryness. The residue was dissolved in 200 ml of water, and 200 ml of 80% phenol was added. The layers were shaken and mixed well in a separatory funnel. The emulsified material was centrifuged at $6,000 \times g$ for 20 min, and the water layer was discarded. B_{12} compounds were transferred into water by shaking the phenol extract with 500 ml of diethyl ether and 250 ml of water in a separatory funnel. The aqueous layer thus obtained was extracted twice with 500 ml of diethyl ether and concentrated to dryness. The residue, containing B_{12} compounds, was dissolved in 30 ml of water and applied to a diethylaminoethyl-cellulose column (2.3 by 12.5 cm) that had been washed with 0.1 M potassium phosphate buffer (pH 7.2) and then with water. The column was eluted with 200 ml of water, followed by 100 ml of 1 M NaCl; 10-ml fractions were collected. The activity of vitamin B_{12} in the microbiological assay (*Escherichia coli* 215, an auxotroph responding to vitamin B_{12} or to methionine) was largely in fractions 4 through 13, eluted with water. These fractions were combined and concentrated. They were applied to an Amberlite XAD-2 column (100 to 200 mesh, 1.8 by 13 cm). An unidentified reddish pigment having no B_{12} activity was eluted with about 100 ml of water. The column was washed with an additional 200 ml of water, and afterwards B_{12} compounds were eluted with 100 ml of 25% ethanol. The concentrated B_{12} fractions were subjected to P-cellulose (Brown Co.) column chromatography (1.8 by 9 cm). Elution was carried out with 0.1 M sodium chloride in 0.5 N acetic acid. Vitamin B_{12} activity was located at two positions, a minor peak (sample 1) and a major peak (crude sample 2) (Fig. 1). Since the B_{12} compound obtained in the major peak still had a trace amount of contaminant, it was further purified on a Sephadex G-25 column (1.8 by 24 cm). The B_{12} compound was eluted with water and found in fractions (10 ml) 6 and 7 (sample 2). Purified samples 1 and 2 weighed 30 and 600 μ g, respectively.

Paper chromatography, paper electrophoresis, and absorption spectroscopy were performed as described in the figure legends.

Identification of base moiety. After sample 2 was heated with 2 ml of 6 N HCl in a sealed tube at 150°C for 20 h, the base moiety was isolated according to the method of Barker et al. (1). Identity of the base moiety was performed by paper chromatography and ultraviolet (UV) spectroscopy. Before its UV spectrum was measured, the base moiety was purified by paper chromatography with 0.1 N acetic acid as developing solvent. The paper had previously been washed with pyridine, ethanol, and 0.5 N acetic acid.

Microbiological assay of vitamin B_{12} compounds. For determining the B_{12} compounds formed by *P. ruber*, 10.0 ml of culture was brought to pH 5.5 to 6.0 with 0.1 N acetic acid. After the addition of potassium cyanide (1 μ g per an estimated 1 ng of vitamin B_{12}), B_{12} compounds were extracted by boiling the suspension for 20 min. The volume of the extract was adjusted to 100 ml, and the extract was centrifuged at $15,000 \times g$ for 20 min to remove cell materi-

FIG. 1. Separation of B_{12} compounds from *P. ruber* on a P-cellulose column (1.8 by 9 cm). The column was eluted with 0.1 M sodium chloride in 0.5 N acetic acid.

als. The content of vitamin B_{12} in the supernatant fluid was assayed with *E. coli* 215 (4).

The forms of B_{12} in the cells harvested at suitable intervals were examined by bioautography after cellulose membrane electrophoresis instead of paper electrophoresis, since filter paper interacted with the coenzyme form of B_{12} and prevented its migration. A SELECTA-electrophoresis membrane, code no. EPI-3 (Carl Schleicher and Schüll 3354 Dassel), was used for the electrophoresis.

To obtain samples for assay and bioautography, cells were cultivated in 500-ml Sakaguchi flasks containing 100 ml of medium. Ten milliliters of the culture was used for each B_{12} assay. B_{12} compounds were extracted with ethanol from the cells of the remaining culture (about 90 ml) and partially purified by the phenol extraction method already described. The amount of cells was too small early in growth (16 h) for bioautography. Therefore, the contents of two flasks were mixed and treated as above after 10 ml was removed for the B_{12} assay.

In the electrophoresis, each membrane (2 by 40 cm) was soaked in 0.5 N acetic acid just before use. The membrane was wiped gently with filter paper and the sample was applied to it. Electrophoresis was conducted at 15 V/cm for 1 h in 0.5 N acetic acid. The membrane was cut into 25 pieces at 0.5-cm intervals toward the anode and 5 pieces towards the cathode. Each piece was put into a test tube with an aluminum cap, and 5 ml of the B_{12} assay medium containing potassium cyanide (20 ng/ml) was added to each tube. After heating at 120°C for 5 min, 1 drop of an *E. coli* 215 suspension was added to each tube, using a syringe as in the B_{12} assay (4). Turbidity after 20 h was measured at 600 nm with a Shimadzu Spectronic 20 spectrophotometer. The form of B_{12} was characterized by comparison with authentic B_{12} .

Preparation of cell-free extracts. About 4.5 g of cells after cultivation for 45 h was ground with 10 g of alumina for 10 min, using a mortar and pestle. When the cell mass became pasty, 20 ml of 0.05 M potassium phosphate buffer (pH 7.5) was added, and

grinding was continued for another 5 min. Cell debris and alumina were removed by centrifugation at $15,000 \times g$ for 30 min. Solid ammonium sulfate was added slowly, with stirring, to 21 ml of the supernatant fluid to achieve 30% saturation at 0°C. After 30 min, the precipitate was separated by centrifugation and ammonium sulfate was added to the supernatant fluid to a final concentration of 60% saturation. After another 30 min, the precipitate was collected by centrifugation. The precipitate was dissolved in 5 ml of 0.01 M potassium phosphate buffer (pH 7.5) and dialyzed overnight against 2 liters of the same buffer. The dialyzed preparation was used for enzyme assay. The supernatant fluid, not treated with ammonium sulfate but dialyzed, was also used as the enzyme source.

Enzyme assay. The concentrations of all B₁₂ compounds used for the enzyme assay were determined spectrophotometrically. B₁₂-dependent transmethylation activity was assayed by measuring the formation of [¹⁴C]methionine from N⁵-[¹⁴C]methyltetrahydrofolate or from [¹⁴C]methyl-B₁₂ (11, 24). Radioactivity measurements were performed by liquid scintillation with Bray solution (2) in a Packard model 3320-515 scintillation counter. The incubation mixtures and incubation conditions are described in the legend for Tables 3 through 5. Formation of methionine from unlabeled methyl-B₁₂ was determined by using a methionine-requiring mutant, *E. coli* 243, as follows. After the reaction was stopped with 0.5 ml of 1 N HCl, the mixtures were added to a Dowex 50 W $\times$ 2 (H⁺ form, 50 to 100 mesh) column (0.6 by 3 cm). The column was washed with 15 ml of distilled water and eluted with 2 ml of 1 M dibasic potassium phosphate. The eluate was used as the sample for the assay of methionine by *E. coli* 243.

Coenzyme activity of sample 2 in the dioldehydrase (dl-1,2-propanediol hydro-lyase, EC 4.2.1.28) assay was determined by using cell-free extracts from *Aerobacter aerogenes* IFO 3318 (ATCC 8724) according to the procedure of Lee and Abeles (6).

Chemicals. N⁵-[¹⁴C]methyltetrahydrofolate was purchased from Japan Radio Isotope Association, and S-adenosylmethionine iodide (SAM) was purchased from Calbiochem. Unlabeled N⁵-methyltetrahydrofolate was synthesized according to the method of Keresztesy and Donaldson (5). Methyl-B₁₂ was synthesized as described by Smith et al. (17). Riboflavine 5'-phosphate, reduced form (FMNH₂), was prepared each day by the catalytic hydrogenation of FMN with PtO₂ (20). Adenosyl-B₁₂ and 5,6-dimethylbenzimidazole (DBI) were kindly supplied by S. Fukui, Kyoto University. Other chemicals were of the highest purity obtainable commercially.

RESULTS

Time course of B₁₂ formation. *P. ruber* produced a considerable amount of B₁₂. The time course of B₁₂ formation, cell growth, and pH of the medium are shown in Fig. 2. Vitamin B₁₂ was formed in parallel with multiplication of the bacterium and reached its maximum (140 ng/ml of culture broth) after about 65 h of cul-

vation. Almost all of the vitamin produced remained in the cells during the stationary phase. The pH of the culture was at its minimum level during the early stationary phase.

Characterization of the B₁₂ compounds from *P. ruber*. Two B₁₂ fractions, samples 1 and 2 in Fig. 1, were subjected to paper chromatography. The *R_f* values of each sample corresponded very well with those of methyl-B₁₂ and adenosyl-B₁₂, respectively (Table 1). The results of paper electrophoresis also showed the methyl-B₁₂-like behavior of sample 1 and adenosyl-B₁₂-like behavior of sample 2 (Fig.

FIG. 2. Time course of growth, pH of the culture, and vitamin B₁₂ formation of *P. ruber* in 2% methanol medium. Growth (O), pH (●), total B₁₂ (Δ), B₁₂ in the cell (▲).

TABLE 1. Paper chromatography of B₁₂ compounds isolated from *P. ruber*^a

B ₁₂ compounds	<i>R_f</i> value	
	Solvent A	Solvent B
Sample 1	0.34	0.42
Sample 2	0.15	0.22
Reference compound		
Cyano-B ₁₂	0.21	0.31
Hydroxo-B ₁₂	0.09	0.18
Methyl-B ₁₂	0.34	0.42
Adenosyl-B ₁₂	0.15	0.22

^a Samples and reference compounds were subjected to ascending paper chromatography for 18 h on Toyo filter paper no. 50 and developed with solvent A (water-saturated *sec*-butanol) or solvent B (*sec*-butanol-water-28% NH₃, 100:36:14).

3A,B,D). Both of the samples were photolabile and decomposed into hydroxo-B₁₂ by irradiation with light (Fig. 3C), which is characteristic of B₁₂ compounds having a carbon-cobalt bond in the upper ligand. At pH 11.5, all the B₁₂ compounds remained at the origin. Since no B₁₂ compound moving toward the anode was detected in paper electrophoresis at pH 11.5, we assumed that no anionic groups (such as carboxylic acid) existed in the samples of B₁₂.

To identify the lower ligand more clearly, the base moiety was isolated and examined by paper chromatography and UV absorption spectroscopy. The *R_f* value of the base moiety isolated from the decomposition of sample 2 was the same as that of authentic DBI (Table 2). It was also confirmed spectrophotometrically (Fig. 4).

FIG. 3. Paper electrophoretic behavior of B₁₂ compounds from *P. ruber*. Paper electrophoresis was carried out for 2 h on Toyo filter paper no. 50 with a Toyo type C-II paper electrophoretic apparatus in (A) 0.5 N acetic acid at 20 V/cm, (B and C) 0.1 N acetate buffer (pH 3.5) at 25 V/cm, and (D) 0.01 M Na₂CO₃ (pH 11.5) at 20 V/cm. The abbreviations used are: Ad-B₁₂, adenosylcobalamin; OH-B₁₂, hydroxocobalamin; CH₃-B₁₂, methylcobalamin; CN-B₁₂, cyanocobalamin; hv, light exposure with a 300-W tungsten lamp for 5 min at 10-cm distance.

Figure 5 shows the absorption spectra of sample 2. Curve a represents the spectrum of sample 2 itself, which is identical to that of adenosyl-B₁₂. The addition of HCl caused a spectral shift to curve b. By light irradiation, a new peak appeared at 351 nm showing the formation of hydroxyl group in the upper ligand (curve c). Treatment of the photolyzed sample with potassium cyanide produced the dicyano form of B₁₂ as in curve d.

To confirm that the upper ligand contains the adenosyl group, we determined whether sample 2 would act as coenzyme in dioldehydrase reaction; to do this, the upper ligand of B₁₂ must contain the adenosyl group. The plots of coenzyme activity of sample 2 in the dioldehydrase system coincide with those of authentic adenosyl-B₁₂ (Fig. 6). The inset shows a double-reciprocal plot of the data, and the *K_m* obtained for the B₁₂ compounds was 8.7×10^{-7} M. Therefore we concluded that sample 2 was adenosyl-B₁₂.

Existence of a B₁₂-dependent methionine synthetase. Because sample 1 behaved like methyl-B₁₂ (Table 1, Fig. 3), we assumed that a B₁₂-dependent methionine synthetase might exist in this culture (Tables 3 and 4). Methionine was formed enzymatically by transmethylation from methyl-B₁₂ to homocysteine; methionine was not formed with a boiled extract (Table 3). Table 4 shows methionine synthesis from N⁵-methyltetrahydrofolate. This reaction was strictly dependent on SAM and a reducing reagent, FMNH₂, but dithiothreitol was not necessary in this reaction. The requirements of SAM and FMNH₂ are characteristic of B₁₂-dependent methionine synthetase as demonstrated in various organisms (21).

Whether sample 1 could function as the substrate of methyl-B₁₂:homocysteine methyltransferase and form methionine like authentic methyl-B₁₂ was examined to confirm that the upper ligand of sample 1 contained a methyl group. Both sample 1 and authentic methyl-B₁₂ formed methionine in almost equal amounts, whereas adenosyl-B₁₂ did not form methionine (Table 5). Therefore, sample 1 was identified as methyl-B₁₂.

The form of B₁₂ at various times. The form of B₁₂ in the cells during growth was examined

TABLE 2. Paper chromatography of the base moiety obtained from sample 2^a

Solvent system	<i>R_f</i> values			
	Adenine	BI ^b	DBI ^c	Sample
0.1 N acetic acid	0.64	0.81	0.61	0.61
sec-Butanol-acetic acid-water (100:3:50)	0.64	0.73	0.76	0.76

^a Samples were subjected to ascending paper chromatography for 3.5 h on Toyo filter paper no. 53.

^b Benzimidazole.

^c 5,6-Dimethylbenzimidazole.

FIG. 4. Absorption spectra of the base moiety obtained from sample 2 in 0.1 N acetic acid and in absolute ethanol. (-----) Authentic DBI; (—) base moiety obtained from sample 2. Spectra were obtained with a Hitachi model 624 digital spectrophotometer.

FIG. 5. Absorption spectra of sample 2 isolated from *P. ruber*. (a) Sample 2 in water (—); (b) the solution in (a) was acidified with about 0.05 ml of 2 N HCl (·····); (c) the solution in (b) was irradiated at 0°C with a 300-W tungsten lamp for 10 min at 15 cm (---); (d) potassium cyanide (1 mg) was added to an irradiated aqueous solution of sample 2 (— · — · —).

by cellulose acetate membrane electrophoresis and assays of B₁₂ activity on the membrane with *E. coli* 215. Figure 7 summarizes the electrophoretic behavior of B₁₂ compounds from *P. ruber* for each cultivation time. More methyl-B₁₂ was formed in the earlier phases of growth. By the late logarithmic phase, a considerable

amount of methyl-B₁₂ was present in the cells. However, in the stationary phase almost all of B₁₂ existed in the form of adenosyl-B₁₂.

DISCUSSION

Although the formation of vitamin B₁₂ by methanol-utilizing bacteria has been reported

FIG. 6. Coenzyme activity of sample 2 (●) and adenosyl- B_{12} (×) in a dioldehydrase system. The inset shows a Lineweaver-Burk plot of the data. S , Molar concentration of sample 2; V , amount of propionaldehyde formed (in micromoles).

TABLE 3. Requirements for methionine formation from methyl- B_{12} ^a

Reaction conditions	Methionine formed (cpm)
Complete system	20,020
Minus homocysteine	110
Minus enzyme	120
Minus enzyme, plus boiled enzyme	310

^a The complete system contained [^{14}C]methyl- B_{12} , 10 nmol (89,000 cpm); homocysteine, 300 nmol; potassium phosphate buffer, 30 μ mol; and crude extract (protein, 4.0 mg) in a total volume of 1.5 ml. The incubation was carried out for 1 h at 30°C in the dark.

TABLE 4. Requirements for methionine synthesis from N^5 -methyltetrahydrofolate^a

Reaction conditions	Methionine formed (nmol)
Complete system	7.38
Minus homocysteine	1.30
Minus SAM	0.48
Minus DTT	7.52
Minus FMNH ₂	0.11
Minus SAM, minus DTT, minus FMNH ₂	0.19

The complete system contained N^5 -[^{14}C]methyltetrahydrofolate, 100 nmol; homocysteine, 500 nmol; SAM, 20 nmol; dithiothreitol (DTT), 100 nmol; FMNH₂, 100 nmol; potassium phosphate buffer, 25 μ mol; and ammonium sulfate fraction (protein, 1 mg) at 30 to 60% saturation in a total volume of 0.4 ml. The reaction mixtures were incubated for 15 min at 30°C under an atmosphere of hydrogen gas.

TABLE 5. Formation of methionine from sample 1 by methyl- B_{12} :homocysteine methyltransferase^a

B_{12} compound added	Amt added (nmol)	Methionine formed (nmol)
None	0.0	0.0
Methyl- B_{12}	7.4	6.1
Methyl- B_{12}	14.8	13.4
Adenosyl- B_{12}	6.4	0.0
Sample 1	7.0	5.4

^a The reaction mixtures contained homocysteine, 3.7 μ mol; potassium phosphate buffer, 100 μ mol; ammonium sulfate fraction (protein, 8 mg) at 30 to 60% saturation; and B_{12} compounds as indicated in a total volume of 2.0 ml. Incubation was carried out for 1 h at 30°C. Methionine formed was assayed with *E. coli* 243.

FIG. 7. Time course of B_{12} form in *P. ruber* cells. Electrophoresis was carried out on cellulose acetate membrane in 0.5 N acetic acid at 600 V for 1 h. Vitamin B_{12} activity on the membrane was assayed with *E. coli* 215.

(9, 10, 18, 22), its physiological significance in such bacteria remains to be studied. Our experiments showed the existence of adenosyl- B_{12} and methyl- B_{12} in the methanol-utilizing bacterium *P. ruber*. Toraya et al. reported the formation of these B_{12} compounds in a methanol-utilizing bacterium, strain FM-02T, but the data were not presented (22). Very recently, adenosyl- B_{12} was isolated from a methanol-utilizing bacterium, *Klebsiella* sp. no. 101 (8). These facts suggested that there were at least

two kinds of B₁₂-dependent enzymatic reaction systems in methanol-utilizing bacteria.

The lower ligand of B₁₂ from *P. ruber* was shown to be DBI by isolation of the base moiety. The upper ligand was identified by determining whether B₁₂ compounds from *P. ruber* could function as coenzyme in adenosyl-B₁₂-dependent dioldehydrase (14) or as the substrate for methyl-B₁₂:homocysteine transmethylase. Confirmation of the methyl group as the upper ligand by the latter enzymatic system was very reliable and overcome the ambiguity of identification by more common methods such as paper chromatography. The time course of B₁₂ formation revealed that more methyl-B₁₂ was produced at earlier phases of growth but that after the late logarithmic phase almost all the B₁₂ consisted of adenosyl-B₁₂. Similar results have been obtained in streptomycetes (15).

From our finding of methyl-B₁₂ formation, especially in the early stages of growth, we presume that one-carbon transfer reactions requiring methyl-B₁₂ exist in this bacterium and play an important role for cell multiplication. It was found here that methyl-B₁₂ participates in B₁₂-dependent methionine synthesis as occurs in many organisms other than plants (11, 19, 21) (Tables 3 and 4). The specific activity of N⁵-methyltetrahydrofolate:homocysteine transmethylase during cultivation corresponded to the rise and fall of methyl-B₁₂ in the cells (unpublished data). On the other hand, methylmalonyl-coenzyme A mutase, for which adenosyl-B₁₂ acts as a coenzyme, was found in the cell-free system of *P. ruber*. The demonstration of methylmalonyl-coenzyme A mutase and the relationship between the B₁₂-dependent enzymatic systems will be reported in another paper.

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LITERATURE CITED

1. Barker, H. A., R. D. Smyth, H. Weissbach, J. I. Toohey, J. N. Ladd, and B. E. Volcani. 1960. Isolation and properties of crystalline cobamide coenzyme containing benzimidazole or 5,6-dimethylbenzimidazole. *J. Biol. Chem.* 235:480-488.
2. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
3. Cooney, C. L., and D. W. Levine. 1972. Microbial utilization of methanol. *Adv. Appl. Microbiol.* 15:337-365.
4. Kamikubo, T. 1956. Microbiological assay procedures for vitamin B₁₂. *Vitamins* 11:328-338.
5. Keresztesy, J. C., and K. O. Donaldson. 1961. Synthetic prefolic A. *Biochem. Biophys. Res. Commun.* 5:286-288.
6. Lee, H. A., and R. H. Abeles. 1963. Purification and properties of dioldehydrase, an enzyme requiring a cobamide coenzyme. *J. Biol. Chem.* 238:2367-2373.
7. Lipinsky, E. S., and J. H. Litchfield. 1974. Single cell protein in perspective. *Food Technol.* 28:16-24.
8. Nishio, N., T. Yano, M. Hayashi, and T. Kamikubo. 1976. Isolation of 5,6-dimethylbenzimidazolylcobamide coenzyme from a methanol utilizing bacterium, *Klebsiella* sp. no. 101. *Agr. Biol. Chem.* 40:1035-1037.
9. Nishio, N., T. Yano, and T. Kamikubo. 1975. Further studies of vitamin B₁₂ production by methanol utilizing bacterium, *Klebsiella* sp. no. 101. *Agr. Biol. Chem.* 39:207-213.
10. Nishio, N., T. Yano, and T. Kamikubo. 1975. Isolation of methanol-utilizing bacteria and its vitamin B₁₂ production. *Agr. Biol. Chem.* 39:21-27.
11. Ohmori, H., K. Sato, S. Shimizu, and S. Fukui. 1971. Studies on the corrinoids and porphyrins in streptomycetes. Part 4. Confirmation of a cobalamin-dependent methionine synthesizing system in *Streptomyces olivaceus*. *Agr. Biol. Chem.* 35:338-343.
12. Poston, J. M., and T. C. Stadtman. 1975. Cobamides as cofactors. Methylcobamides and the synthesis of methionine, methane, and acetate, p. 113-140. In B. M. Babior (ed.), *Cobalamin. Biochemistry and pathophysiology*. John Wiley & Sons, New York.
13. Quayle, J. R. 1972. The metabolism of one-carbon compounds in microorganisms. *Adv. Microb. Physiol.* 7:119-203.
14. Sato, K., S. Shimizu, and S. Fukui. 1968. Studies on the corrinoids and porphyrins in streptomycetes. Part 1. Characterization of 5,6-dimethylbenzimidazolylcobamide coenzyme formed in the cells of *Streptomyces olivaceus*. *Agr. Biol. Chem.* 32:1-6.
15. Sato, K., S. Shimizu, and S. Fukui. 1971. Studies on the corrinoids and porphyrins in streptomycetes. Part 3. Influence of cultural conditions on the forms of corrinoids produced by *Streptomyces olivaceus*. *Agr. Biol. Chem.* 35:333-337.
16. Slater, L. 1974. SCP: the methanol way. *Food Eng.* 46:68-72.
17. Smith, E. L., L. Mervyn, A. W. Johnson, and N. Shaw. 1962. Partial synthesis of vitamin B₁₂ coenzyme and analogues. *Nature (London)* 194:1175.
18. Tanaka, A., Y. Ohya, S. Shimizu, and S. Fukui. 1974. Production of vitamin B₁₂ by methanol-assimilating bacteria. *J. Ferment. Technol.* 52:921-924.
19. Taylor, R. T., and M. L. Hanna. 1973. Relationship between the cobalamin-dependent methyltransferase activities in *Escherichia coli* B and an *E. coli* B (Met H⁻) mutant. *Arch. Biochem. Biophys.* 156:480-492.
20. Taylor, R. T., and H. Weissbach. 1967. N⁵-methyltetrahydrofolate-homocysteine transmethylase. Partial purification and properties. *J. Biol. Chem.* 242:1502-1508.
21. Taylor, R. T., and H. Weissbach. 1973. N⁵-methyltetrahydrofolate homocysteine methyltransferase, p. 121-165. In P. D. Boyer (ed.), *The enzymes*, 3rd ed., vol. 9. Academic Press Inc., New York.
22. Toraya, T., B. Yongsmit, A. Tanaka, and S. Fukui. 1975. Vitamin B₁₂ production by a methanol-utilizing bacterium. *Appl. Microbiol.* 30:477-479.
23. Vogelmann, H., and F. Wagner. 1974. Isolation and chromatographic separation of vitamin B₁₂ and other corrinoids from biological sources. *Biotech. Bioeng. Symp.* 4:969-975.
24. Weissbach, H., A. Peterkofsky, B. G. Redfield, and H. Dickerman. 1963. Studies on the terminal reaction in the biosynthesis of methionine. *J. Biol. Chem.* 238:3318-3324.