ISOLATION OF 5,6-DIMETHYLBENZIMIDAZOLYL COBAMIDE COENZYME FROM *RHODOPSEUDOMONAS SPHEROIDES**

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1. Introduction

A photosynthetic bacterium, *Rhodopseudomonas spheroides*, has often been used for studies of the biosynthesis of porphyrins and bacteriochlorophyll. It is also known that the bacterium can be grown non-photosynthetically, e.g. aerobically in the dark on a medium containing organic acids and natural nutrients such as yeast extract, peptone, etc. This is why the organism has been used for the studies of organic acid metabolism under aerobic as well as anaerobic conditions, photosynthetic reactions and regulation mechanism of porphyrin biosynthesis [1]. Recently, Lascelles et al. [2] reported that vitamin B_{12} activity was found microbiologically in cells of *R. spheroides* grown on medium containing cobalt chloride. However, the forms of vitamin B_{12} produced and the quantitative relationship among the vitamin B_{12} analogues have not been elucidated. The pathway of vitamin B_{12} biosynthesis in *R. spheroides* seems to be the same as that of porphyrins, at least from glycine to porphobilinogen, but this has not been confirmed. It is of interest to elucidate the relationship between porphyrin and vitamin B_{12}-factor formation with special reference to the mechanism of tetrapyrrole biosynthesis. We have tried to identify the forms of vitamin B_{12} produced aerobically in shake culture.

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2. Materials and methods

R. spheroides was generously given by Dr. J.Lascelles [2]. The organism was grown on a medium containing Na-glutamate. H_2O, 3.8 g; DL-malic acid, 2.7 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $(NH_4)_2HPO_4$, 0.8 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 53 mg; nicotinic acid, 1 mg; thiamine-HCl, 1 mg; biotin, 10 μg; yeast extract, 2 g; $MnSO_4.5H_2O$, 1.2 mg; ferric citrate, 2.45 mg; $CoCl_2.6H_2O$, 0.95 mg; in 1 l of distilled water; pH, 6.8. The medium was sterilized at 10 lb for 10 min. Shake cultures were carried out using 500 ml-Erlenmyer flasks containing 200 ml medium on a rotary shaker (180 rotations/min) at 28° for 48 hr, and static culture using special culture bottles of 5 l content containing 5 l medium, at 28° for 15 days. Cells grown anaerobically in the light on a medium free Mn-, Fe-, and Co-salts were used for inoculum. Illuminated incubation was made under 20 W fluorescent light at 30 cm distance. Corrinoids were extracted and purified from centrifuged cells of *R. spheroides* as the cyano forms, for identification of the kinds and forms of the corrinoids in accordance with methods reported previously [3–6], with some modifications. The purified corrinoids were fractionated by paper ionophoresis and ion-exchange column chromatography. The amounts of corrinoids were estimated from the absorbances at 367 nm of their dicyano forms. The corrinoids were identified by paper chromatography [7], paper ionophoresis [8], spectrophotometry and microbiological procedures [9, 10]. The procedures for isolation and identification of the coenzyme forms of corrinoids were almost the same as those for cyanocorrinoids, except that each process

was performed without cyanide and in the dark, and ethanol was used for the extraction in a final concentration of 80%.

Porphyrins were identified and estimated in the culture fluid according to the Lascelles [11] and Falk [12] methods, with some modification [13]. The supernatant fluid and wash water of *Rhodopseudomonas* cells were extracted with ethyl acetate at pH 4, subsequently transferred to 4.2 N HCl and then to ether. The extracted porphyrins were subjected to fractionation extraction with 0.1 N and then 1.37 N HCl. The residual porphyrins after ethyl acetate extraction at pH 4 were transferred to ethyl acetate at pH 3, and then extracted with 0.5 N HCl. The fractionated porphyrins thus obtained, as well as their methyl esters, were identified and estimated by spectrophotometry and paper chromatography. Bacteriochlorophyll [1] was extracted from the washed cells with acetone-methanol (7:2, v/v), identified by partition chromatography and UV-spectrophotometry, [14], and estimated from the absorbance at 755 nm.

3. Results

3.1. *Formation of corrinoids and porphyrins by shaking culture*

Microbiological activities of corrinoids extracted in the presence of cyanide from the washed cells of *R. spheroides* grown in the dark and in the light on an 8 l scale were determined using *Escherichia coli* 215 and *Ochromonas malhamensis*. The results in table 1 indicate that the corrinoids in the cells of *R. spheroides*

Table 1
Microbiological activities of corrinoids formed in cells of *R. spheroides* grown under various conditions.

Culture condition	Dry cell yield (g/l)	Microbiological activity* (μg/g dry cells)	
		E. coli	*O. malhamensis*
Shaking in the dark	2.0	17.8	17.5
in the light	2.1	16.8	14.8
Static in the dark	0.1	340	109
in the light	0.2	129	48

* As cyanocobalamin.

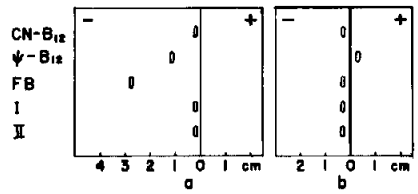

Fig. 1. Paper ionophoretic behavior of corrinoids from *R. spheroides* cells grown aerobically in shake culture. I: Corrinoid from dark-grown cells; II: Corrinoid from light-grown cells; Migration: *a*, at pH 2.5; *b*, at pH 7.5.

consist mainly of cobalamin and that little or no difference was found in the amount of corrinoids between the dark-grown and the light-grown cells. The paper chromatographic and paper ionophoretic behaviors of the purified preparations were the same as of cyanocobalamin, as shown in table 2 and fig. 1, respectively. Further, UV-absorption spectra of the purified preparations in the monocyano- as well as dicyano-forms confirmed that the main corrinoid is cobalamin, namely, 5,6-dimethylbenzimidazolyl cobamide.

In order to investigate the coenzyme forms of corrinoids, about 232 g of *Rhodopseudomonas* cells grown aerobically in the dark were extracted with hot ethanol as described above, purified with *p*-chlorophenol-chloroform and subjected to column chromatography on P- and DEAE-cellulose. Paper chromatographic as well as paper ionophoretic and spectrophotometric behaviors of the purified preparation of the main corrinoid were in good agreement with those of coenzyme vitamin B_{12}, namely, 5'-deoxyadenosyl-5,6-dimethylbenzimidazolyl cobamide, as shown in figs. 2 and 3. Similar investigations on the coenzyme B_{12} preparation obtained after conversion into its cyanoform by

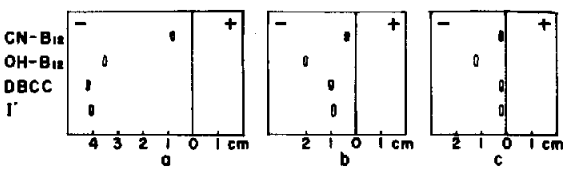

Fig. 2. Paper ionophoretic behavior of the coenzyme form of corrinoid from *R. spheroides* cells grown aerobically in the dark. I': corrinoid; *a*, pH 2.5, *b*, pH 3.5, *c*, pH 7.5.

Table 2
R_f-Values of corrinoids in *R. spheroides* cells grown aerobically.

Solvents*	Authentic B_{12}-preparations			Samples from cells grown	
	DBC**	φ-B_{12}	FB***	in the dark	in the light
I	0.24	0.14	0.39	0.24	0.23
II	0.28	0.17	0.39	0.28	0.28
III	0.40	0.26	0.40	0.41	0.39

* I: Water-saturated butan-2-ol (0.01% KCN)
II: Water-saturated butan-2-ol (1% acetic acid, 0.01% KCN)
III: Water-butan-2-ol-25% NH_4OH (18:50:7)
** DBC = cobalamin = 5,6-dimethylbenzimidazolyl cobamide
*** FB = cobamide

addition of KCN showed that the resultant was cobalamin. The yield of coenzyme B_{12} was 11 μg per g of dry cells.

Spectrophotometric and paper chromatographic behaviors of the fractionated porphyrin solutions in HCl and their methyl ester solutions in chloroform were in good agreement with those in the literature, and the presence of copro(III)-, proto(IX)- and uro(III)-porphyrins were recognized. However, the amount of each porphyrin formed in the culture broth was slight. Total porphyrins formed in the dark and in the light were 19 and 17 pmoles/ml, respectively.

3.2. *Formation of corrinoids and porphyrins by static culture*

Corrinoids were extracted from the cells of *R. spheroides* grown in 20 l culture in the dark as well as in the light. As shown in table 1, higher microbiological activities were found in the cells grown in the dark, and those for *O. malhamensis* were about one-third of those for *E. coli* 215, both in the dark and in the light. In comparison with the results in the shake culture, much higher microbiological activities were found in the dark-grown cells, in contrast to the cell yields. The ratios of corrinoid fractions by paper ionophoresis (pH 2.5) of cyanocorrinoids purified with *p*-chlorophenol-chloroform were 39:22:15:24 for cobalamin, φ-B_{12}, factor A, and cobinamide, respectively. This finding is approximately in agreement with microbiological activities.

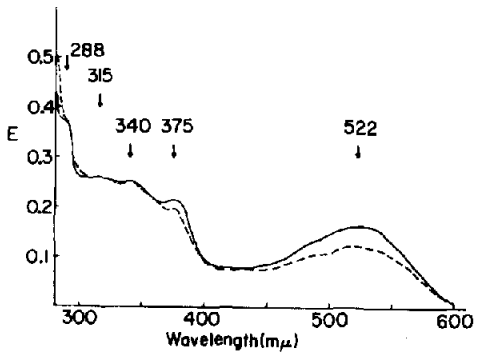

Fig. 3. Absorption spectra of the coenzyme form of corrinoid from *R. spheroides* cells. ——, authentic coenzyme B_{12}; - - - -, coenzyme form of corrinoid from cells grown aerobically in the dark.

In the case of shake culture no difference was found between the porphyrins formed in the dark and in the light, but in static culture the porphyrins formed in the light were four times as much as those formed in shake culture. The acetone-methanol extracts of the cells were fractionated by two-dimensional paper chromatography using light petroleum-propanol (100:1) and light petroleum-chloroform (3:1). The visible and UV-absorption spectra showed that the substance having absorbance at 770 nm was bacteriochlorophyll, and the amount was 7.2 μmoles per g of dry cells.

4. Discussion

The biosynthetic pathway for corrinoids in *R. spheroides* has not yet been as completely elucidated as in propionic acid bacteria. We have made efforts in

this report to identify the types of corrinoids produced by *R. spheroides*. For that purpose, aerobic conditions, in which cell yields are much better, were first investigated. The findings on the microbiological activities, as well as physicochemical investigations, suggested that the major corrinoid formed aerobically might be cobalamin, as the coenzyme form. On the other hand, the corrinoids formed anaerobically or semi-anaerobically were different from those in shake culture. Microbiological activities for *O. malhamensis* were about one-third those for *E. coli* 215 in the dark-grown as well as in the light-grown cells. In agreement with this result, paper ionophoresis (pH 2.5) separated at least four fractions, cobalamin, φ-B_{12}, factor A, and cobinamide. The findings on the formation of corrinoids seem to be similar to the results with *Propionibacterium shermanii*, in that aerobic condition might be suitable or somewhat essential for the formation of complete (nucleotide-containing) corrinoids.

The small accumulation of porphyrins in shake as well as static cultures seems to be due to Fe salt in the medium, as in Lascelles' report [11], and more accumulation was obtained in static culture, the majority of which was coproporphyrin(III), amounting to ca. 93%. The amount of bacteriochlorophyll in the static light culture is about 70 times that in the shaking light culture, and is in agreement with one of the characters of *R. spheroides*. A similar tendency was also observed in corrinoid formation. These findings suggest that the porphyrin pathway seems to be more activated anaerobically or semi-anaerobically than is the corrin pathway among a variety of tetrapyrrole biosynthesis pathways, and is further stimulated by a rate-limiting δ-aminolevulinic acid synthetase induced at a low O_2 pressure.

5. Conclusion

The major corrinoid in cells of *R. spheroides* grown aerobically on cobalt-containing medium is cobalamin, which is supposed to be present in the cells in coenzyme form. The yield of the coenzyme form was 11 μg per g of dry cells grown aerobically in the dark. Corrinoids in cells grown anaerobically both in the dark and in the light consisted of φ-B_{12}, factor A, and cobinamide besides cobalamin, the last of which is about one-third of the total corrinoids. A small amount of porphyrins seems to arise from Fe salt in the medium. Bacteriochlorophyll was markedly greater in amount in static culture.

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References

[1] G.Cohen-Bazire, W.R.Sistrom and R.Y.Stanier, J. Cell. Comp. Physiol. 49 (1957) 25.
[2] S.E.Cauthen, J.R.Pattison and J.Lascelles, Biochem. J. 102 (1967) 774.
[3] K.Bernhauer, E.Becher and G.Wilharm, Arch. Biochem. Biophys. 83 (1959) 248.
[4] T.Kamikubo, H.Narahara, K.Murai and N.Kumamoto, Biochem. Z. 346 (1966) 159.
[5] W.Friedrich and K.Bernhauer, Z. Naturforsch. 9b (1954) 755.
[6] K.Kato, M.Hayashi and T.Kamikubo, Biochim. Biophys. Acta 165 (1968) 233.
[7] H.Dellweg, E.Becher and K.Bernhauer, Arch. Biochem. Biophys. 60 (1957) 74.
[8] H.Dellweg, E.Becher and K.Bernhauer, Biochem. Z. 327 (1956) 422.
[9] T.Kamikubo, Vitamins 11 (1956) 43.
[10] T.Kamikubo and Y.Oguni, J. Vitaminol. Kyoto 5 (1959) 51.
[11] J.Lascelles, Biochem. J. 62 (1956) 78.
[12] J.E.Falk, Porphyrins and Metalloporphyrins (Elsevier, Amsterdam, 1964).
[13] T.C.Chu, S.A.A.Green and E.J.Chu, J. Biol. Chem. 190 (1951) 643.
[14] L.P.Vernon and G.R.Seely, The Chlorophylls (Academic Press, New York, 1966).