BIOSYNTHESIS AND PROPERTIES OF 2-THIOADENYL COBAMIDE AND ITS COENZYME FORM

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1. Introduction

Barker et al. [1–2] first isolated adenyl cobamide coenzyme and subsequently cobalamin coenzyme from cultures of *Clostridium tetanomorphum* and later from *Propionibacterium* sp. Thereafter, many reports have appeared on the roles of the coenzyme forms of cobalamin in a number of important biochemical reactions. Kamikubo et al. [3] reported that biosynthetic 2-chloroadenyl cobamide coenzyme from growing *Propionibacterium arabinosum* cells was active as a coenzyme for propanediol dehydrase. However, no further reports have appeared on the biochemical functions of cobamide coenzymes with purines in the nucleotide moiety.

In this paper, the biosyhthesis of 2-thioadenyl cobamide and its coenzyme form using resting *Pr. arabinosum* cells, their physico-chemical properties and biological as well as biochemical activities are dealt with.

Abbreviations:
DBC, cobalamin, 5,6-dimethylbenzimidazolyl cobamide;
DBCC, cobalamine coenzyme, 5'-deoxyadenosyl 5,6-dimethylbenzimidazolyl cobamide;
FA, factor A, 2-methyladenyl cobamide;
FB, factor B, cobinamide;
2-TA, 2-thioadenine;
2-TAC, 2-thioadenyl cobamide.

2. Materials and methods

2.1. *Preparation of resting cells of Pr. arabinosum*

Pr. arabinosum was grown as described [4] with some modifications, for 6 days. Fourty g of wet cells obtained by centrifugation and washing of the culture were suspended in 400 ml 0.05 M phosphate buffer (pH 7.0) containing glucose 0.25%, FB 5 mg, and appropriate amounts of 2-thioadenine (Mann Res. Labs.) in 500 ml flasks, and incubated at 28° for 24 hr on a reciprocating shaker (Amplitude 6.5 cm, 115 strokes/min). FB was prepared by Ce-decomposition [5] of DBC (Roussel-UCLAF, Paris), purified with *p*-chlorophenol, and further fractionated on an ion-exchange column. Identification of FB thus prepared was by paper ionophoresis.

2.2. *Isolation and identification of cyanocorrinoids*

Corrinoids were extracted from centrifuged and washed *Pr. arabinosum* cells as cyano forms for identification of the base in the nucleotide moiety by methods reported previously [6–9], with some modifications. The corrinoids purified with *p*-chlorophenol were fractionated on an ion-change cellulose and further by ionophoresis on paper (1 N acetic acid, pH 2.5; M/15 phosphate buffer, pH 7.5, each included 0.01% KCN). The corrinoids thus obtained were identified by paper chromatography, paper ionophoresis, spectrophotometry and microbioassay with vitamin B_{12}-requiring *Escherichia coli* 215 and *Ochromonas malhamensis*. Quantitative estimation of corrinoids was also made from the absorbances at 367 nm of the dicyano forms.

2.3. *Isolation and identification of 2-thioadenyl cobamide coenzyme*

Each step in extraction, purification, and identification was almost the same as for the cyanocorrinoids except that cyanide was absent and they were done in the dark. Coenzyme activity was estimated in propanediol dehydration using apoenzyme from *Aerobacter aerogenes* [10].

3. Results

It is known that *Pr. arabinosum* produces mainly pseudovitamin B_{12} (ψ-B_{12}) if no suitable base is added, and a corresponding corrinoid, when it is. Following our previous work [11], incorporation of 2-thioadenine was investigated.

3.1. *Formation of 2-thioadenyl cobamide*

The results in table 1, show that increasing amounts of 2-thioadenine bond little effect on the yield of total corrinoids, but resulted in a decrease of ψ-B_{12} and an increase of the DBC-fraction, as estimated by the densitography of the ionophoregrams. The DBC-fraction on the paper was extracted with water and separated by paper ionophoresis at pH 7.5 into two fractions, one of which we supposed to be a new corrinoid and the other DBC. This new fraction increased with the amount of 2-thioadenine. Subsequently, 100 mg base was added per 40 g of wet cells.

To confirm whether the new corrinoid contained 2-thioadenine in its nucleotide moiety, incubation on a larger scale was carried out using 560 g of washed wet cells and 1.4 g of 2-thioadenine. From this, 58 mg of purified corrinoids were obtained, which were further fractionated by column chromatography on P- and DEAE-cellulose, yielding 21 mg of neutral corrinoids. This fraction behaved the same as DBC on paper ionophoresis at pH 2.5 and it was further

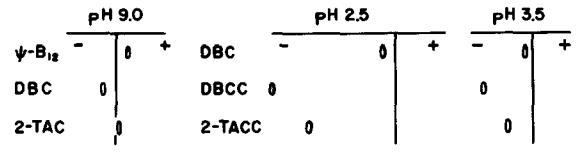

Fig. 1. Paper ionophoresis of 2-thioadenyl cobamide and its coenzyme form.

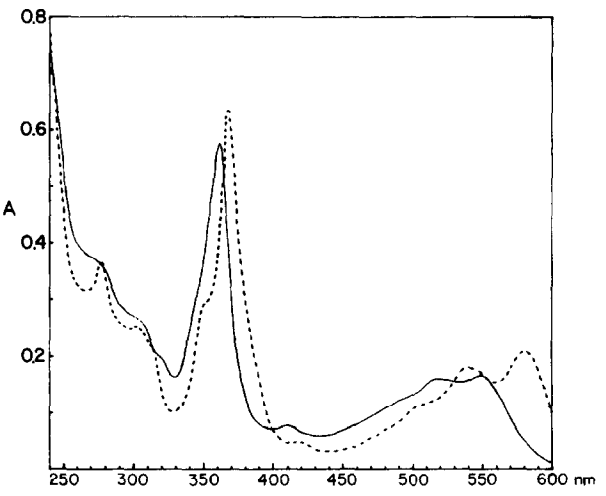

Fig. 2. Absorption spectra of 2-thioadenyl cobamide. —— monocyano form; - - - - dicyano form.

fractionated by paper ionophoresis at pH 9.0 (M/15 phosphate buffer, 0.01% KCN) into two fractions, DBC and a new corrinoid (fig. 1). Further purification of the latter by solvent extraction and chromatography on ion-exchange cellulose gave 11 mg of crystalline material.

Paper chromatography showed that the new corrinoid had the same R_f value in the acidic solvent used as authentic DBC (table 2).

The UV-visible absorption spectrum of the new corrinoid was almost the same as that of DBC except at 278 nm, where there was no maximum, but only a shoulder (fig. 2).

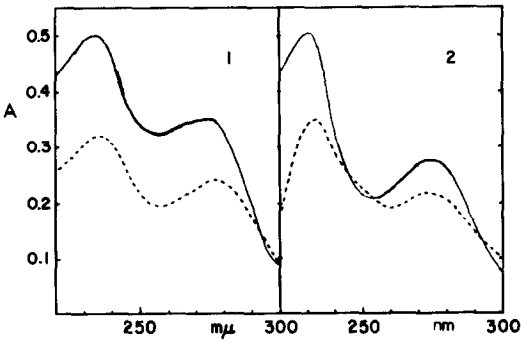

Fig. 3. Absorption spectra of HCl-decomposition product of 2-thioadenyl cobamide. —— base from 2-thioadenyl cobamide; - - - - authentic 2-thioadenine. (a) pH 1.5, (2) pH 10.

Table 1
Effect of 2-thioadenine on the biosynthesis of corrinoids.

2-Thio-adenine added (mg/400 ml)	Yield of corrinoids (mg)	Ratio of corrinoid factors formed (%)				
		FB	FA	ψ-B_{12}	DBC	Unknown factors
0	3.2	15	14	56	15	
10	3.0	11	10	48	31	
50	2.8	13	11	27	43	6
100	3.3	15	11	12	49	13

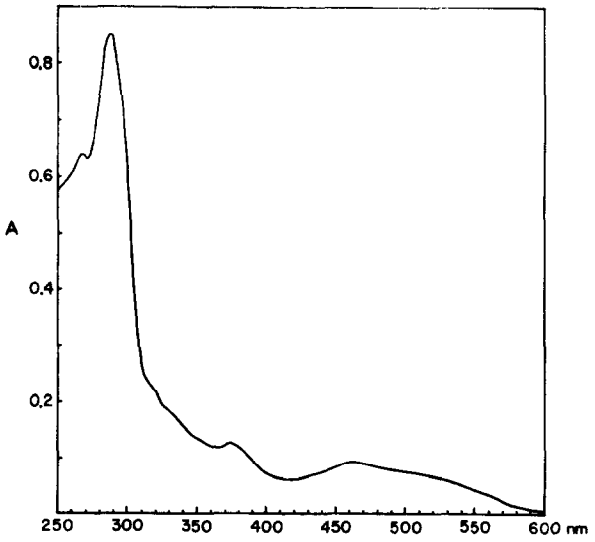

Fig. 4. Absorption spectrum of 2-thioadenyl cobamide coenzyme.

The compound showed 86% and 18% of the microbiological activities of DBC for vitamin B_{12}-requiring *Escherichia coli* 215 and *Ochromonas malhamensis*, respectively.

Table 2
Paper chromatography of 2-thioadenyl cobamide.

Solvents*	R_{DBC}**
Water-saturated 2-butanol	0.79
Water-saturated 2-butanol–1% acetic acid	1.02
2-Butanol–H_2O–NH_3 (28%) = 100:36:14	0.46

* Each solvent contained 0.01% KCN
** $R_{DBC} = Rf(2\text{-TAC})/Rf(DBC)$.

To confirm the nature of the base in the nucleotide moiety of the new corrinoid, the compound was decomposed by heating with 6 N HCl for 1 hr, followed by isolation of the base on ion-exchange cellulose columns. The absorption spectrum of the purified HCl-decomposition product were fairly well coincident with that of similarly-treated authentic 2-thioadenine (fig. 3). The R_f value of the base obtained was the same as that of authentic 2-thioadenine in ascending paper chromatography using butanol saturated with 5% aqueous urea as solvent.

These findings suggest that the added 2-thioadenine had been incorporated into the new corrinoid molecule synthesized by *Pr. arabinosum*.

3.2. *Formation of 2-thioadenyl cobamide coenzyme*

Coenzyme forms of corrinoids were extracted in the dark, in the absence of cyanide, from cells incubated with 2-thioadenine. They were loaded onto P-cellulose and eluted with 0.005 M acetate buffers (pH 4.2 and 5.5). Extictions were estimated with each 7 ml of eluate. Fractions no. 60–100, eluted at pH 4.2, were combined as were no. 101–113, eluted at pH 5.5. The pH 4.2 combined fraction was separated into two fractions, DBCC (R_f 0.24) and a new compound (R_f 0.18), by ascending chromatography with water-saturated 2-butanol, followed by extraction and further purification with *p*-chlorophenol–chloroform. One of the fractions showed the same electrophoretic bahaviour as DBCC, while the other migrated faster towards the anode (fig. 1). In ionophoresis at pH 2.5 and 7.5, after conversion to the cyano forms, the corrinoid corresponding to DBCC and the new one showed the same behaviours as DBC and 2-thioadenyl cobamide, respectively.

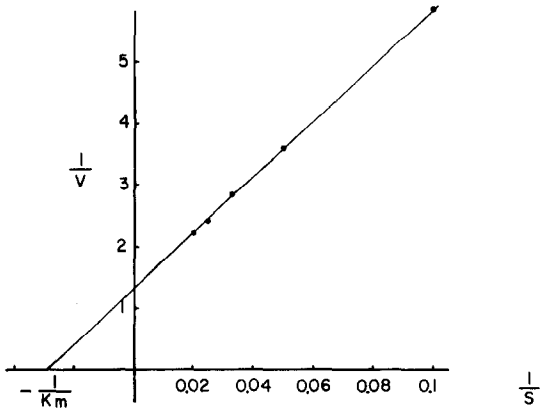

Fig. 5. Coenzyme activity of 2-thioadenyl cobamide coenzyme in propanediol dehydration.

The UV-visible absorption spectrum of the coenzyme form of the new corrinoid showed absorption maxima at 267, 288, 375, and 458 nm (fig. 4).

The pH 5.5 combined fraction was found to be a mixture of adenyl cobamide coenzyme and a small amount of DBCC.

The coenzyme activity of the 2-thioadenyl cobamide coenzyme was estimated by enzymatic 1,2-propanediol dehydration [10]. The Michaelis constant of the coenzyme was 34.5×10^{-7} M, while that of DBCC in the same reaction using the same apoenzyme from *A. aerogenes* was 1.7×10^{-7} M (fig. 5).

4. Discussion

It is known that there are two types of Propionibacteria with respect to corrinoid formation, one, such as *Pr. shermanii*, *Pr. freudenreichii*, etc., produces mainly DBC, and the other, such as *Pr. arabinosum*, *Pr. pentosaceum*, etc., produces mainly ψ-B_{12}, if no suitable base is added, and that the cobamides formed in the cells are their coenzyme forms. The findings suggest that the *Pr. arabinosum* type of bacteria can form little or no 5,6-dimethylbenzimidazole.

It has been suggested that various bases may have different likelihoods of being incorporated into the corrinoid molecule, so that one base may be competitively incorporated into the nucleotide moiety in the presence of another base.

At 100 mg to 40 g of wet cells, 2-thioadenine might compete with adenine for incorporation into corrinoid molecules, suggesting that 2-thioadenine might have a greater likelihood of being incorporated and thus its presence in the amounts tested does not affect the total amount of corrinoids formed.

The UV-visible absorption spectrum of the monocyano form of the new corrinoid resembles that of natural 2-methylthioadenyl cobamide, showing no absorption maxima but shoulders at 278, 308, and 320 nm, where that of ψ-B_{12} had absorption maxima. The absorption spectrum of the dicyano form resembles that of ψ-B_{12}.

It is remarkable that the monocyano form of this new corrinoid shows 18% of the activity of DBC for *O. malhamensis*, while purine-cobamides, other than 2-monochloroadenyl cobamide, have no activity for the Protozoa [3].

It is also interesting that 2-thioadenyl cobamide coenzyme has one twentieth the activity of DBCC in enzymatic 1,2-propanediol dehydration.

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