RELATIONSHIP BETWEEN VITAMIN B_{12} CONTENT AND RATIO OF MONO-UNSATURATED FATTY ACIDS TO METHYL-BRANCHED FATTY ACIDS IN *CORYNEBACTERIUM SIMPLEX* CELLS GROWN ON HYDROCARBONS

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1. Introduction

In the course of our studies on the properties of hydrocarbon-utilizing microorganisms, we found that the ratio of the intracellular monounsaturated fatty acids to the methyl branched-chain fatty acids was dramatically affected by the vitamin B_{12} level in the cells of *Corynebacterium simplex* ATCC 6946. Namely the content of monounsaturated acids was higher than that of branched-chain acids in B_{12}-deficient cells grown on a Co^{2+}-free hydrocarbon medium, while the relative composition was reversed in B_{12}-sufficient cells harvested from a Co^{2+}-containing medium. Experiments using cell-free extracts revealed that the conversion of Δ^9-monounsaturated acids to their corresponding 10-methyl branched-chain acids was not directly dependent on B_{12} but required *S*-adenosylmethionine as the methyl donor. In the organism used, the presence of a B_{12}-dependent N^5-methyltetrahydrofolate: homocysteine methyltransferase (abbreviated as "transmethylase") was confirmed, the activity of which decreased significantly in the cells of low B_{12} level. These results strongly suggest that the reduced activity of methionine-synthesizing system due to the cobalt deficiency causes a lowering in the transformation of monounsaturated fatty acids into methyl branched-chain acids in the microorganism.

2. Materials and methods

The organism used in this study was a hydrocarbon-utilizing bacterium, *Corynebacterium simplex* ATCC 6946, which was found to contain a considerable amount of B_{12} in the form of 5,6-dimethylbenzimidazolylcobamide coenzyme when cultured on a Co^{2+}-supplemented hydrocarbon medium (Fukui, Shimizu and Fujii [1]). The composition of the hydrocarbon salts medium used was as follows: $NH_4H_2PO_4$, 5 g; KH_2PO_4, 2 g; $Na_2HPO_4 \cdot 12H_2O$, 3 g; $MgSO_4 \cdot 7H_2O$, 200 mg; Na_2CO_3, 100 mg; $CaCl_2 \cdot 2H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$ 5 mg; $MnSO_4 \cdot 4-5H_2O$, 2 mg; *n*-alkane of an appropriate chain-length (from *n*-dodecane to *n*-octadecane) 10 ml; distilled water, 1 liter. The pH was adjusted to 7.0 with NaOH. To obtain the cells with different levels of B_{12}, the organism was precultured in the medium described above for 5 days at 30°C, then transferred to the same medium supplemented with or without $CoSO_4 \cdot 7H_2O$ (10 mg/l), and cultured on a rotary shaker at 220 rpm for 5–7 days at 30°C. At the end of the exponential growth phase, the cells were harvested. B_{12} contents of the cells were determined microbiologically using *Lactobacillus leichmannii* ATCC 4797 after the cells had been treated with KCN. Lipids of the cells were extracted with $CHCl_3$-methanol (2 : 1) by the method of Folch et al. [2], saponified, then esterified with freshly distilled diazomethane. Fatty acid methyl esters were identified by gas chromatography and the area under a peak was determined by triangulation. Double bond positional analyses in unsaturated fatty acids were performed by permanganate oxidation (James and Webb [3]). The determination of the position of methyl side chain in branched-chain fatty acids was

carried out by mass spectrometry (Ryhage and Stenhagen [4]). Incorporation of the methyl group of CH_3-^{14}C-methionine into unsaturated fatty acids in the living cells was studied by incubating the washed cells (0.5 g of dry weight), grown on a cobalt-free medium containing *n*-alkane mixture (C_{11}-C_{14}) as carbon sources, with 50 μg of CH_3-^{14}C-methionine (2.0 μCi) at pH 7.0 at 30°C. After 2 hr, the fatty acids were extracted and analyzed as described above. The radioactivity of each fatty acid was measured by the method of Lennarz et al. [5].

For the enzymatic assay of the branched-chain acid syntheses, washed cells were suspended in 0.05 M K-phosphate buffer (pH 7.0), disrupted by ultrasonic disintegrator (20 kc), and the resulting homogenate was centrifuged at 20,000 *g* for 60 min. Cell-free extracts thus obtained were incubated with an appropriate ^{14}C-labeled methyl donor at 30°C in the presence of other additions as noted in table 2. Methyl branched-chain acids formed were determined according to the method of Zalkin et al. [6]. The activity of "transmethylase" was studied in an analogous way to that of Weissbach et al. [7].

3. Results and discussion

The omission of cobalt from the medium resulted in a marked decrease in the bacterial growth and the cellular B_{12} content: the growth rate as well as maximum cell yield in the Co^{2+}-deficient medium was about one-half of those in the complete medium. The vitamin content of the cells harvested from the complete medium was 35–40 (μg/g dry cells), while that from the Co^{2+}-free medium was 6–10. Thus it was confirmed that cells with different levels of B_{12}, i.e. B_{12}-sufficient cells and B_{12}-deficient cells, could be obtained. No qualitative changes occurred in the fatty acids composition between the B_{12}-sufficient and deficient cells, whereas it was significantly affected by the chain-length of the alkane employed as a carbon source. For example, the fatty acids isolated from the cells grown on a long-chain hydrocarbon (C_{16}, C_{17} or C_{18}) contained negligible amounts of fatty acids longer than the initial hydrocarbon. In these cases, the main components were the monounsaturated and saturated fatty acids, whose chain lengths were identical with that of the hydrocarbon used, and the methyl-branched-chain acid having one more carbon atom. When a medium chain-length hydrocarbon (C_{12}-C_{14}) was used, such a correlation was not observed and the C number of the main branched-chain acid formed was C_{19}. The results concerning the fatty acids composition of the cells grown on a various kind of hydrocarbons will be reported elsewhere.

Between the B_{12}-sufficient cells and B_{12}-deficient cells, however, a striking change was observed in the relative amounts of monounsaturated and methyl-branched-chain fatty acids, while the contents of saturated acids were affected very little. As shown in table 1, B_{12}-deficient cells contained more mono-unsaturated acids and less methyl branched-chain acids than B_{12}-sufficient cells. Mass spectrometric

Table 1
Influences of vitamin B_{12}-levels on the main fatty acids composition* of *C. simplex* cells grown on hydrocarbon media.

Fatty acids	B_{12}-deficient cells			B_{12}-sufficient cells		
Growth substrate	18:1	18:0	br 19:0	18:1	18:0	br 19:0
C_{12}	23.2	3.4	18.3	6.9	1.3	49.8
C_{14}	27.8	2.2	8.5	19.3	1.7	28.4
C_{18}	30.3	9.2	8.5	17.5	9.4	30.4
C_{16}	16:1	16:0	br 17:0	16:1	16:0	br 17:0
	39.6	33.3	7.5	5.6	23.5	31.0
C_{17}	17:1	17:0	br 18:0	17:1	17:0	br 18:0
	45.9	32.6	3.2	28.5	35.7	17.5

* (Peak area of each fatty acid/Sum of peak areas of total fatty acids) X 100.

Table 2
Comparison of the activities of various methyl donors in the formation of methyl branched-chain fatty acids.

Methyl donor		Methyl branched-chain fatty acids formed*
CH_3-^{14}C-B_{12},	50 mμmole	0.00 mμmole
N^5-CH_3-^{14}C-FAH_4,	1.0 μmole	0.00 mμmole
AMe-CH_3-^{14}C,	1.0 μmole	7.90 mμmole

Incubation mixture: NADH, 1.0 μmole; FAD, 0.2 μmole; cell-lipid extracted from B_{12}-deficient cells, 5.0 mg; methyl donor, as indicated; cell-free extracts (protein 10 mg); K phosphate buffer (pH 7.0), 150 μmole; final volume, 1.5 ml. Incubation was carried out for 1 hr at 30°C.

* The amount was expressed by the ^{14}C-labeled methyl branched-chain acids formed.

FAH_4; Tetrahydrofolate. AMe; *S*-adenosylmethionine.

Table 3
Comparison of enzyme activities concerning with the syntheses of methionine and branched-chain fatty acids between B_{12}-sufficient cells and B_{12}-deficient cells.

Enzyme source	(1) Methionine formed		(2) Branched-chain acids formed
	mμmole/mg protein/10 min		mμmole/mg protein/hr
	With AMe	Without AMe	
B_{12}-deficient cells	0.15	0.03	0.46
B_{12}-sufficient cells	1.46	0.90	0.49

Incubation mixture:
(1) N^5-CH_3-^{14}C-tetrahydrofolate, 600 mμmole; homocysteine, 1.0 μmole; NADH, 400 mμmole; FAD, 80 mμmole; (*S*-adenosylmethionine (AMe), 50 mμmole); cell-free extracts (protein 2 mg); K phosphate buffer (pH 7.0), 15 μmole; final volume, 0.6 ml.
(2) NADH, 1.0 μmole; FAD, 0.2 μmole; AMe-CH_3-^{14}C, 1.0 μmole; cell-lipid, 5.0 mg; cell-free extracts (protein 10 mg); K phosphate buffer (pH 7.0), 45 μmole; final volume, 1.8 ml.

studies of methyl branched-chain acids (C_{16}-C_{19}) indicated that the methyl side chain was located at C-10 without exception. This was concluded by a relatively strong peak at 199 m/e and 3 peaks at 171 to 173 m/e [4]. On the other hand it was found by oxidative degradation that all the monounsaturated fatty acids belonged to Δ^9-series. These results suggest that B_{12} would play an important role, either directly or indirectly, in the transformation of Δ^9-monounsaturated acids into the corresponding 10-methyl-branched acids.

Experiments using cell-free extracts showed that monounsaturated fatty acids of cell-lipid were converted to ^{14}C-labeled methyl branched-chair fatty acids when *S*-adenosylmethionine-$^{14}CH_3$ was used as a methyl donor. In this case, methyl-B_{12} and N^5-methyltetrahydrofolate did not serve as the methyl donor (table 2). For the methyl acceptor, the endogeneous cell-lipid extracted from the B_{12}-deficient cells was most active and authentic oleyl-CoA was also effective to some extent. Further, resting cell experiments showed that the methyl group of methionine-$^{14}CH_3$ added was exclusive y incorporated into 10-methyl fatty acids in the ce ls irrespective of their B_{12} levels. These results strongly suggest that B_{12} is not involved directly in the formation of 10-methyl fatty acids and the latter are derived from monoenoic fatty acids and *S*-adenosyl-methionine by reductive methylation (Jaureguiberry et al. [8]). The recent work by Akamatsu and Law [9] has indicated that NADPH might be involved in the reduction. In our case, however, the addition of NADPH to the cell-free system was not effective but NADH stimulated the formation of methyl branched-chain acids to some degree.

Enzymatic studies on the synthesis of methionine in *C. simplex* revealed the presence of a B_{12}-dependent "transmethylase", similar to that of *E. coli* (unpublished data). As given in table 3, the B_{12}-level of the bacterial cells had no significant influences on the activity of the methyl branched-chain acids synthesizing system. On the other hand, the activity of methionine synthesizing system of B_{12}-deficient cells was only one-tenth of that of B_{12}-sufficient cells in the presence of *S*-adenosylmethionine, the effector of "transmethylase". Furthermore, the situation was more distinct in the absence of *S*-adenosylmethionine.

From the results obtained here, it would be concluded that a low B_{12} content of the cells of *C. simplex* grown on a Co^{2+}-deficient hydrocarbon medium results in a decreased synthesis of methionine, which cannot fully meet the need for the formation of 10-methyl branched-chain fatty acids from Δ^9-monoenoic acids.

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