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On Vitamins in Sewage Sludge

VIII. Utilization of Some Vitamin B_{12} Factors by an Enrichment Culture of *Methanobacterium omelianskii*

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An enrichment culture of *Methanobacterium omelianskii* was grown in the synthetic medium of Barker [3], with and without the addition of 1 000 mμg/ml of 5,6-dimethyl benziminazole and some vitamin B_{12} factors previously isolated from sewage sludge.

In the absence of 5,6-dimethyl benziminazole, most factors were converted to factor Met [1].

In the presence of 5,6-dimethyl benzimianzole, the incomplete factors B, Z1, Z2, Z3 and a greater part of the factor X4 preparation could easily be converted to cyanocobalamin by the culture.

The complete, nucleotide-containing factor W may also to a certain extent be converted to cyanocobalamin.

In a preceding paper [1], a series of experiments performed in order to investigate the ability of an enrichment culture of *Methanobacterium omelianskii* to produce vitamin B_{12} factors was reported. Using the same enrichment culture and an identical experimental technique, the authors have now investigated the ability of the culture to utilize some vitamin B_{12} factors previously isolated from sewage sludge [2]. The culture was grown in the synthetic medium of Barker [3] containing 2 % ethanol as the only organic compound. The different factors were added to the medium at levels of approximately 1 000 mμg/ml. Two series of experiments were made. Series I was a preliminary investigation and was carried out with a less purified enrichment culture. The inoculum had been prepared by ten successive reinoculations of the raw culture into Barker's medium. In series II, an inoculum was used which had been obtained by 25 successive reinoculations of the raw culture.

RESULTS AND DISCUSSION

Series I

From the results of the preliminary investigation which are shown in Table 1, it seems as though the different factors are converted by the enrichment culture into factor Met. The effectivity of the conversion seems be higher in the simple medium than in the presence of Bacto Peptone. However, when Bacto Peptone was present, cyanocobalamin was formed together with factor Met and this fact may account for the lower activity values obtained in the presence of Bacto Peptone. In plate assay with *E. coli*, several vitamin B_{12} factors are known to cause greater growth zones than cyanocobalamin and this may also apply to factor Met.

When adenine was added together with factor B or factor X4, small amounts of factor B were detected in the fermentation liquor together with factor Met but no ψ-B_{12} was found. Thus, adenine in the presence of factor B is not utilized for the production of ψ-B_{12}. This is in agreement with an earlier observation [1] that the enrichment culture of *Methanobacterium omelianskii* cannot use adenine to produce ψ-B_{12}. It is interesting to observe that the microbiologically inactive factor Z1 was converted in medium a to the microbiologically active factor Met. That a conversion really took place can be

Table 1. Utilization of Vitamin B_{12} factors by an enrichment culture of *Methanobacterium omelianskii;* a — medium according to Barker [3]; b — medium according to Barker + 0.25 % Bacto Peptone; Cy — cyanocobalamin, R_c = 1.0. Brackets mean that the factor in question is present only in small amounts.

Medium	Factor added	*E. coli* activity immediately after the addition mμg/ml *	Beginning of gas evolution h	*E. coli* activity in samples taken after 20 days of fermentation	
				mμg/ml *	Factors present **
a	no	0	40	20	Met
b	no	+	30	80	Met
a	factor B	170	40	130	Met
b	factor B +adenine	170	40	130	Met; (B)
b	factor B	1 000	30	70	Met
a	factor Z1	+	60	130	Met
b	factor Z1	+	30	15	Met; Cy
a	factor Z2	370	60	100	Met
b	factor Z2	1 000	30	70	Met; Cy
a	factor Z3	110	50	70	Met
b	factor Z3	600	30	80	Met; Cy
a	factor X4	60	60	90	Met (and X4?)
b	factor X4 +adenine	60	60	250	Met (and X4?); (B)
b	factor X4	140	30	180	Met (and X4?); (B)

* Calculated as cyanocobalamin in plate assay.
** Identified by chromatography and ionophoresis. Factor Met has R_c-value = 0.7.

seen from *E. coli* activity values: 20 mμg/ml when the fermentation was carried out without factor Z1 and 130 mμg/ml in the presence of it. The corresponding values for medium b cannot be so readily explained.

The conversion of factor B as well as factor X4 to factor Met (in medium a) seems to be more effective than the conversion of the other factors investigated.

The question of whether the enrichment culture of *Methanobacterium omelianskii* can utilize the different factors as precursors for the cyanocobalamin molecule has been more thoroughly investigated in Series II.

Series II

The fermentations of this series were carried out in the simple medium of Barker[3] supplemented with 1 000 mμg/ml of 5,6-dimethyl benziminazole. The different factors were added at levels of approximately 1 000 mμg/ml. A series of 24 tubes was prepared for every factor. Twenty of these were inoculated, the reamaining four serving as non-inoculated controls. A control

Table 2. Utilization of factor B by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml); Cy = cyanocobalamin.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls		
		mμg/ml*	factors present **	pH	*E. coli* activity	
					mμg/ml*	factors present **
0		1 130	B		1 130	B
0.5	7.2	1 130	B			
2	7.2	1 130	B			
3	6.6	750	B; (Cy)			
4	6.1	380	B; Cy	7.3	720	B
5	6.1					
6	6.1	90	Cy			
7	6.2	100	Cy			
9	6.3	110	Cy	7.7	690	B
10	6.3	70	Cy			
11	6.2	100	Cy			
12	6.2	100	Cy			
13	6.2	80	Cy			
14	6.3	60	Cy	7.7	320	B
16	6.2	70	Cy			
17	6.2	90	Cy			
18	6.2	60	Cy			
19	6.2	70	Cy			
20	6.3	70	Cy			
21	6.2	50	Cy			
27	6.1	60	Cy	8.3	410	B

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I[4].

Table 3. Utilization of factor Z1 by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml); Cy = cyanocobalamin; Z = factor(s) Z.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls		
		mμg/ml *	factors present **	pH	*E. coli* activity mμg/ml *	*E. coli* activity factors present **
0		5	Z1		5	Z1
0.5	7.2	5	Z; B; (Cy)			
2	7.1	30	Cy			
3	6.9	60	Cy			
4	6.2	60	Cy	7.4	4	B
5	6.3					
6	6.2	40	Cy			
7	6.4	70	Cy			
9	6.4	50	Cy	7.6	3	B
10	6.3	40	Cy			
11	6.3	50	Cy			
12	6.3	50	Cy			
13	6.3	30	Cy			
14	6.4	30	Cy	7.9	<3	not identified
16	6.1	40	Cy			
17	6.1	30	Cy			
18	6.1	<3	not identified			
19	6.1	<3	» »			
20	6.3	<3	» »			
21	6.2	<3	» »			
27				8.2	<3	not identified

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I[4].

series to which dimethyl benziminazole but no vitamin B_{12} factor had been added was fermented simultaneously with the other series. Samples were taken almost every day by removing a tube from each series. Samples from the non-inoculated controls were taken at 5 day intervals. The results of these experiments are given in Table 2—8.

Factor B. An examination of Tables 2 and 8 leads to the conclusion that factor B in the presence of dimethyl benzimidazole can be converted into cyanocobalamin by the enrichment culture. Taking into consideration the decrease of *E. coli* activity in the non-inoculated controls, a conversion of at least 20 % can be estimated as follows. After fermentation for 7—12 days, the *E. coli* activity had decreased from about 1 000 to about 100 mμg/ml. This would suggest a conversion of factor B to cyanocobalamin of about 10 %. In the non-inoculated controls there was a decrease to about half of the original *E. coli* activity. This means that the conversion of factor B to cyanocobalamin in the fermented series was at least 20 %.

Table 4. Utilization of factor Z2 by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml); Cy = cyanocobalamin; Z = factor(s) Z.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls		
		mμg/ml*	factors present **	pH	*E. coli* activity	
					mμg/ml*	factors present **
0		1 800	Z		1 800	Z
0.5	7.3	1 800	Z			
2	7.1	1 050	Z			
3	6.5	700	Z; Cy			
4	6.2	280	Z; Cy	7.5	410	Z
5	6.2					
6	6.2	80	Cy; (Z)			
7	6.3	60	Cy			
9	6.3	70	Cy	7.7	420	Z
10	6.3	70	Cy			
11	6.3	80	Cy			
12	6.3	70	Cy			
13	6.4	70	Cy			
14	6.4	80	Cy	7.7	370	Z
16	6.1	60	Cy			
17	6.1	70	Cy			
18	6.3	60	Cy			
19	6.3	60	Cy			
20	6.2	60	Cy			
21	6.3	50	Cy			
27	6.2	60	Cy	8.4	360	Z

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I[4].

In reality the yield might be much higher since factor B is known to give much greater growth zones than cyanocobalamin when assayed with *E. coli* in the plate assay. The considerable stability of factor B and of cyanocobalamin upon incubation at 37°C for several days is rather surprising. The reducing character of Barker's medium might be favourable for this stability. The maximum yield of cyanocobalamin was about 100 mμg/ml.

Factor Z1. It can be seen in Table 3 (*cf.* also Table 8) that the microbiologically almost inactive factor Z1 is converted by the enrichment culture into cyanocobalamin. In this fermentation, cyanocobalamin seems to be less stable than in the fermentation with factor B. The maximum yield of cyanocobalamin was 50—70 mμg/ml. Factor Z1 seems to be unstable upon incubation at 37°C and is spontaneously converted into factor B in the reducing medium of Barker (*cf.* values for non-inoculated controls). A partial spontaneous conversion of the factors Z into factor B was observed during their isolation [2].

The conversion of factor Z1 to cyanocobalamin seems to take place more rapidly than that of factor B (*cf.* Table 2).

Table 5. Utilization of factor Z3 by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml). Brackets mean that the factor in question is present only in small amounts; Cy = cyanocobalamin; Z = factor(s) Z.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls		
		mμg/ml *	factors present **	pH	*E. coli* activity	
					mμg/ml *	factors present **
0		400	Z		400	Z
0.5	7.1	400	Z			
2	7.2	350	Z			
3	7.1	190	Z			
4	6.5	90	Cy	7.4	110	Z; (B)
5	6.3					
6	6.3	70	Cy			
7	6.2	50	Cy			
9	6.1	50	Cy	7.6	90	Z; (B)
10	6.4	80	Cy			
11	6.3	60	Cy			
13	6.2	70	Cy			
13	6.1	50	Cy			
14	6.1	50	Cy	7.8	80	Z
16	6.1	70	Cy			
17	6.3	50	Cy			
18	6.2	50	Cy			
19	6.2	60	Cy			
20	6.2	40	Cy			
21	6.1	60	Cy			
27	6.1	60	Cy	8.0	70	Z

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I [4].

Factor Z2. Unlike factor Z1, factor Z2 is fairly stable on incubation at 37°C (see Table 4 and *cf.* Table 8). This factor is also converted into cyanocobalamin by the enrichment culture. The maximum yield of cyanocobalamin was about 80 mμg/ml. The rate and possibly also the extent of conversion of factor Z2 was comparable with that of factor B (Table 2).

Factor Z3. This factor seems to be less stable than factor Z2, but more stable than factor Z1. Upon incubation at 37°C, a small part of it is spontaneously converted into factor B (see Table 5, *cf.* also Tables 4 and 8). The enrichment culture converts it to cyanocobalamin, the maximum yield reaching 90 mμg/ml.

Factor X4 (see Table 6 and *cf.* Table 8). This factor seems to be surprisingly stable during incubation at 37°C. The enrichment culture converts a part of it (~half of the amount) into cyanocobalamin, but the other part remains unconverted or is converted to a factor which is indistinguishable from factor X4 by paper chromatography in solvent system I. The conversion or other-

Table 6. Utilization of factor X4 by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml); Cy = cyanocobalamin.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls		
		mμg/ml*	factors present **	pH	*E. coli* activity	
					mμg/ml*	factors present **
0		1 000	X4		1 000	X4
0.5	7.1	990	X4			
2	7.2	1 040	X4			
3	7.3	870	X4			
4	6.7	990	X4; (Cy)	7.4	1 350	X4
5	6.4					
6	6.4	360	X4; Cy			
7	6.2	240	X4; Cy			
9	6.2	360	X4; Cy	7.5	930	X4
10	6.4	230	X4; Cy			
11	6.3	250	X4; Cy			
12	6.3	210	X4; Cy			
13	6.3	240	X4; Cy			
14	6.3	290	X4; Cy	7.7	1 380	X4
16	6.2	250	X4; Cy			
17	6.2	280	X4; Cy			
18	6.3	350	X4; Cy			
19	6.3	220	X4; Cy			
20	6.2	230	X4; Cy			
21	6.3	340	X4; Cy			
27	6.4	150	X4; Cy	8.0	1 290	X4

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I[4].

wise of this latter part was not further investigated. That conversion of a part of factor X4 really took place (*i.e.*, that the cyanocobalamin produced was not due merely to an independent synthesis in the presence of 5,6-dimethyl benziminazole) was indicated by the decrease in *E. coli* activity values in the fermentation liquor as compared with the practically unchanged values in the non-inoculated controls.

The yield of cyanocobalamin obtained from this factor cannot be estimated since factor X4 is also present in the fermentation liquor and contributes to the *E. coli* activity.

Factor W (see Table 7 and *cf.* Table 8). This factor was not very stable on incubation at 37°C. After only 3—4 days, the *E. coli* activity had decreased to almost one third. In the medium fermented for 3—4 days by the enrichment culture, the activity of the factor was almost unchanged. A pronounced decrease took place only after 6—7 days. However, with a longer fermentation time (> 7 days), the *E. coli* activity values for the fermented medium were always lower than those for the non-inoculated controls, which may suggest some conversion at least.

Table 7. Utilization of factor W by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml); Cy = cyanocobalamin.

Time of fermentation (days)	*E. coli* activity			Non-inoculated controls		
	pH	mμg/ml*	factors present **	pH	*E. coli* activity	
					mμg/ml*	factors present **
0		250	W		250	W
0.5	7.2	250	W			
2	7.2	260	W			
3	7.0	240	W			
4	6.5	220	W; (Cy)	7.3	90	W
5	6.3					
6	6.2	120	W; Cy			
7	6.3	90	W; Cy			
9	6.3	40	W; Cy	7.6	80	W
10	6.3	40	W; Cy			
11	6.3	40	W; Cy			
12	6.3	40	W; Cy			
13	6.3	40	W; Cy			
14	6.3	40	W; Cy	7.8	70	W
16	6.3	40	W; Cy			
17	6.1	40	W; Cy			
18	6.2	30	W; Cy			
19	6.3	30	W; Cy			
20	6.1	30	W; Cy			
21	6.2	30	W; Cy			
27	6.2	30	W; Cy	8.1	70	W

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I [4].

CONCLUSIONS

As already reported [4], factor W behaves as a complete, nucleotide-containing factor whereas the other factors used here behave like incomplete nucleotide-lacking factors. In agreement with this, factors B, Z1, Z2, Z3 can easily be converted into cyanocobalamin by the enrichment culture if 5,6-dimethyl benziminazole is present in the medium. Factor X4, which has also been characterized as an incomplete factor, is converted only partially to cyanocobalamin. This may be explained by the fact that the preparation has now, after 3 years, been shown to separate into two electrophoretic fractions [1]. This investigation suggests that even factor W — being a complete nucleotide-containing factor — may to a certain extent be converted to cyanocobalamin when 5,6-dimethyl benziminazole is present in the medium.

Table 8. Production of vitamin B_{12} factors by an enrichment culture of *Methanobacterium omelianskii*, grown in Barker's medium supplemented with 5,6-dimethyl benziminazole (1 000 mμg/ml), Control series. To be compared with Tables 2—7.
Cy = cyanocobalamin.

Time of fermentation (days)	pH	*E. coli* activity		Non-inoculated controls	
		mμg/ml *	factors present **	pH	*E. coli* activity mμg/ml
0.5	7.3	+			
2	7.1	3	Cy		
3	7.1	3	Cy		
4	6.7	3	Cy	7.4	+
5	6.4				
6	6.3	3	Cy		
7	6.5	3	Cy		
9	6.4	3	Cy	7.6	+
10	6.5	3	Cy		
11	6.4	3	Cy		
12	6.4	3	Cy		
13	6.3	3	Cy		
14	6.5	3	Cy	7.8	+
16	6.5	3	Cy		
17	6.3	3	Cy		
18	6.4	3	Cy		
19	6.4	3	Cy		
20	6.3	3	Cy		
21	6.4	3	Cy		
27	6.5	3	Cy	7.6	—

* Calculated as cyanocobalamin in plate assay.
** Identified by paper chromatography in solvent system I[4].

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