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On Vitamins in Sewage Sludge

IX. Production of Vitamin B_{12} by Methane Bacteria

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Twelve different enrichment cultures of methane-producing bacteria have been prepared and their ability to produce vitamin B_{12} has been studied.

Most of the cultures produced small amounts of vitamin B_{12}. The enrichment cultures grown in the media containing either methanol, acetate or butyrate were shown to produce considerably larger amounts of vitamin B_{12} than those grown in the other media. The amount of vitamin B_{12} produced in the methanol-containing medium seems to be higher than that reported in the literature for many other microorganisms.

It was shown that the kind of factors produced by the enrichment cultures depends upon the composition of the medium. The formation of cyanocobalamin depends upon the supply of 5,6-dimethyl benziminazole.

In the two preceding papers [1,2], the authors have reported investigations on the ability of an enrichment culture of *Methanobacterium omelianskii* to produce vitamin B_{12}. This culture proved to have a rather limited synthetic ability with respect to vitamin B_{12}. The greatest yield of cyanocobalamin obtained using certain modifications of a simple synthetic medium did not exceed 100 mμg/ml. Since the high contents of vitamin B_{12} in sewage sludge evidently could not be explained by only the activity of *Methanobacterium omelianskii*, the synthetic ability of other methane bacteria, active during the digestion of sewage sludge, was investigated. Enrichment cultures of methane-producing bacteria were successfully prepared by many workers already in the beginning of this century (*cf.* Barker: *Bacterial Fermentations*, 1956 [9]). Such cultures are usually characterized by a high degree of morphological and physiological homogeneity. Wikén [3] gives a review of the earlier works and describes the preparation of several enrichment cultures as well as the isolation of some pure strains of methane bacteria. Using the technique described by Wikén, the authors prepared twelve enrichment cultures from digesting sewage sludge and investigated their ability to produce vitamin B_{12}, both in

a simple synthetic medium containing only one low molecular organic compound and also in the same medium modified by the addition of Co^{2+}, 5,6-dimethyl benziminazole and Bacto Peptone.

EXPERIMENTAL

Basal medium. The basal medium used was that described by Wikén [3] — it had the following composition: NH_4Cl, 1.0 g; K_2HPO_4, 0.4 g; $MgCl_2 . 6H_2O$, 0.1 g; tap water, 1 000 ml. To each 100 ml of this medium, a separately sterilized solution consisting of 2 ml of 1 % $Na_2S . 9H_2O$ solution + 8 ml of 5 % $Na_2CO_3 . 10H_2O$ solution was added. The pH of the medium was adjusted with separately sterilized HCl (1:5) to 6.5—7.5.

Elective media. Twelve different elective media were used to obtain the various enrichment cultures. These media were prepared by supplementing twelve portions of the basal medium with the following organic compounds (one compound only was added to each portion of the basal medium): methanol 1 %, ethanol 2 %, *n*-propanol 1 %, *iso*-propanol 1 %, *iso*amyl alcohol 0.5 %, acetone 0.5 %, calcium formate 2 %, calcium acetate 2 %, calcium propionate 2 %, and calcium *n*-butyrate 2 %. The media containing alcohols were further supplemented with 2 % of $CaCO_3$ in order to neutralize the acid formed during the fermentation and to form a sediment which, according to many workers, is very favourable for the growth of methane bacteria. The media containing acids (as salts) were instead supplemented with 2 % fine asbestos powder.

The preparation of the enrichment cultures was carried out by inoculating a few milliliters digesting sludge into the appropriate media. When a vigorous gas evolution was established, a reinoculation was performed into an identical medium. This procedure was repeated until altogether 10 reinoculations had been made in the same medium. To the last (the tenth) reinoculation, Co^{2+} was added (2 500 mμg/ml). After two further reinoculations into Co^{2+}-containing media, a reinoculation was made into a medium containing both Co^{2+} (2 500 mμg/ml) and 5,6-dimethyl benziminazole (1 000 mμg/ml), see Table 1. Finally, the media were also supplemented with Bacto Peptone (0.25 %) as seen in Table 1.

All cultures were grown in conical flasks of 100—300 ml capacity and incubated at 37°C.

Anaerobic conditions. All manipulations with the media and cultures were made in the carbon dioxide chamber described in a preceding paper [1]. The cultures were always covered with a layer of paraffin oil.

Samples were taken from the cultures at the end of the fermentation just before each reinoculation and were analyzed for vitamin B_{12} activity using *E. coli* 113—3 as test organism in a plate assay and by bioautography as described elsewhere [4].

RESULTS AND DISCUSSION

The results of the investigation can be seen in Table 1. The enrichment cultures grown in the media containing methanol, ethanol, *n*-propanol, *n*-butanol, *iso*butanol, *iso*amyl alcohol, acetate or butyrate grew relatively easily and considerable gas evolution usually occurred after incubation at 37°C for 5—15 days. The cultures grown in media containing methanol, acetate or butyrate were especially active — the most intensive gas evolution occurring only 1—2 days after the inoculation. The remaining four media produced much poorer growths and culturing times up to several months were required before a subsequent reinoculation could be made. An examination of Table 1 reveals that, apart from media containing methanol, acetate or butyrate, only small amounts of vitamin B_{12} activity (comparable with those produced in the ethanol-containing medium in which *Methanobacterium omelianskii* is dominant) were produced.

The cultures which were grown in the media containing methanol, acetate or butyrate (especially the first named) provide, however, a striking exception. When supplied with Co^{2+}, 5,6-dimethyl benziminazole and Bacto Peptone, the methanol culture produced vitamin B_{12} activities up to 6 000 mμg/ml estimated as *E. coli* activity in the cup plate assay and calculated as cyanocobalamin. The amounts of *E. coli* activity produced by this culture are thus 80—300 times greater than those produced by the cultures developed in most of the other media and even ~3 resp. 6 times greater than the amounts produced by the butyrate resp. acetate cultures. The yields of vitamin B_{12} activity obtained with the methanol culture seem to be even higher than those reported for *Streptomyces olivaceus* [5].

All three cultures produce sufficient amounts of *E. coli* activity to explain the high vitamin B_{12} activity of digested sewage sludge. Moreover, it seems as if the amount of vitamin B_{12} activity which can be obtained with the three most active cultures, especially when calculated on a dry solids basis, is even higher than the vitamin B_{12} activity of digested sludge. A possible explanation of this phenomenon is that digested sludge, as suggested in a previous work [6], probably contains organisms which consume vitamin B_{12}. Furthermore, the essential substrates, *viz.* methanol, acetate or butyrate may be present in the sludge only in limited quantities or the rate of their formation may not be rapid enough to provide the appropriate organisms with adequate quantities of the substrates. This may also be true for 5,6-dimethyl benziminazole. However, it has been shown in a previous work [1] that an increase above 1 000 mμg/ml in the content of 5,6-dimethyl-benziminazole in the medium fermented by *Mb. omelianskii* does not produce any increase in the yield of vitamin B_{12} and that the addition of 10 000 mμg/ml of benziminazole, *i.e.* a tenfold increase, seems instead to bring about an inhibition of the growth. Similar observations have also been made with the methanol culture — more detailed results will be reported in a separate communication. It can further be seen in Table 1 that the addition of Co^{2+} may stimulate the growth of certain cultures (as judged by the gas evolution), *e.g.* the cultures grown in the media containing methanol, ethanol, acetate or butyrate. This effect was most pronounced in the last two cases — the addition of Co^{2+} seemed to be essential for the production of any appreciable growth. In most cultures, even those in which the Co^{2+} addition had no effect on the gas evolution, it was found that the formation of *E. coli* activity was stimulated by this addition. An exception in this respect was the culture grown on *iso*butanol.

The *E. coli* activity produced by the different cultures could in most cases be separated into at least two spots by the usual chromatographic-bioautographic technique. One of these spots corresponded to cyanocobalamin, R_c (= R_F relative to R_F of cyanocobalamin) = 1.0. Although cyanocobalamin was formed in most cultures, its formation in some cultures was dependent upon the addition of Co^{2+} alone (*e.g.* cultures on ethanol, *n*-propanol, *iso*propanol, acetone, butyrate) while, in others, both Co^{2+} and 5,6-dimethyl benziminazole had to be added in order to obtain the formation of cyanocobalamin (*e.g.* cultures on methanol, *n*-butanol, *iso*amyl alcohol and acetate).

Even in those cultures in which the addition of Co^{2+} alone stimulated the synthesis of cyanocobalamin, a simultaneous addition of 5,6-dimethyl benzi-

Table 1. Production of vitamin B_{12} by enrichment cultures of methane bacteria. Co — addition of Co^{2+}, 2 500 mμg/ml. DMB — addition of 5,6-dimethyl benziminazole, 1 000 mμg/ml. BP — addition of Bacto Peptone (Difco), 0.25 %. + slight gas evolution, ++ medium gas evolution, +++ strong gas evolution, ++++ very strong gas evolution.

Enrichment culture grown in the basal medium, supplemented with	Further additions to the medium	Gas-evolution	*E. coli* activity produced	
			mμg/ml **	chromatographic spots corresponding to factors ***
Methanol 1 %	none	+	traces	not identified
» »	Co	+++	500 — 900	B; W (or III) *; Z
» »	Co + DMB	+++	600 — 2 000	B; Cy; W (or III) *; Z
» »	Co + DMB + BP	++++	4 000 — 6 000	B; Cy; W (or III) *; Z
Ethanol 2 %	none	++	1 — 3	Met
» »	Co	+++	60 — 130	B; Cy; Met
» »	Co + DMB	+++	15 — 30	Cy;
» »	Co + DMB + BP	++++	50 — 70	Cy;
n-Propanol	none	++	1 — 3	not identified
»	Co	++	40 — 80	B; Cy; W (or III) *
»	Co + DMB	++	20 — 30	B; Cy
»	Co + DMB + BP	++	20 — 30	Cy; W (or III) *
*Iso*propanol 1 %	none	++	1 — 3	not identified
» »	Co	++	4 — 6	Cy; W (or III) *
» »	Co + DMB	++	10 — 20	Cy; W (or III) *
» »	Co + DMB + BP	++	40 — 60	Cy; W (or III) *
n-Butanol 1 %	none	++	1 — 3	not identified
» »	Co	++	10 — 30	B; W (or III) *
» »	Co + DMB	++	10 — 20	Cy
» »	Co + DMB + BP	++	10 — 20	Cy; W (or III) *
*Iso*butanol 1 %	none	++	1 — 3	not identified
» »	Co	++	1 — 3	not identified
» »	Co + DMB	++	4 — 6	Cy; W (or III) *
» »	Co + DMB + BP	++	20 — 30	Cy; W (or III) *

minazole directed the synthesis towards the formation of cyanocobalamin at the expense of the other factors. In two cases (ethanol and *iso*amyl alcohol), such a simultaneous addition resulted in the formation of cyanocobalamin as the only factor, although in rather smaller amounts. In these two and certain other cultures, the addition of 5,6-dimethyl benziminazole seemed to decrease the *E. coli* activity estimated in the cup plate assay as compared with a corresponding culture grown with the addition of Co^{2+} only. It cannot, however, be concluded whether this was a real decrease in the vitamin B_{12} activity or merely a reflection of the differences in activity towards *E. coli* in the cup plate assay between the other factors and the cyanocobalamin to which they had been converted due to the addition.

Table 1. Contd.

Enrichment culture grown in the basal medium, supplemented with	Further additions	Gas-evolution	*E. coli* activity produced	
			mμg/ml **	chromatographic spots corresponding to factors ***
Iso-amyl alcohol 0.5 %	none	++	1— 3	not identified
» » »	Co	++	30— 130	W (or III) *
» » »	Co + DMB	++	2— 4	Cy
» » »	Co + DMB + BP	++	10— 20	Cy
Acetone 0.5 %	none	+	traces	not identified
» »	Co	+	»	Cy; W (or III) *
» »	Co + DMB	+	»	not identified
» »	Co + DMB + BP	+	»	not identified
Calcium formate 2 %	none	+	traces	not identified
» » » »	Co	+	»	not identified
» » » »	extremely slow growth			
» » » »				
Calcium acetate 2 %	none	+	traces	not identified
» » » »	Co	++++	50— 250	W (or III) *
» » » »	Co + DMB	++++	600— 700	Cy; W (or III) *
» » » »	Co + DMB + BP	++++	800—1 000	Cy; W (or III) *
Calcium propionate 2 %	none	+	traces	not identified
» » » »	Co	+	2— 3	not identified
» » » »	extremely slow growth			
» » » »				
Calcium *n*-butyrate 2 %	none	+	5— 15	not identified
» » » »	Co	++++	100—1 000	Cy; W (or III) *
» » » »	Co + DMB		not determined	
» » » »	Co + DMB + BP	++++	1 000—2 200	Cy; W (or III) *

* and/or possibly some incomplete factor with R_c-value (R_F-value relative to that of cyanocobalamin) in the range 0.6—0.7; *cf.* discussion and Ref [7].

** calculated as cyanocobalamin in plate assay.

*** identified by paper chromatography in solvent system I [7].

The second chromatographic spot of *E. coli* activity obtained in most cultures had an R_c-value in the range 0.6—0.7. It has been reported earlier [7] that the presence of at least 8 different vitamin B_{12} factors can be attributed to such a spot, *viz.* factor III, (Bernhauer) [8], factor A (Ford, Kon and Porter)[11], pseudovitamin B_{12} (Pfiffner) [12] and the factors X1, X2, X3, X4, and W isolated from sewage sludge in this laboratory [7].

More accurate chromatographic and electrophoretic studies, however, indicated that it was improbable that factor A or (and) pseudovitamin B_{12} were present in the spot.

It has also been shown in a previous work [1] that one of the methane bacteria, *viz. Mb. omelianskii*, cannot utilize adenine in the presence of Co^{2+} or even in the presence of added factor B to produce pseudovitamin B_{12}. It is therefore probable that neither pseudovitamin B_{12} nor factor A was formed by the various enrichment cultures. The ever increasing number of vitamin B_{12} factors reported in the literature makes the identification of any factor by the chromatographic-bioautographic technique quite uncertain. However, certain interesting observations can be made concerning the occurrence of certain factors (chromatographic spots) in certain cultures while not in others. It has been found in an earlier study and confirmed by the present investigation that the enrichment culture grown in the ethanol-containing, but not further supplemented, basal medium produces only one vitamin B_{12} factor, *viz.* factor Met. This factor has an R_c-value ($\sim$ 0.7) close to that for the eight factors mentioned above and its occurrence in sewage sludge has never before been noticed (probably partly due to its very low concentration in this material). It is interesting to note that factor Met was not formed by any other enrichment culture. It seems therefore to be in some way characteristic for the enrichment culture of *Mb. omelianskii* prepared in the ethanol-containing medium. As far as the remaining factors which may be present in the second chromatographic spot (R_c = 0.6—0.7) of *E. coli* activity are concerned, no conclusions could be made about which of these factors were formed by the respective cultures. The results of paper electrophoretic studies indicated that, in most cultures, the spot with R_c = 0.6—0.7 corresponded mainly to factor W (and/or factor III) possibly accompanied by minor quantities of the incomplete factors X4 and (or) X3. In the culture grown on butyrate, these last two factors were formed in considerably greater amounts than in the other cultures.

The culture grown in the methanol-containing medium produced the greatest number of vitamin B_{12} factors. As pointed out above, this culture also produced the largest amount of *E. coli* activity. A chromatographic separation of the activity gave 4 spots when both Co^{2+} and 5,6-dimethyl benziminazole were added to the medium, but only 3 spots when the benziminazole was omitted. The four spots corresponded to factors: B (R_c = 1.3—1.5), Cy (R_c = 1.0), W and (or) factor III + minor quantities of X4 and (or) X3 (R_c = 0.6—0.7) and finally some of the factors Z. When 5,6-dimethyl benziminazole was lacking, but Co^{2+} present, in the medium, the spot corresponding to cyanocobalamin was always absent after chromatographic separation.

It can, in addition, be seen in Table 1 that the enrichment culture grown in the methanol-containing medium was the only one which produced factors Z [7]. This culture was exceptional also with respect to the occurrence of factor B since the chromatographic spot corresponding to this factor was not (or not completely) converted to the cyanocobalamin-spot in the presence of added 5,6-dimethyl benziminazole and Bacto Peptone. In a preliminary study of this problem, we investigated whether greater additions of 5,6-dimethyl benziminazole to this culture cause a more complete conversion of factor B (and possibly also of factors Z) to cyanocobalamin. However, the results indicated that greater additions of this compound ($>$ 1 μg/ml medium) may have an inhibiting effect on the growth of the culture.

It is possible that the spots corresponding to factors B and Z contained also some other factors which could not be converted to cyanacobalamin by the culture.

Microscopic examinations of the enrichment culture grown in the basal medium supplemented with methanol revealed the presence of at least two kinds of organisms. One of them was a coccus of about 1.5 μ diameter, the second was a rod-shaped organism the size of which was greater than that of *Methanobacterium omelianskii*. Both were Gram variable. The coccoidal form was the dominant one. A rough estimation indicated that the coccoidal form was 1 000 times more abundant than the rod shaped one.

The enrichment culture grown in the butyrate-containing medium consisted of rods 3—6 μ long and 1 μ thick, whereas the culture obtained in the acetate-containing medium contained almost exclusively coccoidal forms of 1.5—2 μ diameter. In agreement with our previous experience, the culture grown in the ethanol-containing medium was composed of rods, sometimes slightly bent, which corresponded morphologically to the *Mb. omelianskii* described by Barker [10]. All those forms were Gram variable. Attempts to isolate pure strains form the enrichment cultures have so far failed. As soon as a single colony form a semi-solid medium was transferred to a fresh medium, the growth ceased in spite of great precautions for the maintenance of anaerobiosis.

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