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On Vitamins in Sewage Sludge

VII. Production of Vitamin B_{12} 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 with and without the addition of various compounds. The ability of the culture to produce vitamin B_{12} factors was investigated.

The culture was shown to produce one single vitamin B_{12} factor when grown in the simple Barker medium. This factor has an R_c-value = 0.7 (R_F-value relative to that of cyanocobalamin) and could not be identified with any other factor. It is called factor Met. In the presence of Co^{2+}, factors B and Z were produced simultaneously with factor Met. It is suggested that both these latter factors are precursors of factor Met and cyanocobalamin. When supplied with 5,6-dimethyl benziminazole and Co^{2+}, the culture produced only cyanocobalamin, the yield of which was 80 mμg/ml. The addition of Bacto Peptone stimulated the formation of vitamin B_{12} activity, mainly due to the formation of factor Met. Cyanocobalamin was formed only when 5,6-dimethyl benziminazole was supplied and its yield was not increased by Bacto Peptone. It is suggested that the enrichment culture has a limited synthetic ability with respect to 5,6-dimethyl benziminazole but can produce all the other parts of the vitamin B_{12} molecule and join them together. Unlike 5,6-dimethyl benziminazole, adenine could not be used by the culture to produce $\psi-B_{12}$. The ability of sterilized sewage and sewage sludge to maintain the growth and vitamin B_{12} production of the enrichment culture was also investigated.

The culture did not produce any vitamins of the folic and folinic acid groups. Cu++ was found to have an inhibiting effect upon the growth and vitamin B_{12} production of the culture.

The anaerobic bacterium *Methanobacterium omelianskii* was isolated in pure culture by Barker in 1939[1]. This organism appears to be one of the most abundant bacteria active in the anaerobic digestion of sewage sludge[2]. It has been shown in previous reports from this laboratory[3] that the anaerobic digestion of sewage sludge is accompanied by a considerable production of vitamin B_{12} factors. It was therefore interesting to investigate the ability

of *Methanobacterium omelianskii* to produce vitamin B_{12} factors. A pure strain of *Methanobacterium omelianskii* was received through the courtesy of Professor Barker. However, this strain proved to be very difficult to handle and, in fact, we were not able to obtain an active, quickly fermenting culture from it.

Barker's method was used to isolate a strain of *Methanobacterium omelianskii* from digesting sewage sludge but required, of course, a considerable amount of time. The ability of this strain to produce vitamin B_{12} will be investigated in the near future. In the present work, the authors used enrichment cultures of *Methanobacterium omelianskii* prepared from digesting sludge by successive reinoculations into the medium of Barker [1]. Microscopic examinations of such cultures revealed that they consisted mostly of *Methanobacterium omelianskii*. They proved to be very convenient for large experimental series since, with the chosen, carefully standardized conditions, the gas production started relatively soon after the inoculation and it was found that the agreement between the parallel series was very good. The determination of the gas production is of special importance when culturing methane bacteria since these bacteria tend to form bulky precipitates and occur mostly in the sediment thus making accurate turbidity measurements impossible. The quantity of gas evolved is a good measure of the amount of growth.

Using such an enrichment culture of *Methanobacterium omelianskii*, the authors investigated its ability to produce vitamin B_{12} factors, partly in the simple medium elaborated by Barker [1], which contains ethanol as the only organic compound, and partly in the Barker medium with various additions, *e.g.* organic nutrient extracts, Co^{2+}, Cu^{2+}, 5,6-dimethyl benziminazole, adenine. The ability of the culture to ferment sterilized sewage and sewage sludge was also investigated.

EXPERIMENTAL

The enrichment culture of *Methanobacterium omelianskii* was prepared by successively reinoculating a raw culture obtained from digesting sludge using the medium given by Barker [1]: K_2HPO_4 0.6 %; KH_2PO_4 0.9 %; $(NH_4)_2SO_4$ 0.03 %; $MgSO_4 \cdot 7\ H_2O$ 0.01 %; $FeSO_4 \cdot 7\ H_2O$ 0.001 %; saturated $CaSO_4$ solution 1 %; C_2H_5OH 2 % and tap water. An additional component (2 ml of 1 % $Na_2S \cdot 9\ H_2O$ solution +8 ml of 5 % $Na_2CO_3 \cdot 10\ H_2O$ solution) was sterilized separately and added to every 100 ml of the medium.

A few milliliters of digesting sludge were put into 300 ml substrate in a conical flask and incubated at 37°C. When the gas evolution became considerable, a reinoculation was done in the same way, followed by a second reinoculation and so on. After 10—15 reinoculations the culture was almost totally free from the initial sludge solids.

The inocula for the experimental series were prepared from the enrichment culture by reinoculating into the same medium in conical flasks of 100—300 ml capacity and incubating until a considerable gas evolution was established (usually 2—10 days). During the earlier part of the work, longer periods, 7—10 days, were usually required to obtain an active inoculum whereas, in the later series, an active inoculum could be obtained even after 2—3 days.

The experimental series were performed in test tubes of 15 ml capacity. Each series consisted of several identical tubes which were removed one by one during the course of the experiment, their contents being analyzed for vitamin B_{12} activity. Each tube contained a small gas tube and was filled with 5 ml of the medium. After sterilization, 0.5 ml of the separately sterilized component was put into each tube and thereafter, at an appropriate temperature, 5 drops of the inoculum were released from a pipet.

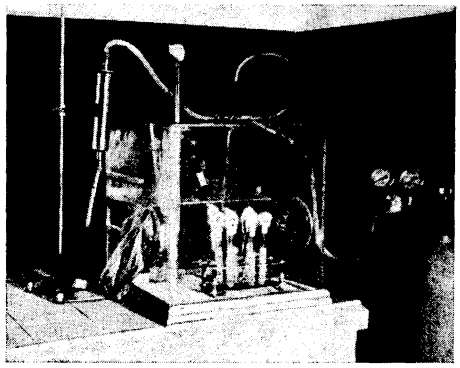


Fig. 1. Carbon dioxide chamber for inoculating methane bacteria. For description see text.

To *ensure anaerobic conditions* all manipulations, *e. g.* necessary additions to the medium, inoculations and reinoculations, were performed in a specially constructed chamber (Fig. 1). The chamber consisted of a box (40 × 40 × 40 cm) five sides of which consisted of plexiglas plates firmly attached to each other whereas the sixth consisted of a wooden base plate provided with tightening arrangements along its edges. The upper (plexiglas) part could be easily removed from the bottom plate so that the various vessels could be put inside. The two parts of the box fitted tightly together. The chamber was provided with gas inlets and outlets, a few openings for the insertion of pipets and two openings with plastic sleeves for the hands. Compressed carbon dioxide or nitrogen could be passed into the chamber at its bottom and removed at the top. Both gases passed a microbiological filter before entering the chamber. The substrate-containing vessels were put into the chamber while still hot after the sterilization in the autoclave, nitrogen being blown through the chamber at 0.5 kg/cm². The extra component was then added to the substrate. When the temperature of the substrate had reached 40 – 30°C, carbon dioxide instead of nitrogen was blown * through (0.5 kg/cm²) and the reinoculations were performed.

All the cultures were incubated at 37°C.

To *maintain the anaerobic conditions after the inoculation* the liquid surfaces in the tubes as well as in the conical flasks were covered with a 0.5 – 1 cm thick layer of sterilized paraffin oil. During the first of these investigations, the test tubes and flasks were also plugged with cotton wool impregnated with an alkaline pyrogallol solution (containing equal parts of 20 % pyrogallol solution and 20 % K_2CO_3 solution). This additional security was found superfluous in later work and the tubes were plugged with ordinary cotton wool.

Sampling was achieved by removing a tube from each series at appropriate time intervals. The samples were autoclaved with cyanide (100 μg KCN/ml sample) and kept at −20°C. Their activity for *E. coli* 113 – 3 and, in a few cases, also for *S. faecalis* and *Leuconostoc citrovorum*, was determined in the cup plate assay. The presence of different vitamin B_{12} factors [4] was revealed by bioautography on *E. coli* plates using chromatography in two solvent systems as described elsewhere [5] and by ionophoresis according to Holdsworth [6]. The R_F-values of the chromatographic spots are always given relative to that of cyanocobalamin and are called R_c. Two groups of experiments were performed. In the first group (expts 1 – 4) an inoculum was used which had been obtained by ten successive reinoculations of the raw culture into the medium of Barker [1]. The second group of experiments was performed several months later and the inoculum was obtained

* Carbon dioxide was not introduced from beginning in order to avoid pH-changes in the substrate.

by twenty five successive reinoculations. The details of the different experiments were as follows:

Experiment 1. Influence of different nutrients. Preliminary investigation. The following additions to Barker's medium were made: Co^{2+} 2 500 mμg/ml; Bacto Vitamin-free Casamino Acids (Difco) 0.25 %; Bacto Yeast Extract Dehydrated (Difco) 0.25 %; Bacto Peptone (Difco) 0.25 %; biotin 0.002 ppm. Samples were taken 3, 10, 17 and 30 days after the beginning of gas evolution. The results are given in Table 1.

Experiment 2. Influence of 5,6-dimethyl benziminazole and adenine. The two bases were added to the medium at the following levels: 100; 1 000 and 10 000 mμg/ml. The simple Barker medium was used both without any addition (a) and also with the addition of 0.25 % Bacto Peptone (b). Samples were taken 3, 10, 17 and 30 days after the beginning of gas evolution. The results can be seen in Table 2 and Fig. 2.

Experiment 3. Influence of Co^{2+}. The following additions of Co^{2+} were used: 0; 1 000; 2 000; 5 000; 10 000 and 25 000 mμg/ml*. The Co^{2+} was added to the medium as $CoCl_2 \cdot 6H_2O$. Barker's medium without (a) and with 0.25 % Bacto Peptone (b) was used. Samples were taken after 3, 10, 17 and 30 days. The results are given in Table 3.

Experiment 4. Influence of Cu^{2+}. The following additions of Cu^{2+} were used: 0; 1 000; 2 000; 5 000; 10 000 mμg/ml. The Cu^{2+} was added to the medium as $CuSO_4 \cdot 5 H_2O$. Barker's medium without (a) and with 0.25 % Bacto Peptone (b) was used. Samples were taken after 3, 10, 17 and 30 days. The results can be seen in Table 4.

Experiment 5. Influence of Co^{2+} and of 5,6-dimethyl benziminazole in the simple medium. Three parallel series were performed with (I) the medium of Barker (a), (II) (a) with added 3 000 mμg Co^{2+}/ml, (III) (a) with added 3 000 mμg Co^{2+}/ml and 1 000 mμg 5,6-dimethyl benziminazole/ml. Samples were taken every day. The results are given in Figs. 2a, 2b and 2c.

Experiment 6. Influence of Co^{2+} and 5,6-dimethyl benziminazole in the presence of Bacto Peptone. This experiment was performed in exactly the same way as expt. 5, except that the Barker medium was supplemented with 0.25 % Bacto Peptone. The results can be seen in Figs. 3a, 3b and 3c.

Experiment 7. In this experiment the ability of five "natural" substrates to maintain the growth of the enrichment culture was investigated. The substrates were taken from a sewage plant where the sewage is sedimented and where the sedimented solids (fresh sludge) undergo digestion in digestion tanks. Liquid separated during the digestion process is withdrawn and is called "digested sludge water". The effluent from the sedimentation tanks is not further treated.

The following five substrates were used in the experiment: incoming sewage, I; effluent, E; digested sludge water, W; fresh sludge, F; digested sludge diluted 100 times, D. The substrates were sterilized by tyndallization. F was diluted 10 times and D 10 times (called Dl). A part of D was alkali treated (called D2). I, E, and W were not diluted. The substrates were buffered with phosphate (total concentration 1.5 %) to pH 7.7.

The final substrates thus obtained had the following dry solid contents and *E. coli* activities:

Substrate	Dry solids mg/ml	*E. coli* activity calc. as cyanocobalamin mμg/ml
I	0.7	0.5
E	0.6	0.5
W	1.9	1.5
F	3.0	0.5
D	0.9	2.5
Dl	0.09	2.0
D2	0.9	—

Conical flasks of 100 ml capacity were filled with the different media, sterilized and inoculated with the enrichment culture. The results are given in Table 5.

* Due to the presence of S^{2-} the actual concentration of Co^{2+} in the fermentation liquor was probably lower than indicated by the additions.

RESULTS AND DISCUSSION

Experiment 1. Yeast extract, peptone and casamino acids stimulated considerably the growth of the culture as deemed by the gas evolution. Yeast extract was most effective in this respect — the gas started to evolve after only 60 h of incubation as compared with about 100 h for the cultures containing the other two organic nutrients. The addition of Co^{2+} (2.5 μg/ml) in the absence of organic nutrients seemed to retard the growth of the culture — the gas evolution starting after ~ 200 h of incubation. This phenomenon, however, could not be observed in later experiments in which a more purified enrichment culture was used. Biotin was without effect upon the growth.

The effect of the nutrient additions upon the formation of vitamin B_{12} factors can be seen in Table 1. The total *E. coli* activity is markedly stimulated by the different additions, except biotin. The stimulating effect depends

Table 1. Influence of different nutrients on vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii;* medium according to Barker; underlined factors occur in the largest amounts and the factors within brackets in the smallest amounts.

Met designates a factor — not yet identified — which is specifically produced by the culture. It has a R_c-value = 0.7 in solvent system I[5].

Additions	*E. coli* activity in samples taken			
	after 3 days of fermentation		after 10 days of fermentation	
	mμg/ml *	factors	mμg/ml *	factors
No addition	30	Met; B; (Cy); (Z)	40	Met
Co^{2+} 2 500 mμg/ml	200	B; (Met); (Z)	120	B; (Met); (Z)
Casaminoacids 0.25 %	50	Met; B; Z (III)	80	Met
Casaminoacids 0.25 % + Co^{2+} 2 500 mμg/ml	60	Met; Z; B	110	Met
Yeast extract 0.25 %	80	Met; III; (Z) (B)	80	Met
Yeast extract 0.25 % + Co^{2+} 2 500 mμg/ml	120	Met; Z; (B)	100	Met
Bactopeptone 0.25 %	90	Met; III; Z; (Cy)	150	Met
Bactopeptone 0.25 % + Co^{2+} 2 500 mμg/ml	90	Met; III; Z; (Cy)	90	Met
Casaminoacids 0.25 % + biotin 2 mμg/ml	30	Z; (B)	70	Met
Casaminoacids 0.25 % + biotin 2 mμg/ml + Co^{2+} 2 500 mμg/ml	70	Z; (B)	90	Met
Biotin 2 mμg/ml	20	Met; (Cy); (B)	50	Met
Biotin 2 mμg/ml + Co^{2+} 2 500 mμg/ml	300	B; (Z); (Cy)		B; (Z)

* Calculated as cyanocobalamin, cup plate assay.

probably upon the better growth of the culture obtained with the active nutrients. It can be seen in Table 1 that, at the beginning of the fermentation, the culture produced, in most cases, smaller amounts of several factors together with a greater amount of one single factor whereas after 10 days of fermentation only this latter factor was produced. It is a striking fact that cyanocobalamin was not produced at all. The very small amounts of cyanocobalamin produced in the beginning of the fermentation could not be found in later investigations with a more purified enrichment culture. The addition of Co^{2+} alone, or together with biotin, markedly stimulated the formation of factor B. It is easily seen in Table 1 that biotin does not contribute to this effect. Since factor B has been shown to be the fundamental part of all B_{12} vitamins [7] so far characterized, the phenomenon observed suggests that the enrichment culture has a limited synthetic ability with respect to the other parts of the vitamin B_{12} molecule. From Table 1, it seems evident that the enrichment culture of *Methanobacterium omelianskii*, grown in the simple Barker medium, produces specifically one single vitamin B_{12} factor, albeit in limited amounts. This has also been proved by all later experiments. So far we have not succeeded in identifying this factor with any other vitamin B_{12} factor hitherto described (*cf.* p. 1165). It is provisionally called factor Met. The culture produces greater amounts of this factor when supplied with some organic nutrient (see Table 3). It cannot, however, be concluded whether these additions stimulate the formation of factor Met *per se* or if the effect depends merely upon the enhanced growth of the culture.

Table 2. Influence of 5,6-dimethylbenziminazole and adenine on vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii;* a — medium according to Barker; b — medium according to Barker + 0.25 % Bactopeptone; DMB — 5,6-dimethyl benziminazole; Ad — adenine.

Medium	Additions mμg/ml medium	Largest *E. coli* activity obtained (usually in samples taken after 20—30 days of fermentation)	
		mμg/ml *	factors present
a	No	15	Met
b	No	30	Met
a	DMB 100	25	cyanocobalamin
a	Ad 100	20	Met
b	DMB 100	20	cyanocobalamin
b	Ad 100	45	Met
a	DMB 1 000	30	cyanocobalamin
a	Ad 1 000	40	Met
b	DMB 1 000	40	cyanocobalamin
b	Ad 1 000	40	Met
a	DMB 10 000	10	cyanocobalamin
a	Ad 10 000	40	Met
b	DMB 10 000	15	cyanocobalamin
b	Ad 10 000	60	Met

* Calculated as cyanocobalamin.

Since Bacto Peptone evidently had the greatest stimulating effect upon the formation of factor Met, we chose this nutrient as a standard addition to the medium in several further experiments.

Experiment 2. This experiment was performed in order to elucidate whether the formation of factor Met by the enrichment culture is to be considered as an individual biochemical feature of the culture or whether it was merely caused by absence of precursors for other vitamin B_{12} factors when the culture was grown in the simple Barker medium [1] or in this medium modified as in expt. 1.

It can be seen in Table 2 that the culture produces cyanocobalamin when supplied with the specific base of this factor — 5,6-dimethyl benziminazole. Adenine, however, which is the specific base of ψ-B_{12}, cannot be utilized by the culture to produce the corresponding factor. Instead of ψ-B_{12}, factor Met is produced as in the case without the addition of adenine. These results suggest that the organism is unable to synthesize 5,6-dimethyl benziminazole but can synthesize all the other parts of the cyanocobalamin molecule and couple them together. Such a suggestion cannot of course be conclusive since only an enrichment culture, and not a pure strain, was used in this experiment. It would be interesting to see if some compounds of adenine, *e.g.* the appropriate nucleoside and nucleotide, can be utilized in a better way by the culture. Such compounds, however, were not available in this preliminary study.

For subsequent experiments, 1 000 mμg/ml of 5,6-dimethyl benziminazole was chosen as a standard addition.

Experiment 3. The highest total *E. coli* activity was obtained with 2 000 —5 000 mμg Co^{2+}/ml of medium a (*cf.* Table 3). The activity values reached,

Table 3. Influence of Co^{2+} on vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii;* a — medium according to Barker [1]; b — medium according to Barker +0.25 % Bactopeptone.

Medium	Amount of Co^{2+} in the medium mμg/ml	*E. coli* activity in samples taken after 10 days of fermentation **	
		mμg/ml *	factors present
a	0	10	Met
b	0	30	Met
a	1 000	480	Met (B)
b	1 000	140	Met; B; Cy; (Z)
a	2 000	550	Met; (B); (Cy)
b	2 000	150	Met; (B); (Cy)
a	5 000	640	Met; B; (Cy); (Z)
b	5 000	160	Met; B; Cy; Z
a	10 000	470	Met; B
b	10 000	110	Met; B
a	25 000	490	Met; (B); (Z)
b	25 000	310	Met; (B); (Z)

* Calculated as cyanocobalamin.

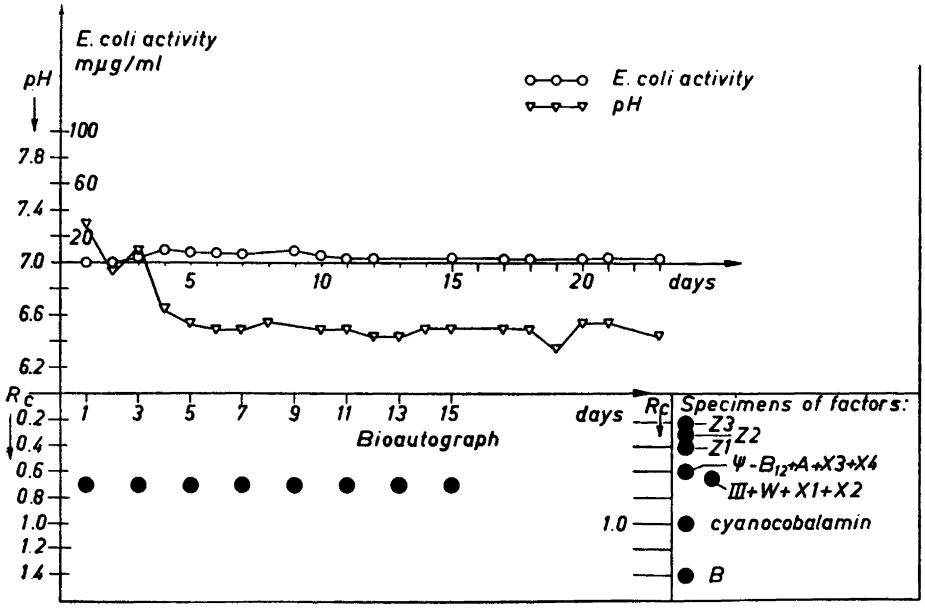
** These samples contained the highest activity obtained in the series.

Table 4. Influence of Cu^{2+} on vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii;* a — medium according to Barker [1]; b — medium according to Barker + 0.25 % Bactopeptone.

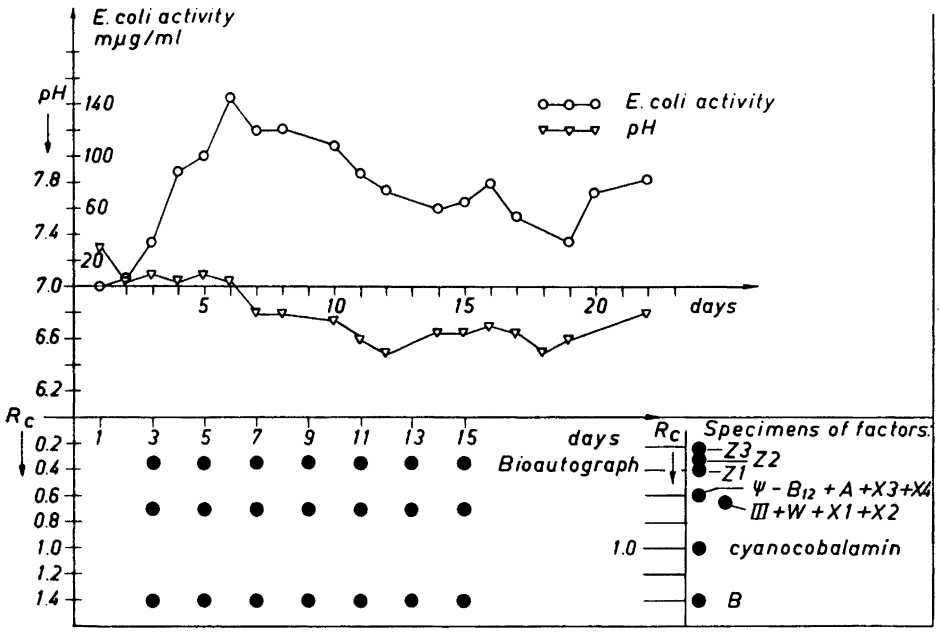
Medium	Amount of Cu^{2+} in the medium mμg/ml	*E. coli* activity in samples taken after 20 days of fermentation **	
		mμg/ml *	factors present
a	0	20	Met
b	0	100	Met
a	1 000	20	Met
b	1 000	30	Met
a	2 000	10	Met
b	2 000	30	Met
a	5 000	9	Met
b	5 000	30	Met
a	10 000	7	Met
b	10 000	30	Met

* Calculated as cyanocobalamin.
** These samples contained the highest activity obtained in the series.

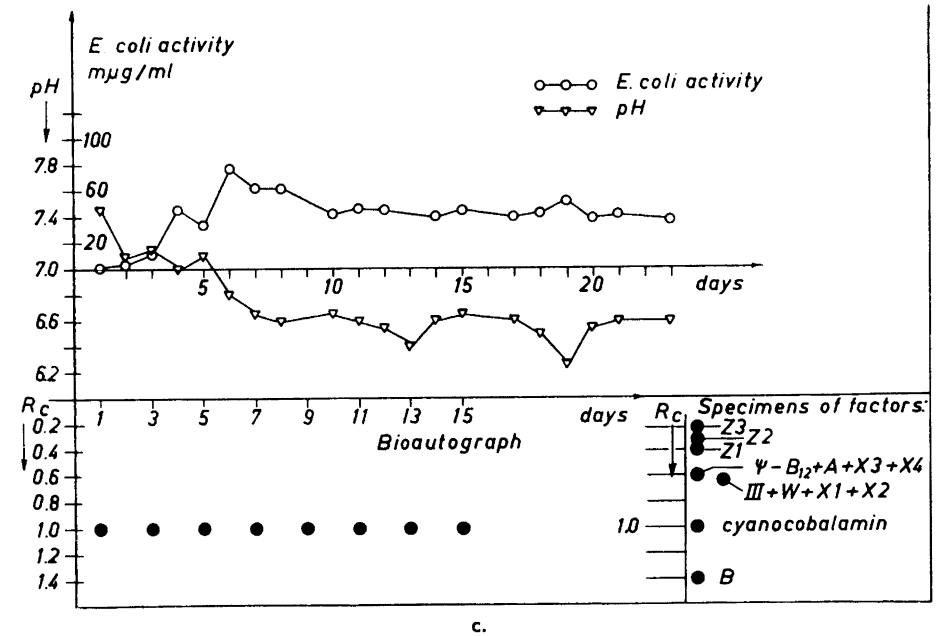
640 mμg/ml (calculated as cyanocobalamin), are much higher than the corresponding values obtained in expt. 1. The latter values were also confirmed in later experiments. The main factor produced was factor Met. With 5 000—10 000 mμg Co^{2+}/ml, considerable amounts of factor B were



a.



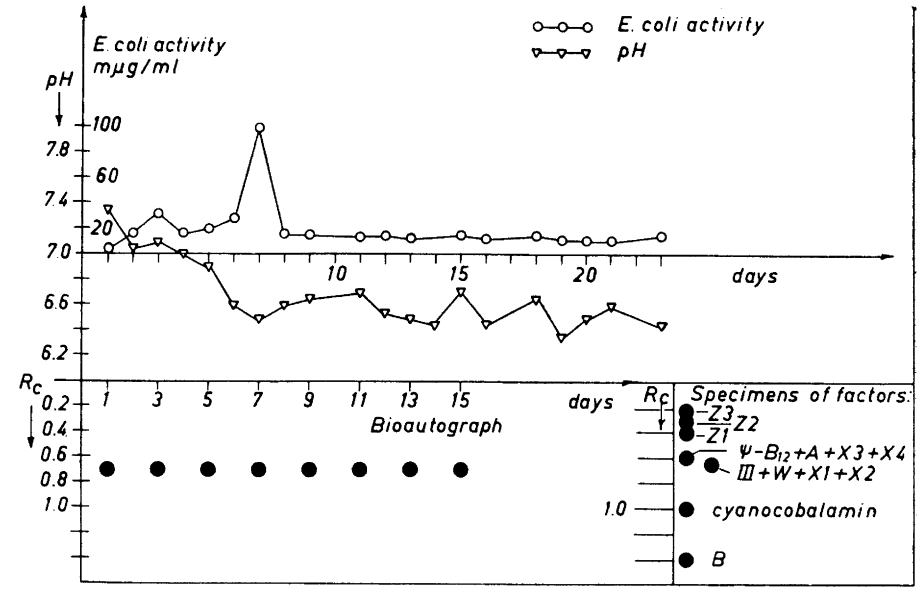
b.



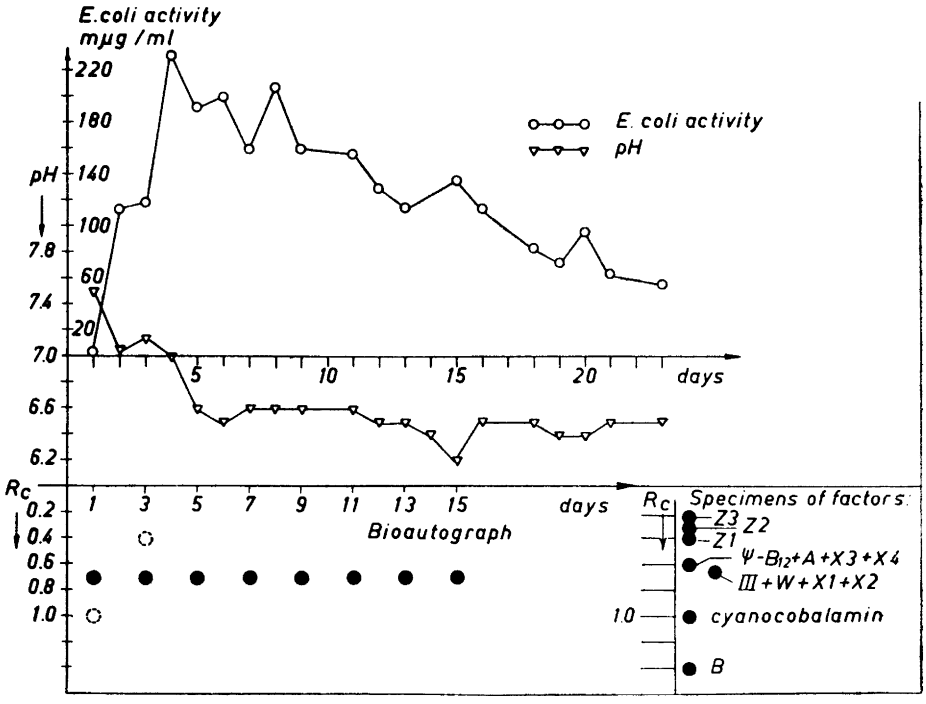
c.

Fig. 2. Fermentation and vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii* grown in the medium of Barker:

a. not supplemented;
b. supplemented with Co^{2+} (3 000 mμg/ml);
c. supplemented with Co^{2+} (3 000 mμg/ml) and 5,6-dimethyl benziminazole (1 000 mμg/ml).


E. coli activity
pH
E. coli activity
mμg/ml
pH
100
60
20
7.8
7.4
7.0
6.6
6.2
10
15
20
days
Rc
0.2
0.4
0.6
0.8
1.0
1
3
5
7
9
11
13
15
days
Bioautograph
Rc
Specimens of factors:
Z3
Z2
Z1
Ψ-B12+A+X3+X4
III+W+X1+X2
1.0
cyanocobalamin
B

a.


E.coli activity
mμg /ml
220
180
140
100
60
20
pH
7.8
7.4
7.0
6.6
6.2
E. coli activity
pH
5
10
15
20
days
Rc
0.2
0.4
0.6
0.8
1.0
1
3
5
7
9
11
13
15
days
Bioautograph
Rc
Specimens of factors:
Z3
Z2
Z1
Ψ-B12+A+X3+X4
III+W+X1+X2
1.0
cyanocobalamin
B

b.

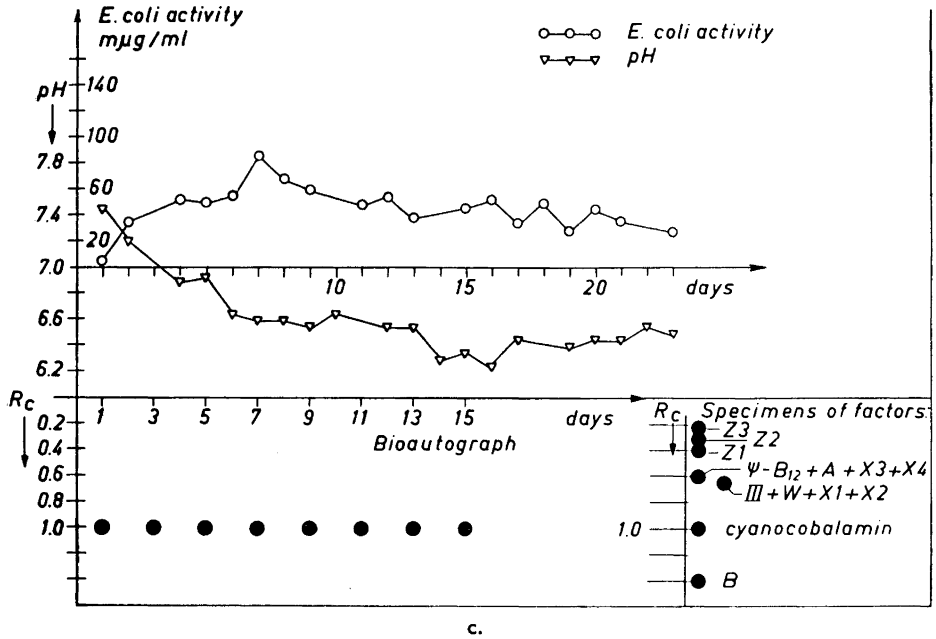


Fig. 3. Fermentation and vitamin B_{12} production by an enrichment culture of *Methanobacterium omelianskii* grown in the medium of Barker modified by the addition of 0.25 % Bacto Peptone:

a. not supplemented;
b. supplemented with Co^{2+} (3 000 mμg/ml);
c. supplemented with Co^{2+} (3 000 mμg/ml) and 5,6-dimethyl benziminazole (1 000 mμg/ml).

also formed. For subsequent experiments, 3 000 mμg/ml of Co^{2+} was chosen as standard addition.

Experiment 4. 1 000—2 000 mμg Cu^{2+}/ml had a retarding effect upon the growth and vitamin B_{12} production of the enrichment culture. 5 000—10 000 mμg Cu^{2+}/ml markedly inhibited both growth and vitamin B_{12} production (*cf.* Table 4).

Experiment 5. The results of this experiment which has been performed more thoroughly and with a more purified enrichment culture than expts. 1—4 confirm the preliminary findings of these early experiments. An examination of Fig. 2a, 2b and 2c leads to the following conclusions:

1) When grown in the simple Barker medium, the enrichment culture of *Methanobacterium omelianskii* produces one single factor Met (with $R_c = 0.7$) as shown by chromatography and ionophoresis (*cf.* p. 1165). The yield of factor Met is rather low and corresponds to at most 10 mμg/ml calculated as cyanocobalamin (*cf.* Fig. 2a).

2) When supplied with Co^{2+} (3 000 mμg/ml), the culture produces factors B and Z together with factor Met suggesting that both the two former factors

are precursors of factor Met and cyanocobalamin. The total *E. coli* activity is at most 140 mμg/ml calculated as cyanocobalamin (*cf.* Fig. 2b).

3) With Co^{2+} (3 000 mμg/ml) and 5,6-dimethyl benziminazole (1 000 mμg/ml), the culture produces only cyanocobalamin, the yield of which is at most 80 mμg/ml (*cf.* Fig. 2c).

Experiment 6. It can be seen from Fig. 4a that the addition of Bacto Peptone to the simple Barker [1] medium stimulates the formation of *E. coli* activity (*cf.* Fig. 3a) but does not influence the kind of factors formed since, as before, factor Met is exclusively produced. With Co^{2+} (3 000 mμg/ml) in the modified medium, only factor Met is again formed (see Fig. 3b) except in the beginning of the fermentation when small amounts of cyanocobalamin and of one of the factors Z are formed. The fermentation in the presence of Bacto Peptone differs in this respect markedly from that without this nutrient (*cf.* Fig. 2b). The total *E. coli* activity in the presence of Bacto Peptone is much higher than in experiment 5 and reaches at most 240 mμg/ml. With additions of Co^{2+} and 5,6-dimethyl benziminazole (see Fig. 3c), cyanocobalamin is formed exclusively as in the corresponding experiment without Bacto Peptone (*cf.* Fig. 2c). The yield of cyanocobalamin is likewise of the same order of magnitude, *viz.* 85 mμg/ml.

Experiment 7. It can be seen in Table 5 that the sterilized and buffered effluent E and digested sludge, diluted 100 times, D, and 1 000 times, D1, could not be fermented by the enrichment culture. However, sterilized and buffered incoming sewage, I, digested sludge water, W, alkali treated diluted digested sludge, D2, and fresh sludge, F, could maintain the growth of the culture. The total *E. coli* activity/ml substrate was largest with fresh sludge (70 mμg/ml) and lowest with incoming sewage and alkali treated digested sludge (~15 mμg/ml. However, calculated on the basis of initial dry solid content, the yields obtained with fresh sludge and incoming sewage were

Table 5. The ability of sterilized sewage to maintain the growth and vitamin B_{12} production of an enrichment culture of *Methanobacterium omelianskii.*

Medium *	Initial pH	Begin. of gas evol. days	pH and *E. coli* activity in samples taken after 7 days of fermentation			15 days of fermentation		
			pH	*E. coli* activity ** mμg/ml	*E. coli* activity ** mμg per g initial solids	pH	*E. coli* activity ** mμg/ml	*E. coli* activity ** mμg per g initial solids
I	7.7	7	6.8	15	21 000	6.7	15	21 000
E	7.7	No evol.						
W	7.6	6	6.5	30	16 000	6.3	20	11 000
F	7.5	3	6.7	70	23 000	6.6	70	23 000
D	7.6	No evol.						
D1	7.7	No evol.						
D2	7.7	6	6.8	15	17 000	6.7	10	11 000

* For the characteristics of the different media, *cf.* p. 1156.
** Calculated as cyanocobalamin.

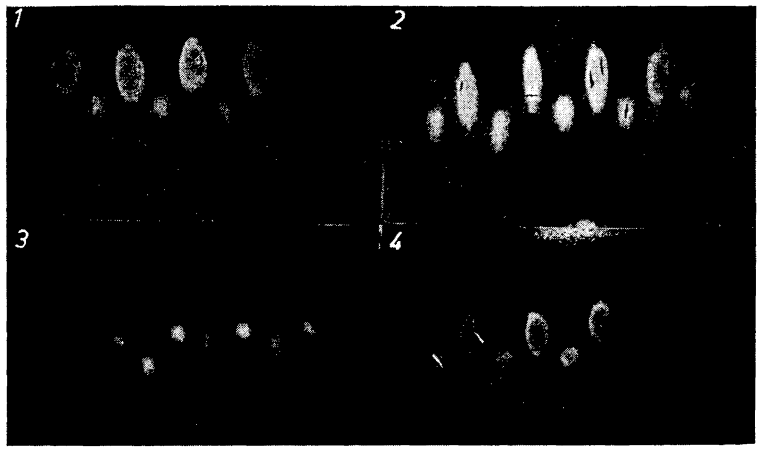


Fig. 4. Experiments to identify factor Met by paper chromatography. Bioautographs on *E. coli* 113—3 plates.
1. Factor A (upper spots) and factor Met (lower spots). 2. Factor X4 (upper spots) and factor Met (lower spots). 3. Factor W (upper spots) and factor Met (lower spots). 4. Factor III (upper spots) and factor Met (lower spots).

comparable ($\sim$ 20 000 mμg/g) and comparable with that of spontaneously digested sewage sludge. These yields are considerably higher than the yield obtained with the other substrates. Paper chromatography revealed two spots, one due to cyanocobalamin, the other due to some factor or factors with R_c in the range 0.6—0.7. As reported above, the factor produced by *Methanobacterium omelianskii* in synthetic substrates, factor Met, has a R_c-value = 0.7 and it might be that this factor is also produced when the enrichment culture is grown in sterilized sewage. This, however, has not yet been more closely investigated.

Experiments to identify factor Met. When chromatographed in solvent system I [5], factor Met moved distinctly quicker than all the other factors with R_c-values in the range 0.6—0.7. Some of the experiments are shown in Fig. 4, which contains bioautographs of some of the factors together with factor Met. Spots of factor Met were put on each chromatogram alternately with spots of the respective factors. The uncertainty due to uneven wandering of the solvent front was thus eliminated.

However, upon ionophoresis in 2 M HAc in the presence of KCN, the mobility of factor Met was found to be almost identical with that of factor W, factor III, cyanocobalamin and of the slower fraction of factor X4.

The latter factor, though found a homogeneous three years ago, has now been found to separate into two electrophoretic fractions, one with a mobility about that of factor B (or A?) (quick-moving) and the other with a mobility about that of factor Met (*cf.* Fig. 2). The solution of factor X4 was kept at —20°C but had been thawed on several occasions.

As has been noticed many times before, the identification of vitamin B_{12} factors on the basis of their chromatographic and ionophoretic mobilities very often seems to give misleading results.

Vitamins of the folic and folinic acid groups. Fermentations, performed with the enrichment culture of *Methanobacterium omelianskii* in the simple Barker medium with and without the addition of various compounds, were tested for *S. faecalis* and *Leuconostoc citrovorum* activity. No such activity could, however, be detected.

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