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On Vitamins in Sewage Sludge

XI. Further Studies on the Production of Vitamin B_{12} by Some Enrichment Cultures of Methane Bacteria

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Fermentations with a methanol-fermenting and vitamin B_{12}-producing enrichment culture of methane bacteria were performed in order to find the most favourable conditions for obtaining high yields of vitamin B_{12}.

Stirring was not favourable for high yields of *E. coli* activity but, on the other hand, it resulted in the formation of only one vitamin B_{12} factor which seems to be the clinically active factor III of Bernhauer [5] or/and factor W[4], which has in earlier investigations been found to be active for *Ochromonas malhamensis*.

The conversion of the different vitamin B_{12} factors formed in non-stirred cultures to cyanocobalamin could not be achieved by adding greater than previously used amounts of 5,6-dimethyl benziminazole at the start or smaller amounts at a later stage of the fermentation. Concentrations of 10 μg benziminazole per ml medium inhibited the growth of the culture.

Under certain conditions, the addition of glucose was found to stimulate both the evolution of gas and the production of vitamin B_{12} activity. As far as could be observed, the carbohydrate had no effect upon the kind of vitamin B_{12} factors formed in synthetic media.

The addition of certain nitrogenous materials, *e. g.* corn steep liquor or casein hydrolyzates together with glucose, or casein hydrolyzates together with glucose and yeast extract, resulted in a greatly diminished synthesis of vitamin B_{12}. The possible reasons for this phenomenon are discussed.

The addition of sterilized sewage to the medium was found to have a favourable effect on vitamin B_{12} synthesis and can successfully replace the Bactopeptone (Difco) used in earlier experiments.

The greatest utilization of methanol was found to take place in media containing sterilized sewage.

It seems that vitamin B_{12} begins to accumulate in the cells when their metabolic activity (juged by gas evolution and methanol utilization) tends to decrease. However, no correlation between the utilization of methanol and the production of gas and vitamin B_{12} activity could finally be concluded, so far.

It has been reported earlier from this laboratory that enrichment cultures of methane bacteria produce varying amounts of vitamin B_{12} activity [1-3]. The enrichment culture producing the highest activity was the one obtained in a methanol-containing medium [3]. The present investigation is concerned with a further study of the conditions under which such a culture should be grown in order to obtain high yields of the vitamin. Fermentations were carried out in two liter vessels. The intensity of gas evolution was used as an indicator for the intensity of growth since other changes in the culture, *e.g.* turbidity, dry solids content, *etc.* were difficult to follow for reasons discussed elsewhere [1]. In one series of experiments, the influence of stirring was studied both with the enrichment culture obtained in the medium containing ethanol and consisting mainly of *Mb. omelianskii* [1], and with the enrichment culture obtained in the methanol-containing medium [3]. In the rest of the experiments, certain modifications of the earlier adopted medium [3] were studied using only the culture obtained in the methanol-containing medium. The following points were studied: 1. addition of greater than earlier used amounts of 5,6-dimethyl benziminazole (DMB) and variation of the time of this addition; 2. addition of organic nutrients, notably casein hydrolyzates, corn steep liquor, sterilized sewage, whey and glucose; 3. adjustment of the pH every day to the optimum value; 4. adjustment of the methanol concentration in the medium several times during the fermentation period.

Using certain modifications of the medium, it has been possible to obtain *E. coli* activities higher than 10 μg/ml calculated as cyanocobalamin in cup plate assay. These high activities represented, however, mixtures of several vitamin B_{12} factors. Attempts to get all these factors converted by the cultures to cyanocobalamin have not yet been successful, even if the yield of cyanocobalamin could be improved. The dominating factor(s) in the cultures was (were) probably factor W[4] earlier isolated from digested sludge in this laboratory and (or) factor III of Bernhauer[5]. The former factor was found to be active for *Ochromonas malhamensis* [4], which indicates that it may also be active for higher animals. The clinical activity of factor III has been proved [6]. It thus seems to be of considerable interest that the enrichment cultures of the methanol-fermenting organism can produce such high amounts of this(these)physiologically active factor(s).

EXPERIMENTAL

The fermentations were carried out in 2 l conical flasks provided with stoppered outlets at the bottom through which samples could be removed by means of a syringe. The tops of the flasks were closed with rubber stoppers provided with glass tubes containing sterile cotton wool filters, by means of which the flasks were connected, using rubber tubing, with devices for collecting and measuring the evolved gas. The gas collecting devices consisted of scaled cylindrical vessels of 1 l capacity, provided with two outlets, one at the bottom and one at the top. The top outlet was connected to the fermentation vessel whereas the bottom outlet was connected to a separating funnel, which functioned as a levelling flask. The gas was collected over a saturated aqueous solution of NaCl. The apparatus is shown schematically in Fig. 1. Up to six fermentations could be run simultaneously. The fermentation flasks were filled with media, only a little space being left above. The basal medium used in all experiments with the methanol-fermenting organism was the one previously used [3] and had the following composition:

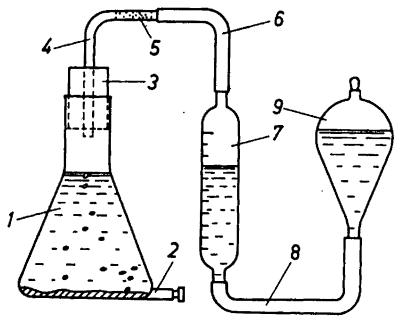

Fig. 1. Apparatus used in fermentations with a methanol-fermenting enrichment culture of methane bacteria. 1 fermentation flask; 2 outlet for taking samples; 3 pierced rubber tap; 4 bent glass tube; 5 cotton wool filter; 6 rubber tubing; 7 calibrated gas collection vessel; 8 rubber tubing; 9 levelling flask.

NH_4Cl	—	1.0 g
K_2HPO_4	—	0.4 g
$MgCl_2 . 6H_2O$	—	0.1 g
$CoCl_2 . 6H_2O$	—	10 mg
CH_3OH	—	10 ml
$CaCO_3$	—	15 g
Asbestos powder	—	5 g
Tap water	—	1 000 ml

Each fermentation flask was filled with 1 600 ml of this medium and, in addition, 160 ml of a separately sterilized solution consisting of 32 ml 1 % $Na_2S . 9H_2O$ solution + 128 ml of 5 % $Na_2CO_3 . 10H_2O$ solution, was added while the medium was still hot after autoclaving. If not otherwise stated, the methanol was autoclaved together with the medium. The pH of the final medium M was adjusted with separately sterilized HCl (1:5) to 6.5—7.5. The flasks were cooled in a nitrogen atmosphere, inoculated with 100 ml of the actively growing enrichment culture and incubated at 37°C for 2—3 weeks. Samples were removed every day and assayed for vitamin B_{12} activity as described elsewhere [7]. The gas evolved and the pH of the medium were also measured daily in all fermentations. The gas evolution is given in the tables and figures as the number of ml evolved from a 2 l vessel in 1 h. The vitamin B_{12} activity is given as the activity for *E. coli* 113-3 calculated as cyanocobalamin in the plate assay. In some experiments, determinations of reducing substances [8] and of methanol according to the *Official Methods of Analysis* [9] were performed. In some experiments, certain fractions of sterilized sewage were used as a substrate after supplementation with the components of medium M.

Further details of the different experiments are given below.

I. *Influence of stirring.* The influence of stirring was studied *a.* with the methanol-fermenting organism, and *b.* with the ethanol-fermenting one. The stirring was achieved by means of a magnete. Media: *a.* (M) supplemented with Bactopeptone, 0.25 %; *b.* Barker's medium (used already in earlier experiments [1]). In some vessels, the fermenting medium was covered with a layer of paraffin oil. The results are given in Tables 1 and 2.

II. *Influence of DMB.* The influence of DMB was studied only with the methanol-fermenting organism.

The purpose of this experiment was to investigate whether concentrations of DMB greater than those previously used or adding the DMB at a later stage of fermentation could lead to greater yields of cyanocobalamin at the expense of the other vitamin B_{12} factors. Medium: M supplemented with 0.25 % Bactopeptone and with *a.* 1 μg or 10 μg DMB/ml medium added at the beginning of the fermentation, or *b.* 1 μg DMB added after 8 days' fermentation. The results are shown in Figs. 2a and 2b.

III. *Influence of carbohydrates.* The influence of carbohydrates was studied only with the methanol-fermenting organism.

a. Glucose (10 g/l) was added to the medium M and its content as well as the pH were readjusted to their initial values four times during the fermentation period. DMB

was present in the medium from the beginning at a level of 1 μg/ml. *b.* Glucose was added to the medium M as in *a*, and its content as well as the pH were readjusted to their initial values every day. Methanol was added aseptically and its content readjusted to 1 % several times during the fermentation period. DMB was added after eight days' fermentation at a level of 1 μg/ml. *c.* Milk whey was mixed with sterilized sewage in the proportion 1:1. Methanol was added aseptically and its content readjusted several times to 1 %. DMB was present in the medium from the beginning.

For comparison parallel fermentations in all three cases, *a*, *b*, and *c*, were performed without the addition of carbohydrate. The results of these experiments are given in Figs. 3a, 3b and in Table 3.

IV. *Influence of organic nitrogen sources and yeast extract.* The influence of these materials was studied only with the methanol-fermenting organism.

In all fermentations of this series, methanol was sterilized separately and its content in the medium adjusted four times during the fermentation period. If not otherwise stated, DMB was added after 8 days' fermentation at a level of 1 μg/ml medium. The different supplements to the medium M were as follows:

a. Bactopeptone (Difco) 0.25 %; DMB.
b. Corn steep liquor added at a level corresponding to 0.4 % total nitrogen or ~0.2 % available nitrogen; DMB added at the beginning of the fermentation.
c. Acid hydrolyzed casein (Casaminoacids Vitaminfree, Difco) added at a level corresponding to 0.11 % total nitrogen; trypsin hydrolyzed casein (Bactogen, Vitrum) added at a level corresponding to 0.16 % total nitrogen; glucose 1 %; DMB added at the beginning of the fermentation.
d. Nitrogen sources and glucose as in *c.*; Yeast Extract (Difco) 0.25 %; DMB.
e. The medium consisted of sterilized sewage supplemented with the components of medium M and with DMB.
f. Medium as in *e*, further supplemented with glucose 1 % and Yeast Extract (Difco) 0.25 %.

The results of this experiment are shown in Figs. 4a, 4b, 4c, 4d, 4e and 4f.

V. *Utilization of methanol.* Fermentations were performed as in the other experiments using the methanol-fermenting organism and six different modifications of the basal medium M. The modifications used are given in Table 4. The amounts of the different materials added to medium M were the same as in corresponding additions applied in

Table 1. The influence of stirring on the gas production and vitamin B_{12} activity of an enrichment culture of a methanol-fermenting methane organism. Experimental details in text.

Time of fermentation h	Gas evolution ml/h *			*E. coli* activity μg/ml **		
	stirring	stirring + oil layer	no stirring	stirring	stirring + oil layer	no stirring
0	0	0	0	0	0	0
20	17	14	34		0.1	
25	22	25	50	0.1	0.1	0.5
28	23		53			
44	32	30	70	0.3	0.3	1.0
53	35		80			
92	30	22	70	0.8	0.8	4.6
116	25	20	60	0.9	0.8	4.6
140	23		50	1.0		4.6
164	17	24	46	1.0	0.9	4.6

* evolved in a 2 liter fermentation flask
** calculated as cyanocobalamin in plate assay

the experimental series III and IV. In all fermentations in this series, DMB was added after 8 days. MeOH was added aseptically to the sterile medium and its content determined three times during the fermentation period. After each determination, the level of the methanol in the medium was readjusted to its initial value (1 %). The results of these experiments can be seen in Table 4.

RESULTS AND DISCUSSION

I. *Influence of stirring.* It seems from Table 1 that stirring is unfavourable for both gas evolution and production of vitamin B_{12} in fermentations with the methanol-fermenting organism. In fermentations with *Mb. omelianskii* (see Table 2) the use of stirring together with a covering oil layer gave higher values for the *E. coli* activity than those obtained with stirring alone. This may be explained by the fact that stirring facilitates the distribution of the oxygen which has diffused in from the surrounding air and which is harmful to the growth and, thus also, to the production of vitamin B_{12}. Since the evolution of gas in this fermentation is very slow, the diffusion of oxygen from the air may be quite important if oil is not used. The *E. coli* activity values obtained in the fermentations with *Mb. omelianskii* are so small that no further conclusions can be made about the influence of stirring.

In the methanol fermentation (see Table 1) an oil layer together with stirring does not give a higher gas evolution than that obtained with stirring alone whereas the stationary fermentation gives the highest gas evolution and vitamin B_{12} activity. Thus the unfavourable effect of stirring upon the production of gas and vitamin B_{12} activity cannot be explained by the distribution throughout the medium of the diffused oxygen. Besides, the evolution of gas is so strong in this fermentation that a diffusion of oxygen into

Table 2. The influence of stirring on the gas production and vitamin B_{12} activity of an enrichment culture of *Mb. omelianskii.* Experimental details in text.

Time of fermentation h	Gas evolution ml/*h* *			*E. coli* activity μg/ml **		
	stirring	stirring + oil layer	no stirring	stirring	stirring + oil layer	no stirring
20	6	1	6			
28	14	7	8			
42	9	4	9	0.001	0.001	0.001
50	7	5	2			
66	4	8	2			
74	1	11	1			
90	0.5	8	1	0.001	0.003	0.001
115	0.1	2	0.6			
137	0.2	0.2	0.2	0.001	0.003	0.001
162	0.2	0.1	0			
166	0.2	0.2	0.1	0.002	0.004	0.001
190	0.2	0.3	0.2			
215	0	0	0	0.002	0.004	0.002

* evolved in a 2 liter fermentation flask

** calculated as cyanocobalamin in plate assay

the medium is not probable since an overpressure in the medium is established very soon after the start of the fermentation. The reasons for the unfavourable effect of stirring could thus not be deduced. Possibly, the stirring causes a disintegration of some complexes or configurations of the medium components which are favourable for the growth of the organism. It would be interesting to investigate whether the same effect is produced at different stirring rates. This, however, could not be done in the present investigation. Still, it cannot be understood why stirring had such distinctly different effects on two different enrichment cultures of methane organisms.

Chromatographic and electrophoretic studies were performed only with the methanol-fermenting culture since the enrichment culture of *Mb. omelianskii* produced such small amounts of *E. coli* activity. The results of these studies indicate that stirring, although not favourable for the production of gas and *E. coli* activity, may cause a different distribution of the activity between the various vitamin B_{12} factors than is the case in a stationary fermentation. Thus stirring seems to favour the formation of vitamin B_{12} substances which upon chromatography and electrophoresis give only one spot. The position of the spot indicates that it may be identical with factor III (Bernhauer) [5] and/or factor W (Neujahr) [4] and possibly other factors with R_c-values in the range 0.6—0.7 (*cf.* Ref.[4]). The phenomenon could be noticed even if DMB (1 μg/ml) was present in the medium. In the stationary fermentations, on the other hand, the *E. coli* activity formed could always be separated by chromatography into three spots if DMB had not been added to the medium and into four spots if it had been added. The four spots corresponded to the following factors: spot 1 - factor B (Ford) [10] spot 2 - cyanocobalamin, spot 3 - factor III (Bernhauer) [5] and/or factor W (Neujahr) [4] and possibly other factors with R_c-values in the range 0.6—0.7 (*cf.* Ref.[4]), spot 4 - factors Z (Neujahr) [4]. The distribution of *E. coli* activity between the different factors

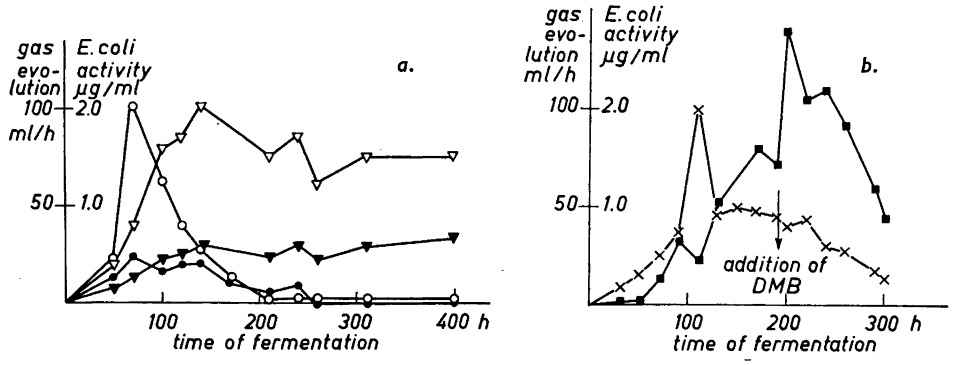

Fig. 2. Influence of 5,6-dimethyl benziminazole (DMB) on the gas production and *E. coli* activity of a methanol fermenting enrichment culture of methane bacteria. a. DMB added at the beginning: O—O—O gas evolution, ▽-▽-▽ *E. coli* activity in a fermentation with 1 μg DMB/ml medium; ●—●—● gas evolution, ▼—▼—▼ *E. coli* activity in a fermentation with 10 μg DMB/ml medium. b. 1 μg DMB/ml medium added after 192 h of fermentation: X—X—X gas evolution, ■—■—■ *E. coli* activity.

as found in this experiment is in agreement with previous experience [3]. Since stirring did not seem to give higher yields of vitamin B_{12}, not even when applied together with a covering oil layer, only stationary fermentations were carried out in the following experiments. For convenience no oil layer was employed.

II. *Influence of DMB.* It can be seen in Fig. 2a that the addition of 10 μg instead of 1 μg DMB per ml medium has a strongly inhibiting effect on both evolution of gas and production of vitamin B_{12} activity. A similar inhibitory effect of DMB on the enrichment culture of *Mb. omelianskii* has been reported earlier from this laboratory [1]. The inhibitory effect of benziminazoles upon the growth of several microorganisms is well known and attributed to its competition with adenine or guanine [11]. The inhibitory effect of benziminazoles may be influenced by vitamin B_{12} [12]. On the other hand, Pawelkiewicz [13] has used amounts of 20 μg DMB per ml medium in his fermentations with *Propionibacterium shermannii* for production of vitamin B_{12} without reporting any inhibition of the bacterial growth. Bernhauer [14] reports the use of amounts up to 40 μg DMB per ml medium in similar fermentations without mentioning any inhibitory effect on growth. In fermentations with *Propionibacterium shermannii* carried out in our laboratory [15], in which large additions of DMB (30 μg/ml) were used together with glucose and different modifications of the conventional medium, no such effect could be noticed. Thus it seems that the effect of DMB on cultures of vitamin B_{12}-producing methane bacteria differs from its effect on cultures of vitamin B_{12}-producing propionic acid bacteria. The following conclusions could be made at this point:

1) DMB seems to inhibit the growth of the methanol-fermenting organism, at least when added at a level of 10 μg/ml.

2) In the presence of DMB, on the other hand, cyanocobalamin is formed in preference to other vitamin B_{12} factors [3].

3) In the presence of DMB, several vitamin B_{12} factors could be converted to cyanocobalamin by an enrichment culture of another methane organism, viz. *Mb. omelianskii.*

Starting from the above three statements, a fermentation was made in which the methanol-fermenting culture was allowed to grow without added DMB for 192 h, after which time DMB was added to the medium at a level of 1 μg/ml. It was hoped that the amply formed vitamin B_{12} factors would then be converted to cyanocobalamin. The course of this fermentation can be seen in Fig. 2b. A comparison of Figs. 2a and 2b leads to the following conclusions: Fermentation without added DMB seems to proceed somewhat more slowly than in the presence of 1 μg DMB/ml medium. This is in agreement with previous experience with the methanol-fermenting organism [3]. Adding 1 μg DMB/ml medium after 192 h of fermentation results in a sudden increase of *E. coli* activity to a value which is about 50 % higher than the highest value in the corresponding fermentation with DMB added at the beginning. However, this higher *E. coli* activity decreases again very rapidly contrarily to what is the case in the fermentation with DMB added at the beginning. Chromatographic studies revealed further that the addition of DMB after 192 h results in the formation of a considerably smaller amount of cyanocobalamin than is the case in the fermentation in which DMB is added at the

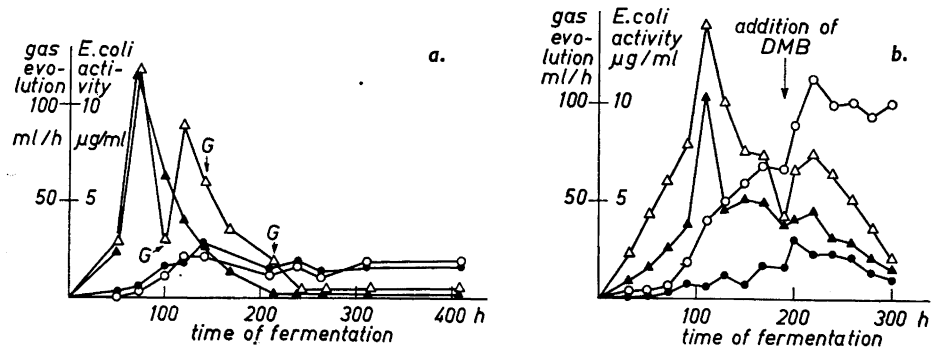

Fig. 3. Influence of glucose on the gas production and *E. coli* activity of a methanol-fermenting enrichment culture of methane bacteria. ▲—▲—▲ gas evolution, ●—●—● *E. coli* activity in the fermentation without glucose; △—△—△ gas evolution, O—O—O *E. coli* activity in the fermentation with glucose. a. DMB added at the beginning, methanol level not adjusted: G readjustment of the glucose level. b. DMB added after 192 h, methanol level adjusted, glucose level and pH readjusted daily.

beginning. Thus the conversion of the different vitamin B_{12} factors to cyanocobalamin cannot be achieved by this method, at least not with the level of DMB used. The factors predominantly produced in such fermentation are again factor III or/and factor W together with only smaller amounts of cyanocobalamin, factor B and factors Z.

It seems from above that smaller amounts of DMB (1 µg/ml) may be favourable for the fermentation, especially in its initial phase, whereas higher amounts of DMB (10 µg/ml) are harmful to the fermentation. This harmful effect depends probably on the known antimetabolic effect of benziminazoles [10,11] whereas the favourable effect of small amounts of DMB may depend on the fact that it is a precursor of cyanocobalamin and at a low concentration — 1 µg/ml — may not produce the antimetabolic effect.

III. *Influence of carbohydrates. a.* The course of two fermentations, in which DMB was added at the beginning and the level of methanol not adjusted during the fermentation time, is shown in Fig. 3a. In one of the fermentations glucose was present and its level adjusted 3 times during the fermentation period. It seems from Fig. 3a that, under the conditions stated above, glucose may stimulate the evolution of gas if added at a relatively early stage of the fermentation. Thus the adjustment of the glucose level (to 1 %) after 100 h results in a considerably increased gas evolution whereas repeated readjustments of glucose after both 140 h and 214 h have no longer such an effect. It should be mentioned that the last two readjustments necessitated the addition of approximately the same amounts of glucose as the first one. The glucose was thus being consumed in the medium all the time.

The production of vitamin B_{12} activity does not seem to be affected by the addition of glucose under the conditions described above.

b. Two other fermentations were performed in which DMB was added after 192 h and methanol added aseptically to the sterilized medium and its

level readjusted to the initial value (1 %) several times during the fermentation. In one of the fermentations, glucose was also present and its level was determined and readjusted to the initial value (1 %) every day. The course of these two fermentations is represented in Fig. 3b. It is evident from Fig. 3b that, under the new and more carefully controlled conditions, the addition of glucose very distinctly stimulates the formation of both gas and vitamin B_{12} activity. The highest *E. coli* activity obtained in this fermentation, *viz.* 11 μg/ml calculated as cyanocobalamin in the plate assay, is higher than any other activity obtained in fermentations of this kind. Moreover, in the fermentation in which the level of glucose was adjusted every day to 1 %, the vitamin B_{12} activity once formed is not destroyed again as is the case in the corresponding fermentation without glucose. Since the favourable effect of glucose appears only when the level of methanol is maintained at a somewhat constant value, it may be assumed that both substances are necessary for the high production of gas and vitamin B_{12}. The favourable effect of low molecular carbohydrates upon the production of vitamin B_{12} in septic methane fermentations of sewage sludge with *Mb. omelianskii* has been reported also by Cserei-Pehany [16].

c. In a separate experiment in this series, an attempt was made to use milk whey instead of glucose as a source of carbohydrate. The results are given in Table 3. It can be seen there that the addition of whey leads to a somewhat lower gas production but a consistently higher production of vitamin B_{12} activity throughout the fermentation than is the case in the corresponding fermentation without added carbohydrate. The highest *E. coli* activity obtained in the fermentation with whey is of the order of 3—4 μg/ml, calculated

Table 3. The influence of milk whey on the gas production and vitamin B_{12} activity of an enrichment culture of a methanol-fermenting methane organism.

Time of fermentation h	Medium supplemented with whey		Medium not supplemented	
	Gas evolution ml/h *	*E. coli activity* μg/ml **	Gas evolution ml/*h* *	*E. coli* activity μg/ml **
38	0		2	
62	0	0.1	16	0.02
90	12		15	
111	12	0.9	28	0.1
158	45	2.4	42	0.6
182	28		42	
209	37	3.1	44	1.5
233	42	3.1	48	1.9
258	33		50	
278	3	3.0	25	2.1
326	2	3.4	22	3.1
350	0	3.9	3	2.6
380	0		3	
405	0	3.2	12	2.8

* evolved in a 2 l fermentation flask

** calculated as cyanocobalamin in plate assay

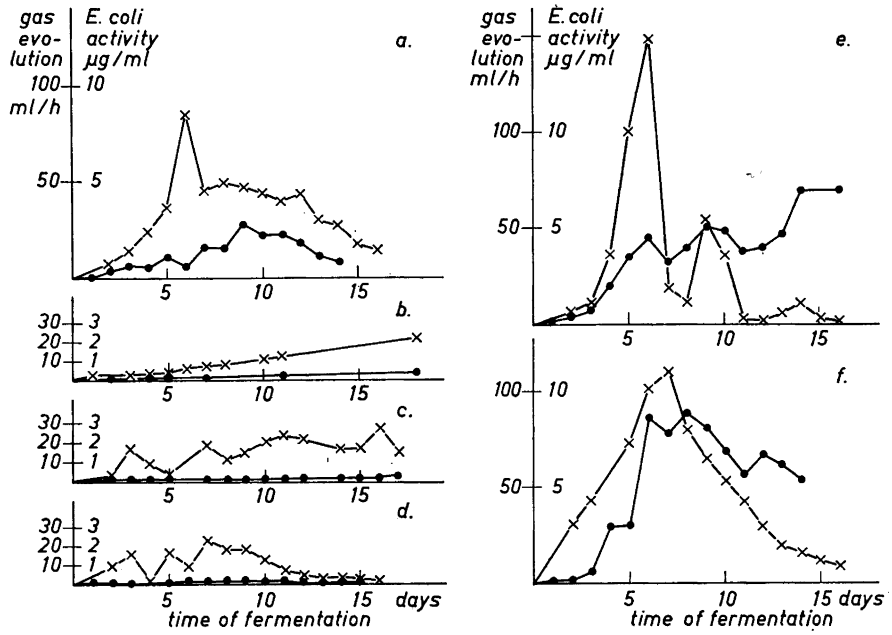

Fig. 4. Influence of certain nitrogenous materials on the gas production and vitamin B_{12} activity of a methanol-fermenting enrichment culture of methane bacteria. X—X—X evolution of gas, ●—●—● *E. coli* activity calculated as cyanocobalamin in plate assay. Basal medium containing. a. Bactopeptone. b. corn steep liquor. c. casein hydrolyzates + glucose. d. casein hydrolyzates + glucose + yeast extract. e. sterilized sewage. f. sterilized sewage + glucose.

as cyanocobalamin in the plate assay. These values are not directly comparable with those of the fermentations with glucose since, as described in Experimental, a different medium was used in the fermentations with whey and only fermentations with DMB added at the beginning were carried out.

Chromatographic and electrophoretic studies of the *E. coli* activity did not reveal any differences between the kind of factors formed in fermentations with and without the addition of carbohydrate to the basal medium. Chromatography gave spots corresponding to the following factors (listed in order of decreasing amounts): factor W[4] and/or III [5], factor B[10], cyanocobalamin, factors Z [4]. In the fermentations performed under more carefully controlled conditions, *i.e.* repeated readjustments of the glucose content, pH and methanol, a certain regularity in the occurrence of factors Z, B and W and/or III could be observed. Thus, during the first two or three days of the fermentation, there occurred a chromatographically almost immobile factor, different from factors Z2 + Z3 [4]. The behaviour of this factor was similar to that of factor C_2[17] (*cf.* Ref.[4]). This factor disappeared simultaneously as factors Z2 + Z3 appeared in the medium. The latter factors could usually be observed for the following two or three days, after which time their content

decreased markedly and they finally disappeared. Simultaneously with the disappearance of factors Z2 + Z3, factor B began to appear. Factors W and/or III were present all the time. A similar sequence for the appearance of factors C_2, C_1[17] (which may possibly be identical with factor Z3) and B has been observed earlier in anaerobically decomposing sewage sludge [18].

IV. *Influence of organic nitrogen sources and yeast extract.* It has been found in an earlier investigation [3] that the addition of Bactopeptone (Difco) stimulated both the production of gas and the vitamin B_{12} activity. An attempt was now made to elucidate whether other nitrogenous sources may have a similar and possibly more pronounced effect since the use of *a.* Bactopeptone, is very expensive. It can be seen in Fig. 4 that the addition of *b.* corn steep liquor (Fig. 4b), *c.* casein hydrolyzates together with glucose (Fig. 4c), and *d.* casein hydrolyzates together with glucose and yeast extract (Fig. 4d) results in a greatly diminished production of gas and vitamin B_{12} activity as compared with the fermentation with Bactopeptone (Fig. 4a). The shape of the gas evolution curves in Figs. 4b, 4c, and 4d is also quite different from the shape of the corresponding curve in Fig. 4a and the corresponding curves in Figs. 2a, 2b, 3a, 3b. Thus, the maximum in the gas evolution, which usually occurs between 70—110 h in the fermentations with the methanol-fermenting organism, is missing in those fermentations in which the above mentioned modifications of the conventional medium were employed. In the fermentations with casein hydrolyzates (Fig. 4c and 4d), the gas evolution curve has instead a "pulsating" character and the maximum gas evolution values are 4—5 times lower than the maximum values in other fermentations of this kind. A suspicion has arisen that, in the fermentations with media modified according to *b*, *c*, and *d*, some other organisms present in the enrichment culture of methane bacteria have developed due to the fact that the modified media might not have been any longer selective for the methanol-fermenting organism. At least in so far as the fermentations with casein hydrolyzates (Figs. 4c and 4d) are concerned, such a suspicion was jutified by the results of microscopic studies, by the putrefactive odour produced in these fermentations and also by the "pulsating" character of the gas evolution. In these fermentations, motile, possibly rod shaped organisms seemed to be present together with the coccoidal forms and the immobile rods usually present in fermentations of this kind [3]. Some of the motile forms may possibly have had the characteristic shape of *Clostridia*. However, the microscopic studies were rendered very difficult by the fact that methane bacteria grow mostly in the sediment [1] (*i.e.* the asbestos powder, calcium carbonate and sediment produced during the fermentation) the particles of which were very difficult to separate from the bacterial cells. Thus, under the microscope, the bacteria always appeared imbedded in a bulky precipitate and no definite conclusions about their shape could be made. There remains, however, the fact that certain motile forms were present in the cultures which was inconsistent with the immobile character of methane bacteria.

Further support for the suspicion that some *Clostridia* developed in the fermentations with casein hydrolyzates came from the results of chromatographic and electrophoretic studies. The *E. coli* activity produced in these fermentations was found to contain ψ-B_{12} together with factors W [4] and/or

III [5], B^{10} and cyanocobalamin. None of the methane bacteria studied so far was found to produce ψ-B_{12}[1,3]. On the other hand, certain strains of *Clostridia* were found, in a separate study, to produce ψ-B_{12}[10].

In the fermentation with corn steep liquor (Fig. 4b), the shape of the gas evolution curve is still more different from the gas evolution curves usually obtained in the fermentations with methane bacteria. Not only are the gas evolution maxima missing — at least during the period investigated — but also the gas evolution is extremely slow (2—3 ml/h) during the first five days of fermentation after which it increases slowly but steadily and, after eighteen days, still shows a tendency to increase. It should further be mentioned that, during the first five days, which were characterized by an extremely slow gas evolution (*cf.* Fig. 4b), the pH of the medium decreased considerably (to 6.5—6.7) after each of the daily readjustments to its optimum value (7.2) whereas, during the following period of time, characterized by a steady increase in gas evolution, the changes of pH were less pronounced and limited to the range 6.7—6.9 even without readjustments which were therefore no longer undertaken after 7 days of fermentation.

Microscopic examinations did not indicate that other organisms than the methanol-fermenting methane bacteria may have developed in the fermentation. Neither could any putrefactive odour be detected in the fermentation flask as was the case in the fermentations with casein hydrolyzates.

Knivett in England who has succeeded in growing a methane organism in a continuous culture [20] suggests that "a starved cell, which has to synthesize cell material from the simplest materials, may need to produce a lot of B_{12} coenzyme to do this whilst a cell adequately supplied with growth factors, amino acids etc., has no need to synthesize them and its coenzyme requirement may be less"[20]. Corn steep liquor is considered to be a source of most essential nutrients. According to the attractive suggestion of Knivett, the addition of corn steep liquor would thus result in a considerably diminished synthesis of vitamin B_{12} until the supply of the nutrients was exhausted in the medium. But can a similar relation apply also to the production of gas? (*cf.* Fig. 4b), *i.e.*, can the adequately supplied cells of the methanol-fermenting methane organism satisfy their growth requirements in a different way than by producing methane gas? (*cf.* also the above mentioned changes of pH!). The results of the present investigation are, however, much too preliminary to permit speculations of this kind. It should further be remembered that an enrichment and *not* a pure culture of the methanol-fermenting organism was used in the experiment. Bactopeptone is also an excellent source of different nutrients but it was found to stimulate both the production of gas and vitamin B_{12} activity in cultures of the methanol-fermenting organism [3]. On the other hand, the mutual relation of gas evolution and vitamin B_{12} activity curves in most fermentations with the enrichment culture can be plausibly interpreted by the suggestion of Knivett [20]. It can be seen for instance in Figs. 2a, 2b, 3a, 3b, 4a that the maximum in *E. coli* activity always comes *after* the maximum in gas evolution. It seems that the vitamin B_{12} content of the cells begins to increase as their metabolic activity — judged by the gas evolution — tends to decrease, probably due to the exhaustion of certain nutrients in the medium.

It can be seen in Fig. 4e that the use of sterilized sewage in the medium greatly stimulates the evolution of gas and production of vitamin B_{12} activity as compared for instance with Bactopeptone. The maximum gas evolution obtained in this fermentation is much greater than with most other modifications of the basal medium and comparable perhaps only with the medium containing glucose, DMB added after 8 days in the fermentation with regularly adjusted pH and methanol content (Fig. 3b). As in the fermentations with casein hydrolyzates (*cf.* Figs. 4c and 4d), the gas evolution curve has three maxima. However, here the second maximum is much smaller than the first and the third much smaller than the second. No indications could be found that other organisms than the methanol-fermenting one had developed in the culture. The curve for *E. coli* activity (Fig. 4e) shows a steady tendency to increase apart from two less pronounced maxima which seem to correspond to the first two maxima of the gas evolution curve. Between the 14th and 16th day of fermentation, the *E. coli* activity remains at the level of 7 μg/ml which is very high compared with the yields obtained in the fermentations in which nitrogenous materials were added (*cf.* Figs. 4a, 4b, 4c and 4d).

A still higher yield of *E. coli* activity, *viz.* 9 μg/ml, was obtained in a similar fermentation with sterilized sewage in which, however, the medium was further supplemented with glucose and yeast extract (Fig. 4f). The maximum value of *E. coli* activity was obtained after only 8 days of fermentation, after which time the activity tended to decrease again.

Chromatographic and electrophoretic studies revealed that the *E. coli* activity produced in the fermentations with sterilized sewage was mainly due to factors W[4] and/or III [5]. Roughly estimated, at least 70 % of the *E. coli* activity was contributed by these factors. Cyanocobalamin was present in much smaller amounts than the above factors. Factors B and Z were present only in trace amounts. In the fermentations with sterilized sewage, in which the medium was further supplemented with glucose and yeast extract, the dominant factors were factor W[4] or/and III[5] and factor B[10]. Cyanocobalamin was present in much smaller amounts than in the fermentations with sterilized sewage only. Thus the higher *E. coli* activity obtained in the further supplemented medium (Fig. 4f), *viz.* 9 μg/ml instead of 7 μg/ml (Fig. 4e) in the medium containing only sterilized sewage, may not reflect a really higher yield of vitamin B_{12} obtained in the richer medium since, according to previous experience [4], factor B gives much greater growth zones than cyanocobalamin when assayed by the plate method. Very small amounts of factors Z[4] and of some not identified factors were also produced in the fermentation with sterilized sewage, glucose and yeast extract.

It seems from the above that sterilized sewage, supplemented in an appropriate way provides a very good medium for obtaining high yields of vitamin B_{12} activity in fermentations with the methanol-fermenting enrichment culture of methane bacteria. It has also been successfully used in this laboratory for the cultivation of other vitamin B_{12}-producing organisms [15]. As described above (*cf.* p. 36), the addition of glucose to the basal medium M may lead to even higher yields of *E. coli* activity (Fig. 3b), *viz.* 11 μg/ml, than is the case in the fermentation with sterilized sewage (Fig. 4e). However, with glucose, greater amounts of factor B are formed and this makes un-

Table 4. Gas evolution, vitamin B_{12} production and utilization of methanol by an enrichment culture of a methanol-fermenting organism grown in different media. Experimental details in text.

Media / ıe of nen-on h	M + BP + DMB			M + BP + DMB + G			M+BP+ +DMB + G +Cas.ac. + Bactogen + YE			SM + YE			SM + DMB			SM + DMB + G + YE		
	Gas	B_{12}	MeOH	Gas	B_{12}	MeOH	Gas	B_{12}	MeOH	Gas	B_{12}	MeOH	Gas	B_{12}	MeOH	Gas	B_{12}	MeOH
0	0	0.2	0	0	0.2	0	0	0.2	0	0	0.2	0	0	0.2	0	0	0.2	0
10	85	0.5	0.09	140	4.6	0.11	10	0.05	0.07	116	4.4	0.15	150	2.0	0.09	104	2.5	0.11
30	36	2.3	0.08	72	10	0.14	5	0.15	0.04	30	4.4	0.05	4	4.4	0.18	25	6.4	0.03
50	0	0.8	0.02	0	10	0.07	0	0.15	0.03	0	7.4	0.08	0	7.0	0	0	5.4	0

M — basal medium described in the text
BP — Bactopeptone (Difco, 0.25 %)
DMB — 5,6-dimethyl-benziminazole,1 μg/ml
G — glucose, 1 %
Cas.ac. — Casaminoacids Vitaminfree (Difco)
Bactogen — trypsin hydrolyzed casein (Vitrum)
YE — yeast extract (Difco)
SM — sterilized sewage, supplemented with components of medium M

Gas — ml/h in a 2 l fermentation flask
B_{12} — μg/ml calculated as cyanocobalamin in plate assay
MeOH — ml methanol used/h in a 2 l fermentation flask

certain, once again, a direct comparison of the *E. coli* activity values obtained in the fermentations with glucose with those obtained in the fermentations with sterilized sewage. It should also be mentioned that the sterilized sewage contained only negligible amounts of reducing sugars.

V. *Utilization of methanol.* It can be seen in Table 4 that the greatest utilization of methanol took place in the fermentations with sterilized sewage in the medium. A somewhat lower utilization of methanol can be noticed in the media to which glucose had been added while the lowest utilization was observed in the media containing Bactopeptone or casein hydrolyzates. The latter finding may provide an interesting contribution to the discussion on p. 39. However, no conclusions concerning the relation between utilization of methanol and an adequate supply of preformed cell material can so far be made for various reasons — *viz.* partly because the values for methanol utilization, given in Table 4, are too few, and also because no sure correlation between utilization of methanol, production of gas and production of vitamin B_{12} activity can be deduced from Table 4 even if in most cases the vitamin B_{12} activity tends to increase when the utilization of methanol begins to decrease.

VI. *Maintainance of optimum pH.* As was described above, certain fermentations were performed with a daily readjustment of the pH to its optimum value for the methanol-fermenting organism (7.2). However, it was found that, in the media not containing glucose, the changes in pH were very small. After a few days of fermentation, these media exhibited in addition a very strong buffering capacity in the range 6.8—7.0. On the other hand, the fermentations in media containing glucose were characterized by considerable daily decreases in pH.

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