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On Vitamins in Sewage Sludge

XII. Production of Vitamin B_{12} by Certain *Clostridia*

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The ability to produce vitamin B_{12} was investigated in three strains of *Clostridia*.

The strains were found to produce amounts of *E. coli* activity ranging from 16—60 mμg/ml medium, calculated as cyanocobalamin in plate assay. These amounts are considerably smaller than those obtained in this laboratory with certain methane bacteria [6,7].

The addition of Co^{2+} was found to have only a negligible effect on the production of *E. coli* activity.

The factors mainly produced by the strains are factor A and ψ-B_{12}. If the medium is supplemented with 5,6-dimethyl benzimidazole, however, cyanocobalamin is produced almost exclusively.

As reported from this laboratory [1] and by several other authors [2–4], digested sewage sludge contains considerable amounts of vitamin B_{12} activity. It has also been reported earlier from this laboratory that the formation of vitamin B_{12} activity during the anaerobic decomposition of sewage sludge proceeds in several stages and seems to be caused by several different microorganisms [5]. The initial stages of the anaerobic sludge fermentation imply the decomposition of high molecular organic matter (proteins, fats and carbohydrates) to low molecular compounds (alcohols and fatty acids). The latter compounds are utilized during the final stages of the anaerobic sludge decomposition by methane bacteria with a simultaneous formation of large amounts of methane and carbon dioxide.

It was found in this laboratory that several of the methane organisms active during the final stages of the anaerobic sludge decomposition produce vitamin B_{12} activity [6]. Some of the methane organisms were found to produce considerable amounts of certain vitamin B_{12} factors when grown in appropriate media [7]. It was therefore considered to be of interest to investigate also whether the organisms active during the initial stages of the anaerobic sludge decomposition produce vitamin B_{12} activity and, if that is the case, which particular vitamin B_{12} factors are synthesized.

According to Enebo [8], the quantitatively most important group of organic materials in municipal sewage are high molecular carbohydrates. The organisms known to decompose such carbohydrates under anaerobic conditions are *Clostridia*. Three available strains of *Clostridia*, which presumably are active also during the anaerobic decomposition of sewage sludge, were grown in a medium containing maize meal, yeast autolyzate and phosphates both with and without the addition of Co^{2+} and 5,6-dimethyl benziminazole. The ability of these strains to produce vitamin B_{12} activity was investigated.

EXPERIMENTAL

The three strains of *Clostridia* investigated were

1. *Clostridium acetobutylicum* Weizmann (AB)
2. *Clostridium butylicum* (B4)
3. *Clostridium butylicum* (B6)

The cultures were maintained in the form of spores in soil.

Basal medium. Maize meal 70 g, yeast antolyzate (12 h at 50–60°C) 25 ml, $NH_4H_2PO_4$ 5 g, KH_2PO_4 1 g, dist. water 1 000 ml. The maize meal was boiled for about 30 min and mixed with the yeast autolyzate and salts. The pH was adjusted to 6 with NaOH and finally with $CaCO_3$ and the medium autoclaved for 1 h at 120°C and steamed the following day.

Preparation of the inocula. 10 ml portions of the sterile medium in test tubes (18 × 200 mm) were inoculated with 2 ml of a soil-spore suspension in a sterile saline solution which had been submitted to a heat shock (2 min in boiling water). The test tubes were incubated at 37°C for 24 h in evacuated vessels containing an alkaline pyrogallol solution. After this time, a considerable gas evolution usually took place.

Fermentations for determining the ability of Clostridia to produce vitamin B_{12}. 20 ml portions of the sterile medium in 50 ml E-flasks were inoculated with 5 ml of inoculum prepared as described above. The flasks were incubated at 37°C for either 7 or 14 days in evacuated vessels containing alkaline pyrogallol solution. Six simultaneous fermentations were performed with each strain according to the following scheme:

No. of flasks	Additions to the basal medium	Time of fermentation days
1	none	7
1	»	14
1	Co^{2+}, 2.5 μg/ml	7
1	» 2.5 »	14
1	Co^{2+}, 2.5 μg/ml + 5,6-dimethyl benziminazole, 1 μg/ml	7
1	—»— —»—	14

The Co^{2+} was supplied as a 0.1 % solution of $CoCl_2 \cdot 6H_2O$.

Vitamin B_{12} activity was determined by the cup plate method using *E. coli 113-3* as described elsewhere [5]. Chromatographic determinations were performed in solvent system I[9] and paper electrophoresis according to Holdsworth [10] using 2 M HAc containing 0.01 % KCN, pH 2.5, 10 V/cm, 18 h at +4°C. In both the chromatographic and electrophoretic determinations, bioautography on *E. coli 113-3* plates was used to reveal the spots obtained.

The results of this investigation are given in Table 1.

Table 1. Production of vitamin B_{12} by certain *Clostridia*

Co, 2.5 μg Co^{2+}/ml medium
DMB, 1 μg 5,6-dimethyl benziminazole/ml medium
AB, *Cl. acetobutylicum* Weizmann
B4, *Cl. butylicum*
B6, *Cl. butylicum*
ψ, ψ-B_{12}
Cy, cyanocobalamin
A, factor A
Brackets mean that the factor in question is present in much smaller amounts than the other factors.

Strain	Additions to the basal medium	Time of ferment. days	pH	*E. coli* activity		
				μg/ml *	Vitamin B_{12} factors present as identified by	
					chromatography	electrophoresis
AB	none	7	4.9	53	A + ψ	A; ψ
»	Co	7	4.9	60	A + ψ	A; ψ; (?)
»	Co + DMB	7	4.9	35	Cy	Cy; (B?)
»	none	14	4.9	46	A + ψ	A; ψ
»	Co	14	4.9	54	A + ψ; (Z?)	A; ψ; (?)
»	Co + DMB	14	4.9	18	Cy; (Z?)	Cy
B4	none	7	4.9	24	A + ψ	A; ψ
»	Co	7	4.9	30	A + ψ	A; ψ; (?)
»	Co + DMB	7	4.9	13	Cy	Cy; (B?)
»	none	14	4.9	33	A + ψ	A; ψ
»	Co	14	4.9	46	A + ψ; (Z?)	A; ψ; (?)
»	Co + DMB	14	4.9	18	Cy	Cy; (B?)
B6	none	7		16	A + ψ	A; ψ
»	Co	7		growth typical for methionine		
»	Co + DMB	7		24	Cy; (Z?)	Cy
»	none	14	5.0	40	A + ψ	A; ψ
»	Co	14	5.0	50	A + ψ	A; ψ; (?)
»	Co + DMB	14	5.0	24	Cy; (Z?)	Cy

* calculated as cyanocobalamin in plate assay.

RESULTS AND DISCUSSION

In all of the flasks, an intensive fermentation must have taken place during the first 4—5 days of incubation judging by the vigorous gas evolution and the decrease of pH from its initial value (6.0) to 4.9—5.0 (see Table 1). It can further be seen in Table 1 that in all flasks, except one, there was a production of *E. coli* activity in amounts ranging from 16 to 60 mμg/ml calculated as cyanocobalamin in plate assay. A prolonged time of fermentation (14 days instead of 7 days) seemed to increase the total *E. coli* activity produced in fermentations with strains B4 and B6 whereas the reverse may be true for strain AB. In one flask, *viz.* the fermentation with strain B6 in the basal medium supplemented with Co^{2+} and interrupted after 7 days, considerable amounts of

methionine seemed to be present instead of vitamin B_{12} activity. The reasons for this discrepancy could not be deduced.

The *E. coli* activity produced in the fermentations of the basal medium, not further supplemented, was due to factor A and ψ-B_{12} as shown by both chromatography and electrophoresis. The situation was the same with all three strains after both 7 and 14 days of fermentation. The addition of Co^{2+} had only negligible effect on the total amount of *E. coli* activity obtained and only a very slight effect on the kind of vitamin B_{12} factors produced which, with all three strains, were once again mainly factor A and ψ-B_{12} together with only trace amounts of a factor neutral on electrophoresis at pH 2.5. After 14 days of fermentation, small amounts of a factor ressembling the factors Z[10] could be detected by chromatography in fermentations with strains AB and B4 but not with strain B6. This factor moved still more slowly, upon chromatography in solvent system I[8], than factor Z3[11]. Its presence could not be confirmed by electrophoresis. The nature of this factor was not further investigated since it was present in much smaller amounts than the other two factors, *viz.* factors A and ψ-B_{12}.

The addition of DMB together with Co^{2+} resulted in a considerable decrease in the values for total *E. coli* activity produced in fermentations with all three strains as compared with the fermentations in the unsupplemented basal medium or this medium supplemented with Co^{2+} alone. The decrease may, however, be only an apparent one since the addition of DMB led the synthesis of vitamin B_{12} activity in all fermentations towards the formation of mainly cyanocobalamin instead of factors A and ψ-B_{12}. As discussed elsewhere [5,12], cyanocobalamin gives much smaller growth zones on *E. coli* plates than several other vitamin B_{12} factors. In certain cases (cf. Table 1), very small amounts of the above mentioned factor Z was formed together with cyanocobalamin as revealed by chromatography but not confirmed by electrophoresis. In other cases, very small amounts of a factor ressembling factor B seemed to be present as judged by the results of paper electrophoresis but not confirmed by chromatography. Again, the nature of these factors was not further investigated since they were present only in very small amounts.

The following conclusions concerning the three strains of *Clostridia* can be made on the basis of the present investigation.

1. *Clostridia* seem to produce amounts of vitamin B_{12} activity which are considerably smaller than those obtained in fermentations with certain methane bacteria [6,7].

2. *Clostridia* seem to produce mainly factor A and ψ-B_{12} in the basal medium not supplemented with 5,6-dimethyl benziminazole. However, with this compound added to the medium they produce almost exclusively cyanocobalamin. It should be mentioned that none of the hitherto investigated methane bacteria was found to produce ψ-B_{12} or factor A[6,13].

3. The presence of centain amounts of ψ-B_{12} and factor A in digested sludge could thus be attributed to ***inter alia*** the activity of *Clostridia*.

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