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On Vitamins in Sewage Sludge

X. Production of Vitamin B_{12} by Sulphate-Reducing Bacteria

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Vitamin B_{12} and some other growth factors were determined in sewage sludge fermented by sulphate-reducing bacteria according to Butlin [1] and in an extract of dry *Desulphovibrio desulphuricans* cells *.

The vitamin B_{12} activity, calculated as cyanocobalamin in *E. coli* cup plate assay, of sludge fermented by sulphate reducers was found to be approximately 0.2—0.3 μg/ml or 10—15 μg/g dry solids which is of the same order of magnitude as the corresponding activity of sludge fermented by methane bacteria. Bioautographic studies revealed the presence of cyanocobalamin and also some factor or factors with an R_c-value (R_F-value of the factor relative to that of cyanocobalamin) = 0.6—0.7 the nature of which was not elucidated.

The fermented sludge contained only small amounts of folic and folinic acid activity.

The dry cell extracts were found to contain 9 μg vitamin B_{12} activity/g dry cell material when assayed by the *E. coli* cup method but 15 μg/g when assayed by the *E. coli* turbidimetric method. The reasons for this discrepancy are discussed and some attempts to elucidate them are described.

The *E. coli* activity of the dry cell extract represents a mixture of at least four factors. Cyanocobalamin is present in small amounts. The predominating factor(s) may be identical with factor A and some unidentified factor(s) which behave slightly basically upon paper electrophoresis at pH 2.5. Factors: B(Ford), [19] aetiocobalamin phosphoribose (Bernhauer) [11], 2-methyl-mercapto-adenin-cobalamin (Bernhauer) [18] and factors Z2 + Z3 (Neujahr) [8] are absent or present only in very small amounts.

Three or four desoxyriboside factors were also found to be present in the cell extracts.

The cell extract was found to contain three factors which had a growth inhibiting activity for *L. leichmannii 313*. Attempts to purify these factors are described and their possible nature is discussed.

Butlin and co-workers [1] have prepared enrichment cultures of sulphate-reducing bacteria which can be successfully used for the production of sulphide from sulphate-enriched sewage sludges. When such sulphide fermentation

* The dry cell material was kindly provided by Dr. John Postgate of National Chemical Laboratory, Teddington, England.

takes place in sewage sludge, the otherwise spontaneously occurring methane fermentation is inhibited and the production of methane gas ceases. Butlin and co-workers have shown that the inhibition of methane fermentation by sulphide fermentation in combined, methane- and sulphide-producing fermentations depends a.o. on the preferential utilization of nutrients or (and) reducing material by sulphate-reducing bacteria [1]. In their subsequent work Butlin and co-workers [21] have found also certain other reasons for the inhibition of the methane fermentation.

Furthermore, the above cited authors have shown that pure cultures of laboratory strains of *Desulphovibrio desulphuricans* inoculated into sterile raw sludge produced negligible sulphide in contrast to crude enrichment cultures which were very active in this respect [1].

It has been reported from this laboratory [2,4] as well as from several others [3,5] that considerable amounts of vitamin B_{12} activity are formed during the methane fermentation of sewage sludge. It has also been reported that many of the organisms active during the methane fermentation of sewage sludge produce vitamin B_{12} activity when cultivated in synthetic media [6]. It was therefore considered to be of interest to investigate if vitamin B_{12} was formed also during the sulphide fermentation of sewage sludge and if it was formed by pure strains of *Desulphovibrio desulphuricans* grown in synthetic media.

EXPERIMENTAL

Using an active sulphide-producing crude culture of sulphate-reducing bacteria kindly provided by Dr. Butlin, the author performed two semicontinuous fermentations of raw sulphated sewage sludge according to the technique described by Butlin and co-workers [1]. Raw sludge was homogenized in a Turmix blendor and sieved through a sieve with a 0.80 mm mesh. The material obtained in this way contained 2 % dry solids. It was sulphated (50 g $CaSO_4$/l) and 1 l batches were inoculated with the active culture. Samples of 50 ml were removed daily and replaced by 50 ml raw sulphated sludge (2.5 g $CaSO_4$/50 ml). Nitrogen gas was blown through the fermentation flask. The H_2S formed was trapped in a solution of $CdCl_2 \cdot 5H_2O$.

The vitamin B_{12} activity of the removed samples was investigated using *E. coli 113—3* in plate assay and by bioautography as described elsewhere [7]. The growth activity for *S. faecalis ATCC 8043* and *L. citrovorum ATCC 8081* was also determined in some samples and calculated as folic acid and folinic acid activity, respectively. The results of the two series can be seen in Tables 1 and 2.

The determination of the ability of a pure strain of *Desulphovibrio desulphuricans* to produce vitamin B_{12} activity was performed on dry cell material *. The cells were autoclaved for 10 min at 121°C in water containing 0.01 % KCN at pH 6.5. The proportion of the dry cell material and water was 1:30. The autoclaved mixture was centrifuged and the supernatant used as cell extract for the different assays. For assaying desoxyriboside factors, the extract was concentrated tenfold by evaporation under reduced pressure at 30—40°C.

The values found for vitamin B_{12} activities, calculated as cyanocobalamin, are given in Table 3.

* *Cf.* footnote on p. 1960.

RESULTS AND DISCUSSION

Vitamin B_{12} activity of sulphate-enriched sludge fermented by the crude culture of sulphate-reducing bacteria

It is seen in Tables 1 and 2 that approximately 0.2—0.3 mg/l of vitamin B_{12} activity was produced in a sludge containing 2 % dry solids. This corresponds to 10—15 mg/kg dry solids which is of the same order of magnitude as in sludge which has undergone methane fermentation (10—15 mg/kg).

Paper chromatography followed by bioautography on *E. coli* plates gave two spots, one of which was identified as cyanocobalamin. The second spot, R_c = 0.6—0.7, could not be conclusively identified since, as reported before [8], it may correspond to several vitamin B_{12} factors having R_c-values (= R_F-values relative to that of cyanocobalamin) in the range 0.6—0.7. It can further be seen in Table 2 that the sludge fermented by sulphate-reducing bacteria exhibited only a small activity for *S. faecalis* and *L. citrovorum*. These activities were considerably higher in fresh sludge. A similar relation has been observed earlier in the methane fermentation of sewage sludge [2,4].

Table 1. Semi-continuos fermentation of raw sulphated sludge (ca 2 % dry solids content) by sulphate reducing bacteria.
R_c-value = R_F of a factor relative that of cyanocobalamin.

Days of fermentation	pH	*E. coli* activity	
		μg/ml *	Factors detected by paper chromatography **
0	8.1	0.03	
1	6.3	0.16	
2	6.3	0.17	Two chromatographic
3	6.3	0.19	spots were always present.
6	6.1	0.22	
7	6.2	0.21	1) R_c = 1.0
9	6.4	0.22	corresponding to cyanocobalamin
11	6.5	0.20	2) R_c = 0.6—0.7
13	6.6	0.20	the nature of which was
15	7.1	0.17	not elucidated
16	7.5	0.15	Factor B (R_c = 1.4) and
17	8.2	0.19	factors Z (R_c 0—0.4)
18	8.2	0.21	could not be detected at all.
20	7.9	0.16	
(21)	(8.1)	(0.11)	
24	7.8	0.20	
25	8.1	0.17	
27	8.0	0.17	
30	7.8	0.17	
31	7.8	0.17	

* calculated as cyanocobalamin in plate assay

** solvent system: butyl alcohol: water: acetic acid = 75 : 24 : 1 containing 0.01 % KCN, 48 h development, descending technique.

Table 2. Semi-continuous fermentation of raw sulphated sludge (ca. 2% dry solids content) by sulphate-reducing bacteria.
R_c-value = R_F of a factor relative to that of cyanocobalamin.

Days of fermentation	pH	*E. coli* activity		*S.f.* ***	*L.c.* ****
		µg/ml *	Factors detected by paper chromatography **	Activity µg/ml	
0	7.5	0.03		0.013	0.021
1	6.6	0.11			
2	6.8	0.13			
3	7.4	0.14	Two chromatographic spots		
4	7.6	0.21	were always present:	<0.003	<0.003
7	7.6	0.21	1) R_c = 1.0		
8	7.8	0.23	corresponding to		
9	7.8	0.21	cyanocobalamin		
10	7.8	0.21		0.004	0.005
11	7.8	0.21	2) R_c = 0.6–0.7		
12	7.8	0.16	the nature of which		
13	7.6	0.21	was not elucidated		
14	7.7				
15	8.0	0.20			
16	8.1	0.18	Factor B (R_c = 1.4) and factors		
17	8.0	0.27	Z (R_c 0–0.4) could not be		
19	8.0	0.22	detected at all.		
20	8.0	0.22		0.007	0.004
21	8.3	0.20			
22	8.0	0.20			
23	8.0	0.21			
24	8.1	(0.64)			
26	8.3	0.30			
27	8.3	0.30			
28	8.4	0.27			
29	8.4	0.20			
30	8.5	0.20			
31	8.4	0.20			
33	8.4	0.21			
34	8.5	0.24			
44	8.5	0.30			
59	8.3				

* calculated as cyanocobalamin in plate assay
** solvent system: butyl alcohol: water: acetic acid = 75 : 24 : 1 containing 0.01 % KCN, 48 h development, descending technique.
*** *S. faecalis* activity calculated as folic acid in plate assay.
**** *L. citrovonum* acitivity calculated as folinic acid in plate assay.

Vitamin B_{12} activity of dry cells of a pure strain of *Desulphovibrio desulphuricans.*

It can be seen in Table 3 that extracts of *Desulphovibrio desulphuricans* cells exhibit a greater vitamin B_{12} activity when assayed with *L. leichmannii* than when assayed with *E. coli*. The difference is expecially pronounced in the cup

plate assay. It was found that the higher activity values obtained with *L. leichmannii* could be accounted for by the presence of certain desoxyribosides which are also active for this organism. Paper chromatography in two solvent systems [7] followed by bioautography on agar plates seeded with *L. leichmannii*, and *L. acidophilus* R-26, ATCC 11506 gave three spots with R_F-values close to those of the desoxyribosides of adenine, uracil and 5-methylcytosine and/or guanine. These substances were present in the cell extracts in amounts of about 5—10 μg/ml as estimated by comparison with spots obtained on the bioautographs with standard solutions, *i.e.* they may constitute about 150—300 μg in 1 g dry cell material. Other desoxyriboside factors, if present, may occur in much smaller amounts than the three (or four) mentioned above since they could not be detected even in the tenfold concentrated cell extract.

Table 3. Vitamin B_{12} activity of *Desulphovibrio desulphuricans* cell extracts.

Test organism	Assay method	Vit. B_{12} activity calculated as cyanocobalamin μg/g dry cell material *
E. coli 113-3	cup plate	9
» » *113-3*	turbidimetry	15
L. leichmannii 313	cup plate	30
» »	turbidimetry	17

* average of three different determinations.

It can further be seen in Table 3 that the extracts of *Desulphovibrio desulphuricans* cells exhibit a greater vitamin B_{12} activity when assayed with *E. coli* by the turbidimetric method than by the cup plate technique. This phenomenon is quite unusual compared with previous experience. The vitamin B_{12} activity of natural materials investigated hitherto was always found to be greater when assayed by the cup plate technique than by the turbidimetric one. This is generally attributed to the presence in natural materials of different vitamin B_{12} factors together with cyanocobalamin.

In his work on the vitamin B_{12} requirement in certain soil bacteria Ford suggests that certain vitamin B_{12}-like factors, the "inactive analogues may slow down the rate at which vitamin B_{12} is absorbed and utilized by the cells" [9]. The presence of the "inactive analogues" in relatively high concentrations together with vitamin B_{12} gives, according to Ford [9], rise to larger and correspondingly less dense zones of growth in the plate assays than those obtained with vitamin B_{12} alone. A similar relation may apply also to *E. coli 113—3* and the reasoning of Ford [9] could thus give a plausible explanation of the often much larger and more diffuse, as compared with pure solutions of cyanocobalamin, growth zones obtained in *E.coli* plates with solutions containing a mixture of several vitamin B_{12} factors. However, a number of vitamin B_{12}-like factors, even those which lack the nucleotide part, *e.g.* factor B, are active for *E.coli*. Many of those "active analogues" give also larger and less dense zones

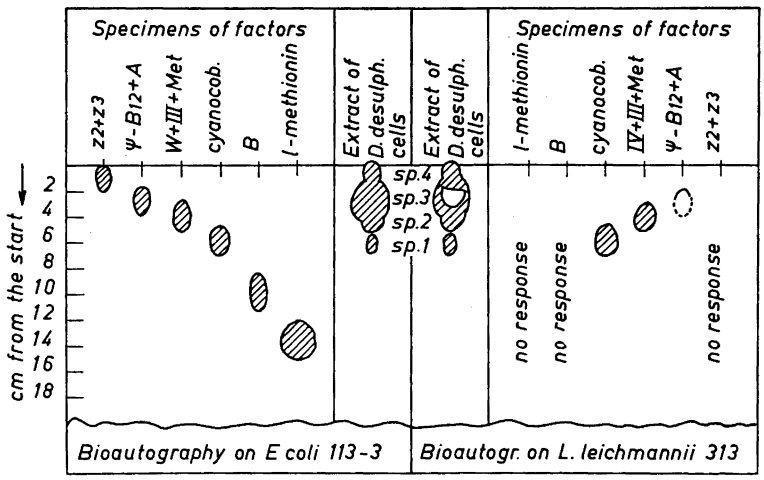

Fig. 1. Chromatographic separation of the vitamin B_{12} activity present in cell extracts of *Desulphovibrio desulphuricans*. Solvent system I [7], development: 18 h at 24°C.

of growth than cyanocobalamin when assayed with *E.coli* by the plate method, even when a solution of a single vitamin B_{12} factor is applied (*cf.* Ref. 8). Coates and Ford [20] suggest that cyanocobalamin may be utilized more rapidly by the *E.coli* cells than the other vitamin B_{12} factors active for this organism. The other factors may thus have time to diffuse further away from the cup until they can be utilized for growth than is the case with cyanocobalamin. This may perhaps explain why the solutions of such factors give rise to larger but less dense growth zones than those obtained with cyanocobalamin.

Reasoning in a similar way may lead to the supposition that the greater *E.coli* activity of *Desulphovibrio desulphuricans* cells exhibited in tube assay than in cup plate assay may depend on the presence in the cell extracts of a vitamin B_{12} factor which is still more rapidly utilized by the test organism cells than cyanocobalamin. Furthermore, this factor may be a dominant one in the cell extract.

Paper chromatography in two-solvent systems [7] combined with bioautography on *E.coli* and *L. leichmannii* plates gave four spots equally active for *E.coli* and *L. leichmannii*. The chromatographic separation after 18 h of development in solvent system I [7] is represented in Fig. 1. A consideration of Fig. 1 leads to following conclusions.

1) factor B is not present in the cell extract.

2) the spot corresponding to cyanocobalamin (spot 1), if identical with this factor, indicates that cyanocobalamin is present only in a small amount.

3) spot 2 and the largest spot of vitamin B_{12} activity (spot 3) cannot be identified after chromatographic separation during 18 h since they have R_c-values which may correspond to several vitamin B_{12} factors.

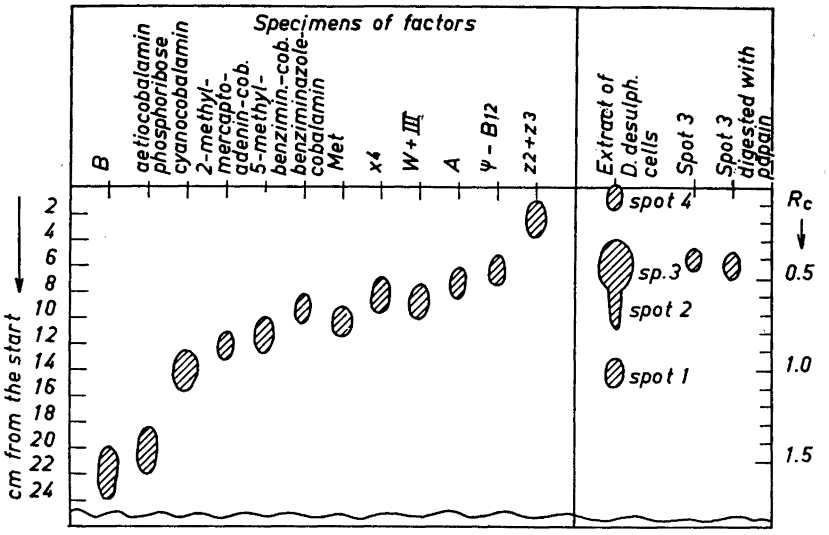

Fig. 2. Chromatographic separation of the *E. coli* activity exhibited by cell extracts of *Desulphovibrio desulphuricans*. Solvent system I [7], development: 48 h at 24°C.

4) spot 4 which corresponds on the *E.coli* bioautogram to factors Z (Z2 + Z3) cannot be identical with these factors since it is also active for *L. leichmannii* which is not the case with specimens of factors Z2 and Z3.

5) the extract of *Desulphovibrio* cells contains a growth inhibitor for *L. leichmannii* with an R_F-value close to that of spot 3.

By electrophoresis in 2 M Hac containing 0.01 % KCN according to Holdsworth [10], the *E. coli* activity of the cell extract could be separated into four spots, one slightly basic or almost neutral, one basic and two acidic. The largest part of the *E. coli* activity was represented by the basic and neutral factors while the acidic factors were present only in lesser amounts.

In order to identify spots 2 and 3, further chromatographic and electrophoretic studies were performed using for comparison specimens of several vitamin B_{12} factors kindly provided by Prof. Bernhauer. Fig. 2 shows the chromatographic separation of the *E. coli* activity after 48 h at 24°C in solvent system I [7].

From Fig. 2, the following conclusions can be drawn:

1) a prolonged period of development gives once again only four spots but provides, on the other hand, a better possibility for characterizing the spots obtained.

2) factor B (Ford) [19], aetiocobalamin phosphoribose (Bernhauer) [11] and factors Z2 + Z3 (Neujahr) [8] are not present in the cell extract.

3) spot 4 thus corresponds to some factor (or factors) which could not be identified with any of the available specimens. Its immobile character upon development with solvent system I [7] ressembles the behaviour of factor C_2 [12]. The possible identity of spot 4 with factor C_2 was not excluded by the results

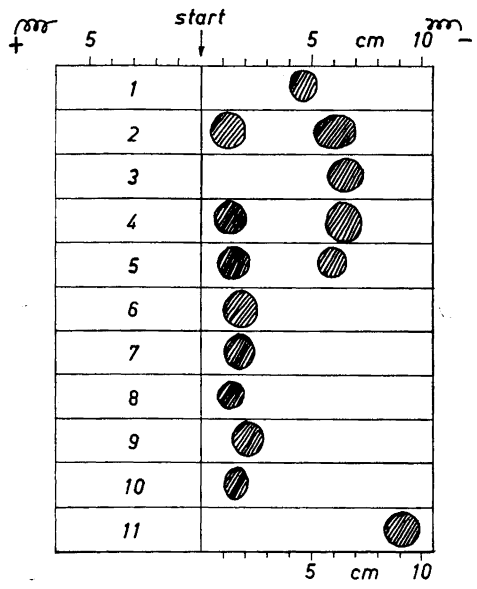

Fig. 3. Electrophoretic separation of the *E. coli* activity exhibited by cell extracts of *Desulphovibrio desulphuricans*. 2 M HAc containing 0.01 % KCN, pH 2.5, 10 V/cm, 18 h at +4°C; 1. ψ $-B_{12}$; 2. cell extract*; 3. factor A; 4. "spot 3" eluted from chromatograms; 5. "spot 3" after digestion with papain; 6. factor III; 7. cyanocobalamin; 8. benziminazole cobalamin; 9. factor W; 10. factor Met; 11. factor B.

of the electrophoretic studies since, as mentioned above minor quantities of two acidic factors could be observed, at pH 2.5, in the cell extract.

4) spot 1 most probably represents cyanocobalamin — this is in agreement with the presence of an electrophoretically almost neutral factor at pH 2.5.

5) Spot 2 is more difficult to identify. It can be seen in Fig. 2 that it may correspond to at least 6 factors, *viz.* benziminazole cobalamin (Bernhauer) [3], factor Met (Neujahr) [13], factor X [4] (Neujahr) [8], factor W (Neujahr) [8], factor 111 (Bernhauer) [3] and possibly others. The elongated character of this spot indicates also that it is a mixture of several factors. Further identification of this spot by means of paper electrophoresis was not possible since the six factors mentioned above behave similarily upon electrophoresis.

6) The main spot of *E. coli* activity (spot 3) seems at first sight to correspond to ψ-B_{12} as judged by its R_c-value. However, this is excluded by the results of electrophoretic studies shown in Fig. 3 where once again factor A seems to be present in the cell extract. Thus the nature of spot 3 could not be conclusively determined.

Due to a suspicion that spot 3 may represent some combined form of vitamin B_{12}, a number of spots 3 was cut out from the chromatograms, eluted with water and concentrated under reduced pressure. A part of the concentrated solution was digested with papain and another part autoclaved with KCN (1 mg/ml) for 15 min. The vitamin B_{12} activity of the resulting solutions was determined in plate assay and tube assay using *E. coli* and *L. leichmannii* and

* With the amount of cell extract applied in this experiment the two acidic factors mentioned in text (*cf.* p. 1966) did not appear. It was, however, couvenient to use this amount in order to identify the two basic factors.

also by chromatography and electrophoresis. The *E. coli* activity of the eluted spots 3 did not show the same relation between cup plate and tube assay values as the whole extracts. On the contrary, the activities found in tube assay were now lower than those found in the cup plate assay. Spots 1, 2 and 4 were also cut out from the chromatograms, eluted with water and assayed for *E. coli* activity alone and together with each other and with spot 3, but no evidence for synenergetic phenomena could be obtained. The problem could not be more thoroughly investigated because of the small amount of material available. The vitamin B_{12} activity values of the eluted spots 3 found with *L. leichmannii* in cup plate assay were once again higher than those found with *E. coli*. Upon electrophoresis in 2 M HAc at pH 2,5, the chromatographically homogenous spot 3 separated into two spots, one almost neutral and one basic with a mobility corresponding to factor A. Autoclaving with KCN (1 mg/ml) destroyed the greater part of the *E. coli* activity. Digestion with papain did not alter the position of spot 3 on the chromatograms noteworthly (Fig. 2) nor the position of the resulting two spots on electrophorograms (Fig 3), although the *E. coli* activity found in tube assay was now once again much higher than the activity found in cup plate assay. It is possible that the discrepancy of the values was at least to some extent due to methionine derived from the papain preparation.

Since the supposition that spot 3 represents some combined form of vitamin B_{12} could not be confirmed, it may be assumed that it is a mixture of factor A and some factor(s) which behave slightly basically upon electrophoresis at pH 2,5.

Inhibition factor.

It was mentioned above and, in addition, can be seen in Fig. 1 that the extract of *Desulphovibrio desulphuricans* cells contains a substance inhibiting the growth of *Lactobacillus leichmannii*. In solvent systems I and II[7], the substance had R_c-values corresponding to those of spot 3 of *E. coli* activity in these solvent systems. On electrophoresis in 2 M HAc at pH 2.5, it was neutral or slightly basic. Attempts were made to purify the substance. 6 g of dry cell material were autoclaved in 180 ml water containing 100 μg KCN/ml. After centrifugation the supernatant was evaporated to dryness under reduced pressure at 40°C. The dry residue was then extracted with 70 % EtOH several times and the combined extracts as well as the residue were assayed for the inhibiting activity using *L. leichmannii* plates for vitamin B_{12} assay but supplemented with cyanocobalamin (1 μg/ml). Almost all the growth inhibiting activity was found in the extracts. The combined extracts were then evaporated to dryness under the conditions described above. The dry residue was dissolved in a small amount of water and chromatographed on Wh 1 paper sheets of 18 × 38 cm for 48 h in solvent system I[7]. After drying, chromatogram strips of 1 cm width were cut at the right and the left side of each sheet and the position of the inhibition factor determined by putting the strips on *L. leichmannii* plates for vitamin B_{12} which contained cyanocobalamin. On this occasion, it was found that not one but three distinctly different inhibition zones were given by the extract. The three zones: Inh 1, Inh 2, Inh 3 had approximately following R_c-values:

Inh 1	1.10
Inh 2	0.65
Inh 3	0.37

These R_c-values are thus closely related to the R_c-values of many vitamin B_{12} factors [8]. Inh 1 seems to be present in much greater amounts than the other two inhibition factors. The three inhibition zones were cut out from chromatograms and eluted with water. The eluates were concentrated by evaporation under reduced pressure at 40°C. Attempts were made to determine absorption spectra of the three solutions thus obtained, but they were not successful.

The solutions were not sufficiently purified and concentrated. The experiments could not be continued due to the small amount of material available. However, even if the solutions had been sufficiently purified and concentrated the determination of their absorption spectra would have been quite uncertain since they contained considerable amounts of vitamin B_{12} activity. The separation of this activity from the inhibitor factors by the conventional chromatographic and electrophoretic techniques may be a rather difficult task as can be understood from the fact that the R_c-values of the inhibitor factors are very closely related to the R_c-values of many vitamin B_{12} factors (*cf.* Fig. 2) and that their neutral or slightly basic electrophoretic character at pH 2.5 ressembles that of cyanocobalamin and several other vitamin B_{12} factors.

The occurrence of bacterial inhibition factors in bacterial cell extracts has been reported by several authors. Bolinder [14] in this laboratory has found that several purine nucleotides, notably adenine ribotides and desoxyribotides, possess a growth inhibiting activity for *L. leichmannii* 313 in agar plates for the assay of vitamin B_{12}, desoxyribotides and desoxyribosides. However, the identity of the inhibitor factors found in *Desulphovibrio desulphuricans* cells with any of the compounds found by Bolinder [14] to have an inhibitory activity is unlikely since they behave quite differently upon chromatography.

Juillard [15] has isolated from a *Bacillus megaterium* mutant a growth inhibition factor for *E. coli 113—3* and identified it chromatographically as the monocarboxylic acid of factor B. This inhibition factor behaved as a competitive antimetabolite for *E. coli 113—3*.

Smith *et al.*[16] and Cuthbertson *et al.* [17] describe the antimetabolic activities for a B_{12}-requiring *E. coli* mutant of several compounds structurally closely related to vitamin B_{12}. In their experiments they used rigorously purified substances obtained by the chemical degradation of cyanocobalamin. As mentioned above, a further purification of the inhibition factors extracted from *Desulphovibrio desulphuricans* cells was not possible. The fact that the preparations did not exhibit any growth inhibiting activity on *E. coli 113—3* plates (*cf.* Fig. 1) may depend upon their high content of vitamin B_{12} which may overcome their possible growth inhibiting activity for *E. coli* whereas in *L. leichmannii 313* plates the growth inhibiting activity could not be overcome. Thus it cannot be decided whether the inhibition factors found in *Desulphovibrio* cell extracts are of the kind described by Juillard [15], Smith *et al.* [16] and Cuthbertson *et al.* [17], although their chromatographic and electrophoretic behaviour points to the suggestion that they may be structurally closely related to vitamin B_{12}.

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