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On Vitamins in Sewage Sludge

V. The Effect of Antibiotics on Vitamin B_{12} Contents of Microbially Decomposing Sewage Sludge

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The effect of the addition of antibiotics to microbially decomposing sewage sludge was studied with respect to the amount of vitamin B_{12} activity formed and its distribution between the different factors. *Aureomycin* was found to stimulate the formation of vitamin B_{12} activity if added at a sludge decomposition stage characterized by pH 6.5–7.0. Under anaerobic conditions the addition of aureomycin caused an enhanced synthesis of factors with R_F-values related to that of factor III, whereas under aerobic conditions the synthesis of cyanocobalamin was stimulated. — Under aerobic conditions *terramycin* was found to have a similar, but less pronounced, effect. — Both active and inactivated *penicillin* stimulated the synthesis of cyanocobalamin and of other vitamin B_{12} factors when incubated with sludge. The effect was more pronounced with the inactivated agent, thus suggesting the possibility that the latter may be the factor stimulating the vitamin B_{12} synthesis. — *Neomycin* stimulated the synthesis of cyanocobalamin and of other vitamin B_{12} factors. Heat and alkali treated (but not inactivated) neomycin had a detrimental effect on the synthesis of cyanocobalamin.

Antibiotics are known to exert a vitamin B_{12} "sparing action" on several animals and are extensively used as "feed supplement" for pigs and chickens. The mechanism of this action has not yet been elucidated and the experimental evidence is contradictory on some points.

Many investigators support the view that antibiotics enhance the growth of young poultry and swine merely through alterations of the normal intestinal microflora, whereas others postulate that the antibiotic molecule or a fragment thereof is involved as a metabolite in the host. The latter hypothesis is based on experiments with inactivated antibiotics, administered perorally and parenterally to chickens [1] and pigs [2].

Peterson and Johanson [3] in their experiments with rats have shown that both the active and inactivated agents, regardless of the route of administration, induced marked changes in the intestinal microflora which, however, were not always accompanied by a growth effect in the host. It has been shown by several workers that feeding with a diet containing antibiotics — but limited in vitamin B_{12} — results in increased synthesis (or contents) of vitamin B_{12} in the intestines of chickens [4] and rats [5].

According to Pawe kiewicz [6] the addition of aureomycin to a culture of propionic acid bacteria increases the formation of cyanocobalamin and cobalamin X at the expense of vitamin B_{12p} (which has been identified as factor B). Mannino and Pipitone [7], however, have reported that in *Bacterium coli* the amount of vitamin B_{12} produced in the presence of antibiotics was less than in their absence.

The process of formation of vitamin B_{12} factors during aerobic and anaerobic fermentation of municipal sewage sludge has previously been studied in this laboratory [8].

The attempt has now been made to investigate in what way this process may be influenced by addition of antibiotics.

Fresh sewage sludge with graded levels of aureomycin, terramycin, penicillin and neomycin was fermented aerobically and anaerobically. Samples were taken at definite time intervals and analyzed for vitamin B_{12} activity by means of microbiological tests; the different vitamin B_{12} factors were identified by bioautographic methods.

EXPERIMENTAL

Fresh sludge from a settling tank of the Stockholm municipal sewage plant was disintegrated in a "Turmix" blendor, mixed with *ca.* 20 % of digested sludge and diluted with water. The substrate thus obtained had a dry solid content of *ca.* 4 % and pH 5.0 – 5.5. It was fermented aerobically and anaerobically at 33°C with addition of some antibiotics. The experimental conditions of the different investigations are summarized in Table 1. Experiments Nos. 2 and 3 were performed in glass fermenters each of 10 litres capacity, provided with stirrers, heating coils, arrangements for inlet of gas and for removal of samples. The appropriate temperature (33°C) was obtained by circulating warm water from constant temperature baths through the coils of the fermenters with simultaneous stirring. In the anaerobic fermentation (expt. No. 2) a gas mixture of CH_4 and CO_2 (6:4) was blown through the sludge until all air was removed, this procedure being used every time the sludge came in contact with air, *e. g.* after removal of a sample. In expt. No. 3 air was blown through the sludge, 2 × 30 min every day.

Experiments Nos. 1, 4, 5, 6, and 7 were performed in conical flasks placed in rooms with thermostatically controlled air temperature. Shaking table was used for the aerobic fermentations, whereas for the anaerobic fermentations no special arrangements were made – the flasks were filled with sludge with only a very small space left above and incubated in the thermostat. Samples were treated as described elsewhere [8]. Vitamin B_{12} activity was estimated with the aid of the cup plate, the tube and the bioautographic methods using *Escherichia coli* 113 – 3. In some few cases ionophoretic estimations in combination with bioautography were carried out.

Bioautography. For chromatography it proved to be more convenient to modify the technique used in the earlier experiments [9]. In the present investigation the following procedure was adapted:

Paper: Whatman 1. Each spot corresponded to 1 μl of the solution to be tested. The paper strips were prepared in duplicate and developed for 48 h in two different solvent systems at 18°C.

Solvent system I: *sec.* butanol: H_2O:HAc (75:24:1) + 0.01 % KCN.
Solvent system II: *sec.* butanol: H_2O:NH_3 (75:24:1) + 0.01 % KCN.
Two standard solutions were used:
Standard solution 1 (S1): cyanocobalamin + factor III + factors (Z2 + Z3) [10].
Standard solution 2 (S2): factor A + factor B.

The resolution of factors Z2 and Z3 could not be achieved by this technique but instead factor III could be separated from factor A in solvent system II.

However, after the chromatographic resolution of the initial spots of the samples, the spots with R_F-values corresponding to that of factor A may not represent this factor or perhaps not only this factor. In an earlier investigation [10] it has been shown that sewage sludge contains at least 6 different factors with R_F-values closely related to that of factor A and of ψ -B_{12}. As only the two latter factors had been obtained in crystalline state [11,12], the author found it appropriate to use factor A as standard instead of a mixture of factors isolated from sewage sludge. In the following the spots corresponding to factor A will be designated as "A". Obviously, "A" may signify a mixture of the following factors: A + ψ-B_{12} + XI + X2 + X3 + X4 + III + W in solvent system I, whereas in solvent system II factors III + W can be resolved from the other factors. The spots corresponding to factor III may also contain factor W.

Paper electrophoresis of the samples in combination with bioautography revealed in several instances that "A" corresponded to more than one factor but it was not possible to obtain sufficient information about the nature of these factors.

Following preparations of antibiotics were used in the different experiments:

Aureomycin:	Aureomycin HCl crystalline, Lederle.
Terramycin:	Terramycin HCl crystalline, Pfizer.
Penicillin:	Benzylpenicillin, AB Kabi.
Neomycin:	Mycifradin sulphate, The Upjohn Company.

Heat and alkali treatment of penicillin and neomycin. The solutions were autoclaved at 121°C for 15 min at pH 12.

Table 1.

Expt. No.	Antibiotic added	Type of fermentation	Apparatus	Amount of sludge fermented ml	Time of addition	
					Days after the start of the ferment.	pH of the sludge
1	Aureomycin	Anaerobic	conical flasks (2 l); no stirring	1 800	0	5.5
2	Aureomycin	Anaerobic	glass fermenter (10 l) filled with a mixture of CH_4 and CO_2 gas (6:4); stirring	5 000	40	6.8
3	Aureomycin	Aerobic	glass fermenter (10 l); intermittent aeration; stirring	5 000	40	6.5
4	Auremycin	Aerobic	conical flasks (300 ml); shaking	100	20	7.5
5	Terramycin	Aerobic	conical flasks (300 ml); shaking	100	20	7.5
6	Penicillin	Anaerobic	conical flasks (300 ml);			
	a) active			280	0	5.5
	b) inactivated *			280	0	5.5
7	Neomycin	Anaerobic	conical flasks (300 ml);			
	a) active		no stirring	280	0	5.5
	b) heat and alkali treated, but not inactivated			280	0	5.5

* by heat and alkali.

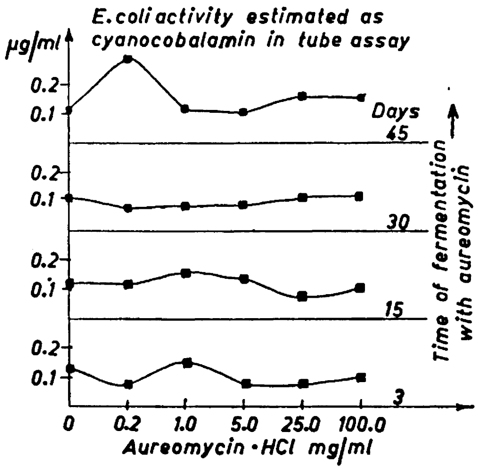

Fig. 1. (Expt. No. 1). Effect of the addition of aureomycin to fresh sewage sludge, subsequently anaerobically fermented.

RESULTS

The pH changes of the fermenting sludges to which antibiotics had been added were essentially the same as the corresponding changes in sludges containing no added antibiotics.

The results of the different experiments are shown in Figs. 1—7 *.

Experiment No. 1. The bioautographic estimation of vitamin B_{12} activity in samples taken 3, 15, 30, and 45 days after the start of the fermentation did not reveal any significant variations in the distribution of *E. coli* activity between the different vitamin B_{12} factors. The estimation in tube assay gave somewhat varying values of the total *E. coli* activity. These values are represented in Fig. 1. A slight maximum in *E. coli* activity can be noticed after 3 days of anaerobic fermentation of the sludge to which 1 mg aureomycin/l sludge had been added. This maximum is still maintained after 15 days of fermentation and it disappears after 30 days while, after 45 days, an unexpected maximum appears in the sludge with 0.2 mg aureomycin/l added. In view of the instability of aureomycin at the actual pH (~ 6) the results can hardly be explained by the antibiotic effect of aureomycin.

Experiments Nos. 2 and 3. The addition of aureomycin (10 mg/l) to sewage sludge which had already undergone certain microbial decomposition (pH ~6.5) resulted in enhanced formation of cyanocobalamin under aerobic condi-

* Abbreviations used in Figs. 1—7.

I	— solvent system I	Z	— factors Z2 + Z3
II	— solvent system II	III	— factor III
t	— time of fermentation	Cy	— cyanocabalamin
S1	— standard solution 1	A	— factor A
S2	— standard solution 2	B	— factor B

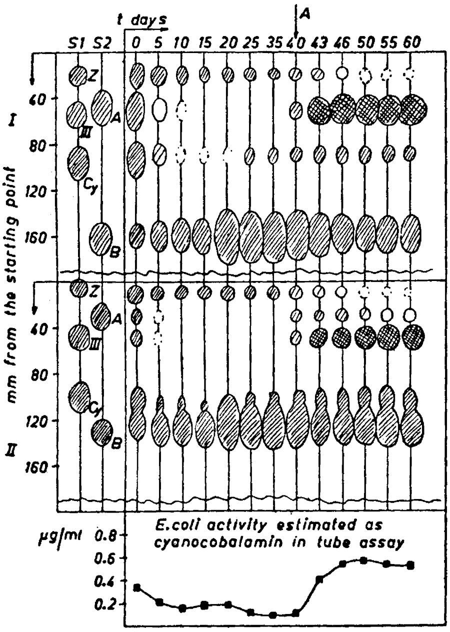

Fig. 2. (Expt. No. 2). Effect of the addition of aureomycin to anaerobically fermenting sewage sludge. Bioautogram *. A — addition of aureomycin . HCl (10 mg/l sludge).

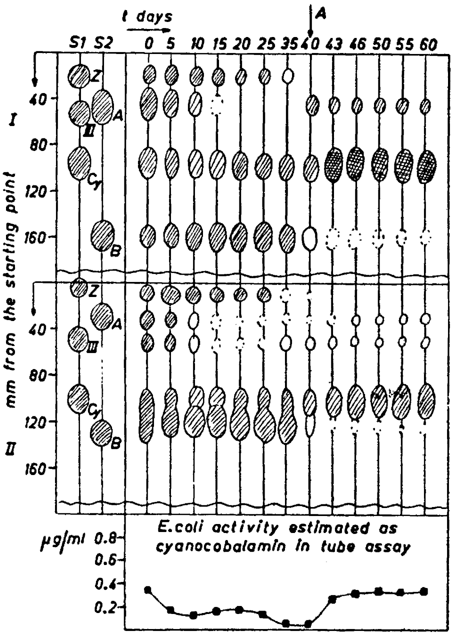

Fig. 3. (Expt. No. 3). Effect of the addition of aureomycin to aerobically fermenting sewage sludge. Bioautogram *. A — addition of aureomycin · HCl (10 mg/l sludge).

tions (*cf.* Fig. 3) and of factor III under anaerobic conditions (*cf.* Fig. 2). This is also clearly reflected in the significant increase of the total *E. coli* activity as estimated in tube assay (*cf.* Figs. 2 and 3).

Experiment No. 4. The results of this experiment further confirm the finding (expt. No. 3) that aureomycin stimulates the formation of cyanocobalamin in aerobically fermented sludge if added at the stage when the pH of the sludge has risen to about 7. The effect was most pronounced with 5—25 mg aureomycin per litre sludge after 40—90 h of fermentation, but was partly abolished by further fermentation as seen in the samples taken after 160 h **. During the first 40 h aureomycin also seemed to stimulate the formation of factors Z, whereas after 90 and 160 h its effect was the opposite. The same phenomenon could be observed with factors "A". The formation of factor III was only slightly stimulated by the addition.

* For abbreviations see footnote on p. 1194.

** This is apparently not fully in agreement with the results of expt. No. 3, where the increased contents of cyanocobalamin persisted for several days (*cf.* Fig. 3). It is possible that the relatively quicker destruction of cyanocobalamin in expt. No. 4 as compared with expt. No. 3 was due to the more vigorous aeration employed in this experiment (*cf.* Table 1).

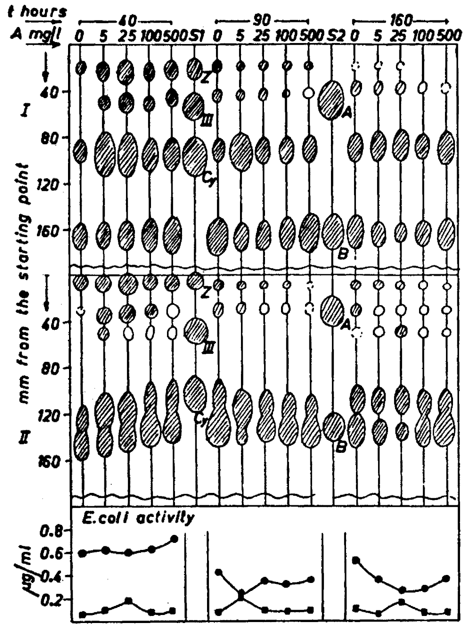

Fig. 4. (Expt. No. 4). Effect of the addition of aureomycin to aerobically fermenting sludge. Bioautogram*. A — aureomycin · HCl, graded amounts. ●—● cup assay; ■—■ tube assay.

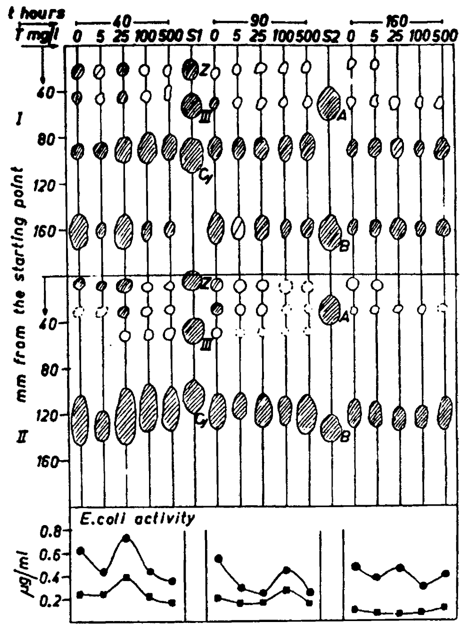

Fig. 5. (Expt. No. 5). Effect of the addition of terramycin to aerobically fermenting sewage sludge. Bioautogram*. T — terramycin · HCl, graded amounts. ●—● cup assay; ■—■ tube assay.

Experiment No. 5. 25—100 mg terramycin per litre aerobically fermented sludge stimulated formation of cyanocobalamin after 40 h of fermentation. This effect was partly abolished during continued fermentation. The effect upon other factors was more obscure. 5 mg terramycin/l sludge seemed to cause a minimum in the amount of factors B and Z, while 25 mg/l caused a maximum in the amounts of these factors as well as in those of factors "A".

Experiment No. 6. a) Addition of active penicillin stimulated the formation of cyanocobalamin; the effect was strongest with 20 mg penicillin/l sludge and could be noted after 10 days as well as after 30 days of anaerobic fermentation. With increasing amounts of penicillin increasing amounts of factors Z could be observed after 10 days of fermentation; the amount of factors "A" and factor III simultaneously decreased. The amount of factor B was almost unaffected. The effect was quite different after 30 days of fermentation. A pronounced maximum in the amounts of factor B, cyanocobalamin and factor III could then be observed with 20 mg/l. Factors Z disappeared.

b) The addition of increasing amounts of inactivated penicillin caused an increased formation of cyanocobalamin. The effect was strongest with 50

* For abbreviations see footnote on p. 1194.

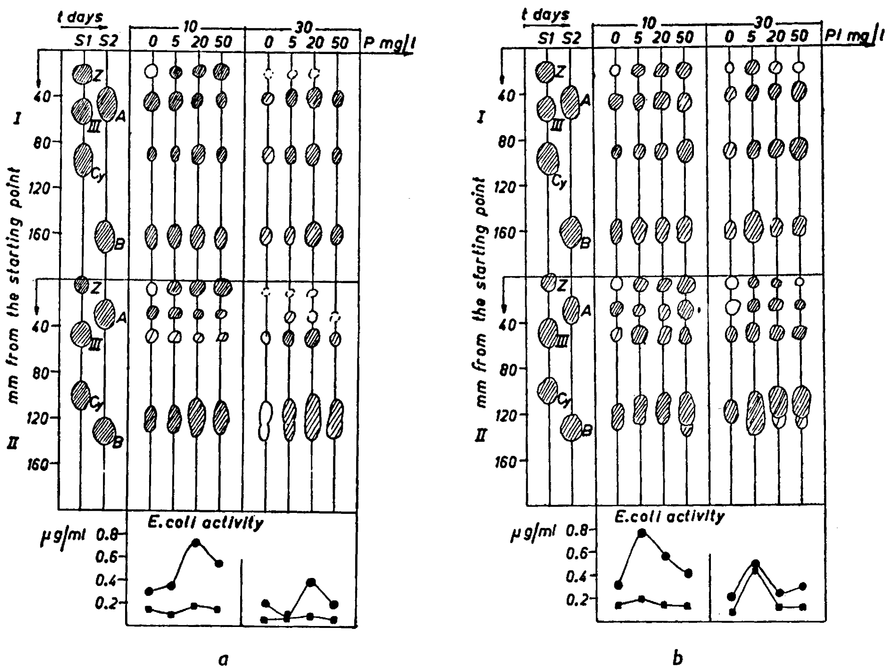

Fig. 6. (Expt. No. 6). Effect of the addition of penicillin to fresh sewage sludge, subsequently anaerobically fermented. Bioautograms *. a) P — active benzyl penicillin; b) Pi — heat and alkali inactivated benzyl penicillin. ●—● cup assay; ■—■ tube assay.

mg/l and could be noted after 10 days of fermentation as well as after 30 days. With increasing amounts of penicillin added, increased amounts of factors Z were observed after 10 days; after 30 days a maximum in the amount of these factors corresponded to the addition of 5 mg/l. The effect on the formation of factors "A" and III was less pronounced although a slight stimulation of their synthesis could be observed also here. The amount of factor B was unaffected after 10 days, but showed a clear maximum after 30 days in the sludge to which 5 mg/l had been added.

Experiment No. 7. a) 5—25 mg neomycin/l sludge caused an increased synthesis of cyanocobalamin and of factors Z, "A" and III after 10 days, but after 30 days the effect of neomycin upon the two latter factors was abolished. The synthesis of factor B was slightly stimulated by the addition of neomycin.

b) Increasing amounts of heat and alkali treated (but not inactivated) neomycin caused a decrease in the vitamin B_{12} activity as estimated in tube assay. The detrimental influence was exerted mainly upon cyanocobalamin, whereas the amount of factor Z showed a slight maximum after 10 days of fermentation in the sludge to which 5 mg neomycin per litre had been added.

The amount of factor B was essentially unaffected by the addition.

* For abbreviations see footnote on p. 1194.

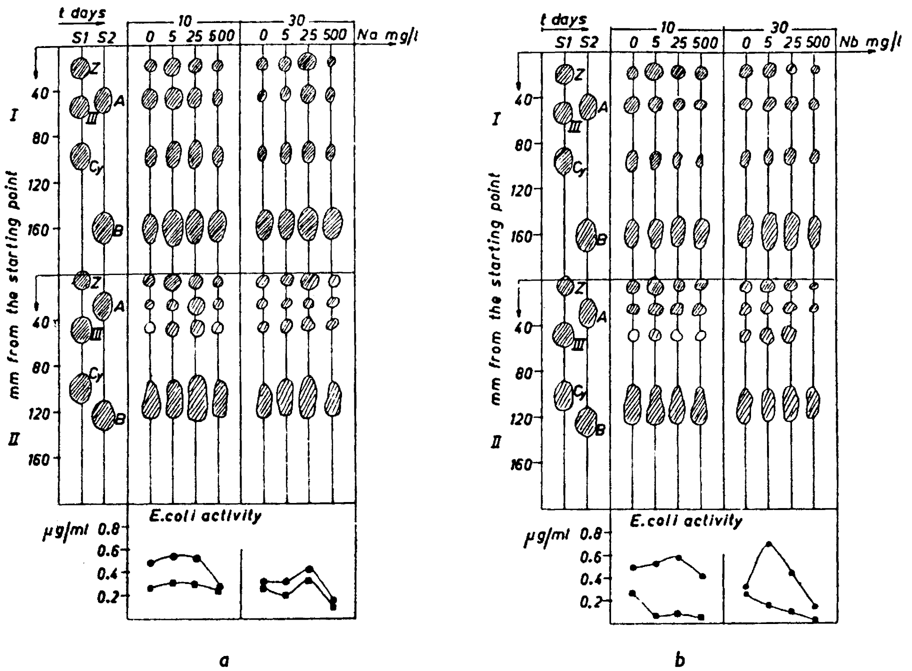

Fig. 7. (Expt. No. 7). Effect of the addition of neomycin to fresh sewage sludge, subsequently anaerobically fermented. Bioautograms *. a) Na — mycifradin sulphate; b) Nb — heat and alkali treated but not inactivated mycifradin sulphate. ●—● cup assay; ■—■ tube assay.

As can be seen in Table 2, antibiotics (except for the heat and alkali treated neomycin) generally increased the total vitamin B_{12} activity of microbially decomposing sewage sludge and most of them caused an increase of that part of the activity which is due to cyanocobalamin. The following circumstances seemed to exert a particular influence on this phenomenon.

1) the type of fermentation: aerobic or anaerobic.

2) the degree of decomposition of the sludge at the moment of addition, as characterized by the pH of the sludge.

Thus, under anaerobic conditions aureomycin had only a very slight effect on the vitamin B_{12} activity of decomposing sludge if added to "fresh" sludge (pH 5.5). Under similar conditions its addition to partly decomposed sludge (pH 6.5—6.8) caused a significant increase in the vitamin B_{12} content mainly due to the synthesis of *factor III*. Corresponding addition to intermittently aerated sludge resulted in an increased synthesis of *cyanocobalamin* accompanied by the disappearance of factor B, whereas with continuous aeration (shaking) a simultaneous formation of cyanocobalamin and of factor B was obtained. It is difficult to decide whether the effect of aureomycin depended on its antibiotic action or whether it was used as a "metabolite" enhancing the synthesis of vitamin B_{12} (*cf.* Pawelkiewicz, 1955[6]). The "immediate"

Table 2. Effect of the addition of antibiotics upon the vitamin B_{12} activity of sewage sludge.

+ = increase; — = decrease; 0 = no change; +,— = slight effect; ++,—— = medium effect; +++,——— = strong effect
+;— = initial increase followed by a decrease.

Antibiotic added mg/l sludge	Type of sludge	Type of fermentation after the addition	Effect on					
			Total vit. B activity, tube assay	Cyano-cobalamin	Factor III	Factors "A"	Factors Z	Factor B
Aureomycin 0.2—5.0	fresh, pH 5.5	anaerobic	+ (?)	0	0	0	0	0
Aureomycin 10	fermenting, pH 6.8	anaerobic	+++	0	+++	0	0	—
Aureomycin 10	fermenting, pH 6.5	aerobic (intermittent aeration)	+++	+++	0	0	0	———
Aureomycin 5—25	fermeuting, pH 7.5	aerobic (continuous aeration)	++	+++	+;—	+;—	+;—	++
Terramycin 25—100	fermenting, pH 7.5	aerobic (continuous aeration)	++	++;—	(+;—)	(+;—)	(+;—)	(—;+;—)
Penicillin								
a) active 5—50	fresh, pH 5.5	anaerobic	+	+++	——	——	+++	0;++
b) inactivated 5—50	fresh, pH 5.5	anaerobic	+++	+++	+	+	+++	+
Neomycin								
a) untreated 5—25	fresh, pH 5.5	anaerobic	++	+++	++	++	++	+
b) heat and alkali treated 5—25	fresh, pH 5.5	anaerobic	———	——	(?)	0	+;—	0

(40 h) effect of the addition of aureomycin to partially decomposed sludge (expts. Nos. 2 and 3) may suggest the latter possibility, or it may indicate that the growth of some strains consuming different vitamin B_{12} factors has been inhibited, thus causing the accumulation of the corresponding factors in the sludge. Probably both mechanisms were involved *.

The results obtained with penicillin differ from those obtained with aureomycin (expt. No. 1) in that the addition to *fresh sludge* of active as well as of inactivated penicillin followed by anaerobic fermentation caused an increase of the vitamin B_{12} activity. Both agents seemed to enhance the synthesis of

* The reservation must here be made that due to some variations in experimental conditions as stirring, shaking, amount of sludge fermented (*cf.* Table 1) not all the experiments are fully comparable.

cyanocobalamin, strikingly accompanied by the synthesis of factors Z which may suggest the possibility of a metabolic relationship between cyanocobalamin and these factors. Active penicillin caused a decrease in the amounts of factors III and "A" and did not affect the amount of factor B in the beginning but increased it during continued fermentation. On the other hand inactivated penicillin increased the amount of all these factors. It seems likely, therefore,, that inactivated penicillin is the agent responsible for an enhanced synthesis of vitamin B_{12} factors in digesting sewage sludge, as the benzylpenicillin used in the experiments must have been inactivated very soon after the beginning of the fermentation.

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