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On Vitamins in Sewage Sludge

II *. Formation of Vitamin B_{12}-, Folic Acid-, and Folinic Acid Factors in Municipal Sludge

HALINA Y. NEUJAHR

Division of Food Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The decomposition of municipal sewage sludge by means of anaerobic and aerobic fermentation was studied with respect to the content and distribution of vitamin B_{12}-, folic- and folinic acid factors.

Factor B was found to represent an intermediate step in the decomposition as well as in the synthesis of other vitamin B_{12} factors.

Factor C_2 seems to occur only during the early stages of the microbial decomposition of the sludge.

The quantitative estimation of vitamin B_{12} activity by different methods and test organisms is discussed in connection with the experiments performed.

A new factor active towards *Streptococcus faecalis* was shown to be present during early stages of the anaerobic fermentation of sludge and was found to be chromatographically identical with a synthetic factor.

The mechanism of the formation of the different vitamin B_{12}-factors during decomposition of sewage sludge is discussed.

It has been previously reported from this laboratory [1] and by other authors [2,2a] that digested sewage sludge contains considerable amounts of 4—6 different vitamin B_{12}-factors. Several folic acid and folinic acid factors have also been found in the sludge [1].

In order to ascertain the necessary statistical basis for the estimation of the quantitative distribution of vitamin B_{12}-activity amongst these factors [3], data have been collected from assays during the last three years. Though the values obtained in this way were fairly constant over long periods of time, considerable deviations could however be noted in a few cases. It happened for instance that in some sludge samples almost all of the vitamin B_{12}-activity was due to the factors B and C, or to the factors A and pseudovitamin B_{12}, and only to a small degree to cyanocobalamin. In order to find some explanation for these phenomenona an attempt was made to investigate in what way the

* The first paper in this series was published by Sjöström, A.G.M., Neujahr, Halina Y. and Lundin, H. *Acta Chem. Scand.* 7 (1953) 1036.

different vitamin B_{12}-factors are formed in the sewage sludge when it is fermented in different manners. For this purpose primary sludge from a settling tank was fermented both aerobically and anaerobically in the laboratory, samples being taken during the fermentation and analyzed for vitamin B_{12}-, folic acid-, and folinic acid-activities in both quantitative and qualitative respects.

Anaerobic digestion of sewage sludge is a very slow process when allowed to develop spontaneously without seeding with previously digested sludge. According to Imhoff and Fair [4] the digestion proceeds in three stages:

1) "Acid fermentation", characterized by intensive acid production (pH drops below 6.0) and putrefactive odours. This stage lasts about 2—3 weeks, large volumes of gas (chiefly CO_2 with some H_2S) being produced.

2) "Acid regression" — a lengthy period (about 3—4 months) with small volumes of gas produced (CO_2 + H_2) and slow rise of pH to about 6.8.

3) "Alkaline fermentation" — large volumes of gas (chiefly CH_4 + CO_2) are liberated. The pH-value may rise above 7.0 and the sludge becomes well buffered.

"Acid fermentation" and "acid regression" constitute a "breaking in" or "ripening" period through which all primary sludge which is not seeded must pass when fermented anaerobically. Once alkaline fermentation is established the well buffered sludge rich in enzymes and possesing proper bacterial flora exerts a controlling influence over the course of digestion of incoming fresh sludge.

In technical practice, therefore, digestion tanks already containing well-digested sludge are charged daily with fresh sludge in amounts that cannot upset the alkaline fermentation. At the sewage plants of Stockholm, for instance, only 1/30 of the digested sludge in the digestion tanks is daily replaced by fresh solids, the total time for sludge digestion being 60 days. Obviously, this method could not be used in our laboratory digestion experiments since the microbiological methods for the determination of vitamin B_{12} have limits of error of ± 20 %. These limits are probably still wider in the experiments where the vitamin B_{12}-activity is due to several different factors. Consequently we limited the amount of "seed sludge" to only 10 % in two experiments and in other experiments no seeding at all was used. The total time of fermentation was thus extremely prolonged.

EXPERIMENTAL

Fresh sludge from a settling tank of the municipal sewage plant of Stockholm was disintegrated in a "Turmix" blendor and placed in glass fermentors each of 10 liters capacity, provided with stirrers, heating coils, arrangements for inlet of gas and for removal of samples. The appropriate temperature (33° C or 55° C) was obtained by circulating warm water from constant temperature baths through the coils of the fermentors with simultaneous stirring. In anaerobic digestions, nitrogen gas 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 removing a sample. In the aerobic fermentation, air was blown through the sludge continously. The gas evolved in anaerobic digestions was collected and measured. Four parallel experiments were performed according to the scheme shown in Table 1.

Samples of about 100 ml were taken off at regular time intervals (3—4 days) and the pH and content of dry solids were determined. 30 ml of each sample was treated as follows:

100 μg NaCN for each ml sludge was added, pH adjusted with sulphuric acid to 5.5, the sample autoclaved at 120° C for 10 min. and centrifuged. The centrifugate was used for the following estimations:

1) vitamin B_{12}-activity with the cup plate, the tube, and the bioautographic methods using *Escherichia coli* 113-3 and *Lactobacillus leichmannii* 313 and 4797, respectively.

2) "folic acid" and "folinic acid" activities with the cup plate and bioautographic methods using *Streptococcus faecalis* and *Leuconostoc citrovorum*, respectively.

Table 1.

Experiment No.	Type of treatment	Temp.	Seeding	pH adjustment	Additions
1	anaerobic	33° C	with digested sludge, 10 % v/v ,at the start	with $Ca(OH)_2$ to 7.0, at the start	crystalline cyanocobalamin after 80 days in an amount corresponding to0.08 μg B_{12}/ml sludge *
2	anaerobic	33° C	none	with $(NH_4)_2CO_3$ to 7.0, after the period of acid fermentation,	no
3	anaerobic	55° C	with digested sludge, 10 % v/v, after 10 days	with $Ca(OH)_2$ to 7.0	no
4	aerobic	33° C	none	no	no

* The reason for this addition is explained on p. 626.

RESULTS AND DISCUSSION

Vitamin B_{12}-factors

In experiments 1, 2 and 4 considerable amounts of cyanocobalamin were formed during the later stages of fermentation. In experiment 3, on the contrary, a destruction of all vitamin B_{12}-factors after about 100 days could be noted. This was probably caused by the relatively high temperature in connection with the prolonged time of the experiment (*cf.* p. 626). In practice where seeding with a sufficient amount of well-digested sludge can be made, such a thermophilic digestion of sewage sludge is performed in only 10—15 days. The course of formation of vitamin B_{12}-factors in the four experiments is represented in Figs. 1 a, b, c, d, respectively, by means of bioautograms with *E. coli* 113-3.

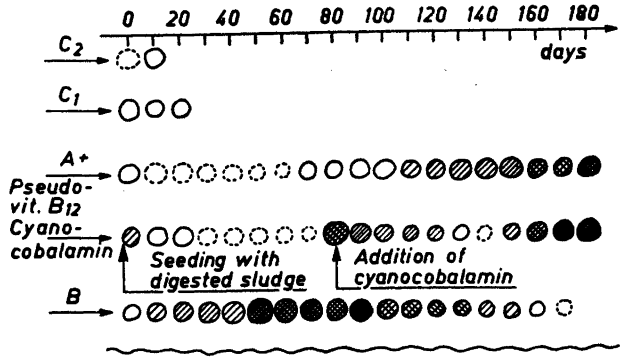

Fig. 1 a. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C (with seeding).

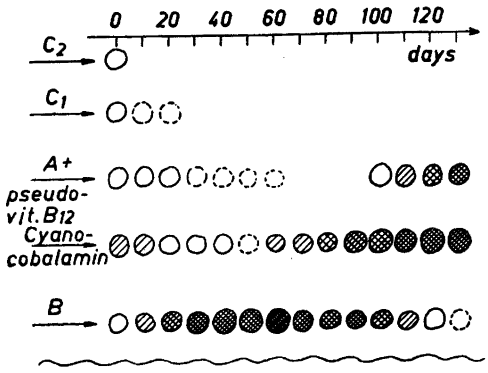

Fig. 1 b. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C (without seeding).

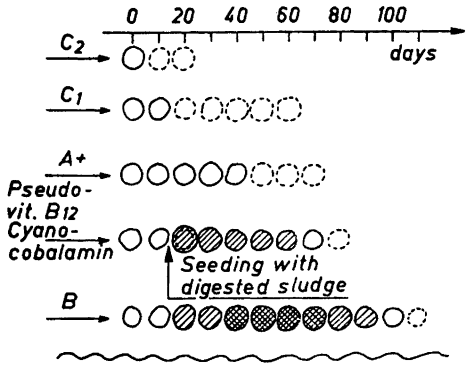

Fig. 1 c. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 55° C (with seeding).

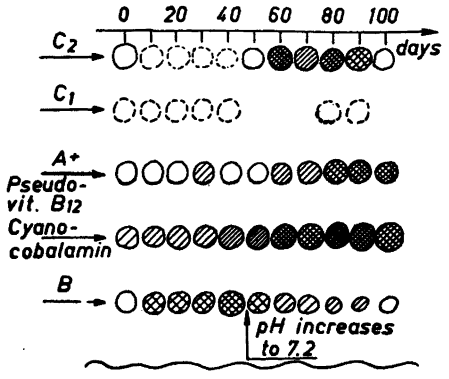

Fig. 1 d. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Aerobic fermentation at 33° C (without seeding).

It can be seen in Figs. 1 a and 1 b that in anaerobic fermentation the amounts of cyanocobalamin and of factors A + pseudovitamin B_{12} — present in the fresh sludge and the seeding — are usually destroyed during the first stage of digestion whilst factor B is simultaneously formed. To confirm this surprising observation, that cyanocobalamin is destroyed instead of being formed, crystalline cyanocobalamin was added after 80 days to the digestion tank of experiment 1 (Fig. 1 a). This amount of cyanocobalamin was also slowly destroyed. In the last stage of the digestion (after about 5—6 months) cyanocobalamin and factors A + pseudovitamin B_{12} were reformed while factor B disappeared. Factor C_1 and factor C_2 were present only at very early stages of the digestion.

Anaerobic digestion at 55° C (Fig. 1 c) had a similar course with initial destruction of cyanocobalamin and formation of factor B. At the later stage of this digestion, all vitamin B_{12}-factors were destroyed, probably due to the high temperature in connection with the prolonged time of the experiment as already mentioned. It can be seen in Fig. 1 c that factor B is comparatively more resistant to this destruction than the other factors which is in good agreement with the observation made above and by other authors[5,6] that this factor is a decomposition product of cyanocobalamin and factors A + pseudovitamin B_{12}.

In the aerobic fermentation of sludge (Fig. 1 d), no destruction of cyanocobalamin could be noted. This factor was instead formed continuously and, after 70 days, became the dominant form of vitamin B_{12}. Notable amounts of factor B were formed during the first 30 days, but after 40 days, when the pH-value increased to 7.0—7.5 (Figs. 1 d and 2 b), this factor slowly disappeared while considerable amounts of cyanocobalamin were formed, and also of factors A + pseudovitamin B_{12} and of factor C_2.

Quantitative estimation of vitamin B_{12}-activity. The quantitative estimation of vitamin B_{12} activity formed during the anaerobic and aerobic fermentation as performed by two different methods with *E. coli* 113-3, revealed a striking discrepancy between the values obtained (Figs. 2 a and 2 b).

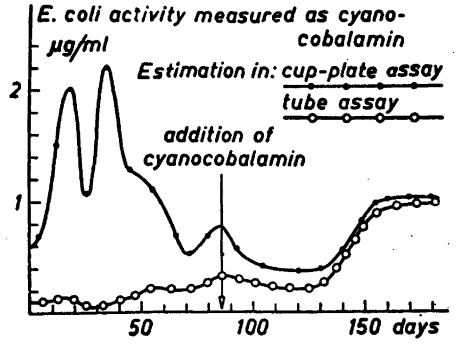

Fig. 2 a. Variations in vitamin B_{12}-activity during microbial decomposition of sewage sludge. Anaerobic fermentation at 33° C.

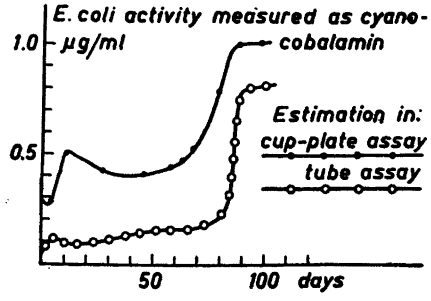

Fig. 2 b. Variations in vitamin B_{12}-activity during microbial decomposition of sewage sludge. Aerobic fermentation at 33° C.

Figs. 2 a and 2 b show that, in the determination of the vitamin B_{12} activity of sewage sludge, the cup-plate method gives much higher values than the turbidimetric method. This is due to the fact that the sludge contains not only cyanocobalamin but also other vitamin B_{12}-factors, many of which in the cup plate method exert a greater activity towards the test organism than in the tube method. The discrepancy in the values is especially pronounced when factors B and C are present in considerable amounts (*cf. e.g.* Figs 1a and 2a). Gregory and Holdsworth [7] have determined the activities of different vitamin B_{12}-factors towards *E. coli* in the tube assay and found them to be 34—92 % lower than that of cyanocobalamin. The corresponding activities in the cup-plate assay as found by Ford [8] are much higher than the activity of cyanocobalamin with differences varying between 274 and 1 000 %. This may account for the fact that the cup-plate method proved to be much less reliable than the turbidimetric method in the estimation of vitamin B_{12}-activity of sewage sludge, which often contains relatively large amounts of factors B, C and pseudovitamin B_{12}. However as soon as cyanocobalamin becomes the main vitamin B_{12}-factor in the sample analyzed, both methods may be of the same degree of reliability, as can be seen, for instance, in Fig. 2 a (activity values after 150 days of digestion) and in Fig. 1 a which shows that after 150 days cyanocobalamin and factors A + pseudovitamin B_{12} dominate.

A large discrepancy has also been found between the values for vitamin B_{12} activity obtained by assaying with *E. coli* 113-3 and *L. leichmannii*, the latter values being much lower. This is in agreement with the findings of Ford [9] that the "vitamin B_{12}-like" factors are much less active towards *L. leichmannii* than towards *E. coli* 113-3, factor B not being active at all towards the first mentioned organism.

Discussion. The formation of cyanocobalamin and other vitamin B_{12}-factors in sewage sludge is apparently a complicated process, proceeding in several stages and being caused by several different microorganisms, anaerobic as well as aerobic. Some of the anaerobic organisms probably consume cyanocobalamin and produce factor B which is then transformed by other organisms to cyanocobalamin and to factors A + pseudovitamin B_{12}. Factor C_2 seems to occur only during the growth period of the microorganisms. It was present during the early stages of both the anaerobic and the aerobic fermentations. In the aerobic fermentation it was also found at a later stage where the pH increases from about 5.0 to about 7.2 probably allowing a new microflora to grow.

However the results obtained until now do not exclude the possibility that during anaerobic fermentation the destruction of factors $C_1 + C_2$ at an early stage and the more slowly proceeding destruction of cyanocobalamin and factors A + pseudovitamin B_{12} during formation of factor B, and the disappearance of factor B under reformation of cyanocobalamin and factors A + pseudovitamin B_{12} during a later stage are independent processes, caused by different microorganisms. The formation of these factors during the different stages of fermentation could thus be caused by successive changes in the sludge, allowing different microorganisms to grow and to produce their characteristic growth factors.

In view of the findings of Armitage *et al.*[5] and of Gant *et al.*[6] that factor B is cyanocobalamin, factor A, or pseudovitamin B_{12} lacking their respective nucleotides, it seems possible that factor B formed during the digestion of sludge may derive from the decomposition of these three factors, or some of them. It is, however, probable that at least some of the organisms active in sludge digestion may be able to directly synthesize factor B. This seems to hold especially for organisms active in the aerobic decomposition of sludge since in this case no destruction of cyanocobalamin occurred during the formation of factor B. The mechanism of formation of factors A + pseudovitamin B_{12} could not be explained with any degree of probability. For the formation of these two factors * the presence of both factor B and cyanocobalamin may be necessary; cf. Fig. 1 a and 1 b.

Probably, the best solution of these problems could be obtained by isolation of the different organisms active in sludge decomposition and by investigating their ability to synthesise the different vitamin B_{12}-factors. This task, however, has proved so difficult and time consuming that we had to postpone it for the time being, especially as we have some reason to suspect that complicated symbiotic and synenergistic phenomenona are involved in the mechanism of vitamin B_{12} formation in the sludge.

Folic acid- and folinic acid-factors.

The course of formation of these factors in the anaerobic fermentation of sewage sludge carried out at 33° C is represented in Fig. 3 a and 3 b.

Fig. 3 a shows that during the digestion the "folinic acid activity" diminishes much quicker than the "folic acid activity" does. Fig. 3 b shows a chromatographic picture of different factors present and active towards *S. faecalis*. At early stages of the digestion the formation of a *new S. faecalis*-factor — PFH — with R_F-value = 0.75 could be noted (see Fig. 3 b). This factor has been chromatographically identified with a synthetic factor obtained by Ericson[9] in this laboratory by formylation and reduction of pteroic acid. This factor could not be found in a wort fermented by an anaerob, *Clostridium thermocellulaseum*[10].

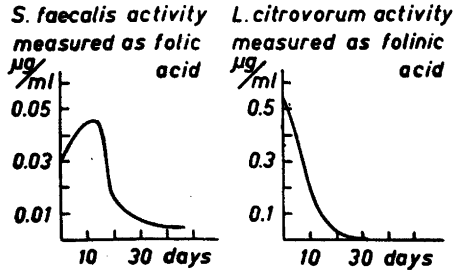

Fig. 3 a. Variations in S. faecalis- and L. citrovorum-activities during microbial decomposition of sewage sludge. Anaerobic fermentation at 33° C.

* After this paper had been prepared for publication, the latest papers of Ford and Holdsworth[9] came to our knowledge. In view of the synthesis of factor A, pseudovitamin B_{12} and cyanocobalamin, performed by these authors from factor B and precursors of different nucleotides, it seems likely that the formation of these three factors at a later stage of sludge decomposition depends on the appearance of the corresponding precursors in the medium.

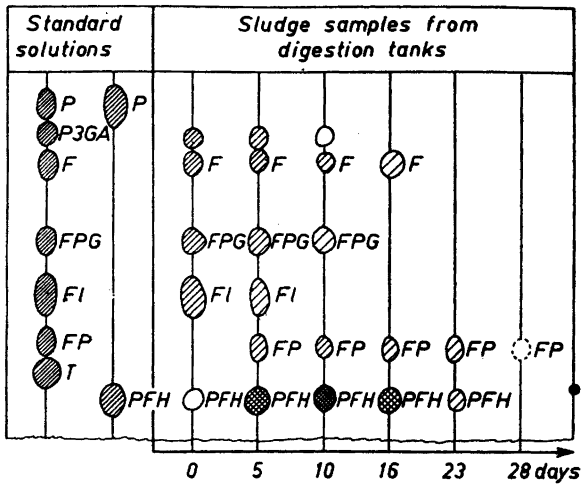

Fig. 3 b. Formation (destruction) of S. faecalis-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C.

P = *pteroic acid*
P3GA = *pteroyltriglutamic acid*
F = *folic acid*
FPG = *formylpteroylglutamic acid*
Fl = *folinic acid (Leucovorin)*
PF = *formylpteroic acid (Rhizopterin)*
PFH = *pteroic acid, formylated, hydrated.*

The appearance of this factor between the 5th and the 23rd day of digestion (Fig. 3 b) may explain the maximum of the *S. faecalis*-activity curve occurring on the 14—15th day of digestion (Fig. 3 a).

Bioautograms with *L. citrovorum* revealed activity mainly due to folinic acid (Leucovorin Lederle) and only to a less degree due to two unidentified factors with R_F-values 0.18 and 0.22. The presence of these factors in sludge has already been reported in a previous work [1]. Nothing of special interest concerning the course of formation or disappearance of these factors could be noted.

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