ACTA CHEMICA SCANDINAVICA 7 (1953) 1036—1040

Vitamin B_{12}, Folic Acid, and Folinic Acid Factors in Digested Municipal Sludge

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Many bacteria and streptomycetes are able to produce vitamin B_{12} * when cultivated under aerobic conditions as reported by many workers. It has also been found that facultative and obligate anaerobic bacteria can produce the vitamin in the absence of oxygen. Pfiffner *et al.*[1] found two vitamin B_{12}-like substances, which they named pseudo-vitamin B_{12} and pseudo-vitamin B_{12b}, in the fermentation broth of an anaerobic bacteria isolated from rumen contents. Lewis *et al.*[2] isolated a similar substance, named vitamin B_{12f}, from rat faeces. Ford *et al.*[3] have isolated three vitamin B_{12}-like factors, named factors A, B, and C, from calf rumen contents and faeces. Leviton *et al.*[4] found that propionic acid bacteria synthesized vitamin B_{12} in very high amounts when cultivated under aerobic or anaerobic conditions. Finally, Hodge *et al.*[5] have reported the production of vitamin B_{12} by certain anaerobic bacteria in pure and mixed cultures.

Hoover *et al.*[6] found significant amounts of vitamin B_{12} in "activated" sludge from municipal sewage treatment plants. They did not show, however, if the vitamin found had been formed during aerobic or anaerobic digestion. In the following it will be shown that vitamin B_{12} is produced in the anaerobic digestion of municipal sewage sludge.

The vitamin B_{12} content of digested sludge from three sewage treatment plants was estimated. The nature of the vitamin B_{12} factors present was determined by chromatography and ionophoresis in combination with bioautography. Crystalline cyanocobalamin was isolated from the sludge and its identity confirmed spectrophotometrically. The nature and amount of desoxyribosides, folic acid and folinic acid factors in fresh and digested sludge were also investigated.

* In this paper the term "vitamin B_{12}" is used as the collective name for cyanocobalamin and other, vitamin B_{12}-like substances.

EXPERIMENTAL

Samples of sludge from the different stages of the digestion process were taken at three municipal sewage treatment plants in Stockholm, *viz.* the Henriksdal, Loudden, and Åkeshov plants, hereafter referred to as plants 1, 2, and 3 respectively. The samples were stored at −20° C.

More than 98 % of the vitamin B_{12} amount present was found to be adsorbed on particles suspended in the sludge. In order to release the vitamin into the water phase before analysing, the samples were autoclaved. The maximum amount of vitamin B_{12} was released by autoclaving at pH 6.0 and 120° C for 15 min. in the presence of 0.01 % potassium cyanide. After centrifugation the clear solution was diluted to an appropriate concentration and applied to the agar cup plate method with *E. coli* 113-3 [7] using the medium described by Diding [8]. Crystalline cyanocobalamin (Merck) was used as a standard.

The same solution was analysed for desoxyribosides, folinic acid and folic acid factors with the agar cup plate method using *Lactobacillus lactis* Dorner (ATCC 8 000), *Leuconostoc citrovorum* (ATCC 8 081), and *Streptococcus faecalis* (ATCC 8 043) as test bacteria. The media used were those described by Bánhidi *et al.* [9] (for *L. lactis* and *L. citrovorum*) and Capps *et al.* [10] (for *S. faecalis*). The last mentioned medium was prepared from Difco folic acid assay medium with 1.6 % agar. Thymidine, synthetic folinic acid (Leucovorin, Lederle), and folic acid (Folvite, Lederle) were used as standards.

The nature of the different growth factors was determined by chromatography or ionophoresis in combination with the bioautographic technique first introduced by Winsten and Eigen [11]. The bacteria and media were the same as in the agar cup plate methods. The paper chromatograms were run at 20° C on Whatman No. 1 filter paper and developed with *sec.* butanol saturated with water and containing 3 % acetic acid and 0.0025 % potassium cyanide. The R_F-values of some of the growth factors showed to be sensitive to the pH and the salt concentration of the samples. The comparison of the growth factors with known substances was, therefore, always performed by chromatography of samples and of known factors on the same strip.

The electrophoretic separation of the vitamin B_{12} factors was made according to Ericson *et al.*[12].

RESULTS

The vitamin B_{12} content of the digested sludge from plant 1 was followed from Jan. 1952 to Febr. 1953. No regular seasonal variations were observed. The concentration of the vitamin varied from 1.2—2.4 μg/ml; the mean value was 1.7 μg/ml or 22 μg/g dry matter. Digested sludge from plants 2 and 3 was analysed on two occasions and was found to contain 1.5 and 1.6 μg/ml vitamin B_{12}, respectively.

In order to investigate whether the vitamin B_{12} found had been synthesized during the digestion process or whether it had been brought into the plant with the sewage, samples from different stages of the sewage treatment process in plant 1 were collected and analysed. It was found that 82 % of the vitamin B_{12} content of the digested sludge was produced during the digestion process; 18 %, already present in the fresh sludge (0.13 μg/ml), possibly originates from human faecal matter.

Paper chromatography with bioautography revealed the presence of four growth factors (called factors I, II, III, IV) for *E. coli* 113-3 in fresh and digested sludge with the R_F-values 0.08, 0.14, 0.20, and 0.28. Factors I, II, III were also found in human faeces. Factor IV had the same R_F-value as factor B [3], factor III the same as cyanocobalamin, and factor II the same as factor A [3], pseudo-vitamin B_{12} [1], and vitamin B_{12f} [2]. Factors I, II, and III

Table 1. L. citrovorum and S. faecalis factors in fresh and digested sludge.

Factor	R_F-value	Fresh sludge		Digested sludge	
		L. c.	*S. f.*	*L. c.*	*S. f.*
Pteroic acid	0.10		+		(+)
Unidentified	0.18	+		(+)	
Unidentified	0.22	++		+	
Pteroyltriglutamic a	0.22		+		(+)
Folic acid	0.27		++		++
Unidentified	0.28	+		+	
Formylpteroylglutamic a.	0.40		+		(+)
Folinic acid	0.47	++	+++	+	+
Formylpteroic acid	0.57		(+)		(+)
Thymidine	0.66	+	+	(+)	(+)

The number of plus signs indicates the intensity of growth of the different factors as observed after chromatographic separation.

were also active towards *L. lactis* Dorner (ATCC 8 000) and *Lactobacillus leichmannii* 313 (ATCC 7 830). By autoclaving samples at 120° C and at different pH for 15 min. it was shown that the four factors were stable in the pH range 3—9. At pH 13.3 the vitamin B_{12} activity of the crude samples was fully destroyed. This is in agreement with the properties of vitamin B_{12} in liver extracts [14].

Paper electrophoresis of the samples showed the presence of six factors; three moving towards the cathode, one immobile, and two moving towards the anode. By comparison with known vitamin B_{12} factors it was found that the three positively charged factors migrated with the same speed as factor B, factor A, and pseudo-vitamin B_{12}, respectively. The immobile factor behaved as cyanocobalamin. Paper electrophoresis of a preparation, which contained mainly factor I, showed that this factor was one of the factors moving towards the anode. This behaviour is typical for factor C [3], as reported by Holdsworth [15].

From the above it is probable that the vitamin B_{12} activity of fresh and digested sludge derives from a mixture of cyanocobalamin *, factor A, pseudo-vitamin B_{12}, factor B, factor C, and a sixth factor (possibly the same as factor C_2 [16]). Factor I (C) seems to be rather labile and is either destroyed or converted to factor II (A and ψ-B_{12}), or factor IV (B), or both on standing in a water solution at room temperature. The bioautograms indicated that more than 50 % of the *E. coli* activity found was due to factor III (cyanocobalamin), 25 % to factor II and 25 % to factors I and IV.

In order to get a better proof for the identity of factors I, II, III, IV with the above mentioned vitamin B_{12} factors an attempt was made to isolate the different factors in a crystalline form from digested sludge.

* Chromatographic analysis without the addition of cyanide showed the presence in fresh and digested sludge of hydroxocobalamin and very little cyanocobalamin.

30 l digested sludge were boiled at pH 6.0 for 15 min. in the presence of 100 mg/l KCN. After centrifugation the solution was acidified to pH 3.0 and filtered with 2 % filter aid. The vitamin B_{12} activity of the filtrate was adsorbed on IRC50 [17] (Rohm & Haas) and thereafter eluted with *iso*propanol-water (60 : 40). The *iso*propanol of the eluate was distilled off *in vacuo* and the remaining liquid treated according to Schindler and Reichstein [21]. Factors I and IV were partly lost in the water phase during the phenol-chloroform extraction and in the methanol-insoluble material. Chromatography of the purified extracts on alumina column separated factors III and II. Factor III was eluted with methanol in a rather pure form while factor II left the column in impure form only after elution with water containing cyanide. The methanol eluate was evaporated and the red material purified by repeated crystallizations in acetone-water. Under the microscope the product was found to consist of red, needleshaped crystals.

A water solution of the pure factor III showed absorption maxima at 278, 323, 361, 523, and 550 mμ. The ratio E_{361}/E_{550} was 3.22. Cyanocobalamin gives the same absorption maxima and ratio [18] and it can therefore be concluded that factor III is identical with cyanocobalamin.

The digestion of municipal sewage sludge, which is carried out at 30° C at the three plants investigated, is believed to be accomplished in two stages by two types of bacteria. In the first stage the sludge is decomposed into simple organic compounds by bacteria with highly developed enzyme systems, *inter alia* cellulose fermenting bacteria. In the second step the degradation products formed are "mineralized" to methane, carbon dioxide and ammonia by methane bacteria. Whether the vitamin B_{12} is formed by one or both of these two types of bacteria has not been investigated. A cellulose thermophile, *Clostridium thermocellulaseum*, produced small amounts of vitamin B_{12} (mostly factor A [3]) when cultivated according to Enebo [13]. This indicates that at least some bacteria of the first type are able to synthesize vitamin B_{12}.

Other growth factors. Chromatography with *L. lactis* Dorner showed the presence of small amounts (less than 200 μg/g dry matter) of three desoxyribosides in fresh and digested sludge. They were identified as the desoxyribosides of thymine, adenine, and guanine by comparison with the corresponding pure compounds.

Fresh and digested sludge possessed 3.8 and 0.9 μg/g dry matter folic acid activity resp., and 7.7 and 1.3 μg/g folinic acid activity resp. It was evident that none of these growth factors was produced during the digestion process. Chromatographic studies revealed that the experimentally determined *L. citrovorum* and *S. faecalis* activities derived from five and seven different growth factors resp. (listed in Table 1). Some of the factors were identified with pure substances. The three unknown are probably identical with factors found in algae and lichens [19,20].

SUMMARY

Considerable amounts of vitamin B_{12} are formed during the anaerobic digestion of municipal sewage sludge. The vitamin B_{12} activity derives from hydroxocobalamin (50 %), factor A and pseudovitamin B_{12} (25 %), and factor B, factor C and factor C_2 (together 25 %).

Desoxyribosides and factors belonging to the folic acid and folinic acid groups have been found in fresh and digested sludge.

This investigation was greatly facilitated by the excellent cooperation of Mr. H. Johansson, Head of the Laboratory, Henriksdals Reningsverk, Stockholm.

The authors wish to express their thanks to Prof. C. A. Elvehjem and to Dr. U. J. Lewis, Department of Biochemistry, University of Wisconsin, Madison, Wis., for samples of vitamin B_{12} and pseudo-vitamin B_{12}, to Dr. J. W. G. Porter, The National Institute for Research in Dairying, Shinfield, Reading, for a solution containing factor A and factor B, and to the Lederle Laboratories Inc., New York, for samples of the different folic acid and folinic acid factors.

We also wish to thank Dr. L.-E. Ericson of this laboratory for the electrophoretic studies and Dr. L. Enebo for samples of *Clostridium thermocellulaseum* fermentation broth.

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Received May 20, 1953.