ACTA CHEMICA SCANDINAVICA 12 (1958) 408—414

On Vitamins in Sewage Sludge

VI. The Effect of Cobalt Chloride and Copper Sulphate on Vitamin B_{12} Contents of Microbially Decomposing Sewage Sludge

HALINA Y. NEUJAHR

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

The effect of the addition of cobalt chloride and copper sulphate to fermenting sewage sludge was studied with respect to *E. coli* activity formed and its distribution between the different vitamin B_{12} factors.

The addition of cobalt chloride was found to stimulate the synthesis of cyanocobalamin and vitamin B_{12}-like factors in aerobically fermenting sewage sludge, whereas under anaerobic conditions its stimulating effect was less pronounced and restricted only to the initial stages of the fermentation.

The addition of copper sulphate had a detrimental effect on the formation of cyanocobalamin and of some vitamin B_{12}-like factors but not of factors Z, the formation of which in the aerobically fermenting sludge was drastically stimulated by the highest additions of copper sulphate used.

Many investigators have observed the growth promoting and vitamin B_{12} sparing effect resulting from the addition of small amounts of antibiotics, arsonic acids, and certain sulfonamides as well as of certain mineral compounds (*i.e.* cobalt, copper and iron salts) to commercial animal rations (*cf. Nutrition Revs 14* (1956) 238). It has been suggested that several of these agents may exert an influence on the intestinal synthesis of vitamin B_{12}, *i.e.* by alteration of the microbial intestinal flora (*cf. Vitamins and Hormones 14* (1956)).

Microbially decomposing sewage sludge is the site of synthesis of different vitamin B_{12} factors[1]. It has been shown in a previous investigation [2] that the addition of small amounts of several antibiotics may exert an influence upon the amounts of the different vitamin B_{12} factors synthesized during the process of microbial decomposition of sludge. A study of a similar effect resulting from the addition of cobalt chloride and copper sulphate is reported in the present paper.

EXPERIMENTAL

Fresh sludge from a settling tank of the Stockholm municipal sewage plant was disintegrated in a "Turmix" blendor, then mixed with ca. 20 % of digested sludge and diluted with water. The material 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 the addition of graded amounts of $CoCl_2 . 6H_2O$ and $CuSO_4 . 5H_2O$, respectively. The experiments were performed in conical flasks of 300 ml capacity placed in rooms with thermostatically controlled air temperature. In the anaerobic fermentations the flasks were filled with 280 ml of sludge and incubated in the thermostat. In the aerobic fermentations the flasks were placed in a shaking machine and the amount of sludge fermented was 100 ml. All additions were made before the start of the fermentations. Samples were autoclaved in the presence of KCN as described elsewhere[1]. Vitamin B_{12} activity was estimated with the aid of the cup plate, the tube and the bioautographic methods using *Escherichia coli* 113—3. Paper chromatograms were developed for 48 h at 18°C in the following two solvent systems:

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 to identify the spots after chromatographic separation.

Standard solution 1 (S1): cyanocobalamin + factor III + factors (Z2 + Z3)[3].
Standard solution 2 (S2): factor A + factor B.

Each spot corresponded to 1 μl of the solution to be tested. The spot corresponding to that of factor A may represent a mixture of several of the following factors:

A + ψ-B_{12} + III + W + XI + X2 + X3 + X4 in solvent system I, whereas in solvent system II factors III + W can be separated from the other factors. The spot corresponding to factor A is in the following designated as "A". The spot corresponding to factor III may contain also factor W.

The following additions to the sludge were made:

% $CoCl_2 . 6H_2O$	corresp. to *ppm* Co^{++}	% $CuSO_4 . 5H_2O$	corresp. to *ppm* Cu^{++}
0.00	0	0.00	0
0.05	118	0.05	127
0.10	236	0.10	254
0.50	1 118	0.50	1 270
1.00	2 360	1.00	2 540

According to Anderson[4] digested municipal sludges may contain 315—1 980 ppm Cu^{++} (average: 643). No published data about the contents of cobalt in native or digested sludge were available. Total copper in the sludge mixtures used for the fermentation experiments was determined absorptiometrically with diethylammonium diethyldithiocarbamate in the dry ashed sludge according to the method of Abson and Lipscomb[5]. The values obtained were 22—25 μg Cu/ml sludge (determined as Cu^{++}) and showed fair constancy in several investigated batches of the sludge. These values are of a quite different order of magnitude from those reported by Anderson[4] for American digested sludge. The difference probably reflects the influence of industrial products entering sewage purification systems in Stockholm and in the American towns.

The cobalt contents in the sludge mixtures were determined absorptiometrically with 1-nitroso-2-naphthol by the method of Middleton and Stuckey[6]. The determinations were carried out on dry ashed sludge as in the case of copper. The values obtained were 0.5—0.6 μg Co/ml sludge (determined as Co^{++}). This represents an amount about ten times as large as the amount of cobalt bound in the vitamin B_{12} factors.

RESULTS

It can be seen in Fig. 1 that the highest additions of copper sulphate (0.5 — 1.0 %) caused a marked decrease in the pH of the sludge in both the aerobic and the anaerobic fermentation thus indicating that the "normal"

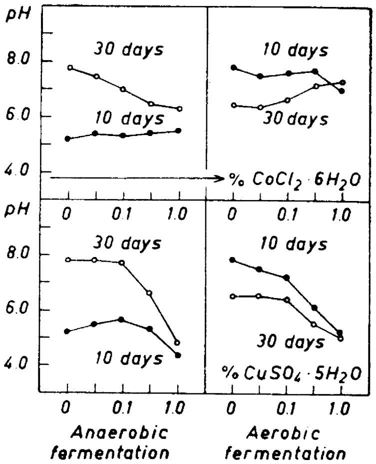

Fig. 1. Effect of addition of cobalt chloride and copper sulphate upon the pH of microbically decomposing sewage sludge.

microbial flora of the decomposing sludge had become modified by the addition. The anaerobic microbial decomposition of the sludge proceeded in two stages; the first stage lasting for at least 10 days was characterized by a lower pH (5.0 — 5.5) and the second stage by a higher pH (7 — 8).

In the aerobic process (continuous shaking) a pH value of 7 — 8 was reached already after 10 days and was followed by a drop to pH 6.5 after 20 more days of microbial decomposition.

Obviously, these differences in pH variations between the aerobic and the anaerobic processes must be taken into account when considering the differences in formation of the different vitamin B_{12} factors during these processes.

Effect of cobalt chloride

a) *Anaerobic fermentation* (Fig. 2a). The addition of 0.1 % $CoCl_2.6H_2O$ corresponding to 236 ppm of Co^{++} slightly stimulated the synthesis of cyanocobalamin and of factor B during the first 10 days of fermentation. The addition of 0.05 — 0.1 % $CoCl_2.6H_2O$, corresponding to 118—236 ppm of Co^{++}, stimulated the synthesis of factors Z in a very pronounced way. With greater amounts of cobalt chloride a detrimental effect on the synthesis of all vitamin B_{12} factors could be observed. After 20 more days of fermentation with 118 ppm Co^{++} a slightly stimulating effect on the synthesis of factors Z and of factors "A" could be noticed whereas the amount of cyanocobalamin remained unaffected by the additions of 118 and 236 ppm and rather diminished by the additions of larger amounts.

b) *Aerobic fermentation* (Fig. 2b). The effect of cobalt chloride was here much more pronounced than under anaerobic conditions. With 118 ppm of Co^{++} a drastic stimulation of the synthesis of cyanocobalamin and of factors Z could be observed after 10 days of fermentation. At the same time the amounts of factors "A" and of factor B decreased with increasing amounts

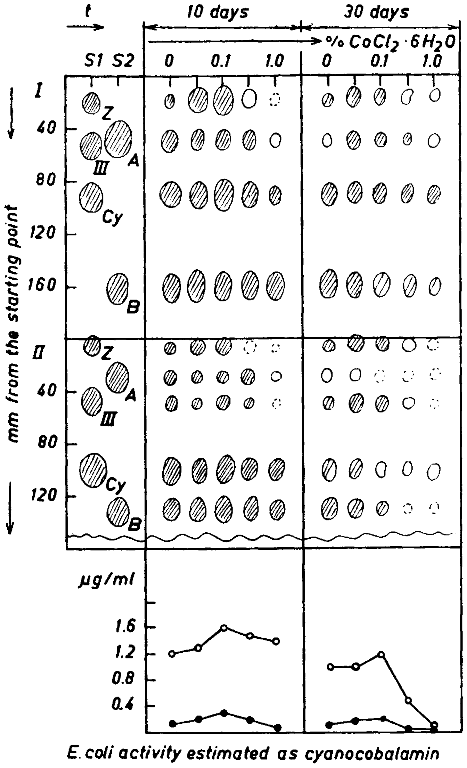

Fig. 2a. Effect of addition of cobalt chloride to fresh sewage sludge, subsequently anaerobically decomposed. Bioautogram.

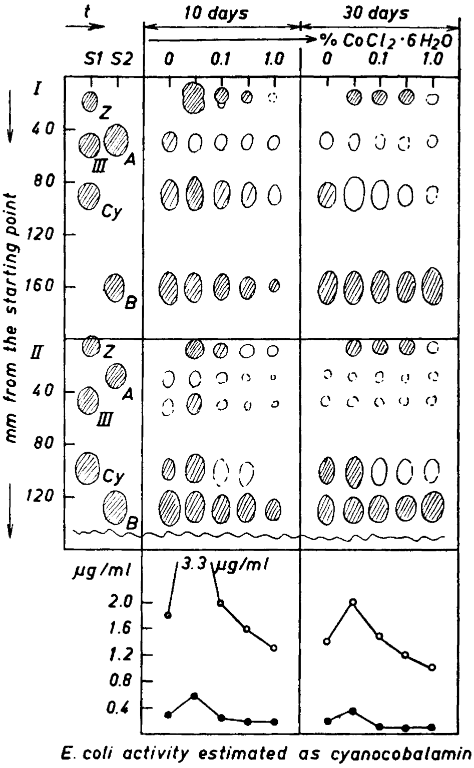

Fig. 2b. Effect of addition of cobalt chloride to fresh sewage sludge, subsequently aerobically decomposed. Bioautogram. ○———○ cup assay; ●———● tube assay.

of Co^{++}. After 20 more days of fermentation the stimulating effect on the synthesis of cyanocobalamin was still very pronounced whereas the effect on the synthesis of factors Z was less pronounced than after 10 days. The addition of 118 — 1 180 ppm of Co^{++} seemed to stimulate the synthesis of the latter factors.

The amount of factor B was greatest in the sludge with the highest addition of Co^{++} (2 360 ppm) which was accompanied by an almost total disappearance of all the other vitamin B_{12} factors.

Effect of copper sulphate

a) *Anaerobic fermentation* (Fig. 3a). The addition of 0.05 % $CuSO_4.5H_2O$ (corresponding to 127 ppm of Cu^{++}) had, after 10 days of fermentation, a slightly stimulating effect on the synthesis of all vitamin B_{12} factors. Higher additions had either no effect or a decreasing one. However, after 20 more

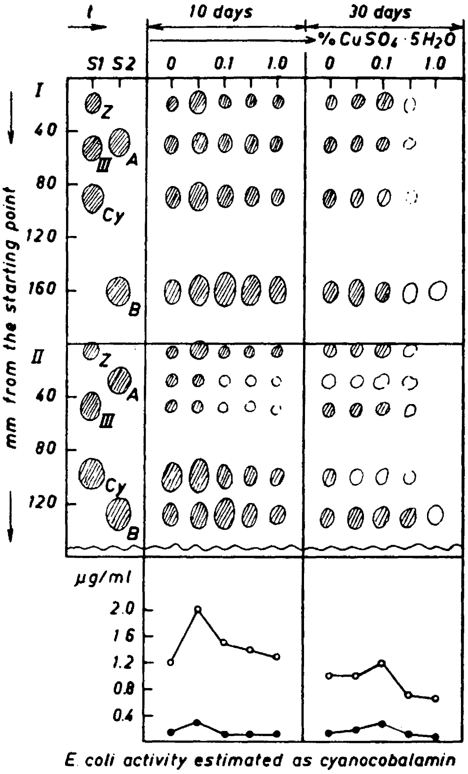

Fig. 3a. Effect of addition of copper sulphate to fresh sewage sludge, subsequently anaerobically decomposed. Bioautogram.

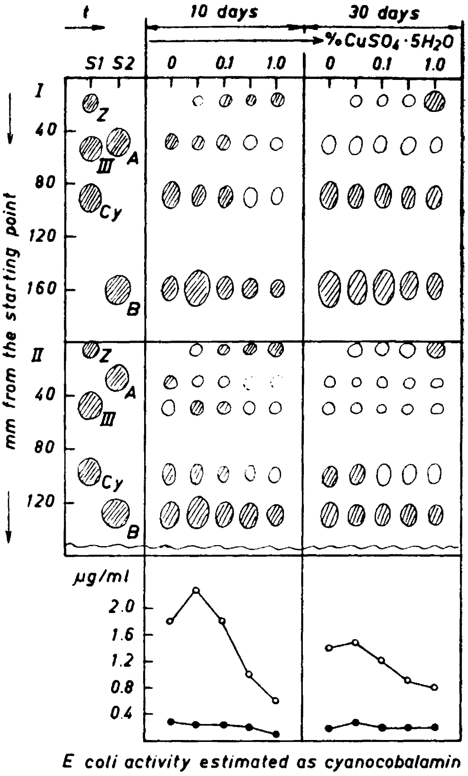

Fig. 3b. Effect of addition of copper sulphate to fresh sewage sludge, subsequently aerobically decomposed. Bioautogram. ○——○ cup assay; ●——● tube assay.

* Abbreviations used in Figs. 1—3.

I — solvent system I
II — solvent system II
t — time of fermentation
S1 — standard solution 1
S2 — standard solution 2

Z — factors Z2 + Z3
III — factor III
Cy — cyanocobalamin
A — factor A
B — factor B

days, the amount of cyanocobalamin decreased in the sludge containing 0.05 % copper sulphate and still more with increasing additions of Cu^{++}, but the synthesis of factors Z and of factor B was slightly stimulated by the additions corresponding to 254 and 127 ppm Cu^{++}, respectively. With higher additions all the vitamin B_{12} factors disappeared.

b) *Aerobic fermentation* (Fig. 3b). Increasing additions of copper sulphate decreased the amount of cyanocobalamin and increased the amount of factors Z. The situation was the same after 10 days as after 20 more days of fermentation. The amount of factor III and of factor B was slightly increased after 10 days in the sludge to which 127 ppm Cu^{++} had been added.

DISCUSSION

It can be concluded from the above that the addition of cobalt chloride to fresh sewage sludge, exerts a stimulating influence on the synthesis of cyanocobalamin and of other vitamin B_{12} factors during the subsequent microbial decomposition. This influence is much more pronounced in aerobic than in anaerobic fermentation. Moreover, under anaerobic conditions the addition of Co^{++} seems to stimulate the synthesis of vitamin B_{12} factors solely during the first stage of the decomposition of the sludge (10 days, pH $\sim$ 5.5) whereas during a latter stage (20 more days, pH $\sim$ 7.5) the amount of cyanocobalamin formed remains unaffected and the synthesis of factors Z and of factors "A" is stimulated only very slightly. In the aerobic fermentation the addition of Co^{++} stimulates the synthesis of cyanocobalamin and of factors Z very markedly both after 10 and after 20 more days of fermentation, which probably may be connected with the faster decomposition rate of the aerobically fermented sludge as reflected by the changes in pH (*cf.* p. 409). In an earlier investigation [1] it has been found that the first stage (pH 5.5) of anaerobic microbial decomposition of sewage sludge is characterized by a disappearance of cyanocobalamin which is probably consumed by some microorganisms active in the sludge at this pH, whereas during the decomposition of the sludge by aerobic organisms cyanocobalamin is synthesized continuously. The present result suggests therefore the possibility that the additon of Co^{++} to sewage sludge with subsequent anaerobic decomposition may exert a stimulating influence on the formation of cyanocobalamin rather by inhibition of the organisms which consume it (or enzymes which destroy it), whereas under aerobic conditions this addition may directly stimulate the synthesis of cyanocobalamin.

The addition of copper sulphate caused a slightly increased formation of cyanocobalamin and of several vitamin B_{12}-like factors during the first stage of microbial anaerobic decomposition of sludge (pH$\sim$5.5), whereas at the later stage (pH$\sim$7.5) the amount of cyanocobalamin was decreased by the addition. In view of the well known antimicrobial properties of copper it is tempting to suggest that its stimulating effect on the synthesis of cyanocobalamin and vitamin B_{12}-like factors during the first stage of anaerobic microbial decomposition of sludge may be due to inhibition of the cyanocobalamin consuming organisms (or enzymes which destroy it) analogously to the mechanism suggested for the effect of cobalt chloride in the first stage of anaerobic fermentation.

The addition of Cu^{++} with subsequent aerobic fermentation of the sludge had a detrimental effect on the formation of cyanocobalamin and of most vitamin B_{12}-like factors but not of factors Z the amounts of which drastically increased with the highest additions of Cu^{++}.

In earlier investigations [1,3] the formation of factors with R_F-values corresponding to those of factors Z was found to accompany very often the formation of cyanocobalamin. Cu^{++} thus seems to have some selective effect on the formation of cyanocobalamin and of these factors.

The amounts of cobalt and copper added to the sludge were chosen with regard to the range of similar additions usually employed in commercial animal rations (*cf. Nutrition Revs. 14* (1956) 238). With a view, however, to the relatively low natural contents of these elements in sludge (*cf.*p. 409) it would be of interest also to investigate the range 0—100 ppm of both additional copper and cobalt. It is thus too early to draw any conclusions about the optimal amounts of these two elements in fermenting sewage sludge for obtaining largest amounts of vitamin B_{12} factors.

Acknowledgements. The author is grateful to Professor H. Lundin, Head of the Division, for his encouraging interest in and valuable advice on the execution of this work. Thanks are due to Miss R. Wolf for technical assistance. The financial support from *Statens Tekniska Forskningsråd* is gratefully acknowledged.

REFERENCES

1. Neujahr, H. Y. *Acta Chem. Scand.* **9** (1955) 622.
2. Neujahr, H. Y. *Acta Chem. Scand.* **11** (1957) 1191.
3. Neujahr, H. Y. *Acta Chem. Scand.* **10** (1956) 917.
4. Anderson, M. S. *Sewage and Ind. Wastes* **28** (1956) 132.
5. Abson, D. and Lipscomb, A. G. *Analyst* **82** (1957) 152.
6. Middleton, G. and Stuckey, R. E. *Clin. Chim. Acta* **1** (1956) 135.

Received October 22, 1957.