# THE INHIBITIVE ACTION OF COBALT CHLORIDE ON MICROORGANISMS

by

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# INTRODUCTION

In the study of inorganic elements and their effect on microorganisms cobalt and its compounds have received much attention in recent years. Carl von Nageli in 1893 found that metals or metallic salts when added in high enough concentration, were toxic to the fresh water alga, Spirogyra. He described these alterations in some detail and assigned the name "oligodynamic action" to this phenomenon. The term "oligodynamic" as defined in those days included morphological changes in the organisms as well as inhibition of growth, while today oligodynamic is defined as follows, "to designate the zone of inhibition which results when a bright piece of metal is placed on the surface of a fresh agar plate uniformly inoculated with a young bacterial culture."

Cobalt has become of use to the farmer and rancher, especially in New Zealand where "lamb sickness" was cured by the use of cobalt drench. As little as 0.29 mgs per week proved to be effective in the treatment of this disease. Sheehy (1947) showed that soils deficient in cobalt caused sheep to pine and die, while cattle showed pronounced ill health.

To the biochemist cobalt has also taken on a new interest since the discovery that cobalt is a component of vitamin $B_{12}$. This vitamin is essential for a large variety of animals and even for microorganisms. When cobalt is fed or injected into rats, as little as 0.04 to 0.05 mg in the entire body is enough

to develop a polycythemia. Cobalt appears in such foods as wholemeal flour (0.01 p.p.m.), milk (0.001 p.p.m.), butter (0.013 p.p.m.) and in white flour (0.003 p.p.m.). With more research being conducted every day on cobalt, this element may take a more prominent position in body chemistry and also in the chemistry of enzymes.

The scarcity of knowledge on the effect of cobalt on microorganisms as compared to other metals initiated this investigation. In this study the author has selected a group of organisms which show variation in respect to staining properties, capsule formation, flagellation, toxin production, and spore formation. In this way a study could be made to see if the above-mentioned features would affect the action of the cobalt ion.

The organisms which were selected for this study were: Pseudomonas graveolens[1]; Pseudomonas putrefaciens; Pseudomonas jaegeri; Aerobacter aerogenes; Alcaligenes faecalis; Proteus morganii; Bacillus subtilis; Escherichia coli; Micrococcus pyogenes var. aureus; Salmonella pullorum; an organism isolated from soil near Manhattan, Kansas; an organism isolated from sour cream from a local farm; an organism isolated from Deep Creek, near Manhattan, Kansas; and an organism isolated from soil 10 miles east of Manhattan, Kansas, beside Kansas Highway Number 29. Basically the research was an attempt to show the effect

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[1] All names of bacteria mentioned in this thesis follow the nomenclature used in Bergy's Manual of Determinative Bacteriology, 6th Edition.

of cobalt chloride on bacterial growth when incorporated into a basal medium and to determine the concentration which would have inhibitory or stimulative action.

## REVIEW OF LITERATURE

Soils deficient in cobalt in various geographic areas have caused sickness of cattle, and Underwood (1940) showed that dosages as small as 1 to 2 mg of cobalt per day effected a cure. The disease may be prevented either by giving 0.1 mg daily to sheep and 0.3 mg daily to cattle or by treating the soil with a dressing of 0.5 lb. of cobalt acetate per acre. The disease has been known by many names; enzootic marasmus, Denmark disease, coast disease, Bush sickness, salt sick, pine, and Lake Shore disease. This disease has been observed in parts of Australia, New Zealand, Scotland, Florida, Michigan, and Wisconsin.

Marston and Lee (1949) showed that cobalt introduced parenterally is of no benefit to sheep pastured on cobalt deficient pastures. Cobalt exerts its influence on the ruminant primarily either in the lumen of the alimentary canal or when passing through its wall, the site being apparently above the level of the duodenum. When cobalt is administered intravenously it is excreted partially in the feces and appears in the intestinal tract below the duodenum. An important function of cobalt in the ruminant may be in altering the activity of the symbiotic flora within the paunch. Since sheep and cattle seem to be the

only animals which suffer from cobalt deficiency when grazed on deficient terrain, it appears that ruminants are unique in having a higher demand for this element than other animals.

McCance and Widdowson (1945) stated that organisms in the rumen of sheep and cattle may synthesize some essential nutrient, presumably vitamin $B_{12}$, but can do so only when cobalt is available.

In an exhaustive study of soil microorganisms Lochhead and Thexton (1952) showed the presence of a well-defined group of organisms dependent on soil extract for maximum growth. Some of these bacteria showed no growth; others, at best, showed only submaximal growth in media containing sugars and inorganic salts supplemented by yeast extract or combinations of amino acids and vitamins (not including vitamin $B_{12}$). For an important proportion of the indigenous bacteria of soil, soil extract could be replaced by vitamin $B_{12}$, though not by cobalt ($Co^{++}$). Various other soil bacteria having simple nutritional requirements were able to synthesize a growth-promoting factor, the nutrilite effect of which was related to the amount of vitamin $B_{12}$ produced. A large proportion of the bacteria for which soil extract or vitamin $B_{12}$ is essential consists of highly pleomorphic organisms.

Burk (1946) found cobalt to be an effective inhibitor of growth and respiration of various aerobic and anaerobic microorganisms, animal tissues, and tumors. Its physiologic action may be overcome reversibly and rather specifically by histidine

among natural amino acids tested. Various other substituted histidine compounds, including carnosine and anserine behaved qualitatively like histidine but $Co^{++}$ was not observed to combine with 5-methylimidazole or N-acetyl glutamate.

In 1923 Hotchkiss made a study of the effect of a series of inorganic compounds, in which different cations were combined with the same anion (chloride), on the growth of Escherichia coli. The salts could be divided into two groups on the basis of their toxicity. The salts in Group I included the following: NaCl, KCL, $NH_4Cl$, $SrCl_2.6H_2O$, $MgCl_2.6H_2O$, $CaCl_2$, $BaCl_2$, $MnCl_4.4H_2O$, $TiCl_3$. $SnCl_4.5H_2O$. Group II comprised the following salts: $NiCl_2$, TlCl, $CuCl_2$, $FeCl_3.12H_2O$, $FeCl_2.4H_2O$, $ZnCl_2$, $CoCl_26H_2O$, $PbCl_2$, $AlCl_3$, $CeCl_3$, $CdCl_2$, $HgCl_2$.

The salts in Group I are common in protoplasm and are considered nontoxic. Results of studies on the chlorides of $Na^+$, $K^+$, $NH_4^+$, and $Li^{++}$ showed that maximum growth occurred at a salt concentration of 0.25 M after an incubation period of three days. Salt concentrations above or below 0.25 M showed a decreased growth of the organisms. The bivalent salts in Group I showed a greater toxicity than the monovalent salts. Manganese was the only notable exception. Studies on the salts in Group II showed that they exhibited a greater degree of toxicity toward Escherichia coli. In more dilute solutions some of the salts including $HgCl_2$, produced a definite stimulating action. The author (Hotchkiss) found a general relationship existed between the toxicity of salts and their solution tensions. This means that

salts that are stimulating in low concentrations exhibit a toxic action in higher concentrations. In concentrations from 0.001 M through 0.005 M, cobalt was found to inhibit growth completely after three days of incubation at 37° C.

The effect of cobalt on the microbial synthesis of LLD-(Lactobacillus lactis Dorner) active substance was tested in an experiment conducted by Hendlin and Ruger (1950). In reviewing the literature they noted that Shorb (1947) reported an unidentified growth factor in liver extract, LLD, which is required by Lactobacillus lactis Dorner.

Crystalline vitamin $B_{12}$, a cobalt coordination complex isolated from liver, was found capable of satisfying the LLD requirement of this organism. During an extensive screening program the authors observed that large number of microorganisms were capable of synthesizing LLD-active substance. They investigated various modifications of the medium in an attempt to increase the microbial synthesis of vitamin $B_{12}$. The medium employed in these studies was composed of one per cent N-A Amine (Type A)[2], 0.3 per cent Difco beef extract, and distilled water to volume. The medium was adjusted to pH 6.8 to 7.0 with sodium hydroxide, dispensed in 40 ml aliquots per 250-ml Erlenmeyer flask, and sterilized by autoclaving at 121° C for 20 minutes. In experiments with Streptomyces griseus, varying concentrations of cobalt in the form of $Co(NO_3)_2.6H_2O$ were added to the N-Z Amine medium

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[2] An enzymatic digest of casein manufactured by Sheffield Farms, Inc.

prior to sterilization. LLD activity was then determined after 2, 3, 4, and 5 days' incubation. Maximum activity was observed between the 3rd and 5th days, with no significant loss in titer after peak production. For this reason the authors assayed all samples on the 5th day. Figure 1 shows the response curve obtained with increased dosage of $Co^{++}$. An approximate three-fold increase in LLD titer was obtained by the addition of $Co^{++}$ to the basal medium. Although LLD activity is not identical with vitamin $B_{12}$, an increase in the $B_{12}$ production was also obtained which more or less paralleled the increase in LLD activity. Maximal activities were obtained with as little as 1 to 2 p.p.m. $Co^{++}$. Toxic manifestations became apparent at levels of 20 to 50 p.p.m. $Co^{++}$. At these levels a marked decrease in growth and LLD activity was observed. It is apparent from these experiments that $Co^{++}$, a structural constituent of the $B_{12}$ molecule, becomes the limiting factor in N-Z Amine medium for microbial synthesis of LLD-active substance. Supplementation of the medium with $Co^{++}$, therefore, gives rise to an increase in LLD activity and vitamin $B_{12}$.

Hendlin and Ruger (1950) in addition to the above study also showed that other microorganisms could synthesize LLD-active substance and that, in these cases as well, supplementation with $Co^{++}$ (2 p.p.m.) gave rise to a significant increase in LLD activity. These experiments throw some light on the biological significance of cobalt in the nutrition of the microbial

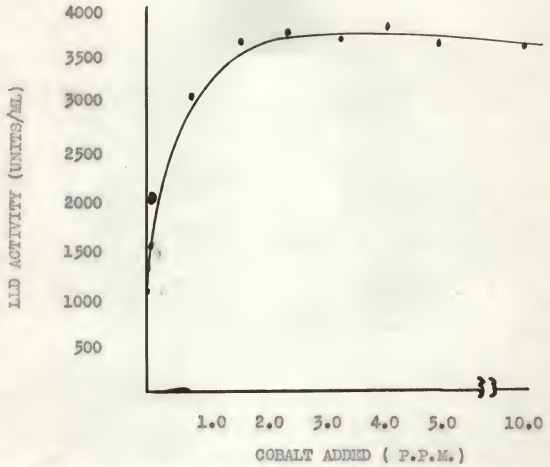


Fig. 1. The effect of cobalt in the synthesis of LLD-(Lactobacillus lactis Dorner) active substance by Streptomyces griseus when grown in a basal medium. (from Hendlin and Ruger, 1950)

cell. One role of cobalt in the bio-synthesis of vitamin $B_{12}$ has been experimentally substantiated with macroorganisms.

In a study on the effect of metallic ions on the growth and morphology of Clostridium perfringens, Shankar and Bar (1952) showed that for optimum growth, $Ca^{++}$, $Mg^{++}$, $Fe^{++}$, $Na^{+}$, and $K^{+}$ are required but not $Zn^{++}$, $Mn^{++}$. $Co^{++}$, or $Cu^{++}$. None of the latter metallic ions can replace those required for growth. In the absence of $Ca^{++}$ cells grow in an aggregate state whereas

$Mg^{++}$ or $K^{+}$ deficiency results in the growth of filamentous cells. In the presence of $Cu^{++}$ which is growth inhibitory, curved cells of normal size are obtained.

Bower (1949) made a study on the inhibitive action of cobalt on Salmonella pullorum (on the basis of antigenic structure he grouped these into "regular", "intermediate" and "variant" strains) and one strain of Proteus species. He employed several of the salts of cobalt among which were, $CoCl_2$, $CoSO_4$, and Co $(CH_3.COO)_2$ (cobalt acetate). Nutrient broth was used as the basal medium. In his experiments the hydrogen ion concentration was shown to have a decided effect on the action of the cobalt ion. The effect became very noticeable as the pH value of the medium was raised from pH 6.0 to pH 9.0. Salmonella pullorum strains seemed to be more tolerant to cobalt the higher the pH level. Cobalt concentrations of 150 p.p.m. and above were sufficient to inhibit growth completely in all cases except with the Proteus strain.

It was shown that one cobalt salt is not significantly different from another salt in its growth-inhibition of Salmonella pullorum. Under anaerobic conditions 150 p.p.m. of cobalt were sufficient to inhibit growth completely. In another phase of his research he showed that histidine and cysteine when employed in the ratio of 2:1 to cobalt would overcome the growth-inhibition exerted by cobalt.

McCulloch and Poe (1952) in research which is still in progress have shown that magnesium in small amounts will reverse

the inhibition of cobalt and nickel on yeast cultures. Both ions are toxic in very dilute concentrations, and neither appear to promote cell proliferation. These ions exert a bacteriostatic effect in very dilute concentrations and a bactericidal effect in higher concentrations. The bacteriostatic effect can be prevented if magnesium ions are added along with the cobalt or nickel ions and can be reversed if magnesium ions are added several days after the other ions. As the concentration of magnesium is increased, no further stimulatory effect is observed until toxic concentrations are reached.

## MATERIALS AND METHODS

### Cultures

Identified microorganisms used in this study of the inhibitive action of cobalt chloride ($CoCl_2.6H_2O$) were: Escherichia coli, ATCC strain 8677; Bacillus subtilis, ATCC strain 102; Aerobacter aerogenes, Bushness strain; Micrococcus pyogenes var. aureus, ATCC strain 9664; Alcaligenes faecalis, Kansas State College strain; Pseudomonas putrefaciens, Oregon State College strain; Pseudomonas graveolens, ATCC strain 4683; Pseudomonas jaegeri, old Kansas State College strain; Salmonella pullorum, strain isolated by Dr. Lester E. Erwin; Proteus morganii strain obtained from Dr. John Harris. In addition four organisms were used which were isolated by the author from the following sources:

Sour cream from a farm near Manhattan, Kansas; soil sample obtained 10 miles east of Manhattan, Kansas; water from Deep Creek, near Manhattan, Kansas; and a soil sample obtained near Manhattan, Kansas.

## Phase I

The basal medium was a modification of nutrient broth which consisted of: Bacto-beef extract 3 grams, Bacto-peptone 10 grams, sodium chloride 5 grams, all of which were added to one liter of distilled water. The medium was then tubed in 10 ml amounts in 150 X 18 mm Pyrex tubes. The final reaction after autoclaving for 15 minutes at 121° C was pH 6.8.

Nutrient broth was inoculated from the agar slant cultures, a small loopful of each of the desired organisms being placed in the broth. The cultures were placed in the incubator at 32° C for 24 hours. The broth cultures were transferred throughout the experiment every 24 hours, with a volume of 1 ml being inoculated into a sterile tube of nutrient broth.

Cobalt chloride ($CoCl_2.6H_2O$) was used in this experiment to test its inhibitive action against the above named organisms. A range of cobalt concentrations was selected which would show complete inhibition in the higher concentrations and no inhibition in the more dilute concentrations. Two series of cobalt media were set up for each organism using cobalt concentrations which progressed geometrically from 1000 p.p.m. through 0.1 p.p.m. for

Series I and 100 p.p.m. through 0.01 p.p.m. for Series II. The calculations for determining these concentrations were as follows:

(a) Molecular weight of cobalt chloride ($CoCl_2.6H_2O$) is 237.95. Then, the amount of cobalt in the chloride salt is $\frac{58.94}{237.95}$ or 0.247 grams of cobalt in one gram of cobalt chloride.

(b) 2000 p.p.m. of cobalt. For a solution to contain 2000 p.p.m. of $Co^{++}$, it must contain

$$\frac{2.000 \text{ (grams per liter)}}{0.247 \text{ (grams of cobalt in cobalt chloride)}}$$

or 8.09 grams of cobalt chloride per liter of solution.

To obtain a concentration of 1000 p.p.m. of cobalt, 10 ml of nutrient broth was mixed with 10 ml of solution containing 2000 p.p.m. of cobalt. Geometric dilutions were made (each half as concentrated as the preceding tube) through 14 tubes, discarding 10 ml from the last tube. These cobalt media were then autoclaved at 121° C for 15 minutes. The procedure for making the cobalt concentrations ranging from 100 p.p.m. through 0.01 p.p.m. was carried out in a like manner by starting with a 200 p.p.m. cobalt solution.

After all tubes had been inoculated with 0.1 ml of 24-hour culture of organism, an initial reading was taken with an Universal Spectrophotometer Model II with a No. 4 filter with the wave length set on 500, after which each test was then placed in the incubator at 32° C. Turbidity was then tested photometrically in the spectrophotometer each hour for a period of 12 hours. The

time consumed in reading 14 tubes was about 5 minutes. Each tube was shaken between the thumb and palm of the hand vigorously before reading. Due to the variation in the bore and thickness of the tubes and also due to the colored cobalt ion in the higher concentrations, the meter had to be set with a reading of 80 per cent transmittance as the standard, which in this case is equal to 100 per cent transmittance of light. The values were later adjusted to 100 as the standard in plotting all graphs. Each organism was tested three times in the above manner. Before reading each tube the meter was adjusted to 80, otherwise fluctuation in the current could cause a considerable degree of error.

Other factors to be taken into consideration when utilizing the spectrophotometer for testing growth of microorganisms are as follows:

(1) Tubes must be read in an uniform manner;

(2) Tubes should be uniform in size and thickness with no visible scratches, otherwise, a certain amount of the light waves will be blocked causing a drop in the transmittance value;

(3) Any line or fleck of cotton dropping into the tube due to shaking will upset the reading;

(4) Tubes should be rinsed in distilled water before tubing the medium;

(5) Tubes should be wiped with a clean towel occasionally during reading as finger marks from one's hands will collect on the tube while shaking and give lowered readings; and

(6) Allow meter to warm up for same length of time before using.

## Phase II

The second phase of this research consisted of isolating organisms from each of the following sources: Sour cream; two soil samples; and a water sample obtained from Deep Creek. The object was to see if organisms from selected natural sources could withstand a high concentration of cobalt. Concentrations of cobalt ranging from 1000 p.p.m. through 0.1 p.p.m. were prepared using nutrient broth as the diluent in the same manner as described in Phase I. The samples which consisted of soil, water, and sour cream were then added in 1 gram amounts to 99 ml of sterile distilled water, and shaken vigorously until an even suspension was obtained. One ml of this suspension was then transferred to 10 ml of nutrient broth and incubated at 32° C for 24 hours. One-tenth ml of this 24-hour broth culture was then inoculated into a tube of nutrient broth which contained 0.1 p.p.m. of cobalt. The cultures were then incubated for 12 hours at 32° C after which they were checked for growth. One-tenth ml of tubes showing growth was then transferred to the next higher concentration of cobalt. This was continued until a concentration was reached in which growth was completely inhibited. These tubes were then streaked on nutrient agar and read after 24 hours. If growth was then apparent one ml of this broth culture was transferred to 99 ml of sterile distilled water. Dilutions were carried out to 1-1,000,000 with each dilution being streaked on nutrient agar. Plates were read after 24 hours. An isolated

colony was then picked from one of the plates and placed in nutrient broth. This was then checked after 24 hours by means of the Gram stain to see if a pure culture was obtained. In the event the tube containing the highest cobalt concentration did not show growth when plated to nutrient agar then the last tube showing growth was selected for isolating an organism.

The organisms after being isolated in pure culture were checked for their rate of growth in nutrient broth with cobalt concentrations ranging from 0.1 p.p.m. through 1000 p.p.m. as described in Phase I, utilizing the spectrophotometer.

## RESULTS

### Phase I

Figure 2 illustrates the growth of Micrococcus pyogenes var. aureus when grown for 12 hours in nutrient broth and incubated at 32° C with varying concentration of cobalt. A cobalt concentration of 25 p.p.m. shows complete inhibition while a concentration of 15.6 p.p.m. of cobalt shows very slight growth at the end of 2 hours followed by a 9 hour period of no growth with a slight increase in growth for the final hour. As no growth was apparent macroscopically this slight growth is believed to be due to experimental error. A concentration of 7.81 p.p.m. of cobalt shows slight growth at the end of 3 hours followed by a gradual decline in the curve throughout the remaining 9 hours.

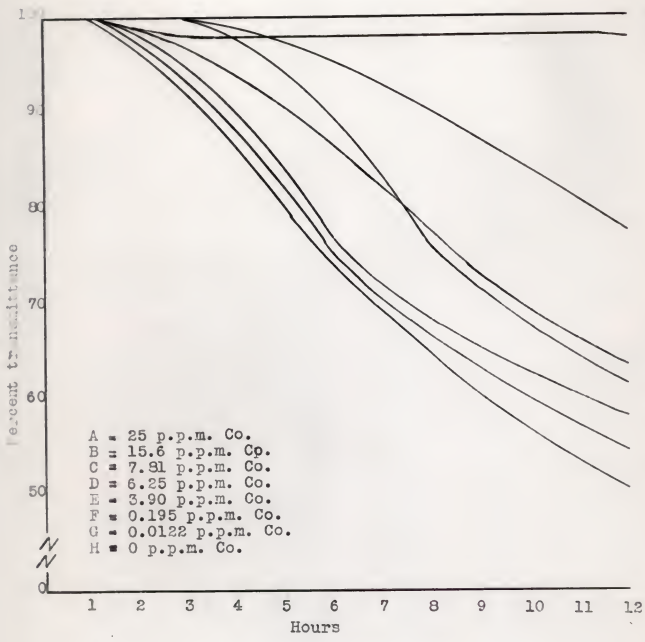


Fig.2. Growth of Staphylococcus pyogenes var. aureus expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically

Concentrations of 6.25 p.p.m. and 3.90 p.p.m. of cobalt showed a gradual increase in growth throughout the 12-hour period while concentrations of 0.195 p.p.m. and 0.0122 p.p.m. of cobalt more nearly approached the growth of the control which contained 0 p.p.m. of cobalt. Cobalt appears to inhibit the growth of Micrococcus pyogenes var. aureus to a slight extent even in the more dilute concentrations while higher concentrations show greater inhibition.

The data given in Figure 3 shows a concentration of 25 p.p.m. of cobalt to inhibit the growth of Escherichia coli during the 12-hour period while 15.6 p.p.m. of cobalt shows slight inhibition up to 4 hours after which there is a gradual increase in growth the remaining 8 hours. A concentration of 7.81 p.p.m. of cobalt was slightly inhibitory for the first hour after which there was a rather sharp increase in growth for the remaining 11 hours. Concentrations of 1.95 p.p.m., 0.122 p.p.m., 0.488 p.p.m. of cobalt more nearly approached the growth of the control which contained 0. p.p.m. of cobalt.

Aerobacter aerogenes as shown by Figure 4 closely parallels the growth of Escherichia coli in the same cobalt concentration. Both organisms were completely inhibited by a cobalt concentration of 25 p.p.m.

As shown by Figure 5 Salmonella pullorum is inhibited completely by a cobalt concentration of 15 p.p.m. while cobalt concentrations of 7.81 and 12.5 p.p.m. show very slight growth at

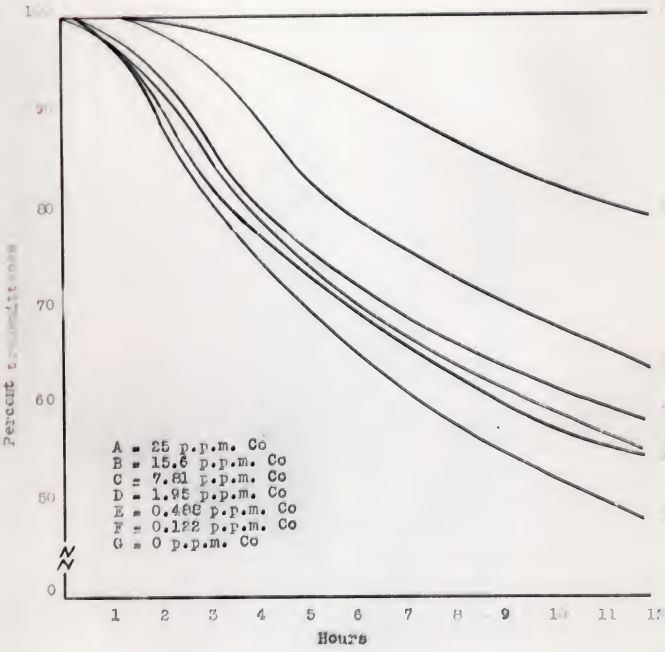


Fig. 3. Growth of Escherichia coli expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

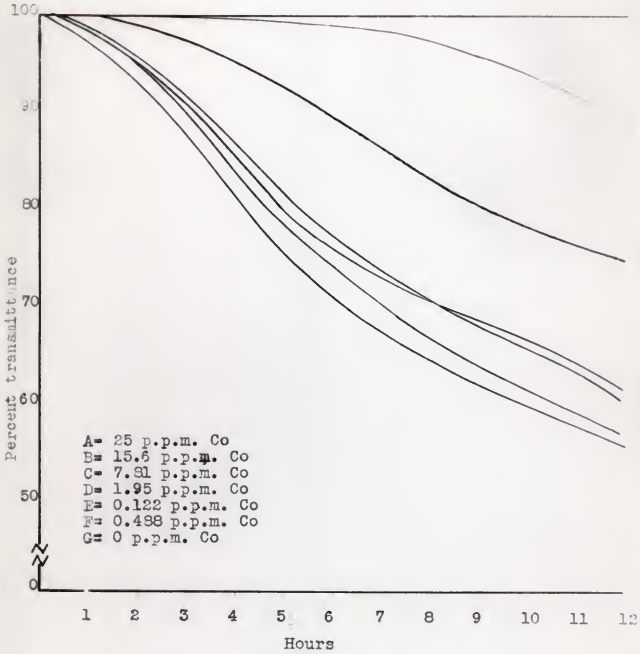


Fig. 4. Growth of Aerobacter aerogenes expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

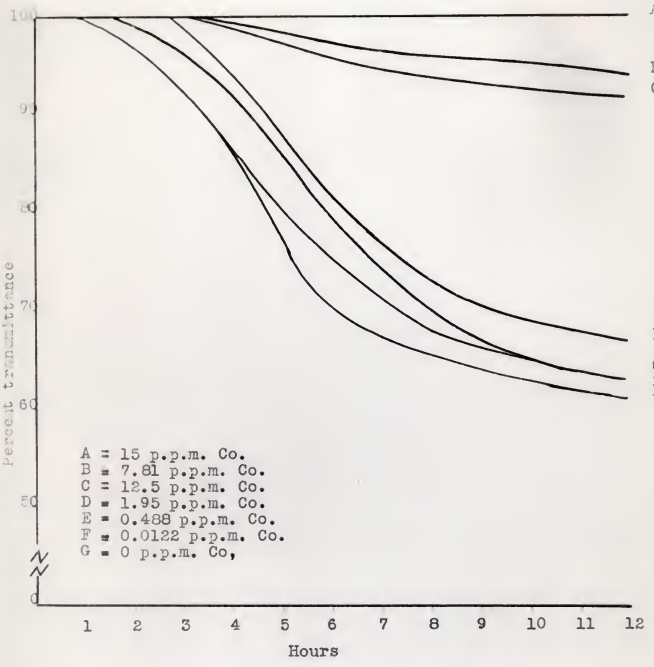


Fig. 5. Growth of Salmonella pullorum expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

the end of 12 hours. Cobalt concentrations of 0.488, 0.0122, and 1.95 p.p.m. closely paralleled each other in growth. As shown by Figure 4, 1.95 p.p.m. and 0.0122 p.p.m. of cobalt showed a slight zone of stimulation as compared to the control. Why Salmonella pullorum grew better in a cobalt concentration of 1.95 as compared to a cobalt concentration of 0.488 and 0. could not be explained. In Figure 6 Proteus morganii was completely inhibited by 25 p.p.m. of cobalt while a concentration of 15.6 p.p.m. showed slight growth after the 12 hour period. A concentration of 12.5 p.p.m. of cobalt was inhibitory for a two hour period after which there was a gradual increase in growth throughout the remaining 10 hours. Cobalt concentration of 3.12 p.p.m., 0.122 p.p.m., 0.0976 p.p.m. and 0 p.p.m. showed a growth response which closely paralleled each other. In these concentrations cobalt did not appear to inhibit growth to any degree.

Bacillus subtilis as shown by Figure 7 did not grow in a concentration of 7.81 p.p.m. of cobalt. A concentration of 3.90 p.p.m. of cobalt showed inhibition up to 4 hours after which there was a gradual decline in the slope of the curve. Concentrations of 1.56, 0.976, 0.390, 0.0122 and 0 p.p.m. of cobalt showed similar growth curves however a cobalt concentration of 0.390 p.p.m. did show a slight stimulation as compared to the control which contained 0 p.p.m. of cobalt.

Figure 8, 15.6 p.p.m. of cobalt shows complete inhibition of Alcaligenes faecalis. A cobalt concentration of 3.90 p.p.m. shows inhibition for a 2-hour period followed by a gradual

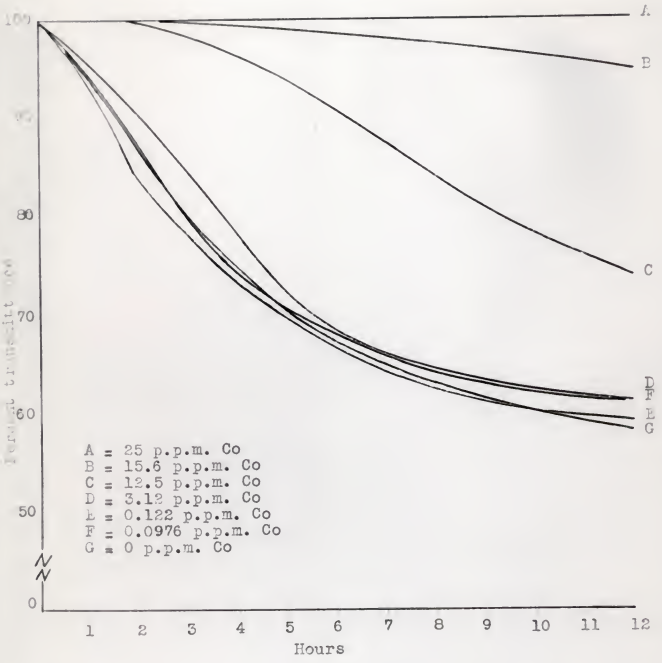


Fig. 6. Growth of Proteus morganii expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

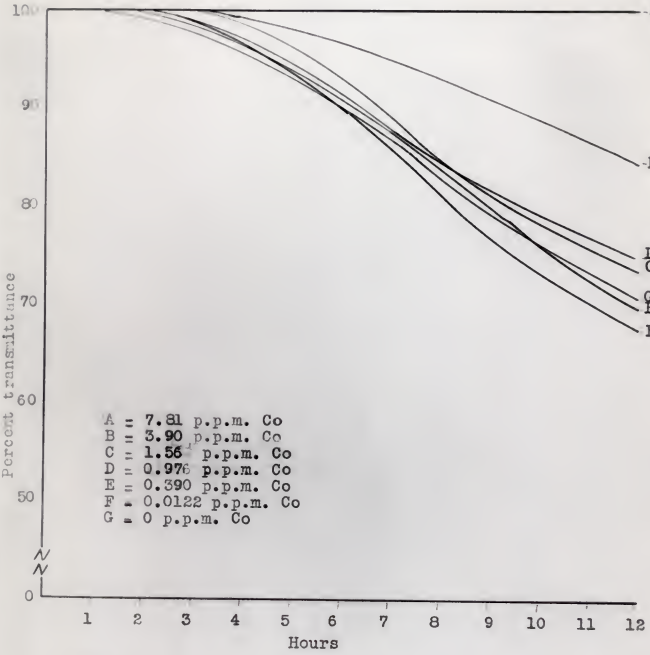


Fig. 7. Growth of Bacillus subtilis expressed in persent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

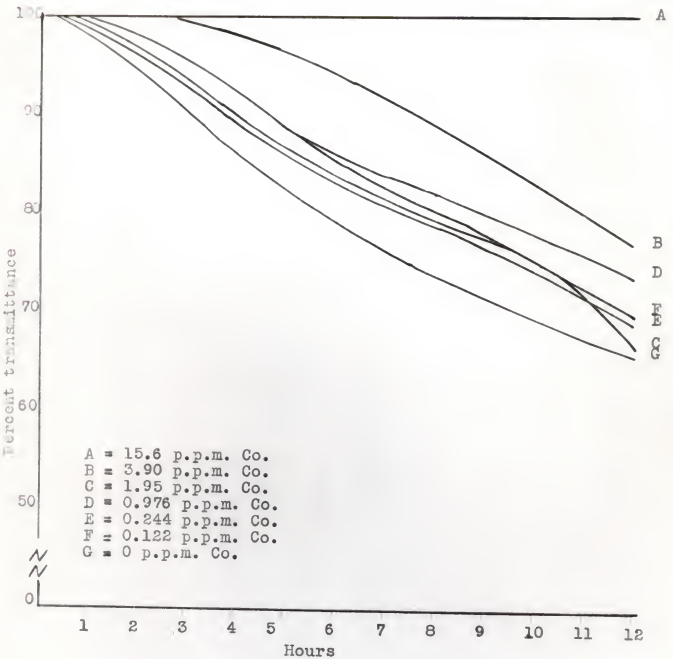


Fig. 8. Growth of Alcaligenes faecalis expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically

increase in growth for the remaining 10 hours. Concentrations of 1.95, 0.976, 0.244, and 0.122 p.p.m. of cobalt closely paralleled each other in growth during the 12-hour period. The control which contained 0 p.p.m. of cobalt showed only slightly more growth response than a cobalt concentration of 1.95 p.p.m. of cobalt.

Two of the Pseudomonas species, were inhibited by the same concentration of cobalt as shown by Figures 9 and 10, respectively. Pseudomonas jaegeri and Pseudomonas graveolens showed similar growth curves when grown in a concentration of 15.62 p.p.m. of cobalt. As shown by Figure 11, Pseudomonas nutrefaciens was completely inhibited at this cobalt concentration. These three organisms showed a similar growth curve when grown in a cobalt concentration of 12.5 p.p.m. A concentration of 3.12 p.p.m. of cobalt showed slight stimulation for Pseudomonas graveolens as compared to the control medium which contained 0 p.p.m. of cobalt. Pseudomonas jaegeri showed complete inhibition at a cobalt concentration of 25 p.p.m., while Pseudomonas graveolens was also inhibited by a cobalt concentration of 25 p.p.m.

## Phase II

Table 1 shows the growth of the organisms naturally occurring in soil, creek water, and cream in nutrient broth with cobalt concentrations ranging from 0.1 p.p.m. through 1000 p.p.m.

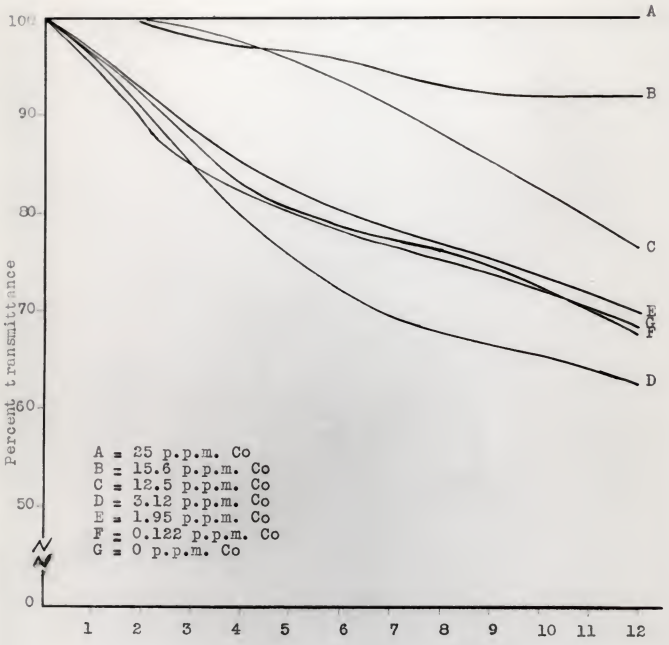


Fig. 9. Growth of Pseudomonas graveolens expressed in percent transmittance in varying concentrations of cobalt incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

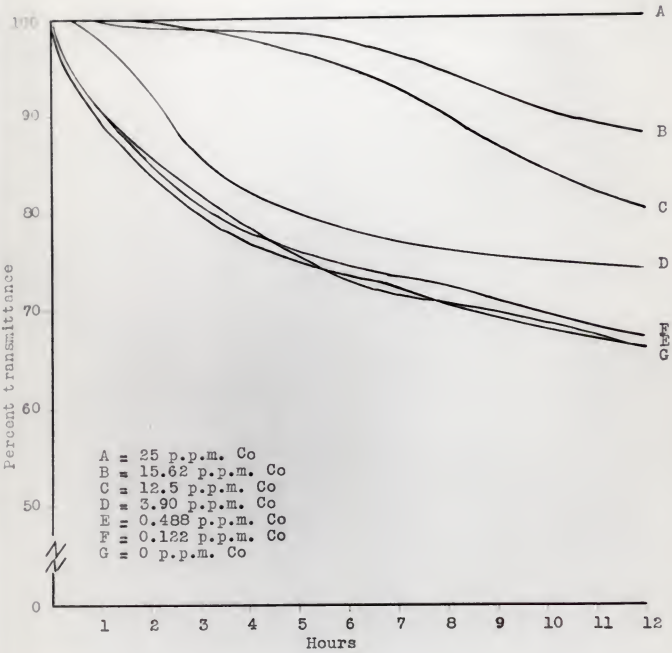


Fig. 10 Growth of Pseudomonas jaegeri expressed in percent transmittance in varying concentrations of cobalt incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

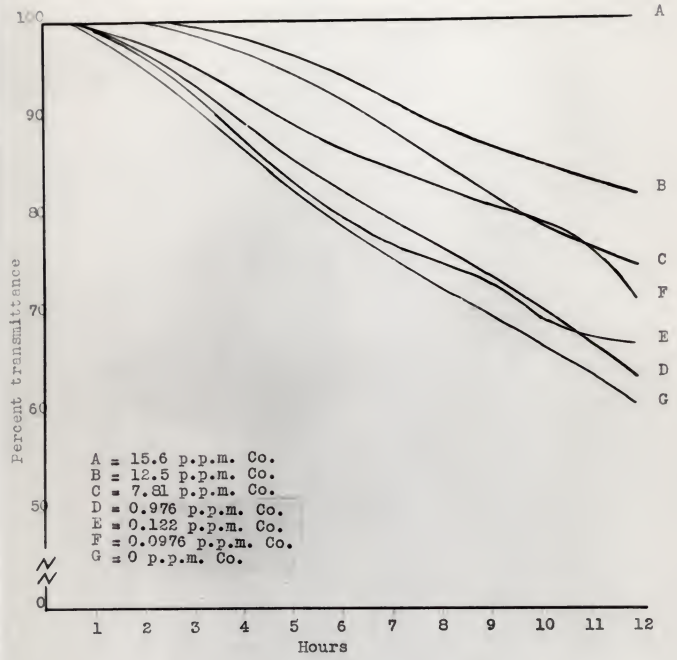


Fig. 11. Growth of Pseudomonas putrefaciens expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

Table 1. Pattern of growth of microorganisms from soil samples, sour cream, and water when first grown in a concentration of 0.122 p.p.m. of $Co^{++}$ in nutrient broth and progressing geometrically through a $Co^{++}$ of 1000 p.p.m. with 0.1 ml of each culture being transferred to a higher concentration of $Co^{++}$ every 12 hours and incubated at 32° C.

| Sample | $Co^{++}$ concentration in p.p.m. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.12 | 0.24 | 0.48 | 0.97 | 1.95 | 3.90 | 7.81 | 15.6 | 31.2 | 62.5 | 125 | 250 | 500 | 1000 |
| Deep Creek water | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 3+ G-R G+R | 3+ G-R G+R | 1+ G-R G+R | Neg * | Neg * | Neg | Neg | Neg | Neg |
| Soil I | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 3+ G-R | 3+ G-R | 2+ G-R | 2+ G-R | 1+ G-R | Neg * | Neg | Neg | Neg |
| Soil II | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 4+ G-R G+R | 3+ G-R | 3+ G-R | 2+ G-R | 2+ G-R | 1+ G-R | Neg * | Neg | Neg | Neg |
| Sour Cream | 4+ G+C G-R | 4+ G+C G-R | 4+ G+C G-R | 4+ G+C G-R | 4+ G+C G-R | 3+ G+C G-R | 1+ G+C G-R | 1+ G+C G-R | 1+ G+C G-R | 1+ G+C | 1+ G+C | Neg * | Neg * | Neg |

Key:
4+ = Heavy growth
3+ = Medium growth
2+ = Light growth
1+ = Very light growth
Neg = No growth in broth or on agar

G-R = Gram neg rod
G+C = Gram positive cocci
G+R = Gram positive rod (spore)
* = Growth when plated to nutrient agar 24 hours

with 0.1 ml being transferred each 12 hour period. In the water sample from Deep Creek the Gram positive organisms were completely inhibited at a cobalt concentration of 62.5 p.p.m., while the Gram negative rods showed growth when plated to nutrient agar. In the two soil samples the Gram positive organisms failed to grow in a cobalt concentration of 3.90 p.p.m., while the Gram negative organisms showed slight growth in a cobalt concentration of 62.5 p.p.m. A concentration of 125 p.p.m. of cobalt was bacteriostatic to the Gram negative organisms after 12 hours' growth at 32° C; however, on plating on nutrient agar, with subsequent incubation at 32° C for a period of 24 hours colonies were evident.

In the sour cream sample as shown by Table 1, the Gram negative organisms failed to grow at a cobalt concentration of 62.5 p.p.m., while the Gram positive organisms showed no growth in concentration of 125 and 250 p.p.m. of cobalt; however, when plated to nutrient agar and incubated at 32° C for 12 hours colonies were present. In this instance a pure culture was obtained, and upon Gram staining, the organism showed all the characteristics of a streptococcus.

Figure 12 shows the rate of growth of the streptococcus which was isolated from sour cream in cobalt media. A concentration of 1000 p.p.m. of cobalt gives complete inhibition while a concentration of 500 p.p.m. is bacteriostatic for the first 5 hours, after which there is a slight growth for the remaining 7 hours. Concentrations from 125 p.p.m. through 0

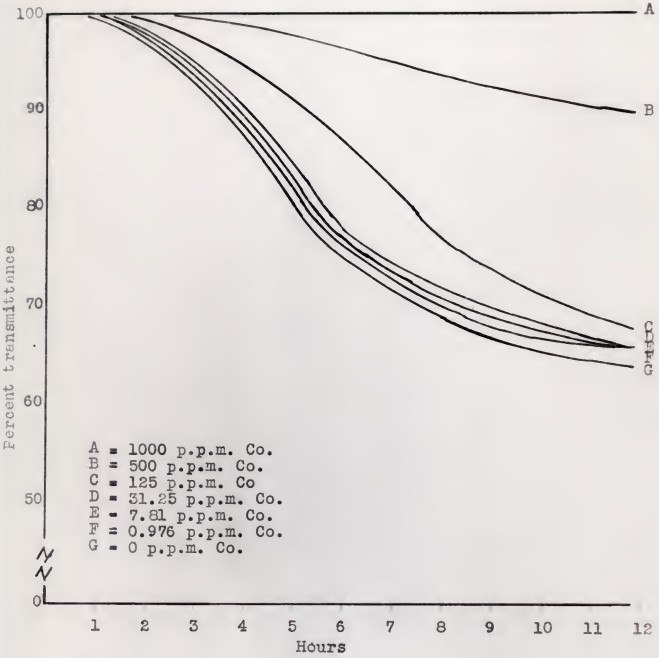


Fig. 12. Growth of the organism isolated from Sour Cream expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically

p.p.m. of cobalt produce a gradual sloping of the curve after the initial lag phase and more nearly approach a normal growth curve as compared to the control.

Figure 13 shows that a concentration of 500 p.p.m. completely inhibited the Gram negative bacillus species which was isolated from a soil sample beside Highway 29 near Manhattan, Kansas. A concentration of 250 p.p.m. was bacteriostatic for a period of 9 hours, after which there was a rather sharp increase in growth. Concentrations of 125 p.p.m., 7.81 p.p.m., 1.95 p.p.m., 0.244 p.p.m., and 0 p.p.m. of cobalt gave approximately the same rate of growth. This is believed to be within experimental error, and further tests would show essentially the same growth rate for the 12-hour period. In Figure 13 the graph shows that 31.25 p.p.m. is more inhibitory than the higher cobalt concentration of 125 p.p.m. This could not be explained. As is apparent from the graph this organism showed submaximal growth in nutrient broth for 12 hour period. Lochhead and Thexton (1952) have found that a well defined group of soil organisms is dependent on soil extract for maximum growth. This might prove true for this organism, if further experiments were carried out.

Figure 14 illustrates the growth zones of the organism isolated from Deep Creek, a Gram negative bacillus species. As is shown by the straight line of the graph, 50 p.p.m. allowed no growth, while a concentration of 31.25 p.p.m. allowed slight growth after 7 hours with a gradual decline up to 9 hours

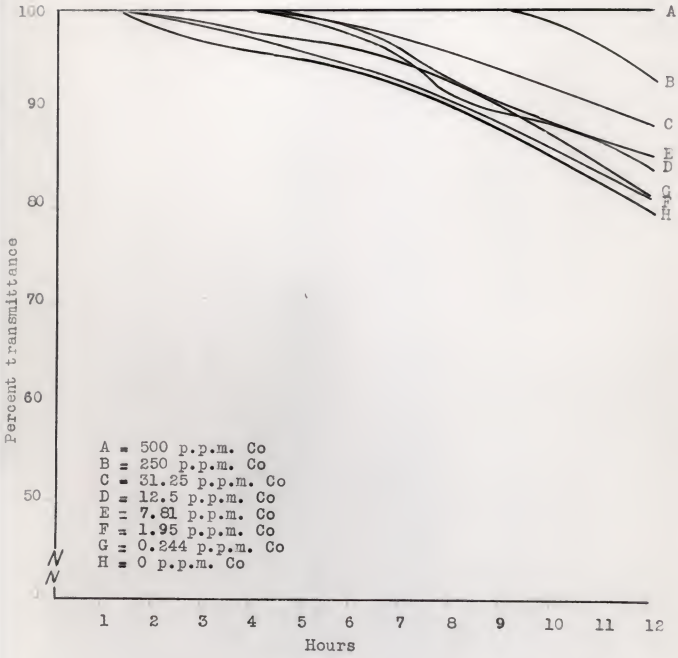


Fig. 13. Growth organism isolated from Soil sample Number II expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

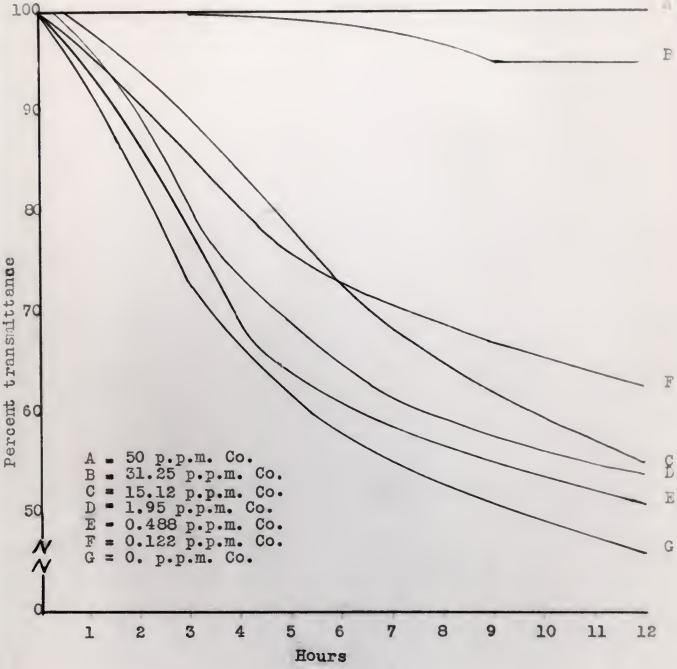


Fig. 14. Growth of organism isolated from Deep Creek expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

after which the curve straightens out with no further growth apparent. A slight stimulation by 15.12 p.p.m. of cobalt was noted as the slope of the curve drops sharply, almost paralleling that of the control which contained 0 p.p.m. of cobalt. This stimulation could not be explained. In Table 1 it was shown that this organism grew at a cobalt concentration of 62.5 p.p.m.; however, it is apparent that this was a mixed culture and that it is possible that other organisms might have inactivated part of the cobalt ion by adsorption, thus making it possible for this organism to grow. The inhibition of the organism in a concentration of 0.122 p.p.m. of cobalt could not be explained. It would appear that at this low a concentration the slope of the curve should more nearly parallel that of the control.

The growth of the organism isolated from soil near Manhattan, Kansas, a Gram negative bacillus species, is shown in Figure 15. A concentration of 250 p.p.m. of cobalt was completely inhibitory, while a concentration of 125 p.p.m. allowed slight growth after 12 hours incubation. After the initial lag period, there was very slight growth with a short lag period up to four hours, then a slight growth with a three hour lag period interspaced, followed by a gradual decline in the slope of the curve. A concentration of 31.25 p.p.m. allowed more rapid growth with a more even slope to the curve; however, this organism does not appear to grow too rapidly within the 12 hour period in the control medium.

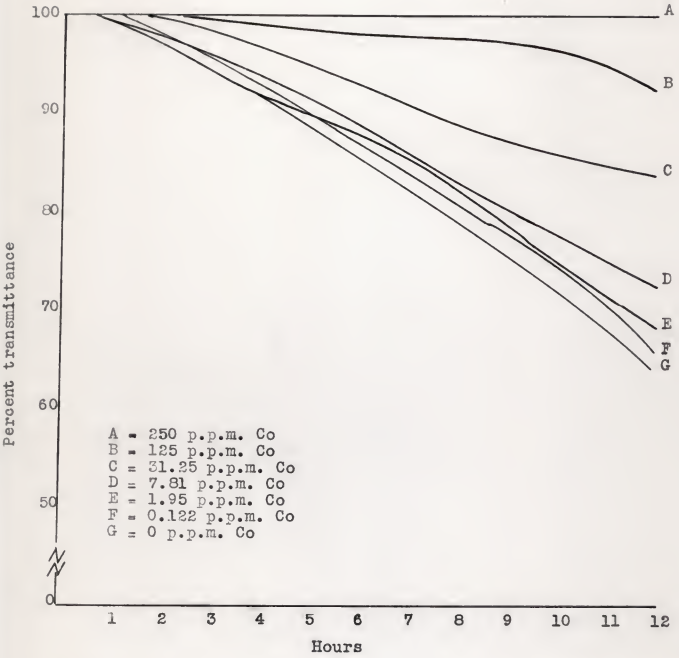


Fig. 15 Growth of organism isolated from Soil sample Number I expressed in percent transmittance in varying concentrations of cobalt, incubated at 32°C for 12 hours with growth measured each hour spectrophotometrically.

## GENERAL DISCUSSION

The effect of the cobalt ion ($Co^{++}$) on the microorganisms used in this research has been determined. Growth was measured with the spectrophotometer each hour for a 12-hour period with the organisms incubated at $32^{o}$ C. The organisms used in this experiment showed variations as to sporulation, capsule formation, flagellation, toxin production, and reaction to the Gram stains. The experiment was divided into two Phases and for clarity each Phase will be discussed separately.

Phase One: The Effect of Cobalt on ten identified bacteria utilizing one cobalt salt and measuring growth with the spectrophotometer. In the initial study of the effect of the cobalt ion ($Co^{++}$) on microorganisms used in this experiment, it was shown that in dilute concentration the ion had variable toxicity while in higher concentrations there was no apparent growth during the 12-hour period. In only three cases did a cobalt concentration show some stimulatory effect as represented by Figures 5, 7 and 9. The possible effect of the hydrogen ion was nil as all cobalt concentrations used in this experiment lay within the pH range of 6.5 to 7.5. All organisms used in this experiment with the exception of the four unknown organisms isolated from soil, water and cream grow equally well within this pH range. Bower (1951) showed that a pH range that lay between 6.5 to 7.5 had no deleterious effect on the growth of Salmonella pullorum. Six organisms were completely inhibited by a cobalt

concentration of 25 p.p.m. as shown by Figures 1, 2, 3, 5, 6, and 10. In a cobalt concentration of 15.6 p.p.m. four of these organisms showed only slight growth, while one, as represented by Figure 2, showed moderate growth at this concentration. Pseudomonas graveolens, as shown in Figure 10, showed stimulation when grown in a cobalt concentration of 3.12 p.p.m. This could not be explained as it would appear that the more dilute concentrations would show stimulation first.

Pseudomonas putrefaciens, Salmonella pullorum, and Alcaligenes faecalis, as shown by Figures 4, 8, and 9, were completely inhibited by a cobalt concentration of 15.6 p.p.m. Salmonella pullorum showed slight stimulation in a cobalt concentration of 0.0122 p.p.m. and also a cobalt concentration of 1.95 p.p.m. As the growth curves for the 12-hour period closely paralleled that of the control this apparent slight stimulation is believed to lie within experimental error. It is of interest to note that one of the three Pseudomonas species was inhibited by a different concentration of cobalt, as shown by Figures 9, 10, and 14. Pseudomonas jaegeri was inhibited by a cobalt concentration of 25 p.p.m., as was Pseudomonas graveolens. Pseudomonas putrefaciens was inhibited by a cobalt concentration of 15.6 p.p.m. The inhibition of Pseudomonas putrefaciens might be partially explained, since 32° C is not the optimum temperature for the growth of this organism. Its optimum temperature is 21° C; however, the control grew as well as or better at 32° C than the controls of the other two Pseudomonas species. It would

appear that inhibition of growth is at least in part due to the cobalt ion. Bacillus subtilis was inhibited by a cobalt concentration of 7.81 p.p.m. A cobalt concentration of 0.390 and 0.0122 p.p.m. showed slight stimulation for this organism. Growth was not apparent in a concentration of 15.6 p.p.m. of cobalt. Of all of the organisms tested, Bacillus subtilis was inhibited by the lowest cobalt concentration. Since Bacillus subtilis is a spore-producer, sporulation may have taken place, and germination was prevented by the cobalt ion. It would appear from this research that such variation in an organism as capsule formation, flagellation, toxin production do not greatly affect the action of the cobalt ion.

Phase Two: The effect of cobalt on four organisms isolated from soil, water and cream. In Phase II of this research organisms from two soil samples, creek water, and sour cream were isolated using the procedure as described in Table 1. This Phase of the research was carried out mainly to see if organisms which were isolated from their nature habitat would be more resistant to the effect of the cobalt ion. As shown in Table 1 the organisms which grew in the highest cobalt concentration from the Deep Creek water sample were Gram negative bacilli. Growth was apparent at a cobalt concentration of 15.6 p.p.m. At this concentration the Gram stain showed Gram negative bacilli and Gram positive bacilli. At a cobalt concentration of 31.2 the Gram stain showed only Gram negative bacilli with no Gram positive organisms being present. Although growth was not evident

macroscopically in the nutrient broth, which contained 31.2 p.p.m. of cobalt upon plating to nutrient agar with incubation at 32° C for 24 hours, heavy growth was noted. As a result of this information 0.1 ml of the culture which contained cobalt in a concentration of 31.2 p.p.m. was transferred to the next higher cobalt concentration which was 62.5 p.p.m. Here again no growth was evident macroscopically; however, on plating to nutrient agar growth was seen. The Gram stain showed only Gram negative bacilli. One-tenth ml of this culture was transferred to broth containing a cobalt concentration of 125 p.p.m. and incubated for 12 hours at 32° C. The Gram stain showed no organisms present and when this culture was plated to nutrient agar there was no growth after 24 hours. Cobalt concentrations of 250, 500 and 1000 p.p.m. were bactericidal for these Gram negative bacilli as shown by a lack of organisms on the Gram smear, and negative growth when plated to nutrient agar.

Table 1 shows that organisms from soil sample I and soil sample II were completely inhibited by a cobalt concentration of 250 p.p.m., while 125 p.p.m. of cobalt allowed heavy growth when plated to nutrient agar and incubated at 32° C for 24 hours. The organism which was isolated in this instance was a Gram negative bacillus. Gram positive bacilli were completely inhibited at a cobalt concentration of 390 p.p.m. In the sour cream sample a Gram positive coccus was isolated in pure culture from a cobalt concentration of 500 p.p.m. This organism as shown by the Gram stain had all the characteristics of a streptococcus. In this

sample Gram negative bacilli failed to grow in a cobalt concentration of 62.5 p.p.m. After an organism was isolated in pure culture from each of the samples described in Table 1, it was decided to test the growth of these organisms in varying concentrations of cobalt with a spectrophotometer. The same protocol for preparing the cobalt concentrations was used as described in Phase I. As is shown in Figure 12 the organism which was isolated from sour cream showed slight growth in a cobalt concentration of 500 p.p.m., while a concentration of 1000 p.p.m. of cobalt completely inhibited growth. Cobalt concentrations of 125, 31.25, 7.8, 0.976, and 0 p.p.m. showed similar growth curves for the 12-hour period. Figure 13 shows that a cobalt concentration of 500 p.p.m. completely inhibited the growth of the organism which was isolated from soil sample II. Growth was apparent after 9 hours with a gradual increase in growth for the remaining three hours in a cobalt concentration of 250 p.p.m. A cobalt concentration of 1.95 p.p.m. showed slight stimulation as compared to the control. Other concentrations closely paralleled the growth of the control. The organism which was isolated from the water sample from Deep Creek was inhibited by a cobalt concentration of 50 p.p.m., as shown by Figure 14. A cobalt concentration of 31.25 p.p.m. showed slight growth at the end of the 12-hour period. Why a cobalt concentration of 0.122 p.p.m. was more inhibitory than a cobalt concentration of 12.12 p.p.m. could not be explained. Figure 15 shows that a cobalt concentration of 250 p.p.m. completely inhibits the growth of the Gram

negative bacillus which was isolated from soil sample number I. Slight growth is shown at a cobalt concentration of 125 p.p.m., while a concentration of 31.25 p.p.m. of cobalt shows increased growth for the 12-hour period. Growth in other concentrations are shown to parallel closely the growth of the control.

As to why cobalt has such an inhibitory action on the growth of bacteria one can only present hypotheses which have been advanced in the past and to quote data which have been recorded by other authors who have done research along similar lines.

Many theories have been advanced as to why various metals are toxic to living protoplasm, among which are: Effect of adsorption on the surface of the cell; altering the surface tension; interference or blocking an essential ion; or action might be similar to the effect a hypertonic and hypotonic sodium chloride solution would have. The so-called "oligodynamic action" is also included among these theories. Since bacteria are known to have a negative charge it would seem possible that since cobalt has a valence of a positive two, ions with lesser charges would be replaced on the cell surface by the more heavily charged cobalt ion. If this were true then ions essential for normal metabolism would be blocked out and the cell would eventually die.

Abelson and Aldous (1950) showed that the ions which interfere most with magnesium, a chemical which is an activator for almost all known enzymes, are $Co^{++}$, $Ni^{++}$, $Cd^{++}$, $Zn^{++}$, and $Mn^{++}$. These ions tend to form highly stable organic complexes with

biologically important compounds, such as folic acid, asparagine, and proline.

The effect of surface tension was well illustrated by Frobisher (1927). He used phenol as a germicide, sodium oleate as a surface-tension depressant and Salmonella typhosa, which is not sensitive to sodium oleate, as the test organism. He showed that within a comparatively narrow range of concentration, the presence of sodium oleate because of its tendency to reduce the surface tension increases the germicidal efficiency of phenol. Smaller amounts delayed the action of phenol and larger amounts very definitely interferred with the ability of the phenol to kill Salmonella typhosa. Since it is known that a substance which reduces the surface tension collects at the interface of the particles in solution (bacteria in this case) and also at the boundary layer in greater proportions than in the liquid, it is possible that cobalt could have acted in this way. If this were the case then cobalt was collected on the bacterial cells and as shown by this research was definitely toxic in very dilute concentrations.

The "oligodynamic action" of metals on bacteria is widely known, however, the exact reason for exhibiting this effect is not known. There are two theories which are much discussed, one, which implies that the metallic ion is acting as a catalyst in the formation of hydrogen peroxide from the dissolved oxygen in the media and the other states that the action is due to the metal being ionized slightly and then is dispersed throughout the

media. However, the latter explanation does not exactly say what the action is on the bacterial cell. The author feels that a better explanation is that the cobalt is tied up in some way with inhibiting or replacing a coenzyme of an enzyme system of the bacteria.

Hotchkiss, as stated earlier in the review of the literature, found that a 0.001 M and 0.005 M cobalt concentration inhibited growth of Escherichia coli after three days of incubation at 37° C. She used visual observation for determining growth and also a longer period of incubation before reading than the research herein reported. She was more interested in total toxicity.

The author found that a 0.000105 M concentration of cobalt inhibited the growth of Escherichia coli for a period of 12 hours when incubated at 32° C; however, the organisms can later recover and grow. The author utilized the spectrophotometer for measurement of growth and it is believed that early growth could best be noted utilizing this method, as the author was more interested in initial inhibition.

The results of this research, although employing other organisms, fall in line with the results obtained by Hendlin and Ruger (1950) with Streptomyces griseus.

## SUMMARY

1. A study has been made on the effect of cobalt chloride on the following organisms: Escherichia coli, Aerobacter aerogenes, Alcaligenes faecalis, Micrococcus pyogenes var. aureus, Bacillus subtilis, Salmonella pullorum, Proteus morganii, Pseudomonas jaegeri, Pseudomonas graveolens, Pseudomonas putrefaciens, and four organisms isolated from soil, water, and cream.

2. The following organisms were completely inhibited at a cobalt concentration of 25 p.p.m.: Proteus morganii, Escherichia coli, Pseudomonas graveolens, Pseudomonas jaegeri, Aerobacter aerogenes, and Micrococcus pyogenes var. aureus, Salmonella pullorum, Alcaligenes faecalis, and Pseudomonas putrefaciens were all inhibited by a cobalt concentration of 15.6 p.p.m. Bacillus subtilis was inhibited by a cobalt concentration of 7.81 p.p.m., however, cobalt concentrations of 0.390 and 0.0122 p.p.m. showed slight stimulation for this organism as compared to the control.

3. Salmonella pullorum was stimulated in growth by a cobalt concentration of 1.95 and 0.0122 p.p.m., while Pseudomonas graveolens showed stimulation when grown in cobalt concentrations of 3.12 p.p.m. and 0.122 p.p.m. Why a cobalt concentration of 3.12 p.p.m. showed more stimulation than a cobalt concentration of 0.122 p.p.m. could not be explained.

4. In Phase 2 of this research a study was made to see if organism isolated from their natural surroundings could withstand a higher concentration of cobalt than those which had been

maintained in stock culture over a period of months. The organism isolated from sour cream had all of the characteristics of a streptococcus and grew in the highest cobalt concentration of any organism tested in this experiment. A concentration of 1000 p.p.m. showed complete inhibition while slight growth was shown in a cobalt concentration of 500 p.p.m.

5. The organism isolated from soil sample I was a Gram negative bacillus and grew in a cobalt concentration of 125 p.p.m. A concentration of 250 p.p.m. of cobalt showed complete inhibition.

6. From the water sample a Gram negative bacillus was isolated which was inhibited by a cobalt concentration of 50 p.p.m. and showed slight growth in a cobalt concentration of 31.25 p.p.m.

7. Cobalt was definitely inhibitory in low concentrations to all of the organisms studied in this research.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. Thomas H. Lord for guidance and valuable assistance in directing this research to its finality. Gratitude is also expressed to Dr. P. L. Gainey and to Dr. John O. Harris for making it possible to use certain equipment and supplies. Appreciation is also expressed to Dr. William A. Miller for the use of his laboratory. Due appreciation is extended to members of the central supply room who were most helpful in keeping a ready supply of clean glassware on hand.

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# THE INHIBITIVE ACTION OF COBALT CHLORIDE ON MICROORGANISMS

by

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AN ABSTRACT OF A THESIS

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The element cobalt has stimulated renewed interest among bacteriologists as to its effect on microorganisms, and as a result many new discoveries are being made. The role of cobalt in the well-being of ruminants has become of economic importance since the discovery that cattle and sheep pastured on land deficient in this element become sick and sometimes die. The disease may be prevented either by giving 0.1 mg daily to sheep and 0.3 mg daily to cattle or by treating the soil with a top dressing of 0.5 lb of cobalt acetate per acre. It is believed that organisms in the rumen of sheep and cattle may synthesize some essential nutrient, presumably vitamin $B_{12}$, but can do so only when cobalt is available.

Shorb (1947) reported an unidentified growth factor in liver extract, which he called LLD-active substance which was required by Lactobacillus lactis Dorner, an organism commonly found in milk. In 1950 Hendlin and Ruger showed that vitamin $B_{12}$, a cobalt coordination complex, could readily satisfy the LLD requirement of this organism. In another phase of their experiment Hendlin and Ruger (1950) showed that when Streptomyces griseus was grown in a basal medium to which varying concentrations of cobalt were added there was in increase in LLD activity and also an increase in vitamin $B_{12}$. Maximal activities were obtained with as little as 1 to 2 p.p.m. of cobalt. That cobalt is toxic to microorganisms was also shown by Hendlin and Ruger as concentrations of 20 to 50 p.p.m. caused a marked decrease in growth of Streptomyces griseus. Other

authors have found cobalt to be toxic to Escherichia coli, Salmonella pullorum yeasts and species of Proteus.

The first phase of this problem was concerned primarily with determining the concentration of cobalt which would be inhibitory or show a stimulative effect toward the bacteria used in this experiment. Growth was measured with the spectrophotometer each hour for a period of 12 hours with the temperature of incubation at 32° C. With the exception of Pseudomonas putrefaciens whose optimum temperature is 21° C, all of the other organisms grow well at 32° C. Cobalt concentrations were set up in nutrient broth in two series, Series I ranging from 1000 p.p.m. and progressing geometrically through 0.1 p.p.m. and Series II starting with a cobalt concentration of 100 p.p.m. and progressing geometrically through 0.01 p.p.m.

Both coliform organisms (Escherichia coli and Aerobacter aerogenes) were inhibited by a cobalt concentration of 25 p.p.m. In cobalt concentrations below 25 p.p.m. Escherichia coli showed better growth than Aerobacter aerogenes. Proteus morganii and Alcaligenes faecalis organisms which are less frequently found in the intestinal tract of man and animals were inhibited by cobalt concentrations of 25 p.p.m. and 15.6 p.p.m., respectively. Salmonella pullorum was inhibited by a cobalt concentration of 15.6 p.p.m.

With these facts as a background the author has proceeded to see how inhibitive cobalt was on some typical bacterial species. A total of ten identified bacteria were employed in

the first phase of this research and included the following: Escherichia coli, Aerobacter aerogenes, Alcaligenes faecalis, Micrococcus pyogenes var. aureus, Bacillus subtilis, Proteus morganii, Salmonella pullorum, Pseudomonas putrefaciens, Pseudomonas graveolens, and Pseudomonas jaegeri. Pseudomonas graveolens and Pseudomonas jaegeri were inhibited by a cobalt concentration of 25 p.p.m. while Pseudomonas putrefaciens was inhibited by a concentration of 15.6 p.p.m. of cobalt. A cobalt concentration of 3.12 p.p.m. gave stimulation of growth for Pseudomonas graveolens, while a concentration of 0.122 p.p.m. of cobalt showed only slight stimulation.

Of the two Gram positive organisms studied in this phase of the research, Bacillus subtilis was inhibited by a cobalt concentration of 7.81 p.p.m., while Micrococcus pyogenes var. aureus was inhibited by a cobalt concentration of 25 p.p.m. Bacillus subtilis showed slight stimulation in the presence of 0.390 and 0.0122 p.p.m. of cobalt.

In the second phase of this research experiments were carried out to see if organisms which were isolated from their natural habitat could withstand higher concentrations of cobalt than those which had been cultured on artificial media over a long period. Four organisms were isolated - two from soil, one from sour cream, and one from creek water. One ml portions of each sample was cultured in nutrient broth for a 24 hour period at 32° C. Concentrations of cobalt were prepared ranging from 1000 p.p.m. and progressing geometrically through 0.1 p.p.m. in

nutrient broth. From the 24-hour culture of each of the samples, 0.1 ml was inoculated into a tube of nutrient broth which contained 0.1 p.p.m. Co. These tubes were then incubated at 32° C for a 12-hour period, after which all tubes showing growth were inoculated to the next higher concentration of cobalt until a concentration was reached in which no growth appeared. Gram stains were made after each 12-hour incubation period of all cultures. In the water sample the Gram positive organisms failed to grow in a cobalt concentration of 31.25 p.p.m. while the Gram negative rods showed no growth macroscopically, but growth was obtained when a portion of this culture was plated on nutrient agar. In a concentration of 62.5 p.p.m. of cobalt, growth only took place after plating to nutrient agar. A concentration of 125 p.p.m. was inhibitory both in broth and when plated to nutrient agar.

In soil sample I the Gram positive organisms failed to grow in a cobalt concentration of 3.90 p.p.m., while the Gram negative bacilli grew on nutrient agar when plated from a cobalt concentration of 125 p.p.m. Growth was not apparent at a cobalt concentration of 250 p.p.m.

In soil sample II the Gram positive organisms were inhibited at a cobalt concentration of 3.90, while the Gram negative bacilli failed to grow in a cobalt concentration of 250 p.p.m. In a cobalt concentration of 125 p.p.m. growth was seen after the culture was streaked onto nutrient agar and incubated for 24 hours at 32° C.

From the sour cream sample an organism (a Streptococcus) was isolated in pure culture which grew on nutrient agar after having been cultured in nutrient broth which contained cobalt in a concentration of 500 p.p.m. The Gram negative bacilli which were present in this sample were completely inhibited at a cobalt concentration of 62.5 p.p.m.

The final step in this phase entailed determining the cobalt concentration which would inhibit the growth of these organisms which had been isolated in pure culture from the above named sources. Growth was tested with the use of the spectrophotometer and cobalt concentrations were set up in the same manner as described in Phase 1 of this research. The organism which was isolated from sour cream and which had the characteristics of a Streptococcus species grew in a cobalt concentration of 500 p.p.m. A cobalt concentration of 1000 p.p.m. was definitely inhibitory.

In soil sample number II the isolated bacteria was inhibited by 500 p.p.m. of cobalt, while there was slight growth in a cobalt concentration of 250 p.p.m. A concentration of 1.95 p.p.m. was slightly stimulative.

The organism isolated from creek water did not grow in a cobalt concentration of 50 p.p.m. Slight growth was recorded at a concentration of 31.25 p.p.m. of cobalt.

The organism isolated from soil sample I did not grow in a cobalt concentration of 250 p.p.m. while there was slight growth in a cobalt concentration of 125 p.p.m.

It would appear from this study that certain organisms which are isolated from natural sources can better withstand the delecterious effects of cobalt than many experimental organisms which have been retained in stock cultures on artifical media over a long period.