THE INHIBITIVE ACTION OF COBALT ON SALMONELLA PULLORUM

by

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INTRODUCTION

The action of organic as well as inorganic chemical compounds on microorganisms has been an important phase of microbial investigation and one which has contributed richly to present knowledge with relation to the field of bacteriology.

Among the lesser studied metals in the chemical system of classification is the element cobalt and its respective salts. Allied with ferrous and ferric iron, aluminum, chromium, zinc, nickel, and manganese in the third group of the qualitative system of cation classification, the metal cobalt has recently become a focal point of interest to the biochemist, as well as the bacteriologist. The biochemist's interest in cobalt was greatly stimulated when it was learned that the element was intimately associated with the vitamin B-12, of the vitamin B-complex group. Cobalt became of interest to the bacteriologist due to the inhibitory effect which this element exerts on bacteria.

Evidence proves that the metals, as a group of chemical elements, have a definite effect on bacteria. One of the earliest bases for this evidence was furnished by Carl von Nägeli who investigated the effect of various quantities of metallic salts on different kinds of fresh water algae.

Since the inhibitory effect of cobalt on bacteria is as yet in an unsettled stage, the author undertook to explore the possibilities of this problem. The selection of a bacterium proved to be relatively simple. A bacterium was selected which has not been extensively studied in its relationship to cobalt. This bacterium was Salmonella pullorum, the

etiologic agent of a disease of poultry often referred to as "pullorum disease".

A specific purpose of this problem was to elaborate upon present knowledge of the inhibitive action of cobalt on growth of this bacterium. It is definitely known that cobalt does exert an inhibitory effect upon this particular species of Salmonella, but the author wished to widen the scope of knowledge with reference to this problem.

The acquirement of knowledge in any of our scientific fields demands meticulous, deliberate, and cautious reasoning. Only through observation, sound judgment, and intelligent forethought is one able to conclude accurately. Moreover, by these processes one is able to forge theory into fact. With this in mind, the author has taken an innovational adventure into the fascinating, yet paradoxically bewildering field of bacteriology.

REVIEW OF LITERATURE

Cobalt has been found to be an effective inhibitor of growth and respiration of various aerobic and anaerobic organisms. Abelson and Aldous (1950) record the toxicity of certain metallic cations in the following order: Ni^{++}, Co^{++}, Zn^{++}, and Mn^{++}. Both Abelson and Aldous studied the toxic effect of high and low magnesium concentration with relation to the ions of nickel, cobalt, cadmium, zinc, and manganese. The microorganisms studied were Escherichia coli, Aerobacter aerogenes, Torulopsis utilis, and Aspergillus niger. To enhance this study, certain of the radioactive tracers were utilized. These were Ni^{57}, Co^{56}, and Mn^{52}. Their experimental results, with special attention to cobalt, are significant in several aspects. (1) The experiments using high concentrations of

magnesium, and also inactive cobalt, lead one to conclude that the cobalt is strongly bound to chemical groups of the organism. (2) The cobalt that is attached to the organism is not readily dissociable. Hence, the amount of cobalt per unit weight can reach very high levels. These authors found that 90 percent of the Co^{56} of the medium had been concentrated in the yeast Torulopsis utilis. This infers that 6 percent of the organism is cobalt. (3) An important possibility is that magnesium, an activator for almost all known enzymes, may be replaced by either of the divalent cations Co^{++} or Ni^{++}, which is incorporated in an enzyme usually employing magnesium as an activator. As a consequence of this, toxic effects may result. The ions which interfere most with magnesium, (Co^{++}, Ni^{++}, Cd^{++}, Zn^{++}, and Mn^{++}), are those ions that tend to form highly stable organic complexes with typical structures of biological significance, such as folic acid, asparagine, and proline.

Burk (1946) found that its physiologic action may be overcome reversibly and rather specifically by histidine. He also found that various other substituted histidine compounds, including carnosine and anserine, behaved qualitatively like histidine but Co^{++} was not observed to combine with 5-methyl imidazole or N-acetyl glutamate.

Burk, Schade, and Hesselbach (1946) noted that respiration in mouse tissue is inhibited by cobalt but regarded this observation as secondary in importance to the reversibility of cobalt inhibition by histidine, in the inhibition of aerobic and anaerobic organisms. Hearon (1948) did extensive investigations in the complexity of the cobalt-histidine system. He noted that a coordination complex formed between histidine and cobaltous ion which possesses the ability to combine reversibly with molecular oxygen.

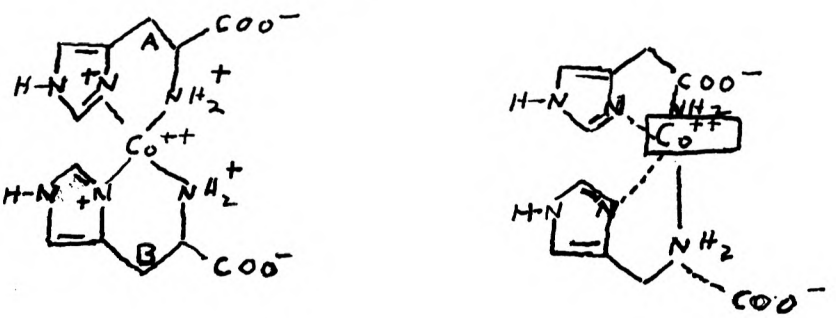
Initial points in consideration of this are that 2 molecules of histidine combine with Co^{++}, yielding a reversible complex that is formed immediately:

$$2 \text{ Histidine} + Co^{++} \rightleftharpoons Co\ (\text{histidine})_2 + 2\ H^+$$

The cobalt-histidine reacts with oxygen, possibly in the following manner:

$$2\ Co\ (\text{histidine})_2 + O_2 \longrightarrow [Co\ (\text{histidine})_2]_2 O_2$$

Hearon (1948) outlines these possibilities as the structural compound of this cobalto-dihistidine complex:



Hearon further points out that the stability of cobalto-dihistidine, relative to that of the cobaltous complexes of other alpha-amino acids, may be explained on the basis of the presence (and nature) of the imidazole ring in histidine. He suggests that the bonds in cobalto-dihistidine are essentially ionic; those in oxy-bis (cobalto-dihistidine) are strictly covalent.

Johnson, Carver, and Harryman (1942), in their investigation, were interested in comparing the effects of diverse bactericidal and bacteriostatic agents, including metal cations, sulfonamide compounds, and narcotics. Their studies may be summarized in effect as follows: (1) The effects of certain metals, metal chlorides, narcotics, and bacteriostatic compounds

on the luminescence of plate cultures of Achromobacter fischeri and Photobacterium phosphoreum were studied by an auxanographic method. (2) Inhibitory effects on growth were caused by the addition of pure metallic copper, cobalt, cadmium, and arsenic; to a slight degree by zinc, lead, and nickel; to a doubtful extent by manganese, magnesium and bismuth; and not at all by aluminum and gold. Pronounced inhibitions were apparent with the chlorides of copper, cobalt, manganese, nickel, and silver; and slight inhibitions by the chlorides of lead and magnesium.

The investigations of Michaleis and Barrow (1929) were lengthy and thorough. Their studies were concerned with the complexes of cysteine formed with metals of the iron group. The results of these extensive investigations may be elucidated with the following statements: (1) Cobalt gives with cysteine, in the absence of oxygen, a cobaltous complex which is usually slightly olive green, but pink when there is a large excess of cysteine. (2) The cobaltous complex is rapidly oxidized by air, organic dyestuffs, or ferricyanide to a brown complex. The ratio of cobalt to cysteine in this oxidized complex is 1:3. (3) The potential of pure cobaltous cysteine at a pH 7.5 practically matches that of the hydrogen electrode for the same pH. Cobaltous cysteine is one of the most powerful reductants at a pH 7.5 to 8.0. (4) Cobalt and nickel are no catalysts for the oxidation of cysteine. (5) The study of the cobalt-cysteine complex is important because the end product of its oxidation is a compound which is analogous to an intermediary compound in the case of iron, and will permit the study of the intermediary state in the iron catalysis.

The investigation of Schade and Caroline (1944), in reference to the raw egg white of the egg of the hen and the role of iron in growth inhibition

of Shigella dysenteriae, Micrococcus aureus, Escherichia coli, and the yeast Saccharomyces cerevisiae, merits attention in that of the metals of the iron group only iron, which was tested with other vitamin factors and 31 elements, overcame the egg white inhibition of growth of these organisms.

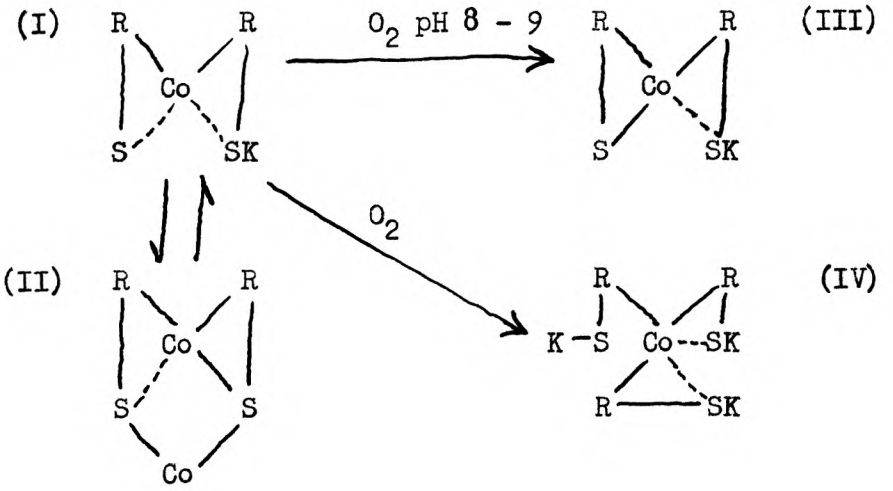
Schade (1949) undertook comprehensive investigations in reference to cobalt and bacterial growth with reference primarily to Proteus vulgaris. It was primarily due to his studies that the author conducted investigations in this problem with respect to Salmonella pullorum. Schade observed that 10 to 20 parts per million of cobalt were adequate to inhibit growth of a certain strain of Salmonella pullorum. Schade also studied the effect of regulation of the hydrogen ion concentration in relation to cobalt inhibitive effect. His results did not disclose any noticeable effect as a result of altering the pH value. Schade further studied the reversibility of cobalt inhibition by histidine with special regard to Proteus vulgaris. In further explorations on the problem, he made a study of cobalt inhibition of certain bacteria under aerobic and anaerobic conditions. The results of this demonstrated that it required three to four times the amount of cobalt to inhibit growth under anaerobic conditions in comparison with that required under aerobic conditions, in study of a given species of bacteria.

Schade (1949) also undertook investigations as to the effect of cobalt on the size and staining ability of cells, with special interest in Proteus vulgaris. It was found that cells inoculated into nutrient broth containing a concentration of cobalt sufficient to inhibit growth failed to show any increase in size or evidence of cell division over a 2-hour period of incubation.

Another interesting phase of Schade's work (1949) was that concerning the effect of cobalt on the viability of cells. Again special emphasis was placed on Proteus vulgaris. It was determined that Proteus vulgaris varied in the degree of sensitivity to a growth-inhibiting concentration of cobalt depending upon the stage of its life cycle at the time of cobalt addition to the growth medium. Schade concluded that cobalt does in time have a significant effect on the cells of Proteus vulgaris in relation to cell viability.

Schubert (1933) did work on the action of carbon monoxide on certain of the iron and cobalt complexes of cysteine. He found that the action of carbon monoxide on the green potassium cobalti-tricysteinate in strong alkali involves oxidation-reduction reactions. The products which are formed are a carbonate and the new complex H $[Co(CO)_4]$. This complex cobalt form on treatment with acid gives cobalt tetracarbonyl. Cobalt tetracarbonyl on treatment with cysteine and alkali gives cobalti-tricysteinate and H $[Co(CO)_4]$.

Scubert (1931) did extensive work with the formation of cobalt complexes with cysteine. These might be best made clear by illustrations. Complex (I) is formed by mixing deaerated solutions of cysteine, potassium hydroxide, and cobalt chloride in an atmosphere of oxygen-free nitrogen.



(I) Potassium cobalto-bis cysteinate.
(II) Cobaltous cobalto-bis cysteinate (grass green in color).
(III) Potassium cobalti bis-cysteinate (olive brown).
(IV) Potassium cobalti tri-cysteinate (red).

Schubert prepared certain cysteine-cobalt crystalline complexes containing cobalt and cysteine in the ratios 1:1, 1:2, and 1:3.

THE MATERIALS AND METHODS

On the basis of antigenic structure, the organism selected for study was Salmonella pullorum, which can be separated into regular, intermediate, and variant strains. Antigenic structure is concerned with the immunologicalchemical constitution of the cells of bacteria. On the basis of antigenic structure, a method is provided for a separation of bacterial species from one another.

A total of 25 cultures was secured of Salmonella pullorum. These 25 cultures are separated into the 3 strains as mentioned previously. Eighteen of the strains were of the regular strain. Five of the strains were of the variant strain, and 2 other strains were of the intermediate type.

One additional species of the genus Proteus of Canadian origin was included; thus, 26 cultures in all were selected for investigation.

Three cobalt salts were selected to be utilized in an investigation of cobalt inhibitory action. These respective cobalt salts were cobalt chloride ($CoCl_2.6H_2O$), cobalt sulfate ($CoSO_4.7H_2O$), and cobalt acetate [$Co(CH_3COO)_2.4H_2O$]. Emphasis should be placed on the fact that the cobalt ion, Co^{++}, is in actuality the active principle exerting an inhibitory effect. Three cobalt salts were selected, however, in an effort to determine whether 1 cobalt salt demonstrated a greater inhibitory effect than any other.

The method of investigation consisted of several parts. The first step was a simple preparatory experimental phase in which an attempt was made to determine the range in cobalt concentration from complete inhibition to no apparent inhibition. Inhibition was based on whether or not growth of the organisms occurred in the cultural medium containing cobalt.

Successive dilutions of cobalt ranging from 200 parts per million to 0 parts per million were made. Arithmetical calculations were utilized to determine the successive cobalt dilutions of 200, 175, 150, 125, 100, 75, 50, 25, and 0 parts per million. In essence, this calculation is as follows:

(a) 400 p.p.m. of cobalt = $\frac{0.400 \text{ grams per liter}}{0.254 \text{ grams of cobalt in cobalt chloride}}$

(b) Molecular weight of cobalt chloride; $CoCl_2.6H_2O$ is 231.84. Thus:

Molecular weight of cobalt = $\frac{58.94}{231.84}$ = 0.254 grams of cobalt in 1 gram of cobalt chloride. Thus: For a solution to contain cobalt in the concentration of 400 parts per million, it must contain 1.574 grams of cobalt chloride per liter of water.

By similar arithmetical calculations, it was possible to determine the number of grams of cobalt sulfate and cobalt acetate necessary in order to make a solution containing 400 parts per million of these respective salts. Four-hundred parts per million of cobalt was an arbitrarily selected figure since it simplified the method of making cultural media with the specified dilutions of cobalt desired. Specifically, a ratio between nutritive medium and cobalt was devised to accomplish the dilutions extending from 200 to 0 parts per million of cobalt. Zero parts per million of cobalt was selected as a standard control employed in every investigation.

The 26 cultures were divided into 5 separate groups in order to facilitate experimental investigation. The experimental results are listed collectively in order to present a more condensed, integrated series of results.

The actual experimental investigation was performed in the following manner: Nutrient broth was mixed with the cobalt solution of 400 parts per million, original strength, to make successive dilutions of 200, 175, 150, 125, 100, 75, 50, and 25 parts per million of cobalt. Cumulatively, this was a total of 8 dilutions for each organism. Ten milliliters of media containing cobalt in the specified dilutions were placed into test tubes. Following sterilization in the autoclave at 121° C. for 25 minutes, each tube was inoculated with 0.05 ml of the Salmonella culture grown in nutrient broth. This pattern was followed for each of the cobalt salts. After inoculation all of the cultures were incubated at 37° C for a total period of 168 hours. At the end of each 24-hour interval, the cultures were observed and results recorded to determine whether growth of the organisms did or did not occur.

The concentration of the hydrogen ion (H^+) is important in any bacteriological investigation. In this problem, the hydrogen ion concentration was also studied to determine any possible influence this might exert on cobalt inhibition. Consequently, 6 pH values were selected for study. These were pH values of 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0. All 26 of the Salmonella cultures were studied at these different hydrogen ion concentrations. In all of the experimental studies, the dilutions of the three cobalt salts remained constant at the values of 200, 175, 150, 125, 100, 75, 50, 25, and 0 p.p.m.

The hydrogen ion study began at the pH value of 6.0 and continued through the pH values previously mentioned. Since data acquired from study of the hydrogen ion concentration at the 2 values of pH 6.5 and 7.5 were not markedly different from results obtained at the pH value of 7.0, the author was advised to omit these from the experimental results. Data are recorded in the experimental results only for 48 and 168 hours, respectively.

The possible effect of cobalt on the viability of the cells was also of interest. To study this, only 1 cobalt salt, cobalt acetate, was employed. In the method of experiment, the dilutions of cobalt acetate ranging from 200 through 125 parts per million with controls were used. Following 72 hours and 168 hours of incubation of Salmonella and Proteus cultures in media containing cobalt, transfers were made from these cultures to nutrient broth enriched one and one-half times the normal ingredients usually contained in the medium. Transfers were made by transference of 3 loopfuls of material from each of the 4 dilutions of cobalt (200, 175, 150, 125) to the enriched nutrient broth medium. These inoculated cultures were observed for growth after an incubation period of 48 hours at 37° C.

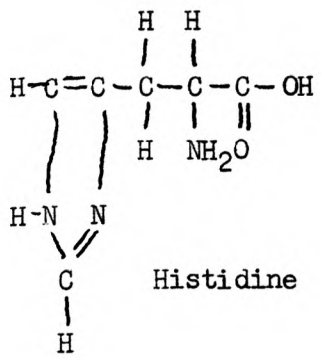
Experimentally, the results are given in accordance with the pH values at which the study was made. These were pH values of 7.0, 7.5, and 8.0. The pH of the enriched media to which the transfers were made, was at the value of pH 7.0 to 7.2.

An investigation as to the growth of the Salmonella cultures under anaerobic conditions comprised a third phase of the problem. The medium selected for anaerobic studies was Brewer's liquid thioglycollate medium, a medium in which anaerobic conditions may be preserved with relative simplicity. The dilutions of cobalt employed were 150, 125, 100, 75, 50, 25, and 0 parts per million. In these anaerobic experiments, only cobalt sulfate was utilized.

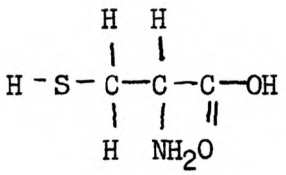
Following the previously set pattern of study, the cultural media containing cobalt in the aforementioned dilutions were inoculated with the Salmonella strains as previously described. The strains were 24 hours old. In each case of inoculation in the experiments, the age of the organisms was kept standard at 24 hours. These inoculated cultures were incubated at 37° C. for a total period of 168 hours. Readings were made and recorded following a 48-hour and 168-hour period of incubation, respectively. In these experiments, the pH was kept at a stationary value of 7.1. The amount of inoculum used in each case was 0.05 ml. of the 24-hour old culture.

The final phase of investigation of the problem has been concerned with the reversibility of growth-inhibition due to cobalt by the 2 amino acids histidine and cysteine. The chemical structures of these 2 amino

acids are:



Histidine



Cysteine

In utilization of these 2 amino acids, their salts were employed. These salts were histidine mono-hydrochloride and cysteine mono-hydrochloride.

Using previous work done along this line as a guidepost, the author determined a scheme of titrating these amino acids against a stationary concentration of cobalt in parts per million, to ascertain the point at which growth of the organisms occurred. In essence, this is the point at which the ratio of each respective amino acid to cobalt was sufficient to overcome the growth-inhibition due to cobalt. The method of arithmetic calculation was on the same basis as that employed for the determinations for cobalt to be present in certain concentrations. Thus:

Molecular weight of histidine mono-HCl = 191.66
Determine the actual amount of histidine in 1 gram of histidine mono-HCl by this calculation;

Molecular weight of histidine = 155.16, thus, $\frac{155.16}{191.66} = 0.809$, the actual part of 1 gram of histidine mono-HCl which is histidine.

Finally, determine the amount of grams of histidine mono-HCl to be added to 1 liter of water to have a concentration of histidine equal to 500 parts per million.

Thus, $\frac{0.500 \text{ gram per liter}}{0.809 \text{ gram histidine per gram of histidine mono-HCl}} = 0.6179$ gram of histidine mono-HCl to be added to 1 liter of water

The same method of calculation was employed for the determination of cysteine to be added to 1 liter of water to give a similar concentration of cysteine in parts per million. After these calculations were made, it was necessary to devise a scheme of ratios of amino acid to cobalt. This scheme was in a series form, the following proportions of histidine to cobalt being selected: 4:1, 3:1, 2.5:1, 2:1, 1.5:1, and 1:1. This same series, with the exception of the ratios 1.5:1 and 1:1, was employed for the proportion of cysteine to cobalt.

Nutrient broth of double strength, as was used in the previous investigations, was employed to provide an ample source of nutrients for the organisms, and to compensate for the dilution of the cultural media in making the different concentrations involved in these experiments. In all of the experiments the hydrogen ion (H^+) was maintained at a constant pH value of 7.0 $\pm$ 0.2. The cultural media in all cases, except the controls, contained cobalt in a concentration of 100 parts per million. The 2 amino acids were present in concentrations of 400, 300, 250, 200, 150, and 100 parts per million. Each amino acid of course was titrated separately against cobalt. The 2 acids were not together in the same media. In the investigation the cultural media were inoculated with 0.10 ml of each respective strain of Salmonella pullorum. These inoculated tubes were then incubated at 37° C. for a total period of 168 hours. In the experimental results, readings are recorded for periods of incubation following 48 and 168 hours, respectively. The control medium contained neither cobalt nor either of the 2 amino acids. It was inoculated, as was the other media with 0.10 ml of the various Salmonella pullorum strains. The amount of cultural media in each test tube was 10 ml. Thus, a constant amount of inoculum was

added to a constant amount of cultural media. This policy was maintained throughout the investigations.

With reference to the maintenance of the hydrogen ion (H^+) at a certain value, it was necessary to employ a buffer system. The buffer system which the author used was an acetate buffer system, since in the early part of the problem the phosphate buffer system proved to be unsatisfactory. Hence, by addition of either dilute acid or alkali to the cultural media, it was possible to maintain the hydrogen ion (H^+) at a specific value. The acetate buffer system was also employed in the first phase of the problem.

Table 1. The inhibitive action of cobalt on Salmonella pullorum.

Cultural organisms	
Culture number	Variety of strain
1. Cal-P 135 (I)	Regular*
2. 3282	Regular
3. 3245	Regular
4. 4002-3	Variant
5. 3433	Regular
6. 4803	Variant
7. B.L. 2394.41	Intermediate
8. 3239	Regular
9. 4016-3	Variant
10. 854422	Regular
11. 85777	Regular
12. 4903-3	Regular
13. 3976-BS	Regular
14. 4903-7	Regular
15. C-30701	Variant
16. 34628-33	Regular
17. 85817	Intermediate
18. 4803-L	Variant
19. 3486	Regular
20. 3558	Regular
21. Proteus 1	-
22. Turkey	Regular
23. 4074	Regular
24. Jones	Regular

Table 1. (concl.).

Cultural organisms	
Culture number	Variety of strain
25. 3933	Regular
26. 3920	Regular

*Separation of the cultures of Salmonella pullorum on the basis of antigenic structure was done by A. J. Luzzio.

(I) In the following tables, for the reason of simplification, the Salmonella pullorum strain varieties are listed by number. In Table 2, for example, the arabic number (1) corresponds to Cal-P 135, number (2) corresponds to strain 3282 and so on, throughout the experimental results.

Table 2. The inhibitive action of cobalt chloride, cobalt sulfate, cobalt acetate on Salmonella pullorum.

pH 6.0									
48 Hours					168 Hours				
Cobalt in parts per million									
100	75	50	25		100	75	50	25	Control 0
Cobalt chloride									
1. –	–	–	–		–	–	–	+++	+++
2. –	–	–	–		–	–	–	–	+++
3. –	–	+	+		–	–	+	+	+++
4. –	–	–	–		–	–	+	+	+++
5. –	–	++	+		–	–	+	+	+++
6. –	–	–	+		–	–	+	+	+++
7. –	–	+	+		–	–	+	+++	+++
8. –	±	+	++		–	±	+	++	+++
9. –	–	+	+		–	–	+	+	+++
10. –	–	–	±		–	–	–	+	+++
11. –	–	–	±		–	–	+	+++	+++
12. –	–	–	–		–	–	–	+++	+++
13. –	–	+	+		–	–	+	+++	+++
14. –	–	+	+		–	–	+	+++	+++
15. –	–	+	+		–	–	+	+++	+++
16. –	+	++	++		±	+	++	+++	+++
17. ±	+	+	++		–	±	++	+++	+++
18. –	+	++	++		–	+	+	+++	+++
19. –	+	+	++		–	+	+	+++	+++

Table 2. (cont.).

	pH 6.0								
	48 Hours				168 Hours				
	Cobalt in parts per million								
	100	75	50	25	100	75	50	25	Control 0
Cobalt chloride									
20.	-	+	++	++	-	±	+	+++	+++
21.	+	+	++	++	++	++	++	+++	++++
22.	-	-	-	+	-	-	±	+++	+++
23.	-	+	+	++	-	+	+	+++	+++
24.	-	-	+	++	-	-	+	+++	+++
25.	-	+	+	++	-	±	+	+++	+++
26.	-	-	-	++	-	-	++	+++	+++
Cobalt sulfate									
1.	-	-	-	+	-	-	-	+++	+++
2.	-	-	-	-	-	-	-	+++	+++
3.	-	-	-	+	-	-	-	+++	+++
4.	-	-	-	+	-	-	-	+++	+++
5.	-	-	-	+	-	-	-	+++	+++
6.	-	±	+	++	-	±	+	++	+++
7.	-	±	+	+++	-	+	+	+++	+++
8.	-	±	++	+	-	±	+	++	+++
9.	-	±	+	+	-	±	+	+	+++
10.	-	-	-	+	-	-	-	+	+++
11.	-	-	±	+	-	-	+	++++	+++
12.	-	-	-	-	-	-	-	+++	+++
13.	-	-	+	+	-	-	+	+++	+++
14.	-	-	±	+	-	-	+	+++	+++
15.	-	-	-	+	-	-	+	+++	+++
16.	-	-	±	+	-	-	+	+++	+++
17.	-	+	±	++	-	+	++	+++	+++
18.	-	+	+	++	-	+	++	+++	+++
19.	-	-	+	+	-	-	±	+++	+++
20.	-	+	+	+	-	+	+	+++	+++
21.	+	+	+	+++	++	++	+++	++++	++++
22.	-	-	-	+	-	-	-	+	+++
23.	-	-	-	+	-	-	-	+++	+++
24.	-	±	+	+	-	±	+	+++	+++
25.	-	±	+	+	-	±	+	+++	+++
26.	-	-	+	++	-	-	++	+++	+++

Table 2. (concl.).

	pH 6.0								
	48 Hours				168 Hours				
	Cobalt in parts per million								
	100	75	50	25	100	75	50	25	Control 0
	Cobalt acetate								
1.	-	-	-	++	-	-	-	+++	+++
2.	-	-	-	+	-	-	-	+++	+++
3.	-	-	-	-	-	-	-	-	+++
4.	-	-	-	-	-	-	-	+++	+++
5.	-	-	++	++	-	-	+++	+++	+++
6.	-	-	+	++	-	-	+	++	+++
7.	-	±	+	+++	-	±	+	+++	+++
8.	-	+	+	+++	±	+	+	+++	+++
9.	-	-	+	++	-	-	+	++	+++
10.	-	-	-	+	-	-	-	+	+++
11.	-	-	+	++	-	-	++	+++	+++
12.	-	-	-	-	-	-	-	+++	+++
13.	-	-	+	++	-	-	++	+++	+++
14.	-	±	+	++	-	-	±	+++	+++
15.	-	-	±	+	-	-	++	+++	+++
16.	-	-	+	+	-	-	+	+	+++
17.	-	-	-	+	-	-	+	+++	+++
18.	-	-	+	++	-	-	+	+++	+++
19.	-	-	-	±	-	-	+	+	+++
20.	-	-	+	++	-	-	+	+	+++
21.	+	+	++	+++	+++	+++	+++	++++	++++
22.	-	-	±	+	-	-	+	++	+++
23.	-	-	-	-	-	-	-	-	+++
24.	-	-	-	++	-	-	-	+++	+++
25.	-	-	±	+	-	-	+	+++	+++
26.	-	-	+	+	-	-	+	+++	+++

Interpretation of the various signs employed in Tables 1 to 7 inclusive are as follows:

A negative sign (-) indicated no growth.
The sign (±) was used to indicate only a trace of growth.
The sign (+) was used to indicate slight growth.
The sign (++) indicated moderate growth.
The sign (+++) indicated pronounced growth.
The sign (++++) indicated very pronounced growth.

Table 3. The inhibitive action of cobalt chloride, cobalt sulfate, and cobalt acetate on Salmonella pullorum.

	pH 7.0								
	48 Hours				168 Hours				
	Cobalt in parts per million								
	100	75	50	25	100	75	50	25	Control 0
Cobalt chloride									
1.	-	+	+	++	-	+	++	++	+++
2.	-	+	+	++	-	+	++	++	+++
3.	-	-	-	++	-	++	++	++	+++
4.	-	+	+	++	-	+	++	++	+++
5.	-	+	+	++	-	+	++	++	+++
6.	-	±	-	++	-	-	++	++	++
7.	-	-	-	+	-	-	-	++	++
8.	-	-	-	++	-	-	-	++	++
9.	-	-	-	++	-	-	+	++	++
10.	-	-	-	+	-	-	++	++	++
11.	-	±	±	++	-	±	++	++	+++
12.	-	+	+	++	-	+	++	+++	+++
13.	±	+	++	++	±	+	++	+++	+++
14.	-	+	+	++	-	+	++	+++	+++
15.	-	-	±	++	-	+	++	++	+++
16.	-	+	+	+++	-	+	+	+++	+++
17.	-	-	-	+++	-	+	+	+++	+++
18.	-	±	++	++	-	+	+	+++	+++
19.	-	-	++	++	-	+	++	+++	+++
20.	-	+	+	++	+	++	++	+++	+++
21.	++	+++	+++	++++	+++	+++	+++	+++	++++
22.	-	-	±	++	±	++	++	++	++
23.	-	-	±	+++	±	++	++	++	++
24.	-	+	+	++	±	±	++	++	++
25.	-	±	++	++	±	±	++	++	++
26.	-	+	++	++	-	±	++	++	++
Cobalt sulfate									
1.	-	±	+	+++	-	++	++	++	+++
2.	-	±	+	++	-	++	±	+	+++
3.	-	-	-	++	-	-	++	++	+++
4.	-	±	+	++	-	++	++	++	+++
5.	-	±	+	++	-	±	++	++	+++
6.	-	-	+	++	-	-	++	++	++
7.	-	-	-	++	-	-	-	++	++
8.	-	-	+	++	-	-	++	++	++
9.	-	-	-	++	-	-	-	++	++

Table 3. (cont.).

	pH 7.0								
	48 Hours				168 Hours				
	Cobalt in parts per million								
	100	75	50	25	100	75	50	25	Control 0
Cobalt sulfate									
10.	-	++	++	++	-	-	++	++	++
11.	-	±	+	++	-	±	++	+++	+++
12.	-	+	++	++	-	+	++	+++	+++
13.	±	+	++	++	±	+	++	+++	+++
14.	-	+	++	++	-	±	++	+++	+++
15.	-	-	±	++	-	+	++	+++	+++
16.	±	+	++	+++	±	+	++	+++	+++
17.	-	-	±	+++	-	+	++	+++	+++
18.	-	+	+	+++	-	+	++	+++	+++
19.	-	-	++	+++	-	+	++	++	+++
20.	±	+	++	+++	-	++	++	++	+++
21.	++	++	+++	+++	++	+++	+++	++++	++++
22.	-	-	+	++	-	++	++	++	++
23.	-	-	+	++	-	±	++	++	++
24.	-	-	-	++	-	±	++	++	++
25.	-	±	+	++	-	++	++	++	++
26.	-	-	+	++	-	++	++	++	++
Cobalt acetate									
1.	-	±	+	++	-	++	++	++	+++
2.	-	-	±	++	-	-	+	++	+++
3.	-	-	-	++	-	++	++	++	+++
4.	-	+	+	++	-	++	++	++	+++
5.	-	±	++	+	-	++	++	++	+++
6.	-	-	±	++	-	-	++	+++	+++
7.	-	-	±	++	-	-	++	+++	+++
8.	-	-	-	++	-	-	-	+++	+++
9.	-	-	-	++	-	-	-	+++	+++
10.	-	-	±	++	-	-	++	+++	+++
11.	-	+	+	+++	-	+	++	+++	+++
12.	-	+	+	+++	-	+	++	+++	+++
13.	±	+	+	+++	±	+	++	++	+++
14.	-	+	+	+++	-	±	++	+++	+++
15.	-	-	±	++	-	+	++	++	+++
16.	-	+	+	++	-	+	++	+++	+++
17.	-	+	++	++	-	±	++	+++	+++
18.	-	+	++	++	-	+	++	+++	+++
19.	-	-	++	++	-	±	++	+++	+++
20.	-	+	++	++	-	+	++	+++	+++

Table 3. (concl.).

	pH 7.0								
	48 Hours				168 Hours				
	Cobalt in parts per million								
	100	75	50	25	100	75	50	25	Control 0
	Cobalt acetate								
21.	++	++	++	++++	+++	+++	++++	++++	++++
22.	-	-	±	++	-	++	++	++	+++
23.	-	-	+	++	-	++	++	+++	+++
24.	-	-	-	++	-	+	++	+++	+++
25.	-	-	++	++	-	+	++	+++	+++
26.	-	-	++	++	-	+	++	+++	+++

Table 4. The inhibitive action of cobalt chloride, cobalt sulfate, and cobalt acetate on Salmonella pullorum.

	pH 8.0								
	48 Hours				168 Hours				
	Cobalt in parts per million*								
	100	75	50	25	125	100	75	50	25	Control 0
	Cobalt chloride									
1.	-	±	+	++	-	+	+	+	+	+++
2.	-	+	+	++	+	+	+	+	++	+++
3.	-	+	++	++	-	-	+	+	++	+++
4.	-	-	++	++	-	-	+	+	++	+++
5.	-	-	+	++	-	+	+	+	++	+++
6.	-	-	+	++	-	+	+	++	++	+++
7.	-	±	+	++	-	-	+	++	++	+++
8.	-	±	+	++	-	-	+	++	++	+++
9.	-	±	+	++	-	-	+	++	++	+++
10.	-	-	±	++	-	+	+	++	++	+++
11.	-	-	±	++	-	-	+	++	++	+++
12.	-	-	+	++	-	-	+	++	++	+++
13.	±	+	+	++	±	+	+	++	++	+++
14.	-	-	+	++	-	-	+	++	++	+++
15.	-	-	+	++	-	-	+	++	++	+++
16.	-	±	+	++	-	+	+	++	++	+++
17.	-	-	+	++	±	+	++	++	++	+++
18.	-	+	++	++	-	-	++	++	++	+++

Table 4. (cont.).

	pH 8.0									
	48 Hours					168 Hours				
	Cobalt in parts per million*									
	100	75	50	25	125	100	75	50	25	Control 0
					Cobalt chloride					
19.	-	+	++	++	-	±	+	++	++	+++
20.	-	+	++	++	-	±	+	++	++	+++
21.	++	++	+++	+++	++	++	+++	+++	+++	++++
22.	+	+	+	++	+	+	+	++	++	+++
23.	+	+	+	++	+	+	++	++	+++	+++
24.	-	-	-	-	-	+	++	++	++	+++
25.	+	+	++	++	+	+	++	++	+++	+++
26.	+	+	++	++	+	+	++	++	+++	+++
					Cobalt sulfate					
1.	-	+	++	++	-	+	+	++	++	+++
2.	+	+	++	++	+	+	+	++	++	+++
3.	-	-	++	++	-	+	+	++	++	+++
4.	-	+	++	+++	-	+	+	++	++	+++
5.	-	+	++	+++	-	±	+	++	++	+++
6.	-	+	+	+++	-	+	+	++	++	+++
7.	-	+	+	+++	-	±	+	++	+++	+++
8.	-	-	+	++	-	-	+	++	+++	+++
9.	-	+	+	+++	-	-	+	++	+++	+++
10.	-	-	+	+++	-	±	+	++	+++	+++
11.	-	-	-	+++	-	+	++	++	+++	+++
12.	-	+	+	+++	-	+	+	++	++	+++
13.	-	+	+	+++	+	+	+	++	++	+++
14.	-	+	+	+++	-	+	+	++	++	+++
15.	-	-	+	+++	-	-	+	++	++	+++
16.	-	+	++	++	-	+	++	++	++	+++
17.	-	+	++	++	-	+	++	++	++	+++
18.	-	+	++	++	-	+	++	++	++	+++
19.	-	+	++	++	+	+	++	++	++	+++
20.	-	+	++	++	-	+	++	++	++	+++
21.	++	++	++	++	++	++	+++	+++	+++	++++
22.	+	+	++	+++	+	+	++	++	+++	+++
23.	+	+	++	+++	+	+	++	++	++	+++
24.	-	+	++	++	+	+	++	++	++	+++
25.	-	+	++	++	+	+	++	++	+++	+++
26.	+	+	++	++	+	+	++	++	+++	+++

Table 4. (concl.).

	pH 8.0									
	48 Hours				168 Hours					
	Cobalt in parts per million*									
	100	75	50	25	125	100	75	50	25	Control 0
	Cobalt acetate									
1.	-	+	+++	+++	-	+	+	++	++	+++
2.	+	+	++	++	+	+	+	++	++	+++
3.	±	+	++	++	-	+	+	++	++	+++
4.	±	+	++	++	-	+	+	++	++	+++
5.	-	±	++	++	-	±	+	++	++	+++
6.	-	-	++	+++	-	-	++	++	+++	+++
7.	-	±	+	+++	-	-	++	++	++	+++
8.	-	±	+	+++	-	-	++	++	++	+++
9.	-	±	+	+++	-	-	++	++	++	+++
10.	-	-	±	+++	-	-	++	++	++	+++
11.	-	-	±	++	-	-	+	++	++	++
12.	-	+	+	+++	-	-	+	++	++	+++
13.	-	+	+	+++	-	-	+	++	++	+++
14.	-	+	+	+++	-	-	+	++	+++	+++
15.	-	-	+	+++	-	-	+	++	+++	+++
16.	-	+	++	++	-	+	+	++	++	+++
17.	+	+	++	++	-	+	++	++	++	+++
18.	-	+	++	++	-	+	++	++	++	+++
19.	±	+	++	++	+	+	++	++	++	+++
20.	±	+	++	++	+	+	++	++	++	+++
21.	++	++	++++	++++	++	++	+++	++++	++++	++++
22.	+	+	++	++	+	+	++	++	+++	+++
23.	+	+	++	++	+	+	++	++	+++	+++
24.	-	+	++	++	+	+	++	++	+++	+++
25.	-	+	++	++	+	+	++	++	+++	+++
26.	+	+	++	++	+	+	++	++	+++	+++

*125 parts per million of cobalt is included at 168 hours reading at the pH values of 8.0 and 9.0 since growth occurred at these concentrations cited.

Table 5. The inhibitive action of cobalt chloride, cobalt sulfate, and cobalt acetate on Salmonella pullorum.

	pH 9.0									
	48 Hours					168 Hours				
	Cobalt in parts per million									
	100	75	50	25	125	100	75	50	25	Control 0
Cobalt chloride										
1.	+	+	+	++	+	+	+	+	+	++
2.	+	+	+	+	+	+	+	+	+	+
3.	+	+	+	+	−	±	±	+	+	+
4.	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	−	+	+	+	+	+
6.	±	+	+	+	+	+	+	+	+	+
7.	±	+	+	+	+	+	+	+	+	+
8.	±	+	+	+	+	+	+	+	+	++
9.	±	+	+	+	+	+	+	+	+	++
10.	±	+	+	+	+	+	+	+	+	+
11.	±	+	+	++	+	+	+	+	+	+
12.	−	+	+	+	−	+	+	+	+	+
13.	±	+	+	+	+	+	+	+	+	+
14.	±	+	+	+	−	+	+	+	+	+
15.	±	+	+	+	+	+	+	+	+	+
16.	−	+	+	+	+	+	+	+	+	+
17.	+	+	+	+	+	+	+	+	+	+
18.	−	+	+	+	−	−	±	+	+	+
19.	+	+	+	+	+	+	+	+	+	+
20.	−	+	+	+	±	+	+	+	+	+
21.	++	++	++	++	++	++	+++	+++	+++	++++
22.	+	+	+	+	+	+	+	+	+	+
23.	+	+	+	+	+	+	+	+	+	+
24.	−	±	+	+	+	+	+	+	+	+
25.	−	−	−	−	−	−	+	+	+	+
26.	+	+	+	+	+	+	+	+	+	+
Cobalt sulfate										
1.	+	+	+	+	+	+	+	+	+	+
2.	+	+	+	+	+	+	+	+	+	+
3.	+	+	+	+	−	−	±	+	+	+
4.	−	+	+	+	−	±	+	+	+	+
5.	+	+	+	+	−	+	+	+	+	+
6.	±	+	+	+	+	+	+	+	+	+
7.	+	+	+	+	−	+	+	+	+	+
8.	+	+	+	+	+	+	+	+	+	+
9.	+	+	+	+	+	+	+	+	+	+

Table 5. (cont.).

	pH 9.0									
	48 Hours					168 Hours				
	Cobalt in parts per million									
	100	75	50	25	125	100	75	50	25	Control 0
	Cobalt sulfate									
10.	+	+	+	+	+	+	+	+	+	+
11.	±	+	+	++	+	+	+	+	+	+
12.	±	+	++	++	-	±	+	+	+	+
13.	+	+	++	+	+	+	+	+	+	+
14.	-	+	++	-	+	+	+	+	+	+
15.	±	+	+	+	++	+	+	+	+	+
16.	-	+	+	+	+	+	+	+	+	++
17.	+	+	+	+	+	+	+	+	+	++
18.	+	+	+	+	-	±	±	+	+	++
19.	+	+	+	+	+	+	+	+	+	++
20.	-	-	+	+	±	+	+	+	+	++
21.	+	++	++	++	++	++	+++	+++	+++	+++
22.	±	±	+	+	±	±	+	+	+	+
23.	±	±	+	+	±	±	+	+	+	+
24.	-	±	+	+	±	±	+	+	+	+
25.	-	-	-	-	-	±	+	+	+	+
26.	±	±	+	+	±	+	+	+	+	+
	Cobalt acetate									
1.	+	+	+	+	+	+	+	+	+	+
2.	+	+	+	+	+	+	+	+	+	+
3.	+	+	+	+	-	±	±	+	+	+
4.	-	+	+	+	+	±	±	+	+	+
5.	-	+	+	+	-	±	+	+	+	+
6.	±	+	+	+	+	+	+	+	+	+
7.	±	+	+	+	+	+	+	+	+	+
8.	±	+	+	+	+	+	+	+	+	+
9.	±	+	+	+	+	+	+	+	+	+
10.	±	+	+	+	+	+	+	+	+	+
11.	±	+	+	++	+	+	+	+	+	+
12.	-	-	+	++	+	+	+	+	+	+
13.	+	+	+	+	+	+	+	+	+	+
14.	+	+	+	+	+	+	+	+	+	+
15.	-	+	+	+	+	+	+	+	+	+
16.	+	+	+	++	+	+	+	+	+	+
17.	-	+	+	+	+	+	+	+	+	++
18.	-	+	+	+	-	-	±	+	+	+
19.	+	+	+	+	-	+	+	+	+	+

Table 5. (concl.).

	pH 9.0									
	48 Hours					168 Hours				
	Cobalt in parts per million									
	100	75	50	25	125	100	75	50	25	Control 0
	Cobalt acetate									
20.	+	+	+	+	+	+	+	+	+	+
21.	++	++	++	++	+	++	+++	+++	+++	+++
22.	+	+	+	+	±	+	+	+	+	+
23.	-	+	+	+	-	+	+	+	+	+
24.	-	-	±	+	±	±	+	+	+	+
25.	-	-	-	-	-	+	+	+	+	+
26.	+	+	+	+	±	+	+	+	+	+

Table 6. The effect of cobalt acetate on the viability of the cells of Salmonella pullorum.

	48 Hours					168 Hours				
	200	175	150	125[1]		200	175	150	125	Control 0
	pH 8.0									
1.	-	++	++	++		++	++	++	++	++
2.	-	-	++	++		-	-	++	++	++
3.	-	++	++	++		-	++	++	++	++
4.	++	++	++	++		++	++	++	++	++
5.	++	++	++	++		++	++	++	++	++
6.	-	-	++	++		-	-	++	++	
7.	-	++	++	++		-	++	++	++	
8.	-	-	++	++		-	-	++	++	
9.	-	-	-	++		-	-	-	++	
10.	++	++	++	++		++	++	++	++	
	pH 7.5									
11.	-	-	++	++		-	-	++	++	
12.	-	-	-	++		-	-	-	++	
13.	-	++	++	++		-	++	++	++	
14.	-	-	++	++		-	-	++	++	
15.	-	-	-	++		-	-	++	++	
16.	-	-	-	++		-	-	-	++	

Table 6. (concl.)

	48 Hours				168 Hours				
	200	175	150	125	200	175	150	125	Control 0
				pH 7.5					
17.	-	-	-	++	-	-	-	++	
18.	-	-	-	-	-	-	-	-	
19.	-	-	-	++	-	-	-	++	
20.	-	-	-	++	-	-	-	++	
				pH 7.0					
21.	-	-	-	+++	-	-	++	++	+++
22.	-	-	++	++	-	-	++	++	++
23.	-	-	++	++	-	-	-	-	++
24.	-	-	++	++	-	-	++	++	++
25.	-	++	++	++	-	-	-	-	++
26.	-	-	-	++	-	-	-	-	++

/1 Original concentrations of cobalt in these experiments were 200, 175, 150, and 125 parts per million.

Table 7. The effect of cobalt sulfate on Salmonella pullorum under anaerobic conditions.

	pH 7.0												
	48 Hours						168 Hours						
	Cobalt in parts per million												
	150	125	100	75	50	25	150	125	100	75	50	25	Cont. 0
1.	-	-	-	+	++	+++	-	-	-	++	++	++	+++
2.	-	-	-	-	++	+++	-	-	-	+	+	+++	+++
3.	-	-	-	+	++	+++	-	-	-	++	++	++	+++
4.	-	-	-	-	++	+++	-	-	-	++	++	++	+++
5.	-	-	-	-	++	+++	-	-	-	+	++	++	+++
6.	-	-	-	+	++	+++	-	-	-	++	++	++	+++
7.	-	-	-	++	++	+++	-	-	++	++	++	++	+++
8.	-	-	-	-	++	+++	-	-	-	+	++	++	+++
9.	-	-	-	+	++	+++	-	-	-	++	+	++	+++
10.	-	-	-	+	+++	+++	-	-	-	+	++	++	+++
11.	-	-	+	+	++	++	-	+	+	++	++	++	+++
12.	-	-	+	+	++	+++	-	-	+	++	++	++	+++
13.	-	-	+	+	++	+++	-	+	+	++	++	++	+++
14.	-	-	-	+	++	+++	-	-	+	++	++	++	+++
15.	-	-	+	+	++	+++	-	-	++	++	++	++	+++
16.	-	-	-	+	++	+++	-	-	-	+	++	++	+++
17.	-	-	-	+	++	+++	-	-	-	+	++	++	+++
18.	-	-	-	+	++	+++	-	-	-	+	++	++	+++
19.	-	-	-	+	++	+++	-	-	++	++	++	++	+++
20.	-	-	+	+	++	+++	-	-	+	+	++	++	+++
21.	-	-	+	++	++	+++	-	+	++	++	++	++	+++
22.	-	-	-	-	++	+++	-	-	-	+	++	++	+++
23.	-	-	-	+	++	+++	-	-	-	+	++	++	+++
24.	-	-	-	+	++	+++	-	-	+	+	++	++	+++
25.	-	-	+	+	++	+++	-	-	+	++	++	++	+++
26.	-	-	+	+	++	+++	-	-	+	++	++	++	+++

Table 8. The effect of histidine in the reversibility of growth-inhibition due to cobalt on Salmonella pullorum.

	pH 7.0										
	48 Hours						168 Hours				
	Ratio of histidine to cobalt										
	4:1	3:1	2.5:1	2:1	1.5:1	1:1[1]	3:1	2.5:1	2:1	1:1	Cont. 0[2]
1.	+++	+++	-	-	-	-	+++	++	++	-	+++
2.	+++	+++	++	++	-	-	+++	++	++	-	+++
3.	+++	+++	-	-	-	-	+++	++	++	-	+++
4.	+++	+++	-	-	-	-	+++	++	++	-	+++
5.	+++	+++	-	-	-	-	+++	-	-	-	+++
6.	++	++	+	±	-	-	++	+	+	-	+++
7.	++	+	+	±	-	-	++	+	+	-	+++
8.	++	+	+	±	-	-	++	+	+	-	+++
9.	++	+	+	+	-	-	++	+	+	-	+++
10.	++	+	+	±	-	-	++	+	±	-	+++
11.	++	+	+	+	-	-	++	+	+	-	+++
12.	++	+	+	+	-	-	++	+	+	-	+++
13.	++	+	+	+	-	-	++	+	+	-	+++
14.	++	+	+	+	-	-	++	+	+	-	+++
15.	++	+	+	-	-	-	++	+	-	-	+++
16.	+++	+++	++	++	-	-	+++	+++	++	-	+++
17.	+++	++	++	++	-	-	+++	+++	++	-	+++
18.	+++	++	++	++	-	-	+++	++	+	-	+++
19.	+++	++	++	++	-	-	+++	++	+	-	+++
20.	+++	++	++	++	-	-	+++	++	+	-	+++
21.	0	0	0	0	0	0	0	0	0	0	0[3]
22.	+++	++	++	++	-	-	++	++	+	-	+++
23.	+++	++	+	+	-	-	++	++	+	-	+++
24.	+++	+	+	-	-	-	++	++	+	-	+++
25.	+++	++	++	+	-	-	++	++	+	-	+++
26.	+++	++	++	+	-	-	++	++	+	-	+++

[1] The ratios of histidine to cobalt of 4:1, and 1.5:1 were omitted since growth did occur strongly at the former ratio, and it did not occur at the latter ratio, after a period of 168 hours of incubation at 37° C.

[2] The control medium contained neither cobalt nor histidine. It consisted simply of nutritive materials for the organisms.

[3] The Proteus strain was not included since this strain grew at concentrations of 100 p.p.m. of cobalt and above.

Table 9. The effect of cysteine in the reversibility of growth-inhibition due to cobalt on Salmonella pullorum.

	pH 7.0								
	48 Hours				168 Hours				
	Ratio of cysteine to cobalt/1								
	4:1	3:1	2.5:1	2:1/2	4:1	3:1	2.5:1	2:1	Control 0
1.	+++	+++	-	-	+++	+++	++	-	+++
2.	+++	+++	-	-	+++	+++	++	-	+++
3.	+++	+++	-	-	+++	+++	++	-	+++
4.	+++	+++	-	-	+++	+++	++	-	+++
5.	+++	+++	-	-	+++	+++	++	-	+++
6.	++	++	++	++	+++	+++	++	++	+++
7.	++	++	++	++	+++	+++	++	++	+++
8.	++	++	++	++	+++	+++	++	++	+++
9.	++	++	++	++	+++	+++	++	++	+++
10.	++	++	++	++	+++	+++	++	++	+++
11.	++	++	++	++	+++	+++	++	++	+++
12.	++	++	++	++	+++	+++	++	++	+++
13.	++	++	++	++	+++	+++	++	++	+++
14.	++	++	++	++	+++	+++	++	++	+++
15.	++	++	++	++	+++	+++	++	++	+++
16.	+	+	+	+	+	+	+	+	+++
17.	+	+	+	+	+	+	+	+	+++
18.	+	+	+	+	+	+	+	+	+++
19.	+	+	+	+	+	+	+	+	+++
20.	+	+	+	+	+	+	+	+	+++
21.	0	0	0	0	0	0	0	0	0/3
22.	++	+	+	+	+++	+++	++	++	+++
23.	++	+	+	+	+++	+++	++	++	+++
24.	++	+	+	+	+++	+++	++	++	+++
25.	++	+	+	+	+++	+++	++	++	+++
26.	++	+	+	+	+++	+++	++	++	+++

/1 In both of these experiments, the concentration of cobalt was 100 parts per million. This was sufficient to inhibit growth of the strains when cobalt alone was employed.

/2 The ratios of cysteine to cobalt did not include those of 1.5:1 or 1:1 since these were included in the experiment using histidine as the amino acid.

/3 The Proteus strain was not included since this strain grew at concentrations of 100 p.p.m. of cobalt and above.

GENERAL DISCUSSION

Specifically, the problem selected has been investigated with 4 particular objectives in mind. The first of these was to analyze the inhibitive action exerted by the cobalt ion (Co^{++}) on 25 strains of Salmonella pullorum and 1 Proteus strain. The second phase consisted of determining the effect of cobalt on the viability of the bacterial cells of these Salmonella strains. The third phase of the problem has been concerned with the effect of growth-inhibition of Salmonella pullorum under anaerobic conditions. The fourth and final phase of the investigation has been concerned with the reversibility of cobalt growth-inhibition by 2 amino acids. These were cysteine, a sulfur-containing amino acid, and histidine.

Phase One: The Effect of Cobalt on Salmonella pullorum Utilizing 3 Cobalt Salts. (Tables 2, 3, 4, and 5)

Referring to the first phase of the investigation, several points may be considered. First, it may be definitely concluded that the element cobalt exerts an inhibition on growth of Salmonella pullorum. Secondly, the inhibitive action of cobalt is most effective at moderately acid conditions. As the pH value is raised, it seems apparent that Salmonella pullorum becomes increasingly tolerant to cobalt. This was true of all three of the cobalt salts studied, that is cobalt chloride, cobalt sulfate, and cobalt acetate. With reference to the experimental results (Tables 2 through 5) centering attention on cobalt chloride, it may be seen readily that at a pH value of 6.0, 4 organisms did not grow at the highest dilution of cobalt employed, namely, 25 parts per million. Nine organisms were unable to grow at a cobalt concentration of 50 parts per million. Only 9 organisms were

able to grow after 48 hours in a cobalt concentration of 75 parts per million. Only 2 organisms were able to grow at a cobalt concentration of 100 parts per million. Considering cobalt chloride at a pH value of 9.0, it is readily discernible that the experimental results are different. During 48 hours of incubation, all of the organisms grew in cobalt concentrations of 25, 50, and 75 parts per million. Only 6 organisms did not grow at 100 parts per million of cobalt after 48 hours. Yet, following 168 hours of incubation, 20 of the organisms grew in a relatively high concentration of cobalt; i.e., 125 parts per million. This makes it apparent that the effect of the hydrogen ion (H^+) is important in the effectiveness of growth-inhibition by cobalt. The differences between the 3 cobalt salts with reference to growth-inhibition were not marked. Consequently, it may be concluded that the cobalt ion (Co^{++}) is the important factor responsible for the inhibition of growth of Salmonella pullorum.

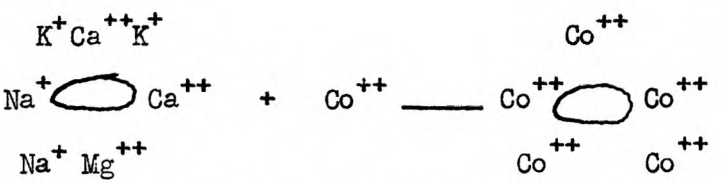
Clarification as to why growth is inhibited by cobalt is difficult. The author suggests that with an increase in the concentration of cobalt, growth may be inhibited due to an unbalanced bacterial metabolism, thereby, interfering directly with the maintenance of life by the bacterial cell. It is certain that cobalt in some manner, as yet not fully explained, exerts a definite inhibition on the growth of Salmonella pullorum. The organisms are able to tolerate cobalt at certain concentrations, and apparently they thrive well. However, when the concentration of cobalt exceeds a certain critical point, growth ceases.

Phase Two: The Effect of Cobalt on the Viability of the Cells of Salmonella pullorum. (Table 6)

The second phase of the investigation has been concerned with the effect of cobalt on the viability of cells of Salmonella pullorum. The point of significance is that an effort has been made to determine whether the inhibitory effect of cobalt was bacteriostatic or bactericidal in nature. A consideration of the experimental results (Table 6) reveals that except for 4 organisms, (15.34 percent of the total number of organisms) a concentration of cobalt of 200 parts per million for 168 hours is definitely bactericidal. At a concentration of 175 parts per million of cobalt, 7 organisms grew after 168 hours of incubation. This represents a percentage of 26.91 of the total number of organisms. It may be concluded that at least for the majority of the Salmonella strains, cobalt at this concentration also is bactericidal. Decreasing the amount of cobalt to 150 parts per million in concentration, it may be observed from the experimental results that only 10 of the organisms, a percentage of 38.46 of the total number, did not grow. Thus, for the majority of the organisms, a cobalt concentration of 150 parts per million is bacteriostatic. The demonstration of bacteriostasis is evident when the experimental results for cobalt at a concentration of 125 parts per million are considered. Here, only 4 organisms, representing 15.38 percent of the total number of organisms, failed to grow after a period of 168 hours of incubation. At this concentration of cobalt for the decided majority of organisms, the effect is bacteriostatic in nature.

Although the exact cause as to the toxic effect of cobalt is unknown, certain theories may be presented. The author would suggest these two:

First, it may be conceivable that in causing a toxic effect to the cell, cobalt might replace certain basic ions, such as Na^{+}, K^{+}, Ca^{++}, and Mg^{++}, on the surface of the cell. Thus, by this base exchange, the cell would be deprived of ions essential to the maintenance of life, in consequence, death of the cell would ensue. Such a reaction may be pictured in an equation form:



(1)

This reaction is believed to typify the reaction of the mercury ion (Hg^{++}) in its toxic effect on cell viability. Perhaps this possibility would explain the reason as to the antagonistic effect exerted by cobalt to cell viability.

A second possibility might also be the disruption of cell metabolism, thereby leading to death of the cell. This possibility is suggested by the work of Abelson and Aldous, as mentioned in the review of literature. It is known that it is largely through the enzyme systems that bacterial cells are able to maintain the metabolic reactions necessary for the continuation of life. If the metabolism of the bacterial cells is disrupted, or impaired, so that it is no longer carried on in a normal manner, death of the cell inevitably would occur. The magnesium ion (Mg^{++}) is a known activator for many enzyme systems in bacteria. It is possible that the cation of cobalt (Co^{++}), also being divalent, may replace magnesium as an activator. If this were true, then the enzyme system would be disturbed

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[1] McCalla, T. M. "Cation Adsorption by Bacteria," Jour. of Bacteriology 40: 23-43, July, 1940.

with a subsequent cessation of metabolic activity, ultimately resulting in the death of the cell. Either of these 2 suggested theories may explain the reason as to the cause of death of the bacterial cell. It may be concluded definitely that as the concentration of cobalt is raised above 150 parts per million, its effect on Salmonella pullorum becomes distinctly bactericidal.

Phase Three: The Effect of Cobalt on Salmonella pullorum Under Anaerobic Conditions. (Table 7)

Salmonella pullorum is a facultative anaerobe. Consequently it seemed desirable to study the influence of cobalt upon Salmonella pullorum under anaerobic as well as aerobic conditions. The selected medium was Brewer's liquid thioglycollate medium, because of its simplicity of handling, and the relative ease with which anaerobic conditions may be preserved. However, mention should be made of a difficulty encountered in this part of the experiment. Brewer's thioglycollate medium contains sodium thioglycollate, the sodium salt of thioglycollic acid, as an active reducing agent necessary to preserve anaerobic conditions. Due to some unknown reason the reducing ability of sodium thioglycollate in the presence of relatively high concentrations of cobalt, that is 100 parts per million and higher, is noticeably affected. Namely, the methylene blue, present in the medium as an indicator to determine whether anaerobic conditions exist, becomes colored. This indicates strongly that in relatively high concentrations of cobalt, anaerobic conditions are lost. It is apparent that cobalt is in some intricate manner involved. The author is unable to explain the "How!" of it. By experimenting it was learned that this loss of reducing ability may be overcome by the

addition of excess sodium thioglycollate to the medium. It is due to this fact that the author is persuaded to believe that cobalt is in some way responsible for the loss of reducing ability by sodium thioglycollate.

Consideration of the experimental results in Table 7 leads to the conclusion that none of the specific strains of Salmonella pullorum or the Proteus strain was able to grow at a cobalt concentration of 150 parts per million under anaerobic conditions. Three strains were able to grow at 125 parts per million (11.54 percent of the total number of strains). Twelve strains were able to grow at a concentration of 100 parts per million of cobalt (46.15 percent of the total number of strains), and all 26 strains grew at cobalt concentrations of 75 parts per million or less. The experimental results obtained under anaerobic conditions, at the pH value of 7.0, are not strikingly different from those recorded for aerobic conditions.

Phase Four: The Effect of Histidine and Cysteine on the Reversibility of Growth-Inhibition of Salmonella pullorum due to Cobalt. (Tables 8 and 9)

The fourth and final phase of the problem has been concerned with the reversibility of growth-inhibition effected by cobalt on Salmonella pullorum by the 2 amino acids histidine and cysteine. This phase of the problem has proved 1 point very conclusively; namely, that it is possible to overcome the growth-inhibition effect of cobalt by either of these 2 amino acids. The data in Table 8 indicate that one provision is strictly necessary to accomplish this; namely, that only when the ratio of histidine to cobalt was approximately 2:1 or wider, on a molecular weight basis, were the strains of Salmonella pullorum able to grow well.

Cobalt does form complex compounds with both of these amino acids. The formation of these compounds may readily explain why the strains were able to grow when the ratio of the amino acid to cobalt was 2:1 or larger. It is possible that by a combination with these amino acids, the cobalt ion (Co^{++}) is removed from the cultural medium so that it is not present to inhibit the growth of these strains. If, however, the ratio of amino acid to cobalt is less than 2:1, then growth does not occur.

SUMMARY

Four phases of the inhibitive action of cobalt on Salmonella pullorum have been studied. These 4 phases have been concerned with different approaches to the selected problem. Each contributes a source of information that is pertinent to the problem as a whole.

The first phase of the problem was concerned with the inhibition of growth of Salmonella pullorum exerted by cobalt. Allied with this was a study made to determine the effect of the hydrogen ion (H^+) upon this inhibitory action of cobalt. The findings concerning this phase of the problem may be briefly summarized as follows: Cobalt definitely exerts an inhibitive effect on growth of Salmonella pullorum. The concentration of cobalt necessary for complete inhibition of growth varies with the pH of the cultural medium. Cobalt concentrations of 150 parts per million and above were sufficient to inhibit growth completely in all cases except with the Proteus strain. It is apparent that 1 cobalt salt is not significantly different from another salt in its growth-inhibition of Salmonella pullorum. The effect of the hydrogen ion (H^+) becomes noticeable as the pH value of the cultural media is raised from a moderately acid pH of 6.0

to a fairly strong alkaline pH of 9.0. At pH of 9.0, the Salmonella pullorum strains seemed definitely to be more tolerant to cobalt and accordingly growth occurred at greater concentrations of cobalt than at the moderately acid pH of 6.0. Thus, it is indicated that altering the pH of the cultural medium influences the growth-inhibitory property of cobalt.

The second phase of the problem was a study to determine whether the inhibitory effect of cobalt on Salmonella pullorum was bactericidal or bacteriostatic in nature. It is definite that cobalt for the majority of these Salmonella strains is bactericidal at a concentration of 200 parts per million. As the concentration of cobalt is decreased, the bactericidal property diminishes steadily. At the cobalt concentration of 125 parts per million, the effect on all except 4 strains of the pullorum organism was only bacteriostatic. Thus, it may be definitely concluded that concentration of the cobalt ion (Co^{++}) is important in effecting bactericidal or bacteriostatic activity.

The third phase of the problem was concerned with a study of the growth-inhibitive effect of cobalt on Salmonella pullorum under anaerobic conditions. It was found that 150 parts per million of cobalt present in the cultural medium were sufficient to inhibit growth completely. With only three exceptions, 125 parts per million of cobalt was adequate to inhibit growth completely. It is important to note that even though growth occurred at the cobalt concentrations of 100 parts per million and less, it definitely was not as good as that obtained in the control medium which contained no cobalt. This suggests that even at fairly low concentrations of cobalt the inhibitive effect is noticeable.

In the final phase of the problem, a study was made concerning the reversibility of the growth-inhibition of Salmonella pullorum due to cobalt by the 2 amino acids, histidine and cysteine. Either of these amino acids is able to overcome the growth-inhibition exerted by cobalt, provided the ratio of amino acid to cobalt is as wide as 2:1 on a molecular weight basis or greater. With few exceptions, the ratio of 2:1 in the case of either amino acid was adequate to overcome this growth-inhibition. It is important to realize that the ratios of 1.5:1 and 1: 1, in the case of the amino acid histidine, were not sufficient to reverse this growth-inhibition due to cobalt.

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THE INHIBITIVE ACTION OF COBALT ON SALMONELLA PULLORUM

by

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Briefly outlining the manner in which the problem was approached in the experimental investigation may best be accomplished by describing each phase of the problem separately. Four phases of the inhibitive action of cobalt on Salmonella pullorum have been studied. These four phases have been concerned with different approaches to the selected problem. Each contributes a source of information that is pertinent to the problem as a whole.

In the first phase of the problem, two aspects were considered; namely, the concentration of cobalt in parts per million necessary for the complete inhibition of growth, and the effect of the hydrogen ion (H^+) exerted on the growth-inhibitive action of cobalt, were the two factors. The concentrations of cobalt in parts per million ranged from 200 to 25. These were devised by arithmetical calculations. The effect of the hydrogen ion depended upon shifting the pH of the cultural medium from one which was moderately acid to one which was fairly alkaline.

The second phase of the problem was concerned with the effect of cobalt on the viability of the cells of Salmonella pullorum. In this phase, the cultures of Salmonella pullorum were subjected to certain concentrations of cobalt in parts per million. Following certain time intervals of incubation, that is 48 and 168 hours, transfers were made to nutrient broth of enriched strength. These inoculated tubes were then incubated and were observed to determine whether growth had occurred.

The third phase of the problem pertained to a study of the effect of cobalt on Salmonella pullorum under anaerobic conditions. This phase consisted of employing Brewer's liquid thioglycollate medium and employing specific concentrations of cobalt ranging from 150 to 25 parts per million.

Interest here was centered in determining the concentration of cobalt necessary to inhibit growth completely. This study was also a comparison with the first phase of the problem, that being an aerobic study.

In finality, the fourth phase of the problem was concerned with a reversal of the growth-inhibition of Salmonella pullorum due to cobalt by two amino acids, histidine and cysteine. This was accomplished by devising arithmetic proportions of amino acid to cobalt ranging from a 1:1 ratio, on a molecular weight basis, to a 4:1 ratio. The concentration of cobalt was high enough to inhibit growth. This concentration was 100 parts per million. In this phase of the problem, interest centered upon that ratio of amino acid to cobalt which would overcome the inhibitive action of cobalt and permit growth to occur.

The first phase of the problem was concerned with the inhibition of growth of Salmonella pullorum exerted by cobalt. Allied with this was a study made to determine the effect of the hydrogen ion (H^+) upon this inhibitory action of cobalt. The findings concerning this phase of the problem may be briefly summarized as follows. Cobalt definitely exerts an inhibitive action on growth of Salmonella pullorum. The concentration of cobalt necessary for complete inhibition varies with the pH of the cultural medium. Cobalt concentrations of 150 parts per million and above were sufficient to inhibit growth completely in all cases except with the Proteus strain. It is apparent that one cobalt salt is not significantly different from another salt in its growth-inhibition of Salmonella pullorum. The effect of the hydrogen ion (H^+) becomes noticeable as the pH of the cultural media is raised from a moderately acid pH of 6.0 to a fairly strong alkaline pH of 9.0. At the pH of 9.0 the Salmonella pullorum strains seemed

definitely to be more tolerant to cobalt, and accordingly, growth occurred at greater concentrations of cobalt than at the moderately acid pH of 6.0. Thus, it is indicated that altering the pH of the cultural medium influences the growth-inhibitory property of cobalt.

The second phase of the problem was a study to determine whether the inhibitory effect of cobalt on Salmonella pullorum was bactericidal or bacteriostatic in nature. It is definite that cobalt for the majority of these Salmonella strains is bactericidal at a concentration of 200 parts per million. As the concentration of cobalt is decreased the bactericidal property diminishes steadily. At the cobalt concentration of 125 parts per million, the effect on all except four strains of the pullorum organism was only bacteriostatic. Thus, it may be definitely concluded that concentration of the cobalt ion (Co^{++}) is important in effecting bactericidal or bacteriostatic activity.

The third phase of the problem was concerned with a study of the growth-inhibitive effect of cobalt on Salmonella pullorum under anaerobic conditions. It was found that 150 parts per million of cobalt present in the cultural medium were sufficient to inhibit growth completely. With only three exceptions, 125 parts per million were adequate to inhibit growth completely. It is important to note that even though growth occurred at the cobalt concentrations of 100 parts per million and less, it definitely was not as good as that obtained in the control medium which contained no cobalt. This suggests that even at fairly low concentrations of cobalt the inhibitive effect is noticeable.

In the final phase of the problem, a study was made concerning the reversibility of the growth-inhibition of Salmonella pullorum due to cobalt

by the two amino acids histidine and cysteine. Either of these amino acids is able to overcome the growth-inhibition exerted by cobalt, provided the ratio of amino acid to cobalt is as wide as 2:1 on a molecular weight basis, or greater. With few exceptions, the ratio of 2:1 in the case of either amino acid was adequate to overcome this growth-inhibition. It is important to realize that the ratios of 1.5:1 and 1:1 in the case of the amino acid histidine were not sufficient to reverse this growth-inhibition due to cobalt.