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The Uptake of Cobalt by *Proteus vulgaris*

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SUMMARY: *Proteus vulgaris*, suspended in meat extract broth or in a defined medium, bound cobalt to an extent dependent on the cell concentration and on the concentration of cobalt in the medium. An increase in concentration of cobalt or a decrease in the number of cells per unit volume resulted in a greater amount of cobalt ion bound per unit of cellular material. The composition of the suspending medium with regard to the cobalt combining groups present, as well as the availability of other metallic ions, markedly affected the amount of cobalt bound by the cells. Magnesium was particularly effective in diminishing the cobalt-combining capacity of the cells. An exposure time of 15 min. was sufficient for the major portion of the ion to be taken up by the cells. In an anaerobic medium, the cells bound approximately one-half as much cobalt as in an aerobic medium. Raising the pH value of the medium from 5.0 to 7.0 resulted in an increased uptake of the metal by the cells. Cobalt taken up by the cells could be removed to an extent of 70–80 % by dilution in distilled water and almost completely by 0.1 N-HCl. A derived strain of *Proteus*, capable of growth in relatively high concentrations of cobalt, bound smaller quantities of the ion at each level tested than did the parent strain. However, at the growth inhibitory level peculiar to each strain, the resistant form took up a much greater quantity of the metal than did the sensitive strain. This fact indicates that resistance to cobalt cannot be explained merely on the basis of the cobalt-concentrating ability of the cells.

The growth inhibitive action of cobalt on various bacteria and especially on *Proteus vulgaris* has been studied as a function of the composition of the growth medium, strain sensitivity, age of culture, size of inoculum, and other cultural conditions (Schade, 1949). Further, the effect of cobalt on the relationship between nucleic acid concentration and growth rate in the nucleic acids of *P. vulgaris* have been determined (Levy, Skutch & Schade, 1949*a, b*). Employing radio-isotope techniques, we have now investigated the uptake of cobalt by the cells of *P. vulgaris* under various environmental conditions in an effort to determine the principal factors involved in the binding of the metal.

METHODS

The organism employed in these studies was a strain of *P. vulgaris* isolated originally from a case of cystitis and maintained on meat extract agar slopes of the composition: 0.3 % (w/v) Difco meat extract; 1.0 % (w/v) Difco peptone; 0.5 % (w/v) NaCl; 3.0 % (w/v) Difco agar; adjusted to pH 7.5 with NaOH.

Cells from 18 to 24 hr. slope cultures were washed with either distilled water or 0.001 M potassium phosphate buffer, pH 7.3. The cell suspensions, made up to the desired turbidity, were then inoculated into the test medium containing cobalt as $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.03–0.07 $\mu\text{C./ml.}$ of radio-active cobalt 60 as

CoCl_2 . Following incubation in a water-bath at 37° , the radio-active cell suspensions were spun down in a Sorvall high-speed centrifuge in weighed 15 ml. glass centrifuge tubes. The supernatants were poured off and saved for determination of radio-activity. The tubes were then drained and weighed. From the known wet weight of cells calculated from the turbidity measurement, the amount of supernatant remaining in the tubes could be determined by difference. The cells were then suspended in 1.0 ml. 0.1N-HCl and determinations of radio-activity made on 0.2 ml. portions of the acid cell suspensions and of their original supernatants. The samples to be assayed were pipetted on to glass cover-slips previously moistened with a drop of detergent (1 % Duponol, Dupont) to ensure even distribution of the radio-active sample. The total quantity of liquid was contained within a ring of Dow Corning heavy silicone stopcock grease. Samples thus prepared were dried under an infra-red lamp and counted with a Geiger-Müller counter.

RESULTS

Cobalt uptake as affected by the concentration of cobalt and cells in the medium

In previous studies of the inhibitive action of cobalt on the growth of *P. vulgaris*, we found that the extent of inhibition was conditioned by the concentration of cobalt in the medium and by the size of the inoculum. In a defined medium, $0.12 \mu\text{g. Co}^{++}/\text{ml.}$ was sufficient to prevent the growth of approximately 1000 cells/ml. At the same level of cobalt concentration, cultures containing 10,000 cells/ml. showed only partial inhibition and those containing 100,000 cells/ml. grew out to a degree equal to control cultures without cobalt.

To determine to what extent the amount of cobalt taken up by *P. vulgaris* was affected by the concentration of cobalt and the number of cells in a given volume of medium, we performed a number of experiments in which both of these factors were varied. In one set of experiments, a given weight of cells was exposed to increasing quantities of cobalt contained in equal volumes of 1:5 meat extract broth (MEB). Under these conditions, a fair degree of correlation was found between concentration of cobalt in the medium and uptake of the metal by the cells. The results of a typical experiment are summarized in Table 1. From these data, it is evident that an increase in the level of cobalt from 1 to $10 \mu\text{g./ml.}$ resulted in close to a tenfold increase in the amount of cobalt bound per mg. dry weight of cells. At the higher concentra-

Table 1. *Uptake of cobalt as affected by concentration of Co^{++} in the medium*

One mg. dry wt. cells suspended in 30.0 ml. of 1:5 MEB, pH 7.3 containing $1.0 \mu\text{c.}$ radio-active $^{60}\text{Co}^{++}$ and varying quantities of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ to give the required concentration. Time of exposure 1 hr. at 37° .

Co^{++} concentration ($\mu\text{g./ml.}$)	Dry wt. of cells (mg./ml.)	Co^{++} uptake ($\mu\text{g. Co}^{++}/\text{mg.}$ dry wt. cells)
1	0.033	0.237
10	0.033	2.12
100	0.033	15.3

tion of $100\mu\text{g. Co}^{++}/\text{ml.}$, the increased uptake by the cells was no longer directly proportional.

When the concentration of cobalt was maintained at a constant level and the number of cells altered by the addition of varying dilutions of a stock-cell suspension, an inverse relationship was found between the amount of cobalt taken up per unit weight of cells and the concentration of cells in the medium. The results of two of such experiments are given in Table 2. The ability of the cells to concentrate high amounts of cobalt per unit of cellular material is particularly evident in those instances where the total cell number was low. The capacity of relatively low concentrations of cells to take up large quantities of cobalt was found also in experiments where variation in cell concentration was achieved in a different manner. In such tests, $0.5\text{ mg. dry wt. of cells}$ exposed to $100\mu\text{g. Co}^{++}/\text{ml.}$ in 6.0 ml. 1:5 MEB took up approximately $19\mu\text{g. Co}^{++}/\text{mg. dry wt. cells}$, while the same weight of cells suspended in 30 ml. of this medium containing $100\mu\text{g. Co}^{++}/\text{ml.}$ took up as much as $68\mu\text{g. Co}^{++}/\text{mg. dry wt. cells}$.

Table 2. *Uptake of cobalt as affected by concentration of cells in the medium*

Varying dilutions of a stock cell suspension were inoculated into $35\text{ ml. of 1:5 MEB, pH 7.3}$ containing $1.4\mu\text{c. radio-active cobalt }^{60}\text{Co}^{++}$ in a final concentration of $100\mu\text{g. Co}^{++}/\text{ml.}$ Time of exposure $1\text{ hr. at }37^\circ$.

Cell conc.	Total dry wt. of cells (mg.)	Co^{++} uptake ($\mu\text{g. Co}^{++}/\text{mg. dry wt. cells}$)
(a) $\times 1$	0.093	86.4
$\times 5$	0.464	10.0
$\times 24$	2.24	4.92
(b) $\times 1$	0.130	51.5
$\times 4.7$	0.612	8.81
$\times 23.3$	3.024	5.14

It can be noted here that in comparing different groups of inocula the actual amount of cobalt bound/unit of cell material varied, even though inocula of comparable age and size were exposed to the same concentration of cobalt. This variability of different inocula in their response to cobalt makes it difficult to obtain quantitative data necessary for elucidating the method whereby cell concentration affects the amount of cobalt bound.

Consideration of the data of Tables 1 and 2 led us to anticipate that if the concentrations of both cobalt and cells were simultaneously increased to the same degree, the observed uptake of cobalt by the cells would be the resultant of these two opposing factors. Such an experiment was set up by exposing equal quantities of cells to a given weight of cobalt contained in different volumes of suspending medium. The results summarized in Table 3 show that a decrease in the amount of cobalt bound/unit of cell material occurs as the concentration of both cobalt and cells is diminished. The amount of cobalt associated with the cells will be affected to a greater extent by a change in the concentration of cobalt in the medium than by an equivalent change in the concentration of cells.

Table 3. *Uptake of cobalt varying both Co^{++} and cell concentration*

2.0, 8.0, 32.0 ml. 0.9 % NaCl in 0.01M sodium acetate, pH 6.0, containing: (a) 32 μg . cobalt, 0.5 μc . radio-active cobalt, 0.196 mg. dry wt./cells; (b) 2 μg . cobalt, 1.0 μc . radio-active cobalt, 0.223 mg. dry wt. cells.

	Co^{++} concentration (μg ./ml.)	Cell concentration (μg . dry wt./ml.)	Co^{++} uptake (μg . Co^{++} /mg. dry wt. cells)
(a)	16	98	0.810
	4	24.5	0.369
	1	6.1	0.298
(b)	1	112	0.102
	0.25	27.9	0.0349
	0.0616	6.97	0.0171

Cobalt uptake as affected by the constituents of the suspending medium

Early in our work we had observed that the amount of cobalt taken up by the cells exposed to 10 μg . Co^{++} /ml. in a growth medium (1:5 MEB) was approximately one-third that taken up by an equal weight of cells in 0.001M potassium phosphate buffer. The possibility that the metabolic state of the cells in a growth medium compared to that of cells in phosphate buffer might be reflected in their cobalt-combining capacity was investigated. Since, however, the MEB has cobalt-combining constituents which could compete with the cells for the free cobalt ions and thus effect a diminished uptake of cobalt by the cells, we employed a defined growth medium free of cobalt-combining substances. In a medium containing 0.1 % (w/v) glucose, 0.1 % (w/v) $(\text{NH}_4)_2\text{SO}_4$, 30 μg ./ml. nicotinamide, 100 μg ./ml. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.001M- K_2HPO_4 pH 7.3 the uptake of cobalt by the cells was again considerably less (one-third to one-quarter) than that shown by cells suspended in phosphate buffer alone. The removal of glucose and/or $(\text{NH}_4)_2\text{SO}_4$ from the complete medium did not significantly alter the amount of cobalt bound by the cells. Further, when an aged inoculum of viability less than 0.001 % was used, a similar difference was again found in the amount of cobalt bound by these cells in the complete defined medium and in 0.001M- K_2HPO_4 . From these results, it appeared doubtful whether the change in metabolic state associated with the ability of the cells to reproduce themselves would account for the wide differences found in cobalt uptake.

Antagonistic action of magnesium

We next investigated the effect of magnesium on the amount of cobalt taken up by the cells. When magnesium was added to the phosphate buffer in a concentration equivalent to that used in the defined medium, 10 μg . Mg^{++} /ml., a marked decrease in the amount of cobalt bound by the cells was observed. Increasing the concentration of Mg^{++} from 10 to 1000 μg ./ml. resulted in a further diminished uptake of cobalt by the cells (Table 4). The extent of the antagonism was such that between the limits of 10 and 1000 μg . Mg^{++} /ml., a tenfold increase in the concentration of magnesium resulted in nearly a fourfold decrease in the amount of cobalt bound by the cells. Independent

studies of the extent of growth inhibition effected by cobalt at increasing levels of magnesium in the defined medium showed that as the concentration of magnesium in the medium was raised, increased concentrations of cobalt were required to prevent growth of the cells.

Table 4. *Cobalt uptake as affected by concentration of magnesium in the medium*

0.618 mg. dry wt. cells inoculated into 10.0 ml. 0.001 M- K_2HPO_4 , pH 7.4, containing 0.4 μc . radio-active cobalt in final concentration of 10 μg . Co^{++} /ml. and varying concentrations of Mg^{++} as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Time of incubation 1 hr. at 37°.

Concentration of Mg^{++} (μg ./ml.)	Conc. of cells (mg. dry wt./ml.)	Co^{++} uptake (μg . Co^{++} /mg. dry wt. cells)
0	0.06	18.3
10	0.06	7.68
100	0.06	1.90
1000	0.06	0.48

Cobalt uptake under anaerobic conditions

The uptake of cobalt by cells of *P. vulgaris* under anaerobic conditions was studied by passing a continuous stream of oxygen-free nitrogen gas through the inoculated medium (1:5 MEB) contained in 50 ml. capacity plastic centrifuge tubes. Oxygen-free solutions of cobalt were added by means of a syringe. Control tubes were treated in an identical manner but with the nitrogen gas replaced by compressed air. At the end of the incubation period the tubes were capped immediately to maintain the gas phase unchanged during time of centrifugation.

Table 5. *Uptake of cobalt under anaerobic conditions*

20 ml. 1:5 MEB containing 1.0 μc . radio-active cobalt in final concentrations of 50 μg . Co^{++} /ml., inoculated with: (a) 2.76 mg. dry wt. of cells; (b) 4.2 mg. dry wt. cells. Incubation period 1 hr. at 37°.

Gas phase	Cell concentration (mg. dry wt./ml.)	Co^{++} uptake (μg . Co^{++} /mg. dry wt. cells)
(a) Air	0.0138	4.15
N_2	0.0138	2.34
(b) Air	0.21	6.6
N_2	0.21	4.7

The results of two such experiments are summarized in Table 5. In each case, sufficient cobalt (50 μg ./ml.) was present in the medium to prevent growth of both aerobic and anaerobic cultures. Under these conditions, cells maintained in a nitrogen atmosphere bound less cobalt/unit of cellular material than did the cells maintained in the presence of air. These results are of interest since it has been previously found that the concentration of cobalt required for growth inhibition of anaerobic cultures of *Proteus* is 2 to 3 times that required to prevent growth of similar inocula cultured in air (Schade, 1949).

Cobalt uptake by a cobalt resistant strain of P. vulgaris

By repeated transfer of inocula of our cobalt sensitive strain of *P. vulgaris* into media (MEB) containing increasing concentrations of cobalt, we obtained a strain capable of growth in the presence of 150 $\mu\text{g. Co}^{++}/\text{ml.}$ The amount of cobalt bound by these adapted cells was compared with that bound by the parent strain. Tests were run in 1:5 MEB at two levels of cobalt concentration, 10 and 200 $\mu\text{g.}/\text{ml.}$, corresponding to growth inhibitory levels for the stock and cobalt-resistant strain, respectively. The results of two experiments are given in Table 6.

Although the cobalt-sensitive strain fixed approximately 1.5 times more cobalt than did the resistant strain at both levels of cobalt concentration used, the resistant strain bound, at 200 $\mu\text{g. Co}^{++}$, thirty times as much cobalt as the sensitive strain bound at its growth inhibitory level of 10 $\mu\text{g. CO}^{++}/\text{ml.}$ From these results, it seems doubtful whether the failure of 150 $\mu\text{g. CO}^{++}/\text{ml.}$ to inhibit growth of the resistant strain can be ascribed merely to failure of the cells to bind significant quantities of the metal.

Table 6. *Cobalt uptake by P. vulgaris, comparison of stock strain with cobalt resistant variant*

10.0 ml. of 1:5 MEB containing $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ in concentration of 10 and 200 $\mu\text{g. Co}^{++}/\text{ml.}$ and 0.49 $\mu\text{c.}$ radio-active cobalt inoculated with approximately 1.0 mg. dry wt. cells of each strain. Time of incubation 1 hr. at 37°.

Cell type	Concentration of cobalt in the medium			
	10 $\mu\text{g.}/\text{ml.}$		200 $\mu\text{g.}/\text{ml.}$	
	Cell conc. (mg. dry wt./ml.)	Co^{++} uptake ($\mu\text{g. Co}^{++}/\text{mg.}$ dry wt.)	Cell conc. (mg. dry wt./ml.)	Co^{++} uptake ($\mu\text{g. Co}^{++}/\text{mg.}$ dry wt. cells)
Co R	0.073	0.977	0.071	43.6
Co S	0.07	1.432	0.076	76.7
Co R	0.121	0.775	0.105	39.1
Co S	0.087	1.361	0.095	59.9

Co R = cobalt resistant; Co S = cobalt sensitive

Cobalt uptake as affected by the time of exposure and the pH of the medium

Employing a concentration of 100 $\mu\text{g. Co}^{++}/\text{ml.}$ of 1:5 MEB, we determined the amount of cobalt bound by cells suspended in this medium for 15, 30, 60 and 120 min. No significant increase in cobalt uptake by cells suspended for periods longer than 30 min. was observed; 70–80 % of the maximum amount taken up could be accounted for in the cells at the end of only 15 min. exposure.

The effect of the pH value of the medium on cobalt uptake by the cells was tested in 0.01M potassium phosphate buffer. At concentrations of 5 $\mu\text{g. CO}^{++}$ and of 0.07 mg. dry wt. cells/ml. medium, the amount of cobalt bound/unit of cellular material was 0.75 $\mu\text{g.}$ at pH 5.0, 1.02 $\mu\text{g.}$ at pH 6.0 and 1.65 $\mu\text{g.}$ at pH 7.0. Efforts to test the effect of higher pH values on cobalt uptake in this medium were complicated by precipitation of insoluble cobalt compounds.

Removal of cobalt from the cells

How much of the cobalt bound to the cell substance was readily removable with water or HCl was investigated. A quantity of cells (1.43 mg. dry wt.) was suspended in 2.0 ml. distilled water containing $1\mu\text{c.}$ radioactive cobalt in a final concentration of $10\mu\text{g. Co}^{++}/\text{ml.}$ After incubation at 37° for 1 hr. the suspensions were centrifuged, supernatants removed, and the cells washed four times by repeated centrifugation and suspension in 10 ml. distilled water. The amount of cobalt taken up by the cells prior to the first wash was determined by summation of the counts found in the supernatant of each wash and the number remaining in the washed cells. Correction for cobalt retained in adhering supernatant was made. Of a total $3.91\mu\text{g. Co}^{++}$ taken up by the cells, 46, 32, 28 and 26 % remained with the cells after the first, second, third and fourth washes, respectively. Hence, it is apparent that 20–25 % of the cobalt originally associated with the cells was bound more or less fixedly to the cellular substance.

When fresh cells exposed to similar concentrations of cobalt were subsequently suspended in 0.1 N-HCl, 95–98 % of the total cobalt taken up appeared in the supernatant.

DISCUSSION

The observation that cells of *P. vulgaris* take up cobalt rapidly and that distilled water or 0.1 N-HCl easily remove it indicates that cobalt is primarily bound with the cell substance in a readily reversible manner. Such binding may be accomplished by the mechanism of ion adsorption with the cells acting in a manner similar to charged colloidal particles, or by direct chemical combination with reactive groups throughout the cell as well as with those found at the cell surface.

Evidence for the existence of amino-acid groups at the surface of bacterial cells has been presented by Cohen (1945). Chelation of such groups with cobalt may represent one mode of attachment of the metal to the cell. Albert (1950) measured the affinity of a number of divalent amino-acids for heavy metal ions, including cobalt, and found a degree of stability for all amino-acids: $\text{Cu}^{++} > \text{Ni}^{++} > \text{Zn}^{++} > \text{Co}^{++} > \text{Cd}^{++} > \text{Fe}^{++} > \text{Mn}^{++} > \text{Mg}^{++}$. The ability of cobalt to form complexes with histidine at physiological pH values has been shown by Hearon, Burk & Schade (1949). The first reaction product formed, cobalt dihistidine, as well as the oxygenated product oxybiscobaltodihistidine, are completely reversible with HCl, but in time, in the presence of oxygen, the latter product is converted to an irreversible complex. It is conceivable that this type of bond may in part account for the small portion of cobalt remaining in the cells of *P. vulgaris* after treatment with HCl. Further, cobalt may be combined irreversibly with cysteine (Michaelis & Barron, 1929).

Reversible uptake of cations from solution by bacteria has been investigated by a number of workers. McCalla (1940) studied the uptake of H^+ , methylene blue and Mg^{++} by a number of soil bacteria and found, for example, that Mg^{++} taken up by *Bact. coli* could be removed to a significant degree by the

subsequent addition of Co^{++} , Ba^{++} , or Mn^{++} . Valko & Dubois (1944) obtained a reversal of the bactericidal action of surface-active cations by detoxification with surface-active anions or by non-toxic surface-active cations. In these instances the uptake of cations was explained as the result of adsorptive forces exhibited by the negatively charged cells.

The ability of magnesium to alter the amount of cobalt taken up by the cells of *P. vulgaris* and to affect the concentration of cobalt required for growth inhibition may be an expression of competition of the two ions for an essential metabolic group on the cell. Abelson & Aldous (1950) reported a similar action of magnesium for nickel as well as cobalt with *Bact. coli* and found that Mg^{++} exerted its effect at the time of metal uptake. The inability of magnesium to replace cobalt already bound by the cell may be due to a difference in the stability of the metal complexes formed with the cell substance.

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