# ACCUMULATION OF RADIOACTIVE COBALT BY DIVIDING YEAST CELLS

by

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## INTRODUCTION

Until recently the literature on the metabolism of cobalt by microorganisms was limited to a few studies wherein it had been included in investigations of the toxicity of a series of cations[1, 2]. While the wide-spread distribution of cobalt in soils, and its general distribution in the tissues of higher plants[3, 4], and in animal tissues[5] are well known, there seems to be no report of the natural occurrence of cobalt in the cells of microorganisms. Spectroscopic examination of the ash of yeast cells failed to reveal the presence of cobalt[6]. In contrast to the findings with some other metals (*i.e.*, Zn, Cu, Fe, Mn, Mo, Ga, and Tl), trace concentrations of which are known to stimulate the growth of different microorganisms, there seems to be no report for any such action on the part of ionic cobalt. Beginning with very dilute concentrations, cobalt is reported to have the effect of progressively decreasing the amount of growth of different microorganisms when added to culture media in increasing concentration[1, 7]. With some animals, on the contrary, cobalt is known to be an essential trace element (for review, see [8]); a deficiency of cobalt in pasturage has been shown to be responsible for the appearance of characteristic symptoms in ruminants feeding thereon, the condition having been recognized in several localized areas throughout the world.

Within the past year work has appeared from different laboratories indicating that cobalt is probably of especial importance in the metabolism of several groups of microorganisms. ALBERT *et al.*[9] have shown that cobalt is specifically active in reversing the inhibitory action of oxine (8-hydroxy quinoline) on Gram positive bacteria, (while zinc and iron have such an action with Gram negative bacteria). In contrast, PRATT AND DUFRENOY[10] have observed that cobalt specifically potentiates the *in vitro* and *in vivo* action of penicillin. NICKERSON AND VAN RIJ[11, 12] have found that cobalt can selectively inhibit cell division in various yeasts without simultaneously inhibiting growth (defined as an irreversible increase in volume) and other metabolic processes. Thus, they have obtained the growth of yeasts as elongated mycelial elements. The selective action of

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cobalt against the enzymatic mechanism of cell division[11] was observed to be specifically antagonized by sulphydryl group compounds.

In the present study we have examined quantitatively the relationship between cobalt concentration and the growth of yeasts (measured as weight increase) in liquid media. Employing radioactive cobalt, the extent to which yeast cells accumulate this ion has been determined. A preliminary fractionation has been made to learn the manner in which cobalt is bound in the yeast cell. A possible mechanism by which yeast cells may concentrate cobalt from dilute solutions is briefly considered.

## MATERIALS AND METHODS

Pure cultures of two different yeasts were employed; *Saccharomyces cerevisiae* (Carlsberg No. 237), and a pathogenic non-sporulating yeast, *Candida albicans*. Stock cultures were maintained in 8% Pilsner wort in Freudenreich flasks at 25°. For experimental purposes the yeasts were grown in a medium (GGY) consisting of: 20.0 g glucose, 10.0 g glycine, 1.0 g yeast extract (Difco), and one liter glass distilled water. To this medium appropriate dilutions of a solution of cobaltous nitrate (Merck, Darmstadt, $Co(NO_3)_2.6H_2O$) were added to give final concentrations of $Co^{++}$ ranging from M/1000 to M/64000. The medium was dispensed (50 ml per 100 ml Erlenmeyer flask), autoclaved (120° for 20 min), cooled, and inoculated with 0.1 ml of a standard density suspension of washed cells obtained from a 24 hour growth. The flasks were then incubated, with or without continuous agitation, as indicated. Growth measurements were made by removing 10 ml aliquots, in duplicate, from duplicate flasks, filtering through tared Schott G–4 fritted glass filters, washing with distilled water, drying at 105° for 16 hours, and weighing. Duplicate determinations by this method agreed to within $\pm$ 0.2 mg.

Radioactive cobalt (as cobaltous nitrate) was incorporated into 500 ml quantities of the GGY medium contained in one liter Florence flasks, inoculated, and incubated with continuous agitation for 17 hours at 20°. Samples of the yeast crop (obtained from the centrifuged, triply-washed, yeast cream), as well as the samples obtained by subsequent fractionation procedures, were placed on aluminum dishes adapted for GEIGER-MÜLLER counting, and measured automatically[13]. The radioactive cobalt was obtained as a purified solution of cobalt nitrate; it comprised a mixture of isotopes of cobalt with an average half-life of about 78 days.

## EXPERIMENTAL

### *Cobalt Concentration* vs *Yeast Growth*

The inhibitory effect of cobalt on the growth of yeast in a natural medium was apparently first studied by BOKORNY[1]. He observed no visible growth of yeast with concentrations of $Co(NO_3)_2.6H_2O$ greater than 0.02% ($7 \cdot 10^{-4}$ M). This concentration limit agrees well with our own findings. Results on the growth of *S. cerevisiae* in two series of experiments with different cobalt concentrations are given in Table I. Culture medium with a cobalt ion concentration greater than $10^{-4}$ M permitted little, or no, growth of the yeast. A rather sharp break in the curve of growth *vs* cobalt concentration in the region of $10^{-4}$ M $Co^{++}$ was apparent. No concentration of cobalt tested was found to increase the growth of *S. cerevisiae*, thus agreeing with the report of MOKRAGNATZ[7], made on *Aspergillus niger*.

BURK *et al.*[14, 15] observed an inhibitory effect of cobalt on the growth and respiration of various microorganisms; this inhibition could be reversibly overcome by the addition of histidine, which was shown[16] to form a chelate complex with cobalt. The cobalt inhibition of yeast growth has been found to be antagonized by oxine[12]; oxine itself was without appreciable effect on the strains of yeasts employed. Another type of cobalt complex (ammine complexes) was shown by BOOIJ[2] to be without inhibitory effect on yeast fermentation, even at a concentration of 0.1 M, whereas $Co^{++}$ caused a very marked

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TABLE I

EFFECT OF COBALT CONCENTRATION ON THE GROWTH OF *Saccharomyces cerevisiae*
DATA PRESENTED AS mg DRY WEIGHT OF YEAST PER 50 ml CULTURE; AVERAGE OF DUPLICATES

| Series | Culture Conditions | Cobalt Concentration | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M/1000 | M/2000 | M/4000 | M/8000 | M/16000 | M/32000 | M/40000 | M/64000 | Zero |
| A | Constant agitation at 20° for 48 hours | < 0.1 | 0.1 | 0.3 | — | - - | — | 26.8 | — | 64.8 |
| B | Constant agitation at 20° for 24 hours | — | — | 1.4 | 4.2 | 10.7 | 17.8 | — | 39.8 | 53.0 |
| C | Stationary culture at 25° for 24 hours | — | — | 1.3 | 3.2 | 16.0 | 40.6 | — | 42.7 | 50.3 |

inhibition of fermentation at 0.01 M. The experiments of BOOIJ were conducted with non-dividing cells, and the concentration of $Co^{++}$ necessary to achieve inhibitory effects was about $10^2$ greater than we have found necessary to cause almost complete inhibition of growth in liquid media. Likewise, the effect of cobalt on yeast cells growing in liquid media is somewhat different from its effect on cells growing on the surface of agar media. Concentrations of $10^{-3}$ M and $5 \cdot 10^{-2}$ M $Co^{++}$ cause no appreciable inhibition of the amount of yeast growth on agar media, but do result in the inhibition of cell division and the consequent growth of the yeast in a filamentous manner. The effect of cobalt on the morphology of yeast grown in liquid media is not so pronounced. The differing sensitivity of yeasts to cobalt when grown in the two types of media may result in part from differences in oxygen tension; reaction of the cobalt with some component of the agar also appears to be a possibility.

*Accumulation of Radioactive Cobalt by Growing Yeasts*

Radioactive cobalt incorporated into the GGY medium was found to be taken up by yeast cells during growth and to become more concentrated in the cells than in their growth medium. The accumulation of cobalt by the cells represented a many-fold concentration of the ion intracellularly compared with the concentration initially present in the medium. Data in this respect, for *S. cerevisiae* and for *C. albicans*, are shown in Table II. The strain of *S. cerevisiae* employed accumulated a cobalt concentration approximately 670-fold that present in the medium, while the cells of *C. albicans* achieved a 25-fold concentration (the *C. albicans* culture was not aerated, however).

The cobalt taken up by the cells was not loosely adsorbed to the cell surface since there was no detectable loss of activity by the cells after shaking them for 20 hours in sterile distilled water at 20° (Table III). If physical adsorption at the cell surface is involved in cobalt retention by these yeasts, the binding forces must not be overcome by the washing procedure employed. It is also evident that the cobalt accumulated by the cells is not free to diffuse out of the cells.

Water-washed yeast was subjected to continuous extraction with ether-acetone (1:1) in a Soxhlet apparatus for 24 hours. Activity measurements were made on the acetone-ether extract (directly, and after concentration), and on the residual yeast mass. The yeast before extraction contained 79.3% water; the extraction removed

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TABLE II

ACCUMULATION OF LABELLED COBALT BY YEASTS DURING GROWTH

| Culture Conditions | Organism and Fraction | Counts per minute | Counts/min/g wet wt | Activity in cells Activity in medium |
|---|---|---|---|---|
| Constant agitation at 20° C for 17 h in 600 ml of medium; 21.0 μg/L initial cobalt conc., 106 mg dry wt yeast measured for activity | *S. cerevisiae* yeast cells | 4360. | 8700. | — |
| | medium-fresh | 65/ml | 65. | 134 |
| | medium-spent | 44.4/ml | 44.4 | 196 |
| Constant agitation at 20° C for 66 h in 1000 ml of medium; 62.5 μg/L initial cobalt conc., 60 mg dry wt yeast measured for activity | *S. cerevisiae* yeast cells | 6243. | 21390. | — |
| | medium-fresh | 190.6/ml | 190.6 | 112 |
| | medium-spent | 31.9/ml | 31.9 | 670 |
| Stationary culture at 25° C for 168 h in 400 ml of medium; 21.0 μg/L initial cobalt conc., 278 mg dry wt yeast measured for activity | *C. albicans* yeast cells | 2083. | 1500. | — |
| | medium-fresh | 70/ml | 70. | 21 |
| | medium-spent | 60/ml | 60. | 25 |

TABLE III

RETENTION OF LABELLED COBALT BY CELLS OF *C. albicans* SUBJECTED TO WASHING WITH DISTILLED WATER FOR 20 HOURS AT 20°

Cells employed were part of the crop grown for Table II.

| Fraction | Dry wt of samples (mg) | Counts/min | Counts/min/g wet wt |
|---|---|---|---|
| Yeast cells (after washing) | 203 | 1660 | 1600 |
| Wash water (60 ml) | — | $<1.5$ | $<1.5$ |

TABLE IV

EXTRACTION OF LABELLED COBALT FROM CELLS OF *S. cerevisiae*

One liter culture incubated 66 hours at 20° with continuous agitation; 20.7 micro-Curies (62.5 micrograms cobalt) added per liter. See Table II for details on total crop.

| Fraction | % Standard | % Standard/gm yeast wet wt |
|---|---|---|
| Whole yeast | 308/60 mg dry wt | 1030 |
| Ether extract | 0.05/ml | zero |
| Yeast (ether extracted) | 531/143 mg dry wt | 745 |
| Trichloracetic acid extract (filtrate after neutralization) | 26/ml | 200 |
| Trichloracetic acid extract (precipitate after neutralization) | 250/19 mg dry wt | 700 |
| Yeast (trichloracetic acid extracted) | 13.2/78 mg dry wt | 35 |
| Wash water used on acid extracted yeast | 2.53/ml | 100 |

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81.4% of the fresh weight, and 10.0% of the dry weight of the yeast. Results of the activity measurements are shown in Table IV; it is clear the cobalt was not removed from the yeasts by the acetone-ether extraction.

The residual yeast mass from the above extractions was subsequently extracted with cold, 10% trichloracetic acid; insoluble material was removed by centrifugation and washed with water. A portion of the acid extract was neutralized with NaOH, and allowed to stand. A flocculant, brownish precipitate settled from the neutralized extract; it was removed by filtration, and both filtrate and precipitate were measured for activity. The residue insoluble in trichloracetic acid, and the water used in washing it by suspension and centrifugation were also measured for activity; results shown in Table IV. It is clear that the trichloracetic acid extraction removed the greater part of the cobalt activity from the yeast, and that most of the activity in the extract was precipitated on neutralization.

Since inorganic cobalt ($Co^{++}$), if present in the acid extract, would be precipitated on neutralization by NaOH, we carried out a neutralization in another experiment with $NH_4OH$, which leads to the formation of soluble ammine-complexes and provides a partial means for distinguishing between the possibilities of an organic or an inorganic binding of the cobalt in the acid extract. In this experiment inactive carrier cobalt was added to a trichloracetic acid extract which was then divided into two equal volumes. One volume was neutralized with NaOH, and the other with $NH_4OH$. A slight, brownish, flocculent precipitate still developed in the extract neutralized with $NH_4OH$; the precipitate with NaOH was in this case heavier and bluish-green, due to the presence of the added carrier cobalt. Activity measurements on the two precipitates (total amount in each case) gave nearly identical values: 55% standard with $NH_4OH$, and 43% standard with NaOH neutralization. Unfortunately, because of lack of sufficient radioactive cobalt, we have been unable to repeat this experiment. We can only conclude that it *indicates* that the cobalt activity in the trichloracetic acid extract was not in ionic form, but probably in some organic combination in which the cobalt is so held that it did not exchange with added inorganic cobalt ions.

## DISCUSSION

The avidity of growing yeast cells for the cobaltous ion, as shown in our experiments, is in marked contrast to the fate of cobalt injected into animals; yet cobalt is toxic for yeasts, and an essential trace element rôle in animals, at least in ruminants. COPP AND GREENBERG[17] found that 90% of the radioactive cobalt injected intraperitoneally into rats was excreted within four days. HEVESY[18] found no uptake of radioactive cobalt by red blood corpuscles. Oral administration of labelled cobalt to rats resulted in 80% elimination in the feces, 10% rapididly eliminated in the urine, and very little retention by tissues; only the liver consistently accumulated significant amounts[19]. Injection of cobalt has been found not to cure "coast" disease in sheep; cure is accomplished only on feeding cobalt[20]; the suggestion has been made that ingested cobalt may act upon microorganisms of the rumen and, not directly, upon the host. Certainly, our own results show that furnishing cobalt to two different microorganisms results in their accumulating and combining it.

The occurrence of cobalt in an organic compound of biological origin was reported[21,22] simultaneously with our first notice[11] of the possible organic combination of the labelled

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cobalt extracted from yeast cells. The demonstration[21, 22] of the presence of cobalt in the anti-pernicious anemia factor raises still more interest in the problem of cobalt metabolism by animals, plants, and microorganisms. The existence of a requirement for an organic cobalt compound (vitamin $B_{12}$, the anti-pernicious anemia factor) by a microorganism, *Lactobacillus lactis*[23, 24], which cannot be satisfied by supplying the cobalt ion[22], is now known. Demonstration of whether this is a true requirement for organically bound cobalt, or merely for the organic part of the molecule is yet to be made. The complete absence of requirement for a cobalt compound or ionic cobalt in the case of a yeast, *S. cerevisiae*, is indicated by the report[6] of the absence of cobalt in yeast ash examined spectroscopically; the absence of cobalt in the ash of the plant extract medium (malt extract) used to support the growth of the yeasts in these experiments[6] should also be noted.

The mechanisms by which microorganisms (including plankton in the sea) are able to accumulate significant concentrations of various cations from extremely dilute solutions are of considerable interest*. An attractive suggestion has recently been made by HUTNER[25] that the cell surface of a microorganisms may include among its architectural features the presence of a variety of compounds capable of acting as metal receptors by reactions of a chelating nature. The effectiveness of many substances, acting by chelation, for quantitative reaction with cations is well known. We have considered the possibility that accumulation of cobalt by yeast cells may be the result of its combination with substances at the cell surface. One substance in yeast cells that is known to form complexes with metals is metaphosphate. A peripheral distribution for this substance in yeast cells is a likely possibility, judging from staining reactions[26], and from the localization of alkaline hexametaphosphatase in yeasts[27]. Cobalt is not precipitated under alkaline conditions when hexametaphosphate is present, indicating suppression of $Co^{++}$ by metaphosphate**. Complex formation by metaphosphate with $Ca^{++}$, $Mg^{++}$, and $Fe^{+++}$ has been discussed by QUIMBY[28]. It is suggested that peripherally located metaphosphate might be of significance in the accumulation of metals by yeasts from dilute solutions.

*Acknowledgments*

The authors are very grateful to Professor NIELS BOHR for the use of facilities at the Institute for Theoretical Physics, and to Professor Ø. WINGE for facilities at the Carlsberg Laboratory. They also wish to express their appreciation to Professor G. DE HEVESY for his advice and interest in the work.

## SUMMARY

Data are presented on the relationship between cobalt concentration in a nutrient medium and the growth of yeast cells. No evidence was found for growth stimulation at dilute cobalt concentrations. The toxicity of cobalt for yeast growth increases with increasing concentration, and becomes markedly toxic in the region of $10^{-4}$ M Co.

* With extremely dilute labelled copper ($10^{-10}$ M), MAZIA AND MULLINS[30] observed an almost quantitative removal of the $Cu^{++}$ by photosynthesizing leaves of *Elodea* (a concentration of $3 \cdot 10^{8}$ of $Cu^{++}$ inside over outside was obtained).

** Systems of $FeCl_3$–$Co(NO_3)_2$-Na hexametaphosphate and $FeCl_3$–$Co(NO_3)_2$-oxine have been examined[29]; it has been found that cobalt weakens the iron-metaphosphate and iron-chelate complexes with the reappearance of $Fe^{+++}$. The possibility of an action of this type being a basis for iron "mobilization" (*e.g.*, action of one trace metal in promoting the formation of an organic complex of another trace metal, *ex.*, action of Cobalt on hemoglobin formation) has been considered.

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Uptake of labelled cobalt by growing yeasts in the accumulation of cobalt by the cells to a level over 600 times greater than the concentration initially present in the medium. The cobalt accumulated by the cells was not free to diffuse out of the cells when subjected to prolonged washing in distilled water. Acetone-ether extraction of water washed cells removed over 80% of the fresh weight, and 10% of the dry weight of the cells but removed no labelled cobalt. The cobalt was largely extracted from the cells by cold 10% trichloracetic acid, from which it precipitated as organically combined cobalt on neutralization of the acid extract.

The ability of yeast cells to accumulate cobalt from dilute solutions during growth is suggested to result from the presence of ion-suppressing, complex-forming substances (such as metaphosphate) at the cell surface which can unite with cobalt to effect a nearly quantitative removal of the ion from a dilute solution.

Some aspects of cobalt metabolism are briefly discussed.

## RÉSUMÉ

Les résultats obtenus concernent les relations existant entre la concentration en cobalt dans un milieu de culture et la croissance des cellules de levure. Il n'apparait aucune stimulation de la croissance aux concentrations diluées en cobalt. La toxicité du cobalt pour la croissance des levures s'accroît avec la concentration et devient très nette pour une concentration de l'ordre de $10^{-4}$ M.

Les levures en voie de croissance accumulent du cobalt marqué jusqu'à une concentration plus de 600 fois supérieure à la concentration initiale du milieu. Le cobalt ainsi concentré dans les cellules ne peut plus diffuser à l'extérieur lorsqu'on lave les cellules d'une façon prolongée par l'eau distillée. Le traitement des cellules par l'acétone-éther élimine plus de 80% du poids frais des cellules déjà lavées par l'eau, et 10% du poids sec, mais n'enlève pas de cobalt marqué. Au contraire le cobalt est extrait pour la plus grande part par l'acide trichloracétique à 10% dont on peut le précipiter sous forme organique par neutralisation de l'extrait acide.

L'aptitude des cellules de levures à accumuler le cobalt à partir de solutions diluées au cours de la croissance semble due à la présence de substances capables de former des complexes (telles que le métaphosphate) à la surface de la cellule de telle sorte que la fixation du cobalt soit pratiquement quantitative. Quelques aspects du métabolisme du cobalt sont brièvement discutés.

## ZUSAMMENFASSUNG

Die beschriebenen Versuchsergebnisse betreffen das Verhältnis zwischen der Kobaltkonzentration im Nährboden und dem Wachstum der Hefezellen. Bei geringen Kobaltkonzentrationen konnte keine Wachstumsförderung festgestellt werden. Die Giftigkeit von Kobalt für Hefezellen nimmt mit der Konzentration zu und wird bei einer Konzentration von $10^{-4}$ M Co sehr deutlich.

Wachsende Hefe sammelt markiertes Kobalt bis zu einer Konzentration die 600 mal grösser ist, als die Anfangskonzentration im Nährboden, in den Zellen an. Das so angesammelte Kobalt kann auch bei langem Waschen mit destilliertem Wasser nicht mehr aus den Zellen herausdiffundieren. Werden die Zellen nach dem Waschen mit Wasser mit Aceton-Äther extrahiert, so werden über 80% des Frischgewichtes und 10% des Trockengewichtes aber kein markiertes Kobalt entfernt. Dagegen wurde das Kobalt durch kalte 10%-ige Trichloressigsäure weitgehend extrahiert. Aus dieser Lösung kann es als organisches Kobalt durch Neutralisation des sauren Extraktes gefällt werden.

Die Fähigkeit der Hefezellen Kobalt aus verdünnten Lösungen während des Wachstums anzusammeln, scheint von komplexbildenden Substanzen (wie Metaphosphat) herzurühren die sich an der Oberfläche der Zelle befinden und sich mit dem Kobalt verbinden so dass dieses Ion nahezu quantitativ aus verdünnten Lösungen entfernt wird.

Einige Aspekte des Kobaltmetabolismus werden kurz auseinandergesetzt.

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Received December 28th, 1948.