Original article

Cobalt binding by *Propionibacterium arabinosum*

Z Schneider [1], K Trojanowska [2], B Jaszewski [2], T Nowak [3]

[1] Department of Biochemistry and Biotechnology;
[2] Department of Food Microbiology, University of Agriculture, 60-637 Poznan, Poland;
[3] Department of Chemistry and Biochemistry, University of Notre Dame,
Notre Dame, 46556 Indiana, USA

Summary — Cobalt occurs in nature usually at very low concentrations and is accompanied by much higher concentrations of various metals. In order to acquire cobalt under such unfavorable conditions, living systems evolved effective uptake mechanisms based on high affinity to cobalt and distinguishing this element from among the others. Taking *Propionibacterium arabinosum* as the bacterial model, which is known for its cobalt requirement, the performance of microorganisms has been examined in this regard. By using radiolabeled cobalt and a filter technique, the uptake capability of these bacteria at the stage of cobalt binding has been characterized in terms of binding capacity, and dissociation and equilibrium constants. Monovalent and divalent ions inhibit cobalt binding to bacteria. Divalent metal ions showed a competitive mode of inhibition, and the inhibitory constants for some of the toxic and biologically important divalent metal ions were determined. The chelating agent EDTA diminishes cobalt binding to bacteria already at concentration 10^{-5} μmol/l. Bacteria exposed to thermal shock at 60°C increased several fold cobalt-binding capacity.

cobalt uptake / B_{12} biosynthesis / propionibacteria / cobalt-binding assay

Résumé — Liaison du cobalt par *Propionibacterium arabinosum*. *Le cobalt se trouve dans la nature habituellement à de très faibles concentrations, et ce parmi des concentrations beaucoup plus élevées d'autres métaux. Pour acquérir le cobalt dans des conditions aussi défavorables, les êtres vivants développent des mécanismes d'acquisition efficaces basés sur une affinité élevée pour le cobalt et distinguant cet élément parmi les autres. En prenant* Propionibacterium arabinosum *comme modèle bactérien, la performance des microorganismes dans ce domaine a été examinée. À l'aide de cobalt marqué radioactivement et d'une technique de filtration, la capacité d'acquisition du cobalt par liaison de ces bactéries a été caractérisée en termes de capacité de liaison et de constantes de dissociation et d'équilibre. Les ions monovalents et divalents inhibent la liaison du cobalt à la bactérie. Les ions métalliques divalents montraient un mode d'inhibition compétitif, et les constantes d'inhibition pour certains des ions métalliques divalents importants sur le plan toxique et biologique ont été déterminées. L'agent chélateur EDTA diminue la liaison du cobalt à la bactérie dès la concentration molaire de 10^{-5}. La bactérie exposée à un choc thermique de 60°C augmentait de plusieurs fois sa capacité de liaison du cobalt.*

acquisition du cobalt / biosynthèse de vitamine B_{12} / bactérie propionique / liaison du cobalt

INTRODUCTION

Cobalt is one of the bioelements essential for the growth of microorganisms and algae (Swift, 1980). It functions mainly in the form of corrinoids, a group of cobalt-containing compounds to which cobalamin, known in medicine as vitamin B_{12}, also belongs. Vitamin B_{12} is also indispensable for humans and animals (Schneider and Stroinski, 1987). The requirement of this vitamin by higher plant forms is less certain; however, inorganic cobalt seems to be the cause of a number of biological responses (Lau and Yang, 1976; Foy *et al*, 1978; Herich and Bobak, 1987; Lipskya, 1988). It has been shown that plants readily take up cobalt and bind it within themselves to a low molecular carrier (Schneider *et al*, 1994a). The requirement of vitamin B_{12} by humans is well established; however, among animals, a particular requirement for nutritional cobalt was demonstrated in ruminants, which metabolize in the liver appreciable amounts of propionic acid in a B_{12}-dependent pathway (Schneider and Stroinski, 1987). A sheep requires up to 0.5 mg Co/d to maintain optimal fermentation in the rumen, in particular for microbial synthesis of corrinoids also acquired from this source by the animal (Marston and Allen, 1972). The supply of cobalt to ruminants on earth at such a rate would use up its whole resources within decades. Thus, it seems that most animals are undernourished with cobalt.

Fortunately, humans require minute amounts of cobalt in the form of vitamin B_{12} (5 µg B_{12}/d = 0.22 µg Co/d). Milk, meat and, in particular, liver are the principal sources of this vitamin for humans. Because of its very low concentration at which it is available in food, the uptake from the digestive system in humans and the transport into the cells is one of the most effective and elaborated processes developed by nature (Lindemans and Kapel, 1979; Schneider, 1987a). The route of cobalt from soil to human cells is long and may proceed in the following main steps:

soil (Co) —> roots of plants (Co) —> green parts of plants (Co) —> ruminant microorganisms (B_{12}) —> animal body (B_{12}) —> human gut (B_{12}) —> blood (B_{12}) —> cells (B_{12})

The uptake is characterized by extremely strong and specific binding of the vitamin by B_{12} carriers at all steps.

The objective of the present study was to determine the firmness and specificity of cobalt binding to the surface of microorganisms and also to evaluate the extent of interference of some heavy metals in this process. Cobalt binding is considered to be the first, still reversible step of its uptake. The application of labeled cobalt in the present study allowed for a more precise approach to cobalt acquirement by bacteria. The rapidity and high sensitivity of the radiolabeled cobalt assay combined with a filter technique elaborated in this laboratory enabled evaluations of the dynamics of cobalt uptake at such low concentrations of this element as it occurs in nature. The bacterial uptake system, framed for very low concentrations of cobalt, must be characterized by sensitive analytical methods as well.

A strain of *Propionibacterium arabinosum* from our collection has been found to be a convenient model in this study. Propionibacteria species known for cobalt requirement have been used extensively in this laboratory since the early 1960s for the investigation of B_{12} biosynthesis and for the production of this vitamin on an industrial scale (Pedziwilk, 1962; Pedziwilk *et al*, 1979; Schneider and Stroinski, 1987).

MATERIALS AND METHODS

Source of labelled cobalt and other chemicals

[^{60}Co]CoCl$_2$ in 0.1 mol/l HCl of specific activity of 1.85 GBq/mg Co and [^{57}Co]CoCl$_2$ in 0.1 mol/l

HCl carrier free sp activity 150 MBq/µg were from Amersham, England. If necessary, the cobalt was diluted with the nonradioactive CoCl$_2$ as follows: CoCl$_2$ salt of analytical grade was dissolved at 60°C in 0.1 mol/l HCl to obtain a concentration of 1 mmol/l and kept for 1 d at room temperature. Next, the solution was mixed with the labelled cobalt in an adequate proportion to obtain the desired specific radioactivity. The mixture was placed gently onto the bottom of a Teflon dish within a desiccator and dried under moderate vacuum in the presence of P$_2$O$_5$ and solid KOH. The dry residue was dissolved in 0.01 mol/l HCl and kept sealed in a quartz container at room temperature at all times. The purpose for such treatment is to obtain a possible uniform arrangement of ligands coordinating around cobalt ions in both labelled and nonlabelled cobalt. Other chemicals were of analytical grade.

Source of bacteria

Propionibacteria are particularly suitable for studies of cobalt uptake because of their documented demand for this element, their facility to grow and maintain and their resistance to infection and to sudden changes of osmotic pressure. Despite their relatively small size, the bacteria are easy to sedimentate by means of centrifugation and to resuspend. For this study, a strain of P arabinosum was chosen from our own collection and grown as described by Pedziwilk (1962) except that no cobalt was added to the growth medium. Cultures 12 to 16 d old suspended in 0.05 mol/l potassium acetate, pH 6.0, were applied for binding experiments. Previously, the bacteria were washed 4 times with the buffer by means of repeated centrifugation followed by suspension. Under conditions using 1 mg of wet bacteria suspended in 1 ml of potassium acetate gave an OD value of 0.64 at 600 nm with 10 mm cuvette.

Cobalt-binding assay

Unless specified, the standard incubation mixture contained 10–100 nmol/l of [60Co]CoCl$_2$ or its 57Co counterpart and freshly washed bacterial suspension, containing 0.5–2.0 mg of bacteria suspended in 1 ml of 0.05 molar potassium acetate at pH 6.0. The mixture was incubated at room temperature (22°C) and stirred gently every

one min. After the given time, 100 µl aliquots of the suspension were applied onto wet diameter 24 mm filter disk BA 85 pore size 0.45 µm (Schleicher and Schüll) placed on a filter holder. Moderate vacuum (water pump suction) was applied. The bacteria on the filter were washed within 3 min with 3 x 1 ml sodium acetate at pH 6.0. The air-dried disks were placed on the bottom of a plastic tube, diameter 15 mm, and counted in a semiautomatic gamma counter (Polon, Poland). The efficiency of counting was 55% for [60Co] and approximately 7% for [57Co]. The binding was expressed in counts or picomoles of Co per mg of wet bacteria. The values shown in figures represent the mean of 3 assays.

RESULTS

Binding and release of cobalt

Pilot experiments revealed that propionibacteria take up efficiently cobaltous ions even if at very low concentrations. Figure 1 shows the progress of cobalt binding and uptake by P arabinosum at a concentration of 0.2 µmol/l.

Two stages of binding were observed: fast binding within 5–8 min, followed by a much slower progress after this time. The fast binding appears to be associated with the adsorption of cobalt to the cell surface and was reversible, since the greater part of cobalt could be desorbed by washing the bacteria on a filter with 1 mmolal EDTA solution. However, an appreciable percentage of cobalt is not removable by this means from those bacteria which were exposed to cobalt ions for longer periods of time (fig 1).

Treatment of the bacterial suspension with 0.1 molar HCl brought about the release of the residual radioactivity from the cell. As will be described elsewhere, the greater part of radioactive cobalt resistant to EDTA wash turned out to be in the form of corrinoids. This is an unequivocal indication that this fraction of cobalt enters the cell interior and
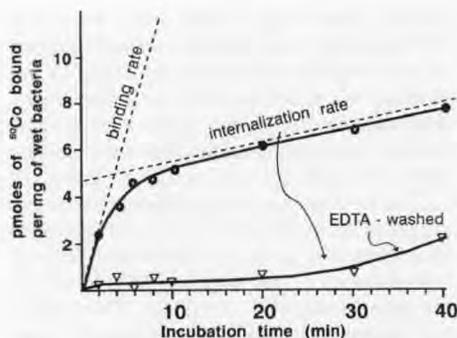
Fig 1. Time-course of Co^{2+} binding by *P arabinosum*. The incubation mixture containing 0.84 mg/ml of bacterial suspension and 200 nmol/l [^{60}Co] $CoCl_2$ in 50 mmolar acetate buffer at pH 6.0 was incubated at 22°C. At time intervals, specified 100 µl aliquots were taken and the binding of cobalt was determined as described in the Materials and Methods section. Simultaneously, another set of 100 µl aliquots of the incubation mixture was washed 4 x 2 ml with 5 mmolar EDTA-Na at pH 6 while still on filter. The filters were next dried and counted as described in the Materials and Methods section.
Liaison du cobalt aux cellules de P arabinosum en fonction du temps. Le mélange d'incubation contenant 0,84 mg/ml de suspension bactérienne et 200 nmol/l de [^{60}Co] $CoCl_2$ dans 50 mmol/l de tampon acétate à pH 6,0 était incubé à 22°C. Des prélèvements de 100 ml étaient effectués périodiquement et la liaison du cobalt était déterminée comme décrit dans «Matériel et méthodes». Simultanément, une autre série de prélèvements de 100 ml du mélange d'incubation étaient déposés sur filtre et lavés avec 4 x 2 ml d'EDTA-Na 5 mmol/l à pH 6. Les filtres étaient ensuite séchés et comptés comme décrit dans «Matériel et méthodes».

is used by the cell for B_{12} synthesis. Propionibacteria readily release corrinoids from the cells at pH < 5 (Schneider, 1987b).

Assessment of cobalt-binding capacity of P arabinosum

Cobalt-binding capacity is one of the essential parameters affecting effective uptake of this element. By using the filter technique (see Materials and Methods section), it has been found that, under saturating conditions, 1 mg of centrifuged bacteria (*ca* 10 x 10^{10} cells) bind on the cell surface, depending on the batch (10–26 pmol of Co).

It was also of interest to learn what binding capacity would be shown by bacteria at cobalt concentrations likely to occur in nature and also being applied in bacterial artificial nutrients. The results obtained with cobalt concentrations from 9 to 100 nmol/l are summarized in table I. The data show that bacteria may concentrate cobalt at their surface several hundred fold. Lower concentrations of the cobaltous ions in the medium result in the occupation of only a fraction of the binding capacity. The concentration factor increases, however, with decreasing concentrations of cobalt.

Determination of the $K_{0.5}$ value

Michaelis-Menten kinetics have been conveniently adapted to assess the $K_{0.5}$ value for cobalt binding to bacteria. There are similar parameters to be determined in the case of cobalt binding to bacteria: *i)* the concentration of cobalt needed for half saturation of binding sites $K_{0.5}$ corresponds to K_m and *ii)* the V_{max} to cobalt-binding capacity. Similar approaches were already exploited in studies of vitamin B_{12} uptake by an alga (Bradbeer, 1971; Sarhan *et al*, 1980), for uptake of some metal ions by yeast cells (Fuhrman and Rothstein, 1963; Okorokov *et al*, 1977; White and Gadd, 1986), and sorption of heavy metals by soil fungi (Mullen *et al*, 1992). In the case of cobalt-binding studies by propionibacteria, care must be taken to measure the binding of cobalt after equilibrium is reached, which requires an incubation for at least 15 min (see fig 1). The incubation of a fixed amount of *P arabinosum* with varied concentrations of cobalt produced data which gave a straight line in the Lineweaver-

Table I. Cobalt-binding by *Propionibacterium arabinosum* cells.
Liaison du cobalt par les cellules de Propionibacterium arabinosum.

Concentration of Co^{2+} in solution (nmol/l)	Concentration of bacteria suspension (mg/ml)	Binding site saturation (%)	Concentration of Co within bacteria (μmol/l)	Concentration factor K_f
9	0.84	14	3.7	640
23	0.84	30	8.0	495
48	0.84	44	11.7	304
71	0.84	58	15.5	264
100	0.84	70	18.7	221

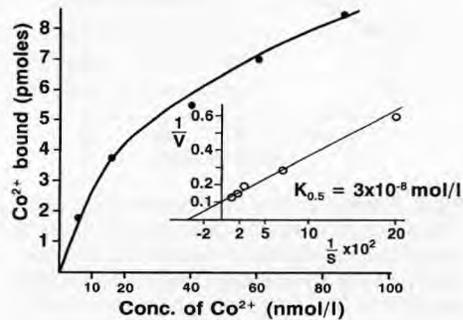The experimental conditions were as described in figure 2. The assessment of binding capacity of *P arabinosum* has been performed by the standard procedure at saturating concentrations of cobalt (250 nmol/l) and by applying a 20 min incubation time.
Les conditions expérimentales sont celles décrites sur la figure 2. L'évaluation de la capacité de liaison de P arabi-nosum *a été réalisée par la procédure standard à des concentrations saturantes de cobalt (250 nmol/l) et après un temps d'incubation de 20 min.*

Burk reciprocal plot, derived from the transformed Michaelis-Menten equation (fig 2). The concentration of cobalt necessary for half saturation of the binding capacity, $K_{0.5}$, was graphically evaluated from the intercept with the abscissa and found to be 3.3×10^{-8} mol/l (insert to fig 2). The maximal binding capacity of the bacteria read from the plot intercept with the ordinate corresponds to that given previously.

Fig 2. Michaelis-Menten plot of cobalt binding. Tubes containing 550 μl of the standard incubation mixture, 0.42 mg of suspended bacteria and [^{60}Co]CoCl$_2$ at concentrations as specified were incubated at 22°C for 15 min followed by standard assay of cobalt binding in 100 μl aliquots. *Représentation de Michaelis-Menten de la liaison du cobalt. Les tubes contenant 550 ml du mélange d'incubation standard, 0,42 mg de suspension bactérienne et les concentrations indiquées de [^{60}Co] CoCl$_2$ étaient incubées à 22°C pendant 15 min puis la liaison du cobalt était mesurée par essai standard dans des échantillons de 100 ml.*

Determination of the apparent equilibrium constant K_c

The interacting partners – cobaltous ions and the binding sites (BS) – are not equally dispersed in the medium. While cobalt is in true solution, the binding sites are condensed on the surface of the suspended bacteria. Thus, in the course of binding, cobalt also gets concentrated on the surface of bacteria. This requires additional expenditure of energy affecting, to some
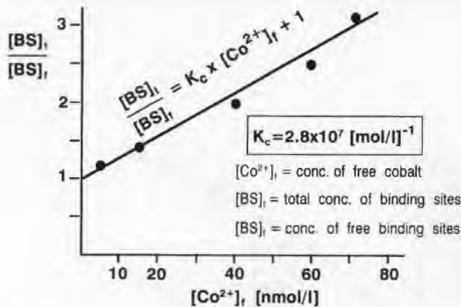
extent, the equilibrium. Under these circumstances, it was of interest to learn whether the equilibrium constant holds over the concentration range of cobalt used in these experiments and also occurring in nature. For this purpose, the equation:

$$K_c = [BS \bullet Co] \,/\, [BS]_f \times [Co]_f$$

was transformed into a function of the type: $y = ax + b$ (the transformed equation is given in the insert of fig 3). Despite the microheterogeneity of the solution, the data points in figure 3 are aligned, more or less within experimental error, along a straight line and the K_c could be determined from the slope of the obtained plot. The K_c value obtained

has rather practical significance, since it might need correction for the concentration of the binding sites on the cell surface.

Determination of the dissociation constant K_d

The experimental data produced for equilibrium constant determination were treated for the assessment of K_d from the Scatchard plot (fig 4). The K_d value obtained from the slope of the plot ($K_d = -1/\text{slope}$) shown was found to be: $K_d = 3.4 \times 10^{-8}$ mol/l.

Inhibition of cobalt binding by divalent metal ions

Metal ions inhibit the binding of cobalt ions to *P arabinosum* to various extents. The inhibition is of the competitive type as has been found for Ni^{2+} and Zn^{2+} by the classic approach using the Lineweaver-Burk plot of 1/V against 1/S (Dawes, 1969). It

$$\frac{[BS]_i}{[BS]_f} = K_c \times [Co^{2+}]_f + 1$$

$K_c = 2.8 \times 10^7 \, [\text{mol/l}]^{-1}$

$[Co^{2+}]_f = $ conc. of free cobalt
$[BS]_t = $ total conc. of binding sites
$[BS]_f = $ conc. of free binding sites

Fig 3. Determination of the equilibrium constant. The procedure was as described in figure 2 except that the incubation mixture of 2.7 ml contained 2.1 mg of bacteria and the incubation time was 20 min. The total binding capacity of bacterial suspension, $[BS]_t$, was determined from the Lineweaver-Burk plot (fig 2). The data obtained were corrected by subtracting the internalized fraction of cobalt measured by running EDTA-washed controls as described in figure 1.
Détermination de la constante d'équilibre. La procédure était celle décrite sur la figure 2 mais avec un mélange d'incubation de 2,7 ml contenant 2,1 mg de bactéries et un temps d'incubation de 20 min. La capacité totale de liaison de la suspension bactérienne [BS]_t était déterminée à partir de la représentation de Lineweaver-Burk (fig 2). Les données obtenues étaient corrigées en soustrayant la fraction de cobalt intracellulaire déterminée sur des témoins lavés à l'EDTA, comme décrit sur la figure 1.
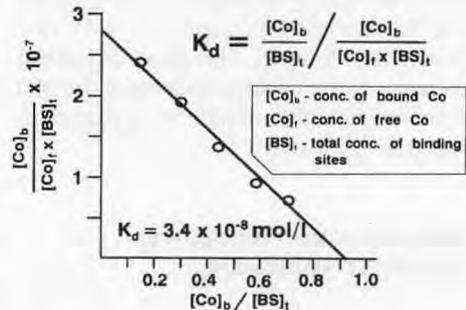
$$K_d = \frac{[Co]_b}{[BS]_t} \Big/ \frac{[Co]_b}{[Co]_f \times [BS]_t}$$

$[Co]_b$ - conc. of bound Co
$[Co]_f$ - conc. of free Co
$[BS]_t$ - total conc. of binding sites

$K_d = 3.4 \times 10^{-8}$ mol/l

Fig 4. Dissociation constant determination from the Scatchard plot. The data from the experiment described in figure 3 were treated as shown in this figure. The K_d value was calculated from the slope of the plot: $K_d = -1/\text{slope}$.
Détermination de la constante de dissociation à partir de la représentation de Scatchard. Les données de l'essai décrit sur la figure 3 sont traitées comme indiqué. La valeur K_d était calculée à partir de la pente de la courbe : $K_d = -1/\text{pente}$.

seems that the other ions listed in table II represent the same mode of inhibition. The Dixon plot (Dawes, 1969) was used to determine the K_i values given in table II. An example is shown in figure 5; the values were calculated from the equation shown in the insert of the figure 5.

As shown in table II, the Ni^{2+} and Hg^{2+} appear to be the strongest inhibitors. Surprisingly, there is a strong inhibitory effect of Mg^{2+} and Mn^{2+}, since these elements are present at appreciable concentrations in bacterial growth media used for industrial production of vitamin B_{12}. Conversely, the inhibition by Pb^{2+} has been found to be much weaker than expected.

Effect of buffers and monovalent ions

Preliminary experiments were done to study the influence of various buffers on cobalt

**Table II.** Inhibition of cobalt binding by divalent metal ions.
Inhibition de la liaison du cobalt par les ions métalliques divalents.

Metal	Inhibitory constant K_i ($\mu mol/l$)
Hg^{2+}	3.0
Ni^{2+}	6.4
Mn^{2+}	12
Mg^{2+}	12
Zn^{2+}	14
Cd^{2+}	150
Ca^{2+}	440
Pb^{2+}	600

The experimental conditions were as described in figure 5 except that the ions specified in the table were used in the form of nitrates (Hg^{2+} Pb^{2+} Ca^{2+} Ni^{2+}) or acetates (Cd^{2+} Mg^{2+} Mn^{2+} Zn^{2+}).
Les conditions expérimentales sont celles décrites sur la figure 5. Les ions spécifiés dans le tableau sont utilisés sous la forme de nitrate (Hg^{2+}, Pb^{2+}, Ca^{2+}, Ni^{2+}) ou acétate (Cd^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}).

binding to bacteria. Some buffers form coordinate bonds with cobalt and thus make it less accessible to binding sites on bacteria. Potassium acetate was chosen as having the least interference, despite its minimal capacity for buffering at pH 6.0. The concentration of the buffer could not be increased above 0.05 mol/l due to inhibition by the potassium present. The addition of

$$K_i = \frac{K_{0.5}}{slope} \times \frac{1}{K_{0.5} + S}$$

$$K_I = 14 \, \mu mol/l$$
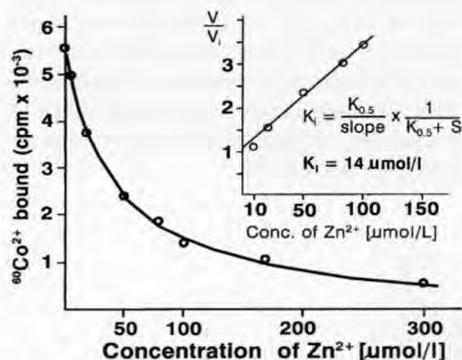
Fig 5. Inhibition of Co^{2+} binding to *P arabinosum* cells by Zn^{2+}. The assay of binding was performed as described in the Materials and Methods section. The incubation mixture contained 30 nmol/l [^{60}Co]$CoCl_2$, 0.92 mg/ml bacteria and $ZnCl_2$ concentration as specified. The mixture of Zn^{2+} and [^{60}Co]Co^{2+} was preincubated for 10 min followed by the addition of bacterial suspension and by incubation for 15 min at 22°C. The $K_{0.5}$ was determined as described in figure 2. The K_i values for other metal ions shown in table II were obtained by an identical procedure except that the particular metal ions were used instead of Zn^{2+}.
Inhibition par Zn^{2+} de la liaison de Co^{2+} aux cellules de P arabinosum. L'essai de liaison était effectué comme décrit dans «Matériel et méthodes». Le mélange d'incubation contenait 30 nmol/l de [^{60}Co] $CoCl_2$, 0,92 mg/ml de bactéries et les concentrations spécifiées de Zn^{2+}. Le mélange Zn^{2+} et de [^{60}Co] Co^{2+} était préincubé pendant 10 min puis la suspension bactérienne était ajoutée et le mélange incubé 15 min à 22°C. La valeur $K_{0.5}$ était déterminée comme décrit sur la figure 2. Les valeurs K_i pour les autres ions métalliques, indiquées dans le tableau II, étaient obtenues par une procédure identique, ces ions métalliques particuliers étant utilisés au lieu du Zn^{2+}.

KCl also greatly enhances the inhibitory effect of the medium. As shown in figure 6, KCl at a concentration of 0.1 mol/l depletes the Co^{2+} binding by 50%. In order to elucidate the mechanism behind this effect, it will be necessary to carry out additional extensive studies with other monovalent ions. The problem seems complex since it has been reported previously (Baes and Mesmer, 1976) as well as observed in our laboratory (Schneider *et al*, 1994) that cobalt ions at high salt concentration or at pH above 7.5 tend to associate with themselves into oligonuclear complexes. Such complexes of various sizes were separated into fractions in our laboratory by column filtration with Sephadex G-10.
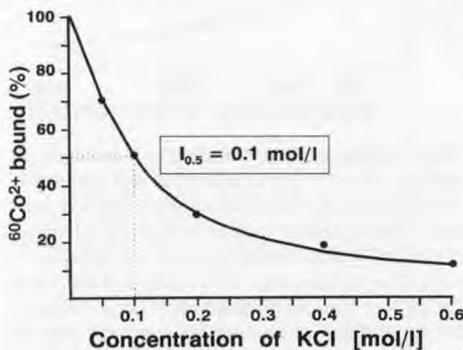

Fig 6. Effect of KCl on binding of Co^{2+} to *P arabinosum* cells. The binding assay was standard. The incubation mixture contained: $[^{60}Co]CoCl_2$ 30 nmol/l, 1.5 mg/ml bacteria and the specified KCl concentration.The incubation was run for 25 min. A pilot experiment was run at the $I_{0.5}$ KCl concentration in order to determine the incubation time needed to reach the equilibrium of binding (see fig 1).
Effet du KCl sur la liaison de Co^{2+} aux cellules de P arabinosum. L'essai de liaison était réalisé selon la procédure standard. Le mélange d'incubation contenait : $[^{60}Co]$ $CoCl_2$ 30 nmol/l, 1,5 mg/ml de bactéries et les concentrations spécifiées de KCl. Le temps d'incubation était de 25 min. Un essai pilote était effectué à la concentration de KCl $I_{0.5}$ de façon à déterminer le temps d'incubation nécessaire pour atteindre l'équilibre de liaison (voir fig 1).

Inhibition of cobalt binding by chelating agents

Chelating agents have been found to inhibit strongly the binding of cobalt to bacteria. The effect of EDTA was studied in more detail in order to find an alternative approach to determine the association constant. A sudden decrease of cobalt binding has been observed already at an EDTA concentration of > 10 µmol/l (fig 7). The EDTA concentration cannot be controlled precisely in this range in bacterial suspension due to
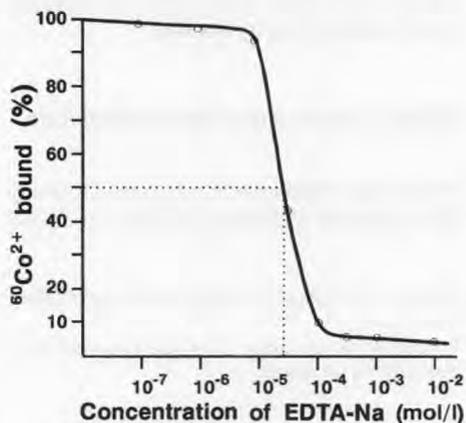

Fig 7. Inhibition of cobalt binding by EDTA. The standard procedure of cobalt-binding assay was applied. The standard incubation mixture contained 0.95 mg/ml bacteria, 30 nmol/l of $[^{60}Co]CoCl_2$ and the specified concentrations of EDTA-Na at pH 6.0. The incubation mixture containing the ingredients except bacteria was preincubated at 22°C for 5 min followed by the addition of bacterial suspension and incubation for 15 min. The binding inhibition was expressed relative to the EDTA free control.
Inhibition de la liaison du cobalt par l'EDTA. La procédure standard de l'essai de liaison du cobalt était utilisée. Le milieu d'incubation standard contenait 0,95 mg/ml de bactéries, 30 nmol/l de $[^{60}Co]$ $CoCl_2$ et les concentrations spécifiées d'EDTA-Na à pH 6,0. Le milieu sans bactéries était préincubé à 22°C pendant 5 min, puis la suspension bactérienne était ajoutée et l'incubation durait 15 min. L'inhibition de liaison était exprimée par rapport au contrôle sans EDTA.

possible interactions with other divalent ions excreted by bacteria. Thus, the association constant obtained by this indirect approach might not be valid and therefore its calculation was postponed.

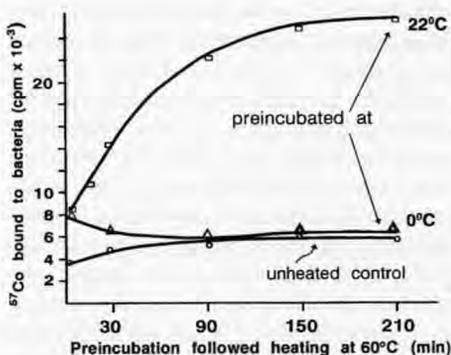## Induction of cobalt-binding capacity by thermal shock

In order to investigate the chemical nature of the cobalt-binding site, the bacterial suspension was exposed to short heating at
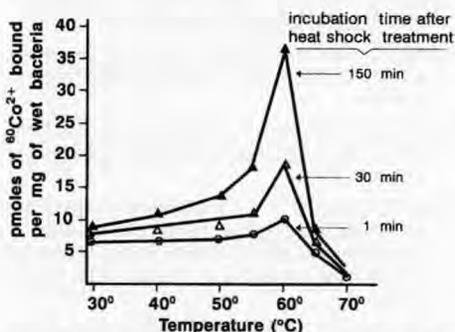
Fig 8. Effect of temperature on binding of cobalt by bacteria. A set of tubes containing bacterial suspension at a concentration of 2 mg/ml in 1 ml of 0.05 molar potassium acetate at pH 6.0 were heated for 2 min in a water bath at the temperature specified, cooled rapidly on bath to room temperature and incubation continued at 22°C for 1, 30 and 150 min. $[^{60}Co]CoCl_2$ was then added to obtain a concentration of 200 nmol/l and the tubes were incubated for 15 min. Finally, the binding of cobalt was assayed by standard procedure.
Effet de la température sur la liaison du cobalt aux bactéries. Une série de tubes contenant la suspension bactérienne à une concentration de 2 mg/ml dans 1 ml d'acétate de potassium 0,05 mol/l à pH 6,0 étaient chauffés 2 min dans un bain d'eau à la température spécifiée, rapidement refroidie dans un bain à température ambiante et l'incubation continuait à 22°C pendant 1, 30 et 150 min. On ajoutait ensuite [^{60}Co] $CoCl_2$ pour obtenir une concentration de 200 mmol/l et les tubes étaient incubés pendant 15 min. Puis la liaison du cobalt était déterminée selon la procédure standard.

various temperatures. Surprisingly, the bacteria increased their cobalt-binding capacity after 2 min of heating at 60°C (fig 8). It was even more surprising that thus treated bacteria, if incubated at room temperature for a longer period of time (up to 3.5 h), continued to increase the binding capacity up to several fold over the unheated control (fig 9). Suspensions of bacteria placed immediately on ice after heating at 60°C lost their capa-

Fig 9. Cobalt-binding capacity induction by thermal shock. Bacterial suspension containing 5 mg of wet bacteria in 5 ml of K-acetate buffer at pH 6.0 was heated for 3 min at 60°C followed by rapid cooling to room temperature. The suspension was then divided into 2 equal portions. The 2 portions were incubated accordingly: 1 at room temperature and the other on ice. At the specified time, 200 µl portions were withdrawn and assayed for cobalt binding by the standard procedure using 200 nmol/l $[^{57}Co]CoCl_2$ concentration. Simultaneously, a control, not exposed to thermal shock, was run.
Induction de la capacité de liaison du cobalt par choc thermique. La suspension bactérienne contenant 5 mg de bactéries (masse humide) dans un tampon de 5 ml d'acétate de potassium à pH 6,0 était chauffée 3 min à 60°C puis rapidement refroidie jusqu'à température ambiante. La suspension était ensuite divisée en 2 portions égales. L'une était incubée à température ambiante et l'autre dans la glace. Au temps spécifié, des échantillons de 20 ml était prélevés et testés pour la liaison du cobalt selon la procédure standard en utilisant une concentration de 20 nmol/l de [^{57}Co] $CoCl_2$. En parallèle, un témoin sans choc thermique était suivi.

bility to increase cobalt-binding capacity. The long-term incubation at room temperature required to produce the effect seems to be an indication that the higher binding capacity induced is due to *de novo* protein synthesis.

DISCUSSION

The data on cobalt-binding affinity and the inhibition of its binding by divalent ions may have practical application. They also drew our attention to the concentration of metal ions in the bacterial growth media used for vitamin B_{12} production. Indeed, it has been found (Schneider *et al*, 1994b; Trojanowska *et al*, 1994a) that magnesium and manganese concentrations used so far in standard bacterial media are affecting the yield of B_{12} synthesis. Conversely, cobalt may interfere effectively with the uptake of these ions. It was observed in our laboratory that cobalt in the growth media at a concentration of > 4 mg Co/l affects the growth of propionibacteria and vitamin B_{12} production (Trojanowska *et al*, 1994b).

The results may give practical clues as to how to avoid this undesired phenomenon which might also appear on an industrial scale. One of the solutions might be the successive addition of mineral nutrients to the medium in portions or added sequentially. To any of these approaches, more information is needed about the mutual interaction of mineral ions in complex media. Production of bacteria on an industrial scale may need continuous monitoring of the ions in the growth medium in order to optimize growth.

The media containing many kinds of amino acids and peptides present themselves as one of the richest mixtures of various chelating agents which obviously interfere with the uptake of the heavier biometals. For practical reasons, it would be useful to have some guidelines to help deal with this problem. Propionibacteria are good models for developing standard procedures in this regard. Such procedures might also, to a great extent, be applicable to other microorganisms.

REFERENCES

Baes CF, Mesmer RE (1976) *The hydrolysis of cations.* Wiley and Sons, New York

Bradbeer C (1971) Transport of vitamin B_{12} in *Ochromonas malhamensis. Arch Biochem Biophys* 144, 184-192

Dawes EA (1969) *Quantitative problems in biochemistry.* Williams & Wilkins, Baltimore, MD

Foy CD, Chaney RL, White MC (1978) The physiology of metal toxicity in plants. *Ann Rev Plant Physiol* 29, 511-566

Fuhrmann GF, Rothstein A (1963) The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells. *Biochim Biophys Acta* 163, 325-330

Herich R, Bobak M (1987) Influence of exogenously added cobalt on the submicroscopical structure of the proplastids. *Acta Facultatis Rerum Naturalium Universitatis Comenianae Physiologia Plantarum* 13, 3-8

Lau OL, Yang SF (1976) Inhibition of ethylene production by cobaltous ions. *Plant Physiol* 58, 114-117

Lindemans J, Van Kapel JA (1979) Adsorptive endocytosis of transcobalamin II-vitamin B_{12} by isolated rat liver parenchymal cells. *In: Vitamin B_{12}* (B Zagalak, W Friedrich, eds) Walter de Gruyter, Berlin

Lipskya GA (1988) Molecular aspects of investigation of the photosynthetic apparatus at chloroplast level and the productivity of plants given different cobalt rates. *Refereativnyi Zhurnal, Biologiya* 1

Marston HR, Allen SH (1972) Production within the rumen and removal from the blood-stream of volatile fatty acids in sheep given a diet deficient in cobalt. *J Nutr* 27, 147-157

Mullen MD, Wolf DC, Beveridge TJ, Bailey GW (1992) Sorption of heavy metals by the soil fungi *Aspergillus niger* and *Mucor rouxii. Soil Biol Biochem* 24, 129-135

Okorokov LA, Lichko LP, Kadomtseva VM, Kholodenko VP, Titovsky VT, Kulaey JS (1977) Energy-dependent transport of manganese into yeast cells and distribution of accumulated ions. *Eur J Biochem* 75, 373-377

Pedziwilk F (1962) Biosynthesis of cobalamin by propionic acid bacteria. *Poznań Soc Friends Sci* 11, 141-191

Pedziwilk F, Skupin J, Trojanowska K, Nowakowska K (1979) Effect of iron ions and 5,6 dimethylbenzimi-

dazole on biosynthesis of corrinoids by *Propioni-bacterium shermanii* 1 on cheese-whey medium. *Acta Aliment Pol* 5, 61-67

Sarhan F, Houde M, Cheneved JP (1980) The role of vitamin B_{12} binding in the uptake of the vitamin by *Euglena gracilis. J Protozool* 27, 235-238

Schneider Z (1987a) Chemistry of cobalamin related compounds. Non-enzymatic vitamin B_{12} binding proteins in man and animals. *In: Comprehensive B_{12}* (Z Schneider, A Stroiński, eds) Walter de Gruyter, Berlin

Schneider Z (1987b) Biosynthesis of vitamin B_{12}. Occurrence and distribution of corrinoids. *In: Comprehensive B_{12}* (Z Schneider, A Stroinski, eds) Walter de Gruyter, Berlin

Schneider Z, Stroinski A, eds (1987) *Comprehensive B_{12}*. Walter de Gruyter, Berlin

Schneider Z, Leszczynska D, Jaszewski B (1994a) Dystrybucja kobaltu i nosników kobaltu u roslin. *In: XXX Congr Polish Biochemical Society,* Szczecin

Schneider Z, Trojanowska K, Leszczynska D, Gorzelańczyk J (1994b) Wiazanie kobaltu przez bakterie propionowe. *In: XXX Congr Polish Biochemical Society,* Szczecin

Schneider Z, Trojanowska K, Jaszewski B (1994c) Inhibicja wiazania kobaltu do bakterii propionowych. *In: XXX Congr Polish Biochemical Society,* Szczecin

Swift DG (1980) Vitamin and phytoplankton growth. *In: The physiological ecology of phytoplankton* (I Morris, ed) Blackwell Scientific Publications, Oxford

Trojanowska K, Jaszewski B, Czaczyk K, Schneider Z (1994a) Wydajnosć syntezy witaminy B_{12} w zalezności od stezenia kobaltu. *In: XXX Congr Polish Biochemical Society,* Szczecin

Trojanowska K, Jaszewski B, Czaczyk K, Schneider Z (1994b) Biosynteza witaminy B_{12} w róznych środowiskach mikroelementowych. *In: XXX Congr Polish Biochemical Society,* Szczecin

White C, Gadd GM (1986) The uptake and cellular distribution of zinc in *Saccharomyces cerevisiae. J Gen Microbiol* 133, 727-737