BBA 26431

THE MECHANISM OF ACQUIRED RESISTANCE TO Co^{2+} AND Ni^{2+} IN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

M. WEBB

Strangeways Research Laboratory, Cambridge (England)

SUMMARY

1. Bacteria rendered resistant to the growth-inhibitory action of Co^{2+} by serial subculture in the presence of increasing concentrations of the cation, are resistant also to Ni^{2+}. Resistance is retained on repeated transfer in the absence of Co^{2+}, provided that the medium contains adequate amounts of Mg^{2+}. Higher concentrations of Mg^{2+} are required for the maintenance of resistance in Co^{2+} resistant strains of the Gram-positive organism, *Bacillus subtilis*, than in those of the Gram-negative bacteria, *Aerobacter aerogenes* and *Escherichia coli*.

2. Studies with *Aerobacter aerogenes* show that resistance to Co^{2+} and Ni^{2+} is not associated with alterations in the rate of oxidative metabolism of glucose or pyruvate, or in the cellular contents of cation-binding components, but with a reduction in the capacity for the energy-linked uptake of the toxic cations. Since these cations are translocated by the Mg^{2+} transport pathway, the resistant bacteria also assimilate Mg^{2+} less efficiently than do the parent organisms. Resistant bacteria regain the normal ability to concentrate Mg^{2+}, but at the same time, lose their resistance to Co^{2+} during one subculture in a Mg^{2+}-limited medium.

INTRODUCTION

The induction of resistance to Co^{2+} in cultures of *Proteus vulgaris* has been described by NEYLAND *et al.*[1]. These authors found that the cobalt-resistant organisms bound less Co^{2+} than cells of the sensitive parent strain both at low (0.17 mM) and high (3.4 mM) concentrations of the cation. No explanation was given, however, to account for the properties of the resistant bacteria, and the distinction between surface binding and transport was not appreciated. Furthermore, much of the work was done with cultures in a meat-extract broth (pH 7.5), the protein components of which would bind considerable but unknown amounts of ionic Co^{2+}.

The results described in the preceding paper[2], which distinguish between surface adsorption and the energy-linked translocation by the Mg^{2+} transport pathway of Ni^{2+} and Co^{2+}, raise a number of questions with regard to the mechanisms involved in the development of resistance to these toxic cations in bacterial cultures. The present paper, which attempts to answer some of these questions, is concerned with a comparison of the properties of parent and Co^{2+}-resistant strains, particularly of *Aerobacter aerogenes*.

MATERIALS AND METHODS

Bacterial cultures

The Gram-negative bacteria (*Aerobacter aerogenes* and *Escherichia coli*) and the Gram-positive organism (*Bacillus subtilis*) were grown at 37° in shaken flasks of M and P media[3] respectively, at pH 6.0, growth being followed turbidimetrically[3]. Cells were harvested from cultures in the stationary phase. Cobalt (as $CoCl_2$), when present, was added as a concentrated aqueous solution to the culture medium at a nominal concentration of 1 mM. After sterilization by filtration, such solutions were found by analysis to contain usually between 0.75 and 0.8 mM Co^{2+}.

Maintenance slopes were prepared by mixing aseptically equal volumes of double strength medium with a 4 % (w/v) solution of agar in distilled water at 40°.

Analytical methods

Metallic ions, DNA, RNA and acetylmethylcarbinol were determined as described previously[2,4,5]. Total "polysaccharide" was measured by the anthrone method[6], and polyphosphate as described by HAROLD[7]. Other procedures were as given in the preceding paper[2].

RESULTS

Growth inhibition by metallic ions and the isolation of metal-resistant strains

As, in initial experiments, the concentration of Co^{2+} for 50 % growth-inhibition of *A. aerogenes* and *E. coli* over a 48 h period was found to increase with the age of the culture and also, but not proportionately, with the size of the inoculum (see also ref. 1), all measurements of growth-inhibitory activities were made with a standard inoculum (20 μg dry wt. organisms per ml culture medium) and a fixed (16 h) incubation time. Under these conditions Ni^{2+} and Co^{2+} were strongly inhibitory to the growth of these organisms, the inhibition, as observed previously by others (see *e.g.* refs. 1 and 8), being dependent upon the Mg^{2+} content of the medium. This antagonism by Mg^{2+} of Ni^{2+} or Co^{2+} toxicity was found to be more effective with the Gram-negative *A. aerogenes* than with the Gram-positive *B. subtilis*, the concentration of Ni^{2+} necessary for 50 % growth inhibition, for example, being increased about 40 times in cultures of the former organism but only about twice in cultures of the latter by a 10-fold increase in the Mg^{2+}-content of the medium.

Resistance to Co^{2+} (1 mM) was induced readily in *A. aerogenes* and *E. coli* by 6–10 serial subcultures in the presence of increasing concentrations of the cation. These Co^{2+}-resistant cells were also resistant to Ni^{2+} (Fig. 1). Resistance was maintained during 26 subcultures at 48 h intervals on agar slopes of the defined medium, transfer being discontinued after this period, but was lost after one subculture in a liquid medium of limited Mg^{2+} content (1 μg/ml;41.2 μM).

With the Gram-positive bacillus, *B. subtilis*, in the normal P-medium it was more difficult to induce resistance, and even with cultures that ultimately tolerated 0.25 mM Co^{2+}, there was a long lag of 12 h or more before growth began. Resistance developed more rapidly when the Mg^{2+} content of the medium was increased 10-fold, and organisms that grew in the presence of 1 mM Co^{2+}, without the extended lag, were obtained after 6–8 subcultures. These Co^{2+}-resistant organisms were resistant also

to Ni^{2+}. Resistance to both cations was lost, however, after 7 subcultures in P-medium of normal Mg^{2+} content.

Properties of the metal-resistant strains

Although the resistant strains tolerated 1 mM Co^{2+}, their rates of both growth and pyruvate oxidation were reduced by about 25–50 % in the presence of this concentration of the cation. In the absence of Co^{2+} the rates of growth and, in stationary-phase cells, the Mg^{2+} contents and RNA to DNA ratios in the parent and resistant organisms were the same. Also the rates of oxidative metabolism of the resistant organisms with either glucose or pyruvate as substrate were similar to those of the corresponding parent strain, but were 50–55 % less susceptible to inhibition by Co^{2+}. With *A. aerogenes* organisms, additional analyses showed also that the contents of polysaccharide and polyphosphate, as well as the rates of production of acetylmethylcarbinol were unaltered by the acquisition of resistance. Growth of the resistant organisms in the presence of 1 mM Co^{2+}, however, reduced the Mg^{2+} content of stationary phase cells from 102 to 73 nmoles Mg^{2+} per mg dry wt. organisms.

Uptake of Ni^{2+} and Co^{2+} by washed suspensions of the Co^{2+}-resistant strains of *E. coli* and *A. aerogenes* at 28° was significantly less than that by the parent organisms (Table I). At 0°, or 28° in the presence of 0.1 mM 2,4-dinitrophenol, however, both the resistant and parent strains bound similar amounts of a given cation (Table I). Thus, as shown by the difference between the uptake values at 28° and 0° (Fig. 2) the energy-linked translocation of Ni^{2+} and Co^{2+} was reduced by 50 % or more in the resistant organisms. After growth of the latter in the presence of 1 mM Co^{2+} the capacities for surface-binding and energy-linked uptake of Ni^{2+} were reduced by 5–8 % (Table I) and by about 45 % (Fig. 2) respectively.

Under the conditions previously defined[2], cells of both the parent and Co^{2+}-resistant strains of *A. aerogenes* at first liberated, and then reutilized Mg^{2+} when

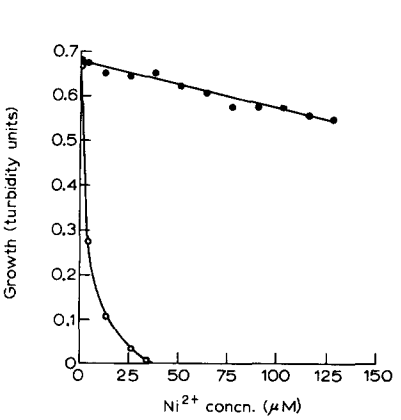

Fig. 1. Effect of Ni^{2+} on the growth of the parent (○—○) and Co^{2+}-resistant (●—●) strains of *E. coli*.

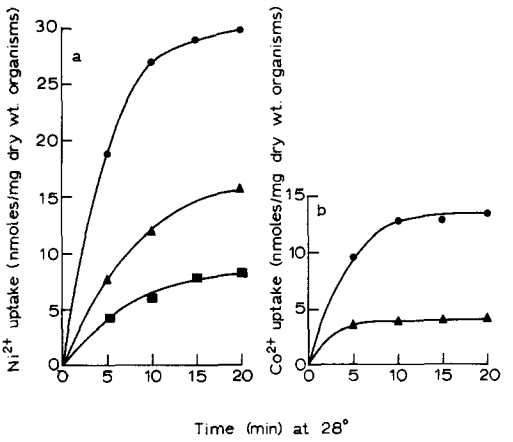

Fig. 2. Energy-linked uptake of (a) Ni^{2+} and (b) Co^{2+} by the parent (●—●) and Co^{2+}-resistant (▲—▲) strains of *A. aerogenes* as determined by the difference between uptake at 28° and 0° under the conditions defined in the legend of Table I. Fig. 2a also shows the energy-linked uptake of Ni^{2+} by the Co^{2+}-resistant strain after growth in the presence of 1 mM Co^{2+} (■—■).

TABLE I

UPTAKE OF Ni^{2+} AND Co^{2+} BY THE PARENT AND Co^{2+}-RESISTANT STRAINS OF *Aerobacter aerogenes*

Cultures of the parent and Co^{2+}-resistant strains of *A. aerogenes* in the normal chemically-defined medium (MATERIALS AND METHODS) and of the Co^{2+}-resistant strain in the same medium, but with the addition of 1 mM Co^{2+}, were harvested after 16 h. at 37°, the cells being washed and resuspended in 154 mM NaCl at 10 mg dry wt. organisms per ml. Portions of these suspensions (1.5 ml) were added to flasks that contained solutions (4.5 ml) of 225 μmoles sodium phosphate, pH 6.0, 15 μmoles sodium pyruvate, 1.5 μmcles $CoCl_2$ or $NiCl_2$ and (when present) 0.6 μmoles 2,4-dinitrophenol. After incubation at the temperature, and for the times given below, the contents of the appropriate flasks were centrifuged at 0°, and the cell-free supernatant fractions analysed for Ni^{2+} or Co^{2+} by atomic absorption.

	Conditions of incubation	*Ni^{2+} uptake nmoles/mg dry wt. organisms)*				*Co^{2+} uptake nmoles/mg dry wt. organisms)*			
		5 min	*10 min*	*15 min*	*20 min*	*5 min*	*10 min*	*15 min*	*20 min*
Control	28°	39.0	47.2	48.5	49.5	21.6	24.8	25.1	25.6
Co^{2+}-resistant	28°	25.0	29.3	32.7	33.0	14.9	15.0	15.0	14.9
Co^{2+}-resistant, grown with Co^{2+}	28°	20.8	21.6	24.4	24.4				
Control	28° + 0.1 mM 2,4-dinitrophenol	20.2	20.2	20.2	20.2	12.9	12.9	12.9	13.5
Co^{2+}-resistant	28° + 0.1 mM 2,4-dinitrophenol	18.1	18.2	18.3	18.2	10.4	10.4	11.7	12.0
Co^{2+}-resistant, grown with Co^{2+}	28° + 0.1 mM 2,4-dinitrophenol	16.2	16.2	16.8	16.8				
Control	0°	20.2	20.2	19.6	19.6	12.0	12.0	12.3	12.3
Co^{2+}-resistant	0°	18.3	18.3	17.9	18.3	11.2	11.2	10.7	10.9
Co^{2+}-resistant, grown with Co^{2+}	0°	16.2	15.6	16.2	16.2				

transferred to a Mg^{2+} -free pyruvate–phosphate medium. In suspensions of the resistant organisms both the initial liberation, and the rate of reutilization were much less than in those of the parent (Fig. 3). Differences in the rates of Mg^{2+} utilization were observed also in media supplemented with low concentrations of Mg^{2+}. At higher concentrations, however, the rate of utilization by the resistant cells was increased disproportionately and, in media with either 60 or 80 μM Mg^{2+}, it was essentially the same as that by the parent organisms (Fig. 3).

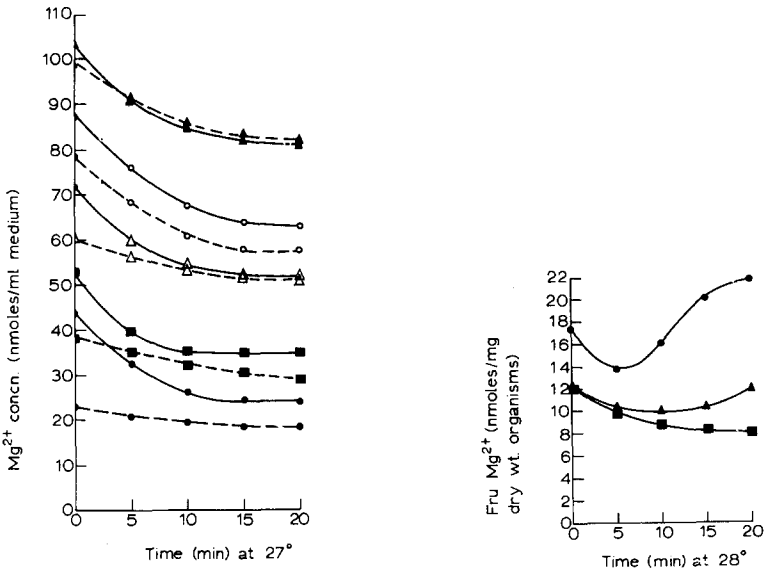

Fig. 3. Magnesium utilization by the parent (—) and Co^{2+}-resistant (- - -) strains of *A. aerogenes* in a pyruvate–phosphate medium, pH 6.0, with 80 (▲), 60 (○), 40 (△), 20 (■) and 0 (●) μM Mg^{2+}. Magnesium analyses were made on the cell free supernatant fractions that were collected at the time intervals shown.

Fig. 4. Effect of 0.25 mM Co^{2+} on the reutilization of Mg^{2+} liberated from *A. aerogenes* cells on transfer to a Mg^{2+}-free pyruvate–phosphate medium pH 6.0. ●—●, parent bacteria; ■—■, Co^{2+}-resistant strain; ▲—▲, Co^{2+}-resistant strain after growth in the presence of 1 mM Co^{2+}. Values for Mg^{2+} liberation at zero time were obtained from measurements made at 0°. Experimental details were as described in the legend of Fig. 3.

In suspensions of the sensitive, parent bacteria in the Mg^{2+}-free medium the reutilization of the liberated cation was inhibited by 0.25 mM Co^{2+} and, after 5 min, efflux of cellular Mg^{2+} occurred progressively with time. Under the same conditions there was a slow, continual reutilization of Mg^{2+} in suspensions of the resistant bacteria (Fig. 4), and even after growth in the presence of 1 mM Co^{2+}, Mg^{2+} utilization by these organisms was less susceptible to Co^{2+} inhibition than was that by the parent bacteria (Fig. 4). Organisms derived from the resistant strain by subculture in a Mg^{2+}-limited medium (41·2 μM), followed by growth in the normal medium, concentrated Mg^{2+} from dilute solution with the same efficiency as cells of the original parent strain, but uptake of this cation was no longer resistant to inhibition by Co^{2+}.

DISCUSSION

Although the growth of Gram-negative bacteria such as *E. coli* and *A. aerogenes*, in a simple chemically-defined medium is very susceptible to inhibition by low concentrations of Ni^{2+} and Co^{2+}, these organisms readily acquire resistance to both cations when serially subcultured in the presence of increasing concentrations of either one of them. This cross resistance is to be expected if, as previously concluded[2], these ions are translocated by the constitutive energy-linked Mg^{2+} transport pathway.

In the absence of Co^{2+}, the metabolism of Co^{2+}-resistant *A. aerogenes* cells is the same as that of the parent organisms, insofar as the rates of oxidation of either glucose or pyruvate, and the rates of acetylmethylcarbinol production during growth, are identical. Furthermore, resistance to Co^{2+} is not associated with increased cellular contents of either potential cation-binding components, such as RNA, polysaccharide and polyphosphate, or antagonists of the toxic cations (*e.g.* Mg^{2+}). Although alterations in the surface properties and cell wall composition of *B. subtilis* in response to environmental changes, have been reported by MEERS AND TEMPEST[9], there is little difference in cation-binding at the cell surface by the resistant and parent strains. The resistant organisms, however, differ from those of the parent strain in their capacity for the energy linked-translocation of Ni^{2+} and Co^{2+}, and it seems that the acquisition of resistance is associated with decreased incorporation of the toxic cat ons. The fact that the energy-linked accumulation of Co^{2+} is reduced but not eliminated, explains the reduced growth-rate of the resistant strain in the presence of Co^{2+}, and the decrease in both Mg^{2+}content and the rate of oxidative metabolism in cells from such cultures.

It seems unlikely that the decreased translocation of Ni^{2+} and Co^{2+} by the resistant bacteria is due to more effective discrimination between Mg^{2+} and the toxic cations. Although as shown in Fig. 4, resistant bacteria continue to utilize Mg^{2+} in the presence of a concentration of Co^{2+} (0.25 mM) that causes complete inhibition of Mg^{2+} uptake in suspensions of the sensitive, parent organisms, this difference probably reflects only the decreased uptake of Co^{2+} by the resistant cells (*cf.* Fig. 7 in ref. 2). A more probable explanation is that resistance is correlated with a decreased ability of the cells to concentrate ions by the Mg^{2+} transport pathway, rather than with an increased selectivity of this pathway. A decrease in "transport efficiency" would be expected to affect the uptake of the less effectively translocated Ni^{2+} and Co^{2+} more than that of normal Mg^{2+}. As shown by the results of Fig. 3, the decreased efficiency of Mg^{2+} transport by the resistant strain of *A. aerogenes* is apparent only at low concentrations of Mg^{2+} and, therefore, is likely to be significant only in cultures that are deficient in this cation. Under such conditions, organisms of the resistant strain regain the ability to concentrate Mg^{2+} from dilute solution with the same efficiency as the parent bacteria. Significantly, however, this reversion to normal Mg^{2+} transport is accompanied by the loss of resistance to Co^{2+} and Ni^{2+}. Acquisition of resistance to Co^{2+} (or Ni^{2+}), therefore, has some similarity with the induction of resistance to arsenite which has been described in yeast[10] and *Pseudomonas pseudomallei*[11], and which is correlated with a decrease in permeability to the toxic anion. In this connection, therefore, it may be significant that BROCK[12] considers that Mg^{2+} deficiency may induce changes in the permeability of the cell membrane of *E. coli*, whilst FIIL AND BRANTON[13] conclude that this

organism is able to vary the structure of its plasma membrane in response to alterations in the environment.

It is probable that the development of resistance to Co^{2+} in *B. subtilis* occurs by the same mechanism as in *A. aerogenes*. As however, Mg^{2+} utilization by the Gram-positive bacillus is already less efficient than that by the Gram-negative bacterium, higher concentrations of Mg^{2+} are necessary for the growth and maintenance of resistant strains.

ACKNOWLEDGMENTS

The technical assistance of Mr G. Payton in the work summarised in this and the preceding paper, is gratefully acknowledged.

REFERENCES

1 M. NEYLAND, P. DUNKEL AND A. L. SCHADE, *J. Gen. Microbiol.*, 7 (1952) 409.
2 M. WEBB, *Biochim. Biophys. Acta*, 222 (1970) 428.
3 M. WEBB, *J. Gen. Microbiol.*, 43 (1966) 401.
4 J. C. HEATH AND M. WEBB, *Brit. J. Cancer*, 21 (1967) 768.
5. M. WEBB, *Biochem. J.*, 106 (1968) 367.
6 R. DREYWOOD, *Ind. Eng. Chem. Anal. Ed.*, 18 (1946) 499.
7 F. M. HAROLD, *J. Bacteriol.*, 86 (1963) 216.
8 P. H. ABELSON AND E. ALDOUS, *J. Bacteriol.*, 60 (1950) 401.
9 J. L. MEERS AND D. W. TEMPEST. *J. Gen. Microbiol.*, 55 (1969) X.
10 J. CERBON, *J. Bacteriol.*, 97 (1969) 658.
11 M. BEPPU AND K. ARIMA, *J. Bacteriol.*, 88 (1964) 151.
12 T. D. BROCK, *J. Bacteriol.*, 84 (1962) 679.
13 A. FILL AND D. BRANTON, *J. Bacteriol.*, 98 (1969) 1320.