

## Aspects of Mycobacterial Response to Beryllate Ions *in vitro*

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Comparative growth measurements of the mycobacterial species *Mycobacterium phlei* and *Mycobacterium tuberculosis* were carried out with liquid cultures supplemented with oxalatoberyllate ions in the concentration range 0–120  $\mu\text{M}$  Be. For both species, the effect of beryllium on specific growth parameters can be represented by a Be concentration-dependent, exponential expression. Evidence for beryllium binding to cellular phospholipids ( $\sim 15\%$  of Be uptake) is provided by  $^{31}\text{P}$  NMR spectroscopy, thin-layer chromatography and atomic absorption spectrometry.

### Introduction

Inhibitory effects of beryllium on mycobacteria, in particular tubercle bacilli, have been known for many decades [1, 2]. However, the assessment of early results was impeded by an incomplete picture of the toxicological and chemical properties of this element. Later investigations, involving various bacterial genera [3–8], were based on the action of the  $\text{Be}^{2+}$  ion in hydrolytically stabilized, *i.e.*, complexed form, and showed that inhibiting concentrations were generally situated at much lower levels than those previously estimated.

In continuation of preliminary work along these lines, we report here a comparative study of effects produced by beryllium on the representative mycobacterial species *Mycobacterium phlei* and *M. tuberculosis* in liquid culture.

### Materials and Methods

Bacterial strains used were *Mycobacterium phlei* 64.29 (Institut Pasteur, Paris) and *Mycobacterium tuberculosis* T864 (Faculté de Médecine, Université Louis Pasteur, Strasbourg). Series of broth cultures (50 ml, pH 5.95) in 1% bacto-peptone medium (*M. phlei*) and in enriched peptone medium (*M. tuberculosis*) were supplemented with Be in the form of a (bis) dicarboxylic acid (usually oxalate) complex in the concentration range 0–120  $\mu\text{M}$  Be. The preparation of these materials and spectrophotometric meas-

urement of cell growth were carried out as described previously [7–9]. Total phospholipid fractions from 0.5 l cultures of normal and Be-treated cells (for *M. phlei*, 10  $\mu\text{M}$  Be; for *M. tuberculosis*, 40  $\mu\text{M}$  Be) were isolated by the method of Brennan and Ballou [10]. A  $\text{Be}^{2+}$ -cardiolipin derivative for comparative measurements was prepared by agitation of a chloroform solution of bovine cardiolipin (Na-salt) with an aqueous solution of  $\text{Na}_2[\text{Be}(\text{C}_2\text{O}_4)_2]$  in molar proportions ( $\text{P}:\text{Be}=2:1$ ). The mixture was treated with the stoichiometric amount of calcium acetate to remove oxalate and the product isolated by azeotropic evaporation [11] of the organic layer. Thin-layer chromatography (TLC) of phospholipids was performed with activated (90 min, 80 °C) silica gel 60F–254 plates using  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$  (25:15:4:2, v/v) as solvent system [12]. Plates were developed in iodine vapour and sprayed with commercial starch aerosol. Be in dried (60 °C) biomass and extracts thereof was determined by flameless atomic absorption spectrometry (AAS) following quantitative solubilization of samples in  $\text{HNO}_3/\text{H}_2\text{SO}_4$  at 100 °C [13].

### Results and Discussion

Turbidimetric measurements of culture development as a function of initial beryllium ion concentration ( $C$ ) in the medium show characteristically different behaviour patterns for the two mycobacterial species studied. Typical growth curves for a representative value of  $C$  (14  $\mu\text{M}$ ) are shown in Fig. 1. In

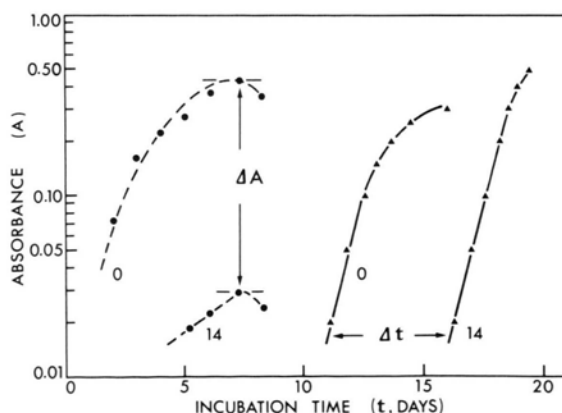


Fig. 1. Typical growth curves for *M. phlei* (●—●) and *M. tuberculosis* (▲—▲) in presence and absence of beryllate ions. Curve numbers indicate micromolar concentrations of Be in the medium.

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contrast with *M. tuberculosis* [8], *M. phlei* does not reveal a substantial Be-dependent variation in the time required to reach a maximum growth level when  $C \leq 110 \mu\text{M Be}$  [7]. Instead, there is a sensitive decrease in growth rate and in the subsequent stationary phase population level as a function of  $C$  in the range  $\sim 0.2$  to  $5 \mu\text{M Be}$ . The general absence of Arndt-Schulz stimulation at such low Be concentrations in the case of *M. phlei* is remarkable in view of the fact that *M. tuberculosis* frequently exhibits growth stimulation up to relatively high concentrations of the order of  $14 \mu\text{M Be}$  [8].

With respect to the major growth parameters affected, culture development in both species can, however, be described by an expression of the form:

$$|\Delta P| = F(1 - e^{-kC}) \quad (1)$$

where  $\Delta P$  denotes the Be-dependent change in a specific growth parameter (decrease in maximum cell population, *i.e.*, absorbance values, for *M. phlei* and increase in lag period for *M. tuberculosis* (Fig. 1)),  $k$  is a numerical constant for the system concerned and  $F$  represents a limiting factor corresponding to the upper boundary condition in inhibitory action of the toxic ion. Optimal values for these constants were determined by least-square fitting of experimental curves based on the dependency  $\Delta P$  vs.  $C$ . Calculated values for  $\Delta P$  are compared with observed data in Table I.

Table I. Concentration-dependent effect of beryllate ions<sup>a</sup> on the growth of *M. phlei* and *M. tuberculosis* T864.

$C$ [ $\mu\text{mol Be/l}$ ]	<i>M. phlei</i> Decrease in maximum cell population ( $\Delta A$ , absorbance)		<i>M. tuberculosis</i> Increase in lag period ( $\Delta t$ , days)	
	obs.	calc. <sup>b</sup>	obs.	calc. <sup>b</sup>
0.44	$0.06 \pm 0.01$	0.07	—	—
2.2	$0.20 \pm 0.01$	0.25	—	—
6.0	—	—	$4 \pm 4$	2.8
8.8	$0.40 \pm 0.01$	0.40	—	—
10	—	—	$4 \pm 1$	4.5
14 <sup>c</sup>	$0.41 \pm 0.01$	0.41	$5.5 \pm 1$	6.1
20	$0.41 \pm 0.01$	0.41	$8 \pm 1$	8.2
40	$0.41 \pm 0.01$	0.41	$14 \pm 1$	13.7
80	$0.42 \pm 0.01$	0.41	$19 \pm 2$	19.9
120	—	—	$23 \pm 3$	22.6

<sup>a</sup> As dioxalato complex

<sup>b</sup> Using Eq. 1 and the following values of computational parameters: *M. phlei*:  $F=0.41$ ,  $k=0.42$ ; *M. tuberculosis*:  $F=24.9$ ,  $k=0.02$

<sup>c</sup> Effective limiting inhibitory concentration for *M. phlei*.

In the case of *M. tuberculosis* it should be pointed out that, although batch series of cultures generally showed coherent growth behaviour, marked differences were occasionally apparent between corresponding cultures of different series, probably owing to varying degrees of bacterial adaptation to the medium.

Although factors which govern the distribution and mode of action of beryllium in microbial cells are manifold and complex, it appears certain that Be can produce inhibitory effects from competitive binding to some enzymes and phosphorylated proteins [14]. It has also been suggested that replacement of Mg by Be in ribosomes may occur [15].

In view of the possibility of beryllium binding to cellular phosphate groups [16], it appeared interesting to examine the phospholipid fractions from beryllium-treated cultures of the two species tested. For *M. phlei*, this was done by  $^{31}\text{P}$  NMR and by atomic absorption measurements [17] of Be in biomass extracts.  $^{31}\text{P}$  NMR of total phospholipid fraction, *M. phlei* ( $\text{CDCl}_3$ , 36.43 MHz; ref. 85%  $\text{H}_3\text{PO}_4$ ): normal culture:  $\delta$   $-2.81$  (2.9 [18]),  $-1.19$  (4.4),  $0.10$  (10),  $1.35$  (5.5); culture with  $10 \mu\text{M Be}$ :  $\delta$   $0.26$  (10) br. AAS: concentration of Be ( $\mu\text{g Be/g}$  of material; std. deviation  $\pm 13\%$ ): in liquid culture medium: 0.09; in biomass, total non lipid fraction: 75.63; in total crude phospholipids: 13.38. Estimated distribution ratio of Be, biomass/total medium:  $(669 \pm 90)$ : 1. The slight, general upfield displacement in  $^{31}\text{P}$  chemical shift of the phospholipid peak envelope for *M. phlei* grown in Be-supplemented culture is consistent with the trend observed in the  $^{31}\text{P}$  NMR spectra of both inorganic and organic beryllium phosphates [6, 16].

TLC separation of phospholipids from the broth cultures of *M. tuberculosis* revealed a small but reproducible increase in  $R_f$  (3.7%) for a main constituent from Be-treated cells (Fig. 2, 2-D). A similar tendency is observed with the model compound "beryllium-cardiolipin" (Fig. 2, lane 4), for which the  $^{31}\text{P}$  NMR spectrum in  $\text{CDCl}_3$  showed two main peaks of equal area ( $\delta$  4.50, 9.43 as opposed to one peak, at  $\delta$   $-1.46$ , for Na-cardiolipin alone), presumably owing to binding of Be to one of the phosphate moieties. The cardiolipin sample used in this preparation contained several constituents, all of which were affected by the presence of Be; analogous treatment with calcium ions produced no significant difference in migration of main spots (Fig. 2, lane 5). The effects thus observed are ascribed to a higher

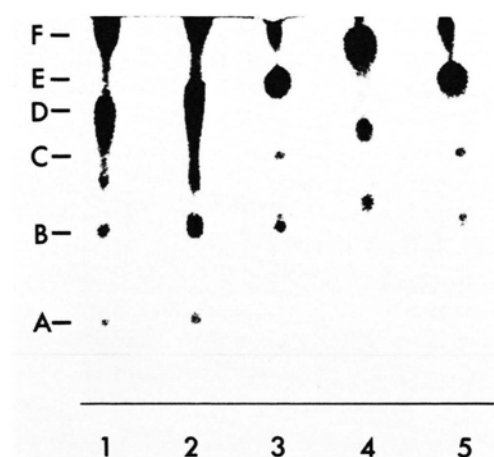


Fig. 2. Thin-layer chromatogram of phospholipids. 1 – From *M. tuberculosis* T864, normal culture; 2 – From T864 grown in presence of 40  $\mu\text{M}$  Be; 3 – Cardiolipin (bovine), Na-salt; 4 – Cardiolipin, Be derivative; 5 – Cardiolipin +  $\text{Ca}^{2+}$ . A weak spot of uncertain nature at 3A is not indicated.

degree of covalent bonding character in Be and its particular ease of association with  $-\text{OH}$  and  $\text{H}_2\text{O}$  ligands [19, 20].

The results obtained indicate that combination of Be ions with intracellular lipid phosphate groups (possibly in a 4-coordinate form such as  $(\text{RO})(\text{R}'\text{O})\text{P}(\text{O})-\text{O}-\text{Be}(\text{H}_2\text{O})_2-\text{OH}$ ) contributes significantly towards the uptake of beryllium in these species. Since there is evidence that phospholipids can be involved in the transport of amino acids *via* terminal chain-binding [21], it is conceivable that beryllium bonding at these sites could interfere with an important stage of the growth process. On the other hand, a relationship between beryllium uptake and the manner in which growth is affected is not immediately apparent. It is, however, of interest that the overall pattern of growth inhibition in both species can be satisfactorily represented by a common mathematical expression involving few computational parameters.

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