PHYSIOLOGICAL STUDIES ON GENTAMICIN: PHOSPHATE REPRESSION OF ANTIBIOTIC FORMATION

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The effect of inorganic phosphate on the fermentative production of gentamicin by *Micromonospora purpurea* has been studied using a chemically defined medium. Phosphate concentrations higher than 5.75 mM (1 g/liter^{-1}) did not inhibit growth but specifically prevented antibiotic formation. Changes in the pH medium and carbon or nitrogen depletion were excluded as the cause of antibiotic underproduction. The use of a phosphate analogue, a protein synthesis inhibitor and the profiles of differential rate of antibiotic production suggested that phosphate itself transiently repressed gentamicin formation. Phosphate affected the formation of 2-deoxystreptamine from 2-deoxyinosose, a none phosphorylated substrate.

Gentamicin is an antibiotic complex containing three major aminoglycoside components referred to as C-1, C-1a and C-2. Among aminoglycosides, gentamicin is the antimicrobial agent of choice for the treatment of several classes of infections caused by *Escherichia coli*, *Proteus* and *Pseudomonas*[1]. Even though gentamicin has an industrial importance, little information is available with respect to its fermentative production and the factors and conditions that control its biosynthesis.

Inorganic phosphate has long been known to suppress the biosynthesis of many antibiotics and other secondary metabolites[2]. In these examples orthophosphate either inhibited or repressed dephosphorylation reactions or synthetases in which orthophosphate is neither a substrate nor a product. During the biosynthesis of other aminoglycoside antibiotics such as streptomycin, neomicin and butirosin A, biologically inactive phosphorylated intermediates are formed which later are enzymatically dephosphorylated to yield bioactive products. In these examples, dephosphorylation reactions are phosphate inhibited and repressed with the subsequent accumulation of streptomycin-6-phosphate, neomicin-6-phosphate and butirosin A-6'-diphosphate respectively[3~5].

Our group have been involved in studies on the factors and conditions controlling gentamicin formation[6]. In this antibiotic, phosphate regulation has been suggested by the use of phosphate-trapping agents in *Micromonospora echinospora* subsp. *echinospora*[7]. Considering that among aminoglycosides antibiotics, gentamicin biosynthesis does not includes dephosphorylation reactions, we endeavored identify the mechanism of phosphate regulation involved in this antibiotic. This paper describes the effect of phosphate on the synthesis of gentamicin and presents data indicating that phosphate transiently represses antibiotic formation by preventing the synthesis of 2-deoxystreptamine from 2-deoxyinosose, a none phosphorylated compound.

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Materials and Methods

Microorganism and Cultivation

M. purpurea NRRL-2953 was kindly supplied by the ARS Culture Collection, U.S. Department of Agriculture, Peoria, IL. U.S.A. Spores of this microorganism were obtained and maintained as previously reported[6]. For antibiotic production, 2 ml of a seed culture previously washed and suspended in sterile distilled water, were inoculated into 250-ml Erlenmeyer flasks containing 50 ml of the following chemically defined medium (CD): 1 g $(NH_4)_2SO_4$, 2 g sucrose, 0.3 g NaCl, 0.002 g $MgSO_4 \cdot 7H_2O$, 0.003 g $FeSO_4 \cdot H_2O$, 1 g $CaCO_3$, 0.003 g $ZnSO_4 \cdot H_2O$, 0.001 g $MnSO_4 \cdot 4H_2O$, 0.0001 g $CoCl_2 \cdot 6H_2O$ and the desired K_2HPO_4 concentration, per 100 ml distilled water. After preparation, the CD medium was adjusted to pH 6.8 with 1 N HCl and autoclaved at 22 atm for 20 minutes. Sucrose was sterilized separately and added before inoculation. Fermentations were carried out at 29°C for 7 days on a rotary shaker at 175 rpm.

Additions to the Fermentation.

After 48 hours fermentation, chloramphenicol, arsenate, antibiotic precursors and phosphate, previously filter sterilized (with Millipore filters type HAWP) were added to cultures growing in the CD medium described above with 1.42 mM phosphate. The cultures were returned to the shaker and at desired times 2 ml samples were withdrawn for further analysis.

Assay of Gentamicin and Phosphate Determination

At specified intervals, the production of antibiotic was determined by an agar disk technique using *Bacillus subtilis* ATCC 6633 as the assay organism[6]. Phosphate consumption was evaluated in the culture medium (50 μl samples), according to SUMMER[8].

Growth Determination

Samples of mycelia were harvested, washed with 2 volume distilled water and placed in 2 ml of 0.3 M trichloroacetic acid. After centrifugation, the pellet was resuspended in 1 ml of 0.4 N NaOH, and its protein concentration determined by the Lowry method using bovine serum albumin as standard.

Reproducibility of Results

The experiments reported were repeated at least once (two independent experiments) in duplicate and the results are mean values. The observed variations were consistently less than 10%.

Results and Discussion

Effect of Inorganic Phosphate Concentration

M. purpurea was able to grow and produce gentamicin in a chemically defined medium containing 1.42 mM inorganic phosphate. Fig. 1 shows maximum growth and specific antibiotic production of this microorganism in fermentations with phosphate concentrations ranging from 1.42 to 17.2 mM. As shown in the figure, with the use of phosphate concentrations higher than 5.75 mM an inverse correlation was found between the maximum antibiotic production and the initial phosphate concentration with a maximum effect (65% decrease) seen at 11.5 mM. Therefore, the addition of 11.5 mM phosphate did not inhibit growth but specifically prevented antibiotic formation. Under these conditions, no changes were observed in the pH of the medium, nor limitations in the carbon and nitrogen sources, even at 144 hours of incubation, thus excluding pH and nutrient depletion as the cause of gentamicin underproduction.

After 96 hours of fermentation, phosphate was totally consumed when added to the medium at 1.44 mM. On the other hand, no more than 65% consumption was observed when phosphate concentrations higher than 5.75 mM were utilized (not shown). Nevertheless, an inverse correlation between phosphate consumed

Fig. 1. Effect of phosphate concentration on maximum growth (□), specific gentamicin formation (△), and final pH of the medium (○).

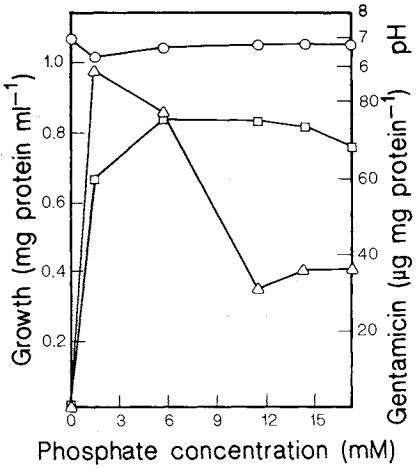
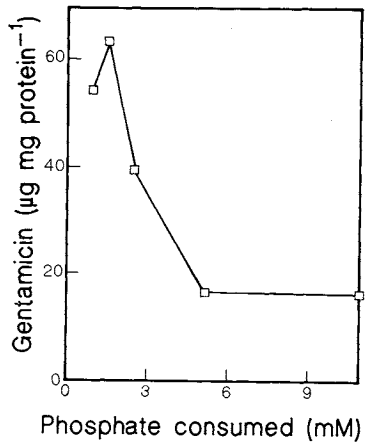


Fig. 2. Relationship between phosphate consumed and gentamicin production at 96 hours of fermentation.



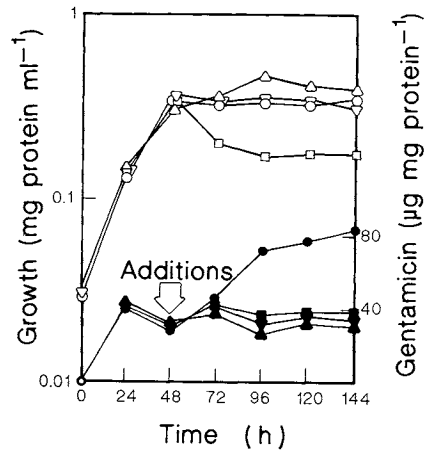
Cultures were grown in fermentation medium with phosphate ranging from 0.86 to 17.2 mM.

and antibiotic formed was observed (Fig. 2).

Addition of Inorganic Phosphate During the Fermentation

In order to characterize the phosphate effect, a high ion concentration was added to cultures growing in low phosphate at 48 hours of fermentation (once the production of gentamicin had started). As seen in Fig. 3, in contrast to the control without additional phosphate, supplementation with 11.5 mM phosphate reduced the rate of antibiotic biosynthesis. As shown in the same figure, the addition of a protein synthesis inhibitor (chloramphenicol) to low phosphate fermentations, also exerted a negative effect on gentamicin production, similar to that observed at a high phosphate concentration, suggesting a repressive rather than an inhibitory action on antibiotic formation.

Fig. 3. Effect of 11.5 mM phosphate (△, ▲), 11.5 mM arsenate (▽, ▼) or 0.15 mM chloramphenicol (■, □) addition, on the course of growth (light symbols) and specific gentamicin formation (dark symbols). Control without further addition (○, ●).

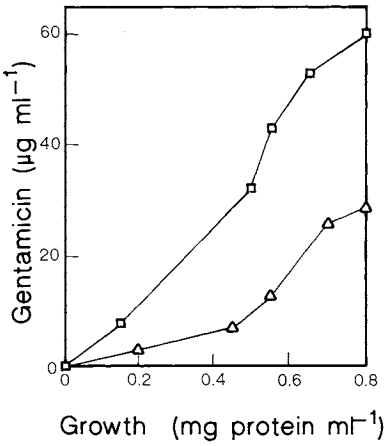


Additions were done in 48 hours cultures grown in fermentation medium with 1.42 mM phosphate.

Effect of Arsenate on Antibiotic Formation

To establish whether or not phosphate *per se* was responsible for this effect, a non-metabolizable phosphate analogue, sodium arsenate[9], was supplied to cultures grown with low phosphate. As revealed in Fig. 3, in contrast to a control without further addition, arsenate significantly decreased antibiotic production, but had no effect on growth. As can be seen in the same figure, the magnitude of this effect was similar to that obtained at a high phosphate concentration. Therefore phosphate, rather than a product derived from its metabolism, seemed to be responsible for the suppression of gentamicin formation.

Fig. 4. Effect of phosphate on the differential rate of gentamicin formation.



Cultures were grown in fermentation medium with phosphate 1.42 (□) or 11.5 (△) mM.

Table 1. Specific antibiotic produced by phosphate repressed cultures supplemented with precursors and intermediates of the gentamicin biosynthetic pathway.

Condition	Concentration (mM)	Specific antibiotic formation (μg mg protein^{-1})
Controls:		
non-repressed		80
repressed		34
2-Deoxystreptamine	5	50
	10	64
D-Glucosamine	15	34
	30	34
L-Glutamine	15	32
	30	32
D-Xylose	66	32
	132	32

Phosphate (11.5 mM) was added at 48 hours of fermentation (repressed cultures). Precursors and intermediates were added to repressed cultures at 96 hours of incubation. After 144 hours of fermentation, gentamicin was quantified in the fermentation broths.

Differential Rate of Antibiotic Synthesis

To gain more information about the phosphate effect, the microorganism was grown with low (1.42 mM) and high (11.5 mM) phosphate concentrations, and growth was plotted *versus* gentamicin production at different times during the fermentation. As can be seen in Fig. 4, a close relationship between these parameters was observed at a low phosphate concentration. However, when a high concentration of the ion was used, this relationship was modified by an increase in the extent of growth and a delay in antibiotic production. The profile observed with high phosphate was comparable to that reported for the transient repression of β-galactosidase and erythromycin elicited by glucose in *Escherichia coli* and *Streptomyces erythraeus* respectively.[10,11] As pointed out by these authors, a transient repressive effect is characterized by a strong but temporary suppression in the formation of a given metabolite. Therefore, a transient repression exerted by phosphate was considered as the cause of its negative effect on antibiotic formation.

Addition of Precursors of the Antibiotic Pathway

In order to define the step in antibiotic formation that is affected by phosphate, we treated repressed cultures with several precursors and intermediates of the gentamicin pathway in an attempt to reverse the negative effect. L-Glutamine, D-glucosamine, D-glucosamine-6-phosphate, 2-deoxystreptamine (ranging from 5 to 30 mM) and D-xylose (66 and 132 mM) were employed for this purpose. These compounds were efficiently taken up by the cells, however, among them, only 2-deoxystreptamine, added at 96 hours of fermentation, was able to overcome the negative effect of high phosphate concentration (Table 1). Therefore, the regulation observed seemed to take place at a step in the gentamicin pathway between deoxyinosose and 2-deoxystreptamine. Considering that phosphate has been reported as a regulator of some glutamine amido transferases[2], there is the possibility that this ion could regulate L-glutamine: ketoscyllo-inositol amino transferase. This enzyme catalyzes the amination of deoxy-inosose and aminodeoxyinosose, precursors of 2-deoxystreptamine[12]. In any case, phosphate repressed gentamicin formation in *M. purpurea*, by a mechanism which does not seems to involve dephosphorylation reactions. As far as we know this

is the first report of aminoglycoside antibiotic synthetases regulated in this way. Diverse none-aminoglycoside antibiotic synthetases catalyzing reactions in which orthophosphate is neither a substrate nor a product have been reported as repressible by phosphate. These include anhydrotetracycline oxygenase in the biosynthesis of tetracycline by *Streptomyces aureofaciens*, *p*-aminobenzoate synthase in the formation of candicidin by *Streptomyces griseus* and β-lactam synthetases in the production of cephalosporin by *Acremonium chrysogenum*[2].

Acknowledgments

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