Gentamicin Formation in *Micromonospora purpurea*: Stimulatory Effect of Ammonium

RINA GONZALEZ†, LAURA ISLAS, ANA-MARIA OBREGON,
LAURA ESCALANTE and SERGIO SANCHEZ

Departamento de Biotecnologia del Instituto de Investigaciones Biomèdicas,
Universidad Nacional Autonoma de Mexico,
Mexico D.F. 04510, Mexico

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The effect of ammonium on the fermentative production of gentamicin in *Micromonospora purpurea* has been studied using a chemically defined medium. Ammonium chloride concentrations ranging from 20 to 150 mM resulted in a proportional stimulation of growth and antibiotic formation. The use of other ammonium salts exerted a similar effect. Among the products of ammonium assimilation, glutamate and glutamine were able to exert the stimulatory effect. In addition, both amino acids reproduced the stimulation in resting cell systems of this microorganism and this result was not modified by the presence of chloramphenicol, eliminating a possible inductive action as the cause of this effect. The use of a glutamine synthetase inhibitor prevented antibiotic formation. This inhibition was reverted only by glutamine, suggesting that this amino acid was responsible of ammonium stimulation. Glutamine stimulation seems to be due to its ability to produce 2-deoxystreptamine and glucosamine, intermediates of the gentamicin biosynthetic pathway.

Gentamicin is a complex of aminocyclitol compounds containing three major moieties referred to as C1, C1a and C2. Among aminoglycosides, this antibiotic is the antimicrobial agent of choice for the treatment of several classes of infections caused by *Escherichia coli*, *Proteus* and *Pseudomonas*[1]. In spite of the industrial importance of gentamicin, little information is available with respect to its fermentative production and the factors and conditions that control its biosynthesis.

Nitrogen sources have long been known to suppress the biosynthesis of a variety of chemically unrelated antibiotics and other secondary metabolites[2]. The most common observation is a decrease in the levels of antibiotic produced in the presence of an excess of nitrogen source. Ammonium salts are the principal nitrogen sources which have been reported to interfere with antibiotic production[3]. Although a possible indirect effect of pH has not always been ruled out, the cases studied in more depth show that repression of enzymes involved in antibiotic production seems to be quite a common effect of ammonium[2].

Our group has been involved in studies on the factors and conditions controlling gentamicin formation in *Micromonospora purpurea*[4,5]. In this regard, the nitrogen content of the antibiotic moiety[1], suggested some type of nitrogen source control. The pattern of nitrogen source utilization by *M. purpurea* has been well established[6,7], however, there is no information concerning their influence on antibiotic production. This paper describes the stimulatory effect of ammonium on the synthesis of gentamicin and presents data indicating that ammonium increases antibiotic formation through its conversion to glutamine.

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[4]. 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): 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 $CoCl_2 \cdot 6H_2O$, 0.1 g K_2HPO_4 and the desired ammonium 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.

† Present address: Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-X, Box 23-181, México, D.F. 16000.

Additions to the Fermentation

After 48 hours fermentation, 1 mM methionine sulfoximine (MS), previously filter sterilized (with Millipore filters type HAWP) was added to cultures growing in the CD medium described above with 10 mM glutamate. Sterile glutamine, D-glucosamine and 2-deoxystreptamine (DOS) were added to the cultures 6 hours after MS addition. The cultures were returned to the shaker and at desired times 2 ml samples were withdrawn for further analysis.

Resting Cell Systems

To measure *de novo* synthesis of gentamicin, cultures were grown to early stationary phase (36 hours), washed with 2 volumes sterile distilled water and resuspended in 25 ml of the following resting cell medium: the salts of the CD medium in 0.05 M MOPS buffer pH 7.4 and the desired nitrogen source. When required, 50 $\mu g\,ml^{-1}$ chloramphenicol was utilized.

Glutamine Synthetase Activity

At 48 and 72 hours fermentation, cell free extracts were prepared and glutamine synthetase (EC 6.3.1.2) activity was measured as reported for *Streptomyces clavuligerus*[2]. Enzyme activities were expressed as units per mg of total cell protein. One unit (U) was defined as the amount of enzyme that produced 1 μmol glutamine per minute at pH 7.4 and 35°C.

Assay of Gentamicin

At specified intervals, the production of antibiotic was determined by an agar disk technique using *Bacillus subtilis* ATCC 6633 as the assay organism[4].

Growth Determination

Samples of mycelia were harvested, washed with 2 volumes 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 Ammonium on Gentamicin Formation

M. purpurea was able to grow and produce gentamicin in a chemically defined medium containing 20 mM NH_4Cl. Fig. 1 shows maximum growth and specific antibiotic production of this microorganism in fermentations with ammonium concentrations ranging from 20 to 150 mM. As shown in the figure, when ammonium concentrations higher than 20 mM were used, an increase in the growth and specific production of gentamicin, proportional to the amount of ammonium present in the culture medium was obtained. That is, although microbial growth was increased, the ability of that biomass to produce gentamicin was also stimulated (2.8 fold augment with the highest ammonium concentration in regard to that produced with 20 mM NH_4Cl). Concentrations higher than 150 mM significantly decreased cell growth and therefore were not utilized. As can be seen in the same figure, changes in the pH medium were not observed, excluding this possibility as the cause of gentamicin stimulation. The use of 150 mM of other ammonium salts (NH_4NO_3 and $(NH_4)_2SO_4$) also stimulated gentamicin formation (not shown).

After 72 hours of fermentation, the ammonium was totally consumed from the medium when added in low

Fig. 1. Maximum values for growth (○), specific gentamicin formation (△) and final pH of the medium (●) by *M. purpurea* grown in the presence of different ammonium concentrations.

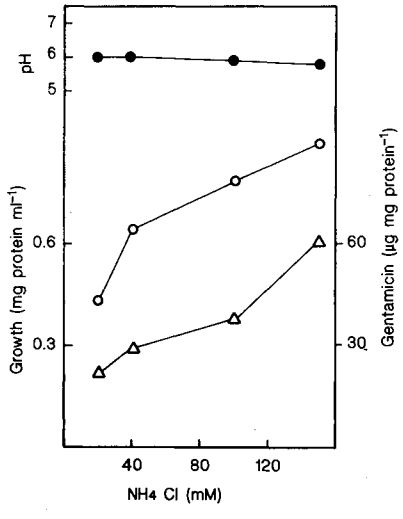
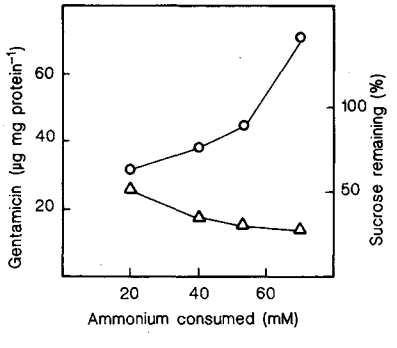


Fig. 2. Relationship between ammonium consumed *versus* gentamicin production (○) and sucrose remaining in the culture medium (△) at 72 hours of fermentation.



Cultures were grown in CD medium with ammonium ranging from 20 to 150 mM.

concentrations (20 and 40 mM). On the other hand, no more than 70% consumption was observed with concentrations higher than 40 mM, although a close relationship between the ammonium consumed and the maximum gentamicin production was maintained (Fig. 2). In addition, more than 25% of the initial sucrose concentration remained in the culture medium at the end of all fermentations, assuring an enough supply of the carbon source for growth and antibiotic formation.

Effect of Alanine, Glutamate and Glutamine on Gentamicin Formation

In order to establish whether or not ammonium itself was responsible of the stimulatory action, the amino acids alanine, glutamate and glutamine, products of ammonium assimilation in actinomycetes[2] were tested on the synthesis of gentamicin. As can be seen in Table 1, in regard to a control with 40 mM ammonium, glutamine and glutamate (10 mM) increased gentamicin formation 37 and 65%, respectively. As shown in the same table, glutamine also stimulated the microbial growth, a situation that was reflected in the antibiotic specific production values obtained with this amino acid. With the exception of alanine (60% consumption), the amino acids were efficiently taken up and consumed by the cells (not shown). On the other hand, alanine inhibited antibiotic biosynthesis and this effect could not be explained in terms of its lower consumption. In this regard, it has been reported that alanine inhibits glutamine synthetase (GS) in *Streptomyces clavuligerus*[8]. This enzyme produces glutamine from glutamate ATP and ammonium. Thus, under inhibitory conditions, one might expect a reduction in glutamine formation, condition which fitted well with the possible participation of this amino acid in the stimulatory effect of ammonium on antibiotic production.

Synthesis of Gentamicin by Resting Cell Systems Incubated with Glutamate and Glutamine

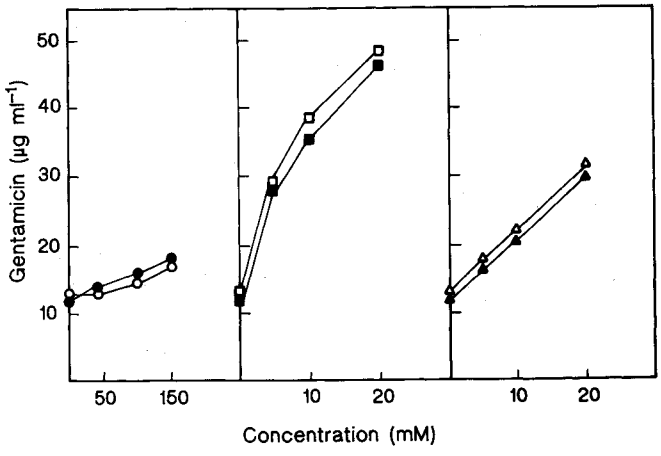
In order to further characterize the effect of glutamate and glutamine, the synthesis of gentamicin was determined using resting cell systems. The use of these systems allowed to distinguish between an induction or activation effect of the amino acids on antibiotic formation. As can be seen in Fig. 3, in regard to a control with ammonium, glutamate and glutamine again exerted a stimulatory effect on antibiotic formation (2.6 and 1.7 fold, respectively). As shown in the same figure, no differences were observed in the synthesis of gentamicin when a protein synthesis inhibitor was added to the resting conditions, thus eliminating an inductive action of these amino acids on the stimulation of antibiotic formation.

Table 1. Specific antibiotic produced by cultures supplemented with products of the ammonium assimilation.

Amino acid	Growth ($mg\,ml^{-1}$)	Specific antibiotic formation ($\mu g\,mg\ protein^{-1}$)
Control	0.45	35
L-Alanine	0.36	28
L-Glutamate	0.37	58
L-Glutamine	0.56	48

Fermentations were carried out during 144 hours in 40 mM NH_4Cl (control) supplemented or not with the amino acids (10 mM).

Fig. 3. Effect of different ammonium (●, ○), glutamate (■, □) and glutamine (▲, △) concentrations on gentamicin production in resting cell systems supplemented (dark symbols) or not with 50 $\mu g\,ml^{-1}$ chloramphenicol (light symbols).



Effect of Methionine Sulphoximine

To establish whether glutamate, glutamine or both were responsible for the stimulatory effect, gentamicin production was tested in the presence of methionine sulfoximine (MS), an irreversible inhibitor of GS activity[9]. For this purpose, cells growing with glutamate were exposed to the action of MS, thus preventing its conversion to glutamine. As revealed in Fig. 4, the addition of 1 mM MS at 48 hours fermentations stopped at once gentamicin formation. As expected, the GS activity was 75% reduced in cells exposed during 24 hours to the inhibitor (from 3.2 to 0.8 U $mg\ protein^{-1}$). Thus, this experiment suggested that glutamine, rather than glutamate, was directly involved in the stimulatory effect of ammonium on gentamicin formation.

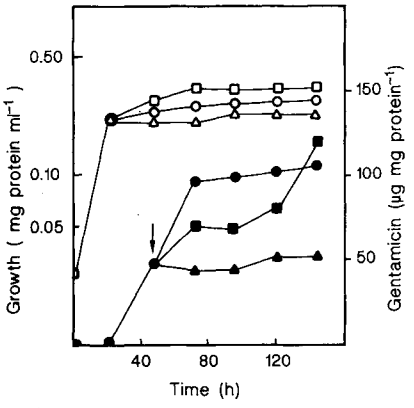
In order to confirm that glutamine was responsible for

the stimulatory effect, cells treated with MS were exposed to this amino acid (10 mM) in an attempt to recover gentamicin formation. As shown in the same figure glutamine stimulated growth and reverted the action of MS. Other amino acids like glutamate and alanine did not revert the effect (not shown). The delay in glutamine reversion was probably due to the stimulatory effect exerted by this amino acid on cell growth. In addition, this experiment suggested that rather than activating one or several steps in gentamicin biosynthesis, glutamine functions as an antibiotic precursor.

Addition of Intermediates of the Antibiotic Pathway

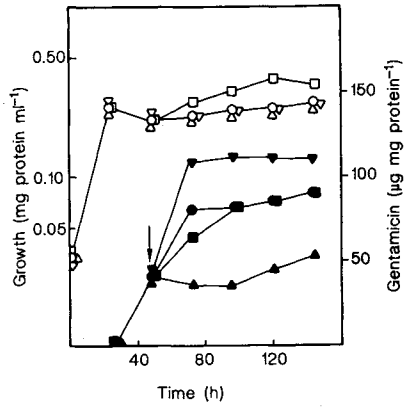
If glutamine works as precursor of either one or several intermediates of the gentamicin biosynthetic pathway, one can expect that the addition of such intermediates to cultures exposed to MS might also revert its inhibitory action. The candidates to test this possibility were D-glucosamine, aminodeoxy-inosose, and 2-deoxystreptamine (DOS), all of them transamination products obtained between glutamine and D-fructose-6-phosphate, deoxy-inosose, and aminodeoxy-inosose, respectively[10]. Due to limitations in the availability of aminodeoxy-inosose, cultures treated with MS were exposed only to DOS and D-glucosamine. These compounds were efficiently taken up by the cells and when added to 48 hours fermentations, both were able to overcome the negative effect of MS (Fig. 5). Therefore, the glutamine stimulation of gentamicin formation seemed to be due at least in part to its ability to transaminate with aminodeoxy-inosose and D-fructose-6-phosphate by the action of L-glutamine : keto-*scyllo*-inositol amino transferase and L-glutamine: D-fructose-6-phosphate amino transferase[11]. Stimulation by ammonium without the use of ammonium-trapping agents, represents an unusual phenomenon in the biosynthesis of antibiotics and other secondary metabolites. A similar effect of nitrogen flow has been reported for neomycin[12] and streptomycin[13] biosynthesis *i.e.* glutamate, glutamine and glucosamine stimulated antibiotic formation. A common feature of these compounds with gentamicine, is that all of them belong to the aminoglycoside group of antibiotics. Although in the neomycin and streptomycin reports, the right mechanism was not elucidated, the stimulatory effect probably takes place also by means of the formation of glutamine, amino acid precursor of DOS, streptamine and D-glucosamine. Therefore there are likely underlying common regulatory mechanism in their formation. In support of this view, we have recently observed a similar effect of ammonium on the synthesis of kanamycin, aminoglycoside antibiotic produced by *Streptomyces kanamyceticus* (unpublished results).

Fig. 4. Effect of 1 mM MS (▲, △) or 1 mM MS plus 10 mM glutamine (■, □) 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 CD medium with 10 mM glutamate.

Fig. 5. Effect of 1 mM MS plus 10 mM 2-deoxystreptamine (▼, ▽) and 1 mM MS plus 10 mM D-glucosamine (■, □) on the course of growth (light symbols) and specific gentamicin formation (dark symbols). Controls with (▲, △) and without MS (●, ○).



Additions were done in 48 hours cultures grown in CD medium with 10 mM glutamate.

Acknowledgments

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