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Regulation of spiramycin synthesis in *Streptomyces ambofaciens*: effects of glucose and inorganic phosphate

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Abstract The production of the 16-membered macrolide antibiotic, spiramycin, in *Streptomyces ambofaciens* is inhibited by glucose, 2-deoxyglucose and inorganic phosphate. The role of intracellular ATP content and phosphorylated metabolites as common regulating signals of both glucose and phosphate inhibitory effects is discussed. Two enzymatic targets of the effect of phosphate on spiramycin biosynthesis were studied. Valine dehydrogenase, the first enzyme of valine catabolism (supplier of aglycone spiramycin precursors), and alkaline phosphatase, which cleaves phosphorylated intermediates, were repressed in the presence of excess phosphate.

Introduction

In microorganisms, the formation of secondary metabolites is frequently inhibited by rapidly assimilated carbon sources, i.e. glucose and several other carbohydrates (Drew and Demain 1977). This phenomenon resembles catabolite repression in bacteria, but the basic mechanism(s) of this effect is not completely understood. Besides carbon catabolite regulation, the formation of many secondary metabolites is inhibited by inorganic phosphate concentration (Weinberg 1978). Among the elements added to fermentation media used in secondary metabolite production, phosphate is one of the most critical (Weinberg 1974). Many antibiotics are only produced at concentrations of inorganic phosphate suboptimal for growth. Phosphate in the range 0.3–500 mM permits excellent cell growth, whereas 10 mM phosphate often suppresses biosynthesis of antibiotics. Several mechanisms have been proposed to explain the effect of phosphate on the production of secondary metabolites (Martin 1977). (a) Phosphate favours primary metabolism; a shift down in primary metabolism derepresses secondary metabolism. (b) Phosphate shifts carbohydrate catabolic pathways. (c) Phosphate limits synthesis of the inducer of the antibiotic pathway. (d) Phosphate inhibits the formation of antibiotic precursors. (e) Phosphate inhibits or represses phosphatases necessary for antibiotic biosynthesis. (f) Phosphate suppresses antibiotic production by depriving the cell of an essential metal. It is clear from the above that phosphate concentration controls the differential expression of growth relative to antibiotic synthesis. It is unclear, however, how the nutritional message is transmitted to those mechanisms that control expression of antibiotic production genes. Is phosphate the ultimate effector, or does it simply regulate the level of an intracellular effector, which in turn controls antibiotic synthesis? Such phosphate-controlled intracellular effectors could include cyclic nucleotides, ATP, the adenylate energy charge, polyphosphates and highly phosphorylated adenine or guanine nucleotides.

In this study, the effects of glucose and phosphate on spiramycin production were investigated for the first time in *Streptomyces ambofaciens*. We attempted to understand, in part, the mechanisms involved in the negative influence on spiramycin biosynthesis caused by excess of glucose and high phosphate concentration.

Materials and methods

Microorganisms

These studies were carried out with *Streptomyces ambofaciens* RP 181110 (Rhône-Poulenc, Vitry-sur-Seine, France), a producer of spiramycin, and *Bacillus subtilis* ATCC 6633 as a control microorganism.

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Medium and culture conditions

Spores of *S. ambofaciens* were maintained in 20% glycerol at − 20° C. A 0.1-ml aliquot of this suspension was used to inoculate 50 ml seed medium and incubated for 48 h. The resulting seed culture was inoculated into the desired fermentation medium (4% v/v). All liquid cultures were conducted in 300-ml conical flasks containing 50 ml medium, at 28° C on a rotatory shaker at 250 rpm.

The basal medium, developed in our laboratory, contained (per liter) 1 g $MgSO_4$, 15 mg $ZnSO_4$, 2 g KH_2PO_4, 5 g $CaCO_3$, 20 g NaCl and 0.3 mg $CoCl_2$. The concentration and nature of the nitrogen and carbon sources added to the basal medium are described in the text.

Glucose and phosphate concentrations were varied as indicated in the text. 3-*O*-Methyl-D-glucose and 2-deoxy-D-glucose were added as sterile aqueous solutions.

Growth measurement

Determination of cell dry weight (CDW) was performed as described by Lebrihi et al. (1987). In the conditions used, 1 g CDW l^{-1} corresponded to an absorbance of 2 at 660 nm.

Dextrin, glucose and phosphate determination

Dextrins, glucose and phosphate in the medium were determined as described by Hanson and Phillips (1981), Miller (1959) and Kuzdzal-Savoie and Lebon (1971) respectively.

Intracellular content of ATP, DNA and RNA determination

ATP was measured by the luciferase assay as described by Jakubczak and Leclerc (1980). DNA and RNA were determined as described by Burton (1956) and Griswold et al. (1951) respectively.

Spiramycin determination

The spiramycin titre was estimated by the conventional disc method using *Bacillus subtilis* as test microorganism (Isaacson and Kirschbaum 1986).

Preparation of crude cell-free extracts

The mycelia obtained by filtration were washed twice with distilled water and then suspended in 15 ml 0.05 M TRIS/HCl buffer (pH 8.0) containing 30% glycerol. The mycelia were disrupted in an ice/water bath with a sonicater (Ultrasonics Inc.; model W225 R) for 5 min at 120 W. Cell fragments were removed by centrifugation at 12000 *g* for 30 min. The supernatant fluid was used as the enzymatic extract. The protein concentration of cell-free extracts was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Valine dehydrogenase

Valine dehydrogenase activity was assayed according to the method described by Schütte et al. (1985), originally described for leucine dehydrogenase. The 2-ml final reaction mixture contained 100 mM glycine/NaCl/NaOH buffer, pH 10.6, 50 mM valine, 0.5 mM NAD^+ and 100 µl enzyme solution. The reaction was started by the addition of cell-free extract and the mixture was incubated at 30° C. The control was performed in the same conditions without addition of valine. The increase in NADH was monitored at 340 nm. Enzyme activity is expressed as nmol product formed min^{-1} mg^{-1} protein.

Measurement of alkaline phosphatase activities

Intracellular alkaline phosphatase activities were measured on the cell-free extract, and extracellular phosphatase activities were measured on the filtered supernatant.

Phosphatase activities were measured as described by Shindelmeister and Pape (1981). Assay mixtures of 750 µl contained 400 µl TRIS/HCl pH 7.4, and 1.0 M and 250 µl 14.5 mM *p*-nitrophenyl phosphate. The reaction was started by addition of cell-free extract or supernatant (100 µl). The variation of the absorbance at 28° C was measured at 405 nm. The control was performed in the same conditions without addition of the enzymatic extract. One unit of enzymatic activity was defined as the quantity of enzyme that leads to a variation of 0.01 absorbance unit/min. The specific enzymatic activity was expressed in terms of UA mg^{-1} protein.

Chemicals

Spiramycin was supplied by Rhône-Poulenc Santé (France); white dextrins (weiß) by Prolabo and other reagents were obtained from Sigma and Fluka.

Results

Comparison of growth and spiramycin production on dextrins and glucose

Cultures of *S. ambofaciens* were grown in basal medium with either dextrins or glucose (30 g l^{-1}) as sole carbon source and valine (50 mM) as the nitrogen source. *S. ambofaciens* grew faster on glucose ($\mu_{max} = 0.075\ h^{-1}$) than on dextrins ($\mu_{max} = 0.04\ h^{-1}$). The final biomass obtained with dextrins and glucose was approximately similar (5 g l^{-1}). Glucose was consumed at a higher specific uptake rate ($q_{Glc} = 0.06$ g h^{-1} g^{-1} DCW) than dextrins ($q_{dex} = 0.03$ g h^{-1} g^{-1} DCW). In both cases, spiramycin production started after 72 h fermentation and continued with a maximum specific production rate (q_{sp}) of 0.4 and 0.2 mg h^{-1} g^{-1} DCW respectively with dextrins and glucose. With glucose, the maximum specific spiramycin production (8 mg g^{-1} DCW) was found to be approximately 66% weaker than that obtained with dextrins (24 mg g^{-1} DCW). With dextrins as carbon and energy source, the intracellular ATP content (measured at the beginning of spiramycin production, 72 h) was 60% weaker than with the rapidly assimilated carbon source, glucose (Table 1).

Effect of increasing glucose concentrations on growth and spiramycin production

Several fermentations with 50 mM valine as nitrogen source and increased concentrations of glucose (5, 20, 30, 40, 50, 100 g l^{-1}) as carbon source were carried out.

Table 1 Effect of carbon source on growth and spiramycin production in the basal medium containing 50 mM valine as nitrogen source and 30 g l^{-1} dextrins or glucose as carbon source. *DCW* dry cell weight, μ_{max} maximum specific growth rate, $q_{carbon\ source}$ specific uptake rate, q_{sp} maximum specific production rate

Parameter	Dextrins	Glucose
DCW_{max} (g l^{-1})	5.0	4.9
μ_{max} (h^{-1})	0.04	0.075
$q_{carbon\ source}$ (g h^{-1} g^{-1} DCW)	0.03	0.06
Volumetric spiramycin production (mg l^{-1})	120	40
q_{sp} (mg h^{-1} g^{-1} DCW)	0.4	0.2
ATP at the start of spiramycin production (pmol g^{-1} DCW)	6	14

Increasing the glucose to 40 g l^{-1} caused an increase in DCW and a decrease in pH. From these concentrations and above, DCW did not increase, indicating that glucose was no longer a growth-limiting factor for final biomass. The specific growth rate increased with carbon source concentration.

The specific spiramycin production rate (q_{sp} = 0.3 mg h^{-1} g^{-1} DCW) and the specific spiramycin production (14 mg g^{-1}DCW) were maximum at 20 g l^{-1} glucose. Above and beyond this concentration, production decreased rapidly. When glucose was increased to 100 g l^{-1}, spiramycin was produced slowly (q_{sp} = 0.05 mg h^{-1} g^{-1} DCW) and the specific spiramycin production decreased by 83% in regard with the control carried out on the basal medium with 30 g l^{-1} dextrins and 50 mM valine (Fig. 1).

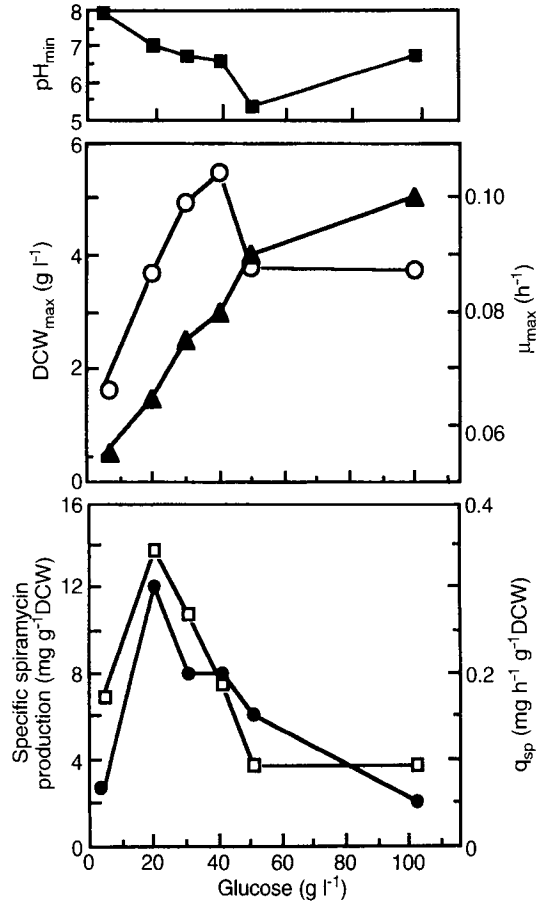

Fig. 1 Effect of increased initial concentrations of glucose on final biomass (O), pH (■), specific growth rate, μ_{max} (▲), specific spiramycin production (□) and specific spiramycin production rate, q_{sp} (●). *DCW* dry cell weight

Effect of glucose addition, at 56 h, on spiramycin production

We noted in the previous experiment that, when the concentration of glucose was increased, the specific growth rate increased and spiramycin production decreased (Fig. 1).

In order to verify that the negative effect exerted by glucose on spiramycin production was related to the carbon source concentration and not only to the increase in specific growth rate, several experiments, with addition of different concentrations of glucose (5, 20, 30, 40 and 60 g l^{-1}) at the end of exponential phase in conditions of nitrogen limitation (56 h) on basal medium with 15 g l^{-1} dextrins and 25 mM valine, were carried out. pH and DCW did not vary significantly. Addition of glucose to 20 g l^{-1} reduced the specific spiramycin production and q_{sp} respectively by 50% and 66% relative to the control (dextrins/valine). Increasing the concentration of glucose to 60 g l^{-1} decreased the specific spiramycin production more severely (a reduction of 75% when compared with no addition) and the fall in q_{sp} remained at approximately 73% (Fig. 2).

Effect of glucose and glucose derivatives on spiramycin formation

It is not known whether glucose or a glucose metabolite exerted this catabolite-repression-like effect. Therefore, the influence of two glucose derivatives, which did not support cellular growth, was studied. A comparison was performed between a fermentation carried out in basal medium containing 15 g l^{-1} dextrins and 25 mM valine (control) and three other fermentations in the same medium to which either 30 g l^{-1} glucose, or 30 g l^{-1} 3-*O*-methylglucose or 10 g l^{-1} 2-deoxyglucose had been added.

2-Deoxy-D-glucose is taken up and phosphorylated, but not metabolized further, by most bacteria. 3-*O*-methyl-D-glucose is transportable, but not phosphorylated (Dietz and Heppel 1971; Weber 1973). Both sugars were added to cultures of *S. ambofaciens* that had been grown in basal chemically defined medium, with 15 g l^{-1} dextrins and 25 mM valine for 56 h. 2-Deoxyglucose suppressed spiramycin synthesis. Glucose inhibited the formation of antibiotic by 46% while the threefold amount of 3-*O*-methylglucose did not

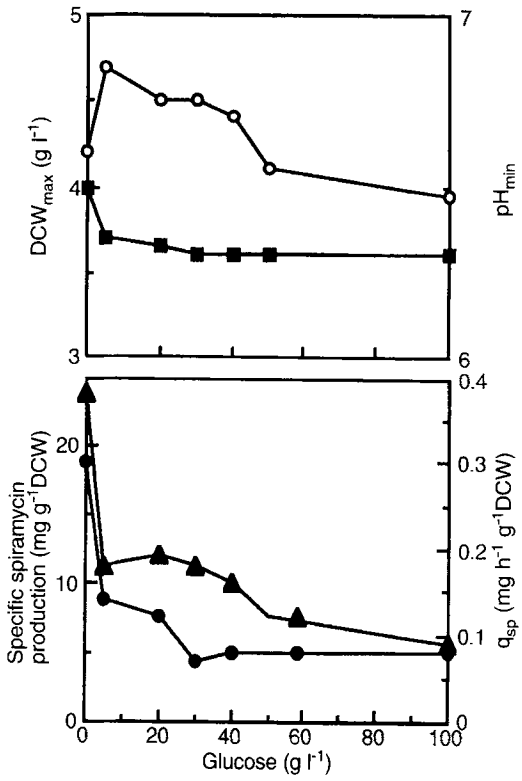

Fig. 2 Effect of addition, at 56 h, of different concentrations of glucose on final biomass (○), pH (■), specific spiramycin production (▲) and specific spiramycin production rate (●)

Table 2 Effect of glucose and glucose derivatives (2-deoxy-D-glucose and 3-*O*-methyl-D-glucose) on spiramycin formation using the basal medium containing 50 mM valine and 30 g l^{-1} dextrins

Additions at 56 h	Specific spiramycin production (mg g^{-1} DCW)
Glucose (30 g l^{-1})	13
3-*O*-Methyl-glucose (30 g l^{-1})	26
2-Deoxyglucose (10 g l^{-1})	0
Control (dextrins/valine)	24

affect spiramycin production. The results indicated that phosphorylation of the sugar appeared to be necessary for the inhibitory action.

Effect of initial phosphate concentration on growth and spiramycin production

Like several other antibiotics, the formation of spiramycin was also regulated by the concentration of inorganic phosphate in the medium. Several fermentations with 25 mM valine as nitrogen source, 15 g l^{-1} dextrins as carbon source and increasing concentrations of phosphate (10, 15, 20, 25, 30, 50 mM) were carried out.

Vegetative growth increased with the initial phosphate concentration up to 5 mM, a further increase of phosphate supply having no significant effect on cell yield. The specific spiramycin production, however, showed a sharp decline at phosphate concentrations above 10 mM. Beyond 10 mM initial phosphate, the concentration of residual phosphate detected was increased as the initial concentration increased (Fig. 3).

Dependence of spiramycin biosynthesis on addition time of inorganic phosphate

The addition of phosphate (100 mM) to *S. ambofaciens* cultures, with 25 mM valine and 15 g l^{-1} dextrins during the active growth phase and before the onset of spiramycin biosynthesis caused an important decrease in antibiotic production (about 60% reduction). However addition of phosphate at 72 h or 96 h, i.e. at or after the onset of antibiotic production respectively, had no significant effect on spiramycin production (Fig. 4).

Spiramycin production, biomass formation, intracellular content of ATP, DNA, RNA and proteins in high- and low-phosphate cultures

Two fermentations carried out on the basal chemical medium with dextrins (15 g l^{-1}) and valine 25 mM in the presence of 14 mM and 100 mM phosphate were compared.

In high phosphate concentrations (100 mM), the growth was accelerated (Fig. 5a) and spiramycin production was suppressed (Fig. 5b).

At the active phase of growth, the intracellular ATP content was higher in the presence of excess phosphate than in the presence of 14 mM. Afterwards the concentration of ATP showed a similar profile in both media. However, the level dropped in the presence of 14 mM phosphate during intensive phase of spiramycin production, while it remained at the same high level in the

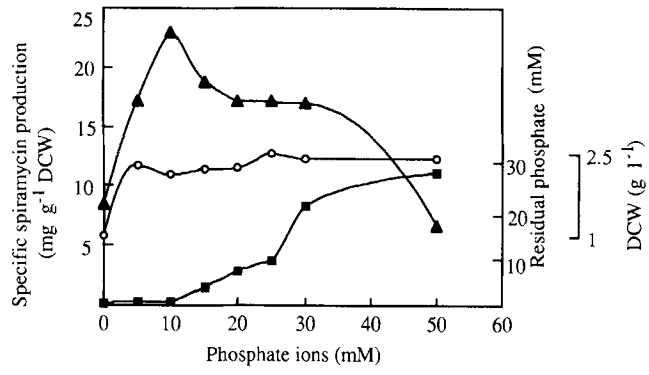

Fig. 3 Effect of increased initial concentrations of phosphate on final biomass (○), residual phosphate (■) and specific spiramycin production (▲)

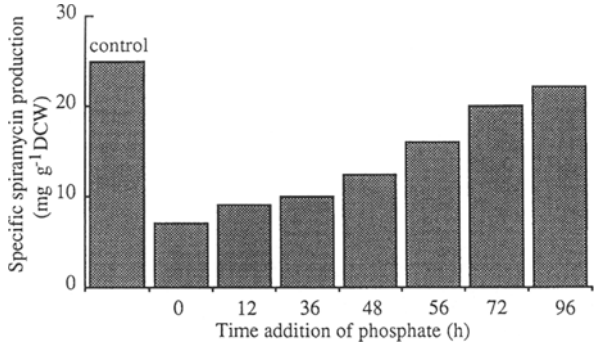

Fig. 4 Dependence of spiramycin biosynthesis on addition time of phosphate

presence of 100 mM phosphate where spiramycin was not synthesized (Fig. 5c). The time course of ATP concentrations observed in our study was similar to the data reported by Madry et al. (1979) and Liras et al. (1977) for *Streptomyces* T59 and *S. griseus* respectively for tylosin and erythromycin biosynthesis. The cellular ATP concentration has been suggested to be an effector of phosphate regulation in candicidin biosynthesis (Martin 1977).

Throughout all the fermentations, extracellular protein evolution was similar in both media, but the concentration of the proteins was higher in the presence of 100 mM phosphate (Fig. 5d).

DNA content was more important in cells cultured in the presence of 100 mM phosphate during the growth phase and was similar at stationary phase in both media (Fig. 5e).

Growth of the culture in medium containing 100 mM phosphate was characterized by a superior synthesis of RNA. RNA content was higher at the active growth phase and dropped at stationary phase (Fig. 5f). The drop of RNA, characteristic of the end of trophophase, appeared to release antibiotic production. These results were in agreement with those obtained by Mertz and Doolin (1973) and Liras et al. (1977).

Effect of phosphate concentration on valine dehydrogenase biosynthesis

The effect of valine dehydrogenase, the first enzyme in the catabolic pathway of valine (supplier of aglycone spiramycin precursors), which catalyzes the deamination of valine to 2-oxoisovalerate, was examined.

The specific activity of the enzyme, measured in basal medium containing dextrins (15 g l^{-1}) and valine (25 mM) with either 14 mM or 100 mM phosphate, is reported in Fig. 5g.

The evolution profils of valine dehydrogenase activity were similar in both media. It increased during the active growth phase and reached a maximum of 80 nmol min^{-1} mg^{-1} protein at 48 h in the presence of 14 mM phosphate, whereas the maximum was only 50 nmol min^{-1} mg^{-1} protein in medium with 100 mM phosphate. At the end of growth, valine dehydrogenase activity fell rapidly and stabilized during the stationary phase at 40 and 20 nmol min^{-1} mg^{-1} protein in 14 mM and 100 mM phosphate-containing media respectively.

Effect of phosphate concentration on alkaline phosphatase activities

Several groups have reported that phosphatase, which cleaves phosphorylated intermediates, could be a potent enzymatic target for the action of inorganic phosphate. So the effect of phosphate on phosphatase activities was studied in *S. ambofaciens*, a producer of spiramycin.

Figure 5h, shows the evolution of intracellular and extracellular alkaline phosphatase activities measured throughout the experiment. Extracellular and intracellular phosphatase activities were detected at low levels at the growth phase and rose to their maximum during the intensive phase of spiramycin production. In medium containing 14 mM phosphate, where the total yield of spiramycin was 24 mg g^{-1}DCW, alkaline phosphatase activities were clearly higher than in phosphate (100 mM)-containing medium where spiramycin production was nil. The maximum extracellular alkaline phosphatase activities were 85 UA mg^{-1} protein in 14 mM phosphate, whereas in 100 mM phosphate they were 15 UA mg^{-1} protein. Intracellular alkaline phosphatase activities evolved to the same pattern in both media until 72 h. Afterwards, these activities reached a maximum of 70 UA mg^{-1} protein in the presence of 14 mM phosphate when spiramycin was produced intensively, whereas they dropped in the presence of 100 mM where spiramycin was not produced.

Discussion

In low-phosphate medium (14 mM), the kinetics of growth and spiramycin production varied according to the energy source. Dextrins, a carbon and energy source slowly assimilated, favoured spiramycin production when compared with glucose. This was probably due to the slow and continuous hydrolysis of dextrins, which created energy-limiting conditions. This view was supported by the intracellular ATP content, which was higher with glucose. In addition, with glucose as sole carbon and energy source, the specific growth rate was higher than that obtained with dextrins. As the initial concentrations of glucose were increased, the specific growth rate increased and spiramycin production dropped. The inhibitory effect of glucose was not

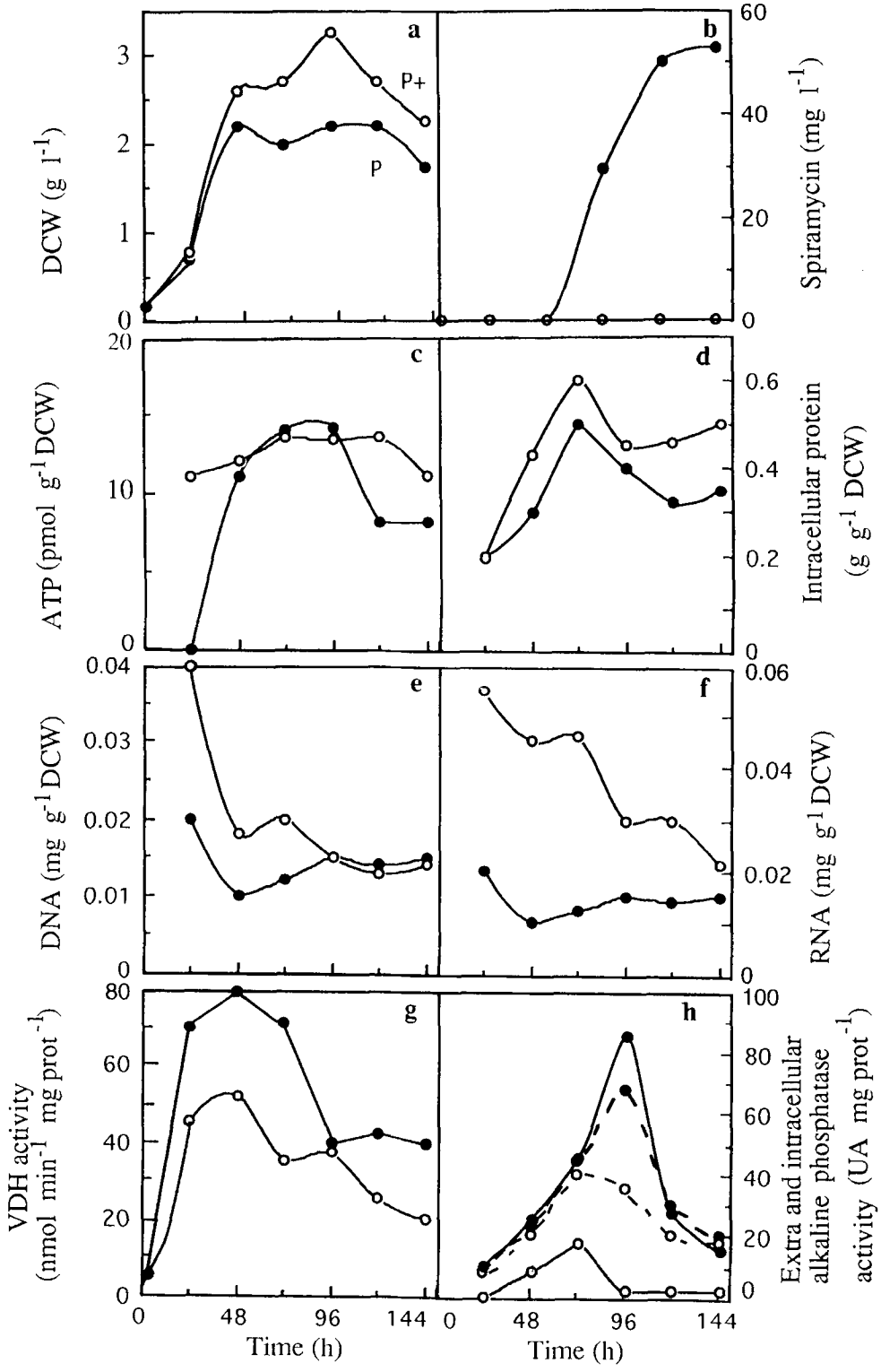

Fig. 5a–h Kinetics of growth (**a**), spiramycin formation (**b**), intracellular ATP content (**c**), intracellular proteins (**d**), DNA synthesis (**e**), RNA synthesis (**f**), valine dehydrogenase specific activity (**g**), extra (——) and intracellular (---) alkaline phosphatase specific activities (**h**) in high- (100 mM) (○) and low-phosphate (14 mM) (●) cultures

due to trivial effects, such as acid production or oxygen limitation, because the pH was relatively constant and because the negative effect occurred at low biomass concentration where oxygen could not have been limiting.

Addition of glucose in conditions of nitrogen limitation (at the end of the exponential phase) showed that the inhibitory effect of glucose on spiramycin biosynthesis was not only a consequence of an increase in specific growth rate. The intensity of the negative effect of glucose on spiramycin biosynthesis depended upon the concentration. It seemed that spiramycin biosynthesis and cell growth are both controlled by energetic metabolism but by different means.

We found that inorganic phosphate also inhibited spiramycin production. Several studies showed that antibiotic production in *Streptomyces* was sensitive to phosphate control (Martin 1977; Vu-Trong et al. 1981; Lebrihi et al. 1987). Payne and Wang (1988) also showed that a low continuous phosphate concentration ($0.1\ g\ l^{-1}\ day^{-1}$) in the culture maintained the metabolic activities of the cell at a good level and cycloheximide production was optimum. By contrast, highest phosphate concentration ($0.2\ g\ l^{-1}\ day^{-1}$) strongly stimulated the glucose pathway metabolism and decreased cycloheximide production.

Our results showed that high concentrations of ATP were detected with the rapidly assimilated carbon source, glucose, and in the presence of excess in phosphate, and this was correlated with reduction or suppression of spiramycin production. A close relationship probably existed between the effect of carbon and energy source and the phosphorylated metabolite. In fact, inhibition of spiramycin formation by glucose (catabolite regulation) required phosphorylation of the sugar. Further metabolism of the sugar phosphate was apparently not necessary; 2-deoxyglucose (which was probably phosphorylated to 2-deoxy-6-phospho-D-glucose but not further metabolized) was a very potent inhibitor of spiramycin synthesis. In contrast, addition of 3-*O*-methyl-D-glucose, which was assimilated but not phosphorylated, had no inhibitory effect on spiramycin biosynthesis. The present data are in agreement with those of Madry et al. (1979) which suggested a role for glucose-6-*P* as a regulating signal of both glucose- and phosphate-inhibitory effects in *Streptomyces* T59, a producer of tylosin.

We have shown that the negative effect of phosphate on spiramycin production acted at different levels of cellular metabolism. In the presence of an inhibitory concentration of phosphate, parallel to an increase of intracellular ATP content, the production of biomass, intracellular proteins, DNA and RNA was increased. Phosphate ions seemed to stimulate the process of primary metabolism at the expense of secondary metabolism.

An enzyme of primary metabolism, valine dehydrogenase, was partially repressed in the presence of high concentrations of phosphate. This type of phosphate effect can be considered as an effect on primary metabolism, leading to altered secondary metabolite formation, probably via a limitation of precursors necessary for the synthesis of the secondary metabolite.

In *S. fradiae*, a producer of tylosin, propionyl-CoA (a precursor of tylosin) is formed by catabolism of amino acids (Dotzalf et al. 1984). In this organism, valine dehydrogenase, which converts valine to 2-oxoisovalerate, is regulated by phosphate (Omura and Tanaka 1986). Madry et al. (1979) suggested that phosphate affected tylosin biosynthesis by inhibiting fatty acid degradation, thereby leading to a decreased supply of precursors for the formation of tylonolide; later studies, however, indicated that phosphate principally affected the activity of enzymes specifically involved in tylosin biosynthesis: methylmalonyl-CoA transcarboxylase (Vu-Trong et al. 1980), propionyl-CoA carboxylase (Vu-Trong et al. 1981) and protylonolide synthetase (Omura et al. 1984).

Several reports have stated that phosphatase could be regulated via feedback inhibition or repression by inorganic phosphate. In *S. ambofaciens* we found that the activity of alkaline phosphatase depended significantly on the concentration of phosphate in the medium. A good correlation could be established between the increase of phosphatase activity and the intensive phase of spiramycin production. A similar effect has been described for *Streptomyces* T59 and for *Serratia marscecens*, where the synthesis of tylosin and prodigiosin, respectively, were regulated by phosphate (Madry et al. 1979). Phosphate inhibition of alkaline phosphatase in *S. ambofaciens* may be responsible for the inhibition of spiramycin formation, although no phosphorylated intermediaites have been reported so far. These results are in agreement with those described by Mertz and Doolin (1973) where a similar effect has been noted in *S. orientalis* as a vancomycin-producing strain. The inhibition of streptomycin biosynthesis by phosphate was attributed to the inhibition of phosphatase participating in the streptomycin pathway (Demain and Inamine 1970; Walker and Walker 1971). A biosynthetic role for phosphatase in the largely unknown pathway to macrolide antibiotics would be highly hypothetical.

On the other hand, we showed that there was a critical phase (between 0 and 48 h), during which an excess of phosphate ions had a maximum inhibitory effect on spiramycin biosynthesis. This phase could correspond to the expression phase of certain genes during cellular differentiation and spiramycin biosynthesis. Thus, addition of phosphate in excess in the growth phase would lead to repression of these genes, provoking an important diminution of spiramycin production.

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