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# *Aspergillus niger* cyclic AMP levels are not influenced by manganese deficiency and do not correlate with citric acid accumulation

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**Summary.** The role of intracellular levels of cyclic AMP in the control of citic acid accumulation by *Aspergillus niger* has been investigated. For this purpose, *A. niger* was grown in media containing either high (14%, w/v) or low (2%, w/v) concentrations of sucrose, supplemented with 10 $\mu M$ $Mn^{2+}$ (manganese-sufficient) or not (manganese-deficient), to obtain conditions leading to variable citrate accumulation. Citric acid accumulation was only observed in high-sugar, manganese-deficient medium. Intracellular levels of cyclic AMP were significantly higher in mycelia grown on low-sugar media, but were not significantly influenced by the absence of manganese ions. When sucrose in the high-sugar medium was substituted by other mono- or disaccharides, similar intracellular concentrations of cyclic AMP were observed. However, citric acid accumulation was only significant with sucrose, glucose and fructose. It is thus concluded that the intracellular level of cyclic AMP is not causally related to the accumulation of citric acid by the fungus, and — noteworthy — is not affected by manganese deficiency (despite adenylate cyclase reputed to be a manganese-requiring enzyme).

## Introduction

Adenosine-3,5-monophosphate (cyclic AMP) has been studied as an intracellular regulator in a wide variety of organisms, including some filamentous fungi. Phenomena that appear to be controlled by internal levels of cyclic AMP include utilization of exogenous carbon sources, conidiation, dimorphism, phototropism and lipid synthesis (Cantore et al. 1980; Pall 1981, 1977; Vaidya and Khuller 1988). In *Aspergillus niger*, cyclic AMP has been postulated to be involved in citric acid accumulation (Al Obaidy and Berry 1980; Wold and Suzuki 1973a). However, evidence for this has been obtained only by studying the effect of addition of exogenous cyclic AMP on *A. niger* citric acid production during growth on media containing a low (0.6%, w/v) concentration of sugar, which does not allow the accumulation of large amounts of citric acid.

We have previously found that addition of cyclic AMP to *A. niger* cultivated on media containing 14% (w/v) sucrose as carbon source was without any effect (Kubicek and Röhr 1986). An explanation for this may be that high concentrations of cyclic AMP are already present under these conditions. However, one of the key conditions for the occurrence of citric acid accumulation is a severe deficiency of manganese ions in the nutrient medium (Kubicek and Röhr 1977, 1986), which contrasts with the fact that adenylate cyclase (ATP pyrophosphate lyase [cyclizing]; EC 4.6.1.1.), the enzyme forming cyclic AMP from ATP, has been reported to require manganese ions (Flawia and Torres 1972; Gomes and Maia 1979; Pall 1981). Hence one might assume that citric-acid-producing mycelia should contain lower amounts of cyclic AMP than those cultivated in a full (manganese-ions-containing) medium.

The present study was undertaken in view of this apparent contradiction. It will be shown that citric acid accumulation in *A. niger* does not correlate with high internal levels of cyclic AMP. Moreover, cyclic AMP levels are unaffected by the concentration of manganese ions in the nutrient medium.

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## Materials and methods

*Strain and culture conditions. Aspergillus niger* ATCC 11414 was grown in shake flasks using Shu-Johnson medium (Shu and Johnson 1948), containing decationized sucrose (Kubicek and Röhr 1977) (20 or 140 g/l, as indicated) as a carbon source; manganese ions (as $MnCl_2 \cdot 2H_2O$) were omitted from one series of flasks, whereas 10 μ*M* (final concentration) were added to a second series.

*Extraction and quantification of intracellular and extracellular cyclic AMP.* Mycelia were harvested at several points during growth, washed with cold (4° C) water and frozen in liquid nitrogen. This procedure was completed in less then 60 s. Cyclic AMP was extracted with acid as described by Cantore et al. (1980), and quantified by the aid of a cyclic AMP test kit (Amersham International, Bucks, UK) according to the suppliers recommendations. Cyclic AMP from the culture fluid was assayed directly without any pretreatment.

*Analytical procedures.* Fungal growth was quantified by measuring the dry weight of carefully washed mycelia as described by Kubicek and Röhr (1977). Citric acid in the medium was quantified as described previously (Xu et al. 1989). The total concentration of sugar in the medium was determined by the phenol-sulphuric acid method (Dubois et al. 1956).

## Results and discussion

In order to determine the levels and time-course of cyclic AMP in *A. niger* under "normal" conditions of cultivation, growth, external and internal concentrations of cyclic AMP were measured for a period of 10 days in the "low-sugar" medium (Fig. 1a–c). Manganese-deficient growth exhibited a somewhat delayed peak of cyclic AMP (at day 4), which however correlated with the slower growth under these conditions. Maximal cyclic AMP levels were comparable in both cultures. The decrease in cyclic AMP during the stationary phase of growth was similar, but occurred 1–2 days later in the manganese-deficient medium. It should be noted that the intracellular concentrations found in *A. niger* during these studies resemble those found in most other fungi (Cantore et al. 1980; Pall 1981; Vaidya and Khuller 1988). Cyclic AMP was also detected in the culture medium, reaching a peak after 3 and 4 days, respectively, whereafter a rapid decline was noted (Fig. 1c).

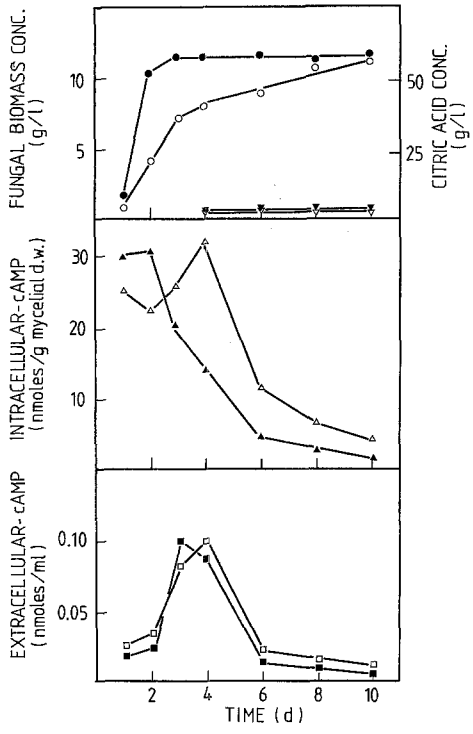


**Fig. 1.** Growth (○, ●), citric acid production (▽, ▼), extracellular (□, ■) and intracellular (△, ▲) cyclic AMP levels during cultivation of *Aspergillus niger* in media containing 2% (w/v) sucrose. The presence or absence of manganese ions is indicated by full and empty symbols, respectively. Values are means of at least two separate experiments that yielded consistent results

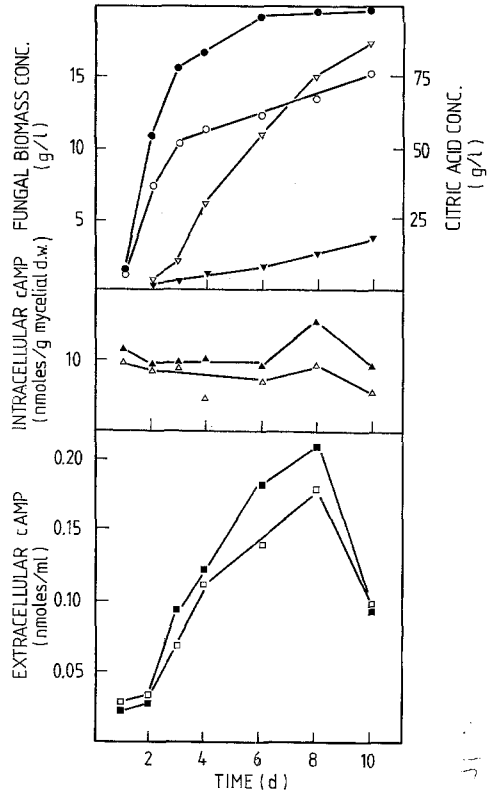


**Fig. 2.** Growth, citric acid production, external and internal levels of cyclic AMP in *A. niger* during cultivation in 14% (w/v) sucrose medium. Symbols as in Fig. 1

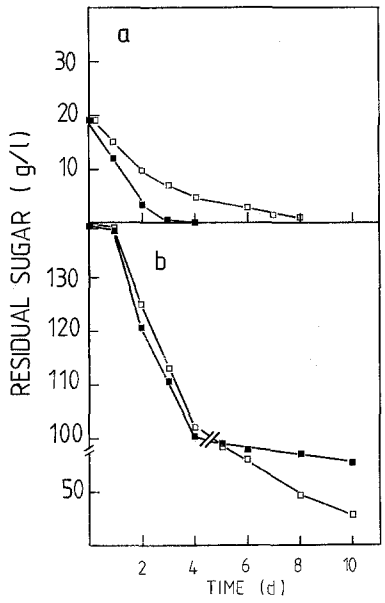


**Fig. 3.** Concentration of total sugar in the medium during cultivation of *A. niger* in media containing 14% (w/v) **(a)** and 2% (w/v) **(b)** sucrose, in the presence or absence of manganese ions (indicated by full and empty symbols, respectively)

In order to assess the concentrations of cyclic AMP under conditions of citric acid fermentation, the experiment shown in Fig. 1 was repeated, but the concentration of the carbon source was increased to 14% (w/v), since citric acid accumulation is only induced when the carbohydrate concentration exceeds 50 g/l (Xu et al. 1989). The results obtained are shown in Fig. 2 a–c. We found a strikingly lower concentration of cyclic AMP present in *A. niger* during the first 6 days of cultivation under these conditions, which was only marginally influenced by the exogenous presence of manganese ions. Unlike the low-sugar cultures, however, its level peaked during the late phases of cultivation. Mycelia grown in the presence of this high sugar concentration continued secreting cyclic AMP also during the stationary phase of growth. Citric acid accumulation, as expected, was strongly reduced in the manganese-containing medium.

**Table 1.** Intracellular concentration of cyclic AMP in *Aspergillus niger* during cultivation on high concentrations (14%, w/v) of various carbohydrates (manganese deficiency)

| Carbohydrate | Intracellular cyclic AMP (nmol/g dry weight) | | Residual sugar (g/l, 96 h) |
|---|---|---|---|
| | 48 h | 96 h | |
| Sucrose | 9.4 | 7.4 | 76 |
| Glucose | 10.2 | 8.7 | 82 |
| Fructose | 8.8 | 6.9 | 93 |
| Maltose | 9.3 | 8.3 | 72 |
| Lactose | 8.4 | 8.0 | 115 |
| Cellulose | 9.1[b] | 8.9[b] | nd[a] |
| Xylose | 8.8 | 6.7 | 108 |
| Galactose | 9.1 | 9.7 | 127 |

[a] Not determined
[b] Values for cellulose are related to 200 mg fungal intracellular protein extracted and determined as described by Kubicek et al. (1979)

In order to find out whether these different concentrations of cyclic AMP reflect different rates of sugar uptake, the concentration of sugars (sucrose, glucose and fructose) in the medium was quantified (Fig. 3 a and b). Coincident with previous data (cf. Kubicek and Röhr 1986), the uptake of sugars was more rapid in the high-sugar medium. Interestingly, the absence of manganese ions accelerated sugar uptake in the high-sugar medium, but retarded it in the low-sugar medium.

Since different sugars exhibit differential effects on citric acid production by *A. niger* (Xu et al. 1989), we have also investigated whether the mycelial levels of cyclic AMP would be different during growth of the fungus on sucrose, glucose, fructose, maltose, lactose, cellulose, xylose or galactose (all at 14%, w/v). Mycelial levels of cyclic AMP were all within the same range of concentrations (Table 1). Also, we have not noted significant differences in growth (except for galactose, which permitted only very poor growth, and slower growth on cellulose and lactose). However, the production of citric acid by the fungus was strikingly different on these different sugars, in accordance with recent reports (Xu et al. 1989).

The results presented are noteworthy for two reasons: firstly, they show that the synthesis of cyclic AMP by *A. niger* is unaffected by the absence of $Mn^{2+}$ ions from the nutrient medium. Since the concentration of manganese ions introduced by the impurities in the inorganic nutrients and from the conidial inoculum was less than $10^{-8}$ *M* (cf. Kubicek and Röhr 1986), it is unlikely that this may have led to the presence of still saturating concentrations of this ion in *A. niger*; the $K_m$ of adenylate cyclase for $Mn^{2+}$ is in the range 0.5–1.5 m*M* (Flawia and Torres 1972; Gomes and Maia 1979).

Unfortunately the adenylate cyclase from *A. niger* has only been marginally described (Wold and Suzuki 1974) and we do not know whether it actually requires $Mn^{2+}$ ions. A lack of requirement for manganese ions has been reported for the enzyme from *Phycomyces blakesleeanus* (Cohen et al. 1980). It is however possible as well,

as Pall (1981) originally suggested, that the in-vitro requirement of adenylate cyclase for manganese ions is an artefact. Cell-free extracts from *Neurospora crassa,* when prepared under certain conditions, exhibit an activity which only requires $Mg^{2+}$ and not $Mn^{2+}$ ions (Pall 1981).

Concerning the postulated relationship between cyclic AMP levels and citric acid accumulation, our experiments failed to obtain support for this. The levels of cyclic AMP were of the same size under conditions of low and high citric acid production (i.e. in the presence and absence of $Mn^{2+}$ ions) and hence cannot be causally related to the mechanism of citric acid accumulation. Such an assumption is further supported by our lack of finding different concentrations of cyclic AMP upon growth of *A. niger* on sugars of different suitability for citric acid production. Hence, a particular mycelial concentration of cyclic AMP is not obligatory for citric acid accumulation. Furthermore, it was observed that in *A. niger* growing on media containing 14% (w/v) sucrose, lower concentrations of cyclic AMP were present in the mycelia than in those growing on 2% (w/v) sucrose. This was not correlated with faster growth or a higher rate of sugar uptake under the latter conditions; sugar uptake and metabolism was actually considerably more rapid in the high-sugar-containing media. The lack of correlation between sugar uptake, citric acid accumulation, and intracellular cyclic AMP levels was also evident from the findings of similar levels of cyclic AMP in mycelia of *A. niger* grown on a variety of sugars that are taken up at different rates and allow different yields of citric acid.

Hence the present data suggest a reciprocal relationship between cyclic AMP levels and the glucose supply in *A. niger*, which is in contrast to Pall's postulation of cyclic AMP as a stimulator of glycolysis in fungi (Pall 1981). This control involves phosphorylation by a cyclic-AMP-dependent protein kinase of phosphofructokinase 2, which forms fructose-2,6-bisphosphate, an activator of one of the key regulatory enzymes of glycolysis, phosphofructokinase 1. It should be noted however that phosphofructokinase 2 is activated by a cyclic-AMP-dependent protein kinase in *Saccharomyces cerevisiae,* but inactivated in higher eukaryotes (Hers et al. 1985). In *N. crassa,* fructose-2,6-bisphosphate levels only weakly correlate with changes in the cyclic AMP levels (Dumbrava and Pall 1987). The relationship between fructose-2,6-bisphosphate levels, carbohydrate supply and citric acid accumulation has recently been studied in our laboratory (E. M. Kubicek-Pranz et al., manuscript submitted), and elevated concentrations were found in mycelia cultivated on media containing high sugar concentrations.

While these data appear to rule out a role for cyclic AMP in citric acid accumulation by *A. niger*, it remains to be explained why the addition of cyclic AMP stimulates the formation of citric acid in *A. niger* when growing on media containing low (0.6%, w/v) sucrose concentrations (Wold and Suzuki 1973a). No conclusive answer can be given at the moment, but one should be aware that the addition of cyclic AMP results in a drastic alteration in the morphology of *A. niger* from filamentous to pellet growth (Wold and Suzuki 1973b, also our unpublished observations). There is a possibility that the cyclic AMP effect is primarily via an effect on morphology, but this clearly requires further investigation.

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