CYCLIC AMP AND CITRIC ACID ACCUMULATION BY ASPERGILLUS NIGER

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Summary

Aspergillus niger accumulated citric acid in the medium under certain conditions. Cyclic AMP concentrations of the order of 10^{-6}M and higher caused an increase in the rate of citrate synthesis. Adenosine, ATP, and cyclic GMP at 10^{-3}M also stimulated, but were ineffective at 10^{-4}M. 5'-AMP had no effect while 5'-GMP and guanosine inhibited slightly. ADP showed a 42% inhibition. Theophylline enhanced the cyclic AMP effect. It is proposed that citric acid accumulation by Aspergillus niger may result from abnormal cyclic AMP metabolism.

The accumulation of extracellular citric acid by fungi was discovered by Wehmer in 1893 (1), and most of the commercial citric acid produced today is by fermentation by Aspergillus niger (2). Although aspects of the fermentation have been extensively investigated (2,3), the etiology of the process is not understood. Citrate is produced only under certain conditions, and it is generally believed that accumulation results from induced abnormalities in the metabolism of the mould.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) is known to be a metabolic regulator in a wide variety of systems (4, 14). In this report we demonstrate that cyclic AMP induces a marked stimulation of citrate accumulation.

Materials and Methods

A. niger NRC A-1-233, obtained from the National Research Council of Canada, was used in these studies.

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The whole cell experiments were performed in 125 ml Erlenmeyer flasks washed in 6N HCl. Each flask was filled with 55 ml of medium consisting of (g/l): sucrose, 8.0; NH_4NO_3, 2.5; KH_2PO_4, 2.5; $MgSO_4$, 0.25. The media was adjusted to pH 3.5 with HCl, and autoclaved. Only triple glass distilled water or Super Q water (Millipore Corp.) was used.

Conidia suspensions were prepared by washing conidia from agar slants with a 0.5% Tween 80 solution. Each flask was inoculated with 6-12 x 10^6 conidia and incubated at 28°C on a rotary shaker at 220 rpm.

Cyclic AMP and other effecters were added directly to the medium after 16-20 hours growth unless otherwise specified. Germination had been completed by this time. Normally the mycelia were harvested after 48-50 hours growth. Media samples were taken and analysed for sucrose and citrate.

Sucrose was determined by the anthrone method (5). Citrate was determined enzymatically by a couple of citratase (prepared from Aerobacter aerogenes) and malic dehydrogenase (6).

The mycelial pellets were filtered on cloth, washed, dried, and weighed. The citrate produced is expressed as specific activity. This is defined as the micromoles citrate produced per gram dry weight of mycelia.

Results

Citrate appeared in the medium after about 30 hours growth and continued to increase at a linear rate until the sucrose was exhausted (Fig. 1). Addition of cyclic AMP at zero hours (not shown) or 16 hours resulted in increased rates of citrate synthesis and sucrose utilization. Addition

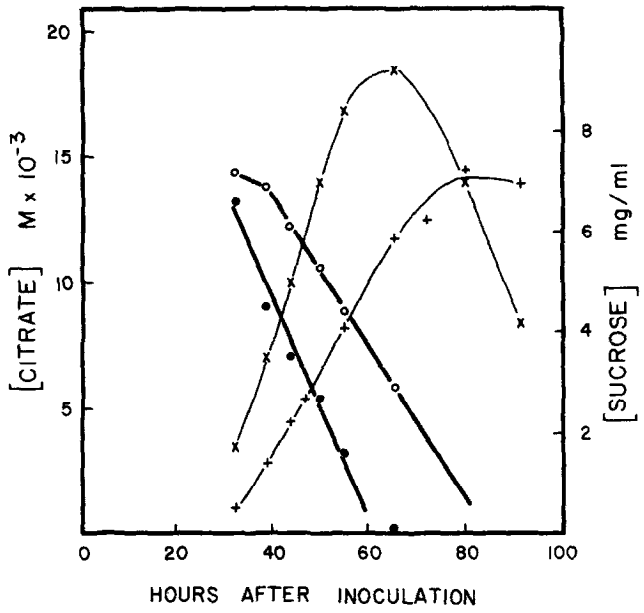

Figure 1 Stimulation of citrate production and sucrose utilization by cyclic AMP (10^{-3}M) when added after 16 hours growth. Cyclic AMP cells: citrate production (x) and sucrose utilization (•). Control cells:citrate production (+) and sucrose utilization. (o)

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of cyclic AMP or theophylline after 40 hours growth also increased synthesis (Fig. 2). Adenosine (1 and 2 x 10^{-3}M) caused a slight stimulation, but 5'-AMP (1 and 2 x 10^{-3}M) had no effect.

The influence of various adenine and guanine nucleotides are shown in Table I. Adenosine, ATP, and cyclic GMP stimulated at 10^{-3}M but were ineffective at 10^{-4}M (not shown). 5'-AMP had no effect while 5'-GMP and guanosine were slightly inhibitory. ADP exhibited marked inhibition.

The cyclic AMP stimulation of citrate production was observed at concentrations of the order of 10^{-6}M (Fig. 3). The graph depicts the compiled data from 5 separate experiments. Each point is the mean of at least 4 flasks and usually 6. The point at 10^{-3}M is the mean of 25 flasks, and

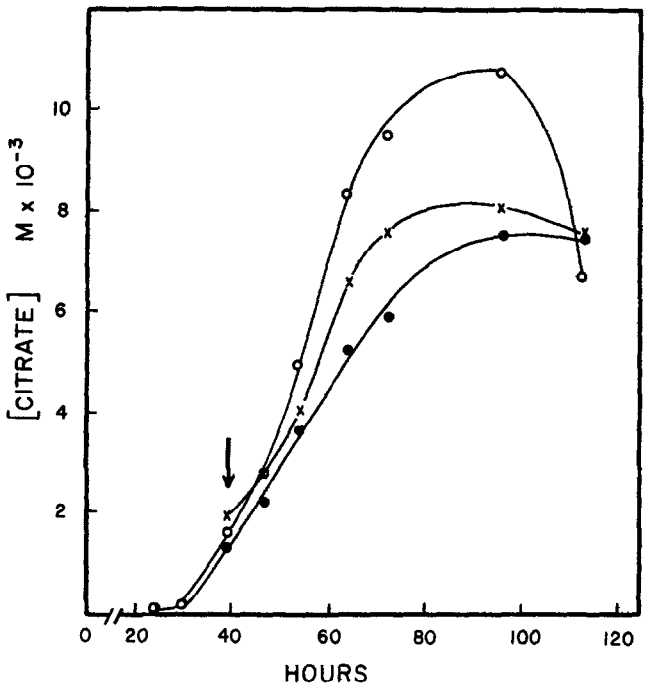

Figure 2 Stimulation of citrate accumulation by cyclic AMP (1.5×10^{-3}M) and theophylline (1.5×10^{-2}M) when added after 40 hours growth. The data represent the means of 2-4 flasks. The final cell weights were: cyclic AMP, 0.103g; theophylline, 0.092 g; control, 0.102 g. Cyclic AMP (o), theophylline (x), and control (•).

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the control line (nothing added) represents 45 flasks. The total number of flasks was 172.

Theophylline exhibited a complex effect when added after 16 hours growth (Fig. 4). When added alone it was ineffective or inhibitory depending on the concentration. High concentrations (2.5×10^{-2}M) inhibited growth as well. When added in combination with cyclic AMP there was an increase in specific activities over control levels and cyclic AMP levels alone. Cyclic AMP was not able to overcome the growth inhibition.

The results with N^6-2'-O-dibutyryl adenosine monophosphate were variable and ambiguous so were not included.

Table I

Influence of adenine and guanine nucleotides on citrate accumulation. The number of flasks used is shown in brackets.

EFFECTER	SPECIFIC ACTIVITY	% OF CONTROL
EXPERIMENT 1		
Control (13)	1372 ± 79*	100
10^{-3}M 3':5'-cyclic AMP (9)	2454 ± 195	179
10^{-3}M 2':3'-cyclic AMP (9)	1363 ± 131	99
10^{-3}M adenosine (9)	1481 ± 122	108
10^{-3}M 5'-AMP (9)	1263 ± 105	92
10^{-3}M ADP (9)	792 ± 125	58
10^{-3}M ATP (9)	1736 ± 122	127
EXPERIMENT 2		
Control (9)	1875 ± 158	100
10^{-3}M 3':5'-cyclic AMP (4)	3082 ± 74	163
2 x 10^{-4}M 3':5'-cyclic AMP (4)	2374 ± 89	127
10^{-3}M 3':5'-cyclic GMP (4)	2831 ± 349	151
2 x 10^{-4}M 3':5'-cyclic GMP (5)	2067 ± 201	110
10^{-3}M 5'-GMP (8)	1694 ± 156	90
10^{-3}M guanosine (9)	1485 ± 196	79
EXPERIMENT 3		
Control (8)	1155 ± 203	100
10^{-3}M 3':5'-cyclic AMP (9)	1808 ± 278	156
10^{-3}M 5'-AMP (9)	1229 ± 169	106
10^{-3}M adenosine (9)	1756 ± 244	152

*standard deviation of the mean

Discussion

A. niger accumulates citric acid in the medium under certain conditions. Addition of cyclic AMP caused an increase in the rate of citrate synthesis and the total amount of citrate produced. Adenosine and ATP (10^{-3}M) enhanced accumulation as well, presumably because they were converted to cyclic AMP inside the cells (7). High concentrations of cyclic GMP (10^{-3}M) mimicked cyclic AMP but not lower concentrations (10^{-4}M). This phenomenon has been observed in other systems, but its significance is not understood (8).

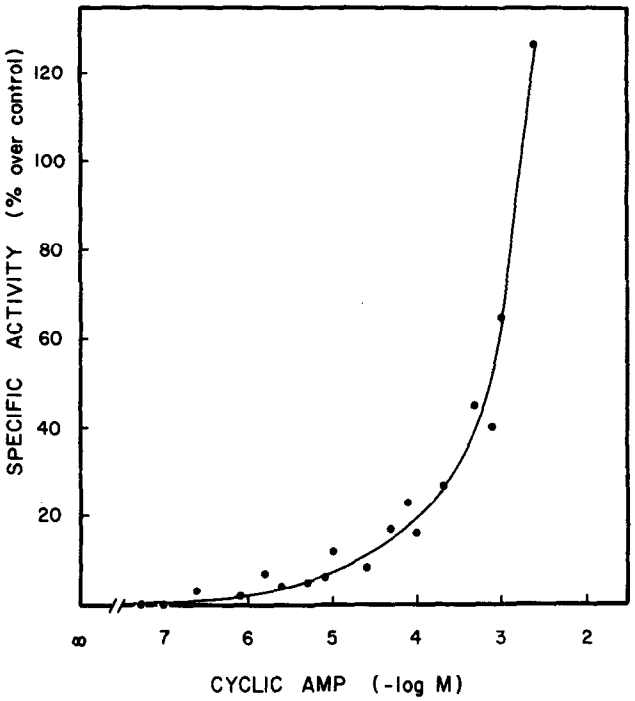

Figure 3 Dose response of citrate accumulation to cyclic AMP. Specific activity is defined as the micromoles citrate produced per gram dry weight mycelia. See text for further explanation.

Cyclic AMP is known to be a regulator of glycolysis in some mammalian tissues, the effect being mediated through phosphorylase, phosphofructokinase (9), fructose diphosphatase (10), and pyruvate kinase (11). Phosphorylase is activated in Neurospora (12), and in yeast there is an increase in NADH oscillations and a shift in the steady state oxidation-reduction level towards oxidation (13).

Abnormal cyclic AMP metabolism in A. niger might result in loss of glycolytic control. Activation of the pathway would produce excess citrate, and this might subsequently spill over into the medium.

Another possibility is that cyclic AMP activates the actual secretion of citrate from the cell. Cyclic AMP involvement in secretion and membrane function is well known (14).

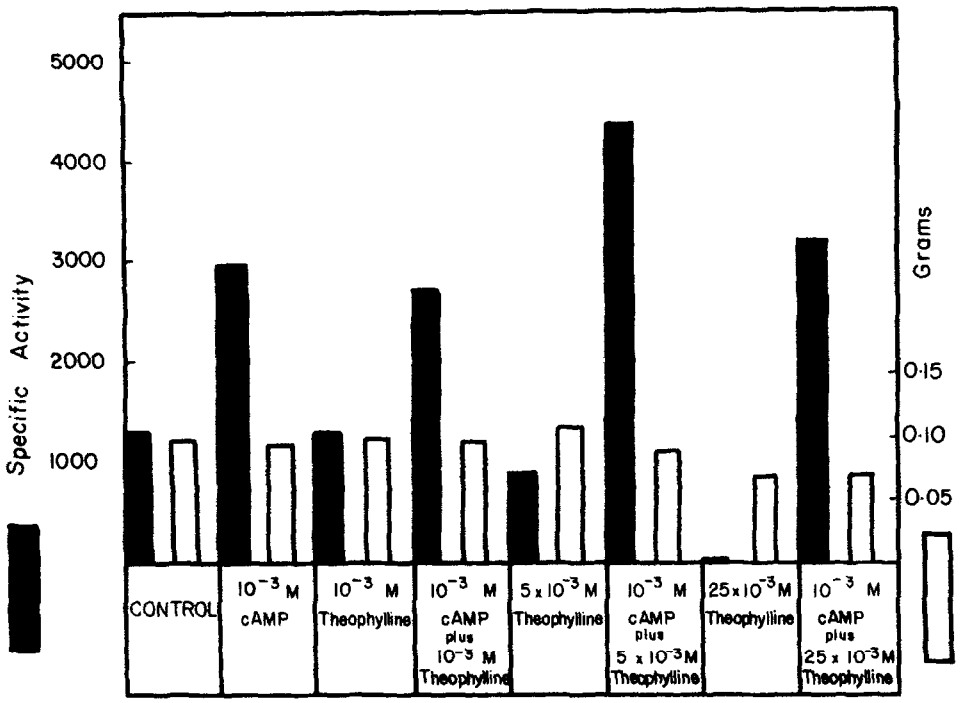

Figure 4 Effect of theophylline alone and theophylline and cyclic AMP combination on citrate production and cell weight. Each test is the mean of 9 flasks.

We propose, therefore, that citric acid accumulation by A. niger may be caused by abnormal cyclic AMP metabolism.

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