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Note

Determination of cyclic nucleotide phosphodiesterase activity in cellular systems by ion-pair reversed-phase liquid chromatography

DIETER TECHEL* and THOMAS BRAUMANN[a]
Department of Biology/Chemistry, FB2/NWII, University of Bremen, D-2800 Bremen 33 (F.R.G.)
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Adenosine 3',5'-monophosphate (cAMP)-dependent protein kinases are considered to play an important role in the differentiation[1] and in the circadian rhythmicity of conidiation of *Neurospora crassa*[2,3]. The intracellular cAMP level is regulated by the activities of cyclic phosphodiesterase (PDE)[4] and adenylate cyclase[5,6].

In order to elucidate the role of cAMP in the molecular mechanism of the conidiation rhythm, it is necessary to determine the activity of the cAMP-hydrolysing PDE and, at the same time, to assess the fate of its hydrolysis product, adenosine 5'-monophosphate (5'-AMP).

Hence a method is required to determine cAMP, 5'-AMP, adenosine, inosine and hypoxanthine simultaneously in cell extracts. Reversed-phase high-performance liquid chromatography (HPLC) is suitable for the simultaneous separation of nucleotides, nucleosides and nucleobases[7–10]. We have recently described a reversed-phase separation system based on methanol–phosphate buffer eluents that is very useful for determining the uptake and metabolism of cAMP during the differentiation of the PC12 clonal cell line[11]. However, a cellular extract from *N. crassa* is much more complex and that method failed to resolve the nucleic acid components completely from other cell constituents.

In this paper, we describe a simple and sensitive reversed-phase ion-pair system that allows the simultaneous determination of cAMP and all of its major metabolites in cellular extracts of *N. crassa*. An example of the application of the method to the study of the circadian rhythm of *N. crassa* is presented.

EXPERIMENTAL

Fifteen discs of *N. crassa* mycelium were ground in pre-cooled mortars with 1.5 ml of cooled Tris [tris(hydroxymethyl)aminomethane] buffer (10 m*M*, pH 7.2). A 1-ml volume of the homogenate was incubated with a final concentration of 1 m*M* cAMP at 21°C for 2 h. The homogenate was then boiled for 3 min at 100°C in a water-bath and centrifuged at 13 000 *g*. The supernatant was filtered through a 0.45-μm SM 1106 membrane filter (Sartorius, Göttingen, F.R.G.) and used for all measurements.

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[a] Present address: M. Brinkmann AG, Dötlinger Strasse, D-2800 Bremen, F.R.G.

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The HPLC equipment consisted of two pumps (Constametric III, LDC Milton Roy, Riviera Beach, FL, U.S.A.), an injection valve equipped with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a detector (LDC, UV monitor D) set at 254 nm and an integrator with printer (UTP, Model 604). Stainless-steel columns (25 cm × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.), were packed by the slurry technique as described previously[12]. The stationary phase was 5-μm LiChrosorb RP-18 (Merck). The mobile phase consisted of a gradient of methanol (solvent A) and 50 m*M* phosphate buffer (pH 6.6) containing 10 m*M* tetrabutylammonium hydrogensulphate (solvent B) from 90 to 55% (v/v) B within 8 min.

Nucleic acid components were identified by comparison of their retention times with those of authentic standards and by their absorption spectra. The quantitative evaluation of the peak areas was performed by the integrating unit. The system was calibrated by injecting five different known amounts of cAMP ranging from 0.5 to 50 μmol and measuring the areas under the peaks. The resulting calibration graph was linear over the entire concentration range. The calibration factor for cAMP thus obtained was related to the molar absorptivities of the cAMP metabolites at 254 nm, the detection wave-length of the UV detector, yielding response factors for 5'-AMP (1.02), hypoxanthine (0.68), inosine (0.84) and adenosine (0.98) relative to cAMP (1.00). The standard error of the peak-area measurements was ±3.6% (n = 12), so that for each sample values of two measurements were averaged. The experiments on the metabolism of cAMP by *N. crassa* cell extracts were repeated twice and gave similar results, with a maximum deviation of 10% with respect to the nucleic acid component concentrations.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of cAMP and its metabolites after injection of the standard solution. Owing to ion-pair formation in the mobile phase, both charged nucleotides elute after the nucleosides and nucleobases. This is partic-

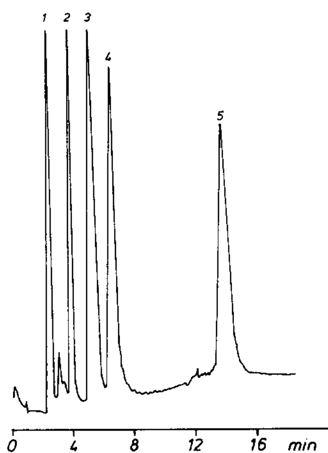

Fig. 1. Chromatogram of 10 μl of a 0.05 m*M* standard solution of cAMP and its metabolites. Peaks: 1 = hypoxanthine; 2 = inosine; 3 = adenosine; 4 = 5'-AMP; 5 = cAMP.

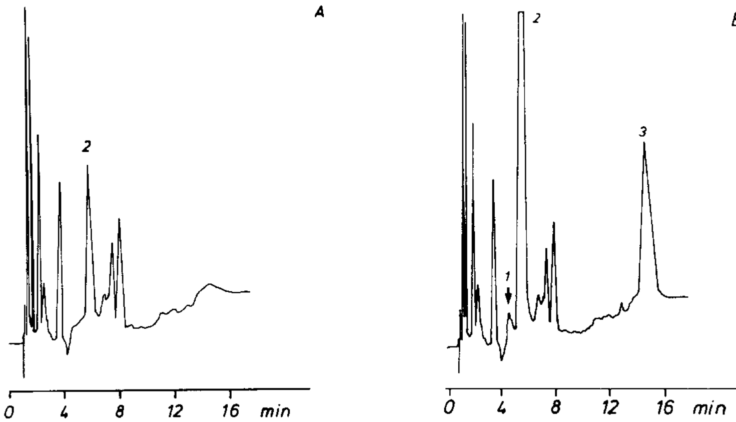

Fig. 2. Chromatograms of *N. crassa* extracts. (A) Without addition and (B) with addition of cAMP to a final concentration of 1 m*M*. Peaks: 1 = inosine; 2 = adenosine; 3 = cAMP.

ularly importent for 5′-AMP, which carries two negative charges under the conditions applied, because this highly polar compound would co-elute with other polar cell constituents under simple reversed-phase conditions[13]. Addition of cAMP to cell homogenates as a substrate of PDE mainly leads to an increase in adenosine and, to a small extent, also inosine. Fig. 2 shows an example in which extremely small concentrations of inosine, at the detection limit of the system, were observed. Usually, up to a factor of ten higher concentrations of inosine could be detected in most samples. No significant changes in the concentration of hypoxanthine and 5′-AMP were detected. Although an increase in hypoxanthine was not expected because of its position in the catabolic cascade of cAMP, it is remarkable that no accumulation of 5′-AMP oc-

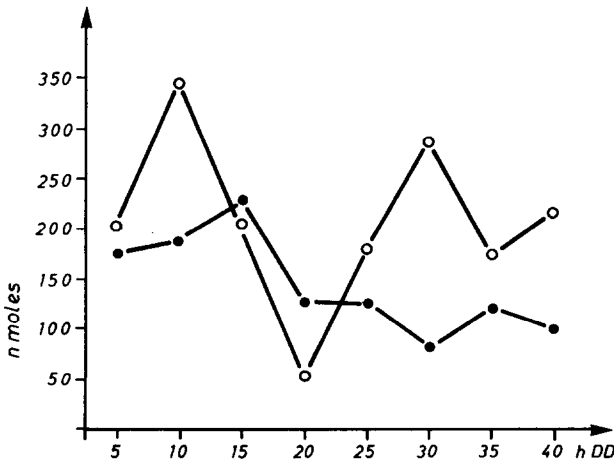

Fig. 3. (●) cAMP and (○) net concentration increases of the metabolites adenosine and inosine. Abscissa, duration of exposure to darkness (hDD = hours in darkness); ordinate, absolute concentration of the nucleic acid compounds in the sample (nmol).

curred. This might be due to the rapid degradation of excess 5′-AMP by a 5′-nucleotide phosphatase.

Fig. 3 shows the increase in the cAMP metabolites adenosine and inosine after addition of cAMP to the cell homogenate as a function of the duration of darkness to which the living mycelium had been exposed before homogenization. The duration of exposure to darkness determines the phase of the circadian cycle at which the cAMP pulse was applied[14]. Maximum increases in metabolite concentration occur at 10 and 30 h after transition of the mycelium from light to dark. As the period of the *N. crassa* (banding strain) conidation rythm is about 20 h, this result strongly suggests rhythmic changes in the PDE activity of the circadian type in this organism.

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