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Note

Two-dimensional thin-layer chromatography–autoradiography of intracellular purine interconversion products

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Many chromatographic methods for the analysis of nucleic acids have been developed, for example paper[1], thin-layer (TLC)[2,3], ion-exchange[4], gas[5] and high-performance liquid chromatography[6]. Most of the proposed separation systems were devised to solve special problems, *e.g.*, the separation of bases from nucleosides[7,8] or the separation of only nucleosides[9] or only nucleotides[10–12]. Some workers, however, separated complex mixtures of nucleobases, nucleosides and nucleotides by using either one-dimensional TLC with a variety of solvent systems or two-dimensional TLC[13–18]. In the course of our studies on purine transport in yeast, we investigated the progress of purine interconversion within the cells. For this purpose, it was desirable to use a separation system that would give an easy and complete characterization of the interconversion process. The two-dimensional TLC system of Pataki[15] first seemed to be the most suitable, as he demonstrated the separation of complex mixtures of nucleic acids and their derivatives. Unfortunately, however, the application of this system to biological material failed in our hands, owing to considerable tailing effects. Therefore, we tried to develop a separation system that would work with our yeast cell extracts. The two-dimensional TLC system combined with autoradiographic detection reported in this paper proved to be especially suitable, because it permits the detection of extremely small amounts of purine derivatives.

EXPERIMENTAL

Materials

Purine and pyrimidine bases, ribonucleosides, and 5′-ribonucleotides were purchased from Boehringer (Mannheim, G.F.R.) and uric acid from Serva (Heidelberg, G.F.R.) [8-^{14}C]hypoxanthine and [2-^{14}C]cytosine were supplied from Amersham-Buchler (Braunschweig, G.F.R.) and [2-^{14}C]xanthine from the Commissariat a l'Energie Atomique (Gif-sur-Yvette, France). Polygram CEL 400 UV_{254} TLC sheets were supplied by Macherey, Nagel & Co. (Düren, G.F.R.). Materials used for autoradiography were Kodirex X-ray film (9 × 12 cm), Kodak D 19 developer and Kodak AL 4 X-ray fixer. Cellulose nitrate filters (pore size 0.45 μm) were obtained from Schleicher and Schüll (Dassel, G.F.R.).

Organisms and culture conditions

Saccharomyces cerevisiae, strain R XII, and *Schizosaccharomyces pombe*, strain 972 h^-, were kind gifts from Dr. A. Kotyk, Prague, and Dr. H. Heslot, Paris. Cells were cultivated to the early stationary phase in a semi-synthetic medium and thereafter incubated in a glucose–citrate buffer [0.05 *M* sodium citrate (pH 5.4), 0.1 *M* glucose] as described elsewhere[19].

Uptake conditions and cell extraction

10^{10} cells pre-treated with glucose were suspended in 10 ml of glucose–citrate buffer, containing 200 μM of labelled base (0.6–0.9 μCi/ml). At intervals, 10^9 cells were separated from the medium by filtration on cellulose nitrate filter-discs and washed with ice-cold water. With reference to the extraction procedure of Nazar *et al.*[20], the cells were suspended in 2 ml of ice-cold 1 *N* acetic acid in ethanol, lyophilized and re-suspended in 500 μl of 0.25% (w/w) ammonia solution. After centrifugation for 5 min at 8,000 *g*, the clear supernatant was used as a pool extract.

Separation of the pool extracts

A 10-μl volume of the pool extracts and 1 μl of a reference mixture were applied to 10 × 10 cm Polygram CEL 400 UV_{254} TLC sheets at the origin, as indicated in Fig. 1. A reference mixture was necessary in order to reveal the separated spots under UV light and consisted of the following solutions (A and B) in the ratio 3:2 (v/v). Solution A: 2.5 mg each of adenine, hypoxanthine, guanine, xanthine and uric acid and 4.0 mg each of adenosine, inosine, guanosine and xanthosine were dissolved in a trace amount of sodium hydroxide solution and diluted to 5 ml with distilled water. The pH was adjusted to 8.0 with hydrochloric acid. Solution B: 5 mg each of AMP, IMP, GMP and XMP, 6 mg each of ADP and GDP, 8 mg each of ATP and GTP and 15 mg each of NAD and NADP were dissolved in 5 ml of distilled water.

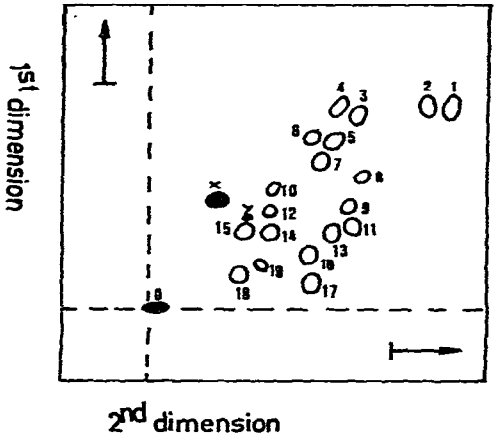

Fig. 1. Two-dimensional separation of purine derivatives. O = Origin; 1 = adenine; 2 = adenosine; 3 = hypoxanthine; 4 = inosine; 5 = guanosine; 6 = xanthosine; 7 = xanthine; 8 = guanine; 9 = AMP; 10 = uric acid; 11 = NAD; 12 = IMP; 13 = ADP; 14 = GMP; 15 = XMP; 16 = ATP; 17 = NADP; 18 = GTP; 19 = GDP; x and y = positions of unidentified autoradiographic spots, transferred from Fig. 2.

In the first dimension, the chromatogram was developed successively with the following solvent systems: (i) methanol, to the top of the sheet; (ii) methanol–2-propanol–25% (w/w) ammonia solution–1% (w/v) EDTA solution (3:9:2:6), to the middle of the sheet; (iii) as (ii), but to the top of the sheet. In the second dimension it was developed with isobutyric acid–25% (w/w) ammonia solution–1% (w/v) EDTA solution (200:9:114), in a single run to the top of the sheet.

To render the labelled spots visible, X-ray films were placed on the chromatograms in the dark and developed after 1 or 2 days.

RESULTS AND DISCUSSION

In order to demonstrate the application of the method to biological extracts, it was used in the study of purine uptake and conversion by two yeast species. One species, *Saccharomyces cerevisiae*, is able to take up adenine, guanine, hypoxanthine and cytosine in the stationary growth phase after pre-treatment with glucose[19], whereas the other, *Schizosaccharomyces pombe*, has been shown to take up xanthine under these conditions[21]. In the cells, the bases may be accumulated to a certain extent, interconverted, metabolized to nucleosides and nucleotides or incorporated into nucleic acids. Fig. 2 shows the autoradiographic patterns of the intracellular conversion products depending on the incubation time with the labelled base.

Comparison of these patterns with Fig. 1 indicates that during the first minute the bases are taken up without significant metabolization. With increasing time, an

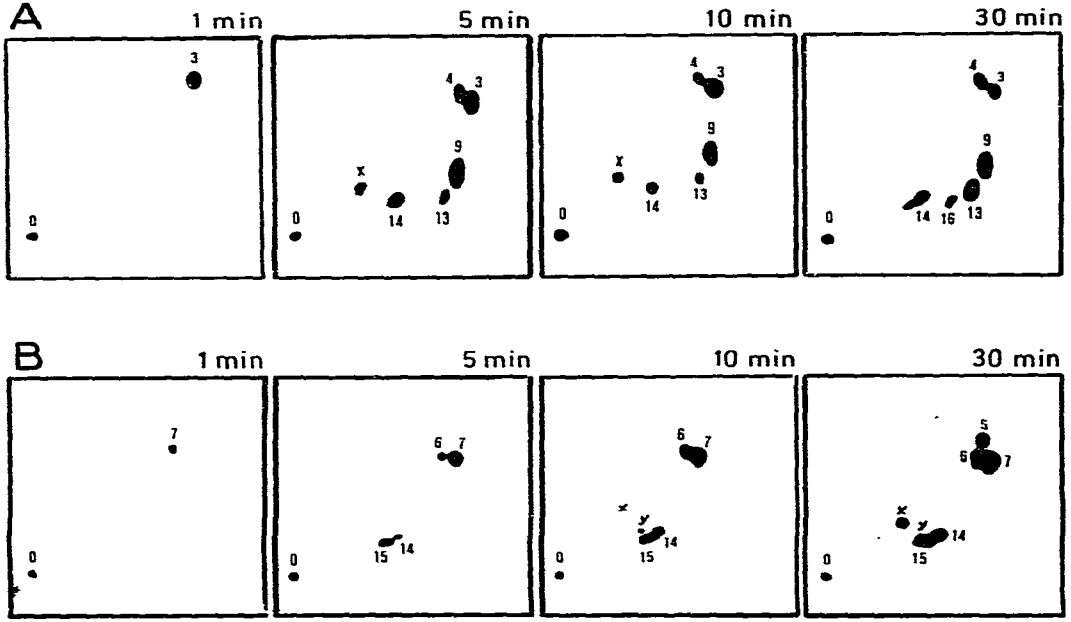

Fig. 2. Progress of intracellular conversion after uptake (A) of hypoxanthine in *Saccharomyces cerevisiae* and (B) of xanthine in *Schizosaccharomyces pombe*. The TLC–autoradiograms were prepared after different times of incubation with the ^{14}C-labelled bases. The origin was marked with ink, because it contained no detectable radioactivity.

increasing conversion into nucleosides and nucleotides is observable. In addition to the identifiable spots, one (Fig. 2A) or two (Fig. 2B) further spots occur that are not visible under UV light. We are not yet able to identify these spots.

CONCLUSION

The separation of at least 20 naturally occurring purine derivatives, including bases, ribonucleosides, and ribonucleotides on a single cellulose-TLC-sheet is described. In combination with TLC–autoradiography the method is especially suited for the analysis of cellular interconversion products after the uptake of labelled purines.

In comparison with the method of Pataki[15] the advantage of our separation system is that it works conveniently with crude biological extracts. The separated material appears under UV light as small distinct spots without tailing effects.

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