ANALYSIS OF BIOLOGICAL ADENOSINE 3′:5′ CYCLIC MONOPHOSPHATE DEGRADATION BY A THIN-LAYER CHROMATOGRAPHIC METHOD

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1. Introduction

Since 3′:5′ cAMP plays a major regulatory role in biological systems, the regulation of its intracellular concentration is of special interest. This concentration is a function of both the rate of formation and the rate of degradation. A thin-layer chromatographic study of 3′:5′ cAMP degradation in a cytosolic fraction of bovine adrenal cortex is presented here. This method displays the complete metabolic pattern within one run. 3′:5′ cAMP is degraded first to 5′AMP, as established [1], then the conversion to adenosine and inosine as an end product is shown. There was no evidence for a second pathway as suggested [2] and reported to exist in toad bladder mucosal cell [3]. However, the results may indicate the presence of a cAMP-degrading multi-enzyme complex.

2. Materials and methods

2.1. *Chemicals*

[U-^{14}C]Adenosine 3′:5′ cyclic monophosphate, ammonium salt (spec. act. 287 mCi/mmol) and [U-^{14}C]adenosine 5′monophosphate, ammonium salt (spec. act. 538 mCi/mmol) were purchased from the Radiochemical Centre Amersham. All unlabelled nucleotides and nucleosides were obtained from Boehringer, FRG and all other chemicals were analytical grade obtained from Merck, FRG. Thin-layer plates, silica gel 60 F_{254} (0.25 mm) and cellulose F_{254} (0.1 mm) were purchased from Merck, FRG.

Abbreviations: 3′:5′ cIMP (inosine 3′:5′ cyclic monophosphate); 3′:5′ cAMP (adenosine 3′:5′ cyclic monophosphate); 2′:3′ cAMP (adenosine 2′:3′ cyclic monophosphate); 3′:5′ PDE (3′:5′ cyclic-nucleotide phosphodiesterase EC 3.1.4 17); 2′:3′ PDE (2′:3′ cyclic-nucleotide 3′phosphodiesterase EC 3.1.4. 37), 5′ AMP (5′ adenosine-monophosphate); 2′ AMP (2′ adenosine-monophosphate); 5′IMP (5′ inosine-monophosphate)

2.2. *Biological material*

Cytosol (100 000 × *g*, 1.5 h) from bovine adrenal cortex was chromatographed on Sephadex G-200 (Pharmacia/Sweden). The elution buffer contained 50 mM Tris–HCl (pH 7.4), 1 mM $EDTANa_2$ and 5 mM $MgCl_2$. Two main peaks of 3′:5′ PDE activity were eluted from the column (2.5 × 100 cm). The first of these which eluted in the void volume, was used for this study.

2.3. *Protein determination*

Protein was determined according to the micro-version of [4], using bovine serum albumin as standard. All samples were pretreated with sodium dodecyl-sulfate (0.4%).

2.4. *Chromatographic system*

2.4.1. Solvent system 1

Ethanol, saturated aqueous $Na_2B_4O_7$ solution, 5 M ammonium acetate, 0.5 mM $EDTANa_2$ (220 : 80 : 20 : 0.5, by vol.) was used on silica gel 60 unless otherwise indicated.

2.4.2. Solvent system 2

Saturated aqueous ammonium sulfate, 1 M sodium citrate, isopropanol (80 : 18 : 2, by vol.) was used on cellulose F.

2.5. *Purification procedure for 3′:5′ c[^{14}C]AMP*

The 3′:5′ c[^{14}C]AMP Amersham) used as tracer in this study contained a significant amount of 2′:3′ c[^{14}C]AMP (approx. 7%). Purification was achieved by incubation with a 2′:3′ PDE from human erythrocyte ghosts [5], prepared by the method [6]. Subsequent separation of the single enzymatic product, 2′AMP, with a chromatographic procedure employing solvent system 1 on cellulose led to very pure 3′:5′ c[^{14}C]AMP suitable for 3′:5′ cAMP degrading studies.

2.6. *3′:5′ cAMP degrading assay proce re*

The incubation mixture contained 40 mM Tris–HCl (pH 7.2), 2.5 mM $MgCl_2$, 0.1% bovine serum albumin, 10 μm 3′:5′ c[^{14}C]AMP (60 nCi). The reaction was initiated by the addition of the cytosolic protein mentioned above to give final vol. 50 μl. It was incubated at 30°C for 30 min, then stopped by the addition of 10 μl 3′:5′ cAMP (5 mM) and immediate heating for 1 min at 100°C. After heating, the samples were cooled in an ice-bath and then centrifuged at 2000 × *g* for 15 min. Assay blanks were prepared with preheated protein. After centrifugation, a 40 μl aliquot was taken from the sample, lyophilised and redissolved in 20 μl aqueous solution containing the reference substances (2 mM). One-half (10 μl) was applied to the 2 cm start line on the thin-layer plate for chromatography system 1, and the other half was applied to the chromatographic system 2.

2.7. *Evaluation*

After the chromatograms had been developed the distribution of radioactivity along the solvent track was detected by a gas-flow proportional counter (Berthold, FRG). The counts were integrated and plotted by *xy*-plotter. The non-radioactive, cochromatographed reference substances were visualized with an ultraviolet lamp at 254 nm to identify the peaks on the scan. For exact quantitative studies the radioactive areas indicated by the peaks in the scan were scraped out, powdered and transferred to counting vials, suspended in water and a gel-forming scintillation mixture and measured by scintillation counting technique (e.g., Instagel (Packard, USA)/water, 10 : 3). The identity of the radioactive peaks was deduced by cochromatography of the radioactive unknown products after 3′:5′ cAMP degradation with pure non-radioactive substances on the basis of chromatographic system 1 and 2. The identity of the radioactive peaks was confirmed by scraping out the radioactive area, eluting it and rechromatographing the unknown on the alternative system together with the suspected nonradioactive pure reference substance. In addition, 2′AMP and 5′AMP were identified by chemical transformation. The scraped and eluted substances were subjected to acidic hydrolysis according to [7]. In both cases the degradaton product, adenine, was chromatographically identified.

3. Results and discussion

The chromatographic system 1 fields complete information about the pathway and extent of 3′:5′ cAMP degradation. The catabolism of 3′:5′ cAMP by a cytosolic fraction of bovine adrenal cortex produces the pattern shown in fig.1.

The following metabolites were identified by chromatography in system 2, rechromatography of the eluted metabolites and chemical transformation, as described under methods: 5′AMP, 2′AMP, adenosine and inosine (fig.1B). The 2′AMP, however, is not likely to be a metabolite of 3′:5′ cAMP. Rather, it must be regarded as a product of enzyme hydrolysis of 2′:3′ c[^{14}C]AMP, which is a significant, but chromatographically undetected impurity of the ^{14}C-labelled 3′:5′ cAMP used as tracer in this study.

This result is included to demonstrate the necessity of purifying the tracer, as well as to document the presence of the 2′:3′ PDE in the cytosolic fraction. Since this enzyme has been described as exclusively membrane-bound [5,8], the finding was surprising. The high activity and specificity of this 2′:3′ PDE explains why the impurity cannot be 'diluted out' of the tracer by the non-radioactive 3′:5′ cAMP, which contains no detectable amount of 2′:3′ cAMP.

As fig.1 demonstrates, 3′:5′ cAMP is converted to inosine as an end product. Transformation proceeds by initial hydrolysis of the 3′-phosphate bond by 3′:5′ PDE, followed by removal of the phosphate by 5′ nucleotidase and deamination by adenosine-deaminase. No additional metabolites could be detected. A second pathway, possibly via cIMP and/or 5′ IMP to the end product inosine, as suggested [2], and reported to exist in other biological material

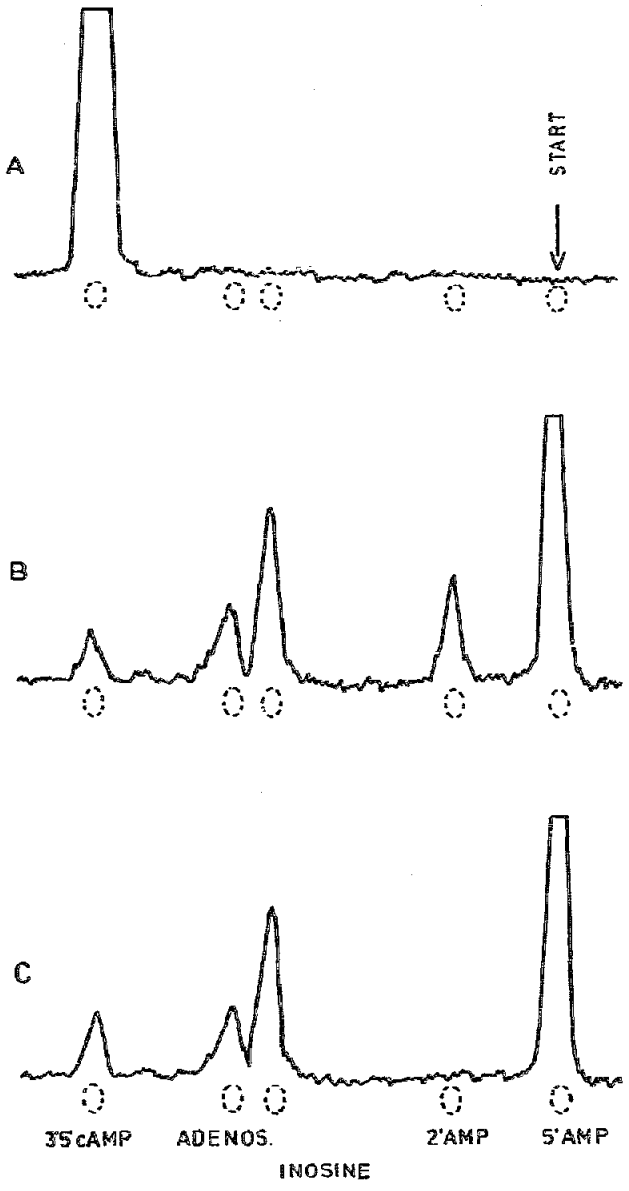


Fig.1. Separation of 3′:5′ cAMP metabolites, generated by a cytosolic fraction of bovine adrenal cortex, on thin-layer chromatographic system 1 (see Materials and methods). (1A) Blank with preheated enzyme material. (1B) Incubation with non-purified tracer, (1C) Incubation with purified tracer. Radioactivity of the tracer 3′:5′ c[^{14}C]AMP is detected by a gas-flow proportional counter and documented by a scan. The whole scale extends from the base-line to the cut-off of the main peak and corresponds to 600 cpm. The spots of non-radioactive, cochromatographed references are represented by dotted areas.

[3], could not be affirmed. Our inability to detect these metabolites might have been due to extremely rapid metabolism; however, addition of large amounts of the conjectured 'cold' metabolites (1 mM) did not change the result. Rather, the proposed conversion of 3′:5′ cAMP via the adenosine derivatives to inosine is confirmed by the experiment starting with the first metabolite, 5′AMP, in the present 3′:5′ cAMP degrading system. As is shown in fig.2, 5′AMP is converted to inosine via adenosine.

In conclusion, on the basis of the thin-layer chromatographic method presented here, which makes it possible to follow the metabolic pathway of cAMP beyond 5′ AMP 3′:5′ PDE activity was detected in the cytosolic fraction, accompanied by 5′nucleotidase and adenosine deaminase activity. It could be accidental, or it might hint at the existence of a partially preserved cAMP-degrading multi-enzyme complex.

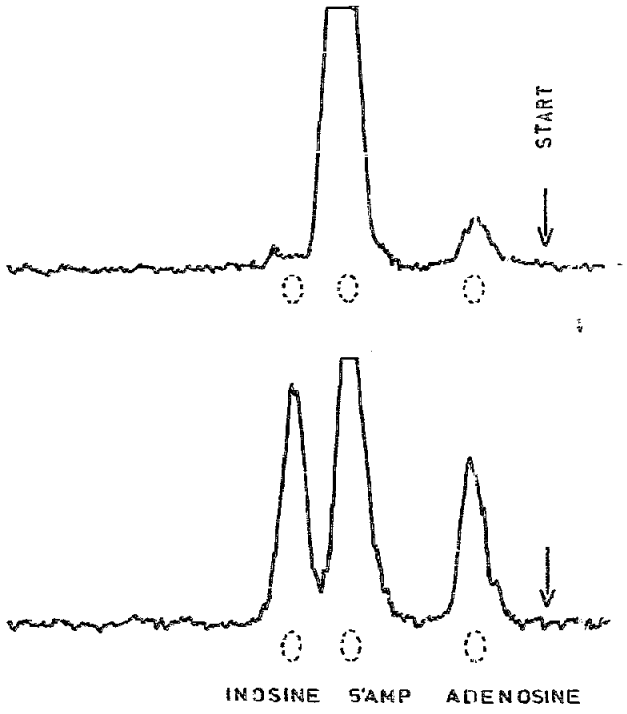


Fig.2. Separation of 5′AMP metabolites generated by the cytosolic fraction of bovine adrenal cortex, on thin-layer chromatographic system 2 (see Materials and methods). Determination of radioactivity as in fig.1. Upper curve, control; lower curve, incubation mixture.

This idea is supported by the observation that cytosolic fraction, studied here, contained only high molecular proteins (approx. mol. wt 600 000 and higher) and the finding that other 3′:5′ PDE preparations, commercially available from Boehringer, FRG and Sigma, USA, also converted cAMP to inosine under our assay conditions.

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