Notes

CHROM. 3955

Separation of cyclic-3′,5′-adenosine monophosphate from other adenine nucleotides

Cyclic-3′,5′-adenosine monophosphate (CAMP), an intermediate and a "second messenger" in most of the hormonal reactions[1], has become the subject of increasing interest. The available paper chromatographic methods[2–4] for its separation from other adenine nucleotides are relatively inconvenient and time consuming*. A solvent system consisting of a mixture of isobutyric acid–ammonia–water (64:3:33, v/v) was found by us to be most suitable for the thin-layer chromatographic separation of adenine nucleotides on silica gel.

Materials

The disodium salt of ATP, monosodium salt of ADP, 5′-AMP and adenosine used were products of Nutritional Biochemical Corporation. Cyclic-3′,5′-AMP was a gift sample from Sigma Laboratories. The isobutyric acid was from Riedel (Germany). Isopropanol, ammonia liquor (chromatographic grade) and acetic acid were obtained from British Drug Houses. Silica Gel G (mesh size, 40–80 μ; E. Merck) was employed for thin-layer chromatography (TLC). Dowex 50 X 8 H^+ cation exchanger (mesh size, 200–400; Baker) was used for the column chromatography. The nucleotide spots were visualized under an ultraviolet monochromatolite lamp (Hanovia, Slough, Great Britain). Hilger–Watt (Model FA 41-1/64463) and Cary (–15) recording spectrophotometers were used to read the concentrations of the nucleotides in eluates either from the column or from the thin layers. Double glass-distilled water was used throughout the experiment.

Methods

Thin-layer chromatography. Aqueous solutions of 100–150 μg each of ATP, ADP, 5′-AMP and adenosine and 10–15 μg of CAMP in 10–15 μl were applied to activated, Silica Gel G coated glass plates for TLC. A mixture containing all the nucleotides (20–30 μg of ATP, ADP, 5′-AMP and adenosine and 2–3 μg of CAMP) was also applied. The plates were developed for about 3.5–4 h in the solvent system isobutyric acid–ammonia–water (64:3:33, v/v) at room temperature. The dried plates were exposed to an ultraviolet light source to localise the separated nucleotides. Each spot was then scraped off and extracted with 3 ml of distilled water. The extract was centrifuged at 2500 × g for 20 min, and the supernatant obtained from the extract of each spot was read at its $\lambda_{max.}$ on the spectrophotometers.

The plates were also sprayed with orcinol–H_2SO_4 (ref. 5) reagent and heated

* A separation on DEAE Cellulose layers was reported by YOSHIMOTO *et al.*[12].

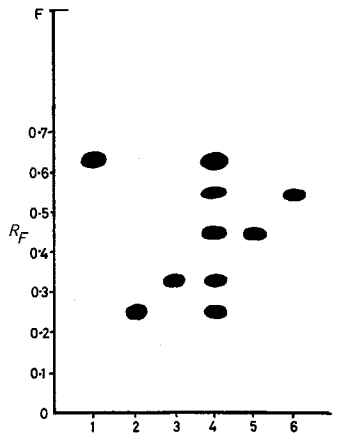

Fig. 1. Thin-layer chromatography on Silica Gel G of adenine nucleotides. 1, Adenosine; 2, ATP; 3, ADP; 4, Mixture of 1, 2, 3, 5, and 6; 5, 5′-AMP; 6, Cyclic-3′,5′-AMP. Solvent system: isobutyric acid–ammonia–water (64:3:33, v/v; pH 4.1). Detection: Monochromatic U.V. light of short wavelength (254 mμ).

in an oven at 110° for 15 min to detect the distinct violet-coloured spots for pentose.

Ion-exchange chromatography. A mixture of ATP, ADP, 5′-AMP, adenosine and CAMP (1250 μg each/ml) was prepared in an aqueous solution containing Tris–HCl buffer (pH 7.3; 8×10^{-2} *M*), $MgSO_4$ (7×10^{-3} *M*) and NaF (2×10^{-2} *M*). Of this mixture 0.2 ml was loaded on the column containing a 50% aqueous suspension of Dowex 50 X 8 H^+ (maximum effective pH, 14.0) plugged with glass wool (resin bed, 0.4–0.6 cm diameter at 8–10 cm resin height, in a total column length of 18–20 cm, with a flow rate of 1 ml/5 min). This was eluted with a total of 9.8 ml distilled water. Three 3-ml eluate fractions were collected separately. On a thin-layer plate, for detection of the nucleotides, 75–100 μl of each was spotted.

Although the $BaSO_4$ adsorption step was not necessary (as seen under *Results*), the eluates were subjected to $BaSO_4$ adsorption according to the method of KRISHNA *et al.*[6]. After $BaSO_4$ treatment the supernatants were spotted on thin-layer plates to check for contamination by other adenine nucleotides. The concentration of CAMP

TABLE I

R_F VALUE OF ADENINE NUCLEOTIDES SEPARATED BY TLC USING SILICA GEL G

Solvent system: isobutyric acid–ammonia–water (64:3:33, v/v; pH 4.1). Detection: Monochromatic U.V. light of short wavelength.

Compound	λ_{max} *(mμ) pH 5.6*	R_F *values*	*Recovery (%)*
ATP	260	0.25 ± 0.01	
ADP	260	0.33 ± 0.01	
5′-AMP	260	0.45 ± 0.01	90 ± 10
Cyclic-3′,5′-AMP	256	0.55 ± 0.01	
Adenosine	259	0.63 ± 0.01	

TABLE II

RECOVERY OF CAMP AFTER ION-EXCHANGE CHROMATOGRAPHY ON A DOWEX 50 X 8 H^+ COLUMN (BEFORE AND AFTER $BaSO_4$ TREATMENT) AS DETECTED BY TLC

Nucleotides	*Effluents from the Dowex column*			*Recovery of CAMP from column (%)*
	I	*II*	*III*	
ATP	—	—	—	—
ADP	—	—	—	—
5′-AMP	—	—	—	—
Cyclic-3′,5′-AMP	—	—	+	80 ± 20
Adenosine	—	—	—	—

in the fractions eluted from the column and from the thin-layer plates was determined by the spectrophotometer at its $\lambda_{max.}$

Results

With the solvent used for TLC, CAMP is distinctly separated from other adenine nucleotides (Fig. 1). Table I represents the R_F values obtained for individual nucleotides in such a system. Percentage recovery of the nucleotides from the thin-layer plates as determined spectrophotometrically was 90 ± 10.

In order to check the recovery of the nucleotides, a known amount of the mixture of adenine nucleotides (1250 μg/ml each) was processed according to the method of KRISHNA *et al.*[6], involving separation of the mixture on a Dowex-50 X 8 H^+ column followed by $BaSO_4$ adsorption. The eluates from the column (as described in *Methods*) before and after treatment with $BaSO_4$ were scanned after TLC for nucleotide content (Table II). It is evident from Table II that only CAMP is eluted from the column, while other adenine nucleotides were not detected when eluates were checked on thin-layer plates. Almost 80 ± 20% of the CAMP could be recovered. Since other nucleotides were found to be absent from the column eluates, the $BaSO_4$ adsorption step used in KRISHNA's method could conveniently be omitted. However, to confirm our findings, the three fractions obtained from the column were treated with $BaSO_4$, and the supernatant from each was analysed for CAMP. Again, only the third fraction was CAMP positive. This spot on thin-layer plates, when eluted with water and read at 256 mμ on the spectrophotometer, gave the same recovery (90 ± 10%).

Discussion

In a series of publications from different laboratories[7,8] radioactive material was used for the assay of adenyl cyclase in terms of CAMP formed from the reaction mixtures. CAMP was also quantitated by the degree of conversion of inactive phosphorylase *b* to active phosphorylase *a*[9,10].

Paper and column chromatographic methods[2–4,6] have also been used for the isolation and quantitation of CAMP in the reaction mixtures.

The available paper chromatographic methods have employed the following solvent systems for the separation of CAMP:

isopropanol–ammonia–water (70:20:10, v/v)
isopropanol–acetic acid–water (60:30:10, v/v)
isobutyric acid–ammonia–water (66:1:33, v/v)
isobutyric acid–0.5 *M* NH_4OH (10:6, v/v).

All these solvent systems were tried on paper as well as on thin layers, but were found to be time consuming and unsatisfactory.

According to our procedure, CAMP can be eluted from the Dowex 50 X 8 H^+ column without the interference of other nucleotides in the reaction mixture. The $BaSO_4$ adsorption step is therefore unnecessary. This is probably due to the weak chelating property of CAMP as compared to other adenine nucleotides[11].

Moreover, the thin-layer chromatographic system separates all the nucleotides very distinctly, and hence CAMP could be assayed conveniently from the biological system within a short period.

Further work on quantitation of CAMP using Bial's reagent (orcinol–HCl) is in progress.

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