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Note

Thin-layer electrophoretic separation of the degradation products of cyclic adenosine-3′,5′-monophosphate

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A method of thin-layer electrophoresis (TLE) developed by Pastuska and Trinks[1] and by Honegger[2] has been successfully applied to the separation of several classes of compounds, including amino acids[2], alkaloids[3], indoles[4] and carbohydrates[5]. However, the TLE of cyclic adenosine-3′,5′-monophosphate (cAMP) and its degradation products has not hitherto been investigated.

The separation of cAMP from its degradation products is important because it is involved in many assays for of cAMP phosphodiesterases, where it is accomplished by using tedious chromatographic methods[6–9]. These methods are mostly concerned with the separation of cAMP from other compounds and not with the separation of the degradation products from each other. This latter separation, however, is important because it can be of use in characterizing a particular biopharmacological situation. It was therefore of interest to develop a simple and rapid method for separating the degradation products of cAMP, and we describe here the use of TLE for this purpose.

MATERIALS AND METHODS

Chemicals and equipment

[8-^{3}H]cAMP (ammonium salt; 21 Ci per mmole) was obtained from the Radiochemical Centre (Amersham, Great Britain). Inosine, hypoxanthine, buffer components, silica gel DC60-F254 TLC plates (200 × 200 mm; 0.1 mm thick) and scintillation chemicals were purchased from E. Merck (Darmstadt, G.F.R.). cAMP, adenosine 5′-monophosphate (AMP) and adenosine were obtained from Boehringer und Soehne (Mannheim, G.F.R.).

A power supply with a 0–600-V facility and a chamber from Desaga (Heidelberg, G.F.R.) were used for TLE.

Thin-layer electrophoresis

Borate buffer solution was made up from solutions of 7.22 g/l of boric acid and 17.6 g/l of sodium carbonate monohydrate, and phosphate buffer from 9.08 g/l of potassium dihydrogen phosphate and 11.8 g/l of disodium hydrogen phosphate dihydrate.

The compounds being investigated were dissolved in twice-distilled water to

give solutions of the following concentration: cAMP and adenosine, 1 mg/ml; inosine and AMP, 1.5 mg/ml, and hypoxanthine 2 mg/ml; these solutions were applied to the thin layers in amounts of 2 μl.

A potential difference of 400 V was applied across the plate; a typical current for 90% phosphate–10% borate combined buffer of pH 6.5 was 40 mA.

After electrophoresis for 60 min, the dried plate was viewed under UV radiation (254 nm), and migration distances towards the cathode were measured.

Thin-layer electrophoresis of [^{3}H]cAMP and its metabolites

A 200-g female Sprague-Dawley rat was decapitated, and the brain was removed immediately into ice-cold 0.16 *M* Tris buffer of pH 7.5 containing, per 100 ml, 100 mg of magnesium chloride trihydrate. The tissue (1 part by weight) was homogenised with the buffer solution (10 parts by weight) for 60 sec in an all-glass system, then the homogenate was centrifuged at 20,000 *g* for 30 min at 5° and stored at 2° until required. This preparation was characterised by biuret protein determination (Lowry *et al.*[10]), and the cAMP phosphodiesterase activity was determined by the method of Poech[11] as follows. A 200-μl portion of the supernatant was incubated with 200 μl of Tris–magnesium chloride buffer solution (pH 7.5) containing 70 nmoles of [^{3}H]cAMP for 10 min at 37°, then the mixtures were stabilised by adding 5 μl of 25% acetic acid and heated in boiling water for 3 min.

A blank comprising 200 μl of the buffer and 200 μl of the [^{3}H]cAMP buffer was used. After centrifugation at 2000 *g* at room temperature for 10 min, 10-μl portions of the supernatant were applied to the silica gel TLE plates, to which marker substances had been added as mentioned above. Electrophoresis was performed for 1 h in 90% phosphate–10% borate buffer medium at pH 6.8. After drying and identification of the substances under UV radiation, the gel from each zone was scraped into a scintillation counting phial. Then 400 μl of twice-distilled water were added to each phial, followed by 20 ml of scintillation fluid, and the radioactivity was counted in a Packard liquid scintillation counter (Tri-Carb 2420), quenching being measured with use of an internal standard. The scintillation fluid used consisted of 150 mg of 2,2′-*p*-phenylenebis-(4-methyl-5-phenyloxazole), 6 g of 2,5-diphenyloxazole, 1000 ml of toluene, 100 ml of dioxan and 400 ml of ethanol.

RESULTS

The effects of phosphate buffer solutions ranging in pH from 5.0 to 7.8 are shown in Fig. 1, which shows that adenosine and hypoxanthine, and cAMP and AMP, were not resolved; hypoxanthine migrated the furthest. A range of borate buffer solutions (pH 5.5 to 10.5) was also investigated (results not shown); migration distances were small, and cAMP, AMP and hypoxanthine were not separated. In this instance, at each pH value, inosine migrated the furthest.

The effects of phosphate and borate buffer mixtures at pH 6.8 are shown in Fig. 2. Separation of the individual substances improved as the content of phosphate increased up to a maximum of 90%. Complete separation of cAMP, AMP, adenosine, hypoxanthine and inosine was achieved with a combined phosphate–borate buffer of pH 6.8 containing 85 to 95% of phosphate. Optimum separation was with 90% phosphate–10% borate buffer at pH 6.8 in 1 h at 400 V; under these conditions, the

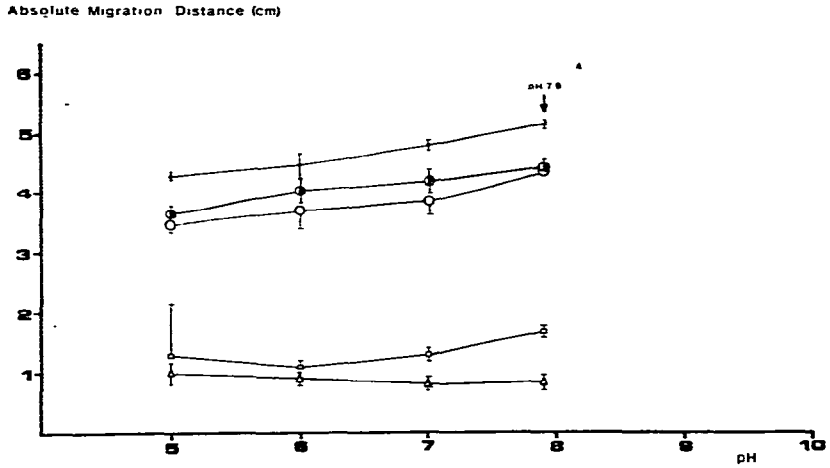

Fig. 1. Migration values of cAMP and degradation products in phosphate buffer solution of pH 5.0 to 7.8 on silica gel layers. Each result is the average of five experiments ± the standard deviation. □ = cAMP; △ = AMP; ○ = adenosine; ● = inosine; ◐ = hypoxanthine.

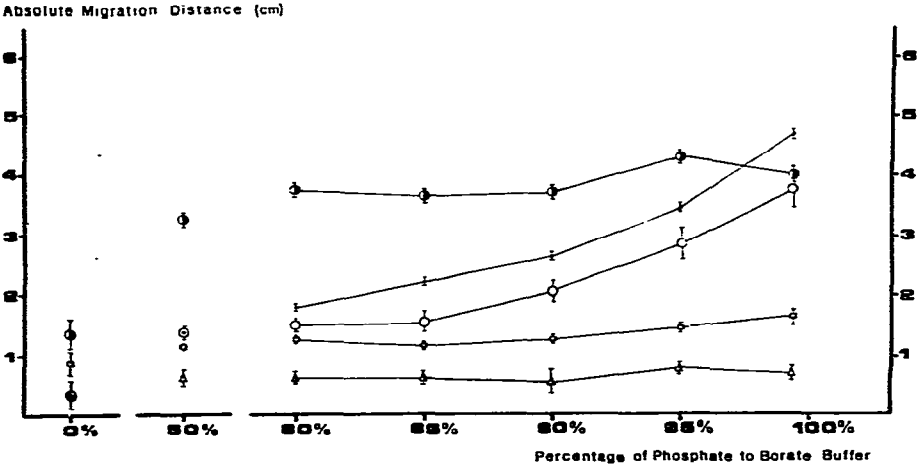

Fig. 2. Migration values of cAMP and degradation products in various combinations of phosphate and borate buffer solutions at pH 6.8 on silica gel layers. Each result is the average of five experiments ± the standard deviation. Symbols as in Fig. 1.

absolute migration distances (± the standard deviation) were 0.8 ± 0.01 for AMP, 1.5 ± 0.05 for cAMP, 2.9 ± 0.025 for adenosine, 3.5 ± 0.025 for inosine, and 4.35 ± 0.025 for hypoxanthine (each value being the mean of five determinations). The developed plate from a typical experiment is shown in Fig. 3.

The final pH values of the buffer solutions remaining in the anode and cathode compartments were 7.27 and 5.77, respectively; the final zone diameter was consistently in the region of 3.5 mm.

The results of the separation of the degradation products of cAMP after hydrolysis with the supernatant fluid from a rat-brain homogenate are presented in Table I.

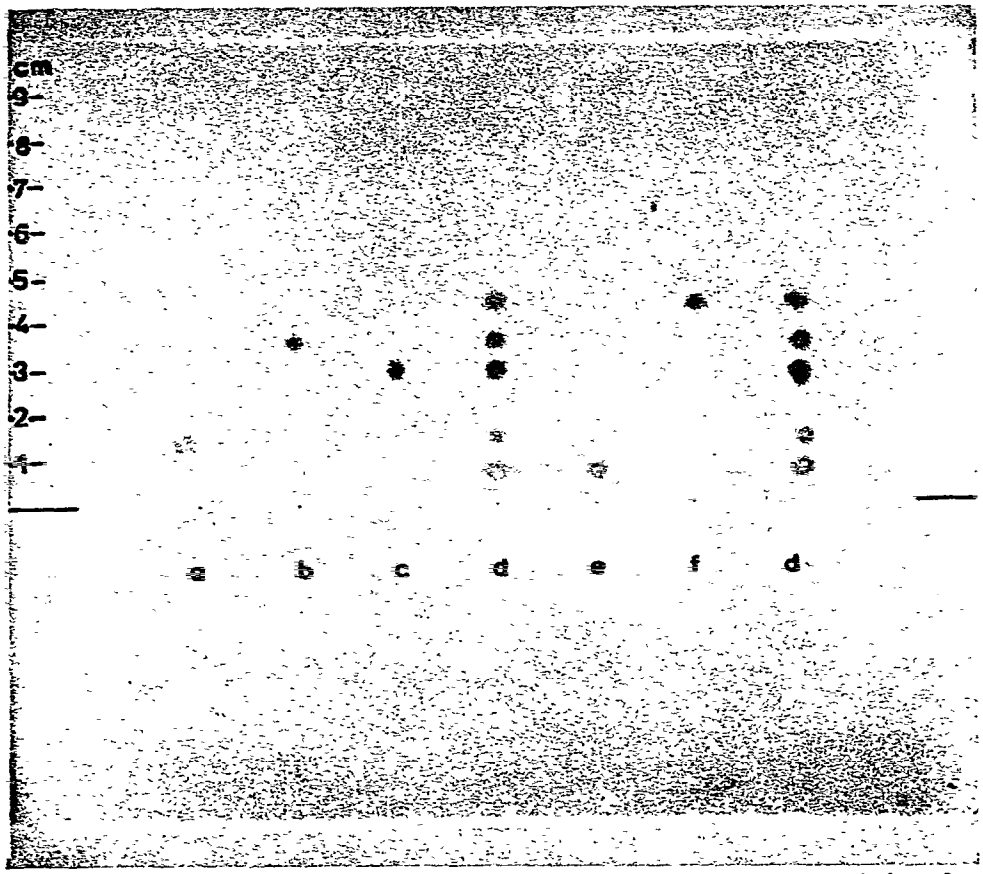

Fig. 3. Photograph (Polaroid Land film) of a developed plate under 254-nm radiation after TLE in 90% phosphate–10% borate buffer solution of pH 6.8; TLE for 1 h at 400 V. a = cAMP; b = inosine; c = adenosine; d = cAMP, inosine, adenosine, AMP and hypoxanthine; e = AMP; f = hypoxanthine.

TABLE I

ANALYSIS OF INCUBATION MIXTURE OF [^{3}H]cAMP WITH RAT-BRAIN HOMOGENATE SUPERNATANT AFTER SEPARATION BY TLE AND SCINTILLATION COUNTING

Results are expressed as the percentage of radioactivity per sample, and each is the average of eight experiments (± the standard deviation). The cAMP phosphodiesterase activity of the supernatant was 1.05×10^{-7} mg of cAMP per mg of protein per min at a cAMP substrate concentration of *0.125 μM*.

Substance	*Incubation with supernatant*	*Incubation blank*
AMP	1.1 ± 0.3	2.3 ± 0.8
cAMP	1.83 ± 0.3	94.7 ± 2.1
Hypoxanthine	15.1 ± 0.5	0.1 ± 0.05
Adenosine	25.9 ± 1.8	1.0 ± 0.8
Inosine	54.1 ± 2.0	0.5 ± 0.3
Origin	1.8 ± 0.2	1.3 ± 0.2

Our results demonstrate that this method can be used for the separation and subsequent determination of radioactive labelled cAMP and its degradation products. We suggest that this method could be usefully applied to the study of the degradation pattern of cAMP in various physiological and pharmacological situations.

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