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Note

Use of cation-exchange thin layers in adenyl cyclase assay

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In the routine determination of adenyl cyclase activity using chromatographic techniques, it is essential to separate cyclic 3',5'-adenosine phosphate (cAMP) from adenosine 5'-triphosphate (ATP) and other adenine metabolites and from possible radiochemical impurities present in commercial ATP preparations labelled with ^{3}H or ^{14}C. Usually, separations on columns of Dowex 50 or Dowex 2 resin[1,2], alumina[3,4] or DEAE-Sephadex[5], thin layers of silica gel[6–8], alumina[8] or PEI-cellulose[9,10], silica gel glass microfibre sheets[11], paper chromatography[12–16] or a combination of paper chromatography with paper ionophoresis[17] have been employed. A combination of Dowex 50 column chromatography with precipitation by inorganic compounds has also been reported[18–20].

Each separation, however, has drawbacks. Several methods result in only a partial resolution of the compounds to be separated, while others are too tedious and time consuming. For this reason, a method that combines an efficient separation with a rapid result is desirable. It has been found that a thin-layer chromatographic system described earlier for the separation of 5'-ribonucleotides[21] fulfils both requirements.

EXPERIMENTAL

Reagents

Nucleobases, nucleosides and nucleotides were commercial products (Sigma, St. Louis, Mo., U.S.A.) and their purities were checked by paper chromatography[22] and UV absorption[23]. [8-^{3}H]cAMP (27.5 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, Great Britain). [2,8-^{3}H]ATP (34.2 Ci/mmole) and [8-^{14}C]ATP (49.3 mCi/mmole) were obtained from New England Nuclear (Boston, Mass., U.S.A.). Creatine phosphate and creatine phosphokinase from rabbit skeletal muscle were obtained from Calbiochem (San Diego, Calif., U.S.A.). Cyclic inosine phosphates were prepared by amyl nitrite deamination according to the literature[24].

Thin-layer chromatography

Thin-layer chromatography was performed on Polygram Ionex-25 SA chro-

matoplates (Macherey, Nagel & Co., Düren, G.F.R.); identical results were obtained by using the same type of plate produced under the name Fixion 50-X8 (Chinoin, Nagytétény, Hungary). Plates in the sodium form were converted into the acidic form before use by continuous development[25] with 1.0 *N* hydrochloric acid and then with deionized water for 16 h with both solvents. Layers in the acidic form must be kept in the dark and it is advisable to use them within a few days. Stock solutions were prepared from bases in 1.0 *N* hydrochloric acid and from nucleosides and nucleotides in deionized water (5 mg/ml). For qualitative purposes, aliquots of 1 μl from these solutions were deposited 3 cm from the bottom edge of the chromatoplates in the form of small spots. Layers were immersed in deionized water at a depth of about 1.5 cm, and were developed for a distance of 16 cm from the origin. The time of the run was about 1 h.

The spots were rendered visible under a short-wave (254 nm) UV lamp (Desaga Uvis).

Elution

A known amount of [8-^{3}H]cAMP (about 250,000 cpm/ml of stock solution) was added to the appropriate stock solution. Aliquots of 5 μl of this sample solution were applied to a chromatoplate and the spots were eluted directly and after development.

In direct elution, the spots were cut out as squares of area 1.5 × 1.5 cm and were shaken with 3 ml of 1.0 *N* ammonia solution in a gyrotory shaker (Vibroterm, Labor-MIM, Esztergom, Hungary) at room temperature for 10 min.

In elution after development, the area of the chromatoplate between starting point and solvent front was divided into eleven equal sections and the individual sections were cut and eluted.

A volume of 1 ml of the eluate was added to 9 ml of Bray solution[26] and the radioactivity was measured in a Nuclear Chicago Isocap/300 liquid scintillation counter. The reference value was the radioactivity of 1 ml of a solution prepared from 5 μl of the sample solution and 3 ml of 1.0 *N* ammonia solution in the presence of a 1.5 × 1.5 cm piece of unloaded layer.

Recovery of cAMP

A rat liver particulate fraction (100 μg of protein)[27] was incubated for 10 min at 37° (final volume 0.2 ml) in a medium that contained 1 m*M* ATP, 1 m*M* cAMP, 10 m*M* magnesium chloride, 10 m*M* theophylline, 0.3% bovine fraction V albumin, 40 m*M* Tris–hydrochloric acid, pH 7.4, 12 m*M* creatine phosphate and 30 μg (6.3 I.U.) of creatine phosphokinase. At the end of the incubation, 0.1 ml of a recovery mixture consisting of 1 mg of cAMP, 1 mg of adenosine 2′(3′)-phosphate [2′(3′)-AMP] and 10 mg of ATP per millilitre in Tris–hydrochloric acid, pH 7.4, was added. The reaction was stopped by the addition of 0.1 ml of 5% zinc sulphate solution and 0.1 ml of 0.3 *N* barium hydroxide solution at room temperature[28]. A known amount of [8-^{3}H]cAMP (12,500 cpm per millilitre of reaction mixture) was added. After centrifugation for 10 min at 2000 *g*, 50-μl aliquots of supernatant were applied to an Ionex 25-SA (H^+) chromatoplate. The chromatographic separation and the measurement of radioactivity present in the cAMP spot were performed as described above.

Determination of blank value

The incubation mixture was prepared with heat-denaturated enzyme and with [8-^{14}C]ATP or [2.8-^{3}H]ATP (specific activity 20 μCi/μmole). The reaction was stopped as before and, after centrifuging for 10 min at 2000 *g*, 50-μl aliquots of the supernatant were separated. In a separate experiment, the mixture was centrifuged before adding the zinc sulphate and barium hydroxide solutions. In this case, 30-μl aliquots of the supernatant were chromatographed. The elution and the measurement of radioactivity were performed as described above.

Separation of guanosine 5'-phosphate (GMP) from cAMP

The incubation mixture was prepared with labelled ATP. In addition, the recovery mixture contained GMP (1 mg/ml). The reaction was stopped and, after centrifuging, 50-μl aliquots of the supernatant were subjected to two-dimensional separation on Ionex 25-SA (H^+) chromatoplates. The developing solvents were deionized water (first dimension) and 2 *N* hydrochloric acid (second dimension). The spots of GMP and cAMP were cut out, eluted and measured.

RESULTS AND DISCUSSION

As shown in Table I, the thin-layer chromatographic system, consisting of an Ionex 25-SA (H^+) chromatoplate and deionized water as developing solvent, is very suitable for the separation of cAMP from the most important interfering substances that may be present in a system for the assay of adenyl cyclase activity*.

The complete separation of all of the compounds listed in Table I could not be achieved, but a sharp and distinct resolution of cAMP from the other substances was attained, with the exception of GMP. The time of development was fairly short (1 h) and special precautions (chamber saturation, constant temperature, etc.) were unnecessary. The presence of protein and inorganic salts in moderate amounts did not interfere in the separation. This rendered possible the direct application of aliquots of an adenyl cyclase assay system to the chromatoplates. GMP ran together with cAMP, but the probability of transfer of radioactivity from ATP to GMP seemed small. It was justified further by separating the two compounds in the second dimension by developing with 2 *N* hydrochloric acid. As expected, no radioactivity was observed in the spot of GMP. However, it must be pointed out that the other 3',5'-cyclic nucleotides, which are the main interfering substances in protein binding assay[30], are well separated from cAMP in our system.

The applicability of this thin-layer chromatographic system to the determination of adenyl cyclase activity depends on whether the radioactivity of the cAMP, produced from a labelled ATP, can be measured at the picomole level with adequate reproducibility. In general, this measurement is performed by the liquid scintillation technique.

The direct measurement of radioactivity on chromatoplates in such a way, however, is not feasible owing to the high self-absorption and quenching effect of the layer material. Thus, the elution of cAMP was required, prior to measurement.

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* Some of these interfering compounds were separated on a Dowex 50-X4 (H^+) column with 0.01 *N* hydrochloric acid as eluent[29].

TABLE I

R_F VALUES OF ADENINE DERIVATIVES ON IONEX 25-SA(H^+) CHROMATOPLATES IN DEIONIZED WATER

Each figure is the average of six determinations.

Compound	R_F	*Compound*	R_F
Adenine	0.00	2'(3')-GMP	0.31 ± 0.03*
Adenosine	0.00	2',3'-cyclic-GMP	0.31 ± 0.03*,**
Hypoxanthine	0.00	3',5'-cyclic-GMP	0.64 ± 0.05
Guanine	0.00	ADP	0.90 ± 0.05§
Guanosine	0.00	ATP	>0.90§
Inosine	0.06 ± 0.01*	2'(3')-IMP	>0.90§
5'-AMP	0.07 ± 0.01*	5'-IMP	>0.90§
3'-AMP	0.10 ± 0.01*	2',3'-cyclic-IMP	>0.90**,§
2'-AMP	0.14 ± 0.01*	3',5'-cyclic-IMP	>0.90§
2',3'-cyclic-AMP	0.10; 0.14*,**	IDP	>0.90§
3',5'-cyclic-AMP	0.24 ± 0.02*	ITP	>0.90§
	(0.37 ± 0.02)***	GDP	>0.90§
5'-GMP	0.25 ± 0.02*	GTP	>0.90§
	(0.63 ± 0.05)***		

* Small, round spots with no tailing.

** Compounds were hydrolyzed quantitatively to a mixture of the isomeric monophosphates by the acidity of the layer.

*** Values in parentheses are those observed in 2 *N* HCl.

§ Diffuse spots.

As shown in Table II, cAMP could be recovered quantitatively from the chromatoplates by elution with 1.0 *N* ammonia solution at room temperature for 10 min. After development, an average of 85% of the total radioactivity applied was found in the spot of cAMP, while an additional 10% was detected elsewhere on the chromatoplate (Fig. 1).

In order to determine the overall recovery of cAMP, an adenyl cyclase assay system was prepared with cold ATP. A known amount of [8-^{3}H]cAMP was added to the incubation mixture after stopping the reaction (see below). Aliquots of the

TABLE II

RECOVERY OF [8-^{3}H]cAMP FROM IONEX 25-SA(H^+) CHROMATOPLATES

Results are given as counts per minute. Average amount applied: 1056 cpm.

	Recovered directly	*Recovered after development**	
		a	*b*
	1050	935	945
	1059	902	912
	1058	876	877
	1103	879	890
	1087	930	923
	1032	918	901
Average:	1065	905	908

* a, Spotting from deionized water; b, spotting from an assay system containing cold ATP.

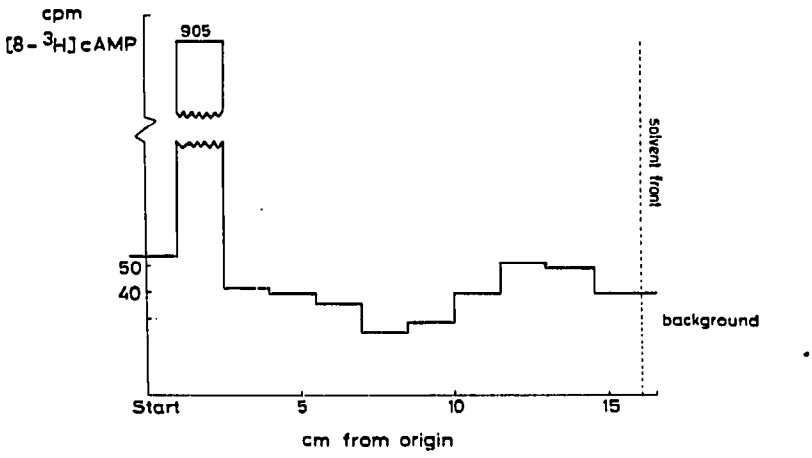

Fig. 1. Distribution of radioactivity in an [8-³H]cAMP preparation after chromatography on Ionex 25-SA(H^+) chromatoplates in deionized water.

incubation mixture, containing the same amount of radioactivity as above, were subjected to separation. Again, 85% of the total radioactivity was found in the spot of cAMP. In view of the above experiment, this represents a quantitative recovery (Table II).

In order to establish the amount of unknown radioactive impurities in commercial ^3H- and ^{14}C-labelled ATP preparations, which migrated together with cAMP, *i.e.*, the blank value, heat-denaturated enzyme was used in the preparation of the assay system. About 0.05% of the total radioactivity of ATP appeared at the position of cAMP, which corresponded to an average of 200 cpm per spot. This value was rather high, as the specific activity of ATP was about 20 pCi/pmole, and thus only blank values at least at the picomole level could give an adequate accuracy. Adequate blank values (average 80 cpm per spot) could be attained, however, when the reaction was stopped by the zinc sulphate–barium hydroxide precipitation technique[28]. Using this method, most of the ATP was precipitated before chromatography and, simultaneously, the radioactivity co-chromatographed with cAMP decreased by approximately 60% without any loss in the cAMP recovered (Table II).

The aim of this paper is to draw attention to the applicability of chromatoplates coated with strong cation-exchange resin in the routine assay of adenyl cyclase activity, which is based on the use of ^3H- or ^{14}C-labelled ATP as substrate and the measurement of the radioactivity of the product, cAMP, by the liquid scintillation technique. The use of this type of chromatoplate could improve the assay, as it offers a better separation of cAMP from interfering substances than that obtained by earlier chromatographic methods. Some additional advantages are the simplicity of the chromatography, the short time required and the adequate and reproducible recovery.

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