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Isolation of cyclic 3′,5′-adenosine monophosphate on a neutral silicic acid–glass microfiber matrix

The isolation of cyclic 3′,5′-adenosine monophosphate (cyclic-AMP) has concerned an increasing number of investigators. The multitude of published methods attests to the difficulties encountered with such an isolation[1–10]. This nucleotide is formed from adenosine 5′-triphosphate (ATP) by the enzyme, adenyl cyclase, and apparently functions as an intracellular mediator for a large array of hormones and neurotransmitters[11].

The isolation of cyclic-AMP on a silicic acid–glass microfiber matrix, as outlined in this communication, suggests a combination of advantages over other published procedures: (1) rapidity of development (approximately 50 min); (2) no significant interference by trichloroacetic acid (TCA), salts or Tris buffer; (3) ease of spot removal and dispersal for liquid scintillation counting; (4) separation from inosine, hypoxanthine, adenine, adenosine, as well as other nucleotide phosphates; (5) good recovery (95 %) from 40 nmoles to less than 0.01 pmole of cyclic-AMP when eluted following chromatography. Our chromatographic techniques have been applied to the assay of adenyl cyclase activity in tissue (manuscript in preparation) and are suited also to quantitative isolation of endogenous cyclic-AMP.

Materials and methods

Chemicals. Non-radioactive cyclic 3′,5′-adenosine monophosphoric acid was obtained from PL Biochemicals, Inc. N^6-2′-O-Dibutyryl cyclic 3′,5′-adenosine monophosphate was purchased from Schwarz Bioresearch, Inc. Cyclic 3′,5′-guanosine monophosphate was purchased from Boehringer Mannheim Corporation. All other non-radioactive purine bases, nucleosides and nucleoside mono-, di-, and triphosphates

were obtained from Sigma Chemical Company. [^{3}H]Cyclic-AMP (2350 mCi/mmole) was purchased from Schwarz Bioresearch, Inc. Anhydrous theophylline and ultra-pure trihydroxymethylaminomethane (Tris) were purchased from Mann Research Laboratories. Ethylene glycol-bis(β-aminoethylether) N,N-tetraacetic acid (EGTA) was purchased from Eastman Organic Chemicals. Dithiothreitol was purchased from Calbiochem, and bovine albumin (crystalline) was purchased from Pentex, Inc. Chemicals for scintillation counting were obtained from Packard Instrument Company, Inc. All other reagents were Mallinckrodt analytical grade.

Chromatography. Ascending chromatography was accomplished in a sandwich chamber (Brinkman Instruments, Inc.) on 18.0 cm × 18.5 cm sections of 70 % neutral silicic acid–30 % glass microfiber matrix 500 μ in thickness (ChromAR-500, Mallinckrodt Chemical Works). Untreated matrix sheets were used without activation. For some separations, the silicic acid-glass microfiber matrix was pretreated by development with dimethylpolysiloxane (Dow Corning Corp.), (dimethylpolysiloxane–chloroform-acetone, 1:4:15). The pretreated matrix was activated at 110° for 30 min prior to spotting. The 4-mm-thick Teflon spacers of the commercial sandwich chromatographic chamber were replaced, usually, by 7-mm-thick spacers. Thus, it was convenient to develop two matrix sheets simultaneously within a single chamber. Such sheets were separated from each other and from the chamber walls by Teflon matting (Bel Art Products). Some chromatograms were also developed by supporting 2-cm-wide strips of the matrix sheet between strips of Teflon matting in covered 100-cc graduated cylinders.

The following solvent systems were used for chromatography: (A) 2-Propanol–ethyl acetate–13.6 *N* ammonium hydroxide (55:29:16). Development time was approximately 50 min at 35°. (B) 2-Propanol–ethyl acetate–8.5 *N* ammonium hydroxide (30:44.5:25.5). Development time was approximately 55 min at 35°. (C) Disodium ethylenediaminetetraacetic acid (EDTA, 0.05 *N*) in 0.5 *N* ammonium hydroxide. This system, developed in 12 min at room temperature, approached to within 3.5 cm of the top of the sheet. Upon removal of the chromatogram and allowing it to air dry, the front moved to near the top of the sheet. (D) *tert.*-Butanol–ethyl acetate –8.2 *N* ammonium hydroxide (37:26:37). Unless this system was made up a few hours prior to use, streaking would result. Development time was approximately 80 min at 35°. (E) Ammonium sulfate (saturated aqueous at 25°, pH 3.0)–2-propanol (98:2). Development time was approximately 55 min at 35°.

Unless otherwise specified, all compounds which were first chromatographed with solvent system A or B were dissolved in an adenyl cyclase incubation medium which contained 62 mmoles Tris–HCl buffer pH 7.5, 3 mmoles $MgSO_4$, 50 mmoles KCl, 10 mmoles theophylline, 0.1 mmoles EGTA, 20 mmoles dithiothreitol, 3 mmoles cyclic-AMP carrier, and 0.8 mg /ml bovine albumin. Solutions of the compounds dissolved in this adenyl cyclase incubation medium were then mixed 2:1 with 15 % trichloroacetic acid (TCA). Aliquots (20 μl) of the final mixture were made basic with 1 μl of concentrated ammonium hydroxide and spotted 2.5 cm from the bottom of a 18.5 cm × 18.0 cm matrix sheet. Eight such spots could be placed along an 18-cm edge of the sheet. Developed spots were visualized under short-wave UV. Ribose could be located by charring on a hot plate.

Radioactive spots were cut out, dispersed in 1 ml of 0.5 *N* ammonium hydroxide, and then mixed with 14.0 ml of scintillation mixture. The scintillation mixture[12]

contained 25 % of Triton X-100, 2,5-diphenyloxazole (5.36 mg/ml) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.536 mg/ml) dissolved in toluene. Counting was done in a Nuclear Chicago Mark I scintillation counter (counting error $< 5\%$). Corrections were made for counting efficiency (approximately 40% for ^3H) based on the external standard quench ratios.

Results

As seen in Fig. 1, solvent system A gives adequate separation of cyclic-AMP from the common adenine and hypoxanthine derivatives found in tissue extracts. Fig. 2 shows the effects which the variations in ammonium hydroxide, ethyl acetate and aqueous content have upon the R_F values of these compounds. Preliminary experiments indicated that increasing the percentage of ethyl acetate from 0 up to 15 decreased the R_F values of cyclic -AMP, inosine, and 5'-AMP, but had much less effect upon adenine and adenosine. Such an increase in the percentage of ethyl acetate (from 0 up to 15) also increased the degree of separation between cyclic-AMP and inosine.

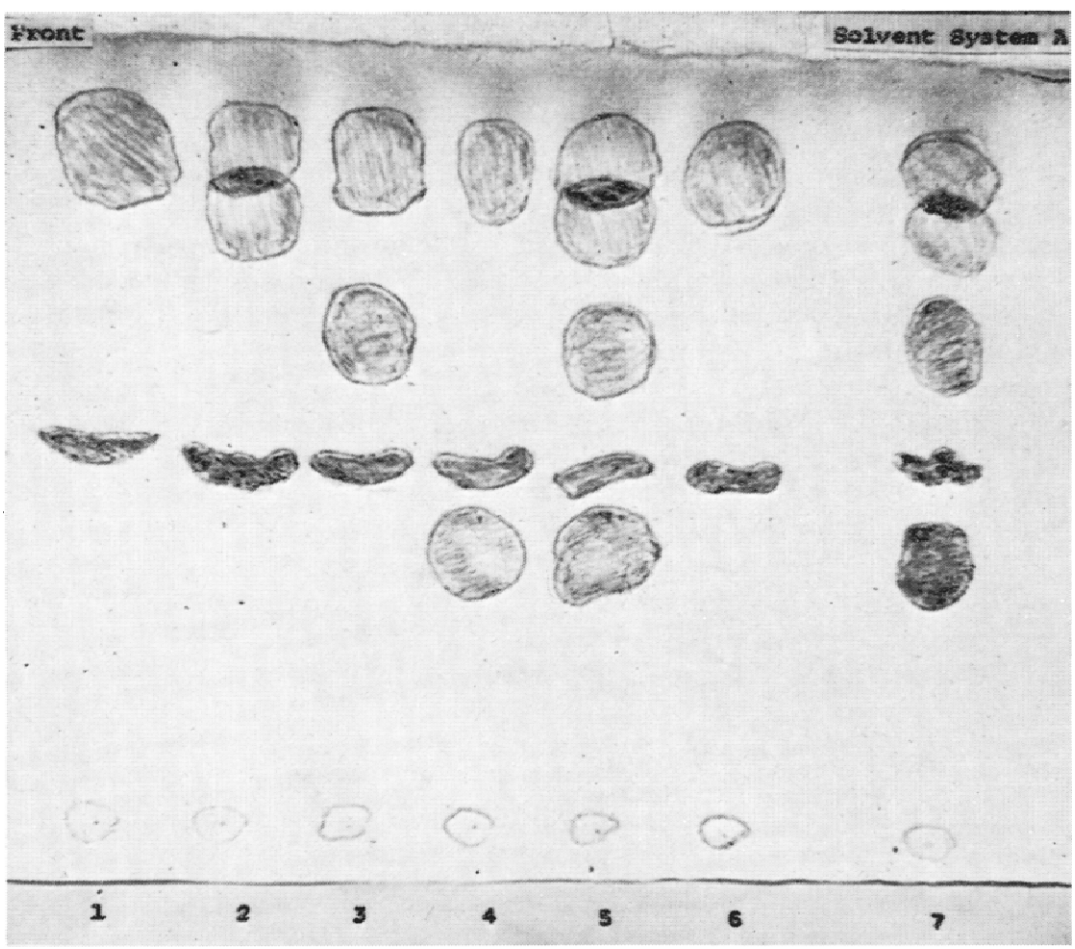

Fig. 1. Development of neutral silicic acid–glass microfiber matrix in solvent system A. In channel 6, 20 μl of adenyl cyclase incubation medium was spotted containing theophylline (upper spot) and cyclic-AMP (lower spot). All channels contain these two compounds but, in addition, contain adenine superimposed on theophylline (1), adenosine (2), hypoxanthine (3), inosine (4), and a mixture of adenine, adenosine, hypoxanthine and inosine (5). Channel 7 is the same as channel 5 except the spotting volume was only 10 μl.

Further increases in ethyl acetate concentration reduced the separation of cyclic-AMP from inosine, but gave greater resolution from ribose and hypoxanthine (Fig. 2b). Increasing the ammonium hydroxide content from 1.0 *N* up to 2.2 *N* decreased the R_F values of cyclic-AMP and inosine while simultaneously increasing the resolution (Fig. 2a). However, the higher concentrations of ammonium hydroxide and ethyl acetate induced some streaking of adenosine and inosine. In this latter situation, a small increase in the total aqueous content of the solvent increased the R_F values and prevented streaking.

If TCA and Tris were present when solvent system A, B, or D was used (Table I), it was necessary to make the spotting solution basic with ammonium hydroxide; otherwise, a significant portion of the sample was retained at the origin. The use of solvent system C with pretreated matrix or of solvent system E with the untreated matrix has the disadvantage that the presence of TCA, Tris or salts will interfere with separation. Materials in the presence of these substances were, therefore, always chromatographed first with either solvent system A or B on the untreated matrix prior to reversed-phase chromatography.

The order of R_F values could be partially reversed by development with solvent system E. It seemed desirable, however, to be able to move cyclic-AMP ahead of adenine and adenosine. This latter was accomplished when solvent system C was used in conjunction with a silicic acid-glass microfiber matrix which had been pretreated with 5% dimethylpolysiloxane (Table I). Solvent system C is useful, therefore, in verifying the purity of cyclic-AMP which has been isolated by chromatography with solvent system A. When re-chromatography is planned, the compounds are visualized

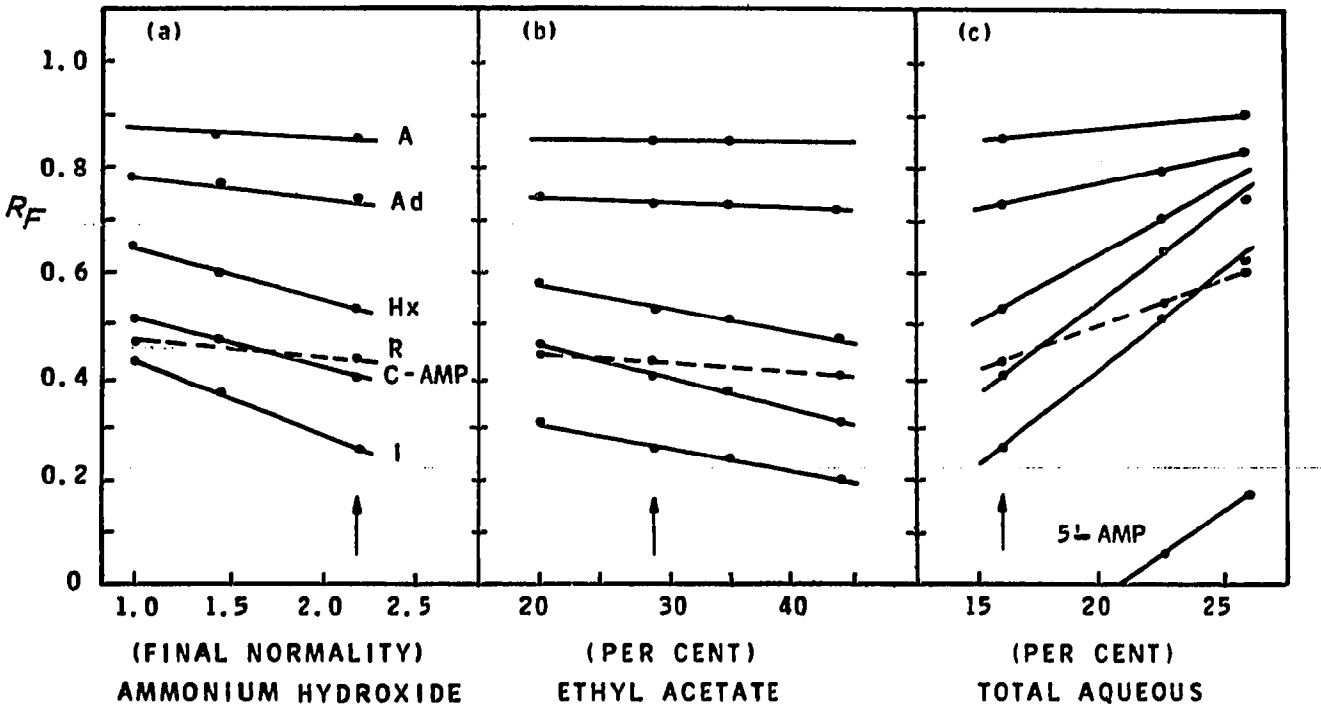

Fig. 2. Variations in R_F values produced by varying the concentration of a single component of solvent system A. The arrows indicate the concentrations present in solvent system A. Variations in a specified component were always in lieu of 2-propanol. Compounds were spotted in 20-μl aliquots of adenyl cyclase incubation medium mixed 2:1 with 15% TCA.

TABLE I

R_F VALUES OF COMPOUNDS SEPARATED BY CHROMATOGRAPHY ON A SILICIC ACID—GLASS MICROFIBER MATRIX

Procedures and solvent systems are described in *Methods*. Solvent system C was used with 5% dimethylpolysiloxane-pretreated matrix and the compounds were spotted on an attached strip of untreated matrix as described in *Methods*. All other solvent systems were with the untreated matrix. Values in parentheses were from samples dissolved in the adenyl cyclase incubation medium mixed 2:1 with 15% TCA. All other samples were spotted in water. The R_F value for cyclic-AMP with solvent system A was 0.39 ± 0.01 S.D. Reproducibility was similar for other R_F values.

Compound	*Solvent system*				
	A	*B*	*C*	*D*	*E*
Theophylline	— (0.86)	—	—	—	0.42
Adenine	0.80 (0.85)	(0.90)	0.45	0.92	0.65
Adenosine	0.70 (0.73)	(0.78)	0.50	0.85	0.68
Dibutyryl cyclic-AMP	0.73	—	—	0.93	—
Hypoxanthine	0.53 (0.53)	0.73 (0.73)	0.77	—	0.82
D-Ribose	— (0.43)	0.53 (0.53)	0.95	0.75	—
Cyclic 2′,3′-AMP	0.53 (0.47)	0.69 (0.68)	0.80	0.81	—
Cyclic 3′,5′-AMP	0.42 (0.39)	0.69 (0.68)	0.67	0.89 (0.81)	0.53
Inosine	0.25 (0.26)	0.51 (0.52)	0.85	0.75 (0.58)	0.88
Cyclic-GMP	0.12	—	—	0.78	—
3′-AMP	0 (0)	0.30 (0.31)	0.91	0.66	—
2′-AMP	0 (0)	0.33 (0.33)	0.87	0.57 (0.57)	—
5′-AMP	0 (0)	0.19 (0.19)	0.92	0.51 (0.47)	0.66
ADP	0 (0)	0.09 (0.09)	—	0.36 (0.35)	—
ATP	0 (0)	(0)	—	0.25 (0.25)	—

under short-wave UV following development in solvent system A. Then a horizontal strip containing the compound of interest is cut from the matrix and placed against the bottom edge of a sheet pretreated with 5% dimethylpolysiloxane. There should be a 0.5-cm overlap and essentially no untreated matrix sheet left above the leading edge of the spots. The spots are held in good contact by the slight pressure of the Teflon matting on the front and back of the sheets. Using this procedure, re-chromatography with solvent system C gives good resolution and very little additional spreading of the spots. The use of solvent system C with the dimethylpolysiloxane-treated matrix sheet also provided a means for moving 5′-AMP ahead of cyclic-AMP. Such a separation is desirable to verify the purity of 5′-AMP which has been isolated on untreated matrix sheets with solvent system B or D. These chromatographic systems have been utilized primarily for evaluation of cyclic-AMP phosphodiesterase activity.

Commercial [^{3}H]cyclic-AMP (without purification) was used to check the chromatographic recovery of low concentrations of the nucleotide. Aliquots (20 μl) of 32 samples were spotted in quantities ranging from 0.01 pmoles to 40 pmoles with or without subsequent development with solvent system A. Spots were then removed, dispersed in the standard scintillation mixture and counted. Recoveries of the impure material were 82.1% (± 0.9 S.E.) based upon the undeveloped spots. There was no indication that the percentage recovery of radioactive material varied with the quantity of cyclic-AMP applied, even when an additional 40 nmoles of authentic cyclic-AMP carrier were present. Neither was recovery significantly different when the spots were placed in disposable glass Pasteur-type pipettes with glass wool plugs and eluted into counting vials with 1 ml of 0.5 *N* ammonium hydroxide. When eight 20-μl aliquots of puri-

fied [^{3}H]cyclic-AMP (2.8 pmoles each) were chromatographed with solvent system A and eluted, the recoveries were 94.9% (± 1.0 S.E.). Elution with 50% ethanol is recommended for a greater non-destructive recovery of nucleotides, but unless the ethanol is removed prior to scintillation counting there is considerable quenching.

Discussion

In general, the purpose of cyclic-AMP isolation has been to facilitate the quantitation of the extremely small concentrations of the nucleotide which are endogenously present in tissue, and/or to make possible the estimation of adenyl cyclase activity in rather crude tissue preparations. The isolation procedures described in this paper can be used to accomplish both of these purposes with a combination of greater convenience, rapidity, and specificity than afforded by other techniques[1–10].

Variations of the technique published by KRISHNA *et al.*[7] have been employed by many investigators for estimation of adenyl cyclase activities. This procedure used a $Ba(OH)_2$ and $ZnSO_4$ precipitation to remove the majority of contaminants. There is only a slight loss of cyclic-AMP by this precipitation and adenosine is not removed. Removal of adenosine and more extensive purification from other contaminants is effected by passage of the samples through Dowex 50 cation-exchange resin. Although apparently satisfactory in some instances, low rates of cyclic-AMP formation cause unreliable results with this technique[8]. It is likely, also, that trace amounts of barium and zinc would preclude the quantitation of cyclic-AMP by enzymatic means. These disadvantages of $Ba(OH)_2$–$ZnSO_4$ precipitation may be overcome with further purification by thin-layer[10] or paper chromatography[13].

There are several isolation procedures which do not include $Ba(OH)_2$–$ZnSO_4$ precipitation. The original method of BUTCHER *et al.*[1] employs fractionation on a series of three columns. The acid extract is first passed through an anion-exchange column, then through a cation-exchange resin, and finally through a second anion exchanger. Some workers have used paper chromatography alone[3,6]. Others have used unsupplemented thin-layer chromatography on either MN 300 cellulose[4] or Silica Gel G[9].

Each of the published procedures discussed above requires either more time and manipulation or would seem to be less specific for cyclic-AMP than the method described in this paper. Chromatography on neutral silicic acid–glass microfiber matrix sheets using solvent system A (for adenyl cyclase assays which employ radioactive ATP as substrate) and solvent system B (for phosphodiesterase assays which use radioactive cyclic-AMP as substrate) has been used extensively in this laboratory during the past year (details of these procedures are being prepared for publication). It is a simple matter for one technician to perform the assay incubation for 32 samples in a morning and then to complete the chromatography so that the samples can be counted the evening of the same day.

A recent publication[14] shows the isolation of cyclic-AMP on silica gel-impregnated glass–microfiber sheets, but gives no indication as to whether separation from inosine and hypoxanthine occurs and indicates that salts and other tissue constituents reduce separation. In contrast, in our chromatographic method, the presence of TCA, salts or Tris buffer caused only a slight reduction in R_F values with solvent systems A, B or D (see Table I). Also, extracts of rabbit ciliary process and renal tissue which contained up to 1.2 mg of TCA-precipitable protein, caused no significant alteration in these values.

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Identification of drugs of abuse in urine

I. A study of the Dole technique

DOLE *et al.*[1] developed a thin-layer chromatographic method for the detection in urine of narcotic drugs, quinine, barbiturates, amphetamines and some tranquilizers. The drugs are first absorbed on ion-exchange paper and then extracted at controlled pH values into an organic phase. An aliquot of the organic phase is concentrated and chromatographed. A series of spray reagents were developed to provide detection and confirmation. This report describes a study of the DOLE technique.

Experimental procedure

The urine samples used in this study were selected at random from the samples routinely received for analysis from the NIMH methadone treatment out-patient