Assay of Cyclic 3′,5′-Nucleotide Phosphodiesterase in Tissue Homogenates[1] (35577)

PAUL F. GULYASSY[2] AND RICHARD L. OKEN[3]
(Introduced by John H. Peters)

Medical Service, San Francisco General Hospital and Department of Medicine and Cardiovascular Research Institute, University of California, San Francisco, California 94110

Adenosine-3′,5′-monophosphate (cyclic AMP) is the intracellular mediator of the actions of most peptide hormones and biogenic amines. The concentration of cyclic AMP in cells is controlled by adenyl cyclase, the enzyme which catalyzes its synthesis from ATP, and by cyclic 3′,5′-nucleotide phosphodiesterase, which catalyzes its inactivation by hydrolysis to adenosine-5′-monophosphate (5′-AMP) (1, 2). The assay of phosphodiesterase in crude homogenates at low concentrations of cyclic AMP presents several problems. We had difficulty using the indirect method of Butcher and Sutherland (3) because of the high background of phosphate. Direct assay of cyclic AMP by the original method of Butcher and Sutherland (3) or even by newer methods (4, 5) is extremely complex and subject to interference.

In a previous study we made limited use of chromatographic separation with thin layers of cellulose mixed with polyethyleneimine (PEI) in studies of the effect of theophylline on phosphodiesterase activity (6). In that work, residual labeled cyclic AMP was isolated but the amounts of specific metabolites formed were not measured. To determine whether simpler methods of assay of phosphodiesterase (7, 8) could be used for our purposes, we need to have detailed knowledge of which labeled metabolites are formed from cyclic AMP-^{3}H in tissue homogenates. In the present study we perfected the chromatographic separation on PEI-cellulose of isotopically labeled cyclic AMP from labeled products as a means to assay phosphodiesterase. To reduce contamination on chromatograms of the residual labeled cyclic AMP by remote derivatives, we evaluated two approaches: limitation of the time of incubation and "trapping" of the initial product, 5′-AMP, by adding a large excess of unlabeled 5′-AMP. The advantages and limits of these approaches are described. Chromatographic analysis of the pattern of formation of metabolites of cyclic AMP indicates possible sources of error in the assay of phosphodiesterase by other simpler methods.

Materials and Methods. Purification of nucleotides. Cyclic AMP-^{3}H (obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y.) contained a small amount of impurity (1–5%), which was removed by column chromatography on Dowex-50 (Bio-Rad Laboratories, Richmond, Calif.). Similarly 5′-AMP-8-^{14}C (Schwarz BioResearch, Inc.) was purified before use by column chromatography, followed by desalting with charcoal (9) to remove the ammonium formate used in elution from the column.

Thin-layer chromatography. We prepared thin layers of cellulose (Macherey, Nagel & Co., Duren, Germany) impregnated with 0.5% undialyzed PEI on glass plates according to the method of Randerath and Randerath (10). PEI of 30,000–40,000 molecular weight was obtained initially from Chemirad Corp., East Brunswick, New Jersey and later from Alcolac Chemical Corp., Baltimore, Maryland. Clear separation of the most important metabolites known to be formed in toad bladders (6) was obtained by developing the plates first in distilled water

[1] Supported by U.S. Public Health Service Grant No. AM-12638.

[2] Recipient of NIH Career Development Award (HE-11350).

[3] Recipient of NIH Student Fellowship (HE-05251-11).

to 4–5 cm beyond the origin and then to the front (16–17 cm) with 0.3 *M* LiCl–5 m*M* tris(hydroxymethyl) aminomethane (Tris) chloride buffer (pH 8.4). The relative mobilities of the metabolites were essentially the same whether run as simple aqueous standards or mixed with 5% trichloroacetic acid (TCA) extracts of toad bladders or rat kidneys. During most of these studies, using PEI obtained from Chemirad Corp., it was possible to obtain clear separations of standards added to 5% TCA extracts of tissues when applied as such to the thin-layer plates (see Results). In more recent studies, using PEI obtained from Alcolac Chemical Corp. (who purchased the Chemirad Corp.), the slower moving nucleotides often exhibited prohibitive degrees of bowing and 5′-AMP overlapped with cyclic AMP. This artifact was completely eliminated (without loss of radioactivity) by extracting the TCA supernates 3–4 times with 1.5 vol of diethyl ether before applying samples to the thin-layer plates.

Homogenates of toad bladder epithelial cells. The epithelial cell layer lining the urinary surface of toad bladders (*Bufo marinus* from Tarpon Zoo, Silver Springs, Fla.) was scraped off as previously described (6). The scrapings were washed into a solution containing 120 mEq of KCl and 5 mEq of $MgCl_2$/liter and homogenized with a Polytron homogenizer (Brinkman Instruments, Inc.) at the 3 setting for 10–20 sec. The following solutions were pipetted into four pairs of small test tubes: (a) 0.15 ml of 160 m*M* Tris chloride (pH 9.0) into one, Set A, and 0.15 ml of 160 m*M* Tris chloride (pH 9.0) with 20 m*M* 5′-AMP (Calbiochem, Los Angeles, Calif.) into the second, Set B; (b) 0.05 ml of cyclic AMP-^{3}H (5 μCi, 20 mμmoles) obtained from Schwarz BioResearch, Inc.; and (c) 0.4 ml of the homogenate of the bladder. The concentration of cyclic AMP in the incubation mixture was 3×10^{-5} *M*. One pair of tubes, designated the "0 time" sample, contained either 0.05 ml of 50% TCA or the same volume of TCA was added immediately after addition of the homogenate. The other three pairs of tubes were incubated for 10, 20, and 30 min before addition of 0.05 ml of 50% TCA. The precipitate was removed by centrifugation at 5000 rpm for 5 min. Protein content of the precipitate was assayed by the method of Lowry *et al.* (11). Mean protein concentration ranged from 0.60 to 0.75 mg/ml. Ten μl of the TCA supernate, along with reference solutions of inosine, adenosine, cyclic AMP, and 5′-AMP were spotted on the PEI-cellulose thin-layer plates and developed as described earlier. The areas occupied by the reference standards were outlined under ultraviolet (UV) light. The outlined areas plus 1-cm margins were scraped off separately (forming small scrolls) and placed into individual liquid scintillation counting vials. The radioactivity from the areas of the reference standards was eluted in the scintillation vials by extraction with 1 ml of 1.0 *N* NaOH. The remaining PEI-cellulose from the front to 1 cm below the origin was placed into a test tube. The unidentified radioactivity on the PEI-cellulose in the test tubes (designated "other" in Tables II and III) was extracted with 5 ml of 1.0 *N* NaOH and 1 ml of this extract was transferred to a counting vial. Fifteen ml of the liquid scintillation solution described previously (12) were added and the samples were assayed in a liquid scintillation spectrometer. Corrections for quenching were made by using internal standards.

Parallel sets of studies were conducted to determine the rate of metabolism of 5′-AMP-8-^{14}C. The protocol was identical to that outlined in the study of cyclic AMP-^{3}H except that in the latter studies 5′-AMP-8-^{14}C (0.4 μCi, 9 mμmoles) was the labeled nucleotide added. The remaining aspects of the incubation of the homogenates, separation of metabolites on PEI-cellulose and radioassay were as outlined in the studies of the metabolism of cyclic AMP-^{3}H.

Results and Discussion. The recovery of radioactivity for each phase of the procedure was evaluated. The TCA extracts contained over 99% of the original ^{3}H (or ^{14}C) added and only 0.3–0.4% was lost during extraction with ether. Losses during the remainder of the procedure were also extremely small. The sum of cpm (after correction for quenching and aliquot size) for the five segments of PEI-cellulose (four zones with "cold" standards and *all* remaining material, designated

"other" in Tables II and III) in 13 studies was 98.5 ± 1.3% (mean ± SEM) of the quantity in the original ether-extracted TCA supernates. The 1.5% loss was accounted for as residual material inside or around the tip of the 10-μl pipette used for applying the sample to the thin-layer plates.

Among the many solutions and combinations of solutions we tried for development of the PEI-cellulose, the sequence of water and 0.3 *M* LiCl provided the best results for the present purposes. Table I shows the separation of cyclic AMP from potential metabolites was excellent, such that a margin of at least 1.0 cm (beyond the visible area of absorption under UV light) was always available during scraping of each standard.

The amount of metabolism as a function of time and the pattern of distribution of radioactivity among major metabolites are shown in Tables II and III. Studies in Table II show progressive increase in the percentage of cyclic AMP-^{3}H metabolized by the toad bladder preparation. In the absence of added

TABLE I. Mobilities (R_f) of Nucleotides and Nucleosides During Chromatography on Thin Layers of PEI-Cellulose.

Compounds were visualized under ultraviolet light.

Compounds	TCA extract	
	Toad bladder	Rat kidney
Inosine	0.80 ± 0.01[a]	0.80 ± 0.01[b]
Adenosine	0.61 ± 0.01	0.60 ± 0.02
Cyclic AMP	0.45 ± 0.01	0.42 ± 0.01
5′-AMP	0.30 ± 0.01	0.26 ± 0.01
ADP, ATP[c]		

[a] Means ± SEM for 25 separate runs.

[b] Means ± SEM for 18 separate runs.

[c] ATP and ADP did not separate and moved only a short distance (R_f: 0.05–0.10).

TABLE II. Metabolism of Cyclic AMP-^{3}H by Homogenates of Toad Bladder Epithelial Cells in the Absence (Set A) and Presence (Set B) of Added 5′-AMP in Paired Studies.

The initial concentration of cyclic AMP in both groups was 3×10^{-5} *M*.

Compounds	Duration of incubation (min)			
	0	10	20	30
		1. Distribution of radioactivity (%)[a]		
		A. No added 5′-AMP		
Cyclic AMP	95.8 ± 2.0	79.5 ± 3.2	64.7 ± 6.3	50.4 ± 7.5
5′-AMP	1.8 ± 1.3	1.5 ± 0.3	1.6 ± 0.3	1.3 ± 0.2
Adenosine	1.3 ± 1.3	13.7 ± 2.7	19.4 ± 5.6	24.4 ± 5.6
Inosine	0.4 ± 0.3	4.0 ± 0.7	13.2 ± 2.2	22.5 ± 2.6
Other	0.5 ± 0.4	0.9 ± 0.5	0.7 ± 0.4	1.0 ± 0.5
		B. 5′-AMP added (5×10^{-3} *M*)		
Cyclic AMP	96.4 ± 1.6	81.3 ± 3.1	68.1 ± 5.6	57.3 ± 6.2
5′-AMP	2.1 ± 1.6	16.8 ± 2.4	29.1 ± 4.6	37.9 ± 4.7
Adenosine	0.1 ± .04	0.6 ± 0.2	1.6 ± 0.6	3.1 ± 1.1
Inosine	0.1 ± .02	0.1 ± .04	0.2 ± .08	0.6 ± 0.2
Other	1.0 ± 0.8	0.7 ± 0.5	0.6 ± 0.5	0.8 ± 0.5
		2. Cyclic AMP-^{3}H metabolized (%)[b]		
A	—	17.0	33.1	48.1
B	—	15.7	30.6	41.5
B—A	—	—1.3 ± 0.7[c]	—2.5 ± 0.2	—6.5 ± 1.3
p value		NS	<0.01	<0.05

[a] Mean ± SEM in 3–4 paired studies for percentage of total ^{3}H found in each zone of chromatograms.

[b] % Metabolized = $100 \times [(\text{cyclic AMP-}^3\text{H})_{0'} - (\text{cyclic AMP-}^3\text{H})_{t'}/(\text{cyclic AMP-}^3\text{H})_{0'}]$.

[c] Mean ± SEM of difference (B—A) for each pair.

TABLE III. Metabolism of 5′-AMP-8-^{14}C by Homogenates of Toad Bladder Epithelial Cells in the Absence (Set A) and Presence (Set B) of Added 5′-AMP.

Compounds	Duration of incubation (min)			
	0	10	20	30
	1. Distribution of radioactivity (%)[a]			
	A. No added 5′-AMP			
5′-AMP	92.7 ± 0.7	7.8 ± 4.6	1.9 ± 0.9	0.7 ± 0.3
Cyclic AMP	1.0 ± 0.2	3.0 ± 0.8	2.8 ± 1.0	4.2 ± 1.4
Adenosine	2.3 ± 0.7	60.3 ± 5.7	46.6 ± 4.0	38.8 ± 4.1
Inosine	0.3 ± 0.1	25.2 ± 6.9	44.9 ± 4.0	52.9 ± 3.6
Other	3.0 ± 0.4	3.1 ± 0.4	3.4 ± 0.9	2.9 ± 0.5
	B. 5′-AMP added (5×10^{-3} *M*)			
5′-AMP	92.5 ± 0.7	84.5 ± 3.7	80.6 ± 4.5	77.0 ± 5.5
Cyclic AMP	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.2 ± 0.1
Adenosine	2.4 ± 0.7	8.8 ± 3.2	11.9 ± 3.9	14.5 ± 4.7
Inosine	0.4 ± .04	1.9 ± 0.6	2.9 ± 0.8	3.9 ± 1.0
Other	3.0 ± 0.3	3.1 ± 0.3	2.8 ± 0.4	3.0 ± 0.2
	2. 5′-AMP-^{14}C metabolized (%)[b]			
A	—	91.6 ± 4.9[c]	97.8 ± 1.0	99.1 ± 0.3
B	—	8.6 ± 3.7	12.8 ± 4.5	16.3 ± 5.3

[a] Mean ± SEM in 3–4 paired studies for percentage of total ^{14}C found in each zone of chromatograms.

[b] % Metabolized = $100 \times [(5'\text{-AMP-8-}^{14}\text{C})_{0'} - (5'\text{-AMP-8-}^{14}\text{C})_{t'}/(5'\text{-AMP-8-}^{14}\text{C})_{0'}]$.

[c] Mean ± SEM for each set.

5′-AMP there was total failure of 5′-AMP-^{3}H to accumulate at any time between 10 and 30 min of incubation.

Without added 5′-AMP the first labeled product formed from cyclic AMP-^{3}H was adenosine, followed by inosine (Table II, Set A). Failure to detect adenosine-^{3}H in our earlier studies (6) could have been due either to the much longer time of incubation or deamination during column chromatography. Accumulation of such large quantities of adenosine-^{3}H and inosine-^{3}H, which are secondary and tertiary derivatives of cyclic AMP-^{3}H, leads to the distinct possibility that even more remote derivatives may be formed, which could contaminate the cyclic AMP region in this chromatographic system. To reduce this risk when assaying phosphodiesterase activity in homogenates or in the crude materials of initial stages of enzyme purification, short periods of incubation and a total metabolism of cyclic AMP of under 20% are advisable. We tried another approach to minimizing this risk, *i.e.*, product trapping (and isotope dilution) with the initial product of hydrolysis, 5′-AMP (Table II, Set B). However, this approach led to a significant depression of overall metabolism beyond 10 min of incubation. Thus, product trapping can be utilized with impure preparations of phosphodiesterase only if one limits the time of the reaction to the period before an inhibitory factor has appeared. With crude homogenates, the safer approach would be to limit the time of incubation, rather than to add 5′-AMP.

Failure of 5′-AMP-^{3}H to accumulate in the absence of added 5′-AMP (Table II, Set A) resulted from a low initial concentration of 5′-AMP in the medium and the presence of potent 5′-nucleotidase activity as shown in Table III. In separate studies with homogenates of the rat kidney (data not presented), the patterns of distribution of metabolites of cyclic AMP-^{3}H and 5′-AMP-8-^{14}C with, and without, added 5′-AMP, as well as the delayed inhibition by 5′-AMP, were very similar to the results in the studies on the toad bladder.

Knowledge of the pattern of formation of

metabolites of cyclic AMP revealed to us potential errors in several methods used to measure phosphodiesterase. Cheung attempted to follow the rate of breakdown of cyclic AMP by measuring the rate at which 5′-AMP was appearing in the incubate (7). His technique may have underestimated the rate of metabolism of cyclic AMP by crude extracts of rat brain. Another assay of phosphodiesterase is that which was developed by Krishna *et al.* (8). However, they pointed out that adenosine behaves like cyclic AMP in their assay. This technique (without prior chromatographic purification) has been utilized (13) apparently without consideration of whether adenosine has accumulated, particularly when relatively crude preparations are used. Thus the method of both Cheung and Krishna, although reliable for assay of highly purified preparations of phosphodiesterase, may not be reliable for studies on tissue homogenates or impure subcellular fractions.

Summary. Factors are described that affect the assay of cyclic 3′,5′-nucleotide phosphodiesterase in tissue homogenates. Enzyme activity was measured by chromatographic separation of cyclic AMP-^{3}H from labeled derivatives using thin-layer plates of PEI-cellulose. The method separates cyclic AMP, 5′-AMP, and secondary metabolites formed in significant quantities by homogenates of toad bladders. Recovery of radioactivity for all phases of the procedure was excellent —only 0.3 to 0.4% was lost during ether extraction and 98% of radioactivity applied to the thin-layer plates was recovered in the five zones scraped from the plates. In the absence of added 5′-AMP, no 5′-AMP-^{3}H accumulated; the major labeled products were adenosine-^{3}H and inosine-^{3}H. Thus methods for assay of phosphodiesterase that depend solely on the rate of accumulation of 5′-AMP or do not separate cyclic AMP from adenosine would not be valid under these circumstances. Formation of remote labeled derivatives can be avoided by use of short periods of incubation and isotope trapping with unlabeled 5′-AMP, the product of the phosphodiesterase reaction. Use of 5′-AMP, however, is safe only during short periods of incubation because of gradual formation of an inhibitor (or removal of an activator) in the presence of added 5′-AMP.

The excellent technical assistance of May Pahland is gratefully acknowledged.

1. Sutherland, E. W., Robison, A., and Butcher, R. W., Circulation **37**, 279 (1968).

2. Robison, G. A., Butcher, R. W., and Sutherland, E. W., Annu. Rev. Biochem. **37**, 149 (1968).

3. Butcher, R. W., and Sutherland, E. W., J. Biol. Chem. **237**, 1244 (1962).

4. Aurbach, G. D., and Houston, B. A., J. Biol. Chem. **243**, 5935 (1968).

5. Goldberg, N. D., Larner, J., Sasko, H., and O'Toole, A. G., Anal. Biochem. **28**, 523 (1969).

6. Gulyassy, P. F., J. Clin. Invest. **47**, 2458 (1968).

7. Cheung, W. Y., Biochem. Biophys. Res. Commun. **23**, 214 (1966).

8. Krishna, G., Weiss, B., and Brodie, B. B., J. Pharmacol. Exp. Ther. **163**, 379 (1968).

9. Tsuboi, K. K., and Price, T. D., Arch. Biochem. Biophys. **81**, 223 (1959).

10. Randerath, K., and Randerath, E., J. Chromatogr. **16**, 111 (1964).

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

12. Gulyassy, P. F., and Edelman, I. W., Biochim. Biophys. Acta **102**, 185 (1965).

13. Levey, G. S., Skelton, C. L., and Epstein, S. E., J. Clin. Invest. **48**, 2244 (1969).

Received Nov. 6, 1970. P.S.E.B.M., 1971, Vol. 137.