0273-2289/93/4103—0189$07.00

Assays of Cyclic Nucleotides

A Review of Current Techniques

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Received December 2, 1992; Accepted December 18, 1992

ABSTRACT

Cyclic nucleotides are recognized as important second messenger molecules, and many assay formats exist for their quantification. This article critically reviews these different approaches. For measurement of cAMP or cGMP in biological fluids or tissue extracts, immunoassay is effective. For other purposes, such as measurement of cyclic nucleotide phosphodiesterase activity, methods that separate nucleotides from their cyclic counterparts are best, and offer a variety of means of detection.

Index Entries: Cyclic nucleotides; cyclic nucleotide phosphodiesterases; immunoassay; affinity chromatography; capillary electrophoresis; HPLC; TLC.

INTRODUCTION

As is now well-recognized, the cell detects signals from its environment and produces the appropriate responses by mechanisms involving cell surface receptors and an array of intracellular signalling molecules. Adenosine 3′-5′ cyclic monophosphate (cAMP) has been studied as a second messenger for over three decades. It is synthesized from ATP by adenylyl cyclases in response to extracellular signals mediated by surface-bound receptors and their associated G-proteins (*1,2*). The cAMP activates cAMP-dependent kinases (3) and cAMP-gated ion channels (*4*), and can also interact with other second messenger systems (*5,6*). cAMP therefore elicits a wide range of effects within the cell. Clearly, it is important for the cell to be able to attenuate an increase in concentration of a potent

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molecule such as cAMP. This is done by cyclic nucleotide phosphodiesterases, which convert cAMP to adenosine 5′ monophosphate (AMP). The phosphodiesterases are a group of related enzymes whose activities are found (in various proportions) in every eukaryote cell (*7,8*). The balance of adenylyl cyclase and phosphodiesterase activities therefore regulates intracellular cAMP concentrations, and thus many cellular functions. Various pharmacological agents act by stimulating production of cAMP within the cell—for instance isoproterenol (isoprenaline) or prostaglandin E_1 are receptor agonists that stimulate adenylyl cyclase. Forskolin acts on adenylyl cyclase directly to have the same effect. Other agents are known that may inhibit phosphodiesterases and so exert an effect equivalent to stimulation of cAMP synthesis. Theophylline is one such example.

In addition to cAMP there are other naturally-occurring cyclic nucleotides. Guanosine 3′,5′-cyclic monophosphate (cGMP) is another important second messenger, a component of a signalling system equivalent to that which uses cAMP, consisting of a similar system of guanylate cyclases, cGMP-dependent kinases, cGMP-gated ion channels, and cyclic nucleotide phosphodiesterases, of which some are cGMP-specific and others not (i.e., they use cAMP and/or cGMP as substrates) (*7,9*). Cytidine 3′,5′-monophosphate (cCMP) and a cCMP phosphodiesterase are known to exist but their significance is still uncertain (*10*). Nucleotide 2′3′-cyclic monophosphates (and phosphodiesterases) also exist, and are possibly involved in the formation and maintenance of myelin (*11*).

The cyclic nucleotides have attracted a lot of scientific attention for many years, not only by virtue of interest in their involvement in so many biological processes, but also by the possibility of interference with cyclic nucleotide turnover. The aim of the latter is to bring about an effect that would be beneficial in the clinic, for treating cardiovascular and inflammatory disorders, for instance. This interest has generated a large volume of literature. The assay methods used by workers in this field are numerous, but fall into two broad classes. For measurement of unlabeled nucleotides (such as in cell or tissue extracts), the method of choice has proven to be an assay in which the nucleotide to be quantified competes with labeled, exogenous nucleotide for a binding protein. On the other hand, the action of cyclases and phosphodiesterases may be studied in vitro by use of their natural substrates, which are readily available in either labeled or unlabeled forms. The means to separate and quantify substrate and product are various. These methods for cyclic nucleotide quantification are the subject of this review, which compares their relative strengths and weaknesses. For the purposes of exemplification we have concentrated on cAMP/AMP assays, but assays of other nucleotides are similar in principle. Details of the experiments that precede the final nucleotide quantification (for instance, incubation of adenylyl cyclase with ATP in order to generate cAMP, as described in ref. *12*) are not covered here since these will vary according to the purpose of each experiment. References quoted below stand as examples of different experiment designs.

IMMUNOASSAY

Immunoassay offers a method of high sensitivity for analyte determination. There are now several commercially available immunoassay kits available for the measurement of cAMP and cGMP (e.g., Amersham International, Arlington Heights, IL; DuPont, Boston, MA) but not, to our knowledge, specific for other cyclic, or any noncyclic nucleotides, such as ATP or AMP. As such these assays have not gained utility in the measurement of PDE activity in vitro where the measurement of product appearance rather than substrate consumption is necessary for maximum precision. However, these assays are particularly suited to the measurement of cAMP in tissue extracts, cells, and other biological fluids, such as urine.

These assays are competitive, being based on the competition between the unlabeled cAMP present in the sample and a fixed quantity of labeled cAMP for a limited concentration of a cAMP specific antibody. The amount of labeled cAMP bound by the antibody is inversely proportional to the concentration of the sample cAMP. The antibody bound label is then separated from the free label, quantified, and the concentration of unlabeled cAMP in the sample is determined by interpolation from a standard curve. The label employed is frequently ^{125}I but recently assays utilizing nonisotopic labels, such as enzymes, have become available (from Amersham International). The potential advantage of immunoassay lies in its sensitivity, typically 0.25 n*M* cAMP, with a working range extending nearly 2 orders of magnitude (i.e., 0.25–25 nM). This makes immunoassay ideally suited to monitoring cyclic nucleotide changes occurring in the intact cell.

We have used immunoassay routinely to monitor the efficacy of PDE inhibitors in potentiating the cAMP elevating ability of β_2 adrenergic receptor agonists in leukocytes. By way of example, human neutrophils (10^6) are stimulated with isoproterenol (10 μM) in the presence of various concentrations of the PDE IV inhibitor rolipram. The stimulation is terminated after 2 min incubation at 37°C by placing in a boiling water bath for 5 min. The cell debris is sedimented by centrifugation and a 100-μL aliquot of the supernatant removed and assayed for cAMP using the Rianen cAMP radioimmunoassay kit (DuPont).

A typical dose response curve for rolipram is shown in Fig. 1 and the standard curve and precision profile for the assay are illustrated in Fig. 2. Rolipram potentiates the ability of the β_2 adrenergic receptor agonist to elevate cellular cAMP in a dose responsive manner with 50% of the maximal elevation occurring at 1000 n*M* rolipram.

One potential disadvantage in the use of the commercially available cyclic nucleotide immunoassay kits is their cost. A cheaper alternative utilizes the cAMP binding subunit of protein kinase A instead of an antibody. In this method, sample cAMP competes with ^3H-cAMP (available from Amersham International) for the limited concentration of cAMP binding protein (Sigma, St. Louis, MO). The separation of protein-bound

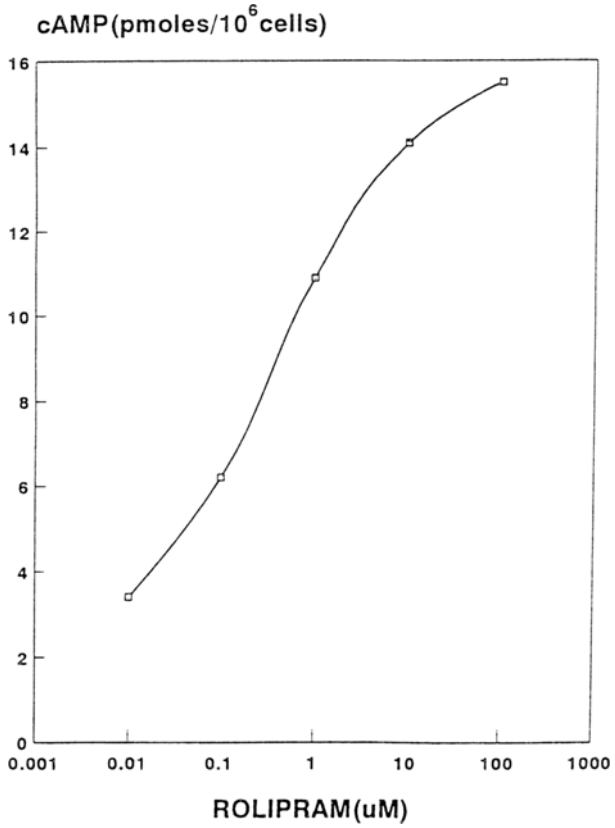

Fig. 1. The effect of rolipram, an inhibitor of cAMP phosphodiesterase type IV, on the cAMP content of human neutrophils. Assay: competition assay using DuPont's Rianen kit.

cAMP from free cAMP is achieved by addition of charcoal, which binds the free cAMP and is subsequently sedimented by centrifugation leaving the protein-bound cAMP in the supernatant ready for quantification by scintillation counting. A method for optimizing this assay system is given by Gilman (*13*) and although somewhat less sensitive (range 5–400 n*M*) than the immunoassay techniques, we have found it to offer acceptable precision. A representative standard curve and precision profile obtained in our laboratories is given in Fig. 3.

SNAKE VENOM/ION EXCHANGE

The lack of a robust method to separate 5′ nucleotides and cyclic nucleotides led to the development by Brooker et al. (*14*) of a two step radioisotopic approach that has become one of the most cited assays for phosphodiesterase. In this method the 5′ nucleotide product of phosphodiesterase

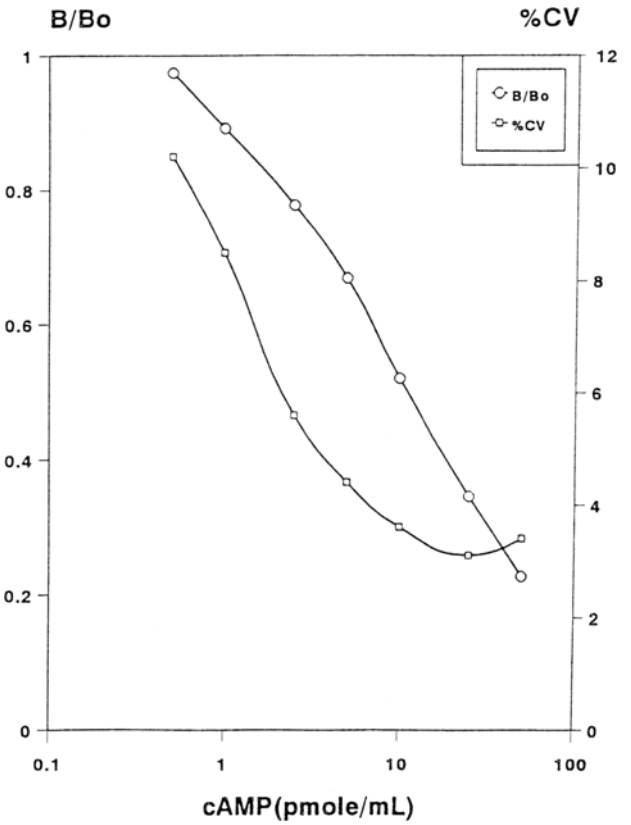

Fig. 2. cAMP Immunoassay. Example standard curve and precision profile obtained by use of DuPont's Rianen kit. % CV = percentage coefficient of variation. B/Bo = cpm bound in the presence of cAMP divided by cpm bound at zero cAMP.

action on a cyclic nucleotide is dephosphorylated to the corresponding nucleoside using a 5′ nucleotidase from snake venom. Subsequent application of the reaction mixture to small columns of an appropriate anion exchange resin permits ready separation, with the cyclic nucleotides retained on the column and nucleosides eluted, to be quantified by scintillation counting.

By way of example, in the assay of phosphodiesterase a common reaction mixture has a total volume of 100 μL and contains 20 m*M* Tris HCl (pH 7.4), 10 m*M* $MgCl_2$, 0.1 μ*M* [^{3}H]-cAMP, and 0.1 μ*M* [^{14}C]-5′-AMP. The assay is initiated by addition of 50 μL of enzyme and terminated after 30 min at 30°C by heating for 1 min in a boiling water bath. The solution is cooled on ice before the second reaction is initiated by addition of 50 μL of 5′ nucleotidase (10–30 μg) (*Crotalus atrox* or *Crotalus adamenteus* snake venom). After 15 min at 30°C the reaction is terminated by boiling as above and after cooling on ice the [^{3}H]-adenosine derived from cAMP

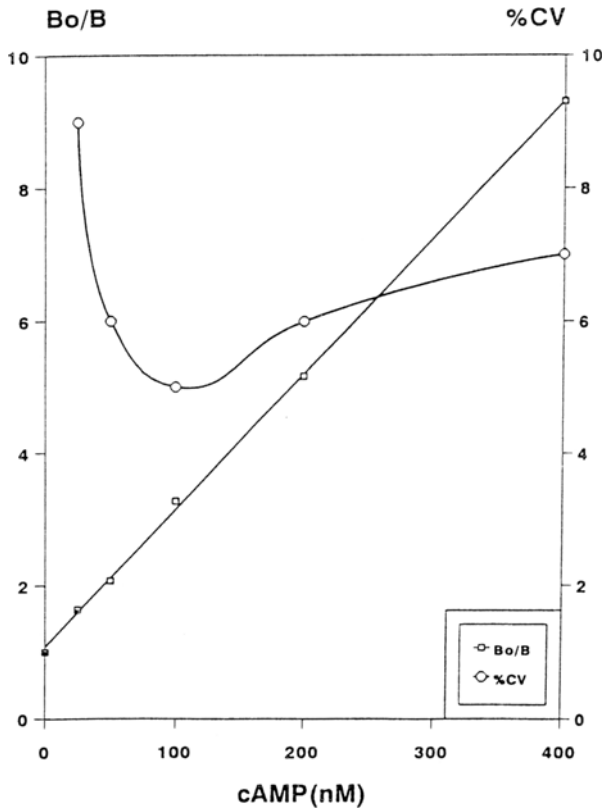

Fig. 3. Example standard curve and precision profile obtained with a method using a cAMP binding protein. % CV = percentage coefficient of variation. Bo/B = cpm bound at zero cAMP divided by cpm bound in the presence of cAMP.

is separated on 1 mL columns of QAE-Sephadex previously equilibrated with 20 m*M* ammonium formate. The sample is applied, washed through with 2 mL of 20 m*M* ammonium formate, and the whole eluate added to 10 mL of scintillation fluid for determination of [^{3}H]-nucleoside, and these values are corrected for blank values obtained in the absence of added phosphodiesterase. The correct ^{3}H dpm for the nucleoside are expressed as a percentage of the initial total [^{3}H]-cyclic nucleotide to give the percentage substrate utilization.

The assay gives good precision and sensitivity (< 100 p*M* 5′ nucleotide). Contamination of the phosphodiesterase enzyme with nucleosidases, phosphatases, or nucleotidases does not compromise the assay since the products arising from the action of these enzymes on the 5′ nucleotide (e.g., nucleosides and free bases) are not retained by the anion exchange resin. A practical disadvantage of the method is that it requires a second

reaction step. Further, the method is not particularly robust. It is important when investigating unknowns (for example pH, divalent cation, or inhibitor activator) to include extensive controls to verify that changes in the apparent hydrolysis of cyclic nucleotides are owing to primary effects on phosphodiesterase activity and not to secondary effects on the 5′ nucleotidase. In addition, the elution characteristics of the dephosphorylated product and unreacted cyclic nucleotide substrate can be altered by variations in the composition of the assay mixture (e.g., pH, salt). These variations are frequently introduced in the diluent used for the phosphodiesterase or inhibitor/activator, especially when the assay is being used to monitor their purification.

ALUMINA CHROMATOGRAPHY

The problems of the method described in the previous section were obviated by White and Zenser (*15*) who described a method for obtaining excellent nucleotide separation using small columns of alumina. The basis of this technique is that the 5′ nucleotides are strongly bound by the alumina and retained whereas the cyclic nucleotides are readily eluted at neutral or slightly alkaline pH (optimum pH=7.6). Subsequently, the method was refined by Ramachandran (*16*) and more recently by Alvarez and Daniels (*17*). The latter authors observed improved reproducibility with the use of ammonium acetate as the eluting buffer irrespective of the source or type of alumina employed. This method was thus of good utility in the assay of adenylyl cyclase. However, the inability to recover 5′ nucleotides in good yield was a severe limitation in the assay of phosphodiesterase since measurements were confined to the consumption of substrate, i.e., cyclic nucleotides.

This is acceptable for those assays requiring lower precision, e.g., monitoring purification procedures, but less acceptable in detailed kinetic studies where accuracy is required at low substrate consumption. Thus, although precision could be improved by the use of multiple replicates, (we found $n=6$ to be acceptable) a method that enabled estimation of product formed was required for greater precision and reduced need of sample replication. Accordingly, we investigated the method further and discovered that the 5′ nucleotide could be recovered in excellent yield (>90%) by elution with 2*M* NaOH (M. Perry, M. Wales, J. Jappy, and B. Smith, manuscript submitted for publication). In addition, the use of an appropriate scintillation fluid, such as Ultima Gold XR, enables the eluate to be counted for ^{3}H and ^{14}C directly without prior neutralization. The remainder of this section is given over to the description of our experiences with this method.

The disposable columns used to contain the alumina are readily available commercially and for economy may be washed and reused. For convenience they should be narrower at the lower end with a column diameter of about 0.5 cm and a wider top end that acts as a reservior for up to 2 mL. We find those supplied by Bio-Rad (Richmond, CA) excellent for the purpose. Neutral Alumina is dispensed dry into the columns. No more than 0.3 g is required per column and this can be readily dispensed using a spoon made from the well of a disposable microtiter plate. An aliquot (50–200 μL) of the reaction mixture containing the 3′5′-cyclic and 5′-nucleotides is added directly to the dry alumina.

The methodology is particularly robust with regard to the reaction buffer, with the only requirement being a sample pH of ≤8.5. The reaction can thus be stopped by addition of TFA, TCA, or by boiling and the presence of additives, such as high salt (up to 2*M* NaCl or KCl), detergents (2% Triton X-100 or SDS), or urea (6*M*), do not interfere with the subsequent separation process. The 3′5′-cyclic nucleotides are eluted in good yield (~90%) by the application of 2 mL 0.1*M* TES-NaOH, pH 8.0. A further 10 mL of the same buffer is passed through the column to elute the remaining cyclic nucleotides before the 5′ nucleosides are eluted using 2 mL of 2*M* NaOH.

The elution profiles of a variety of nucleotides and nucleosides is shown in Table 1. These data were obtained by elution of the columns with successive 2-mL aliquots of the appropriate buffer and determination of the recovery in each eluate. As can be seen, all the 5′ nucleotides irrespective of the base were retained on the column (>90% retention) whereas the corresponding cyclic nucleotides, again irrespective of base, were eluted with good recovery (>90% in the first 4 mL). All the 5′ nucleotides were eluted from the alumina by application of 2*M* NaOH with approx 90% being recovered in the first 2 mL irrespective of the base. The di- and triphosphate nucleotides of adenosine and guanosine exhibited similar chromatography to their monophosphate counterparts. The importance of the noncyclized phosphate moiety in the interaction with alumina was evident by the nonretention of the equivalent nucleosides and free bases.

The coelution of the nucleotides and free bases with the cyclic nucleotides has to be considered as a problem when assaying for phosphodiesterase activity in crude extracts that contain nucleotidases, phosphatases, and/or nucleosidases. These latter enzymes can convert the 5′ nucleotide product of phosphodiesterase action to the nucleoside or free base which, coeluting with the cyclic nucleotide substrate, will obviously lead to an underestimation of the phosphodiesterase activity. However, phosphodiesterases are not end-product inhibited so the problem of contamination by phosphatase, nucleosidase, or nucleotidase can be overcome by the addition of excess 5′ nucleotide into the reaction mixture. Typically, we have found that the inclusion of 50–100 μ*M* 5′ nucleotide in the reaction mixture is sufficient to permit reliable estimation of phosphodiesterase activity in crude tissue extracts. If necessary, such as when working with

Table 1
Recovery of Purine and Pyrimidine Derivatives from Alumina Chromatography

Derivative	0.1*M* TES-NaOH, pH 8.0					2*M* NaOH
	1	2	3	4	5	1
*cAMP	96	1.8	0.5	0.4	0.4	0.43
*cGMP	97	2.8	0.3	0.1	0.1	0.4
cIMP	99	3.1	0	0	0	0.5
cCMP	99	0.2	0	0	0	0.5
*AMP	0.9	0.2	0.2	0.2	0.1	91
*ATP	1.5	0.4	0	0	0	89
ADP	7	0	0	0	0	91
*5′GMP	1.4	0.3	0	0	0	89
5′IMP	0.8	0.5	0.2	0	0	90
5′CMP	8	0	0	0	0	91
*Adenosine	97	1.2	0	0	0	0.4
*Guanosine	86	4.1	0	0	0	0.6
Inosine	86	15	0.4	0	0	0.6
Adenine	99	0	0	0	0	0.3
Guanine	99	0	0	0	0	1.1
Cytidine	99	0.6	0	0	0	0.6
Hypoxanthine	99	0	0	0	0	0.4
*GTP	2.3	0	0	0	0	90
GDP	3.1	0	0	0	0	88

The derivatives were dissolved in 50 m*M* TES-NaOH, pH 7.6, containing 10 m*M* $MgCl_2$ and 100 μL applied to the column. The percentage recovery of each derivative eluted by successive 2 mL aliquots of 0.1*M* TES-NaOH pH 8.0 and then 2*M* NaOH was determined by radioactivity (*) or absorbance at 260 nm.

enzymes of high K_m that require the use of high cyclic nucleotide concentrations (up to 200 μ*M*), the 5′ nucleotide can be increased accordingly. Indeed, this illustrates the very high capacity of the alumina for the 5′ nucleotides with columns containing 0.3 g alumina being able to retain more than 1 μmol of 5′ nucleotide. A recent report by Alvarez and Daniels (*18*) describes how the relative contribution of any of these contaminating enzymes may be assessed. The authors described the separation of nucleosides and bases from their corresponding nucleotides using microcolumns of acidic alumina. Thus, the nucleosides and bases were eluted first by the application of 0.005*M* HCl and then the corresponding 3′5′-cyclic nucleotide were eluted with 0.1*M* ammonium acetate. We have not observed the same efficiency of separation using neutral alumina, with approx 60% of the nucleoside/base being eluted by 0.005*M* HCl, but have demonstrated that the recovery of noncyclic nucleotides from acid alumina by elution in 2*M* NaOH is equivalent to that reported herein for neutral alumina.

Thus the method can be used to quantify both adenylyl cyclase activity by eluting the cAMP from the column with 4 mL of 0.1*M* TES-NaOH, pH 8.0, while the ATP is retained on the column and phosphodiesterase activity by eluting the cAMP from the column with 10 mL of buffer followed by elution of the product AMP with 2*M* NaOH. In addition it is of use in determining nucleotidase and combined phosphatase/nucleosidase since the products of such enzymatic activity, i.e., nucleosides and free bases, are readily eluted from the alumina with 4 mL of 0.1*M* TES-NaOH, pH 8.0, leaving the substrate retained on the alumina.

By way of illustrating the method, the following describes the determination of the K_m for cAMP and an inhibitor IC_{50} for the low K_m, rolipram-sensitive phosphodiesterase isolated from HL60 cells. For the determination of rolipram IC_{50} the reaction mixture comprized [^{3}H]-cAMP (0.1 μM), plus rolipram (0–10 μM) whereas for the K_m measurement the rolipram was replaced with unlabeled cAMP (0–20 μM). The reactions were initiated by the addition of phosphodiesterase. All dilutions were in 50 m*M* TES-NaOH, pH 7.6, 10 m*M* $MgCl_2$. After 30 min at 30°C the reactions were terminated by the addition of [^{14}C]-AMP (0.1 μM) in trifluoracetic acid (0.5%). All figures in parentheses indicate final concentration. Aliquots (75 μL) were applied to alumina columns containing 0.3 g alumina and the cAMP eluted with 10 mL of 0.1*M* TES-NaOH, pH 8.0. The [^{3}H]-AMP was then eluted with 2 mL of 2*M* NaOH directly into scintillation vials containing 10 mL Ultima Gold XR scintillant.

Estimates of totals for both [^{3}H] and [^{14}C] nucleotides are made by dispensing 75 μL of the nonseparated reaction mixture and 2 mL 2*M* NaOH into 10 mL of scintillant. All vials were then counted for 1 min for simultaneous determination of ^{3}H and ^{14}C. The separated [^{3}H]-AMP was then back-corrected for recovery using the separated and nonseparated ^{14}C values and expressed as a fraction of the total [^{3}H]-cAMP in order to determine the fraction of substrate hydrolyzed. Reaction conditions were adjusted to keep this value below 20% in order to remain in the linear part of the reaction curve. Background contamination of the 2*M* NaOH eluate with [^{3}H]-cAMP was less than 0.1% of the total ^{3}H dpm. If required, this can be corrected for by inclusion of a boiled enzyme or "no-enzyme" control. Typical results for the cAMP K_m and rolipram IC_{50} are shown in Fig. 4. As can be seen from the precision profile (Fig. 5) the method gave excellent precision throughout the working range of the experiments, i.e., from 400 to 0.4 n*M* [^{3}H]-AMP.

We have found that this alumina chromatography method can be converted to a microtiter plate format that retains its reliability while reducing labor and assay time by allowing use of multiple tip pipets. For this, we use membrane-bottomed microtiter plates, each well of which is effectively made into an alumina column by addition of alumina powder to fill $^1/_4$ to $^1/_3$ of the well.

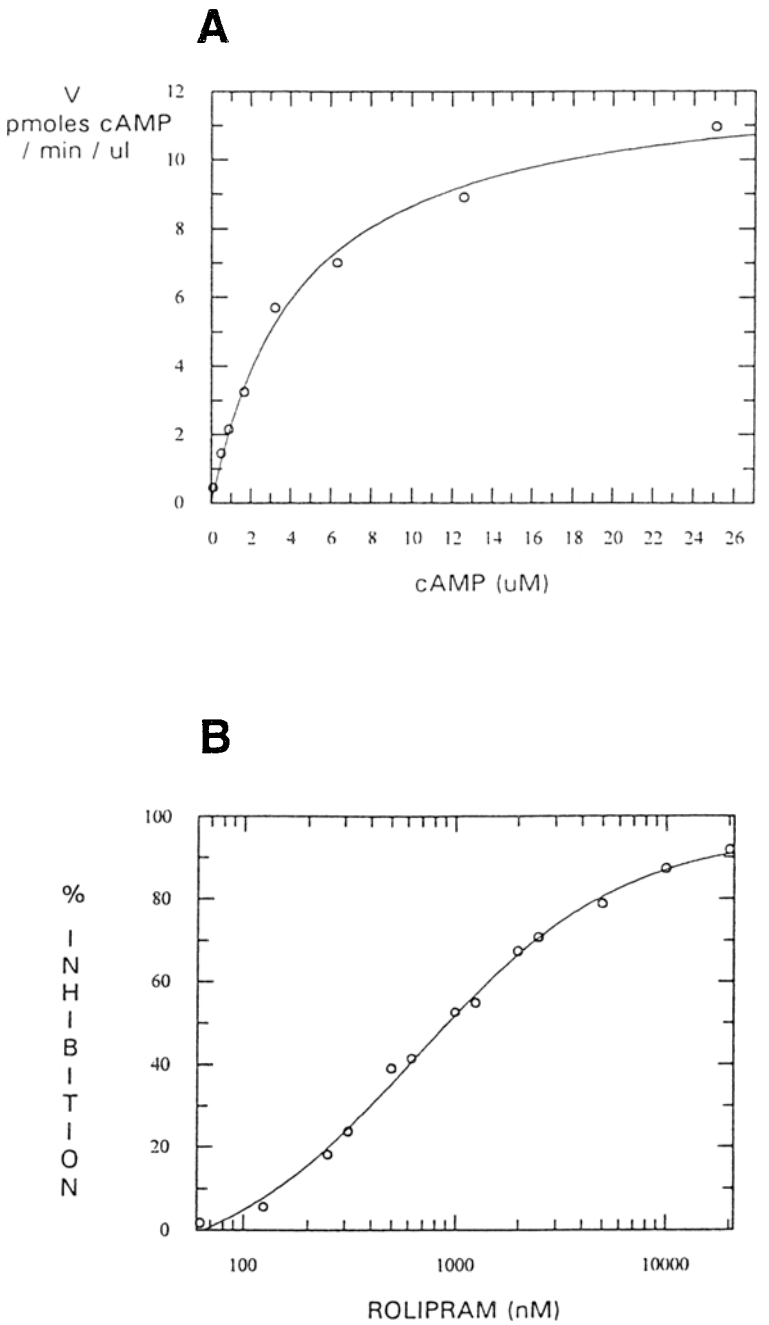

Fig. 4. Type IV phosphodiesterase from HL60 cells. (a) Estimation of K_m (4.3 μM) and V_{max} (12.3 pmol cAMP hydrolyzed min^{-1} μL^{-1} of reaction mixture). (b) Dose response curve for type IV inhibitor rolipram. Assay: alumina chromatography with 2*M* NaOH elution of AMP.

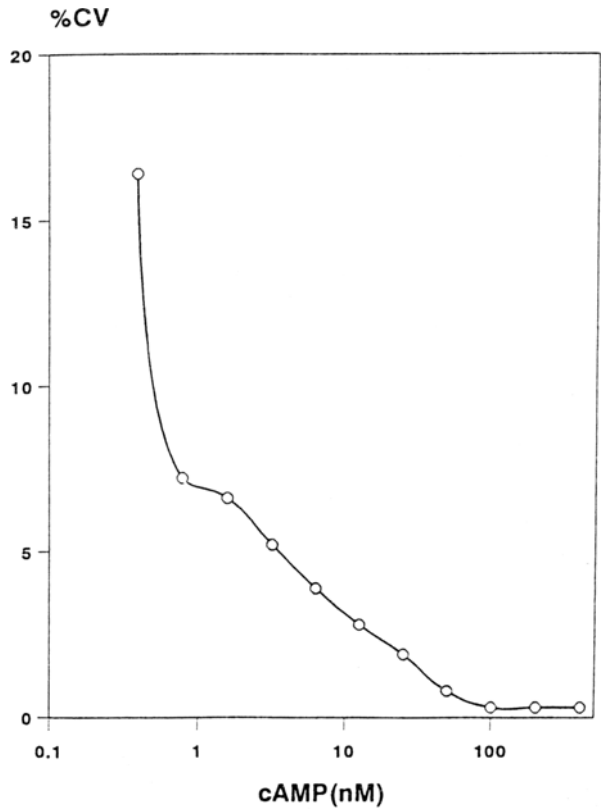

Fig. 5. Precision profiles of estimation of AMP. Assay: alumina chromatography with 2*M* NaOH elution of AMP.

We have investigated various plates for (non) binding of nucleotides, for liquid flow characteristics, and for lack of crosscontamination between neighboring samples, and have found the "Silent Monitor" plate with 1.2 μm Loprodyne membrane (Pall Corporation, Portsmouth, UK) excellent for this purpose. Typically, 75 μL of stopped reaction mixture of the type described above is applied to each "column" and then washed with TES-NaOH buffer described above. 3 mL buffer per "column" (applied in lots of 200–250 μL) is more than sufficient to elute cAMP. To make liquid flow through the plate, vacuum is applied beneath it using an appropriate type of manifold, such as that sold by Millipore (Bedford, MA) for this purpose. Bound nucleotide is eluted by 4×150 μL lots of 2*M* NaOH per "column," this being collected sequentially into two microtiter plates beneath the membrane-bottomed plate, and then transferred to scintillant for measurement of radioactivity.

"No enzyme" controls are always run and routinely show an apparent background of cAMP eluting in the NaOH (regardless of TES-NaOH buffer

wash volumes) of 1–3% of total substrate. This background is subtracted from sample results. Duplicates of samples are routinely used. The results (accuracy and precision) from the two formats (large column and microtiter plate) are equivalent and the two methods are equally robust in practice. The more compact format and simpler liquid handling in the microtiter plate approach gives a saving in assay time (of approaching two-fold), and a saving in costs (principally of plasticware—one 96-well plate costs between 5–10% that of 96 BioRad disposable columns).

In summary, the alumina method offers a reliable estimation for both adenylyl cyclase and phosphodiesterase activity. It is cheap, convenient in that the alumina columns are prepared dry and require no equilibration, and is particularly robust. It also posesses good analytical specificity, being able to rapidly separate bases/nucleosides, cyclic nucleotides, and 5′ nucleotides. It does require the use of labeled substrate (usually ^3H) but accordingly is sensitive down to substrate concentrations of 1 n*M* (given typical specific activities for [^3H]-ATP or cAMP of 20–50 Ci/mmol). In addition, 2′–0 anthraniloyl and 2′0-(*N*-methyl) anthraniloyl derivatives of cAMP and cGMP (discussed further under "Use of Fluorescent Cyclic Nucleotide Analogs") exhibit similar chromatographic properties on neutral alumina to the nonfluorescent, native nucleotides. However, the kinetics of their hydrolysis by phosphodiesterases may be significantly different from those of the native cyclic nucleotides.

This sensitivity is particularly useful when dealing with low K_m enzymes where maintaining the substrate at levels $^1/_{10}$ of the K_m permits inhibitor IC_{50} to approximate K_I. The one disadvantage of the method is that it is prone to interference with phosphatases/nucleosidases and nucleotidases although, as discussed above, this can be minimized by the inclusion of excess 5′-nucleotide into the incubation mixture.

BORONATE CHROMATOGRAPHY

This column chromatographic method of separation of cyclic and 5′ nucleotides is a form of *cis*-diol affinity chromatography, hence its ability to bind 5′ nucleotides that are *cis*-diols but not cyclic nucleotides. Structurally, boronate affinity gels consist of a solid support (e.g., aminoethylcellulose or polyacrylamide) to which a boronate derivative, generally *m*-aminophenylboronic acid, is linked. The boronate reacts with a free hydroxyl group, forming a charged hydroxyl boronate species, which then reacts with the free *cis*-diol (5′ nucleotide) to form a five membered ring structure, i.e.:

$$R.B(OH)_2 + OH^- \rightleftharpoons R.B(OH)_3^- + R''OH \rightleftharpoons R.B(OH)^- OR' OR''$$

This binding occurs generally above pH 7.5 and can be enhanced by the presence of magnesium ions in some cases. Elution can be accomplished below pH 6.5, or by using other *cis*-diols, such as sorbitol. Therefore, reactions cannot be stopped by addition of acid because the 5′ nucleotide product of reaction would not stick to the column. Also, it should be noted that some buffers, such as Tris, can alter binding characteristics adversely.

Columns of about 4 mL (0.4 g of gel) are used generally (*19*) although smaller columns can be used. Slightly different buffers are used in separation of different nucleotides. Separation of cAMP and AMP by boronate chromatography was described first by Davis and Daly (*19*) and is now a popular method in PDE assays (e.g., *20*). Generally, sample is applied to the column in 0.1*M* HEPES, pH 8.5, 0.1*M* NaCl, and cAMP is eluted fully with about 5 column vol of buffer. Subsequently, AMP can be eluted in 2–3 column vols of 0.1*M* sodium acetate, pH 4.8 (*19*) or 0.25*M* acetic acid (*21*). This method has been reported as giving between 98 and 99% recovery of cAMP and 93–94% recovery of AMP in the HEPES and the acetate buffers respectively (*19*).

However, in our hands it has frequently given as low as 50% recovery of 5′ AMP in the acetate buffer, the rest coeluting with the cAMP. We have found that binding of AMP to the boronate matrix can be improved by inclusion of Mg^{2+} (e.g., 20 m*M* $MgCl_2$) in the HEPES loading and washing buffer. Also, Hageman and Kuehn (*22*) report the use of 5 m*M* magnesium acetate (in 0.1*M* HEPES, pH 8.5) for binding of ATP in the course of its separation from cAMP in adenylyl cyclase assays. It has been reported that cGMP and 5′GMP can be separated using a slighly altered method (*19*) involving application of sample and elution of cGMP in 0.1*M* HEPES, pH 8.5, and elution of 5′ GMP by 0.1*M* sodium phosphate, pH 6.5 (*23*).

There are a number of attractions in using this method. It has a high capacity for *cis*-diols, binding 130 μmol sorbitol per mL gel (*23*), and is relatively sensitive, binding and eluting 10 pmol AMP (*21,22*), parameters that are more than sufficient for those substrate concentrations used generally. There is a problem in assaying 5′ nucleotidases because 5′ nucleotides and nucleosides (e.g., adenosine, guanosine) coelute under those conditions described above. Conversely, this means that there is no 5′ nucleotidease contamination problem in assaying phosphodiesterases. The gel is easy to use, being stable for 2 mo in the hydrated form (*23*) and the ability to regenerate the used gel by washing it with 0.1*M* acetic acid, then re-equilibrating the gel in starting buffer, means that once the columns are poured, there is a turnaround time of minutes in preparing them for more separations and this also lowers the cost of using this method. The flow rate of buffer through the gel under gravity is rapid, taking few minutes for a column vol to pass through; the low M_r exclusion limit of the gel (normally ~6 kDa) ensures a rapid equilibration, binding, and elution of nucleotides.

In order to reduce the labor involved in working a large number of columns (such as might be the case in screening procedures or in the study of enzyme kinetics) we have adapted the boronate chromatography method to the microtiter plate format, just as is described in the previous section for the alumina method. The method is merely a scaled-down version of the standard method with a "column" consisting of 200–250 μL of boronate gel placed in a well in a 96-well filterplate. Buffer is drained through by applying a vacuum beneath the filterplate and discarded, or collected, into a 96-well microtiter plate. A column of about 200 μL is sufficient for samples containing a few tens of picomoles of nucleotide.

The drawbacks to this method would appear to be:

1. The apparent variability of 5′ nucleotide binding mentioned above although the use of magnesium ions in the buffer appears to get around this;
2. The large volumes of buffer necessary to elute the AMP, larger than those used in alumina chromatography, and of increased volumes of scintillant required for accurate counting. However, as the microtiter plate method shows, it is possible to use a reduced column size to that originally reported (*19*) therefore reducing the elution volumes of buffer required; and
3. The cost of the gel is an order of magnitude greater than that of alumina, although recycling of the gel reduces costs.

In summary, boronate chromatography is a reasonably cheap, quick, and sensitive method of separation of 5′ and cyclic nucleotides although some minor alterations to the buffers used may be necessary to maximize recovery.

HPLC

High performance liquid chromatography (HPLC) offers the advantages of ready automation, sample quantification, and data handling, married to relatively rapid separation techniques, some of which are peculiar to HLPC. Both ion exchange and reverse phase methods have been described for separation of nucleotides by HPLC. Thus, Watterson et al. (*24*) described the use of a Whatman Partisil-10 SAX anion exchange column with phosphate buffer eluants. Isocratic elution by KH_2PO_4 (25 m*M*, pH 4) at 2 mL, min^{-1} gave rapid separation of cAMP from AMP, with retention times of 3.7 and 4.9 min, respectively. Using a stepped gradient of phosphate buffers they also accomplished separation of guanosine, inosine, and cytosine 5′ monophosphates (GMP, IMP, and CMP) from their cyclic counterparts, with the slowest (GMP) eluting at 10.34 min. Monitoring elution by absorption at 254 nm, the lower limit of detection was given as 3 pmole, delivered as a sample of 20 μL of a 150 n*M* nucleotide solution.

Reverse phase HPLC can achieve a similar speed and sensitivity to that just described. Reported methods have used octadecylsilane matrices from various manufacturers (*25–27*). Typically, methanol gradients in phosphate buffer have been used in 30 min runs to separate AMP from cAMP, for example. The quickest run time achieved was that by Spoto et al. (*27*) using a 5-μm Lichrosorb RP18 column (250×4 mm) with isocratic elution in 15% (v/v) acetonitrile in NaH_2PO_4 (200 mM, pH 6.0) buffer containing 25 m*M* tetrabutyl ammonium hydroxide (as an ion pairing agent). At a flow rate of 1 mL/in^{-1}, the retention times for AMP and cAMP were 6 and 4.5 min, respectively. Using detection by absorbance at 254 nm, the lower limit of sensitivity was given a 10 pmol (equivalent to 100 μL of a 100 n*M* nucleotide solution).

We have evaluated the reverse phase HPLC approach to nucleotide separations, particularly for the purposes of assay of PDE activity. The choice of reverse phase in preference to ion exchange chromatography was made because column lifetime was likely to be longer. In order to avoid problems such as pump wear, which can arise from use of salt buffers in HPLC, we have used an acetonitrile gradient in trifluoroacetic acid (0.1% v/v in water). The 3–5% acetonitrile gradient (over 5.8 min) was preceded by 0% acetonitrile (2 min) to remove buffer constituents, and followed by a 1.3-min wash in 90% acetonitrile to ensure that the column was clean and so maintain consistent chromatography. Total run time was 10 min. Flow rate was 1 mL, min^{-1} at 40°C. Elution was monitored by absorbance at 256 nm. The column used was a TSK ODS 120T (250×4.6 mm).

In this system, AMP eluted at 6.76 min with a coefficient of variation (CV, standard deviation×100%/mean; $n=103$) of 0.5%. The AMP was completely separated from cAMP, which eluted at 9.20 min (CV=1.1%; $n=103$). Various other C18 HPLC columns were inspected, and found to give a generally similar performance, with some variation in retention times and peak shapes. Day-to-day variation of AMP retention time was 4.0% ($n=6$) and of cAMP, 2.4% ($n=5$). Other nucleotides could be chromatographed, e.g., GMP eluted about 0.7 min later than AMP and cGMP about 0.2 min earlier than cAMP. The detector response was linear at least over the range tested, of 25–12500 pmol of AMP or cAMP, whether delivered in 2.5-, 25-, or 250-μL sample volumes. The signal: noise ratio was approx 3:1 at loadings in the range 25–50 pmol, although smaller peaks were readily visible.

A stringent criterion was applied in order to test the efficacy of the system as a routine assay—that of a maximum CV of 5% of peak integration. Integration, especially of very small peaks, was affected by baseline noise and nonnucleotide peaks. The AMP peak, being about twice as broad (and half as tall) as the cAMP peak was affected slightly worse in this respect. By analyzing 129 samples of various loadings of cAMP it was found that a loading of about 250 pmol was required to meet this criterion —equivalent to 250 μL of a 1 μ*M* solution. For AMP solutions this lower

limit for reliable integration was 1–2 μM. If studying PDE activity and measuring 10–20% conversion of cAMP to AMP (for first order rates), reliable measurements (CV ≤ 5%) of AMP required 10–20 μ*M* substrate. For many purposes this concentration is unacceptably high. The alternative of measurement of loss of substrate is possible in the few μ*M* range of cAMP concentration.

This real, practical limit on the sensitivity of quantification of nucleotides is an issue for all the methods discussed, using UV absorbance for detection. Sensitivity could be improved slightly by use of a microbore column (e.g., a 2.1 mm id column would in theory improve sensitivity about fourfold). Greater improvement could be achieved by use of radiolabeled nucleotides and on-line radiochemical detectors. An alternative approach is that described by De Petrillo et al. (*28*) involving conversion of nucleotides to fluorescent etheno derivatives, followed by separation by HPLC on a C18 column. Detection and quantification was by fluorimetry, the fluorimeter response being linear in the range 0.1 to 1000 pmol injected sample. Although being a feasible way of measuring relatively small numbers of samples, especially if radioactive labeling is not possible (e.g., measuring cAMP concentration in cells), the extra sample handling required by the derivatization procedure is a drawback when there are many samples to process.

Depending on the circumstances in which it is to be used, the HPLC method can show various advantages. If sensitivity is not an issue, the hazards and costs of radiochemicals may be avoided by use of nonlabeled compounds detected by UV absorbance. Also, running costs (of solvents and occasional columns) are low. A major advantage is that systms of the type described can be adapted for analysis of a variety of substrates and products of enzyme activity that may not be available in radiolabeled form. Lang and Rizzi (*29*) have described a column-switching technique that allows combination of reverse phase and ion exchange HPLC for resolution of complex mixtures of bases, nucleosides, and nucleotides. The major disadvantages are the issue of the sensitivity of quantification, and the fact that samples are analyzed serially, such that a batch of a large number of analyses may require a long time to complete.

THIN LAYER CHROMATOGRAPHY

In recent years, the popularity of thin layer chromatography (TLC) has waned slightly in the face of developments of other methods of chromatographic separation, notably HPLC. Nevertheless, one advantage that TLC has over HPLC is that samples can be resolved in parallel. This can lead to a reduction in time needed to complete a large set of samples.

This feature, together with the development of instruments for automatic TLC sample application and quantitative plate scanning, made TLC worthy of consideration for use in resolution and quantification of nucleotide mixtures. Detection of spots of nucleotides on TLC by virtue of their absorption at 254 nm is too insensitive for the present application, the lower limit being of the order of 100 pmol, mm^{-2}) (e.g., 5 μL sample of 50–100 μM solution of AMP). Jacks and Jones (*30*) describe a method whereby the nucleotide sugar is charred by spraying the TLC with 5% sulfuric acid in methanol and heating to 110°C. The nucleotides are then quantified by reflectance scanning and image analysis. From their data, however, it appeared that the lower limit of detection was relatively high, of the order of 1 nmol, mm^{-2}, and further compromised by fading of the brown/black color from about 15 min after the heating step. It is possible, however, to analyze nucleotides at concentrations in the low nM range by resorting to radioactivity. The design of one scanner for radioactivity quantification on TLC (the Imaging Scanner by Bioscan) is described by Rock et al. (*31*). Lablogic's Vanguard and Berthold's LB284 linear scanners are of similar design, and we have used the last-mentioned in the work described below. The detector in each of these machines is windowless and placed very close to the TLC plate surface to measure the ionization caused by β- or γ-rays emitted by the radioactive sample. To do this, it is necessary to flood the area with gas, such as methane 10% (v/v), in air or argon and this constitutes the largest of the running expenses other than the TLC plates themselves. The whole length of the TLC plate is scanned at once and the position of each decay event on the plate is determined by the position of the detection event along the length of the detector. The width of the track sampled on the TLC plate is governed by the width of a collimator. The detector can be stepped across the plate to read separate tracks and so allow the running of multiple samples on one TLC plate.

Jost and Hauck (*32*) have described separation of AMP, cAMP, and other nucleotides on the HPTLC NH_2 254s precoated TLC plate (E. Merck, Rahway, NJ), using, for instance, an eluant of 0.2M NaCl in a mixture of ethanol : water :: 30 : 70 (v/v). We have similar results with this type of plate, with cAMP running nearer the solvent front than did AMP. To avoid problems of a large cAMP spot (appearing as a peak on scanning) smearing backward over a smaller AMP peak, we wished to reverse the order of elution of these nucleotides. Furthermore, to increase sensitivity, we wished to load large sample volumes. We achieved this by using the wettable reverse-phase plate, RP18 WF254s, of 20×10 cm (E. Merck). This plate had a 2 cm-wide loading and concentrating zone that allowed us to load as much as 50 μL (5×10 μL) of sample. Each sample was run in a 9 mm-wide track scribed into the plate, separated from its neighbors by 1 mm-wide track on each side. This was to eliminate crosstalk when scanning with a 1-cm-wide collimator. Normal free flow ascending develop-

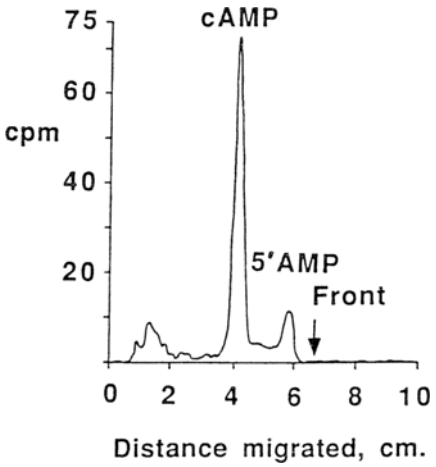

Fig. 6. TLC of a mixture of cAMP and AMP resulting from the action of mammalian phosphodiesterase (PDE) type IV on 0.1 μM cAMP. The PDE reaction was carried out in 100 μL of a buffer of 20 mM TES, pH 7.5, 5 mM $MgCl_2$, at 30°C. Reaction was terminated by addition of 10 μL of 11% trifluoroacetic acid (v/v in water). 5×10 μL lots of sample were loaded onto the concentration zone (from 0 to 2 cm on axis in figure). The TLC plate was RP18 WF254S, and the eluant was 1% acetic acid in H_2O : acetonitrile :: 95.5 (v/v), as described in "Thin Layer Chromatography".

ment was used with an eluent of 1% acetic acid in water : acetonitrile : 95: 5 (v/v), run in a saturated atmosphere (although this was not a crucial consideration). At T_r, the solvent front took about 20 min to approach close to the top of the plate. Before counting, plates were thoroughly dried to eliminate quenching by evaporating solvent. The samples were counted one at a time for up to 10 min each, although a 1 or 2 min count was usually found to be sufficient.

R_f values were calculated from distances migrated from the bottom edge of the TLC plates to the solvent front or peak of radioactivity for a sample peak. The R_f value for cAMP was 0.63 (CV=9.4%, where $n=23$) and the R_f AMP was 0.89 (CV=3.2%; $n=15$). The R_f for adenosine was close to that of cAMP, at a value of about 0.32, such that the two peaks were not completely resolved.

Although cAMP samples gave good, symmetrical peaks, AMP samples gave peaks that tailed backward somewhat. No alternative eluent was found that improved AMP peak shape, or that fully resolved adenosine and cAMP. A further problem was encountered associated with the use of TFA in the sample (to stop reaction of phosphodiesterase, for example). It was found that in these circumstances AMP, particularly, did not move completely from the point of sample loading. The reason for this is not known. These problems are illustrated in Fig. 6. They led to difficulty in accurately and directly quantifying the AMP produced by the action of phosphodiesterase on cAMP, particularly if the quantity of AMP

produced was small relative to cAMP. Despite the problem of cAMP quantification over a small background generated by AMP tailing, in order to quantify phosphodiesterase reaction, recourse was made to quantification of the cAMP remaining (i.e., ratio of counts detected in the cAMP : total counts in the whole sample). This was compared to results from an experiment carried out in parallel but using the alumina separation method (see "Alumina Chromatograpy" section) to quantify cAMP remaining after phosphodiesterase action. With a sufficient number of replicates, the two methods were approximately equivalent. For one reaction, TLC scanning gave a result of 28.3% cAMP used (CV = 6.6%, $n = 6$); for a replication reaction, alumina-cAMP estimation gave a result of 25.4% cAMP used (coefficient of variation = 17.1%, $n = 16$).

A nonradioactive method that employs TLC was described by Hiratsuka (33). Fluorescent analogs of cAMP and cGMP could be separated on silica gel, and fluorescent spots cut out for quantification in solution. A figure of about 8 pmol of nucleotide is mentioned as a lower limit. Assuming a sample loading of 10 μL, this limit is equivalent of 800 n*M* nucleotide. If this represents 20% conversion of substrate, then 100% substrate is 4 μ*M*. The method is therefore less sensitive than radioactive methods, but more importantly the analogs are unnatural substrates for PDEs and results may therefore be compromised.

The TLC method described has the advantage of speed over HPLC, but the latter has better resolving powers. The TLC method has no advantage over the alumina method of nucleotide separation, especially in the form whereby nucleotide monophosphate is eluted by alkali. Improvement of resolution and peak shape of nucleotides in TLC would, however, improve accuracy and precision of this method for nucleotide quantification.

CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE), a relatively new technology, is based on the migration of charged particles in an electric field, but unlike other forms of electrophoresis CE does not require any stabilizing medium (such as polyacrylamide gel). The problem of zone broadening, which can occur during electrophoresis in free solution, is avoided by effecting the migration and separation in a capillary, which is commonly 25–200 μ*M* id. The total volumes of such capillaries are of the order of a few μL, and sample volumes are measured in nL. In this sense, at least, CE could be considered a sensitive analytical technique. CE also offers the potential of rapidity of analysis and of automation. Among the many compounds that have been analyzed by CE are nucleotides (as reviewed in reference 34). In order to explore the potential of CE for cyclic nucleotide assay we have studied the separation of cAMP and AMP.

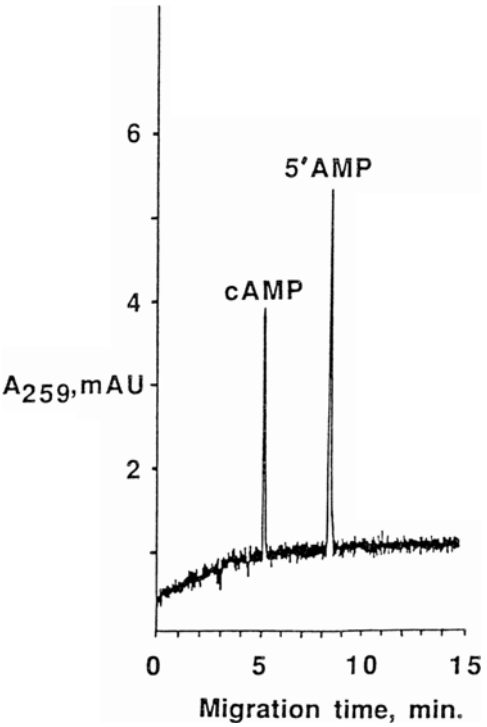

Fig. 7. Capillary electrophoresis of cAMP and AMP. Sample : 50 μM of each nucleotide in Tris-HCl (50 m*M*, pH 7.4), $MgCl_2$ (5 m*M*) and dithiothreitol (1 m*M*). Conditions were as described in "Capillary Electrophoresis".

The system used was an Applied Biosystems (Foster City, CA) 270A, the performance of which has been illustrated by Moring et al. (*35*). An unmodified glass capillary of 72 cm length and 50 μm id was used. Sample migration was monitored by absorbance at 259 nm, at a position approx 50 cm from the anode, where the sample was loaded by a 5 s (approx 10–20 nL) vacuum injection. A buffer of sodium phosphate (20 mM, pH 9.3) was used with an applied potential of 30 kV. The whole system was maintained at 50°C. Sample analyses were interspersed with capillary washes of phosphoric acid (100 m*M*, 3 min) and electrophoresis buffer (3 min).

AMP and cAMP were resolved, each giving sharp peaks (*see* Fig. 7), but migration speeds were variable between runs. For example, cAMP was detected (on successive runs) at 5.1, 5.6, and 5.8 min. On the same runs, AMP was detected at 8.4, 9.3, and 9.7 min (respectively). GMP and cGMP were not resolved from their adenosine counterparts. Sample buffer components were not detected, but organic solvent or acid added to the sample could affect migration speeds (dependent on their concentration), addition of methanol or trifluoroacetic acid slowing migration, for instance.

Thus, to allow for some variation in electrophoretic mobility, a run time of about 15 min was required (plus time for wash steps). This run time is similar to that reported by Tsuda et al. (*36*), using a glass capillary of 42 cm by 80 μm id, with a voltage of 9.4 kV and a buffer of 0.02*M* phosphate (pH 7.0) containing 0.5% ethylene glycol. In that system cAMP emerged at about 7 min and AMP at about 9 min. This time is also approximately equivalent to HPLC run times.

It was estimated that to obtain a signal : noise ratio of 3:1 or better, a minimum sample nucleotide concentration of about 5 μ*M* was required. This is in agreement with the lower limit of detection of UV-absorbing substances being in the μ*M* range, given as a general rule by Gordon et al. (*37*). The apparent lack of sensitivity in this respect is because although the sample is concentrated in just a few nL, the sample path length at the detector is only 50 μm. Although only nL of sample were injected into the capillary for each analysis, 20–60 μL samples were used in sample vials in order to reduce the effect of sample concentration owing to solvent evaporating into the vial head space. Thus, economy of sample utilization in CE is less than the nL loading volumes imply. As with HPLC, use of fluorescence, radiochemical, or other detection systems could increase the sensitivity of the method (as reviewed in ref. *37*), possibly by the same degree as for HPLC or in the case of fluorescence, possibly by less, as discussed by Perrett and Ross (*38*).

Although it is true that our system was not optimized, our experiences of variable migration rates, somewhat lengthy run times, and lack of concentration sensitivity have been shared by others (*38*). Improvements in equipment design and sample loading techniques may go some way to improve these aspects of CE in the future. Future developments may also improve quantification by CE, which has been discussed in the literature (*39*). Use of an internal standard, such as pyridoxamine (*36*), may go some way to overcome this problem. It is clear, however, that each CE system requires thorough validation for regular use in assay procedures.

CONTINUOUS SPECTROPHOTOMETRIC ASSAYS

A continuous spectrophotometric assay of PDE activity is possible using a linked assay. The 5′ nucleotide is converted to some other moiety enzymatically, by means that allow a continuous spectrophotometric measurement of PDE activity. A number of such assays have been described in the literature but have not gone into popular use in 5′ nucleotide measurement.

5′AMP is converted to 5′IMP by addition of exogenous adenylate deaminase (an enzyme that is specific for 5′AMP, not cAMP). Measurement of the subsequent decrease in absorbance at 265 nm (the difference

in absorption spectra of cAMP and 5'IMP being greatest at this wavelength; Dedman and Means (*40*)) was first used to measure PDE activity in rabbit tissues (*41*). The rate of decrease of A_{265} was linear between 0.8 and 0.4 absorbance units but little attempt was made to quantify the method with respect to determining the amount of cAMP hydrolyzed/min and it was necessary to purify the deaminase used from muscle (*41*). Therefore, Dedman and Means (*40*) examined the method in more detail in order to examine PDE activities in rat tissues. They determined the molar extinction coefficient at 265 nm to be 8650 $M^{-1}cm^{-1}$ and used 5'AMP deaminase from Sigma as the coupling enzyme, including it in the assay mix at 100–400-fold excess of activity over PDE. This method gives good agreement of kinetic coefficients with published values (determined by radioactive means) at pH 7.0. However, 5'AMP deaminase shows too low activity above pH 7.0 to be used at higher pH values (*40*). An alternative assay that avoids this problem is to use 5'AMP nucleotidase and adenosine deaminase, each in 100-fold excess, which results in inosine production that can be monitored at A_{265} also, in a similar fashion (*40*).

High concentrations of cAMP give easily measurable absorbances at 265 nm (*42*), which interferes with the assay and, therefore, limits the maximum concentration of substrate that can be present in the assay mixture to 0.2 m*M*. This limitation can be avoided in one or two ways: first, by measuring absorbance at 240 or 280 nm, i.e., wavelengths at which cAMP absorbance is negligible, but this decreases the sensitivity of the assay and second, by addition of glutamine acid dehydrogenase, α-ketoglutarate, and NADH to the reaction mixture containing the 5'AMP deaminase and measuring change in A_{340} (*40*). This works because ammonium ions are released in the deamination of 5'AMP and the subsequent condensation of the ammonium ions and 2-oxoglutarate by glutamic acid dehydrogenase can be followed by monitoring the concomitant oxidation of NADH at 340 nm. In order to allow for the low endogenous ammonium ion concentration it is necessary to either include the appropriate "no enzyme" control assays or to preincubate the assay mixture in the absence of cAMP to remove the endogenous ammonium ions. All of these three continuous spectrophotometric methods give results in good agreement with each other when bovine heart cAMP PDE is assayed (*40*).

One possible problem with all these methods is that contaminating endogenous enzyme activities, such as 5'AMP nucleotidase, 5'AMP deaminase, adenosine deaminase, or myokinase, may affect the measured change in absorbance. However, as long as the exogenously added enzymes are in sufficient excess the steady state concentration of 5'AMP should be negligible, therefore contaminating activities should not affect the measured rate of PDE activity.

The major limitation to use of such assays as those described is that their sensitivity is limited to low μM amounts of 5'AMP, the molar extinction coefficients of 5'IMP and NADH being 8650 and 6300 $M^{-1}cm^{-1}$ respectively. However, relatively simple adaptations of the methods should

allow fluorimetric monitoring of 5'IMP or NAD(H) (e.g., excitation of NAD(H) at 340 nm and measurement of fluorescence at 440 nm), which would increase the sensitivity of the assay by two orders of magnitude, making it suitable for most kinetic measurements of PDE activity.

A continuous method has been described for assay of another type of phosphodiesterase—namely 2',3'-cyclic nucleotide-3'-phosphodiesterase (*43*). The assay is based on an acid-base indicator monitoring the amount of acid produced in the hydrolysis of the substrate, 2',3'-cyclic AMP. It was necessary to have the indicator (bromothymol blue) matched with a buffer of identical pKa (imidazole–HCl, pKa=6.9 at 30°C) so that the amount of buffer neutralized by the acid product was proportional to the amount of basic indicator neutralized. Hexadecyltrimethylammonium bromide was required to prevent precipitation of the indicator dye with the imidazole, as well as to activate the enzyme. A dye absorbance increase of 1.0 corresponded to hydrolysis of 11.1 μmol of substrate when monitored at 420 nm, or a decrease of 1.0 was equivalent to hydrolysis of 7.5 μmol if monitored at 630 nm. The assay was clearly very dependent on control of pH of reactant solutions, and also on temperature since a small increase in temperature simulated acid formation. Further, Jacks and Jones (*30*) have commented on the danger (in experiments in which enzyme inhibition is being studied) of inhibition of the chromophore producing an artificial change in absorbance. Such an assay would require careful validation before routine use.

OTHER METHODS

A variety of other possible ways of assaying PDEs have been described in the literature. A discontinuous method that has received some attention recently involves using fast atom bombardment/mass-analyzed ion kinetic energy spectrophotometry (FAB/MIKE) to measure amounts of cAMP and 5'AMP in a reaction stopped by heating reaction mixture (*44,45*). FAB/MIKE analysis of rat brain cAMP PDE activity resulted in the accurate determination of K'_m (181 μM cf 205 μM) and V'_{max} (28.6 nmol min^{-1} cf 33.2 nmol min^{-1}) compared with values obtained using a standard radioactive assay (*44*). In this method it was necessary to spike the stopped reaction mixture with an internal standard in order to relate individual FAB spectral peaks to nucleotide concentration (*44*). Even then, calibration curves did not pass through the origin and in order to obtain a good correlation it was necessary to consider the intensity of the three peaks within a spectrum that correspond to the important fragment ions of the nucleotide (*44*).

More recently, this approach has been extended to assay cCMP dependent PDE activity using FAB/tandem mass spectrometry (*45*). Again, good agreement of kinetic parameters was obtained with those determined by

more traditional radioactive-type assays but it was still necessary to use internal standards and to consider the intensities of the four major peaks within a spectrum in order to obtain a good linear correlation of nucleotide concentration with peak size. Such assays are discontinuous but analysis time is brief (1.5–5 min) so it should be relatively straightforward to develop an essentially continuous assay by frequent sampling of the assay mixtime during incubation (*45*). The major advantages of such a system are its sensitivity, speed, and reproducibility. However, the capital cost of purchasing such equipment would make this method prohibitively expensive in the absence of easy accessibility to an existing facility.

Another, more conventional, method of separating radiolabeled cAMP and 5′ AMP involves using a fluorosil (activated magnesium silicate) column (*46*). This column (0.4×4 cm) is reported to bind cAMP, adenine, and adenosine but not AMP at pH 7.0. AMP could be washed off the column in 3 vol of pH 7.0 buffer. The column could be regenerated by washing with 10 column vol of 4*M* HCl then re-equilibrating with 10 vol of start buffer. This method would appear to be a quick, easy, inexpensive method of separation of cAMP and AMP. However, the method is not very robust, giving incomplete separation at even slightly acid pH values. It is sensitive to 5′ nucleotidase contamination and any adenosine produced will bind to the column giving an inaccurate result for 5′ AMP determination, although this could be avoided by using ^{32}P-labeled cAMP or spiking the reaction with cold 5′ AMP (*46*). Furthermore, cGMP hydrolysis cannot be measured using fluorosil, cGMP and 5′ GMP not being separated fully by this method. The limitations of this method of separation probably outweigh the advantages for most uses.

Measurement of the inorganic phosphate released after conversion of AMP to adenosine by 5′ nucleotidase provides a discontinuous assay of low sensitivity. Methods of measuring the absorbance of a phosphate-molybdate complex at 450 nm are well established (*47*). This method has been used to measure PDE activity in beef heart extracts and cAMP levels (adding exogenous beef heart PDE) in urine (*48*). It is necessary to include the appropriate control in order to allow for background inorganic phosphate levels and this method is accurate to a lower limit of about 20 μM inorganic phosphate (*48*).

A novel continuous method of PDE assay involves measurement of protons released during hydrolysis of cAMP using a pH stat. This method has been used successfully to measure bovine brain phosphodiesterase activity, for example (*19*). The rate of addition of alkali (NaOH) to maintain neutral pH is proportional to the rate of cAMP hydrolysis. The assay is reproducible (duplicate titrations being within 5% of each other) but not very sensitive, the lower limit being 10 nmol of cAMP hydrolyzed per minute in an assay volume of 1.5 mL. Another major drawback to this method is the necessity to maintain the reaction vessel under nitrogen owing to the sensitivity of the assay (i.e., proton concentration) to atmospheric CO_2.

Fluorescence has been employed to develop an elegant method of measuring cAMP levels in single cells in a nondestructive fashion. It involves the use of cAMP-dependent protein kinase (cA-PK) as a sensor, introducing it into whole cells by microinjection (*50*). It is well established that cAMP binding to the R (regulatory) subunit of cA-PK causes it to dissociate from the C (catalytic) subunit. Labeling of R and C with different fluorescent labels, i.e., C with fluorescein (which gives a blue green fluorescence) and R with rhodamine (which gives an orange fluorescence), leads to differential fluorescence patterns depending on whether C and R are associated or dissociated. At low cAMP concentrations C and R are associated with each other and an orange fluorescence occurs (owing to the effects of fluorescence resonance energy transfer). At higher cAMP concentrations C and R are dissociated and a blue-green fluorescence results. These fluorescence patterns can be quantified, allowing for background, and the method is sensitive for cAMP concentrations of between 0.01 and 1 μM. Although the method is technically demanding it has been used successfully in a number of studies, e.g., showing that intracellular cAMP determines the direction of movement in melanophores (*51*).

USE OF FLUORESCENT CYCLIC NUCLEOTIDE ANALOGS

In order to avoid the hazard and inconvenience associated with the use of radiochemicals but retain sensitivity of detection, fluorescent cyclic nucleotide analogs have been explored. We discuss these separately here.

2′-O-anthraniloyl (ANT) and 2′-O-(*N*-methyl) anthraniloyl (MANT) cAMP and cGMP derivatives (*see* Fig. 8) are fluorescent compounds that have been used in the assay of phosphodiesterases by either continuous (*52*) or discontinuous methods (*53*). Excitation of these derivatives and their 5′ equivalents at 320–360 nm results in fluorescence at 410–450 nm (*33*).

These cyclic nucleotides were developed primarily for use in discontinuous type assays, in order to avoid the use of radioisotopes. In this type of method, assays are carried out in standard conditions except that 8–10 μ*M* fluorescent analog is used instead of the "natural" cyclic nucleotide (*33*). Reactions are stopped by boiling and the substrate and product separated by either ion exchange on DEAE Sephadex, eluting with a 0–2*M* NaCl gradient in pH 7.0 (*54*), or by TLC with 1-propan-1-ol: NH_4OH: water :: 6:3:1 (v/v) on silica gel (*33*). Usually, more than 95% of all sample applied can be recovered using either separation method. Fluorescence of the product can be selectively enhanced by addition of 8 m*M* zirconyl chloride and this increases the sensitivity of the assay such that 0.4 nmol of ANT5′AMP can be detected accurately in a 5 mL elution volume (*54*). Alternately, fluorescence of both substrate and product can be enhanced to a greater extent by addition of *N*,*N*-dimethylformamide (*33*). This allows accurate detection of 8 pmol of the ANT and MANT derivatives (in 2.3 mL final volume).

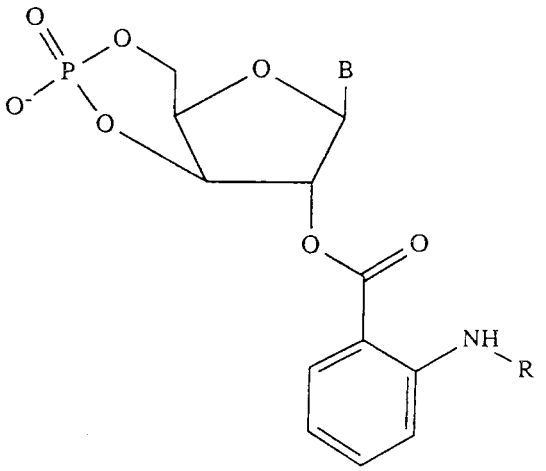

Fig. 8. Structures of 2′-0-anthraniloyl (ANT) and 2′-*N*-Methylanthranilayl (MANT) derivatives of cAMP and cGMP. In: ANT-cAMP, B = adenine and R = H; ANT-cGMP; B = guanine and R = H; MANT-cAMP, B = adenine and R = CH_3; MANT-cGMP, B = guanine and R = CH_3.

Discontinuous methods have been used in assay of Ca^{2+}/Calmodulin dependent PDEs (type 1). Purified enzyme from bovine brain has similar or slightly higher K'_m values for cAMP and ANTcAMP ($\sim 90\ \mu M$), but the V_{max} value for the fluorescent analog is only 1–15% of that for the cAMP (*53,54*). Purified bovine heart enzymes gives similar results also (*53*). Studies on a partially purified mixture of phosphodiesterases from bovine heart showed that both ANT and MANT cGMP gave similar K'_m values to native cGMP (*33*). However, two values were obtained for the K'_m values of the fluorescent derivatives of cAMP, one low (3–4 μM) and one high (36–72 μM) presumably reflecting the affinity of different PDE isozymes in the mixture for the fluorescent substrates.

Conversion of MANT cGMP to MANT 5′GMP leads to the 45% decrease in fluorescence at 450 nm and this fact has been used in development of a continuous assay for Ca^{2+}/Calmodulin dependent PDE (I) from rabbit and from bovine brain. Comparison of substrate disappearance as measured by HPLC and by change in fluorescence over time as the enzyme was incubated with fluorescent substrate showed a strong correlation ($R = 0.996$) over complete substrate conversion to product, i.e., the method showed high sensitivity. Again the K_m values of the natural and fluorescent substrates were similar, although, in this case V_{max} data were not reported (*52*). Furthermore, inhibition of the enzyme with an inhibitor of Calmodulin was measured accurately by this method (*52*).

Despite its comparatively low sensitivity, this method may prove attractive in some cases because it is nonradioactive. The time necessary to carry out PDE assays using fluorescent analogs varies depending on whether a continuous or a discontinuous method of assay is used, the former being faster. Also, the cost is not prohibitive, the reagents necessary

for synthesizing these derivatives being inexpensive. Alternatively, some fluorescent labels are available commercially from Calbiochem (La Jolla, CA). However, a serious deficiency is the limitation in that the fluorescent labels have different, generally much lower, V_{max} values (and possibly slightly different K'_m values) compared to their equivalent native nucleotides therefore limiting kinetic analysis of the enzymes by these methods. Also, it may be that the fluorescent analogs are not substrates for some PDE isozymes, such as PDE IV and V, which are more specific than the others, accommodating only one cyclic nucleotide.

SUMMARY

Quantification of cyclic nucleotides is an important part of the study of receptor function and second messenger systems. We have reviewed the various ways by which this may be achieved. For quantification of cAMP and cGMP at low concentrations (such as in cell extracts) the competition assay method stands alone. To our knowledge, however, there is no readily available equivalent for other cyclic nucleotides or their noncyclic counterparts. The method for quantifying cAMP concentrations in individual cells is an interesting development which, although technically demanding and limited to cAMP, allows study of cAMP levels within compartments in an individual cell, as opposed to a population of cells.

Where in vitro studies of enzyme activities are concerned there are other, and in some circumstances better, alternatives. Generally, the need for sensitivity has dictated the use of radiolabeled substrates. Attempts to use fluorescent analogs have been compromised by them being unnatural substrates, or by the necessity for extra manipulation (derivatization). Continuous assays have not proven generally acceptable and most experiments involve stopped reaction samples, and consequently many samples to analyze. The accent has therefore been on simplicity, speed, and robustness in the assay method, as well as accuracy and precision. Assays that can handle samples in parallel (for at least a significant part of the method) are best suited to this purpose. Of the methods discussed we have certainly found the alumina method, particularly with elution by 2*M* NaOH, to be the best, as well as being the least expensive overall (including hidden costs of recycling of used materials in other methods).

There may well be cases where the methods mentioned above are inappropriate—for instance, in investigating substrate analogs that are not available in radiolabeled form. In such cases the analytical chromatographic methods come into their own, and HPLC is probably the best of these. Although being limited in sensitivity and in assay turnaround time it offers (and optimation of any particular system) complete separation, characterization (by elution time), and quantification of all reactants. The key to good progress in any experiment is a good assay. It is owing to the efforts

of many workers over the years that we now have a wide choice of cylic nucleotide assay formats. Among them is a suitable one for any purpose in cyclic nucleotide research.

ACKNOWLEDGMENTS

We would like to thank our colleagues, especially J. Jappy, M. Merriman, M. O'Driscoll, and M. Reece-Ford, for their help with the study of cyclic nucleotide assay techniques and M. Day for her typing. Thanks are also due to Berthold Instruments, Canberra Packard, Lab Logic, BDH/Merck, Pharmacia Wallac, and Poly Filtronics for access to equipment and advice during the course of the work described here.

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