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COMPARISON OF BATCH AND CHROMATOGRAPHIC ASSAYS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES*

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SUMMARY

An improved method for the assay of cyclic nucleotide phosphodiesterases (CNPases) is described in which residual ^{3}H-cAMP or ^{3}H-cGMP is separated from products by chromatography on thin layers of PEI-cellulose. Comparison of CNPase activity in crude or purified kidney enzyme assayed by the PEI-cellulose method with a batch anion-exchange resin method showed serious underestimation of activity by the latter method. The batch method underestimated CNPase activity by 34–82%. The error was due to substantial binding of nucleosides (adenosine and guanosine) to the resin, which is assumed not to occur by those using this method.

INTRODUCTION

The cellular levels of the cyclic nucleotides, cAMP and cGMP, are controlled not only by the cyclases which catalyze their rates of formation but by several cyclic nucleotide phosphodiesterases (CNPases) which hydrolyze them to 5'-AMP or 5'-GMP[1]. The rapid assay originally used to study CNPase by Butcher and Sutherland[2] was based on the measurement of inorganic phosphate released from 5'-AMP by snake venom. As this method was too insensitive to measure CNPase activity at the low (μmolar) substrate levels present in tissues, the enzymes have been assayed in recent years using isotopically labelled substrates. The initial nucleotide product (or the nucleoside formed by the action of snake venom) is separated from residual substrate by several means. Some investigators have used tedious but well defined chromatographic methods for the separation of the substrate from all possible primary, secondary, or remote products[3–5]. The capability of the analytical method to separate residual substrate from remote products is particularly important in studies on the enzyme content of crude homogenates, subcellular fractions, and partially purified enzymes.

In recent years many investigators have used the batch resin assay method of Appleman and Terasaki[1] because of its simplicity and rapidity. In this method a

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sample of the reaction solution is mixed with a slurry of anion-exchange resin, centrifuged and an aliquot of the supernatant fluid (assumed to contain all the [^{3}H]-adenosine or [^{3}H]guanosine) is assayed for radioactivity. The residual ^{3}H-cAMP or ^{3}H-cGMP is assumed to bind to the resin. Although the recovery of cAMP was shown to be high in the original publication of this method[6], there have been no data published on the behavior of adenosine, guanosine, inosine, xanthosine, etc., in this system.

In studies on crude and partially purified CNPases from rat kidney, we noted anomalous time courses of apparent enzyme velocity, especially when ^{3}H-cGMP was the substrate. Comparisons to the assay of reaction mixtures by our PEI-cellulose thin-layer chromatographic method[5] showed serious underestimation by the batch resin method of the hydrolysis of cAMP and cGMP. We, therefore, undertook a systemic comparison of the two assay methods and evaluated the adsorption of [^{3}H]-adenosine and [^{3}H]guanosine to the anion-exchange resin. We also modified our PEI-cellulose method for the assay of ^{3}H-cAMP hydrolysis such that it also gave excellent results with ^{3}H-cGMP as substrate.

MATERIALS AND METHODS

Assay 1

For Assay 1 we prepared 0.5-mm layers of PEI-cellulose on glass plates as previously described[7] with minor modifications. After drying at room temperature the plates were arranged in the "sandwich" form. The plates were washed once with distilled water by ascending flow. The band of yellow material at the front was scraped off (it increased the background cpm) and plates were wrapped in aluminum foil and stored in the refrigerator until used. Plates kept in the refrigerator showed no signs of deterioration even after up to six months of storage.

To facilitate subsequent scraping of scrolls of the PEI-cellulose for the assay of tritium, six channels were cut per plate by scraping off a 1-mm band between the channels. Samples were applied (in a volume of 5–20 μl) along with unlabelled "cold" standards using a jet of cold air to ensure that the spots were less than 1 cm in diameter. Solvent systems used to develop the plates are described under Results. To recover tritiated substrates and products the cold markers were detected with a UV light and appropriate sections were scraped off the plates. Radioactivity was eluted in a test tube using 5 ml of 0.05 *N* NaOH. A 3-ml aliquot of the extract was neutralized with 0.2 ml of a 1:25 dilution of concentrated H_3PO_4, added to 10 ml of Insta-Gel and counted in a Packard Tricarb Liquid Scintillation counter. The validity of this method of elution of ^{3}H-labeled cyclic nucleotides and derivative nucleotides and nucleosides and quantitation are described elsewhere[7].

Assay 2

For Assay 2 the anion-exchange resin (AG-1-X2, 200–400 mesh from Bio-Rad Labs., Richmond, Calif., U.S.A.) was washed extensively with NaOH, HCl, and distilled water to pH 6.0 before use. A 100-μl aliquot of the final supernatant from the enzyme reaction was mixed with 200 μl of a 1:3 (settled resin:total volume) resin slurry in distilled water. The mixture was agitated several times over 15 min and then centrifuged for 10 min at 5,000 *g*. (At much lower centrifugal force, *e.g.*, in a clinical table top centrifuge, resin beads remained on the surface, contaminating the sampling

in the next step.) A 50 μl aliquot from above the packed resin was added to 5 ml of Insta-Gel and counted in a liquid scintillation spectrometer.

Whole rat kidneys were minced, homogenized with a glass–PTFE device and centrifuged as indicated for individual studies in an isotonic solution (160 m*M* KCl or 10.9% sucrose). Partial purification of CNPase was achieved as described elsewhere by passage of the soluble fraction over 0.5 *M* agarose gel and/or DEAE-cellulose columns. The enzyme reaction of 0.4 ml total volume contained 2.5 m*M* $MgCl_2$, 15 m*M* Tris·HCl (titrated to pH 8.2) and tissue protein and ^{3}H-cAMP or ^{3}H-cGMP as indicated for individual studies. The mixture was incubated (except for time–course studies) for 20 min at 30° and then inactivated by heating for 1 min at 80°. After cooling, 100 μl of a 2 mg/ml solution of *Crotalus atrox* venom was added, and the mixture was re-incubated for 10 min and again heated at 80° for 1 min.

RESULTS

PEI-cellulose thin-layer chromatography

When we applied our previous system of separation of cAMP from its metabolites[5], we did not achieve adequate separation of cGMP from its principal metabolites. We explored numerous variations in the concentration of PEI, the types of cellulose, the pH and types of buffers and the concentrations of LiCl in an attempt to develop separation methods for both substrates. Excellent separation of cAMP from most possible metabolic derivatives[8] was achieved by first developing the plates (for about 90 min) with water to the front. (Nucleosides and bases moved 2/3–3/4 of the distance to the front, while cAMP, 5′-AMP, and ATP stayed at the origin.) After the plates had been dried (overnight at room temperature or in a few minutes using a fan), they were developed again in 0.15 *M* LiCl. The second run produced wide separation of cAMP from the more mobile nucleosides and bases as well as the less mobile 5′-AMP and ATP (Table I). Although cIMP is barely separated from cAMP, no cIMP was formed in our studies even on prolonged incubation with crude enzymes.

For the separation of cGMP the best solvent for the first run was 0.4 *M* acetic acid, since mobility of guanosine and guanine were too slow in water. Drying and the second development with 0.15 *M* LiCl was as for cAMP. Different lots of PEI and types of cellulose required slight adjustment of the concentration of LiCl, which was done empirically such that cAMP and cGMP were maximally separated from the

TABLE I

R_F VALUES OF cAMP, cGMP, AND RELATED COMPOUNDS

Compound	R_F	*Compound*	R_F
ATP	0.01	GTP	0.01
ADP	0.01	GDP	0.02
5′-AMP	0.10	XMP	0.04
cAMP	0.47	5′-GMP	0.06
cIMP	0.59	cGMP	0.46
Adenine	0.70	Xanthosine	0.73
Hypoxanthine	0.82	Guanine	0.87
Adenosine	0.84	Guanosine	0.89
Inosine	0.93		

TABLE II

COMPARISON OF APPARENT HYDROLYSIS OF cAMP OVER TIME BY PEI-CELLULOSE CHROMATOGRAPHIC *vs.* BATCH ANION-EXCHANGE RESIN ASSAY METHODS

The crude enzyme source was 12.6 μg of protein from a 15,000 *g* supernatant of whole rat kidney homogenate. Concentrations in the assay were 1.0 μM ^{3}H-cAMP, 2.5 mM $MgCl_2$ and 15 mM Tris·HCl (pH 8.2). The results represent the percentages of ^{3}H-cAMP hydrolyzed.

Time (min)	*Assay 1 (PEI-cellulose)*	*Assay 2 (batch-resin)*	*Assay 2/1*
5	18.4	10.8	0.59
10	29.7	17.6	0.59
20	55.0	29.5	0.54
40	70.4	32.9	0.47
60	86.1	27.0	0.31
120	91.9	22.0	0.24

TABLE III

COMPARISON OF APPARENT HYDROLYSIS OF cAMP BY TWO ASSAY METHODS AS A FUNCTION OF THE AMOUNT OF CRUDE ENZYME PROTEIN

The crude enzyme source was a 15,000 *g* supernatant of whole rat kidney homogenate. The amount of protein was varied by dilution (1:50 to 1:500) with a 3 mg/ml solution of bovine albumin. Concentrations in the assay were 1.0 μM ^{3}H-cAMP, 2.5 mM $MgCl_2$ and 15 mM Tris·HCl (pH 8.2). The results represent the percentages of ^{3}H-cAMP hydrolyzed.

Protein (μg)	*Assay 1 (PEI-cellulose)*	*Assay 2 (batch resin)*	*Assay 2/1*
16.7	56.2	33.6	0.60
8.3	33.3	20.7	0.62
4.2	20.0	11.3	0.57
3.3	13.9	8.9	0.64
1.7	8.1	4.2	0.52

TABLE IV

COMPARISON OF APPARENT HYDROLYSIS OF cGMP BY TWO ASSAY METHODS AS A FUNCTION OF THE AMOUNT OF CRUDE ENZYME PROTEIN

The crude enzyme source was a 15,000 *g* supernatant of whole rat kidney homogenate. The amount of protein was varied by dilution with a 3 mg/ml solution of bovine albumin. Concentrations in the assay were 1.0 μM ^{3}H-cGMP, 2.5 mM $MgCl_2$ and 15 mM Tris·HCl (pH 8.2). The results represent the percentages of ^{3}H-cGMP hydrolyzed.

Protein (μg)	*Assay 1 (PEI-cellulose)*	*Assay 2 (batch resin)*	*Assay 2/1*
2.0	15.9	2.9	0.18
2.9	21.6	5.1	0.24
3.9	21.2	7.5	0.35
4.9	26.7	8.8	0.33
6.5	32.8	12.3	0.38
9.8	43.5	15.7	0.36
19.6	61.1	25.1	0.41

nucleoside (ahead) and the nucleotide (behind) on the plate. The remainder of the procedure was completed as given under Materials and methods.

On prolonged incubation of ^{3}H-cGMP with crude preparations, the activity in the guanosine region first increased and then declined, while a new band appeared between cGMP and guanosine. Unless the product region was cut from the front to within 1 cm of the cGMP spot, this unknown product would contaminate the cGMP region. Comparison with numerous possible remote products indicated that this material moving just ahead of cGMP was xanthosine.

Crude enzyme reactions

In our studies on physiological or pharmacological effects on CNPases it is necessary to perform assays of enzyme content in whole homogenates or crude particulate fractions. A comparison of the apparent rate of hydrolysis by a crude tissue fraction at variable incubation times is shown in Table II. Over the first 20 min the assay results were proportional, with the batch resin data being 54–59% of the PEI-cellulose data. Beyond 20 min, hydrolysis proceeded logically toward completion with Assay 1 but the reaction appeared to reverse with Assay 2, a very unlikely possibility.

We next compared the assays when incubation time was kept constant but the amount of protein in the assay was varied (Table III). Under these circumstances both assays showed progressive slowing of the average velocity as substrate was depleted with an average ratio of Assay 2:Assay 1 of 0.59.

Similar studies were done using cGMP as substrate. As shown in Table IV, the discrepancy between the assays was most severe at low rates of substrate consumption which was, however, at the level one usually attempts to keep hydrolysis (under 30% by Assay 1) so that product formation is linear. With Assay 2, this limit was, in fact, exceeded when apparent hydrolysis was only 12.3%.

Similar discrepancies were found when assays were compared using partially purified CNPases. Incubation of an aliquot from a pool of the region of the high K_m CNPase from a DEAE-cellulose column[9] with 0.5 μM ^{3}H-cGMP for 10, 20, 40, and 80 min yielded 5, 10, 13, and 14% apparent hydrolysis by Assay 2 and 39, 59, 86, and 92% by Assay 1, an even more serious discrepancy than with the crude soluble preparation of Table IV.

Finally, we tested our most highly purified low K_m enzyme preparation, which had been eluted from a DEAE-cellulose column and further purified by gel filtration[9]. Variation in enzymic activity as a function of magnesium concentration was studied (Table V). Again the results with Assay 2 were 62% of that by Assay 1 over a wide range of hydrolysis.

Binding of [^{3}H]adenosine and [^{3}H]guanosine

The most likely explanation for the discrepancies between the two assays seemed to us that the batch resin method did not fulfill the untested assumption that [^{3}H]-adenosine and [^{3}H]guanosine remained completely unadsorbed in the presence of resin. We tested this possibility by setting up "mock" enzyme assays under conditions identical to those given under Materials and methods and in Tables II and IV for cAMP and cGMP, except that no enzyme was present and [^{3}H]adenosine, [^{3}H]-guanosine, ^{3}H-cAMP, or ^{3}H-GMP were present in separate tubes. The concentration of resin was varied to test binding by resin of both substrate and product. These re-

TABLE V

COMPARISON OF APPARENT HYDROLYSIS OF cAMP BY PURIFIED ENZYME WITH TWO ASSAY METHODS AS A FUNCTION OF MAGNESIUM CONCENTRATION

Enzyme was purified from a 100,000 *g* supernatant of whole rat kidney homogenate using DEAE-cellulose and agarose column chromatography (see ref. 9). Concentrations in the assay were 3.3 μM ^{3}H-cAMP, 15 m*M* Tris·HCl (pH 8.2) and variable concentrations of magnesium.

Magnesium (mM)	*Assay 1 (PEI-cellulose)*	*Assay 2 (batch resin)*	*Assay 2/1*
0.05	17.0	9.5	0.56
0.15	21.4	12.7	0.63
0.30	23.9	14.0	0.59
0.50	25.1	15.0	0.59
1.00	27.5	17.8	0.65
2.50	31.8	20.5	0.65
10.00	32.1	21.2	0.66

sults are shown in Tables VI and VII. Increasing the per cent resin in the slurry above the standard concentration (33%) did lower the blank slightly by reducing the amount of unbound substrate. However, even with the most dilute resin slurry a large percentage of each nucleoside was bound and the amount bound increased steeply as the per cent resin was increased.

TABLE VI

MOCK BATCH RESIN ASSAY OF ^{3}H-cAMP AND [^{3}H]ADENOSINE

200 μl of resin slurry was mixed with 100 μl of 0.1 μM ^{3}H-cAMP or [^{3}H]adenosine in the Tris·HCl/magnesium buffer (pH 8.0). After centrifugation 50 μl of the supernate were counted. The assay blanks for ^{3}H-cAMP represent the percentages of total cpm not bound by the resin. The [^{3}H]adenosine bound to resin represents the percentage of the total cpm which failed to remain in the supernate.

Resin slurry (%)	*Assay blank for ^{3}H-cAMP (%)*	*[^{3}H]Adenosine bound to resin (%)*
20	5.9	26.9
30	3.5	32.2
40	3.0	42.8
50	2.6	45.0
60	2.6	50.9

TABLE VII

MOCK BATCH RESIN ASSAY OF ^{3}H-cGMP AND ^{3}H-GUANOSINE

200 μl of resin slurry was mixed with 100 μl of 0.1 μM ^{3}H-cGMP or [^{3}H]guanosine in Tris·HCl/magnesium buffer (pH 8.0). After centrifugation 50 μl of the supernate were counted. The assay blanks for ^{3}H-cGMP represent the percentages of total cpm not bound by the resin. The [^{3}H]guanosine bound to resin represents the percentage of the total cpm which failed to remain in the supernate

Resin slurry (%)	*Assay blank for ^{3}H-cGMP (%)*	*[^{3}H]Guanosine bound to resin (%)*
20	5.4	56.1
30	4.8	68.7
40	4.4	70.9
50	4.6	70.7
60	4.6	73.5

Several lines of evidence support the validity of the PEI-cellulose thin-layer chromatography method as a tedious but highly reliable reference method for measuring CNPases, especially in crude enzyme preparations. The recovery of ^{3}H-cAMP, ^{3}H-cGMP, and their principal metabolites has been shown to be excellent[7]. Secondly, the method clearly separates residual substrate from even very remote products (Table I). Finally, on prolonged incubation, the consumption of substrate approaches 100% in curvilinear fashion.

The underestimation of reaction velocity by the batch resin method was most serious when crude preparations were used, especially with ^{3}H-cGMP as substrate. The error in this assay corresponded very closely to the degree of binding of the nucleoside to the resin (Tables VI and VII). Thus the assumptions originally made did not prove to be correct in our hands, under conditions very similar to those in the original publication on the method. We attempted several methods in an effort to reduce non-specific binding of products. Acidification of the reaction medium over a range from pH 8.0 down to pH 3.4 did not improve the results. Addition of a large excess of nucleoside likewise did not decrease binding of labeled product by the resin.

The batch resin method, because of its simplicity and speed, remains useful for rapid location of enzyme peaks in column effluents. The exact number of CNPase peaks, however, may be deceiving. We have in several instances found that what appeared to be two closely located peaks of cGMPase activity were, in fact, a single peak when the assay was repeated with the PEI-cellulose method. Likewise, the batch method will falsely underestimate $V_{max.}$ values even of purified enzymes.

We have not evaluated other methods[10,11] of intermediate speed, using small columns of various ion-exchange materials. When these methods are used to determine enzyme activity of crude preparations, rigorous evaluations as above should be conducted before assuming that the method gives valid results.

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