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Note

Sources of error in counting tritium-labelled adenosine and guanosine derivatives on chromatograms

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Because tissue levels of the cyclic nucleotides adenosine-3′,5′-monophosphate (cyclic AMP, cAMP) and guanosine-3′,5′-monophosphate (cyclic GMP, cGMP) are below micromolar concentrations[1], radioactively labelled forms of these nucleotides are now almost always used in assays of the enzymes for which they are the substrates, the cyclic nucleotide phosphodiesterases. To determine the rate of product formation during hydrolysis, it is necessary to separate labelled products from substrate. The most reliable methods for separation of these compounds is by some form of chromatography, using either paper or thin layers of PEI-cellulose as the supporting medium[2–4]. In an effort to simplify what is a time-consuming procedure, we and other investigators have used unlabelled standards of substrate and product as markers, cut out or scraped off the zones containing the markers and simply added the paper sections or PEI-cellulose scrapings to a liquid scintillation "cocktail" in a counting vial with or without an extracting solvent and determined the radioactivity in the mixture. We have reported using such an approach to assay hydrolysis of cyclic AMP[3]. In attempting to use the same technique to assay hydrolysis of cyclic GMP we encountered a number of sources of error. This report describes these errors and methods for circumventing them.

METHODS AND MATERIALS

Thin-layer plates of 0.5 mm thickness were prepared by spreading on glass plates a well-stirred mixture of 25 g of MN-300 cellulose (Macherey, Nagel and Co., Düren, G.F.R.) and 150 ml of a 0.5% solution of polyethyleneimine (Alcolac Chemical Corp., Baltimore, Md., U.S.A. or BASF Corp., Charlotte, N.C., U.S.A.), which was titrated to pH 6.0 with HCl. After drying at room temperature these PEI-cellulose plates were washed once with distilled water by ascending flow, dried and stored at refrigerator temperature until used.

For purification of labelled materials and studies of recoveries from paper chromatograms we used Whatman SFC No. 40 paper. [^{3}H]Toluene (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) was used as an internal standard. Tritium-labelled nucleotides and nucleosides (obtained from Schwartz/Mann, Orangeburg, N.Y., U.S.A. or New England Nuclear, Boston, Mass., U.S.A.) in some cases contained appreciable amounts of impurities (especially volatile tritium). Therefore,

we purified the labelled nucleotides by descending paper chromatography in the system isoamyl alcohol–5% Na_2HPO_4 (1:1) and nucleosides in the system 95% ethanol–0.5 *M* ammonium acetate (5:2), pH 7.5. The purified materials were eluted from the paper with water.

As scintillation solution we used either Insta-Gel (Packard Instrument, Downers Grove, Ill., U.S.A.) or a modified Bray solution, which contained 0.5 g 1,4-bis-2-(5-phenyloxazolyl)-benzene, 10 g 2,5-diphenyloxazole, 80 g naphthalene, 428 ml ethylene glycol monoethyl ether, 428 ml *p*-dioxane and 143 ml xylene. Tritium was assayed using a Packard Tri-Carb liquid scintillation counter.

RESULTS AND DISCUSSION

In studying the elution of labelled substrates and products from chromatograms we applied pmole amounts, comparable to quantities used in enzymatic studies. To determine the "apparent" dpm of tritium applied to the chromatograms, we counted aliquots added directly to counting solutions in glass or plastic vials and corrected for quenching using [^{3}H]toluene as internal standard. With some tritiated materials, especially when glass vials and the modified Bray solution were used, the apparent dpm was substantially below the expected value based on the manufacturer's data. This artifact was found to result from quenching by adsorption to the vial, which was not corrected for because the non-polar internal standard ([^{3}H]-toluene) did not behave similarly. This problem was circumvented by addition of 1.0 ml of 1.0 *N* NaOH to the counting solution (15 ml) containing the tracer nucleotide or nucleoside. Such an effect is illustrated for the counting of 45 pmoles of ^{3}H-labelled 5'-AMP (in 10 μl of water) which yielded an apparent dpm in the solvent alone of 126,000 (at an efficiency for [H^3]toluene of 34.6%) but a value of 180,000 dpm in solvent plus NaOH (efficiency of 18.8%).

The requirement for NaOH to prevent quenching by adsorption of tracers to the counting vial led us to re-evaluate our previous technique for counting ^{3}H-labelled cAMP and its products, in which the PEI-cellulose was left in the counting vial after elution with NaOH and addition of the scintillation solution[3]. In that study we determined recovery of tritium by comparing the sum of "apparent" dpm (after correction for quenching using [^{3}H]toluene) recovered from five scraped zones with the apparent dpm recovered from a comparable-sized single rectangle of PEI-cellulose on which was spotted a volume of the enzyme supernatant equal to that which was developed chromatographically. The five scraped zones and the single reference section of PEI-cellulose were then each eluted with NaOH in counting vials as just described. This method provided a control for any quenching effects due to PEI but did not consider possible adsorption of the original tritiated substrate or labelled products to the PEI-cellulose, even in the presence of NaOH and counting solution. This artifact does occur, especially for ^{3}H-labelled cGMP. As shown in Table I, recovery of ^{3}H-labelled cyclic nucleotides was incomplete in all cases, especially for cGMP, when the PEI-cellulose was left in the counting vial. In similar studies the recovery of ^{3}H-labelled 5'-AMP was 33–34% and that of [^{3}H]adenosine was 87–90%. Comparable levels of incomplete recovery were also found from paper chromatograms.

We next attempted to use the more polar scintillation mixture Insta-Gel to improve recoveries. Extraction of the PEI-cellulose was attempted with 1 ml of either

TABLE I

RECOVERY OF CYCLIC NUCLEOTIDES WHEN PEI-CELLULOSE IS EXTRACTED IN A COUNTING VIAL FOLLOWED BY THE ADDITION OF THE MODIFIED BRAY SOLUTION

Nucleotide	*Extractant**	*Dpm added*	*Dpm found***	
[^{3}H]cAMP	None	21,200	11,200	4,630
[^{3}H]cAMP	Water	21,200	16,700	17,000
[^{3}H]cAMP	NaOH	21,200	16,600	16,400
[^{3}H]cGMP	None	23,100	5,790	3,570
[^{3}H]cGMP	Water	23,100	16,900	17,000
[^{3}H]cGMP	NaOH	23,100	8,300	8,260

* 1 ml of water or 1.0 *N* NaOH.
** Two separate experiments.

water, 1.0 *N* NaOH, 1.0 *N* HCl, or 100 m*M* Tris–HCl (pH 8.0)–50 m*M* $MgCl_2$. Even the best extracting agent (HCl) gave only 82–86% recovery for ^{3}H-labelled cGMP.

It appeared likely that the tracers were being adsorbed and quenched by the PEI-cellulose in the scintillation solvent. We, therefore, attempted to extract the tracer from the PEI-cellulose (using 5 ml of aqueous solvents) in a separate first step, followed by centrifugation and addition of 3 ml of the extract to Insta-Gel. Extraction with water of ^{3}H-labelled cGMP gave only 7–8% recovery. Extraction with 1.0 *N* NaOH gave 94% recovery for ^{3}H-labelled cGMP in terms of the manufacturers' data or a 10-μl sample counted in 3 ml NaOH plus 10 ml Insta-Gel. Therefore, this procedure was tested for other nucleotides and nucleosides. The concentration of NaOH used for extraction was reduced to 0.05 *N* because concentrations over 0.1 *N* yielded a slow loss of counts (5% over 24 h). Up to 3 ml of 0.05 *N* NaOH could be mixed with 10 ml of Insta-Gel with the mixture staying a single phase. This mixture was opaque and viscous after cooling but nevertheless yielded consistent results at a good efficiency for tritium (30%) with counts remaining stable over a long time (observed up to 88 h). There was no quenching effect of any PEI extracted by the NaOH solution, as shown by addition of tracers to 3 ml of a NaOH extract of a blank segment of PEI-cellulose.

Table II shows that the above method yields excellent recoveries for the original cyclic nucleotides, the nucleotide products of enzymatic hydrolysis and the main nucleoside secondary derivatives[5]. The simple additional step of extraction of the PEI-cellulose thus enabled us to continue to use this very versatile method for chromatographic separation of substrate from product in studies of enzymatic hydrolysis of both cAMP and cGMP.

The size of the PEI-cellulose section eluted (from 2 to 5 cm by 3.5 cm) had essentially no effect on either blank counts or efficiency of counting tritium. Older (several months) PEI-cellulose plates and Insta-Gel solutions gave moderate levels of chemiluminescence with the NaOH extracts. If immediate counting was desired this artifact was immediately corrected by addition of acid (0.2 ml of a 1:25 dilution of concentrated H_3PO_4). We attempted using commercial organic bases instead of the NaOH. Since efficiency for tritium was only slightly less in the NaOH extract, we abandoned further studies with the organic bases because of their considerably greater cost.

TABLE II

RECOVERY OF NUCLEOTIDES AND NUCLEOSIDES FROM PEI-CELLULOSE AND PAPER CHROMATOGRAMS

The tracers were spotted on a section of either surface, eluted with 5 ml of 0.05 *N* NaOH and after centrifugation 3 ml of clear extract was counted in Insta-Gel.

Tritiated tracers	*Recovery (%)*	
	PEI-cellulose	*Paper*
cAMP	99.6*	102.5
5'-AMP	101.3	106.8
Adenosine	99.9	96.2
cGMP	95.5	98.4
5'-GMP	94.3	97.0
Guanosine	96.3	94.0

* Mean values for four experiments.

Errors comparable to the above have been encountered by Davison and Andersson in determining counting rates of basic tritiated compounds[6].

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