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Application of solid-phase extraction on anion-exchange cartridges to quantify 5′-nucleotidase activity

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ABSTRACT

The enzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) catalyzes a critical reaction in intermediary metabolism, the phosphohydrolysis of nucleoside 5′-monophosphates to their corresponding nucleosides. We have evaluated solid-phase extraction on pre-packed anion-exchange cartridges as a chromatographic technique with which 5′-nucleotidase activity may be detected and quantified. Chromatographic conditions were established whereby substrate nucleotide was rapidly and completely separated from its corresponding nucleoside by solid-phase extraction. Both analytes were recovered quantitatively, without loss or degradation. This chromatographic system was integrated into a discontinuous radiochemical assay for 5′-nucleotidase which enabled both substrate utilization and product formation to be assessed simultaneously. Enzyme reaction samples could be analyzed directly for 5′-nucleotidase activity without any pre-chromatography preparation. The high capacity of the solid-phase cartridges and the inability of 5′-nucleotidase to enter the packing bed during analyte elution facilitated termination of the enzyme reaction by applying the entire reaction mixture to the cartridge. Loaded cartridges could then be stored at 4°C prior to chromatography and subsequently batch-eluted. The excellent resolution between substrate and product in solid-phase extraction and the sensitivity of radioisotopic counting enabled detection/quantification of low tissue levels of 5′-nucleotidase in conjunction with ancillary assays for secondary enzyme reactions with the potential to elicit the artifactual loss of 5′-nucleotidase substrate/product when crude biological preparations are examined for 5′-nucleotidase activity. Our results demonstrate that solid-phase extraction on anion-exchange cartridges with elution solvents of appropriate pH offers several unique advantages for 5′-nucleotidase determination.

INTRODUCTION

Dephosphorylation of nucleoside 5′-monophosphates in eukaryotic cells is regulated by the enzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) [1]. At least three enzyme subtypes have been described based upon their intracellular localization and enzymatic properties. The most active 5′-nucleotidase is a plasmalemmal ectoenzyme in plant [2] and animal [3] cells which acts as an adenosine "translocase" by dephosphorylating cytoplasmic adenosine 5′-monophosphate (AMP) and liberating adenosine to the extracellular milieu. Two soluble 5′-nucleotidases of lesser activity have also been purified, one of which exhibits a preference for inosine 5′-monophosphate (IMP) [4–7]. Both the substrates and the products of 5′-nucleotidase play important homeostatic roles in regulating tissue function and intermediary metabolism [8], and 5′-nucleotidase is a potential site of pharmacological intervention against tissue nucleotide depletion and cellular de-energization [9]. These considerations have made 5′-nucleotidase the subject of intense current research and place great importance on the detection and quantification of 5′-nucleotidase activity.

The assays routinely used for 5′-nucleotidase monitor spectrophotometrically the formation of one of the enzyme products (inorganic phosphate, nucleoside) [10]. Such microchemical determinations are suitable for highly active, purified enzyme, but are of limited, if any, use when assaying dilute preparations or crude biological samples. In these latter instances, detector sensitivity and discrimination are problematic, and tissue constituents can cause interference and high backgrounds. A recent study [11] has employed

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high-performance liquid chromatography (HPLC) with ultraviolet detection in an attempt to overcome some of these analytical obstacles, but the procedure demands specialized equipment and has restricted appeal due to low sample capacity (*i.e.*, a 10-min HPLC run per sample).

An alternative approach to 5′-nucleotidase assay involves radioisotopic monitoring of product formation from nucleoside 5′-monophosphate containing either ^{32}P or a ^{3}H- or ^{14}C-labeled nucleoside moiety [10,12]. Such radioisotopic assays are sensitive and facilitate quantification of 5′-nucleotidase activity regardless of enzyme purity, concentration, or the presence of tissue substances whose spectral properties resemble those of the nucleoside product. However, they require analytical separation of radiolabeled product from identically labeled substrate. This separation requirement has most often been addressed through paper [10] or thin-layer [13] chromatography (TLC), although these approaches are cumbersome, time-consuming, of limited sample capacity, and demand pre-chromatography sample preparation (*e.g*, deproteinization). Column chromatography on either neutral aluminum oxide [14] or Dowex anion-exchange resin [15,16] has been employed to isolate the nucleoside product of the 5′-nucleotidase reaction based on the fact that substrate nucleotide is anionic in alkaline solution, whereas the nucleoside product is uncharged. The column chromatographic techniques which have been described evidence the limitations of paper chromatography and TLC and have the additional disadvantage that quantitative nucleoside elution can require large solvent volumes prohibitive to radiochemical analysis of the entire eluate, thereby restricting the sensitivity of the assay.

These methodological considerations prompted evaluation of solid-phase extraction with disposable, pre-packed anion-exchange cartridges to assay for 5′-nucleotidase. We report here integration of solid-phase extraction into a discontinuous radiochemical assay for 5′-nucleotidase. The resultant 5′-nucleotidase assay offers ease, rapidity, and high sample capacity without the need for pre-chromatography sample preparation. The assay advanced has the further advantage of enabling simultaneous, facile monitoring of both substrate utilization and product formation. The chromatographic properties of the solid-phase extraction system described and the sensitivity of radioisotopic counting allow detection and reliable quantification of low 5′-nucleotidase levels. The data exemplify how solid-phase extraction can be a valuable chromatographic aid to the analysis of intermediary metabolism.

EXPERIMENTAL

Materials and reagents

[2,8,5-^{3}H]Adenosine (50.3 Ci/mmol, sp. act.) and [^{3}H(G)]AMP (29.2 Ci/mmol, sp. act.) were from New England Nuclear (Boston, MA, USA). [8-^{14}C]Inosine (57.0 mCi/mmol, sp. act.) and [8-^{14}C]IMP (56.0 mCi/mmol, sp. act.) were purchased from Amersham (Arlington Heights, IL, USA). Ready-flow III liquid scintillation fluid was from Beckman (Fullerton, CA, USA). Adenosine, AMP, inosine, IMP, imidazole, glycine, *p*-nitrophenylphosphate (PNPP), trichloroacetic acid (TCA), and 5′-nucleotidase (analytical preparation from snake venom; 320 U/mg of protein) were from Sigma (St. Louis, MO, USA). Coformycin was purchased from Calbiochem (San Diego, CA, USA). Fluka (Buchs, Switzerland) was the source of analytical-grade H_3PO_4, and HPLC-grade water was obtained from a Milli-Q-Plus reverse-osmosis system (Millipore, Bedford, MA, USA). Solvents for HPLC were from Burdick and Jackson (Muskegon, MI, USA), and trioctylamine was from Aldrich (Milwaukee, WI, USA). Sep-Pak Accell Plus QMA cartridges (prepacked with 400 mg strong anion-exchanger; 0.7 ml void volume) were from Waters Chromatography (Milford, MA, USA). A Beckman LS 9800 liquid scintillation spectrometer was used to quantify sample radioactivity; the counting efficiencies were $>50\%$ for ^{3}H and $>80\%$ for ^{14}C.

Tissue preparation

Heart muscle from rabbit left ventricle was rapidly homogenized at 4°C with a Waring blendor in 55 m*M* potassium phosphate buffer, pH 6.5, in the ratio 0.5 g tissue per ml buffer. After removal of debris by brief low-speed centrifugation (500 *g*, 5 min, 4°C), the myocardial homog-

enate was filtered through four-ply cloth gauze, and the filtrate was assayed immediately for protein content and 5'-nucleotidase activity (below).

Sep-Pak anion-exchange chromatography

Samples in a volume of $\leq$ 500 μl were loaded onto Sep-Pak Accell Plus QMA cartridges by using a micropipette whose tip was placed at the upper surface of the packing bed. After adsorption of the sample by gravity, syringes were sequentially connected to each cartridge for elution; the syringes contained various water-based solvent systems whose composition is detailed in the text, tables, and figures. The columns were eluted manually with a flow-rate of about 15 ml/min at either ambient temperature (22°C) or 4°C.

Evaluation of resolution, cartridge capacity, and analyte recovery in solid-phase extraction

For analysis of the utility of Accell Plus QMA cartridges to nucleoside and nucleotide chromatography, 500 m*M* stock solutions of adenosine, AMP, inosine, and IMP were prepared in 0.1 *M* glycine–NaOH buffer (pH 8.5) containing 10 m*M* $MgCl_2$. These solutions also contained a trace amount of either [^{3}H]adenosine ($5 \cdot 10^5$ cpm; 10.0 pmol/ml), [^{3}H]AMP ($5 \cdot 10^5$ cpm; 17.0 pmol/ml), [^{14}C]inosine ($5 \cdot 10^5$ cpm; 8.8 pmol/ml), or [^{14}C]IMP ($5 \cdot 10^5$ cpm; 9.0 pmol/ml), respectively. A known quantity of each nucleotide or nucleoside was loaded onto Sep-Pak Accell Plus QMA cartridges which had been pre-equilibrated with 10.0 ml of the glycine–NaOH buffer. The columns were eluted with the solvents specified in the text, tables, and figures. Sample load was varied either by altering the volume of stock solution applied to the cartridge (within the range 10–500 μl) or by diluting the stock solutions with glycine–NaOH buffer containing $MgCl_2$ and loading a fixed volume ($\leq$ 500 μl) of each of the resulting solutions. The cartridge eluates were collected, and aliquots ($\leq$2.0 ml) were mixed with 10.0 ml Ready-flow scintillant and analyzed by liquid scintillation spectrometry. Replicate aliquots of each radioactive analyte solution chromatographed were counted in parallel. The resultant data enabled quantitative assessment of the behavior, resolution, and recovery of the nucleosides/nucleotides chromatographed and the capacity of the solid-phase extraction column for each analyte. Mixtures of adenosine plus AMP or inosine plus IMP containing either the respective radioactive nucleoside or the respective radioactive nucleotide as internal standard were likewise chromatographed and analyzed to investigate potential nucleoside–nucleotide interactions during solid-phase extraction.

Evaluation of the integrity of analytes subjected to solid-phase extraction

HPLC was used to verify the molecular integrity of the nucleosides and nucleotides subjected to solid-phase extraction on Accell Plus QMA cartridges. To this intent, one volume of each cartridge eluate to be analyzed by HPLC was diluted with nine volumes of 7.0 m*M* ammonium phosphate, pH 3.8. The resulting mixture was injected into a 500-μl stainless-steel sample loop and analyzed by HPLC on a Partisil-SAX anion-exchange column (Whatman, Maidstone, UK). The HPLC conditions, at ambient temperature, involved co-development of phosphate and pH gradients, as detailed in ref. 17. On-line spectrophotometric detection at 254 nm was used, and for samples containing radioactive internal standard(s) the detector flow-through was collected in scintillation vials at 20-s intervals. Each HPLC eluate fraction (1.0-ml volume per fraction) was mixed with 10.0 ml of Ready-flow and counted for radioactivity. A mixture of radioactive standards ([^{3}H]adenosine, [^{3}H]AMP, [^{14}C]inosine, [^{14}C]IMP; 500 cpm/μl each) was subjected to HPLC as a check on the column. The HPLC behavior of these analytes was verified by chromatographing each one separately and determining its elution volume and retention time in comparison to published values [17].

Quantification of non-specific phosphomonoesterase ("phosphatase") activity

Phosphatase (orthophosphoric-monoester phosphohydrolase; EC 3.1.3.1/2) activity was determined colorimetrically as the hydrolysis of PNPP under two different incubation conditions. The first assay was conducted under the conditions used to assay 5'-nucleotidase (below); the reaction mixture contained (final concentrations)

0.1 *M* glycine–NaOH buffer (pH 8.5), 10 m*M* $MgCl_2$, 50 m*M* PNPP, and test sample. Phosphatase activity was also determined with the standard assay of Berry *et al.* [18] at pH 7.0. Both assays were conducted in the linear response range with respect to sample protein and time; spontaneous hydrolysis of PNPP in either assay was negligible.

5′-Nucleotidase assay

Incubation conditions for the assay of 5′-nucleotidase were modified from those detailed [10,15]. The assay mixture contained (final concentrations) 0.1 *M* glycine–NaOH buffer (pH 8.5), 10 m*M* $MgCl_2$, 50 m*M* AMP, a trace amount ($5 \cdot 10^5$ cpm/ml; 17.0 pmol/ml) of [^{3}H]AMP internal standard, and test sample (isolated enzyme, tissue homogenate). The reaction was initiated upon addition of test sample and was carried out by incubating the reaction mixture for 15 min at 37°C in a shaking water bath. The enzyme reaction was terminated either by plunging the sample into ice-water or by loading the reaction mixture onto an Accell Plus QMA cartridge which had been pre-equilibrated with 10.0 ml of ice-cold 0.1 *M* glycine–NaOH buffer (pH 8.5) and placing the cartridge into a refrigerator at 4°C. A 2-ml volume of H_2O was passed through the loaded cartridge to elute the nucleoside product of the reaction, after which 2.0 ml of 30.0 m*M* H_3PO_4 were passed through to elute the bound nucleotide substrate. Each 2.0-ml eluate was mixed with 10.0 ml of Ready-flow and analyzed by liquid scintillation spectrometry. Samples of the ongoing enzyme reaction were analyzed by anion-exchange HPLC [17] with collection and counting of the HPLC eluate for independent verification of the analyte recovery and identity observed with solid-phase extraction. The reaction "blank" consisted of the complete assay mixture without enzyme/tissue.

Evaluation of potential secondary deamination reactions as influences upon analyte recovery in the assay of myocardial 5′-nucleotidase

Utilization of 5′-nucleotidase substrate (*i.e.*, [^{3}H]AMP) or product (*i.e.*, [^{3}H]adenosine) by secondary deamination reactions in cardiac homogenate during the standard 5′-nucleotidase assay (above) was evaluated by HPLC such that the AMP substrate and adenosine product of 5′-nucleotidase along with IMP and inosine could be resolved simultaneously. To this intent, a 5′-nucleotidase assay mixture containing (final concentrations) 0.1 *M* glycine–NaOH buffer (pH 8.5), 10 m*M* $MgCl_2$, 50 m*M* AMP, 17.0 pmol/ml [^{3}H]AMP, and 8 mg/ml myocardial homogenate protein was incubated at 37°C. After 15 min, the assay mixture was divided into two portions. One portion was subjected to Sep-Pak anion-exchange chromatography and radiochemical analysis of the eluate fractions (above). The remainder was extracted with TCA (final TCA concentration, 0.6 *M*) and neutralized with trioctylamine [19]. The neutralized extract was then subjected to ion-pair reversed-phase HPLC on an Ultrasphere-XL ODS column (Beckman) in order to resolve simultaneously the major nucleotides, nucleosides, and bases in the extract, as detailed in ref. 20. After on-line spectrophotometric detection at 254 nm, the detector flow-through was collected in 150-μl fractions, and each fraction was mixed with 10.0 ml of Ready-flow and counted for ^{3}H label. Identities of the substances eluted from the HPLC column were established by their spectral properties, their coelution with internal standards, and enzymatic peak-shift techniques [21].

Protein determination

Protein was quantified with a dye-binding microassay [22].

RESULTS AND DISCUSSION

As a nucleoside 5′-monophosphate phosphomonoesterase, 5′-nucleotidase generates a nucleoside (usually, adenosine or inosine) from the respective nucleotide (AMP or IMP) [1]. In alkaline solution, the nucleosides carry no net charge, whereas the nucleotides are anions. Consequently, we initially investigated the ability of the recently introduced Sep-Pak Accell Plus QMA cartridge to bind AMP or IMP and the conditions required for efficient anion-exchange and quantitative resolution of each nucleotide from its corresponding nucleoside (Table I). Quantitative elution of 25 μmol of adenosine in 0.5 ml of aque-

TABLE I

ELUTION AND RECOVERY OF ADENOSINE AND AMP FROM SEP-PAK ACCELL PLUS QMA CARTRIDGES

A 500-μl sample of either 50 mM adenosine or 50 mM AMP (*i.e.*, 25 μmol analyte) in 0.1 M glycine–NaOH buffer (pH 8.5) containing 10 mM $MgCl_2$ and $5 \cdot 10^5$ cpm of the corresponding, ^{3}H-labeled internal standard was loaded onto a Sep-Pak cartridge which had been pre-equilibrated with 10.0 ml of the glycine–NaOH buffer. The indicated volumes of each solvent were then passed through the cartridge, and the radioactivity in the eluate was quantified by liquid scintillation spectrometry.

Eluent	Volume (ml)	Recovery[a] (mean ± S.D., $n \geq 5$) (%)	
		Adenosine	AMP
Water	1.0	86.4 ± 1.1	0
	2.0	99.4 ± 1.0	0
	10.0	99.5 ± 0.6	< 1.0
15 mM H_3PO_4	1.0	83.2 ± 2.2	45.8 ± 1.8
	10.0	99.7 ± 1.3	97.3 ± 2.9
30 mM H_3PO_4	1.0	81.6 ± 1.4	60.8 ± 6.5
	2.0	99.2 ± 2.0	99.3 ± 2.2
	10.0	101.3 ± 1.5	98.3 ± 3.7

[a] Results signify analyte recovery as assessed by the recovery of radiolabeled internal standard with respect to the known quantity loaded onto the cartridge. Comparable results were obtained for inosine and IMP, and neither the elution profiles nor the recoveries were affected with sample volumes $\leq$ 500 μl.

ous solution at pH 8.5 from Accell Plus QMA cartridges pre-conditioned with 10.0 ml of 0.1 M glycine–NaOH could be accomplished with 2.0 ml of water. A volume of water in excess of 2.0 ml was not required for quantitative adenosine elution, yet volumes of water several-fold greater than the cartridge void volume did not disturb the excellent retention of AMP by the anion-exchange packing. The elution profiles were unaffected by temperature over the range 4–22°C.

Aqueous acid (H_3PO_4) was tested as protonating solvent for elution of the bound AMP. Recovery of AMP during solid-phase extraction was critically dependent on both the volume and the acidity of the eluent passed through the cartridge (Table I). Although aqeuous H_3PO_4 at less than 30.0 mM could elute the AMP, volumes several-fold in excess of the cartridge void volume were required, making these procedures unattractive for routine analytical use. A 2-ml volume of 30.0 mM H_3PO_4 afforded quantitative AMP recovery in a sample volume small enough to be counted in its entirety for radioactivity. An identical elution profile was obtained when inosine and IMP were subjected to solid-phase extraction (data not shown). It is worthy of note that aqueous H_3PO_4 solutions were capable of eluting nucleoside from the Accell Plus QMA cartridge (Table I) and that variation of sample loading volume (between 10 and 500 μl) did not affect the chromatographic results (data not shown).

The capacity of Accell Plus QMA cartridges to bind nucleoside 5'-monophosphate was investigated by loading various amounts of adenosine (5.0 pmol to 500 mmol) and AMP (8.5 pmol to 500 mmol) (separately as well as in mixtures) onto the cartridges and eluting them sequentially with 2.0 ml of water and 2.0 ml of 30.0 mM H_3PO_4 to recover, first, the unbound nucleoside and then the bound nucleotide. The results (Table II) demonstrate that adenosine, over a wide range of concentrations well below and above those reported in assays for 5'-nucleotidase [6,7,9–12,14–16], could be eluted quantitatively with 2.0 ml of water. Likewise, < 100 mmol of AMP could be eluted quantitatively with 2.0 ml of 30.0 mM H_3PO_4. But at AMP loads of $\geq$ 100 mmol, a significant portion of the nucleotide did not bind to the column and emerged in the nucleoside fraction with 2.0 ml of water. Identical results were obtained for inosine and IMP (data not shown). Co-chromatography of adenosine and AMP in mixtures from 5 pmol adenosine–50 mmol AMP to 500 mmol adenosine–0.8 pmol AMP did not alter the selective elution profile and quantitative recovery of these analytes as compared to when they were chromatographed individually (Table II and data not shown). Thus, there is no adverse nucleoside–nucleotide interaction during solid-phase extraction under the conditions employed.

Investigation was next made to ascertain whether the integrity of the nucleoside and nucleotide analytes was compromised during chromatography on Accell Plus QMA cartridges with sequential water and H_3PO_4 elutions. To this intent, cartridge eluates from applied samples containing nucleoside, nucleotide, or both were subjected to an anion-exchange HPLC procedure [17] having both ionic and pH gradients strong

TABLE II

NUCLEOSIDE AND NUCLEOTIDE SAMPLE CAPACITIES OF SEP-PAK ACCELL PLUS QMA CARTRIDGES

Each specified sample in 500 μl of 0.1 *M* glycine–NaOH buffer (pH 8.5) containing 10.0 m*M* $MgCl_2$ and $5 \cdot 10^5$ cpm of the corresponding ^3H-labeled internal standard(s) was loaded onto a Sep-Pak cartridge which had been pre-equilibrated with 10.0 ml of the glycine–NaOH buffer prior to chromatography. Fraction 1 was first eluted in 2.0 ml of water; fraction 2 was then eluted in 2.0 ml of 30 m*M* H_3PO_4.

Sample load	Recovery[a] (mean ± S.D., $n \geq 5$) (%)	
	Fraction 1	Fraction 2
Adenosine		
5 pmol	97.9 ± 4.8	0
25 mmol	99.4 ± 4.0	< 1.0
50 mmol	94.1 ± 6.0	0
500 mmol	99.8 ± 2.3	< 1.0
AMP		
8.5 pmol	0	98.7 ± 3.3
25 mmol	0	95.1 ± 5.0
50 mmol	0	99.3 ± 2.2
100 mmol	56.7 ± 3.7	42.0 ± 3.1
Adenosine (5 pmol) + AMP (50 mmol)	98.7 ± 3.2	99.6 ± 3.3
Adenosine (500 mmol) + AMP (8.5 pmol)	98.9 ± 3.1	98.6 ± 2.6

[a] Results signify analyte recovery as assessed by the recovery of the radiolabeled internal standards with respect to the known quantity loaded onto the cartridge. The identity of the eluted products was verified by HPLC (*cf.* Fig. 1A). Comparable elution profiles were obtained for inosine and IMP, and neither the elution patterns nor the recoveries were affected with sample volumes ≤ 500 μl.

enough to resolve not only nucleotides, but also their nucleoside and base moieties. The HPLC analyses (Fig. 1) demonstrated that neither the nucleosides nor their corresponding nucleoside 5′-monophosphates were altered when subjected to solid-phase extraction and elution from Accell Plus QMA cartridges with water or 30.0 m*M* H_3PO_4, respectively. These HPLC data also confirmed the quantitative nature of the nucleoside and nucleotide recoveries suggested earlier by the discrete elution profiles of these analytes from the solid-phase extraction cartridges (Table II). It is particularly important to note that the nucleotide solutions subjected to solid-phase extraction contained internal standards radiolabeled at specific positions in the base substituent of the nucleoside moiety. Thus, any nucleotide degradation as a consequence of solid-phase extraction would be immediately and unambiguously detected as the appearance on HPLC of radiolabeled degradation products.

The chromatographic behavior of nucleosides and their corresponding nucleoside 5′-monophosphates on Accell Plus QMA cartridges (Tables I and II) and the integrity of both analytes after elution (Fig. 1) encouraged investigation of the applicability of this solid-phase extraction methodology to detecting and quantifying 5′-nucleotidase activity. To this intent, a commercial preparation of 5′-nucleotidase essentially free of other phosphomonoesterases as purchased was used initially, for it enabled precise titration of nucleotide conversion to nucleoside over a wide range of enzyme activity, and standard incubation conditions for the expression of this activity were known [10,15]. As summarized in Fig. 2, solid-phase extraction allowed for quantitative analysis of the progress and kinetics of the 5′-nucleotidase reaction in a radioisotopic enzyme assay based on the conversion of [^3H]AMP substrate to [^3H]adenosine product. Both substrate utilization and product formation could be reliably and simultaneously quantified by applying the entire enzyme reaction mixture directly onto the solid-phase extraction cartridge without the need for pre-chromatography sample prepara-

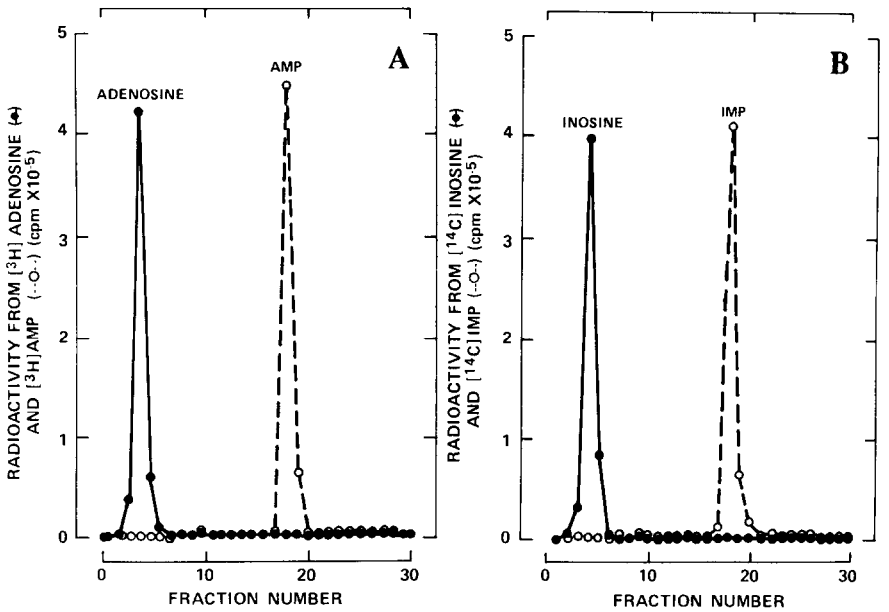

Fig. 1. Molecular integrity of adenosine, AMP, inosine, and IMP after solid-phase extraction. A mixture of 25 mmol adenosine and 25 mmol AMP containing ^{3}H-labeled internal standards ($5 \cdot 10^{5}$ cpm each in 500 μl) (A) and a mixture of 25 mmol inosine and 25 mmol IMP containing ^{14}C-labeled internal standards ($5 \cdot 10^{5}$ cpm each in 500 μl) (B) were subjected to solid-phase extraction on Accell Plus QMA cartridges which had been pre-equilibrated with 10.0 ml of 0.1 *M* glycine–NaOH buffer, pH 8.5. Each cartridge was eluted sequentially with 2.0 ml of water and 2.0 ml of 30 m*M* H_3PO_4, as detailed in Experimental. A 500-μl aliquot of each resulting eluate was analyzed by anion-exchange HPLC [17] for its nucleoside and nucleotide content. The HPLC column eluate was collected in 20-s intervals over a 60-min HPLC run and counted by liquid scintillation spectrometry. The HPLC elution profile of the material in each water (●) and H_3PO_4 (○) fraction from solid-phase extraction is plotted here against the total radioactivity associated with that fraction. The elution profiles given are representative of three independent experiments, and the recovery of each analyte was >97% in all cases. Since no material emerged from the HPLC column after fraction 21, only the initial portion of the entire 180-fraction, 60-min HPLC run is shown here.

tion. In contrast, the limited capacities of both TLC [13] and HPLC [11] would restrict analysis to a portion of the reaction mixture only. Solid-phase extraction also obviated diluting the sample, as would have occurred, for instance, by adding reagents necessary for colorimetric product (*i.e.*, phosphate) determination [23,24]. Only if the enzyme reaction were carried out at a pH below 8.5 would a minimal volume of concentrated base need to be added to the reaction mixture prior to Sep-Pak loading for alkalinization of the sample.

As demonstrated by the data in Fig. 3, 5'-nucleotidase could be quantified over a wide activity range because of the excellent chromatographic resolution between substrate and product and the sensitivity of radioisotope counting. Under the standard assay conditions employed, as little as 5 nmol (*i.e.*, 50 cpm) substrate loss and/or product formation could be quantified. This level of sensitivity is at least 10- to 100-fold greater than presently attainable with microchemical analyses for inorganic phosphate [23,24] or nucleosides [25]. The sensitivity could be extended into the sub-nanomole range by increasing the specific radioactivity of the [^{3}H]AMP substrate. Since the trace amount of [^{3}H]AMP in the reaction mixture is negligible relative to non-labeled substrate, augmenting the [^{3}H]AMP content of the reaction in this manner would not affect subsequent solid-phase extraction.

An additional procedural benefit of using solid-phase extraction to analyze 5'-nucleotidase ac-

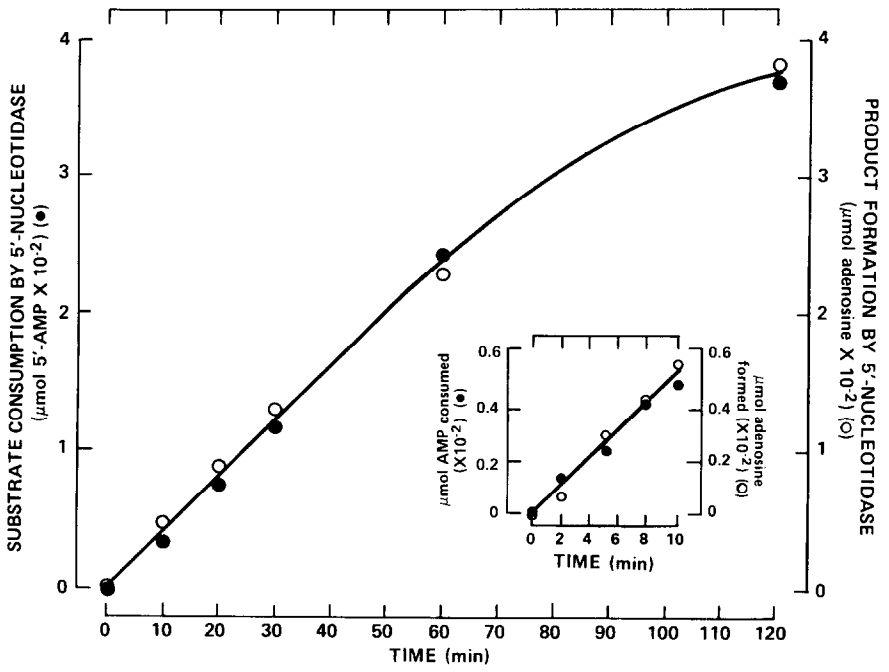

Fig. 2. Application of solid-phase extraction to 5′-nucleotidase determination. A reaction mixture containing 500 μmol AMP, 5 U 5′-nucleotidase, $5 \cdot 10^5$ cpm (17 pmol) [^{3}H]AMP, and 10 m*M* $MgCl_2$ in 0.1 *M* glycine–NaOH buffer (pH 8.5) (15 ml final volume) was incubated at 37°C in a shaking water bath. At various times during the ongoing enzyme reaction, 500-μl aliquots were removed and subjected to the solid-phase extraction procedure outlined in Table II to isolate any adenosine product formed and AMP substrate remaining. The eluates from solid-phase extraction were counted by liquid scintillation spectrometry, and the radioactivity in these eluates, along with the known specific radioactivity of the [^{3}H]AMP in the enzyme reaction mixture, was used to calculate the mass amount of substrate consumed (●) and product formed (○) during the 5′-nucleotidase reaction over a 120-min incubation. Data representative of three independent enzyme reactions are plotted; each point is the average of three replicate aliquots whose individual values did not vary by more than 5%. The inset gives the progress of the 5′-nucleotidase reaction during the first 10 min of incubation.

tivity stems from our observation that enzyme reaction samples loaded onto pre-equilibrated cartridges remained stable for at least 8 h at 4°C. This feature, along with the commercial availability of an instrument for simultaneous elution of multiple Sep-Pak cartridges, can be used to make the 5′-nucleotidase assay proposed herein a "batch" procedure. Such rapid, multi-sample analysis is not possible with existing radiochemical 5′-nucleotidase assays: although several enzyme reaction samples may be co-chromatographed on paper or thin layers to isolate product [10,13], these separation techniques are far more laborious and time-consuming than solid-phase extraction and are also very sensitive to sample load. The flexibility and facile nature of the solid-phase system using radiolabeled 5′-nucleotidase substrate do not, however, make it an "on-line" assay, since chromatographic separation and analyte detection/quantification are not contemporaneous; others [26] have developed an on-line assay procedure for 5′-nucleotidase using HPLC and a fluorescent AMP analogue.

The sensitivity of radioisotopic 5′-nucleotidase assay and the facile separation of substrate and product by solid-phase extraction methods described herein prompted analysis of 5′-nucleotidase activity in a biological sample matrix. Cardiac tissue was chosen, since heart muscle 5′-nucleotidase has been extensively studied [3,5,9,15]. Our results (Table III) demonstrate that low levels of 5′-nucleotidase could be reliably and reproducibly quantified in a crude myocardial homogenate either as substrate consumption or product formation. The apparent 5′-nucleotidase activity in rabbit heart is comparable to that recently quantified in pigeon heart [27] with a radiochemical assay dependent upon anion-exchange chro-

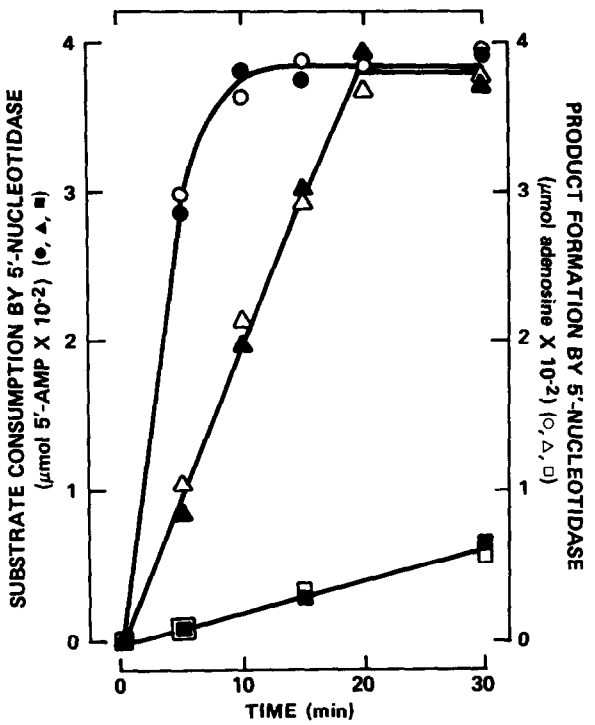

Fig. 3. Utility of solid-phase extraction in the quantitative analysis of 5'-nucleotidase activity. Reaction mixtures containing 500 μmol AMP, $5 \cdot 10^5$ cpm (17 pmol) [^{3}H]AMP, 10 m*M* $MgCl_2$, and either 2.5 U (■, □), 25 U (▲, △), or 75 U (●, ○) 5'-nucleotidase in 0.1 *M* glycine–NaOH buffer (pH 8.5) (20 ml final volume) were incubated at 37°C in a shaking water bath. At various times during the ongoing enzyme reactions, 500-μl aliquots were removed from each and subjected to the solid-phase extraction procedure outlined in Table II to isolate any adenosine product formed and AMP substrate remaining. The eluates from the solid-phase extraction cartridges were counted by liquid scintillation spectrometry, and calculation was made as to the mass amounts of substrate consumed (solid symbols) and product formed (open symbols) in the total reaction mixture as summarized in the legend to Fig. 2. Each data point is the average of three replicate aliquots whose individual values did not vary by more than 5%. The experiment shown is representative of three independent enzyme reactions.

matography on Dowex columns, a more laborious and time-consuming procedure than the solid-phase extraction method detailed here. It should be noted, in line with the results of other investigators (*cf.* ref. 10), that spectrophotometric and microchemical assays for product formation yielded inconsistent and therefore unreliable results at these 5'-nucleotidase levels (data not shown).

In crude biological samples such as myocardial homogenate, assessment of 5'-nucleotidase activity based upon the conversion of [^{3}H]AMP to [^{3}H]adenosine may be complicated by reactions of intermediary metabolism secondary to 5'-nucleotidase [28]. As outlined in Fig. 4, three secondary reactions have been identified in cardiac tissue which have at least the potential to utilize directly either the [^{3}H]AMP substrate or the [^{3}H]adenosine product of 5'-nucleotidase and in so doing to interfere with the assessment of 5'-nucleotidase activity as either [^{3}H]AMP loss or [^{3}H]adenosine accumulation (*cf.* Table III) [29–31]. Non-specific phosphomonoesterases ("phosphatases") share with 5'-nucleotidase an ability to convert AMP and IMP into their corresponding nucleosides, although phosphatases do not display the high affinity and specificity for nucleoside 5'-monophosphates as does 5'-nucleotidase [32–34]. Cardiac AMP deaminase (5'-AMP nucleosidase; EC 3.5.4.6) and adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) could also potentially affect the recoveries of [^{3}H]AMP substrate and [^{3}H]adenosine product of the primary 5'-nucleotidase reaction by catalyzing, respectively, their deamination to [^{3}H]IMP or [^{3}H]adenosine [29–31]. Consequently, the activity of each of these secondary reactions was determined in cardiac homogenate under the standard incubation conditions detailed (Table III) for 5'-nucleotidase.

Direct spectrophotometric assay of myocardial phosphatase with the high-affinity chromogenic substrate PNPP [35] revealed a tissue phosphatase activity of 13.8 ± 1.8 nmol PNPP consumed/min/mg cardiac protein (mean ± S.D.; $n = 4$) under the 5'-nucleotidase assay conditions detailed herein. A standard literature phosphatase assay at pH 7.0 [18] gave a specific activity of 11.9 ± 1.0 nmol PNPP consumed/min/mg cardiac protein (mean ± S.D.; $n = 4$). These data demonstrate that the level of phosphatase activity in cardiac homogenate could potentially constitute some 8% of the total apparent 5'-nucleotidase activity of ~180 nmol AMP consumed/min/mg cardiac protein (Table III). Furthermore, this percentage is a maximal figure, for non-specific phosphomonoesterases also act on soluble tissue constituents aside from the [^{3}H]AMP provided as substrate in our 5'-nucleotidase assay system [32,34]. Thus, phosphatase

TABLE III

QUANTIFICATION OF APPARENT 5'-NUCLEOTIDASE ACTIVITY IN MYOCARDIAL TISSUE

Left-ventricular myocardium was homogenized in 55 m*M* potassium phosphate buffer, pH 6.5, and clarified by low-speed centrifugation. The supernatant, after filtration through cheese cloth, was assayed for apparent 5'-nucleotidase activity at 37°C in a mixture containing (final concentrations) 0.1 *M* glycine–NaOH buffer (pH 8.5), 10 m*M* $MgCl_2$, 50 m*M* AMP, and trace ($5 \cdot 10^5$ cpm; 17.0 pmol/ml) [^{3}H]AMP. Aliquots (500 μl) of the 5'-nucleotidase reaction mixture were removed over a 15-min reaction period and loaded onto Accell Plus QMA anion-exchange cartridges pre-equilibrated with 10.0 ml of 0.1 *M* glycine–NaOH buffer, pH 8.5. Solid-phase extraction was performed by sequential elution of the [^{3}H]adenosine product with 2.0 ml of water and the unreacted [^{3}H]AMP substrate with 2.0 ml of 30 m*M* H_3PO_4 (*cf.* Fig. 1A). The samples were counted for ^{3}H label, and the known specific radioactivity of [^{3}H]AMP was used to calculate the apparent 5'-nucleotidase activity as both substrate consumption and product formation. The sample without protein represents a reaction "blank" lacking myocardial tissue.

Tissue protein (mg)	Apparent 5'-nucleotidase activity (mean ± S.D., $n = 4$)	
	nmol AMP consumed/min	nmol adenosine formed/min
0	<0.01	<0.01
4	720 ± 36	735 ± 32
8	1400 ± 68	1382 ± 73

contribution to the apparent myocardial 5'-nucleotidase activity (Table III) may be considered slight, if not negligible.

Deamination of [^{3}H]AMP and/or [^{3}H]adenosine during the incubation of cardiac homogenate with the [^{3}H]AMP substrate for 5'-nucleotidase under the standard 5'-nucleotidase assay conditions was evaluated as [^{3}H]IMP and [^{3}H]-inosine formation, respectively (Fig. 4). An ion-pair HPLC procedure [20] was used to resolve simultaneously AMP, adenosine, IMP, inosine, and lower purines (such as hypoxanthine and xanthine). Analysis of the 5'-nucleotidase reaction mixture by HPLC after a 15-min incubation of myocardial homogenate (8 mg tissue protein per ml) in the assay system detailed in Table III revealed that 0.9% of the total ^{3}H label originally in AMP was present as IMP, 0.2% was present as inosine, 42% was present as adenosine, and 57% remained as AMP; no ^{3}H-labeled lower purines were detected. Formation of [^{3}H]IMP and [^{3}H]inosine could be blocked by including coformycin in the incubation mixture at a concentration (250 μM) sufficient to inhibit both AMP dea-

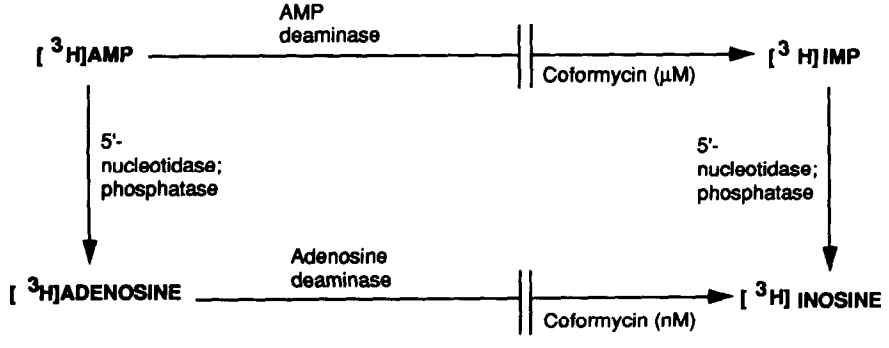

Fig. 4. Metabolic reactions secondary to 5'-nucleotidase which could directly contribute to a loss of [^{3}H]AMP substrate or [^{3}H]adenosine product from the primary 5'-nucleotidase reaction in a non-homogeneous biological sample matrix, such as the myocardial homogenate used in Table III. These potential secondary reactions are catalyzed by non-specific phosphomonoesterases ("phosphatases"), AMP deaminase, and adenosine deaminase. The enzymatic deamination of AMP may be prevented by micromolar concentrations of coformycin, and deamination of adenosine is blocked by nanomolar coformycin [36]; coformycin does not inhibit 5'-nucleotidase [37] or phosphatases (data not shown).

minase and adenosine deaminase [36] while not affecting 5′-nucleotidase [37] or phosphatases (data not shown). Consequently, the influence of enzymatically catalyzed deamination reactions in myocardium on the recovery of the [^{3}H]AMP and [^{3}H]adenosine analytes from the 5′-nucleotidase incubation is negligible.

Our finding that secondary reactions (*i.e.*, nonspecific phosphomonoesterases, AMP deaminase, adenosine deaminase) having the potential to utilize directly either the [^{3}H]AMP substrate or the [^{3}H]adenosine product of 5′-nucleotidase do not appreciably interfere with the assessment of 5′-nucleotidase activity in myocardial homogenate as either [^{3}H]AMP loss or [^{3}H]adenosine accumulation cannot automatically be extended to other sample matrices. For instance, skeletal muscle displays a much greater capacity for AMP deamination than does cardiac muscle [38], and the activities of enzymes of adenine nucleotide catabolism in a given tissue may vary widely under pathological conditions [39] or with development [40]. Consequently, the importance of recognizing the potential influence of secondary reactions on the quantification of 5′-nucleotidase activity in a given heterogeneous sample matrix by the solid-phase extraction system detailed herein must be emphasized. The data presented for myocardial homogenate serve as a practical illustration of at least one experimental route for evaluating secondary reactions which might cause direct interference when crude biological preparations are assayed for 5′-nucleotidase as AMP loss and/or adenosine formation.

In conclusion, a chromatographic system has been developed and characterized for quantitative nucleotide–nucleoside separation and recovery using solid-phase extraction on pre-packed Accell Plus QMA cartridges. The optimized system has been integrated into a radiochemical assay for 5′-nucleotidase such that nucleotide substrate and nucleoside product can be separated and quantified with ease, economy of time and materials, excellent analyte recovery, and modest solvent volumes. Coupling of solid-phase extraction to a radiochemical 5′-nucleotidase assay allowed reliable quantification of low enzyme levels in dilute or crude samples without pre-chromatography sample preparation (*e.g.*, deproteinization) or elaborate post-chromatography work-up. The high capacity of solid-phase extraction for nucleosides and nucleoside 5′-monophosphates and the relative insensitivity of the cartridges to analyte load over a wide range (Table II) contrast sharply with other methods (*e.g.*, paper chromatography and TLC) presently used to monitor 5′-nucleotidase activity radiochemically. The attractiveness of our solid-phase extraction system can perhaps best be appreciated from the considerable enhancement of sample throughput afforded: multiple 5′-nucleotidase determinations can be made within a few minutes in one chromatographic step with no sample preparation prior to chromatography. By contrast, microchemical 5′-nucleotidase assays or radiochemical assays coupled to paper, thin-layer, or column chromatographic isolation of product may inherently require many hours and multiple cumbersome manipulations. The facility with which both nucleotide enzyme substrate and nucleoside product may be quantified is particularly advantageous when studying 5′-nucleotidase activity in heterogeneous biological samples, for the stoichiometric relationship between nucleotide consumption and nucleoside formation can readily be examined and may suggest potential complications due to analyte transformation by secondary reactions. The analytical advantages attained by coupling solid-phase extraction on pre-packed anion-exchange cartridges to the radiochemical analysis of 5′-nucleotidase activity invite further application of solid-phase extraction chromatography to the enzymological analysis of intermediary metabolism.

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