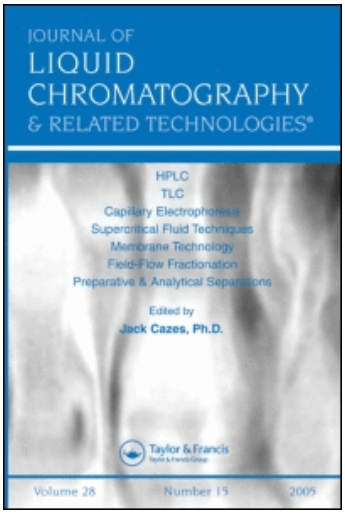

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Superiority of HPLC to Assay for Enzymes Regulating Adenine Nucleotide Pool Intermediates Metabolism: 5'-Nucleotidase, Adenylate Deaminase, Adenosine Deaminase, and Adenylosuccinate Lyase-A Simple and Rapid Determination of Adenosine

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SUPERIORITY OF HPLC TO ASSAY FOR ENZYMES REGULATING ADENINE NUCLEOTIDE POOL INTERMEDIATES METABOLISM: 5'-NUCLEOTIDASE, ADENYLATE DEAMINASE, ADENOSINE DEAMINASE AND ADENYLOSUCCINATE LYASE—A SIMPLE AND RAPID DETERMINATION OF ADENOSINE

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ABSTRACT

A new application of HPLC analysis to assay for all the enzymes involved in regulation of adenine nucleotide pool metabolism is described. In vitro, enzymatic reactions were carried out in buffered reaction media containing appropriate concentrations of metal ions, specific substrate, inhibitors and enzymes. Following an incubation period,

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the enzymatic reactions were terminated and extracted with cold trichloroacetic acid. The soluble acid extracts were neutralized and injected in a HPLC system. Using a C18-Nova Pak reverse phase column, we were able to separate, identify and quantify the substrate, products and their possible catabolites and UV-detectable inhibitors. A complete separation and quantitation of metabolites was accomplished within 16-18 minutes. However, rapid and simple HPLC runs were also developed which can be routinely used to determine adenosine levels within 3-4 minutes, using a single solvent HPLC system. This procedure is extremely reproducible and very reliable as demonstrated in assaying for 5'-nucleotidase, adenylate deaminase, adenosine deaminase and adenylosuccinate lyase activities.

INTRODUCTION

Adenine nucleotides play an essential role in any living organism. About 90% of the energy produced during oxidative metabolism of carbohydrate, protein, and lipid is stored in the ATP molecule as chemical energy. Adenine nucelotide metabolism is very well balanced in normoxic well-perfused hearts (1,2). However, rapid utilization of ATP with concomitant cessation of its synthesis, occurs during hypoxia and ischemia triggering a cascade of degradative reactions, catalyzed by key regulatory enzymes producing diffusible adenine nucleosides and purine bases (3-6). Since adenine nucleotides (ATP, ADP, AMP) are not diffusible through plasma membranes, dephosphorlyation of intracellular AMP to adenosine

catalyzed by the regulatory enzyme 5'-nucleotidase, results in rapid depletion of intracellular adenine nucleotides. Adenosine is a potent vasodilator (7-9) in all organs except the kidney, where it is a potent vasoconstrictor (10,11). This nucleoside plays an important role in regulation of blood flow (7-9). Accurate determination of adenosine production and metabolism is a prerequisite step for characterization of the mechanism(s) of adenosine production and compartmentalization. Pharmacological interventions modulating blood flow necessitate understanding the role of the key regulatory enzymes in adenosine production and metabolism. Several methods (12-20) have been commonly used to assay for 5'-nucleotidase activity none of which provide HPLC unique features. Therefore, we employed a HPLC procedure that separates, identifies and quantitates adenine nucelotide pool intermediates produced during the assay for enzyme activities. In this manuscript we describe a new application of the step gradient elution of adenine nucleotides, nucleosides and purine bases recently described by Hull-Ryde et al (21). We determined the enzyme activity of 5'-nucleotidase, adenylate deamindase, adenosine deaminase and adenylosuccinate lyase as a few examples of all enzymes involved in adenine nucleotide pool metabolism (Fig 1) using standard enzymes, isolated rat cardiomyocytes, and myocardial subcellular preparations.

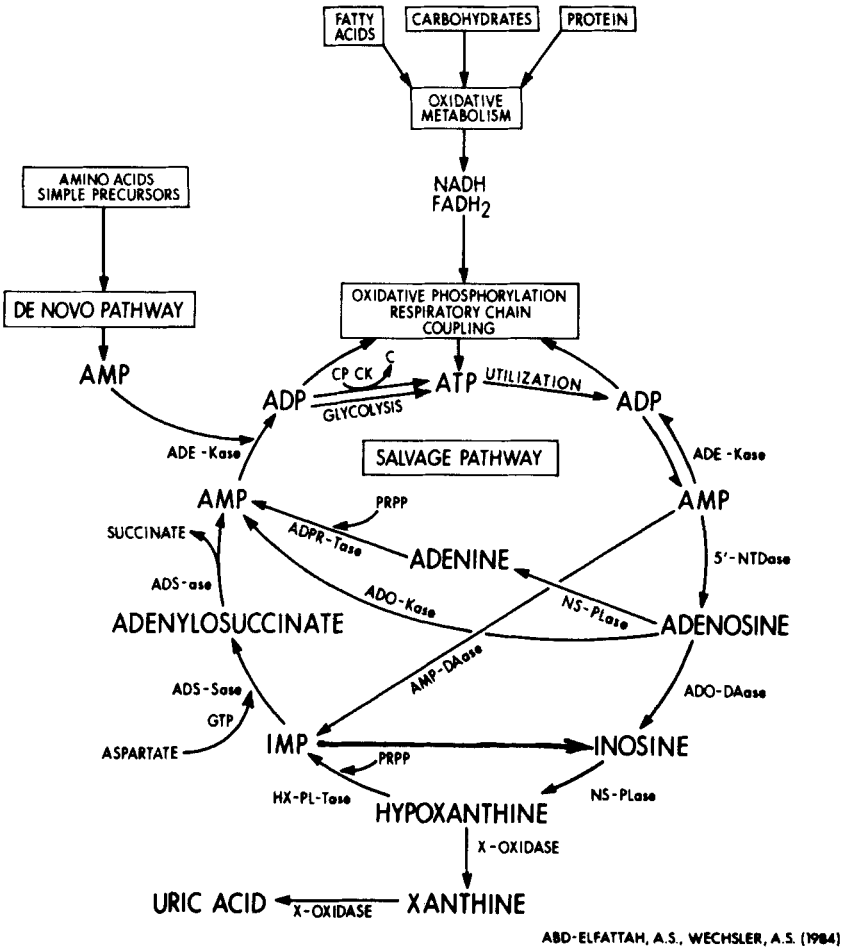


Fig (1) Adenine nucleotide pool metabolism. Ado-Kase= adenosine kinase; 5'-NTDase= 5'-nucleotidase; Ado-DAase= adenosine deaminase; AMP-DAase=Adenylate Deaminase; NP-lase=nucleoside phosphorylase; X-oxidase= xanthine oxidase, Ado-STase=adenosylsuccinyl; transferase; and AdeS-Lyase=adenylsuccinate lyase.

MATERIALS AND METHODS

Chemical Reagents:

Standard enzymes and ultrapure biochemical, buffers and reagents were purchased from Sigma Chemical Company (ST. Louis, MO). HPLC grade ammonium phosphate and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Organic-free deionized water was obtained from a Milli-Q system(Millipore, Bedford, MA).

HPLC System:

Standard solutions of a mixture of adenine nucleotides, nucleosides , purine bases, NAD+ and cAMP or unknown samples were injected by a Waters Intelligent Sampler Processor (WISP) Model 710B, (Waters Associates, Milford, MA). A solvent selector Model 101 (Alltech Association, Deerfield, Il) was used in conjunction with a solvent delivery system Model 6000A (Waters Associates, Milford, MA). Separation of these metabolites was accomplished through a NOVA-Pak-A (C18, 5um particle size, 10X8 mm) chromatographic column inside a Radial Compressor Module Model RCM 100 (Waters Associates, Milford,

MA). A multiwavelength four channel visible/ultraviolet detector Model 490 and a data module Model 730 (Waters Associate, Milford, MA) were used to detect, integrate and quantitate eluted metabolites using external standards.

HPLC Anaylsis:

A step gradient elution of adenine nucleotides, nucleosides, purine bases, NAD+ and cAMP was performed as previously described (21). Briefly, HPLC grade ammonium phosphate buffer, pH 5.5 (adjusted with 0.6 N ammmonium hydroxide) elutes sequentially ATP, ADP, hypoxanthine, xanthine and AMP. As a second solvent, 7% methanol elutes NAD+, while a third solvent , 40% methanol elutes inosine, cAMP and adenosine. Switching the solvent selector is programmed in the data module.

New Applications of HPLC to Assay for Enzyme Activities:

Enzymes were assayed in a reaction medium (500 ul) containing 10 mM N-2-hydroxyethylpiperazine-N-2-ethan sulfonic acid- Tris-hydroxymethyl)-aminoethan (Hepes-Tris) buffer, pH 7.5, 1 mM MgCl2, 1.5 mM

substrate in the presence or absence of specific inhibitors. The reaction was initiated by the addition of enzyme protein and incubated at 37°C. At the end of a specified incubation period, the enzymatic reaction was terminated by the addition of 1:1 volume of 24% TCA, and extracted at 4°C for 30 minutes. Denatured protein was separated by centrifugation (8000g for 10 min) and the soluble acid extracts were transferred and neutralized with 1:2 volumes of tri-n-octylamine: Freon mixture (1:3 v/v). Neutralized aqueous extracts were separated from the organic phase by centrifugation (8000g for 5 minutes), transferred into small vials and frozen immediately in liquid nitrogen and stored at -70°C. Blank runs were also performed exactly as mentioned above except that the enzyme protein was added following the addtion of TCA solution. Aliquots of standard solution or samples (20 ul) were automatically injected by the WISP. The solvent delivery rate was 1.5 ml/min and the chart recorder of the data module was run at a speed of 1.25 cm/min. Retention time, peak area and amounts of each eluted metabolite were recorded in each chromatogram.

Subcellular Fractionation of Myocardial Homogenates:

Cardiac muscle obtained from anesthetized adult dogs was homogenized in buffered sucrose solution containing

0.32 M sucrose, 10 mM Hepes-Tris, pH 7.5, (1:10 w/v), 1 mM EDTA using a blender for 15 sec intervals, at 4°C. Further homogenization was performed using a Polytron (Model PCU-2, Brinkmann, Switzerland). Whole myocardial homogenates were centrifuged at 600g for 10 minutes and the pellets were discarded. Each supernatant fraction was then recentrifuged at 16,000g for 20 minutes and the pellet, containing mainly mitochondria, was suspended in 10 mM Hepes-Tris-sucrose solution, frozen in liquid nitrogen and stored at -70°C as a crude mitochondrial fraction. The supernatant fraction was also recentrifuged at 100,000g for 2 hours and the pellet was suspended in the same buffered solution, frozen and designated as a crude microsomal fraction. The latter supernatant fraction containing the cytosolic pool of enzymes and substrates was treated with a cold saturated solution of ammonium sulfate to bring the final concentration to 50% saturation and kept at 4°C for 30 minutes. Salted-out proteins were separated by centrifugation at 18,000g for 20 minutes. The cytosolic fraction in the pellet was resuspended in sucrose-Hepes-Tris solution and frozen and stored at -70°C. Protein was determined as described by Lowry et al (22).

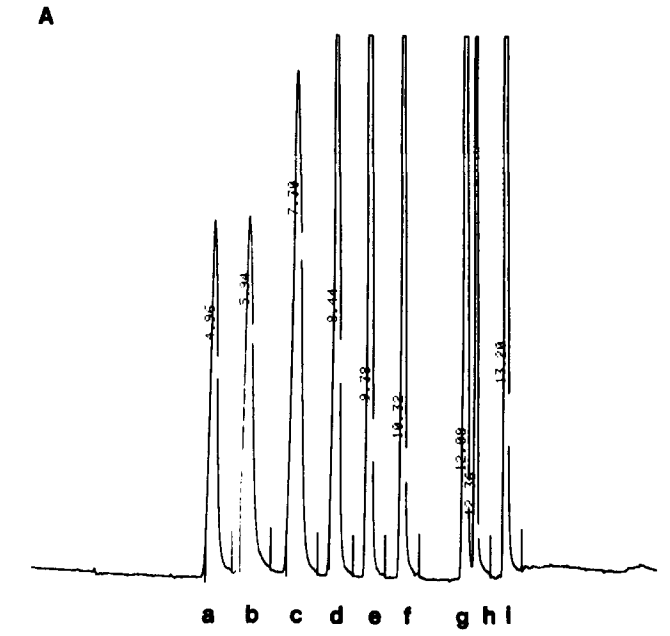
RESULTS

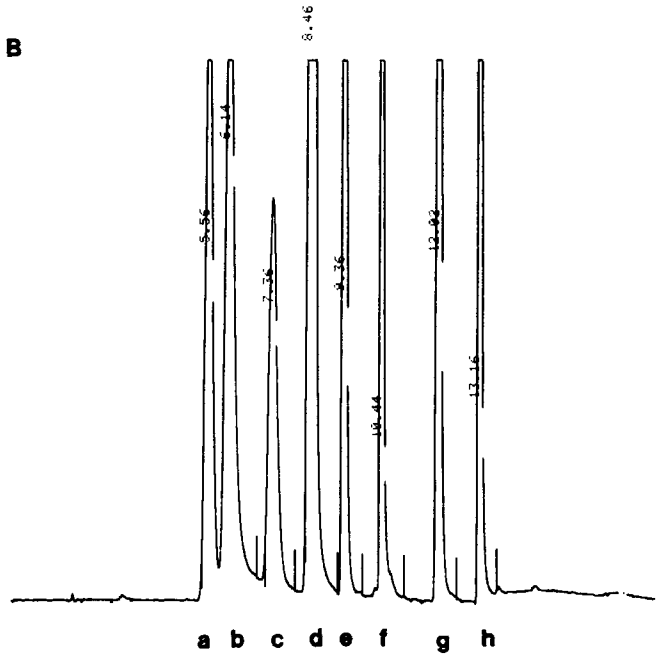
Fig (2) illustrates typical chromatograms for standard metabolites. Fig (2A) shows separation of adenine nucleotides (ATP, ADP, and AMP), adenine nucleosides (adenosine and inosine) and purine bases (hypoxanthine and xanthine) in addition to NAD+ and AMP. Fig (2B) illustrates separation of inosine-5'-monophosphate (IMP), AMP, adenosine, inosine, hypoxanthine, xanthine, as well as inhibitors,i.e. , -methylene-adenosine-5'-adenosine diphosphate, (, - M-ADP) and bromo-adenosine- 5'-monophosphate (Br-AMP). Percent recovery of acid extraction and HPLC analysis for all standard metabolites ranged from 98-100%.

Determination of the Standard Enzyme Activity Using HPLC:

A. AMP-Specific-5'-Nucleotidase:

Fig (3) illustrates the activity of 5-nucleotidase assayed in a reaction medium containing 10 mM Hepes-Tris buffer, pH 7.5, 1 mM MgCl2, 6 mM dithiotheritol (DTT), 1.5 mM AMP and incubated for 2 minutes at 37°C. Fig (3A) depicts the blank run where





the non-enzymatic degradation of AMP and contaminants can be detected. The total activity of 5'-nucleotidase and non-specific phosphatases (77.75±5.5 nmol/min/ml incubation medium) are shown in Fig (3B). In the presence of 100 uM α,β-M-ADP, the activity of 5'-nucleotidase was inhibited. The remaining activity designated as α,β-M-ADP-insensitive activity of the non-specific phosphatases (Fig 3C). We calculated the activity of α,β-M-ADP-sensitive-5'-nucleotidase to represent the specific activity of the enzyme (Table 1). It is important to note that about 10 % of the total AMP-phosphatase activity was not inhibited by , -M-ADP.

Fig (2) Typical HPLC chromatograms of standard metabolite mixture using a step gradient elution. The solvent system consists of 1) 100 mM ammonium phosphate buffer, pH 5.5, followed by 2) 7% methanol and 3) 40% methanol. Nova-Pak-A column (C18, 5 um particle size, 10X8 mm), flow rate 1.5 ml/min, detector sensitivity was 0.05 absorbance unit full scale at 254 nm. Peak identification: Fig 2A: A= ATP; B= ADP; C=Hypoxanthine; D=xanthine; E=AMP; F=NAD=; G=inosine; H=cAMP; I=adenosine; Fig 2B: A=IMP; B= , -M-ADP; C= HX; D=X; E=AMP; F=BrAMP; G=INO; and H=Ado

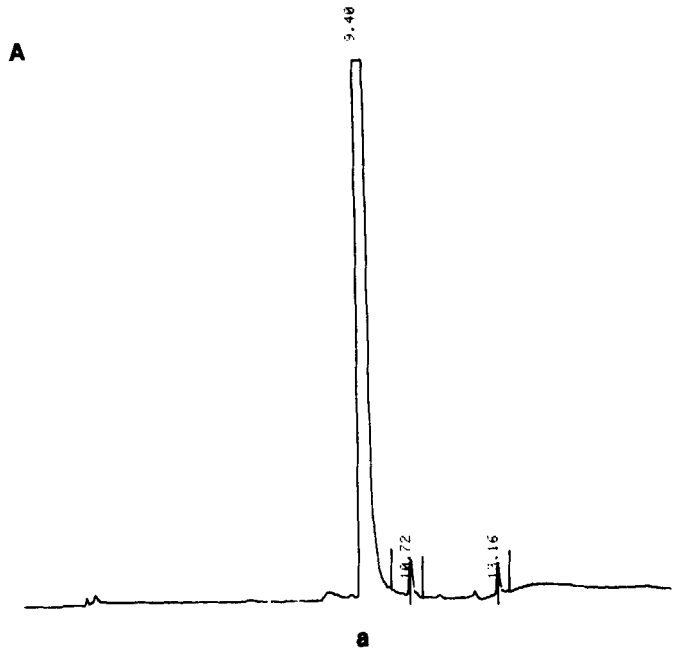


Fig (3) HPLC chromatograms of standard AMP-5'-nucleotidase assay.
A) Blank run showing the substrate (a) and traces of adenosine,; B) Total activity, the enzyme consumed 1.5 mM AMP within 2 minutes and the only peak is adenosine (b);
C) Inhibition of 5'-nucleotidase by α,β-M-ADP (c) in the presence of AMP (a). Adenosine (b) is produced by inhibitor insensitive phosphatases.

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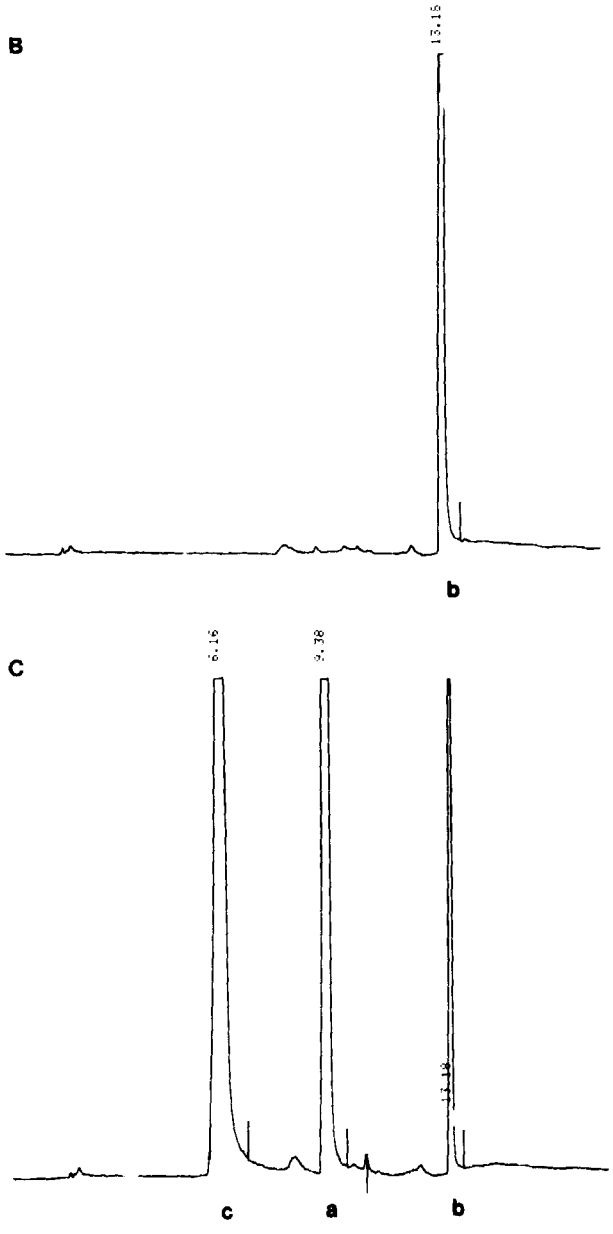

Fig 3 (continued)

B. IMP-Specific-5'-Nucleotidase:

5'-Nucleotidase catalyzes dephosphorylation of AMP and IMP to produce adenosine and inosine, respectively. To assay for IMP-5'-nucleotidase we used 1.5 mM IMP as a specific substrate. Reaction media and conditions were exactly similar to those in (A). Fig (4A) shows the blank run which demonstrates the presence of hypoxanthine as a background contaminant. The total activity of the enzyme as determined by inosine formation is depicted in Fig (4B). α,β-M-ADP (100 uM) strongly inhibited the enzyme activity as shown (Fig 4C). About 6% of the total activity was insensitive to the inhibitor.

C. Adenylate Deaminase:

Adenylate deaminase utilizes AMP as a Specific substrate to produce IMP. The enzyme activity was assayed in 10 mM Hepes-Tris buffer, pH 7.5, 1 mM MgCl2, 1.5 mM AMP and incubated for 2 minutes at 37°C. Fig (5) illustrates the blank run (Fig 5A), total enzyme activity (Fig 5B) and and the activity of the enzyme in the presence of 100 uM adenosine deaminase inhibitor [erythro-6-amino-9(2-hydroxy-3-nonyl)-purine], known as EHNA (Fig 5C). As demonstrated

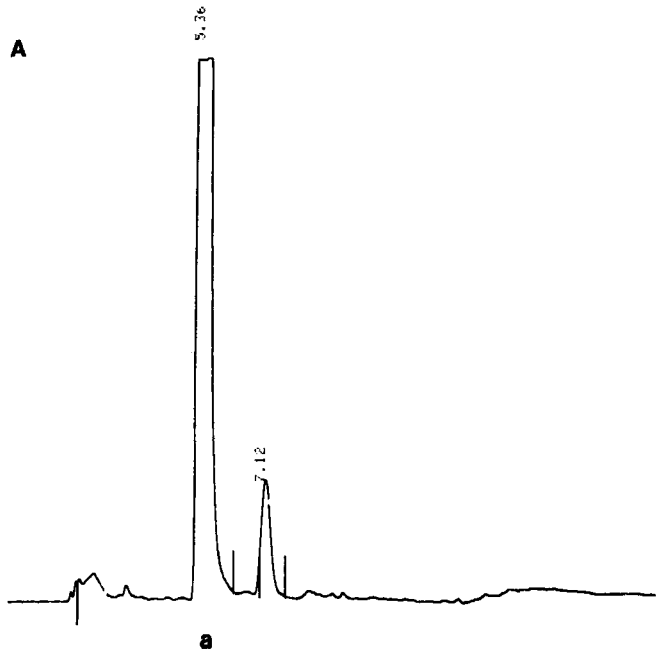

Fig (4) HPLC chromatograms of standard IMP-5'-nucleotidase assay.
A) Blank , (a)= 1.5 mM IMP; B) Total activity, (a)= IMP and (b)=inosine; C) Inhibitor effect, (a)=IMP, (b)=inosine, (c)=α,β-M-ADP.

(continued)

in Fig (5), about 95% of adenylate deaminase activity was insensitive to EHNA (Table 1).

D. Adenosine Deaminase:

Adenosine deaminase catalyzes deamination of adenosine to produce inosine. Fig (6) illustrates the

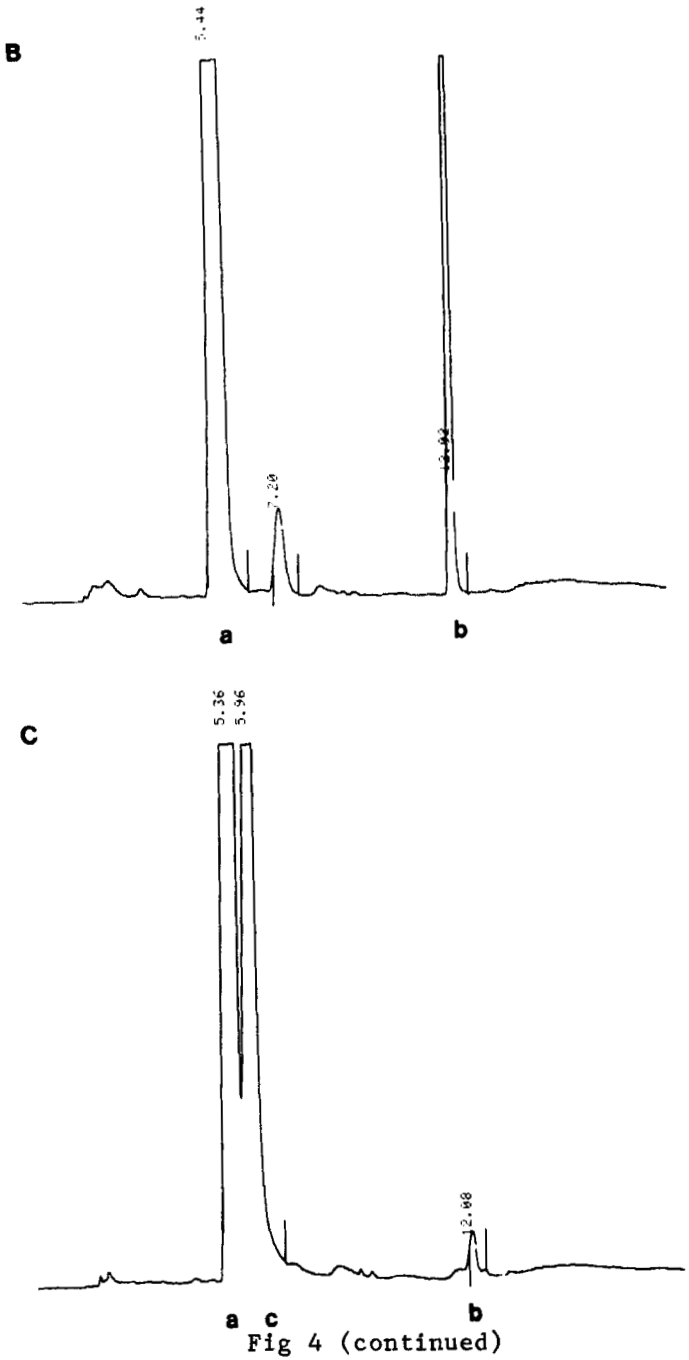

Fig 4 (continued)

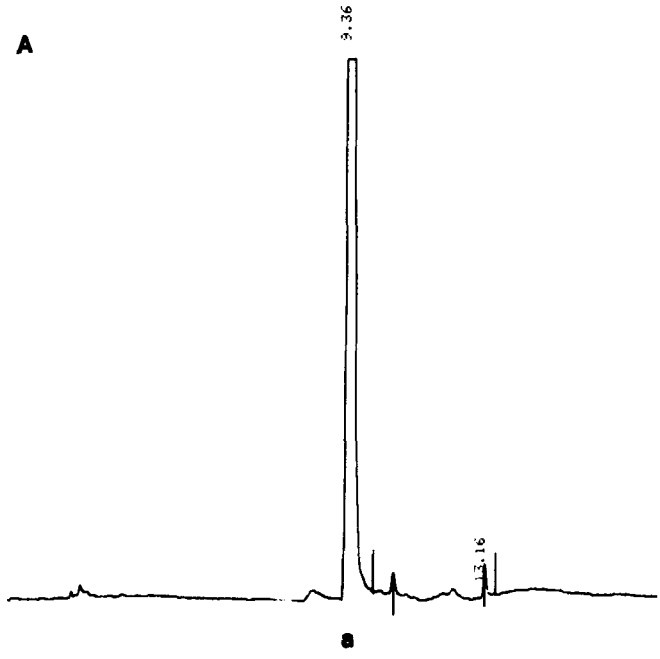

Fig (5) HPLC chromatograms of standard adenylate deaminase assay.
A) Blank, (a)=AMP; B) Total activity (a)=AMP, (b)=IMP; C) Effect of EHNA (100 uM), (a)=AMP, (b)=IMP.

(continued)

assay for adenosine deaminase and the effect of ENHA on its activity. Unlike adenylate deaminase, adenosine deaminase was totally sensitive to 100 uM EHNA (Fig 6C).

E. Adenylosuccinate Lyase:

Adenylosuccinate lyase catalyzes the formation of AMP and fumarate from adenylosuccinate. This enzyme

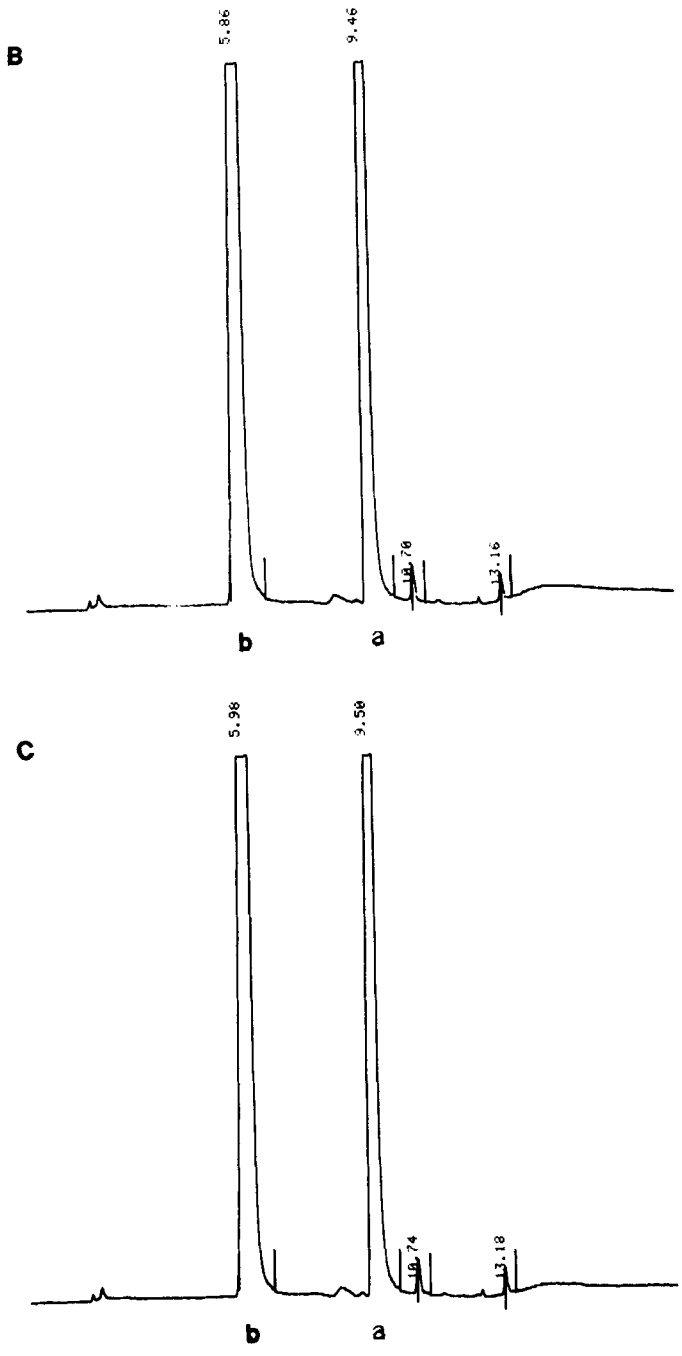

Fig 5 (continued)

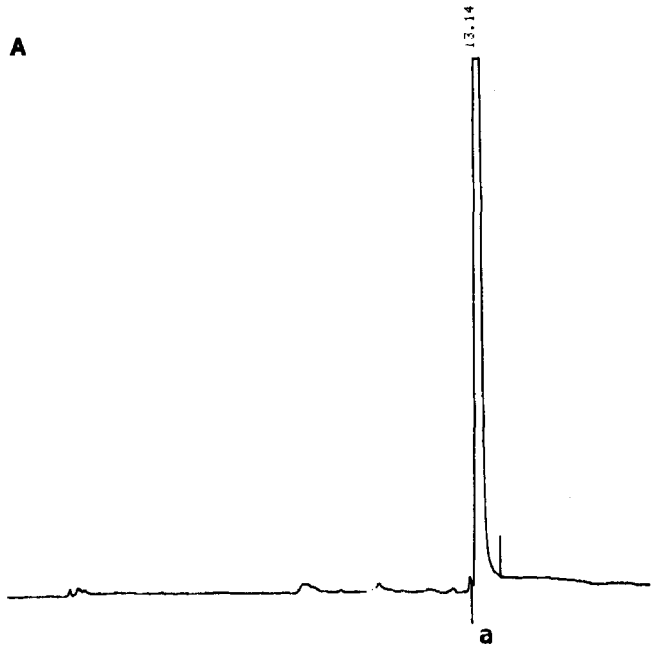

Fig (6) HPLC chromatograms of standard adenosine deaminase assay.
A) Blank, (a)=adenosine; B) Total activity, (a)=adenosine, (b)=inosine; C) Effect of EHNA (100 uM), (a)=adenosine.

(continued)

plays an important role in the salvage synthetic pathway of AMP (Fig 1). Fig (7) depicts typical chromatograms for adenylosuccinste lyase activity. In the present study, we did not investigate the effect of inhibitors on adenylsuccinate activity (Table 1).

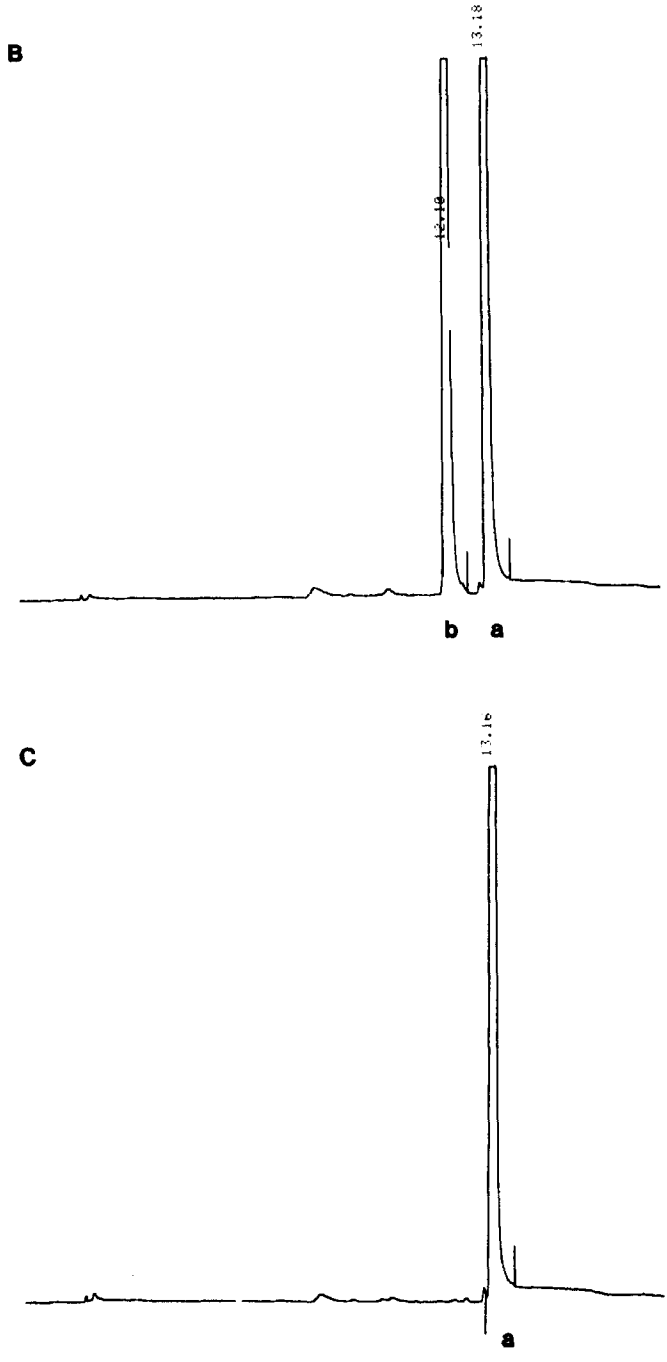

Fig 6 (continued)

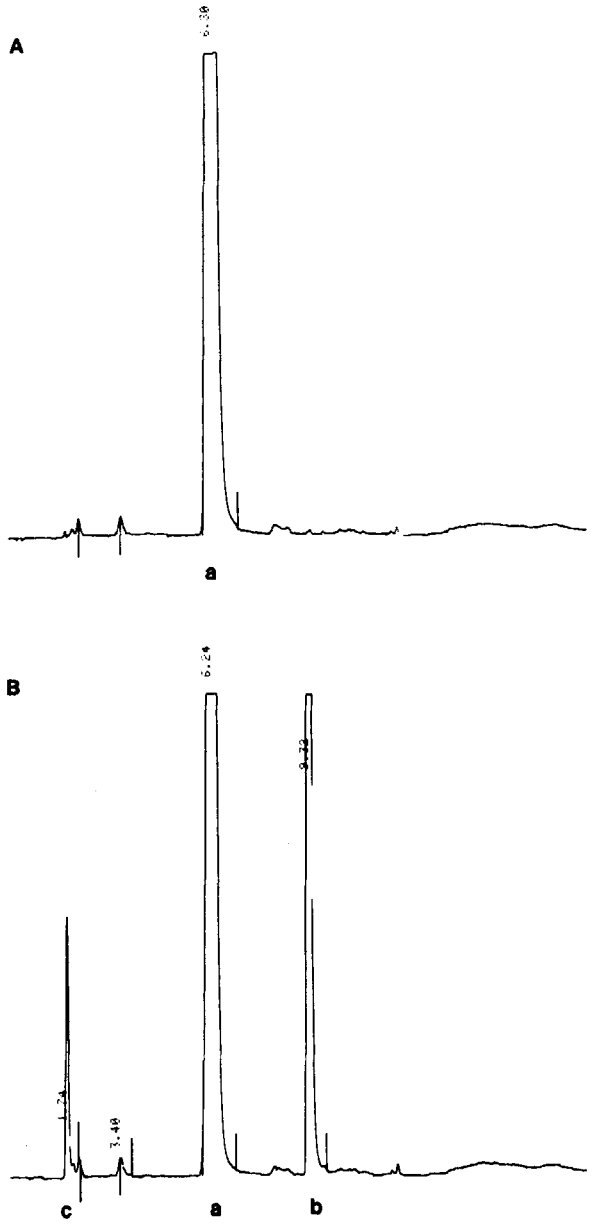

Fig (7) HPLC chromatograms of standard adenosylsuccinate lyase assay.
A) Blank, (a)=adenylsuccinate; B) Total activity, (a)=adenylsuccinate, (b)=AMP, (c)=fumarate.

Table (1): Activities of Standard Enzymes Using HPLC Anaylsis.

	Enzyme Activity a (nmol/min/ml assay medium)		
	Total	Inhbitor-sensitive	Inhibitor-insensitive
AMP-5'-Nucleotidase	77.75±5.50	70.25±5.75 b	7.50±0.50 b
IMP-5-Nucleotidase	37.75±3.75	30.50±1.50 b	2.25±0.25 b
AMP-Deaminase	154.25±3.25	3.00±1.50 c	145.50±6.00 c
Adenosine Deaminase	75.50±5.75	75.50±5.75 c	0 c
Adenylosuccinate Lyase	133.75±0.20	ND d	ND d

a Enzyme assays were carried out in vitro as described in the text and analyzed by HPLC. The reaction was intiated by the addition of 1-2 ug standard enzyme protein. Results are presented as mean±SEM (n=4).

b 5'-Nucleotidase inhibitor α,β-Methylene-ADP (100 uM).

c Adenosine deaminase inhibitor EHNA (100 uM).

d ND = not determined.

The Linearity of Assay Procedure for 5'-Nucleotidase Activity using HPLC:

The time course of 5'-nucleotidase activity in rat heart microsomal fractions is shown in Fig (8). The enzyme was assayed in a 10 mM Hepes-Tris buffer, pH 7.5, 1 mM MgCl2, 6 mM DTT, 1.5 mM AMP and 75 ug protein preparation. The enzymatic reaction was allowed to proceed at 37°C for 25 minutes. The relationship between the enzyme activity and incubation period was linear.

Determination of Sarcolemmal 5'-Nucleotidase in Cardiomyocytes:

Cardiomyocytes were prepared by enzymatic infusion and treatment of rat hearts as previously described (23). The effect of α,β-M-ADP on sarcolemmal 5'-nucleotidase activity in isolated cardiomyocytes is shown in Table (2). The inhibitor strongly inactivated about 80% of the total AMP-phosphatase activities.

, -M-ADP-sensitive-AMP-phosphatase activity was taken to represent the specific 5'-nucleotidase activity while the remaining activity was cosidered to be mediated by AMP hydrolysis by means other than 5'-nucleotidase, e.g. non-specific-AMP-phosphatases.

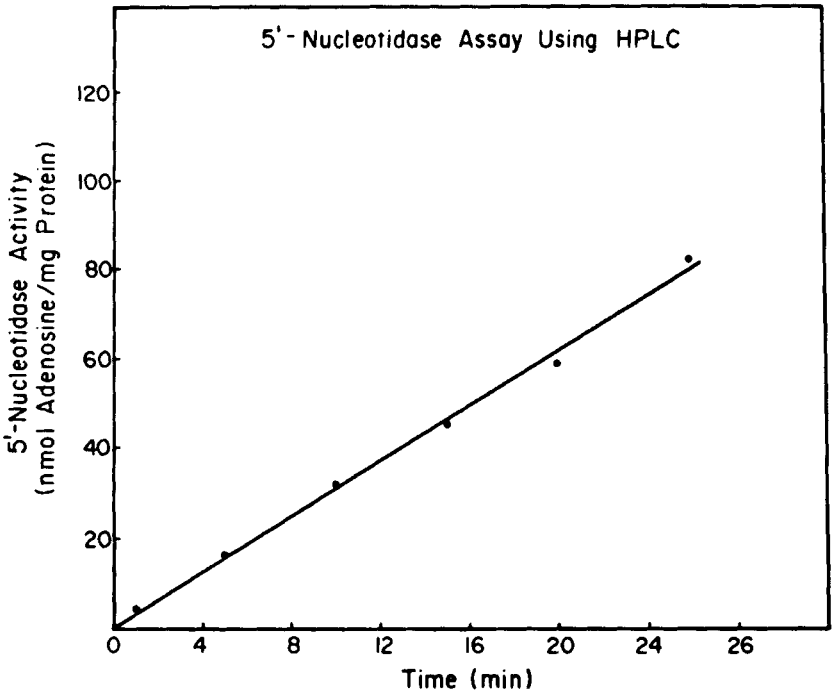

Fig (8) Linearity of HPLC assay of microsomal 5'-nucleotidase of rat myocardium. The enzyme activity was assayed in 10 mM-Hepes-Tris, pH 7.5, containing 1mM MgCl2, 6 mM DTT, 1.5 mM AMP, 75-100 ug protein, in the presence or absence of 100 uM α,β-M-ADP. Data presented as nmol adenosine produced/mg protein, mean±SEM (n=4).

Table (2) Sarcolemmal 5'-Nucleotidase Activity in Isolated Rat Heart Cardiomyocytes Using HPLC Anaylsis.

	5'-Nucleotidase Activity * (nmol/min/mg protein)	
Control	79.43±2.36	
α,β-Methylene-ADP(100 uM)	22.98±1.65	**

* The enzyme activity was assayed using HPLC analysis.
** $P<0.001$ vs control (n=8), t-test.

Determination of 5'-Nucleotidase and Adenylate Deaminase Activities in Myocardial Subcellular Fractions Using HPLC:

We also determined 5'-nucleotidase in subcellular preparations of canine myocardium. Figures (9, 10, 11) illustrate the activity of 5'-nucleotidase and possibly other enzymes that exist in mitochondrial, microsomal and cytosolic fractions, respectively. As shown in these figures most of AMP-phosphatase activity is sensitive to α,β-M-ADP (100 uM) in the microsomal fractions. The activity is less sensitive to the same

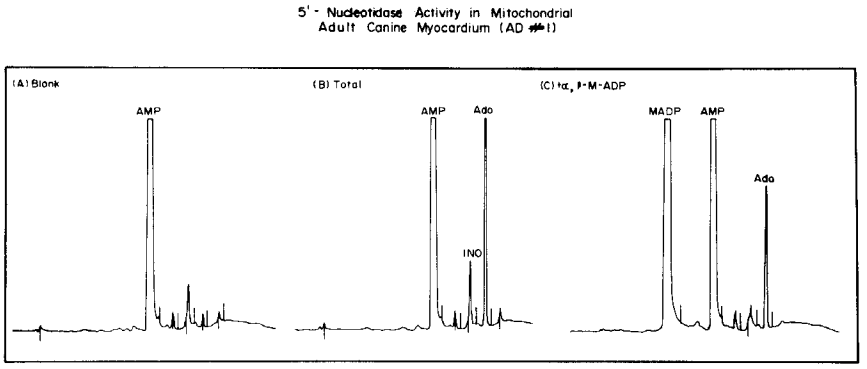

Fig (9) HPLC chromatogram of mitochondrial 5'nucleotidase, adenylate deaminase and adenosine deaminase assays in canine myocardium. A) Blank; B) Total activity; C) Inhibitor-insensitive activity.

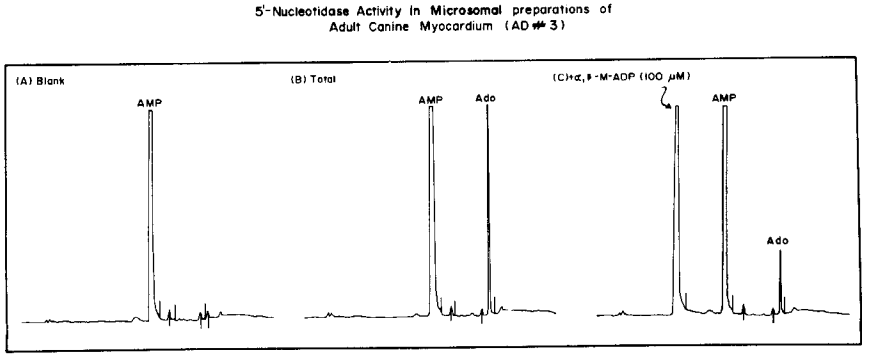

Fig (10) HPLC chromatograms of microsomal 5'-nucleotidase, adenylate deaminase and adenosine deaminase assays in canine myocardium. A) Blank; B) Total activity; C) inhibitor-insensitive activity.

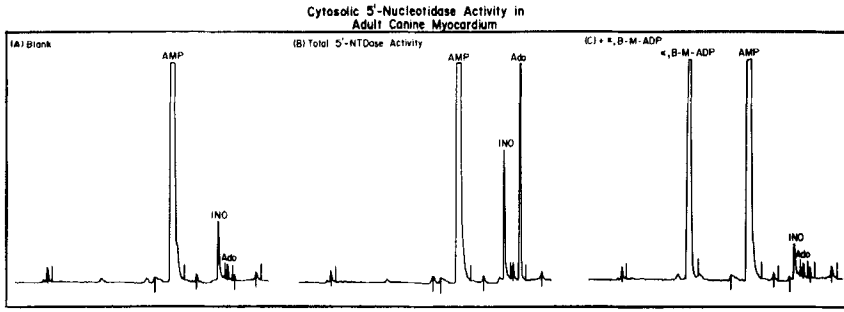

Fig (11) HPLC chromatograms of cytosolic 5'-nucleotidase, adenylate deaminase and adenosine deaminase assays in canine myocardium. A) Blank; B) Total activity; C) Inhibitor-insensitive activity.

inhibitor in mitochondrial and cytosolic fractions suggesting differences in subcellular compartmentation of 5'-nucleotidase isoenzyme. The addition of EHNA (100 uM) to each assay medium prevented further degradation of adenosine to inosine which is catalyzed by adenosine deaminase. This intervention allowed precise determination of the absolute activity of 5'-nucleotidase, i.e. adenosine formation.

Fig (12) shows HPLC assay of 5'-nucloetidase in cytosolic fractions of adult (Fig 12A) and fetal (Fig 12B) myocardium. The cytosloic fraction of the adult myocardium exhibited 5'-nucleotidaes activity as

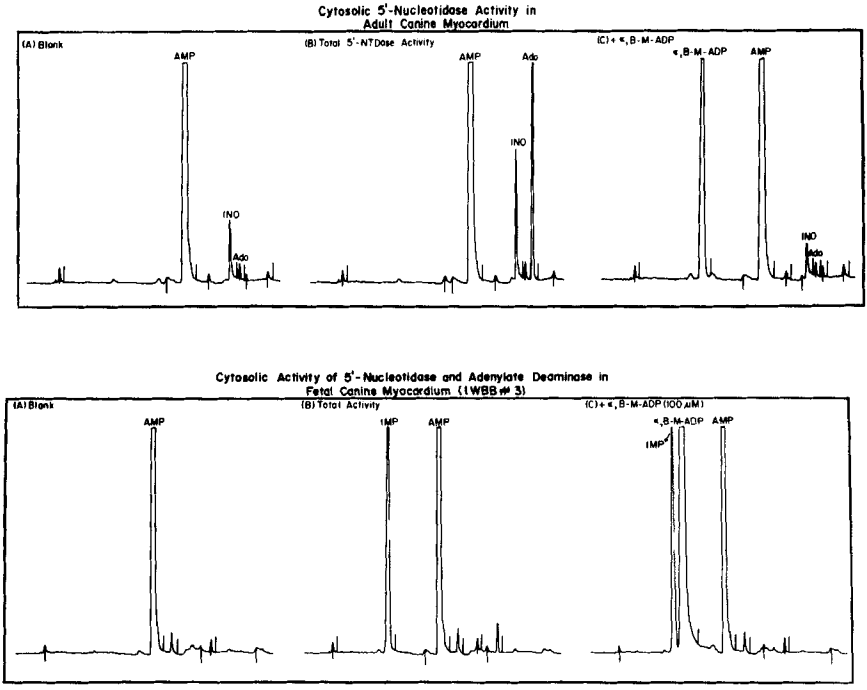

Fig (12) HPLC chromatogram of cytosolic 5'-nucleotidase and adenylate deaminase assays in adult and fetal myocardium. Fig (12A) shows 5'-nucleotidase in the cytosolic fraction of normal adult myocardium. Fig (12B) illustrates that the cytosolic fraction of fetal myocardium lacks 5'-nucleotidase activity, instead it exhibits adenylate deaminase activity which is not inhibited by α,β-M-ADP (100 uM).

well as adenosine deaminase activity as demonstrated by the formation of adenosine and inosine, respectively. On the other hand, unlike the adult myocardium, fetal cytosolic fractions exhibited adenylate deaminase activity rather than 5'-nucleotidase activity [detailed studies will be published elsewhere (24)].

A simple and rapid determination of adenosine:

By inhibition of adenosine catabolism, we were able to shorten the time required to elute adenosine from 22-26 minutes to only 3-4 minutes with an isocratic HPLC analysis using a single solvent (30%-40% methanol). Fig (13) illustrates a standard adenosine peak (A); a blank run indicating that no adenosine was produced by non-enzymatic hydrolysis of AMP (B); total activity of AMP-phosphatases (C); α,β-M-ADP-insensitive-AMP-phosphatase activity (D); and the effect of Br-AMP on 5'-nucleotidase activity (E). The specific activity of 5'-nucleotidase is calculated by subtracting the inhibitor-insensitive activity from the total AMP-phosphatase actvities. This simple and extremely rapid detection and identification of adenosine makes this procedure more attractive to assay for 5'-nucleotidase activity using HPLC analysis.
By changing the concentration of methanol from 40% to

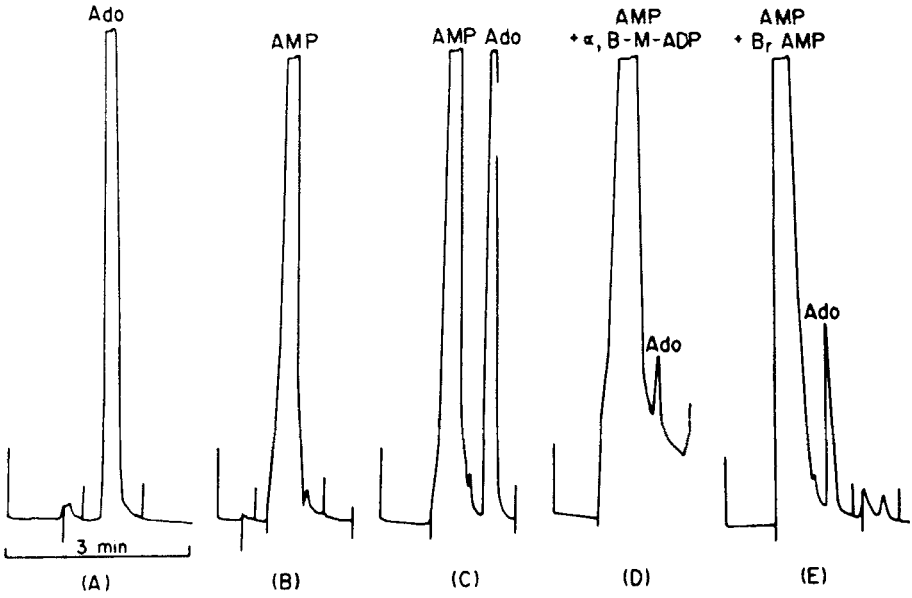

Fig (13) Simple and Rapid Determination of 5'-nucleotidase activity and Adenosine using HPLC.
A) Standard adenosine elution; B) Standard AMP; C) Total 5'-nucleotidase activity; D) α,β-M-ADP-insensitive phosphatase activity; and E) Br-AMP-insensitive phosphatase activity.

20% (or 15%) one can detect inosine as well as adenosine in a short chromatographic run of 5-8 minutes. Inhibition of 5'-nucloetidase activity by Br-AMP (25) will be published elsewhere.

DISCUSSION

Assaying for 5'-nucleotidase activity using conventional colorimetric, spectrophotometric, flurometric and radiometric techniques (12-20) lacks direct separation and identification of reaction products and their possible catabolites. This may generate inaccurate assessment of the enzyme activity and, by inference, its physiologic role. This manuscript describes a new application of HPLC analysis in assaying for enzyme activity in crude and purified preparations. Although our investigation focused on those enzymes involved in adenine nucleotide pool metabolism, this HPLC approach can be applied to any other enzyme assays using appropriate detectors. One of the most important advantages of this procedure is not only monitoring the enzymatic products but also determining utilization of the substrate by other enzymes that exist in the same preparation, and identifying further degradation of the products. For example, AMP is the specific substrate for 5'-nucleotidase and adenylate deaminase producing adenosine and IMP, respectively. Subsequently, adenosine deaminase and IMP-5-nucleotidase catalyze the formation of inosine from adenosine and IMP,

respectively. All these derivatives of adenine nucleotide pool metabolites absorb within a narrow range of the ultraviolet spectrum (245-265 nm). This unique characteristic, although limiting the spectrophotometric assays of 5'-nucleotidase, is advantageous for HPLC-enzyme assay. Using HPLC procedure, we were able to disclose developmental differences in subcellular compartmentaion of 5'-nucleotidase and adenylate deaminase isoenzyme in relation in AMP metabolism and adenosine production (25). This has been demonstrated in Fig (12). Unlike adult myocardium , fetal myocardium exhibited adenylate deaminase activity instead of 5'-nucleotidase or adenosine deaminase in cytosolic fractions (Fig 12A vs 12B).

The intention of the present report was not directed to compare our HPLC method with those procedures widely used to assay for 5'-nucleotidase. Nevertheless, it would be beneficial to discuss advantages and limitations of these methods. Early methods described for assaying 5'-nucloetidase are based on the colorimetric determination of inorganic phosphate released from 5'-AMP (12-14). Generally, colorimetric determinations of the released inorganic phosphate are not as sensitive and specific as newly developed methods. The spectrophotometric assay for

5'-nucleotidase is based on the indirect determination of adenosine by monitoring the change in absorbance of inosine formation in the presence of adenosine deaminase as a coupled enzyme (14,15). Although this method was thought to be more sensitive than that of inorganic phosphate determinations, it was noted that adenosine deaminase activity was influenced by divalent cations and accumulation of inosine (15). Radiometric assays for 5'-nucleotidase were developed using radiolabelled substrate (15-20). The release of ^{32}P- inorganic phosphate from ^{32}P-AMP has been used to assay for 5'-nucleotidase (15). Other methods required separation of the radiolabelled AMP from the product by either ion-exchange column chromatography (15-19), or its preciptation as zinc and barium salts and separated by centrifugation (20,26). Klaushofer et al (20) described a double labelled radioassay for the determination of 5'-nucleotidase activity. In addition to [8-14C]-AMP as a substrate, [2-^{3}H]-adenosine was added as a tracer to determine the efficiency of separation and recovery of [8-14C]-adenosine formation catalyzed by 5'-nucleotidase.

All previously described methods provided a great deal of information, but lacked a direct separation, identification and quantification of products and their

metabolites (12-20). This may account for the existing discrepancies among available data. An important source of disagreement is related to the often obscure definition of so called " non-specific AMP- phosphatase activity". Several studies have used β-glycerophosphate, p-nitrophenylphosphate or phenylphosphate as substrates for acid and alkaline phosphatase activities. These activities have been assayed in a different reaction media and pH (12) yet the authors subtracted this activity from the total AMP-phosphatase activity which had been determined under optimal conditions that yielded a maximal activity of 5'-nucleotidase. It is well estabished that crude and purified preparations of ATPases catalyze the hydrolysis of β-glycerophosphate, p-nitrophosphate and phenylphosphate, in addition to the natural substrate, i.e. ATP. For example, purified Na+-K+-ATPase (27-31) exhibit about 5-10% of the total activity of the enzyme as K+-stimulated p-nitophenylphosphatase activity. In our experience, purified 5'-nucleotidase catalyzes the hydrolysis of non-nucleotide p-nitrophenylphosphates, and this activity accounts for about 5-10% of the total 5'-nucleotidase activity which was determined under the same experimental conditions (Fig. 14). These observations argue against the use of these phosphates as specific substartes to saturate the non-specific

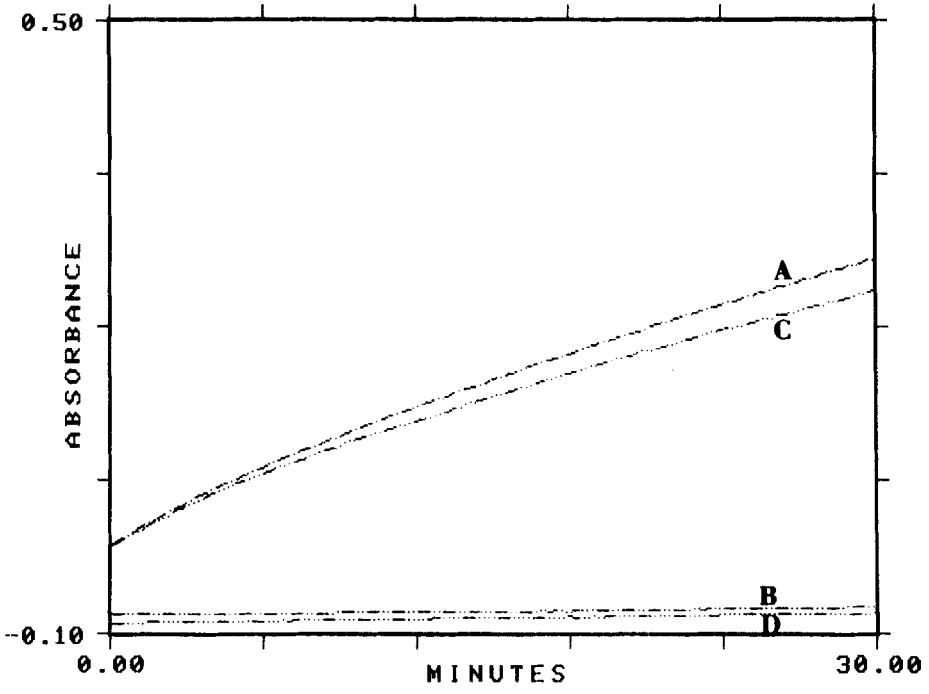

Fig (14) p-Nitrophenylphoshatase activity of a standard 5'-nucleotidase.
5'-Nucleotidase was incubated (37°C) with 3 mM p-nitrophenylphosphate in a reaction medium containing 10 mM Hepes-Tris, pH 7.5, 1 mM MgCl2 and in absence (A) or presence (C) of 10 mM KCl. α,β-M-ADP (100 uM) was added to both incubation media in absence (B) or presence of 10 mM KCl (D). The release of p-nitrophenol was monitored at 410 nm using a Gilford Response.

catalytic sites (10,19,20). We (25) and others (15,32,33) have used α,β-M-ADP, an unhydrolyzable analogue of ADP, as a potent and specific inhibitor (Ki=2 nM) of 5'-nucleotidase activity (15,32,33). The common adenine moeity in both AMP and this inhibitor made the latter a structurally more suitable configuration to block the catalytic side of the enzyme. Results from our laboratory demonstrated that 80-90% of the total AMP-phosphatase activity in crude and purified 5'-nucleotidase is sensitive to α,β-M-ADP (Table 1 and 2). The remaining activity (10-20%) was insensitive to α,β-M-ADP (100 uM) similar to that noted for ATPases (27-31). Klaushofer et al (20) reported inhibition of "5'-nucleotidase activity" by 20 mM , -M-ADP in the presence of 20 mM -glycerophosphate, 12 mM AMP and 4 mM MgCl2 in 50 mM Tris-HCl buffer, pH 7.6. The concentration of the substrates and inhibitors in the latter report are 10-fold greater than those we have used and what other studies have reported (12-19). These observations again raise several questions related to the determination of non-specific phosphatase activities. However, according to these reports , 10% of the total AMPase activity was related to non specific phosphatases (20,26). Determination of 5'-nucleotidase using HPLC analysis is not affected by turbidity of tissue homogenates which

limits spectrophotometric assays. Furthermore, we did not experience any loss in adenosine recovery during the extraction procedure, separation and HPLC analysis. Subsequently, this procedure does not require a tracer metabolite to monitor the yield during extraction and analysis. Finally, age-related differences in subcellular compartmentalization of 5'-nucleotidase and adenylate deaminase isoenzymes were disclosed only by employing the HPLC assay (25). HPLC assay is simple to perform and very reproducible and reliable, as demonstrated by determining the activity of 5'-nucleotidase, adenylate deaminase, adenosine deaminase, adenylosuccinate lyase and S-adenosyl-homocystein hydrolase as well as other enzymes regulating the adenine nucleotide pool metabolism.

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