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DETERMINATION OF ADENINE NUCLEOTIDES IN BIOLOGICAL SAMPLES BY ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

A simple, fast and sensitive, isocratic high performance liquid chromatography technique is developed for the determination of adenine nucleotides (ATP, ADP, AMP) in biological tissues. In conjunction with this technique, a new method of dissolving biological samples and extraction of nucleotides is introduced, using a tissue solubilizer, TMAH (Tetramethylammonium hydroxide). The solubilizing agent not only dissolves the sample and releases the adenine nucleotides effectively, but also preserves other metals and organometals in their authentic forms suitable for speciation. The HPLC technique has sensitivity in the picomol level and is suitable for nucleotide pool and adenylate energy charge calculations.

RÉSUMÉ

Une méthode de chromatographie liquide haute performance en régime isocratique qui est simple, rapide et sensible est en voie d'élaboration pour le dosage des nucléotides à base d'adénine (ATP, ADP, AMP) dans les tissus biologiques. En même temps que cette méthode, on introduit une nouvelle méthode pour dissoudre les échantillons biologiques et extraire les nucléotides; cette méthode fait appel à un agent de solubilisation des tissus, le TMAH (hydroxyde de tétraméthylammonium). Non seulement l'agent de solubilisation dissout l'échantillon et libère les nucléotides à base d'adénine de manière efficace, mais il conserve les autres métaux et organométaux dans leur forme originale, ce qui permet leur caractérisation. La technique de LCHP a une sensibilité de l'ordre de la picomole et elle convient pour les calculs du pool nucléotidique et de la charge énergétique en adénylate.

MANAGEMENT PERSPECTIVE

Adenine nucleotides are biologically active components in the anabolic and catabolic pathways. The nucleotide pool and the adenylate energy charge can be used as biochemical indicators for the vitality of a living organism, hence the well-being of the organism. It is a powerful biochemical parameter for studying environmental health.

Existing techniques for adenine nucleotide determination are enzymatic, fluorimetric and HPLC. Enzymatic reactions are difficult to control and extremely variable. Fluorimetric measurements are also based on enzyme reactions to produce fluorescence. Available HPLC techniques are primarily developed for medical investigations which are lengthy, and not suitable for environmental studies. A simple, sensitive and fast method is in demand.

A simple, fast and sensitive, isocratic high performance liquid chromatography technique is developed for the determination of adenine nucleotides (ATP, ADP, AMP) in biological tissues. In conjunction with this technique, a new method of dissolving biological samples and extraction of nucleotides is introduced, using a tissue solubilizer, TMAH (Tetramethylammonium hydroxide). The solubilizing agent not only dissolves the sample and release the nucleotides effectively, but also preserve other metals and organometals in their authentic forms suitable for speciation. The HPLC technique has sensitivity at the picomol (500×10^{-12}g) level and is suitable for nucleotide pool and adenylate energy charge calculations.

PERSPECTIVE-GESTION

Les nucléotides à base d'adénine sont des constituants biologiquement actifs des voies de l'anabolisme et du catabolisme. Le pool nucléotidique et la charge énergétique en adénylate peuvent être utilisés comme des indicateurs biochimiques de la vitalité d'un organisme vivant, et par conséquent, du bien-être de cet organisme. Il s'agit d'un paramètre biochimique puissant pour l'étude de la santé environnementale.

Parmi les méthodes actuellement utilisées pour le dosage des nucléotides à base d'adénine figurent la méthode enzymatique, le méthode fluorimétrique et la LCHP. Les réactions enzymatiques sont difficiles à maîtriser et extrêmement variables. Les mesures fluorimétriques reposent également sur des réactions enzymatiques pour produire une fluorescence. Les techniques de LCHP actuelles, mises au point principalement pour des applications médicales qui sont longues, ne conviennent pas aux études environnementales. Nous sommes donc à la recherche d'une méthode simple, sensible et rapide.

Une méthode de chromatographie liquide haute performance en régime isocratique qui est simple, rapide et sensible est en voie d'élaboration pour le dosage des nucléotides à base d'adénine (ATP, ADP, AMP) dans les tissus biologiques. En même temps que cette méthode, on introduit une nouvelle méthode pour dissoudre les échantillons biologiques et extraire les nucléotides; cette méthode fait appel à un agent de solubilisation des tissus, le TMAH (hydroxyde de tétraméthylammonium). Non seulement l'agent de solubilisation dissout l'échantillon et libère les nucléotides à base d'adénine de

manière efficace, mais il conserve les autres métaux et organométaux dans leur forme originale, ce qui permet leur caractérisation. La technique de LCHP a une sensibilité de l'ordre de la picomole (500 x 10^{-12} g) et elle convient pour les calculs du pool nucléotidique et de la charge énergétique en adénylate.

Determination of Adenine Nucleotides in Biological Samples by Isocratic High Performance Liquid Chromatography

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Introduction

The application of biochemical indicators in studying stress, toxic effects and environmental health has been one of the major topics of interest. The measurement of adenine nucleotides in living organisms - adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), leading to the calculation of Adenylate Energy Charge (AEC) has been applied to a wide range of organisms, such as bacteria (Chapman et al. 1971), marine worms (Verschraegen et al. 1985), bivalves (Wijsman, 1976; Zaroogian et al. 1982; Giesy et al. 1983; Gies, 1986; Sylvestre and Gal, 1987), microplankton (Romano and Daumas, 1981) and fish (Reinert and Hohreiter, 1981).

Several techniques are available for the determination of adenine nucleotides. ATP, ADP and AMP were determined spectrophotometrically after enzymatic assays with hexokinase (Lamprecht and Trautschold, 1974; Jaworek et al. 1974), pyruvate kinase and myokinase (Adam, 1963) respectively with sensitivity in the 10-100 nmole/ml level. Sensitivity is much enhanced by fluorometric measurement of the bioluminescence formed in the reaction of ATP with luciferin-luciferase which is not only sensitive but also specific in the 0.1 - 80 pmole/assay range (Strehler, 1974; Lust et al. 1981). Radioenzymatic technique (Gonzalez and Garcia-Sancho, 1981) following the phosphorylation of a labelled sugar can be applied to quantify ATP at 3 pmole/assay level. Most of the analytical methods used in the past are based on enzymatic reactions which give variable results and are difficult to control. More accurate and reproducible analysis can be obtained with high-performance liquid chromatography (HPLC) techniques by using ultra-violet detector or fluorescence detector (Taylor et al. 1981; Ramos-Salazar and Baines, 1985) after derivatization. Many of the existing HPLC techniques are lengthy in gradient operation modes designed for analyzing homologs of nucleotides for biochemical investigations (Gies, 1986; Werner et al. 1987). There are, however, only a few simple and fast HPLC techniques available for environmental studies (Walker et al. 1986).

The present method describes a simple, sensitive, and rapid, isocratic HPLC technique for the determination of adenine nucleotides in environmental samples, using phosphate buffer and water as the mobile phase. The major adenine nucleotides are separated in about 15 minutes with picomolar sensitivity, suitable for adenylate energy charge and nucleotides pool calculations for aquatic biota. The method has a wide dynamic range covering a concentration range of 4 orders of magnitudes which minimizes the necessity of sample size adjustment for analysis. The

procedures have been used extensively in our laboratory and in the field in conjuction with other biochemical indicators in studies of environmental stress and pollutant effects. Results of these studies will be reported elsewhere.

Materials and Methods

A HPLC model 600 and a WISP 712 Auto-sampler (Waters Associates, U.S.A.) were used with a Hewlett Packard 1046A variable-wavelength UV detector. The column was a u-Bondapack C-18, reversed-phase, 30cmx4mm (Waters Associates). The mobile phase consisted of a buffer of 0.15M potassium dihydrogen phosphate and disodium hydrogen phosphate (pH=6.54), and water at 60:40 % ratio. The flow rate was 1.0 ml/min operated in the isocratic mode, at room temperature (20°C).

The UV detector wavelength was set at 254 nm, and sensitivity at 0.05 Absorbance Unit Full Scale (Aufs), optimal for measurement of all three adenine nucleotides. Peak areas were integrated by a Hewlett-Packard 3392A Electronic Integrator.

Chemicals

The adenine nucleotides (AMP,ADP,ATP) were obtained in the highest quality from Sigma (St. Louis, MO). Disodium hydrogen phosphate and potassium dihydrogen phosphate were HPLC grade from Fisher Scientific. After dissolving the appropriate amounts (0.15 mol of each) in 1 L of water, the pH of the buffer solution was adjusted to 6.54 (±0.10) with H_3PO_4 or NH_4OH. Other solvents were HPLC grade. Water was distilled and purified in a Milli-Q-water system. All solutions were filtered through a 0.45 um membrane and degassed with nitrogen for 20 min in the solvent reservoirs before use.

Procedure

Before use, the HPLC column was conditioned first by passing with water at 1 ml/min for 20 min to displace the methanol in which it was stored, followed by the eluent buffer/water, 60:40 mixture, for 30 min at 1 ml/min. Methanol must always be displaced before the buffer solution is let into the HPLC system, otherwise precipitation will occur and will cause severe damage to the column. After use, the column was first washed with water for 20 min and then with methanol for 20 min and stored in the same medium until next use.

Prepared samples (20-50ul) were injected into the HPLC through the WISP Auto-sampler.

Extraction of nucleotides from mussels

Frozen tissue samples (mussels, fish, 1g) were digested in 5 ml of 20% Tetramethyl-ammonium Hydroxide (TMAH) aqueous solution at 60°C for 20 min to a straw-yellow color clear solution. The solution was cooled and

neutralized with 50% HCl to pH 7-7.5, recording the final volume of the solution. The sample was centrifuged and 0.5 ml of the supernatant was taken out and diluted to 10 ml with the buffer solution. An aliquot (ca. 2 ml) of the diluted sample was filtered through a syringe filter (0.45 um, hydrophilic membrane) into an Auto-sampler vial. A 20-50 ul aliquot was injected into the HPLC.

Nucleotide standard stock solution was prepared as 1 mM solution in water. A working standard was prepared by adding 0.2 ml of each of the nucleotide stock solution to 5 ml of 20% TMAH (total vol = 5.6ml) and processed in parallel with the samples. After digestion, 0.2 ml of the mixed standards was diluted to 2 ml with the phosphate buffer, then filtered through a syringe filter (hydrophilic membrane, 0.45um) into the Auto-sampler vial. There was no need to neutralize the TMAH solution as the buffer itself has sufficient capacity to overcome the strong alkalinity and to maintain the required pH. The standards so diluted have a concentration of 3.57 pmol/ul of each nucleotide. The absolute amounts (pmol) of nucleotide injected into the HPLC were:

ATP = 3.57 x (actual mM of stock solution) x ul injected
ADP = 3.57 x (actual mM of stock solution) x ul injected
AMP = 3.57 x (actual mM of stock solution) x ul injected

In normal practice, 20 ul of the mixed standards (containing ca. 80 pmol of each nucleotide) was injected into the HPLC, the averaged peak areas from replicate analysis (n=3) were used for calculation of the sample concentrations.

Stock standard solutions maintained at -20^{0}C can be kept for a period of up to 3 months without noticeable deterioration. Diluted standards were kept in refrigerator and were stable for a period of at least up to two weeks.

Results and Discussion

Extraction of nucleotides from biological samples

Extraction of analytes from samples without altering their chemical forms is one of the major challenge in trace speciation techniques. The digestant used must be strong enough to release all the analytes from the sample matrix to achieve quantitative recovery, and yet "mild" enough not to cause any destruction of the authentic form of the analytes.

Several extracting agents were investigated for their efficiency in releasing the adenine nucleotides from tissues. Phosphoric acid (cold and warm), boiling tris buffer, and sulfuric acid (50%) did not dissolve the sample to yield reasonable recovery and were not at all suitable. Two other extracting agents were investigated in detail with mussels and fish. The first one is perchloric acid reported to be efficient for oyster and mollusc (Sylvestre et al. 1987). The other one, TMAH, is a tissue solubilizer, which has been used successfully for dissolving biological samples for speciation of alkyllead and butyltin compounds (Chau 1988). It is non-oxidizing and dissolves the complex biological tissues without rigorous heating.

Both reagents released the adenine nucleotides effectively. While perchloric acid occasionally gave higher but variable results for the three nucleotides, TMAH digestion gave more consistent and reproducible results, and had no destructive effects on the authenticity of the nucleotides, nor interference with the UV detection. TMAH was selected because the resulting digest solution could also be used for speciation of other components, such as metals, etc.

HPLC techniques

Analytical techniques based on enzymatic reactions are difficult to reproduce because of the difficulties in obtaining high purity enzymes, and in controlling the reaction time. Reaction time is crucial in enzymatic reactions for reproducible results. From our experience, we have not been able to purchase commercial enzyme kits that have low blank values, and to obtain reproducible results with standards even though procedures were followed with scrupulous care.

HPLC techniques basically involve a physical separation and detection system with very simple, if any, chemical reactions. There are pre-column or post-column derivatizations if fluorescence detection is to be used. For UV detection, it is based on the intrinsic UV absorption properties of the compounds and no chemical reaction is involved. Once the analyte compounds are isolated from the sample, the analysis part is highly reproducible. Thus the major difficulties in the analysis of adenine nucleotides are in the extraction of these compounds from the living organisms. Adenine nucleotides are biologically active but chemically stable. As long as the organisms are still living, these compounds are active components in the anabolic (energy-utilizing) and catabolic (energy-forming) biochemical pathways. After isolation from the living organism, these compounds are stable in the buffer solution at -20^{0} for a period of at least two weeks.

All three nucleotides have UV absorbance maxima at 210-211 and 259 nm. The wavelength 254 nm was chosen for analysis for a more stable baseline without much sacrifice in sensitivity.

The Solvent system

Several buffer systems were investigated in an attempt to achieve better separation of the nucleotides on the C-18 column using UV absorbance detection. Ammonium phosphate, potassium phosphate and a combination of sodium and potassium phosphate were tried at different pH values. The best result obtained was with a buffer system consisting of 0.15M of potassium dihydrogen phosphate and disodium hydrogen phosphate. This method was further investigated with varying compositions of eluent, flow rates, pH, until the best sensitivity and resolution were achieved.

At a ratio of 60:40, buffer to water ratio, the three nucleotides were well separated in clear and well-formed peaks on a stable baseline. Retention times and peak areas were identical for standards prepared in buffer solution and in biological matrix. There was no interference effect of the biological matrices.

Reproducibility, recovery and detection limit

The reproducibility of the method was evaluated by replicate analysis (n=6) of a standard mixture, which has been taken through the TMAH digestion, and the complete analytical procedure. The standard solution containing 80 pmol of each was injected into the HPLC. The percentages of variation of the method for ATP, ADP and AMP were 1.19, 0.56 and 1.38 respectively at this level. No deterioration nor decomposition effect was observed on the nucleotides by the action of TMAH. The absolute detection limits for the nucleotides are 2 picomols for a signal 5 times the noise level at a detector sensitivity of 0.005 Aufs. When 1 g sample is used according to the present procedure, the detection limit is 2 pmol of nucleotide per gram tissue, which is far more sensitive than is required for aquatic organisms. In ordinary practice, a detector sensitivity in the range of 0.10 - 0.02 Aufs is adequate.

The calibration curves for the three nucleotides are shown in Figure 1. The signal responses are almost identical for each nucleotide and are linear over a dynamic range of 4 orders of magnitudes (8 to 20,000 picomols) as indicated by their coefficients of correlation (0.99 for all three nucleotides). AMP has a 20% higher signals all over than those of ATP and ADP. This is purely due to chromatogarphic effects because AMP is the last peak in the chromatogram. Peaks with longer retension times tend to be slightly broader than the earlier ones and hence producing larger areas. With the present sensitivity, very small sample (mg) is required for analysis.

For analytes which are biologically active and labile, it is extremely difficult to evaluate their recoveries from biologicl materials. The sample-spiking technique commonly used for recovery evaluation cannot be applied here. However, from the fact that the concentration of nucleotide standards did not change after going through the complete analytical procedure and had no matrix effect when these standards were spiked to a biological digest for analysis, plus the fact that the tissues were totally dissolved in the digestion, it is reasonable to assume satisfactory recovery of the procedure.

There is no interference from a few other homologs of nucleotides. Guanosine diphosphate and guanosine triphosphate gave peaks at retention times of 4.95 and 4.65 min respectively under the present chromatographic conditions. There was no peak for 2,3 cyclic AMP and 3,5 cyclic AMP, adenine, and adenosine.

HPLC chromatograms of the adenine nucleotides standards and of samples are illustrated in Fig.2A and 2B. Table 1 shows the application of the present technique in the determination of adenine nucleotides in mussels.

Preservation of sample

Biological samples were kept in liquid nitrogen immediately after sampling and remained there until analysis. After thawing, samples were immediately dissected and the desired tissue was digested in TMAH accordingly. The final worked up samples in buffer solution, if not

immediately analyzed, can be stored in freezer for future use.

Table 2 summarizes some analyses of adenine nucleotides in aquatic organisms published in the literature. The disparity of values is obviously the result of different analytical techniques and sample varieties. There is indeed a need for a simple and reproducible procedure for nucleotide measurement in environmental studies.

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Table 1. Adenine nucleotides in mussel (*Elliptio complanata*)

Sample wt. (g)	ATP	ADP	AMP	AEC
0.299	1.045	1.096	0.809	0.540
0.208	1.270	1.533	0.866	0.550
0.285	1.156	1.303	0.835	0.549
0.242	0.964	1.187	1.050	0.486

Live mussel was dissected, and the foot muscle was immediately weighed and analyzed.
Units in umol nucleotide/g wet tissue. AEC (Adenylate Energy Charge is a ratio defined as (ATP + 0.5 ADP) / (ATP + ADP + AMP)

Table 2. Concentration of adenine nucleotides in aquatic biota.

Species	ATP	ADP	AMP	AEC	Method	Reference
M. edulis	2.82	0.97	0.09	0.85	1	Zaroogian, 1982
M. edulis	3.63	0.93	0.14	0.87	1	Zaroogian, 1989
Crassostrea virginica	0.94	1.49	1.59	0.42	1	" "
M. edulis	22.83[a]	6.00	0.28		2	Gies, 1986
Nereis diversicolor	1.30				3	Verschraegen, 1985
Nephtys sp.	2.66					" "
Pimephales promelas	3.8[b]			0.770	1	Reinert & Hohreiter, 1984
Homarus americanus tail	4.67	0.75	0.12	0.911	1	Haya & Waiwood 1983
gills	0.74	0.09	0.08	0.866		

Analytical Methods: 1- enzymatic, using spectrophotometry.
2- HPLC
3- enzymatic, firefly bioluminescence reactions.

All nucleotide concentrations in umol/g tissue wet wt. except
a - umol/g protein.
b - umol/g dry wt.

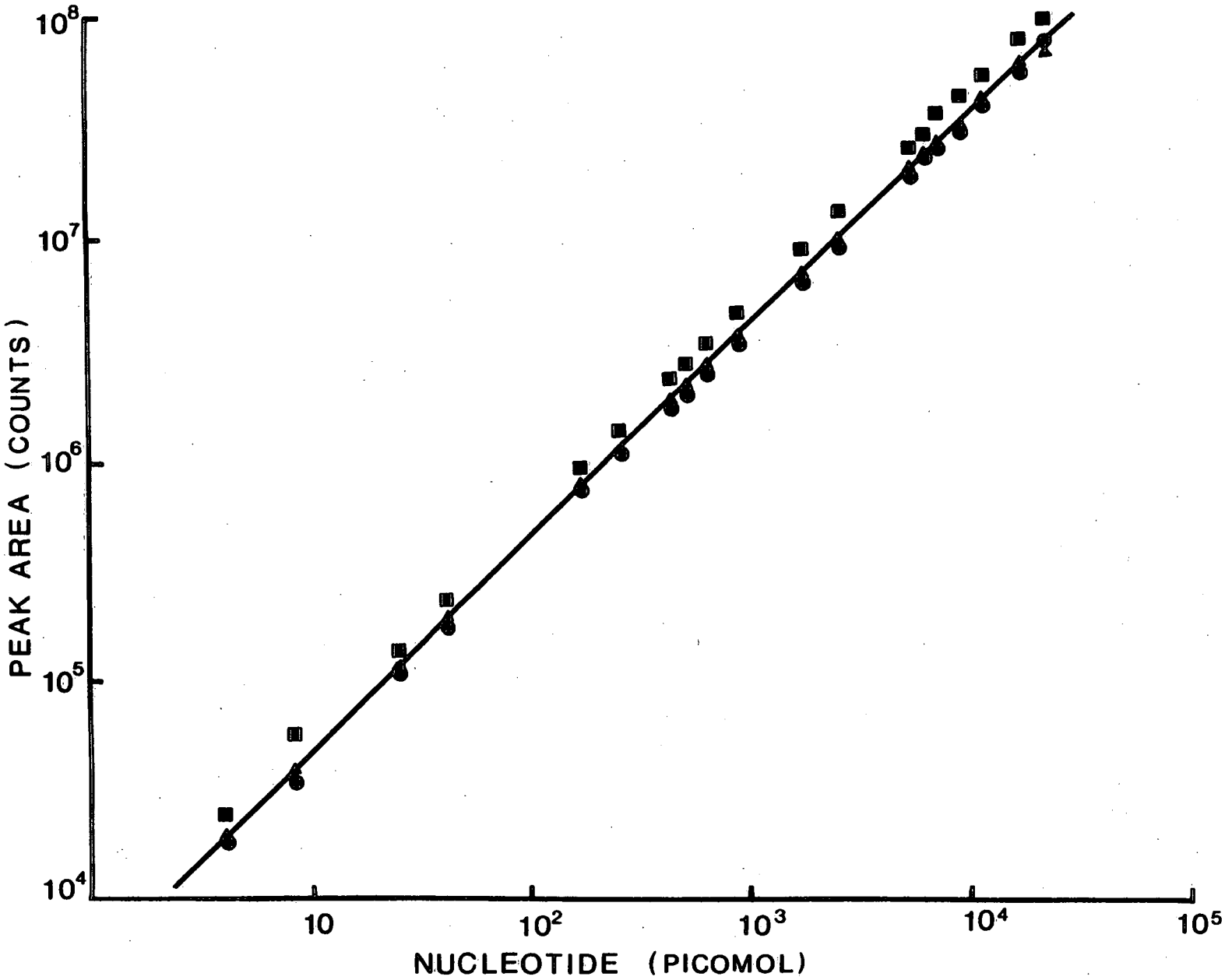
Figure Legends

Fig. 1. Calibration curves for ATP, ADP and AMP.
▲ - ATP ● - ADP ■ - AMP

Fig. 2A. HPLC chromatograms of a standard mixture of adenine nucleotides. Measured at 254 nm, 0.01 Aufs; Each peak contained 60 pmol of nucleotide.

Fig. 2B. HPLC chromatograms of adenine nucleotides in the foot muscle of Elliptio complanata.

Sample (0.280 g) dissolved in 5 ml TMAH, diluted 10X with phosphate buffer; 30 ul injected into HPLC. Instrument parameters same as in Fig. 2A.


10^8
10^7
10^6
10^5
10^4
PEAK AREA (COUNTS)
10
10^2
10^3
10^4
10^5
NUCLEOTIDE (PICOMOL)

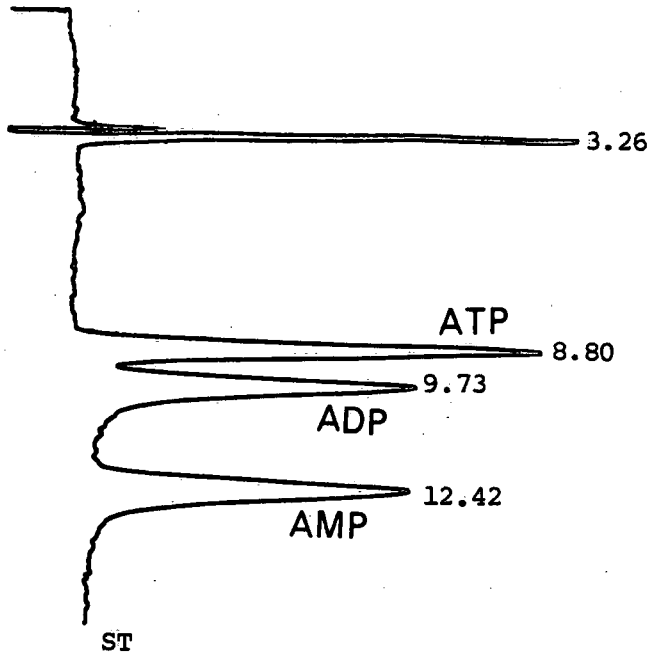

3.26
ATP
8.80
9.73
ADP
12.42
AMP
ST

Fig. 2A

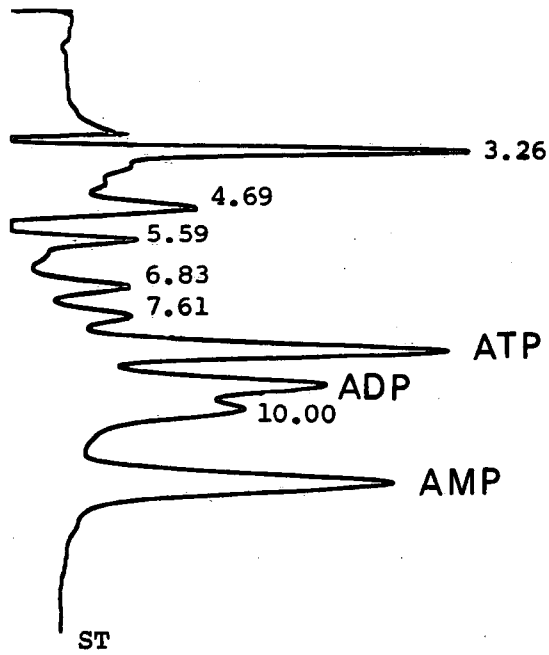

3.26
4.69
5.59
6.83
7.61
ATP
ADP
10.00
AMP
ST

Fig. 2B