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A rapid HPLC method for determination of adenylate energy charge[1]

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Summary. A simple procedure is described for separation and analysis of adenine nucleotides in tissue extracts, utilizing anion exchange HPLC. Determination of AMP, ADP, and ATP takes 10 min per sample.
Key words. HPLC adenine nucleotide evaluation; adenylate energy charge; stress index.

Several biochemical indices for the assessment of sublethal stress in marine organisms have been described with the aim of evaluating the biological impact of pollutants[3]. Among these is the Adenylate Energy Charge (AEC), defined by Atkinson and Walton[4] as the ratio:

$$AEC = (ATP+0.5\ ADP)/(ATP+ADP+AMP)$$

where ATP, ADP, and AMP are the molar concentrations of adenosine-5'-triphosphate, -diphosphate, and -monophosphate. Advantages and limitations of AEC determinations in pollution monitoring have been reviewed[5]. It appears that the most important limitations are related to the methodology, in particular to the lack of analytical procedures allowing an accurate determination of the three nucleotides and suitable for routine analysis of a large number of samples. We describe a simple method which utilizes ion-exchange HPLC for rapid separation of adenine nucleotides, followed by spectrophotometric determination at 260 nm. This procedure was successfully employed in the evaluation of AEC in tissues of *Mytilus galloprovincialis* Lam. and *Palaemon elegans* L., organisms widely utilized in environmental studies.

Materials. Adenine nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and purchased from Riedel & De Haene (Hannover, West Germany). The liquid chromatograph was a Hewlett-Packard, model 1084, equipped with a high-speed spectrophotometric detector, model 1040, and facilities for data processing. The 100 × 4.6 mm analytical column was protected by a 30 × 4.6 guard column. Both were packed with 10 µm Aquapore anion exchanger (Brownlee Labs, Santa Clara, CA).

Sample preparation. The animal (*Mytilus galloprovincialis* Lam. or *Palaemon elegans* L.) was killed and immediately dissected: required organs or tissues were rapidly excised and pressed in liquid nitrogen. After evaporation of the nitrogen, the wet weight was determined and the material homogenized with 3 vols of ice-cold 7% (w/v) trichloroacetic acid (TCA) in a refrigerated homogenizer. After centrifugation at 35,000 × g for 10 min at 0 °C, the supernatant was removed. The pellet was resuspended in TCA, centrifuged again and the washing added to the first supernatant. The extract was neutralized with 5 M K_2CO_3 and filtered (Milliporefilter type Millex-HV, 0.45 µm). Some aliquots of the TCA extracts were also extracted five times with diethylether to remove the acid. This procedure which has been utilized by various authors[6], gave the same chromatographic results as the simplified procedure that was routinely adopted for the analysis.

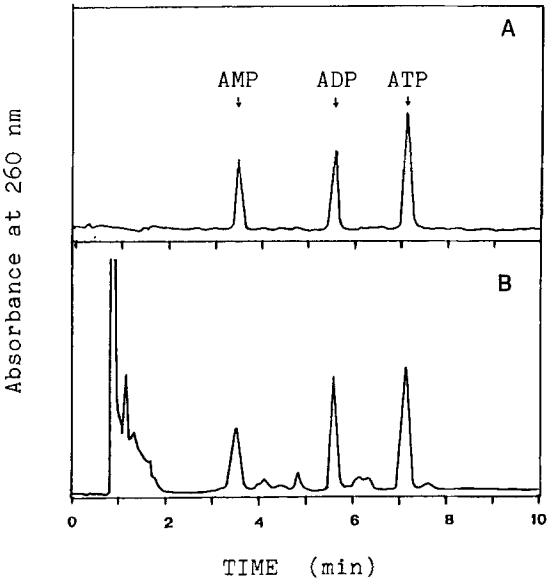
Chromatographic procedure. Separation of AMP, ADP, and ATP was carried out using a gradient of phosphate buffer at pH 8.0. The mobile phase consisted of distilled water (A) and 200 mM phosphate buffer (B). Both solutions were filtered through 0.22 µm prior to use and the column was equilibrated with 3% B. After injection of the sample, the column was eluted with 3% B for 1.5 min, then B was increased up to 95% at 8 min and held up to 10 min. The gradient was then returned to 3% B and the initial conditions were restored in 5 min. Flow rate was 2 ml/min. Buffers and column temperatures were 35 °C. Detection was performed at 260 nm. Peak identities were confirmed by coelution with standards. Peak purity was verified by recording the UV-spectra at the apex and at the inflection points on the up- and down-slopes of each peak: after normalization, the three spectra should be superimposable. Quantitative measurements were carried out by injection of standard solutions at known concentrations. A molar absorption coefficient of 15,400 cm^{-1} M^{-1} was assumed for adenine nucleotides.

Results and discussion. Figure A shows the separation of a standard mixture of AMP, ADP, and ATP: the nucleotides are completely separated in 10 min, and thus there is an improvement with respect to reported HPLC separations[7], often obtained by reverse-phase or ion-paired chromatography. Furthermore, ion-exchange separation does not requires high-purity organic solvents or chemical modifiers. A chromatogram of an extract of the digestive gland of *Mytilus galloprovincialis* Lam. is reported in figure B: the nucleotides are completely separated from other components and the gradient used does not cause an appreciable baseline drift. It should be pointed out that the peaks corresponding to AMP, ADP, and ATP, extracted from several organs or tissues of the mussel *Mytilus galloprovincialis* Lam. and the shrimp *Palaemon elegans* L. were absolutely pure when checked spectrophotometrically as described under Meàthods. To ensure a long lifetime for the analytical column, the guard column was replaced every 150 injections, when analyzing 10-µl samples.

Reproducibility was tested by injecting the same extract ten times; the variation coefficients of retention times and nucleotide concentrations are reported in the table. Stability of adenine

Reproducibility of HPLC determination of adenine nucleotides. Mean values, standard errors, and coefficients of variation for retention times and concentrations obtained from 10 determinations carried out on the same extract of muscular tissue of *Palaemon elegans* L.

	AMP		ADP		ATP	
	R.T. min	Conc. µM	R.T. min	Conc. µM	R.T. min	Conc. µM
Mean value	3.75	374.10	5.82	359.9	7.52	1852.1
Standard error	0.03	20.87	0.007	3.71	0.007	13.20
Coeff. variation	0.008	0.056	0.001	0.010	0.001	0.007



Separation of adenine nucleotides from a standard mixture (A) and from an extract of digestive gland of mussel (B). Injection volume: 10 µl. Experimental conditions as described in the text.

nucleotides in the TCA extract was checked by analyzing the same extract, maintained at −20 °C over a period of two months several times; no significant variation was observed.

The values of AEC calculated from the results obtained utilizing this procedure (Mytilus digestive gland AEC = 0.67; Palaemon muscular tissue = 0.78) are in good agreement with the data reported in the literature[5,8].

In conclusion, the method described here, based on TCA extraction and anion exchange HPLC, allows a simple, rapid, and accurate determination of adenine nucleotides. Therefore, it represents a very useful tool for routine determination of the adenylate energy charge in marine organisms, and thus for the assessment of pollution stress in environmental monitoring programs.

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Comparative effects of ouabain, natriuretic factor and ammonium chloride in the toad urinary bladder

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Summary. Electrical changes and direct effects on Na-K ATPase activity induced by an endogenous digitalis-like natriuretic factor (NF), NH_4Cl and ouabain were studied in toad bladders. NF inhibited the SCC and the Na-K ATPase activity in a similar manner to ouabain, but induced a greater increase in calculated direct current resistance (R) ($p < 0.05$). NH_4Cl was a weak inhibitor of Na-K ATPase activity, although it produced steeper SCC inhibition slopes than those observed with ouabain or NF ($p < 0.01$). The data suggested the same mechanism of action of NF and ouabain on the sodium pump, with an additional effect of the former on apical sodium permeability of the cells and/or closure of paracellular routes leading to an increased tissue resistance. In contrast, the effects of NH_4Cl were mostly compatible with intracellular inhibition of apical sodium entry into the cell.
Key words. Bladder; cell membrane permeability; Na-K ATPase; digitalis; natriuretic factor; ouabain; ammonium chloride.

In the 1970s, NH_4Cl and a natriuretic factor (NF) isolated from the chromatographed urine of salt-loaded normal men were reported to have ouabain-like electrical properties in frog skin preparations[1]. Ouabain is known to be a specific and powerful inhibitor of the sodium pump[2] and the Na-K activated ATPase of cell membranes, and to inhibit the short-circuit current (SCC) in a toad bladder preparation mounted in an Ussing-chamber, and the transepithelial sodium flux through various epithelia and cells.

NH_4Cl was shown to inhibit the SCC in toad bladder[3] and frog skin preparations[4], and to suppress ouabain-binding to isolated Na-K ATPase receptors of rabbit kidney preparations at $5 \cdot 10^{-1}$ M (unpublished observations). NF inhibited the SCC in toad bladder[5] and rat colon[6] in vitro preparations, and induced a natriuresis when injected into rats[5]; its activity was shown to be closely related to the sodium balance and the urinary sodium elimination in the rat[7]; it displaced ouabain bound to Na-K ATPase receptors of rabbit kidney at concentrations thought to be physiological[8].

Our purpose was to compare the respective modes of action, at the cellular level, of NF and NH_4Cl on those cellular events which are known to be induced by ouabain.

To do so, we first studied the electrical effects of NF and NH_4Cl in terms of short-circuit current (SCC), potential difference (PD) and calculated direct-current resistance (R) obtained in toad bladders mounted in chambers. Secondly, we examined the direct effects of NF and NH_4Cl on the activity of the Na-K ATPase isolated from toad bladder cells. The phenomena observed in the presence of NF and NH_4Cl were compared with those produced by ouabain.

Material and methods. SCC, PD, and R measurements. Hemibladders from Dominican *Bufo marinus* female toads were mounted in a conventional Ussing-chamber[5] with an exposed surface area of 2 cm^2. Both surfaces of the bladders were bathed with 5 ml of Ringer's solution (110 mM, NaCl; 2.5 mM, KCl; 2.0 mM, $MgCl_2$; 1.5 mM, $CaCl_2$; 10 mM, glucose; 5.0 mM, $NaHCO_3$). The solutions were gassed with an O_2 95%–CO_2 5% mixture. SCC and PD values were automatically recorded at 5-min intervals[8].

No membrane was employed for experiment unless the SCC and