CSIRO
Division of Fisheries and Oceanography

REPORT 118

A Method for Extraction and Analysis of Adenine Nucleotides for Determination of Adenylate Energy Charge in Molluscan Tissue

A. M. Ivanovici

1981

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION
DIVISION OF FISHERIES AND OCEANOGRAPHY
P.O. BOX 21, CRONULLA, NSW 2230

National Library of Australia Cataloguing-in-Publication Entry

Ivanovici, A. M. (Angela Maria)

A method for extraction and analysis of adenine nucleotides for determination of adenylate energy charge in molluscan tissue.

Bibliography.

ISBN 0 643 02568 5.

1. Nucleotides—Measurement. 2. Mollusks. I. Commonwealth Scientific and Industrial Research Organization (Australia). Division of Fisheries and Oceanography. II. Title. (Series: Report (Commonwealth Scientific and Industrial Research Organization (Australia). Division of Fisheries and Oceanography); no. 118).

594'.087'3280287

Printed by CSIRO, Melbourne

81.166-600

A METHOD FOR EXTRACTION AND ANALYSIS OF ADENINE NUCLEOTIDES FOR DETERMINATION OF ADENYLATE ENERGY CHARGE IN MOLLUSCAN TISSUE

A.M. Ivanovici

CSIRO Division of Fisheries and Oceanography
P.O. Box 21, Cronulla, NSW 2230

Present address: Department of Biochemistry
John Curtin School for Medical Research
Australian National University
P.O. Box 334, Canberra City ACT 2601

Aust. CSIRO Div. Fish. Oceanogr. Rep. 118 (1981)

Abstract

The application of adenylate energy charge as a monitoring tool for the assessment of physiological condition of marine and estuarine organisms requires standardized procedures for the quantitative extraction of ATP, ADP and AMP from the tissues of the test organisms and accurate determination of concentrations of these nucleotides for subsequent calculation of values of adenylate energy charge and other nucleotide ratios and totals.

This report provides a guide to analytical procedures suitable for the determination of *in vivo* levels of the three nucleotides, and consequently of adenylate energy charge and other nucleotide ratios and totals. The procedures and equipment needed for extraction and analysis of these nucleotides from molluscan tissue are described in detail. Experiments recommended for inclusion early in any experimental program to ascertain that results represent values as close as possible to *in vivo* levels are described. These experiments are illustrated with data obtained from the estuarine molluscs *Pyrazus ebeninus* and *Trichomya hirsuta*.

INTRODUCTION

The adenylate energy charge is a measure of the amount of energy potentially available for metabolism in an organism (Atkinson 1968), and is calculated from measured concentrations of ATP*, ADP and AMP as follows: ([ATP] + ½ [ADP])/([ATP] + [ADP] + [AMP]). Values range from 0 to 1, and, in a diverse range of organisms, correlate with growth state or physiological condition and various environmental conditions (e.g. microorganisms: Chapman *et al.* 1971, Montague and Dawes 1974, Wiebe and Bancroft 1975, Falkowski 1977; plants: Simmonds and Dumbroff 1974; molluscs: Ivanovici 1974, 1977a,b, 1980,

*Abbreviations used are in Appendix 1.

Wijsman 1976; mammals: Ridge 1972). This correlation of high values (0.8 to 0.9) with optimal environmental conditions, and lower values with less favourable conditions has resulted in the suggestion that measurements of adenylate energy charge may provide a concise, integrated and objective measure of the physiological state of an organism (Ivanovici 1974, 1980, Wiebe and Bancroft 1975, Ivanovici and Wiebe 1980).

Reliable estimates of adenylate energy charge approximating *in vivo* values, however, depend on careful extraction and accurate determinations of the concentrations of ATP, ADP and AMP. Newsholme and Start (1973), Bergmeyer (1974) and Beis and Newsholme (1975) have emphasized the importance of including suitable precautions and controls at all stages of sample processing, to ensure that measured concentrations of nucleotides are as close as possible to *in vivo* levels. Three procedural stages require close attention: i) the effect of manipulation of organisms prior to and during excision of tissue; ii) the effect of processing and storage of the tissue before, during and after extraction of the adenine nucleotides; iii) analysis of the nucleotides. When procedural effects on concentrations of the nucleotides occur, controls allow the determination of correction factors which are applied as necessary.

Differences in methodology at any of these stages and lack of adequate controls probably account for much of the variation in nucleotide concentrations and energy charges measured in various molluscan species (Horstmann 1965, Iwanowski and Hutny 1966, Hiltz and Dyer 1970, Zs.-Nagy and Ermini 1972, Beis and Newsholme 1975, Wijsman 1976). For example, although extraction of ATP and other metabolites from tissues of animals using cold perchloric acid is widely and successfully used (Hiltz and Dyer 1970, Faupel *et al.* 1972, Drews and Gilboe 1973, Criss 1973, Behm and Bryant 1975, Beis and Newsholme 1975, Burlington *et al.* 1976), with recovery rates of added nucleotides close to 100% (Beis and Newsholme 1975), Wijsman (1976) found this acid unsuitable as an extractant of ATP from mussel tissues. Recovery was low (value not given) and variability between individual mussels was high (coefficient of variation = 59%).

This report, in two parts, was written to provide a foundation for standardization of methodology. First, it describes in detail methods and equipment that yielded consistent and minimally variable results with two molluscan species *Pyrazus ebeninus* Brugiere and *Trichomya hirsuta* Lamark (Ivanovici 1976, 1977a, 1980). Second, it draws attention to the types of controls and preliminary experiments that ensure values as close as possible to *in vivo* levels by presenting data from experiments which examined the effects of various procedural aspects.

Several points should be emphasized at this stage:

i) no methods are available to determine the absolute levels of nucleotides in tissue; current methods allow only for checks on degradation or enhancement affects, while statistical analyses of data measure precision;

ii) some of the procedures established for the two species studied may be unsuitable for others (see Wadley *et al.* 1980);

iii) aspects such as effects of capture, manipulation and dissection procedures may present problems that are unique to the species studied.

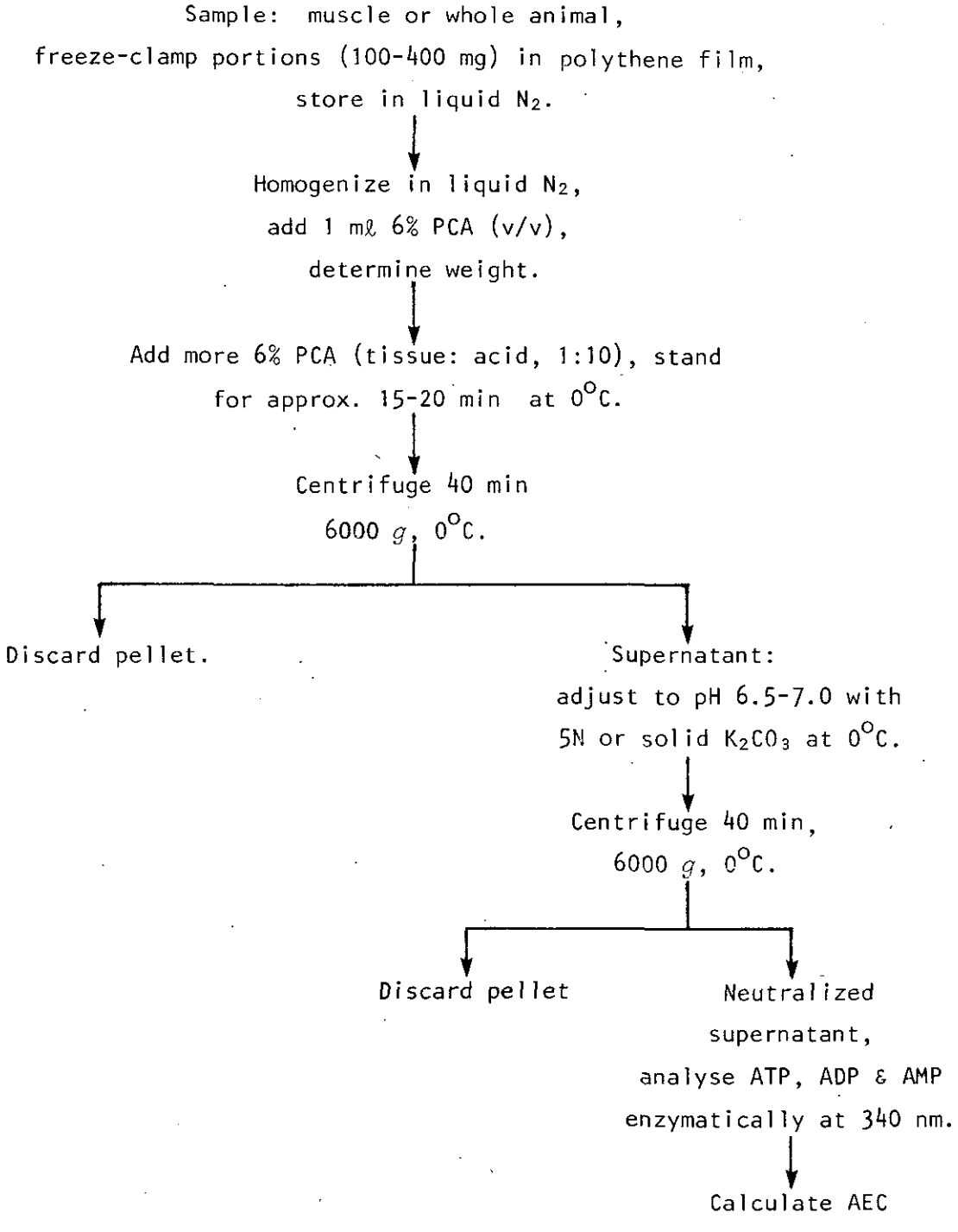


Fig. 1. Summary of procedure for the determination of adenine nucleotides and adenylate energy charge in molluscan tissues.

THE METHOD

Section 1. Procedures for extraction and analysis of adenine nucleotides

This section describes in detail the methods and equipment used for extraction and analysis of ATP, ADP and AMP from molluscan tissue. These procedures gave highly reproducible results (Ivanovici, 1977a, 1980). The methods were derived from Bergmeyer (1974) and Newsholme and Start (1973). A summary of the procedure after collection of the animals is presented in Fig. 1.

A. Extraction

1. Sampling of Tissue

(a) Procedure

Excess surface water was removed from the outside of the shell with tissue paper. The shell of *P. ebeninus* was broken at the penultimate whorl by applying steady pressure with a 10 cm vice, thus giving access to the columella muscle. This was rapidly dissected out, blotted dry, placed on a labelled polythene* strip and freeze-clamped with aluminium blocks (described in next section). These were cooled by frequent additions of liquid nitrogen to the clamp-box. An example of times taken at each main step for *P. ebeninus* is shown in Table 1. Tissue samples were, on average, removed and freeze-clamped within 1 min. Timing could not be controlled exactly, because variations in shell thickness determined how fast the shell could be broken. Significant correlations between total time and either adenine concentrations or AEC were, however, never found.

*This type of polythene film is used to prevent dehydration of food. The Gladwrap brand was found to be superior to other brands because it did not shatter as easily during handling.

The shell of *T. hirsuta* was also cracked with the vice. The attachment muscles (retractor and adductor) were severed as quickly as possible to free the body from the shell. Either the whole animal was used, or the posterior adductor muscle was dissected out and freeze-clamped within 1.5 min. The labelled samples were then stored in liquid N_2 until analysis. Samples were collected from animals in the field and the laboratory with equal ease.

(b) Equipment

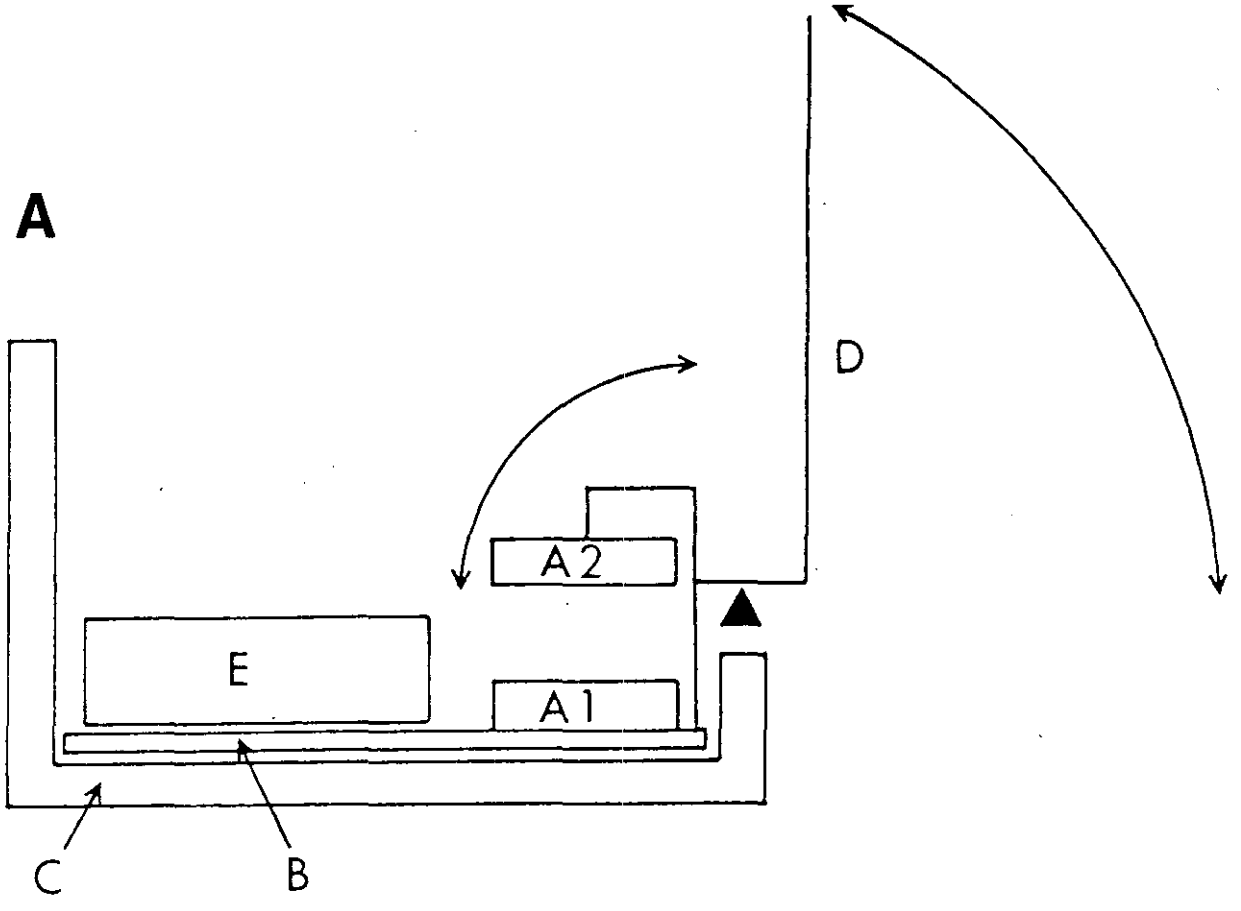
Freeze-clamps cooled to -180°C by liquid N_2 provide one of the fastest methods for stopping metabolic reactions in animal tissue and allow determination of *in vivo* concentrations of adenine nucleotides and other metabolites (Kretzschmar and Wilkie 1969, Hess and Brand 1974). Tissue sampled this way is cooled to about -160°C in less than 0.5s (Wollenberger *et al.* 1960).

The flat-tong design of Hohorst *et al.* (1959) was modified for operation by one person (Fig. 2). One 10 cm x 10 cm x 2.5 cm aluminium block (A1) was fixed to an aliminium plate (B), which in turn was fixed to the bottom of an expanded polystyrene box (C). An identical aluminium block (A2) was attached to a toggle lever (D). An expanded polystyrene block (E) was fixed inside the box to reduce its internal volume. The blocks were cooled by frequent additions of liquid N_2 during the sampling procedure. Block A2 was moved to a vertical position as the sample was brought into position, then rapidly closed. This procedure compressed the sample between the cold blocks into a thin layer of 1-2 mm (Fig. 3). The 2.5 cm thickness for the aluminium blocks is recommended because the cold is retained longer than by thinner blocks.

Table 1. Typical times for each step of the dissection procedure for columella muscle of *P. ebeninus*.

	Procedure	Time (s)
1.	Start break shell to start of dissection of tissue	12.8 ± 1.2[a]
2.	Start dissection of tissue to placement of tissue on Gladwrap	31.1 ± 1.9
3.	Fold Gladwrap to end of freeze-clamp	6.6 ± 0.5

a, mean of 18 experimental molluscs ± standard error.



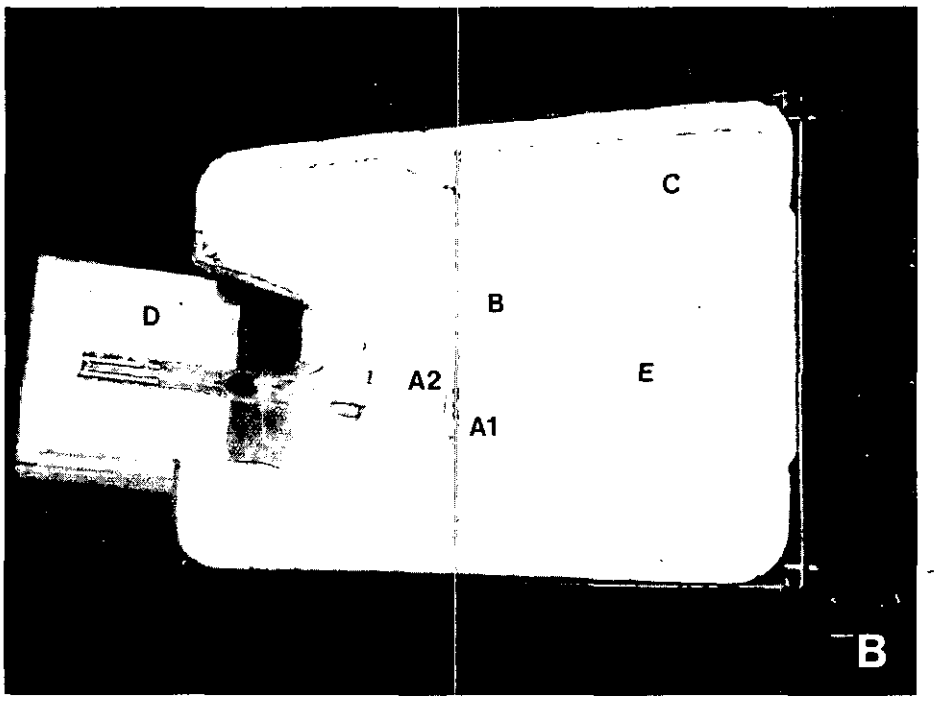


Fig. 2. A. Diagram showing design of freeze-clamps. A1, A2, 10 cm^2 x 2.5 cm aluminium blocks; B, aluminium plate; C, polystyrene; D, handle of lever; E, polystyrene block. Arrows indicate direction and extent of movement; ▲ indicates the fulcrum.

B. Photograph of freeze-clamps.

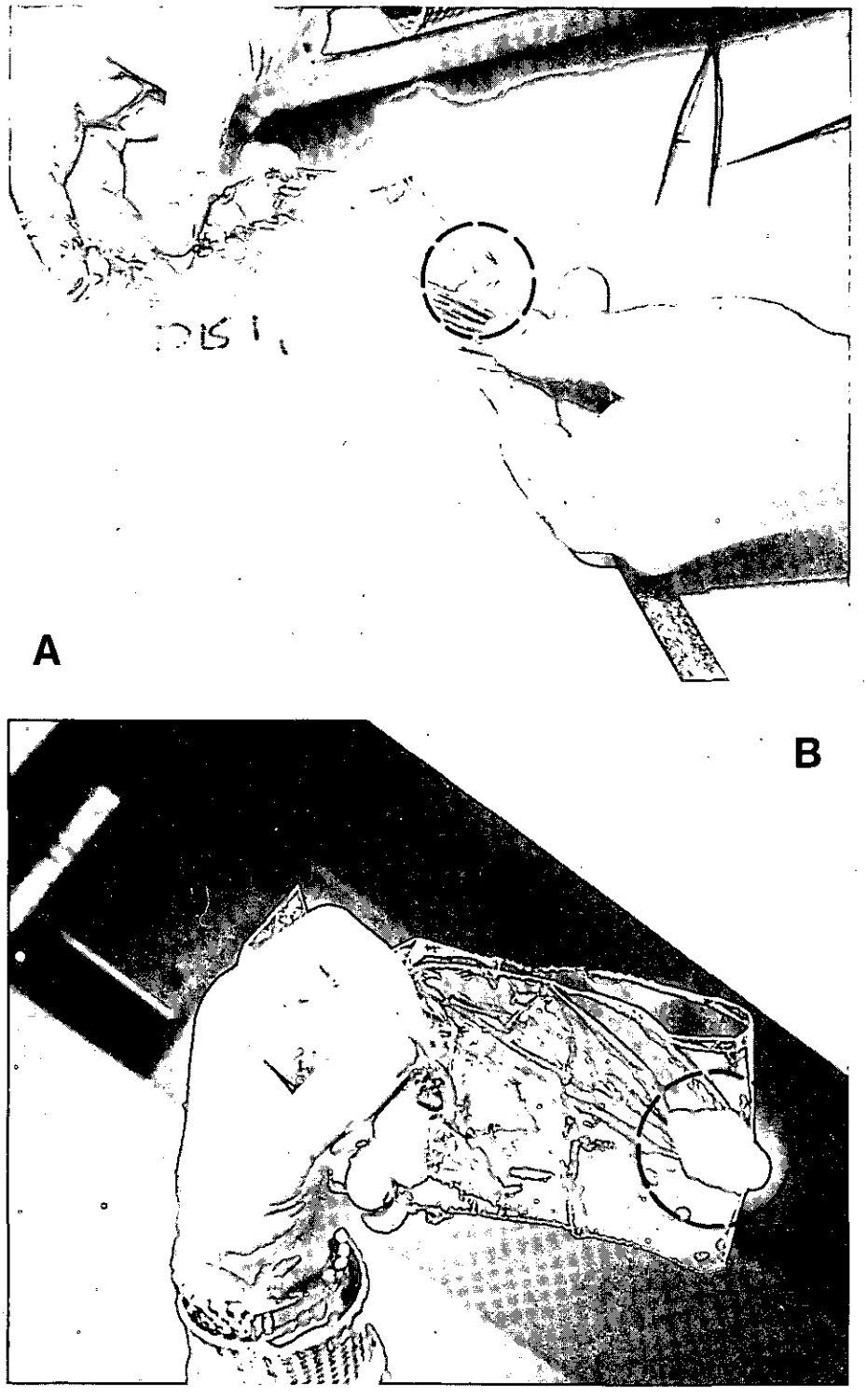


Fig. 3. A piece of columella muscle prior to (A) and after (B) freeze-clamping.

Dewar flasks recommended for collection and storage of samples, and for dispensing and storage of liquid N_2 are listed in Table 2. A design for a lid for the sample carriers is shown in Fig. 4. Its use is recommended to prevent samples floating into the dewar flask during storage.

2. *Extraction of adenine nucleotides*

(a) Procedure

The freeze-clamped tissue was quickly transferred from its wrapping (with forceps) to a stainless steel homogenizer (Fig. 5A) and ground to a fine powder. Both forceps and homogenizer were cooled in liquid N_2. For reasons of safety, the homogenization was done on the bench out of the cooling bath. The lower part of the homogenizer and the pestle were frequently placed in the cooling bath to maintain temperatures well below -90°C throughout the homogenizing procedure. PCA (1 mℓ, 6% v/v) was added to the homogenizer, allowed to freeze, then ground to powder and mixed with the sample. Grinding PCA with the tissue sample ensures that enzymes are immediately inactivated as the sample thaws (Bergmeyer 1963, Newsholme and Start 1973). The homogenate was loosened with a pre-cooled round-end spatula, then quickly tipped into a pre-weighed plastic centrifuge tube. Samples were not allowed to thaw until this step had been reached, and were subsequently kept on ice. Tissue weight was estimated by subtracting the known tube and PCA weights from the total weight (i.e., tube plus 1 mℓ PCA plus sample).

Alternatively, a homogenizing tube (Fig. 5B) may be cooled in liquid N_2, placed in a polyurethane insulator and weighed. The tissue may then be added to the homogenizer and a direct tissue weight estimated before the first addition of PCA. This procedure is suitable for samples as small as ~ 50 mg. Equipment for smaller samples (to 10 mg) has special requirements (Lush *et al.* 1979).

Further ice-cold PCA was added (the ratio of tissue to PCA was 1:10, w/v) and thoroughly mixed with a spatula. The samples were centrifuged in a Servall RC-2 centrifuge, HS4 rotor, at 0°C and 6,000*g* for 40 min. The supernatant was transferred with a Pasteur pipette to a tube containing 5-10 µℓ of Universal indicator (British Drug House), and adjusted to pH 6.5 (pale yellow to colourless) with either 5N K_2CO_3 or solid K_2CO_3. Tubes were left on ice for approximately 15 min, then centrifuged as before. The supernatant was carefully and quickly transferred to a graduated glass centrifuge tube and its volume measured. The sample was either assayed immediately or stored at -30°C. Universal indicator had no apparent inhibitory effect on the analysis of these molluscan adenine nucleotides. This does not preclude possible inhibitory effects on samples from other sources. If an effect is apparent, a pH meter with a single combined electrode may provide a suitable alternative. Care must be taken to keep the temperature of the supernatant low at all times.

(b) Equipment

A homogenizer (Fig. 5A) was made from corrosion and temperature resistant stainless steel (Type 316). All joints were silver soldered. Alternatively, homogenizers with detachable handles could be used as centrifuge tubes (Fig. 5B), but at least 18 to 36 tubes are needed if this design is selected. The internal diameter of the homogenizer should not exceed 1.9 cm. A larger diameter (with a correspondingly larger pestle) is not as efficient because the tissue is not pulverized as finely.

Table 2. List of dewar flasks recommended for the extraction procedure. The number of each type required is indicated in [ ]. A good combination of containers is marked by *.

Capacity	Material	Function	Brand
1ℓ [2]*	Nalgene[a] or stainless steel	Short term storage (<1h) and sample collection	Nalgene
2 or 4ℓ [1]*	As above	As above	As above
7 to 10ℓ [1 if large reservoir available; 2 if not]	Stainless steel, evacuated, with sample carriers	Collection of field samples; liquid N_2 supply for working up samples	Varies between countries
25ℓ [1]	As for 7ℓ, without sample carriers	Storage of N_2 supply	Varies between countries
30 to 35ℓ	As for 7ℓ, with sample carriers	Long-term storage of samples	Varies between countries
200ℓ or larger [1]	Metal	Storage of liquid N_2	This can usually be rented from a liquid gas company

[a] Nalgene containers are light, virtually unbreakable and well suited for laboratory and field use. Glass thermos flasks are not recommended because of their fragility.

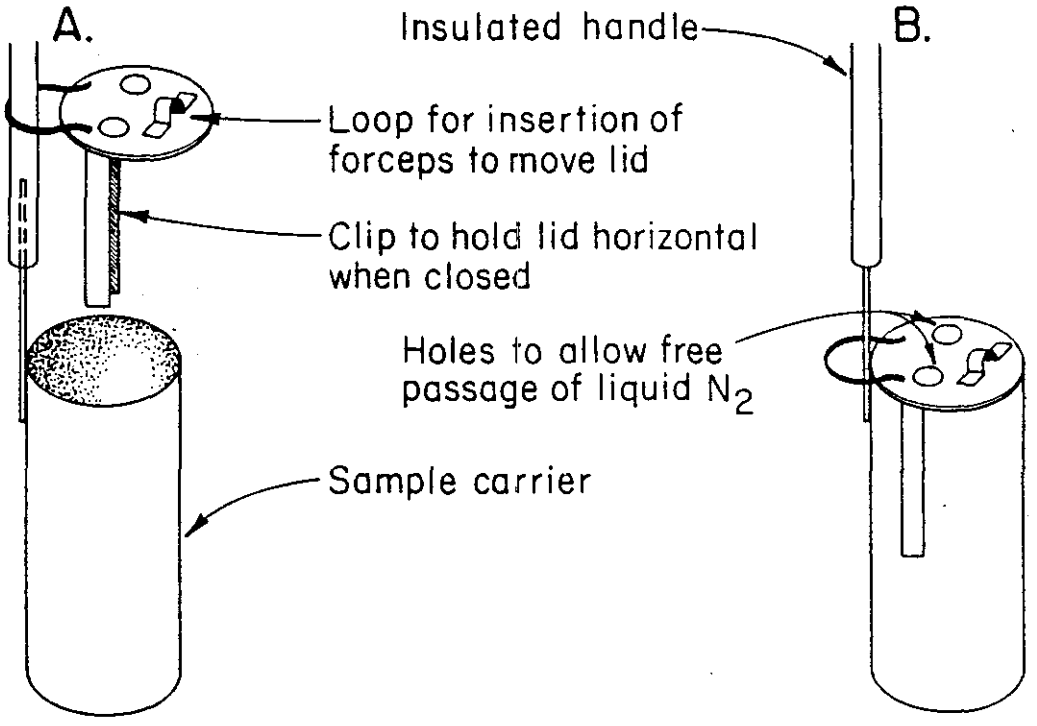


Fig. 4. Design of lid for sample carrier of tissue samples stored in liquid N_2 dewar. A. Lid in open position. B. Lid in closed position.

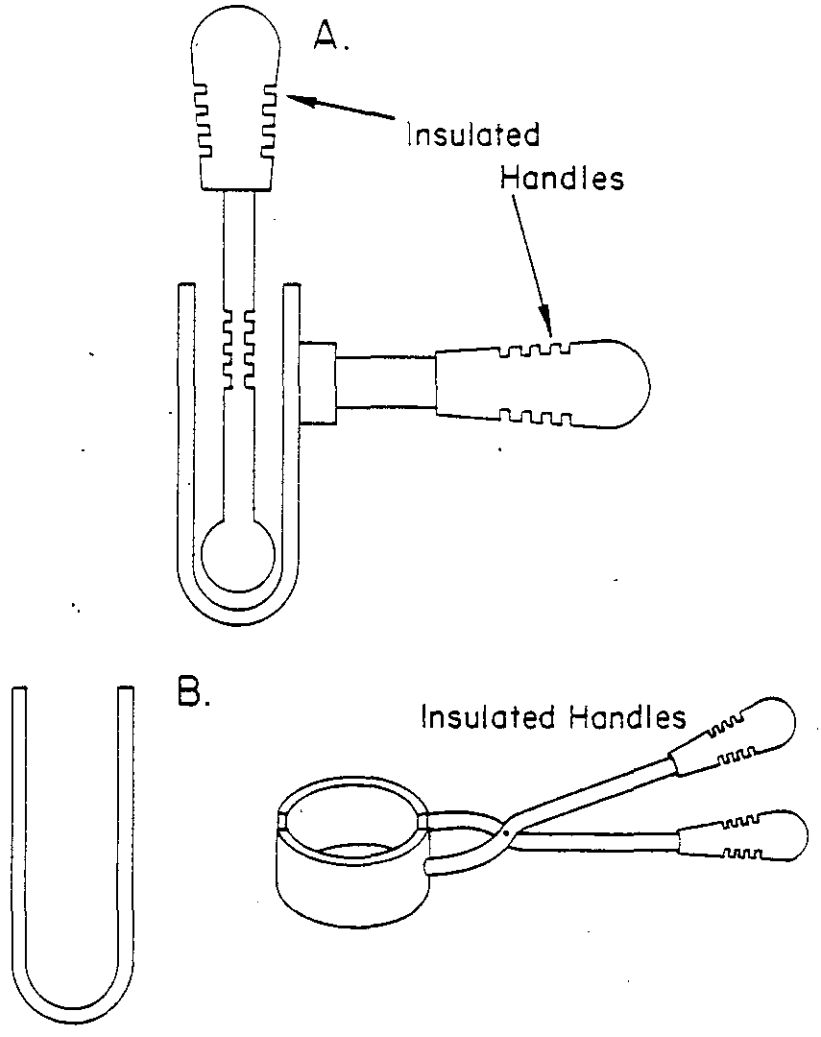


Fig. 5. Diagram of longitudinal sections of 2 types of homogenizers. A. handle attached to tube; B. handle and tube separated.

Wooden handles and leather gloves provided adequate insulation from the low temperatures. The liquid N_2 bath was an expanded polystyrene box that supported the mortar, pestle and an insulated spatula between the periods that the tissue was ground. Standard 10 to 15 cm spatulas were insulated at one end with suitable material.

B. Determination of adenine nucleotides

Methods of Lamprecht and Trautschold (1974) and Jaworek *et al.* (1974) were modified slightly as described in this section, to measure the concentrations of ATP, ADP and AMP. The luciferase assay (Strehler 1974) was not considered necessary, as concentrations of adenylates exceeded 10^{-9}M in *P. ebeninus* (Ivanovici 1974).

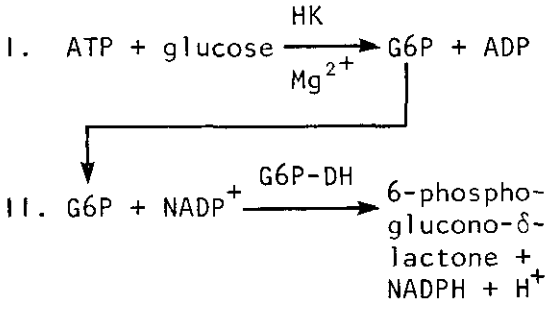
1. General principles

The sensitivity of spectrophotometric measurements depends on indicator compounds that have specific absorption maxima at given wavelengths. The enzymatic determination of ATP uses NADPH, while that of ADP and AMP uses NADH. NADPH and NADH absorb maximally at 340 nm. Their extinction coefficient at this wavelength (6.22 cm^2 $\mu mole^{-1}$) is used to calculate the ATP, ADP and AMP concentrations.

2. ATP

(a) Principle of assay

Glucose is phosphorylated by ATP to G6P with HK (reaction I). G6P then reacts with $NADP^+$ to form 6-phosphoglucono-δ-lactone and NADPH. This reaction is catalysed by G6P-DH (reaction II).



Thus, for every μmole of ATP, 1 μmole of NADPH is formed and causes an increase in absorbancy at 340 nm.

(b) Solutions

Reagents were made up in double distilled water, while the enzymes were diluted from the concentrated stocks with 3.2M ammonium sulphate. All enzymes and biochemical reagents were purchased from Boehringer Mannheim.

Stock solutions: 50 mM TEA, pH 7.6, (add 4.65g to about 200 mℓ water, adjust pH with 1N NaOH, make up to a final volume of 500 mℓ); 0.5M $MgCl_2$ (10.17g $MgCl_2.6H_2O$, make up to 100 mℓ; 0.4M D-glucose (7.21g, make up to 100 mℓ); 20 mM ATP* (ATP-$Na_2H_2.3H_2O$, 12.10 mg $mℓ^{-1}$, dilute 1 in 40 for use in assay).

Assay buffer for 50 assays: TEA, 142.5 mℓ; $MgCl_2$, 1.5 mℓ; NADP (15.75 mg $mℓ^{-1}$, make up on day of assay; no pH adjustment), 1.5 mℓ.

This buffer was stored at -40°C. It was stable for one week. There was no change in the response to 0.5 mM ATP standard over this period. A change in absorbancy (ΔA) of 0.096 ± 0.003 (95% confidence level) was obtained with 0.05 μmole of standard (i.e. 0.1 mℓ of the working standard 0.5 mM ATP; see below).

Enzymes (for 50 assays): 0.6 mg $mℓ^{-1}$ G6P-DH (Grade II, from yeast; 60 μℓ of 5 mg $mℓ^{-1}$ plus 440 μℓ 3.2M ammonium sulphate), 0.5 mℓ; 2.0 mg $mℓ^{-1}$ HK (from yeast, 200 μℓ of 10 mg $mℓ^{-1}$ plus 800 μℓ 3.2M ammonium sulphate), 1.0 mℓ. These enzyme solutions are stable indefinitely at 0-4°C, according to Bergmeyer (1974). No loss of activity was found over four months.

---

*These reagents can be stored indefinitely at -20°C and thawed as required.

Working Standard: A working standard of a mixture containing ATP, ADP and AMP (0.5 mM of each) was made up daily from stock solutions of 20 mM. The stock solutions were stored at -30°C for up to six months, and were thawed up to six times without significant breakdown of ATP or ADP.

(c) Procedure

The procedure for the ATP assay is summarized in Fig. 6. The cuvettes containing samples were compared with a reagent blank (water + buffer), to correct for any changes in absorbency that might be due to enzyme additions.

The assay buffer was brought to room temperature (20-25°C) and dispensed into numbered cuvettes (2.85 mℓ per cuvette). Aliquots (0.1 mℓ) of samples or standard were kept on ice until they were dispensed, in duplicate, into the cuvettes. G6P-DH (10 µℓ) was added to each cuvette, and the absorbancy (A1) read after 5 to 10 min. This allowed sufficient time for any endogenous G6P to be oxidized. Glucose and HK (20 µℓ of each) were added to each cuvette, readings (A2) taken when the reaction was complete (10-20 min at 20-25°C).

When new enzymes are obtained, the assay should be checked with 0.5 mM standards (G6P and ATP) to check their activity.

One or two cells containing the standard solution of ATP, which normally exceeded the ATP level in any sample, were examined first. In early assays, it was found that the reactions in all the other cells were complete by the time the reaction in the standard cell was complete. It was unnecessary, therefore, to scan the other cells for completion of reaction for any further length of time other than to obtain readings of absorbancy.

(d) Calculation of concentration of ATP

ATP (µmole g^{-1} wet weight tissue) =

$$\frac{\Delta A_{ATP} \times AV \times EV}{6.22 \times SV \times TW \times CPL}$$

ΔA_{ATP} = A2 - A1

AV = volume of solution in cuvette (3 mℓ)

EV = volume of neutralized super natant (mℓ)

6.22 = extinction of coefficient: i.e., absorbancy at 340 nm and pH 7.6 of a solution of NADPH containing 1 µmole $mℓ^{-1}$

TW = tissue weight (g)

SV = volume of neutralized super natant used in cuvette

CPL = cell path length, usually 1 cm.

ADP and AMP

(a) Principal of assay.

PK catalyses the phosphorylation of 1 µmole of ADP by PEP to form 1 µmole of ATP and pyruvate (Reaction II). Pyruvate in turn is converted to lactate by LDH. Thus, 1 µmole of ADP results in the formation of 1 µmole of NAD^+ (Reaction III). The decrease in absorbancy at 340 nm caused by the formation of NAD^+ from NADH is, therefore, proportional to the amount of ADP present in the sample. After this absorbance change (ΔA_{ADP}) has been measured in a sample, MK is added. This enzyme catalyses the formation of 2 µmoles of ADP from 1 µmole each of AMP and ATP (Reaction I). In turn, 2 µmmoles of NAD^+ are formed (Reactions II and III).

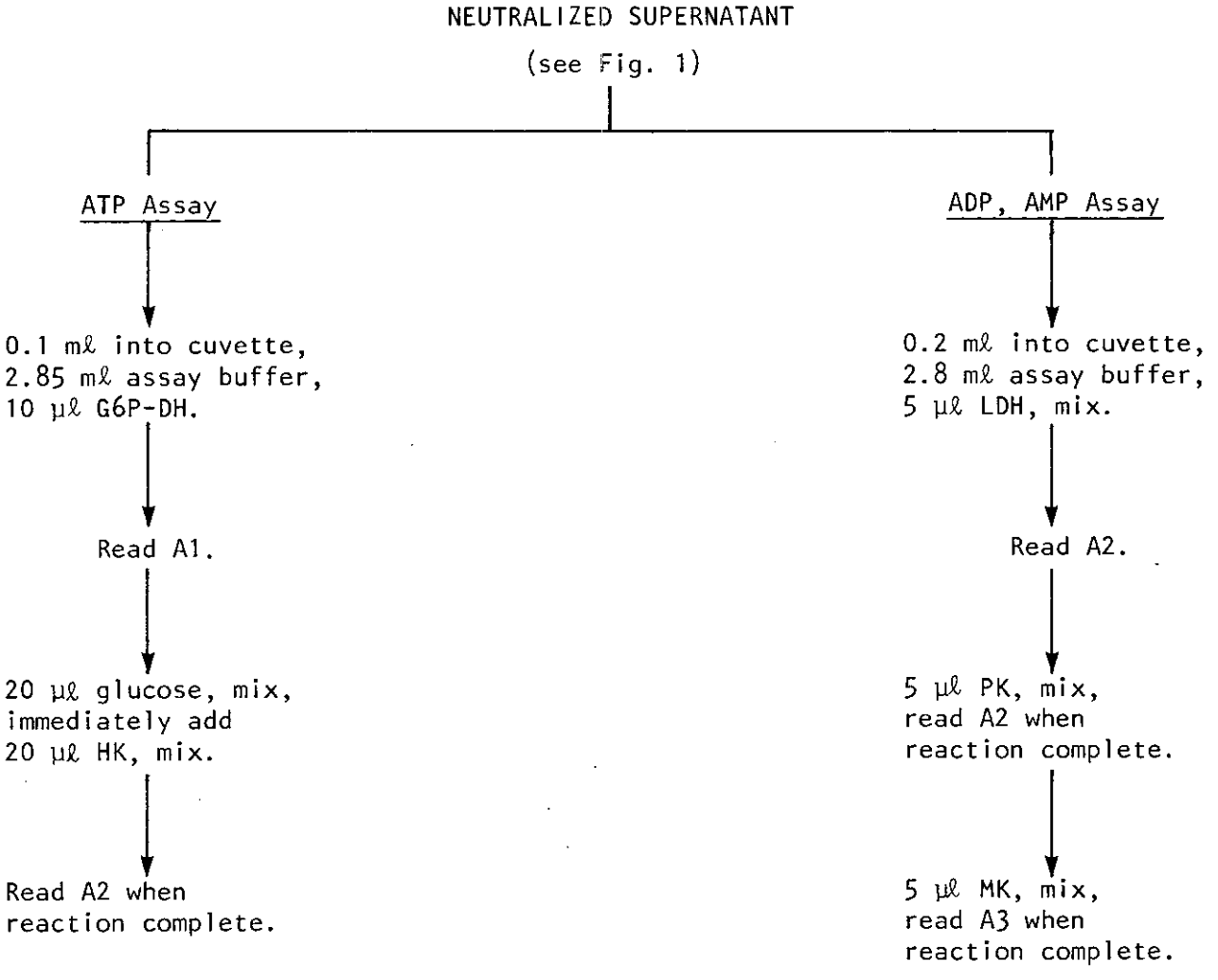


Fig. 6. Summary of procedures for analysis of ATP, ADP and AMP.

I. $AMP + ATP \underset{}{\overset{MK}{\rightleftharpoons}} 2\ ADP$

II. $2\ ADP + 2\ PEP \overset{PK}{\rightleftharpoons} 2\ ATP + 2\ pyruvate$

III. $2\ pyruvate + 2\ NADH = 2H^+ \overset{LDH}{\rightleftharpoons} 2\ lactate + 2\ NAD^+$

(b) Solutions

Reagents and enzymes were made up as described in Section 2 (b), for ATP.

Stock solutions: 50 mM TEA, pH 7.5; 0.5M $MgSO_4$ ($MgSO_4$ anhydrous: 6.02g, make up to a final volume of 100 mℓ; $MgSO_4.7H_2O$: 12.32g, make up to 100 mℓ); 2M KCl (14.91g, make up to 100 mℓ; 100 mg $mℓ^{-1}$ EDTA; 40 mM PEP* (PEP-$Na.H_2O$, 8.32 mg $mℓ^{-1}$); 10 mM NADH (disodium salt, Grade II, 7.09 mg $mℓ^{-1}$, make up to 5% $NaHCO_3$ solution); 10 mM ATP* (diluted from 20 mM stock solution); 20 mM ADP† (9.42 mg $mℓ^{-1}$); 20 mM AMP* (disodium salt $.6H_2O$, 9.98 mg $mℓ^{-1}$).

The following amounts of reagents were mixed to provide buffer for 50 assays: TEA, 130 mℓ; $MgSO_4$, 1.75 mℓ; KCl, 3.75 mℓ; EDTA, 0.25 mℓ; 40 mM PEP, 2.0 mℓ; 10 mM NADH, 1.5 mℓ; 10 mM ATP, 0.5 mℓ. This buffer was stable for one week. The working standard was a solution of ATP, ADP and AMP. The concentration of each nucleotide was 0.5 mM. Changes in absorbance (ΔA) for 50 µmole of ADP and AMP (i.e. 0.1 mℓ of 0.5 mM solutions) were 0.081 ± 0.005† and 0.194 ± 0.003 (95% confidence limit), respectively.

The following enzymes were diluted, the volumes indicated being sufficient for 50 assays: 1 mg $mℓ^{-1}$ LDH (from rabbit muscle; 60 µℓ of 5 mg $mℓ^{-1}$ plus 240 µℓ of 3.2M ammonium sulphate), 0.3 mℓ; 2 mg $mℓ^{-1}$ PK (from rabbit muscle; 60 µℓ of 10 mg $mℓ^{-1}$ plus 240 µℓ of 3.2 M ammonium sulphate), 0.3 mℓ; 1.25 mg $mℓ^{-1}$ MK (from rabbit muscle; 75 µℓ of 5 mg $ℓ^{-1}$ plus 225 µℓ of 3.2M ammonium sulphate), 0.3 mℓ.

(c) Procedure

The procedure for the ADP and AMP assay is summarized in Fig. 6. Assay buffer (2.8 to 2.85 mℓ, depending on sample size) at 20-25°C was pipetted into numbered cuvettes. Samples (0.15 to 0.20 mℓ were dispensed into cuvettes, then LDH (5 µℓ). After 5-10 min, A1 was read for each cuvette. PK (5 µℓ) was added to all cuvettes, and A2 read after completion of the PK reaction (5 min). Finally, MK (5 µℓ) was added, and A3 was read after 15-30 min. The working standard was used to monitor the completion of the PK and MK reactions, as already described for the ATP assays.

(d) Calculation of concentrations

$$\text{ADP (µmole g}^{-1}\text{ wet weight tissue)} = \frac{\Delta A_{ADP} \times AV \times EV}{6.22 \times SV \times TW \times CPL}$$

$$\text{AMP (µmole g}^{-1}\text{ wet weight tissue)} = \frac{\Delta A_{AMP} \times AV \times EV}{6.22 \times SV \times TW \times 2 \times CPL}$$

$\Delta A_{ADP} = A1 - A2$

$\Delta A_{AMP} = A2 - A3$

6.22 = absorbancy at 340 nm and pH 7.6 of a solution of NADH containing 1 µmole $mℓ^{-1}$. Other terms are as described for the ATP assay.

4. Equipment

Absorbancy was measured in the visible range at 340 nm with a Varian split-beam spectrophotometer (Model

---

*These reagents can be stored indefinitely at -20°C and thawed as needed.

†Boehringer ADP is only 80% active and thus gives a ΔA 20% less than ATP or AMP.

365). The enzyme reactions were followed with a National recorder (Model VP-652B, 2 pen). Sarstedt plastic cuvettes (light path 1 cm) were used for the assays. Enzymes were mixed into the cuvettes with plastic paddles (plumpers: Boehringer Mannheim or Calbiochem).

5. Calculations

The AEC and ATP/ADP were calculated from the mean value of duplicate determinations of concentrations of ATP, ADP and AMP.

Section 2. Optimization of methods

This section presents results of experiments that were useful in evaluating the effects of extraction and analytical procedures on the determinations of the adenine nucleotides. Three areas were examined:

i) effects of delays in the processing procedure;

ii) stability of extracts under various storage conditions;

iii) recovery of added standards and stability of the assays.

A fourth area - the suitability of extractants and freezing procedures for samples from various species - is described elsewhere (Wadley *et al.* 1980).

The procedures outlined below do not determine *absolute* levels of adenine nucleotides extracted from tissue. However, the consistency of values measured in samples collected at different times suggests that they may closely approximate absolute values. Despite limitations, the procedures are useful because they show where losses occur, and thus where corrections are necessary. Details of methods used are in the legends of Tables 3-12 (pages 19-23).

1. Delays in processing

a) Delays between collection of animals and sampling of tissue.

When samples are collected in the field, delays of up to 4h may occur between collection of the test animal and dissection and clamping of tissues. The effects of such delays on the adenylate energy charge, and adenylate pool of *P. ebeninus* were negligible for up to 6h (Table 3) as values did not change significantly over this period. These data indicated that sampling could be delayed for several hours if necessary without a change in values.

This lack of effect may not apply to species which are more active than the example presented here, or to species of other phyletic groups. The effect of delays of this type should therefore be examined before extensive sampling.

b) Delays occurring before and during dissection.

The effects of delays that occur during the handling of the animal or during dissection of samples on levels of adenine nucleotides need to be determined for each selected species, since such effects may result in erroneous determinations of nucleotide levels (Faupel *et al.* 1972, Newsholme and Start 1973, Beis and Newsholme 1975).

Experiments with *P. ebeninus* showed that energy charge, ATP and total nucleotides were unaffected by delays in handling prior to dissection in less than 90s (Table 4) and during the dissection itself (Table 5). Correlations between delay-times and adenylate energy charge and other nucleotide measurements were not significant. Although these data suggest that delays during dissection have less effect than during handling in *P. ebeninus*, other species may respond differently and thus need to

be checked thoroughly before extensive experiments are done.

c) Delays between freeze-clamping and analysis.

Freeze-clamped tissues were stored in liquid N_2 until analysed. Delays of up to 15 weeks occurred before some samples of *P. ebeninus* could be analysed. Since information about the stability of nucleotides in tissue stored in this manner was unavailable, the stability of the nucleotides was tested (see legend of Table 6).

The effect of a delay of up to 15 weeks on adenylate energy charge and ATP levels was negligible (Table 6). No significant changes occurred during the test period. Although not tested here, longer storage would probably have minimal effects. Freeze-drying of samples may provide a suitable alternative for stable, long-term storage, but was not examined here.

2. Stability of nucleotides in neutralized extracts stored under various conditions.

Opinion varies so much about the stability of nucleotides stored under differing conditions at or below $0^{o}C$ (Table 7), that it was considered necessary to test extracts of *P. ebeninus*.

Data in Table 8 indicate that either $-30^{o}C$ or $-180^{o}C$ were suitable storage temperatures for neutralized supernatants for up to 4 weeks. While not significantly different, the lower value of adenylate energy charge in samples kept on ice for 48h (Table 9) suggested that the better procedure is to freeze samples if they cannot be analysed immediately.

3. Recovery of nucleotide standards.
a) Recovery from molluscan tissue.

Using the method of extraction and analysis of ATP described earlier in this Report, recovery of added soluble ATP was determined in *P. ebeninus* and *T. hirsuta* by comparing concentrations of spiked and unspiked tissue samples (see details in legend of Table 10).

Mean recoveries were greater than 90% for both species (Table 10), and are similar to levels found by Ridge (1972). Wijsman (1976) found PCA unsuitable as an extractant for mussel tissue (recovery low and precision of 59% coefficient of variation), but this may have been due to his method of homogenizing at $0^{o}C$ instead of well below freezing. Beis and Newsholme (1975) obtained consistent recoveries and concentrations of ATP in *Helix pomatia* (gastropod), *Pecten maximus* and *M. edulis* (bivalves) using a method similar to that used here (coefficients of variation: 10-20%). The high rates of recovery in Table 10 and high precision showed that PCA was a suitable extractant for these species (see also Wadley *et al.* 1980). Correction was considered unnecessary as recoveries were consistently better than 90%.

b) Inhibitory effects of neutralized tissue-extracts on the nucleotide assay systems.

Known amounts of ATP, ADP and AMP were added to neutralized extracts as internal standards and assayed to check for inhibitory (or enhancement) effects by the extract.

Results (Table 11) indicated virtually no inhibition, and hence no need for correction factors with the extracts from these species.

c) Stability of the assay systems.

Since up to 30 samples were analysed at one session, the times recommended by Lamprecht and Trautschold (1974) and Bergmeyer (1974) as adequate for the reactions to reach completion were exceeded. Although Bergmeyer (1974) suggested that absorbancies may not remain stable if recommended

times were exceeded, this did not occur here. Lack of significant changes in absorbance (Table 12) indicated that delays in reading absorbance did not introduce artifacts into the data.

DISCUSSION

The experiments described here were intended as examples of the types of procedures that are suggested for optimization of extraction and analysis of the adenine nucleotides for calculation of adenylate energy charge. While these procedures help to obtain consistent and reproducible values, they do not guarantee the determination of *absolute* nucleotide values, but rather, *apparent* and approximate *in vivo* values. Difficulty in measuring bound nucleotides prevents estimates of absolute values. An estimate of *in vivo* levels may be approximated by comparing the results obtained with different extraction methods (e.g. Wadley *et al.* 1980).

The data described here showed the following features for the two species used as examples:

i) delays of various types had little effect on measurements of adenylate energy charge;

ii) nucleotide levels were stable when samples were stored in liquid N_2 (unextracted) or at $-30^{o}C$ (extracted);

iii) extraction of nucleotides by PCA was efficient and reproducible, and had no effect on the analytical systems used.

While delays in the dissection procedures (within certain limits) did not affect the nucleotides, this may well result from the sessile nature of the two example species. Dissection timing and handling may be more critical for highly mobile organisms (e.g. crabs, fish). Particular care should be taken to evaluate and minimize responses for such organisms.

If the above procedures do not yield consistent results with good precision, variations of the procedures are recommended. For example, Wadley *et al.* (1980) varied the dissection procedure to improve nucleotide determinations in two bivalve species. Extraction media may be varied, e.g. boiling bicarbonate or cold H_2SO_4 (0.6N) instead of PCA (see comparison in Wadley *et al.*, 1980). The size of the organism may require more sensitive analytical procedures such as fluorimetry, use of luciferase (Strehler 1974) or high performance liquid chromatography (Lush *et al.* 1979).

In conclusion, it is reiterated that the procedures which were established for the two molluscan species described here do not necessarily apply to other organisms. It is important, therefore, that adequate tests be conducted in the early stages of experiments on the particular species of interest. While variation from the methods outlined in this paper may prove to be necessary, they provide a guideline which should yield *in vivo* values. This will ensure the accuracy and precision of subsequent results.

ACKNOWLEDGEMENTS

This research was part of a Ph.D. program at the School of Biological Sciences (University of Sydney) during 1975 and 1976. Financial support was provided by a Commonwealth Postgraduate Scholarship, University of Sydney Research Grants and the CSIRO Division of Fisheries and Oceanography, Cronulla. I wish to thank Drs A.J. Underwood, W.J. Wiebe and M.D. Montague for their helpful criticisms and discussions.

Table 3. The effect of delay on energy charge and nucleotide concentration (µmole/g wet tissue weight) between collection of *P. ebeninus* from a field site and freeze-clamping of tissue. Twenty-four molluscs were collected from the estuary at low tide, then kept out of water for up to 6h. Six were freeze-clamped within 30 min of collection, and subsequent groups of 6 at 2, 4 and 6h.

Time of delay(s)	Adenylate energy charge	ATP	(ATP + ADP + AMP)
0	0.62 ± 0.09[a]	2.72 ± 0.86	5.73 ± 0.80
2	0.66	3.15	5.90
4	0.66	3.04	5.42
6	0.68	3.12	5.38
F-ratio (df: 20,3)	0.56 ns	0.35 ns	0.64 ns

a, means of 6 molluscs ± 95% confidence limits (calculated from the mean-square-within from analysis of variance); df, degrees of freedom; ns, not significant, $p > 0.05$.

Table 4. Effect of delay on energy charge and nucleotide concentration (µmole/g wet tissue weight) prior to dissection of columella muscle for freeze-clamping from *P. ebeninus*. Dissection was delayed for varying periods after the mollusc's shell was broken to give clamp times shown.

Time of delay(s)	Adenylate energy charge	ATP	(ATP + ADP + AMP)
60	0.78[a] ± 0.16	3.63 ± 1.29	5.62 ± 0.84
90	0.76	4.10	6.69
120	0.63	2.76	5.73
180	0.62	2.58	5.75
240	0.53	2.22	5.28
F-ratio (df: 4,15)	1.66 ns	1.64 ns	1.79 ns

a, mean of 4 animals ± 95% confidence limit (as in Table 3); all other abbreviations as in Table 3.

Table 5. Effect of delay on energy charge and nucleotide concentration (μmole/g wet tissue weight) after dissection and prior to freeze-clamping of columella muscle from *P. ebeninus*. After removing the muscle, portions of it were freeze-clamped at the indicated times.

Time of delay(s)	Adenylate energy charge	ATP	(ATP + ADP + AMP)
59	0.86[a] ± 0.08	2.64 ± 0.98	3.59 ± 0.21
92	0.82	3.06	4.52
122	0.88	2.86	4.30
180	0.80	2.65	3.84
F-ratio$_{(df: 3,11)}$	3.25 ns	1.02 ns	<1.0 ns

a, means of 5 animals ± 95% confidence limit: as in Table 3; all other abbreviations as in Table 3.

Table 6. The effect of liquid N_2 storage on levels of ATP and the adenylate energy charge of freeze-clamped columella muscle of *P. ebeninus*. Muscle was freeze-clamped and stored in liquid N_2. Portions were removed and assayed at the times indicated. Care was taken to prevent thawing of tissues when subsamples were removed.

Storage time (weeks)	ATP μmole g^{-1} wet tissue weight	Adenylate energy charge
0	3.39[a] ± 0.71	0.84 ± 0.05
3	4.01	0.83
12	4.65	0.84
15	3.94	0.86
t-test	ns	ns

a, means of 6 molluscs ± 95% confidence limit (calculated from the pooled variance).

Table 7. A selection of storage conditions for neutralized and acid-extracted samples assayed for ATP, ADP and AMP.

	Storage time	Storage Temperature	Reference
1.	Not mentioned	0°C, < 0°C	Lamprecht and Trautschold, 1974
2.	8 days	-30°C	Jaworek *et al.*, 1974
3.	Not mentioned	-20°C	Strehler, 1974
4.	48 hours	< 0°C	Montague and Dawes, 1974
5.	3 weeks	< 0°C	Montague (1975, pers. comm.).
6.	1.5 months	< 0°C	Karl and LaRock, 1975
7.	1 year	-80°C	Dietzler *et al.*, 1974
8.	2 days	0 to 3°C	Ball and Atkinson, 1975

Table 8. Stability of adenine nucleotides, expressed as adenylate energy charge, in neutralized extracts, stored for one month. Neutralized supernatant from each mollusc was divided into five aliquots and stored as indicated below. Supernatants were discarded after analysis,not refrozen.

Time and temperature of storage	Adenylate energy charge
< 4h, 0°C	0.88[a] ± 0.017
2 wks, -180°C	0.89
2 wks, -30°C	0.89
4 wks, -180°C	0.88
4 wks, -30°C	0.87
F-ratio$_{(df:4,25)}$	0.98 ns

a, mean of 6 individuals ± 95% confidence limit (as in Table 3); all other abbreviations as in Table 3.

Table 9. Stability of adenylate energy charge in supernatants extracted from *T. hirsuta* and stored for 48h.

Time (h) and temperature of storage	Adenylate energy charge
< 4h, 0°C	0.74[a] ± 0.062
48h, 0°C	0.69
48h, -30°C	0.76
F-ratio$_{(df:2,9)}$	1.58 ns

a, mean of four molluscs ± 95% confidence limit (as in Table 3); all other abbreviations as in Table 3.

Table 10. Percentage of added ATP recovered from, and concentrations of ATP in muscle tissue of *P. ebeninus* and *T. hirsuta*. 0.1 ml 20 mM ATP was added to 0.2g frozen tissue or 0.2 ml distilled water in a homogenizer and processed. ATP was also measured in 0.2g tissue. Percentage recovery was calculated by:

$$\frac{([ATP_{Tissue + Standard}] - [ATP_{Tissue}])}{[ATP_{Standard}]} \times 100$$

Species	% recovered ATP[a]	ATP µmole g^{-1} wet weight
P. ebeninus	92.0 ± 3.7%	3.39 ± 10.7%
T. hirsuta	109.4 ± 12.8%	1.27 ± 17.6%

a, mean of six animals ± coefficient of variation

Table 11. Percentage recovery of nucleotide standards from neutralized supernatants, determined as in Table 10, for the addition of 23×10^{-3} µmoles ATP, ADP or AMP. Values are the means of four estimations ± 95% confidence limits.

Standard added	Percentage recovery
ATP	93.8 ± 10.5
ADP	95.5 ± 8.4
AMP	101.9 ± 9.0

Table 12. Mean absorbance of duplicate samples read at selected times after addition of sample or enzyme.

Assay and Additions		A_{340} Time of Reading (min) 0	30	45	60	120
ATP: Sample		0.001	0.002	n	-0.001	0.003
G6P-DH		0.005	0.004	0.003	0.002	
HK		n	0.102	0.102	0.100	
ADP and AMP:	Sample	0.005	0.006	n	0.003	0.006
	LDH	0.005	0.002	0.006	0.005	
	PK	n	-0.080	-0.080	-0.078	
	MK	n	-0.250	-0.252	-0.249	

n, reading not taken

REFERENCES

Atkinson, D.E. (1968). Citrate and the citrate cycle in the regulation of energy metabolism. In 'Metabolic Roles of Citrate' (Ed. T.W. Goodwin) *Biochem. Soc. Symp.* 27, 23-40. (Academic Press: New York).

Ball, W.J. and Atkinson, D.E. (1975). Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. *J. Bacteriol.* 121, 975-982.

Behm, C.A. and Bryant, C. (1975). Studies of regulatory metabolism in *Moniezia expansa*: the role of phosphofructokinase (with a note on pyruvate kinase). *Int. J. Parasit.* 5, 339-346.

Beis, I. and Newsholme, E.A. (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. *Biochem. J.* 152, 23-32.

Bergmeyer, H.U. (1963). Experimental techniques. In 'Methods of Enzymatic Analysis'. (Ed. H.U. Bergmeyer.) pp. 14-42. (Academic Press: New York).

Bergmeyer, H.U. (Ed.). (1974). Methods of enzymatic analysis. Vols. 1 to 4. (Academic Press: New York).

Burlington, R.F., Meininger, G.A. and Thurston, J.T. (1976). Effects of low temperatures on high energy phosphate compounds in isolated hearts from a hibernator and a non-hibernator. *Comp. Biochem. Physiol.* 55B, 403-407.

Chapman, A.G., Fall, L. and Atkinson, D.E. (1971). Adenylate energy charge in *Escherichia coli* during growth and starvation. *J. Bacteriol.* 108, 1072-1086.

Criss, W.E. (1973). Control of the adenylate charge in the Morris "minimal-deviation" hepatomas. *Cancer Res.* 33, 51-56.

Dietzler, D.N., Lais, C.J. and Leckie, M.P. (1974). Simultaneous increases of the adenylate energy charge and the rate of glycogen synthesis in nitrogen-starved *Escherichia coli* W4597(K). *Arch. Biochem. Biophys.* 160, 14-25.

Drews, L.R. and Gilboe, D.D. (1973). Cerebral metabolite and adenylate energy charge recovery following 10 minutes of anoxia. *Biochim. Biophys. Acta* 320, 701-707.

Falkowski, P.G. (1977). The adenylate energy charge in marine phytoplankton: the effect of temperature on the physiological state of *Skeletonema costatum* (Greve.) Cleve. *J. Exp. Mar. Biol. Ecol.* 27, 37-45.

Faupel, R.P., Seitz, H.J., Tarnowski, W., Thiemann, V. and Weiss, C.H. (1972). The problem of tissue sampling from experimental animals with respect to freezing techniques, anoxia, stress and narcosis. *Arch. Biochem. Biophys.* 148, 509-522.

Hess, B. and Brand, K. (1974). Cell and Tissue Disintegration. In 'Methods of Enzymatic Analysis' (Ed. H.U. Bergmeyer.) pp. 396-413. (Academic Press: New York).

Hiltz, D.F. and Dyer, W.J. (1970). Principal acid-soluble nucleotides in assuctor muscle of the scallop *Placopecten magellanicus* and their degradation during postmortem storage in ice. *J. Fish. Res. Bd Can.* 27, 83-92.

Hohorst, H.J., Kreutz, F.H. and Bucher, T.L. (1959). Uber Metabolitgehalte und Metabolitkonzentrationen in der Lever der Ratte. *Biochem. Z.* 332, 18-46.

Horstmann, H.J. (1965). Untersuchungen zum Galaktogen-Stoffwechsel der Weinbergschnecke (*Helix pomatia* L.). *Biochem. Z.* 342, 23-30.

Ivanovici, A.M. (1974). *Pyrazus ebeninus*, responses to salinity and measurement of stress. *Aust. Mar. Sci. Bull.* 47, 10.

Ivanovici, A.M. (1976). The adenylate energy charge as an estimator of stress in an estuarine mollusc. *Aust. Mar. Sci. Bull.* 55, 5-6.

Ivanovici, A.M. (1977). Adenylate energy charge and physiological stress in the estuarine gastropod, *Pyrazus ebeninus*. Ph.D. thesis, Univ. of Sydney.

Ivanovici, A.M. (1977b). Characterization of adenylate energy charge in the estuarine molluscs, *Pyrazus ebeninus* and *Trichomya hirsuta*, under a range of environmental conditions. *Proc. Biochem. Soc.* 7, 44.

Ivanovici, A.M. (1980). The adenylate energy charge in the estuarine mollusc, *Pyrazus ebeninus*. Laboratory studies of responses to salinity and temperature. *Comp. Biochem. Physiol.* 66A, 43-55.

Ivanovici, A.M. and Wiebe, W.J. (1980). Towards a working definition of "stress": a review and critique. In 'Stress effects on natural ecosystems' (Ed. G.W. Barrett and R. Rosenberg). John Wiley and Co. (in press).

Iwanowski, H. and Hutney, J. (1966). Chromatographic analysis of phosphate esters of sugars and nucleotides in the sphincter muscles of sweet water clams (*Unio* sp.). *Arch. Immun. Therap. Exp.* 14, 508-515.

Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974). Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In 'Methods of Enzymatic Analysis'. (Ed. H.U. Bergmeyer.) 4, 2127-2131. (Academic Press: New York).

Karl, D.M. and Larock, P.A. (1975). Adenosine triphosphate measurements in soil and marine sediments. *J. Fish. Res. Bd Can.* 32, 599-608.

Kretzschmar, K.M. and Wilkie, D.R. (1969). A new approach to freezing tissues rapidly. *J. Physiol.* 202, 66 p.

Lamprecht, W. and Trautschold, I. (1974). Adenosine-5'-triphosphate: determination with hexokinase and glucose-6-phosphate dehydrogenase. In 'Methods of Enzymatic Analysis'. (Ed. H.U. Bergmeyer.) 4, 2101-2110. (Academic Press: New York).

Lush, C., Rahim, Z.H.A., Perrett, D. and Griffiths, J.R. (1979). A microprocedure for extracting tissue nucleotides for analysis by high-performance liquid chromatography. *Anal. Biochem.* 93, 227-232.

Montague, M.D. and Dawes, E.A. (1974). The survival of *Peptococcus prevotii* in relation to the adenylate energy charge. *J. Gen Microbiol.* 80, 291-299.

Newsholme, E.A. and Start, C. (1973). Regulation in Metabolism. John Wiley and Sons: London, 349 pp.

Ridge, W. (1972). Hypoxia and the energy charge of the cerebral adenylate pool. *Biochem. J.* 127, 351-355.

Simmonds, J.A. and Dumbroff, E.B. (1974). High energy charge as a requirement for axis elongation in response to gibberellic acid and kinetin during stratification of *Acer saccharum* seeds. *Plant Physiol.* 53, 91-95.

Strehler, B.L. (1974). Adenosine-5'-triphosphate and creatine phosphate: determination with lucerifase. In 'Methods of Enzymatic Analysis'. (Ed. H.U. Bergmeyer.) 4, 2112-2121. (Academic Press: New York).

Wadley, V.A., Ivanovici, A.M. and Rainer, S.F. (1980). A comparison of techniques for the measurement of adenosine phosphates in three species of estuarine mollusc. Aust. CSIRO Div. Fish. Oceanogr. Rep. 129.

Wiebe, W.J. and Bancroft, K. (1975). Use of the adenylate energy charge ratio to measure growth state of natural microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 72, 2112-2115.

Wijsman, T.C.M. (1976). Adenosine phosphates and energy charge in different tissues of *Mytilus edulis* under aerobic and anaerobic conditions. *J. Comp. Physiol.* **107**, 129-140.

Wollenberger, A., Ristau, O. and Schoffa, G. (1960). Eine einfache Technik der extrem schnellen Abkuhlüng großerer Gewebestücke. *Pfluegers. Arch. Eur. J. Physiol.* 270, 399-412.

Zs.-Nagy, I. and Ermini, M. (1972). ATP Production in the tissues of the bivalve *Mytilus gallo-provincialis* (Pelecypoda) under normal and anoxic conditions. *Comp. Biochem. Physiol.* 43B, 593-600.

APPENDIX 1: ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
EDTA	ethylenediaminetetracetic acid
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49)
HK	hexokinase (D-hexose 6-phosphotransferase, EC 2.7.1.1)
LDH	lactate dehydogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27)
MK	myokinase (ATP:AMP phosphotransferase, EC 2.7.4.3)
NAD	β-nicotinamide-adenine dinucleotide, oxidized form
NADH	β-nicotinamide-adenine dinucleotide, reduced form
NADP	β-nicotinamide-adenine dinucleotide phosphate, oxidized form
NADPH	β-nicotinamide-adenine dinucleotide phosphate, reduced form
PCA	perchloric acid
PEP	phosphoenolpyruvate
PK	pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40)
TEA	triethanolamine hydrochloride buffer

CSIRO
Division of Fisheries and Oceanography

HEADQUARTERS
202 Nicholson Parade, Cronulla, NSW
P.O. Box 21, Cronulla, NSW 2230

NORTHEASTERN REGIONAL LABORATORY
233 Middle Street, Cleveland, Qld
P.O. Box 120, Cleveland, Qld 4163

WESTERN REGIONAL LABORATORY
Leach Street, Marmion, WA 6020
P.O. Box 20, North Beach, WA 6020