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A COMPARISON OF TECHNIQUES FOR THE MEASUREMENT OF ADENINE NUCLEOTIDES IN THREE SPECIES OF ESTUARINE MOLLUSC

V.A. Wadley, A.M. Ivanovici, and S.F. Rainer*

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

*Present address:

Dept Biochemistry, John Curtin School of Medical Research
Australian National University, PO Box 334, Canberra, ACT 2601

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Abstract

Adenine nucleotides, of central importance in energy metabolism, were measured simultaneously in muscle tissue from three species of estuarine mollusc: the gastropod *Pyrazus ebeninus* and the bivalves *Anadara trapezia* and *Saccostrea commercialis*. Comparisons were made between various methods of tissue dissection and subsequent nucleotide extraction in order to select the most effective methods for meauring *in vivo* levels of adenine nucleotides in the three molluscs. The comparisons were based on the total concentration of the three adenine nucleotides extracted from tissue with each method and on energy charge, a measure of the ratio of the adenine nucleotides.

Significant differences in the total concentration of adenine nucleotides extracted from tissues were found when different types of muscle were sampled from the bivalves and when different extractants were used. In energy charge, however, significant differences were found with various methods of collection of tissue, and with differences in the extractant and homogenizing technique used.

Optimal procedures for the measurement of *in vivo* levels of adenine nucleotides were, for *P. ebeninus*, freeze-clamping the muscle in the laboratory with less than 60 s delay during dissection. For *A. trapezia* and *S. commercialis*, the optimal method was freezing the animal whole in the field and subsequently dissecting the frozen muscle. The 'catch' region of the adductor muscle was preferable to the 'quick' region in the bivalves. For all three species, the chosen method of extracting adenine nucleotides was the manual homogenization of tissue with perchloric acid.

INTRODUCTION

The ubiquitous distribution of adenine nucleotides in living organisms and the importance of the nucleotides in the coupling of energy-producing and energy-utilizing reactions has caused considerable attention to be focused on their levels in different organisms and environments (Wiebe and Bancroft 1975; Falkowski 1977; Karl and Holm-Hansen 1978). A variety of methods has been used to extract adenine nucleotides from tissues. As part of an experiment to determine the effect of reduced salinity on the levels of adenine nucleotides in

three species of sessile molluscs from an estuary (Rainer *et al.* 1979), we compared a range of methods of tissue collection and adenylate extraction, to assess their effectiveness in determining *in vivo* levels of adenine nucleotides.

For comparison with other workers, the three adenine nucleotides, adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP) and adenosine-5'-monophosphate (AMP) were often considered together in the form of the energy charge ratio of Atkinson and Walton (1967), defined as:

energy charge = $(ATP + \frac{1}{2}ADP)/(ATP + ADP + AMP)$.

The ratio is typically stabilized between 0.8 and 0.9 in unstressed organisms which are actively growing and reproducing (Ball and Atkinson 1975; Chapman *et al.* 1971). Recent studies on muscle tissue from unstressed molluscs, in which liquid nitrogen was used to stop enzymatic degradation of ATP, have generally given energy charge values within this range. Wijsman *et al.* (1976) obtained an energy charge value of 0.91 for *Mytilus edulis*, Beis and Newsholme (1975) obtained a value of 0.95 for *Pecten maximus*, and Ivanovici (1980) obtained a value of 0.88 for *Pyrazus ebeninus*.

Adenine nucleotides are potentially affected by any disturbance of the animal's normal physiological state, including the disturbances involved in the collection and assay procedure. We aimed to select methods which produced the adenine nucleotide levels that were closest to the published values of energy charge for unstressed muscle tissue of molluscs. Larsson and Olsson (1979, p.153) adopted a similar approach, stating that "it is likely that methods combining high energy charge with high total extraction also reflect the 'true' levels of adenine nucleotides". In some organisms the energy charge may remain relatively constant with alteration in physiological state, while the concentration of total adenine nucleotides is reduced (Chapman and Atkinson 1973; Dietzler *et al.* 1974). Consideration was therefore given in the selection of optimal procedures to changes in total adenine nucleotide levels.

The three species chosen for study were the mud whelk, *Pyrazus ebeninus* (Brugière 1792) (Gastropoda: Potamididae), the cockle, *Anadara trapezia* (Deshayes 1840) (Bivalvia: Arcidae) and the rock oyster, *Saccostrea commercialis* (Iredale and Roughley 1933) (Bivalvia: Ostreidae). Several criteria were used in the selection of these species for the experiments. We wished to use animals which were hardy (to minimize the effect of unavoidable disturbances inherent in assay of adenine nucleotides) and which inhabitated areas affected by rapid reductions in salinity. The species chosen were additionally suitable in being prolific and easy to collect in areas of Port Hacking near the laboratory. Procedures for determining adenine nucleotide levels had been thoroughly investigated for *P. ebeninus* (Ivanovici 1977, 1980), and data were available on variation in its energy charge in a variety of environmental conditions; no previous work had been done on *A. trapezia* or *S. commercialis*. The distribution of *S. commercialis* in the field indicates a greater tolerance of exposure and changes in salinity than *P. ebeninus*, whilst such factors are attenuated for *A. trapezia* by its mainly subtidal distribution. The differences between the bivalves and *P. ebeninus* in physiology, phylogeny, and distribution in the field suggested that they may possess different means of metabolic adjustment and thus may also differ in their sensitivity to particular methods of handling and preparation for assay of adenine nucleotides.

Five experiments are reported here, of which three were designed to examine different methods of collecting animals from their natural environment and the removal and freezing of muscle tissue, as such disturbance has been shown to affect adenine nucleotide levels (Faupel *et al.*1972). A further two experiments examined aspects of the protocol for extraction of adenine nucleotides from tissue: the efficiency of different extractants, and methods of homogenizing the tissue. These were the aspects of extraction considered most likely to be affected by differences in type of muscle tissue or species from which tissue was obtained. The experiments, summarized in Table 1, examined changes in adenine nucleotide levels with:

1. three methods of tissue inactivation by freezing,
2. different time periods between commencing dissection and freeze-clamping of tissue,
3. the use of 'quick' or 'catch' muscle tissue from the two bivalves,
4. four commonly-used extractants, and
5. two methods of homogenizing the tissue in the extractant.

METHODS

Animals

Animals were collected from intertidal areas on the southern shore of Port Hacking, N.S.W., between Cabbage Tree Basin and Costens Point (34°05'S, 151°07'E). In order to minimize variation between individuals selected for experiments, one location typical of the habitat and distribution of each species was chosen, and animals were sampled at the same stage of the tidal cycle (usually on the ebb) within each experiment. Adult animals were selected, using *P. ebeninus* of 50-70 mm length, *A. trapezia* of 50-65 mm length and *S. commercialis* of 50-75 mm in longest dimension.

Collection

Collection procedures were designed to minimize the disturbance of the animals from their *in situ* conditions. Animals were transferred from their habitat to a bucket without water and returned to the laboratory before being frozen in liquid nitrogen. This was always completed within 2 h and involved little disturbance to the animals. Animals were collected on different occasions for each experiment, and although care was taken to standardize collection procedures and to minimize handling, differences in field conditions from one experiment to another were unavoidable. The alternative, acclimation of animals in laboratory aquaria prior to experiments, was avoided in order to minimize extraneous effects such as starvation, of which the implications for adenine nucleotide metabolism were unknown.

Freezing and dissection of muscle

Two methods of obtaining frozen muscle samples from the molluscs were adopted, both of which could be used in either field or laboratory conditions. The first method was to transfer the whole, live animal within 60 s from its habitat (in the field) or a bucket (in the laboratory), to a large container of liquid nitrogen. Freezing of the whole animal to -180°C occurred within 90 s of immersion in liquid nitrogen. The shell was subsequently broken in a vice and muscle dissected from the frozen animal. The second method involved more disturbance of the live animal, but the sample of muscle was frozen more rapidly. The procedure was to break the shell of the live animal in a vice, dissect a sample of muscle and freeze-clamp the tissue between blocks pre-cooled in liquid nitrogen.

Table 1. Summary of species and treatment differences from experiments, based on total adenine nucleotides and on energy charge. Significant differences are indicated by a tick and their absence by a cross.

Experiment	Basis of comparison	
	Total adenine nucleotides	Energy charge
1. Method of collecting tissue		
-significant differences among 3 species?	✓	✓
-significant differences among 3 treatments?	X	✓
2. Delay in dissection time		
-significant differences among 3 species?	X	✓
-significant differences among 3 treatments?	X	X
3. Muscle type		
-significant differences among 2 species?	✓	✓
-significant differences among 2 treatments?	✓	X
4. Extractant type		
-significant differences among 3 species?	X	✓
-significant differences among 4 treatments?	✓	✓
5. Homogenizing method		
-only 1 species considered		
-significant differences among 2 treatments?	X	✓

Tissue was dissected from the columellar muscle of *P. ebeninus* and the 'catch' region of the posterior adductor muscle in *A. trapezia* and of the adductor muscle of *S. commercialis* (Fig. 1). After initial freezing, whole animals and tissue samples were kept well below $0^{o}C$ by storage in liquid nitrogen and frequent immersion in liquid nitrogen during subsequent handling and dissection. Tissue was weighed after freezing, and samples were always in the range of 100-500 mg.

Sampling design

Generally, six animals were processed for each treatment in each experiment, as studies by Ivanovici (1977) indicated that this number of replicates was sufficient to detect significant differences in energy charge between treatments. In experiments 1 and 5, these six individuals were retained as separate replicates throughout, but in experiments 2, 3 and 4, muscle from three animals was pooled at the grinding stage and processed as one replicate thereafter.

Materials

Chemicals and reagents used in these experiments were analytical grade, and were purchased from Boehringer Mannheim, British Drug Houses (Australia) Pty Ltd or, in the case of glucose-6-phosphate dehydrogenase (E.C.1.1.1.49), from Sigma, St. Louis. Hexokinase (E.C.2.7.1.1), lactate dehydrogenase (E.C.1.1.1.27), myokinase (E.C.2.7.4.3), pyruvate kinase (E.C.2.7.1.40), ATP, ADP, AMP, reduced β-nicotinamide-adenine dinucleotide (NADH) and oxidized β-nicotinamide-adenine dinucleotide phosphate (NADP) were obtained from Boehringer, Mannheim.

Preparation of extracts

Adenine nucleotides were extracted from muscle samples as outlined below, except in experiments 4 and 5, for which the procedures are given separately.

Adenine nucleotides were extracted from samples of tissue by homogenization with at least two volumes (v/w) of 6% perchloric acid solution, using a pestle in a stainless-steel centrifuge tube cooled in liquid nitrogen. Additional perchloric acid solution was added to make 10 times the tissue weight (v/w) and the mixture allowed to stand on ice until thawed. Samples were centrifuged at $-2^{o}C$ at 4500 *g* for 45 min (IEC RP-6000 refrigerated centrifuge). The supernatants were neutralized to pH 6.5 with 5N K_2CO_3, allowed to stand on ice for 10-20 min, and then centrifuged at $-2^{o}C$ at 5200 *g* for 30 min. After their volumes had been measured, the supernatants were kept on ice until duplicate aliquots of each were assayed for adenine nucleotides. Sample volumes used for ATP determinations were 0.1 mℓ in 2mℓ buffer and, for both ADP and AMP determinations, 0.15 mℓ in 2 mℓ buffer.

Determination of metabolites

The methods for enzymatic assay of ATP, ADP and AMP followed Adam (1963) and Lamprecht and Trautschold (1974), as modified by Ivanovici (in press). The optical density of NADPH, for ATP assay, and of NADH, for ADP and AMP assay, was measured at 340 nm in 10 mm cells using a Zeiss PM4 spectrophotometer.

Experiment 1

Three treatments involving inactivation of tissue by freezing were investigated, as outlined below:

1. Frozen whole in field. The whole animal was gently removed from its habitat and, within 25 s of removal, was frozen in liquid nitrogen at the field site.

Figure 1. Tissue used in experiments.

(a) Columellar muscle (arrowed) in *Pyrazus ebeninus*.

(b) 'Catch' region (arrowed) of posterior adductor muscle in *Anadara trapezia*.

(c) 'Catch' region (arrowed) of adductor muscle in *Saccostrea commercialis*.

2. Frozen whole in laboratory. Live animals were collected from the field and transported to the laboratory in buckets, as detailed above. In the laboratory, whole animals were dropped into liquid nitrogen. Samples of muscle were then dissected from the frozen nimals and returned to liquid nitrogen.

3. Freeze-clamped in laboratory. Live animals were collected from the field as in 2. Samples of muscle were dissected from the live animals in the laboratory and freeze-clamped.

Experiment 2

Samples of muscle were dissected from *P. ebeninus* and *S. commercialis* and freeze-clamped in the field within seconds of removal of the animals from their habitat. This procedure was difficult under field conditions and, since specimens of *A. trapezia* were completely submerged at midtide when animals for this experiment were collected, these were carefully transported to the laboratory under 0.2 m of water, for more convenient dissection and freeze-clamping. Time from breakage of shell in a vice to freeze-clamping of dissected muscle tissue was recorded. Shell breakage times could not be precisely controlled, so dissection times were divided into three categories: 40-60 s, 60-110 s and 110-135 s. Mean dissection times for each species, within each category respectively, were: *P. ebeninus*, 47 s, 91 s, 116 s; *A. trapezia*, 48 s, 99 s, 121 s; *S. commercialis*, 51 s, 89 s, 119 s.

Experiment 3

Six animals of each bivalve species were frozen whole in the laboratory. A sample of 100-200 mg of both the 'quick' and the 'catch' region of the adductor muscle was dissected from each frozen animal.

Experiment 4

Muscle was dissected from live animals and freeze-clamped in the laboratory. The tissue was ground to powder at -180°C and homogenized in either perchloric acid, trichloroacetic acid, sulphuric acid or boiling bicarbonate buffer. The procedure involved addition of 1 mℓ of either 6% perchloric acid (v/v), 7% trichloroacetic acid (w/v) or 0.6 N sulphuric acid to the homogenizing tube. When this had frozen, the tissue and extractant were pulverized. Additional extractant to make 1:10 dilution (tissue:acid) was added and again pulverized at -180°C. For the boiling buffer extraction (Bancroft *et al.* 1976), the powdered tissue at -180°C was quickly transferred from the homogenizing tube to 5 mℓ boiling 0.1 M sodium bicarbonate, pH 8.5. Boiling for 30 s was resumed after addition of the tissue. The sample was placed on ice and centrifuged to remove particulate matter.

Experiment 5

Muscle samples from 10 *P. ebeninus* were freeze-clamped in the laboratory. Five were individually processed in the usual way by grinding the tissue to powder with perchloric acid in a homogenising tube and pestle which was frequently cooled in a liquid nitrogen bath. Five samples were individually placed in 7 mℓ ice-cold perchloric acid and homogenized for 120 s at 16 000 rpm in a Sorvall micro-macro blade homogenizer (du Pont), fitted with a 50 mℓ stainless steel chamber.

Treatment of data

The concentrations of ATP, ADP and AMP which were determined in experiments 1-5 are given in Appendix Tables 1-5 respectively. From these were calculated the mean energy charge, and concentration of total

adenine nucleotides for each combination of species and treatment in each experiment. All values for total adenine nucleotides were reported per unit wet weight of tissue.

A balanced sampling design was used for each experiment to facilitate analysis of variance of significant differences between treatments and species. The energy charge data from experiments 1-4 were analysed by two-way analysis of variance and the energy charge data of experiment 5 by one-way analysis of variance. Similar analyses of variance were performed using the total adenine nucleotide data from each experiment to check for any depletion of total adenine nucleotide pool which may have been associated with a decrease in energy charge.

On each data set for analysis of variance, the value of Bartlett's χ^2 (Steel and Torrie 1960) was calculated to detect heterogeneity of variance in experiments with five or more degrees of freedom (d.f.). In experiment 1, where a significant interaction occurred between energy charge for species and treatment, a comparison of means was performed using Newman-Keul's test (Zar 1974). Other means were compared by least significant difference (LSD) values, calculated in analysis of variance.

RESULTS

The results for total adenine nucleotide concentrations are presented first, arranged under totals for each species and then for each treatment in the five experiments. Results for energy charge are presented second, arranged by species and then by treatment. A brief summary of the results is provided in Table 1.

Total adenosine phosphates

Mean total adenine nucleotide concentrations for experiments are given in Tables 2 and 3. Mean totals for *P. ebeninus* ranged between 3.6 to 6.0 mmol kg^{-1}, for *A. trapezia* between 2.1 to 5.6 mmol kg^{-1}, and for *S. commercialis* between 3.0 to 5.3 mmol kg^{-1}. Mean values for the three species were significantly different when the tissue for analysis had been collected by different methods (experiment 1), with mean total adenine nucleotide levels of 4.1 mmol kg^{-1} in *P. ebeninus*, 2.1 mmol kg^{-1} in *A. trapezia* and 3.0 mmol kg^{-1} in *S. commercialis*. A significantly higher level of total adenine nucleotides was found in *A. trapezia* than in *S. commercialis*, when comparing different types of muscle (experiment 3). Significant differences between species were not found in the other experiments comparing more than one species (experiments 2, 4).

Significant differences in total adenine nucleotides were found between the two muscle types examined in the bivalves and with the use of different extractants. 'Quick' muscle contained more adenine nucleotide; the difference in total adenine nucleotides was particularly marked in *S. commercialis*, where 'quick' muscle contained 5.12 mmol kg^{-1}, compared with values in the 'catch' muscle of 3.31 mmol kg^{-1}. Of the four extractants tested, the lowest yield was found with trichloroacetic acid, 3.22 mmol kg^{-1}, while perchloric acid yielded a mean of 4.39 mmol kg^{-1}, sulphuric acid 5.21 mmol kg^{-1} and boiling buffer 5.36 mmol kg^{-1}. No significant differences were found with various dissecting times, methods of collecting tissue or homogenizing techniques.

Energy charge

Energy charge varied significantly between the three species, with *P. ebeninus* consistently having the highest values and *S. commercialis* the lowest (Tables 4, 5). The range of mean energy charge values for *P. ebeninus* was 0.72-0.90, for *A. trapezia* 0.51-0.83, and for *S. commercialis* 0.40-0.77.

Table 2. Variance table for total adenine nucleotide data, all experiments. Significance at $0.05 \geq P > 0.01$ is indicated *, at $0.01 \geq P > 0.001$ indicated ** and at $P \leq 0.001$ indicated ***

Source of variation	Degrees of freedom	Mean square	Variance ratio
Experiment 1 - method of collecting tissue			
Species	2	18.78	20.9**
Collecting methods	2	2.30	2.6
Spexies x methods	4	0.75	0.8
Within-cell	44		
(Bartlett's χ^2 = 11.4, with 8 d.f.)			
Experiment 2 - delay in dissection time			
Species	2	0.59	1.2
Delays	2	0.76	1.5
Species x delays	4	0.33	0.7
Within-cell	9	0.51	
(Bartlett's χ^2 = 5.8, with 8 d.f.)			
Experiment 3 - muscle type			
Species	1	3.94	7.1*
Tissue	1	3.78	6.8*
Species x tissue	1	1.44	2.6
Within-cell	8	0.55	
Experiment 4 - extractant type			
Species	2	0.57	1.8
Extractant	3	5.80	18.5***
Species x extractant	6	1.28	4.1
Within-cell	10	0.31	
(Bartlett's χ^2 = 8.7, with 9 d.f.)			
Experiment 5 - homogenizing method			
Methods	1	0.25	1.0
Within-cell	8	0.24	

Table 3. Mean concentrations of total adenine nucleotides (mmol kg^{-1}) in all experiments. Least significant differences (P=0.05) are included for each experiment.

Treatment	Species			Treatment
	P. ebeninus	*A. trapezia*	*S. commercialis*	Mean
Experiment 1 - method of collecting tissue				
Frozen whole in field	4.52	2.16	3.18	3.29
Frozen whole in lab	3.62	1.39	2.98	2.67
Freeze-clamped in lab	4.22	2.67	2.97	3.28
Species mean	4.12	2.07	3.04	3.08
(LSD = 0.64 for species or treatment means, 1.10 for species x treatment means)				
Experiment 2 - delay in dissection time				
Delay - 40-60 s	6.00	6.03	5.20	5.74
60-90 s	5.44	5.48	4.86	5.26
90-135 s	6.47	5.41	5.98	5.95
Species mean	5.97	5.64	5.34	5.65
(LSD = 0.93 for species or treatment means, 1.62 for species x treatment means)				
Experiment 3 - muscle type				
'Quick' muscle	-	5.58	5.12	5.35
'Catch' muscle	-	5.15	3.31	4.23
Species mean	-	5.36	4.22	4.79
(LSD = 0.99 for species or treatment means, 1.40 for species x treatment means)				
Experiment 4 - extractant type				
Perchloric acid	5.23	3.98	3.96	4.39
Trichloraecetic acid	2.10	3.06	4.50	3.22
Sulphuric acid	5.10	4.87	5.66	5.21
Boiling buffer	5.22	5.58	5.28	5.36
Species mean	4.41	4.37	4.85	4.55
(LSD = 0.63 for species means, 0.72 for treatment means, 1.25 for species x treatment means)				
Experiment 5 - homogenizing method				
Manual	3.72	-	-	-
Blender	3.41	-	-	-
Species mean	3.57	-	-	-
(LSD = 0.71 for treatment means)				

Table 4. Variance table for energy charge values, all experiments. Significance at $0.05 \geq P > 0.01$ is indicated *, at $0.01 \geq P > 0.001$ indicated ** and at $P \leq 0.001$ indicated ***

Source of variation	Degrees of freedom	Mean square	Variance ratio
Experiment 1 - method of collecting tissue			
Species	2	0.21	23.8***
Collecting methods	2	0.06	6.3**
Species x methods	4	0.04	4.5*
Within-cell	44	0.01	
(Bartlett's χ^2 = 2.9, with 8 d.f.)			
Experiment 2 - delay in dissection time			
Species	2	0.28	141.8***
Delays	2	0.01	1.8
Species x delays	4	0.01	1.7
Within-cell	9	0.01	
(Bartlett's χ^2 = 9.6, with 8 d.f.)			
Experiment 3 - muscle type			
Species	1	0.01	7.1*
Tissues	1	0.01	4.7
Species x tissues	1	0.01	1.2
Within-cell	8	0.01	
Experiment 4 - extractant type			
Species	2	0.10	16.4***
Extractants	3	0.17	29.9***
Species x extractants	6	0.01	2.4
Within-cell	10	0.01	
(Bartlett's χ^2 = 11.4, with 9 d.f.)			
Experiment 5 - homogenizing method			
Methods	1	0.08	5.4*
Within-cell	8	0.13	

Table 5. Mean energy charge values for all experimental groups. Least significant differences are given for experiments 2-5, at P=0.05; vertical lines adjacent to the species x treatment means for experiment 1 indicate means not significantly different at < P 0.05, from a Neuman-Keuls test.

Treatment	Species			Treatment
	P. ebeninus	*A. trapezia*	*S. commercialis*	Mean
Experiment 1 - method of collecting tissue				
Frozen whole in field	0.71	0.75	0.62	0.69
Frozen whole in lab	0.74	0.65	0.59	0.66
Freeze-clamped in lab	0.76	0.61	0.38	0.58
Species mean	0.74	0.67	0.53	0.65
Experiment 2 - delay in dissection time				
Delay - 40-60 s	0.92	0.76	0.49	0.72
60-90 s	0.89	0.65	0.49	0.67
90-135 s	0.89	0.73	0.42	0.68
Species mean	0.90	0.71	0.47	0.69
(LSD = 0.06 for species or treatment means, 0.10 for species x treatment means)				
Experiment 3 - muscle type				
'Quick' muscle	-	0.79	0.76	0.77
'Catch' muscle	-	0.87	0.78	0.83
Species mean	-	0.83	0.77	0.80
(LSD = 0.06 for species or treatment means, 0.08 for species x treatment means)				
Experiment 4 - extractant type				
Perchloric acid	0.86	0.67	0.50	0.68
Trichloroacetic acid	0.36	0.34	0.28	0.33
Sulphuric acid	0.83	0.59	0.49	0.64
Boiling buffer	0.43	0.46	0.34	0.41
Species mean	0.62	0.51	0.40	0.51
(LSD = 0.09 for species means, 0.10 for treatment means, 0.17 for species x treatment means)				
Experiment 5 - homogenizing method				
Manual	0.82	-	-	-
Blender	0.63	-	-	-
Species mean	0.72	-	-	-
(LSD = 0.18 for treatment means)				

Significant variation of energy charge occurred with differences in the method of collecting tissue, the extractant used and with the homogenizing technique, but not with differences in dissection time or type of muscle tissue.

Of the three methods of collecting tissue for analysis, freezing immediately after collection in the field gave the highest overall mean energy charge and freeze-clamping in the laboratory the lowest except in *P. ebeninus*. A significant difference in response by the three species is indicated by the presence of significant interaction. A Neuman-Keuls test of the species-treatment means indicated a significant decrease in energy charge with *S. commercialis* tissue freeze-clamped in the laboratory, but that differences in the mean energy charge for the other two species were not significant.

The energy charge values for 'quick' and 'catch' muscle in the two bivalves were lower for 'catch' muscle in both species, particularly for *S. commercialis*. The variance ratio for differences between muscle types was 4.7, close to significance at $P<0.05$.

Much greater treatment effects were found with the use of different extractants ($P<0.001$) than within the other experiments, with two of the extractants being much less efficient than the other two. Trichloroacetic acid and boiling bicarbonate yielded low energy charge values, with mean values of 0.33 and 0.41 respectively, and gave consistently low values for all species. Perchloric acid and sulphuric acid yielded much higher energy charge values, with mean values of 0.68 and 0.64 respectively. Extraction with perchloric acid gave consistent and slightly higher values for each species than did sulphuric acid. The values obtained for individual adenine nucleotides, and consequently for total adenine nucleotides and energy charge, were more precisely determined with some extractants and some species. Determination of the endpoint of changes in optical density was difficult with the two bivalves extracted with trichloroacetic acid and sulphuric acid. In these samples, the endpoint due to reactions by adenine nucleotides was in part masked by the presence of large amounts of slow-reacting compounds, probably other nucleotides. When using boiling buffer, a problem with boiling over occurred when the tissue was added to the buffer.

In the comparison of homogenizing methods, using *P. ebeninus*, manual grinding with tissue cooled to $-180^{\circ}C$ yielded significantly higher energy charge values than the use of mechanical blending with ice-cold extractant, at 0.82 and 0.63 respectively.

DISCUSSION

Total adenine nucleotide concentrations

Differential recovery of individual adenine nucleotides would be suspected only if a systematic pattern between the concentration of total adenine nucleotides and energy charge were evident, e.g., if high (or low) concentrations were regularly associated with low (or high) energy charge. Variation in the concentrations of total adenine nucleotides between species and experiments was not systematically related to the variation of energy charge values. This suggests that differential recovery of individual nucleotides was not important in these experiments and, accordingly, internal standards of each nucleotide were not performed.

Standard ATP which was homogenized with tissue was recovered, after extraction and assay in the usual manner, in concentrations of at least 90% of its original concentration in these experiments (unpublished data) and with *P. ebeninus* (Ivanovici 1980) and *Trichomya hirsuta* (Ivanovici 1977). With such high recoveries of ATP, the possibility of breakdown of ATP to ADP or AMP seems unlikely. This, coupled with the lack of evidence of differential recovery of adenine nucleotides, suggests that the methods yielding the highest values of energy charge in these experiments would provide reasonable approximations to *in vivo* concentrations of the adenine nucleotides in the tissues tested.

Individual species showed variable concentrations of total adenine nucleotides with the same extractant in different experiments; the greatest range, from 1.4 to 6.0 mmol kg^{-1}, occurred in *A. trapezia*. These differences in concentration of total adenine nucleotides seem to be related to particular experiments. For example, in Experiment 1, total adenine nucleotide concentrations in each treatment were consistently low (2.7 to 3.3 mmol kg^{-1}), while those in Experiment 2 were consistently high (5.3 to 6.0 mmol kg^{-1}). No difference in the protocol of the experiments themselves seem likely to account for the consistent differences observed, and the most obvious source of such variability lies in the preparation of extracts and determination of metabolites.

One step in the extraction process, that of manual homogenization of tissue with extractant, was not amenable to precise control. Muscle tissue from *P. ebeninus* was consistently easier to homogenize than muscle from *A. trapezia* and *S. commercialis*. This could have contributed to the consistently higher values for the concentration of total adenylates from *P. ebeninus*. relative to the other two species. If the upper values found are taken to be the best indicators of *in vivo* levels of total adenine nucleotides, then the three species each have similar levels in muscle tissue, i.e., 6.5 mmol kg^{-1} in *P. ebeninus* and 6.0 mmol kg^{-1} in *A. trapezia* and *S. commercialis*. Further work to clarify this point is desirable.

Additional evidence of variation in the concentration of individual adenine nucleotides is provided by Larsson and Olsson (1979), who compared the efficiency of six extractants over four species of algae. Great variations in the proportions between ATP, ADP and AMP were found with different extractants, although the total adenine nucleotides varied less. Variation in individual and total adenine nucleotides between species was also marked.

Optimal procedures

The procedures determined by Ivanovici (1977) to be satisfactory for the determination of *in vivo* levels of adenine nucleotides in *Pyrazus ebeninus* included freeze-clamping of columellar muscle within 90 s of dissection in the laboratory of animals collected up to 6 h earlier, manual grinding at -180°C and extraction with perchloric acid. The results of the present work confirmed that these procedures were satisfactory for *P. ebeninus*, but that some modifications were necessary for the bivalves *A. trapezia* and *S. commercialis*. For the purposes of these experiments, optimal procedures were those which maximized the concentration of total adenine nucleotides and energy charge for all three species. Within these constraints, rapid and efficient methods were obviously advantageous.

In particular, the use of freeze-clamping gave low energy charge levels in the bivalves, while dissection after freezing in liquid

nitrogen in the field gave higher levels, significantly so in *S. commercialis*. The latter procedure was convenient in practice, as the dissection of frozen muscle tissue from the bivalves was simple and rapid, while the preparation of muscle tissue by dissection of unfrozen *P. ebeninus* was more convenient than the dissection of frozen animals.

The two muscle types in the bivalves differ in their total adenine nucleotide concentrations, with the higher concentration being found in the 'quick' muscle. A higher energy charge level was found with 'catch' muscle; this was with a relatively small number of animals, and was not significant, but suggested that 'catch' muscle may be a better tissue to use for the measurement of optimal energy charge levels than 'quick' muscle. If the differences in energy charge observed in different types of muscle reflect real differences between muscle of different species and between types of muscle within a species, it would be interesting to relate the muscle function to energy charge; however, this is beyond the scope of the present paper.

The extractants compared in Experiment 4 were selected because they have been used elsewhere on a wide variety of tissues. Perchloric acid is a widely-used extractant for adenine nucleotides from a variety of tissues (Jaworek *et al.* 1974) and was demonstrated by Ivanovici (1980) with molluscan tissue to give consistent recovery of over 90% of added ATP standard. However, Wijsman (1976) reported low recovery and precision for perchloric acid extracts of *Mytilus edulis* tissue and found trichloroacetic acid more suitable. Sulphuric acid is commonly used as an extractant for plant tissue. The use of boiling buffer as the extractant had advantages in saving time because tissue and extractant do not have to be homogenized by hand. Bancroft *et al.* (1976) found boiling bicarbonate buffer, which gave the least variable results for extraction of ATP from marine sediments, the most acceptable extractant. Sodium bicarbonate was recommended as one of the more suitable buffers for animal tissue to use at boiling point. Boiling tris hydrochloride buffer may also have been a suitable extractant (Bamstedt and Skoldal 1976), but the boiling buffer technique was not investigated further after a boiling-over problem was encountered with bicarbonate buffer. On the basis of the energy charge values obtained with the four extractants tested, perchloric acid was the preferred extractant for all three mollusc species.

The method of choice for disintegrating tissue which has been frozen in liquid nitrogen is pulverization pulverization using a pestle and mortar (Hess and Brand 1974) but this is tedious and subject to variations with different types of operation. Disintegration in a blade homogenizer is faster, less tedious and more amenable to precise replication. Mechanical blending at 0°C was reported as a suitable method for *M. edulis* by Wijsman (1976) but was not satisfactory with tissue from *P. ebeninus* in Experiment 5. This may have been a result of local heating around the blades of the blender, but it was also noted that the muscle tissue did not break up completely, even after prolonged blending. This would have been even more of a problem with tissue from the bivalves, which were more resistant to grinding than *P. ebeninus*, and the method was not investigated further. Our results with this method are supported by the report of Karl *et al.* (1978) that large losses of ATP occurred when multicellular organisms were extracted in boiling buffers.

Using the optimal techniques for each species, energy charge values of 0.8-0.9 predicted for unstressed

organisms were obtained only for *P. ebeninus*, while values for the other two species were usually somewhat lower, 0.5-0.8. It was not determined whether the greater variability in the two bivalves reflected greater natural variability or whether these species were sensitive to particular aspects of the experimental procedure that were not considered here.

CONCLUSIONS

A significantly greater concentration of total adenine nucleotides was extracted from tissue when the 'quick' rather than the 'catch' region of the posterior adductor muscle from *S. commercialis* was assayed. A significantly greater concentration was also extracted, over all three species, when either sulphuric acid or boiling buffer was used as the extractant, rather than perchloric or trichloroacetic acid. There were, however, some differences in the effectiveness of the extractant in different species.

Significantly greater values of energy charge occurred in *S. commercialis* when the tissue was collected by freezing the animals whole either in the field or in the laboratory, rather than by freeze-clamping. Significantly greater values of energy charge were also obtained in three species, when perchloric or sulphuric acid was used, rather than trichloroacetic acid or boiling buffer, as the extractant. In *P. ebeninus*, significantly greater values of energy charge were obtained when tissue was homogenized by hand rather than in a blender. The differences in values of energy charge, with the various species and methods tested here, were apparently not associated with differential recovery of adenine nucleotides or depletion of the total concentration of adenine nucleotides.

Considering these results and the efficiency of the techniques tested, recommendations are made for measuring *in vivo* levels of adenine nucleotides in the three molluscs. The method of choice for collecting tissue from *P. ebeninus* was freeze-clamping in the laboratory with less than 60 s delay during dissection, whilst that for *A. trapezia* and *S. commercialis* was freezing the animal whole in the field. 'Catch' posterior adductor muscle was preferable to 'quick' muscle in the bivalves. For all three species, the chosen method of extraction of adenine nucleotides was homogenization of tissue by hand in perchloric acid.

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APPENDIX Table 1. Concentrations of ATP, ADP and AMP (mmol kg^{-1}) measured in experiment 1.

Species	Method of Collection								
	Frozen whole in field			Frozen whole in lab			Freeze-clamped in lab		
	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
P. ebeninus	1.83	1.93	0.72	3.40	0.98	<0.01	1.17	0.98	0.01
	3.77	2.43	0.25	1.31	1.16	0.44	3.28	1.77	0.14
	2.98	1.46	0.20	1.78	1.48	0.30	3.22	1.05	0.16
	1.78	1.35	0.41	1.51	1.65	0.62	3.43	0.89	0.05
	1.73	1.78	0.65	2.20	1.16	0.11	2.08	1.01	0.39
	1.76	0.71	0.28	2.24	1.17	0.20	1.88	1.87	0.83
A. trapezia	1.50	0.99	0.10	0.99	1.34	0.21	0.92	1.14	0.53
	0.19	0.20	0.05	0.55	0.91	<0.01	1.42	1.56	0.48
	0.68	0.67	0.17	0.07	0.37	<0.01	1.42	1.13	0.17
	0.03	0.66	1.14	0.54	0.57	0.18	1.11	1.50	0.35
	1.88	0.55	<0.01	0.32	0.41	0.07	0.52	0.64	0.28
	1.06	1.80	0.77	0.01	0.46	<0.01	0.73	1.50	0.59
S. commercialis	1.09	1.23	0.38	0.94	1.76	0.90	0.68	1.60	0.86
	1.48	0.96	0.05	0.93	0.76	0.43	0.46	1.32	0.71
	1.11	2.73	1.29	0.95	1.26	0.32	0.46	1.87	1.27
	1.09	1.59	0.23	0.79	1.13	0.30	0.58	1.34	1.10
	2.10	2.43	0.69	0.80	1.29	0.42	0.11	1.13	1.23
	0.18	0.74	0.08	1.14	2.12	1.08	0.08	1.59	1.46

APPENDIX Table 2. Concentrations of ATP, ADP and AMP (mmol kg^{-1}) measured in experiment 2.

Species	Dissection Time								
	40-60 s			60-110 s			110-135 s		
	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
P. ebeninus	4.94	1.06	0.06	3.98	0.84	0.05	6.09	1.32	0.15
	5.12	0.77	0.06	4.56	1.28	0.17	4.34	0.87	0.17
A. trapezia	3.78	2.21	0.31	2.56	2.24	1.06	2.85	1.78	0.55
	3.07	2.24	0.45	2.35	2.02	0.74	3.27	1.82	0.54
S. commercialis	1.18	2.41	1.96	1.16	2.17	1.84	1.67	2.55	2.30
	1.49	2.26	1.09	1.51	2.02	1.03	1.05	2.12	2.26

APPENDIX Table 3. Concentrations of ATP, ADP and AMP (mmol kg^{-1}) measured in experiment 3.

Species	Muscle Type					
	'Quick' Muscle			'Catch' Muscle		
	ATP	ADP	AMP	ATP	ADP	AMP
A. trapezia	3.36	2.14	0.47	4.61	1.64	0.34
	4.21	1.44	0.13	3.58	0.78	0.05
	3.13	1.56	0.30	3.43	1.02	0.00
S. commercialis	2.97	2.01	0.30	2.28	1.47	0.06
	3.28	1.97	0.22	2.01	0.96	0.09
	2.45	1.89	0.29	1.60	1.31	0.14

APPENDIX Table 4. Concentrations of ATP, ADP and AMP (mmol kg^{-1}) measured in experiment 4.

Species	Extractant											
	Perchloric acid			Trichloroacetic acid			Sulphuric acid			Boiling bicarbonate		
	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
P. ebeninus	3.93	1.13	0.17	0.32	1.01	0.41	3.25	1.21	0.38	0.80	3.67	1.17
	-	-	-	0.06	1.11	1.29	4.10	0.98	0.27	0.67	2.41	1.74
A. trapezia	1.88	1.57	0.51	0.11	2.09	1.23	1.51	2.19	1.26	1.51	2.74	1.76
	1.78	1.70	0.53	0.00	1.84	0.85	2.11	2.09	0.59	1.05	2.35	1.75
S. commercialis	0.72	1.88	1.00	0.00	2.52	1.97	2.45	2.59	1.47	0.50	2.28	2.39
	1.28	2.08	0.96	-	-	-	0.57	2.74	1.50	0.50	2.83	2.07

APPENDIX Table 5. Concentrations of ATP, ADP and AMP (mmol kg^{-1}) measured in experiment 5.

Species	Method of homogenization					
	Manual grinding with liquid nitrogen			Blender, with ice-cold perchloric acid		
	ATP	ADP	AMP	ATP	ADP	AMP
P. ebeninus	2.33	1.01	0.12	0.00	0.41	0.08
	3.28	1.08	0.07	2.06	1.24	0.16
	2.13	0.75	0.07	1.62	1.19	0.40
	1.55	1.43	0.44	1.97	1.18	0.43
	2.73	1.34	0.26	1.46	1.46	0.86
	2.89	0.59	0.01	0.82	0.82	0.43

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