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Adenylate Energy Charge (AEC) Response to Stress and Extraction Technique in the Louisiana Swamp Crayfish, *Procambarus clarkii*

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The adenylate energy charge (AEC) ratio* represents the amount of metabolically available energy stored in the adenine nucleotide pool. Theoretically, a cell maintains a ratio of ATP to ADP to AMP which varies depending upon the organism's physiological vigor and state of health (Atkinson 1971). High AEC ratios indicate a high ATP content relative to the total adenylate pool, high rates of biosynthetic processes, and a physiologically 'healthy' cell. As the cell is subjected to growth-limiting conditions, the ratio of high energy to low energy adenylates decreases.

Since the accurate measurement of the AEC depends upon the successful and complete extraction of the adenine nucleotides, choice of extraction method is extremely important. Although a number of studies have evaluated the AEC ratio as a measure of energy status in relation to stress (Chapman et al. 1971; Ching and Kronstad 1972; Bachi and Ettlinger 1973; Ball and Atkinson 1975; Gadkari and Stolp 1975; Wiebe and Bancroft 1975; Ivanovici 1976; Karl et al. 1978; Geisy et al. 1981; Mendelssohn and McKee 1981), few have investigated the relative efficiency of the numerous extraction methods described in the literature. Before the AEC can be used to measure stress in aquatic habitats, a suitable extraction technique must be developed and tested with a suitable indicator organism.

The swamp crayfish, Procambarus clarkii, was chosen for study because of its high commercial value (de la Bretonne et al. 1969), its widespread distribution in freshwater wetlands (Huner and Avault 1976; Pennak 1978; Paille 1980), and the likelihood of its habitat being subjected to a wide variety of agricultural and industrial pollutants (Meyers et al. 1981).

The purpose of this study was to determine the response of the AEC ratio of the swamp crayfish to stress in relation to the efficiency of two different procedures for the extraction of adenine nucleotides.

* $$AEC = \frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

METHODS AND MATERIALS

Crayfish were collected from an impounded Lac des Allemands cypress-tupelo swamp, Louisiana, where inputs and outputs of water in the impoundment are carefully controlled by a system of pumps and wiers by local fishermen to produce optimum yields of crayfish and to prevent crop contamination from agricultural and industrial runoff. Crayfish were collected during February of 1980 by fencing in a 3 m by 3 m quadrat with a seine and fishing two 6.4 mm mesh traps in each seine for 36 hours (Paille 1980).

Crayfish were acclimated in the laboratory for nine months in static 5 gallon aquaria, each partitioned in half by a sheet of perforated PVC. Tanks were lined with clear polyethelene plastic bags (Cheah 1978) and were aerated and cleansed with an underground filter composed of crushed oyster and Rangia shells which also served as a buffer (de la Bretonne et al. 1969) and refuge for the crayfish. Dechlorinated artesian well water (see Doucette 1973 for composition) plus 50 ppm CaCl (de la Bretonne et al. 1969) was the medium. Crayfish were fed between 1 and 2 g per week of a high protein pellet food developed by the LSU Food Science Department (Huner et al. 1974). Water temperatures were generally in equilibrium with room temperatures (25°C). Crayfish were maintained in low light throughout the acclimation and experimental period. The volume of each tank satisfied the requirements set by the American Public Health Association (Standard Methods 1976) and the recommendations of previous studies (Stone 1970; Goyert and Avault 1978).

Non-molting males of approximately equal size were exposed in static systems to a lethal dosage of formaldehyde which was added to the aquarium water to produce a 0.5% solution. Three replicate crayfish treated with formaldehyde and three controls were harvested after one hour.

Whole tails were excised from the main body of the crayfish; the exoskeleton and intestine were dissected away and the tails halved. Each half was placed in a labeled polyethylene bag and immersed in liquid nitrogen (-196°C). Time from harvest to freezing did not take longer than 10 seconds. After freezing, one half of the sample tail halves was lyophilized in a LabConco Freeze Drier and the other half remained frozen in the liquid nitrogen.

For EDTA extraction, 1 mg of the freeze-dried tissue was ground in a mortar and pestle, boiled in 10 ml of a 1 mM EDTA (ethylenediamine tetraacetic acid) solution (pH 6.5) for 30 seconds, cooled on ice, and centrifuged at 4°C for 15 min at 20,000 g.

For PCA extraction, approximately 1 mg of frozen tissue and 3 ml of a 6% perchloric acid solution were ground in a stainless steel mortar cooled in a liquid nitrogen bath. An additional 3 ml of PCA were used to wash the contents of the mortar into a centrifuge tube which was then placed on ice for 30 minutes. The samples

were centrifuged at 4°C for 15 minutes at 20,000 g. Tissue weight was determined by subtracting centrifuge tube plus PCA weight from the total weight. The supernatant was neutralized with a known volume of 5 N K_2CO_3 to pH 6.5, left on ice for 15 minutes, and centrifuged as before.

Three replicate extractions were made on each crayfish tail half. The crayfish extracts from the PCA and EDTA extractions were immediately assayed for adenine nucleotides.

The adenylate assay was accomplished by the enzymatic conversion of AMP and ADP to ATP and has been described previously (Mendelssohn and McKee 1981). Adenosine triphosphate was measured using the ATP-dependent light yielding reaction of the firefly-lantern luciferin luciferase (Sigma, FLE-50) complex with a Model 230 Beckman liquid scintillation counter. ATP was determined directly, while ADP and AMP were converted enzymatically to ATP and determined by subtraction.

RESULTS AND DISCUSSION

Subdividing the formaldehyde-treated crayfish and their controls by two extraction techniques showed that the EDTA method resulted in adenylate values significantly lower than those measured after PCA extraction (Table 1). The total adenylates extracted from both treatment and control crayfish by the PCA method were four to five times greater than that extracted by the EDTA method (Table 1). PCA extracted ca. 250 μmoles g^{-1} d wt of ATP, 80 μmoles g^{-1} d wt of ADP, and 50 μmoles g^{-1} d wt of AMP, amounts comparable to that reported previously for crayfish (Giesy et al. 1981; Dickson et al. 1984).

Boiling buffers have often been used for adenylate extraction instead of acids because the latter have been found to inhibit bioluminescence (Strehler 1968), do not always completely inactivate adenylate-degrading enzymes (Ikumah and Tetley 1976), and are generally more difficult to perform. Boiling EDTA was reported to be preferable to PCA as an extraction medium by researchers working with lyophilized plant tissue; recoveries of added ATP, ADP, and AMP were 90 to 100% with EDTA compared to 50 to 80% with PCA (Mendelssohn and McKee 1981). Other researchers have also extracted high levels of adenine nucleotides from lyophilized plant tissue using boiling solutions (Guinn and Eidenbock 1972; Sobczyk and Kacperska-Palacz 1978; Delistraty 1982).

Boiling buffers, however, have been shown to be unsuccessful in completely inactivating adenylate-degrading enzymes (Bieleski 1964). Since the PCA extracted significantly more adenine nucleotides from the crayfish tissue then did the boiling EDTA, it is likely that the latter method allowed the degradation of the adenylates at some point during the extraction procedure.

Table 1. Comparison of two methods for the extraction of adenine nucleotides from crayfish stressed with formaldehyde and their controls (n = 3).

	EDTA (μmoles . g dry wt.[1] ± S.E.)		Perchloric Acid* (μmoles . g dry wt.* ± S.E.)	
	Control	Treatment	Control	Treatment
ATP	42.35±2.86	47.59±1.88	250.67±17.76	154.55±10.29
ADP	13.78±1.39	23.90±1.59	80.94±12.61	68.64± 5.52
AMP	14.47±1.40	5.43±0.69	49.84± 8.13	66.98±10.87
TOTAL ADENYLATES	70.60±0.90	76.93±1.17	381.41±30.28	290.17±10.49
AEC	0.70±0.03	0.78±0.01	0.76± 0.02	0.65± 0.038

*Values from the perchloric acid extraction were converted from a wet weight to a dry weight basis (1 g d wt = 2.84 g w wt, Sklar unpublished data).

It is possible that the boiling EDTA solution did not completely stop all enzyme activity. However, since recoveries of added adenylates have been shown to be high with this method (Mendelssohn and McKee 1981; Delistraty 1982), adenylate degradation during and after boiling extraction may not have been great. A more likely point at which degradation could have occurred was when the lyophilized tissue was ground with a mortar and pestle prior to extraction. The lyophilized crayfish tissue appeared to be hygroscopic and oily and adhered easily to surfaces. If the tissue absorbed sufficient atmospheric moisture to rehydrate it, then degrading enzymes could have been reactivated. If this was the case, then the loss of adenylates occurred before extraction. Thus, the use of lyophilization may not be suitable for any tissue which is highly hygroscopic. It is important to note that this method has been used successfully with several species of plants (Mendelssohn and McKee 1981; Delistraty 1982; Dionigi et al. 1984) and may be satisfactory for less hygroscopic tissues.

These results have demonstrated that the accurate determination of adenine nucleotides can greatly depend on the use of a method which has been matched to the test organism. For the extraction of adenine nucleotides from crayfish tail muscle, the PCA extraction of frozen tissue was the superior method. However, since PCA may not completely inactivate adenylate-degrading enzymes (Ikumah and

Tetley 1976) and sometimes extracts fewer adenine nucleotides than boiling buffers (see Pradet and Raymond 1983), further work needs to be done to optimize this method for use with crayfish.

The AEC ratios determined by the PCA method in this study were lower (0.76) than that reported for crayfish in other studies (0.82-0.95) (Giesy et al. 1981; Dickson et al. 1984). However, the formaldehyde treatment produced a significant lowering of the AEC to 0.65 relative to that of the controls (Table 1). Many studies have demonstrated a decrease in the AEC in organisms under stress (see Chapman et al. 1971; Atkinson 1977; Ivanovici 1980), and two (Skjoldal and Bakke 1978; Giesy et al. 1981) have demonstrated that the AEC of crustaceans decreased in response to stress.

Lyophilization and extraction with EDTA not only resulted in fewer nucleotides than the PCA method, but produced AEC ratios which were significantly higher in the formaldehyde-treated crayfish (Table 1). The reason for this reversal of the AEC ratio during EDTA extraction is not apparent. Examination of the differences between EDTA-extracted and PCA-extracted adenylate concentrations when calculated as a percentage of the PCA-determined adenine nucleotide levels (AN) levels revealed a pattern to the degradation of the AN pools with the EDTA method (Table 2). The loss of high energy compounds (ATP and ADP) was proportionally greater in the controls than in the treatment crayfish, while the loss of low energy compounds (AMP) was proportionally greater in the treatment than the control crayfish. The net result of this differential reduction in adenine nucleotides was a higher AEC ratio in the formaldehyde-treated crayfish.

Table 2. Per cent difference between EDTA-extracted and PCA-extracted adenine nucleotide (AN) concentrations.*

	Control	Treatment
ATP	83	69
ADP	83	65
AMP	71	92

* $\%\ \text{difference in [AN]} = \frac{[AN_{PCA}] - [AN_{EDTA}]}{[AN_{PCA}]} \times 100$

Since formaldehyde is a metabolic poison, the incorporation of a small amount into the crayfish tissue may have partially prevented the degradation of ATP and ADP by inhibiting the activity of the enzymes that were reactivated in the control crayfish during the

grinding of the lyophilized tissue. If so, then the rate of degradation would have been relatively greater in the controls compared to the treated crayfish, and the resultant AEC ratio would have been lower in the controls. These results have demonstrated that when adenylate degradation occurred, the rates of degradation of the individual adenylate pools were not equal and may have been influenced by the treatment itself.

This study has shown that the choice of extraction method was crucial to the accurate measurement of adenine nucleotides and to the determination of the AEC ratio in crayfish. Extraction of lyophilized tissue with boiling EDTA was inadequate for use with crayfish and may also be unsuitable for other hygroscopic animal tissues. The cold PCA technique appeared to be better suited for the crayfish, Procambarus clarkii, and could prove to be a useful tool for the development of an AEC-Crayfish monitoring technique for the detection of environmental perturbations in a wide variety of aquatic habitats.

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