INTERNAL REPORT 142

EFFECT OF FREEZING ON SAMPLES TO BE ASSAYED FOR ELECTRON TRANSPORT ACTIVITY: A METHOD FOR SAMPLE STORAGE

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INTRODUCTION

Measurement of the electron transport system (ETS) activity provides a useful index of the oxygen consumption rate of either the phytoplankton or zooplankton (Packard 1969, Packard, Harmon and Boucher in press). However, samples to be assayed for ETS activity must be processed as soon as possible to avoid changes in the concentrations of ETS enzymes which may occur as a response to alterations of the organism's environment induced by sampling procedure and storage (i.e., changes in light level, temperature, etc.). In a time frame of several hours induction-repression, feedback mechanisms operating on protein synthesis will adjust enzyme concentrations to levels suitable for the new environment (Harrison 1972). Therefore, samples to be assayed for ETS activity should be processed as soon as possible after collection.

Although the assay itself is simple and straightforward, electrical power and a considerable amount of equipment are required. Because of these power and equipment requirements and because of the above-mentioned sampling problems, it has been difficult if not impossible to obtain meaningful data from remote mountain lakes and streams. Therefore, the possibility of freezing samples has been investigated.

METHODS

Sample Procedure and Storage

A 15-liter sample of Portage Bay (Seattle, Wa.) water was taken and prefiltered, first through 212-µm nylong net to remove larger zooplankton. Three 0.5-ℓ aliquots were then filtered through Gelman Type-A glass fiber filters and assayed immediately for ETS Activity (see following section) while three more 0.5-ℓ aliquots were filtered, put in serum-stoppered test tubes and frozen at -196°C (liquid nitrogen). These frozen samples were then thawed at 0°C for one minute and assayed for ETS activity. Twenty-four additional 0.5-ℓ aliquots were filtered and frozen immediately, as described above. Twelve were then removed from the liquid nitrogen and stored at -15°C, while the remaining 12 were maintained at -196°C.

ETS activity

ETS activity was determined using a Triton X-100 modification [King, unpublished manuscript of the ETS assay of Packard (1971)]. Samples were removed from frozen storage and allowed to thaw for 2 minutes in 5 ml of cold (0-4°C) 0.1-m phosphate buffer (pH = 8.0) containing 1.5% (W/V) polyvinlpyrrolidone, 0.2% (W/V) Triton X-100^R and 0.7-mm $MgSO_4$. Samples

were then clarified by centrifugation (~5000 RPM) at room temperature after which the supernatant fluid was decanted, thoroughly mixed and assayed for ETS activity as such. The assay consisted of 1 ml homogenate, 3 ml 0.1-m phosphate buffer (pH = 8.0) containing 0.2 percent (W/V) Triton X-100, 1.5-mm NADH and 0.25-mm NADPH, and 1 ml of 2.0-mg/ml solution of 2(p-iodophenyl)-3-(p nitrophenyl) 5 phenyltetrazolium chloride (INT). Turbidity blanks (750 mm) consisting of 1 ml supernatant fluid and 4 ml 0.1-m phosphate buffer were determined for each homogenate. A non-enzymatic substrate blank containing all components of the assay, except that 1 ml of grinding buffer was substituted for the crude homogenate, was also determined for each set of reagents. Assays and blanks were incubated at 15°C for 20 minutes and the reaction terminated by adding 1 ml of 50 percent formalin-50 percent formate solution buffered to pH = 3.5. Optical densities were then determined @ 490 mm in a Beckman Acta-II spectrophotometer. Pyridine nucleotides were obtained from Sigma Chemical, St. Louis, Missouri. INT was obtained from NBC Biochemicals, Cleveland, Ohio.

RESULTS AND DISCUSSION

The experimental results are presented graphically in Figure 1. The samples stored at -196°C are depicted by solid circles, while the samples maintained at -15°C are represented by plus signs. The activity of the fresh, unfrozen samples is indicated by an arrow on the ordinate. Each data point is the average of three ETS determinations and the error associated with each point is approximately equal to the width of the point.

Figure 1 clearly demonstrates that there is no loss of ETS activity when samples are frozen at -196°C. Furthermore, it is evident that samples frozen at -196°C and stored at either -196°C or -15°C for up to 50 hrs, retain activity levels that are within 10 percent of that of the fresh sample.

The technique of freezing samples to preserve enzyme activity is not new. Syrett (1973) and Hipkin and Syrett (1972) have demonstrated that for whole cells of *Chlorella fusca*, nitrate reductase activity could be maintained in cells frozen at both -15°C and -196°C. Other workers (Ahmed and Campbell 1973, Giles, Case, Partridge and Ahmed 1967) have demonstrated that the activity of various enzymes can be maintained over periods of up to six months by freezing whole cells at -60°C. However, Packard (1969) has shown previously that samples to be assayed for ETS activity suffer approximately a 50 percent loss in activity if frozen slowly at -15°C.

It is tempting to speculate here as to why samples to be assayed for ETS activity can be frozen at -196°C and stored at -15°C for up to 50 hours without any significant change in activity while freezing at -15°C for up to 50 hours without any significant change in activity while freezing at -15°C incurs such a great loss. Since the ETS is embedded in a lipo protein matrix in the convoluted cristae of the mitochondria and function as a unit both *in vitro* and *in vivo* (Packard 1971), slow freezing at -15°C which produces large water crystals, may disrupt the continuity of the ETS, consequently diminishing its activity. On the other hand, rapid freezing at -196°C, with the concomittant formation of small crystals, may not damage the functionality of the ETS. This line of reasoning is similar to

that of Syrett (1973), who found freezing at -15°C renders cell walls of *Chlorella fusca* permeable to NADH, whereas rapid freezing at liquid nitrogen temperatures does not.

CONCLUSIONS

If an apparatus for filtering is available in the field, either over-pressure or vacuum, filtered ETS samples may be frozen at -196°C and stored at -15°C for up to four days before analysis.

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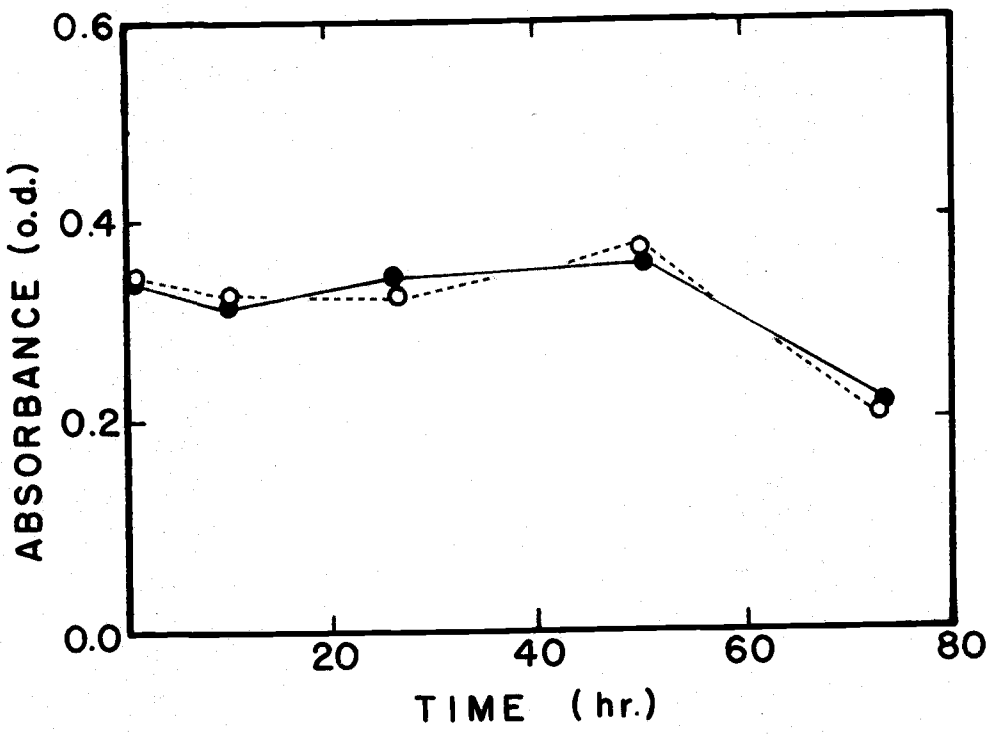

Figure 1. The effect of storage time at -196°C (---0---) and -15°C (---0---) on the ETS assay absorbance for quick frozen (-196°C) filtered Portage Bay water samples. The arrow indicates the activity of the fresh unfrozen sample. Each point is the average of three determinations.