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Journal of Plankton Research Vol.20 no.7 pp.1205–1218, 1998

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Abstract. The optimal conditions for storing and assaying mesozooplankton aspartate transcarbamylase (ATCase) (EC 2.1.3.1.) are defined in order to enable the specific activity of this enzyme to be used as an index of mesozooplankton productivity. ATCase activity was found to be stable for more than 2 weeks when stored in liquid nitrogen, with a negligible loss of activity, whatever the state of frozen material (cell-free homogenate or whole cells). As an alternative, –20°C, –90°C freezers and dry ice (–80°C) can give more flexibility for storing and transporting material, provided initial freezing has been carried out at –196°C. ATCase specific activity was stable over a large range of total protein concentrations (2–55 mg ml^{-1}) for most mesozooplankton samples, and the enzyme activity was linear between 0.8 and 80 nmol carbamyl aspartate min^{-1} mg^{-1} of total protein. The optimal incubation time (30 min), temperature (35°C) and pH range (9–9.5) for assay of ATCase were similar for *Calanus helgolandicus* (Crustacea: Copepoda) (G.O.Sars) and three mesozooplankton assemblages sampled off Plymouth (UK) in May, August and November. However, the K_m values for both substrates were variable: from 3.9 to 79.1 for aspartate, and from 0.83 to 3.46 for carbamyl phosphate. Such variations are likely to be due to the presence of different ATCase catalytic potential, rather than changes in ATCase assemblage.

Introduction

There has long been a need to develop a reliable method for measuring instantaneous zooplankton productivity (i.e. somatic and germinal growth rate), which when combined with biomass would give an estimate of zooplankton production. The methods which are generally used for measuring zooplankton growth rate [cohort method (Aksnes and Magnesen, 1988), physiological methods (Bougis, 1974) and egg production method (Hirche, 1989; Stottrup and Jensen, 1990)] deal with a single species or single developmental stages, and then apply the given information to the whole community, which might lead to misinterpretation of the total zooplankton productivity. In addition, these methods are quite tedious and difficult to perform on board ship because they necessitate the incubation of living organisms.

In order to solve some of these problems, over the past 15 years the use of biochemical methods has been investigated for measuring growth processes, e.g. the measurement of DNA and RNA (Sutcliffe, 1965; Båmstedt and Skjoldal, 1980; Ota and Landry, 1984; Nakata *et al.*, 1994), and enzymes such as DNA polymerase (Sapienza and Mague, 1979) and ATCase (aspartate transcarbamylase) (EC 2.1.3.1.) (Bergeron, 1983, 1986, 1995). ATCase is an enzyme present in prokaryotic and eukaryotic cells, and specifically catalyses the second step of *de novo* pyrimidine nucleotide biosynthesis (Jones, 1980). The measurement of

maximal ATCase activity *in vitro* is directly related to the amount of enzyme present. The control of both the amount of enzyme and its activity are the two basic ways in which rates of enzyme catalysis can be controlled and allow the organism to adapt to endogenous or environmental factors (Hochachka and Somero, 1973). The measurement of maximal ATCase activity should provide an estimate of the rate of nucleic acid synthesis (DNA and RNA), which is essential for protein formation and cellular multiplication. A number of studies have established that maximal ATCase activity is correlated with cellular division and growth rate in plant and animal tissues (Nordman *et al.*, 1964; Stein and Cohen, 1965; Hertzfeld and Knox, 1972; Bergeron and Alayse-Danet, 1981; Koueta and Boucaud-Camou, 1992) and whole organisms (Söderholm and Schwartz, 1975; Fausto-Sterling, 1977; Alayse-Danet, 1980; Bergeron, 1982; Erickson and Selivonchick, 1987), but it has not yet been demonstrated that ATCase activity is correlated with copepod growth (Hernández-León *et al.*, 1995). In addition, the authors who used the measurement of ATCase activity on a single species of copepod, such as *Acartia clausi* (Hernández-León *et al.*, 1995), or mesozooplankton communities (Bergeron, 1983, 1986, 1995), did not establish the optimal assay conditions for this enzyme.

As a first step towards the investigation of the use of ATCase activity as an index of mesozooplankton productivity, the optimal conditions for ATCase assay *in vitro* were determined in this study and their stability in mesozooplankton communities investigated. The following points were addressed: (i) identify the best storage conditions for the enzyme; (ii) check the stability of ATCase kinetic properties between three zooplankton assemblages sampled off Plymouth (UK) at different times of the year: mid-spring, midsummer and mid-autumn; (iii) establish that the kinetic properties are those characteristic of copepods by first identifying the species composition of each sample and, second, comparing the optimum conditions of assay with those of a defined species: *Calanus helgolandicus*.

Method

Sample collection and storage

When not otherwise specified, zooplankton were collected off Plymouth (UK) (Station L4: 50°15'N, 4°13'W) on 8 May 1996, 7 August 1996 and 13 November 1996, by vertical tows from 50 m to the surface, using a 200-μm-mesh WP2 net of 0.56 m diameter. The contents of the cod ends were poured into 2 l plastic bottles and maintained at sea temperature for 2–3 h until return to the laboratory. The samples were then immediately sieved through a 2 mm mesh and the mesozooplankton collected on a 200 μm mesh. For estimation of the species composition, a similarly size-fractionated sample was preserved in 10% buffered formaldehyde. For the assays using different protein concentrations, the mesozooplankton were immediately frozen in cryovials, in liquid nitrogen (LN_2) (–196°C). The samples were defrosted for 10–30 min according to the sample size (~350–15 000 adult copepods) and homogenized at different concentrations.

For each kinetic study, the remaining mesozooplankton were homogenized and distributed into cryovials, to be maintained in LN_2 until further analysis.

Calanus helgolandicus was collected regularly off Plymouth, from June to September 1996, with a WP2 net of 500 µm mesh size. The contents of the cod ends were poured into plastic buckets and diluted into 5 l of sea water. In the laboratory, *C.helgolandicus* (adults and copepodites V) were gently sorted under the microscope and placed in filtered sea water. The animals were then quickly transfered to cryovials and immediately plunged into LN_2. Preceding each series of ATCase assays, 1000 *C.helgolandicus* were defrosted in ice for a few minutes and homogenized.

Preparation of enzyme extract

Preparation of the enzyme extract followed Alayse-Danet (1980), who obtained a more stable ATCase activity by homogenization with distilled H_2O, when compared to homogenization at a range of Tris-buffered pHs. The mesozooplankton and *C.helgolandicus* were homogenized in double-distilled water, for 2 min, on ice, using a Potter glass homogenizer. The mesozooplankton and *C.helgolandicus* concentrations were 1 g wet weight ml^{-1} and 250 individuals ml^{-1}, respectively, when not otherwise specified. Different sizes of homogenizer were used, according to the amount of material.

Aliquots of crude extracts were taken as the source of enzyme and for determination of total protein.

Enzyme assay

ATCase catalyses the following reaction: carbamyl phosphate + L-aspartate → carbamyl aspartate + phosphate. The assay of ATCase activity followed the radiochemical method of Bresnick and Mossé (1966) as modified by Bergeron and Alayse-Danet (1981). All components of the assay medium were defrosted at room temperature and maintained at 4°C before mixing. The assay conditions, described below, were kept identical unless otherwise specified. A 0.2 ml volume of crude extract was incubated for 30 min at 35°C in a total volume of 0.5 ml, with the two ATCase substrates: [^{14}C]L-aspartic acid (ASP) (ICN Biomedicals) (12.62 mM, 111 kBq ml^{-1}) and carbamyl dilithium salt (CAP) (Sigma) (12.6 mM). The substrates were diluted with a Tris (hydroxymethyl methylamine) buffer (Sigma) (pH 9.5), in order to have a final Tris concentration of 240 mM. The enzymatic reaction was stopped with 0.1 ml cold 1 N HCl. The mixture was clarified by centrifugation at 4°C for 20 min at 2000 *g*. Then 0.5 ml of the supernatant was added to an ion-exchange column (0.6 × 6 cm) of freshly prepared Dowex 50 resin (X8; H^+ form; 100–200 mesh) (Merck Ltd). Addition of 3.5 ml of double-distilled water to the column eluted the radiolabelled product [^{14}C]carbamyl aspartate (CAA), with the substrate [^{14}C]L-aspartic acid remaining retained by the column. The radioactivity of a 0.5 ml aliquot of eluate (total eluate 4 ml) was estimated in a 1219 Rackbeta (LKB Wallak) liquid-scintillation spectrophotometer with the addition of 5 ml of Picofluor Packard 15 (Canberra Packard)

scintillation liquid. The liquid scintillation counting efficiency was determined with an external standard, channels ratio method. Chloroform was the quenching agent.

For each assay, controls were included to account for any CAA formed non-enzymatically, by replacing the crude extract with double-distilled water. Moreover, each crude extract and control assay was performed in duplicate or triplicate, except for the study on the influence of substrate concentrations on *C.helgolandicus*, where no replicates were made.

Protein assay

Total protein was estimated using a modification of the Lowry *et al.* (1951) method described by Markwell *et al.* (1978), where sodium dodecyl sulphate (SDS) was added to the alkaline reagent. This method allows the measurement of membrane proteins and lipoproteins in addition to soluble proteins. Bovine serum albumin (BSA) (Merck Ltd) was used as a standard.

Results

Storage conditions

After an initial loss of activity of ~5, 10 or 15% (Figure 1), the activity of ATCase remained constant over a period of at least 15 days, when frozen and stored at –196, –90 and –20°C, respectively. However, a fresh sample left for 30 min on ice or at room temperature before incubation undergoes the same loss of activity (5%) as a sample which has been previously frozen in LN_2 and left to thaw at room temperature for an equivalent time.

The potential to use a small LN_2 container in conjunction with –90°C and –20°C, freezers or dry ice (–80°C) gives greater flexibility for storing and transporting material, as only an additional ≤3% loss of activity was found when compared with the single use of LN_2.

Finally, as we used zooplankton homogenates made up before or after storage in LN_2, it was important to test the stability of enzyme activity between the two procedures, which gave results of 0.171 ± 0.003 and 0.172 ± 0.004 nmol CAA min^{-1} mg^{-1} protein, respectively. The two means were found to be not significantly different ($t = 0.77$, $P < 0.05$).

Kinetic studies of ATCase

Figure 2 presents the proportionality between ATCase activity (nmol CAA min^{-1} ml^{-1}) and the protein concentration of seven mesozooplankton and *C.helgolandicus* homogenates. The proportionality was verified for most of the homogenates over a large range of total protein concentration: ~2–55 mg ml^{-1}. Outside this range, ATCase activity was difficult to obtain and the replicates highly variable. Finally, for two mesozooplankton samples collected on 25 March and 23 April 1996, respectively, the very high catalytic rate decreased around 80 nmol CAA min^{-1} ml^{-1}, corresponding to a protein concentration of 15 mg ml^{-1}.

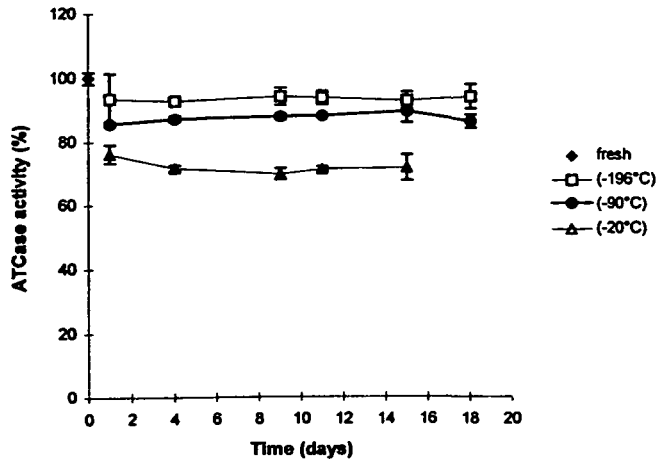

Fig. 1. Stability of ATCase activity from mesozooplankton homogenate, frozen and stored at different temperatures: -196, -90 and -20°C. Each assay was performed in triplicate and the error bars represent the SE.

For the three mesozooplankton samples studied, the kinetics were linear until 40 min (May), 60 min (August) and 80 min (November), and *C.helgolandicus* showed linear kinetics until at least 120 min (Figure 3A). The highest activity was obtained at 35°C, and with an optimum assay pH between 9 and 9.5 (Figure 3B and C).

The influence of substrate concentrations (ASP and CAP) on each of the four samples is displayed in Figure 4. Two main trends can be observed. Firstly, the mesozooplankton samples from May and November reached their maximal velocity (measured by the Hanes–Woolf method) for ASP of 18 and 12 mM, and CAP of 6.4 and 12.6 mM, respectively. Each of these two samples seemed to be inhibited by one (November) or both (May) substrates. Secondly, the mesozooplankton sample from August and the *C.helgolandicus* sample seemed to respond very differently: with no detection of inhibition by any of the substrates, and the maximal velocity was not reached for the highest ASP concentrations (36 mM) (Figure 4). Finally, the dissociation constants of ATCase for each substrate, K_m ASP and K_m CAP, were obtained by the graphical method of Hanes–Woolf (Price and Stevens, 1995). The constants were different for each sample, but were lower for CAP than for ASP (Table I).

Species composition

The species composition of each of the three mesozooplankton samples (Table II) reveals that they were largely dominated by copepods, with 95.3, 91.4 and 97.3% of the total zooplankton counts in May, August and November,

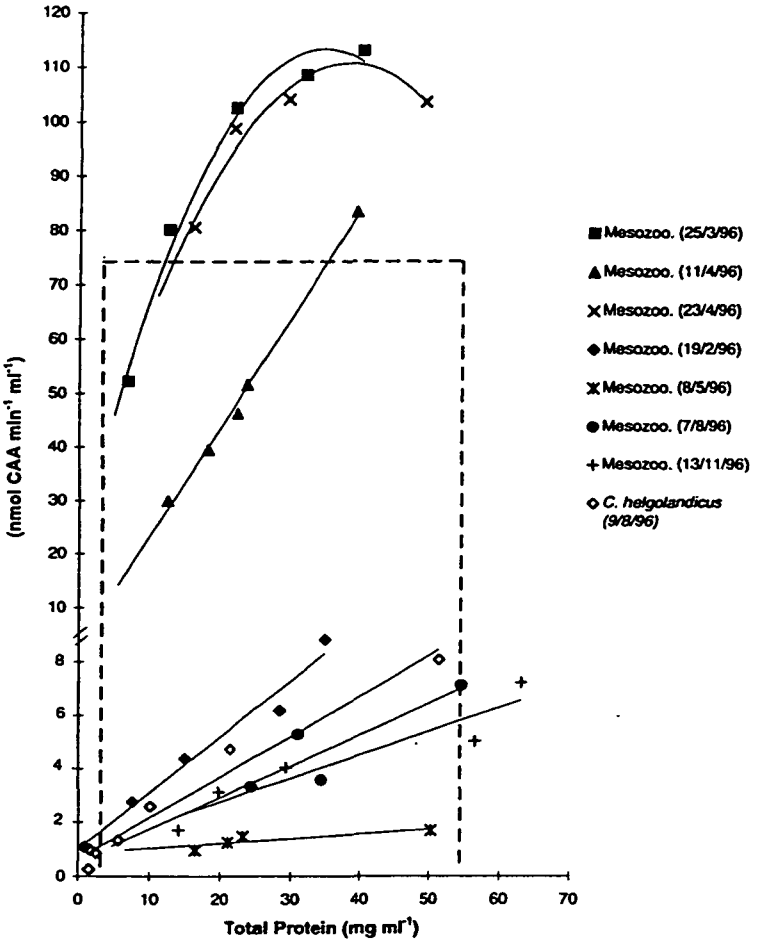

Fig. 2. Stability and limit of interpretation (dashed area) of ATCase activity (nmol CAA min^{-1} ml^{-1}) and protein concentrations. The samples were collected on the dates indicated on the figure. The measurements were made in duplicate and their mean is presented. The lines which join the different points are the lowest order polynomials that give the best fit.

respectively. The copepod assemblages for each month were dominated by only 2–3 species, with *Pseudocalanus elongatus* comprising 30.5% of the total count in May, 48.8% in August and 23.3% in November. A significant proportion (43.3%) of the mesozooplankton count in May was composed of undetermined copepod nauplii.

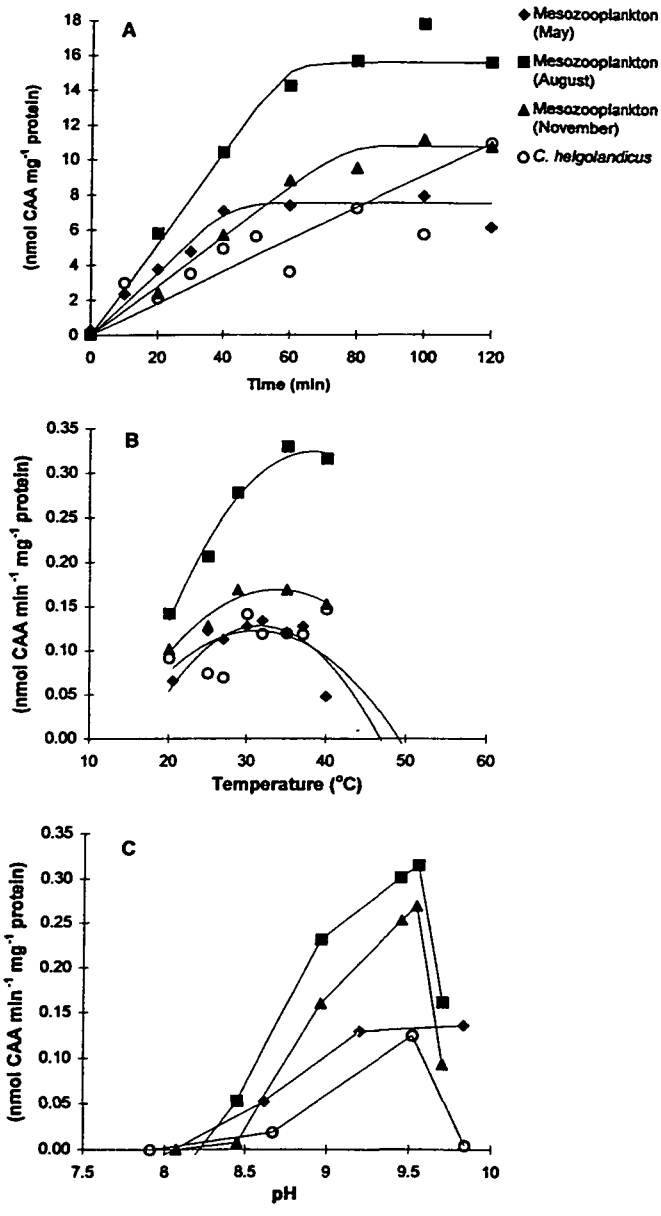

Fig. 3. Influence of the time of incubation (**A**), temperature (**B**) and pH (**C**) on ATCase kinetics for *C.helgolandicus* and mesozooplankton communities collected in May, August and November 1996, respectively. The other assay conditions are kept constant, as described in Method. The measurements were made in duplicate and their mean is presented.

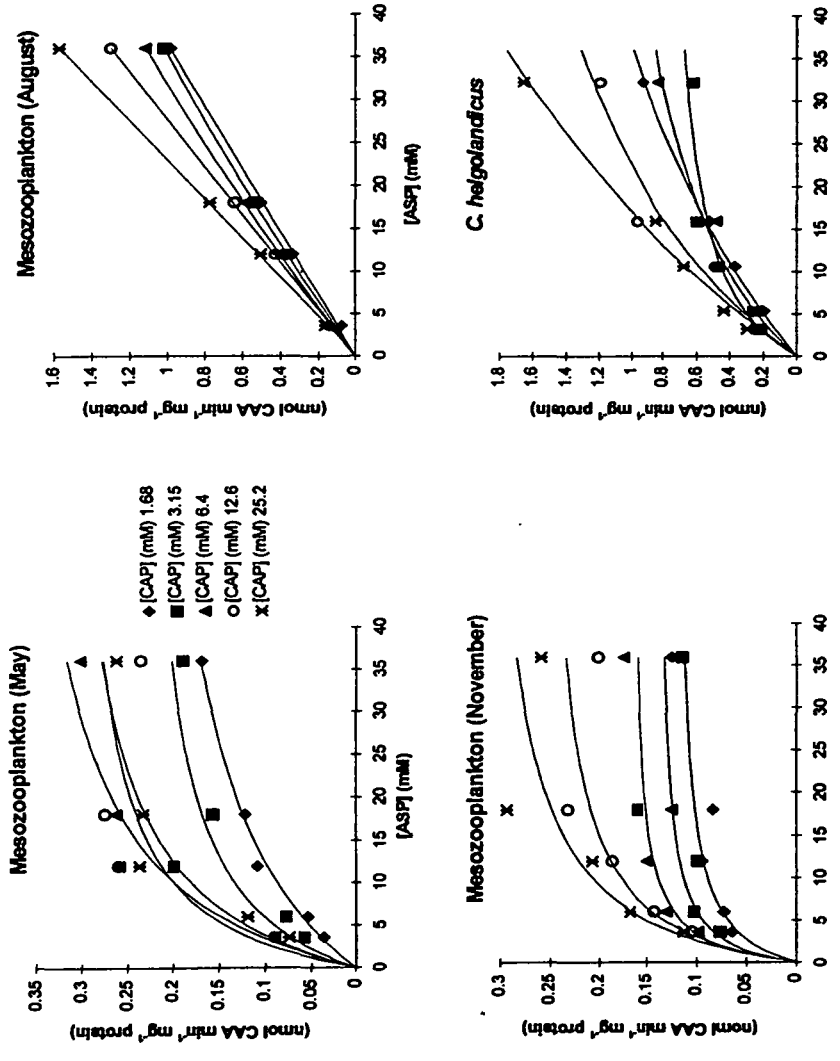

Fig. 4. Influence of substrate concentrations [carbamyl phosphate (CAP) from 1.68 to 25.2 mM and L-aspartic acid (ASP) from 3.2 to 36 mM] on ATCase kinetics. The other assay conditions are kept constant, as described in Method. The measurements were made in duplicate, apart from *C.helgolandicus* for which no replicates were made, and their mean is presented. The lines which join the different points are rectangular hyperbolae.

Table I. Literature review of ATCase kinetic characteristics from various species and zooplankton communities

Species	Kinetic linearity (min)	Optimum T (°C)	Optimum pH	K_m CAP (mM)	K_m ASP (mM)	Authors
Escherichia coli	0–≥30	(37)	7.5	0.45	65	Reichard and Hanshoff, 1956
Lettuce seedlings	(10)	(37)	9–10	2	0.6	Neumann and Jones, 1962
Mytilus edulis	(90)	35–37	8.5–8.9	7	18	Mathieu, 1985
	0–≥120	35	8.6	22	100	Mathieu *et al.*, 1982
Pecten maximus	0–≥80	35–37	9	0.8	4.6	Bergeron and Alayse-Danet, 1981
Crassostrea gigas	0–≥120	39.5	9.3–9.4	–	–	Erickson and Selivonchick, 1987
Sepia officinalis	0–120	37	9–9.1	1.9	5.1	Koueta *et al.*, 1987
Artemia salina	0–≥90	37–45	8.8–9.2	1.6	7	Alayse-Danet, 1980
Drosophila melanogaster	(30)	(30)	–	0.5	5	Söderholm and Schwartz, 1975
	0–45 (60)	(28)	–	2	1	Fausto-Sterling, 1977
Acartia clausi	(20)	(35)	(9)	–	–	Hernández-León *et al.*, 1995
Mesozooplankton	30–180 (50 &110)	(35)	(9)	–	–	Bergeron, 1986, 1995
Swiss–Webster mouse (tumour cells)	(10)	(37)	(8.8)	0.003	2.5	Shoaf and Jones, 1973
Rat liver	–	(37)	9.2	0.038 or 0.022* or 0.054*	3 or 5.5* or 4.3*	Bresnick and Mossé, 1966
Beef liver	0–90	38	7–7.4	–	–	Cygan and Zak, 1967
Mesozooplankton (May)	0–40	25–37	9–9.7 (9)	0.83	10.6	This work
Mesozooplankton (August)	0–60	35	9–9.7 (9)	2.1	79.1	This work
Mesozooplankton (November)	0–80	30–40	9–9.8 (9)	3.46	3.9	This work
Calanus helgolandicus	0–≥120	30–40	9–9.5 (9)	2.7	38.7	This work

The data in parentheses indicate the conditions used by the author. The asterisk means that the data have been obtained with a different method than the previous data mentioned. The dashes imply that the data have not been mentioned by the author. K_m CAP and K_m ASP are the apparent dissociation constants for carbamyl phosphate and aspartate.

Discussion

Optimum storage conditions

Alayse-Danet (1980) and Bergeron (1986) maintained their samples frozen at –18°C (*Artemia* sp.) and –20°C (zooplankton), respectively. However, Koueta *et al.* (1987) showed that the ATCase activity of cephalopod gonad was not stable when the samples were stored at –20°C, but was stable when LN_2 was used. A similar result was found when the stability of ATCase activity was investigated for different storage temperatures (–20, –90 and –196°C) (Figure 1). The initial loss of activity constantly observed can have several reasons: one could be the action of proteases which are still active in the homogenate, if the storage temperature is not low enough (–20 and –90°C) to immobilize the molecules instantly. Another factor, which is inherent in the freezing process, is caused by the 30 min thawing of these samples. Although LN_2 is the best storage condition for ATCase, –20°C, –90°C freezer and dry ice (–80°C) can give a good alternative, providing that a preliminary freezing at –196°C is performed.

Mayzaud (1986) stressed that one should never store cell-free homogenates, which lost activity even at –196°C, when tests were done on the ETS (electron transport system) and GDH (glutamate dehydrogenase) of *Calanus pacificus*. However, in this study, the crude extract shows no loss of activity whatever the state of the frozen material, cell free or whole cells. The same result has been found by Ahmed *et al.* (1976) with ETS and GDH activities when measured on the diatom *Skeletonema costatum*.

Optimal conditions of ATCase assay

Dependence of ATCase activity on protein concentration. For a given homogenate, it should be determined that the ATCase specific activity remains constant at each protein concentration. However, the ATCase activity/protein ratio may vary for each homogenate and thus give a different specific activity. Positive correlations between ATCase activity and protein concentration were found for most mesozooplankton and *C.helgolandicus* homogenates presented in Figure 2, as well as *Pecten maximus* (Bergeron and Alayse-Danet, 1981), *Artemia salina* (Alayse-Danet, 1980) and *Sepia officinalis* (Koueta *et al.*, 1987). The latter two papers mentioned a lack of proportionality when the soluble protein concentration was either <1 mg ml^{-1} or >25 mg ml^{-1}, which corresponds to ~2–55 mg ml^{-1} total protein (Figure 2, x-axis). Below 2 mg ml^{-1}, the method of protein detection is not sensitive enough to give reliable replicates. Approaching 55 mg ml^{-1}, the crude homogenate is likely to be too viscous.

In addition, ATCase specific activity is only constant between 0.8 and 80 nmol CAA min^{-1} ml^{-1} (Figure 2, y-axis). In this study, we found that homogenates constantly absorbed a small portion of radioactivity, which gave a residual activity of 0.8 nmol CAA min^{-1} ml^{-1}. Bergeron (1986) stated that two incubation times were necessary for assaying ATCase activity, to overcome the presence of residual activity in the homogenate, but the constant residual activity observed showed that the use of two incubation times is not necessary. Also, mesozooplankton samples

which had activity >80 nmol CAA min^{-1} ml^{-1} did not show any linearity with the protein concentration. It is possible that the reaction was substrate limited, and after 30 min of incubation the velocity of the enzymatic reaction is no longer at its initial rate. ATCase activity is then underestimated. Such samples should be diluted or a smaller incubation time used.

Optimum incubation time. It is only during the initial reaction period, when the kinetics are linear, that the conditions are accurately known and that the influence of other incubation parameters can be studied (Dixon and Webb, 1979). The velocity of the reaction catalysed by ATCase is linear for 40 to at least 120 min of incubation when tested on zooplankton homogenates and on *C.helgolandicus* (Figure 3), which is consistent with the literature (Table I). However, Bergeron (1986) mentioned that ATCase activity in mesozooplankton was linear between 30 and 150 min. Such results have probably been found for samples having either a very low natural activity, or which could have lost their activity when stored at –20°C, as the author specified. The choice of optimum incubation time was taken as 30 min, this being the best compromise between having a detectable product and maintaining the initial reaction velocity.

Optimum temperature and pH. The range of optimum temperature for zooplankton homogenates ranges between 25 and 37°C, which is consistent with the literature cited in Table I. Above 40°C, the decrease in enzyme activity is very abrupt (Figure 3B), which can be explained by a heat protein denaturation combined with the presence of non-enzymatic reaction. The range of optimum pH for ATCase cited in Table I appears variable according to species (from pH 7 to 10), but the optimum pH for zooplankton ATCase was found to be constantly between 9 and 9.5 (Figure 3C). Although 9.5 appears to be the pH which gives the highest ATCase activity, a pH approaching 9 is appropriate because of the blank's stability. Above 9.5, the level of activity in the blank increases suddenly, probably due to the hydrolysis of CAP.

Optimum substrate concentrations. The four samples tested showed very different maximum velocities (V_{max}) and Michaelis constants (K_m) of the enzymatic reaction (Table I and Figure 4). The first reason for such K_m differences between samples could be the different assemblage of ATCase. Table II reveals, however, that the samples were dominated by one species, *P.elongatus* (23–49% of the total counts), and one class, copepods (91–97%). In addition, according to Jones (1980), it is likely that no evolution of the gene which codes for ATCase occurred from Diptera to mammals. It is, therefore, unlikely that this gene will change from one species of copepod to another. On the other hand, Mathieu *et al.* (1982) and Mathieu (1985) obtained different K_m values on the same species *Mytilus edulis* (Table I). We can therefore propose a second possible explanation for K_m variation in copepods: the coupling between K_m fluctuation and environmental temperature (Hochachka and Somero, 1973). Even if the number of samples analysed is not sufficient to draw a clear conclusion, it is interesting to remark that the two samples which had the highest activities and K_m were from the same

Table II. Proportion of the major species of three mesozooplankton assemblages sampled in May, August and November 1996

Dominant species	Mesozooplankton (May) (% of total zooplankton count)	Mesozooplankton (August) (% of total zooplankton count)	Mesozooplankton (November) (% of total zooplankton count)
Pseudocalanus elongatus	30.5%	48.8%	23.3%
Paracalanus parvus	–	–	30.5%
Temora longicornis	–	18%	4.2%
Oncaea spp.	–	2.7%	30.7%
Oithona spp.	13.2%	5.4%	2%
Acartia clausi		12.6%	2%
Copepod nauplii	43.4%	–	–
Evadne nordmanni	2%	–	–
Oikopleura spp.	2%	–	–
Others	4.2%	4%	4.6%
Total copepods	95.3%	91.4%	97.3%

The dashes imply that the counts were <2%. The term 'Others' takes into account all the species with a percentage of count of <2%.

month, August, where the sea temperature is the highest of the year (~18°C), rather than in May and November (~13°C). Further work should be performed to investigate the importance of temperature for ATCase activity.

Because, at high substrate concentration, inhibition was observed for May and November samples, it is not suitable to use constant CAP and ASP saturation concentrations. An alternative would be the use of substrate concentrations described in Method (12.62 mM ASP and 12.6 mM CAP) as all the other incubation conditions have been defined with those concentrations. However, in order to measure enzyme activity at maximal rate, care must be taken to use samples diluted to the correct protein concentration, because the conditions described in Method can be close to, or even lower than, K_m.

The optimum conditions of assay determined in this study allow the measurement of maximal *in vitro* ATCase activity in copepods. This method can be applied to investigate the relationship between ATCase activity and mesozooplankton productivity (i.e. somatic and germinal growth rate). It is worth to specify that if *in vitro* measurement is appropriate for detecting relative changes in the amount of active enzyme in different physiological states, when comparisons with metabolic rate are needed, *in vivo* enzymatic assays need to be thought of as the next step in enzyme assay (Hernández-León and Gómez, 1996; Packard *et al.*, 1996a,b; Fell, 1997; Hernández-León and Torres, 1997).

Acknowledgements

We especially thank the crew of the RV 'Sepia' and RV 'Squila' for collecting the samples; P.Course for analysing the species composition of the samples; Dr

G.R.Smerdon and Dr D.R.Livingstone for their advice on biochemistry and enzymology; and Dr R.P.Harris, Dr S.A.Poulet, Dr J.A.Berges and Dr P.Mayzaud for helpful comments on the manuscript. This research was partially supported by a European Human Capital and Mobility Programme Fellowship (no. ERB4001GT940181), an IFREMER grant (no. 96064) and the Ministry of Agriculture Fisheries and Food (MAFF).

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Received on July 9, 1997; accepted on December 8, 1997