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ADENYLATE COMPARTMENTATION AND STORAGE IN COELOMIC CELLS OF THE POLYCHAETE *NEREIS VIRENS*

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Summary

The concentrations of adenine nucleotides (AMP, ADP, ATP) were determined in coelomic cells (eleocytes) of the polychaete *Nereis virens*. In cells of immature and male animals, total ADP and AMP contents (each 10–15 μmol ml^{-1} packed cell volume) greatly exceeded that of ATP (0.8 μmol ml^{-1} packed cell volume). ^{31}P-nuclear magnetic resonance (NMR) studies of living eleocytes showed that the high concentrations of both AMP and ADP are free in solution. Comparisons of *in vivo* NMR spectra with those obtained from metabolite extracts of eleocytes suggest that the adenylate pools are compartmentalized, with a large pool being in an environment with a pH<6.0 and a small pool being in a domain where pH>6.7. This indicates that eleocytes are capable of storing high concentrations of ADP and AMP without inhibiting energy metabolism by sequestering these compounds into an acidic compartment. The large acidic vacuole characteristic of eleocytes may function as this compartment.

Key words: adenylates, ADP, AMP, NMR, compartmentation, coelomic cells, polychaete, *Nereis virens.*

Introduction

In nereid polychaetes, eleocytes represent a specialized type of coelomic cells which have phagocytotic activities (Defretin, 1949; Dhainaut, 1984) and are responsible for the synthesis of vitellogenin, the primary building block for yolk material in oocytes (Fischer and Rabien, 1986; Baert and Slomianny, 1987). These cells are produced in large numbers in both sexes shortly before the production of germ cells (U. Hoeger and C. Märker, unpublished observation). Determinations of the nucleotide content from perchloric acid extracts of eleocytes (Hoeger *et al.* 1992) revealed extremely high concentrations of both AMP and ADP, greatly exceeding those of ATP. This finding strongly contrasts with the situation normally in most living cells (see Beis and Newsholme, 1975), which maintain the steady-state ATP content above that of ADP to ensure a favourable free energy change for ATP hydrolysis to support biological functions. This unusual observation led us to the question of how eleocytes survive when the ADP and AMP contents are maintained above that of ATP. One possibility is the binding of nucleotides to intracellular structures. In muscular tissues of animals, for instance, most of the ADP is bound to myofibrillar structures and free ADP concentrations are in the micromolar range, while total ADP concentration is approximately 1 mmol l^{-1} with respect to wet mass (Veech *et al.* 1979). ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy can detect only free forms of ADP and AMP (Gadian, 1982). If these nucleotides are largely bound, then the corresponding areas under the signals of an *in vivo* NMR spectrum of a tissue will be smaller than those from a tissue extract obtained by standard metabolite extraction techniques. In this study, we applied ^{31}P-NMR spectroscopy to living eleocyte suspensions to determine whether the high concentrations of AMP and ADP are bound to intracellular structures or whether they are free in solution. NMR spectra of isolated eleocyte suspensions were compared with those obtained from perchloric acid extracts of these cells. The cell extracts were also analyzed for nucleotide content by high-performance liquid chromatography. Part of the results have been presented in abstract form (Hoeger *et al.* 1992).

Materials and methods

Animals

Nereis virens Sars were collected in 1991 in the mudflats of the Oosterscheldt Bay, at Yerseke, the Netherlands. The animals were transferred on ice to the laboratory within 8 h of capture and kept at 8–12 °C in 40 l trays with artificial sea water and a sandy bottom. Three different groups of animals were investigated in this study: immature animals (characterized by

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the absence of germ cells in the coelomic fluid) were collected in January, while both male and female animals (identified by the presence of spermatogonia and oocytes, respectively) were collected in July.

Isolation of eleocytes

Eleocytes were isolated from the coelomic fluid of anaesthetized animals as described previously (Hoeger, 1991). From the purified suspensions, the packed cell volume (PCV) was determined using a haematocrit centrifuge, and appropriate volumes of the cell samples were then stored in liquid nitrogen.

Metabolite determinations

Metabolites were extracted by ultrasonic disintegration of the frozen cells in a ninefold volume of ice-cold perchloric acid (0.4 mol l^{-1}). The centrifuged extracts (3 min at 15 000 ***g***) were neutralized by adding a twofold volume of 0.5 mol l^{-1} tri-*n*-octylamine in chloroform (1:3.55 v/v; Dobson *et al.* 1991). Nucleotide contents were measured by anion-exchange high-performance liquid chromatography (HPLC; Abe and Okuma, 1991). In addition, ADP and AMP contents were also determined enzymatically as described by Lowry and Passoneau (1972). Inorganic phosphate was determined enzymatically as described by Pörtner (1990).

Total inorganic ion determination

Eleocytes were isolated as described previously (Hoeger, 1991), diluted 1:200 (w/v) with HPLC-grade water, heat-deproteinized (10 min at 95 °C) and centrifuged at 15 000 ***g***. Total magnesium, potassium and sodium contents of the eleocytes were determined by capillary electrophoresis using indirect ultraviolet detection (Scholz and Zerbst-Boroffka, 1994).

^{31}P-nuclear magnetic resonance spectroscopy

Coelomic cells were isolated from male and female animals as described above. 0.7–1.5 ml packed cell volume of eleocytes was diluted to 3 ml with artificial sea water (Hoeger, 1991) containing 10 % Ficoll (w/v) to prevent sedimentation and transferred into a 10 mm NMR tube. The temperature of the cell suspension was kept between 4 and 7 °C by blowing a stream of cold air onto the sample. NMR spectra were obtained on a 9.4 T vertical bore magnet with a Bruker AMV-400 spectrometer. Spectra were proton-decoupled, and the magnetic field homogeneity was modified by optimizing the proton-free induction decay. Phosphorus spectra were collected in 64 scan blocks with a sweep width of 8K and with 8000 data points. A 90 ° pulse angle and an interpulse delay of 15 s were used for the phosphorus spectra to obtain relaxed spectra where peak areas are proportional to free metabolite concentrations. A methylene diphosphonate solution in a centrally placed capillary tube was used as a chemical shift reference. This was calibrated against a solution of phosphocreatine in 20 mmol l^{-1} EDTA. Perchloric acid (PCA) extracts were prepared as described above, then freeze-dried and reconstituted in 3 ml of 20 mmol l^{-1} EDTA, pH 8.5. Total lipids were extracted from the PCA precipitates by adding 3 ml of chloroform/methanol (2:5; v/v) and 1 ml of 1 mol l^{-1} ammonium carbonate per ml of initial cell volume. After phase separation, the upper (aqueous) layer was discarded and the lipid extracts were evaporated under vacuum. The dried residues were resuspended in 5 % sodium cholate, 50 mmol l^{-1} EDTA, pH 8.5, and used for NMR. The reference values used to calculate the pH-dependent chemical shift of the NMR signals were obtained from Robitaille *et al.* (1991). Calibration solutions were prepared in 0.3 mol l^{-1} glycine, 0.15 mol l^{-1} potassium acetate, 40 mmol l^{-1} NaCl, 10 mmol l^{-1} EGTA, 20 mmol l^{-1} glycerol, 1 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes buffer, pH 8.5. For quantification of the metabolites in the NMR spectra, the sizes of the peak areas were determined after cutting out and weighing.

Staining of living eleocytes

A sample of coelomic fluid containing coelomic cells was obtained by puncturing the coelomic cavity with a glass capillary and mixed with a 0.1 % (w/v) solution of Acridine Orange in artificial sea water to give a final concentration of about 0.01 %. The cell suspension was mounted on a microscope slide and observed under an epifluorescence microscope (λ_{exc}=500 nm, λ_{em}=530 nm).

Determination of the vacuole size

Eleocyte suspensions were compressed between a microscope slide and coverslip, adjusting the fluid thickness to about 4–5 μm. After observation under a microscope, video recordings of the cells were taken using an attached camera. For four different eleocyte samples, the video images of 50–80 cells were digitized using a PC-based image-analysis system, and the area occupied by the vacuole was determined and calculated as percentage of the whole cell area. The mean values were used as a measure for the relative vacuole volume of the eleocyte population.

Results

Metabolite concentrations

Eleocytes isolated from immature animals showed a high content of both AMP (10–13 μmol ml^{-1} PCV) and ADP (15–25 μmol ml^{-1} PCV) and a relatively low content of ATP (0.5–1.0 μmol ml^{-1} PCV) as determined by HPLC (Fig. 1). The ADP and AMP contents determined enzymatically in some samples gave very similar results. In animals containing germ cells, the eleocytes of female animals had a reduced content of ADP and AMP by two orders of magnitude, values more commonly observed in living tissues (around 0.1 μmol ml^{-1} PCV; Fig. 1), while male eleocytes maintained the high nucleotide concentrations (Fig. 1). Total inorganic phosphate (P_i) determined enzymatically was also higher in male (37.3±9.0 μmol ml^{-1} PCV; mean ± S.D., *N*=6) than in female (17.0±7.7 μmol ml^{-1} PCV; *N*=3) eleocytes. Total cell

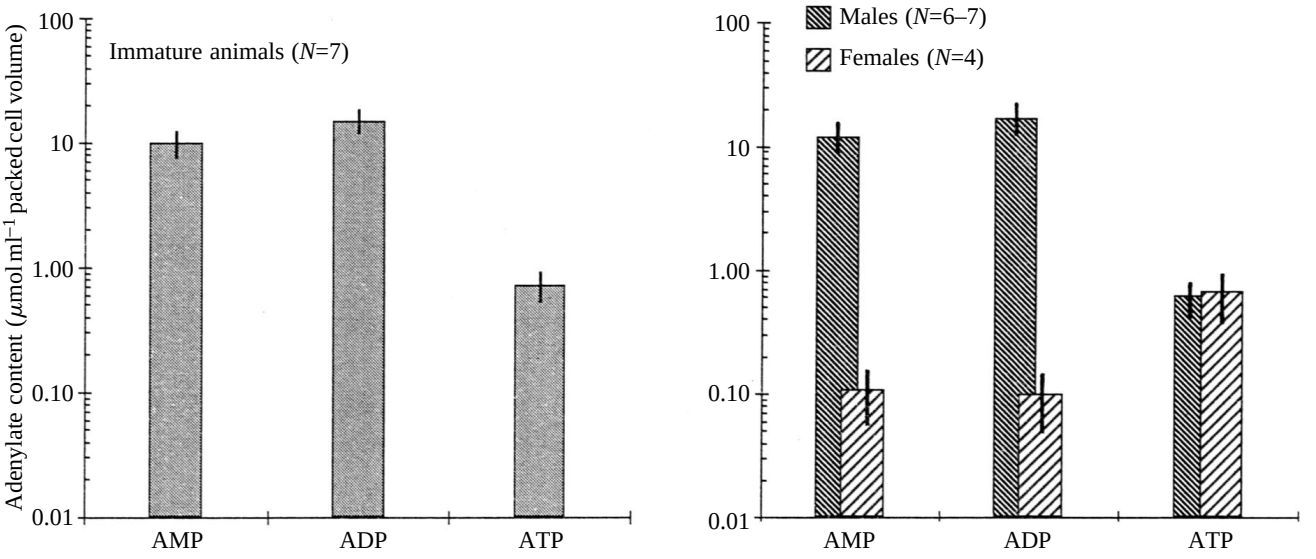

Fig. 1. Adenylate content (expressed as μmol ml^{-1} packed cell volume) in eleocytes of immature, male and female *Nereis virens*. Values are given as mean ± S.D. The ordinate is a logarithmic scale in order to illustrate the low content of AMP and ADP in female eleocytes relative to that in immature and male eleocytes.

magnesium determined by capillary electrophoresis ranged between 1.4 and 4.4 μmol ml^{-1} PCV, with a mean value of 2.3±1.2 μmol ml^{-1} PCV (N=6). Total sodium and potassium concentrations were 132±11 μmol ml^{-1} PCV (N=6) and 26.3±5.3 μmol ml^{-1} PCV (N=6), respectively.

^{31}P-NMR spectroscopy

NMR spectra of both living cells and perchloric acid extracts are shown in Fig. 2. The *in vivo* spectrum from male eleocytes (Fig. 2A) showed large resonances in the location of the α- and β-phosphates of ADP, which are not present in the spectrum of female eleocytes (Fig. 2C). The β-ATP resonance had too low a signal-to-noise ratio to be observed in the *in vivo* spectra. The spectrum of the male cell extract (Fig. 2B) showed only a small resonance from β-ATP. These observations indicated that most of the ADP measured by perchloric acid extraction (see Figs 1, 2B) was free in solution and that the total concentration was far in excess of the concentration of free ATP.

As the frequency difference between the α- and β-ADP resonances is sensitive to the local pH environment, comparison of the shift of these resonances in the *in vivo* spectrum with published values of the chemical shift titration curve (Robitaille *et al.* 1991) provided an estimate of the pH environment of the ADP. This analysis indicated that most of the free ADP was in an environment with a pH of around 6.0 (Table 1), much less than would be expected in the cytoplasm of a living cell.

In male eleocytes, most of the signal from the four peaks from the 5–8 p.p.m. region in the *in vivo* spectrum (Fig. 2A) coalesced into two peaks in the extract (Fig. 2B) which titrated as AMP and P_i. There were no peaks in this region in the lipid extracts, excluding the possibility that part of these resonances in the *in vivo* spectra arose from phospholipids. Titration analysis of the extracts indicated that these two peaks consisted of inorganic phosphate (P_i) and AMP. As both P_i and the phosphate group of AMP also titrate in the chemical shift dimension with changes in pH (Robitaille *et al.* 1991), this indicated that *in vivo* the total pools of these metabolites were split into two major compartments of differing pH. The chemical shift changes of AMP and P_i indicated that one of the compartments was acidic (pH 5.7–6.0) and similar in pH to that obtained using the chemical shift of ADP (pH 5.9) and that the other was more characteristic of a cytoplasmic pH (6.7–7.0; Table 1). For the acidic compartment, the pH values calculated from the chemical shifts of AMP, ADP and P_i were not significantly different (t-test, P>0.01, 6–7 paired observations). For the cytoplasmic compartment, the mean pH calculated from the P_i signal (7.0) was significantly different from that obtained for AMP (6.7; t-test, P<0.01, eight paired observations).

Comparison of the metabolite concentrations determined from the NMR spectra with those obtained by HPLC showed a good agreement for several, but not for all, samples (Fig. 3). The NMR measurements yielded generally higher values; especially for AMP. The overall correlation coefficient was 0.89 (N=19).

Acridine Orange staining and vacuole size determinations of eleocytes

The fluorescence spectrum of acridine orange is pH-dependent (Zelenin, 1966). For both males and females, staining of living eleocytes with Acridine Orange revealed a bright red fluorescence located in the vacuole, indicating an acidic milieu of this compartment (Fig. 4), while the surrounding cytoplasm showed a weaker and more greenish fluorescence. The mean vacuole volume determined in

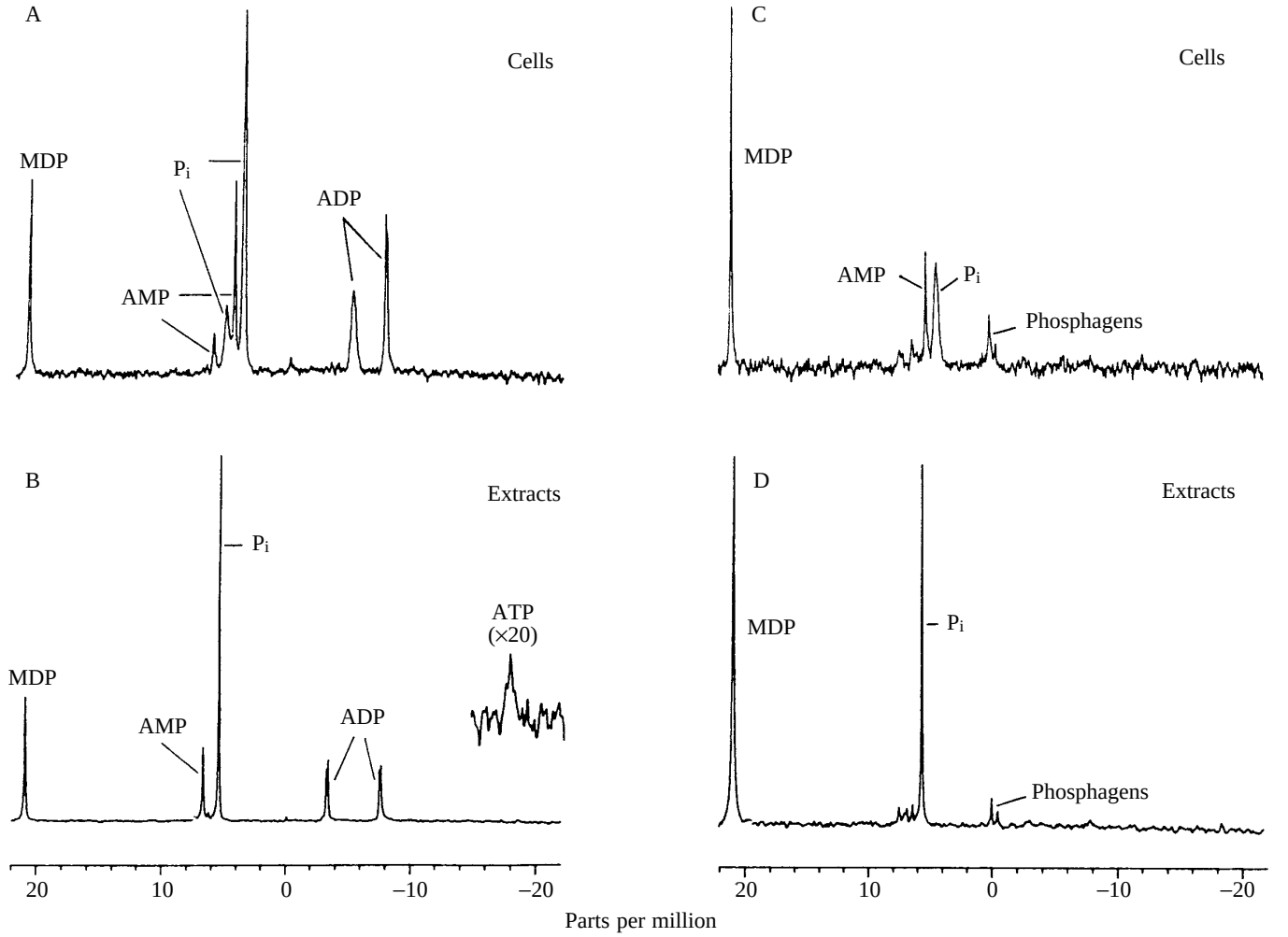

Fig. 2. ^{31}P-NMR spectra of isolated *Nereis virens* eleocytes. (A) Isolated cells from male eleocytes; (B) perchloric acid (PCA) extracts from male eleocytes; (C) isolated cells from female eleocytes; (D) PCA extracts from female eleocytes. The methylene diphosphonate (MDP) peak was from the same MDP standard used in each experiment and provides a reference peak area.

eleocyte populations of four different animals ranged between 41 and 51 % of the total cell volume; the standard deviation within a sample of 50–80 cells was ⩽10 %.

Discussion

Our results indicate that living eleocytes of *Nereis virens* contain unusually high total concentrations of both AMP and ADP which are far in excess of those of free ATP concentration and of the concentrations of AMP and ADP free in solution as revealed by ^{31}P-NMR. Although the signals of the α- and γ-phosphates of ATP, which potentially overlap those of the α- and β-phosphates of ADP, could also contribute to the resonances in this region, the additional peak area would have to be equivalent to the area of the β-phosphate of ATP (located at approximately 16 p.p.m. from the reference phosphocreatine). In the *in vivo* spectrum of living eleocytes, however, the resonance of β-ATP has too low a signal-to-noise ratio to be observed, although the spectrum of the tissue extract shows a small resonance from β-ATP (Fig. 2).

The chemical shift of the NMR signals obtained for the large pools of both ADP and AMP indicated that they were located in an acidic compartment. The chemical shift and, hence, accuracy of the pH determination is influenced by several variables including the ionic strength of the environment (Roberts *et al.* 1981) and the degree of magnesium complexation of nucleotides, which influences the chemical shift of both ATP (Williams *et al.* 1993) and ADP (Kushmerick *et al.* 1986). Assuming that most of the ADP and AMP are

Table 1. *The pH estimates of the two compartments in eleocytes calculated from the chemical shifts of AMP, ADP and inorganic phosphate (P_i)*

	AMP	ADP	P_i
Cytoplasm	6.7±0.2 (9)	–	7.0±0.2 (9)
Acidic compartment	5.7±0.2 (9)	5.9±0.1 (6)	6.0±0.2 (8)

Values are means ± S.D. (*N*).

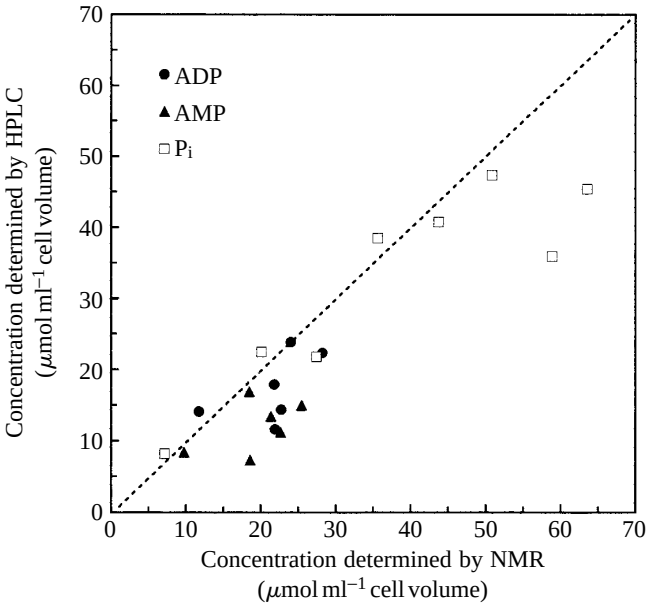

Fig. 3. Correlation between the concentrations of AMP (triangles), ADP (circles) and inorganic phosphate (squares) obtained from ^{31}P-NMR measurements and HPLC (inorganic phosphate: enzymatic) determinations. The dotted line indicates a slope of 1.

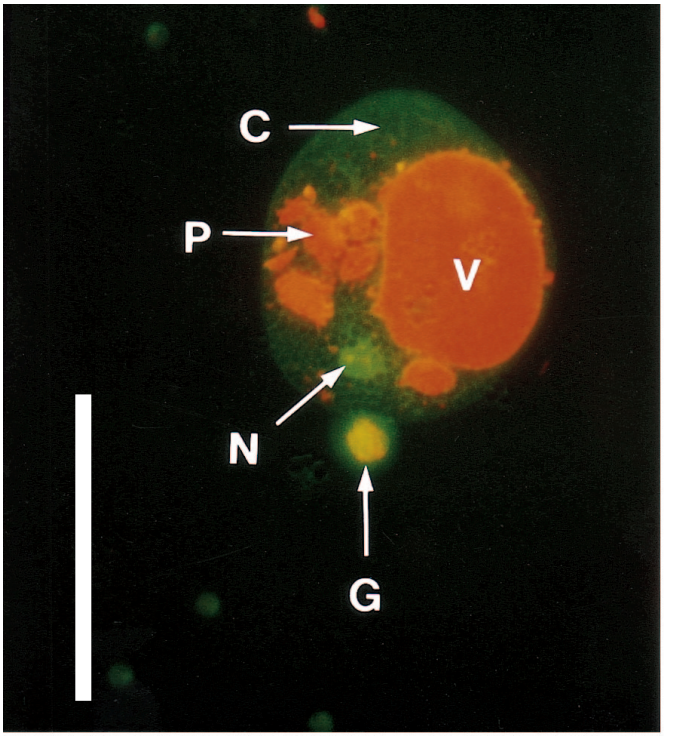

Fig. 4. Fluorescence photomicrograph of an eleocyte after *in vivo* staining with Acridine Orange. In the vacuole (V), the dye shows a strong red fluorescence under acidic conditions. The cell is slightly compressed to demonstrate the large vacuole size. C, cytoplasm containing lipid droplets; P, phagocytized material; N, nucleus; G, attached granulocyte. Scale bar, 50 μm.

stored in the acidic vacuole, their combined concentrations calculated on a total cell volume basis (25–40 μmol ml^{-1} PCV; see Fig. 1) would be almost twice as high in the vacuole given the vacuole volume of about 50 % of the cell volume (see Results section). Although we do not know the distribution of magnesium between the two compartments, the total magnesium concentrations measured (1.4–4.4 μmol ml^{-1} PCV; see Results) are comparatively low. This suggests that little magnesium would be available for complexation and therefore that magnesium would not significantly influence the chemical shift values.

The ionic strength of the medium used for calibration solutions (Robitaille *et al.* 1991) is about 0.5 mol l^{-1} (see Materials and methods) and compares well with that of sea water. Although differences in the ionic composition may exist between the vacuole and the cytoplasm of the eleocytes, a similar ionic strength can be expected in both compartments in order to maintain isotonic conditions with respect to the artificial sea water used as an incubation medium. The coelomic fluid, the ambient medium *in vivo*, is isotonic to sea water (Oglesby *et al.* 1982). Total sodium and potassium concentrations (the main inorganic ions in the eleocytes) accounted for about 150 mmol l^{-1}. Free amino acids probably balance the ionic strength of the ambient medium, as has been shown for the tissues of a variety of marine invertebrates including polychaete annelids (Preston, 1990).

On the basis of the calibration conditions, the calculated pH values for ADP, AMP and P_i were not significantly different for the acidic compartment and gave values between 5.7 and 6.0 (Table 1). For the cytoplasmic compartment, however, the calculated pH values for P_i (7.0) and AMP (6.7) were different, which suggests that there is a limitation in the accuracy of the pH determination for this compartment. Although the calibration constants (obtained at 22 ˚C) were not corrected for temperature, this is expected to have little effect on the precision of measurements as a 10 ˚C change would affect the dissociation constants of P_i, AMP and ADP only by approximately 0.05 units (Phillips *et al.* 1963). In turn, this would change our calculated values by less than 0.1 pH units. The pH-dependent chemical shift of inorganic phosphate measured at 10 ˚C in reconstituted tissue extracts of another polychaete, *Arenicola marina*, agreed with our measurements within 0.1 pH units (Kamp and Juretschke, 1989).

Eleocytes have a large vacuole (Romieu, 1923) reminiscent of plant and fungal vacuoles, which are characteristically acidic (Klionsky *et al.* 1990). Staining of living eleocytes with Acridine Orange showed that their vacuole is also acidic (Fig. 4). The presence of this acidic compartment could explain how eleocytes survive with such high concentrations of ADP and AMP: they compartmentalize these metabolites, in addition to P_i, in the acidic vacuole. Although we have not yet succeeded in isolating the vacuole to confirm the presence of both AMP and ADP in this compartment, this organelle is the most likely storage site for these nucleotides. Lysosomes, the other possible acidic compartment, are unlikely to contain AMP and ADP because of their ample content of hydrolytic

enzymes. The large size and acidic pH of the eleocyte vacuole is reminiscent of the vacuole in yeast cells, where this organelle acts as a store for a variety of metabolites such as amino acids and polyphosphates as well as inorganic ions (Klionsky *et al.* 1990). However, the storage of such high concentrations of ADP and AMP in the vacuole has not been reported.

As shown in Fig. 3, metabolite levels measured using the *in vivo* NMR technique were in good agreement with the HPLC determinations in several, but not in all, samples. The Ficoll concentration used did not always prevent the cell suspensions from settling, which might have influenced the signal areas obtained. However, for most of the samples in which higher NMR values were obtained for the concentrations of AMP, it is also the case that ADP and P_i values differed from the HPLC determinations by the same factor, suggesting that the relative amounts of the metabolites were comparable for both determinations.

Since eleocytes are integrated into the processes of gametogenesis in *Nereis virens*, as shown by their proliferation prior to the formation of germ cells (see Introduction) and their synthesis of yolk protein (vitellogenin), the possible function of nucleotide storage needs to be addressed. In the light of this specific function of eleocytes, we have made the following observations (U. Hoeger, C. Märker and G. Geier, in preparation), which may hint at the further fate of the stored nucleotides: (1) in female eleocytes, the decline of the high nucleotide concentrations during maturation is accompanied by a transient increase in the levels of intracellular nucleosides, especially of inosine and guanosine; (2) eleocytes from both sexes release both inosine and guanosine into the medium under culture conditions; (3) both nucleosides are present in the coelomic fluid, the compartment common to both eleocytes and germ cells, and (4) both male and female germ cells incorporate ^{14}C-labelled inosine in culture. On the basis of these observations, we suggest that the stored ADP and AMP may be degraded to the corresponding nucleosides, released into the surrounding coelomic fluid and taken up by the growing germ cells to supplement nucleic acid synthesis. Thus, supplying the germ cells with purine compounds would be another specific function of eleocytes related to gametogenesis. The requirement for nucleic acid precursors seems obvious for the production of spermatozoa, which requires the synthesis of large amounts of DNA. The DNA content of *Nereis virens* oocytes exceeds that of somatic tissues by over 300-fold (Sidorova, 1984). A similar situation is found in amphibian oocytes, where the high DNA content is the result of a large amplification of RNA-coding genes (Stark and Wahl, 1984). In addition, oocytes of many species, including polychaetes, store large amounts of ribosomal RNA. In this respect, nucleic acid precursors would be of importance for both male and female germ cells.

But why does the eleocyte choose to store purine compounds in phosphorylated form? In yeast cells, one role of polyphosphates stored in the vacuole may be to function as a cation trap for basic storage compounds such as arginine, thus retaining those metabolites by complexation (Dürr *et al.* 1979). Such a function has also been proposed for the high ATP concentrations (100–125 mmol l^{-1}) in the granules of medullar chromaffin cells (Blaschko *et al.* 1956; Njus *et al.* 1978), where it may bind catecholamines (Blaschko *et al.* 1956). However, the major part of the inorganic phosphate, which is present in the eleocytes in concentrations comparable to those of the nucleotides, was also located in the acidic compartment as revealed by NMR. With regard to the storage of purine compounds in the vacuole, however, the phosphorylation of purine nucleosides (i.e. adding a negative charge) could serve to retain them in the vacuole by reducing their diffusion through the vacuole membrane. Hydrolysis of the phosphate bond, in turn, may lead to the transfer of the resulting adenosine out of the vacuole and eventually to a release of purines into the surrounding coelomic fluid.

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