The Journal of Experimental Biology 203, 2907–2920 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2783



# NITROGEN METABOLITES AND RELATED ENZYMATIC ACTIVITIES IN THE BODY FLUIDS AND TISSUES OF THE HYDROTHERMAL VENT TUBEWORM *RIFTIA PACHYPTILA*

MARIE-CÉCILE DE CIAN*, MICHÈLE REGNAULT AND FRANÇOIS H. LALLIER

*Equipe Ecophysiologie, Observatoire Océanologique de Roscoff (UPMC, CNRS, INSU), Station Biologique, BP 74, F-29682 Roscoff Cedex, France*

*e-mail: decian@sb-roscoff.fr

*Accepted 29 June; published on WWW 7 September 2000*

**Summary**

**The distribution of nitrogen metabolism end-products and the associated enzyme activities, free amino acids and purine base catabolites were investigated in all the body compartments (circulating fluids and tissues) of the hydrothermal vent tubeworm *Riftia pachyptila* to acquire a general overview of nitrogen metabolism in this symbiotic organism. There were striking differences between the symbiont-containing trophosome tissue and other host tissues. High concentrations of ammonia, creatinine and, in particular, urate were found in all tissues, but they were present at consistently higher concentrations in the trophosome, which also contained large amounts of urea. Uric acid crystals were present at the periphery of trophosome lobules. The urea cycle appears to be fully functional in this tissue, which also uses creatine phosphate for phosphagen storage, while arginine phosphate or a combination of both phosphagens occurs in other tissues. The amino acid patterns are dominated by sulphated compounds in all tissues except the trophosome, which has high levels of aspartate and glutamate. Although no definitive conclusions could be drawn regarding the nitrogen regime of *Riftia pachyptila*, this *in vitro* study gives several indications for future research in this area.**

Key words: hydrothermal vent, *Riftia pachyptila*, Vestimentifera, nitrogen metabolism, urea, urate, creatinine, ammonia, uricase, urease, phosphagen kinase.

## Introduction

The giant tubeworm *Riftia pachyptila* is the most conspicuous organism associated with deep-sea hydrothermal vents along the East Pacific Rise and the Galapagos Rift. To support its large size and high growth rate, this invertebrate has an unusual mode of existence, relying entirely upon its chemosynthetic bacterial symbionts for nutrition. These symbiotic bacteria have been shown to be chemolithoautotrophic, fixing inorganic carbon ($CO_2$) by using the chemical energy of sulphide ($H_2S$) oxidation (Nelson and Fisher, 1995). The specificity of *Riftia pachyptila* lies in the remote location of these symbiotic bacteria, which are housed in specialised cells (bacteriocytes) of the internal trophosome. It is therefore an absolute necessity for the host to acquire the inorganic molecules necessary for bacterial metabolism from the environment and to transport them to the bacteria. Inorganic carbon, oxygen and hydrogen sulphide are effectively taken up from the environment across the plume (Childress et al., 1993; Goffredi et al., 1997a,b) and are transported in the body fluids, either bound to the vascular and coelomic haemoglobin ($O_2$, $H_2S$) or in solution ($CO_2$) (Arp et al., 1987; Toulmond et al., 1994; Zal et al., 1998).

The source and metabolism of nitrogen by the worm and its symbionts are much less well documented, and there is little information about whether this symbiotic association derives its nitrogen compounds from inorganic sources or about the processes involved. The most common sources of nitrogen for primary producers are ammonia and nitrate, often present at limiting concentrations in surface sea water. In the hydrothermal vent environment, however, nitrate concentrations can reach $40\,\mu mol\,l^{-1}$ (Johnson et al., 1988) because of the high nitrate concentration in deep sea water. Conversely, high levels of ammonia, up to millimolar concentrations, have been measured in vent fluid (Lilley et al., 1993). In the mixing zone around tubeworms, the ranges measured are $18.3–37.5\,\mu mol\,l^{-1}$ for nitrate and $0.1–2.7\,\mu mol\,l^{-1}$ for ammonia (Lee and Childress, 1996). In contrast, free amino acids, another potential source of organic nitrogen, appear to be present at a much lower concentration than inorganic compounds.

Bacterial symbionts have been shown to reduce nitrate to nitrite, thus using nitrate as the final acceptor of electrons in the respiratory chain under hypoxic conditions (Hentschel and Felbeck, 1993). Moreover, studies on the entire organism have shown that nitrate uptake is much greater than ammonia loss

(P. R. Girguis and J. J. Childress, personal communication). High nitrite concentrations have also been reported in the blood and coelomic fluid of *Riftia pachyptila*, which exceeded by 30-fold (in blood) and threefold (in coelomic fluid) the concentrations found in deep sea water (Hentschel and Felbeck, 1993). Only a few enzymes involved in nitrogen metabolism have been found and characterised. Carbamoyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC) activities have been quantified in the trophosome (Simon et al., 2000), suggesting that the urea cycle was, fully or partly, functional in *R. pachyptila*. Activities of both glutamine synthetase (GS) and glutamate dehydrogenase (GDH), ammonia assimilation enzymes, have been detected in both symbiont-containing and symbiont-free tissues of several vent symbiotic organisms including *Riftia pachyptila* (Lee and Childress, 1996; Lee et al., 1997). The use of molecular approaches to resolve the distribution and activity levels of host and symbiont GS and GDH has indicated that host GS activity is repressed and that ammonia assimilation is, therefore, attributable to the symbiont alone (Lee et al., 1999). Other studies of the nitrogen metabolism of hydrothermal vent species have concerned the abundance of sulphur-containing amino acids in symbiotic mytilid bivalves and vestimentiferans (Alberic, 1986; Alberic and Boulegue, 1990) and the potential role of these compounds in the detoxification of sulphur (Pranal et al., 1995; Pruski et al., 1998).

The purpose of this study was to explore nitrogen metabolism pathways in *Riftia pachyptila* through a survey of nitrogenous end-product concentrations and associated enzyme activities. The relative abundance of the main nitrogen catabolites, ammonia, urea, urate and creatinine, was investigated in circulating fluids (vascular blood and coelomic fluid) and tissues (plume, vestimentum, body wall and trophosome, see Fig. 2). The distribution of 21 free amino acids and the main products of purine catabolism were also investigated in the four tissues to define the primary characteristic of nitrogen metabolism in *Riftia pachyptila* and whether it leads to ammoniotelism, ureotelism or uricotelism (see Fig. 6). Furthermore, the activities of several enzymes involved in the biosynthesis and breakdown of these compounds were measured in the same tissues. Our results emphasize the functional differences that exist between the trophosome (the symbiont-containing tissue) and the symbiont-free tissues.

## Materials and methods

### *Animals*

Specimens of *Riftia pachyptila* Jones were collected in the hydrothermal vent fields during the November 1997 cruise ('HOT 97') at 9°50′N on the East Pacific Rise (EPR) at a depth of approximately 2600 m. Three sites were sampled: V Vent, South Riftia and Riftia Field. Coelomic fluid and blood from ventral ('arterial') and dorsal ('venous') vessels were sampled immediately after arrival on board, and samples were frozen in liquid nitrogen. Animals were then dissected on ice, and individual tissue samples were also frozen in liquid nitrogen. Samples were kept in liquid nitrogen during transport and stored at −80 °C in the laboratory. Whole worms of small size were fixed in neutralized10 % formalin and preserved in 70 % ethanol for histological studies.

### *Preliminary treatment of samples*

For analysis of metabolites and amino acids, circulating fluids and tissue samples were thawed on ice for 15–30 min. Tissues samples were homogenised in ice-cold twice-distilled water using a polytron (Kinematica GmbH, Kriens-Luzern, Switzerland); the ratio of wet mass to water volume was 1:8 for the plume, the vestimentum and the body wall and 1:10 for the trophosome. Circulating fluids and tissue homogenates were centrifuged at 10 000 ***g*** and 4 °C for 15 min, and the supernatant was ultrafiltered using a Centricon 50 (Millipore Amicon, 50 000 molecular mass cut-off). Ultrafiltrates were either analysed immediately or frozen at −20 °C.

For enzymatic activities and purine analysis, tissue samples were freeze-dried, and the dry powder was finely ground. For analysis, 20 mg of dried tissue was rehydrated in 500 µl of the appropriate buffer. After extraction for 1 h at 4 °C, the solution was sonicated on ice at 20 kHz for 15 s periods with a Vibra Cell 72446 sonicator (Bioblock Scientific). The homogenates were then centrifuged at 10 000 ***g*** at 4 °C for 20 min. Supernatants were separated from pellets and analysed immediately.

Fig. 6 summarises the relevant cycles and pathways studied in this paper, with an indication of substrates, products and enzymes actually assayed.

### *High-performance liquid chromatography analysis*

All the solvents and salts were of analytical reagent grade. Standard amino acids were purchased from Sigma Chemical Co. and purine compounds from Merck. Concentrations of amino acids and purines were measured by reverse-phase high-performance liquid chromatography (RP-HPLC).

#### *Amino acids*

Concentrations of amino acids were measured according to the method of pre-column derivatization described by Lindroth and Mopper (1979). Original supernatants of circulating fluids and tissues were diluted in twice-distilled water, 100-fold for circulating fluids and 500-fold for tissues. Tissue samples were run a second time at a dilution of 16 000–20 000 because of high concentrations of some amino compounds such as hypotaurine, alanine and glutamic acid. Derivatization was carried out using *o*-phthaldialdehyde and 2-mercaptoethanol for 3 min at room temperature (20 °C); 100 µl of the derivatized sample was injected into the HPLC column. Separation of amino acids was achieved by a mobile binary phase system (gradient flow 1 ml $min^{-1}$). The two eluents used for a C18 column (Ultratech 5 ODS) at 30 °C were (A) 100 % methanol and (B) 10 % methanol/90 % citrate buffer, pH 7.25 (Harris and Andrews, 1985). The gradient was used according to Durand et al. (1999). Primary amino acids were detected by

fluorescence (detector LDC Fluoromonitor III, Milton Roy, 330 nm for excitation and 400–700 nm for emission).

The areas under amino acid absorbance peaks were recorded and integrated using Boreal chromatography package software. Amino acids were identified according to their elution time and quantified against a daily calibration using a standard solution containing 20 amino acids.

*Purines*

Nucleosides and related compounds, uric acid, xanthine, hypoxanthine, inosine and guanosine, were separated and quantified in a single isocratic run (Wynants et al., 1987). The separation was achieved with 0.3 mol $l^{-1}$ ammonium dihydrogen phosphate (pH 4.0) and a mixture of methanol, acetonitrile and tetrahydrofuran (100:1:1:0.4, v/v). Detection of purine compounds was carried out with a variable-wavelength ultraviolet detector at 254 nm (Intelligent-Pump L-6200A, UV-Vis detector L-4200) at a flow rate of 0.7 ml $min^{-1}$. Samples (20 µl) of a standard mixture (60 µmol $l^{-1}$ final concentration of each component: uric acid, xanthine, hypoxanthine, inosine and guanosine) or tissue samples (20 µl) (dilution 1:5–1:20 in perchloric acid) were injected.

*Measurement of ammonia*

A flow-injection method based on gas diffusion through a Teflon membrane into a stream of pH-sensitive indicator (Bromothymol Blue, pH 6.8) was used (Clinch et al., 1988). The colour change induced by $NH_3$, resulting from the dissociation of $NH_4^+$ at high pH, was detected using a double-beam photometric detector incorporating red light-emitting diodes and photodiodes. The magnitude of the colour change was proportional to the concentration of ammonia in the sample. Circulating fluids and tissue samples diluted in twice-distilled water were injected (500 µl) into a 0.01 mol $l^{-1}$ NaOH flow (0.3 ml $min^{-1}$), and the signal was monitored on a chart recorder. Standard solutions of $(NH_4)_2SO_4$ in twice-distilled water were run between every test sample. The response was linear from 10 to 50 µmol $l^{-1}$ ammonia.

*Biochemical analysis*

Measurements of urate and creatinine concentrations in circulating fluids and tissues were performed using enzymatic assays (Sigma Chemical kit no. 685 for urate and kit no. 555 for creatinine). Urea concentrations were determined using the diacetyl monoxime method based on the formation at 70 °C of a coloured complex with maximal absorbance at 520 nm (Newell et al., 1967; Price and Harrison, 1987).

*Enzyme activity measurements*

Freeze-dried tissues (20 mg) were rehydrated on ice for 30 min in 500 µl of appropriate buffer: 100 mmol $l^{-1}$ Tris buffer, pH 8.5, 1 mmol $l^{-1}$ dithioerythritol (DTE) for uricase assays; 50 mmol $l^{-1}$ Tris buffer, 0.4 mmol $l^{-1}$ phenylmethylsulphonylfluoride (PMSF) and 0.5 mmol $l^{-1}$ DTE, pH 8.5, for arginine kinase and arginase assay; the same buffer at pH 7.5 for the creatine kinase assay and at pH 7.8 for the AMP deaminase assay; 25 mmol $l^{-1}$ potassium phosphate buffer, pH 7.5, 1 mmol $l^{-1}$ DTE for the urease assay; and 50 mmol $l^{-1}$ potassium phosphate, pH 7.0, 1 mmol $l^{-1}$ DTE for the xanthine oxidase and xanthine dehydrogenase assays. The PMSF and DTE were dissolved separately, prior to use, in twice-distilled water for DTE and in absolute ethanol for PMSF. The homogenates were then sonicated and centrifuged at 10 000 ***g*** for 20 min. The pellets were discarded, and the supernatants were analysed immediately. All extractions were carried out at 4 °C. Enzymatic activities were assayed spectrophotometrically using 1 cm path length quartz cuvettes at room temperature (24 °C) with specific conditions given below for each assay.

*AMP deaminase (E.C. 3.5.4.6)*

Adenylate deaminase activity was assayed by measuring the disappearance of AMP at 265 nm in the presence of the homogenate over 10 min. The standard assay mixture contained 1150 µl of 50 mmol $l^{-1}$ Tris buffer, 6.6 mmol $l^{-1}$ AMP, 3.3 mmol $l^{-1}$ ATP and 66 mmol $l^{-1}$ KCl. All substrates were adjusted to the appropriate pH with Tris buffer. After 15 min at 24 °C, the AMP deaminase reaction was initiated by adding 50 µl of the homogenate.

*Xanthine oxidase (E.C. 1.2.3.2)*

A sample of the homogenate (100 µl) was first incubated with 1 ml of 50 mmol $l^{-1}$ phosphate buffer, pH 7.8, and 50 µl of uricase (10 units $ml^{-1}$, Sigma 9375) for 10 min at 24 °C to remove uric acid from the sample. The xanthine oxidase reaction was then initiated by adding the substrate (20 µl of 1.5 g $l^{-1}$ hypoxanthine or 1.7 g $l^{-1}$ xanthine solution; 0.2 mmol $l^{-1}$ final concentration) and was followed continuously by monitoring the disappearance of hypoxanthine at 250 nm or xanthine at 270 nm.

*Xanthine dehydrogenase (E.C. 1.2.1.37)*

Endogenous uric acid was removed from the homogenate as described for the xanthine oxidase assay. The reaction was started by adding 100 µl of 5 g $l^{-1}$ $NAD^+$ solution (0.6 mmol $l^{-1}$ final concentration) and 20 µl of 0.2 mmol $l^{-1}$ xanthine or hypoxanthine solution, and was followed at 340 nm to monitor the appearance of NADH.

*Urate oxidase (uricase; E.C. 1.7.3.3)*

Uricase activities were assayed by adding 50 µl of homogenate to 1300 µl of uricase assay buffer (0.1 mol $l^{-1}$ Tris buffer, pH 8.5) and 150 µl of 0.3 mmol $l^{-1}$ uric acid solution to initiate the reaction. The uricase reaction was monitored continuously at 293 nm for 20 min at 24 °C to follow the disappearance of uric acid.

*Urease (E.C. 3.5.1.5)*

A sample of the homogenate (100 µl) was incubated with 740 µl of 25 mmol $l^{-1}$ phosphate buffer, pH 7.5, and 100 µl of 10 mmol $l^{-1}$ urea solution for 20 min at 24 °C. Blanks without urea substrate contained 100 µl of the homogenate and 840 µl

of urease assay buffer. At the end of the incubation, 50 µl of 0.6 mmol $l^{-1}$ $HClO_4$ was added to precipitate the enzyme, and then 10 µl of 2.5 mmol $l^{-1}$ $K_2CO_3$ to neutralise the suspension and stop the reaction. Precipitated proteins were removed by centrifugation at 10 000 ***g*** for 20 min. Ammonia concentrations in the supernatant of tests and blank tubes were then measured (Sigma Chemicals kit no. 640). The amount of ammonia produced in 20 min by urease activity in the samples was calculated from the difference in ammonia content between the test and blanks assay systems.

*Arginase (E.C. 3.5.3.1)*

Arginase in the homogenates was first activated by incubating 270 µl of homogenate with 30 µl of 0.1 mol $l^{-1}$ $MnCl_2$ (0.01 mol $l^{-1}$ final concentration) for 5 min at 55 °C. A 100 µl sample of the activated homogenate was then mixed with 950 µl of 250 mmol $l^{-1}$ L-arginine, pH 9.7, 10 µl of 0.1 mol $l^{-1}$ $MnCl_2$ and 10 µl of 1 mol $l^{-1}$ acetohydroxamic acid solution (urease-specific inhibitor) for 10 min at 37 °C. In blank tubes, L-arginine substrate was replaced by 950 µl of twice-distilled water. The arginase assay was stopped by adding 2.5 ml of 0.5 mol $l^{-1}$ perchloric acid. The amount of urea produced in 10 min by arginase activity was measured using the diacetyl monoxime method (Price and Harrison, 1987).

*Arginine kinase (E.C. 2.7.3.3)*

Arginine kinase activity was measured at 340 nm by monitoring the disappearance of NADH. The reaction mixture consisted of 100 µl of the homogenate, 2550 µl of 50 mmol $l^{-1}$ Tris buffer, pH 7.5, 0.2 mmol $l^{-1}$ NADH, 10 mmol $l^{-1}$ $MgCl_2$, 100 mmol $l^{-1}$ KCl, 2 mmol $l^{-1}$ phosphoenolpyruvate, 10 mmol $l^{-1}$ ATP, excess pyruvate kinase and lactate dehydrogenase and 17.5 mmol $l^{-1}$ L-arginine (omitted from controls).

*Creatine kinase (E.C. 2.7.3.2)*

Creatine kinase activity was measured continuously at 340 nm by monitoring the appearance of NADH (Sigma Chemicals kit no. 47).

*Histological study*

Urate deposits in the trophosome were examined from animals fixed in alcohol–formol and embedded in paraffin wax. Semi-thin sections (7 µm) were stained for urate using methenamine silver staining (Gabe, 1968) and counterstained with Picro Indigo Carmine (PIC). All the reagents used were purchased from Sigma Co. (Accustain silver stain no. HT-100). Sections on slides were incubated in the methenamine silver solution for 30 min at 37 °C. Controls were incubated in 0.5 % $LiCO_3$ solution (to dissolve urate deposits) at 37 °C for 30 min before staining. Following staining, sections were dehydrated and mounted in resin medium (LMR histolaque). Urate deposits could be readily identified by light microscopic examination.

*Presentation of results and statistical analyses*

The ammonia, urea, creatinine, amino acid and purine contents of circulating fluids are expressed as µmol $l^{-1}$, and as µmol $l^{-1}$ of bound water in tissues, assuming a mean percentage of 80 % of interstitial and intracellular water in the four tissues studied (Fisher et al., 1988). Free amino acids (FAAs) are the sum of the 21 amino acids measured. Non-essential free amino acids (NEFAAs) are aspartate, asparagine, glutamate, glutamine, serine, glycine, taurine+thiotaurine, hypotaurine, alanine, tyrosine and ornithine. The essential amino acids (EFAAs) are β-alanine, arginine, histidine, threonine, tryptophan, valine, phenylalanine, isoleucine, leucine and lysine. Enzymatic activities in tissues are expressed as µmoles of product formed (or substrate disappearing) per minute per gram of tissue dry mass (µmol $min^{-1}$ $g^{-1}$ dry mass). Purine contents and enzymatic activities are expressed as mean values of five pools of three animals each. Values are expressed as means ± the standard error of the mean (S.E.M.) The significance of differences between means was assessed ($P \leqslant 0.05$) using the Wilcoxon Mann–Whitney *U*-test.

## Results

*Distribution of nitrogenous end-products*

Ammonia concentrations in tissues and circulating fluids were found to be similar to those commonly found in marine invertebrates, with concentrations 10-fold higher in tissues than in circulating fluids (Fig. 1). To reveal a putative ammonia excretion pathway, we analysed the ammonia content of blood samples from the ventral (‘arterial’) and dorsal (‘venous’) vessels of the same individual. We found a slight difference between the ammonia content in the ventral (0.17±0.05 mmol $l^{-1}$; $N$=14) and dorsal (0.20±0.05 mmol $l^{-1}$) vessels, but the difference was not significant ($P$>0.05). The ammonia content of muscle tissues was 1.9±0.1 mmol $l^{-1}$ ($N$=12) in the vestimentum, 2.3±0.3 mmol $l^{-1}$ ($N$=8) in the body wall and 2.4±0.4 mmol $l^{-1}$ ($N$=8) in the plume, but it reached a value of 5.8±0.5 mmol $l^{-1}$ ($N$=12) in the trophosome.

Urea was found in all the body compartments studied but generally at very low levels of approximately 20 µmol $l^{-1}$ (Fig. 1). Circulating urea concentration was in equilibrium with the aposymbiotic tissue, the highest value being recorded in the vestimentum (46±6.9 µmol $l^{-1}$, $N$=12) and the lowest in the plume (13.2±2.5 µmol $l^{-1}$). Only one body compartment, the trophosome, exhibited a high urea concentration (870±89 µmol $l^{-1}$).

Creatinine may originate either from the urea cycle or from the breakdown of creatine phosphate (see Fig. 6). The creatinine quantification assay used in this study was not able to discriminate between the proportion of creatine and creatinine since, at low pH, all the creatine is transformed into creatinine. The distribution pattern of creatinine was similar to that for urea, although higher levels were observed, with an equilibrium between the circulating and the aposymbiotic tissue concentrations: mean creatinine levels ranged from 153.7±17.8 µmol $l^{-1}$ in the plume to 259.5±28.4 µmol $l^{-1}$ in the body wall (Fig. 1). A much higher creatinine level, 1128.1±171.8 µmol $l^{-1}$, was recorded in the trophosome.

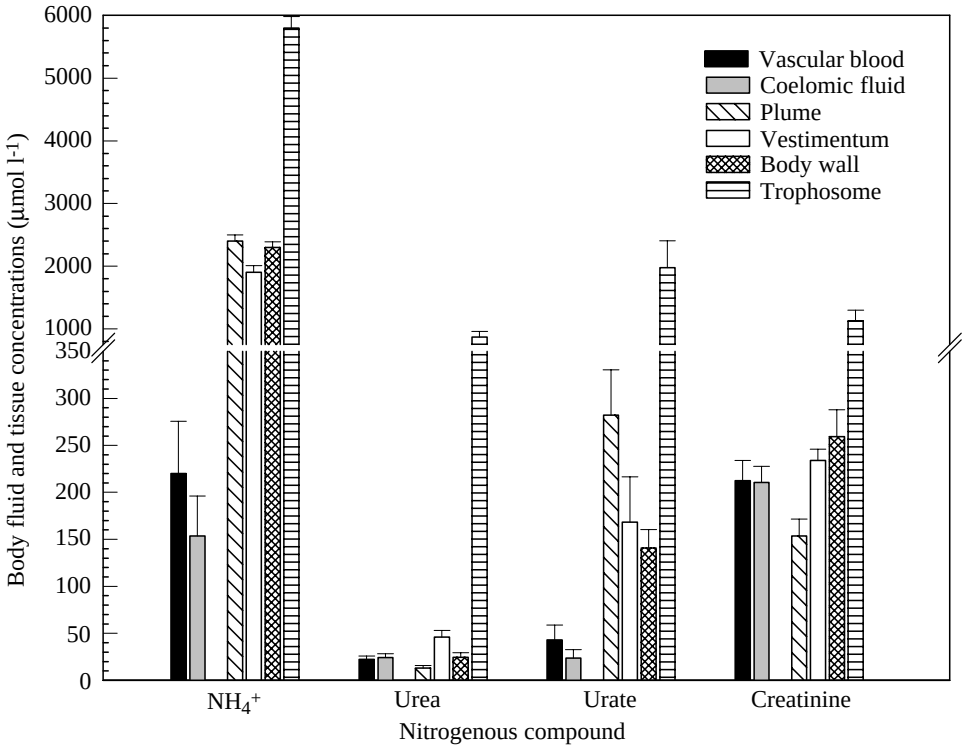


Fig. 1. Distribution of ammonia, urea, urate and creatinine, four important compounds of nitrogen catabolism, in the circulating fluids and tissues of *Riftia pachyptila*. Values are expressed as μmol $l^{-1}$ in both circulating fluids and tissues, with a mean percentage wet mass of water of 80 % for the plume, the vestimentum, the body wall and the trophosome. Values are means + S.E.M. (*N*=12).

The distribution of urate was unusual for a marine invertebrate, so much so that we used two different methods to determine urate contents in the different body compartments of *Riftia pachyptila*, an enzymatic and an HPLC assay. Both gave similar results and corroborated the large discrepancy between the amounts of urate accumulated in the trophosome and those found in the aposymbiotic tissues. The circulating fluids exhibited very low urate contents, less than 50 μmol $l^{-1}$ (Fig. 1). Concentrations were 168.2±48.2 μmol $l^{-1}$ in the vestimentum and 140.8±19.7 μmol $l^{-1}$ in the body wall, and a higher value of 282.3±48.1 μmol $l^{-1}$ (HPLC determination, *N*=5 pools of three individuals each) was recorded in the plume. Urate concentration in the trophosome was as high as 2 mmol $l^{-1}$ (1977.6±428.2 μmol $l^{-1}$, *N*=5). This peculiar urate distribution caused us to carry out further tissue analysis (biochemical and histological) to determine the relative abundance of four potential precursors of urate (guanosine, inosine, hypoxanthine and xanthine) and to investigate the biosynthesis and storage pathways for urate in the tissues of the symbiotic tubeworm (see below).

To summarise these results, to evaluate the total amount of nitrogen accumulated in excretable form in the various body compartments of *Riftia pachyptila* and to determine the relative importance of compounds containing 1–4 nitrogen atoms per molecule, the above data were expressed as μg N $ml^{-1}$ and μg N $g^{-1}$ tissue wet mass (Fig. 2). The trophosome appears to be the favoured tissue for storage of nitrogenous end-products, especially urate and ammonia. In other tissues, ammonia is the main form of nitrogen stored but, unexpectedly, the major form of nitrogen waste in the circulation appears to be creatinine.

*Purines*

The concentration of the hypoxanthine/xanthine fraction was 4102.9±468.1 μmol $l^{-1}$ in the vestimentum, 4101.8±124.9 μmol $l^{-1}$ in the trophosome and 4676.6±214.4 μmol $l^{-1}$ in the body wall (*N*=5). In contrast, the plume exhibited low concentrations of hypoxanthine/xanthine (535.2±64.6 μmol $l^{-1}$, *N*=5) (Fig. 3). The guanosine content, resulting from the catabolism of GTP (see Fig. 6), was very low in all tissues. A similar inosine:guanosine ratio (I:G) was observed in both muscle tissues: I:G=23.5 in the vestimentum (431.5±64.7 μmol $l^{-1}$/18.3±3.4 μmol $l^{-1}$, *N*=5) and 28.9 in the body wall (335.9±37.4 μmol $l^{-1}$/11.6±2.6 μmol $l^{-1}$, *N*=5). This ratio appeared to be totally different in the trophosome (I:G=2.5), where much higher concentrations of both components, 913.3±106.5 μmol $l^{-1}$ for inosine and 360.8±30 μmol $l^{-1}$ for guanosine (*N*=5), were recorded.

These high purine concentrations in the trophosome were surprising, especially since urate was over its solubility threshold, and this prompted us to perform a preliminary histological study of this tissue. The trophosome consists of numerous lobules, each of these being a functional unit (Gardiner and Jones, 1993) (Fig. 4A). A lobule is provided with an axial efferent blood vessel, several afferent vessels situated on the surface of the lobule, and many capillaries joining them. Four cell types make up the trophosomal lobules: in the external layer immediately adjacent to the axial vessel are the non-bacteriocyte cells, either unspecialised cells or muscle cells; filling the whole central part of the lobule are the bacteriocytes, which are specialised cells containing symbiotic chemosynthetic bacteria; and the outermost region is occupied by one or two layers of peritoneal cells.

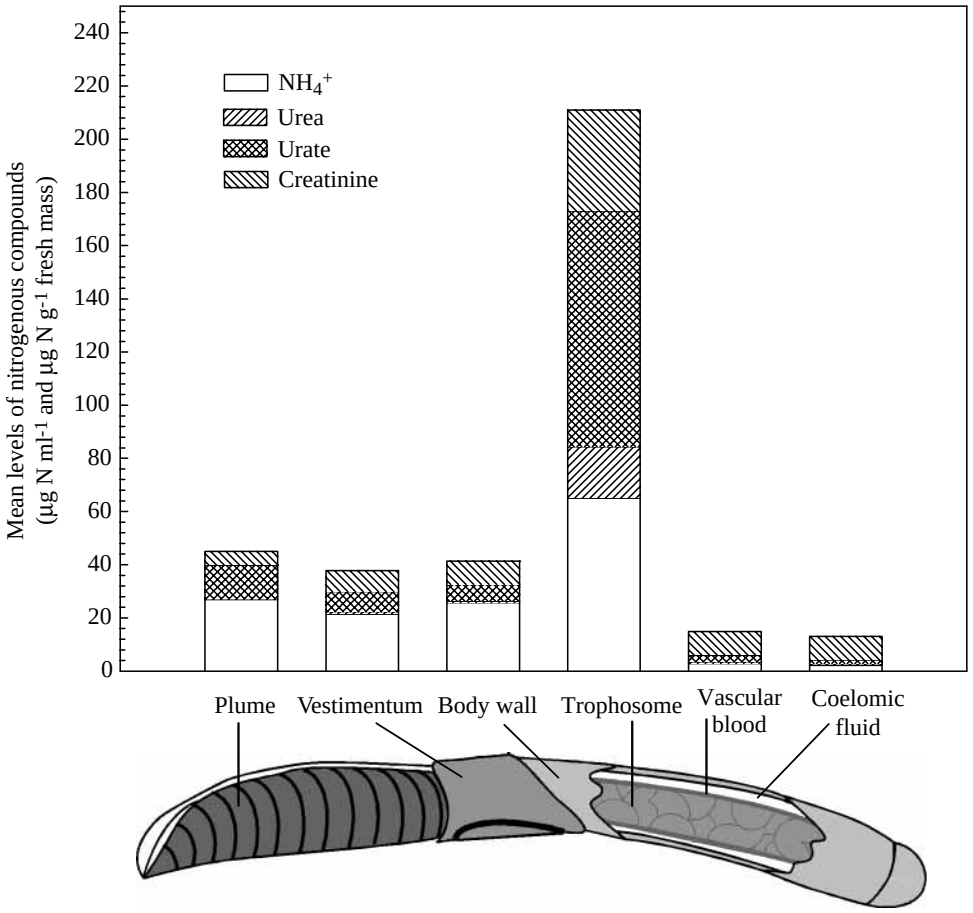


Fig. 2. Relative importance of four nitrogen metabolites in the body compartments of *Riftia pachyptila* (*N*=12). Values are expressed as µg N $g^{-1}$ fresh mass for the tissues and as µg N $ml^{-1}$ for the circulating fluids.

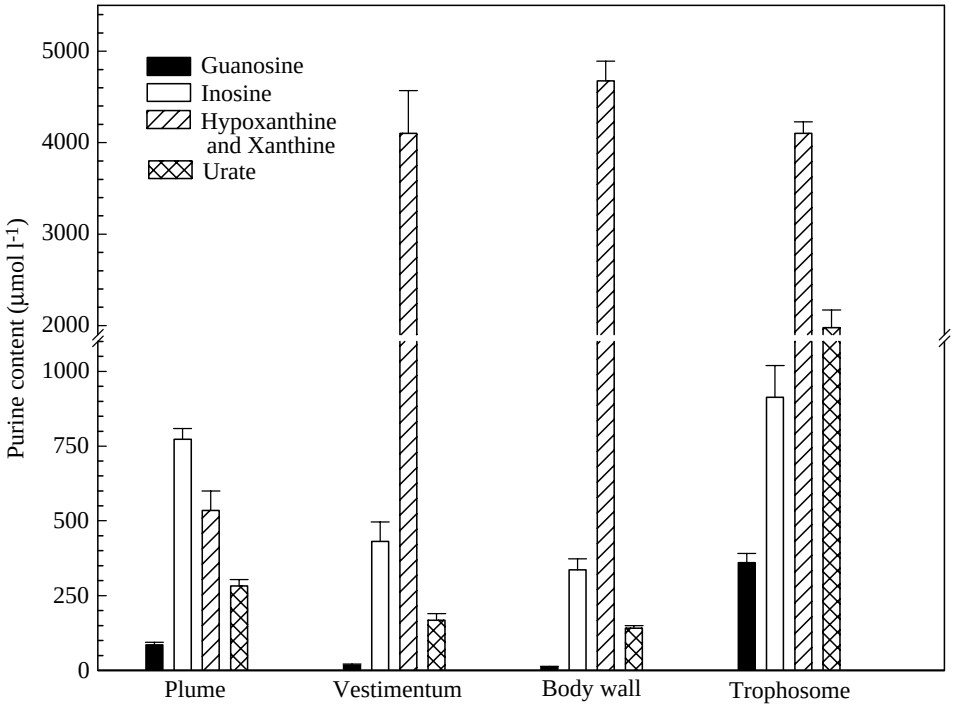


Fig. 3. Distribution and mean content of four intermediate compounds in the purine catabolism pathway in the tissues of *Riftia pachyptila*. Values are mean + S.E.M. (*N*=5 pools of three individual samples each).

Methenamine silver staining showed that all the lobules of the trophosome were positively stained for urate at their periphery (Fig. 4A). Urate deposits in the lobules appeared to be intracellular and specifically located in distal bacteriocytes, at the periphery of the lobules (Fig. 4C). Urate crystals (1–3 µm in diameter) seemed to be organised in increasing levels of

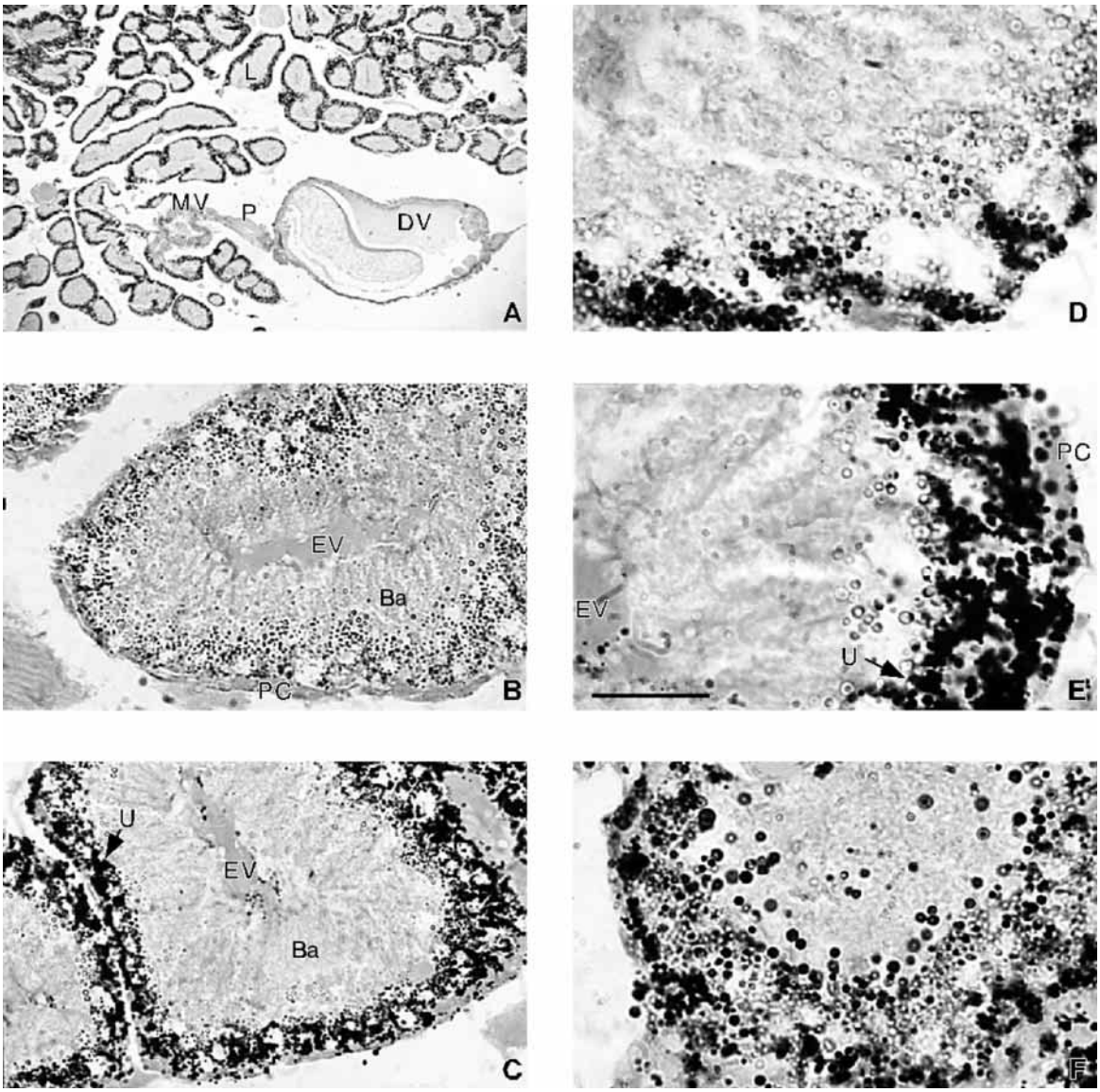


Fig. 4. Light micrographs of trophosome lobules from *Riftia pachyptila* stained with methenamine silver and counterstained with Picro Indigo Carmine (PIC). (A) Cross section of trophosome lobules (L) organised around the dorsal vessel (DV), which is connected to the mesenteric vessel (MV) through mesenteric plexus (P). Solid urate deposits appear as dark spherical precipitates at the periphery of each lobule. (B,D) Cross sections showing the structure of a lobule stained with PIC, D representing a detail of the periphery of B. The efferent vessel is shown at the centre of the lobule (EV), surrounded by bacteriocytes (Ba) and peritoneal cells at the extreme periphery of the lobule (PC). (C,E) Detail of a lobule stained with methenamine silver to show urate deposits (U) and counterstained with PIC. (F) Cross section of a trophosome lobule pre-treated with $LiCO_3$ before methenamine silver staining. Urate deposits have been dissolved and melanin-like pigments remain. Scale bar, 400 μm in A; 40 μm in B and C; 16 μm in D–F.

concentration from the inner part of the lobule, containing young bacteriocytes, to the outer part, where the oldest bacteriocytes are located (Fig. 4E). The intense dark staining observed for urate was confirmed by treating slides with lithium carbonate solution to remove urate crystals before staining (Fig. 4F). The intensity of staining decreased significantly compared with natural lobule slides (Fig. 4B,D) and slides stained specifically for urate (Fig. 4C,E). The remaining stained vesicles probably correspond to melanin-like pigments (Hand, 1987).

*Free amino acids*

The total free amino acid (FAA) concentrations ranged from 2.61±0.97 mmol $l^{-1}$ ($N$=6) in blood and 2.22±0.71 mmol $l^{-1}$ in coelomic fluid to 306.05±40 mmol $l^{-1}$ in the body wall, with 191.34±35 mmol $l^{-1}$ in the vestimentum and 152.9±19.8 mmol $l^{-1}$ in the plume. The trophosome exhibited the lowest level of FAAs of all tissues studied at 125.24±43.4 mmol $l^{-1}$. The composition of the FAA pool in the six body compartments studied was sufficiently characteristic to establish a specific FAA profile for each body compartment

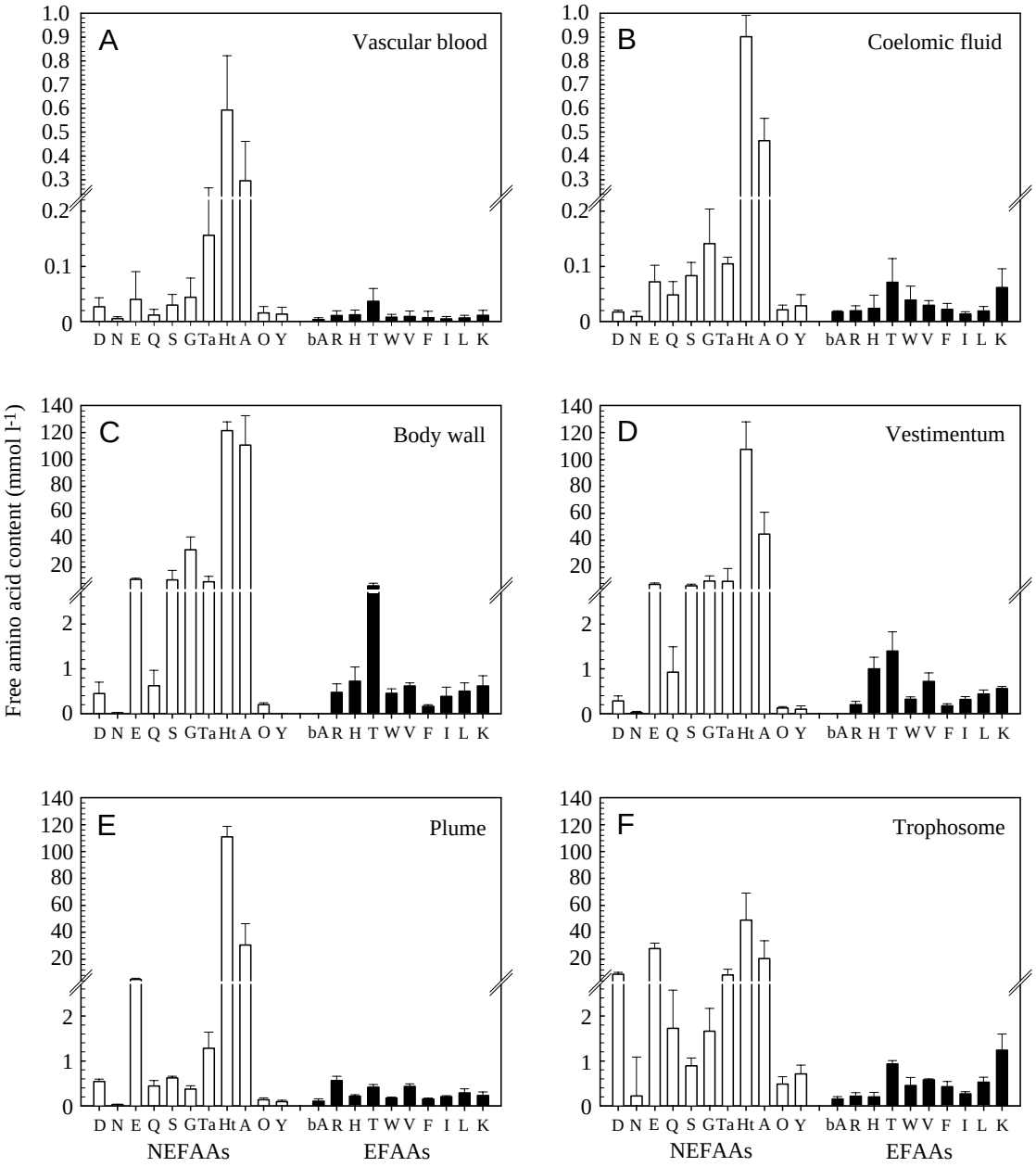


Fig. 5. Free amino acid (FAA) pattern (mmol $l^{-1}$) in circulating fluids (A,B) and tissues (C–F) of *Riftia pachyptila*. Values are means + S.E.M. (*N*=6) of 11 non-essential amino acids (NEFAAs, open columns) and 10 essential amino acids (EFAAs, filled columns). D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; S, serine; G, glycine; Ta, taurine; Ht, hypotaurine; A, alanine; Orn, ornithine; Y, tyrosine; bA, β-alanine; R, arginine; H, histidine; T, threonine; W, tryptophan; V, valine; F, phenylalanine; I, isoleucine; L, leucine; K, lysine.

of the worm (Fig. 5). The non-essential FAA (NEFAA) composition of circulating fluids and tissues was characterised by the predominance of the sulphated amino acid fraction (taurine+thiotaurine and hypotaurine). In the tissues, this fraction accounted for 73.4 % of the total FAAs in the plume (of which hypotaurine represented 72.5 %), 61.2 % in the vestimentum, 42.5 % in the body wall and 45.7 % in the trophosome. This last compartment exhibited the lowest concentrations of hypotaurine, only 50±23.8 mmol $l^{-1}$ compared with 107.5±23.6 mmol $l^{-1}$ in the vestimentum, 110.9±9.2 mmol $l^{-1}$ in the plume and 121.4±7.8 mmol $l^{-1}$ in the body wall. The fact that there was a higher level of the taurine+thiotaurine fraction compared with other tissues to some extent balanced this relatively low hypotaurine level.

Alanine, glutamate, glycine and serine were the main non-sulphated NEFAAs present. Alanine predominated, making up 16–23 % of the total FAAs in circulating fluids and tissues and a maximum of 36 % of the total FAAs in the body wall. The trophosome contained the largest amounts of glutamate (27.8 mmol $l^{-1}$) and of aspartate (8.82 mmol $l^{-1}$). In comparison, the aposymbiotic tissues contained lower levels; the plume had 5.02 mmol $l^{-1}$ glutamate and 0.54 mmol $l^{-1}$

Table 1. *Relationship between enzymatic activities and the concentrations of the nitrogen compounds studied in the tissues of* Riftia pachyptila

| | Plume | Vestimentum | Body wall | Trophosome |
|---|---|---|---|---|
| Enzymes and metabolites of nitrogen breakdown | | | | |
| Urate (μmol $l^{-1}$) | 282.35±48.12 | 168.17±48.22 | 140.79±19.67 | 1977.59±428.16 |
| Uricase (μmol $min^{-1}$ $g^{-1}$) | ND | ND | ND | 2.87±0.26 |
| Urea (μmol $l^{-1}$) | 13.19±2.52 | 46.01±6.92 | 24.52±4.95 | 869.65±89.04 |
| Urease (μmol $min^{-1}$ $g^{-1}$) | 0.38±0.08 | 0.43±0.23 | 0.50±1.27 | 1.24±0.4 |
| Arginine (μmol $l^{-1}$) | 562.86±57.41 | 199.55±36.46 | 472.36±100.20 | 217.03±73.26 |
| Ornithine (μmol $l^{-1}$) | 136.79±24.09 | 127.92±16.78 | 200.67±22.24 | 485.45±86.00 |
| Arginase (μmol $min^{-1}$ $g^{-1}$) | 17.19±2.6 | 6.10±3.11 | 5.03±1.27 | 109.63±16.73 |
| Phosphagen kinases | | | | |
| Arginine kinase (μmol $min^{-1}$ $g^{-1}$) | 31.44±5.92 | 25.44±3.16 | 26.53±3.69 | 9.53±1.33 |
| Creatinine (μmol $l^{-1}$) | 153.66±17.78 | 233.96±11.95 | 259.47±28.37 | 1128.09±171.79 |
| Creatine kinase (μmol $min^{-1}$ $g^{-1}$) | 1.37±1.03 | 16.72±1.84 | 29.67±10.37 | 175.66±20.66 |

Enzyme activities are given as μmol $min^{-1}$ $g^{-1}$ tissue dry mass and nitrogen compound concentrations as μmol $l^{-1}$.
Concentrations are expressed as mean values ± S.E.M. ($N$=12–15) and enzyme activities as means ± S.E.M. ($N$=5 pools of three animals each).
ND, not detected.

aspartate, the vestimentum had 7.26 mmol $l^{-1}$ glutamate and 0.28 mmol $l^{-1}$ aspartate and the body wall had 10.9 mmol $l^{-1}$ glutamate and 0.45 mmol $l^{-1}$ aspartate. The relatively low levels of these compounds could be correlated with the increase in alanine content of tissues *via* anaerobic metabolism. In addition, it is worthy of note that the trophosome also differed from the aposymbiotic tissues in its glutamate:glutamine and aspartate:asparagine ratios: the proportion of neutral forms, asparagine and glutamine, was low in the plume and vestimentum (approximately 0.02 mmol $l^{-1}$ for asparagine and 0.62 mmol $l^{-1}$ for glutamine), while asparagine reached 0.22 mmol $l^{-1}$ and glutamine 1.71 mmol $l^{-1}$ in the trophosome. In both vestimentum and body wall, which are collagen-rich tissues, glycine content was important (9.61 mmol $l^{-1}$ in the vestimentum and 33.05 mmol $l^{-1}$ in the body wall), and the serine pool seemed to be well correlated with the glycine pool, serine being the principal precursor of glycine.

In both aposymbiotic and symbiotic tissues, all the essential amino acids (EFAAs) that could be detected through our protocols were found. These amino acids generally displayed the same pattern in the four tissues and the two circulating fluids, except for the order of magnitude of the concentration. Threonine represented a large proportion of the total EFAAs, especially in the body wall (6.17 mmol $l^{-1}$); free arginine content was quite low in the trophosome (0.22 mmol $l^{-1}$) and much higher in the plume (0.56 mmol $l^{-1}$). Ornithine and tyrosine showed the opposite pattern, with only 0.14 mmol $l^{-1}$ ornithine and 0.09 mmol $l^{-1}$ tyrosine in the plume, and 0.48 mmol $l^{-1}$ ornithine and 0.71 mmol $l^{-1}$ tyrosine in the trophosome.

### *Enzymatic activities*

The maximum activities of eight of the main enzymes related to the biosynthesis and the catabolism of the nitrogen compounds studied (Fig. 6) were investigated in the trophosome and the three aposymbiotic tissues, the plume, vestimentum and body wall. One should keep in mind that the activities reported here are maximal activities, according to the respective assay conditions used (see Materials and methods). Inferred pathway activities must, therefore, be regarded as potential rather than actual.

Linked to the urea cycle, and more particularly to the production and degradation of urea (Fig. 6), arginase and urease activities were detected in all the tissues studied. When correlated to the arginine, ornithine and urea contents of each tissues, arginase and urease appeared to turn over at a very low rate in the muscular tissues (vestimentum and body wall): less than 7 μmol $min^{-1}$ $g^{-1}$ dry mass for arginase and less than 0.5 μmol $min^{-1}$ $g^{-1}$ dry mass for urease (Table 1). Although the plume exhibited a relatively more active arginase (17.2 μmol $min^{-1}$ $g^{-1}$ dry mass), its urease activity was similar to that of the muscles and, consequently, could not explain the very low urea content of this tissue. Both enzymes appeared to be much more active in the trophosome: 109.6 μmol $min^{-1}$ $g^{-1}$ dry mass for arginase and 1.24 μmol $min^{-1}$ $g^{-1}$ dry mass for urease, which is twice that in the plume and muscles, but is still relatively low. These results corroborated the substantial levels of urea and ornithine and the low arginine concentration measured in this tissue (Table 1).

Similar enzymatic studies were carried out on the purine base catabolism pathway, with measurements of AMP deaminase, xanthine oxidase and xanthine dehydrogenase activities, and on the uricolytic pathway, where the activities of uricase and urease, the first and last enzymes of this pathway, were measured (Fig. 6). The trophosome, the symbiotic compartment, was the only tissue where uricase activity could be detected (2.87 μmol $min^{-1}$ $g^{-1}$ dry mass; Table 1). Urease activity (1.24 μmol $min^{-1}$ $g^{-1}$ dry mass) was half that of uricase and this, together with the occurrence of urate deposits in this tissue (Fig. 4), could explain, to some extent,

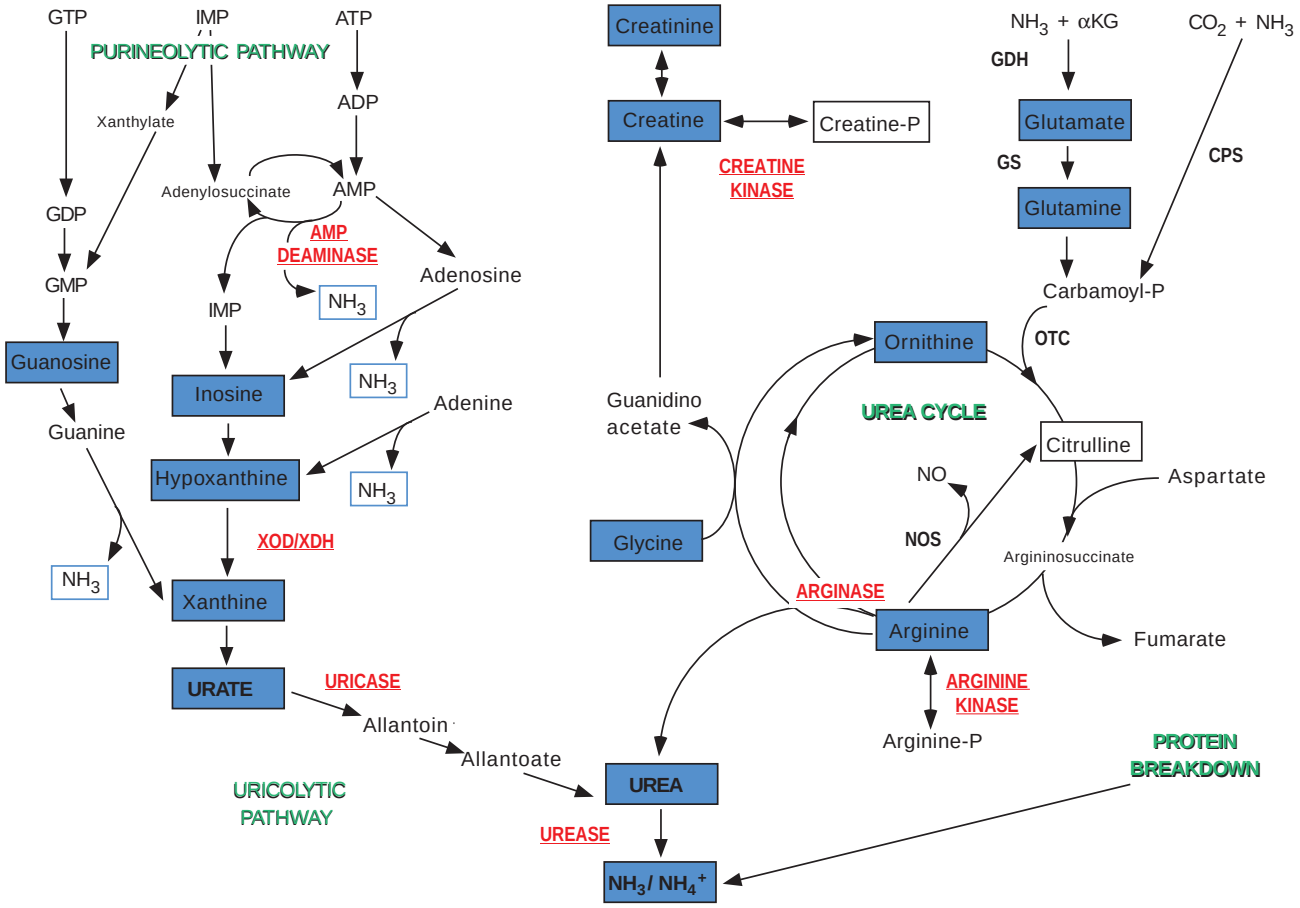


Fig. 6. Nitrogen metabolism and pathways leading to the main end-products (creatinine, urea, urate and ammonia). Shaded boxes indicate the compounds measured in this study, and underlined enzyme names indicate enzymes whose activity has been measured. αKG, α-ketoglutarate; GDH, glutamate dehydrogenase; GS, glutamine synthase; CPS, carbamoyl phosphate synthase; OTC, ornithine transcarbamylase; NO, nitric oxide; NOS, nitric oxide synthase; XOD, xanthine oxidase; XDH, xanthine dehydrogenase; creatine-P, creatine phosphate; carbamoyl-P, carbamoyl phosphate; arginine-P, arginine phosphate.

the very high urate content (1978 μmol $l^{-1}$) observed. The activities of xanthine oxidase, xanthine dehydrogenase and AMP deaminase could not be quantified in our assay conditions (determined according to Gibbs and Bishop, 1977; Lallier and Walsh, 1991; Linton and Greenaway, 1998).

Arginine kinase and creatine kinase activities were also investigated in the four tissues studied to obtain further indications of the potential phosphagen(s) of *Riftia pachyptila*. The two enzymes had similar activity in the vestimentum and body wall (17–30 μmol $min^{-1}$ $g^{-1}$ dry mass) (Table 1). Yet, in the plume, arginine kinase activity was much higher than creatine kinase activity; the reverse situation was observed in the trophosome, where creatine kinase activity was predominant (Table 1). These results must be considered in parallel with the respective measurements of arginine and creatinine levels in the tissues (Table 1). A very high creatinine level (1128.1 μmol $l^{-1}$) could be associated with the high creatine kinase activity in the trophosome and, in contrast, the very low creatine kinase activity in the plume was correlated with a creatinine concentration which was, at approximately 160 μmol $l^{-1}$, the lowest value measured in any of the tissues in this study.

## Discussion

This is an exploratory study that, by analysing a number of nitrogenous metabolites and some of the related enzymatic activities, aimed to depict the main traits of nitrogen metabolism in the symbiotic tubeworm *Riftia pachyptila*. It should be kept in mind that, because of its symbiotic mode of life, *Riftia pachyptila* does not ingest anything. The first question we considered was whether *Riftia pachyptila* is ammoniotelic, ureotelic or uricotelic (Fig. 6).

Our observations show that ammonia levels in the blood of *Riftia pachpytila* are similar to those currently reported in marine ammoniotelic invertebrates, ranging from 100 to 300 μmol $l^{-1}$. The high ammonia content of all the tissues (Fig. 2) indicates a high rate of ammonia production, but no significant difference in ammonia distribution could be found between dorsal (‘venous’) and ventral (‘arterial’) blood. This could indicate that, in *Riftia pachyptila*, either the rate of ammonia excretion is low and its assimilation rate is high, or that $NH_4^+$ is not excreted through the plume and therefore that the body wall could take part in the excretion process, as in the nephridia of annelids. Nitrogenous excretion needs further *in*

*vivo* investigation since the only indication of it we have is the actual existence of an ammonia efflux (P. R. Girguis and J. J. Childress, personal communication). Even if this efflux is of the same order of magnitude as that observed in marine ammoniotelic invertebrates under resting conditions, other possible end-products have not been investigated. Thus, the hypothesis of a non-ammoniotelic metabolism cannot be totally ruled out in the case of *Riftia pachyptila*.

Urea, produced *via* the ornithine–urea cycle, is another common nitrogen end-product in vertebrates and several groups of invertebrates, but the urea cycle has been demonstrated to be either incomplete or non-functional in a number of annelid species. This cycle also allows efficient elimination of excess $HCO_3^-$ and $NH_4^+$ through carbamoyl phosphate synthesis and thus participates in internal pH regulation. Carbamoyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC), the enzymes at the entry to the cycle, were found to be functional in *Riftia pachyptila*, carbamoyl phosphate synthetase being most active in the trophosome while ornithine transcarbamylase was not tissue-specific (Simon et al., 2000). Our study showed that both arginase and urease were present and active in all the tissues studied. The activities of these two enzymes varied according to the tissue, but were generally in good agreement with the respective concentrations of arginine, ornithine and urea found in each tissue. A high arginase activity in the trophosome corresponded to high ornithine and urea concentrations and relatively low arginine concentrations. Urea, which unlike ornithine is not recycled, accumulates in the trophosome because of the low urease activity. It is noteworthy that urea is potentially toxic through cell membrane dissociation and protein denaturation. Excess urea in the trophosome should, therefore, be excreted, recycled for protein synthesis and/or bacterial metabolism, or stored in a neutral form. Given the low concentrations of urea in the circulating fluids and the three other tissues (Fig. 1), urea does not seem to be transported in any other body compartment, nor is it excreted. Recycling would imply a noticeably higher urease activity than that reported in this study (Table 1).

To avoid urea toxicity in the trophosome, this leaves us with the last alternative: storage of urea in a neutral form. Urea production through the ornithine–urea cycle in the aposymbiotic tissues seems much more anecdotal. Both arginase activity and urea concentration are very low, while ornithine content appears to be 10 times higher than urea content (Table 1). These observations could indicate the predominance of another ornithine biosynthesis pathway in the muscles and the plume, independent of the urea cycle, such as the regeneration pathway of ornithine *via* glycine and arginine, an intermediate reaction in creatine and phosphagen biosynthesis.

Surprisingly high urate levels were recorded in the trophosome (2 mmol $l^{-1}$), well above the solubility limit of this compound (approximately 300 μmol $l^{-1}$). Such a high trophosome urate level suggested that urate accumulation in this tissue was the result of inhibition of urate breakdown rather than an enhancement of uricogenesis. Uricase, which had a very low level of activity in the trophosome (2.87 μmol $min^{-1}$ $g^{-1}$ dry mass), is known to be inhibited when oxygen availability is reduced and, consequently, urate would accumulate in the tissue under hypoxic conditions (Dykens, 1991). Microaerophilic conditions have indeed been described in the trophosome to define the symbiotic bacteria microenvironment (Hentschel and Felbeck, 1993). However, we have also found high concentrations of xanthine/hypoxanthine in muscle tissues and trophosome, but xanthine dehydrogenase, xanthine oxidase and AMP deaminase activities were barely detectable in any tissues (activities could be detected in some of the samples but the level was so low that it was unquantifiable). These enzyme activities are reportedly difficult to measure (Lallier and Walsh, 1991), and the assay conditions should be further optimised before any conclusion can be drawn.

Some invertebrate taxa, especially insects and terrestrial crustaceans, are known to synthesise large amounts of urate, but far fewer data are available concerning annelids, in which the occurrence of urate synthesis seems more anecdotal (Needham, 1970). Intracellular accumulation of solid urate in conjunctive cells has been correlated with a nitrogen-rich diet in a terrestrial crab, *Gecarcoidea natalis* (Linton and Greenaway, 1997a), and a *de novo* purine synthesis pathway has been demonstrated in the same species (Linton and Greenaway, 1997b). In the case of *Riftia pachpytila*, purines cannot originate from the diet and must, therefore, be synthesized *de novo*. The necessary amino acids, glutamine, glycine and aspartate, are present in the trophosome tissue (Fig. 5) and could be used for the synthesis of inosine monophosphate (IMP). This compound may, in turn, feed either synthetic or degradative pathways, the latter leading to urate synthesis. Although the role of urate storage is still under study, it has been proposed that urate synthesis contributes to ammonia detoxification (Linton and Greenaway, 1998), and a remobilization of urate through prokaryotic or eukaryotic uricase activation has been demonstrated in two species with symbiotic organisms, a molgulid tunicate *Nephromyces* (Saffo, 1990), and the cockroach *Periplaneta americana* (Donnellan and Kilby, 1967). Furthermore, the flatworm *Convoluta roscoffensis*, which harbours photosynthetic algae, accumulates urate when photosynthesis is absent (aposymbiotic juveniles) or inhibited (Douglas, 1983), and this urate reserve is used during the initiation of the symbiosis or in conditions of low nitrogen. Given the amounts and distribution of spherical crystals of urate in the lobules of the trophosome of *R. pachyptila*, a similar strategy could be proposed, with a bacterial contribution to urate catabolism under low-nitrogen conditions; in active bacteriocytes, urate would be utilised by bacteria, whereas in peripheral bacteriocytes, ‘dying’ bacteria would no longer use it and it would accumulate. Since *Riftia pachpytila* symbionts cannot be cultivated at present, the hypothesis that these bacteria could use urate can only be tested on freshly prepared bacterial suspensions during the course of a scientific cruise.

Our investigation of nitrogenous metabolites in *Riftia pachyptila* continued with the examination of the amino acid profiles in the different tissues. The amino acid signature was different for each of the body compartments studied. Muscle tissues were mainly characterised by very high levels of hypotaurine and alanine and by a significant content of glycine and serine. The plume exhibited the highest concentrations of hypotaurine and an alanine content similar to that of muscles, but it differed from these tissues in having low concentrations of serine and glycine. The FAA profile of the trophosome can be distinguished from the FAA profile of the aposymbiotic tissues on several grounds: (i) a relatively low level of hypotaurine in contrast to the high concentration of the taurine+thiotaurine fraction; (ii) low concentrations of glycine and serine; (iii) the highest levels of glutamate and glutamine, and (iv) a considerable content of aspartate. In addition, the trophosome appears simultaneously to exhibit the lowest total FAA content and the highest $NH_4^+$ concentrations. These observations support the idea that the major use for amino acids is in gluconeogenesis (as demonstrated in vertebrate liver by Campbell, 1991), and a low FAA content in general could indicate a high level of protein synthesis in this tissue.

A general study of the preponderence of sulphated FAAs in the total FAA pool of *Riftia pachyptila* has already been made on the trophosome and the plume of the vestimentiferan (Alberic, 1986; Alberic and Boulegue, 1990). Our study showed that sulphated FAAs, mainly taurine, hypotaurine and thiotaurine, represented 45–70 % of the total FAA pool in the six body compartments of the vestimentiferan. Hypotaurine, taurine and thiotaurine could be involved in the processes of acquisition of energy substrates in a thiotrophic symbiosis such as that found in *Riftia pachyptila*, i.e. phosphagen synthesis, and could be used by some bacterial species as carbon, sulphide or nitrogen sources after oxidation (Huxtable, 1992). In addition, all three of these unusual sulphated amino acids have been found in the sulphide-based symbioses studied in hydrothermal vent animal communities (Pranal et al., 1995; Pruski et al., 1998). The occurrence of sulphated FAAs must, therefore, be the result of a general mechanism primarily involved in the local transport of reduced sulphur to thiotrophic symbionts and seems to be among the adaptations that enable *de facto* detoxification of these compounds (Pruski et al., 1998).

*Riftia pachyptila* appears to possess all the 10 so-called essential amino acids: arginine, threonine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan and valine. It is noteworthy that the position of arginine aa an essential or non-essential amino acid is questionable since high concentrations have been found in the tissues of the Vestimentifera; but since its synthesis by the worm has not been directly demonstrated, we chose to consider it as an essential amino acid. However, the occurrence of all EFAAs, as well as high levels of taurine, an amino acid known to be synthesised at very low rates from methionine and cysteine breakdown in marine invertebrates (Baginski and Pierce, 1977), argues in favour of a possible external source of this amino acid and/or a translocation of amino acids from the symbiont to the host. Indeed, bacteria are not likely to synthesise taurine directly, but could provide cysteine and methionine in sufficient amounts to allow taurine synthesis by their host. In addition, taurine synthesis through endogenous ammonia has been observed in *Solemya reidi*, a symbiotic bivalve living in a reducing environment (Lee et al., 1997), and such a biosynthetic pathway deserves a detailed study in *Riftia pachyptila*.

A third type of amino acid, glutamate, could be produced by bacterial metabolism. Glutamate concentrations are maximal in the symbiotic compartment, and it is known to be the first amino acid synthesised from $NH_3$ and leads to the synthesis of all other non-essential amino acids. The relative importance of aspartate, glutamate and alanine could indicate the occurrence of active pathways of anaerobic energy metabolism in *R. pachyptila*, with aspartate being used for carbohydrate fermentation, which would explain the low concentrations recorded, and alanine, which is present at very high levels, being a well-known product of anaerobiosis in annelids. It should be noted that the worms used in this study were sampled upon recovery on board and were probably using anaerobic metabolism as a result of this stressful treatment (at least 2 h in a closed box). To test normal, aerobic conditions, the animals should have been repressurized. Indeed, previous studies have shown that, under repressurization, blood pH rise from 7 to 7.4 within 2 days (Childress et al., 1984, 1991).

Another interesting issue arising from this study concerns the occurrence and distribution of phosphagens in the tissues of *R. pachyptila*. The predominance of creatinine in circulating fluids is rather unusual (and could eventually be shown to be the result of recovery stress), but it suggests the presence of high levels of phosphocreatine in most tissues. Creatine kinase activity was found in all tissues studied, but arginine kinase was also active, especially in plume tissue, suggesting the co-occurrence of phosphoarginine and phosphocreatine. The former would be preferentially used in the plume, whereas the latter would be prevalent in the trophosome. A recently published study of phosphorus compounds in *Riftia pachyptila* tissues using $^{31}P$ nuclear magnetic resonance (NMR) also showed the simultaneous presence of two phosphagens in the vestimentum and body wall, whereas ‘trunk’ (the trophosome plus body wall) tissue showed only one peak (Thebault et al., 1999). However, the most abundant and ubiquitous peak was identified as either phosphoarginine or phosphotaurocyamine (which have indistinguishable $^{31}P$-NMR signatures), and the other phosphagen peak was not identified although it did not match with phosphocreatine.

The absence of phosphocreatine in trophosome tissue seems contradictory when considering the high activity of creatine kinase and the high level of creatinine found in this tissue. However, if phosphocreatine is the primary phosphagen in *Riftia pachyptila* trophosome, it may well have been utilised completely during recovery stress and thus be absent from $^{31}P$-NMR spectra. Obviously, given the high diversity of possible phosphagens in annelids (Needham, 1970) and the abundance of possible precursors of phosphagens (i.e. arginine, creatinine,

taurine and hypotaurine) in the tissues of *Riftia pachyptila*, a much more detailed study of substrates, enzymes and phosphagens, using repressurised animals to eliminate stress, would be necessary.

In conclusion, this study has emphasized the peculiarity of the trophosome of *Riftia pachyptila* in relation to nitrogen metabolism: the abundance of uric acid, the presumed predominance of phosphocreatine and the probable activity of a complete urea cycle clearly set this tissue apart from other host tissues. Unfortunately, the nitrogen metabolism of the bacterial symbionts is not yet fully characterised, mainly because this strain cannot yet be cultivated. The excretion product of this symbiotic association, if any, therefore remains elusive, and physiological experiments performed on living, preferably repressurised, animals are needed to elucidate this pathway during forthcoming cruises.

We would like to thank the captain and crew of the *Atlantis* and the *Alvin* team, and the chief scientists, Jim Childress (UCSB) and Doug Manahan (WHOI), for allowing us to obtain our samples. We are greatly indebted to Franck Zal for collecting and preserving the samples on board. We also thank Manfred Grieshaber (University of Düsseldorf) for his help with HPLC purine determination. This study was partly funded by Ifremer URM 7.

## References

**Alberic, P.** (1986). Présence de quantité importantes de thiotaurine et d'hypotaurine dans les tissus de *Riftia pachyptila*. *C.R. Acad. Sci. Paris* **302**, 503–508.

**Alberic, P. and Boulegue, J.** (1990). Unusual amino compounds in the tissues of *Calyptogena phaseoliformis* (Japan Trench): possible link to symbiosis. *Prog. Oceanogr.* **24**, 89–101.

**Arp, A. J., Childress, J. J. and Vetter, R. D.** (1987). The sulfide-binding protein in the blood of the vestimentiferan tube worm, *Riftia pachyptila*, is the extracellular haemoglobin. *J. Exp. Biol.* **128**, 139–158.

**Baginski, R. M. and Pierce, S. K.** (1977). The time course of intracellular amino acid accumulation in the tissues of *Modiolus demissius* during high salinity adaptation. *Comp. Biochem. Physiol.* **57**, 407–412.

**Campbell, J. W.** (1991). Excretory nitrogen metabolism. In *Environmental and Metabolic Animal Physiology* (ed. C. L. Prosser), pp. 277–324. New York: Wiley-Liss.

**Childress, J. J., Arp, A. J. and Fisher, C. R.** (1984). Metabolic and blood characteristics of the hydrothermal vent tubeworm *Riftia pachyptila*. *Mar. Biol.* **83**, 109–124.

**Childress, J. J., Fisher, C. R., Favuzzi, J. A., Kochevar, R., Sanders, N. K. and Alayse, A. M.** (1991). Suflide-driven autotrophic balance in the bacterial symbiont containing hydrothermal vent tubeworm *Riftia pachyptila*, Jones. *Biol. Bull.* **180**, 135–153.

**Childress, J. J., Lee, R. W., Sanders, N. K., Felbeck, H., Oros, D. R., Toulmond, A., Desbruyères, D., Kennicut II, M. C. and Brooks, J.** (1993). Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental $P_{CO_2}$. *Nature* **362**, 147–149.

**Clinch, J. R., Worsfold, P. J. and Sweeting, F. W.** (1988). An automated spectrophotometric field monitor for water quality parameters. Determination of ammonia. *Analyt. Chim. Acta* **241**, 401–407.

**Donnellan, J. F. and Kilby, B. A.** (1967). Uric acid metabolism by symbiotic bacteria from the fat body of *Periplaneta americana*. *Comp. Biochem. Physiol.* **22**, 235–252.

**Douglas, A. E.** (1983). Uric acid utilization in *Platymonas convolutae* and symbiotic *Convoluta roscoffensis*. *J. Mar. Biol. Ass. UK* **63**, 435–447.

**Durand, F., Chausson, F. and Regnault, M.** (1999). Increases in tissue free amino acid levels in response to prolonged emersion in marine crabs: an ammonia-detoxifying process efficient in the intertidal *Carcinus maenas* but not in the subtidal *Necora puber*. *J. Exp. Biol.* **202**, 2191–2202.

**Dykens, J. A.** (1991). Purineolytic capacity and origin of haemolymph urate in *Carcinus maenas* during hypoxia. *Comp. Biochem. Physiol.* **98**B, 579–582.

**Fisher, C. R., Childress, J. J., Arp, A. J., Brooks, J. M., Distel, D., Favuzzi, J. A., Macko, S. A., Newton, A., Powell, M. A., Somero, G. N. and Soto, T.** (1988). Physiology, morphology and biochemical composition of *Riftia pachyptila* at Rose Garden in 1985. *Deep-Sea Res.* **35**, 1745–1758.

**Gabe, M.** (1968). *Techniques Histologiques*. Paris: Masson.

**Gardiner, S. L. and Jones, M. L.** (1993). Vestimentifera. In *Microscopic Anatomy of Invertebrates*, vol. 12, *Onychophora, Chilopoda and Lesser Protostomata* (ed. S. L. Gardiner), pp. 371–460. New York: Wiley-Liss.

**Gibbs, K. L. and Bishop, S. H.** (1977). Adenosine triphosphate-activated adenylate deaminase from marine invertebrate animals. *Biochem. J.* **163**, 511–516.

**Goffredi, S. K., Childress, J. J., Desaulniers, N. T. and Lallier, F. H.** (1997a). Sulfide acquisition by the vent worm *Riftia pachyptila* appears to be *via* uptake of $HS^-$, rather than $H_2S$. *J. Exp. Biol.* **200**, 2609–2616.

**Goffredi, S. K., Childress, J. J., Desaulniers, N. T., Lee, R. W., Lallier, F. H. and Hammond, D.** (1997b). Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external $P_{CO_2}$ and upon proton-equivalent ion transport by the worm. *J. Exp. Biol.* **200**, 883–896.

**Hand, S. C.** (1987). Trophosome ultrastructure and the characterization of isolated bacteriocytes from invertebrate–sulfur bacteria symbioses. *Biol. Bull.* **173**, 260–276.

**Harris, R. R. and Andrews, M. B.** (1985). Total NPS pool and ammonia net efflux rate changes in *Carcinus maenas* during acclimation to low environmental salinity. *Comp. Biochem. Physiol.* **82**A, 301–308.

**Hentschel, U. and Felbeck, H.** (1993). Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*. *Nature* **366**, 338–340.

**Huxtable, R. J.** (1992). Physiological actions of taurine. *Physiol. Rev.* **72**, 101–163.

**Johnson, K. S., Childress, J. J., Hessler, R. R., Sakamoto-Arnold, C. M. and Beehler, C. L.** (1988). Chemical and biological interactions in the Rose Garden hydrothermal vent field. *Deep-Sea Res.* **35**, 1723–1744.

**Lallier, F. H. and Walsh, P.** (1991). Activities of uricase, xanthine oxidase and xanthine dehydrogenase in the hepatopancreas of aquatic and terrestrial crabs. *J. Crust. Biol.* **11**, 506–512.

**Lee, R. W. and Childress, J. J.** (1996). Inorganic N assimilation and ammonium pools in a deep-sea mussel containing methanotrophic endosymbionts. *Biol. Bull.* **190**, 373–384.

**Lee, R. W., Childress, J. J. and Desaulniers, N. T.** (1997). Effects of ammonia exposure on ammonia and taurine pools of the symbiotic clam *Solemya reidi*. *J. Exp. Biol.* **200**, 2797–2805.

**Lee, R. W., Robinson, J. J. and Cavanaugh, C. M.** (1999). Pathways of inorganic nitrogen assimilation in chemoautotrophic bacteria–marine invertebrate symbioses: expression of host and symbiont glutamine synthetase. *J. Exp. Biol.* **202**, 289–300.

**Lilley, M. D., Butterfield, D. A., Olson, E. J., Lupton, J. E., Macko, S. A. and McDuff, R. E.** (1993). Anomalous $CH_4$ and $NH_4^+$ concentrations at an unsedimented mid-ocean-ridge hydrothermal system. *Nature* **364**, 45–47.

**Lindroth, P. and Mopper, K.** (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Analyst. Chem.* **51**, 1667–1674.

**Linton, S. M. and Greenaway, P.** (1997a). Intracellular purine deposits in the gecarcinid land crab *Gecarcoidea natalis*. *J. Morph.* **231**, 101–110.

**Linton, S. M. and Greenaway, P.** (1997b). Urate deposits in the gecarcinid land crab *Gecarcoidea natalis* are synthetised *de novo* from excess dietary nitrogen. *J. Exp. Biol.* **200**, 2347–2354.

**Linton, S. M. and Greenaway, P.** (1998). Enzymes of urate synthesis and catabolism in the gecarcinid land crab *Gecarcoidea natalis*. *Exp. Biol. Online* **3**, 5.

**Needham, A. E.** (1970). Nitrogen metabolism in Annelida. In *Comparative Biochemistry of Nitrogen Metabolism*, vol. 1, *The Invertebrates* (ed. J. W. Campbell), pp. 207–287. London: Academic Press.

**Nelson, D. C. and Fisher, C. R.** (1995). Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps. In *Microbiology of Deep-Sea Hydrothermal Vents* (ed. D. M. Karl), pp. 125–167. Boca Raton, FL: CRC Press Inc.

**Newell, B. S., Morgan, B. and Cundy, J.** (1967). The determination of urea in seawater. *J. Mar. Res.* **25**, 201–202.

**Pranal, V., Fialamedioni, A. and Colomines, J. C.** (1995). Amino acid and related compound composition in two symbiotic mytilid species from hydrothermal vents. *Mar. Ecol. Prog. Ser.* **119**, 155–166.

**Price, N. M. and Harrison, P. J.** (1987). Comparison of methods for the analysis of dissolved urea in seawater. *Mar. Biol.* **94**, 307–317.

**Pruski, A. M., Fiala-Medioni, A., Boulegue, J. and Colomines, J.-C.** (1998). Sulfur amino acids in symbiotic species from the hydrothermal vents and cold seeps. *Cah. Biol. Mar.* **39**, 321–324.

**Saffo, M. B.** (1990). Symbiosis within a symbiosis: intracellular bacteria within the endosymbiotic protist *Nephromyces*. *Mar. Biol.* **107**, 291–296.

**Simon, V., Purcarea, C., Sun, K., Joseph, J., Frebourg, G., Lechaire, J. P., Gaill, F. and Herve, G.** (2000). The enzymes of synthesis and utilization of carbamyl-phosphate in the deep-sea tube worm *Riftia pachyptila. Mar. Biol.* **136**, 115–127.

**Thébault, M. T., Kervarec, N., Pichon, R., Nonnotte, G. and Le Gal, Y.** (1999). A $^{31}P$ nuclear magnetic resonance study of the hydrothermal vent tube worm *Riftia pachyptila*. *C.R. Acad. Sci. Paris* **322**, 537–541.

**Toulmond, A., Lallier, F. H., De Frescheville, J., Desbruyères, D., Childress, J. J., Lee, R. and Sanders, N. K.** (1994). Unusual carbon dioxide-combining properties of body fluids in the hydrothermal vent tubeworm *Riftia pachyptila*. *Deep-Sea Res.* **41**, 1447–1456.

**Wynants, J., Petrov, B., Nijhof, J. and van Belle, H.** (1987). Optimization of a high-performance liquid chromatographic method for the determination of nucleosides and their catabolites. Application to cat and rabbit heart perfusates. *J. Chromatogr.* **386**, 297–308.

**Zal, F., Leize, E., Lallier, F. H., Toulmond, A., Van Dorsselaer, A. and Childress, J. J.** (1998). S-sulfohemoglobin and disulfide-exchange: The mechanisms of sulfide-binding by *Riftia pachyptila* hemoglobins. *Proc. Natl. Acad. Sci. USA* **95**, 8997–9002.