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Isolation and characterization of granules from the kidney of the bivalve *Mercenaria mercenaria*

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Abstract

Intracellular and extracellular granules are found in most bivalve kidneys. We examined the composition of kidney granules from the infaunal bivalve *Mercenaria mercenaria* (L.) collected in 1985 from Cataumet Bay, Bourne, and Waquoit Bay, Falmouth, Massachusetts, USA. Small granules are numerically dominant, but large granules dominate the samples on the basis of weight. Large granules (estimated diam. $>15\ \mu m$) are composed primarily of metals (28% by weight) with Mn (8%), Ca (8%), Zn (4%) and Fe (4%) as the major contributors. Non-metal elements (P, C, H, N, S) together constituted 21% of the granule dry weight, though organic content (0.02% protein, 0.3% carbohydrate and 0.5% lipid) was low. Small kidney granules ($\leq 10\ \mu m$ diam.) and digestive gland granules from *M. mercenaria* had significantly higher C, H, and N contents than larger kidney granules. Our results, taken together with those from other bivalve species, suggest that kidney granule formation and subsequent increase in size is a continuous process of lysosomal maturation, residual body release and extracellular accumulation of predominately inorganic elements.

Introduction

Three types of metal-rich granules are frequently observed within bivalve tissues (copper-, calcium-, and iron-containing granules; Brown 1982). Although all three may be present in bivalve tissues, calcium-containing granules have received the most attention. These calcium-containing granules have been described in the bivalve mantle (Neff 1972), digestive gland (Ballan-Dufrançais et al. 1985), gill (Silverman et al. 1987) and hemocytes (Ballan-Dufrançais et al. 1985). Most reports, however, have examined the granules that occur in the bivalve kidney (=paired glandular coelomoducts or organs of Bojanus) (Doyle et al. 1978, Carmichael et al. 1979, Rheinberger et al. 1979, George et al. 1980, Gold et al. 1982). Since the kidney has been shown to be one of the major metal-accumulating organs of the bivalve body (Robinson et al. 1985, Lobel 1986, Robinson and Ryan 1986), a knowledge of the composition of kidney granules is a prerequisite to understanding pathways of metal metabolism.

Kidney granules containing calcium vary greatly in size and may occur intracellularly and extracellularly (Brown 1982). Small intracellular granules ($\leq 15\ \mu m$ diam.) are apparently present in all bivalve species and are thought to originate within the lysosomal system (George 1983a, b). They are usually observed within membrane-limited vacuoles (Fowler et al. 1981, Brown 1982). Accumulation of soluble metal-binding proteins within the lysosomal system of the bivalve kidney has been postulated as a mechanism of cellular metal detoxification (George 1983a, Simkiss and Mason 1983).

Large, extracellular granule accumulations have been observed in many species including *Mercenaria mercenaria* (Morse et al. 1985), *Macrocallista nimbosa* (Tiffany et al. 1980), *Pinna nobilis* (Ghiretti et al. 1972) *Donax trunculus* (Mauri and Orlando 1982), and *Cyclosunetta menstrualis* (Ishii et al. 1986). Extracellular granule size is probably a function of the length of time that the particles are retained in the kidney lumen (Brown 1982). Large extracellular granules may be produced by aggregation of smaller granules (Tiffany et al. 1980, Brown 1982, Ishii et al. 1986) or by layering of intraluminal material in concentric rings around extruded intracellular granules (Doyle et al. 1978, Carmichael et al. 1979, 1980, Tiffany et al. 1980, Simkiss et al. 1982). The actual mechanisms of metal incorporation, detoxification and granule formation are not fully understood.

An extensive profile of the chemical composition of *Mytilus edulis* kidney granules (presumably intracellular granules) has been reported by George et al. (1982). However, preliminary analyses of the extracellular granule composition in *Mercenaria mercenaria,* as well as reported values for

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some other bivalve species, indicate that *M. edulis* granules are not necessarily typical of bivalve kidney granules. Kidney granules isolated from *M. edulis* are smaller (1 to 3 μm diam.; George et al. 1982) and contain less Ca and P (by weight) than granules isolated from the kidney of *Pecten maximus* (George et al. 1980), *Argopecten* spp. (Carmichael et al. 1979), *Tridacna* spp. (Hignette 1979, Reid et al. 1984), *Donax trunculus* (Mauri and Orlando 1982), *Pinna nobilis* (Ghiretti et al. 1972, Hignette 1979), and *Mercenaria mercenaria* (Rheinberger et al. 1979). The granules from all these latter species, however, have not been extensively characterized.

This study was therefore undertaken to fully characterize the calcium-containing granules from the kidney of an infaunal eulamellibranch, the quahog *Mercenaria mercenaria* (L.). We compared three granule separation techniques which we expected would isolate granules from the surrounding kidney tissue with differing efficiencies. The composition of kidney granule samples was compared to that of granules isolated from the digestive gland of the same species and to previously published descriptions of kidney granules from other bivalve species.

Materials and methods

Materials

Mercenaria mercenaria (average length ca. 90 mm) were collected during 1985 from Cataumet Bay, Bourne, and Waquoit Bay, Falmouth, Massachusetts, USA by commercial shellfishermen. The bivalves were maintained in a running seawater system, at 10° to 15 °C until utilized in the experiments. All reagents used were analytical grade.

Isolation methods

Kidney tissue was dissected from surrounding tissues and kept chilled (4 °C) during processing. Granules were isolated from pooled kidneys using one of three methods: (1) differential settling over sucrose; (2) protease digestion; or (3) hand-selection. Emphasis was placed on obtaining granule preparations of maximum purity, not on achieving maximum yield.

To isolate granules by differential settling, an enriched pellet of kidney granules was first prepared (Sullivan et al. 1988). Kidney tissue was homogenized in 10 vols of 15% sucrose buffer (440 m*M* sucrose, 190 m*M* NaCl, 10 m*M* Tris-HCl pH 7.5, 1 m*M* DTT) using a Ten-Broeck homogenizer. The homogenate was sieved through Nitex screen (200 μm pore size) and allowed to settle at unit gravity (1 × g) for 15 min in a conical centrifuge tube. This enriched granule fraction (1 × g pellet) was diluted, layered onto 65% sucrose buffer (2.5 M sucrose, 190 m*M* NaCl, 10 m*M* Tris-HCl pH 7.5, 1 m*M* DTT) and allowed to settle at 1 × g for 60 min. The granule pellet was washed three times in distilled deionized water, and sonicated for 2 min in 100 m*M* DTT to detach organic material adhering to the settled granules. Sonicated granules were relayered onto 65% sucrose, and allowed to settle at 1 × g for an additional 60 min. The settled granules (subsequently referred to as "differentially-settled" granules) were rinsed six times with distilled deionized water.

For the protease digestion procedure (after George 1983a), 10 vols of 0.07% protease solution (Type XIV from *Streptomyces griseus*, in 100 m*M* Tris-HCl, pH 7.5) were added to coarsely chopped *Mercenaria mercenaria* kidney or digestive gland tissues. The mixture was incubated with constant agitation for a minimum of 18 h at 37 °C. The granules (subsequently referred to as "protease-isolated" granules) were sedimented at unit gravity and rinsed several times with distilled deionized water to remove the solubilized tissue.

Kidney granules were also isolated by manually removing them from the surrounding tissue. These large, extracellular granules ("hand-selected" granules) were rinsed several times with distilled deionized water.

Isolated granule preparations were kept in distilled water for light microscopic analysis. Subsamples for marker enzyme analysis were used immediately and mixed with the appropriate assay buffer. Granules to be used for thermogravimetric, elemental, amino acid, carbohydrate, lipid, and histochemical analyses were lyophilized and stored in a vacuum desiccator until needed. Samples for histochemistry were resuspended in distilled water just prior to analysis.

Purity assessment

Kidney granule preparations were observed under fluorescent, light and scanning electron microscopes to determine the purity of the preparations and the visual characteristics of the granules. Unstained kidney granules were examined for autofluorescence under an Olympus Vanox-T AH-2 epifluorescence microscope (UV and blue excitation light). Contamination of granule fractions by nuclei and debris was visually assessed following staining with the fluorescent dyes acridine orange and Hoechst 33258. Contamination of granule preparations was also assessed with marker compounds deoxyribonucleic acid (DNA), succinate dehydrogenase (SDH), 5'nucleotidase (5'N) and acid phosphatase (AP) using procedures described in Sullivan et al. (1988). For scanning electron microscopy (SEM), kidney granules were dehydrated in acetone, mounted on aluminum stubs using double-sided adhesive tape, sputter-coated with gold-palladium and observed using an AMR-1000 microscope.

Granule analysis

Kidney granules isolated using the protease digestion procedure were measured (maximum diameter) under the light microscope using an ocular micrometer. Standard histochemical tests (Pearse 1968, Humason 1972, Lillie and Fullmer 1976) were performed on granules isolated from the kidney (differentially-settled and protease-isolated) as well as granules isolated from the digestive gland (protease-isolated). Protease-isolated kidney granules were analyzed

colorimetrically for total carbohydrate (Brink et al. 1960) and gravimetrically for lipid based on the difference in dry weight following chloroform extraction.

Kidney granules (differentially-settled and protease-isolated) and digestive gland granules (protease-isolated) were digested in 40% HNO_3 for determination of metal content. Samples were heated to near dryness twice, then diluted to a final concentration of 10% HNO_3 and 0.4% lithium spectrochemical buffer. Metal concentrations were measured using a Spectrametrics Spectrospan III B (Applied Research Laboratories) d.c. plasma emission spectrometer (DCP) calibrated with matrix-matched standards. Detection limits (2σ) for Ba, Cr, Cu, Mg, Mn, Ni and Zn were $\geq 0.01\ \mu g\ ml^{-1}$ for each element in solution; for Ca, P and Pb, $\geq 0.1\ \mu g\ ml^{-1}$; for Cd, Fe and K, ≥ 0.02, 0.04, and 1.5 $\mu g\ ml^{-1}$, respectively. Samples of National Bureau of Standards oyster tissue (SRM 1566) were analyzed as a check of accuracy.

Carbon, hydrogen and nitrogen contents of kidney granules (from all three isolation procedures) and digestive gland granules (protease-isolated) were determined on a Perkin-Elmer 240 CHN analyzer using acetamide as a standard. Protease-isolated kidney granules $\leq 10\ \mu m$ in diam. (separated by wet sieving protease-isolated granules from over 700 quahogs through a 10 μm pore size Nitex screen prior to lyophilization) were also analyzed for CHN content. These small granules were considered to be either intracellular granules or granules which had recently been extruded from kidney cells (Morse et al. 1985). This granule sample would also contain any small fragments of extracellular kidney granules which had broken during dissection and initial processing.

Sulfur content was analyzed by high temperature combustion followed by IR detection on a Leco Sulfur analyzer using various organic compounds (e.g. sulfamic acid) as standards (Standard Method E16-2, Galbraith Laboratories, Knoxville, Tennessee). Duplicate samples were analyzed by ion chromatography (Dionex column) using H_2SO_4 as a standard (Standard Method ME16-4, Galbraith Laboratories).

Infra-red spectral characteristics of protease-isolated kidney granules embedded in a potassium bromide pellet were determined with a Perkin-Elmer IR spectrophotometer. Thermogravimetric analysis (TGA) of kidney granules (protease-isolated) was conducted on a Perkin-Elmer TGS-2 thermogravimetric analyzer.

Amino acid contents were determined with a Hewlett-Packard 1084B HPLC using an Altex 3-μm ODS C-18 column. Samples of kidney granules isolated by differential settling and protease digestion were completely digested in 6N HCl in sealed glass vials under inert gas for 24 h at 110 °C, then derivatized with phenylisothiocyanate (PITC) prior to injection (Bidlingmeyer et al. 1984). Results were not corrected for possible loss of serine or threonine. Separate samples were digested in the presence of dimethylsulfoxide (DMSO) for subsequent analysis of cysteic acid. Total amino acid content was used as an estimate of protein content.

Results

Visual examination of purified *Mercenaria mercenaria* kidney granule preparations, with and without acridine orange and Hoechst 33258 staining, revealed no contamination by tissue fragments or kidney cell nuclei. Analysis of marker compounds within the differentially-settled granule preparation indicated that nuclei, mitochondria, lysosomes, and plasma membranes were either not present or only present as trace contaminants (Table 1). Marker assays were not conducted on the protease-isolated granules since the proteolytic enzyme used to digest the tissue surrounding the granules would presumably have destroyed these enzyme markers (SDH, AP and 5′N). Large kidney granules ($>10\ \mu m$) were black in color, while smaller granules (and fragments of the larger granules) appeared either brown or black. All granules were autofluorescent (yellow) under ultraviolet light, but were not birefringent in polarized light and did not exhibit ferromagnetic properties.

Differentially-settled granules were relatively large (estimated mean diameter ca. 150 μm) since most of the smaller intracellular granules did not settle at 1 × g. Many appeared to have jagged edges (Fig. 1 A), indicating that the largest granules were broken during homogenization. In contrast, the granules separated using the protease digestion procedure contained numerous small, rounded particles in addition to large extracellular granules (Fig. 1 B). These small particles did not stain with acridine orange or Hoechst 33258, and are therefore considered to be either intracellular granules or very small extracellular granules. The larger granules from the protease digestion procedure had smoother edges than the granules isolated by the differential settling procedure, indicating that the protease-isolated granules may not have been broken during processing. Smoother granule contours may be more representative of the *in situ* shape of the granules.

Hand-selected extracellular granules were large, highly irregular in shape and often contained numerous protuber-

Table 1. *Mercenaria mercenaria*. Marker compounds in differentially-settled kidney granule fraction. Maximum possible % activity is an estimate based on detection limit for each marker substance. ND = not detected

Marker	Content in granule fraction	Content in unfractionated homogenate	Maximum possible % activity in granule fraction
DNA[a]	ND	21.8 ± 2.1	< 0.3%
SDH[b]	ND	2 400 ± 200	< 2%
AP[c]	ND	6.7 ± 1.3	< 1.5%
5′N[d]	ND	347 ± 11	< 0.3%

[a] DNA content as μg DNA ($n = 2$); detection limit = 0.05 μg DNA ml^{-1}

[b] SDH content as enzyme activity per 20-min incubation ($n = 3$); detection limit = 50 units ml^{-1}

[c] AP content as μg alpha-naphtol produced per 30-min incubation ($n = 2$); detection limit = 0.1 $\mu g\ ml^{-1}$

[d] 5′N content as snake venom equivalents per 20-min incubation ($n = 3$); detection limit = 1 unit ml^{-1}

Fig. 1. *Mercenaria mercenaria.* Isolated kidney granules. (A) Light micrograph of granules isolated by differential settling procedure. Note jagged edges of granules (artifact of preparation). Scale bar = 200 μm. (B) Granules isolated using the protease digestion procedure (scale bar = 200 μm). Extracellular granules have rounded edges; numerous small particles are presumptive intracellular granules. (C) SEM of an exceptionally large extracellular granule (scale bar = 200 μm). Closer examination of surface of this granule indicates that it may have been formed by concentric layering of material around a central particle [(D) area at small arrow in (C); scale bar = 20 μm] as well as aggregation of smaller particles [(E) area at large arrow in (C); scale bar = 20 μm]

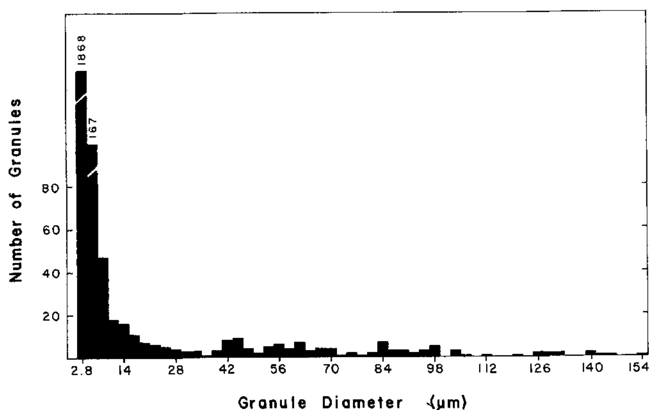

Fig. 2. *Mercenaria mercenaria.* Size distribution of protease-isolated kidney granules. Size presented as diameter (μm) of particle across its widest point (n = 2 283 granules). Detection limit = 1.4 μm at 430 × magnification

ances, as evidenced by SEM examination. The extracellular granule depicted in Fig. 1 C for example, although exceptionally large (1.9 mm across), was apparently made up of aggregates of smaller particles (Fig. 1 E). The fractured face of a protuberance (Fig. 1 D) displayed a central core surrounded by concentric layers of granule material. This exposed core was too large to have formed intracellularly and presumably was itself formed from accretion of layered materials and agglutination of smaller particles while within the kidney lumen.

A broad size-distribution of kidney granules (from ca. 12 individuals) was isolated using the protease-digestion procedure (Fig. 2). Most granules (> 89%) were less than 5.6 μm diam. (probably intracellular granules), although sizes ranged as high as 420 μm in the particular subsample examined. Since more of the small granules may have been lost during the isolation procedure than the larger granules, the

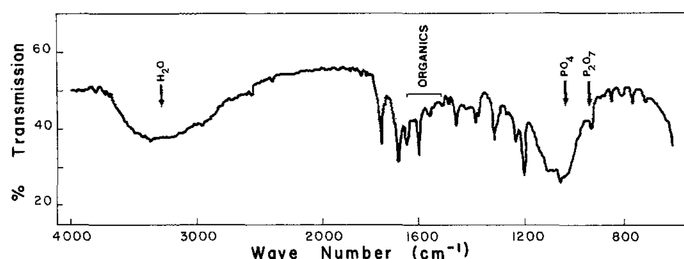

Fig. 3. *Mercenaria mercenaria.* Infrared spectroscopic profile of protease-isolated kidney granules

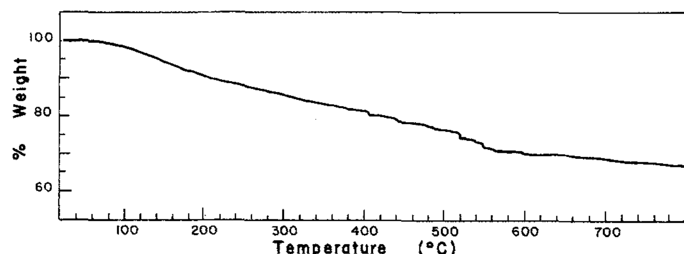

Fig. 4. *Mercenaria mercenaria.* Thermogravimetric profile of protease-isolated kidney granules

actual proportion of intracellular granules would be far greater. Very large granules (e.g. Fig. 1 C) were rare.

Biochemical assays revealed little organic material to be present in quahog kidney granules (Table 2). Combined carbohydrate and lipid concentrations amounted to no more than 0.8% of kidney granule composition. Protein, determined as the sum of detected amino acids, was $\leq 0.02\%$ of the granule weight. Of the amino acids present (Table 3), phenylalanine was the most abundant, followed by leucine and valine. Although it could be argued that the protease digestion may have removed protein associated with the granules, this is unlikely since the amino acid content of differentially-settled granules was not greater than that of protease-isolated granules.

The infra-red spectrum of kidney granules (Fig. 3) corroborated the presence of organic material (peak at 1 500 to 1 650 cm^{-1}). Water (3 000 to 3 750 cm^{-1}) was also present although carbonate (1 425 cm^{-1}) and ammonium (2 900 cm^{-1}) were not detected. A peak ascribed to phosphate (PO_4^{3-}) was present at 1 050 cm^{-1}, as well as a minor peak, possibly attributable to pyrophosphate ($P_2O_7^{4-}$) at 925 cm^{-1}.

Lyophilized kidney granule samples lost ca. 33% of their dry weight upon heating (20 °C min^{-1}) (Fig. 4), although few abrupt weight losses were observed. The most rapid weight loss occurred over the temperature range of 65° to 200 °C (0.07% weight loss °C^{-1}. Less weight was lost between 200° and 600 °C (0.05% weight loss C^{-1}), and even less still between 600° and 800 °C (0.02% weight loss °C^{-1}). Since the decline in weight was gradual over the whole temperature range, there was apparently no preponderance of organic material or individual hydrated compounds in the kidney granules. After 3 min at 800 °C, no additional weight loss was observed over the course of 7 min.

Table 2. *Mercenaria mercenaria.* Biochemical constituents of kidney granules. n = number of pooled samples; 7 to 13 individuals per pool

Constituent	Mean concentration ($\mu g\ mg^{-1}$ granule)	Mean percentage of granule weight
Protein[a] ($n=2$)	0.12–0.24	0.01–0.02%
Carbohydrate[b] ($n=5$)	2.8 ±1.0	0.3%
Lipid[b] ($n=5$)	5.1 ±5.4	0.5%

[a] Protease-isolated and differentially-settled
[b] Protease-isolated

Table 3. *Mercenaria mercenaria.* Amino acid content of granules isolated by differential settling and protease digestion procedures. ND = not detected

Amino acid	Concentration ($\mu g\ g^{-1}$ granule)	
	Differentially-settled	Protease-isolated
ala	7.4550	20.1217
arg	4.0706	9.0527
asp	1.4882	4.9792
cys	6.2034	13.8728
glu	3.9460	9.5515
gly	4.4533	12.7237
his	ND	1.8517
ile	9.7301	19.0377
leu	13.1360	25.8634
lys	0.4957	0.5620
met	2.9666	7.0554
phe	39.0900	43.6876
ser	3.4764	10.6443
thr	6.4662	18.1828
tyr	6.0081	16.0770
val	12.4281	28.0067
Total	121.41	241.27
Total % amino acid in granules	0.012%	0.024%

The concentrations of selected metals within the kidney and digestive gland granules of *Mercenaria mercenaria* are presented in Table 4. Approximately 30% of the kidney granule weight was accounted for by the analyzed elements. Manganese and calcium were the most abundant metals by weight, each having concentrations of ca. 80 to 88 mg g^{-1} of granules. Zn (43 mg/g) and Fe (29–37 mg/g) were also major constituents of kidney granules. There were no significant differences in metal concentrations between the granules isolated using the protease digestion and differential settling procedures ($P > 0.05$; Kruskal-Wallis nonparametric two-factor analysis of variance; Mann-Whitney U Test, Zar 1984). The most abundant metal in the digestive gland granules was Ca (8.5 mg g^{-1}) followed by Mg (1.2 mg g^{-1}), Fe (0.6 mg g^{-1}) and Zn (0.5 mg g^{-1}). Mn (0.03 mg g^{-1}) was present in the digestive gland granules at a significantly

lesser proportion than it had been in the kidney granules.

Of the non-metal elements (C, H, N, S, and P; Table 5), phosphorus occurred in the highest concentration (114 to 129 mg g^{-1}) and was the most abundant of all elements (including metals) in the kidney granules. Carbon (45 to 59 mg g^{-1}), N (9 to 12 mg g^{-1}) and H (21 to 26 mg g^{-1}) together constituted approximately 8% of the kidney granules' weight. Digestive gland granules contained more C (583 mg g^{-1}), H (93 mg g^{-1}) and N (60 mg g^{-1}), but less P (8 mg g^{-1}) than kidney granules. Due to the low atomic weights of these non-metal elements, H (23 mmole g^{-1} granule), C (4.1 mmole g^{-1}) and P (3.8 mmole g^{-1}) were actually the most numerous elements in quahog kidney granules on a molar percent basis.

Table 4. *Mercenaria mercenaria.* Metal concentration in kidney and digestive gland granules. Results presented as mg metal g^{-1} dry weight ± standard deviation. ND = not detected; $n=4$ for protease-isolated, $n=3$ for differentially-settled, and $n=2$ for digestive gland granules

Element	Kidney		Digestive gland
	Protease-isolated	Differentially-settled	Protease-isolated
Ba	0.39±0.10	0.30±0.20	0.03±0
Ca	82.18±12.56	87.70±10.48	8.50±3.93
Cd	0.09±0.04	0.05±0.04	0.01±0
Cr	ND	0.01±0.01	0.01±0
Cu	1.37±0.17	1.17±0.19	0.14±0
Fe	37.34±6.03	28.56±2.65	0.58±0
K	12.65±2.19	13.48±0.93	0.21±0.30
Mg	15.38±2.51	17.49±2.99	1.24±0
Mn	79.80±3.94	87.02±3.59	0.03±0.01
Ni	0.15±0.02	0.17±0.01	0.01±0
Pb	1.94±0.17	1.66±0.01	ND
Sr	4.13±0.31	4.19±0.41	0.05±0.02
Zn	43.43±4.22	43.32±3.61	0.45±0.01
Sum	278.85	285.12	11.26
Total % metal in granules	27.9%	28.5%	1.1%

The C, H, and N content of the small kidney granule sample (≤10 μm) was quite different from that of the extracellular kidney granule pool and the digestive gland granules (Table 5). Small (intracellular) kidney granules contained more C, H, and N than extracellular granules, but less than digestive gland granules. The difficulty in obtaining a sample of sufficient size precluded additional analysis of elemental composition of intracellular kidney granules.

Histochemical tests on protease-isolated and differentially-settled kidney granules and protease-isolated digestive gland granules gave similar reactions indicating the presence of lipofuscin-like materials (Table 6). Kidney granules were strongly basophilic, staining with Alcian blue 8GX, Nile blue A and basic fuchsin (Long Ziehl-Neelsen reaction), but not staining with acid fuchsin. Little or no stainable protein was present, as indicated by the lack of staining with either mercuric bromophenol blue or ninhydrin. The granules were strongly colored by Schiff's reagent, even without initial oxidation by periodic acid. Schiff's staining was not affected by prior amylase digestion. Some kidney granules exhibited positive reactions to Turnbull's blue (47% of the granules examined) and Schmorl's (19%) stains. In addition, the granules exhibited a positive reaction for phospholipids (Sudan black B).

Some staining reactions exhibited by the kidney granules were not characteristic of lipofuscin material. Most of the kidney granules stained with Prussian blue indicating the presence of Fe^{3+}, while digestive gland granules reacted negatively. Mixed results were also obtained for kidney granules stained with argentaffin. However, the natural dark coloration of the kidney granules made interpretation of the results from these histochemical tests difficult. Digestive gland granules did not react to argentaffin staining.

Discussion

The uniformity of results from metal, non-metal, amino acid, marker enzyme and histochemical analyses indicates that the differential settling, protease digestion and hand-

Table 5. *Mercenaria mercenaria.* Contents of selected non-metal elements in tissue granules. Results expressed as mg element per gram purified granules (± standard deviation). NA = not analyzed; n = number of pooled samples

Granule preparation	Element				
	C	H	N	P	S
Kidney, hand-selected	58.5±1.6 ($n=5$)	22±4 ($n=5$)	12.3±0.7 ($n=5$)	NA	NA
Kidney, differentially-settled	45.0±4.4 ($n=5$)	21±4 ($n=5$)	8.7±0.6 ($n=5$)	128.5±4.9 ($n=3$)	NA
Kidney, protease >10 μm extracellular	52.6±3.6 ($n=6$)	26±0 ($n=6$)	11.9±0.7 ($n=6$)	113.9±10.6 ($n=4$)	7.2±3.0 ($n=6$)
Kidney, protease <10 μm intracellular	240.1 ($n=1$)	44 ($n=1$)	37.9 ($n=1$)	NA	NA
Digestive gland, protease	583.2±2.8 ($n=5$)	93±2 ($n=5$)	59.8±0.6 ($n=5$)	8.3±0.2 ($n=2$)	NA

Table 6. *Mercenaria mercenaria.* Histochemical analysis of granules. Staining reactions assessed as +++, very strong; ++, moderately strong; +, detectable; −, absent. Percentages based on $n > 400$ granules (range 405−490). Results from George et al. (1982) on *Mytilus edulis* kidney granules are presented for comparison. NA = not analyzed

Histochemical test	Interpretation of positive	*Mercenaria mercenaria*		*Mytilus edulis* kidney
		kidney	dig. gland	
Mercuric bromophenol blue	protein amino groups	−	−	+ or −
Ninhydrin	protein and peptide amino groups	−	−	NA
Periodic acid-Schiff's reagent (PAS)	vicinol glycols of carbohydrates	+++	+ to +++ (>95%) − (<5%)	+ or −
PAS − amylase control	glycogen	+++	+ to +++ (>95%) − (<5%)	−
Schiff's reagent alone	vicinol glycols	++	+ to +++ (>95%) − (<5%)	NA
Oil red O	neutral lipid	+ (8%) − (92%)	++	+
Sudan black B	phospholipids	++	++	+
Alcian blue (pH 2.5)	mucosubstances, basophilic substances	+++	+++	NA
Alcian blue (pH 1.0)	sulfated mucosubstances, basophilic substances	+++	+++	NA
Acid fuchsin	acidophilic substances	−	−	NA
Prussian blue (Perls)	Fe^{3+}	++ (51%) − (49%)	−	+++
Turnbull's blue	Fe^{2+} lipofuscin	++ (47%) − (53%)	−	NA
Schmorl	lipofuscin, melanin, S-amino acids	+ (19%) − (81%)	++ (98%) − (2%)	++
Long Ziehl-Neelsen	acid-fast lipofuscin	+++	+++	NA
Nile blue A (pH 1.0)	lipofuscin, basophilic substances	+++	+++	+++
Nile blue A (+10% H_2O_2; 24 hours)	lipofuscin	+	+++	+++
Argentaffin	melanin, ascorbic acid, o-diphenols	+ (68%) − (32%)	−	−
Autofluorescence	lipofuscin	+ (yellow)	+++ (yellow)	gr.-yellow

selection methods separate *Mercenaria mercenaria* kidney granules at the same high level of purity. Visual examination of the kidney granule preparations, however, suggests that only the protease-isolation procedure isolates a significant percentage of intracellular and small extracellular granules (based on particle diameter) along with larger extracellular granules from the kidney tissue. Small, intracellular granules may numerically comprise 90% of the granule population, yet offer only a negligible contribution to quahog kidney volume and weight. Therefore, the overall chemical characteristics of the kidney granules from each of the isolation procedures are predominantly influenced by the larger, extracellular granules. In the one instance where small (≤ 10 μm) granules were specifically analyzed (i.e., C, H and N), these presumptive intracellular granules had a significantly different composition from the large extracellular granules.

We have accounted for 49.0% of kidney granule dry weight (calculations based on protease-isolated kidney granules). Metals comprised 27.8%, and the other elements analyzed (C, H, N, S, P) constituted 21.2%, leaving 51.0% of the granules' composition undetermined. The major part of this unidentified portion is probably attributable to oxygen since a wide variety of compounds that contain oxygen, such as phosphates and bound water, were detected qualitatively (by colorimetric, microscopic, IR, and/or thermogravimetric analyses). For example, if all the phosphorus present in kidney granules was attributable to phosphate (PO_4^{3-}) compounds, then oxygen would comprise 23.5% of the granule dry weight. Similarly, if all of the H present in kidney granules were in the form of bound water and all the S were present as sulfate (SO_4^{2-}) compounds, the associated oxygen would contribute an additional 20.6% and 1.4% of the granule dry weight respectively. The total contribution of

Table 7. Summary (% dry weight granules) of kidney granule compositions of various bivalves. NA = not analyzed; ND = not detected; + = detected qualitatively

Element	Present study (protease isol.) *M. mercenaria*	Rheinberger et al. (1979) *M. mercenaria*	George et al. (1982) *M. edulis*	George et al. (1980) *P. maximus*	Ishii et al. (1986) *C. menstrualis*	Carmichael et al. (1979) *A. irradians*
Ba	0.04	NA	NA	NA	0.01	NA
Ca	8.2	7–11	0.42	10.1	13.1	25.1
Cd	0.01	0.0003–0.002	<0.001	0.01	ND	0.002
Cr	ND	NA	NA	NA	0.003	0.005
Cu	0.1	0.03–0.5	0.17	0.04	0.01	0.001
Fe	3.7	1–6	3.0	0.02	0.6	0.06
K	1.3	NA	0.1–0.7	1.1	0.2	NA
Mg	1.5	NA	0.1	1.4	3.1	3.7
Mn	8.0	4–12	0.004	10.6	4.4	2.4
Ni	0.02	NA	NA	NA	0.01	NA
Pb	0.2	0.1–0.2	0.24	NA	0.03	NA
Sr	0.4	NA	NA	NA	0.3	NA
Zn	4.3	3–6	1.0	10.2	2.3	1.7
Sum metals	27.8	15–35	5.0–5.6	33.5	24.1	33.0
C	5.3	2–4	46.27	1.2	NA	NA
H	2.6	NA	6.66	6.7	NA	NA
N	1.2	NA	7.06	2.1	NA	NA
S	0.7	NA	0.74	4.1	NA	NA
P	11.4	NA	0.1 or 1.0	20.7	11.9	14.5
Size (μm)	1–1 900	390–485	1–3	5–15	0.1–200	5–20

oxygen from these estimates would be 45.5% (a value close to the 51.0% of granule weight left undetermined).

A molar ratio of 0.56:1 (Ca:P) is calculated for the protease-isolated *Mercenaria mercenaria* kidney granules of this study. This Ca : P ratio is similar to the values reported by Carmichael et al. (1979) for the kidney granules of *Argopecten gibbus* (0.65:1) and *A. irradians* (0.60:1) using electron probe analysis, but is less than the expected ratio (1.5:1) if Ca and P exist primarily as $Ca_3(PO_4)_2$ (Gold et al. 1982). More phosphorus is present in the kidney granules than can be accounted for simply by this form of calcium phosphate. The excess phosphorus is probably complexed in a number of additional insoluble divalent metal-phosphate compounds (e.g. $Zn_3(PO_4)_2$, $Mn_3(PO_4)_2$). Metal:PO_4 or metal:P ratios may therefore be more meaningful than a Ca:P ratio (George et al. 1980). Since the calculated metal:P ratio for quahog kidney granules is 1.58:1, it is apparent that all of the P present could be accounted for as metal-PO_4 complexes. The slight molar excess of metals to phosphorus could be attributable to metal complexes containing S, C, N, etc. (e.g. metal-SO_4, metal-CO_3) that are likely to be present.

As indicated by the infra-red spectral analysis, phosphorus is present within the kidney granules of *Mercenaria mercenaria* primarily as phosphate (PO_4^{3-}) which may occur in organic or inorganic compounds. Granules from gastropod hepatopancreas frequently contain pyrophosphate compounds which are highly insoluble and thought to function in cellular detoxification (Howard et al. 1981, Mason and Nott 1981, Simkiss et al. 1982). Some pyrophosphate ($P_2O_7^{4-}$) compounds may also be present in quahog kidney granules, although this form of phosphorus has not been previously reported for bivalves.

The results of the present study fall within the range of metal concentrations previously presented for *Mercenaria mercenaria* (Rheinberger et al. 1979). As compared to the kidney granules from other bivalves (Table 7), the kidney granules of *M. mercenaria* are more similar in composition to granule samples taken from species that accumulate large extracellular granules in addition to producing intracellular granules (e.g. *Cyclosunetta menstrualis*, Ishii et al. 1986, *Pinna nobilis*, Ghiretti et al. 1979, Brown 1982). The composition of *Mytilus edulis* kidney granules differs in several respects from the compositions of the other bivalve kidney granules presented in Table 7. There is a greater percentage of organic material in *M. edulis* granules (based on C, H, and N contents) and a lower proportion of metals (notably calcium) and phosphorus. The organic content of kidney granules of *M. edulis* is more like the small (≤ 10 μm) kidney granules and digestive gland granules of *M. mercenaria*. However, an obvious difference between *M. mercenaria* digestive gland granules and *M. edulis* kidney granules is in the accumulation of metals (e.g. Cu, Fe, Zn).

It is likely that bivalve kidney granule production and subsequent increase in size is a continuous process of lysosomal maturation, residual body release, and extracellular accumulation. In *Mytilus edulis*, for example (Table 7), isolated kidney granules are quite small and their composition probably reflects that of intracellular granules (George 1983a). In scallops such as *Pecten maximus* (Table 7), however, granules are slightly larger 5 to 20 μm) possibly reflecting a longer residence in the kidney lumen and concomitant acquisition of material. In general, the organic content of kidney granules decreases as the kidney granule size increases (Table 7). For example, the kidney granules of *M. edulis* (1 to 3 μm diam.) contain more organic material than

the small kidney granules ($\leq 10\ \mu$m) of *Mercenaria mercenaria*, which in turn, contain more organic material than larger kidney granules (*M. mercenaria, P. maximus*). Although metal and P data are unavailable for small ($\leq 10\ \mu$m) quahog granules, the trend for higher metal and P content in large granules (e.g. *M. mercenaria, Cyclosunetta menstrualis*) as granule size increases is apparent. Why granules are retained and continue to grow in the kidney lumen of some species, while in others they do not grow and are quickly excreted, has yet to be explained.

Granules from *Mercenaria mercenaria* are apparently retained for some period of time in the kidney lumen (Rheinberger et al. 1979) and further accumulate additional organic and inorganic materials. Because the intracellular ($\leq 10\ \mu$m) granules contain a higher proportion of organic material than the larger extracellular granules, inorganic material must have been added to the granules once they were extruded into the kidney lumen. This does not preclude addition of organic materials to the granules while in the lumen, but implies only that a higher percentage of the added material was inorganic, thereby reducing the overall organic content. The SEM results of the present study support observations that these extracellular granules are formed both from aggregations of smaller granules (Brown 1982, Ishii et al. 1986) as well as by concentric layering of material on existing granules (Carmichael et al. 1979).

Mercenaria mercenaria kidney and digestive gland granules both contain lipofuscin (the lysosomal breakdown product of membranes; Ivy et al. 1984). Clokey and Jacobson (1986) have recently described the lipofuscin granules of the nematode (*Caenorhabditis elegans*) intestine as secondary lysosomes, while George (1983a, b) defines *M. edulis* kidney granules as tertiary lysosomes (residual bodies). The lipofuscin-like qualities of *M. mercenaria* kidney and digestive gland granules suggests that both particle types are derived from the lysosomal system. The kidney granules may differ only in the amount of additional inorganic material.

The metal accumulating characteristics of the bivalve kidney in conjunction with the presence of metal-rich granules may partially explain why the bivalve kidney accumulates far more metal than other organs, including the digestive gland (George and Pirie 1979, Robinson et al. 1985, Robinson and Ryan 1986). It is likely that the large extracellular granules found in many species of bivalves are more important to metal sequestration and regulation within the kidney than are the small, intracellular granules.

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