# COPPER TRANSPORT BY LOBSTER HEPATOPANCREATIC EPITHELIAL CELLS SEPARATED BY CENTRIFUGAL ELUTRIATION: MEASUREMENTS WITH THE FLUORESCENT DYE PHEN GREEN

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## Summary

The hepatopancreas of the American lobster (*Homarus americanus*) possesses four types of epithelial cells arranged along blind-ended tubules. At the distal tips of these tubules, stem cells termed E-cells differentiate into three other cell types, R-cells, F-cells and B-cells, each of which have different absorptive and secretory roles in the biology of the overall organ. This investigation uses centrifugal elutriation to separate the individual hepatopancreatic epithelial cell types of *Homarus americanus* and to investigate their plasma membrane copper transport properties using the copper-sensitive fluorescent dye Phen Green. Results show highly dissimilar endogenous concentrations of copper in each cell type and within the vacuoles (vesicles) released from these cells during the centrifugation process ([copper] in vacuoles>E-cells>R-cells>F-cells≈B-cells). All four cell types were able to absorb copper from external concentrations ranging from 0.01 to 8 $\mu$mol l$^{-1}$, but considerable differences in transport rates occurred between the cell types. External calcium (0–10 mmol l$^{-1}$) stimulated the uptake of external copper in a saturable fashion, suggesting the occurrence of carrier-mediated metal uptake. Addition of the Ca$^{2+}$ channel blocker verapamil (30 $\mu$mol l$^{-1}$) to the external medium reduced the uptake rate of copper by all four cell types, but to different extents in each type of cell. External zinc (0–1000 nmol l$^{-1}$) was a competitive inhibitor of copper influx in E- and R-cells, suggesting that the two metals shared the same binding and transport mechanism. A model is proposed which suggests that copper may enter all hepatopancreatic epithelial cell types by a divalent cation antiport process that exchanges intracellular Ca$^{2+}$ (or other cations) with either external copper or zinc. Verapamil-sensitive Ca$^{2+}$ channels may allow access of external calcium to cytoplasmic exchange sites on the antiporter or to activator sites on the same transport protein. The results suggest that elutriation is an excellent technique for the separation of complex invertebrate organ systems into their separate cell types and for analyzing the physiological properties of each cell type in isolation.

Key words: American lobster, *Homarus americanus*, copper transport, Phen Green, hepatopancreas, epithelial cell, ion uptake, heavy metal.

## Introduction

Large-bodied members of the phyla Arthropoda, Mollusca and Echinodermata possess gastrointestinal diverticula (digestive glands, hepatopancreas, pyloric ceca, etc.) composed of several distinct epithelial cell types that play a variety of roles in the biology of the overall organ system. Many of these invertebrate functions are similar to physiological properties studied in depth in vertebrate tissues. The arthropod hepatopancreas is composed of four major cell types, E-cells, F-cells, B-cells and R-cells, and each is believed to contribute to the overall functions of digestion, absorption, secretion, osmoregulation and detoxification. Hepatopancreatic E-cells are mitotic and function much like mammalian intestinal crypt cells, giving rise to two distinct cell lines, an absorptive line generating new R-cells that act much like intestinal columnar cells, and a secretory line composed of F-cells manufacturing and secreting digestive enzymes like pancreatic acinar cells or stomach chief cells (Al-Mohanna and Nott, 1987; Al-Mohanna et al., 1985; Paquet et al., 1993). A second type of digestive cell, B-cells, are derived from F-cells and appear to play a significant role in the intracellular digestion and excretion of digestive breakdown products and xenobiotics (Al-Mohanna and Nott, 1986).

Much of the information obtained about the function of invertebrate gastrointestinal diverticula and their associated epithelial cell types has been derived from histology, electron microscopy and elemental microprobe analysis (Al-Mohanna and Nott, 1985; Al-Mohanna and Nott, 1989; Hopkin and Nott, 1979; Johnson, 1980; Loizzi, 1971; Paquet et al., 1993). To understand more fully the physiological role of each cell type in these complex organ systems, methods for isolating the individual cell types from the intact tissues are needed. In 1983, Percoll-gradient centrifugation was applied to lobster hepatopancreas to separate R-cells from F-cells and to investigate differences between the cell types in nutrient absorption as monitored by L-[$^3$H]alanine transport (Ahearn et al., 1983). This study clearly indicated that both cell types were capable of transporting this particular amino acid by carrier-mediated transfer mechanisms, but R-cells exhibited a sixfold higher influx rate and a fivefold greater intracellular amino acid concentration at steady state than F-cells. More recently, this cellular isolation procedure was used by another laboratory to investigate the mechanism of urate transport across plasma membranes of lobster hepatopancreatic R-cells in suspension (Nies et al., 1995). While allowing physiological distinctions to be addressed between cell types, Percoll-gradient centrifugation does not generate large numbers of cells for study and its utility is, therefore, limited.

In the present investigation, centrifugal elutriation was used to isolate pure suspensions of all four lobster hepatopancreatic epithelial cell types in relatively large numbers for subsequent investigation. In this procedure, a mixed suspension of hepatopancreatic cells was perfused into a spinning rotor, and the combination of centrifugal force and peristaltic force, working in opposite directions, was used to isolate each cell type cleanly for later study. This technique was used in combination with a metal-sensitive fluorescent dye, Phen Green, to investigate the mechanisms of copper uptake by individual hepatopancreatic epithelial cell types.

## Materials and methods

Live intermolt Atlantic lobsters (*Homarus americanus* H. Milne Edwards) were obtained from a local commercial dealer in Hawaii and maintained in a chilled seawater aquarium until used for experimentation. Animals were fed frozen mussel tissue up to 24 h prior to use in experiments. The hepatopancreas was removed and minced into small pieces which were added to a citrate buffer consisting of (in mmol l$^{-1}$): 27 sodium citrate, 440 NaCl, 5.0 NaH$_2$PO$_4$, 5.6 Na$_2$SO$_4$, 3.0 KCl, 10 D-glucose, 1.0 EDTA and 1 phenylmethylsulfonylfluoride (PMSF), pH 7.1, osmolality 900 mosmol kg$^{-1}$. The mixture was agitated on an orbital shaker at 5 °C to dissociate the epithelial cells from underlying tissues. The resulting cell suspension was filtered through a 200 μm nylon mesh to remove undissociated tissue and large debris, and centrifuged (Sorval RC5C) at 100 *g* for 10 min. The pellet was resuspended in a lobster physiological elutriation buffer consisting of (in mmol l$^{-1}$): 467 NaCl, 1.0 NaH$_2$PO$_4$, 8.4

Fig. 1. Centrifugal elutriation scheme used to produce pure cell suspensions of each hepatopancreatic cell type. Two separate centrifugation cycles were needed to isolate hepatopancreatic cells and vacuoles (vesicles). A heterogeneous mixture of cell types was introduced into the spinning rotor with a perfusion pump. In the first cycle (A), a perfusion rate of 25 ml min$^{-1}$ was used to separate vesicles, E-cells and R-cells as a separate fraction from the remaining cell types. F-cells and B-cells remained in the centrifuge until the perfusion rate was increased to 45 and 75 ml min$^{-1}$, at which point these remaining cell types were individually collected (B). The first fraction was reintroduced into the spinning rotor for a second cycle, and each fraction was collected at a different perfusion rate as shown. Throughout the entire procedure, a constant centrifugation rate of 1100 revs min$^{-1}$ (100 *g*) was maintained. C gives an overview of the process.
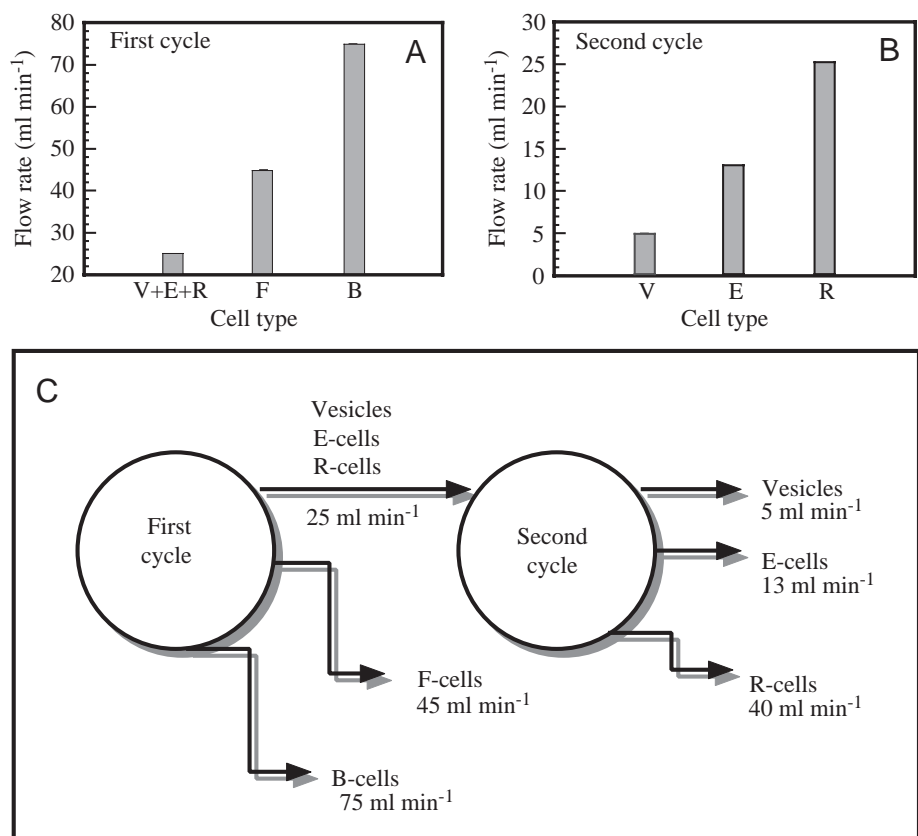
Na$_2$SO$_4$, 4.0 NaHCO$_3$, 10 KCl, 10 D-glucose, 30 Hepes, 1.0 EDTA, pH 7.1, osmolality 900 mosmol kg$^{-1}$.

Further cell separation was performed by centrifugal elutriation (Fig. 1) (McEwen et al., 1968), which has previously been reported as a fast and gentle procedure to separate E-cells from the Atlantic lobster hepatopancreas (Duerr and Ahearn, 1998). A concentrated epithelial cell suspension of all four hepatopancreatic cell types was injected into a rotating Beckman JE-6B elutriator rotor adapted to a Beckman J2-21 high-speed centrifuge at a flow rate of 25 ml min$^{-1}$ and a rotor speed of 1100 revs min$^{-1}$ (100 $g$). At this flow rate and rotor speed, B- and F-cells were retained in the rotor separation chamber, and intracellular vesicles (produced from disrupted cells), E-cells and R-cells were passed out of the rotor and collected in two 50 ml Falcon tubes. The cells in these tubes were subsequently centrifuged at 1100 revs min$^{-1}$ and resuspended in elutriation buffer. The F-cells remaining in the rotor were collected as a single cell suspension by increasing the flow rate to 45 ml min$^{-1}$, and the B-cells were collected by further increasing the flow rate to 75 ml min$^{-1}$. The mixed cell fraction initially collected containing vacuoles, E- and R-cells was next reinjected into the rotating rotor at a flow rate of 5 ml min$^{-1}$. At this flow rate, vesicles were collected from the rotor in Falcon tubes. The E-cells were subsequently collected from the rotor by increasing the flow rate to 13 ml min$^{-1}$, and the R-cells exited the rotor when the flow rate was increased to 40 ml min$^{-1}$.

Cells were morphologically differentiated by light microscopy at 400× magnification (Fig. 2). The vesicles were considerably smaller than all cell types, uniformly white in appearance and irregular in shape. The E-cells were approximately half the size of the other cell types and did not exhibit any specialized intracellular morphology or inclusions. The R-cells contained a large number of lipid droplets and endosomal organelles, the F-cells were characterized by a large amount of endoplasmic reticulum and granular cytoplasm, and the B-cells all contained a single, large, intracellular vacuole occupying most of the intracellular compartment. Cell suspension purity (>90 % pure suspensions of each cell type) and total population size were determined using an Improved Neubauer hemocytometer slide.

The fluorescent dye Phen Green and its acetoxymethyl ester (Molecular Probes, Inc.) were used in this study. Dye calibration at different concentrations of copper was performed with 1.5 µmol l$^{-1}$ Phen Green-AM. To estimate the responsiveness of cytoplasmic Phen Green to heavy metals and the activation time of the dye by intracellular enzymes, a cytosolic solution was prepared by sonication from preparations of each hepatopancreatic cell type. Different concentrations of dye were incubated with different concentrations of heavy metal, and the resulting fluorescence quenching was analyzed as a decrease in the fluorescence signal per milligram of cell protein within 120 s of addition of the metal (i.e. $\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$). Phen Green fluorescence (excitation 490 nm; emission 520 nm) was measured in a Perkin Elmer LS-5B spectrofluorometer

equipped with a magnetic stirrer. Experiments were conducted at room temperature (24 °C). Data were recorded with a GP-100 Graphics Printer (Perkin Elmer).

Cells were equilibrated with the dye at room temperature in an orbital shaker at 100 revs min$^{-1}$ for 60 min. The preparation was then centrifuged at 1100 revs min$^{-1}$ (i.e. 100 $g$) for 10 min, and the resulting pellet was resuspended in buffer and maintained on ice for further analysis. During an experiment, a sample of cell suspension was injected into a cuvette housed in the spectrofluorometer which contained a physiological buffer without copper or another metal ion so that maximal fluorescence would be obtained. Once the dye signal was stable (at 100 %), copper or other metals were added at different concentrations, and the increase in fluorescence quenching (as $\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$) was monitored as an indication of metal transport across the cell plasma membrane. Dissociation constants ($K_d$) of Phen Green for Cu$^+$ ($K_d$=0.5 µmol l$^{-1}$) and Cu$^{2+}$ ($K_d$=4.0 µmol l$^{-1}$) have been published previously (Kuhn et al., 1995) and suggest that, at the copper concentrations used in this investigation, much of the copper reacting with the dye was in the divalent state (Cu$^{2+}$). Cellular protein was estimated using the Bio-Rad method with albumin standards in a Beckman DU-640 recording spectrophotometer. Curve-fitting and the production of the resulting copper influx kinetic constants were performed using Sigma Plot 4.01 software (Jandel). All values reported in this investigation were obtained from triplicate samples, and results are expressed as means ± 1 S.E.M.

## Results

### *Effects of different external metals on Phen Green fluorescence quenching*

To determine the heavy metal specificity of the Phen Green fluorescence quenching response, changes in dye fluorescence were determined in the presence of various external metal ions. Fig. 3 shows the effects of external 125 µmol l$^{-1}$ Cu$^{2+}$, Fe$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Li$^+$, Ca$^{2+}$, Zn$^{2+}$ and Mg$^{2+}$ (added as chloride salts) on the percentage of initial Phen Green fluorescence produced by heterogeneous hepatopancreatic cell suspensions after 120 s of incubation in the respective metal. Only three external metals produced any significant ($P$<0.05) fluorescence quenching of the dye: Cd$^{2+}$, Fe$^{2+}$ and Cu$^{2+}$. Copper produced the largest quenching response, with approximately 95 % fluorescence quenching occurring in the presence of this metal. Iron was the only other metal that was highly effective in reducing the dye signal, generating an approximately 70 % reduction in the initial fluorescence during the incubation period. The other metals produced very little change in dye fluorescence, and therefore the possible presence of Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$ or Mn$^{2+}$ within hepatopancreatic cells would not be expected to interfere with the quenching signal produced by copper.

### *Quantitative Phen Green fluorescence quenching induced by external copper*

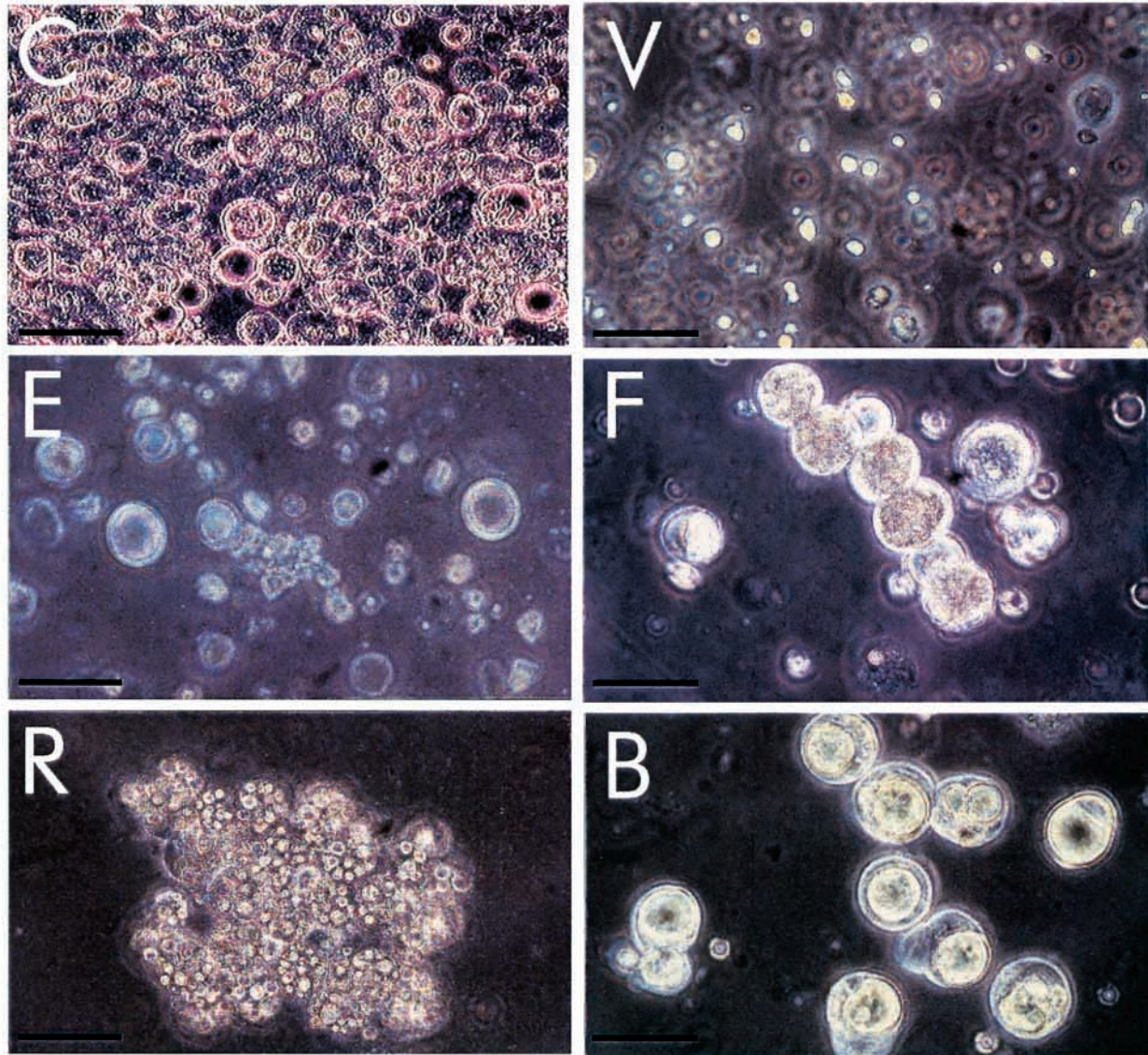Cu$^{2+}$ induced the greatest fluorescence quenching of Phen

Fig. 2. Lobster hepatopancreatic epithelial cells and intracellular vacuoles (vesicles) separated by centrifugal elutriation following the scheme shown in Fig. 1. (C) Heterogeneous dissociated cell suspension of all four cell types. Scale bar, 100 μm. (V) Intracellular vacuoles (vesicles) released from broken cells. Scale bar, 25 μm. (E) E-cell suspension. Scale bar, 25 μm. (F) F-cell suspension. Scale bar, 50 μm. (R) R-cell suspension. Scale bar, 50 μm. (B) B-cell suspension. Scale bar, 50 μm.

Green, so the quantitative extent to which this cation was able to quench the dye signal was examined over a range of metal concentrations through the use of a calibration curve. Fig. 4 shows the effects of increasing external concentrations of $Cu^{2+}$ on the magnitude of fluorescence quenching exhibited by Phen Green reacting with a mixed population of osmotically lysed hepatopancreatic cells in the presence of this cation. As displayed in Fig. 4, $0.025\,mmol\,l^{-1}$ $CuCl_2$ produced

Fig. 3. Percentage change in Phen Green fluorescence (quenching) per milligram cell protein per 120 s incubation induced by various metals in solution. Metals were added to the buffer at a concentration of $125\,\mu mol\,l^{-1}$. The excitation wavelength was 490 nm, and emission of the dye was recorded 520 nm. Values are means + S.E.M., $N=3$.
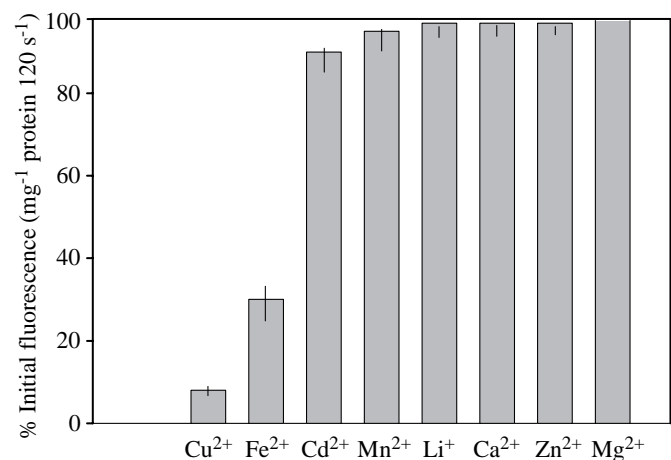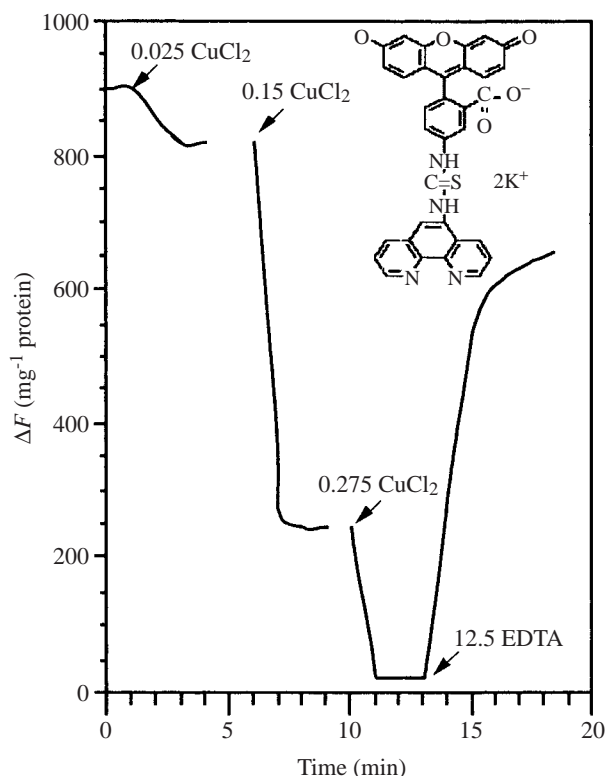
Fig. 4. Quantitative Phen Green fluorescence quenching (as $\Delta F\,mg^{-1}$ protein, where $\Delta F$ is fluorescence quenching) induced by increasing concentrations of copper (as $CuCl_2$) as a function of incubation time. A mixed population of hepatopancreatic cells was osmotically lysed in ultrapure water and reacted with $1.5\,\mu mol\,l^{-1}$ Phen Green in solution in the presence of several concentrations of $CuCl_2$ (given in $mmol\,l^{-1}$) for the times indicated after a stable fluorescence signal had been obtained. Fluorescence quenching is shown as a reduction in the fluorescence signal following the addition of each concentration of copper. Recovery of fluorescence occurred with the addition to the buffer of $12.5\,mmol\,l^{-1}$ EDTA, which chelated copper in solution. Excitation of the dye was at 490 nm, and dye emission was recorded at 520 nm. The inset is the chemical structure of Phen Green as provided by Molecular Probes, Inc.

approximately 10 % fluorescence quenching of Phen Green per milligram of lysed cell protein per 120 s. Increasing the metal concentration to $0.15\,mmol\,l^{-1}$ resulted in an 80 % reduction in the dye signal, and almost total elimination of Phen Green fluorescence was observed at a $CuCl_2$ concentration of $0.275\,mmol\,l^{-1}$. Addition of the cation chelator EDTA to the incubation medium following total fluorescence quenching by $0.275\,mmol\,l^{-1}$ $CuCl_2$ led to significant recovery in the fluorescence signal because of the formation of complexes between the chelator and the copper in solution. These results show that the fluorescence signal from Phen Green after activation by intracellular enzymes can be quantitatively quenched by external copper ions and that this quenching can be reversed by removing the ions from solution with an effective chelator.

Table 1. *Copper concentrations in different cell types and in vesicles separated by centrifugal elutriation*

| Cell type | Copper concentration (ng mg$^{-1}$ cell protein) |
|---|---|
| Vesicles | 425.0±21.3 |
| E-cells | 264.3±13.2 |
| R-cells | 91.0±4.6 |
| F-cells | 30.8±1.6 |
| B-cells | 28.9±1.4 |

Intracellular copper concentrations were measured with a Mass ICP spectrophotometer as described in the text following the release of stored metal by cell lysis using sonication. ICP emission units were converted to ng copper ml$^{-1}$ solution using standard curves.

Samples were prepared in triplicate, and protein concentrations were measured using the Bio-Rad method.

Values are means ± 1 S.E.M.

### Endogenous intracellular copper concentrations in hepatopancreatic epithelial cells and vesicles

Hepatopancreatic epithelial cells and intracellular vesicles released by cell disruption were separated by elutriation as described above. Pure single-cell and vesicle suspensions were individually osmotically ruptured with ultrapure MilliQ water with sonication to release stored copper to solution. Controls contained only MilliQ water without added cells. The concentrations of endogenous intracellular copper present in each cell type and in the released vesicles were quantified using a Leeman Labs inductively coupled plasma optical emission spectrophotometer (Mass ICP). Standard curves were generated for each cell type and vesicle sample following lysis so that emission units could be converted to nanograms of copper per milligram of cell protein. Protein was determined using the Bio Rad method. Table 1 shows that intracellular vesicles displayed the greatest concentration of copper ions, while purified B-cells contained the smallest amount of the metal relative to the protein content of each fraction. Surprisingly, the E-cells had a significantly ($P<0.01$) greater metal content than did any of the other three isolated cell types. Of the three differentiated cell types, the R-cells had three times more intracellular copper than did either of the other two types of hepatopancreatic epithelial cell.

### Effects of external calcium on copper uptake by isolated hepatopancreatic cell types

Hepatopancreatic epithelial cells were individually separated by elutriation and equilibrated with Phen Green as described above. The change in fluorescence intensity per milligram protein was then observed with each cell fraction for 120 s following the addition of selected concentrations of copper and calcium to the external medium. Fig. 5 shows the increase in fluorescence quenching of Phen Green (i.e. copper uptake) in E-cells, R-cells, F-cells and B-cells ($\Delta F\,mg^{-1}$ protein $120\,s^{-1}$) as a function of external copper concentration ($0–8\,\mu mol\,l^{-1}$) at different external concentrations of calcium ($0, 0.1, 2$ and $10\,mmol\,l^{-1}$). Fig. 5 indicates that copper uptake
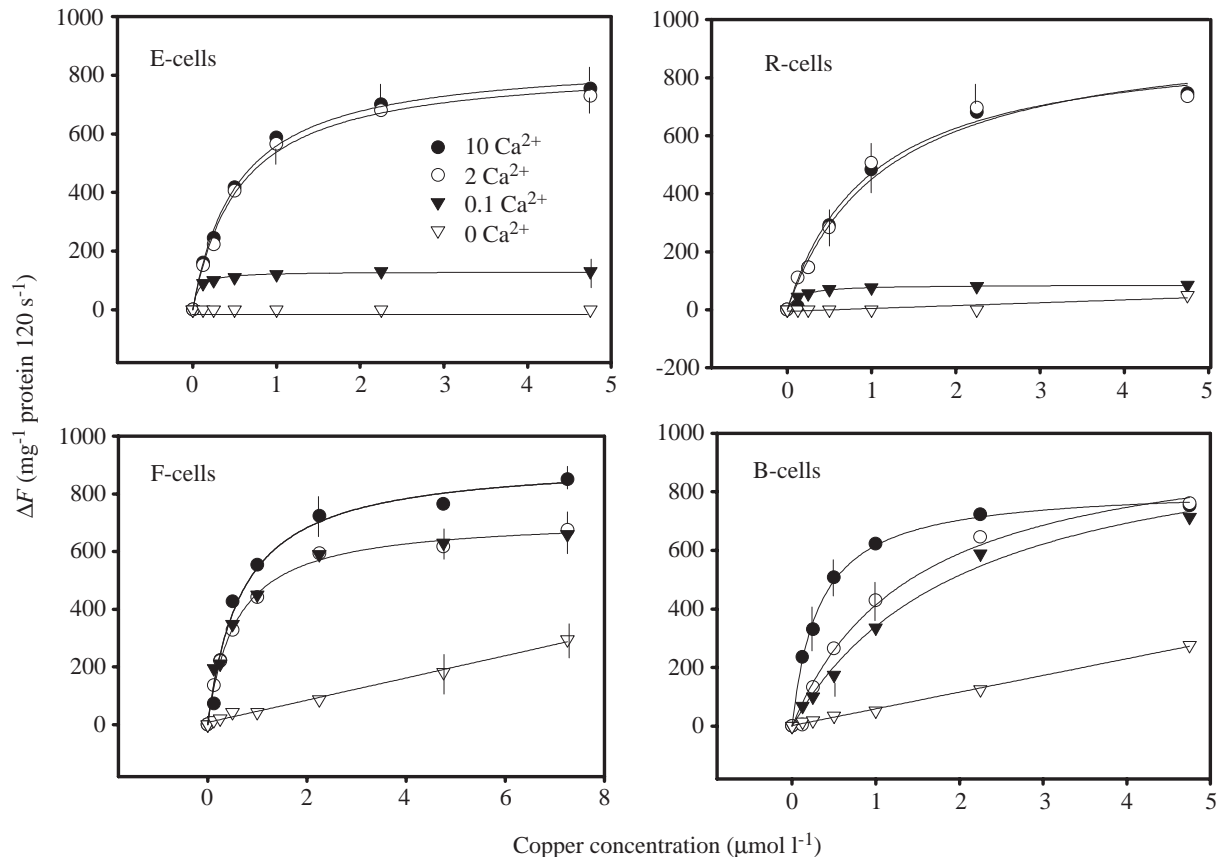
Fig. 5. Effects of external $Ca^{2+}$ concentration on $Cu^{2+}$-induced Phen Green fluorescence quenching ($\Delta F\,mg^{-1}$ protein $120\,s^{-1}$, where $\Delta F$ is fluorescence quenching) in purified hepatopancreatic cell suspensions over a range of external copper concentrations (given in mmol l$^{-1}$). An increase in fluorescence quenching was measured after addition of dye-equilibrated cells to buffers containing selected $Ca^{2+}$ and $Cu^{2+}$ concentrations. Copper uptake was quantitatively related to the decrease in fluorescence intensity (i.e. quenching). Control experiments showed that $Ca^{2+}$ alone had no significant effect on Phen Green fluorescence as determined under these experimental conditions (see also Fig. 3). Best-fitting lines were drawn using Sigma Plot software, and the resulting kinetic constants and their standard errors are presented in Table 2. Values are means ± S.E.M., $N$=3.

by each cell type increased in a hyperbolic fashion as the external metal concentration was elevated. However, the presence of calcium in the external medium had a significant effect on the magnitude of copper uptake by all hepatopancreatic fractions. When calcium was not added to the medium bathing the cell fractions (0 mmol l$^{-1}$ calcium), copper uptake (e.g. fluorescence quenching) was negligible in E- and R-cells, but did occur at a slow rate in F- and B-cells. As external calcium concentration was increased from 0.1 to 10 mmol l$^{-1}$, copper uptake by all fractions increased markedly and approached asymptotic transport rates at the highest calcium levels used. These results suggest that both calcium-independent and calcium-dependent transport processes for copper are present in the cells isolated from lobster hepatopancreas. The lack of copper uptake in the absence of calcium in E- and R-cells, however, suggests that calcium-independent uptake processes are absent from these cell types. The significant copper uptake in the absence of calcium in F-cells and B-cells suggests the presence of copper transfer pathways that were independent of calcium. The asymptotic increase in copper uptake rate as external calcium

concentration was increased in all cell fractions suggests the presence of a saturable, carrier-mediated, transport mechanism for this metal that is stimulated by calcium and follows the Michaelis–Menten relationship:

$$\Delta F_{Cu} = \Delta F_{max}[Cu]/K_{Cu} + [Cu], \qquad (1)$$

where $\Delta F_{Cu}$ is calcium-dependent copper influx as fluorescence quenching ($\Delta F\,mg^{-1}$ protein $120\,s^{-1}$), $\Delta F_{max}$ is maximal copper influx by this process, $K_{Cu}$ is the apparent affinity constant of the transporter for copper and [Cu] is the external copper concentration. Table 2 presents the copper influx kinetic constants, $K_{Cu}$ and $\Delta F_{max}$, for calcium-dependent copper uptake by each cell type at different external calcium concentrations. It is apparent from Table 2 and Fig. 5 that each type of hepatopancreatic cell responds differently to varying copper and calcium concentration. However, the pattern of response to calcium is similar between E- and R-cells, and this response differs from that shown by both F- and B-cells. While similar $\Delta F_{max}$ values were obtained from all four cell types, a higher calcium concentration was needed to reach asymptotic transport rates in E- and R-cell types than was required by F-

Table 2. *Stimulatory effects of $Ca^{2+}$ on influx kinetic constants for copper transport by purified hepatopancreatic cell types in suspension*

| Cell type | $Ca^{2+}$ (mmol l$^{-1}$) | Apparent affinity constant, $K_{Cu}$ (µmol l$^{-1}$) | Apparent maximal influx rate, $\Delta F_{max}$ ($\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$) |
|---|---|---|---|
| E | 10 | 0.54±0.05 | 859±23 |
| E | 2 | 0.56±0.06 | 838±27 |
| E | 0.1 | 0.07±0.01 | 130±3 |
| R | 10 | 1.16±0.28 | 972±89 |
| R | 2 | 0.99±0.19 | 936±65 |
| R | 0.1 | 0.13±0.01 | 86±1 |
| F | 10 | 0.69±0.10 | 920±37 |
| F | 2 | 0.58±0.04 | 717±14 |
| F | 0.1 | 0.50±0.06 | 701±22 |
| B | 10 | 0.32±0.02 | 817±14 |
| B | 2 | 1.46±0.29 | 1020±84 |
| B | 0.1 | 2.10±0.34 | 1057±78 |

Values are means ± 1 S.E.M.; three replicates per mean.

and B-cells. In general, all four cell types illustrated mixed-type responses to varying calcium concentration: both $K_{Cu}$ and $\Delta F_{max}$ changed with an alteration in external calcium concentration. These results suggest that calcium had more than one effect on the carrier-mediated transfer of copper across the plasma membranes of the four cell types. The linear rate of uptake of copper at 0 mmol l$^{-1}$ calcium by both F- and B-cells suggests either the possible presence of a diffusional uptake process or a low-affinity carrier mechanism for the metal in the membranes of these cell types.

*Effects of external verapamil on calcium-dependent copper uptake*

To investigate the mechanism by which external calcium stimulated copper uptake by isolated epithelial cell suspensions of lobster hepatopancreas, an experiment was conducted using the calcium channel blocker verapamil, added at 30 µmol l$^{-1}$ to the external medium in the presence of 10 mmol l$^{-1}$ calcium chloride. In this experiment, several external concentrations of copper (0.14, 0.28, 0.55, 1.14, 2.53 and 5.30 µmol l$^{-1}$ Cu$^{2+}$) were added to the external medium, and $\Delta F$ mg$^{-1}$ protein was monitored over 120 s uptake intervals. Fig. 6 shows that, for



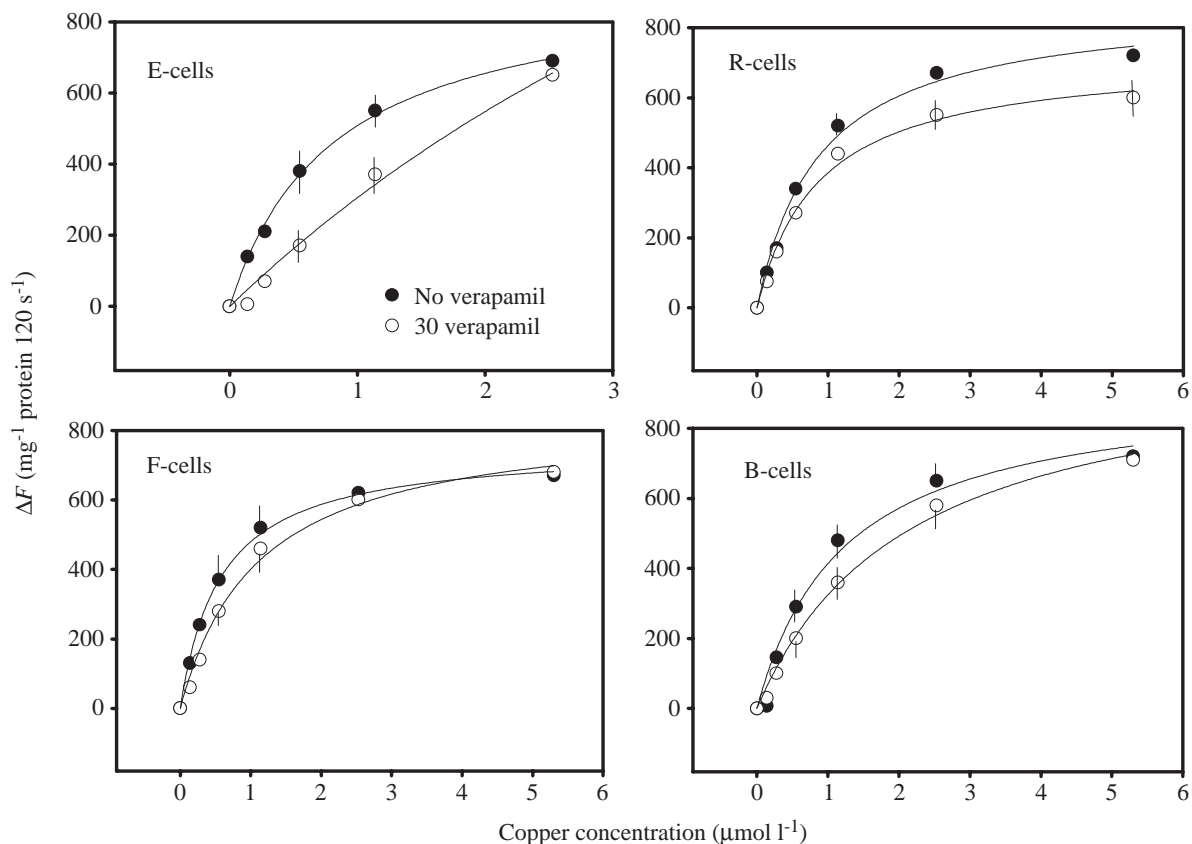Fig. 6. Effects of 30 µmol l$^{-1}$ verapamil on Cu$^{2+}$ influx into purified cell suspensions from lobster hepatopancreas in the presence of 10 mmol l$^{-1}$ Ca$^{2+}$. Control experiments showed that Ca$^{2+}$ alone had no significant effect on Phen Green fluorescence as determined under these experimental conditions (see also Fig. 3). Cells were equilibrated with Phen Green and were subsequently added to a buffer containing Ca$^{2+}$, verapamil (30 µmol l$^{-1}$) and selected Cu$^{2+}$ concentrations for 120 s. Increases in dye fluorescence quenching were measured as $\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$, where $\Delta F$ is fluorescence quenching. Best-fitting lines were determined using Sigma Plot software and yielded the kinetic constants and standard errors presented in Table 3. Values are means ± S.E.M., $N=3$.

pure suspensions of E-, R-, F- and B-cells in the absence of verapamil, an increase in external copper concentration, in the presence of $10 \, \mathrm{mmol \, l^{-1}}$ calcium, universally led to a hyperbolic increase in $\Delta F \, \mathrm{mg^{-1}}$ protein $120 \, \mathrm{s^{-1}}$ that followed the Michaelis–Menten equation (equation 1) previously described by the data in Fig. 5 and Table 2. The addition of $30 \, \mathrm{\mu mol \, l^{-1}}$ verapamil to the external medium of each cell type shifted the copper influx curve to the right in all instances, and each universally retained the hyperbolic character of the uptake curves. The influx data displayed in Fig. 6 were curve-fitted using Sigma Plot software (Jandel) and the Michaelis–Menten equation shown above. The resulting copper influx kinetic constants for metal uptake in the presence and absence of $30 \, \mathrm{\mu mol \, l^{-1}}$ verapamil are displayed in Table 3. Except in R-cells, addition of the channel blocker to the external medium containing calcium led to significant ($P<0.001$) decreases in apparent copper influx binding affinity (i.e. increased $K_{Cu}$), suggesting a marked reduction in apparent binding affinity between the transport system and metal ion when calcium uptake was blocked. Except for E-cells, only minor and inconsistent effects of verapamil were observed on maximal copper influx ($\Delta F_{max}$). The implication of this experiment is that $Ca^{2+}$ channels in the plasma membranes of hepatopancreatic epithelial cells may be involved in metal accumulation by allowing access of external calcium to the cytoplasmic face of the copper transport protein, where $Ca^{2+}$ may serve as either an antiport substrate for an exchange reaction between cations or as an activator of an exchange event involving the metal ion and some other intracellular cation.

### Effects of zinc on $Ca^{2+}$-dependent, carrier-mediated copper influx into hepatopancreatic E- and R-cells

To gain an understanding of the substrate specificity of the $Ca^{2+}$-dependent, carrier-mediated copper influx mechanism in the plasma membranes of hepatopancreatic epithelial cells, two experiments were conducted to determine whether zinc would influence the rate of copper influx if both metals were simultaneously present in the incubation medium. In the first experiment, increases in fluorescence quenching were observed in the presence of increasing copper concentrations ($21–1627 \, \mathrm{nmol \, l^{-1}}$), a fixed concentration of calcium ($10 \, \mathrm{mmol \, l^{-1}}$) and variable concentrations of zinc (0, $500 \, \mathrm{nmol \, l^{-1}}$ and $1000 \, \mathrm{nmol \, l^{-1}}$). As displayed in Fig. 7, increasing the concentration of external zinc with suspensions

Table 3. *Inhibitory effects of verapamil and zinc on influx kinetic constants for copper transport by purified hepatopancreatic cell types in suspension*

| Cell type | Test substance | Apparent affinity constant, $K_{Cu}$ ($\mu \mathrm{mol \, l^{-1}}$) | Apparent maximal influx rate, $\Delta F_{max}$ ($\Delta F \, \mathrm{mg^{-1}}$ protein $120 \, \mathrm{s^{-1}}$) |
|---|---|---|---|
| E | −verapamil | 0.82±0.07 | 922±35 |
| E | +verapamil | 7.61±2.31 | 2629±1178 |
| R | −verapamil | 0.88±0.12 | 871±40 |
| R | +verapamil | 0.87±0.11 | 721±31 |
| F | −verapamil | 0.58±0.04 | 756±14 |
| F | +verapamil | 1.11±0.16 | 845±44 |
| B | −verapamil | 1.23±0.29 | 924±83 |
| B | +verapamil | 2.14±0.29 | 1018±62 |
| E | 0 zinc | 0.75±0.09 | 1185±76 |
| E | 500 zinc | 1.75±0.22 | 1498±128 |
| E | 1000 zinc | 4.49±0.59 | 2074±22 |
| R | 0 zinc | 0.30±0.04 | 1166±57 |
| R | 500 zinc | 0.65±0.09 | 1122±78 |
| R | 1000 zinc | 0.73±0.08 | 1103±67 |

Values are means ± 1 S.E.M.; three replicates per mean.
Verapamil was added at $30 \, \mathrm{\mu mol \, l^{-1}}$.
Zinc concentrations are given in $\mathrm{nmol \, l^{-1}}$.
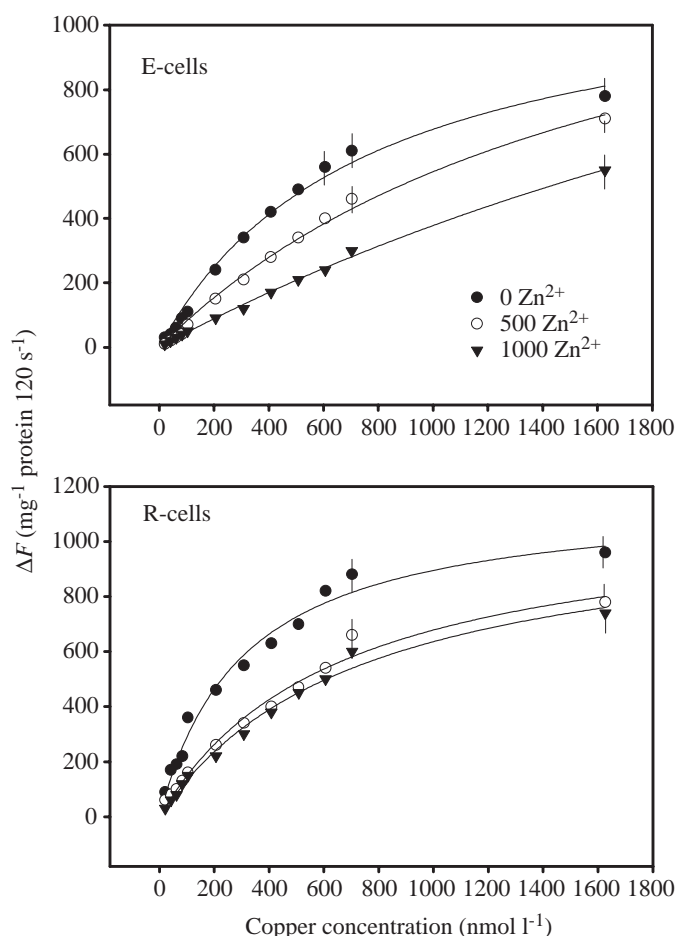


Fig. 7. Effects of variable external concentrations of zinc (as $ZnCl_2$) on calcium-dependent copper influx. Changes in fluorescence quenching (as $\Delta F \, \mathrm{mg^{-1}}$ protein $120 \, \mathrm{s^{-1}}$, where $\Delta F$ is fluorescence quenching) were measured at each external copper concentration in the presence of $10 \, \mathrm{mmol \, l^{-1}}$ calcium and zinc concentrations of 0, 500 or $1000 \, \mathrm{nmol \, l^{-1}}$. Values are means ± S.E.M., $N=3$. Lines are best-fitting curves obtained using Sigma Plot software, and the resulting kinetic constants are displayed in Table 3.

of E- and R-cells led to a significant ($P<0.01$) reduction in hyperbolic copper influx kinetics for both cell types. Table 3 presents copper influx kinetic constants, $K_{Cu}$ and $\Delta F_{max}$, for E- and R-cells at each of the three external zinc concentrations. The most significant effect of zinc on copper influx kinetics was the marked increase in the magnitude of the apparent binding affinity ($K_{Cu}$) of the transporter for copper. In E-cells, the apparent affinity was reduced (apparent $K_{Cu}$ was increased) by a factor of 6, while that for R-cells was reduced more than twofold in the presence of the inhibitory metal. Zinc had smaller effects on the maximal rate of copper transport ($\Delta F_{max}$). These findings are consistent with zinc acting mainly as a competitive inhibitor of copper influx into both E- and R-cells.

In the second experiment using zinc, the external concentration of the inhibitory metal was varied from 0 to 1000 nmol l$^{-1}$ while changes in fluorescence quenching were observed for two concentrations of copper (104 and 508 nmol l$^{-1}$). Fig. 8 shows that increases in zinc concentration at both copper concentrations led to marked reductions in fluorescence quenching for both E- and R-cells. The resulting data were best-fitted with Sigma Plot software using the following two-parameter hyperbolic decay curve:

$$\Delta F = (\Delta F_{max}K_i)/(K_i + [Zn]), \qquad (2)$$

where $\Delta F$ is the change in fluorescence quenching per milligram of protein per 120 s ($y$-axis), $K_i$ is the concentration of zinc that gives half-maximal inhibition, $\Delta F_{max}$ is maximal quenching and [Zn] is the external zinc concentration. Data for both E- and R-cells obtained in Fig. 8 were plotted as Dixon plots in Fig. 9 to obtain the zinc inhibitory constant ($K_i$) for both cell types. When this graphical analysis was performed, zinc was shown to act as a competitive inhibitor of copper uptake in both cell types and displayed $K_i$ values of 780±25 nmol l$^{-1}$ for E-cells and 1015±23 nmol l$^{-1}$ for R-cells (means ± S.E.M., $N=3$).
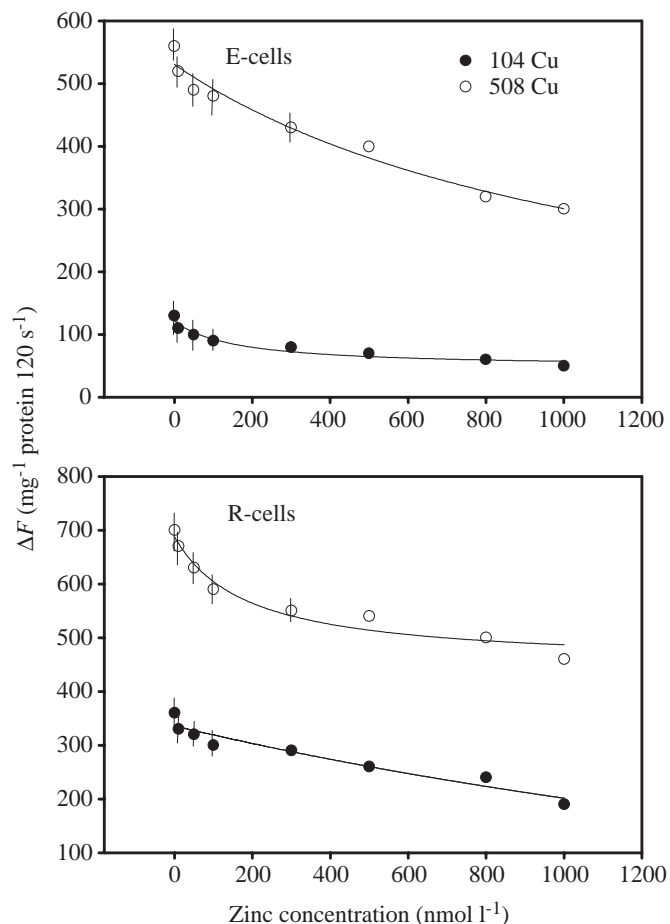


Fig. 8. Effects of variation in external zinc concentration at two fixed external concentrations of copper on calcium-dependent copper influx. Changes in fluorescence quenching (as $\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$, where $\Delta F$ is fluorescence quenching) were measured at each zinc concentration in the presence of 10 mmol l$^{-1}$ calcium and a copper concentration of either 104 or 508 nmol l$^{-1}$. The lines are best-fitting curves obtained using Sigma Plot software. Values are means ± S.E.M., $N=3$.

## Discussion

This paper reports the results of the first attempt to use centrifugal elutriation to separate a complex invertebrate epithelial organ into its separate cell types and to investigate differences between the distinct cell types in aspects of their copper biology. The data suggest that this laboratory method is useful for isolating specific cell types into highly purified suspensions from the crustacean hepatopancreas and, in combination with the fluorescent dye Phen Green, is a powerful tool for investigating the transport properties of ionic copper across hepatopancreatic cellular membranes. Following the protocol illustrated in Fig. 1, highly purified suspensions of E-, R-, F- and B-cells (Fig. 2) were produced in the present investigation. The fluorescent dye Phen Green was a highly specific (Fig. 3) and quantitative (Fig. 4) monitor for copper ions, although the dye did show some reactivity with ionic iron as well. Because most copper concentrations (as $CuCl_2$) used in this investigation were above 0.5 μmol l$^{-1}$, the $K_d$ for the

monovalent form of the ion (Kuhn et al., 1995), it is assumed that the fluorescence quenching of Phen Green reported here resulted mostly from interactions with the divalent form of the metal.

Endogenous concentrations of intracellular copper were greatest in the vesicles released by disruption of hepatopancreatic cells and isolated by elutriation (Table 1). It is well known that crustacean hepatopancreatic R-cells contain vacuoles that sequester and detoxify a variety of heavy metals in combination with sulfur and phosphorus (Al-Mohanna and Nott, 1985; Al-Mohanna and Nott, 1987; Al-Mohanna and Nott, 1989; Roldan and Shivers, 1987). These published reports suggest that the copper-containing vesicles isolated from lobster hepatopancreas by elutriation in the present study may have been derived from R-cells disrupted during the centrifugation process. Intact R-cells contained three times more copper than did the other mature hepatopancreatic cell
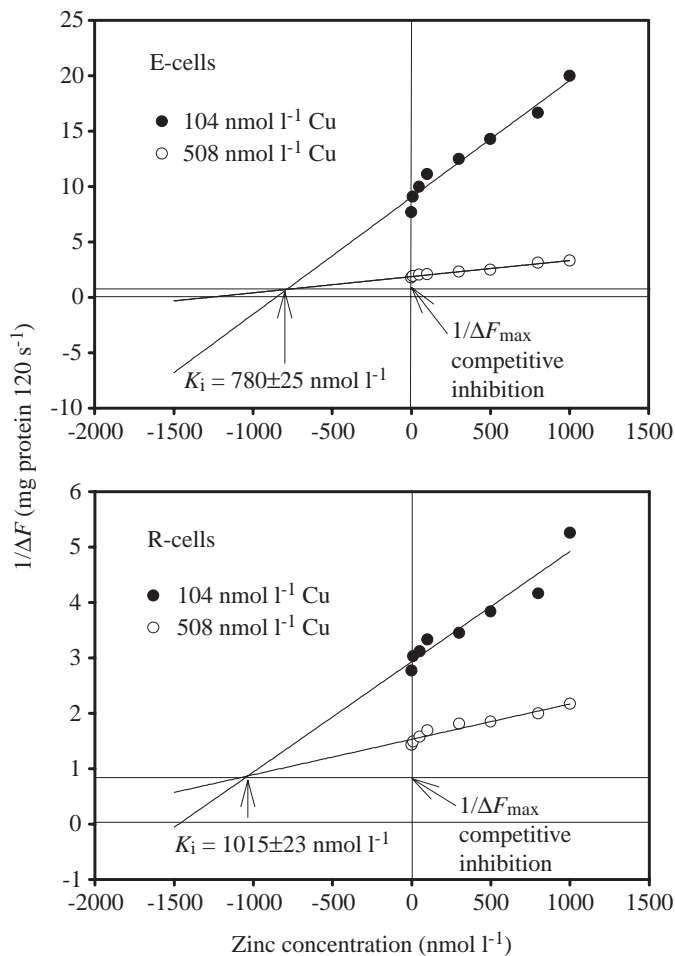
Fig. 9. Dixon plots of mean values from Fig. 8 giving values of the inhibitory constant of zinc on copper influx ($K_i$ values). Lines are best-fitting regression curves drawn using Sigma Plot software. The intersection of the lines above the $x$-axis suggests competitive inhibition between the metals during copper transport. $\Delta F$ is fluorescence quenching. Values of $K_i$ are means $\pm$ S.E.M., $N=3$.

types (Table 1), which supports the suggestion that they are the major metal storage cell type in gastrointestinal diverticula in invertebrates (Al-Mohanna and Nott, 1987). Interestingly, hepatopancreatic stem cells (i.e. E-cells) contained several times more stored copper than did the other cell types from this organ. The E-cells generally possess few, if any, vacuoles that could be used in metal storage, so the chemical nature of copper (ionic, complexed with divalent anions such as sulfur or phosphorus, bound to metallothionein, etc.) in this cell type is not known at present, nor is it known why the stem cells contain so much metal compared with the other cell types. However, the high concentration of copper in these cells is consistent with previous confocal localizations of calcium and heavy metals in stem cells of isolated intact lobster hepatopancreatic tubules (Dice et al., 1995a; Dice et al., 1995b). In these previous studies, a Bio-Rad MRC-600 confocal microscope in combination with fluorescent dyes, Fluo-3 to localize calcium and BTC-5N to localize heavy

metals, was used in lobster tubules. The findings of these studies showed high uniform concentrations of both types of divalent cation in distal E-cells and in punctate intracellular R-cell and F-cell sites along the full length of isolated tubules.

The finding of high concentrations of endogenous copper in lobster hepatopancreatic epithelial cells provides strong justification for using the concentration range selected for this metal in the present study (10 nmol l$^{-1}$ to 8 µmol l$^{-1}$ copper). Lobsters and other crustaceans are scavengers and feed on many sources of food, including other crustaceans and invertebrates. Most of these animals store heavy metals such as copper, zinc and iron in intracellular, membrane-bound concretions within gastrointestinal epithelial cells, as indicated in Table 1. If an invertebrate with stored heavy metal is consumed by a lobster, the hepatopancreatic luminal concentrations of metals during a meal may be considerable because the metals are released from their cellular storage sites during digestion. The quantitative values of the apparent affinity constants for copper binding to the carrier-mediated transport system described in this paper ($K_{Cu}$=0.1–2.0 µmol l$^{-1}$, Table 2) and the apparent inhibitory constant of zinc acting as a competitive inhibitor of copper transport ($K_i$=0.78–1.02 µmol l$^{-1}$; Fig. 9) suggest that these metals may reach micromolar concentrations in the lumen during digestion and, therefore, represent physiologically relevant values for an investigation of this nature.

The results in Fig. 5 suggest that copper uptake by epithelial cells of lobster hepatopancreas occurred by a combination of calcium-independent and calcium-dependent transport processes. Calcium-independent copper uptake appeared minimal in E- and R-cells, while a significant transfer of the metal occurred in the absence of Ca$^{2+}$ in F-cells and B-cells. All four cell types appeared to possess calcium-dependent, saturable transport processes for the uptake of copper. At physiological calcium concentrations (e.g. 10 mmol l$^{-1}$), the apparent binding affinities ($K_{Cu}$; range 0.32–1.16 µmol l$^{-1}$) and maximal transport velocities ($\Delta F_{max}$; range 817–972 $\Delta F$ mg$^{-1}$ protein 120 s$^{-1}$) of the four cell types for copper were similar to one another (Table 2). Calcium appeared to affect both kinetic constants for copper transport, suggesting that both metal binding and its rate of transport across the membrane were influenced by Ca$^{2+}$. Zinc was a competitive inhibitor of copper transport in E- and R-cells (Figs 7–9; Table 3). It is likely, therefore, that both copper and zinc use the same calcium-dependent, carrier-mediated, transport system for uptake into all hepatopancreatic epithelial cell types.

Previous work with lobster hepatopancreatic brush-border membrane vesicles has demonstrated the presence of two saturable carrier-mediated transport proteins transferring both monovalent and divalent cations from the gastrointestinal lumen to the epithelial cytosol (Ahearn et al., 1994; Ahearn and Zhuang, 1976; Zhuang and Ahearn, 1976). One of these transport systems was electrogenic (transferring two monovalent cations or one divalent cation for one monovalent cation) and amiloride-sensitive, while the other was
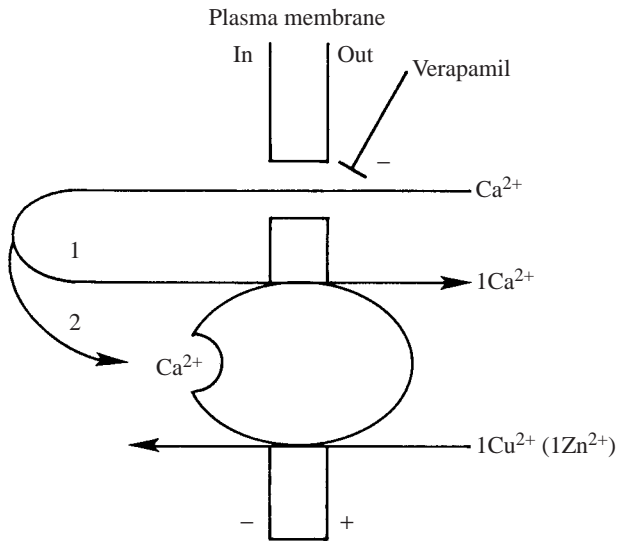
Fig. 10. Proposed model for the involvement of external $Ca^{2+}$ and $Zn^{2+}$ in copper influx into lobster hepatopancreatic epithelial cells. As suggested by the results of this study, external $Ca^{2+}$ may enter E-, R-, F- and B-cells across the plasma membrane through a verapamil-sensitive cation channel that has previously been identified. Once in the respective cells, $Ca^{2+}$ may then either serve as an antiport substrate, exchanging (process 1) with external $Cu^{2+}$ in an electroneutral fashion, or allosterically activate (process 2) the antiport of external $Cu^{2+}$ with $Ca^{2+}$ or another intracellular cation (e.g. $Mg^{2+}$, $Na^+$, $H^+$, etc.). External $Zn^{2+}$ acts as a competitive inhibitor of $Cu^{2+}$ influx in the presence of external $Ca^{2+}$ and, therefore, probably shares the external metal binding site on the transport protein with $Cu^{2+}$.

electroneutral, amiloride-insensitive and exchanged one divalent cation for two monovalent cations. Both zinc and cadmium inhibited the uptake of $Ca^{2+}$ by these carrier processes, but a detailed study determining the specific nature of this inhibition at each site was not made (Ahearn et al., 1994). A hepatopancreatic brush-border transport system catalyzing the exchange of extracellular iron for intracellular calcium has recently been characterized in lobster (A. G. Aslamkhan and G. A. Ahearn, in preparation). In this study, ionic iron in the $Fe^{2+}$ state was translocated into epithelial cells across hepatopancreatic brush-border membrane vesicles by antiport with $Ca^{2+}$ (or other divalent cations such as $Zn^{2+}$ or $Cd^{2+}$) derived from the extracellular compartment by a parallel transporter or channel in the same membrane. It is likely that the $Ca^{2+}$-dependent, carrier-mediated copper transport protein in isolated hepatopancreatic epithelial cells identified in the present investigation is one of the previously characterized brush-border membrane divalent transporters, but its exact identity remains to be determined.

Other studies with isolated lobster hepatopancreatic brush-border membrane vesicles revealed the presence of a verapamil-sensitive $Ca^{2+}$ channel in this location (Ahearn, 1996; Ahearn et al., 1994; Zhuang and Ahearn, 1996). Although detailed studies describing possible heavy metal transport through this $Ca^{2+}$ channel have not been published,

recent investigations of divalent cation transport by this membrane preparation have suggested that $Cd^{2+}$ (Ahearn et al., 1999) and $Zn^{2+}$ (Monteilh-Zoller et al., 1999; A. G. Aslamkhan and G. A. Ahearn, in preparation) may employ a channel-like protein for uptake from the hepatopancreatic lumen in these animals. These studies indicated that, once inside hepatopancreatic epithelial cells, $Cd^{2+}$ serves as an apparent intracellular regulator for ^{59}Fe uptake and that $Zn^{2+}$ regulates L-[^{3}H]proline uptake on independent carrier systems.

The data shown in Fig. 6 and Table 3 suggest that the $Ca^{2+}$ channel blocker verapamil had a significant inhibitory effect on copper uptake by all hepatopancreatic epithelial cell types in the present investigation. While significantly less copper was accumulated by each cell type in the presence of the inhibitor than in its absence, the hyperbolic character of each uptake curve in both control and test conditions was retained. This inhibitory effect of verapamil on copper influx indicates that extracellular calcium plays a role in copper uptake across the plasma membranes of these epithelial cells.

Previous work with lobster hepatopancreatic brush-border membrane vesicles has indicated that $Ca^{2+}$ crosses this cell border by a combination of two carrier-mediated transport mechanisms and by diffusion through a verapamil-sensitive cation channel (Ahearn and Zhuang, 1996; Zhuang and Ahearn, 1996). The model presented in Fig. 10 suggests two potential roles for extracellular calcium in the copper (or zinc) uptake processes described in the present investigation. As displayed in Fig. 10, extracellular calcium may enter hepatopancreatic epithelial cells through verapamil-sensitive $Ca^{2+}$ channels, diffusing down both concentration and electrical gradients. Once inside the respective cells, $Ca^{2+}$ may be involved in copper transport by acting as an antiport substrate exchanging with the metal in what is probably an electroneutral fashion (transport stoichiometery $1Cu^{2+}/1Ca^{2+}$). Alternatively, intracellular $Ca^{2+}$ may stimulate copper uptake allosterically by binding to a regulator site independent of the cation antiport binding site. In this instance, copper antiport may take place using a different intracellular substrate such as $Mg^{2+}$, $Na^+$, $H^+$, etc. It is possible that $Ca^{2+}$ may act both as an antiport substrate and as an allosteric activator in the same cell. Both these mechanisms involve $Ca^{2+}$ and may be sharply curtailed by addition of the $Ca^{2+}$ channel blocker verapamil. Future studies will be directed at further clarifying the nature of heavy metal transport across the hepatopancreatic epithelial brush-border membranes and the role that both monovalent and divalent cations have in this process.

Leeman Labs Mass ICP spectrophotometer to measure the intracellular copper concentrations of hepatopancreatic cells.

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