Frequencies of complementation of recipient protoplasts by isolated *Aspergillus nidulans* nuclei in three separate experiments.

Experiment no.	1	2	3
Number of recipient protoplasts	10^9	10^9	$3 \cdot 10^9$
Frequency of complementation due to karyoduction	$5.0 \cdot 10^{-7}$	$5.3 \cdot 10^{-7}$	$8.1 \cdot 10^{-7}$
Number of colonies from karyoduction	5	6	11

conidia and irregular growth (fig. 2). They exhibited a typical heterokaryotic morphology, the character of which was confirmed by biochemical and genetic analyses. The frequency of diploid formation from these heterokaryons was similar to the frequency seen with the products of protoplast fusion.

With a PEG-Ca^{2+} system for induction, nuclear uptake may have occurred as a result of an ordinary protoplast fusion. In such a case, entrapment of nuclei by the aggregates of the recipient protoplasts could provide a way for nuclear uptake to occur without damage to the isolated nuclei. Experiments using fluorescent-stained nuclei for karyoduction also indicated that this was the mechanism. Following PEG treatment, brightly stained nuclei with good morphological characteristics could be observed inside the aggregates of the recipient protoplasts.

It may be interesting to note that recent observations indicated that bacterial cells could be taken up into *Aspergillus* protoplasts by using PEG-mediated protoplast fusion [12].

The crucial condition for karyoduction proved to be the quality of the nuclear preparation. Isolated nuclei had to be free of cytoplasmic contamination, which caused nuclear clumping and especially of viable protoplasts. As a control, in each experiment the same amount of nuclear fraction as had been used for karyoduction was spread in a top layer onto osmotically stabilized complete medium to check for the presence of protoplasts able to regenerate. In all cases, these control experiments indicated that the complemented colonies had indeed originated from the transfer of isolated nuclei.

The frequency of nuclear uptake was low, but reproducible (table). The maximum complementation achieved was one nutritionally-complementing colony from 5×10^7 isolated nuclei.

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In vitro protein synthesis and α amylase activity in F cells from hepatopancreas of *Palaemon serratus* (Crustacea; Decapoda)

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Abstract. In crustaceans, all the steps in the assimilation of food take place in the hepatopancreas. To facilitate the study of this organ, a method for the dissociation of cell types was developed. The hepatopancreas of the prawn *Palaemon serratus* was mechanically dissociated and the cells separated by Percoll density-gradient centrifugation. The E and R cells had similar densities of around 1.05 g/ml. The F cells were separated into two distinct fractions with densities of 1.075 and 1.082 g/ml. The B cells sedimented at a density of 1.12 g/ml. The ratio between the two populations of F cells was found to vary during the intermolt cycle while B cells disappeared after the molt. When the density gradient fractions were incubated with ^{3}H-leucine, incorporation was highest in the F cell fractions. Measurements of α-amylase activity, indicated that the two populations of F cells may be derived from the same cell type.

Key words. Crustacean; prawn; hepatopancreas; cell suspension; protein synthesis; α-amylase activity.

It is well established that the crustacean hepatopancreas consists of four histologically and functionally different cell types[1]. These cell types were classified by Jacobs[2] and Hirsch and Jacobs[3], from histological data, as E (Embryonalenzellen), R (Resorptionzellen), F (Fibrillenzellen) and B (Blasenzellen) cells. Recently, a fifth cellular type has been described in unfed animals at intermolt, the M or Midgut cells[4,5].

The E cells are undifferentiated and show mitotic activity[6,7]. All the cell types are derived from the embryonic E cells. It has been proposed by Stanier et al.[8] that B cells evolve from F cells, and this view is accepted by a number of authors[9–13]. The R cells are the most abundant hepatopancreatic cell type[14,15] and are involved in lipid and glycogen storage and in nutrient absorption.

Owing to the fragile nature of the hepatopancreatic tissue, many of the physiological activities have been inferred from indirect histochemical and cytochemical observations.

The F cells contain digestive enzymes, which are sequestered within one or several supranuclear vacuoles[6,9,13,16]. It was shown by immunocytochemistry that these vacuoles contained α amylase[17]. Evidence for the role of F cells in enzyme synthesis is provided by studies of their ultrastructure. They have an extensively developed rough endoplasmic reticulum and Golgi apparatus. Moreover, Davis and Burnett[1] have demonstrated, by autoradiography, the rapid accumulation of radiolabelled uridine in the F cell nuclei of *Procambarus*, suggesting the active involvement of F cells in protein synthesis.

The formation of a supranucleolar vacuole in F cells by pinocytotic intake of luminal-nutrients and fluids, as suggested by Loizzi[9] and supported by Barker and Gibson[10,11], is contradicted by Ahearn et al.[15] and Mohanna et al.[4]. Gibson and Barker[6] concluded that the B cells have a secretory function but Hopkins and Nott[18] and Al-Mohanna et al.[19] propose that only the F cells are involved in the secretion of digestive enzymes into the lumen before their ultimate differentiation into B cells. The B cells which contain a single vacuole bounded by a thin ring of cytoplasm may be involved in digestion and assimilation[20].

Direct measurements of the absorptive capacities of R and F cells of *Homarus americanus* were made by Ahearn et al.[15], using cell suspensions separated by density gradient centrifugation. Devillez and Fyler[21], using the same methods, have provided evidence for the presence of trypsin and amylase in the B cells of *Orconectes rusticus*.

In the present work, to clarify the role of F, B and R cells, the protein synthesis and the α amylase activity of hepatopancreatic cells from the marine decapod prawn *Palaemon serratus* (Pennant) were studied after the separation of the cells by density gradient centrifugation. Changes in the proportion of each cell type during the intermolt cycle were followed.

Materials and methods

Animals. Experimental animals were collected with shrimp pots in Concarneau Bay (Brittany, France) and maintained in running seawater. The animals were fed ad libitum and sacrificed some days after their capture. The determination of the molting stages was done according to Drach and Tchernigotvzeff[22].

Preparation of hepatopancreatic cell suspensions. Two saline media were used throughout. The first was a physiological saline based on that developed by Ahearn et al.[15] for *Homarus* and modified to take into account the ionic concentrations of *Palaemon* hemolymph reported by Parry[23]. This physiological saline had the following ionic constitution in mM: Na^+, 446.5; K^+, 15.7; Ca^{++}, 30; Mg^{++}, 13; Cl^-, 527.2; $SO4^{--}$, 8.4; $HCO3^-$, 4.2. The osmolarity was 1100 m Osmoles. The second saline medium was used in the dissociation steps. This divalent cation free saline had the following ionic constitution in mM: Na^+, 488.8; K^+, 15.7; Cl^-, 483.5; $SO4^{--}$, 8.4; $HCO3^-$, 4.2

After removal, the hepatopancreas was rinsed several times in cold divalent cation-free saline solution and minced with scissors. Cold divalent cation-free saline media containing 10 mM EDTA and 0.1 mM PMSF (first dissolved in 100% isopropanol) was added to the mixed tissue (1 ml/g wet wt). Dissociation was accomplished by mild mechanical agitation with a magnetic stirrer for 20–25 min at 4 °C. This method was used because preliminary experiments showed that *P. serratus* hepatopancreatic cells, particularly the R cells, did not survive collagenase dissociation. Cell viabilities were determined using the Trypan blue (0.2%) dye exclusion test; cell counts were made using a hemocytometer. Crude suspensions were filtered twice through 100-µm nylon mesh.

Density gradient centrifugation. The dissociated cell suspension was centrifugated at 400 × g (5 °C) for 10 min. The pellet was suspended in 1 ml physiological saline solution and layered on a preformed Percoll gradient of 50% normal saline-50% Percoll (density = 1.130 g/ml, Sigma) stock solution. The stock solution of the density gradient medium was prepared using a normal saline containing salts at 10 times the normal concentration (9 parts Percoll/1 part 10 times normal saline). Continuous density gradients were preformed from Percoll (Pharmacia) suspensions in physiological saline (starting density = 1.13 g/ml) by centrifugation of duplicate aliquots at 21,500 × g for 13 min in a Beckman L8-70 ultracentrifuge (50 Ti rotor). One gradient was monitored with Density Marker Beads (Pharmacia). The cells were centrifuged on the other gradient at 400 × g for 10 min.

Fractions of 0.6 ml were collected from the top by injection of a high density liquid (Fluorinert FC-40, density = 1.85 g/ml) into the bottom of the tube. Sixteen fractions were thus obtained. The fractions were washed twice by centrifugation at 400 × g for 5 min and resuspend in 3 ml physiological saline to remove Percoll. The

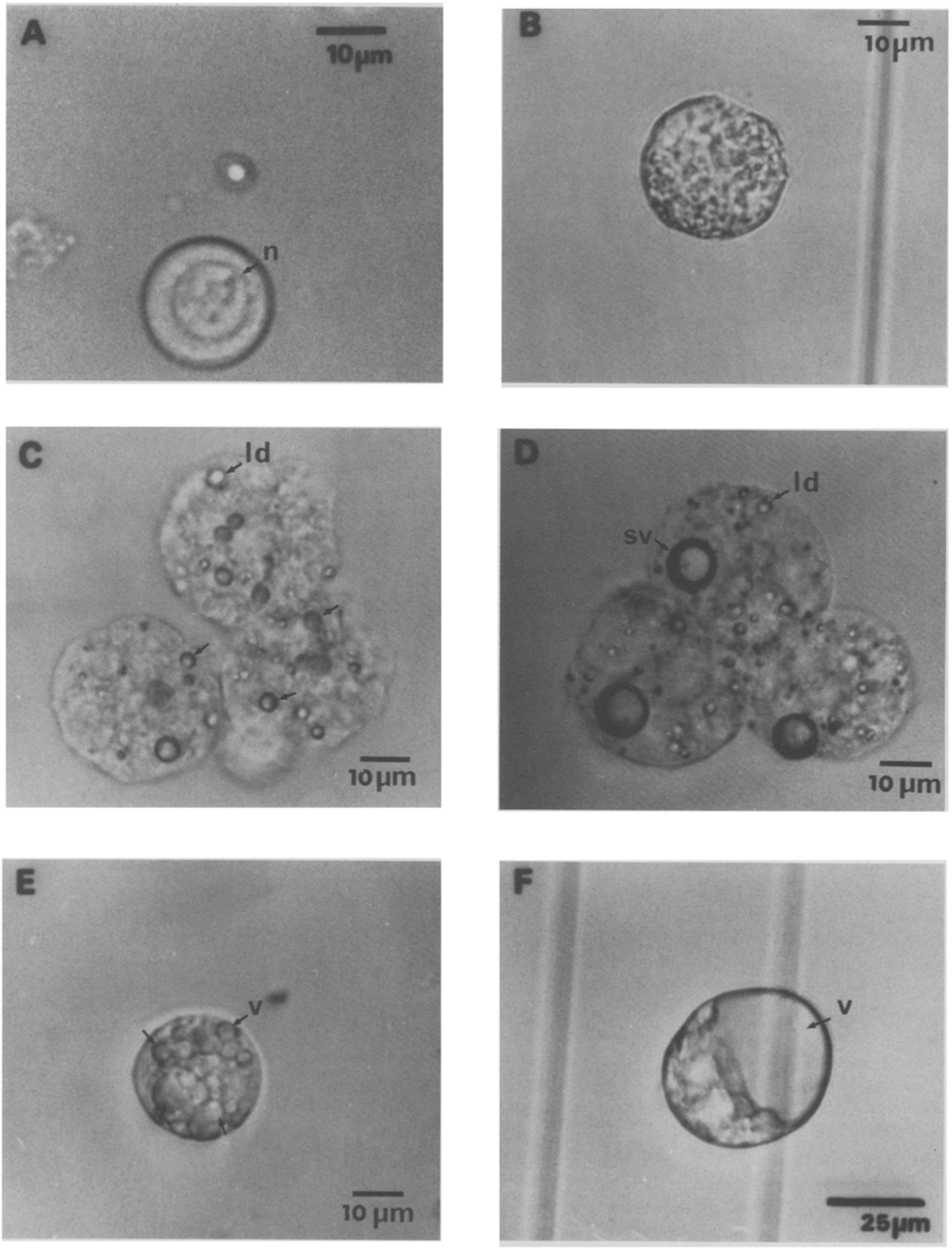

Figure 1. *Palaemon serratus* hepatopancreatic cells from Percoll gradient. *A*, E cell; *B*, F cell; *C*, young R cell; *D*, old R cell with supranucleolar vacuole; *E*, B cell in differentiation; *F*, B cell. n, nucleus; ld, lipid droplet; sv, supranuclear vacuole; v, vacuole.

washed cells were resuspended in 0.6 ml normal saline. Cell counts were made with a Malassez hemocytometer using 0.1 ml of suspension. Assays were made with the remaining 0.5 ml.

α-Amylase activity. α-Amylase activity was determined after hydrolysis of glycogen (oyster from Sigma) by the method of Bernfeld[24].

Incubation with labelled leucine. The incubations were made in 10 ml sterile tubes. 2 μCi of tritiated leucine (sp. act. = 1 mCi/mmol) were added to each aliquot of cell suspension. According to Richard[25] the hepatopancreatic content of leucine was about 5 μmol/10^6 cells and was globally comparable to the pool of ^{3}H-leucine. The tubes were agitated throughout the incubation at 15 °C and in darkness. The reaction was stopped with 500 μl ice-cold 10% trichloroacetic acid. After total precipitation overnight at 4 °C, the pellets were resuspended and 400 μl filtered on a Whatman filter (GF-C) under vacuum. The filters were washed immediately with 20 ml of 5% trichloroacetic acid followed by 10 ml ethanol and finally 10 ml ethanol/ether (2:1 v/v) to remove non-incorporated radioactivity. The filters were dried at 37 °C. The dry filters were added to Ready-Solv HP scintillation cocktail (Beckman Instrument) and their radioactive content measured in a liquid scintillation spectrometer (Intertechnic SL 3000).

Results

Characterization of cell types. Mechanical dissociation yielded an average of $5 \pm 0.5.10^6$ cells/g of hepatopancreatic tissue (n = 9). Cell discrimination was based on different characteristics (fig. 1). The E cells were the smallest cells and had large nuclei. The B cells had numerous vacuoles which, in mature cells, fused to form a single large vacuole that compressed the nucleus into an oval shape at the cell border. The F cells were characterized by dense cytoplasm and a few vacuoles of variable size and shape. Some of these may have represented early stages in the formation of the vacuole found in the B cells. The R cells were very fragile and were characterized by large numbers of lipid droplets. The droplets seemed to be larger in mature cells which also presented a single supranuclear vacuole. The staining of these cells with Sudan Black showed a reaction with the lipid droplets (which do not appear to coalesce) and also with the supranuclear vacuoles.

Gradient separation. The gradient had three bands. The first one contained only cellular fragments. The second contained numerous R cells, E cells and F cells. The third contained a large number of F cells and a few E cells. The B cells were isolated at the bottom of the tube but they were not numerous enough to form a specific band.

Recovery was different for each cell type: E cells = 65 ± 9%, R cells = 55 ± 8%, F cells = 86 ± 12%, B cells = 72 ± 13%. The greater part of the cell mortality was among the R cells. These cells accounted for 50 ± 5% of the total cell numbers in fractions.

Near the top of the tube the R cells containing lipid droplets were present with the E cells at a density of 1.05 g/ml (fig. 2). Two peaks of F cells were observed: the first (F_1) with a density of about 1.075 g/ml, the second (F_2) with a density of 1.082 g/ml. No difference was visible between the two cell categories under the microscope. At the bottom of the tube, B cells were the most numerous with a density of 1.12 g/ml.

Variation of the cell types during molting cycle. Just after the molt (AB), the number of B cells was very low (table); F_1 cells were more abundant than F_2 cells. During stage C, F_1 cells decreased while F_2 cells increased. The same variations were measured between the F_1 and F_2 cells during the premolt stages D_1 to D_2. The variations in numbers of R cells were more limited whereas no variation of E cells was detected.

Incorporation of ^{3}H-leucine. Viabilities were greater than 90% after 3 h of incubation, both in the crude suspension and in the fractions. Tritiated leucine was incorporated linearly with time into the protein fraction of the hepatopancreatic cell suspensions (fig. 3a), and this incorporation was proportional to cell number (fig. 3b). When cell fractions were incubated for 1 h with labelled leucine, specific activity, expressed as cpm per 10^6 cells, was higher in fractions 10–13 which contained mainly F_2 cells, and lower in fractions 2–6 which contained a majority of R and E cells, or fractions 14 and 15 containing B cells (fig. 4). The protein synthesizing activity of R and B cells was limited; this was especially marked for R cells, which showed the highest densities (fractions 2–5)

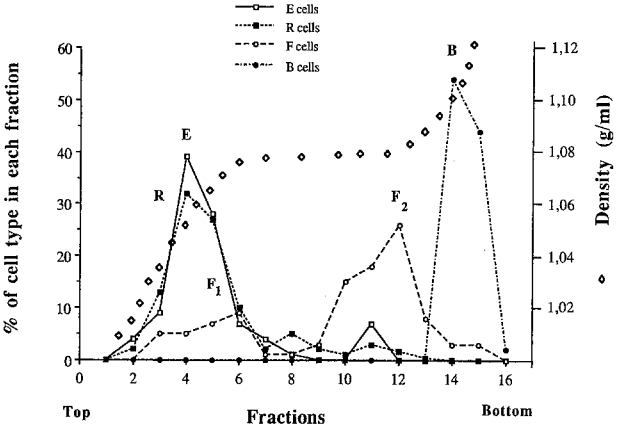

Figure 2. Frequency distribution of the four hepatopancreas cell types of *P. serratus* (stage C) separated by Percoll density gradient centrifugation.

Percentage of each type of hepatopancreatic cell during the molting cycle of the prawn *Palaemon serratus*

Intermolt stages	Cellular types (%) E	R	F_1	F_2	B
A–B	14	50	25.5	9	0.7
C	14.8	35	10	31	8.9
D_1	15.8	48.5	23	8.5	4.2
D_2	12.3	56	8	18	5

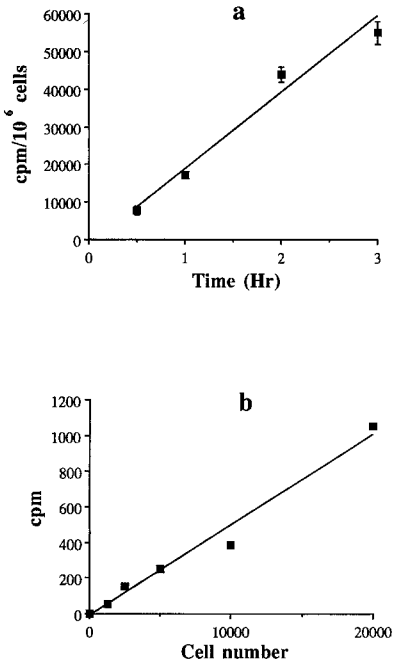

Figure 3. *a* The effect of incubation time on the incorporation of ^{3}H-leucine into the protein fraction of dissociated *P. serratus* (stage C) hepatopancreas cells. *b* The effect of cell numbers on the incorporation of ^{3}H-leucine into the protein fraction of dissociated *P. serratus* (stage C) hepatopancreatic cells.

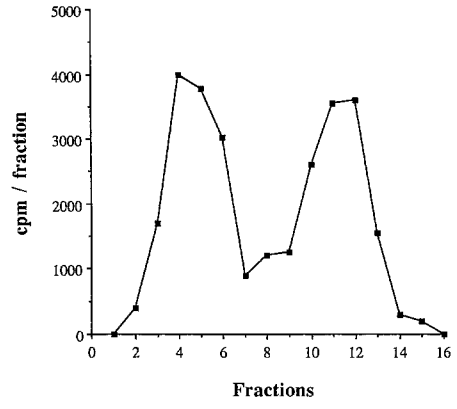

Figure 4. ^{3}H-leucine incorporation into the protein fraction of cell suspensions from *P. serratus* (stage C) separated by density gradient centrifugation.

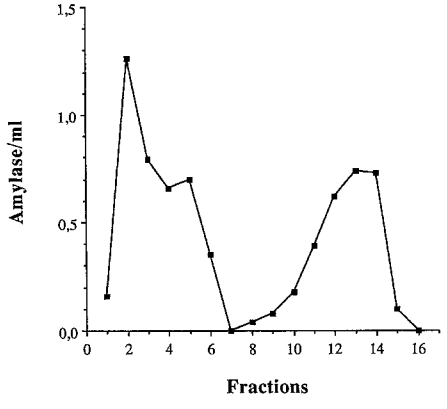

Figure 5. α-Amylase activity in cell fractions after the Percoll density gradient separation of a hepatopancreas cell suspension from *P. serratus* (stage C).

(fig. 4). E cells did not seem to have an important role in protein synthesis.

Partition of α-amylase activity in the gradient. α-Amylase activity was maximal in the fractions 2–5 and 12–14, containing the two populations of F cells, but low in fractions 15 and 16, containing B cells (fig. 5). In the fractions 1 and 2, α-amylase activity did not correspond to cell distribution; this was probably the consequence of some cell disintegration after centrifugation.

Discussion

The distribution of cell types on gradients was comparable to that found with cells of the lobster *Homarus americanus*[15], the crayfish *Orconectes rusticus*[21] and the crab *Carcinus maenas*[26]. The absolute densities were greater in the lobster but were smaller in the crayfish and in *Penaeus vannamei*[27]. The distribution profile was not very different from that of crayfish cells obtained by mechanical disruption. The R cells were at the top of the gradient, the B cells at the bottom and the E and F cells were divided into two peaks.

The E cells are undifferentiated[6,7] and are generally believed to be involved in mitotic activity for the production of the other cell types. The duration of mitotic activity is restricted to a short period after feeding[19]. The prawns were fed ad libitum and sacrificed randomly and variations in density might be accounted for by DNA duplication.

Variations in F cell densities were consistent with their gradual differentiation into B cells and therefore with feeding according to Devillez's observations[21] and Al-Mohanna's studies[19]. The proportion of F cells changed according to the molting stages. F_1 cell numbers were maximal just after the molt, when the prawns were not able to eat. The same results were obtained after a long period of starvation. Ahearn[15] found only one type of F cell using unfed animals, which could have been correlated with feeding rather than resulting from a synchronized transformation of the F cells as proposed by Devillez and Fyler[21].

The two F cell categories could represent the two principal steps of the differentiation from E cells to B cells. The F_1 cells would be the young F cells, and the F_2 cells the last step before the B cell state. The role of the F_1 cells present in the first fractions was difficult to define because of the number of different cell types in these fractions. However, in fractions 4–6, the incorporation per 10^6 cells was half of that found in fractions 11 and 12, although the F_2 cell numbers were three times greater in these last fractions. This indicates that F_1 cells have a higher level of synthesis than F_2 cells. The amount of incorporation was not correlated with the proportion of

F cells. The R cells are probably also involved; however, the low incorporation observed in fractions 4 and 5, where R cells were numerous, seems to indicate that their role in protein synthesis is limited. The same conclusion applies to E cells. It is also possible that some R cells of the type found in fractions 8 and 9, with very few lipid vacuoles and higher density, synthesize protein more actively than old R cells. Taken together, the results indicate that the F cells are the major cell type implicated in protein synthesis in the decapod hepatopancreas (fig. 4) and support for the first time in vitro all the deductions made from ultrastructural studies on F cells [1, 9, 19].

Our results concerning the protein synthesis capacities of the various cell populations do not appear to correlate with those of Ahearn et al.[15], showing that the time course of ^{3}H alanine uptake into purified R cells exhibits a sixfold higher rate of influx than that shown in F cell suspensions. However, the measured rates could be influenced by other factors. For example, the concentration of cold leucine in R cells may be higher than that in F cells. The intracellular pools in different cell types have to be determined.

The F cells are also known to store digestive enzymes in vacuoles before secretion[17]. Measurement of α-amylase activity confirms its presence in both of the F cell types. The difference of density between the two categories may correspond to the evolution of these cells toward B cells. Provided that care is taken only to pool animals which are all in the same physiological condition, dissociation and cell suspension techniques could offer a powerful tool for studying hormonal control of protein synthesizing activities, even in complex organs like the crustacean hepatopancreas.

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Enzymatic basis for protection of fish embryos by the fertilization envelope

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Abstract. The mechanism by which the fertilization envelope (FE) is able to protect the embryo of fish until hatching is almost unknown, except for its function as a physical barrier. FE extract from activated or fertilized eggs of the fish *Salmo gairdneri* was demonstrated to contain enzyme activities using an agar plate enzyme assay. The enzymes apparently active were carboxymethylcellulase (cellulase; EC 3.2.1.4), laminaranase (endo-1,3(4)-β-glucanase; EC 3.2.1.6), carboxymethylchitinase (chitinase; EC 3.2.1.14), xylanase (endo-1,4-β-xylanase; EC 3.2.1.8), mannanase (mannan 1,2-(1,3)-α-mannosidase; EC 3.2.1.77), dextranase (EC 3.2.1.11), a protease and lysozyme (EC 3.2.1.17). The FE extract exerted an antifungal or fungicidal action on the fungus *Saprolegnia parasitica,* whereas an extract from the vitelline envelopes (VE) has no apparent enzyme activity nor antifungal or fungicidal action. Enzymes acquired by the FE through the cortical reaction may have an important defensive role, protecting the embryo against invaders or pathogens.
Key words. Fertilization envelope; enzyme activities; antifungal action.