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Hydrolysis of G6P by a microsomal aspecific phosphatase and glucose phosphorylation by a low K_m hexokinase in the digestive gland of the crab *Carcinus maenas:* variations during the moult cycle

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Summary. The hydrolysis of glucose-6-phospate in the digestive gland of the crab *Carcinus maenas* is carried out by an aspecific phosphatase. This enzyme possesses the following features: (1) insensitivity to acid treatment; (2) absence of inhibition when exposed to citrate at low pH; (3) similar affinity for G6P as the acid phosphatase for Na-β-glycerophosphate (K_m 2.3 and 2.0 m*M*, respectively). Glucose-6-phosphate and Na-β-glycerophate hydrolysis reactions seem to be catalysed by the same enzyme, since both activities exhibit the same distribution in a subcellular fractionation of the gland. Furthermore, as these activities are principally recovered in the subcellular fraction enriched in calcospherites (or calcium phosphate granules), it is proposed that the aspecific G6P-phosphohydrolase could play a major role in the formation of these granules. The phosphorylation of glucose is made by two "low K_m" hexokinases (230 and 64 μ*M*, respectively). As their level of activity shows significant changes over the moult cycle, these enzymes could be considered as having a regulatory role in the storage of glucose in the digestive gland.

Key words: Digestive gland – G6P-phosphohydrolase – Hexokinase – Glucose homeostasis – Crab, *Carcinus maenas*

Abbreviations: Acid Pase, aspecific acid phosphatase; ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetate; G, calcium phosphate granules fraction; G6P, glucose-6-phosphate; G6Pase, hepatic glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; K_m, Michaelis-Menten constant; MI, mitochondria and intermediate post-mitochondrial particles; N, nuclei fraction; NADH, nicotineamide adenine dinucleotide; P, microsome fraction; Pi, inorganic phosphate; PMSF, phenylmethylsulphonylfluoride; STI, soybean trypsin inhibitor; βglyP, Na-β-glycerophosphate; $T_{1,2,3}$, transport protein 1,2,3; TCA, trichloroacetic acid

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Introduction

In mammals G6Pase (E.C. 3.1.3.9.) is principally localized in the liver where it is mainly associated with the endoplasmic reticulum (Hers et al. 1951). This microsomal enzyme is a multi-component system consisting of at least five different polypeptides: the G6Pase (with its active site positioned on the luminal side of the reticulum), a Ca^{2+}-binding protein termed stabilizing protein (Burchell and Burchell 1985), and three transport proteins, T_1, T_2 and T_3, which facilitate the movement of G6P, pyrophosphate and glucose, respectively, across the endoplasmic reticulum membrane. The liver G6Pase catalyses a key step in the regulation of blood glucose homeostasis (Ashmore and Weber 1959). It has also been suggested that G6Pase plays a role in the regulation of cytosolic Ca^{2+} (Benedetti et al. 1985, 1986, 1988). The presence of this enzyme has also been demonstrated in the kidney (Cori and Cori 1952) and has more recently been identified in microsomal fractions isolated from pancreatic islets (Waddell and Burchell 1988) where it could regulate glucose-stimulated insulin release. To yield G6P (a "mobilized" form of glucose in the cell), most tissues possess "low K_m" hexokinase isoenzymes, which are saturated throughout the physiological range of glycemia and are strongly and allosterically inhibited by low concentrations of G6P [for review see Wilson (1985)]. The mammalian liver possesses a fourth isoenzyme, commonly known as glucokinase (E.C. 2.7.1.2.) (Di Pietro and Weinhouse 1960; Vinuela et al. 1963; Walker 1963), which is only partially saturated in the physiological range of glucose concentration because it exhibits a much higher K_m and is only slightly affected by G6P. This enzyme is usually considered as the entry point for glucose in a hepatic tissue during the period following the ingestion of a carbohydrate-rich meal. The existence of a glucokinase was also demonstrated in the β-cells of the mammalian pancreas (Bedoya et al. 1986; Meglasson et al. 1986; Sener et al. 1986; Shimizu et al. 1988; Malaisse-Lagae and Malaisse 1988).

It was of particular interest to check the presence of these key enzymes in the crustacean digestive gland which has long been considered as a liver analogue. This concept must, however, be viewed with some caution. Indeed, the digestive gland is a complex organ which has not only hepatic-like properties but also some characteristics of the vertebrate pancreas and small intestine (Vonk 1960; Hohnke and Scheer 1970). Furthermore, there is some evidence of its capacity to store carbohydrates (Sedlmeier 1987) and other inorganic substances [reviewed by Gibson and Barker (1979)], although little is known of the enzymatic key steps controlling the release and uptake of glucose, i.e. those corresponding to mammalian G6Pase and glucokinase. It is also of interest to follow the evolution of these enzymatic activities over the moult cycle of the crab. Indeed, moult is characterized, among other things, by a succession of fasting and feeding periods which could have an impact on carbohydrate metabolism within the digestive gland and consequently on the control of its key enzymes.

Materials and methods

Reagents. ATP, D-G6P (monosodium salt), *p*-nitrophenylthymidine-5'-phosphate, NAD^+, PMSF and STI were obtained from Sigma, St Louis, MO, USA. DTT, benzamidine and EDTA were obtained from Janssen Chimica, Beerse, Belgium. Glycyl-L-phenylalanine 2-naphthylamine was from Biochemical Co, Cleveland, OH, USA. G6PDH from *Leuconostoc mesenteroides* was obtained from Boehringer, Mannheim, Germany. All other chemicals were of analytical grade.

Animals. Male green crabs *(Carcinus maenas)* weighing approximately 50 g were purchased from the Biological Station of Roscoff, France, kept at room temperature in filtered, aerated synthetic sea water (Vitakraft; salinity 37.5 mg · ml^{-1}) and fed with mussels twice a week. Except when specified, only specimens at intermoult stage and fasted for 48 h were selected for this study. The moulting stages were determined according to Drach and Tchernigovtzeff (1967) and Tchernigovtzeff (1965).

Subcellular fractionation of the digestive gland. All the experimental steps including dissection, homogenization and centrifugation were performed at 4 °C. The tissue was homogenized at a final dilution of 1 : 5 w/w (tissue weight: homogenization medium weight) in an isotonic medium with 0.45 *M* NaCl, 50 m*M* KCl, 10 m*M* EDTA, 30 m*M* trisodium citrate pH 6.8, containing 1 mg DTT · ml^{-1}, 1 m*M* PMSF (prepared from a 1 *M* stock solution in ethanol just before use in order to avoid its aqueous inactivation), 5 m*M* benzamidine and 1 mg STI · ml^{-1}. The homogenate was made with a Dounce homogenizer (three strokes with the loose piston followed by two strokes with the tight piston) and fractionated by differential centrifugation according to a method which is a combination of that of Tartakoff and Jamieson (1974) for the mammalian pancreas and those of Becker and Chen (1974) and Guary and Negrel (1981) for the crustacean digestive gland. Four successive centrifugations were made at $200 \times g$ (1.5 min), $500 \times g$ (10 min), $23000 \times g$ (5 min) and $100000 \times g$ (60 min), respectively, yielding four pellets and one supernatant referred to (in accordance to their probable composition) G, N, MI and P. All pellets were resuspended in the homogenization medium. The first two pellets were rinsed twice before resuspension.

Standard assays. The activity of G6P-phosphohydrolase is assayed at 37 °C by measuring the Pi released after 60 min in 0.2 ml medium containing 50 m*M* G6P, 1 m*M* EDTA and 50 m*M* imidazole buffer, pH 6.5. Pi is determined according to the method of Fiske and Subbarow (1925). As G6P-phosphohydrolase activity is classically attributed to G6Pase in the mammalian liver, the specificity of this activity was tested in all the subcellular fractions except G. This verification involves preincubation of the enzymatic preparation at pH 5; this acid pretreatment inhibits vertebrate G6Pase without affecting the aspecific acid phosphatase (de Duve et al. 1949; Beaufay and de Duve 1954). Consequently, G6Pase activity is calculated by substracting the activity obtained at pH 6.5 after acid pretreatment, from the total activity measured directly at pH 6.5. As the G particles of the digestive gland are rapidly solubilized at acid pH releasing large amounts of Pi in the assay medium, direct measurement of Pi (as indicator of G6P-phosphohydrolase activity) in the G fraction is not possible. For this reason, the assay was modified as follows: G was discarded from the incubation medium by a short centrifugation ($300 \times g$, 1.5 min) before adding the stopper acid reagent (10% TCA). It is important to note that the acid solubility of G prevents the specificity of their G6P-phosphohydrolysis capacity being tested by the usual acid pretreatment procedure.

Acid Pase activity is measured at 37 °C after a 60-min incubation in 0.2 ml medium containing 50 m*M* βglyP as substrate and buffered with 50 m*M* imidazole at pH 6 [this is the optimal pH for βglyP-phosphohydrolysis (see Fig. 1), avoiding problems associated with the acid solubility of G]. The Pi released is revealed spectrophotometrically as described for G6Pase. For both enzymes, one unit of activity is defined as 1 μmol Pi released per min.

Hexokinase and glucokinase were determined on fresh preparations by the continuos spectrophotometric method of Davidson and Arion (1987). One unit of activity is defined as 1 μmol NADH produced per min. Alkaline phosphodiesterase activity was determined according to Trausch et al. (1988), using *p*-nitrophenylthymidine 5'-phosphate as substrate. For cathepsin C measurement the method described by Jadot et al. (1984) was used, except for the determination of the released naphtylamine which was performed via the colorimetric assay described by Barrett (1977).

The linear increase of the enzymatic activity as a function of the time of incubation was verified for each enzyme. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumine as standard.

Statistical analysis. Results are expressed as mean ± standard error (SEM). When the homogeneity of variances is established (after logarithmic transformation of data), groups of values are compared via one-way analysis of variance and the mean values via contrasts of either Scheffé or Student.

Results

G6P-phosphohydrolase activity in a tissue extract of the digestive gland: evolution during the moult cycle

Since G6P-phosphohydrolase activity of the mammalian liver is predominantly in the endoplasmic reticulum (Hers et al. 1951), assays are generally made on a low centrifugation supernatant ($300 \times g$ for 1.5 min.). This procedure is of particular importance when using crustacean tissue because it discards G and so avoids the following major inconveniences: an opalescence which prevents spectrophotometric assay, and a high solubility in acid solutions that disturbs the colorimetric determination of Pi produced by phosphatases.

In contrast to the vertebrate liver G6Pase, acid pretreatment of digestive gland extracts does not affect G6P hydrolysis (Fig. 1). This suggests that the crustacean digestive gland does not contain any specific G6Pase. This property is verified between pH 5 and 7 (Fig. 1). Moreover, in the crustacean gland the optimal pH for the

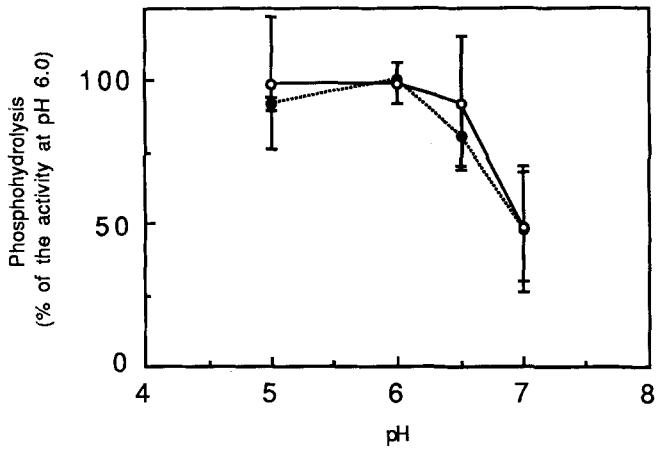

Fig. 1. Effect of an acid pretreatement (-○-) on the total G6P-phosphohydrolytic activity (···●···) of an extract of *Carcinus* digestive gland, measured between pH 5 and 7. Results are expressed as percentage of the activity measured at pH 6. Mean ± SE ($n=4$)

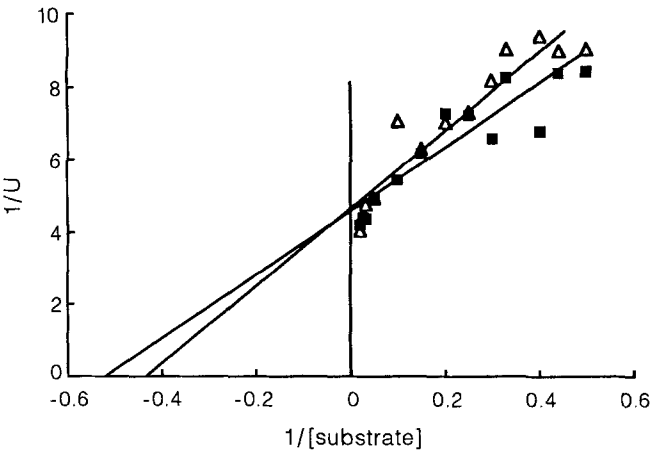

Fig. 3. Lineweaver-Burk plots for G6P-phosphohydrolase (-△-) and βglyP-phosphohydrolase (Acid Pase; -■-) activities of the digestive gland of *Carcinus*. G6P: $y = 4.6360 + 10.701x$; $r=0.89$; $K_m=2.3$ mM; βglyP: $y = 4.5021 + 8.4643x$, $r=0.84$; $K_m=1.9$ mM

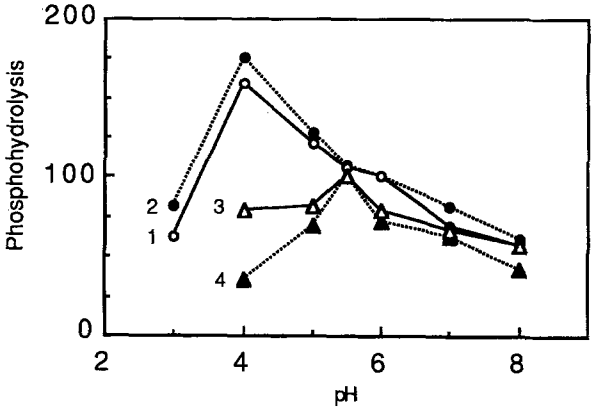

Fig. 2. *Curves 1 and 2:* pH-dependent phosphohydrolysis (% of the activity at pH 6.0) for G6P (···●···) and βglyP (-○-) by an extract of the crustacean digestive gland in imidazole buffer (50 mM) between pH 6 and 8 and in citrate buffer (20 mM) between pH 5 and 3; *Curves 3 and 4:* pH-dependent phosphohydrolysis (% of the activity at pH 6.0) for G6P (···▲···) and βglyP (-△-) by an extract of the crustacean digestive gland in imidazole buffer (50 mM) between pH 6 and 8 and in acetate buffer (10 mM) between pH 4 and 5

hydrolysis of G6P (and of βglyP) is approximately 6, whereas they have different optimal pH (6.5 for G6P and 5.0 for βglyP) in the vertebrate liver. Also, the maximal activity of crustacean G6P-phosphohydrolase (only ± 0.3 units per g fresh tissue at pH 6) is at least 30 times less than that of liver G6Pase. In addition, when assayed in a 20 mM citrate medium at a pH below 6, the phosphohydrolytic activity of a crustacean extract on either βglyP or G6P is markedly increased (Fig. 2), whereas mouse liver G6Pase is inhibited (Nordlie and Lygre 1966). It can also be seen (Fig. 3) that the apparent K_m of each phosphohydrolase is similar (2.3 mM for G6P and 1.9 mM for βglyP), indicating no particular substrate preference.

The influence of physiological events associated with the moult cycle on calcospherite loading was assessed by following variations in Pi content of the total tissue (Fig. 4B), together with the development of G6P-phosphohydrolase activity during the same period (Fig. 4A). It might be expected that the succession of periods of starvation (from the end of the premoult up to the early postmoult) and feeding (almost all stages C, including the intermoult) would induce changes in the activity of the key enzyme controlling the release of glucose by the digestive gland. However, since these experiments were begun before it was observed that G6P-phosphohydrolase is mainly localized in calcospherites (see below), the results presented in Fig. 4B reflect only the microsomal activity which accounts for a maximum of 20% of the total activity. Nevertheless these preliminary results are of interest. Indeed, the inverse relationship between the development of the "microsomal" G6P-phosphohydrolase activity (Fig. 4A) and the total Pi concentration (Fig. 4B) suggests that the endoplasmic reticulum is mobilized for the formation of calcospherites (see Fig. 4A, B in which maximal enzymatic activity in the microsomes corresponds with a minimal amount of calcospherites).

Distribution of G6P-phosphohydrolase and acid phosphatase activities in subcellular fractions of the digestive gland

Before analysing the results, it is important to recall that the identification of a phosphatase activity in the subcellular fraction enriched in G requires a technical adaptation of the standard method (see Materials and methods). The distribution of some marker enzymes is presented in Fig. 5. As might be expected (see Figs. 1, 2, 3), both phosphatases exhibit a similar subcellular distribution (Fig. 5A, B), suggesting that they belong to a unique enzyme. They are also distributed similarly to Pi (Fig. 5D), which indicates the presence of G. Moreover, in a calcospherite-free tissue, crustacean G6P-phosphohydrolase (Table 1) follows the distribution of a liver G6Pase; by contrast the crustacean Acid Pase, since it is localized in microsomes, differs markedly from

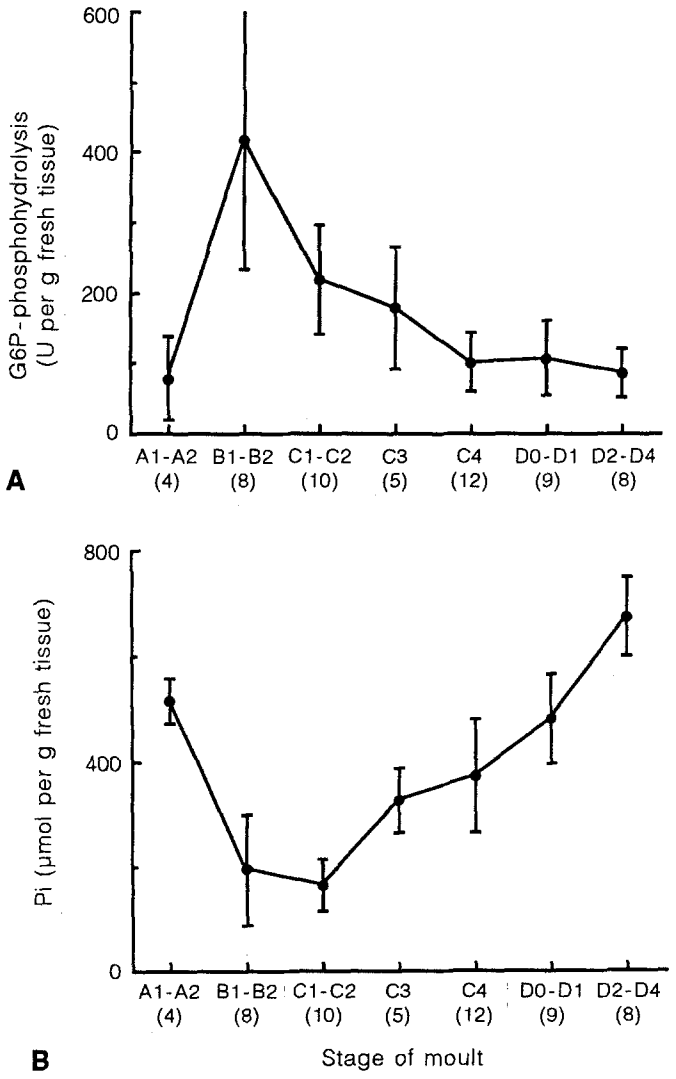

Fig. 4A, B. Total Pi (**A**) and microsomal G6P-phosphohydrolase (**B**) of *Carcinus* digestive gland during the moult cycle. Mean ± SE (*m*). Statistical analysis indicates the following: a negative correlation between these two biochemical parameters ($r=0.55$; $t=4.84$, $P<0.01$); G6P-phosphohydrolase of stages B_1–B_2 is significantly higher than at other stages (comparison by the contrasts of Scheffé, $P<0.01$); Pi at stages D is significantly higher, and at stages B and C_1–C_2 is significantly lower than at other stages (comparisons by the contrast of Scheffé, $P<0.01$)

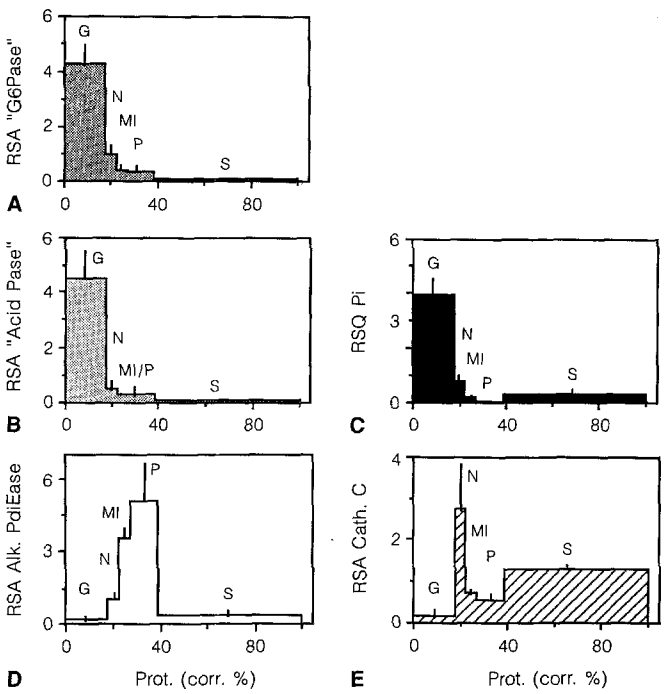

Fig. 5. Distributions of the relative specific activity (RSA) of (**A**), G6P-phosphohydrolase, (**B**) acid phosphatase, (**C**) alkaline phosphodiesterase and (**E**) cathepsin C in subcellular fractions of *Carcinus* digestive gland in comparison to the distribution of (**D**) inorganic phosphate expressed as its specific relative quantity (RSQ). Mean ± SE ($n=5$). The subcellular fractions, obtained from differential centrifugation, are: *G* (phosphate calcium granules, $200\times g$, 1.5 min); *N* (nuclei, $500\times g$, 10 min); *MI* (mitochondria and intermediate postmitochondrial particles, $23000\times g$, 5 min) and *P* (microsomes, $100000\times g$, 60 min). *S* (cytosol) is the supernatant from the last centrifugation

Table 1. Distributions of the relative specific activities (RSA) of G6P-phosphohydrolase (G6P), acid phosphatase (βglyP), alkaline phosphodiesterase (Alk. PdiEase) and cathepsin C (Cath. C) in subcellular fractions of a *Carcinus* digestive gland almost devoid of calcium phosphate granules (in postmoult)

	Enzymes			
Fraction	G6P	βglyP	Alk. PdiEase	Cath. C
G	n*	n*	n*	0.32
N	2.50	2.69	2.88	2.21
MI	2.46	2.27	1.60	0.82
P	4.39	4.47	5.33	0.24
S	0.41	0.47	0.44	1.12

n*: negligible values. The subcellular fractions are obtained as described in Fig. 5. G: calcium phosphate granules; N: nuclei; MI: a mixture of mitochondria and intermediate postmitochondrial particles; P: microsomes; S: cytosol

its liver analogue [mainly lysosomal; for review see Wattiaux (1971)]. The same unusual distribution pattern of Acid Pase has also been obtained with another calcospherite-poor tissue; the macruran digestive gland (Trausch et al. 1988). However, contamination of the microsomal fraction by lysosomes is unlikely since cathepsin C (another lysosomal enzyme) and Acid Pase are quite differently distributed.

General properties of glucose phosphorylation and its evolution during the moult cycle

Phosphorylation of glucose is assayed at two glucose concentrations: 0.5 m*M* [for the measurement of the "low K_m" (or hexokinase) activity and 50 m*M* (for the simultaneous measurement of glucokinase and hexokinase activities). The results obtained at both glucose concentrations were very similar, suggesting the absence of specific glucokinase (Fig. 6). The small fraction (± 5%) of the total activity which could be attributed to this enzyme is strongly reduced in the presence of G6P. In

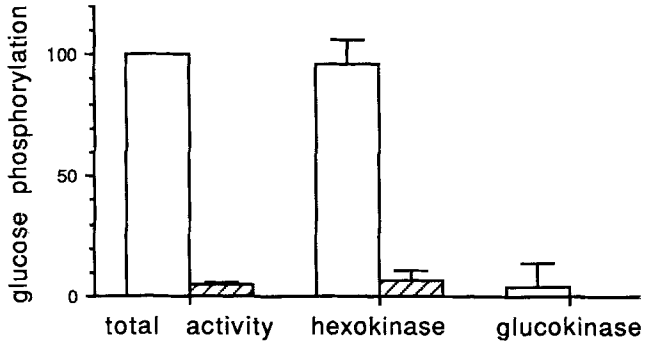

Fig. 6. Glucose phosphorylation by extract of *Carcinus* digestive gland at two concentrations of substrate: glucose 50 m*M* (total activity); glucose 0.5 m*M* (hexokinase activity), without G6P 10 m*M* (□) or with G6P 10 m*M* (▨). The activity of the specific glucokinase corresponds to the difference between the total and hexokinase activities. In each case, the results are expressed as percentage of the total activity measured at glucose 50 m*M* and without G6P. Mean ± SE ($n=4$).

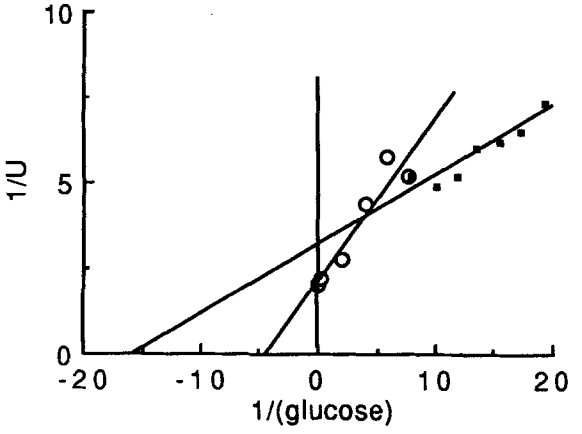

Fig. 7. Lineweaver-Burk plots of hexokinases activities (Ha: ○ and Hb: ●) in a tissue extract of *Carcinus* digestive gland.
Ha: $y = 2.0933 + 0.4908x$, $r = 0.92$; $K_m = 0.23$ m*M*;
Hb: $y = 3.166 + 0.2016x$, $r = 0.94$; $K_m = 0.064$ m*M*

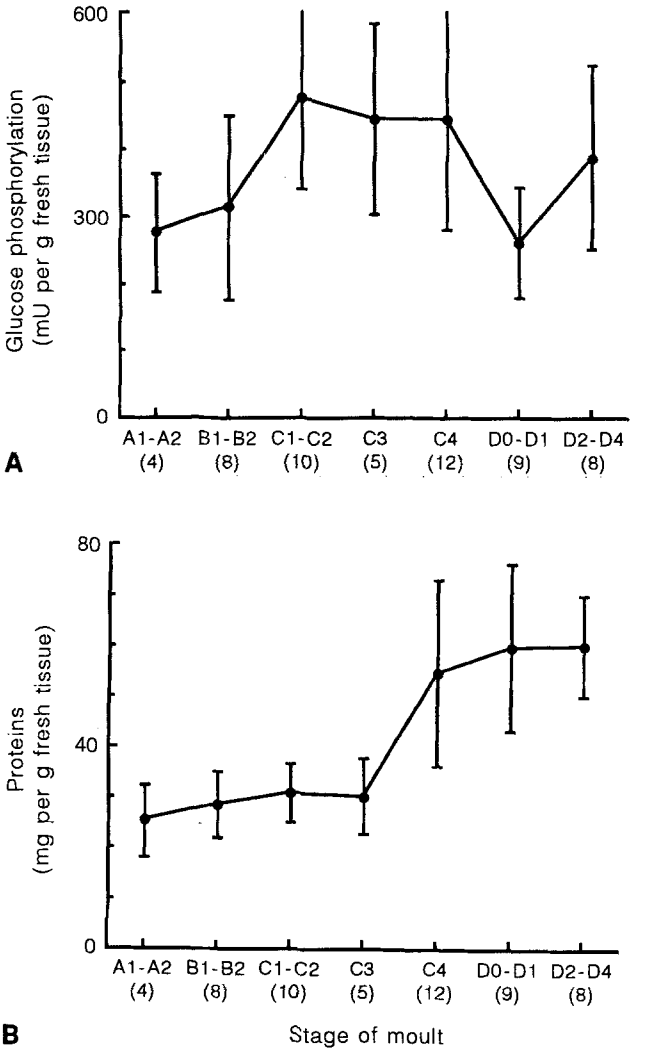

Fig. 8A, B. Development of (**A**) hexokinase activity and (**B**) protein content of *Carcinus* digestive gland during the moult cycle. Mean ± SE (n). Statistical analysis (contrasts of Student for hexokinase and contrasts of Scheffé for proteins) revealed the following: the glucose phosphorylating activity is significantly higher during stages C ($P < 0.05$), and the protein content increases significantly ($P < 0.01$) between the postmoult (stages A and B) and the premoult (stages D), which expresses a storage capacity of the gland during stages C

accordance with these observations, the saturation curve of hexokinase as a function of glucose concentration shows two components (Fig. 7) characterized by a low K_m (230 and 60 μ*M* respectively).

When measured at seven distinctive stages of the moult cycle (Fig. 8A) it appears that this aspecific phosphorylation of glucose is significantly higher at stages C, which correspond to the feeding period of the crab. This is visualized by the increase of protein content from stages C_1–C_2 up to the early premoult (stages D_0–D_1, Fig. 8B).

Discussion

G6Pase activity has previously been studied in invertebrates, principally crustaceans, insects and molluscs [for detailed references see Surholt and Newsholme (1981), Weirich and Adam (1984), Livingstone and Farrar (1984) and Viarengo et al. (1986)]. Unfortunately, in most cases the reports failed to establish whether the measured activity was due to a microsomal G6Pase or merely to an aspecific phosphohydrolase. Moreover, some descriptions of G6Pase reveal unusual features in comparison to the mammalian enzyme, i.e. a cytosolic localization and an insensitivity to citrate exposure (Surholt and Newsholme 1981; Weirich and Adams 1984). The criteria used for the characterization of the enzyme specificity often appear to be inadequate. Livingstone and Farrar (1984), for example, considered the enzyme as specific when the ratio of activity at pH 5.5: activity at pH 6.5 was less than 1. Taken alone, this criterion is not satisfactory, since it should recognize a specific G6Pase where we see an aspecific enzyme using biochemical techniques. In accordance with our observa-

tions on the microsomal enzyme, Viarengo et al. (1986) were unable to detect any specific G6Pase in the microsomes of the mussel digestive gland, whereas they found an aspecific phosphatase in this fraction.

The K_m of the G6P-phosphohydrolase for G6P reported here is close to values given for mammalian liver and pancreas (Beaufay and de Duve 1954; Giroix et al. 1985; Schultz 1988; Waddell and Burchell 1988). This similarity between crustacean G6P-phosphohydrolase and liver G6Pase for G6P suggests a similar key role for both enzymes in the regulation of glucose homeostasis. However, the very low total phosphohydrolase activity present in the digestive gland compared to the liver (at least 30 times lower), as well as the aspecificity of the activity in the crustacean microsomes, lead to the conclusion that the digestive gland is a poor homeostasis regulator. In fact, an efficient mechanism of glucose homeostasis seems to be unnecessary in crustaceans (as well as in other marine invertebrates) since they can tolerate large variations in blood glucose levels (Florkin 1960; Dean and Vernberg 1965; Parvathy 1972; Lynch and Webb 1973).

Despite the apparent aspecificity of *Carcinus* glucose phosphorylase, an adaptation of the glucose phosphorylation seems to occur during the moult stages of the crab, probably as a consequence of food availability. This feature, which is also a property of vertebrate liver glucokinase [for review see Niemeyer et al. (1975), Weinhouse (1976), Iynedjian et al. (1989) and Watford (1990)] but not of vertebrate hexokinase (Reyes et al. 1984), confers on the crustacean enzyme a glucokinase-like function and thereby a hepatic-like function on the digestive gland (i.e. the uptake and storage of glucose when carbohydrate level is high in the serum). In this respect, it is interesting to note that a similar kind of modulations has been observed in the mantle of *Mytilus edulis* (Livingstone and Clark 1983), attributed to two season-dependent sources of glucose: food intake and glycogen reserves.

In order to emphasize the subcellular localization of the G6P phosphohydrolase, a study of its distribution was undertaken. The major part of G6P phosphohydrolysis is associated with the calcium phosphate granules, indicating that aspecific properties described for this enzyme in this study (see Figs. 1, 2, 3) refer only to the microsomal enzyme. Whether these aspecific properties also apply to the activity associated with the calcospherites remains to be established; it is likely, since Acid Pase closely follows G6P-phosphohydrolase in distribution. However, this observation does not constitute an unequivocal demonstration and definitive conclusions require work on purified enzymes.

The surprising distribution of activity attributed to cathepsin C also needs to be considered in any interpretation of subcellular phosphohydrolase fractionation. Its cytosolic localization can be explained as follows: in the B cells (= secretory cells) of the digestive gland the proteolytic enzymes are enclosed in large vacuoles which are extruded at each digestive cycle (Gibson and Barker 1979). The homogenization process likely disrupts them so that their contents are recovered in the cytosol. Whether or not the residual activity of cathepsin C seen in N represents an analogue of pancreatic zymogen granules cosedimenting with the nuclei remains to be established.

The "granular" localization of both phosphatases is surprising. Indeed, in the mammalian liver Acid Pase is mainly associated with lysosomes while G6Pase is principally localized in microsomes [for review see Wattiaux (1971)]. In addition, it must be emphasized that with subcellular fractionation of a gland almost completely devoid of granules (i.e. an organ of a postmoult crab), both phosphohydrolase activities are due to a microsomal enzyme. In such a gland the localization of both activities agrees well with the distribution of alkaline phosphodiesterase, a microsomal enzyme (Table 1). Moreover, having obtained two types of subcellular distribution for both phosphatase avtivities [i.e. granular in calcospherite-rich and microsomal in calcospherite-poor digestive gland; see this study and Trausch et al. (1988)] and an inverse relationship between calcospherite content and microsomal G6P-phosphohydrolase activity during the moult cycle, we propose that calcium phosphate granules could arise from the endoplasmic reticulum. More precisely, they may have budded from the endoplasmic reticulum after its intraluminal overloading in calcium phosphate. In support of this assumption, Loret and Devos (1992) observed membranes probably originating from the endoplasmic reticulum surrounding calcospherites. It has also been reported that free Ca^{2+}, when applied at specific concentrations, can activate liver G6Pase (Yamagushi et al. 1989; Mithieux et al. 1990; Waddell and Burchell 1990).

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