Comp. Biochem. Physiol. Vol. 74B, No. 4, pp. 691–702, 1983
Printed in Great Britain
0305-0491/83/040691-12$03.00/0

SEASONAL CHANGES IN HEXOKINASE FROM THE MANTLE TISSUE OF THE COMMON MUSSEL *MYTILUS EDULIS* L.

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(*Received* 17 *August* 1982)

Abstract—1. Hexokinase from the mantle tissue of *M. edulis* was investigated over almost 2 years for changes in specific activity and in the kinetic characteristics of the glucose concentration vs initial velocity curves.

2. Specific activity changed markedly, peaking in late Spring.

3. Depending upon the time of the year and the sex of the mussel, the substrate versus velocity data were best fitted either to a Michaelis–Menten or a Hill model. The apparent K_m or $K_{0.5S}$ values changed seasonally, being low during the first part of the year and high for most of the rest of the year.

4. The changes in hexokinase are discussed in relation to the two possible seasonally-variable sources of glucose production, food intake and the utilization of stored glycogen.

INTRODUCTION

The metabolism of many marine invertebrates, particularly intertidal species, is seasonally variable. In the common mussel, *Mytilus edulis*, changes have been observed for most major areas of metabolism, both in terms of molecular levels e.g. glycogen, lipid, protein and free amino acids (Zandee *et al.*, 1980), nucleic acids (Thompson, 1972), total free sugars (Bayne, 1973), free glucose (Zaba, 1981), glycolytic intermediates (Ebberink and De Zwaan, 1980) and phosphoarginine (Zurburg & Ebberink, 1981) and in terms of rates of processes, e.g. nitrogen excretion (Bayne & Scullard, 1977), osmoregulation (Livingstone *et al.*, 1979), anaerobic metabolism (Ahmed & Chaplin, 1979; Zandee *et al.*, 1980), pentose phosphate pathway activity and glucose utilization (Zaba *et al.*, 1981). The changes are regular and often large over a seasonal cycle and must involve marked alterations in the direction and/or magnitude of the metabolic fluxes. As part of a study of the enzymatic mechanisms of the seasonal regulation of metabolism (see Livingstone, 1975, 1981), hexokinase (EC 2.7.1.1) was examined from the mantle tissues of *M. edulis*; this tissue functions both in metabolic storage and in reproduction.

Hexokinase catalyses the phosphorylation of glucose (and other sugars) to yield glucose-6-phosphate (the "mobilized" form of glucose) which can then enter the pathways of carbohydrate metabolism. Hexokinase has been shown to be a regulatory enzyme in certain tissues (Newsholme & Start, 1973) and its tissue specific activity under conditions of saturating substrate is taken as a measure of the maximum potential rate of tissue glucose utilization (Crabtree & Newsholme, 1972; Zammit & Newsholme, 1976). Isoenzymes of hexokinase exist throughout the animal kingdom and in certain organisms and tissues respond differentially to factors such as nutritional intake (Ureta, 1975). The hexokinase reaction of the mantle tissue of *M. edulis* is far displaced from equilibrium (Zaba, 1981), indicating a regulatory enzyme and given the seasonal nature of carbohydrate metabolism and glucose utilization in the tissue, seasonal changes in the enzyme might be expected. In this study hexokinase was examined from the mantle of male and female mussels over a period of 2 years. Measurements carried out were tissue specific activity at high (saturating) and low concentrations of glucose, the apparent Michaelis constant (K_m) or $K_{0.5S}$ values for glucose and the effect of the inhibitor *N*-acetylglucosamine on enzyme activity. Haemolymph and tissue glucose concentrations and soluble protein were also determined.

MATERIALS AND METHODS

Chemicals and commercial enzymes

D-glucose, *N*-acetyl-D-glucosamine, NAD^+, ATP, $NADP^+$ (from Torula yeast) and NAD^+ (from *Leuconostoc mesenteroides*) dependent glucose-6-phosphate dehydrogenases (EC 1.1.1.49) and hexokinase (from yeast) were obtained from the Sigma Chemical Co. (London) Limited. Uranyl acetate solution and the GOD/PERID diagnostic kit were obtained from the Boehringer Corporation (London) Limited and the Sephadex G-25 PD-10 columns from Pharmacia (Great Britain) Limited. All other chemicals were obtained from BDH Limited, UK.

Animals and sample collection

Mussels of uniform size (5–5.5 cm in length) were collected at low tide from the estuary of the River Erme near Plymouth (Mothecombe Bay) at approx 7-week intervals. About 50 animals were taken and treated as follows: 8 mussels were immediately sampled on the shore for haemolymph and tissue glucose concentrations and 8–12 mussels were returned to the laboratory and used immediately for the determination of hexokinase specific activity, *N*-acetylglucosamine inhibition and total soluble protein; the remaining mussels were returned to the laboratory and either used immediately or after being kept overnight in a

system of recirculating ambient seawater (see Bayne & Thompson, 1970 for details of the system) for the determination of the substrate concentration vs initial velocity characteristics of hexokinase.

Haemolymph and tissue glucose concentration

A sample of blood was taken from the posterior adductor muscle sinus of each of 8 mussels by hypodermic syringe, deproteinized by the addition of 1 ml of uranyl acetate to 100 μl of blood and placed on ice. The two mantle lobes of each of the 8 mussels were also dissected out: one lobe was immediately frozen and retained in liquid-nitrogen and the other lobe was damp-dried and placed on ice. All samples were then returned to the laboratory.

The deproteinized blood samples were clarified by centrifugation at 3000 *g* for 10 min at 5°C and the resulting supernatants used for the determination of haemolymph glucose by the glucose oxidase method using the GOD PERID Boehringer diagnostic kit. The frozen mantle lobes were either stored at −70°C or analysed immediately. The glucose metabolite was extracted in 6% perchloric acid using the procedure described in Livingstone *et al.* (1981) and assayed by the hexokinase/glucose-6-phosphate dehydrogenase method (Bergmeyer *et al.*, 1974). The assay conditions were as described for the mantle hexokinase assays (see later) except that glucose was omitted, $NADP^+$ replaced NAD^+, $NADP^+$-dependent glucose-6-phosphate dehydrogenase replaced the NAD^+-dependent enzyme and excess commercial hexokinase was added.

Internal standards were added and the percentage conversion exceeded 80%. The second mantle lobe of each mussel was used for sex-determination (where possible) by examining a smear of the tissue under a light microscope for the presence of sperm or oocytes.

Tissue hexokinase specific activity, N-acetylglucosamine inhibition and protein determination

The sexes of 8–12 mussels were established and their mantle tissues then excised, damp-dried on filter-paper and placed on ice. All subsequent procedures were carried out at 4°C. The mantle tissues of individual mussels (4 males and 4 females or 8 indeterminates depending on the time of the year) were weighed and homogenized in 10 vol (w/v) of 10 mM Tris–HCl pH 7.6 containing 1 mM EDTA, 1 mM dithiothreitol, 4 mM $MgSO_4$, 0.15 M KCl and 0.5 M sucrose using an Ultra-Turrax homogeniser. The homogenates were centrifuged at 10,000 *g* for 15 min and the resulting supernatants centrifuged at 50,000 *g* for 30 min. Aliquots of the high-spin supernatants were taken for the determination of protein. A second aliquot was passed down small Sephadex G-25 columns (Pharmacia PD 10 columns, bed volume 9 ml; equilibration and elution buffer were the same as the homogenisation buffer but with sucrose omitted) to remove the low molecular weight fraction (mol. wt 5000 and below) which previously had been found to inhibit the hexokinase assay. The high molecular weight fraction eluted from the columns was immediately used as the source of enzyme.

Hexokinase was assayed spectrophotometrically at 334 nm by a modification of the method of Crane & Sols (1955). Glucose-6-phosphate production was measured by coupling the reaction to the reduction of NAD^+ to $NADH^+$ using NAD^+-dependent glucose-6-phosphate dehydrogenase. NAD^+ was used instead of $NADP^+$ to minimise interference from endogenous glucose dehydrogenase (EC 1.1.1.47) (the glucose dehydrogenase catalyses a reaction between glucose and $NADP^+$ but shows little activity with NAD^+—Livingstone, unpublished data). The optimal assay conditions were determined in preliminary experiments and the assay contained, in a final volume of 1.0 ml, 100 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 1 mM glutathione, 5 mM ATP, 0.2 mM NAD^+, 1 unit of glucose-6-phosphate dehydrogenase and varying concentrations of glucose. An aliquot of the sample and all the reagents except ATP were preincubated for 5 min at 25°C. The background rate (when present) was measured and the reaction proper was then initiated by the addition of ATP and followed at 25°C. The rates were linear for several minutes and under the conditions of the assay there was no detectable interference from endogenous phosphogluconate dehydrogenase (EC 1.1.1.43), ATPase (EC 3.6.1.3) and glucose-6-phosphatase (EC 3.1.3.9). Hexokinase activity was determined in duplicate for each sample in the presence of the following concentrations of substrate and inhibitor: (a) 20 mM glucose; (b) 0.5 mM glucose; (c) 20 mM glucose in the presence of 1 mM *N*-acetylglucosamine and (d) 0.5 mM glucose in the presence of 1 mM *N*-acetylglucosamine. Two concentrations of glucose were used to give an indication of the presence of a glucokinase-type enzyme (EC 2.7.1.2) (Viñuela *et al.*, 1963); 20 mM glucose was chosen because substrate inhibition was occasionally observed at higher concentrations. Similarly, the effect of *N*-acetylglucosamine was examined because it is a strong inhibitor of glucokinase but has a much reduced effect on hexokinase (Newsholme & Start, 1973). Soluble protein was determined by the method of Lowry *et al.* (1951).

Hexokinase glucose concentration vs initial velocity characteristics

The procedures employed for sample preparation were different for male and female mussels (indeterminates were treated as for females). The Sephadex G-25 treated high-spin supernatants of male mussels, when kept on ice, showed no change in apparent $K_{0.5S}$ for glucose for up to a day at least. In contrast, the extracts of female mussels were stable for a few hours but then an increase in this parameter (or in apparent K_m) was observed: the increase was unaffected by the presence of 2 mM of the protease inhibitor phenylmethylsulfonylfluoride. In consequence, whereas the male samples were prepared simultaneously and then subsequently analysed, the female samples were prepared individually and analysed within 1 hr. On each seasonal sampling occasion, with minor variations (see Fig. 3), 4 male and 4 female samples or 4 indeterminates were prepared, each sample containing the pooled mantles of 4 mussels. The extraction procedure was the same as for the determination of specific activity. The assay conditions were also the same except that 10 concentrations of glucose (0.01–60 mM) were employed in duplicate to obtain the substrate concentration vs velocity data. The latter were tested as to whether they best fitted a Michaelis-Menten or a Hill kinetic model (see Appendix for the details of the fitting procedure) and the apparent K_m or $K_{0.5S}$ values for glucose were derived. An example of the (s, v) (substrate concentration vs velocity) results for two contrasting samples is given in Fig. 1.

Statistical treatment of seasonal data

The seasonal data were analysed by one-way analysis of variance between two groups of values or for several groups of values over time. A probability of 0.05 or less was accepted as significant. Mean values, where quoted, are expressed as ± SEM.

RESULTS

Seasonal changes in haemolymph and mantle tissue glucose concentrations

Haemolymph glucose concentration was highest during the Summer, from August to October and lowest in the two Winter periods (Fig. 2C). No significant differences between sexes were observed. The mean Summer concentration was 1.48 ± 0.15 mM (n = 16)

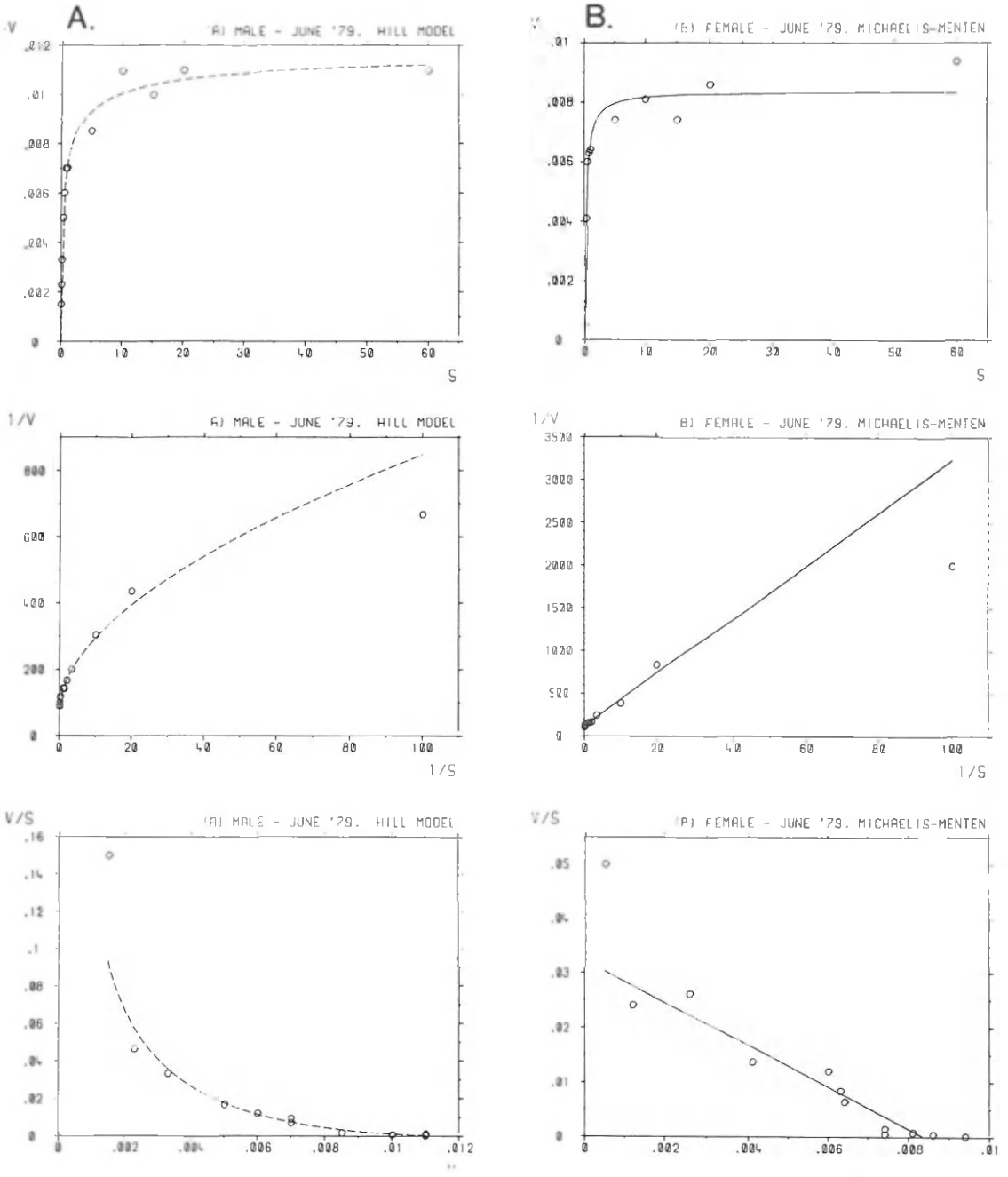

Fig. 1. Computer plots of velocity (v) vs glucose concentrations (s), $1/v$ vs $1/s$ (Lineweaver–Burk plot) and v/s vs v (Eadie–Scatchard plot) (see Appendix, Table 1) for Hill fitted (A) and Michaelis-Menten fitted (B) models of hexokinase of the mantle of *M. edulis*. (A) male sample collected June 1979, (B) female sample collected June 1979.

compared with 0.71 ± 0.04 mM ($n = 64$) for the rest of the time; the lowest sample value recorded (December, 1978) was 0.34 ± 0.10 mM ($n = 8$). Greater variability was seen for mantle glucose concentrations (Fig. 2D). The highest concentrations occurred in Winter at the beginning of 1979 and subsequently declined to a minimum value in early Summer. A similar trend was observed at the beginning of 1980 although the changes were smaller and not statistically significant and the minimum occurred earlier. Significant differences between the sexes occurred at certain times of the year, the concentrations being higher in females. Using pooled data for various times of the year, the minimum and maximum concentrations were, respectively, 0.27 ± 0.05 ($n = 21$) and 1.54 ± 0.32 ($n = 15$) μmol/g fresh weight with the value for the rest of the time being 0.69 ± 0.09 ($n = 30$).

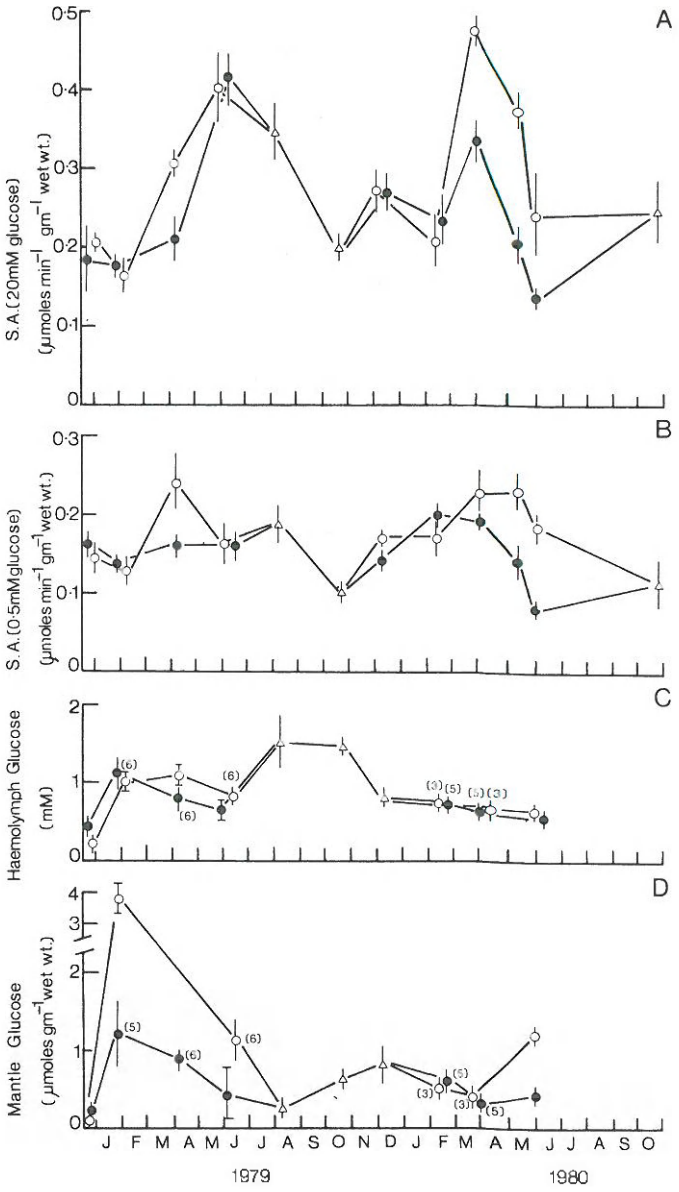

Fig. 2. Seasonal changes in the specific activity (μmol/min/g wet wt) of hexokinase measured at 20 mM (A) and 0.5 mM (B) glucose and in the concentration of glucose in the haemolymph (C) and mantle tissue (D) of *M. edulis*. ●: male, ○: female, △: indeterminate; values are means ± SEM, $n = 4$ (sexed samples) or 8 (indeterminates) unless indicated otherwise, bar lines = range where 2 values only.

Seasonal changes in hexokinase specific activity, total soluble protein and N-*acetylglucosamine inhibition*

Marked changes occurred in specific activity under conditions of saturating substrate (20 mM glucose) and in terms of per gram wet weight (Fig. 2A). Activity increased during the Spring and declined during the Summer with generally low values in Autumn and Winter. The maximum specific activity was earlier in 1980 than in 1979 i.e. March/April compared with May/June. Significant differences were seen between the sexes with higher values for female mussels during the Spring. The changes in specific activity at 0.5 mM glucose were less marked but in some respects similar to those at 20 mM glucose, at least in female mussels, with maximum values tending to occur in Spring and minimum values in Autumn (Fig. 2B). The values for female mussels were similarly higher than those for males in the Spring. Total soluble protein was generally lower in males than females, as has before observed before (Livingstone, 1981), with the result that specific activity in terms of per mg protein was either the same in the sexes or higher in males than in females (Table 1). Despite some variation in protein over time, the pattern of changes in specific activity was essentially similar to that expressed in terms of

Table 1. Seasonal changes in total soluble protein and the specific activity (S.A.) (in terms of nmol/min mg protein), *N*-acetylglucosamine (NAG) inhibition (in terms of the percentage activity of the control) and Hill coefficient of hexokinase of the mantle tissue

Date	Soluble protein (mg gm^{-1} wet wt)	S.A. (20 mM glucose)	S.A. (0.5 mM glucose)	NAG inhibition (20 mM glucose	(0.5 mM glucose)	Hill coefficient
December 1978	73.3 ± 3.3	2.84 ± 0.11	1.99 ± 0.34	–	–	–
	67.3 ± 1.8	2.89 ± 0.55	2.41 ± 0.27	–	–	–
January 1979	64.3 ± 9.3	2.56 ± 0.39	2.01 ± 0.32	67.8 ± 6.6	34.3 ± 7.0	–
	37.6 ± 4.7	4.68 ± 0.31	3.68 ± 0.34	87.2 ± 5.4	51.4 ±11.4	0.63 (0.74, 0.51) (n = 2)
April 1979	67.6 ± 8.8	4.55 ± 0.21	3.56 ± 0.63	80.4 ± 7.1	83.8 ±12.3	M.M.
	23.2 ± 1.2	9.28 ± 1.22	7.05 ± 0.44	90.8 ± 5.4	87.7 ± 8.0	0.57 (0.71, 0.42) (n = 2)
June 1979	–	–	–	94.6 ± 3.2	86.7 ± 8.1	M.M.
	–	–	–	90.6 ± 5.5	93.8 ± 6.2	0.59 ± 0.03
August 1979	49.1 ± 3.2(8)	7.03 ± 0.81(8)	3.96 ± 0.50(8)	90.5 ± 4.7(8)	69.8 ± 6.0(8)	0.51 ± 0.05
October 1979	68.4 ± 3.0(8)	2.91 ± 0.28(8)	1.48 ± 0.15(8)	97.8 ± 2.2(8)	93.1 ± 2.1(8)	0.49 ± 0.04
December 1979	26.5 ± 2.1	10.40 ± 0.94	6.46 ± 0.25	97.7 ± 2.3	54.7 ±20.4	0.39 ± 0.06
	26.9 ± 3.1	10.20 ± 0.76	5.35 ± 0.65	94.9 ± 3.2	67.1 ± 4.1	0.53 ± 0.06
February 1980	66.0 ± 3.4	3.17 ± 0.22	2.57 ± 0.30	100 ± 0	74.0 ±12.5	M.M.
	36.5 ± 1.8	6.41 ± 0.42	3.07 ± 0.21	99.2 ± 0.9	83.4 ± 1.1	0.59 ± 0.11
April 1980	51.5 ± 3.3	9.39 ± 0.79	4.41 ± 0.66	87.2 ± 4.6	95.8 ± 4.2	M.M.
	30.8 ± 3.3	10.91 ± 0.91	6.16 ± 0.18	100 ± 0.18	85.3 ±14.8	0.54 ± 0.09
June 1980	85.2 ±10.1	2.86 ± 0.71	2.18 ± 0.24	93.1 ± 4.2	91.6 ± 4.2	0.37 ± 0.09
	32.0 ± 1.4	4.35 ± 0.37	2.49 ± 0.22	93.1 ± 4.6	86.2 ± 6.3	0.46 ± 0.04
October 1980	–	–	–	–	–	0.51 ± 0.03

Values are means ± SEM; $n = 4$ unless stated otherwise in brackets; where two values are given the upper is for females and the lower for males; single values are for indeterminates and M.M. signifies the enzyme shows Michaelis–Menten kinetics; —, not determined.

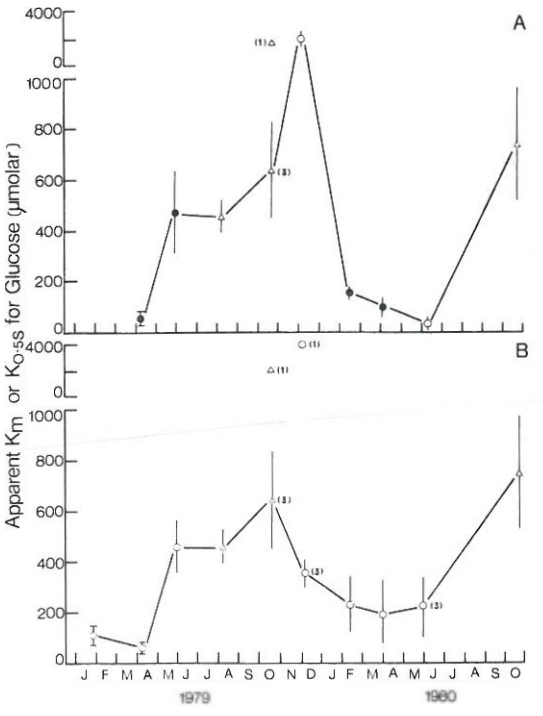

Fig. 3. Seasonal changes in apparent K_m (●) and $K_{0.5S}$ (○) for glucose of hexokinase of the mantle of *M. edulis*. (A) female mussels, (B) male mussels, △: indeterminates (indeterminates are all $K_{0.5S}$). Values are means ± SEM and $n = 4$ unless indicated otherwise, bar lines = range where 2 values only; on occasions high values have been noted individually.

wet weight. Inhibition of hexokinase activity by *N*-acetylglucosamine was low at 20 mM glucose but greater at 0.5 mM glucose: the latter was constant throughout the 2 years with the exception of significantly lower values (greater inhibition) in January 1979 and December 1979 (Table 1). Inhibition at 0.5 mM glucose tended to be greater in females than males but was not statistically significant.

Seasonal changes in the apparent K_m *and* $K_{0.5S}$ *for glucose of hexokinase*

Analysis of the seasonal data for the Hill or Michaelis–Menten model as best fit revealed a complicated picture. Whereas the hexokinase of male and indeterminate mussels always showed Hill-type kinetics with respect to glucose as substrate with Hill coefficients of about 0.5, the enzyme of female mussels varied showing mainly Michaelis–Menten kinetics but occasionally Hill-type kinetics; the latter did not correlate with any particular time of the year or magnitude of $K_{0.5S}$ for glucose (Table 1 and Fig. 3). A detailed analysis of the substrate concentration versus velocity curves also revealed that in addition to the two types of curves typical of the Michaelis–Menten and Hill models (Figs 4A and B), a third type of curve was evident which was characterized by a rapid increase in velocity between about 1 and 10 mM glucose and substrate inhibition at higher concentrations of glucose (Fig. 4C). Fitted to a Hill model with the substrate inhibition removed, i.e. the inhibited velocities were set equal to the observed maximum velocity, the third type of curve had low Hill coefficients of 0.3–0.4 and apparent $K_{0.5S}$ values for glucose between about 400 and 3.4 mmolar. This type of curve was shown by 12 out of the 58 samples in the seasonal study, including male, female and indeterminate samples, and the only consistent feature was that they were absent during January to March when apparent $K_{0.5S}$ or K_m values were low. The overall seasonal picture was as follows: The apparent $K_{0.5S}$ or K_m values of both sexes were low during the beginning of the year and high for the rest of the year, particularly in October and November. A sex difference was observed in December 1980, with the $K_{0.5S}$ value of males declining and that of the females increasing to a very high value (Fig. 3). In contrast to the increases in specific activity at 20 mM glucose which occurred earlier in 1980 than in 1979 (Fig. 2A), the reverse was observed for the increases in apparent $K_{0.5S}$ or K_m (compare May/June 1979 and 1980).

DISCUSSION

The haemolymph glucose concentrations were of the same order as for the freshwater bivalves *Anodonta cygnea* and *Unio pictorum* (Plisetskaya *et al.*, 1978) and the increase in levels in the Summer has also been observed for the total carbohydrate glucose equivalents of the haemolymph of *M. edulis* (Mulvey & Feng, 1981). The concentrations and decreases dur-

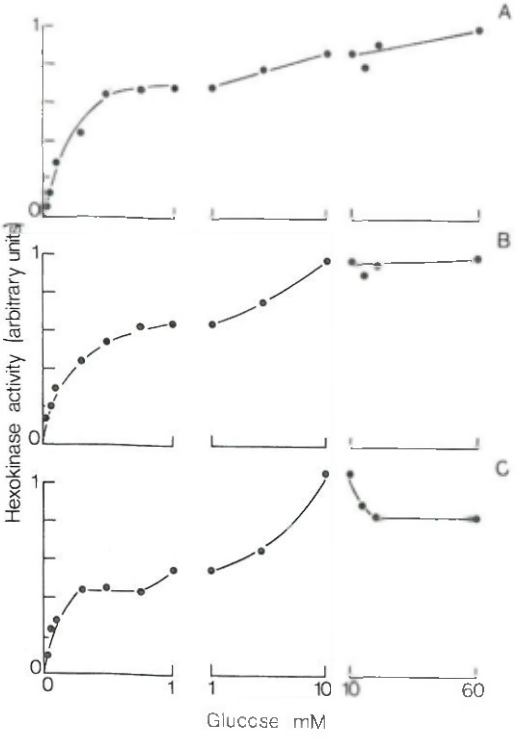

Fig. 4. Changes in hexokinase activity (arbitrary units) with glucose concentration. (A) female sample collected June 1979 (Michaelis–Menten kinetics); (B) male sample collected June 1979 (Hill-type kinetics); (C) female sample collected December 1979.

ing January to March of the tissue glucose concentration of male mussels were similar to those recorded by Zaba (1981) on the same population of animals. Information on the activity of hexokinase in bivalves is limited with the exception of muscular tissues (Zammit & Newsholme, 1976) where the specific activities in μmol/g wet wt were similar to those recorded in this study; the specific activity of hexokinase of the mantle tissue is similar to that of the gills kidney and digestive gland of *M. edulis* but about twice that of the posterior adductor muscle, at least at certain times of the year (Ebberink & De Zwaan, 1980; Widdows *et al.*, 1982). The specific activity of hexokinase of the hepatopancreas of *Mytilus californianus* (Bennett & Nakada, 1968), expressed in terms of per milligram protein, was an order of magnitude higher than that observed for the mantle of *M. edulis*.

Rates of glucose uptake and utilization by mantle preparations of *M. edulis* have been determined by several methods. Zaba & Davies (1980) and Zaba *et al.* (1981) measured ^3H_2O production from 2-^{3}H-glucose and also the incorporation of uniformly- and positionally-labelled ^{14}C-glucose into glycogen, amino acids, organic acids and lipid and obtained rates of approx 0.01–0.03 μmol/min/g wet wt. Madar *et al.* (1979) measured the rate of removal of glucose from the medium into the tissue and obtained rates of approx 0.04–0.05 μmol/min/g wet wt. The temperature and glucose concentrations of the medium were about 20°C and 5 mM for both studies. The measured rates of glucose utilization are therefore less than the specific activities of hexokinase but given the differences in assay conditions and the possibility of modulators affecting enzyme activity *in vivo*, the disagreement is not unreasonable. Rates of glucose utilization in the Spring were higher in the mantles of female than of male mussels (Zaba & Davies, 1980), correlating with the observed higher specific activities of hexokinase of females at that time of the year.

The seasonal alterations in certain properties of hexokinase were marked. The major changes were that specific activity at 20 mM glucose (= maximal specific activity at saturating substrate) increased during the early part of the year reaching a maximum in middle or late Spring and subsequently declined and that the apparent K_m or $K_{0.5S}$ values for glucose were low during the Winter and early Spring and high for most of the rest of the year. Two possible sources of seasonally-variable glucose production appear evident to which the enzyme changes could relate. One is food ingestion which is at a maximum in Summer and Autumn (Widdows *et al.*, 1979) and presumably results in the higher haemolymph glucose concentrations; the other is the suggested breakdown of endogenous glycogen stores by hydrolytic mechanisms giving rise to free glucose (Bayne *et al.*, 1982). The latter pathway occurs in other organisms where it either complements or replaces the phosphorylase enzyme system e.g. respectively, the eggs of various sea urchin species (Hino *et al.*, 1978) and the liver of the carp *Cyprinus carpio* (Murat, 1976). Although there is no direct biochemical evidence for the existence of this pathway in the mantle of *M. edulis*, the necessary enzymes such as amyloglucosidase (E.C. 3.2.1.3) are present (Zaba, 1981) and histochemical, ultrastructural and physiological observations strongly indicate that it could be operational during Winter and Spring when the glycogen stores are being rapidly utilized for the generation of maintenance energy and the synthesis of gametes (Bayne *et al.*, 1982); the high tissue levels of glucose in the early Winter and their subsequent decline (Fig. 2D) possibly support this suggestion.

Against this background an interpretation of the enzyme changes is possible. The timing of the increase in maximal specific activity and the parallel decrease in tissue glucose concentration seem to indicate that these enzyme changes are involved in, or are a response to, endogenous glycogen breakdown. The peaking of maximal specific activity coincides with the maximum observed rates of mantle glucose utilization (see Zaba *et al.*, 1981) and both events occur before the Summer increases in haemolymph glucose concentrations; this was particularly evident in 1980 when maximal specific activity declined to a low value before any increase in haemolymph glucose had occurred (Figs 2A and C). In contrast, the apparent K_m and $K_{0.5S}$ values were high for most of the rest of the year and the highest values coincided with low values of maximal specific activity, possibly suggesting a role for these changes in glucose uptake from the haemolymph. That is, the enzymes in the Summer and Autumn, with high apparent K_m and $K_{0.5S}$ values, would be less easily saturated with substrate and would therefore be poised to "take-up" any sudden influxes of glucose that might result from a variable food intake, a situation similar to that of the role of glucokinase in mammalian liver (see Newsholme & Start, 1973). The actual turnover of mantle tissue glucose from the late Summer onwards, however, is indicated to be low relative to Spring-time (as was observed by Zaba *et al.*, 1981) by the observations of low maximal specific activities, low tissue glucose concentrations and high apparent K_m and $K_{0.5S}$ values. The possible role of the high K_m and $K_{0.5S}$ enzymes in the endogenous glycogen breakdown is not clear. There was an overlap of the high values with high maximal specific activities in 1979 but in 1980 the increase and decrease in the latter were clearly separated from the subsequent increase in K_m and $K_{0.5S}$ (Figs 2A and 4). The interpretation of all the enzyme changes, however, is speculative: for example, increases in steady-state substrate concentrations need not necessarily reflect increased turnover-rates.

Little can be said regarding the molecular basis of the enzyme changes. However, assuming that the Sephadex G-25 treatment was effective in removing the low molecular weight fraction, it is clear that molecular variants of mantle hexokinase are produced at different times of the year; also judging from the complex velocity versus substrate concentration curves (Fig. 4C), molecular variants may also occur at the same time of the year. The production of isoenzymes of hexokinase in response to environmental or biological factors is common in most living organisms; e.g. in animals (Ureta, 1975), the fungus *Neurospora crassa* (Lagos & Ureta, 1980), the bacterium *Streptococcus mutans* (Potter *et al.*, 1980) and the yeast *Saccharomyces cerevisiae* (Muratsubaki & Katsume, 1979). In vertebrates, the isoenzymes of hexokinase are of two main types, either having low apparent K_m values for glucose of 0.01–0.1 mM and

broad sugar specificity or high apparent K_m values of 1–10 mM and high specificity for glucose (this isoenzyme is glucokinase) (Newsholme & Start, 1973). An indication of the presence of the two types of isoenzyme in vertebrates is therefore given by the ratio of hexokinase activity at high and low glucose concentrations (usually 100 and 0.5 mM) (Viñuela *et al.*, 1963). This approach probably has only limited application for *M. edulis* because the range of apparent K_m or $K_{0.5S}$ values is less but nevertheless it is interesting that the seasonally-variable ratios for mussels (seasonal range: 1.20–2.61; calculated from Figs 2A and B) span the range between the ratios for vertebrates possessing a glucokinase (lower reptiles: 2.63 ± 0.40; amphibia 2.80 ± 0.31) and those lacking the isoenzyme (higher reptiles: 1.13 ± 0.06; birds: 1.18 ± 0.04) (calculated from the data of Ureta *et al.*, 1973, 1975, 1978). The properties of a number of invertebrate hexokinases differ significantly from those of the rat liver enzymes (e.g. see Komuniecki & Robers, 1977; Stetten & Goldsmith, 1981) and the same is indicated for *M. edulis* e.g. *N*-acetylglucosamine inhibition was greatest for the low apparent $K_{0.5S}$ enzyme of the mantle [male mussels (January 1979)—Table 1 and Fig. 3B] while the reverse is seen for the rat liver isoenzymes (Newsholme & Start, 1973).

Different kinetic characteristics with respect to glucose are also observed for various hexokinases, varying from Michaelis–Menten behaviour for most enzymes to positive co-operativity of binding for the glucokinase of rat liver (Storer & Cornish-Bowden, 1976) and negative co-operativity of binding for type L_1 hexokinase of wheat-germ (Meunier *et al.*, 1974) and hexokinase A of yeast (Williams & Jones, 1976). The occurrence of both Michaelis–Menten behaviour and an apparent negative cooperativity (Hill coefficients less than one) for the hexokinase of female mussels is perhaps surprising and possibly should be viewed with caution regarding the limited stability of the extracted enzyme (see Materials and Methods). The negatively cooperative Hill plots, however, were consistent observed for males and for mussels of indeterminate sex and represent an interesting phenomenon indicating that at times of the year and in the absence of any other regulatory factors, the mantle will tolerate tissue glucose concentration fluctuations over a certain range but above an upper concentration the hexokinase activity will greatly increase and the enzyme will function to reduce the glucose concentration (also see Fig. 4C). Comparison of the values for tissue glucose concentration and the apparent K_m and $K_{0.5S}$ give a reasonable picture for the Summer and Autumn with ratios of the former to the latter of 0.4–2.3 (calculated from Figs 2D and 3). In contrast, in Winter and early Spring glucose concentrations were very high and ratio values in excess of 10 are obtained. Again some caution may be required with this result as Wijsman & Maaskant (1982) have recently identified methodological problems in measuring glucose concentrations in tissues which contain active hydrolytic enzyme systems; however, the techniques used in this study and the study of Zaba (1981) should have been adequate to prevent any interference in the glucose assay.

The changes in mantle hexokinase of *M. edulis* are another example of seasonal alterations in the kinetic characteristics of a regulatory enzyme linked to an observed or indicated change in metabolic flux. Hexokinase can be added to the list of pyruvate kinase (EC 2.7.1.40) (Livingstone, 1975), glucose-6-phosphate dehydrogenase (EC 1.1.1.4) (Livingstone, 1981) and glycogen synthetase (EC 2.4.1.11) (Gabbott *et al.*, 1979) as seasonally variable regulatory enzymes, adding strength to the argument that such changes are an integral part of seasonal metabolic regulation (Livingstone, 1981). The changes in hexokinase are particularly interesting because they were different at the two times of the year when an increased glucose production is anticipated. The differences presumably relate to the fact that while the increased glucose production of the Spring is probably endogenously determined, that of the Summer and Autumn is dependent on the environment.

Acknowledgements—P. Fieth is thanked for technical assistance and Dr B. L. Bayne is gratefully acknowledged for critically reading the manuscript. This work forms part of the estuarine and nearshore research programme of the Institute for Marine Environmental Research. It was supported in part by the Department of the Environment (Contract No. DGR/480/47).

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APPENDIX

The Michaelis–Menten (1) and Hill (2) models, for fitting to the experimental data on velocity (v) vs substrate concentration (s), are parameterised in the form:

$$(1)\ v = Vs/(K + s)$$

$$(2)\ v = Vs^n/(K^n + s^n)$$

where V denotes the asymptote of the curve and K is the value of s at which $v = V/2$. For the Hill model, the Hill coefficient (n) is assumed to be a continuous unknown parameter; for $n = 1$, the Hill model reduces to the Michaelis–Menten model and values of n greater than or less than one indicate positive and negative co-operativity, respectively.

Though various linearizing transformations of models (1) and (2) are possible (see Appendix, Table 1), it is well known that these are not satisfactory for model fitting by simple linear regression (e.g. Colquhoun, 1971). The direct linear plot, described for Michaelis–Menten fitting by Cornish-Bowden & Eisenthal (1978), is a more attractive technique but it cannot be extended to the 3-parameter Hill model and, even if another robust method were to be used, no framework would exist for testing the adequacy of model (1) compared with model (2). The most satisfactory solution to the estimation of the two models is—conceptually—the simplest one, namely to fit the non-linear relations directly using a non-linear least squares regression algorithm. For example, in model (2), the unknown parameters K, V and n are chosen to minimise the sum of squares

$$S = \sum_i (v_i - Vs_i^n/(K^n + s_i^n))^2$$

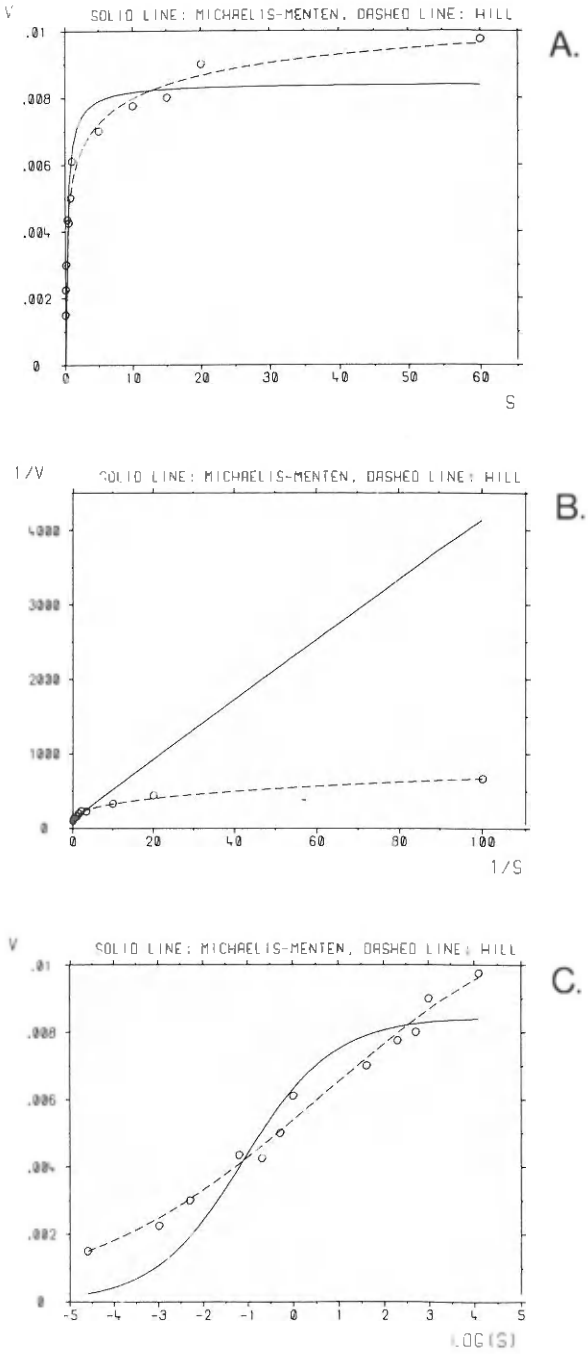
where (s_i, v_i) denotes the ith data pair. The minimisation is performed iteratively by computer program. In our studies we used the modified Marquadt algorithm discussed by Nash (1979), which combines Newton and steepest descent methods, with derivatives estimated numerically. This sum of squares, S, is appropriate to unweighted least squares but replication of s_i values for the experimental determination indicates that a constant variance assumption for v_i is adequate here (there is no difficulty of principle in extending the estimation to non-constant variance cases, using weighted non-linear least squares). Using these procedures, large sample maximum likelihood theory also provides approximate standard errors for the 2 (or 3) model parameters estimated. In particular, if the resulting approximate 95% confidence interval for n in model (2) contains the value $n = 1$, then it can be concluded that the Hill model does not provide a significantly better fit to the data than the Michaelis–Menten kinetics.

Although it is desirable to fit the models on the untransformed (s, v) scales, the various possible transformations to linearity still serve two important functions. Firstly, the iterative non-linear estimation algorithm requires starting values for the parameters which can be provided by the transformation procedures. Thus, for model (1), estimates obtained from the simple linear regression of $1/v$ on $1/s$ (Lineweaver–Burk plot) provide starting values for the iteration. Although these values may often be poor, the modified Marquadt algorithm is quite robust in this case, converging correctly even from very inaccurate starts. Similarly, for model (2), a regression of $\log [v/(V-v)]$ on $\log s$ (Hill plot) provides initial estimates for n and K where the starting V is taken as 1.1 times the largest observation. Both starting procedures can be fully automated, so the whole fitting sequence can be incorporated into a single computer program which then requires no input additional to the original (s, v) data pairs.

The second important use of the transformations is in graphical checking of the adequacy of fit of the estimated

Appendix, Table 1. Equations for Michaelis–Menten and Hill models, under four possible transformations used in graphical assessment of model fit

Transformation name	Transformed axes		Model equations	
	x	y	Michaelis–Menten	Hill
–	s	v	$y = V x / (K + x)$	$y = Vx^n/(K^n + x^n)$
Lineweaver–Burk	1/s	1/v	$y = (1/V) + (K/V)x$	$y = (1/V) + (K^n/V)x^n$
Eadie–Scatchard	v	v/s	$y = (V/K) - (1/K)x$	$y = (1/K)x^{1-(1/n)}(V-x)^{1/n}$
Hill	log s	log(v/(V-v))	$y = (-\log K) + x$	$y = (-n\log K) + nx$
Semi-log	log s	v	$y = V/(1 + e^{(\log K)-x})$	$y = V/(1 + e^{(n\log K)-nx})$



Appendix, Fig. 1. Plots of (A) velocity (v) vs glucose concentration (s), (B) $1/v$ vs $1/s$ and (C) v vs log s. Superimposed are curves for Michaelis–Menten fitted (continuous line) and Hill fitted (dashed line) models of hexokinase of the mantle of *M. edulis*; the sample is of indeterminate sex collected October 1979.

models by superimposition of the fitted curves onto the transformed scatter plots (see Appendix, Table 1 for the relevant equations). Using the untransformed (s, v) scales, it is often difficult to judge the fit for small s values (Appendix, Fig. 1A). The converse applies to the Lineweaver–Burk plot (or Eadie–Scatchard plot) where the fit is easier to observe for small s values than for large ones (Appendix, Fig. 1B). A suitable compromise is often the plot of v vs log s which allows the fit to be judged across the entire range of s (Appendix, Fig. 1c). The plot retains the desirable feature of an untransformed v axis so that deviations from the fitted line can be assessed for skewness and constancy of variance (on these scales the fitted curves are logistic with the point of inflection at $s = K$). The main purpose of the graphs is to judge the adequacy of fit of the Hill model. The latter will always fit the data at least as well as the Michaelis–Menten model since equation (1) is a special case of equation (2). Thus, given that the Hill model is

adequate, a test of whether the Michaelis–Menten equation is also consistent with the data should not be based on graphical assessment but on the confidence interval for the Hill coefficient, discussed earlier. However, the Lineweaver–Burk and Eadie–Scatchard plots can usefully serve to illustrate the results of this test (see Fig. 1 of the main text).