MEASUREMENT OF CRUSTACEAN HYPERGLYCAEMIC HORMONE LEVELS IN THE EDIBLE CRAB *CANCER PAGURUS* DURING EMERSION STRESS

S. G. WEBSTER

School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW, UK

Summary

The effects of emersion stress upon circulating hyperglycaemic hormone (CHH) levels in the edible crab *Cancer pagurus* were investigated using a highly specific and sensitive radioimmunoassay, with an antiserum directed against HPLC-purified *C. pagurus* CHH. Emersion resulted in hyperglycaemia and immediate hypoxia, as shown by rapid hyperlactaemia. CHH levels increased dramatically during the first hour of emersion, from almost undetectable levels to around $17\,\mathrm{pmol\,l^{-1}}$, thereafter increasing to around $30\,\mathrm{pmol\,l^{-1}}$ after 4 h of emersion. Short-term air exposure experiments demonstrated that significant increases in CHH levels (up to $3.5\,\mathrm{pmol\,l^{-1}}$) could be detected during the first 15 min of emersion. Although CHH appears to be fairly stable in haemolymph *in vitro*, injected CHH was cleared extremely rapidly from the haemolymph *in vivo*. The results suggest that emersion results in rapid, massive and prolonged exocytosis of CHH from the sinus gland. The sensitivity of the assay and the utility of this crab model may be useful in further studies to elucidate the control of CHH release in crustaceans.

Key words: crustacean hyperglycaemic hormone (CHH), emersion stress, radioimmunoassay, *Cancer pagurus*.

Introduction

Crustacean hyperglycaemic hormones (CHHs) are without doubt the best-known crustacean neuropeptides, their defining physiological function being the maintenance and control of blood glucose levels (see Keller, 1992, for a review), notwithstanding a proposed role as a secretagogue, controlling amylase release by the midgut gland (Sedlmeier, 1988), a possible role in the regulation of ecdysteroid synthesis (Webster and Keller, 1986) or in osmoregulation (Charmantier-Daures *et al.* 1994). It has long been known that injection of CHH leads to a sustained hyperglycaemia, lasting a few hours (Keller and Andrew, 1973), and a role as an adaptive hormone has been inferred, on the basis of several studies showing that hyperglycaemia occurs during stress such as emersion, anoxia, salinity and temperature changes (Dean and Vernberg, 1965; Salminen and Lindquist, 1975; Keller and Orth, 1990). In nocturnal crustaceans, hyperglycaemia corresponds with periods of increased activity (Gorgels-Kallen and Voorter, 1985), apparently with an endogenous circadian component (Kallen *et al.* 1990).

Whilst measurement of blood glucose levels during experimental manipulation is a simple and valuable technique, little is known regarding changes in circulating CHH levels during hyperglycaemic episodes, and it is possible that these fluctuate extremely rapidly. Thus, a knowledge of temporal changes in CHH levels would be advantageous, particularly when investigating possible feedback control mechanisms or the influence of other neuropeptides and neurotransmitters upon CHH release. Using a radioimmunoassay (RIA), Keller and Orth (1990) have shown that CHH levels are transiently elevated during hypoxia, show sustained elevation following temperature shock, and show variation on a circadian basis in the crayfish *Orconectes limosus*. Recently, Santos and Keller (1993*a*) have shown (using an enzyme immunoassay, EIA) that CHH levels in the crab *Carcinus maenas* may be controlled by positive and negative feedback loops modulated by lactate and glucose respectively.

In the present study, crustacean hyperglycaemic hormone levels were determined in the edible crab *Cancer pagurus* following emersion stress. Since existing immunoassays using available CHH antisera raised against *C. maenas* CHH (Dircksen *et al.* 1988) cross-reacted poorly with *C. pagurus* CHH in competitive immunoassays such as RIA, this study necessitated the development of a highly specific antiserum directed against *C. pagurus* CHH. Furthermore, since initial experiments revealed that the circulating titres of CHH were extremely low, a highly sensitive RIA, coupled with partial purification of haemolymph samples, was developed. Nevertheless, *C. pagurus* is a useful model for such experiments, since repeated, large haemolymph samples can be taken from individuals.

Materials and methods

Animals

Adult male *Cancer pagurus* (L.) weighing 0.5–0.75 kg were obtained off the coast of Anglesey, UK, by local fishermen and maintained in a recirculating seawater system at 12–15 °C prior to use. Experimental animals were subsequently housed in individual aquaria (35 l) in a recirculating seawater system, at 12 °C and 12 h:12 h L:D for 1–2 weeks prior to experimentation. During this period, animals were fed with squid, but feeding was discontinued 48 h before each experiment.

Purification of CHH and antiserum production

Eyestalks were removed from crabs anaesthetised by chilling, and sinus glands (SGs) were dissected from the optic ganglia in batches of 100 and extracted in ice-cold $2 \, \text{mol} \, l^{-1}$ acetic acid, as described previously (Webster and Keller, 1986). Dried extracts were reconstituted in $2 \, \text{mol} \, l^{-1}$ acetic acid and purified by HPLC using a Waters µBondapak Phenyl column (3.9 mm×300 mm) with gradient elution at a flow rate of $1 \, \text{ml} \, \text{min}^{-1}$. Solvent A was 0.11 % trifluoroacetic acid (TFA); solvent B was 0.1 % TFA in 60 % acetonitrile; the gradient was 30 % to 80 % B over 1 h. Under these conditions, material previously identified as CHH by homologous bioassay eluted as two prominent peaks with retention times of around 50 min. Material representing the larger (main) peak was collected, dried and rechromatographed using a Bakerbond WP C_{18} column (4.6 mm×250 mm), using the same solvents, but with a gradient of 40 % to 80 % B over 45 min. Material from this main CHH peak was manually collected, dried and quantified by amino acid analysis of vapour-phase hydrolysed (Tarr, 1986) *o*-phthaldialdehyde-derivatised material as described previously (Webster, 1991). Purity and mass were verified by electrospray mass spectrometry. The relative molecular mass (M_r) of *C. pagurus* CHH (main peak) is 8415.73. The minor CHH peak has an M_r of 8432.84. Purification of approximately 1000 SGs yielded 75 µg of pure CHH. One New Zealand white rabbit was used for immunisation, following the procedure detailed in Dircksen *et al.* (1988), except that two injections of 25 µg of CHH were followed with a final booster injection of 20 µg. The rabbit was terminally exsanguinated under anaesthesia 90 days after the initial immunisation. The specificity of the antiserum was tested by immuno-dot-blotting of HPLC-fractionated *C. pagurus* SG as described by Dircksen *et al.* (1987).

Radioiodination of CHH and radioimmunoassay

Small quantities of *C. pagurus* CHH (approximately 0.3 nmol) were iodinated with 18 MBq of $[^{125}\text{-I}]$NaI (Amersham) using chloramine-T as detailed by Bolton (1989). Separation of labelled product from unreacted iodide was carried out by gel chromatography (PD-10, Pharmacia), eluting with $50 \, \text{mmol} \, l^{-1}$ phosphate buffer, pH 7.4, containing $0.2 \, \text{mol} \, l^{-1}$ NaCl and 0.1 % bovine serum albumin (BSA). Specific activities of approximately $40 \, \text{TBq} \, \text{mmol}^{-1}$ were routinely obtained. For the RIA, standard tubes containing 100 µl of CHH in the range 5000–1.25 pg (in phosphate buffer, pH 7.4, containing 0.1 %

BSA; PB) were used. Antiserum (100 µl) at an initial concentration of 1:10000 diluted in $50 \, \text{mmol} \, l^{-1}$ sodium phosphate buffer, pH 7.4, was added, and tubes were incubated for 6 h at room temperature before addition of 100 µl of PB containing $15\,000 \, \text{disints} \, \text{min}^{-1}$ radioligand. After overnight incubation at 4 °C, separation of bound from free CHH was performed by adding 50 µl of solid-phase second antibody (donkey anti-rabbit IgG adsorbed to microparticulate cellulose, Immunodiagnostic Services), incubated for 30 min at room temperature, followed by addition of water (1 ml), centrifugation (2000 *g*, 5 min) and aspiration of the supernatant. Counting was carried out using an LKB 1275 gamma counter.

Purification of haemolymph samples

Initial experiments using unpurified haemolymph (200 µl) indicated that basal levels of CHH in *C. pagurus* were extremely low, below the sensitivity limit of the assay, i.e. less than 0.2 pmol per tube. Accordingly, it was necessary to develop methods for isolating peptides from larger (>1 ml) volumes of haemolymph. The procedure which gave the highest recoveries and the most consistent results is as follows. Samples of haemolymph (2 ml) were removed from the hypobranchial sinus and rapidly frozen in liquid nitrogen. After gentle thawing, the haemolymph was centrifuged (2000 *g*, 10 min, 4 °C) and placed on ice. The supernatant was slowly applied ($1 \, \text{ml} \, \text{min}^{-1}$) to a Sep-Pak C_{18} cartridge (Waters), previously conditioned with 5 ml of isopropanol, followed by 20 ml of water. The column was washed with 10 ml of water, and the peptide fraction containing CHH was eluted into a Minisorp tube (NUNC Gibco) with 3 ml of 40 % isopropanol, discarding the first 0.5 ml (void volume). The eluate was dried in a vacuum centrifuge. The residue was redissolved by sonication in 200 µl of PB and divided to give duplicate tubes in the RIA. This purification procedure resulted in recoveries of between 60 and 70 %, determined using haemolymph samples spiked with varying amounts of CHH. Application of larger amounts of haemolymph resulted in lower recoveries. To purify sufficient circulating CHH from haemolymph for identification by HPLC and RIA, crabs were stressed by emersion for 2 h, followed by exsanguination (up to 30 ml of haemolymph could easily be withdrawn from a large crab). The haemolymph (35 ml) was then purified on Sep-Pak cartridges (2 ml per cartridge). The resultant eluates were dried, reconstituted in $2 \, \text{mol} \, l^{-1}$ acetic acid, pooled and dried. The resultant (yellow) pellet was redissolved in $2 \, \text{mol} \, l^{-1}$ acetic acid and chromatographed using a phenyl column using the conditions described above. Fractions were collected at 1 min intervals, dried, reconstituted in PB and assayed in duplicate. A new column was used to avoid breakthrough of previously adsorbed CHH from SG purifications. A standard (10 ng CHH) was chromatographed immediately after the sample.

Determination of glucose and lactate

Glucose was determined using the glucose oxidase method (Boehringer-Mannheim) in a microplate format. Before assay, haemolymph samples (50 µl) were deproteinised in an equal volume of $0.6 \, \text{mol} \, l^{-1}$ perchloric acid, and 50 µl of the

supernatant was added to 450 μl of 0.2 mol l^{-1} sodium phosphate buffer, pH 7.4. Samples (50 μl) of this solution were used in the assay with 200 μl of the enzyme chromogen reagent (diluted according to the manufacturer's instructions, but with 0.2 mmol l^{-1} phosphate buffer).

Lactate was determined using the lactate oxidase method (Sigma). Haemolymph samples were diluted fivefold and 10 μl was applied to microtitre plates; 100 μl of reagent was subsequently added. Absorbance was read on a Bio-Tek EL340 microplate reader equipped with proprietary data analysis software.

Results

Radioimmunoassay

The antiserum directed against *C. pagurus* CHH was of high specificity and titre: a final dilution of 1:30 000 resulted in 50 % binding of radioligand (at a specific activity of 40 TBq mmol^{-1}, 15 000 disints min^{-1} tube^{-1}) and, optimally, the sensitivity was about 1–2 pg per tube; the ED$_{50}$ was 50 pg (Fig. 1). The antibody appeared to have remarkable specificity for the major form of CHH; assays using quantified doses of the minor isoform as the competing ligand resulted in nonparallel, shallow dose–response curves with an ED$_{50}$ of around 8 ng per tube. Despite the sensitivity of the assay, levels of CHH could not be measured in haemolymph using volumes compatible with the assay (200 μl), without purifying much larger samples. A suitable purification procedure was developed, using Sep-Pak cartridges, which gave acceptable (60–70 %) recovery. When large volumes of haemolymph from stressed crabs were purified and assayed, logit/log dose–response curves were parallel to those for authentic hormone (Fig. 1, inset). Furthermore, when such extracts were further fractionated by HPLC, the retention time of the purified material was identical to that of authentic CHH (Fig. 2). Thus, both experiments supported the contention that the immunoreactive material was indeed CHH.
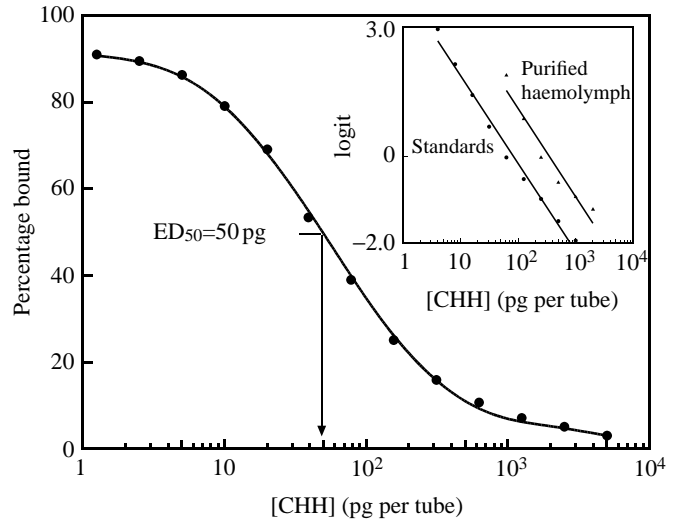

Fig. 1. Typical standard curve for the *Cancer pagurus* crustacean hyperglycaemic hormone (CHH) radioimmunoassay. The ED$_{50}$ (the quantity of CHH necessary to displace 50 % of the corresponding radioligand) was about 50 pg per tube (6 fmol). logit/log analysis of haemolymph samples purified using C$_{18}$ cartridges (inset) demonstrated parallelism with pure CHH standards. Points showing doubling dilutions were from a purified extract containing an equivalent of 8 ml of haemolymph per 100 μl of assay buffer.

Glucose and lactate levels following emersion

Samples of haemolymph taken from emersed, emersed eyestalkless and control (immersed) crabs and analysed for glucose clearly demonstrated that emersion resulted in rapid hyperglycaemia, which was not evident in animals without eyestalks (Fig. 3A). When haemolymph samples were taken at 30 min intervals and assayed for glucose and lactate, it was clear that emersion was accompanied by immediate hyperlactaemia, indicative of hypoxia, whereas hyperglycaemia was only observable after about 30 min of air exposure (Fig. 3B).
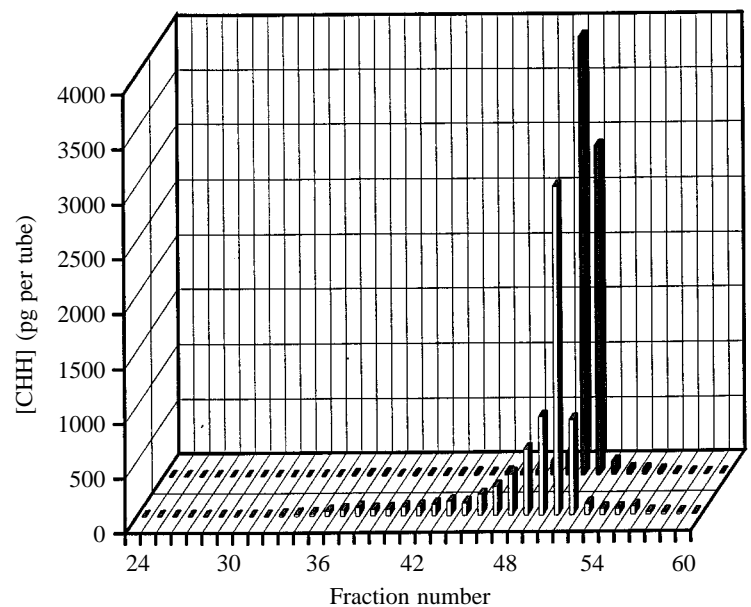

Fig. 2. Histogram showing CHH immunoreactivity of an HPLC-purified sample of an extract obtained by C$_{18}$ cartridge purifications of 35 ml of *C. pagurus* haemolymph (open bars). Fractions (1 ml) were dried and reconstituted in 100 μl of assay buffer prior to radioimmunoassay. Shaded bars show the immunoreactivity of a 10 ng sample of pure CHH, chromatographed immediately after the haemolymph sample.

Hyperglycaemic hormone levels following emersion

Levels of CHH in large (approximately 0.75 kg) *C. pagurus* were determined following haemolymph removal at 30 min intervals over a 4 h period, for both emersed and control crabs. During the first hour of emersion, mean CHH levels increased rapidly, from almost undetectable levels to around 150 pg ml^{-1} (17 pmol l^{-1}), thereafter increasing more slowly to around 250 pg ml^{-1} (30 pmol l^{-1}) after 4 h of emersion (Fig. 4A). In the control group, levels did not rise above 10 pg ml^{-1}. In emersed

crabs without eyestalks, CHH was never detected (results not shown).

Considerable variability between crabs was observed; the responses of two crabs are shown in Fig. 4B. Since these experiments suggested that CHH was released soon after air exposure, experiments were performed to measure CHH and contemporaneous glucose levels over a 1 h period (Fig. 5A,B). CHH levels increased from almost undetectable levels to around 30 pg ml^{-1} (3.5 pmol l^{-1}) within the first 15 min of air exposure, rising to around 60 pg ml^{-1} (7 pmol l^{-1}) after 30 min. Thereafter, little increase was observed. For the control group, some increase in CHH levels were observed, but these increases were individually extremely variable; thus, a non-parametric test (Welch's *t*-test) did not show any significant increase in CHH level during this period. In the emersed group, glucose levels began to increase after 30 min and became significantly higher than initial levels after 45 min, whilst in the control group there was a pronounced and significant increase at around 60 min (Tukey–Kramer multiple-comparison test) (Fig. 5B).
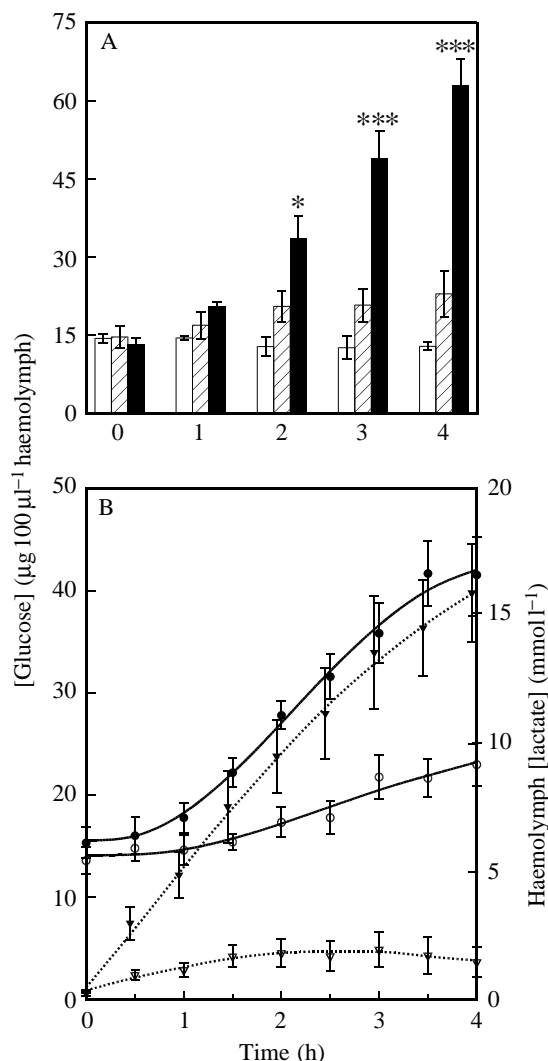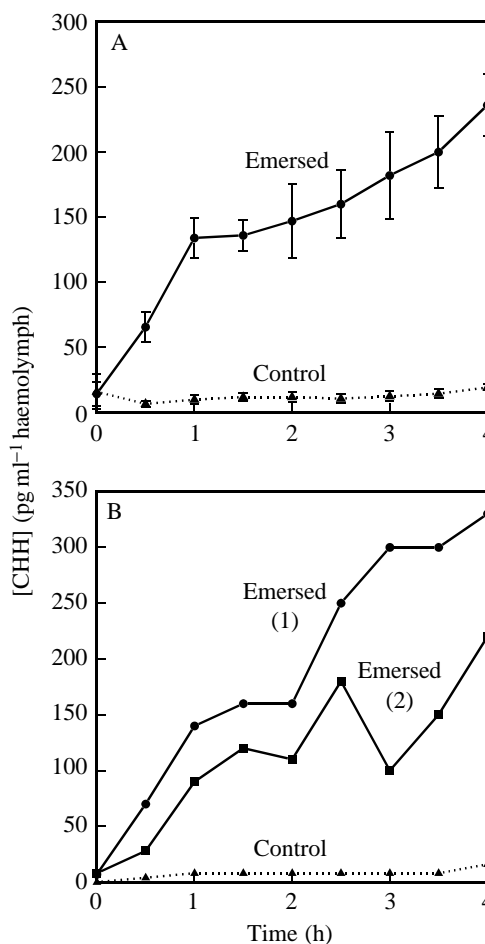

Fig. 3. (A). Haemolymph glucose levels in emersed intact (filled bars, *N*=4), emersed eyestalkless (open bars, *N*=4) and control (immersed) intact *C. pagurus* (hatched bars, *N*=5). Blood samples were taken hourly for 4 h. Tukey–Kramer multiple-comparison tests showed significant increases in haemolymph glucose levels after 2 h of emersion. Eyestalkless emersed animals and intact controls showed no significant changes in haemolymph glucose levels. *P<0.05, ***P<0.001 compared with initial values. (B) Haemolymph glucose and corresponding lactate levels in emersed and control (immersed) crabs. Samples were taken at 30 min intervals for 4 h. Solid lines show glucose levels; lower line, control values. Dashed lines show lactate levels; lower line, control values. *N*=5 for each treatment. Bars show S.E.M.

Fig. 4. (A) Haemolymph CHH levels in emersed and control (immersed) *C. pagurus* over 4 h. *N*=5 for each treatment. Bars show S.E.M. (B) CHH levels in two emersed *C. pagurus* showing the variation in response. Control (immersed) crabs generally exhibited CHH levels below 10 pg ml^{-1} in these experiments.
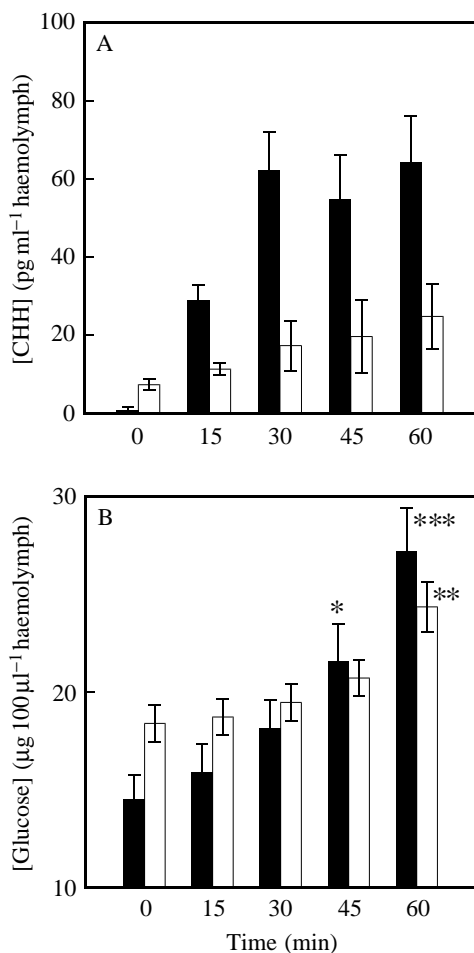
Table 1. *Experiment to determine possible degradation of CHH in haemolymph*

| | Concentration of CHH ($pg\,ml^{-1}$) | | | |
| | 0 °C | | 20 °C | |
Sample time (h)	Crab 1	Crab 2	Crab 1	Crab 2
0	62.5	210	80	200
1	80	170	80	175
2	85	125	75	150
3	82	175	74	120
4	80	200	75	110
Mean ± S.E.M.	77.9±3.9	176±14.7	76.8±1.3	151±16

Two crabs were stressed by emersion for 2 h and large samples (20 ml) of haemolymph removed. Samples were divided and incubated for up to 4 h on ice (0 °C) or at 20 °C, followed by purification and radioimmunoassay.

on Fig. 6. From this figure, is suggested that the half-life of circulating CHH is in between 5–10 min.

Discussion

The present study has used a sensitive and specific radioimmunoassay to measure circulating CHH levels in the edible crab *Cancer pagurus* following air exposure. The immunoreactive material isolated from the haemolymph seems likely to be authentic CHH in view of the parallel dose–response relationships observed when compared with authentic CHH and, more particularly, in view of the identical retention time of this material with CHH when separated by HPLC (Fig. 2). It should be noted, however, that a rather prominent shoulder was seen on this chromatogram. This was probably due to the presence of an isoform of CHH. In all brachyuran SGs thus far examined by HPLC, the prominent peak (main-CHH) is preceded by a four- to fivefold smaller peak of an equally biologically active hyperglycaemic neuropeptide (pre-CHH) (S. G. Webster, unpublished observation; see also Keller and Kegel, 1984; Webster and Keller, 1986). The antibody raised in this study cross-reacted against the pre-CHH peak with extremely nonparallel dose–response relationships: at around 20 pg per tube, both cross-reacted equally, but at higher concentrations the dose–response asymmetry was acute. The ED_{50} for main-CHH was around 50 pg per tube, that for pre-CHH was around 8 ng per tube. The overall effect of this phenomenon would be to emphasize a long shoulder of immunoreactivity, rather than a peak prior to the main immunoreactive peak.

Whilst the levels of glucose found in the haemolymph following emersion stress in *C. pagurus* are similar to those recorded in other crustaceans (Santos and Colares, 1990; Santos and Keller, 1993b), levels of circulating CHH seem to be much lower than the currently published values. For example, in crayfish *Orconectes limosus* undergoing hypoxia, CHH titres of around 120 $pmol\,l^{-1}$ are reached within 15 min and decline rapidly thereafter (Keller and Orth, 1990). In the crab *Carcinus maenas*, basal levels of CHH are around 20 $pmol\,l^{-1}$ (Santos and

Fig. 5. (A) Haemolymph CHH levels in emersed (filled bars, *N*=8) and control (open bars, *N*=6) *C. pagurus* over 1 h, sampling every 15 min. Statistical analysis (Welch's *t*-test) showed that the slight increase in CHH levels during the experiment was not significant for the control group. (B) Corresponding haemolymph glucose levels in these animals. Bars show S.E.M. Tukey–Kramer multiple-comparison tests showed significant increases in haemolymph glucose content compared with initial values after 45 min of emersion, and in the immersed controls after 60 min. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Degradation of CHH in vitro *and* in vivo

To determine the stability of CHH *in vitro*, two crabs were stressed by emersion for 2 h and large samples of haemolymph (20 ml) were removed from each. The samples were divided, one half of each was left on ice, whilst the other was incubated at 20 °C. Subsamples (2 ml) were removed and purified every hour for 4 h. The results are shown in Table 1. For crab 1, the mean (±S.E.M.) haemolymph CHH titre over this period was 77.9±3.9 $pg\,ml^{-1}$ when incubated at 0 °C and 76.8±1.3 $pg\,ml^{-1}$ when incubated at 20 °C. For crab 2, the corresponding values were 176±14.7 $pg\,ml^{-1}$ and 151±16 $pg\,ml^{-1}$. To estimate the rate of degradation of CHH *in vivo*, four crabs (approximately 750 g) were injected with 100 ng of CHH, dissolved in saline. Haemolymph was taken after 2 min (*T*=0, to ensure even distribution of the CHH bolus) and subsequently after 5, 10, 15, 30 and 60 min. The results of this experiment are shown
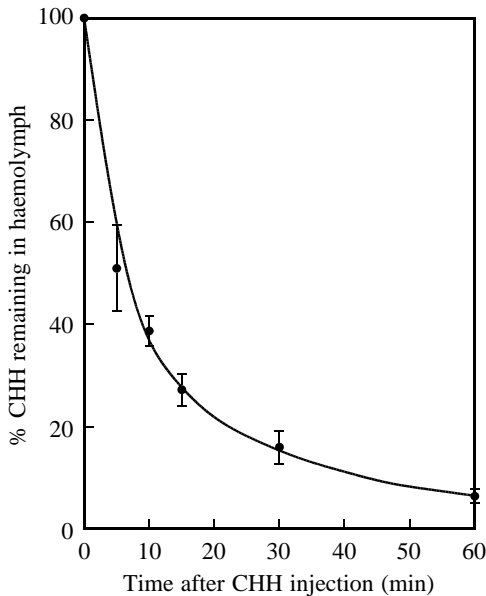
Fig. 6. CHH immunoreactivity, expressed as a percentage of the initial level, in haemolymph of *C. pagurus* following injection of 100 ng of CHH. 2 min elapsed before taking the first sample ($T=0$) to ensure distribution of the hormone bolus. $N=4$. Bars show S.E.M.

Keller, 1993*a*), whereas in the present study, basal levels were frequently less than 1 pmol l^{-1} and maximal levels were only about 30 pmol l^{-1}. In previous studies, it should be noted that CHH levels were measured only once from individuals. Since *C. pagurus* is a very large crustacean, it was possible to sample individuals repeatedly. The results from these experiments clearly demonstrated the rapidity (and variability) of changes in CHH level. For example, in 4 h experiments where haemolymph was taken every 30 min, rapid increases in CHH level were observed during the first hour of emersion (Fig. 4A). The slow increase in level after this period was, to a large extent, a reflection of a subsequently variable response between individuals, as shown by the large error bars (Fig. 4B). Short-term emersion experiments further illuminate this variability: a rapid increase in CHH titre was observed in the first 15–30 min of emersion, but the titres thereafter became variable (Fig. 5A). It was interesting to note that CHH titres also increased during the experiment in the control group, although these increases were individually variable and not significantly different from initial values. It is also readily apparent that whilst blood glucose levels began to increase in the emersed group within the first 30 min, becoming significantly higher than initial values after 45 min, a rather rapid and significant increase in blood glucose level was also seen in the controls 60 min after the first haemolymph sample was taken. In these short-term experiments, there is probably an element of handling-induced stress and resultant hyperglycaemia, which is not highly correlated with increases in CHH levels in every crab. Nevertheless, taking the results of the emersed group into account, it is reasonable to suggest that significant increases in CHH levels result in measurable hyperglycaemia within 15–30 min, a result which is in accord with injection experiments (Keller and Andrew, 1973).

To determine the stability of CHH, experiments were performed to measure possible degradation *in vivo* and *in vitro*. These experiments were designed with an awareness of possible erroneous results. Without exacting purification of the native molecule, for example by HPLC, it is quite possible that an immunoassay might not measure degradation, if the sequence undergoing proteolysis was distant from the epitopes recognised by the antibodies. Somewhat surprisingly, *in vitro* experiments in which haemolymph from stressed crabs was incubated on ice and at room temperature for periods of 0–4 h, prior to purification and assay, suggested that degradation was not occurring in the haemolymph, although some (considerable) reduction in CHH concentration was seen in one of the samples incubated at 20 °C. In contrast, when crabs were injected with CHH *in vivo*, and haemolymph samples were removed at short intervals thereafter, followed by purification and assay, there was convincing evidence for rapid removal from the circulation and/or degradation of the hormone. From the graph shown on Fig. 6, the half-life of CHH can be estimated to be between 5–10 min.

With regard to other studies on neuropeptide stability in arthropods, whilst it has been shown that the haemolymph of cockroaches possesses significant proteolytic activity towards hypertrehalosaemic hormone (HTH) (Lui and Steele, 1994), most studies have indicated that neuropeptides are degraded by cell-surface endopeptidases (Turner, 1990; Isaac, 1987, 1988). In particular, detailed studies on adipokinetic hormone (AKH) inactivation in locusts (Rayne and O'Shea, 1992) have shown that the fat body, Malpighian tubules and skeletal surfaces are important sites for hormone degradation.

In view of the rapid removal of CHH from the haemolymph, and the sustained increases in CHH levels concomitant with emersion hypoxia, it seems likely that, during emersion stress, exocytosis of CHH from the sinus gland is a massive and possibly continuous phenomenon. Assuming that a large *C. pagurus* has a haemolymph volume of a least 100 ml and that a level of 20 pmol l^{-1} CHH is maintained, the total instantaneous amount of CHH in the haemolymph would be around 2 pmol. Assuming a half-life of circulating CHH of 5–10 min, then 6–12 pmol would need to be secreted per hour to maintain a haemolymph concentration of 20 pmol l^{-1}, i.e. 24–48 pmol would be secreted over 4 h. The total amount of CHH contained in both sinus glands of a large *C. pagurus* is about 250 pmol. Thus, between 10 and 20 % of the total store of CHH would be released during this period. Whilst this seems rather large, it is probable that long periods of hypoxia or emersion are probably infrequently experienced by this crab.

Whilst CHH seems to have a major role in controlling hyperglycaemia in crustaceans, evidence has accumulated suggesting that neurotransmitters such as serotonin may induce hyperglycaemia independently from CHH (Lüschen *et al.* 1993) and that dopamine and Leu-enkephalin may decrease CHH release acting *via* the CHH neurosecretory system (Lüschen *et al.* 1993; Rothe *et al.* 1991). Indeed, it is perhaps possible that the lack of a close correlation between hyperglycaemia and increases in CHH levels in the control group where haemolymph

was removed every 15 min might reflect changes in circulating serotonin levels associated with handling stress. A recent finding of particular interest and significance has been reported by Santos and Keller (1993*a*), who have found evidence for a dual feedback mechanism for CHH release in which glucose inhibits and lactate promotes CHH release. Much has yet to be learnt about the control of CHH release: sensitive and specific assays, coupled with suitable model organisms, where repeated samples can be taken from individuals, such as reported here, should help elucidate some of the complex control mechanisms operating in carbohydrate homeostasis in crustaceans.

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