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Circadian Rhythmicity of the Crustacean Hyperglycemic Hormone (CHH) in the Hemolymph of the Crayfish

JANINE L. KALLEN, S. L. ABRAHAMSE, AND F. VAN HERP

Zoölogisch Laboratorium, Faculteit Natuurwetenschappen, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Abstract. The crustacean hyperglycemic hormone (CHH) is involved in the regulation of endogenous blood glucose metabolism. In this paper we describe the daily rhythmicity in the blood glucose and the blood CHH content of the crayfish *Orconectes limosus*. Both blood CHH and blood glucose levels increase during the first hours after the beginning of darkness. The bioactivity of released CHH is far higher than that of CHH stored in the sinus gland. Moreover, the released hyperglycemic material shows an affinity for high molecular weight proteins in the hemolymph. Preliminary results suggest that subunits of hemocyanin may act as potential carrier-proteins for bioactive CHH.

Introduction

The neuroendocrine system producing the crustacean hyperglycemic hormone (CHH) of decapod crustaceans forms part of the medulla terminalis ganglionic X-organ (MTGX). The MTGX lies at the outer edge of the medulla terminalis—the most proximal optic ganglion in the eyestalk—and contains several hundred neuroendocrine cells. In crayfish, such as *Astacus leptodactylus* and *Orconectes limosus*, about 35 to 40 CHH-producing cells form a distinct group located latero-ventrally on the MTGX. Neurosecretory granules containing CHH are transported via a tract that leads across the neuropil of the medulla terminalis to a neurohemal region, the sinus gland. Immunocytochemical and morphometric research indicates that about 40% of the sinus gland axon terminals are filled with neurosecretory granules containing CHH, which is released into the hemolymph by exocytosis. The hyperglycemic hormone in the blood regulates blood sugar levels to meet physiologically required metabolic energy needs (Strolenberg and Van Herp, 1977; Strolenberg *et al.*, 1977; Van Herp and Van Buggenum, 1979; Gorgels-Kallen and Van Herp, 1981; Gorgels-Kallen *et al.*, 1982; for a review see Kleinholz, 1985).

The glucose level in the hemolymph of decapod crustaceans reveals a day/night rhythmicity, characterized by a low basal level during the light period, and a peak in glucose content appearing several hours after the onset of darkness (Hamann, 1974; Strolenberg, 1979; Reddy *et al.*, 1981). The basal level during the day, as well as the height and duration of the nocturnal peak, are species-dependent and are affected by seasonal influences (unpub. obs.) as well as physiological events, such as molting (Kallen, 1985). Physiological research in crayfish strongly indicates an endogenous circadian blood glucose rhythm entrained by the light/dark schedule (Kallen *et al.*, 1988).

In previous studies, we investigated the secretory dynamics of the CHH-system of *Astacus leptodactylus*. Immunocytochemical staining combined with morphometric analyses at the light and electron microscopic level revealed a daily rhythmicity in the synthetic activity of the perikarya, the transport of CHH-material to the sinus gland, and the release of CHH into the hemolymph (Gorgels-Kallen and Voorter, 1984, 1985). In this study, we report and discuss the immunochemical detection of circulating bioactive CHH in the hemolymph during a 24-h period. We present a preliminary molecular characterization of the bioactive CHH present in the blood and

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compare it to the molecular form of the hormone stored in the sinus gland[1].

Materials and Methods

Animals

Crayfish (*Orconectes limosus*) were obtained from a commercial fisherman, who had collected them in the river Meuse. In the laboratory, the animals were kept in running tap water (13–15°C) and fed weekly with ground meat or fish. Experiments were performed with adult intermolt female and male crayfish of equal size and weight (about 20 g). The animals were kept under constant light/dark conditions (LD 12:12; light on 8:00 a.m.).

Antisera

Immunochemical quantification was carried out with an enzyme-linked immunosorbent assay (ELISA); the double antibody-sandwich (DAS) method was followed. The first antiserum was a polyclonal anti-CHH serum raised in rabbits against a biologically active CHH preparation from sinus glands of the crayfish *Astacus leptodactylus*. Details about the purification of the antigen, the production of antiserum, and tests of specificity have been described previously (Gorgels-Kallen and Van Herp, 1981). The second antiserum was a polyclonal anti-CHH serum raised in mice against a biologically active CHH preparation from sinus glands of the crayfish *Orconectes limosus*. The production of this antiserum is described below.

The CHH was isolated from dissected sinus glands that had been collected in 0.1 *N* HCl, homogenized, and extracted overnight in 0.1 *N* HCl. The extracts were centrifuged and the supernatants lyophilized. This crude extract was further purified by preparative polyacrylamide gel electrophoresis (according to Davis, 1964), followed by gel filtration on a calibrated Sephadex G-50 superfine (sf) column. Quantitative protein determination was carried out by absorption at 280 and 220 nm. The purified fractions were tested in a bioassay (according to Leuven *et al.*, 1982), and immunoreactivity was tested by a direct ELISA using the anti-*Astacus*-CHH serum mentioned above. The resulting purified material was a biologically and immunologically active peptide with a MW of around 6500 Da, which is in agreement with former results (*e.g.*, Keller, 1977; Kallen *et al.*, 1986). Female Swiss mice received a first intraperitoneal injection comprising 5 μg purified CHH material buffered with 0.03 *M* ammonium acetate, pH 8.5, and emulsified in Freund's complete adjuvant (1:1). After 10 and 17 days, booster injections containing, respectively, 3 and 2 μg in buffer and emulsified 1:1 in Freund's incomplete adjuvant, were given intraperitoneally. After 27 days, blood was collected by heart puncture. The specificity of the sampled mouse serum was tested in a dilution series by a direct ELISA, carried out in microtiter wells coated with 10 ng of the purified CHH material. Immunoreactivity of the antiserum disappeared after absorption with purified CHH and crude sinus gland extract. Pre-immune serum was immunochemically negative with purified CHH and crude sinus gland extract.

Hemolymph sampling

Hemolymph was sampled over a 24-h period; the sample times were chosen based on former results (Gorgels-Kallen and Voorter, 1985). During this sampling period, the animals were kept in a series of tanks, each tank containing five crayfish. At each sample time, the hemolymph from one group of five crayfish was collected, as described by Gorgels-Kallen and Voorter (1985) for the crayfish *Astacus leptodactylus*. From each animal, two 50-μl samples of hemolymph were aspirated into a calibrated capillary pipet that had been inserted between coxa and basis of the left cheliped. The first hemolymph sample was diluted immediately in an equal volume of buffer (0.1 *M* phosphate solution containing 0.01 *M* sodium citrate and 0.01 *M* EDTA, pH 7.5, to prevent clotting). This sample was used for ELISA. The duplicate sample was frozen immediately, and the blood glucose level was determined by the Gluco Quant Test Combination (Boehringer Mannheim GmbH). For each time point, we report the mean and standard error of the five determinations of CHH and glucose, respectively.

Immunochemical analysis of blood samples

The DAS-ELISA technique was carried out according to Voller *et al.* (1979). After pretreatment with 1% glutaraldehyde, microtiter plates (Nunc) were coated with 100 μl *Astacus*-CHH serum (rabbit serum: optimal dilution 1/1000 in 0.05 *M* sodium carbonate buffer, pH 9.4; incubation for 1 h at 37°C followed by incubation overnight at 4°C). After nine washes in ELISA-buffer (0.02 *M* phosphate buffer, pH 7.4, containing 0.05% Tween 20 and 0.9% NaCl), the wells were adsorbed with 100 μl bovine serum albumine (BSA) and 1% normal goat serum (NGS) dissolved in ELISA-buffer (30 min at room temperature). Each well was then incubated with 100 μl of the different hemolymph samples diluted 1:1 in ELISA-buffer (1 h at 37°C). One hundred microliters of anti-*Orconectes*-CHH serum (mouse serum: optimal dilution 1/200 in ELISA-buffer, 1.5 h at 37°C) was then added, followed by peroxidase-labeled rabbit-anti-mouse (RAM)

[1] Results were presented at the 14th Conference of European Comparative Endocrinologists, Salzburg (4–9 September 1988): Kallen, J. L., and Abrahamse, S. L. (1989).

serum containing 1% NGS (diluted 1/2000 in ELISA-buffer, 1 h at 37°C). The wells were washed, as described above, between each incubation. The enzymatic activity was initiated by the addition of orthophenyl-diamine acid (250 μl: 1 mg/ml in 0.1 *M* Mac-Ilvaine buffer, pH 5.2, containing 1 μl/ml 40% H_2O_2). The reaction was stopped by the addition of 250 μl 4 *N* H_2SO_4. The optical density was measured at 492 nm with an EAR-400 ELISA-reader (Austria Instruments). The detection range of the DAS-ELISA was determined by assaying a dilution series of purified CHH. The lower limit of detection is about 60 pg purified CHH (10–15 fmol).

Biochemical analyses of hemolymph samples

Hemolymph samples were analyzed by gel filtration on a calibrated Sephadex G-200 superfine (sf) column (diam: 1.5 cm; length: 45 cm) eluted with 0.1 *M* phosphate buffer pH 7.5 (containing 0.01 *M* sodium citrate and 0.01 *M* EDTA to prevent blood clotting). The flow rate was 5 ml/h, and 1-ml fractions were collected. For each analysis, 750 μl of hemolymph from one animal was sampled. The sample was taken at the start of the dark to coincide with the exocytosis, expected during that period, of CHH into the blood (Gorgels-Kallen and Voorter, 1985). After gel filtration, the immunological activity of each column fraction was tested by DAS-ELISA.

The hyperglycemic activity of each column fraction was also tested with a bioassay for hyperglycemia, as described by Leuven *et al.* (1982). As controls, animals were injected either with physiological saline (Van Harreveld solution), or with 5 μg bovine serum albumin (BSA) dissolved in saline. The latter control was performed to test the effect on glucose levels of injecting large amounts of high molecular weight proteins. The amount of BSA corresponded with the amount of protein (after Lowry *et al.*, 1951) in the injected blood fractions.

Crude hemolymph samples and fractions obtained after gel filtration were further studied using sodium dodecyl-sulphate-polyacrylamide-gel electrophoresis (SDS-PAGE), according to Laemmli (1970). The fractions to be analyzed were diluted in 0.01 *M* Tris/HCl buffer, pH 6.8, containing 10% glycerol and 1% SDS (crude hemolymph and gel fractions diluted respectively 1:20 and 1:10 in buffer). Each fraction contained 1.5–2 μg protein measured according to Lowry *et al.* (1951). Electrophoresis was carried out with a 3% stacking gel (0.1% SDS in 0.125 *M* Tris/HCl buffer, pH 6.8) and a 10% separating gel (0.1% SDS in 0.375 *M* Tris/HCl buffer, pH 8.8); a 0.05 *M* Tris/0.384 *M* glycine buffer containing 0.1% SDS, pH 8.3, was used. The gels were either stained with Coomassie Brilliant Blue R 250, or immunochemically analyzed by immunoblotting, performed with a Biorad trans blot cell and a Biorad 160/1.6 power supply. Proteins were electrophoretically blotted on nitrocellulose paper: overnight at 30 V, and then for 1 h at 60 V (Schleicher and Schüll BA 85; 0.45 μm); a 0.025 *M* Tris/HCl buffer, pH 8.3, containing 0.192 *M* glycine and 10% methanol was used. After electrophoresis, the nitrocellulose paper was stained immunochemically as follows. Blots were washed for 5 min in TBS (0.05 *M* Tris/HCl buffer, pH 7.4, containing 0.9% NaCl and 0.1% Tween 20). After washing, blots were blocked for 15 min with 3% BSA and 1% NGS in TBS (referred to as blocking solution: BS). They were then incubated for 1 h with anti-*Orconectes*-CHH (mouse serum, dilution 1/1000 in BS). After four washes in TBS, blots were incubated for 1 h in rabbit-anti-mouse peroxidase (RAM-PO: dilution 1/2000 in BS). After four washes in TBS, blots were stained with 3.3′-diaminobenzidine-4 HCl (DAB: 1 mg per 8 ml TBS, containing 0.3% H_2O_2). All incubations were performed at room temperature.

Results

Rhythmicity in the blood glucose and CHH level

During a 12 h L:12 h D period, the blood glucose level of the crayfish *Orconectes limosus* shows a clear rhythmicity (Fig. 1A). During the light period, a rather low basal level is observed, but during the first 3 to 4 h after onset of darkness, blood glucose levels increase by two- to fourfold. We measured 24-h blood glucose rhythms in animals four weeks after their arrival at the laboratory, and also in animals that had been kept under

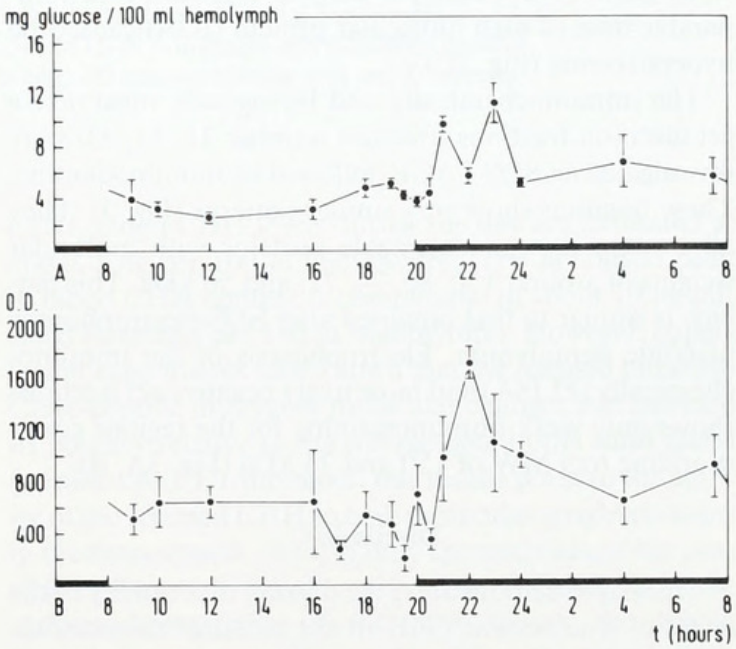

Figure 1. Hemolymph glucose and CHH levels during a 24-h period, determined for crayfish (*Orconectes limosus*) kept under constant 12 h light/12 h dark conditions; light on at 8:00 a.m. (a) Hemolymph glucose levels; means ± SEM; n = 5. (b) Hemolymph CHH levels in the same samples; means ± SEM; n = 5.

experimental conditions for up to a year. The majority of measured 24-h blood glucose cycles show two successive peaks. Hamann (1974) reported 24-h blood glucose rhythms for *Orconectes* with a single glucose increase at the beginning of the dark period. However, he took fewer blood samples at 4-h time intervals. Our results show that the two glucose peaks appear within the first 4 h of darkness. They are only detectable when blood samples are taken more frequently. The physiological significance of this phenomenon is not yet clear to us.

Figure 1B presents the levels of immunodetectable CHH in the duplicate hemolymph samples, measured in a DAS-ELISA. These results show a comparable pattern. The CHH positive fraction in the hemolymph remains on about the same level during the light period and then, during the first 3 h after the onset of darkness, increases steeply threefold.

Biochemical analysis of hemolymph

Analysis of hemolymph samples on a calibrated Sephadex G-200 sf column revealed a broad absorption peak between fractions 25 and 38 (Fig. 2A). Thus, most of the detectable proteins in the hemolymph vary in molecular weight between 60 and 150 kDa. CHH immunoreactivity was found in fractions 29 to 35, with a maximum in fraction 32 (Fig. 2B). When this fraction, obtained from 750 μl of hemolymph, was injected into five crayfish, it produced a level of hyperglycemic activity higher than that of any other fraction. The activity was comparable to the blood glucose increase induced by the injection of 0.5 sinus gland equivalents per animal. Injection of a comparable dose of high molecular protein (BSA) caused no hyperglycemia (Fig. 2C).

The immunochemically and biologically most active gel filtration fractions (fraction number 31, 32, 33) were investigated on SDS-PAGE, followed by immunoblotting. These fractions show very similar patterns (Fig. 3). They all contain immunodetectable proteins with molecular weights of around 150, 80, 74, 72, and 56 kDa. This pattern is similar to that obtained after SDS-electrophoresis of crude hemolymph. Electrophoresis of the immunochemically (ELISA) and bioactively negative gel fractions shows only weak immunostaining for the regions corresponding to a MW of 150 and 74 kDa (Fig. 3A, B).

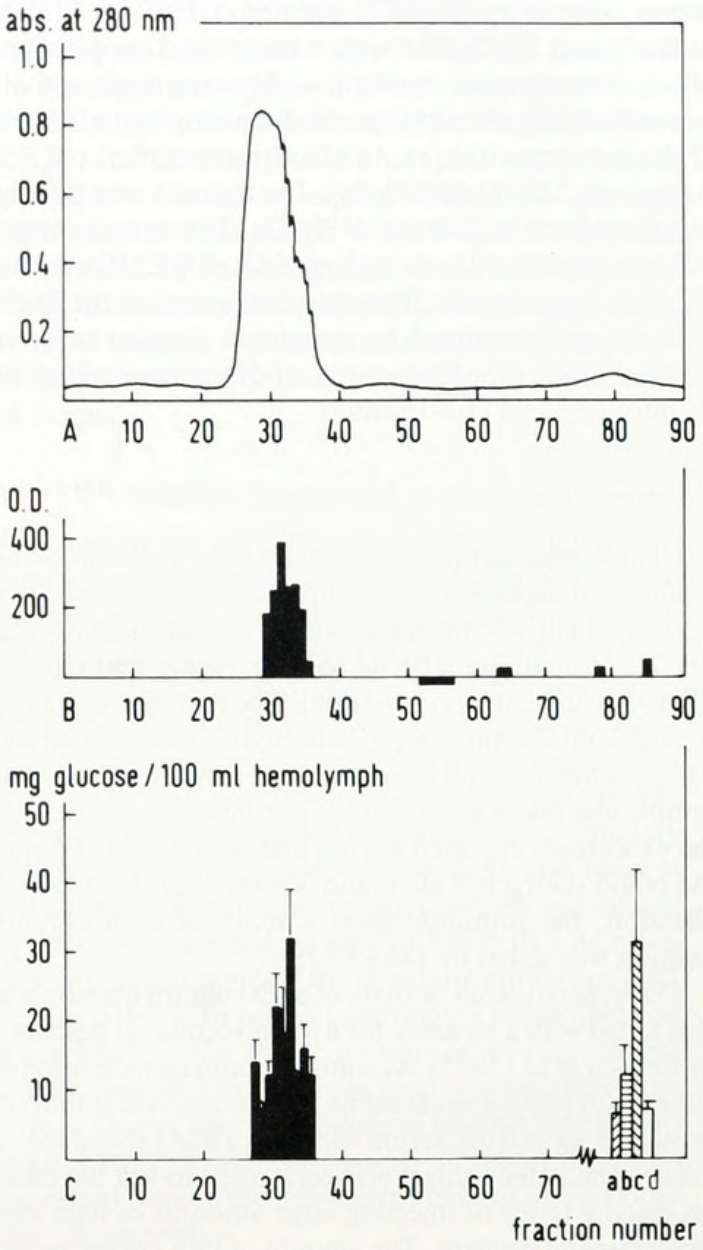

Figure 2. Gel filtration of the hemolymph of the crayfish (*Orconectes limosus*). (a) Gel filtration of 750 μl hemolymph on a Sephadex G-200 sf column. (b) Immunological response determined by a DAS-ELISA of the column fractions. (c) Hyperglycemic response after injecting the column fractions; means ± SEM; n = 5. a = pre-injection value; b = control injection with elution buffer; c = control injection of 0.5 sinus gland equivalents per animal; d = control injection of 5 μg BSA per animal.

Discussion

This paper demonstrates the diurnal rhythmicity in the levels of glucose and CHH in the blood of the crayfish, *Orconectes limosus*. The rhythm in blood glucose emerges as a low basal level during the day and an increase during the night—a day/night pattern comparable to that described previously for *Astacus leptodactylus* (Strolenberg and Van Herp, 1977; Gorgels-Kallen and Voorter, 1985). In *Astacus*, however, the blood glucose peak appears 3 to 4 h after the onset of darkness and remains elevated for most of the night, whereas in *Orconectes* it only increases during the first 3 h of the night period, declining thereafter to day-time levels. Furthermore, although the experimental procedure for both species was identical, *Astacus* has a single period of glucose increase, whereas the hyperglycemic period of *Orconectes* comprises two successive peaks. We are still puzzled about the physiological significance of the latter phenomenon.

The circadian rhythm of the blood CHH levels shows a pattern similar to that found for the blood glucose: basal

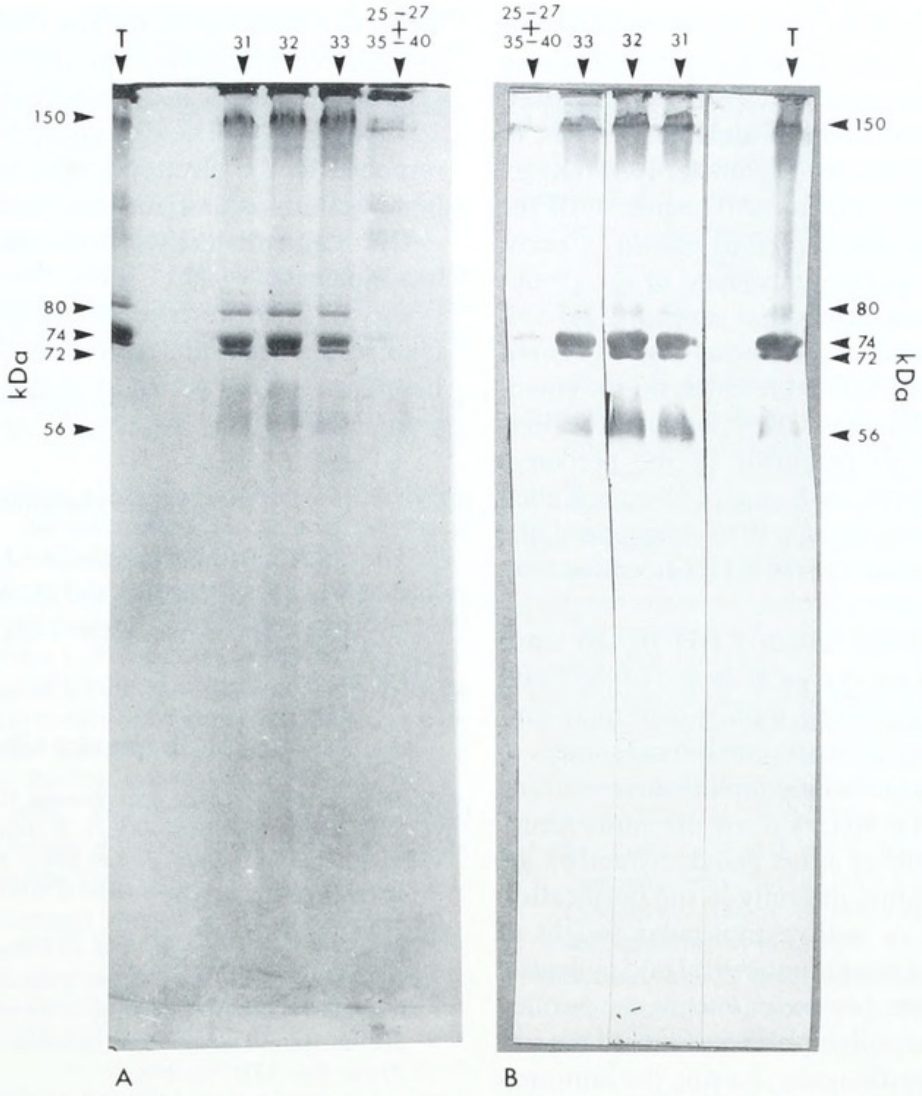

Figure 3. Results obtained after SDS-PAGE followed by immunoblotting of crude hemolymph and hemolymph gel filtration fractions from the crayfish (*Orconectes limosus*). T = total hemolymph; 31, 32, 33 = immuno- and bioactive hemolymph fractions; 25–27 and 35–40 = immuno- and bioactively negative hemolymph fractions. (A) Coomassie Brilliant Blue staining. (B) Immunoblotting with anti-*Orconectes*-CHH mouse serum.

levels during daytime and an increase of blood CHH content during the first hours of darkness. In previous studies on the diurnal cycle of the CHH cells in *Astacus*, we gathered information on the secretory dynamics of the perikarya and the rate of exocytoses of CHH granules, both events preceding nocturnal hyperglycemia (Gorgels-Kallen and Voorter, 1985). The increased blood CHH content described in this paper for *Orconectes* occurs in the period of expected high exocytosis of CHH into the hemolymph.

Our results show further that the application of a DAS-ELISA is a suitable method for determining hormone levels in crustacean hemolymph. Previously, the ELISA-technique was successfully applied by Quackenbush and Fingerman (1985) to determine the level of black pigment dispersing hormone (BPDH) in the blood of the fiddler crab. Our ELISA results are presented as optical densities. If we compare those with a standard curve of purified CHH, blood CHH levels during the day are estimated at about 1 ng per 100 μl hemolymph. The nocturnal peak in blood CHH content is comparable to about 10 ng purified hormone per 100 μl hemolymph. However, superfusion experiments have shown that the released bioactive CHH-peptide undergoes molecular changes that increase its potency relative to the storage pool in the sinus gland (unpub. obs.). Furthermore, our results point to the affinity of the released CHH to high molecular weight proteins in the hemolymph. We can only speculate about the possible effect of these molecular changes on the immunodetectability of the hormone in the blood. Therefore we cannot presently draw any conclusions about the actual quantity of bioactive hormone in the hemolymph.

Previous research on the chemical nature of CHH has focussed on the isolation, characterization, and physio-

logical effects of CHH material in the sinus gland. In this neurohemal organ, the agent causing hyperglycemia in various species of decapod crustaceans has been described primarily as a neuropeptide with a molecular weight of around 7000 Da (for a review see Kleinholz, 1985). Kegel *et al.* (1989) described the amino acid sequence of the CHH (8524 Da) from the crab *Carcinus maenas*. Recent work in our laboratory on the sinus gland of the lobster *Homarus americanus* has shown that several CHH and CHH-like molecular forms occur in this organ (Tensen *et al.*, 1989). Furthermore, limited research on the chemical nature of newly synthesized CHH points to the presence of a prohormone or precursor in the perikarya (Stuenkel, 1983; Van Wormhoudt *et al.*, 1984a, b; Kallen *et al.*, 1986). Weidemann *et al.* (1989) sequenced the cDNA encoding a precursor for the CHH from the crab *Carcinus maenas*.

Although we know much about CHH in the sinus gland, our knowledge of the chemical nature of the CHH material released into the hemolymph is extremely limited. Our results have frequently pointed to substances of high molecular weight in the hemolymph that show strong affinity for hyperglycemic factors from the sinus gland. For instance, purification of sinus gland extract by gel filtration has always resulted not only in the purification of a hyperglycemic factor with a molecular weight of around 6500 Da, but also in immunological and biological activity in the void volume. Moreover, mixing the purified 6500-Da material with hemolymph always caused the low molecular weight form to disappear, leaving the immuno- and bioactivity exclusively in the high molecular weight void volume fraction (unpub. obs.). These observations, together with the strong immunopositive reaction in the hemolymph, encouraged us to search for more information about the molecular characteristics of the circulating hyperglycemia-producing material in the blood.

Our preliminary results presented in this paper show that, after gel filtration of hemolymph on Sephadex G-200 sf, high molecular weight proteins are detectable with both a CHH-immunopositive reaction and a strong hyperglycemic activity. Moreover, the bioactivity of released CHH is far higher than that of the CHH stored in the sinus gland. This might be caused by molecular changes in the hyperglycemic hormone just before or after release. Stuenkel and Cooke (1988) suggested that only small amounts of neurohormones must be released into the blood to meet the physiological needs. These authors suggest the presence of a "readily releasable pool" that is distinguishable from the bulk of stored material. Our results are consistent with this idea. They also point to the existence of a large non-active storage pool, as opposed to a small amount of bioactive neurohormone in the neurohemal organ. The high bioactivity of the CHH in the blood could also be caused by binding of the released factor to a carrier-protein. The results of SDS-PAGE and immunoblotting show immunoreactivity associated with several proteins of high molecular weight: 150, 80, 74, 72, and 56 kDa. The pattern of this electrophoresis corresponds to the electrophoretic behavior of crustacean hemocyanins as described by Markl *et al.* (1979).

Our standard analytical methods did not reveal any low molecular weight CHH-active proteins, but the possibility of their presence should not be excluded. We intend to continue the search for the role of subunits of hemocyanin as potential carrier-proteins for bioactive CHH.

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