Expression of the crustacean hyperglycaemic hormones and the gonad-inhibiting hormone during the reproductive cycle of the female American lobster *Homarus americanus*

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Abstract

Crustacean reproduction is regulated by a complex chain of hormonal interactions in which the crustacean hyperglycaemic hormones A and B (CHH-A and CHH-B) and the gonad-inhibiting hormone (GIH) play a primary role. These neurohormones are produced in the same neuroendocrine cells of the X-organ sinus gland complex, situated in the eyestalks of the American lobster, *Homarus americanus*. In order to obtain more information on the synthesis, storage, release and function of these three neuropeptides during the reproductive cycle, we studied the levels of their mRNAs in the X-organ, their peptide storage in the sinus gland and their concentration in the haemolymph at different stages of the female reproductive cycle. A high CHH-A mRNA level was found only in the previtellogenic stage, while elevated mRNA levels were determined for CHH-B in the mature as well as the previtellogenic stage. High CHH storage levels in the

sinus gland were found during previtellogenesis. The total amount of CHH (CHH-A plus -B) in the haemolymph was significantly higher during maturation. A low level of GIH mRNA in the X-organ and a low amount of the GIH I isoform in the sinus gland were found only in the immature stage. In contrast, GIH haemolymph levels were high during the immature and previtellogenic stages. We conclude that CHH-A and -B are involved in triggering the onset of vitellogenesis and that CHH-B in particular is responsible for stimulating oocyte maturation before spawning, while GIH prevents the start of vitellogenesis in the ovary. Moreover, our results show that the balance between the haemolymph levels of the CHHs and GIH may tune the synchronization of reproduction and molting during the biannual reproductive cycle of the American lobster.

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Introduction

As reflected by their names, the crustacean hyperglycaemic hormones (CHHs) are primarily involved in the regulation of carbohydrate metabolism while the gonad-inhibiting hormone (GIH), also called vitellogenesis-inhibiting hormone (VIH), is important for the inhibition of vitellogenesis. Together with the molt-inhibiting hormone (MIH), these neurohormones belong to the CHH/MIH/VIH neuropeptide family (Keller 1992). They are all produced in the neuroendocrine cells of the medulla terminalis X-organ, localized in the crustacean eyestalks, and are transported to the clustered axon endings of the cells that form the aneurohemal organ, the sinus gland. Detailed *in situ* hybridization and immunocytochemical studies revealed a frequent colocalization of the two neurohormones in the eyestalks of lobster (Kallen & Meusy 1989, De Kleijn *et al.* 1992).

HPLC analysis of sinus gland extracts showed that CHH and GIH are present in different isoforms. For example, in lobster, CHH is eluted as two immunoreactive groups (CHH-A and CHH-B) and GIH as one immunoreactive group (Tensen *et al.* 1991, Meusy & Soyeux 1991). Each of the three groups consists of two isoforms with identical amino acid sequences and molecular masses (Tensen *et al.* 1991, Soyeux *et al.* 1991). It was recently established that the presence of a D-phenylalanine residue in one of the CHH isoforms of both CHH-immunoreactive groups is responsible for the difference in HPLC elution time (Soyeux *et al.* 1994). The CHH isoforms are therefore characterized as [L-Phe³]- and [D-Phe³]-CHH-A and [L-Phe³]- and [D-Phe³]-CHH-B.

The function of GIH in female lobsters may be concerned with the inhibition of the onset of vitellogenesis, as shown by Soyeux *et al.* (1987) using a heterologous bioassay. However, since GIH is also present in male lobsters

(De Kleijn *et al.* 1992), it must also have another role. After determination of the amino acid sequence of pre-proGIH and its alignment with the sequence of crab preproMIH, we found that not only do the mature hormones have a high degree of amino acid identity but the primary structures of their preprohormones are also nearly identical (De Kleijn *et al.* 1994). Therefore we suggested that GIH may be an important modulator of synthesis or release of hormones involved in molting as well as the reproductive process (De Kleijn *et al.* 1994).

The multifunctionality of CHH has already been demonstrated. While all CHH isoforms have a hyperglycaemic effect, CHH-B can also stimulate oocyte growth (Tensen *et al.* 1989) and CHH-A may display molt-inhibiting activity (Chang *et al.* 1991). Moreover, the effects of eyestalk ablation and implantation of thoracic/abdominal ganglia indicated the presence of a vitellogenic-stimulating hormone (Otsu 1963, Gomez & Nayar 1965, Aiken & Waddy 1980). These results, in combination with recent studies showing that CHH-A and -B mRNAs are present in parts of the nervous system other than the optic ganglia, indicate that CHH may have an additional role in the control of reproduction and molting (De Kleijn *et al.* 1995).

Studies on the (neuro)endocrine regulation of crustacean reproduction and molting in general have revealed that both processes are controlled by the interaction of several neuropeptides, juvenoids and steroids and that their control is more complex than believed previously (see reviews by Chang (1997), Charmantier *et al.* (1997) and Van Herp & Soye (1997)). The existence of several groups and isoforms of the CHH and GIH neuropeptides in lobster, in combination with the fact that the gonad-inhibiting activity of lobster GIH has only been tested in a heterologous bioassay with prawns and that lobsters seem to use CHH and/or GIH for inhibition of their molting process, suggests that further studies on the physiological role of these neuropeptides are required. We therefore investigated the synthesis, storage and release of CHH-A and -B and GIH during different stages of the female reproductive cycle in the lobster *Homarus americanus*.

Materials and Methods

Animals

Specimens of the American lobster *Homarus americanus* were obtained commercially at Miminegash, Prince Edward Island, Canada. Experimental female animals were selected in September 1993 based on their molting stage (stages C1–C4) and their carapace length (81–85 mm). As the lobster has a biannual reproductive cycle, these females would be mature and would spawn normally in the summer of 1995. However, artificial induction of spawning by the method of Waddy & Aiken (1992) permitted the different reproductive stages to be obtained

at an earlier period. The reproductive condition of each female was confirmed by examining the pleopodal cement glands in early spring 1994, and all animals selected were kept in vertically stacked tanks with plexiglass front panels so that spawning could be observed without disturbing the animals.

In March 1994, a first group of animals was put into the artificially changed environmental conditions (12 °C, 8 h light:16 h darkness) to induce spawning at the beginning of April 1994. These animals were used to study the different parameters during the reproductive stages after spawning. At the beginning of April 1994, a second group was put into the same changed environmental conditions in order to study the different parameters during maturation and the period before spawning.

The reproductive stage of each animal was determined by the method of Aiken & Waddy (1980), in which a combination of weight, colour and developmental stage of the ovary is used as the criterion.

Isolation of RNA

Total RNA was isolated by the method of Chomczynski & Sacchi (1987), using acid guanidinium thiocyanate–phenol–chloroform extraction. Medulla terminalis tissue from the two eyestalks of each animal was pooled and homogenized in an all-glass homogenizer containing 500 µl guanidinium thiocyanate solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 5% sarcosyl; 0.1 M 2-mercaptoethanol). Sequentially, 50 µl 2 M sodium acetate (pH 4.1), 500 µl water-saturated phenol and 100 µl chloroform–isoamyl alcohol (49:1) were added, the sample was mixed vigorously and the RNA was recovered after ethanol precipitation. RNA preparations were used in an RNase protection assay.

Labelling and purification of RNA probes

RNA probes were synthesized as run-off transcripts from 200 ng linearized plasmid DNA of pBluescript KS⁺ plus insert. Labelling was performed in a final volume of 10 µl containing 50 µCi [³²P]UTP, 15 units T3/T7 RNA polymerase in transcription buffer (Promega), 17.5 U RNasin (Promega) and 1 mM ribo(r)ATP, rCTP and rGTP. After an incubation of 30 min at 37 °C, the probe was separated from the template by electrophoresis on a 4% polyacrylamide gel. RNA was eluted from the gel in 500 µl 2.5 M ammonium acetate, pH 4.6, over 2 h at 55 °C and then precipitated with ethanol using 20 µg tRNA as carrier.

Quantification of CHH-A, CHH-B and GIH mRNA levels in the eyestalk by RNase protection assay

For CHH-A, CHH-B and GIH, cDNA fragments (CHH-A, nucleotides 183–395; CHH-B, nucleotides

675–865 (De Kleijn *et al.* 1995); GIH, nucleotides 527–850 (De Kleijn *et al.* 1994)) were cloned into pBluescript and used as templates for specific cRNA probes. After synthesis and purification, each cRNA probe was dissolved in the protection-assay hybridization mix (80% formamide, 400 mM NaCl, 40 mM 1,4-piperazinediethanesulphonic acid, pH 6.4, 1 mM EDTA). Separately, total RNA samples obtained from the collected eyestalks were dissolved in 23 µl protection-assay hybridization mix. After addition of 2 µl RNA probe and incubation for 10 min at 80 °C, hybridization was performed for 16 h at 55 °C. Next, 300 µl RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, 300 mM NaCl, 25 µg RNase A/ml, 500 U RNase T1/ml) was added, the mixture was incubated for 30 min at 37 °C, 8 µg/ml proteinase K and 0.1% SDS were added, and the incubation was continued for another 30 min at 37 °C. Samples were phenol-extracted, ethanol-precipitated, dissolved in 5 µl formamide loading mix (80% formamide, 1 mM EDTA, pH 8.0, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue) and loaded on to a 4% polyacrylamide gel. After electrophoresis, the gel was fixed by drying and exposed to an X-ray film using an intensifying screen at –70 °C.

The radioactivity present in the protected band was estimated by scintillation counting of the corresponding gel fragment. The amount of CHH mRNA was calculated from a dilution series of sense cRNA.

MicroHPLC analysis

Both sinus glands of one animal were dissected and collected in a glass–glass homogenizer cooled with dry ice, immediately homogenized in 350 µl 0.1 M HCl, heated at 80 °C for 5 min and then freeze-dried. Before chromatography, the dried samples were dissolved in 45 µl solvent A (0.1% trifluoroacetate in water) and centrifuged at 12 000 g for 30 min. The supernatant was then injected on to a microHPLC system (SMART, Pharmacia LKB, Uppsala, Sweden) using a reversed-phase column (type µRPC C₂/C₁₈; 2.1 × 100 mm; particle size 3 µm; gel volume 350 µl). The peptides were eluted with a gradient using solvent A and solvent B (80% acetonitrile/20% water/0.1% trifluoroacetate): 100% solvent A, 5 min; 0% B to 35% B, 5 min; 35% B to 65% B, 30 min; 65% B to 100% B, 15 min. The flow rate was 200 µl/min. UV detection was performed at 214 nm using a UV-MII µ Peak Monitor (Pharmacia LKB).

To calculate the relative amounts of the different CHH and GIH isoforms in both sinus glands of individual lobsters, the chromatographic data were evaluated with the SMART Manager 1.31 software enabling comparison and integration by area and height of the peaks corresponding to the elution time of the respective CHH and GIH isoforms. Details on the identification of the peptides

and their elution pattern in a microHPLC analysis, as used in this study, are described by Van Herp (1992) and Soyez *et al.* (1994).

Determination of CHH and GIH haemolymph levels by a double-sandwich ELISA

Haemolymph sampling Haemolymph samples were taken from the tail with a 1 ml syringe. The haemolymph was divided into five fractions of 200 µl, each diluted 1:1 with haemolymph buffer (PBS (10 mM sodium phosphate, pH 7.2, 0.1% KCl, 0.8% NaCl), 0.03% EDTA, pH 8.0, 2% BSA and 0.02% sodium azide). After centrifugation (1 min at 12 000 g), the supernatants were stored at –20 °C.

ELISA Based on earlier findings (Klein 1989), a double-sandwich ELISA was developed to measure CHH and GIH haemolymph levels during the reproductive cycle. For this purpose three antisera were used: a polyclonal rabbit anti-*Astacus leptodactylus* CHH serum (Gorgels-Kallen & Van Herp 1981), a polyclonal guinea pig anti-*Homarus americanus* CHH serum against HPLC-purified CHH (Meusy & Soyez 1991) and a polyclonal rabbit anti-*Homarus americanus* GIH serum against HPLC-purified GIH (gift from Dr Soyez, Ecole Normale Supérieure, Paris, France).

The antibodies were isolated from the serum with a Protein A-Sepharose CL-4B column equilibrated with PBS. In order to be used as secondary antibody, anti-*Homarus* CHH and anti-*Homarus* GIH were labelled with biotin using a biotinylation kit (Sigma).

The ELISA was carried out as follows. After pretreatment with 1% glutaraldehyde, microtitre plates (Nunc, Roskilde, Denmark) were coated for 16 h at 4 °C with 100 µl anti-*Astacus* CHH IgG (20 µg/ml in 0.1 M sodium phosphate buffer, pH 8.0) for the quantification of CHH or with 100 µl anti-*Homarus* GIH (20 µg/ml in 0.1 M sodium phosphate buffer, pH 8.0) for the determination of GIH. After being coated with the primary antibody, the wells were washed five times with 0.1 M sodium phosphate buffer, pH 8.0, and successively blocked with 400 µl 2% BSA in PBS, pH 7.2, for 8 h at 4 °C. After being blocked, the plates were incubated with 100 µl of each collected haemolymph sample for 16 h at 4 °C and washed seven times with 400 µl 0.1% Tween 20 in PBS (PBS-T). After addition of the respective biotinylated secondary antibody (anti-*Homarus* CHH for CHH and anti-*Homarus* GIH for GIH detection, both 5 µg/ml in PBS containing 2% BSA), the plates were incubated for 6 h at 37 °C and washed seven times with PBS-T. Finally, 100 µl streptavidin–peroxidase conjugate (1:5 dilution; Histomark KPL) was added and the sample was incubated for 1 h at 37 °C and washed seven times with 400 µl PBS-T. The enzymic reaction was then initiated by the addition of 200 µl 1,2-benzenediamine (1 mg/ml 0.1 M MacIlvaine

Table 1 Specificity and cross-reactivity of secondary antibodies

	Anti- <i>Homarus</i> GIH	Anti- <i>Homarus</i> CHH
CHH-A isoforms	±	+
CHH-B isoforms	±	+
GIH isoforms	+	—
<i>C. maenas</i> hemolymph	—	—

buffer, pH 5.2, containing 0.8 µl/ml 40% H₂O₂) and stopped by addition of 100 µl 4 N H₂SO₄. The optical density was measured at 492 nm with an EAR-400 ELISA reader.

Specificity and cross-reactivity of the antibodies To determine the specificity and cross-reactivity of the antibodies in the double-sandwich ELISA, HPLC-purified lobster GIH and CHH isoforms were used as antigens. Furthermore, to eliminate possible cross-reactions with crustacean haemolymph proteins, *Carcinus maenas* haemolymph samples were tested in the ELISA as well. Selection of crab haemolymph was based on an introductory immunochemical study (P V de Kleijn, K P C Janssen, S L Waddy, R Hegeman, W Y Lai, G J Martens & F Van Herp, unpublished observations), which evaluated the utility of antibodies to detect GIH and CHH in sinus gland material of crustaceans other than lobster. No immunoreactivity with crab samples was found, indicating that CHH and putative GIH from crab is not immunologically related to lobster CHH and GIH. Therefore possible positive immunoreactions in the cross-reactivity assay due to the presence of CHH and GIH in the blood samples could be excluded. The specificity and cross-reactivity of the *Homarus* antisera are summarized in Table 1.

These data are in agreement with the results of Meusy & Soye (1991), who show that anti-*Homarus* CHH recognizes both lobster CHH-A and -B isoforms and that anti-*Homarus* GIH also has a low immunoreactivity with both CHH isoforms. With this knowledge in mind, the ELISAs were carried out to obtain an impression of the relative variations in the total CHH and GIH in the haemolymph samples.

Results

Experimental set up

Female lobsters, in different stages of ovary development, were obtained by inducing the reproduction cycle in 70 animals. These animals were divided into ten groups and at each time point seven animals were killed. To exclude the influence of diurnal rhythms, haemolymph samples of individual animals were taken at the same time of the day. The eyestalks were then ablated for extirpation of the

X-organ and the sinus gland. Finally the ovaries were dissected and their exact developmental stage was determined using weight, colour and ovary factor (Aiken & Waddy 1980) as parameters.

The first samples were taken just before the experimental animals were placed in the environmental conditions designed to induce reproduction, 28 days before spawning. The other time points for sampling were 16 days, 7 days and 1 day before spawning, and 1 day, 3 days, 10 days, 21 days, 31 days and 40 days after spawning. The various time points were selected to represent characteristic physiological stages in the female reproductive cycle: three stages of immature ovaria (1, 3 and 10 days after spawning), three stages of previtellogenesis (21, 31 and 40 days after spawning), three stages of vitellogenesis (28, 16 and 7 days before spawning) and one mature stage (1 day before spawning).

This approach made it possible to interpret the individual variations of the data and to evaluate them statistically (Student's *t*-test) according to the different reproductive stages.

Expression, storage and release of CHH during the reproductive cycle (Fig. 1)

Figure 1 shows that the expression of CHH-A mRNA changes during the different stages of the reproductive cycle. In particular, the increase in mRNA during previtellogenesis is marked and significantly different from the levels measured for the other stages. This increase in mRNA levels is also reflected by the higher amount of CHH-A peptide stored in the sinus gland. For all stages, no differences were found between the amounts of [L-Phe³]- and [D-Phe³]-CHH-A (results not shown).

For CHH-B, a significant increase in CHH-B mRNA levels was found in the previtellogenesis and mature stages when compared with the level in the immature stage. A significant increase in the storage level of CHH-B was found during previtellogenesis. As for the CHH-A isoforms, no difference was observed between the amounts of neuropeptide isoform [L-Phe³]- and [D-Phe³]-CHH-B (results not shown).

In contrast with the mRNA and peptide storage levels, the highest amounts of total CHH (CHH-A and CHH-B) in the haemolymph were found in the mature stage. They were significantly different from those of the other three stages. Lower increasing levels could be related to the stage of vitellogenesis.

Expression of GIH during the reproductive cycle (Fig. 2)

Figure 2 shows that the level of GIH mRNA in the immature stage is low and significantly different from the higher levels during previtellogenesis and vitellogenesis. No significant differences were found in the total GIH storage levels in the sinus gland. However, the amount of

Figure 1 Levels of mRNA expression in the medulla terminalis ganglionaris X-organs, neuropeptide storage in the sinus glands, and haemolymph levels of CHH-A and CHH-B during the reproductive cycle of female American lobsters. Levels of mRNA are in percentages of the maximum value. Total storage of the CHH-A and CHH-B isoforms and total CHH haemolymph levels are in arbitrary units (AU). * indicates a significant difference of: $P < 0.02$ between CHH-A mRNA levels in the previtellogenic stage and the immature and vitellogenic stages; $P < 0.001$ between CHH-A neuropeptide levels in the previtellogenic stages and the other three stages. For CHH-B, * indicates a significant difference of: $P < 0.02$ between its mRNA levels in the previtellogenic and mature stages and the immature and vitellogenic stages; $P < 0.001$ between CHH-B neuropeptide levels in the previtellogenic stages and the other three stages. The significance between total CHH haemolymph levels (CHH-A+CHH-B) in the mature stage and the three other stages is $P < 0.005$. 1=immature; 2=previtellogenesis; 3=vitellogenesis; 4=mature.

the GIH I isoform changed dramatically in the immature stage (results not shown). During vitellogenesis, the total GIH level in the haemolymph was significantly lower than in the other stages.

Discussion

This study reveals that the changes in the levels of CHH-A and -B mRNAs and their respective amounts of stored peptide during the previtellogenic stage are not related to changes in the level of total CHH in the haemolymph. This finding indicates that there is no relation between intracellular mRNA and peptide levels and the release of the CHH peptides in the haemolymph at that stage.

Figure 2 Levels of mRNA expression in the medulla terminalis ganglionaris X-organs, neuropeptide storage in the sinus glands, and haemolymph levels of GIH during the reproductive cycle of female American lobsters. Levels of mRNA are in percentages of the maximum value. Total neuropeptide storage of the two GIH isoforms and haemolymph levels are in arbitrary units (AU). * indicates a significant difference between GIH mRNA levels in the immature stage and the pre- and vitellogenic stages ($P < 0.02$), and between GIH haemolymph levels in the vitellogenic stage and the three other stages ($P < 0.001$). 1=immature; 2=previtellogenesis; 3=vitellogenesis; 4=mature.

Whereas CHH-B mRNA and total haemolymph CHH peptide are increased in the mature stage, no differences are found in peptide storage, illustrating that synthesis and release are not related to storage level. This may be because the sinus gland can store large amounts of peptides and that only a small portion is released, as postulated by Stuenkel (1983). The increased CHH-B mRNA levels and the elevated total CHH haemolymph levels during the mature stage may suggest that CHH-B in particular is involved in oocyte maturation. The higher CHH-A and -B neuropeptide storage levels in the sinus gland during previtellogenesis than during vitellogenesis was not reflected in the total CHH content of the haemolymph and may therefore indicate that the peptides are released during late previtellogenesis or at the onset of vitellogenesis. Such a higher release of CHH-A and -B would trigger vitellogenesis during autumn and would be in agreement with the hypothesis of Waddy & Aiken (1992) that, before winter, females only rarely respond to environmental changes. However, in early November, most females respond to artificially changed day length and temperature by spawning 3 months later. In this respect, an experiment in which more precise time points are taken would give more conclusive results.

Figure 3 Diagram showing the expected CHH-A, CHH-B and GIH haemolymph levels during the biannual reproductive cycle in female lobsters *Homarus americanus* according to all our results and their interpretation. A new cycle starts when the female has spawned and incubates her eggs of the last cycle. Reading inward from the periphery, the diagram shows: the biannual cycle (outer circle) in which spawning, hatching of the 'last cycle' larvae and the consecutive molt of the adult animal are indicated by arrows; the developmental stages of the ovary (second circle); the variations in respectively the GIH, CHH-A and CHH-B titres in the haemolymph (inner circles).

To exclude the possibility that our data are due to circadian rhythmicity in the release of the neuropeptides during the reproductive cycle, total CHH, GIH and glucose levels were measured in a 24 h pilot experiment with sampling intervals of 2 h. No differences were found, indicating that there was no circadian rhythm in the release of the hormones (data not shown).

The higher haemolymph levels of GIH at the immature and previtellogenic stages are in agreement with the proposed inhibitory function of this peptide. These higher

levels may inhibit the onset of vitellogenesis, while its higher level at maturation may reflect the restart of inhibition after vitellogenesis. However, as the GIH levels are mostly high when CHH levels decrease, we cannot exclude the possibility that these large amounts of GIH at maturation are due to cross-reactivity with CHH in the ELISA (see Table 1 and Meusy & Soyeux 1991).

The results of our study did not allow us to unravel some aspects of the intracellular regulation of biosynthesis of the different neuropeptides in the same neuroendocrine cell.

For this purpose, the use of combined *in situ* hybridization and immunocytochemical studies, as described by De Kleijn *et al.* (1992), during a reproductive cycle may be more powerful.

Finally, when the presumed molt-inhibiting function of GIH (De Kleijn *et al.* 1994) and the reported MIH effects of the hyperglycaemic hormone (Chang *et al.* 1991) are taken into account, the lower blood levels of both peptides at late previtellogenesis may be responsible for induction of the molting process. Such a combined effect would be ideal for synchronization of the molt and reproductive cycles because molting is then inhibited in the immature ovary stages by GIH and in mature stages by the CHHs.

All the aforementioned interpretations of our results are visualized in Fig. 3. It illustrates the changes in haemolymph levels of CHH-A, CHH-B and GIH during a molt/reproductive cycle as we expect them based on all our results. High levels of CHH-A and -B after molting and at the onset of vitellogenesis are followed by a lower CHH level in winter after which the CHH-B level in particular increases during the last stages of vitellogenesis. The haemolymph level of GIH is mostly high when CHH is low and vice versa. Levels increase after spawning and remain high until they decrease before molting, possibly during hatching of the larvae.

In conclusion, the variations in CHH and GIH activity during the reproductive and molting cycle of the lobster suggest that both are involved in the synchronization of these two cycles. Both biological cycles are controlled by the interaction of several neuropeptides, juvenoids and steroids, as recently reviewed by Chang (1997), Charmanier *et al.* (1997) and Van Herp & Soye (1997). Therefore monitoring of complete cycles by using specific antisera against the different forms of the CHH and GIH neuropeptides should be useful in determining the precise roles of CHHs and GIH in these cycles.

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