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Structure and Significance of Mandibular Organ-inhibiting Hormone in the Crab, *Cancer pagurus*

INVOLVEMENT IN MULTIHORMONAL REGULATION OF GROWTH AND REPRODUCTION*

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Current evidence indicates that methyl farnesoate is the crustacean equivalent of the juvenile hormones of insects. This putative hormone is produced by the mandibular organs and is negatively regulated by a neuropeptide produced and secreted by the X-organ-sinus gland complex of the eyestalk. To identify this neuropeptide, a bioassay was developed which measures the inhibition of methyl farnesoate synthesis by mandibular organs exposed to fractionated sinus gland extracts from the crab, *Cancer pagurus.* Two neuropeptides, named mandibular organ-inhibiting hormones (MOIH-1 and -2) repressed methyl farnesoate synthesis. MOIH-1 was fully sequenced by automated Edman degradation of endoproteinase-derived fragments and further characterized by mass spectrometry. This peptide consisted of 78 residues (M_r 9235.6), with unblocked termini and three intrachain disulfide bridges. MOIH-2 appeared to be almost identical to MOIH-1 with the exception of a Gln for Lys substitution at position 33. Comparison with previously sequenced crustacean neuropeptides shows that these MOIHs are members of the ever expanding crustacean hyperglycemic hormone family, with significant sequence similarity to molt-inhibiting hormones (MIHs). It is possible that these two structurally similar peptides (MIH, MOIH) may control mutually exclusive physiological phenomena (somatic and gonadal growth), suggesting a complex hormonal integration of these processes in crustaceans.

It is established that the mandibular organs (MO)[1] of decapod crustaceans synthesize and secrete methyl farnesoate (MF), the unepoxidized precursor of insect juvenile hormone III (JH III) (1–4). MF has been detected in the hemolymph of many crustaceans (2, 5–7), where it appears to be carried by a specific binding protein (8–10). Nevertheless, the physiologically relevant roles of MF have not yet been unequivocally defined. By analogy to the established roles of JH III in insect reproduction (11, 12), and in an attempt to unify common themes in arthropod endocrinology, it has been suggested that MF has a key role in reproduction (13). MF biosynthesis and circulating titer have been shown to be positively correlated with ovarian maturation in some crustaceans (3, 14), and morphological changes in ovarian follicle cells reminiscent of JH-stimulated patency in insects (15), have been observed in crustaceans following injection of JH mimics (16). A further role of MF as an ecdysiotropin has been suggested, since MO homogenates profoundly stimulate molting *in vivo* (17, 18), and MF stimulates ecdysteroid synthesis *in vitro* (19).

With regard to the control of MF synthesis, it has long been known that eyestalk removal leads to a rapid hypertrophy of the MO (20, 21). This procedure also results in increases in synthesis and circulating titer of MF (3, 14), which can be reduced by injection of sinus gland extract (22). These results clearly suggest that MF synthesis is modulated by a neuropeptide produced and released from the X-organ sinus gland complex of the eyestalk. This phenomenon is particularly significant in view of the established roles of the allatostatins in repressing JH synthesis by the corpora allata in insects (23, 24). Thus, in both insects and crustaceans, juvenoid synthesis appears to be regulated by inhibitory neuropeptides. It therefore, seemed opportune, to identify the neuropeptide(s) regulating MF synthesis in crustaceans. We report here the complete amino acid sequence of a neuropeptide and a variant which profoundly inhibit MF synthesis in the edible crab *Cancer pagurus*, and demonstrate that these peptides are further members of the molt-inhibiting hormone (MIH) group within the crustacean hyperglycemic hormone (CHH) neuropeptide family. Consideration of structural and functional relationships between these neuropeptides, together with the proposed roles of MF, suggest that complex interendocrine mechanisms may control growth and reproduction in crustaceans.

EXPERIMENTAL PROCEDURES

Animals

Edible crabs, *C. pagurus*, were obtained locally from commercial fishermen and briefly stored in a recirculating seawater system under ambient light and temperature conditions prior to use.

Mandibular Organ Bioassay

MO were dissected in pairs from ice-anesthetized, intermolt (Stage C_4) female *C. pagurus* (noting stage of vitellogenesis) and preincubated on ice in a drop of modified Medium 199 (25) for 1 h. MO were subsequently cultured pairwise (control MO *versus* treated contralateral MO) in microtiter plates with 200 μl of the same medium, supplemented with 37 kBq L-[^{3}H-*methyl*]methionine (2.92 TBq/mmol; Amersham Corp.) for upto 24 h at 12 °C, removed, and rapidly frozen in liquid N_2 prior to extraction and analysis. MF was extracted from MO by homogenization in 700 μl of CH_3CN, 4% NaCl: 5/2 v/v, centrifuged (1000 × *g*, 10 min, 4 °C), and partitioned (three times) against 500 μl of hexane.

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[1] The abbreviations used are: MO, mandibular organ; MF, methyl farnesoate; CHH, crustacean hyperglycemic hormone; MIH, molt-inhibiting hormone; VIH/GIH, vitellogenesis-/gonad-inhibiting hormone; SG, sinus gland; SGE, sinus gland equivalent; JH, juvenile hormone; HPLC, high performance liquid chromatography.

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The dried hexane extract was assayed for [^{3}H]MF using HPLC and an on-line radioactivity flow monitor (Flo-one/Beta, Canberra Packard Ltd.). [12-^{3}H]all-*trans*-MF (0.19 TBq/mmol) was used as a standard. Chromatographic conditions were: Novapak C_{18} (Waters) 100 × 8-mm column, isocratic elution with 80% MeOH, flow rate, 2 ml min^{-1} (retention time (R_t) of MF, 21.6 min). The identity of the labeled product was confirmed by co-chromatography with authentic all-*trans*-MF on a silica column (4.6 × 250 mm, 5 μm; Rainin Instruments) eluted isocratically with 2% diethyl ether in 50% water-saturated hexane at 1 ml min^{-1}, by measuring radioactivity and absorbance at 214 nm (R_t of MF, 7.0 min). Under our culture conditions, very little (<5%) of the synthesized MF was released into the culture medium. To identify active fractions obtained by HPLC of sinus gland (SG) extracts (see below), fractions were pooled, initially dividing the chromatogram into six zones, dried, and reconstituted in culture medium to 2 SG equivalents (SGE)/200 μl. This material was bioassayed, using the conditions described above, but for 4-h culture periods, and the resultant active zone further fractionally assayed to determine the bioactive peptide peaks. Since the inhibition of MF synthesis was highly dependent on the vitellogenic stage of the donor crab (see "Results"), data were expressed as an "MF inhibition index," calculated as the percent inhibition of MF synthesis in the test sample divided by the mean maximum percent inhibition of MF synthesis (2SGE) for a crab at the same vitellogenic stage.

Peptide Purification and Identification

Eyestalks were removed from ice-anesthetized, intermolt (Stage C_4) *C. pagurus*. Sinus glands were dissected in batches of approximately 200, and neuropeptides were extracted in 2 M acetic acid as detailed previously (26). Extracts were reconstituted in 2 M acetic acid, centrifuged (14,000 × *g*, 5 min) and purified by HPLC. Conditions were: column, Waters μBondapak phenyl 3.9 × 300 mm; solvent A, 0.11% trifluoroacetic acid and solvent B, 60% acetonitrile containing 0.1% trifluoroacetic acid, gradient 30–80% solvent B over 60 min, 1 ml min^{-1}. Eluate was monitored at 210 nm, and fractions were collected every minute or manually for identified peptides. Fractions were immediately dried in a vacuum centrifuge and stored at −20 °C.

Peptide Sequencing

1–2-nmol portions (100–200 SG) of the most abundant, bioactive peptide (hereafter referred to as MOIH-1) were *S*-carboxymethylated with iodoacetic acid (27) and purified by HPLC (Bakerbond WP C_{18}, 4.6 × 250 mm, 40–80% solvent B over 45 min, 1 ml min^{-1}). Nanomole quantities of *S*-carboxymethylated peptide (S-CM-MOIH) were cleaved using one of the following endoproteinases: Lys-C, Asp-N, trypsin (Boehringer Mannheim, sequencing grade), purified by HPLC, and small portions of cleaved peptides analyzed for amino acid composition, using reported procedures (28). Material of high yield and unequivocal amino acid composition was sequenced on an automated pulsed liquid-phase sequencer (Applied Biosystems 471A). Additionally, 1–2 nmol samples of S-CM-MOIH-1 and -2 were N-terminally sequenced to the limits of unambiguous residue assignment (residue 32).

Mass Spectrometry

Molecular weight determinations from 100–500 pmol quantities of peptide fragments generated by Lys-C endoproteinase digestion, native MOIH-1 and -2, and S-CM-MOIH-1 were obtained using a variety of MS techniques.

Electrospray MS—Samples dissolved in 400 μl of 50% CH_3CN containing 0.1% HCOOH were introduced into a VG Quattro MS at 10 μl min^{-1} using a Waters 600MS HPLC system. Samples were analyzed in the positive ion mode (source temperature 70 °C; capillary voltage, 3.53 kV; lens voltage, 0.13 kV; scan range, *m/z* 500-2000; calibration with equine heart myoglobin).

Matrix-assisted Laser Desorption Ionization-MS—Sample was dissolved in 50% CH_3CN and added to an equal volume of matrix solution (1% α-cyano-4-hydroxycinnamic acid in 50% CH_3CN containing 1% citric acid). 1 μl of this mixture was analyzed using a Vestec Laser desorption MS (acceleration voltage, 25 kV; detector voltage, 3.4 kV; laser at 6% maximum; calibration with insulin internal standard).

Fast Atom Bombardment-MS—Samples were analyzed on (*a*) a VG Quattro MS (positive ion mode; acceleration voltage, 5 kV, cesium ion gun, 25 kV; mass range, 800-3500 Da; sample was dissolved in 50% CH_3CN, 2 μl mixed with an equal volume of 3-nitrobenzyl alcohol matrix and smeared on the probe tip), (*b*) a VG Autospec MS (positive ion mode, acceleration voltage 8 kV; cesium ion gun, 8 kV; mass range, 4500 Da); sample was dissolved in 50% CH_3CN, 2 μl mixed with an equal volume of glycerol/thioglycerol matrix, including 0.5 μl of 1% HCl and smeared on the probe tip.

MIH Bioassay

Pooled fractions from HPLC purifications of sinus glands, containing material previously determined as MOIHs, and material identified as MIH (29), were assayed at an approximate dose of 0.75 SGE, using an *in vitro* Y-organ bioassay (30), but pairs of halved Y-organs were used

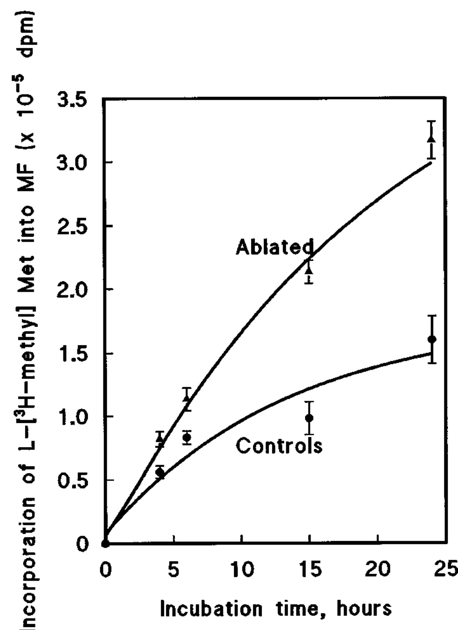

FIG. 1. **Time course of incorporation of [^{3}H-*methyl*]methionine by MO into MF for eyestalk-ablated and intact crabs (controls).** *n* = 8–10 MO pairs for each point); *Bars* = S.E. Lines of best fit were calculated using a curve fitting program (Fig.P, Elsevier Biosoft).

TABLE I
Incorporation of L-[^{3}H-methyl]methionine into MF by MO pairs

Pairs of MO (L,R) were incubated for between 4–24 h at 12 °C in culture medium supplemented with 37 kBq L-[^{3}H-*methyl*]methionine. Incorporation of radiolabel into MF was measured as detailed in the text. Four to five pairs of MO were used for each experiment. Eyestalks were ablated 24 h prior to experiments. Values are means ± S.E.

	Incubation time							
	4 h		6 h		15 h		24 h	
Intact crabs								
Replicates	5		5		5		3	
	L	R	L	R	L	R	L	R
Conversion to MF, dpm × 10^{-3}	52.3 ± 6.7	57.8 ± 5.5	67.1 ± 14.3	81.0 ± 9.4	75.7 ± 24.7	89.8 ± 9.1	167.1 ± 8.9	160.3 ± 31.0
Eyestalk-ablated crabs								
Replicates	5		5		4		5	
	L	R	L	R	L	R	L	R
Conversion to MF, dpm × 10^{-3}	68.8 ± 10.1	67.4 ± 19.9	113.8 ± 11.0	113.0 ± 15.6	219.7 ± 11.4	207.4 ± 15.6	370.0 ± 24.7	379.3 ± 24.3

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TABLE II
Inhibition (percent) of MF synthesis in MO by sinus gland extract: effect of donor vitellogenic stage upon inhibition of MF synthesis

MO pairs from early, mid-, and late vitellogenic crabs (three to five pairs at each stage) were cultured for 4 h using the bioassay procedures detailed in the text. Treated MO were exposed to 2 SGE of a crude sinus gland extract. Values are means ± S.E.

	Mean ± S.E.	*n*
Early vitellogenic ovary	89.7 ± 6.1	5
Mid-vitellogenic ovary	53.4 ± 11.7	5
Late vitellogenic ovary	17.4 ± 8.7	3

instead of the pairwise comparison originally described. Ecdysteroid radioimmunoassay (31) used an antiserum (Code H2A) raised against the 22-succinyl derivative of ecdysone (32).

RESULTS

During culture periods between 4 and 24 h, MO synthesized MF in an asymptotic manner, and it was significant to note that in animals that had been eyestalk-ablated 24 h prior to assay, MF biosynthesis more than doubled compared with eyestalk-intact animals when MOs were cultured over a 24-h assay period (Fig. 1). Analysis of MF synthesis by pairs of glands over this period (Table I) showed that MO pairs taken from eyestalk-ablated crabs produced very similar quantities of MF; pairwise comparison by matched-paired *t* test showed no significant difference in means between MO pairs and highly significant pairing correlations (0.8–0.99). For eyestalk-intact crabs, rather greater variation was evident. Nevertheless, significant differences in MF synthesis could not be observed between means of replicates of gland pairs. Thus, since pairs of MO produce similar quantities of MF, this assay could be used for the further identification of MOIH, where one gland acted as a control and the contralateral gland as a treatment. When MO were exposed to crude sinus gland extract, MF synthesis was profoundly inhibited, but an intriguing finding was that the degree of inhibition was highly dependent on the stage of vitellogenesis of the MO donor; maximal in early vitellogenic animals and minimal in animals that had completed vitellogenesis (Table II). Since vitellogenic crabs were found throughout the year, the (preferable) use of only early vitellogenic crabs would have been impracticable in view of the much larger numbers required. Thus, inhibition of MF synthesis was calculated as an index as detailed above. Since MO rapidly converted significant amounts of radiolabeled methionine to MF, the incubation period for the bioassay was usually 4 h; during this period MO from eyestalk-ablated donors incorporated around 80,000 dpm of radiolabel from L-[^{3}H-*methyl*]methionine to MF (Fig. 1, Table I). Dose-response experiments demonstrated dose dependence of MF synthesis to inhibitory effects of various amounts of SG extracts (Fig. 2), although considerable variability in inhibition was observed at doses below 0.05 SGE.

Bioassay of HPLC-purified SG showed that two prominent, closely eluting neuropeptides (MOIH-1 and -2) inhibited MF synthesis (Fig. 3). Since MOIH-1 was at least twice as abundant in the SG (approximately 10 pmol/SG) compared with MOIH-2, this material was first fully sequenced. Digestion of peptide with Lys-C endoproteinase gave highly reproducible cleavage patterns (Fig. 4), and the fragments were sequenced by Edman degradation and their M_r determined by mass spectrometry (Table III). In every instance, agreement was obtained between masses predicted by sequence and mass spectrometric analysis, within the limits of accuracy of the latter techniques. Overlaps were obtained by selective sequencing of peptide fragments obtained by digestion with Asp-N, and trypsin, and from N-terminal sequencing of S-CM-MOIH-1, as detailed in Fig. 5. The predicted molecular weights of native and S-CM-MOIH-1 were in complete agreement with mass spectrometric determinations (Table III).

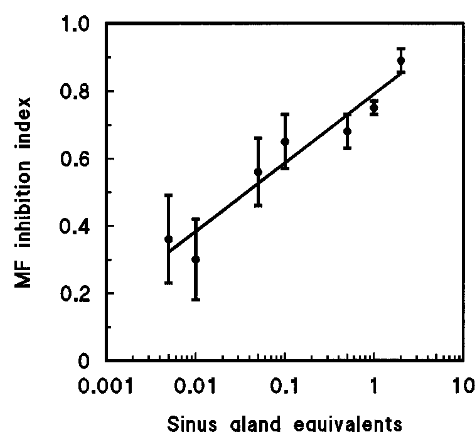

FIG. 2. **Dose-response relationship showing inhibition of MF synthesis in MO exposed to homogenates of SG (from intermolt crabs).** MO were taken from intermolt crabs previously eyestalk-ablated (24 h) and cultured for 4 h. *n* = 5–9 MO pairs for each point. *Bars* = S.E.

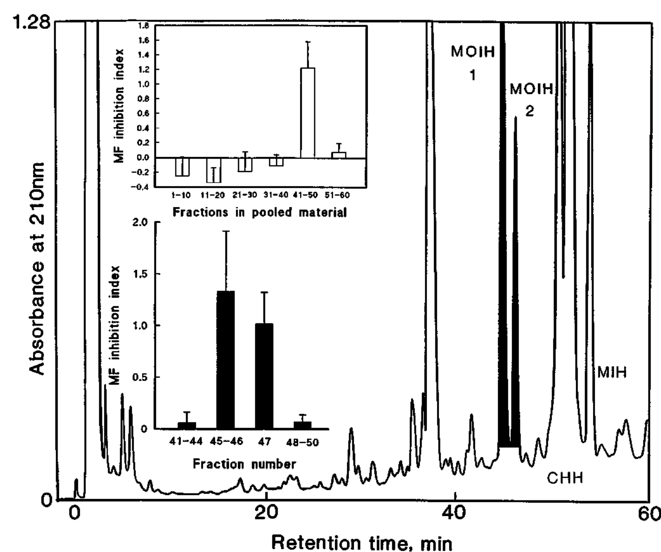

FIG. 3. **HPLC profile of 215 *C. pagurus* SG.** Chromatographic conditions were: column, Waters μBondapak phenyl, 3.9 × 300 mm; solvent A, 0.11% trifluoroacetic acid; solvent B, 60% acetonitrile containing 0.1% trifluoroacetic acid, gradient 30–80% solvent B over 60 min, 1 ml min^{-1}. The *upper inset* shows bioassay results from pooled fractions (6) covering the entire chromatogram. The *lower inset* shows bioassay results for fractions within the active zone (tubes 41–50). *n* = 6–8 pairs of MO for each assay. MO were taken from intact crabs and cultured for 4 h. *Bars* = S.E. Active peaks (MOIH-1 and -2) are *shaded*. Other peptides (*CHH, MIH*) are indicated.

MOIH-2 displayed an amino acid composition very similar to that of MOIH-1 (Table IV). Within the limits af accuracy of the technique used, the only significant difference was a molar excess of glutamic acid and deficit of lysine, and the estimated molecular weight was very similar (9236.9 and 9235.6 for MOIH-2 and -1, respectively, accurate by ES/MS to ~1 Da). N-terminal sequencing showed that the first 32 amino acids were identical. Concurrent digestions of both (*S*-carboxymethylated) peptides, followed by HPLC (Fig. 4) and amino acid analysis (data not shown), demonstrated that fragments L2, L3, and L5 were most likely identical for both peptides. A single amino acid difference at position 33 from lysine to glutamate or glutamine would account for the differences in amino acid composition of the complete peptide and the cleavage pattern after Lys-C digestion of MOIH-2 (absence of L1 and L4, presence and amino acid composition of L1 and L4). Limited se-

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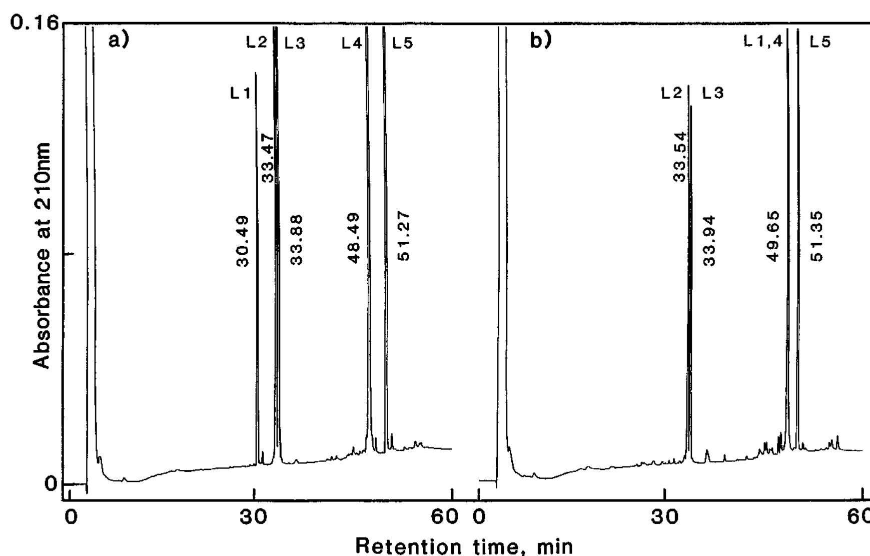

FIG. 4. **HPLC profiles of Lys-C digestions of S-CM-MOIH-1 (*a*) and -2 (*b*).** Chromatographic conditions were: Bakerbond WP C_{18} (4.6 × 250 mm) column, gradient elution 10–80% solvent B over 1 h, flow rate 1 ml min^{-1} (solvents were as detailed in the text). Retention times are given on the peaks. Identification codes are referred to in text.

TABLE III
*Mass spectrometry of endoproteinase-derived fragments of MOIH-1, native MOIH-1 and -2, and S-*carboxymethylated MOIH-1

100–500 pmol of peptide was used for each measurement. S-CM-, *S*-carboxymethyl; **C** = S-carboxymethylcysteine; L1–L5, Lys-C-derived fragments of S-CM-MOIH-1; FAB, fast atom bombardment-MS; MALDI, matrix-assisted laser desorption ionization-MS; ES, electrospray MS; ND, not determined.

Code	Sequence	Calculated M_r	(FAB) MALDI	ES
L1	D**C**ANIFRK	1024.3	(1023.5)	1023.9
L2	RRINNDD**C**QNIFGNRAMYEK	2400.7	ND	2398.9
L3	VDWI**C**K	820.9	(820.6)	820.4
L4	DGLLNN**C**RSN**C**FYNTEFLW**C**IDATENTRNK	3730.0	ND	3729.1
L5	EQLEQWAAILGAGWN	1685.9	1685.7	ND
S-CM-MOIH-1		9589.7	ND	9589
MOIH-1		9235.6	9238	9235.6
MOIH-2		9235.7	ND	9236.9

quencing of L1 and L4 confirmed that residue 33 of MOIH-2 was glutamine rather than glutamate.

HPLC fractions containing both MOIHs and those containing MIH were assayed for MIH activity (repression of ecdysteroid synthesis by Y-organs *in vitro*) at an approximate dose of 0.75 SGE. MIH repressed ecdysteroid synthesis to the maximum possible extent in this assay (mean ± S.E.: 61.85 ± 4.4% repression, n = 11), while MOIH showed much less activity, with considerable variability in response (24.75% ± 14.7%, n = 10).

DISCUSSION

This investigation has identified a neuropeptide from the sinus gland of *C. pagurus*, which represses synthesis of the crustacean juvenile hormone, MF, by the mandibular organ and is, thus, named MOIH. With respect to the action of this neuropeptide, two features are of interest. First, the synthesis of MF was higher in eyestalk-ablated crabs compared with controls, suggesting that the synthesis of MF is continuously modulated by MOIH and that the MO are rapidly activated in the absence of the inhibitory influence of this hormone. A similar phenomenon has been reported for the spider crab, *Libinia emarginata* (3), and the lobster, *Homarus americanus* (33). Second, the degree of inhibition of the MO by MOIH is related to the vitellogenic stage of the MO donor crab, being maximal during early stages of vitellogenesis and minimal in animals that had completed vitellogenesis.

Two SG neuropeptides, MOIH-1 and -2 inhibited MF synthesis *in vitro*. The former was fully sequenced and was found to be a 78 residue peptide (average M_r 9235.6), with unblocked N and C termini. Consideration of sequence and mass spectrometric results suggested three intrachain disulfide bridges. Although MOIH-2 was not completely sequenced, the first 32 residues were identical, and Lys-C digestions, amino acid analysis of fragments, limited sequencing of L1 and L4 and molecular weight determination demonstrated that Lys was replaced by Gln at position 33. This difference could be accounted for by a single base substitution (A-C). While this might suggest allelic polymorphism, preliminary results obtained from HPLC analysis of SG peptides from individual crabs show that both MOIHs are always present.

The sequence of MOIH clearly shows significant similarities (50–60%) to members of the CHH group and in particular to members of the MIH and vitellogenesis-inhibiting hormone/gonad-inhibiting hormone (VIH/GIH) neuropeptides (Fig. 6). While an early study to identify neuropeptides involved in regulation of MF synthesis suggested that the chromatophorotrophins, red pigment-concentrating hormone and pigment-dispersing hormone, respectively, stimulated and inhibited MF synthesis in the crayfish *Procambarus clarkii* (34), we have been unable to confirm this result in *C. pagurus*. However, recent studies using *P. clarkii* (35) have indicated that a CHH-like molecule inhibits MF synthesis in this species, a result at least reconcilable with our results. Furthermore, a recent preliminary report[2] has indicated that in the spider crab, *L. emarginata*, three peptides that inhibit MF synthesis, and that also have hyperglycemic activity, have been isolated; the primary structure of one of the peptides indicates that it is a CHH-like molecule. In *C. pagurus,* material identified by homologous bioassay as CHHs elute in HPLC separations of SGs as two prominent, incompletely separated peaks with retention times

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of around 50 min. While CHH is moderately active in repressing ecdysteroid synthesis by the Y-organs, in addition to MIH, in *C. pagurus* (29) and *Carcinus maenas* (26), it should be stressed that neither of these peptides exhibited any inhibitory activity on MO *in vitro* (see Fig. 3). Thus, in *C. pagurus* it is clear that CHH does not act as an MOIH and that MF synthesis is inhibited by distinct MOIHs.

In a broader context, it is of interest to note that the MOIH neuropeptides identified in this study, and those inferred from studies on *P. clarkii* (34) and *L. emargina¹ta*[2] show no sequence similarities with the allatostatins identified in insects (23, 24). Thus, an important dichotomy in neurohormonal control of juvenoid synthesis (JH, MF) in arthropods is now evident.

The relationship between MIH and MOIH is of particular interest, since these neuropeptides, respectively, inhibit ecdysteroid (26) and juvenoid synthesis. HPLC fractions corresponding to MIH were inactive in the MOIH bioassay, and MOIH had a somewhat limited and variable activity in the MIH bioassay. Thus, despite sequence similarities, MIH and MOIH appear to be functionally distinct. Nevertheless, consideration of a recent report (19), which demonstrated that ecdysteroid synthesis by Y-organs of *Cancer magister* could be promoted in the presence of MF- or MO-conditioned medium, in addition to reports which clearly demonstrate that injection of MO homogenates profoundly accelerate molting *in vivo* by heterologous bioassay in a variety of crustaceans (17, 18), suggest that MF may generally act as an ecdysiotropin. Whilst the direct inhibitory influence of MIH and CHH on Y-organs is well established (for a recent review, see Ref. 36), our findings clearly show that MOIH represses MF synthesis. Thus, considering the reports detailing the ecdysiotropic ativity of MF, it seems entirely reasonable to suggest that synthesis of ecdysteroids by the Y-organ, and consequently molting, may be directly controlled by MIH and CHH, and indirectly by MOIH, clearly suggesting complex, essentially inhibitory regulatory mechanisms, without precedent in the arthropods.

With respect to establishing physiologically relevant roles for

[2] L. Liu and H. Laufer (1995) Poster presented at the Sixth International Conference on the Juvenile Hormones, Woods Hole, MA (September 10–15, 1995).

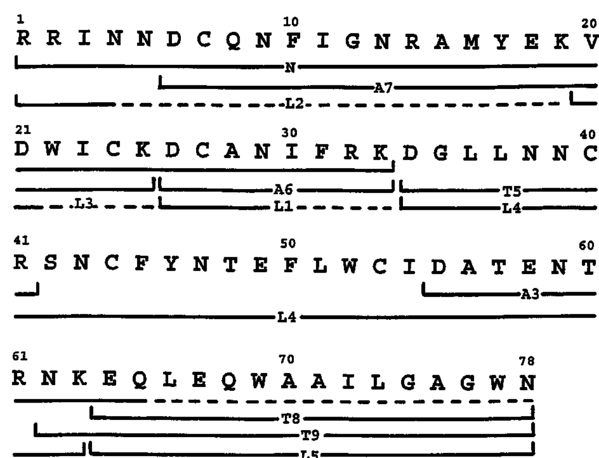

FIG. 5. **Amino acid sequence of MOIH-1.** *Solid lines* refer to completely sequenced material and *solid* and *dashed lines* to sequences inferred by partial sequencing and amino acid analysis. L1–L5 = fragments from Lys-C digestion; A3, A6, and A7 = fragments from Asp-N digestion; T5, T8, and T9 = fragments from trypsin digestion, *N* = N-terminally sequenced fragment.

TABLE IV
Amino acid analysis of MOIH-1 and -2

200–500 pmol of peptides were hydrolyzed in 6 M HCl in the gas-phase for 1 h at 150 °C. Dried hydrolysates were analyzed by *o*-phthaldialdehyde precolumn derivatization and HPLC. Left column refers to amino acid composition derived from sequence. ND, not determined.

Amino acid	MOIH-1 sequence	MOIH-1 analysis	MOIH-2 analysis
Asx	17	14.8	15.0
Glx	8	8.7	9.7
½Cys	6	ND	ND
Ser	1	1.3	1.5
Pro	0	ND	ND
His	0	0	0
Arg	6	6.3	6.3
Gly	4	5.1	5.1
Thr	3	3.0	3.1
Ala	6	6.0	6.1
Tyr	2	2.2	2.4
Trp	4	ND	ND
Met	1	2.2	1.8
Val	1	1.5	1.7
Phe	4	4.0	3.8
Ile	6	6.2	6.1
Leu	5	5.6	6.1
Lys	4	4.4	3.5

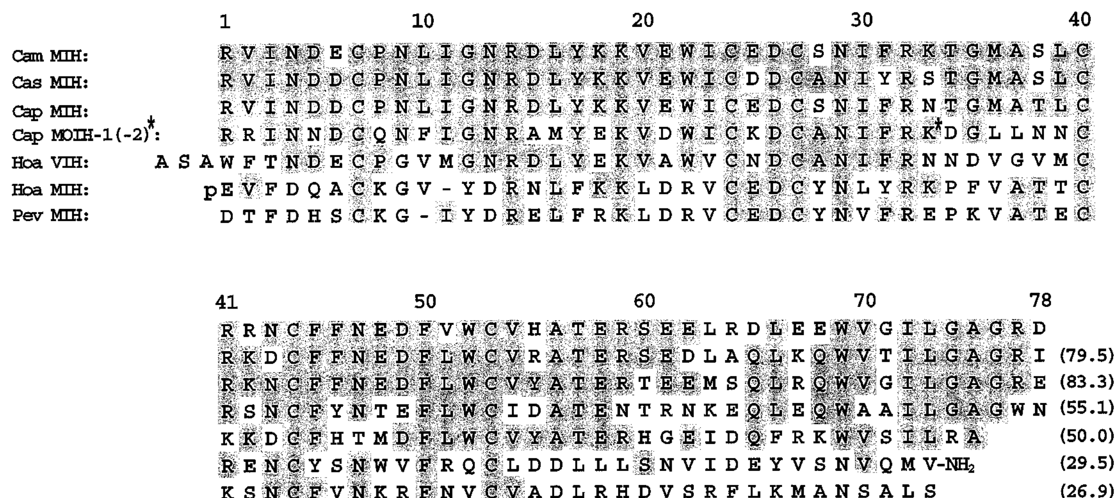

FIG. 6. **Sequence alignment and comparison of MOIH-1 and -2 with MIH neuropeptides.** *Shading* indicates identical residues. Abbreviations and references: *Cam MIH* = *C. maenas* MIH (28), *Cas MIH* = *Callinectes sapidus* MIH (42), *Cap MIH* = *C. pagurus* MIH (29), *Hoa VIH* = *H. americanus* VIH (40, 43), *Hoa MIH* = *H. americanus* MIH (27), *Pev MIH* = *Penaeus vannamei* MIH-like peptide (44). Sequence similarities (percent), compared with the archetype MIH (*Carcinus*) are shown in *brackets*. Gaps (–) have been introduced to maximize sequence similarity. The *asterisk* indicates substitution of K in MOIH-1 by Q in MOIH-2.

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MOIH in the control of reproduction, the somewhat equivocal role of MF in this process is of critical importance. Whilst the balance of evidence suggests that MF has a key role (see Ref. 13 for review) in stimulating gonadal development, and in view of correlative relationships between MF titer and ovarian maturation in some crustaceans (3, 14), firmly established roles for MF in defined physiological processes during gonadal maturation remain to be defined, in contrast to well established roles of JH III in insect reproduction (37). Despite such shortcomings, two aspects of this study may relate to the proposed involvement of MF and MOIH in reproduction. First, the variation in inhibitory action of MOIH, which was found to be highly dependent on the ovarian stage of the donor. For crabs in very early vitellogenic stages, MOIH inhibition was maximal, and the inhibitory activity of MOIH declined quite markedly during subsequent stages of vitellogenesis. This phenomenon suggested that MOs become insensitive to MOIH during vitellogenesis. A further observation that may be of consequence with respect to the initiation of vitellogensis is that a marked, but transient, rise in MF levels is seen in *C. pagurus* hemolymph at the onset of secondary vitellogenesis (38). The second observation relates to the similarity of structure between MOIH and the VIH/GIH. A classical tenet in crustacean endocrinology concerns the action of VIH. This sinus gland neuropeptide apparently inhibits vitellogenesis, as evidenced by many studies reporting accelerated gonadal development in sexually mature eyestalk-ablated female crustaceans (for review, see Ref. 39). The only fully characterized (sequenced) VIH (40) was identified from *H. americanus* sinus glands using a heterologous *in vivo* bioassay (41). Thus, it is entirely feasible that the action of this hormone was indirect in that its action could involve repression of MF synthesis in the donor, *i.e.* VIH/GIH is equivalent to MOIH. Indeed, the evident sequence similarity between MOIH and VIH might support this view.

While this study has identified new peptides, which are likely to be of key importance in controlling reproduction and growth, it should be stressed, if further reiteration is indeed necessary, that very little is known about the physiological significance of any of the CHH or MIH group peptides, and it is evident that complex interendocrine interactions will be discovered. We are currently continuing our work on MOIH, MIH, and MF using molecular, biochemical, immunological, and physiological approaches to address these problems.

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REFERENCES

1. Laufer, H., Borst, D., Baker, F. C., Carrasco, C., Sinkus, M., Reuter, C. C., Tsai, L. W., and Schooley, D. A. (1987) *Science* **235,** 202–205
2. Borst, D. W., Laufer, H., Landau, M., Chang, E. S., Hertz, W. A., Baker, F. C., and Schooley, D. A. (1987) *Insect Biochem.* **17,** 1123–1127
3. Laufer, H., Landau, M., Homola, E., and Borst, D. W. (1987) *Insect Biochem.* **17,** 1129–1131
4. Ding, Q., and Tobe, S. S. (1991) *Insect Biochem.* **21,** 285–291
5. Laufer, H., and Borst, D. W. (1988) in *Invertebrate Endocrinology, Endocrinology of Selected Invertebrate Types* (Laufer, H., and Downer, R. G. H., eds) Vol. 2, pp. 305–313, A. R. Liss Inc., New York
6. Borst, D., and Laufer, H. (1990) in *Recent Advances in Comparative Arthropod Morphology and Development: Morphogenetic Hormones of Arthropods* (Gupta, A. P., ed) pp. 35–60, Rutgers University Press, New Brunswick, NJ
7. Sagi, A., Homola, E., and Laufer, H. (1991) *Comp. Biochem. Physiol.* **99B,** 879–882
8. Prestwich, G. D., Bruce, M. J., Ujváry, I., and Chang, E. S. (1990) *Gen. Comp. Endocrinol.* **80,** 232–237
9. Li, H., and Borst, D. W. (1991) *Gen. Comp. Endocrinol.* **81,** 335–342
10. King, L. E., Ding, Q., Prestwich, G. D., and Tobe, S. S. (1995) *Insect Biochem. Mol. Biol.* **25,** 495–501
11. Koeppe, J. K., Fuchs, M., Chen, T. T., Hunt, L. M., Kovalick, G. E., and Briers, T. (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G. A., and Gilbert, L. I., eds) Vol. 8, pp. 165–204, Pergamon Press, Oxford
12. Riddiford, L. M. (1994) *Adv. Insect Physiol.* **24,** 213–274
13. Laufer, H., Ahl, J. S. B., and Sagi, A. (1993) *Am. Zool.* **33,** 365–374
14. Laufer, H., Landau, M., Borst, D. W., and Homola, E. (1986) in *Advances in Invertebrate Reproduction 4* (Andries, J. C., and Dhainaut, A., eds) pp. 135–143, Elsevier Science Publishers B. V., Amsterdam
15. Sevala, V. L., and Davey, K. G. (1989) *Experientia* (*Basel*) **45,** 355–356
16. Hinsch, G. W. (1981) *Int. J. Invertebr. Reprod.* **3,** 237–244
17. Taketomi, Y., and Kawano, Y. (1989) *Cell Biol. Int. Rep.* **9,** 1069–1074
18. Yudin, A. I., Diener, R. A., Clark, W. H., Jr., and Chang, E. S. (1980) *Biol. Bull.* **159,** 760–772
19. Tamone, S. L., and Chang, E. S. (1993) *Gen. Comp. Endocrinol.* **89,** 425–432
20. Bazin, F. (1976) *C. R. Acad. Sci.* (*Paris*) **282D,** 739–741
21. Hinsch, G. W. (1980) *Trans. Am. Microsc. Soc.* **99,** 317–322
22. Tsukimura, B., and Borst, D. W. (1992) *Gen. Comp. Endocrinol.* **86,** 297–303
23. Tobe, S. S., Yu, C. G., and Bendena, W. G. (1994) in *Perspectives in Comparative Endocrinology* (Davey, K. G., Peter, R. E., and Tobe, S. S., eds) pp. 12–19, National Research Council of Canada, Ottawa
24. Stay, B., Tobe, S. S., and Bendena, W. G. (1994) *Adv. Insect Physiol.* **25,** 268–337
25. Brody, M. D., and Chang, E. S. (1989) *Invertebr. Reprod. Dev.* **16,** 141–147
26. Webster, S. G., and Keller, R. (1986) *J. Comp. Physiol. Biochem. Syst. Environ. Physiol.* **156,** 617–624
27. Chang, E. S., Prestwich, G. D., and Bruce, M. J. (1990) *Biochem. Biophys. Res. Commun.* **171,** 818–826
28. Webster, S. G. (1991) *Proc. R. Soc. Lond. B* **244,** 247–252
29. Chung, J. S., Wilkinson, M. C., and Webster, S. G. (1996) *Neuropeptides* **30,** 95–101
30. Webster, S. G. (1986) *Gen. Comp. Endocrinol.* **61,** 237–247
31. Borst, D. W., and O'Connor, J. D. (1974) *Steroids* **24,** 637–657
32. Horn, D. H. S., Wilkie, J. S., Sage, B. A., and O'Connor, J. D. (1976) *J. Insect Physiol.* **22,** 901–905
33. Borst, D. W., Tsukimura, B., Laufer, H., and Couch, E. F. (1994) *Biol. Bull.* **186,** 9–16
34. Landau, M., Laufer, H., and Homola, E. (1989) *Invert. Reprod. Dev.* **16,** 165–168
35. Laufer, H., Lui, L., and Van Herp, F. (1994) in *Insect Neurochemistry and Neurophysiology* (Borkovec, A. J., and Loeb, M. J., eds) pp. 203–206, CRC, Boca Raton, FL
36. Webster, S. G. (1996) in *Marine Biology-A Port Erin Perspective* (Hartnoll, R. G., and Hawkins, S. J., eds) Immel, London, in press
37. Wyatt, G. R., Braun, R. D., Edwards, G. C., Glinka, A. N., and Zhang, J. (1994) in *Perspectives in Comparative Endocrinology* (Davey, K. G., Peter, R. E., and Tobe, S. S., eds) pp. 202–208, National Research Council of Canada, Ottawa
38. Wainwright, G. (1995) *Hormonal Control of Ovarian Development in the Edible Crab Cancer pagurus.* Ph.D. thesis, University of Liverpool
39. Meusy, J.-J., and Payen, G. G. (1988) *Zool. Sci.* **5,** 217–265
40. Soyez, D., Le Caer, J.-P., Noël, P. Y., and Rossier, J. (1991) *Neuropeptides* **20,** 25–32
41. Soyez, D., Van Deijnen, J.-E., and Martin, M. (1987) *J. Exp. Zool.* **244,** 479–484
42. Lee, K. J., Elton, T. S., Bej, A. K., Watts, S. A., and Watson, R. D. (1995) *Biochem. Biophys. Res. Commun.* **209,** 1126–1131
43. De Kleijn, D. P. V., Sleutels, F. J. G. T., Martens, G. J. M., and Van Herp, F. (1994) *FEBS Lett.* **353,** 255–258
44. Sun, P. S. (1994) *Mol. Mar. Biol. Biotechnol.* **3,** 1–6

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