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A Senior Research Honors Paper Presented
by

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Abstract

Methyl farnesoate (MF) is a crustacean compound that is structurally similar to juvenile hormone, a substance that regulates insect growth and reproduction. This similarity has led to the suggestion that MF may have juvenile hormone-like effects in crustaceans. If this is so, regulation of MF levels might be an important mechanism in the control of crustacean molting and reproduction. The understanding of, and ability to manipulate, the regulation of MF production could be a powerful tool in the aquaculture of economically important crustaceans.

In the lobster *Homarus americanus*, MF synthesis occurs in the mandibular organ (MO). *In vivo* studies have shown that MF synthesis is negatively regulated by the sinus gland (SG), a structure located in the crustacean eyestalk and known to produce neuropeptides. To determine the effect of SG peptides on MF synthesis, fragments of MO tissue were incubated in culture medium supplemented with radiolabeled methionine (a precursor of MF). I used this *in vitro* bioassay to compare the activity of a crude SG extract with that of two peptides purified from the extract. The crude SG extract produced a substantial decrease in MF synthesis by MO fragments when compared with controls. One of the purified peptides also inhibited MF synthesis, but the other had no effect. The data suggest that the first peptide is the MOIH (mandibular organ inhibiting hormone) for the lobster. However, its inhibitory effect on MF production is not as complete as that of the crude SG extract, suggesting that this peptide may act synergistically with another compound from the SG to produce full inhibition.

Another compound assayed for its effects on MF production was serotonin, and this biogenic amine had no inhibitory effect.

Introduction

Regulation of reproduction and metamorphosis in insects

The reproduction and metamorphosis of all arthropods is controlled by a number of endocrine compounds. These hormones circulate in the hemolymph (blood in an open circulatory system) and serve as chemical messengers between organs. In insects, two groups of hormones have central roles in both reproduction and metamorphosis: the ecdysteroids and the juvenile hormone (JH) analogues. Ecdysteroids are steroid hormones produced by the prothoracic glands, paired organs found in close association with the brain. JH is a sesquiterpenoid hormone produced by the corpora allata, another pair of endocrine glands close to the brain.

Insects grow by a process called molting. During molting, the older exoskeleton is shed and replaced by a newer exoskeleton. This periodic shedding of the exoskeleton results in a stepwise pattern of growth. Metamorphosis, which involves gross morphological changes (e.g., from larva to adult) occurs at one of these molts and is therefore partly controlled by ecdysteroids (Hampshire and Horn, 1966).

Ecdysteroids control ecdysis (the replacement of the exoskeleton at molting) and promote advancement to the next developmental stage. In doing so, these steroids stimulate the expression of genes for this next developmental period. In contrast, JH regulates the type (developmental stage) of exoskeleton made at the time of the molt and the presence of JH above a threshold titer promotes retention of the current developmental stage (Laufer et al., 1986). JH also plays a role in the endocrine regulation of reproduction in insects (Laufer et

al., 1986). In female insects, JH stimulates separation of the new follicles from the germarium, previtellogenic growth of the oocytes, and vitellogenesis (production of this yolk protein). In males, JH stimulates spermatogenesis, sexual maturation, and mating activity. These roles of JH can be demonstrated by removing the corpora allata, which inhibits oogenesis in most species as well as male reproductive activity. Treatment with JH or an analogue of JH can restore these activities to individuals lacking a corpora allata (Raabe, 1989).

The key factors in the control of the circulating titer of JH are the rate of its production by and immediate release from the corpora allata. However, control of JH synthesis is neither well understood, nor similar among all insect species studied. In many species, the corpora allata are directly connected to the brain by neurosecretory axons. In addition, the corpora allata appear to be regulated by other hormones in the hemolymph. The level of JH in the hemolymph is reduced and further regulated by esterases which break down the MF. The complete removal of JH from the hemolymph appears to be necessary in many species for metamorphosis to occur.

Regulation of reproduction and metamorphosis in crustaceans

Ecdysteroids and JH-like hormones are also found in crustaceans. Because of the close phylogenetic relationship of insects and crustaceans, it is not surprising to find that they share similar hormone systems. Ecdysteroids have been detected in all crustaceans studied and are produced by the Y-organs, paired endocrine glands found in close association with the brain. The major ecdysteroid in both insects and crustaceans is 20-OH ecdysone (Laufer et al., 1986). A compound that is chemically related to JH, methyl farnesoate

(MF), has also been detected in crustaceans (Laufer et al., 1987a). This sesquiterpenoid is similar to JH III, an analogue of JH, except that MF contains a double bond rather than an epoxide between carbons 10 and 11 (Figure 1) (Laufer et al., 1987a). Crustacean hemolymph contains MF levels that are similar to the JH levels found in insect hemolymph (Laufer et al., 1987a). While the exact functions of MF are not yet known, it is produced by the mandibular organs (MOs), a pair of glands that resemble the insect corpora allata (Borst and Laufer, 1990; Laufer et al., 1986).

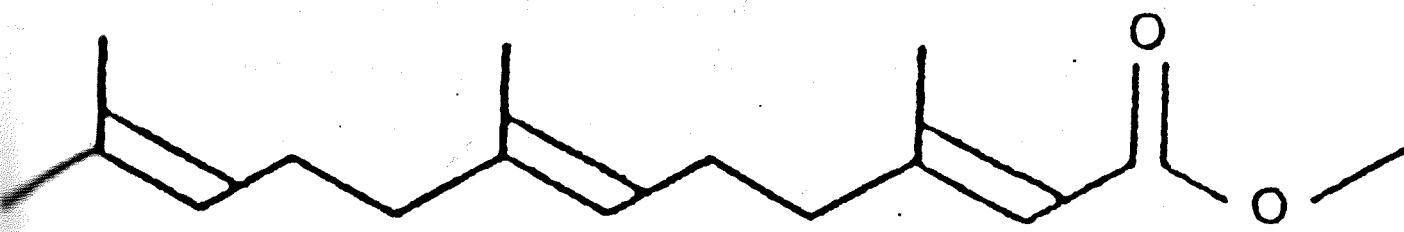
The presence of MF delays metamorphosis in the larvae of the lobster, *Homarus americanus* (hereafter referred to as *Homarus*), as JH does in insects (Borst et al., 1987). MF synthesis is correlated with vitellogenesis and the ovarian cycle. High levels of MF synthesis coincide with periods of active vitellogenesis (Laufer et al., 1987b). Also, ovarian growth is promoted by the implantation of MOs (Borst et al., 1987b). MF also seems to stimulate reproductive behavior in males (Laufer et al., 1993).

Regulation as a way to understand function

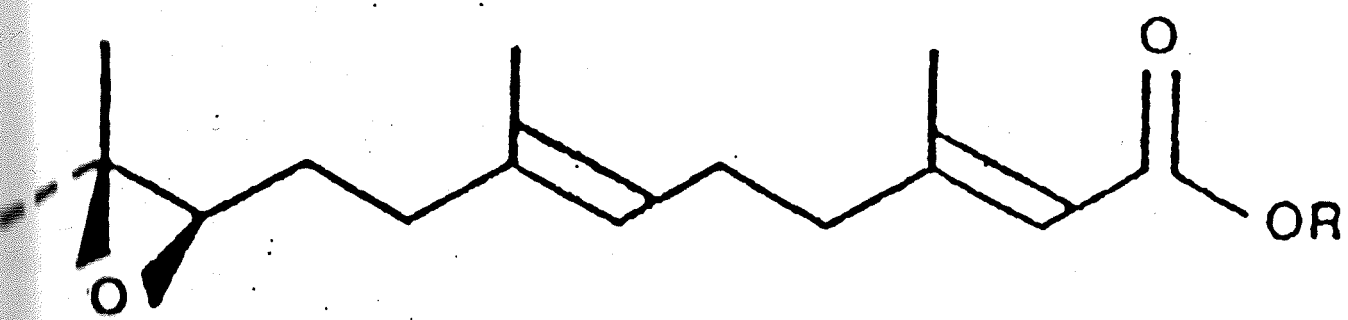
In spite of the observations mentioned above, the roles of MF are still poorly defined. One approach to investigating these roles is the manipulation of MF levels *in vivo*. The discovery and identification of factors that regulate MF would be a useful step toward that goal.

The crustacean eyestalk is a source of many neuroendocrine peptides, most of which are stored and released by the sinus gland (SG), a neuroendocrine gland in the eyestalk of decapod crustaceans (De Kleijn, 1995). The effects of the eyestalk on reproductive and metamorphic functions have

Figure 1. Chemical structures of juvenile hormone III (JH III) and methyl farnesoate (MF). MF differs from JH III in that MF contains a double bond rather than an epoxide between carbons 10 and 11 (Laufer et al., 1987a).



Methyl (2*E*,6*E*)-farnesoate



(10 *R*) Juvenile hormone III (R = CH_3)

(10 *R*) Juvenile hormone III acid (R = H)

been known for some time. Reproduction is inhibited by an eyestalk factor and metamorphosis is disrupted by eyestalk ablation (removal) (Laufer et al., 1986). Neuropeptides synthesized in the SG of the eyestalk regulate many crustacean functions, including glucose levels (through CHH, crustacean hyperglycemic hormone) and ecdysteroid levels (through MIH, the molt inhibiting hormone).

The SG of the eyestalk is also an apparent regulator of MF production. Eyestalk removal increases both MF synthesis by the MO and circulating MF levels in several crustaceans, including the spider crab *Libinia emarginata* (Laufer et al., 1987b) and *Homarus* (Tsukimura and Borst, 1992). Crude SG extracts inhibit MF production both *in vivo* and *in vitro*, indicating that this tissue may contain a peptide that inhibits mandibular organ function (MOIH, mandibular organ inhibiting hormone).

Several SG peptides have been reported to influence MF production. In the crayfish *Procambarus clarkii*, pigment dispersing hormone (PDH) inhibits MF synthesis. PDH may be related to MOIH (Tsukimura et al., 1993). Another crayfish hormone, red pigment concentrating hormone (RPCH) doubles MF synthesis and may be similar to compounds that stimulate MF production (Landau et al., 1989).

Some MOIHs appear to have hyperglycemic effects. The crustacean hyperglycemic hormone (CHH) family includes CHHs, vitellogenic inhibiting hormone (VIH) and molt inhibiting hormone (MIH). This hormone family is defined based on amino acid sequence, molecular weight, number of amino acids and cysteine content of its members. In some species, substances defined as MOIHs based on their regulatory effect on the MO also fit these criteria. These studies suggest that MOIH may have multiple roles in

crustaceans. Both CHHs and MOIHs also have a high degree of species specificity (Liu and Laufer, 1996).

Purified *Libinia* MOIH gave a maximum of 70% inhibition of MF production by disaggregated *Libinia* MO cells when compared to the response of these cells to crude SG extract (Homola, 1989). Because inhibition by *Libinia* MOIH is not complete, it is thought MF production may be regulated cooperatively by a group of compounds. This type of control would allow for the precise regulation of MF production (Liu and Laufer, 1996). Biogenic amines are one type of compound that may act synergistically with the SG neuropeptides (Homola et al., 1989). Biogenic amines are produced and released by neurons. In *Libinia*, inhibition of MF synthesis by biogenic amines alone has been observed. Octopamine, epinephrine and serotonin had inhibitory effects on MF synthesis by dispersed *Libinia* MO cells, in the case of serotonin, at physiological concentrations (Homola, 1989). Serotonin has also been known to act as a crustacean neurohormone affecting behavior and morphology (Homola, 1989).

MOIHs have also been isolated and sequenced in the cancer crab, *Cancer pagarus*. These peptides are SG products, fractionated from the crude SG extract, which inhibit MF production *in vitro*. In sequence they resemble members of the CHH family of crustacean hormones, especially the molt-, vitellogenesis- and gonad inhibiting hormones of *C. pagarus* and other crustaceans (Wainwright, 1996).

Identification of regulation of MF synthesis in the lobster

It has been demonstrated in *Homarus* that MF production by the MO is controlled by the sinus gland in the eyestalk. Eyestalk ablation results in

hypertrophy of the cells of the MO and an increase in MF production; in other words, ablation relieves inhibition by the eyestalk. Unilateral ablation results in an increase in MF production to a lesser extent than bilateral ablation does (Tsukimura and Borst, 1991). The injection of a crude homogenate of the SG into lobsters that have been eyestalk ablated results in a decrease of MF levels to almost undetectable levels. MF levels return to the pre-treatment elevated levels after at least 12 h (Tsukimura and Borst, 1992).

In defining the Homarus MOIH it is helpful to determine the chemical nature of this substance. Evidence that the SG product that regulates MF production is a peptide hormone is that cyclic nucleotides seem to be second messengers for the MOIH in *Homarus*. Cyclic nucleotides (e.g. cAMP and cGMP) are known to be second messengers for peptide hormones including CHH (Tsukimura et al., 1993). Cyclic nucleotides seem to be second messengers for the MOIH in *Homarus* indicating that this SG product is a peptide hormone.

In this study, we designed an *in vitro* assay to study compounds that directly control MF production by the *Homarus* MO. In this assay fragments of the MO were incubated in the presence of possible regulators of MF production. The levels of MF synthesis were measured by supplementing the culture medium with methyl-labeled radioactive methionine. The methyl group of methionine is transferred to farnesoic acid to produce MF (Claerhout et al., 1996). The radiolabeled MF can then be quantified. Two methionine preparations, each radiolabeled with a different isotopes (^{3}H or ^{14}C) were used to measure the synthesis of MF at two different periods. During an initial

labeling period, the tissue was incubated in culture medium containing methionine labeled with one radioisotope. Incorporation of this radioisotope into MF allowed the initial, baseline, level of MF synthesis to be calculated. After treating the tissue with a possible regulator of MF synthesis, the tissue was incubated in culture medium containing methionine labeled with the second radioisotope. Incorporation of this second radioisotope into MF allowed the level of MF synthesis after treatment to be calculated. A comparison of MF synthesis during these two periods made it possible to determine the effect of the compound on MF synthesis.

Materials

Animals: American lobsters (*Homarus americanus*) were obtained from the Massachusetts State Lobster Hatchery (Martha's Vineyard, MA) or from local commercial suppliers in Massachusetts or Illinois. At the Marine Biological Laboratory (MBL), animals were kept in fresh running sea water at ambient temperature until needed. At Illinois State University, the animals were kept in chilled (13C) tanks containing sea salts. In most cases, animals were eyestalk ablated (using a piece of monofilament fishing line) 5 to 10 days prior to the experiment. Bleeding was controlled by treatment with Avitene (MedChem, Woburn, MA).

Radiochemicals: [^{14}C-*methyl*]- and [^{3}H-*methyl*]-labeled methionine (with specific activities of 200 mCi/mmol and 55 mCi/mmol respectively) were purchased from DuPont / NEN (Boston, MA).

Saline and culture medium: *Homarus* saline (Welsh and Smith, 1960) was prepared using deionized water and contained 463 mM NaCl, 15.7 mM KCl, 19.5 mM $CaCl_2$, 3.9 mM $MgCl_2$, 8.36 mM Na_2SO_4 and 8.94 mM H_3BO_3 (pH 7.6). Culture media (DMEM and Medium 199) were obtained from GibcoBRL (Gaitersburg, MD) as dry powder. DMEM (Dulbecco's modified Eagle's medium) was prepared in a 1.5-fold concentration in pyrogen-free H_2O and supplemented with 2.81 mM $NaHCO_3$, 266.4 mM sucrose, 19.43 mM glucose, 25 mM HEPES, 100 U/ml Penicillin and 0.169 mg/ml Streptomycin. The final osmotic pressure of the DMEM was approximately 800 milliosmoles. After adjusting the pH to 7.4,

the medium was filter sterilized (0.22 , Millipore). A second culture medium used in this work was Medium 199 (M199). The salt concentration of M199 was adjusted to those found in *Homarus* saline (343 mM NaCl, 10.3 mM KCl, 18.6 mM $CaCl_2$-H_2O, 7.47 mM $MgSO_4$, and 4.17 mM $NaHCO_3$). The pH was adjusted to 7.4.

Putative Regulators: Several putative regulators of the MO were tested, including sinus gland extract (SGE), mandibular organ inhibiting hormone (MOIH), crustacean hyperglycemic hormone (CHH) and serotonin. SGE was prepared from sinus glands dissected from *Homarus* eyestalks and homogenized in *Homarus* saline. Unless otherwise noted, culture media were supplemented with the equivalent of 0.25 sinus glands per ml.

MOIH and CHH were generously provided by Dr. Ernie Chang (Bodega Marine Laboratory, University of California, Davis, CA). These compounds were isolated by Dr. Chang using reverse phase high performance liquid chromatography (rpHPLC) from SGE. The eluting peaks were identified by their *in vivo* biological activity. For the trials in which these putative inhibitors were tested, the culture medium was supplemented with the amount of peptide found in either 0.25 sinus glands or 0.75 sinus glands per ml of culture medium, depending on the treatment.

Serotonin (5-hydroxytryptamine, 5-HT) was purchased from Sigma Chemical Co. (St. Louis, MO) and stored in a stock solution of 10 mM in 0.1 N perchloric acid.

Methods

Dissection

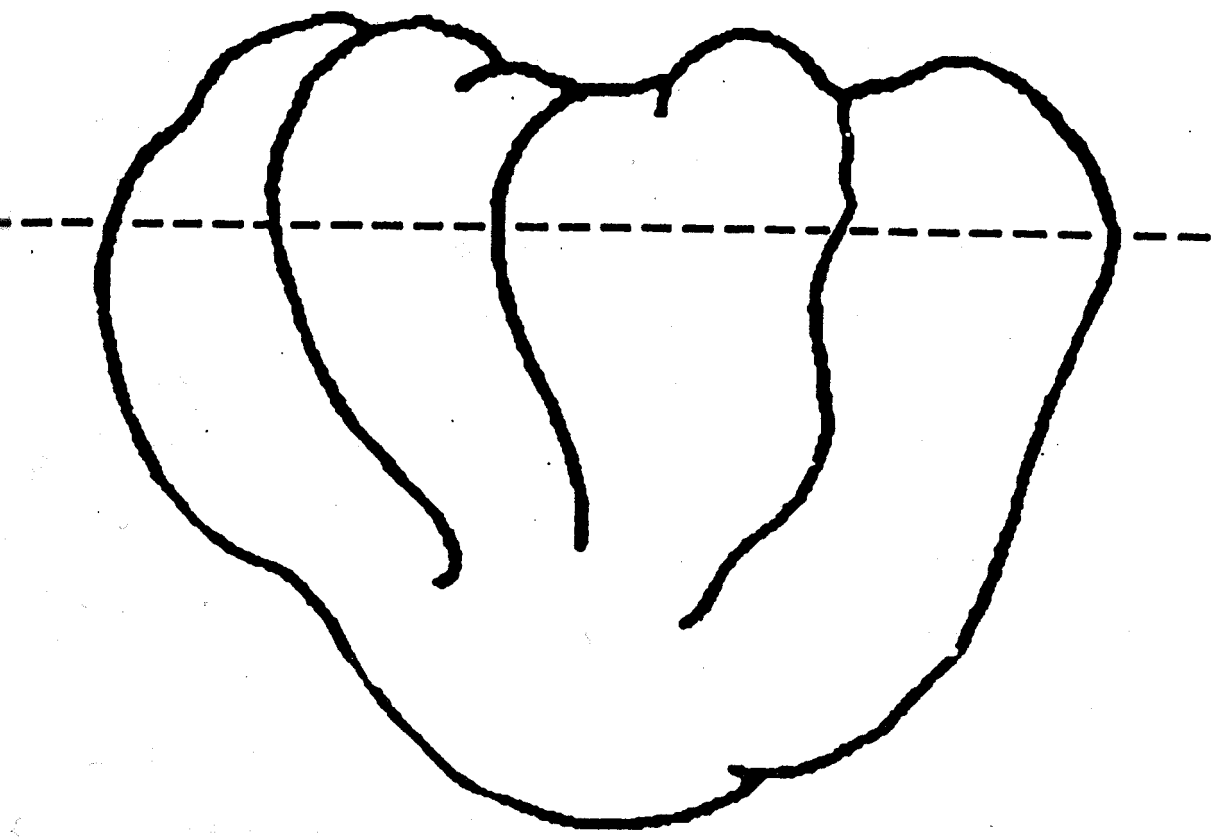
The lobster mandibular organ (MO) is a soft, beige tissue with a fan-folded edge (Figure 2; Borst et al., 1994). It is located ventrally in the lobster, wrapped around and loosely attached to the apodeme of the mandibular abductor which anchors the mandibles to the dorsal carapace (Borst et al., 1994). The fan-folded edge projects anteriorly and it is this fan-folded edge of the MO where MF synthesis occurs (Borst et al., 1994). The dissection was performed as in Borst et al. (1994). Access to the MO was gained by removing the dorsal carapace, clipping the mandibular abductor and removing the cephalic lobes of the hepatopancreas. The MO was then removed from the animal by detaching it from the mandibular abductor (by running a sharp tool under it) and lifting it out of the animal.

Once removed, MOs were kept in *Homarus* saline on ice. Under a dissecting microscope, the MOs were teased so that the fan-folded edge was fully extended. A strip of tissue about 3 mm wide was cut from each MO along this edge (see dashed line in Figure 2). The two strips were subdivided into a total of 70 to 80 fragments for use in the *in vitro* assay.

Double Label *In vitro* Assay

Three to four fragments of the MO were randomly picked and placed in each well of a 24-well tissue culture plate (Falcon, Becton Dickinson, Lincoln Park, NJ). Each treatment was tested in quadruplicate. All tests in an experiment contained MO fragments from a single lobster.

Figure 2. Cartoon of the mandibular organ (MO) of *Homarus* showing the fan-folded edge. The region of the gland above the dashed line across fan-folded edge is the portion used for in vitro studies (Borst et al., 1994).



Unless otherwise noted, the synthesis of MF by the MO fragments was tested using a double-label procedure. This involved incubating tissue fragments for a total of 5 h. MO fragments were incubated on an orbital shaker set at a medium speed (approximately 60 rpm). During the first two hours (T = 0-2), the fragments were incubated in 400 µl culture medium supplemented with 5 µCi/ml [^{14}C-*methyl*] methionine. At the end of this first incubation the fragments were rinsed with unlabeled medium and then transferred to a second well containing 400 µl culture medium without radiolabeled methionine. This second incubation period lasted one hour (T = 2-3). The fragments were then moved to a third well containing 400 µl culture medium supplemented with 40 µCi/ml [^{3}H-*methyl*] methionine. Compounds being tested for their effects on MF synthesis were added during both the second and third incubations (T = 2-5). After two hours (T = 3-5), the MO fragments were removed from the ^{3}H-labeled medium, rinsed, and placed in ice-cold culture medium to prevent additional incorporation of radiolabeled methionine into MF. In some experiments the order of the radiolabels was reversed; however, we have no reason to expect that this reversal had an effect on our results (see Figure 4).

The MO fragments were then extracted by homogenizing in 0.5 ml *Homarus* saline with a 2-ml tissue grinder (Potter-Elvehjem, Fisher Scientific, Pittsburgh, PA). The homogenate was transferred to a screw-capped culture tube with a teflon-lined cap containing 2.5 ml acetonitrile. The homogenizer was rinsed three times with 0.5 mL *Homarus* saline or 2% NaCl, and the rinses were added to the culture tube. The culture tubes were vortexed, 1 ml of hexane was added to each tube to extract the MF, and the tubes were again vortexed. After chilling on ice for ten minutes, the tubes were centrifuged (2000

rpm x 5 min, 6°C) to separate the phases. The hexane phase that contained the MF and was analyzed by scintillation counting and/or HPLC.

Analysis of MF synthesis by scintillation counting

It was necessary to determine the amount of radioactivity in the medium and the specific activity of the methionine in each culture well to calculate the amount of MF synthesized by the MO fragments in each well. Therefore, after each of the three incubations, 5 µl of culture medium was placed in a 4 ml scintillation vial. Three ml of scintillation cocktail (Scintiverse, Fisher Chemical Co., Pittsburg, PA) were added to each vial. The samples were analyzed on a scintillation counter (Packard Tri-Carb 2200CA, Packard Instruments, Downers Grove, IL) to determine the amount of each radioisotope (DPM: disintegrations per min) in the incubation fluid.

The amount of radiolabeled MF synthesized during each labeling period was calculated in the following way. First, the final specific activity (a value expressed in units of Curies/mol) of the methionine in the culture medium in each culture well was calculated. The amount (in moles) of radioactive methionine present in each well was calculated by dividing the DPM of the culture medium (determined from the DPM for the 5 µl aliquot) by the known specific activity of the radioactive compound. The amount of radiolabeled methionine was then added to the amount of unlabeled methionine (from the catalogue description of DMEM) in the culture medium to determine the molarity of the methionine (labeled and unlabeled) present. The amount of radioactivity in each well was then divided by the total methionine present to determine the adjusted specific activity.

Two aliquots (200 μl each) of the extracted MO samples were also analyzed by scintillation counting. The adjusted methionine specific activity in the samples was used to convert the DPM of MF in each sample to amount (moles) of MF synthesized by that tissue. By making these corrections for both the [^{3}H]-methionine and the [^{14}C]-methionine, it was possible to compare the amount of MF synthesized during the first incubation period with the amount synthesized during the second incubation period. Levels of inhibition of MF synthesis are expressed as percentages of MF production during the second, treated period as compared to MF production during the first, control period.

HPLC Analysis

An aliquot of the hexane extract of some samples was analyzed by HPLC to confirm that radioactivity in the extract detected by scintillation counting was MF. Normal phase HPLC was performed using a LKB Bromma 2185 HPLC with the detector wavelength of 206 nm. Sample extracts of homogenized MOs incubated *in vitro* were separated with a silica column (5μ, 4.6 mm x 25 cm) using a solvent of 1% ether in hexane (2 ml/min). Fractions were collected every 30 sec as they eluted from the HPLC. The samples were analyzed in the scintillation counter to identify fractions containing radiolabeled compounds. The time at which these samples eluted off the column was compared with the retention time of the MF standard to determine whether the fractions containing radioisotopes coeluted with MF.

Results

Optimizing Conditions for the Double Label Experiment

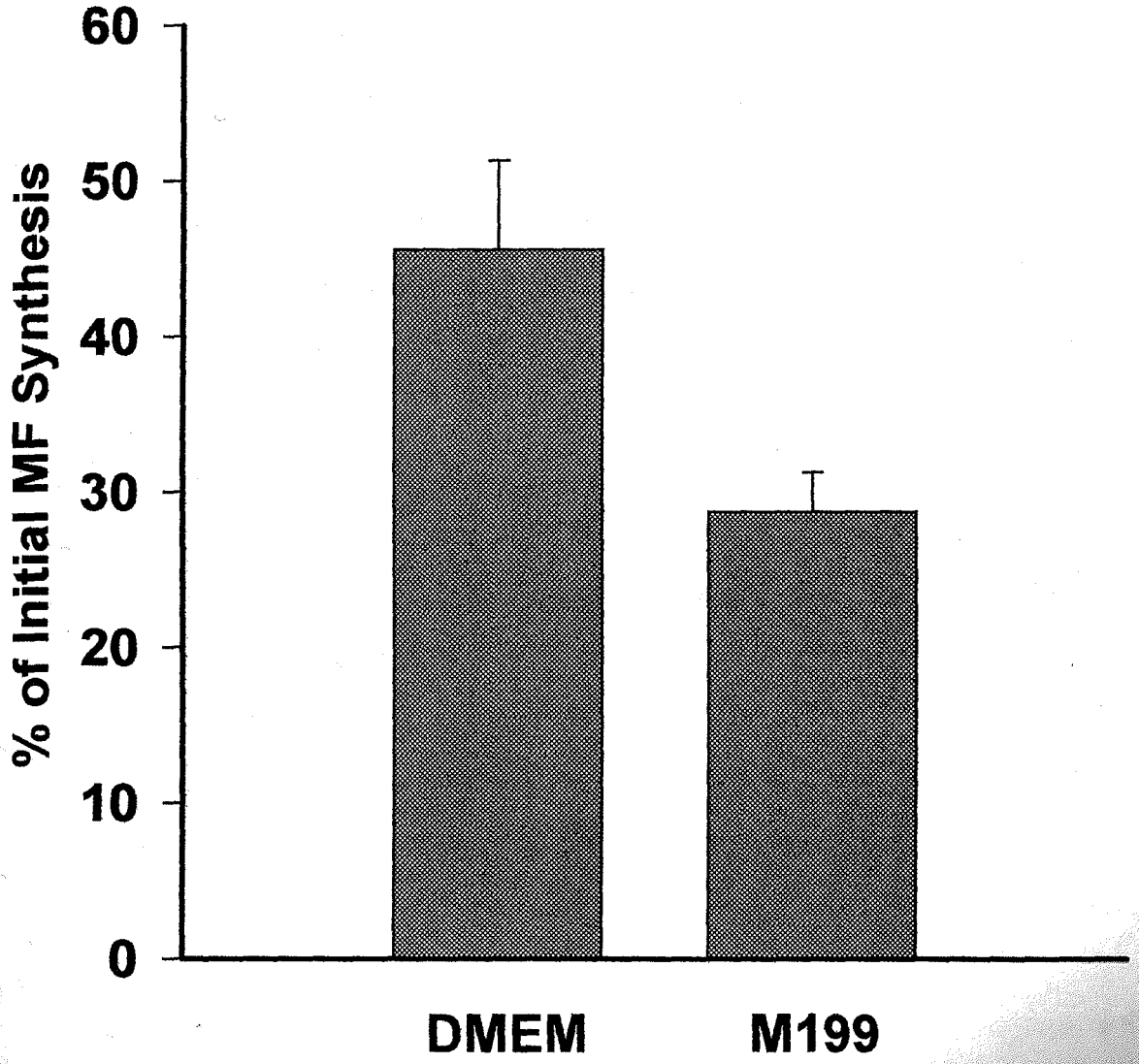
MF synthesis was measured in MO fragments incubated in two culture media (DMEM and M199). When DMEM was used, MF synthesis during the second labeling period was 46% of the level observed in the first period (Figure 3). When M199 was used, synthesis during the second labeling period was 29% of that observed in the first period. DMEM was used in subsequent experiments because MF synthesis in the second labeling period was higher in this medium than in M199.

Ideally, the MO tissue would synthesize similar amounts of MF during both labeling periods. However, even in the DMEM, MF synthesis was reduced considerably in the second labeling period. The basis for this decrease was investigated in several ways. One possible reason for this difference was that the [^{14}C-*methyl*]- and [^{3}H-*methyl*] methionine preparations were different in some way, or that there was an error in the method used to calculate MF synthesis. To test for these possibilities, MO fragments were incubated for 2 h in culture medium containing a methionine mixture labeled with both radioisotopes. The amount of MF synthesized during the incubation was calculated using the incorporations of each radiolabel after adjusting its specific activity to reflect the total methionine in the culture medium. The level of incorporation of ^{3}H into MF was 83% (0.4 SE, n=4) of the incorporation of methionine labeled with ^{14}C into MF.

An additional test for possible differences in the two radioactive methionine preparations was performed by switching the order of these labels in

Figure 3. Comparison of culture media, M199 and DMEM. Levels of MF synthesis during the second labeling period are compared to levels of MF synthesis during the first labeling period. This value is expressed as a percent. Each bar represents mean ± SE (n = 4).

Effect of Culture Media on MF Synthesis



the double label experiment. The order in which the two methionine preparations were used had a minimal effect on the synthesis of MF by the MO fragments (Figure 4).

Finally, the identity of the radiolabeled material produced by the MO fragments was determined to confirm that MF was the labeled product. Hexane extracts of tissue labeled with both [^{3}H-*methyl*]- and [^{14}C-*methyl*] methionine were analyzed by normal phase HPLC. As can be seen in Figure 5, scintillation analysis of fractions separated by HPLC showed a single peak of radioactivity for both radioisotopes that eluted at the same time (about 5.0 min) as the MF standard (Figure 6). This peak represents 84.5% (3.4, SE, n=17) and 84.4% (1.2, SE, n=17) of the [^{3}H]-labeled and [^{14}C]-labeled material, respectively.

Double Label Experiment

SGE inhibited MF production by MO fragments *in vitro*. When the culture medium for the 1-h rinse incubation and the second 2-h incubation was supplemented with SGE, MF synthesis by MO fragments treated with SGE was lower than that of tissue untreated with SGE (Figure 7). Two different doses of SGE were tested to determine whether the inhibition of MF production by SGE was a dose-responsive effect. Within the range from 0.01 to 0.1 SG equivalents, the level of inhibition did not significantly differ (Student's t-test, $p < 0.01$).

MOIH is an HPLC-separated fraction of crude SGE. This peptide also has an inhibitory effect on MF levels in *Homarus* when used in *in vivo*. MOIH partially inhibited MF production by MO fragments (Figure 8), but did not depress MF synthesis as much as SGE did. To determine if the partial

Figure 4. Label reversal trials. Tissue culture was supplemented with [^{3}H-*methyl*]- or [^{14}C-*methyl*] methionine (40 Ci/ml and 5 Ci/ml, respectively). Bars are labeled with the radioisotope with which culture medium was supplemented for the first labeling period. Levels of MF synthesis during second labeling period are compared to levels of MF synthesis during the first labeling period. These values are expressed as a percent. Each bar represents mean ± SE (n = 4).

Effect of the Order of Radiolabel Exposure on MF Synthesis

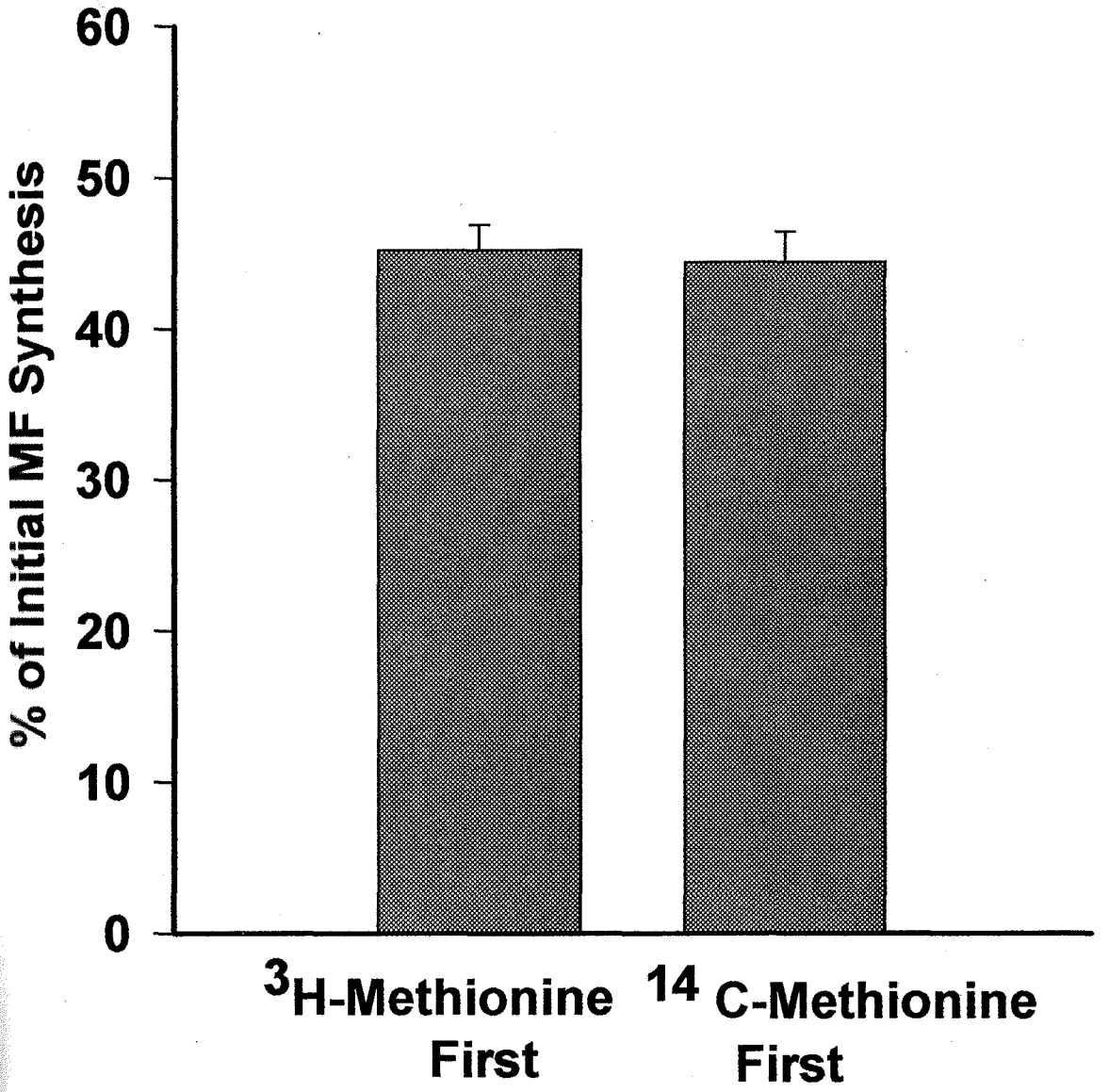


Figure 5. Scintillation analysis of fractions collected from an HPLC separation of the hexane phase of an MO tissue homogenate. Approximately 84% of the radioactivity coeluted with the MF standard (see Figure 6) and is contained in the fractions collected around 5.0 min.

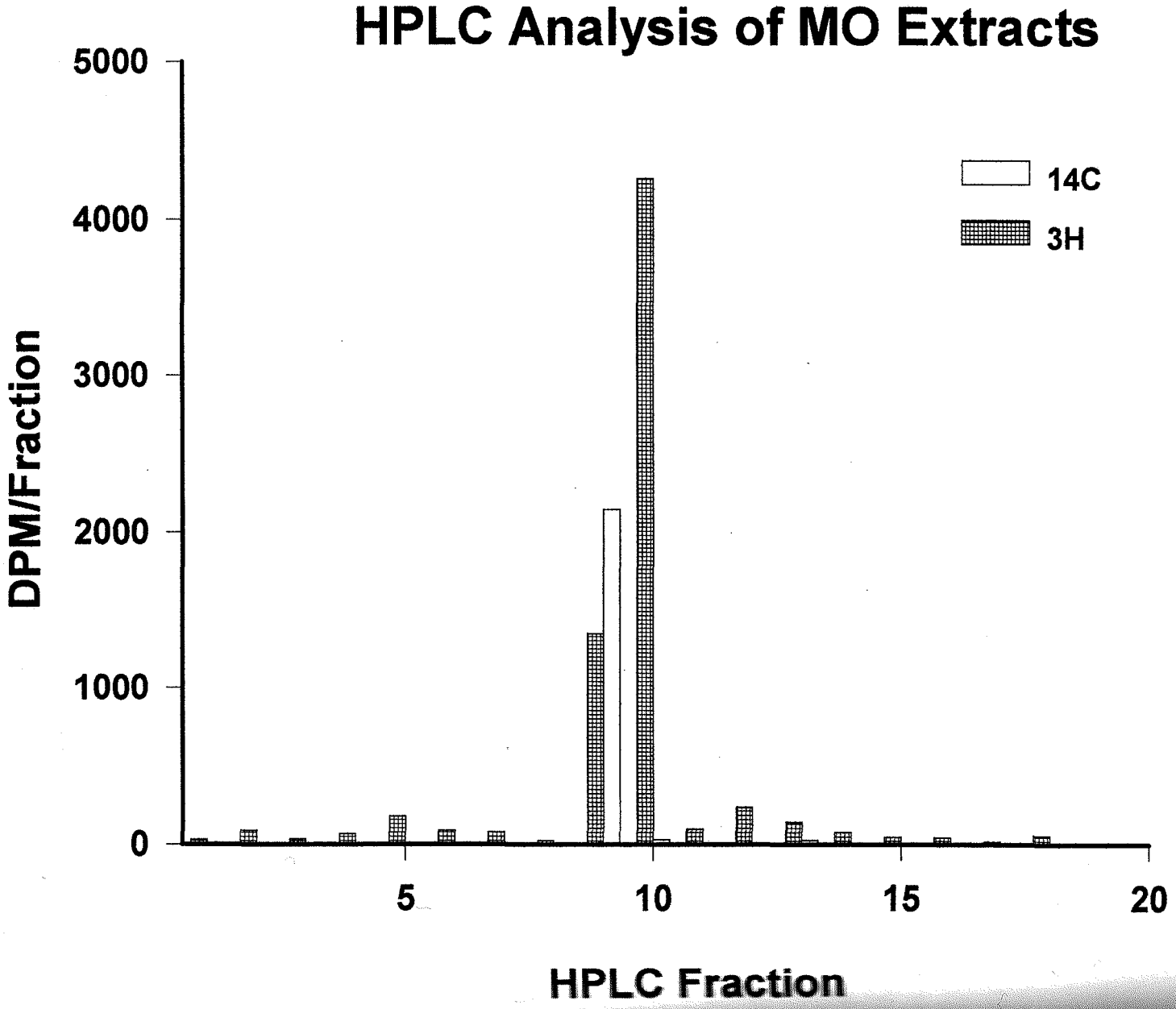

HPLC Analysis of MO Extracts
5000
4000
3000
2000
1000
0
DPM/Fraction
14C
3H
5
10
15
20
HPLC Fraction

Figure 6. Chromatogram of normal phase HPLC separation of the MF standard using the standard HPLC conditions defined in the text. Graph shows absorbance as a function of time as the standard was separated and eluted from the column.

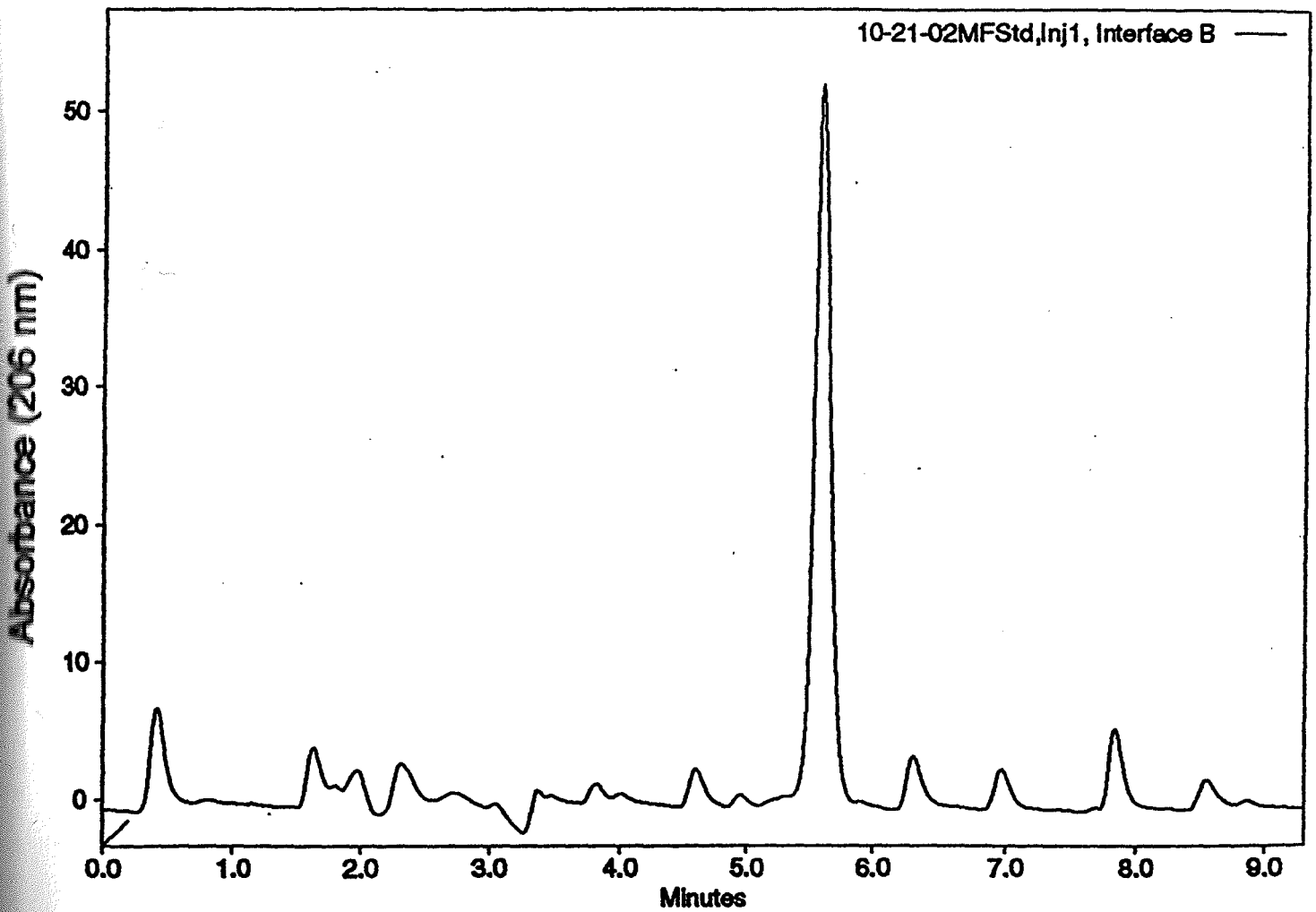

10-21-02MFStd,Inj1, Interface B
Absorbance (206 nm)
50
40
30
20
10
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Minutes

Figure 7. Trials testing dose response to crude *Homarus* sinus gland extract (SGE). Culture medium was supplemented with the equivalent of 0.01 or 0.1 sinus glands per well. Each bar represents mean ± SE (n = 4).

Effect of Sinus Gland Extract on MF Synthesis

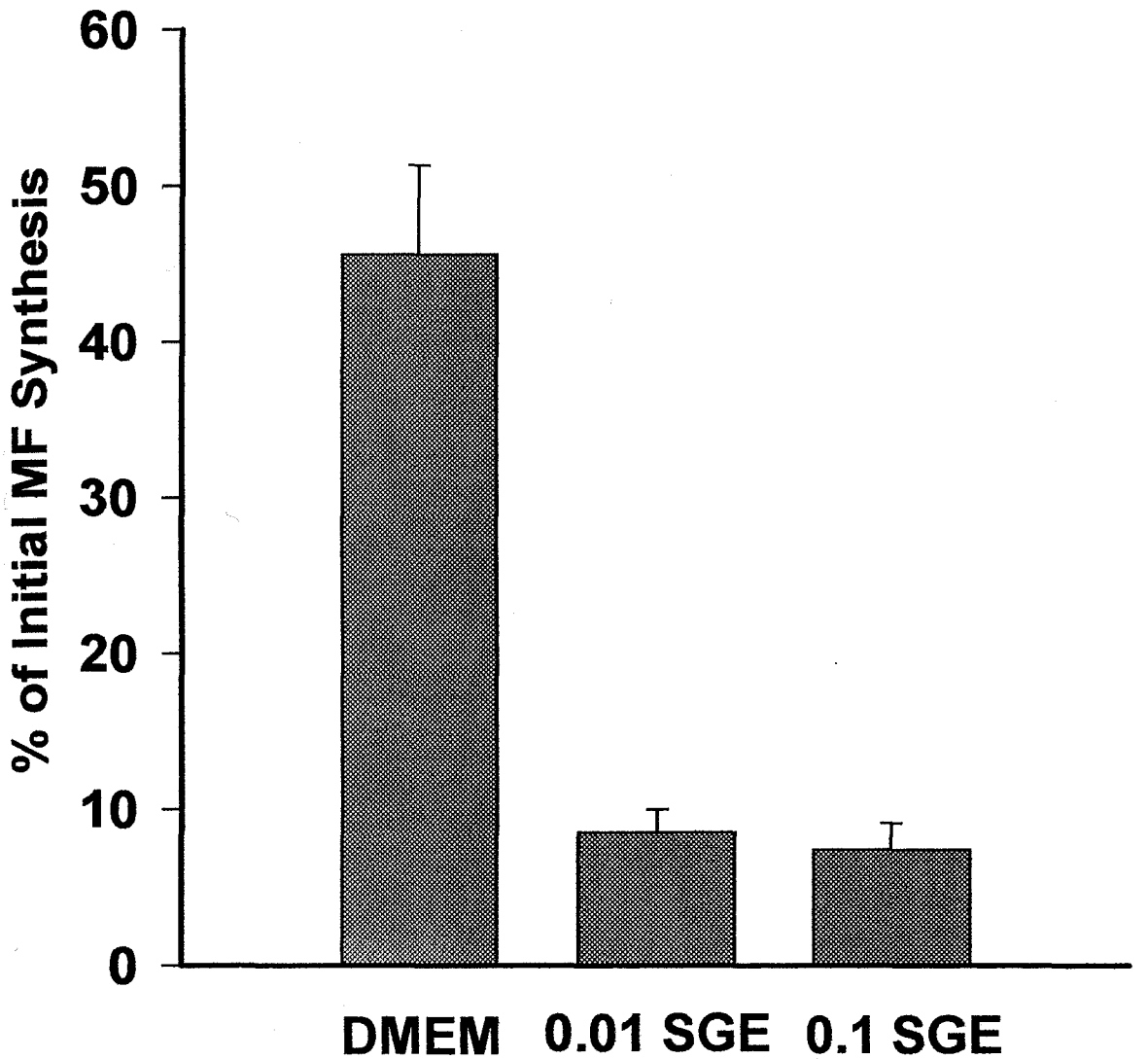
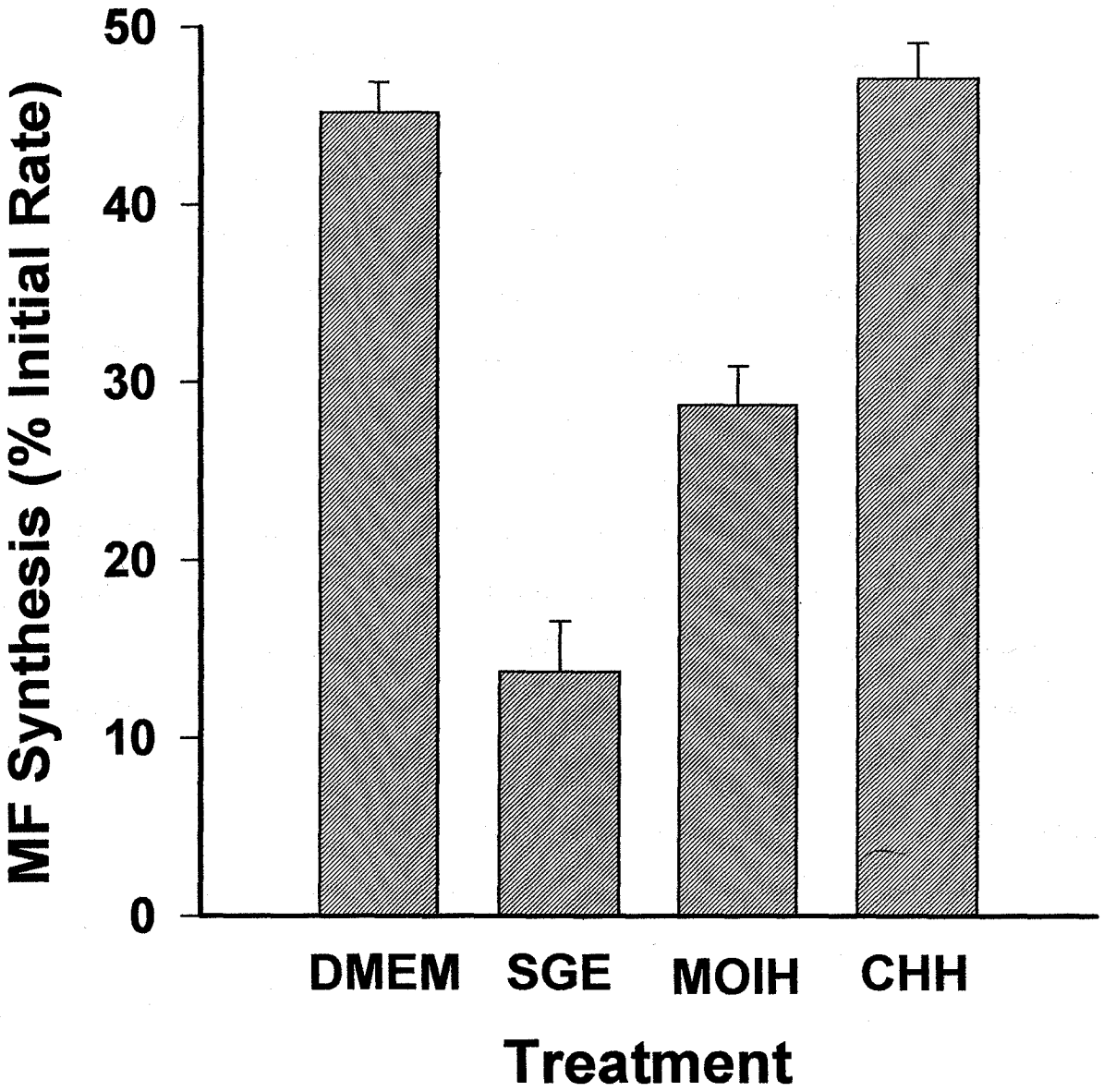


Figure 8. Testing effect on MF synthesis by possible inhibitors. SGE, MOIH (mandibular organ inhibiting hormone) and CHH (crustacean hyperglycemic hormone) were applied in 0.1 SG equivalent aliquots. Each bar represents mean ± SE (n = 4).

Effect of Sinus Gland Peptides on MF Synthesis *in vitro*



response to MOIH reflected an inadequate amount of the peptide, MO fragments were tested with two doses of MOIH (0.1 SG and 0.3 SG equivalents) (Figure 9). These two doses gave equivalent responses, suggesting that the partial response to MOIH was not due to insufficient peptide.

The crustacean hyperglycemic hormone, CHH, was also isolated by HPLC separation of the SGE crude extract. CHH is just one member of a family of crustacean hormones with the same name. MOIH is thought to be part of the CHH family of hormones, all of which have similar structures and may share functions (Liu and Laufer, 1996). CHH did not have any inhibitory effect on MF production *in vitro* (Figure 8). To test whether the 0.1 SG equivalent dose of this peptide was simply too low to illicit a response, a three-fold dose (0.3 SG equivalents) was tested and also failed to give an inhibitory effect (Figure 9).

Serotonin (5-HT, 5-hydroxytryptamine), which inhibited MF production by disaggregated *Libinia* MO cells (Homola, 1989), was tested in the *in vitro* experiment of *Homarus* MOs. No inhibition of MF production was seen by serotonin (Figure 10). This level is above the reported physiological level for *Homarus* (10^{-8}) (Livingston et al., 1980). To test whether serotonin generated any enhancement of the inhibitory effect of SGE (which was short of 100%), additional experiments were performed in which SGE was combined with serotonin for the last 3 h of incubation for some trials. This combination did not have any greater inhibitory effect on MF production than did SGE alone.

Figure 9. Trials testing dose response to different levels of MOIH (mandibular organ inhibiting hormone) and CHH (crustacean hyperglycemic hormone). Culture medium was supplemented with the equivalent of either 0.1 or 0.3 sinus glands per well. Each bar represents mean ± SE (n = 4).

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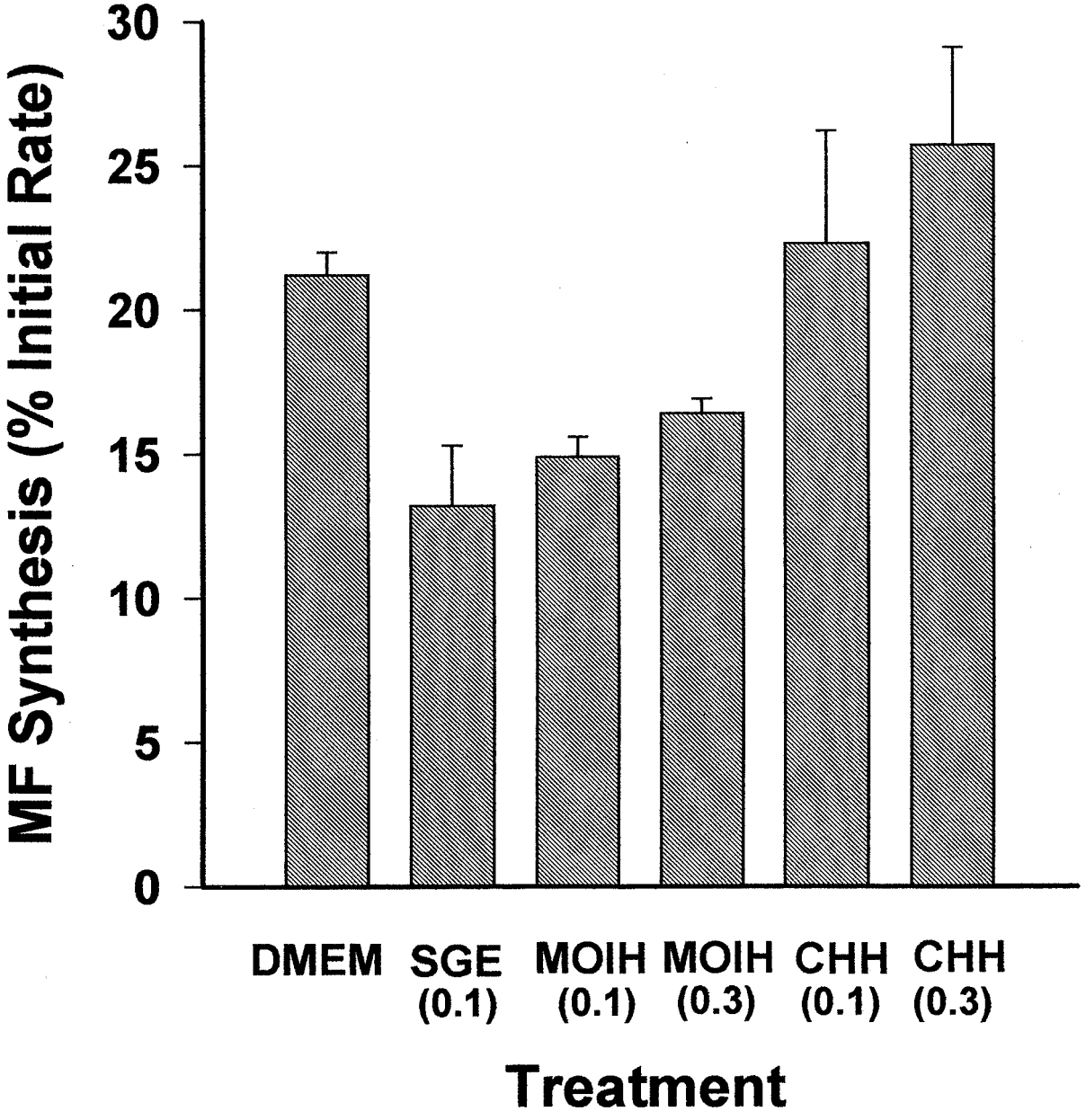
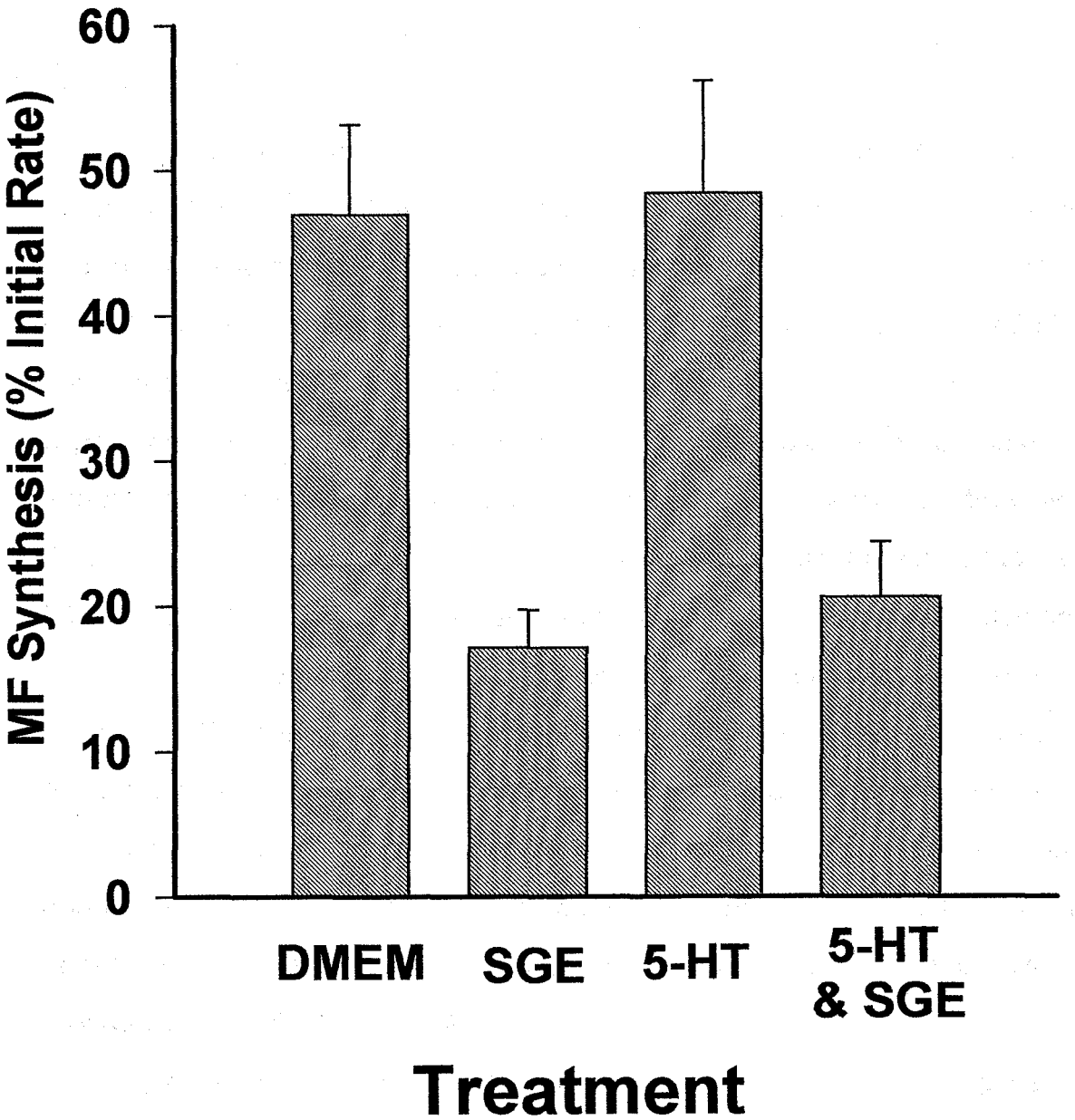


Figure 10. The effect of serotonin on levels of MF synthesis. Culture medium was supplemented with either 0.25 SG equivalents per ml SGE or 10^{-5} M serotonin (5-HT or 5-hydroxytryptamine), or both of these treatments combined. Each bar represents mean ± SE (n = 4).

Effect of Serotonin (5-HT) and Sinus Gland Extract on MF Synthesis



Discussion

The *in vitro* assay of the lobster MO proved to be a satisfactory system with which to study the direct control of MF synthesis by the gland. The MO is large and easily dissected from the animal. In addition, despite using only small amounts of tissue, the levels of MF generated are quantifiable by the methods used here (scintillation analysis of radiolabeled MF).

Levels of MF synthesis by lobsters vary among MOs even from a single individual (Borst et al., 1994). Because of this variability, no control or baseline level of MF synthesis could be defined for the lobster. As a result, the effects of possible inhibitors of MF synthesis had to be examined with respect to controls performed on the MO fragments from the same animal during the same experiment. The double label experiment was devised to give an individual, internal standard for the untreated level of MF synthesis for each quantity of MO tissue assayed. That is, MF synthesis by the fragments was normalized to the initial level of synthesis. Comparisons of percent initial MF synthesis could not be made across experiments. By using two different radiolabels, the relative amount of MF synthesized before and after the treatment could be compared. As stated previously, the results of the double label experiments are expressed as a ratio of MF synthesis during the second labeling period to the MF synthesis during the first labeling period (expressed as a percentage). Thus, these values of percent of initial MF synthesis provide a relative measure of MF synthesis regardless the initial levels of MF synthesis for that tissue sample.

Even for the control trials, for which there was no treatment during the second labeling period, the tissue was not as active during the second period as

during the first. In other words, MF synthesis during the second period was never 100% of the initial synthesis (Figures 3, 4 and 7-10). This reduction of synthesis during the second labeling period could be the result of a shortage of MF precursors, including farnesoic acid, or of other components of the MF synthesis pathway. The cells also may be dying. Studies are currently being done using microscopy to look for morphological changes in MO tissue that has been incubated in DMEM in these conditions for 5 h (Ernest Couch, work in progress). Because of this consistent decrease in MF synthesis over time, the effects of putative inhibitors could not be understood by comparing them with 100% initial MF synthesis. Instead, to assess inhibition by the treatment compounds, the levels of percent initial MF synthesis were compared among control and treatment groups.

An optimal level of MF synthesis during hours 3-5, averaging approximately 50% of initial, was achieved consistently with concentrated DMEM and MOs from eyestalk ablated lobsters. Through the label reversal experiment, it was determined that the order of the radiolabels did not have a significant effect on the rate of incorporation of the radiolabeled methyl group into MF (Student's t, $p < 0.01$). For some experiments, the culture medium was supplemented with [^{3}H-*methyl*] methionine during hours 0-2 and with [^{14}C-*methyl*] methionine for hours 3-5. For others, this order was reversed. This was not considered to have any effect on the experimental conditions.

The *in vitro* assays of crude SGE gave the expected results. SGE inhibited MF production sufficiently for us to consider it a direct effect (in Figure 7, percent initial MF synthesis was 7.5% with SGE compared with 45% for the control). We hypothesize that this is as complete an effect as can be observed

in vitro; therefore, the degree of inhibition by SGE was used as a standard measure of complete inhibition for these studies.

MOIH did not have as great an inhibitory effect as did SGE. This substance, isolated from the crude SGE by Chang, was identified by its effects *in vivo* in *Homarus* where it generated complete inhibition of MF synthesis. The partial inhibitory effect *in vitro* suggests that it may work synergistically with another substance *in vivo*. Since MOIH completely inhibits MF synthesis *in vivo* in animals whose SGs have been removed by eyestalk ablation, it is unlikely that the synergistic substance is a product of the SG. Instead, the substance with which the MOIH could work synergistically could be a biogenic amine delivered to the MO by neuronal contacts and therefore not dependent on the presence of the eyestalk. The MO may be innervated by neurosecretory axons, as are the homologous corpora allata of insects (Raabe, 1989), but this has not been determined for *Homarus*.

CHH had no inhibitory effect on the rate of MF synthesis by MO fragments in this *in vitro* study. CHH seemed a likely candidate for a MOIH in *Homarus* because some *Homarus* CHH-isoforms have been shown to act as gonad stimulators and molt inhibitors, roles described for the MOIH in some other species (De Kleijn, 1995). However, this HPLC fraction of *Homarus* SGE did not have MF-inhibiting activity. The CHH was identified by Chang by its activity *in vivo* (as was the MOIH); therefore, the lack of MF-inhibiting activity *in vitro* of the CHH appears not to be due an absence of overall activity.

Serotonin alone had no inhibitory effect on MF synthesis by the MO fragments. The concentration used in this study, 10^{-5}M, is higher than the circulating titer in *Homarus*; it is not a physiologically significant titer (Livingston

et al., 1980). It follows that at lower concentrations, closer to those found *in vivo,* serotonin would also not have an inhibitory effect on MF synthesis.

The doses of SGE, MOIH and CHH used in this study should have been sufficient to elicit a response. The 0.1 or 0.3 sinus gland equivalent aliquots of the peptides applied to the tissue in the experiment are in excess of the amount of peptide circulating *in vivo* relative to the amount of tissue used in the assays. Thus, the lack of an inhibitory effect by some substances tested here is not due to an ineffectively small dose of these compounds.

While serotonin does not affect MF synthesis alone or in combination with SGE, it is possible that this biogenic amine may act synergistically with a fraction of the crude SGE. In future studies a treatment of MOIH and serotonin together will be applied to the MO tissue to determine whether this combination inhibits MF production completely. Other future studies include the evaluation of the SGE, MOIH, CHH and serotonin on MF synthesis by dispersed *Homarus* MO cells. The isolation of MO cells would control for effects of substances which may be delivered to the MO by neurons (which may remain in the fragment preparation used in these *in vitro* studies).

Conclusions

Under the conditions of this *in vitro* assay, *Homarus* MO tissue actively synthesized MF. When exposed to SG extract, which inhibits MF production *in vivo*, MF synthesis levels were depressed. MOIH, a peptide isolated from the SGE by HPLC which inhibits MF production *in vivo*, also had an inhibitory effect on levels of MF synthesis *in vitro*. CHH, another peptide isolated from the SGE, thought to have some functional homology with MOIH because of structural similarities, did not have an inhibitory effect on MF synthesis *in vitro*. Serotonin, which inhibits MF synthesis by dispersed *Libinia* MO cells (Homola, 1989), did not have an effect on MF synthesis by MO tissue in this *in vitro* study. The partial inhibition of MF synthesis by MOIH suggests that it may act synergistically with a non-SG substance *in vivo*. Future studies will involve combining possible synergistic substances with MOIH in these *in vitro* conditions.

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