Determination of the sub-cellular location of sex pheromone biosynthesis in the yellow mealworm beetle, *Tenebrio molitor*

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Abstract

Pheromones are chemical signals used for intraspecies communication. The yellow mealworm beetle, *Tenebrio molitor*, produces several pheromones, including the sex pheromone (4R)-(+)-4-methyl-1-nonanol. Previous studies in this laboratory localized the site of pheromone production to the ovaries. The main goal of this project was to determine the sub-cellular location of the enzyme that catalyzes the last step of the biosynthesis. This step is of particular interest because it is stimulated during maturation and inhibited after mating. The focus of this study was the conversion of 4-methylnonanoyl CoA to 4-methylnonanol by 4-methylnonanoyl CoA reductase. The ovaries were homogenized and the mitochondrial, microsomal, and soluble fractions were separated by differential centrifugation. Marker enzymes were used to confirm the identity of the subcellular fractions. *In vitro* radio-assays were used to quantify the enzyme activity in each fraction. Most of the 4-methylnonanoyl CoA reductase activity was localized to the "mitochondrial fraction" isolated from ovary cells. Further research is required to determine if the enzyme activity is truly in the mitochondria, or, rather, is located in microsomal "impurities" associated with this fraction. The localization of the subcellular site of pheromone production will help elucidate the mechanism by which pheromone production is regulated in *T. molitor*.

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1.0. INTRODUCTION

Pheromones are chemical signals that many organisms, ranging from bacteria (Lazazzera and Grossman, 1998) to elephants (Rasmussen et al., 1996), use to communicate with others of the same species (Vanderwel, 2003). These chemicals are often, though not always, airborne and can be very effective at small concentrations (Rantala et al., 2003; Vanderwel, 2003). Pheromones are often used to mediate such processes as foraging, mating, aggregation/anti-aggregation, alarm signaling and trail making (Bacala, 2000). The potential for the exploitation of these chemicals to control pest populations of insects has drawn much commercial interest to the identification of insect pheromones. More recently, studies have been expanded to explore the biosynthetic processes involved. The biosynthesis of moth pheromones has been extensively studied, while the biosynthesis of beetle pheromones has only recently garnered attention (Bacala, 2000).

Beetles take advantage of the versatilities of chemical signaling at their disposal to convey very specific and/or complex messages (Vanderwel, 2003). For over two decades, the research on beetle pheromones has concentrated mostly on aggregation pheromones (Seybold and Vanderwel, 2003). These studies have primarily examined the aggregation pheromones of scolytid, curculionid, and cucujid beetles. However to gain a better understanding of the biochemical nature of the coleopteran pheromones, it is important to also take into consideration the production of sex pheromones (Islam *et al*., 1999). There is very little information known about sex pheromone biosynthesis in beetles.

Our laboratory has selected the yellow mealworm beetle, *Tenebrio molitor*, as a model species for sex pheromone studies. *T. molitor* is a pest insect of wheat bran, flour and stored grains in general (Carpita *et al.*, 1989; Rantala et al., 2003). The mealworm is used as a study model for a variety of reasons: it is commercially available, it is easily maintained in colonies and it has a well-described physiology. In addition to the wealth of data on the beetles' behavioural and chemical ecology, *T. molitor* adults are also large enough to allow surgical procedures.

To date, six different sex pheromones of *T. molitor* have been characterized on the basis of bioassays. There are at least two chemicals that act as sex attractants: one produced by females to attract males, and one produced by males to attract females (Vanderwel, 2003). In addition there are two "copulation-release pheromones" (one female- and one male- produced), which induce copulatory behaviour (Vanderwel, 2003). The female produced copulatory release pheromone is only active in the presence of 4-methylnonanol (Tanaka *et al.*, 1986; Vanderwel, Hasting and Currie, unpublished). The fifth pheromone is produced by the male and is an anti-aphrodisiac (Vanderwel, 2003). Though we have knowledge of the existence of these pheromones, more research is needed to identify them. Additionally, further studies are necessary to learn about the regulation and interplay of *T. molitor*'s sex pheromones.

The only pheromone of *T. molitor* that has been identified to date is the male-attractant, (4R)-(+)-4-methyl-1-nonanol (Tanaka *et al.*, 1986; Tanaka *et al.*, 1989), which is only produced by females. Newly eclosed (immature) females produce very little sex pheromone, but production increases as females mature. Juvenile Hormone (JH) III is responsible for the stimulation of increased pheromone production (Valentine, 1931;

Happ and Wheeler, 1969; Menon, 1970). Among other effects, JH III mediates ovary development as well as sex pheromone production (Judy *et al.*1975). The reduction of 4-methylnonanoyl coenzyme A (CoA) to the pheromone is one of the enzymatic steps stimulated by JH III (Bacala, 2000).

Mating causes a transient decrease in the level of 4-methylnonanol (Griffith, Vakili, Currie and Vanderwel, unpublished). Neither the reason for the drop in pheromone titre, nor the mechanism by which this is accomplished, have been elucidated. It is known that the last male to mate with a female incurs a high level of sperm precedence, so males may cause the lowered attractant pheromone after mating (Gage, 1992; Siva-Jothy *et al.*, 1996; Drnevich *et al*., 2000). By inhibiting the production of 4-methylnonanol, the last male to mate reduces the possibility of other males mating afterwards and thus retains sperm precedence. Another possibility is that the female may herself lower the titre of sex attractant pheromone thereby allowing her to lay her eggs without being "harassed" by other males.

The transient inhibition of pheromone production after mating in beetles has only been reported in the boll weevil (*Anthonomus grandis*) (Dickens and Wiygul, 1987). The temporary or permanent inhibition of sex pheromone production has been well documented in several other insects including some species of flies (Miyatake *et al.*, 1999), cockroaches (Schal *et al*., 1997) and moths (McNeil *et al.*, 1997; Raina, 1997; Karube and Kobayashi, 1999; Delisle *et al.*, 2000; Delisle and Simard, 2002). The decrease of 4-methylnonanol is only beneficial if it is transient. Females must mate at least every two days in order to sustain the production of numerous eggs (Drnevich *et al*., 2001).

The biosynthetic route to 4-methylnonanol has been elucidated (Islam et al., 1999). The pathway is a modification of normal fatty acid biosynthesis. The production of 4-methylnonanol begins with one unit of propionate, a three-carbon compound, which results in an uneven number of carbons in the pheromone product (Islam et al., 1999). The addition of a second molecule of propionate is responsible for the methyl branch on the nine-carbon chain (Islam et al., 1999). A 4-methylnonanoyl derivative (most likely a CoA derivative) is then reduced to the pheromone product, an alcohol. This reduction is of particular interest, since it is the regulated step in the pathway (Bacala, 2000). The reduction of fatty acid derivatives to alcohols generally proceeds via an aldehyde intermediate (Riendeau and Meighen, 1985). The 4-methylnonanoyl derivative must undergo reduction to the alcohol with the aid of two enzyme activities: acyl CoA reductase and aldehyde reductase, which may or may not be located on the same polypeptide chain.

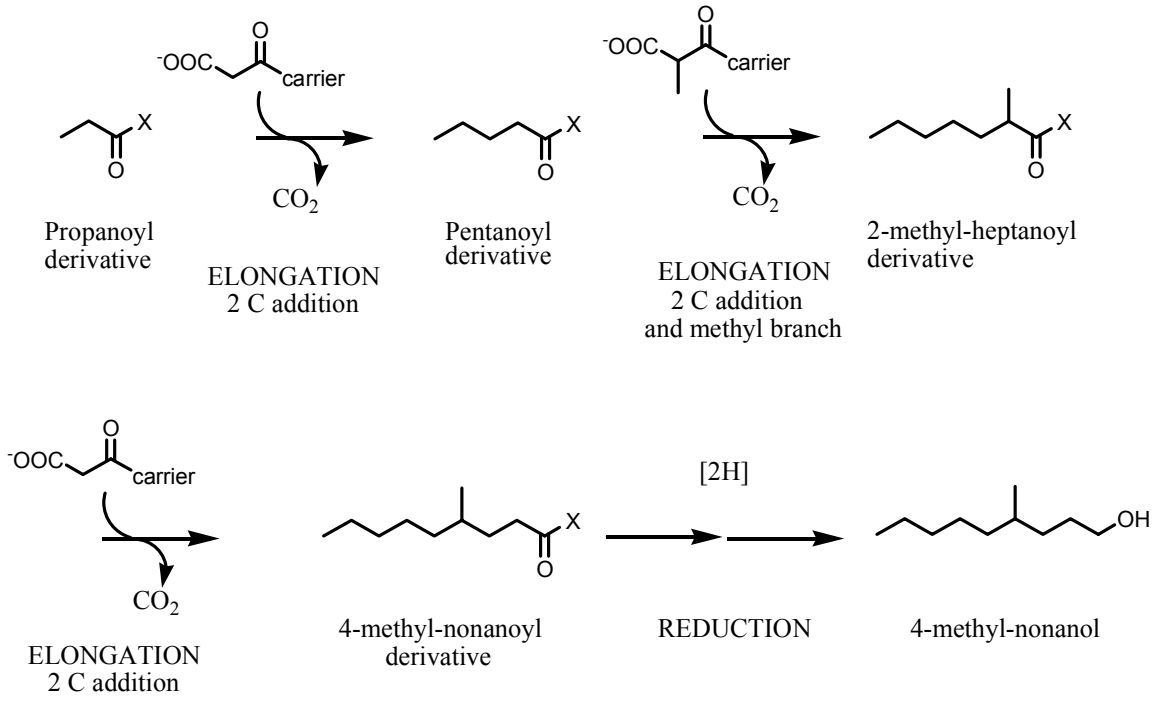

Figure 1: Biosynthetic route to 4-methyl-1-nonanol in female *T. molitor* (Islam *et al.*, 1999). The X is an activated derivative such as Acyl Carrier Protein (ACP) or CoA.

An earlier study in this laboratory by Bacala (2000) yielded confounding results as to the specific sub-cellular location of the acyl CoA reductase. The results were puzzling for several reasons. First, Bacala observed no reducing cofactor dependence: activity was observed in the absence of cofactors. However, the conversion of an acyl CoA derivative to an alcohol is a reduction, and so must be accompanied by a reducing cofactor (Lehninger *et al.*, 1993). Second, Bacala (2000) determined that NADH is the preferred reducing cofactor. This is surprising since most known eukaryotic acyl-CoA reductases have a strong preference for NADPH (Riendeau and Meighen, 1985; Wang and Kollatukudy, 1995; Vioque and Kollantukudy, 1997). Third, Bacala determined that the location of pheromone production was in the mitochondria. Mitochondria usually have an abundance of NADH, while NADPH is not present in high concentrations (Van

den Bergh, 1965). Finally, mitochondria require transport systems for the entry of most material into the organelle. This causes some complications associated with the possibility of a fatty acid entering the mitochondria, a fatty alcohol leaving the mitochondria, and exogenously supplied NADH supporting pheromone production *in vivo*. At the time of the study by Bacala (2000), the tissue location of pheromone production was not known, so he used homogenate of the whole beetle for his experiments. It is possible that the presence of large amounts of cofactors and enzymes unrelated to pheromone production may have confounded the study.

The goal of this project was to clarify the results obtained by Bacala (2000), using homogenate prepared from the ovaries, the tissue involved in pheromone biosynthesis (McCormick, 2003). This entails studying the precise subcellular location of the enzyme 4-methylnonanoyl CoA reductase. The approach for the study of the location of this enzyme consists of the removal of the ovaries, homogenization, centrifugation, *in vitro* radio assays, separation of products and quantification by scintillation counting. Enzyme activity was determined by radioassay (studying the conversion of a radioactive precursor to a radioactive product), to allow for increased specificity and selectivity of the assays. Thus it was necessary to synthesize the CoA derivative of the tritiated precursor, [^{3}H]-4-zmethylnonanoic acid. Preliminary marker enzyme assays were also performed, to help confirm the content of the subcellular fractions.

Mangat and Vanderwel (unpublished) determined that the reduction of the 4-methylnonanoyl moiety uses a coenzyme A (CoA) derivative. In previous studies, the enzyme homogenates were supplied with free fatty acid (solubilized with detergent), ATP and CoA. Endogenous acyl CoA synthetase was relied upon to convert the free fatty acid

to the CoA derivative, the actual substrate for pheromone biosynthesis. However, once the cellular compartments are separated by differential centrifugation, it can no longer be assumed that acyl CoA synthetase will be present in the same fraction as 4-methylnonanoyl CoA reductase. For this reason, it was necessary to use the actual substrate for the acyl CoA reductase in the *in vitro* experiments. A method for large-scale synthesis of short chain fatty acyl CoA by Padmakumar *et al.* (1997) was adapted in order to synthesize the medium chain [^{3}H]-4-methylnonanoyl CoA on a small scale.

Marker enzyme analysis was used to confirm that the fractions thought to contain a certain subcellular fraction actually did contain that subcellular fraction. Previous results obtained by Bacala (2000) demonstrate that the "mitochondrial" and "supernatant" fractions did indeed primarily contain the mitochondrial and supernatant marker enzymes, respectively. Thus the experimental procedures were not repeated for this project. The data collected by Bacala is presented in the "Data and Results" section in order to visualize the results. Cytochrome *c* oxidase was assayed as the mitochondrial marker and alcohol dehydrogenase was assayed as the soluble, supernatant marker (Bacala, 2000). The marker enzyme for the microsomal fraction in Bacala's project yielded confounding results and thus another enzyme marker, cytochrome *c* reductase, was assayed in order to determine the location of the microsomes.

The approach used in this project allowed for assays that were sensitive and selective for the isolation of 4-methylnonanoyl CoA reductase and for the quantification of the pheromone produced. The elucidation of the subcellular fraction containing the enzyme that catalyzes the production of 4-methylnonanol in female beetles will allow for a better insight into the regulation of pheromone production in beetles.

2.0. MATERIALS AND METHODS

2.1. Insect cultures

Tenebrio molitor larvae were obtained from Northwest Scientific Supply Ltd. The insects were maintained in an incubator at 26 ^{o}C (± 0.5) with a 12 hour light/12 hour dark photoperiod and a humidity of 20-30%. Moist paper towels were spread over the colonies and the larvae were occasionally fed potatoes in order to provide liquid moisture to the insects. The larvae were fed a diet of wheat bran, rolled oats and wheat germ (20:10:5 by volume). The pupae were removed from the colonies and separated by sex using the method described by Bhattacharya et al. (1970). The developing genital structures are visible behind the 7th visible abdominal sternite. Males have a small swelling, which has a pair of short and blunt papillae along the midline. In females the swelling is larger and has a pair of widely diverging papillae (Bhattacharya et al., 1970). Female adult virgins between 4 and 7 days post-emergence were used for the experiments. The photoperiod was reversed so that the experiments could be run during the day, or the beetles' night. The beetles produce more pheromone during their dark photoperiod (Vanderwel, unpublished).

2.2. Purchased Chemicals

All solvents were purchased from VWR Canlab and were of spectophotometric grade or better. Adenosine 5'-triphosphate disodium salt (ATP, 98% purity), coenzyme A lithium salt (CoA, 94% purity), nicotinamide adenine dinucleotide disodium salt (reduced form, β-NADH, 98% purity), mammalian protease inhibitor cocktail, N-octyl-N,N-dimethyl-3-ammonio-1-propanesulphonate (Zwittergent® 3-8, 97%) and Florisil®

activated magnesium silicate (column chromatographic stationary phase 100-200 mesh) were obtained from Sigma-Aldrich. Liquid scintillation fluid ScintiSafeTM Econo 2 and ScintiSafe Plus 50% were obtained from Fischer Scientific. All purchased chemicals were used without further purification. [^{3}H]-4-Methylnonanal was generously donated by Aaron Larsen (Dr. Vanderwel's lab, the University of Winnipeg).

2.3. Buffers

A phosphate buffer system was used to run the *in vitro* assays. It consisted of 50 mM of potassium dihydrogen phosphate, 1 mM DTT, 50 mM KCl, 10% glycerol (v/v) and 2mM $MgCl_2$, adjusted to pH 7.4. A "MITO" buffer system, established by Bacala (2000), was used to optimize the isolation of intact mitochondria. This MITO buffer was used for the homogenization and subsequent subcellular fractionation of the ovary tissue. This buffer consists of HEPES (5 mM), potassium dihydrogen phosphate (5mM), sucrose (300mM), EGTA (1 mM), DTT (1mM) and $MgCl_2$ (2mM), adjusted to pH 7.4.

2.4. Synthesis of Required Chemicals

[^{3}H]-4-Methylnonanoyl CoA was synthesized by adapting the large-scale method of Padmakumar *et al.* (1997) to the small scale required to work with radioactive substrates. Approximately 40 mL of [^{3}H]-4-methylnonanoic acid in hexanes [that is approximately 2.64 x 10^8 counts (DPM)] was concentrated to 200 µL, and then 200 µL of dimethylformamide (DMF) was added to the acid. The solution was concentrated again to remove all of the hexanes to 300 µL. One microliter of thiophenol was also added to this mixture. In another conical vial dicyclohexylcarbodiimide (DCC, 0.0025 g, i.e. 24.4

mmol) was added to 200 µL of DMF. The DCC-DMF solution was added drop wise to the DMF with [^{3}H]-4-methylnonanoic acid and thiophenol over a period of 5 minutes. The solution was then magnetically stirred at 0^{o}C for two hours. The reaction was stopped by the addition of 2 mL of cold water. The solution was extracted three times with 1 mL diethyl ether. The organic layers were combined and washed with 2 x 1 mL of water (always keeping the product on ice) and dried with anhydrous sodium sulfate. The product was concentrated to approximately 100-200 µL and 1.5 mL of tetrahydrofuran (THF) was added under nitrogen. Approximately 77mg of CoA was dissolved in 1 mL of 0.1 M $NaHCO_3$, and the pH adjusted to 8. This was added to the product of the first step. The solution was stirred vigorously and the reaction was kept on ice for 15-30 minutes. At this point, thin layer chromatography (TLC) was used to monitor the progress of the reaction. The TLC was run in small closed chambers with a solvent system consisting of 80:20 hexanes: diethyl ether (v/v). The reaction was terminated by dropping the pH to 6. The unreacted free fatty acid was extracted with hexanes (4 x 1 mL), leaving the desired CoA product in the aqueous phase.

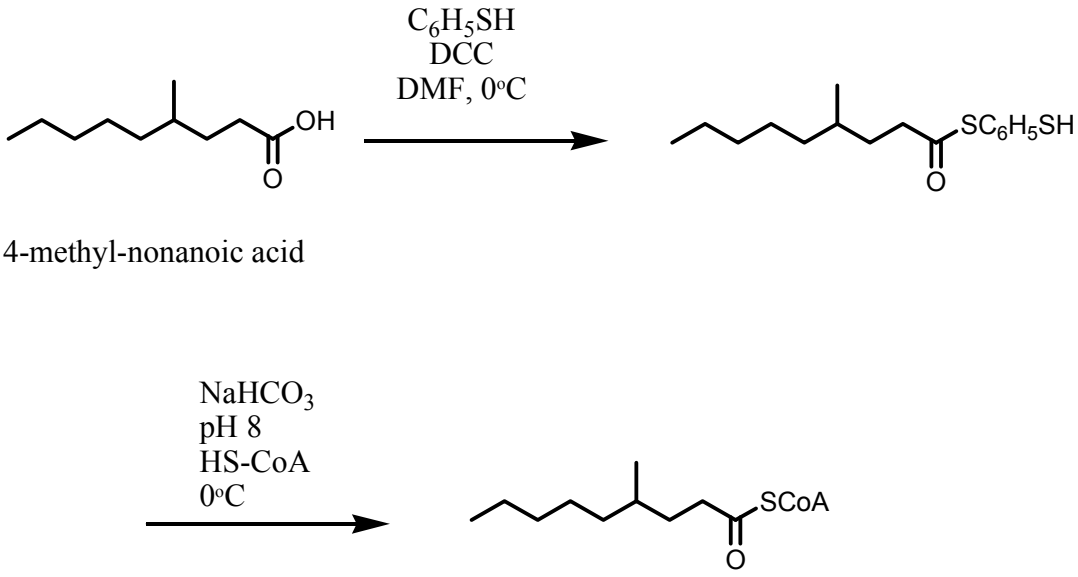

Figure 2: Synthetic route from 4-methyl-1-nonanoic acid to 4-methyl-1-nonanoyl CoA.

2.5. Dissections

The reproductive tract is the site of production of the female sex pheromone 4-methylnonanol (McCormick, 2003). Therefore in order to minimize potential side reactions or complications due to other tissues, the ovaries of *T. molitor* were removed and assayed separately. The adult female beetles were cold anesthetized, and then the legs, the wings and the elytra were removed. The beetles were then dissected in a Petri dish containing the MITO buffer. The ovaries take up a large proportion of the abdominal cavity and were easily removed. Once removed, the ovaries were placed in a specific MITO buffer with mammalian protease inhibitor cocktail (5 µL per ovary).

2.6. Preparation of Homogenate

The ovaries in the MITO buffer were then homogenized for 2 minutes by hand with a ground glass homogenizer (Kontes Glass Co. Duall R 22). Then the homogenizer rod was attached to a 0.33 Amps Laboratory Stirrer and homogenized for 5 minutes at a speed of approximately 3000 RPMs or until there were no deposits at the bottom of the homogenizing tube.

2.7. Subcellular Fractionation

The homogenized ovary mixture was then centrifuged (Beckman model L3-5-ultracentrifuge, with a Ti45 rotor type). The first spin was at 1,000 x g for 10 minutes, the second spin was at 15,000 x g for 10 minutes and the third spin was at 150,000 x g for 90 minutes (Bacala, 2000). The first spin yielded unbroken cells in the pellet, the second spin yielded the mitochondrial pellet and the third spin yielded the microsomal pellet, which was then separated from its supernatant. The three pellets, the supernatant and the controls were then assayed separately in triplicate.

2.8. *In vitro* Enzyme Assays

The incubation mixture using the acid precursor consisted of the following co-factors and reagents (final concentrations): ATP (2mM), CoA (60 μM), NADH (1 mM), zwittergent (50 mM) and 5 μL of protease inhibitor cocktail for each set (from one beetle) of ovaries. Each tube was also given 500 000 counts of [^{3}H]-4-methylnonanoic

acid as a starting substrate. This incubation mixture was optimized for one "beetle equivalent"[1] per mL of incubation mixture.

In order to refine the localization of pheromone, [^{3}H]-4-methylnonanoyl CoA was synthesized, and the incubation mixture was altered to account for the new substrate. Since the new starting substrate was [^{3}H]-4-methylnonanoyl CoA there was no longer a requirement for ATP or CoA in the incubation mixture (instead these two cofactors were used to synthesize the CoA derivative *in vitro*). Thus the new incubation mixture when using 500 000 counts (DPMs) of [^{3}H]-4-methylnonanoyl CoA, consisted of NADH (1 mM), and 5 μL of protease inhibitor cocktail for each set (from one beetle) of ovaries.

One preliminary experiment was also performed to isolate the enzyme activity for aldehyde reductase (the next step in the reduction pathway). As was the case for the 4-methylnonanoyl CoA, there was no need to add ATP or CoA, because [^{3}H]-4-methylnonanal was synthesized previously. The *in vitro* assay conditions for the aldehyde reductase trials were identical to the ones described for the isolation of acyl CoA reductase, except that Zwittergent was required to help solubilize [^{3}H]-4-methylnonanal in the incubation mixture.

To each tube the required cofactors and substrates were added: NADH, 500 000 DPM of [^{3}H]-4-methylnonanoyl CoA and 0.1 "beetle equivalents". The test tubes containing the reaction mixture are then incubated at 30^{o}C and after ten minutes the addition of 0.5 mL of 30 % trichloroacetic acid to each test tube terminated the reaction.

[1] A "beetle equivalent" is one pair of ovaries or one whole beetle.

2.9. Extraction, purification and quantification of [^{3}H]-4-methylnonanol

The pheromone product and other organics were extracted from the aqueous assay mixture using 4 x 2 mL of hexanes. The four organic extracts from each assay tube were combined and dried over granular anhydrous Na_2SO_4. The dried organic extracts were then subjected to bench-top column chromatography. Mini-columns were packed with 5.0 g of Florisil™ (100-200 mesh). Hexanes were used to wet pack the column, and then the organic fractions were loaded onto the columns. The solvent system used to elute the alcohol from the column was 90:10 hexanes: diethyl ether (v/v). Twenty-five, five milliliter fractions were collected (not including the loading fraction). Scintillation fluid (6 mL) was added to each fraction collected and the fractions were analyzed for radioactivity content by liquid scintillation counting (Beckman LS 6500 multi-purpose scintillation counter). The chromatographic elution profile of each trial was then compared to the elution profiles of [^{3}H]-4-methylnonanoic acid and [^{3}H]-4-methylnonanol standards. The alcohol usually eluted in fractions 14 to 20. The quantity of radioactivity in these fractions was added together in order to determine the total quantity of [^{3}H]-4-methylnonanol produced.

2.10. Protein Assay

The protein concentration in each enzyme preparation was determined by the Bradford method using Bovine Serum Albumin (BSA) as the standard (Bradford, 1976). One hundred microliters of BSA (containing 0.1, 0.2, 0.3, 0.4 or 0.5 mg/mL) was added to 3.00 mL of Bradford reagent to construct the standard curve. The enzyme extracts were too concentrated to fit within the range of the BSA standard, therefore the enzyme

extracts were diluted so that the concentrations would fall within the appropriate range. Thus for the crude, the unbroken cells pellet, the mitochondrial pellet and the supernatant, 98 µL of distilled water and 2 µL of enzyme extract was added to the 3.0 mL of Bradford reagent (95 µL of distilled water and 5 µL of enzyme extract was used for the microsomal pellet). This method was used to analyze the concentration of the protein used in the *in vitro* assays. The absorbance was measured at 592 nm after 20 minutes using an HP Diode Array Spectrophotometer to analyze the concentration of the protein used in the *in vitro* trials. Protein concentrations were deduced from the standard curve obtained using the BSA standard.

2.11. Marker Enzymes Analysis

The microsomal enzyme marker used in this experiment was NADPH-cytochrome c reductase from a method adapted from Weirich and Adams (1984). The assay mixtures contained 100 µL of protein, 10 mM KCN, 3mM NADPH, 1.5 mM of cytochrome *c* and 2.6 mL of MITO buffer. The total volume was 3 mL. The reaction was initiated by the addition of NADPH. The reduction of cytochrome *c* at 30^{o}C was measured as an increase in absorbancy at 550 nm and comparing to a blank with no NADPH. The enzyme activities were then calculated using18.5 $mM^{-1}cm^{-1}$ as the absorption coefficient for reduced cytochrome *c* (Weirich and Adams, 1984). The assays for the mitochondrial marker and the soluble marker were previously performed by Bacala (2000), and are provided in the "Data and Results" section for inspection.

2.12. Statistical analysis

The means were compared using the Analysis of Variance Test ($\alpha = 0.05$).

3.0. DATA AND RESULTS

3.1. SYNTHESIS OF REQUIRED CHEMICAL

[^{3}H]-4-Methylnonanoyl CoA was synthesized with a 12.4 % yield by an adaptation of the method described by Padmakumar *et al*. (1997). One reason that accounts for the low percent yield obtained for this reaction is that the last step that converts the thiol to the acyl CoA occurs in an aqueous environment. Since the solvent was present in a much higher concentration then the CoA reagent, it could outcompete the CoA for nucleophilic attack on the carbonyl carbon. However unreacted [^{3}H]-4-methylnonanoic acid could be recovered, so the low yield was not especially problematic.

3.2. BIOCHEMICAL STUDIES

3.2.1. Preliminary *In Vitro* Experiment

After the synthesis of [^{3}H]-4-methylnonanoyl CoA, a preliminary experiment was performed to determine if the synthesized product would serve for *in vitro* studies of pheromone production. The results of the trials are represented in Figure 3. The results depicted in this Figure demonstrate a high conversion rate of [^{3}H] 4-methylnonanoyl CoA to [^{3}H] 4-methylnonanol. The amounts of final pheromone product are not comparable to the results of later experiments due to the fact that whole female beetles were used in these trials.

3.2.2. Aldehyde *In Vitro* Experiments

The abilities of the microsomal, mitochondrial and supernatant fractions of the ovary to reduce the aldehyde intermediate to the alcohol were determined (Figure 4). The buffer was optimized for the isolation of mitochondria (MITO buffer) (Bacala, 2000). The activity of the aldehyde reductase enzyme was apparently ubiquitous: there was no statistically significant difference in activity between the subcellular fractions.

3.2.3. Fatty acyl CoA *In Vitro* Experiments

The subcellular location of the enzyme 4-methylnonanoyl CoA reductase was examined in two experiments. The first experiment was a preliminary trial to compare the activity of 4-methylnonanoyl CoA reductase in the three subcellular fractions (mitochondrial, microsomal and supernatant). The results of the first experiment are represented in Figure 5. This graph demonstrates that the majority of the enzymatic activity was present within the "mitochondrial" fraction.

The second experiment also compared the activity of 4-methylnonanoyl CoA reductase in the three subcellular fractions. The concentration of the enzyme in this assay was decreased to one-tenth the original substrate concentration in the previous experiment. This allowed an even better comparison of the activity present within the subcellular fractions. This second experiment confirmed the results of the first: the majority of the activity for the enzyme 4-methylnonanoyl CoA reductase was present in the "mitochondrial" fraction. The results of the second experiment are depicted in Figure 6.

3.2.4. Protein Curve

In order to determine the concentration of protein in the various subcellular fractions, a protein curve was established based on bovine albumin standard. The concentrations of protein used in the fatty acyl CoA *in vitro* experiment with 0.1 beetle equivalents of protein are represented in Table 1. A second protein curve was established for the marker enzyme analysis; these data are represented in Table 1.

3.2.5. Marker Enzyme Assays

Figure 7 represents the data Bacala (2000) collected during his experiments. Figure 7 A illustrates the 25 000x g (or "mitochondrial") subcellular fraction (Bacala, 2000). This fraction contained 96.9% of the total cytochrome *c* oxidase activity (Bacala, 2000). Figure 7B on the other hand reveals that the majority of the alcohol dehydrogenase activity is localized in the soluble or supernatant fraction (Bacala, 2000).

The procedure used to assay cytochrome *c* reductase activity was adapted from an assay used in moth midgut tissue (Weirich and Adams, 1984). The preliminary results for the cytochrome *c* reductase experiment are represented in Figure 8. Cytochrome *c* reductase is a microsomal marker (Weirich and Adams, 1984). In order to confirm that the enzyme assay would function in *T. molitor* tissue, a first trial was performed for only the microsomal subcellular fraction. Based on the graph in Figure 8 the level of activity in the microsomal fraction can be calculated as 6.98×10^{-2} nmol/min/mg. The specific activity for cytochrome *c* reductase for this experiment was approximately in the same range as the literature values (Weirich and Adams, 1984).

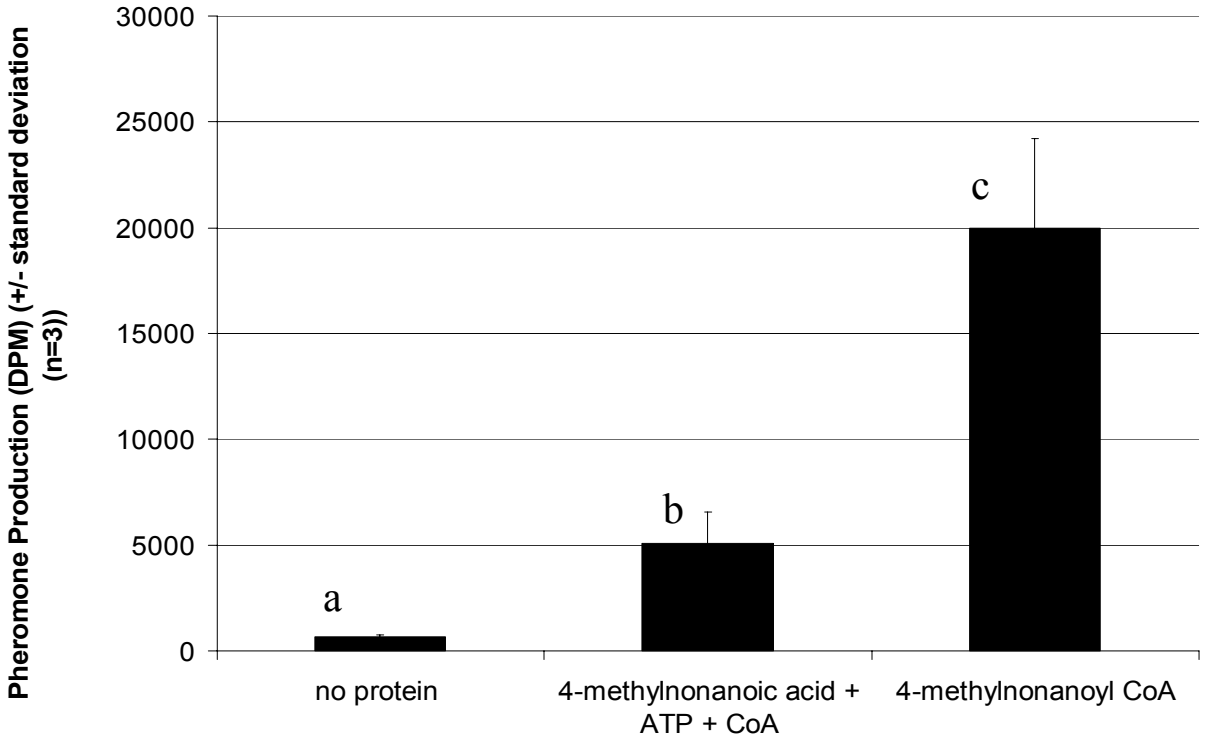

Figure 3: Efficacy of using 4-methylnonanoyl CoA compared to 4-methylnonanoic acid. Assay tubes each contained a whole homogenized beetle, the reducing cofactor NADH and phosphate buffer (pH 7.4). The substrate for the no protein control was 4-methylnonanoic acid. Products were separated by florisil column with 90:10 hexanes: diethyl ether. Differently labeled means are significantly different ($p < 0.05$).

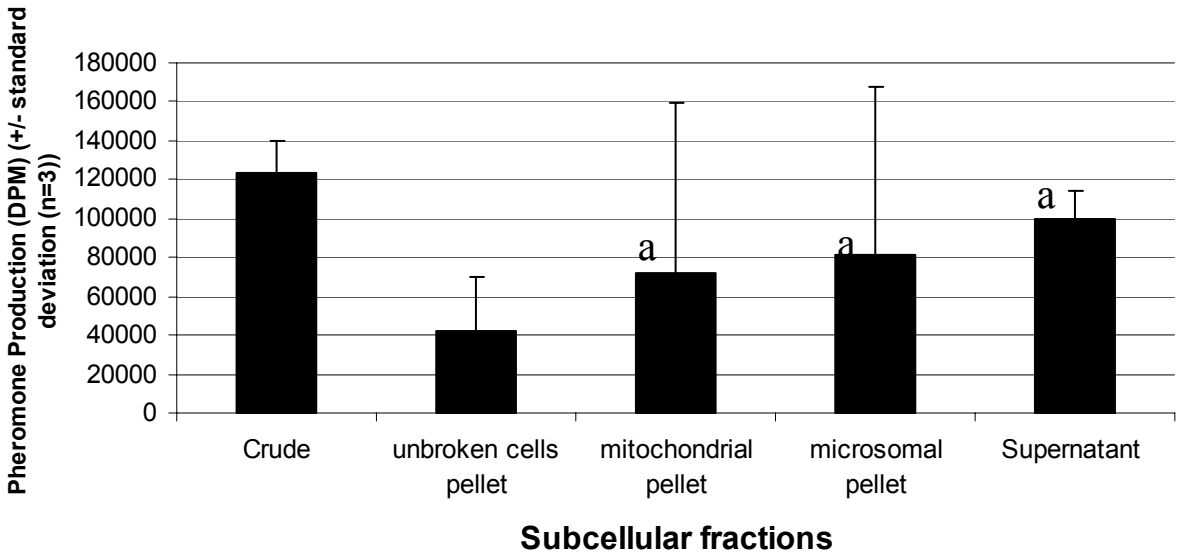

Figure 4: Subcellular localization of Aldehyde Reductase. Assay tubes each contained 1.0 "beetle equivalent" of ovary protein, [^{3}H] aldehyde precursor and phosphate buffer (pH 7.4). Products were separated by florisil column with 90:10 hexanes: diethyl ether. Differently labeled means are significantly different ($p < 0.05$).

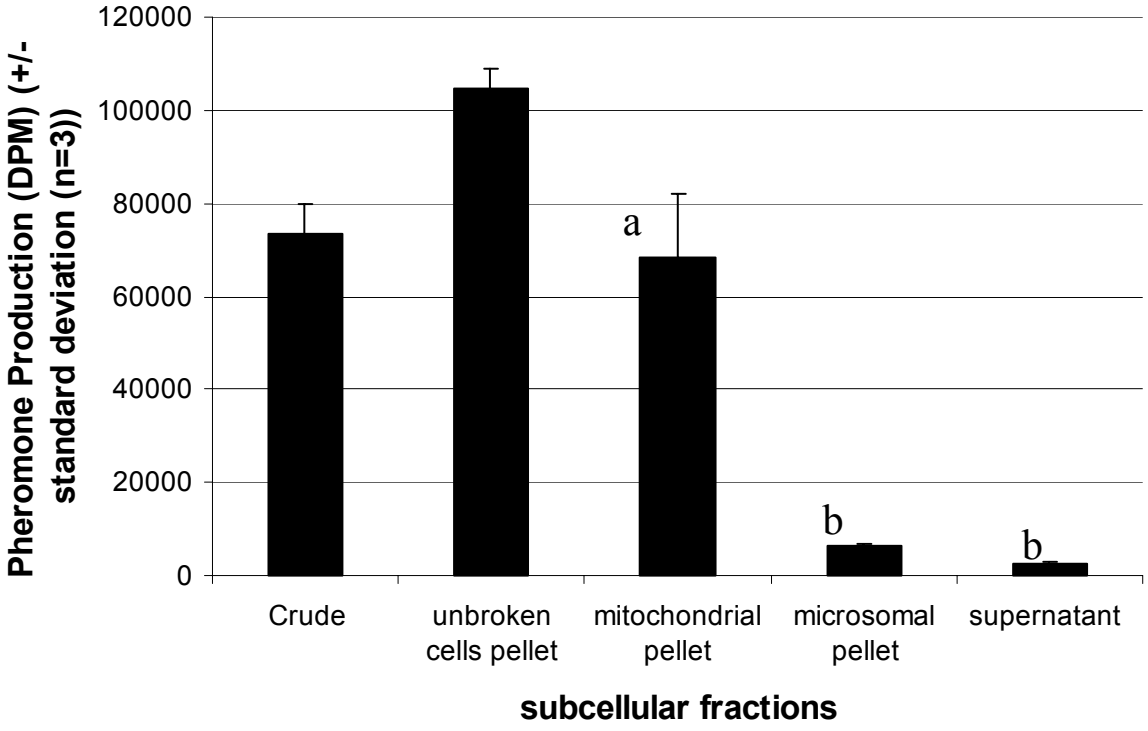

Figure 5: Subcellular localization of CoA Reductase. Assay tubes each contained 1.0 "beetle equivalent" of ovary protein, [3H] 4-methylnonanoyl CoA precursor and NADH in 50 mM phosphate buffer (pH 7.4). Products were separated by florisil column with 90:10 hexanes: diethyl ether. Differently labeled means are significantly different ($p < 0.05$).

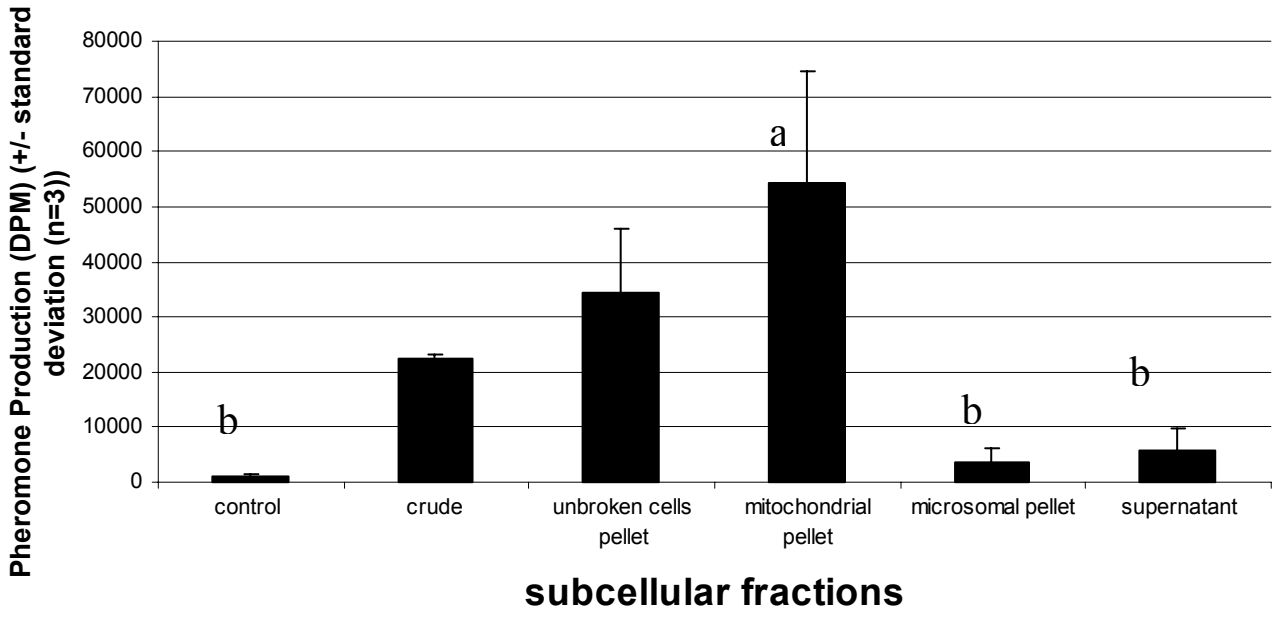

Figure 6: Subcellular localization of the CoA Reductase. Assay tubes each contained 0.1 "beetle equivalent" of ovary protein, [3H] 4-methylnonanoyl CoA precursor and NADH in 50 mM phosphate buffer (pH 7.4). Products were separated by florisil column with 90:10 hexanes: diethyl ether. Differently labeled means are significantly different ($p < 0.05$).

Table 1: Standard Bradford 3.1 mL protein concentration curve, with bovine albumin standard. Calculated concentrations for all five cellular fractions the protein based of the fatty acyl CoA *in vitro* experiment with 0.1 beetle equivalents of protein (Figure 6).

Results from the Protein curve		
fraction	concentration (mg/ µL)	% total Protein
crude	0.01132	22.0
unbroken cells pellet	0.01058	20.5
mitochondrial pellet	0.00899	17.4
microsomal pellet	0.00391	7.6
supernatant	0.01683	32.6

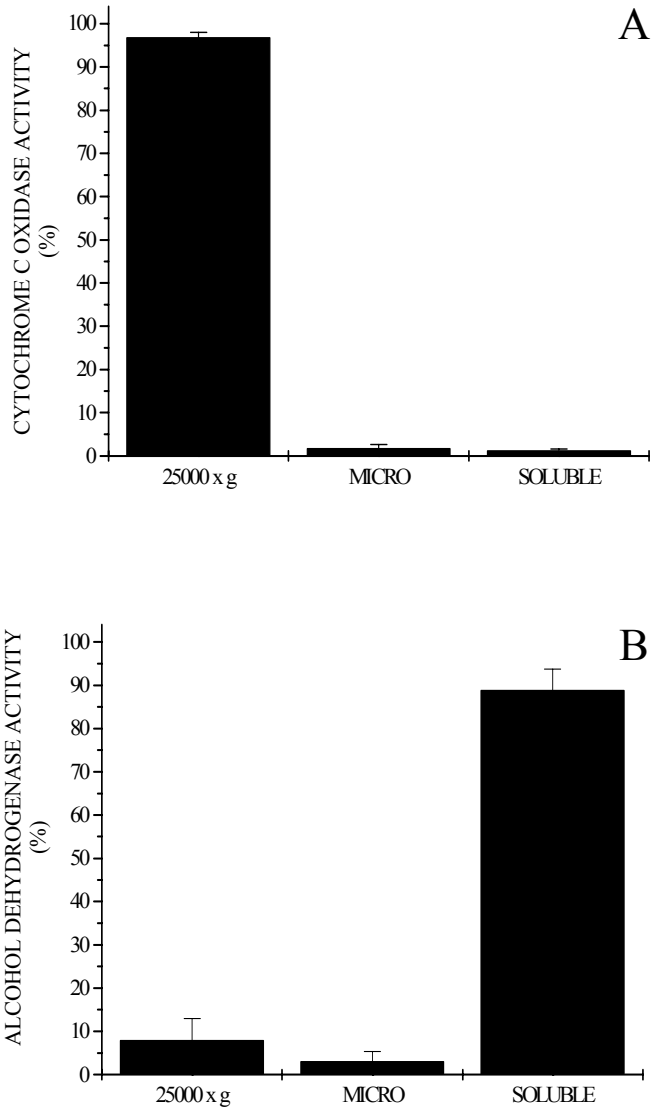

Figure 7: Sub-cellular distribution of marker enzyme activities. A: Cytochrome C oxidase (mitochondrial marker). For each treatment, n=2. B: Alcohol dehydrogenase (soluble marker). For each treatment, n=3. Activity was assayed using 10µl, 100µl or 500µl of protein from the respective sub-cellular fraction. Activity was normalized to sub-cellular fraction volume for comparison (Bacala, 2000).

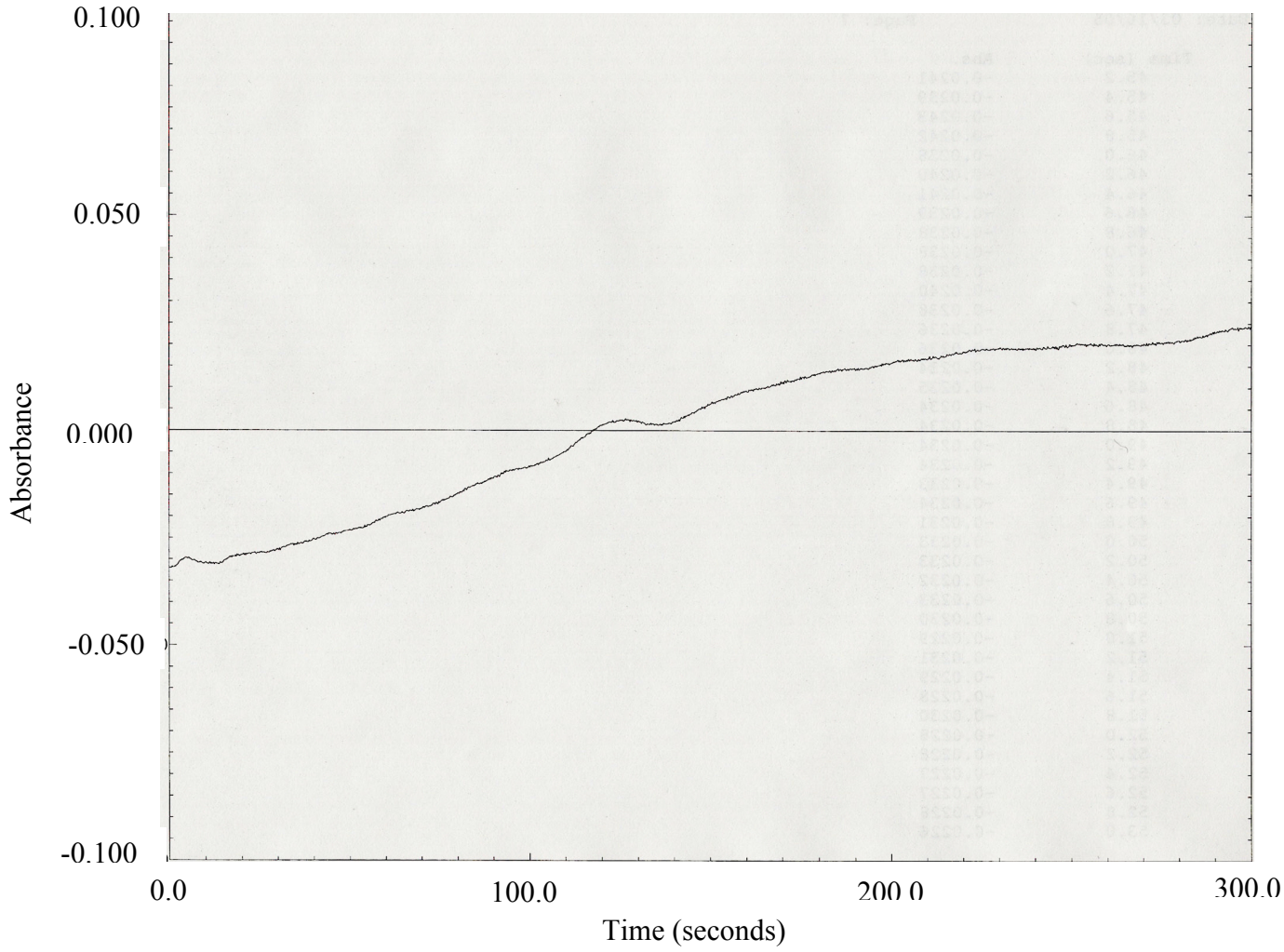

Figure 8: Assay of Cytochrome *c* reductase activity on microsomal fraction of ovary homogenate measuring the increase in absorption of Cytochrome *c* as it is being reduced over time.

4.0. DISCUSSION

4-Methylnonanoyl CoA reductase, the key regulatory enzyme of pheromone biosynthesis in *T. molitor,* was localized in the mitochondrial fraction of the ovary homogenate. This is a highly unusual finding because generally the acyl CoA reductases are located in either the microsomes or the soluble fractions (Riendeau and Meighen, 1985). The elucidation of the location of 4-methylnonanoyl CoA reductase will allow a better understanding of the biochemical pathway that leads to the production of 4-methylnonanol.

4.1. Preliminary Experiments

The first step of this project was to ensure that the synthesized [^{3}H] 4-methylnonanoyl CoA could be used as a substrate by 4-methylnonanoyl CoA reductase. *In vitro* reduction of the synthesized tritiated 4-methylnonanoyl CoA to the pheromone was successful (Figure 3). Not surprisingly, [^{3}H] 4-methylnonanoyl CoA was a superior precursor to [^{3}H] 4-methylnonanoic acid. Use of the acid precursor relied on endogenous acyl CoA synthetase activity to convert the precursor to the CoA derivative. In contrast 4-methylnonanoyl CoA can be used as a substrate directly. For this project, it was important to present the enzyme with its actual substrate, the CoA derivative, since the endogenous acyl CoA synthetase may not have been present in the same subcellular fraction as 4-methylnonanoyl CoA reductase.

Riendeau and Meighen (1985) indicated that distinct enzymes are usually involved in the reduction of fatty acids to aldehyde and from aldehyde to alcohol. However, it is possible that one protein might have both activities. The first step is

catalyzed by the enzyme 4-methylnonanoyl CoA reductase, which most likely forms an aldehyde intermediate. The second step, catalyzed by an aldehyde reductase, reduces the aldehyde intermediate to the alcohol (i.e. the final product). Figure 4 displays the results of the experiment that was devised to try to determine the subcellular location of the enzyme aldehyde reductase. As can be seen by the bar graph, the activity of the enzyme does not seem to be restricted to one subcellular location. The results obtained in this experiment therefore correspond to the normal distribution of aldehyde reductases in cell systems. Aldehydes are dangerous products when they are free in a cell system, thus enzymes that oxidize or reduce aldehydes are omnipresent within a cell system (Burcham *et al.* 2002). Reducing or oxidizing enzymes are specific for certain chain lengths. Since the 4-methylnonanol is of intermediate length, many enzymes may act on this substrate, causing non-specific reduction of the 4-methylnonanal.

4.2. Isolation of 4-Methylnonanoyl CoA reductase

4-Methylnonanoyl CoA reductase activity was localized to the mitochondrial fraction, confirming the earlier study by Bacala (2000). Figures 5 and 6 both show significantly more activity in the mitochondrial pellet, as compared to the microsome pellet and the supernatant.

Surprisingly, the activity present in the three subcellular fractions does not add up to the total crude fraction peak. The crude homogenate contains and extremely active thiolase, which hydrolyzes 4-methylnonanoyl CoA to the (unreactive) free acid (Anders and Vanderwel, unpublished). This thiolase may not be present in the mitochondrial fraction, allowing it to have a much higher level of activity (since more substrate would

be available). The activity in the mitochondrial fraction is unexpectedly high as compared to the crude homogenate, which is most likely due to the absence of acyl CoA hydrolase in the mitochondrial fraction.

The pathway to pheromone production starts with the reduction of the acyl CoA to the aldehyde and the aldehyde is then reduced to the alcohol. The elution profiles for Figures 5 and 6 did not have an aldehyde peak. If no aldehyde reductase was present within the mitochondrial fraction, no alcohol should have been present on the elution profiles. The only product present should have been the aldehyde. Since no aldehyde was present, the aldehyde reductase must therefore be present in the mitochondrial fraction. These experimental results help confirm the ubiquitous presence of aldehyde reductases within the mitochondria and most likely throughout the cell. Further research is needed to clarify the mechanism by which the aldehyde is reduced to the alcohol in the beetle ovaries.

All previously reported eukaryotic acyl CoA reductases have been microsomally localized (Riendeau and Meighen, 1985; Wang and Kollatukudy, 1995; Vioque and Kollatukudy, 1997). Thus the results obtained from this study are quite novel. In addition to the fact that a mitochondrially located acyl CoA reductase has not previously been characterized, there are several questions that arise. Was there damage to the mitochondria? Why does the enzyme utilize NADH? What transport systems allow for the entry of fatty acids into the mitochondria? What else can be done to ensure that these results are accurate?

Insect mitochondria are particularly fragile. Therefore several precautions as described by Van den Bergh (1965) should be taken to ensure proper isolation and

maintenance of the mitochondrial fractions. These precautions were taken while handling the mitochondrial fractions throughout this project.

The fact that 4-methylnonanoyl CoA has been localized within the mitochondria does concur with previous experimental results. A discrepancy resulting from the unconventional use of NADH by the enzyme 4-methylnonanoyl CoA reductase, when most acyl CoA reductases use NADPH, can be understood in the context that the enzyme resides in the mitochondria. Mitochondria have high concentrations of NADH. NADPH is also present in the mitochondria but in lower concentrations (Van den Bergh, 1965). Thus the type of reducing co-factor is also consistent with the evidence that 4-methylnonanoyl CoA is localized to the mitochondria of the ovary cells.

The mitochondrion is a self-sufficient organelle, it contains its own DNA and replicates independently from the cell. While the external mitochondrial membrane is quite permeable, transport systems are required to shuttle material in and out of the inner membrane. Fatty acid biosynthesis does not generally occur in the mitochondria, so the substrate for the enzyme 4-methylnonanoyl CoA reductase would need to be transported into the inner membrane to come into contact with the enzyme. Fatty acid breakdown does occur in the mitochondria, but the results of a previous study by Islam *et al.* (1999) indicates that the mechanism for the biosynthesis of 4-methylnonanol is a modification of the normal fatty acid biosynthesis and not a product of the breakdown of a long chain fatty acid. Therefore the mitochondria seem like an unlikely candidate for the location of the enzyme 4-methylnonanoyl CoA reductase.

Recent studies have discovered new information that could help elucidate the transportation issue of fatty acyl CoAs from their place of origin (the cytoplasm) to the

inner mitochondrial membrane. Classical methods of subcellular fractionation result in a mitochondrial fraction containing a sub-compartment of endoplasmic reticulum membranes or mitochondrial-associated membranes (MAMs) (Vance, 1990; Beauseigneur *et al.,* 1999; Bionda *et al.* 2004). Beauseigneur *et al.* (1999) determined that mitochondrial reactions using acyl CoAs as substrates are supplied from previous endoplasmic reticulum reactions. Vance (1990) revealed that when the mitochondria were further purified, the organelles lost the phospholipids biosynthetic enzyme activities characteristic of the endoplasmic reticulum. Therefore, the mitochondrial fractions obtained by the method of differential centrifugation outlined in this study could contain MAMs. The presence of attached portions of the endoplasmic reticulum, within the mitochondrial fraction, could explain many of the questions that have arisen from the isolation of the enzyme 4-methylnonanoyl CoA reductase in the mitochondria.

4.3. Further Studies

Marker enzymes indicate that the mitochondrial fraction definitely contained mitochondria, while the "soluble" and "microsomal" fractions did not (Bacala, 2000). The "soluble" fraction contained virtually all of the cytosolic marker enzyme activity. Bacala (2000) was unable to develop a working microsomal enzyme marker. However, this is clearly key to determining whether of not the "mitochondrial fraction" did indeed contain MAMs. The cytochrome *c* reductase marker enzyme selected for this study appears to be suitable. It has been shown to be the microsome marker enzyme of choice for insect tissue (Weirich and Adams, 1984), and was functional in *T. molitor*. The experiment remains to be performed for all three subcellular fractions.

The complete enzyme marker analysis will elucidate whether or not there are MAMs in the mitochondrial fraction. If the mitochondrial fraction is indeed contaminated with endoplasmic reticulum, further studies should be undertaken to promote a better purification of the subcellular fractions. If, on the other hand, no contamination is observed, *in vitro* assays will be performed with transport proteins, to determine how the 4-methylnonanoyl CoA enters the mitochondria.

A Percoll[2] density gradient will be utilized to establish the purity of the fractions that are presently obtained by normal centrifugation. This procedure will help elucidate more accurately the location of the enzyme 4-methylnonanoyl CoA reductase. The studies reporting that mitochondria are often associated with segments of the endoplasmic reticulum membrane indicates the possibility of contamination that could only be separated by a better method of differential centrifugation. The Percoll gradient allows for a better resolution in the separation of the subcellular location.

Purification and characterization of the enzyme 4-methylnonanoyl CoA reductase will lead to a better understanding of the biosynthesis and regulation of sex pheromone production. In the long term, studies such as determining the mechanism of action of regulatory events (e.g. maturation, mating) will be possible. Extrapolation from this model beetle system could lead to a greater understanding of the biochemistry of pheromone production in other beetle systems.

[2] A Percoll gradient is a method used to separate subcellular fractions based on the density of the fractions. This allows for further purification of the fractions obtained by centrifugation alone.

REFERENCES

Bacala, R. 2000. *In vitro* studies of sex pheromone biosynthesis in the yellow mealworm beetle*, Tenebrio molitor* (*Coleoptera: Tenebrionidaea*), MSc thesis. University of Manitoba.

Beauseigneur, F., Tsoko, M. Gresti, J., and P. Clouet. 1999. Reciprocal enzymatic interference of carnitine palmitoyltransferase I and glycerol-3-phosphate acyltransferase in purified liver mitochondria. Advances in Experimental Medicine and Biology. 466: 69-78.

Bhattacharya, A.K., Ameel, J.J., and G.P. Waldbauer. 1970. A method for sexing living pupal and adult yellow mealworms. Annals of the Entomological Society of America. 63: 1783.

Bionda, C., Portoukalian, J., Schmitt, D., Rodriguez-Lafrasse, C., and D. Ardail. 2004. Subcellular compartmentalization of ceramide metabolism: MAM (mitochondria-associated membrane) and/or mitochondria? Biochemical Journal. 382: 527-533 Part 2.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 72: 248-254.

Burcham, P.C., Kaminskas, L.M., Fontaine, F.R., Petersen, D.R., and S.M. Pyke. 2002. Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products. Toxicology. 181-182: 229-236.

Carpita, A., De Magistris, E., and R. Rossi. 1989. The racemic form and the two enantiomers of 4-methyl-1-nonanol, a sex attractant of the yellow mealworm, *Tenebrio molitor* L. Gazetta Chimica Italiana. 119: 99-105.

Delisle, J., Picimbon, J., and J. Simard. 2000. Regulation of pheromone inhibition in mated females of *Choristoneura fumiferana* and *C. rosaceana*. Journal of Insect Physiology. 46: 913-921.

Delisle, J. and J. Simard. 2002. Factors involved in the post-copulatory neural inhibition of pheromone production in Choristoneura fumiferana and C-rosaceana females. Journal of Insect Physiology. 48 (2): 181-188

Dickens, J.C. and G. Wiygul. 1987. Conspecific effects on pheromone production by the boll weevil, *Anthonomus grandis* Boh. (Col., Curculionidae). Journal of Applied Entomology. 104: 318-326.

Drnevich, J.M., Hayes, E.F. and R.L. Rutowski. 2000. Sperm precedence, mating interval, and a novel mechanism of paternity bias in a beetle (*Tenebrio molitor* L.). Behavioral Ecology and Sociobiology. 48 (6): 447-451

Drnevich, J.M., Papke, R.S., Rauser, C.L., and R.L. Rutowski. 2001. Material benefits from multiple mating in female mealworm beetles (*Tenebrio molitor* L.). Journal of Insect Behavior. 14 (2): 215-230

Gage, M. J. G. 1992. Removal of rival sperm copulation in a beetle, *Tenebrio molitor*. Animal Behaviour. 44: 587-589.

Happ, G. M. and J. Wheeler. 1969. Bioassay, preliminary purification, and effect of age, crowding and mating on the release of sex pheromone by female *Tenebrio molitor.* Annals of the Entomological Society of America. 62: 846-851.

Islam, N., Bacala, R., Moore, A., and D. Vanderwel. 1999. Biosynthesis of 4-methyl-1-nonanol: female-produced sex pheromone of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). Journal of Insect Biochemistry and Molecular Biology. 29: 201-208.

Judy, K. J., Scooley, D.A., Troetscler, R.G., Jennings, R.C., Bergot B.J. and M.S. Hall. 1975. Juvenile hormone production by corpora allata of *Tenebrio molitor in vitro.* Life Sciences. 16: 1123-1139.

Karube, F. and M. Kobayashi. 1999. Combinative stimulation inactivates sex pheromone production in the silkworm moth, *Bombyx mori*. Archives of Insect Biochemistry and Physiology. 42 (2): 111-118.

Lazazzera, B.A. and A.D. Grossman. 1998. "The Ins and Outs of Peptide Signaling", Trends in Microbiology. 6: 288-294.

Lehninger, A.L., Nelson, D.L. and M.M. Cox. 1993. Principles of Biochemistry. Worth Publishers, New York. p.390.

McCormick, P. 2003. *Localization of Sex Pheromone Production in the Female Yellow Mealworm beetle,* Tenebrio molitor (*Coleoptera: Tenebrionidaea*), BSc Thesis. University of Winnipeg.

McNeil, J. M., Delisle, J., and M. Cusson. 1997. Regulation of pheromone production in Lepidoptera: the need for an ecological perspective. In: Carde, R.T., and A.K. Minks (Eds). Insect pheromone research: new directions. Chapman and Hall, New York. Pp. 31-41.

Menon, M. 1970. Hormone-pheromone relationships in the beetle, Tenebrio molitor. Journal of Insect Physiology. 16: 1123-1139.

Miyatake, T., Chapman, T. and L. Partridge. 1999. Mating-induced inhibition of remating in female Mediterranean fruit flies *Ceratitis capitata*. Journal of Insect Physiology. 45 (11): 1021-1028.

Padmakumar, R., Padmakumar, R., and R. Banerjee. 1997. [24] Large-scale synthesis of coenzyme A esters. Methods in Enzymology. 279: 220-224.

Raina, A. K. 1997. Control of pheromone production in moths. In: Carde, R.T., and A.K. Minks (Eds). Insect pheromone research: new directions. Chapman and Hall, New York. Pp. 21-30.

Rantala, M.J., Kortet, R. Kotiaho, J.S., Vainikka, A. and J. Suhonen. 2003. Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor.* Functional Ecology. 17: 534-540.

Rasmussen, L.E.L, Lee, T.D., Roelofs, W.L., Zhang, A. and G.D. Daves Jr. 1996. "Insect Pheromone in Elephants." Nature. 379: 684.

Riendeau, D. and E. Meighen. 1985. Enzymatic reduction of fatty acids and acyl CoAs to long chain aldehydes and alcohols. Experientia. 41: 707-712.

Schal, C., Liang, D., and G. J. Blomquist. 1997. Neural and endocrine control of pheromone production and release in cockroaches. In: Carde, R. T., and A. K. Minks (Eds). Insect pheromone research: new directions. Chapman and Hall, New York. Pp. 3-20.

Seybold, S. and D. Vanderwel. 2003. Biosynthesis and endocrine regulation of pheromone production in Coleoptera. pp. 137-200 in Insect Pheromones: Biochemistry and Molecular Biology (edited by Gary Blomquist and Richard Vogt). Academic Press.

Siva-Jothy, M. T., Blake, D. E., Thompson, J., and J. J. Ryder. 1996. Short- and long-term sperm precedence in the beetle *Tenebrio molitor*: a test of the 'adaptive sperm removal' hypothesis. Physiological Entomology. 21: 313-316.

Tanaka, Y., Honda, H., Ohsawa, K., and I. Yamamoto. 1986. A sex attractant of the yellow mealworm, *Tenebrio molitor* L., and its role in the mating behavior. Journal of Pesticide Science. 11: 49-55.

Tanaka, Y., Honda, H., Ohsawa, K., and I. Yamamoto.1989. Absolute configuration of a sex attractant of the yellow mealworm, *Tenebrio molitor* L. Journal of Pesticide Science. 14: 197-202.

Vance, J.E. 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. The Journal of Biological Chemistry. 265 (13):7248-7256.

Valentine, J. M. 1931. The olfactory sense of the adult mealworm beetle Tenebrio molitor (Linn.). The Journal of Experimental Zoology. 58: 165-225.

Van den Bergh, S.G. 1965. Insect mitochondria. Methods in Enzymology. 12: 117-122.

Vanderwel, D. 2003. Sex and the Single Beetle. Canadian Chemical News. February.

Vioque, J. and P. E. Kollatukudy. 1997. Resolution of an Aldehyde-Generating and Alcohol-Generating Fatty Acyl-CoA Reductase from Pea Leaves (*Pisum savitum* L.). Archives of Biochemistry and Biophysics. 340: 64-72

Wang, X. and P. E. Kollatukudy. 1995. Solubilization, Purification and Characterization of Fatty Acyl-CoA Reductase from Duck Uropygial Gland. Biochemical and Biophysical Research Communications. 208: 210-215.

Weirich, G F. and J.R. Adams. (1984) Microsomal Marker Enzymes of *Manduca sexta* (L) Midgut. Archives of Insect Biochemistry and Physiology. 1 :311-321.