An *In Vitro* Assay for Monitoring Prenyl Transferase Activity in Lepidopteran Corpora Allata

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The prenyl transferase, farnesyl pyrophosphate (FPP) synthase, which catalyzes the head-to-tail couplings of dimethylallyl pyrophosphate (DMAPP) and geranyl pyrophosphate (GPP) with isopentenyl pyrophosphate (IPP), is a key enzyme in juvenile hormone (JH) biosynthesis. While moderate FPP synthase activity has been found in whole-body insect preparations, enzymatic activity in the corpus allatum, the gland responsible for JH biosynthesis, has been elusive. A new procedure for examining corpora allata prenyl transferase activity in the lepidopteran *Manduca sexta* is described. In contrast to most other short-chain prenyl transferases, the larval enzyme requires detergent for activity. Optimum conversion of DMAPP and GPP to FPP is obtained with corpora allata homogenates containing 2% Triton X-100, 0.15% bovine serum albumin, and 25% glycerol. The enzyme is activated in the presence of Mn^{2+} or Mg^{2+} and is inactivated by the addition of *N*-ethylmaleimide.

INTRODUCTION

The sesquiterpene juvenile hormone (JH) is vital to normal insect growth and development (Gupta, 1990). While the later steps in JH biosynthesis are unique to insects, formation of the farnesyl skeleton is common to all terpene biosynthetic pathways and is the result of the stepwise addition of isoprene building blocks that are formed by the condensation of three molecules of acetyl-CoA. For lepidopteran insects, JH is a series of five structurally related compounds (Scheme 1) that differ only in their carbon skeletons (Schooley et al., 1984). These homologous structures possess additional carbon units at the *cis*-vinyl methyl and C-4 methylene positions of the farnesyl chain.

It has previously been assumed that the formation of the farnesyl (or farnesyl homologue) skeleton in insects is catalyzed by the specific short-chain prenyl transferase farnesyl pyrophosphate synthase (FPP synthase, EC 2.5.1.10), a dimeric protein that has been purified from yeast, bacteria, plants, and porcine, avian, and human liver (Poulter and Rilling, 1983). This enzyme catalyzes the head-to-tail coupling of dimethylallyl pyrophosphate (DMAPP, **1a**) with two molecules of isopentenyl pyrophosphate (IPP, **2a**) to generate geranyl and farnesyl pyrophosphate (GPP and FPP, **3a** and **4a**, respectively, Scheme 1). Because of the additional homologues present in lepidopteran insects, the coupling pattern of allylic and homoallylic substrates for the corresponding insect prenyl transferase is more complex (Schooley and Baker, 1985). For example, the biosynthesis of methyl JH I requires the sequential coupling of homodimethylallyl pyrophosphate (HDMAPP, **1b**) with homoisopentenyl pyrophosphate (HIPP, **2b**) and (*E*)-3-methyl-3-pentenyl pyrophosphate (**5**) (Koyama et al., 1987).

Few researchers have investigated prenyl transferase activity in insects (Koyama et al., 1985a,b; Baker et al.,

1981). While moderate FPP synthase activity has been found in whole-body preparations (Koyama et al., 1985a), the enzymes specifically related to JH biosynthesis, located in the corpora allata, have proved elusive. Herein, we report an *in vitro* assay for examining prenyl transferase activity in larval corpora allata homogenates of the lepidopteran *Manduca sexta*. In contrast to most short-chain prenyl transferases, including all known eukaryotic farnesyl pyrophosphate synthases, the insect enzyme requires detergent for activity.
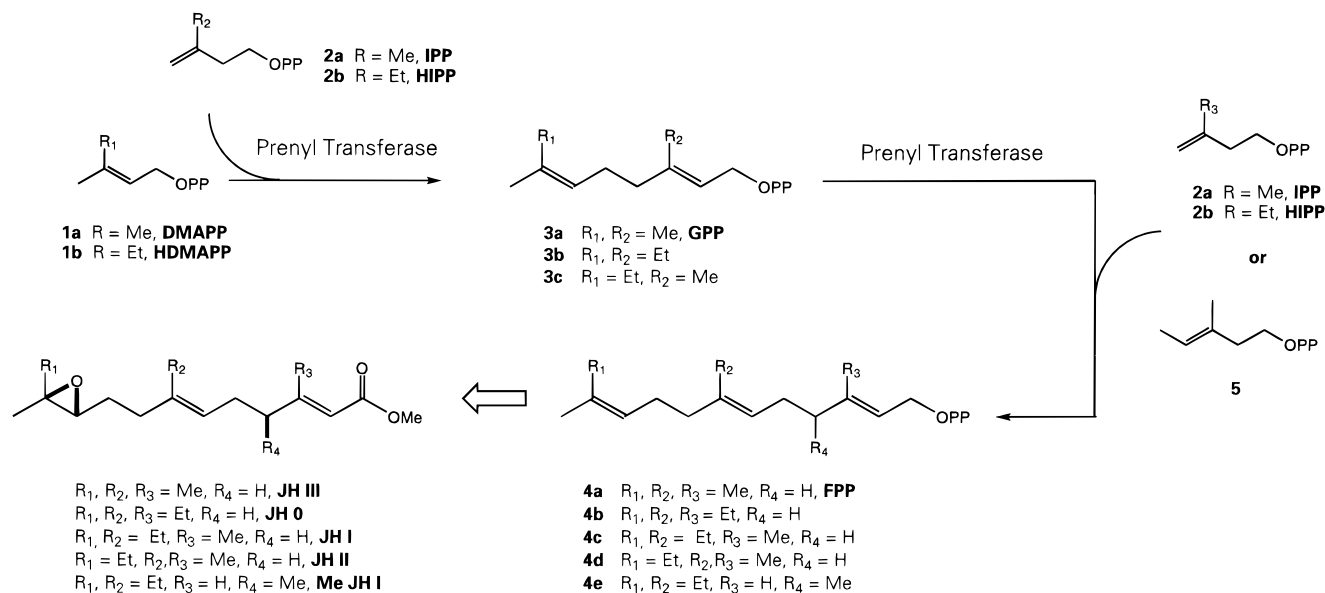
MATERIALS AND METHODS

Chemical Sources. Glycerol, bovine serum albumin (BSA), alkaline phosphatase, deoxycholate, 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), phenyl-methanesulfonyl fluoride (PMSF), and geranylgeraniol (GGOH) were obtained from Sigma Chemical Co. Solvents, buffer salts, Triton X-100, mercaptoethanol, and Scintiverse BD were obtained from Fisher Scientific Co. DMAPP and [4-^{14}C]IPP were purchased from American Radiolabeled Chemicals and DuPont New England Nuclear, respectively. All other chemicals were obtained from Aldrich Chemical Co.

Insects and Enzyme Source. *M. sexta* larvae were reared on an artificial diet and maintained under constant humidity (70%) and photoperiod (18 h light/6 h dark) using previously described procedures (Bell and Joachim, 1976). Animals were staged prior to molting at third and fourth stadia by observing head capsule detachment (Vince and Gilbert, 1977). Corpora cardiaca−corpora allata (CC−CA) complexes from day 0, 1, or 2, fifth stadium larvae (V/0−V/2) were removed following previously established procedures (Bhaskaran and Jones, 1980).

Optimum Enzyme Assay Conditions. Corpora cardiaca−corpora allata complexes were removed from fifth instar *M. sexta* larvae and immediately placed in a cold solution of 20 mM Tris-HCl buffer (pH 7) containing 25% glycerol, 0.15% BSA, 2% Triton X-100 (all w/v), 2.5 mM $MgCl_2$, 10 mM KF, and 0.5 mM mercaptoethanol. The glands were homogenized using a Duall glass homogenizer (Kontes) and sonicated on ice for 5 min. After removal of cellular debris by centrifugation at 16000*g* for 10 min, 25 μL aliquots of supernatant (containing

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Scheme 1. Formation of Farnesyl and JH Homologue Skeletons by Insect Prenyl Transferase

2a R = Me, **IPP**
2b R = Et, **HIPP**

Prenyl Transferase

1a R$_1$ = Me, **DMAPP**
1b R$_1$ = Et, **HDMAPP**

3a R$_1$, R$_2$ = Me, **GPP**
3b R$_1$, R$_2$ = Et
3c R$_1$ = Et, R$_2$ = Me

Prenyl Transferase

2a R = Me, **IPP**
2b R = Et, **HIPP**

or

5

R$_1$, R$_2$, R$_3$ = Me, R$_4$ = H, **JH III**
R$_1$, R$_2$, R$_3$ = Et, R$_4$ = H, **JH 0**
R$_1$, R$_2$ = Et, R$_3$ = Me, R$_4$ = H, **JH I**
R$_1$ = Et, R$_2$,R$_3$ = Me, R$_4$ = H, **JH II**
R$_1$, R$_2$ = Et, R$_3$ = H, R$_4$ = Me, **Me JH I**

4a R$_1$, R$_2$, R$_3$ = Me, R$_4$ = H, **FPP**
4b R$_1$, R$_2$, R$_3$ = Et, R$_4$ = H
4c R$_1$, R$_2$ = Et, R$_3$ = Me, R$_4$ = H
4d R$_1$ = Et, R$_2$,R$_3$ = Me, R$_4$ = H
4e R$_1$, R$_2$ = Et, R$_3$ = H, R$_4$ = Me

Table 1. Activity Enhancement in the Presence of Triton X-100, Glycerol, BSA, and PMSF

assay conditions	FPP formation, % (normalized)	assay conditions	FPP formation, % (normalized)
2% Triton X-100, 25% glycerol, 0.15% BSA	100	25% glycerol, 0.15% BSA	35
no additives	6	2% Triton X-100, 25% glycerol	55
25% glycerol	9	2% Triton X-100, 0.15% BSA	43
0.15% BSA	19	2% Triton X-100, 25% glycerol, 1 mM PMSF	40
2% Triton X-100	73	2% Triton X-100, 25% glycerol, 0.15% BSA, 1 mM PMSF	73

approximately 2 gland pair equiv) were placed into siliconized 500 μL plastic microcentrifuge tubes (Fisher Scientific Co.).

Prenyl transferase activity was determined using [4-^{14}C]IPP (specific activity 58 mCi/mmol) and either DMAPP or GPP as substrates. Geranyl pyrophosphate was prepared by reaction of the corresponding allylic chloride with tris(tetra-*n*-buty-lammonium)pyrophosphate (Davisson et al., 1985). Radioactively labeled IPP and cold allylic pyrophosphate were added as stock solutions to each enzyme aliquot to give final concentrations of 12 and 400 μM, respectively. The homogenate was incubated at 35 °C in an enclosed rotary shaker for 2 h, at which time the enzyme was denatured by heating the solution to 100 °C for 1 min. Under these assay conditions, further metabolism to farnesal, farnesoic acid, epoxyfarnesoate, or JH was not observed, as determined by HPLC analysis. The pyrophosphates were hydrolyzed to the corresponding terpenols by reaction with alkaline phosphatase (8 units in 15 μL of 500 mM Tris-HCl, pH 9.5, overnight at room temperature) and then quenched by the addition of acetonitrile (40 μL) containing isopentenyl alcohol, dimethylallyl alcohol, geraniol, farnesol, and GGOH standards.

Product Analysis. Terpenol formation was determined by radio-HPLC. A portion of the aqueous acetonitrile solution from each assay tube (approximately 40 μL) was injected onto a C$_{18}$ reversed phase column (2.5 × 15 cm, Rainin) and eluted with a two-step CH$_3$CN/H$_2$O gradient (1 mL/min, 60−70% CH$_3$CN over 10 min, 70−80% CH$_3$CN over 20 min, then isocratic at 80% CH$_3$CN for 15 min). Under these conditions, geraniol (GOH), farnesol (FOH), and GGOH were cleanly separated from the C$_5$ terpenols (retention times were as follows: isopentenyl alcohol and dimethylallyl alcohol (IPOH and DMOH, respectively), 3.3 min; GOH, 6.5 min; FOH, 22 min; GGOH, 35 min). The eluents were collected directly into scintillation vials (1 mL fractions) and then analyzed by liquid scintillation counting (Beckman LS1801, using Scintiverse BD).

Detector Requirement. To determine the amount of detergent required for optimum activity, CC−CA pairs were
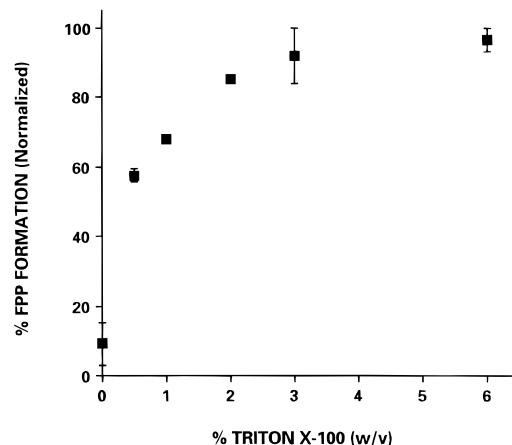
Figure 1. Effect of Triton X-100 detergent concentration on FPP synthase activity.

homogenized in assay buffer containing no detergent (4 CC−CA pair/25 μL) and sonicated on ice for 5 min. After centrifugation to remove cellular debris, aliquots were dispensed into microcentrifuge tubes and diluted with an equal volume of a 2× detergent buffer. The solutions were then assayed for prenyl transferase activity as previously described. Activity is expressed as a normalized percentage of FPP formation, using the average of duplicate experiments.

Effect of Additives. Corpora allata−corpora cardiaca pairs were homogenized in 20 mM Tris-HCl buffer (pH 7), containing 10 mM KF, 0.5 mM mercaptoethanol, and 2.5 mM MgCl$_2$. Aliquots (12.5 μL, 2 gland pair equiv) were diluted with the appropriate 2× buffers containing various combinations of glycerol, Triton X-100, BSA, and PMSF and then assayed for prenyl transferase activity using GPP and IPP as substrates. For each set of experiments, the effect of additives was compared to prenyl transferase activity for homogenates containing 2% Triton X-100, 25% glycerol, and 0.15% BSA. The
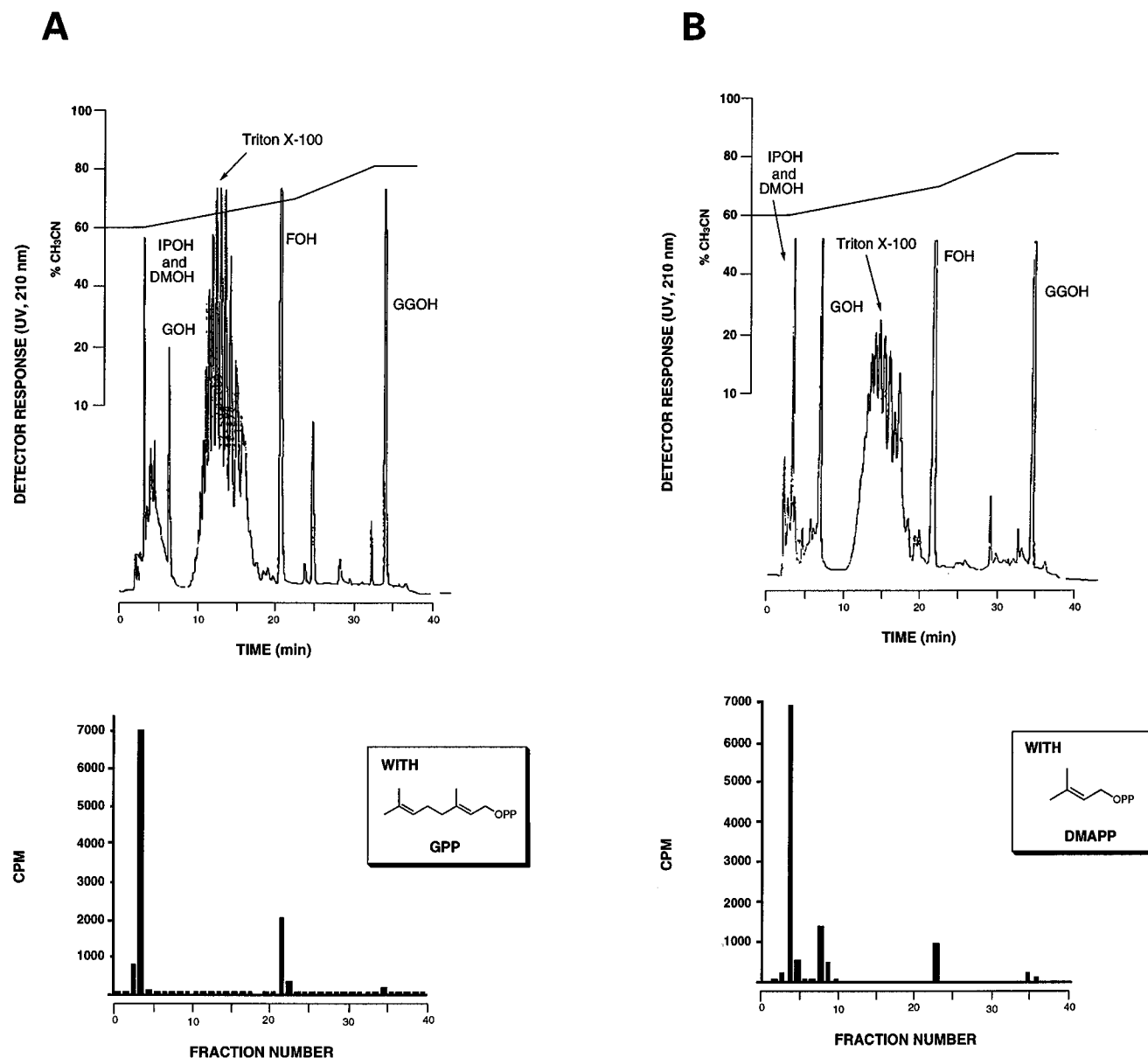
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A

B

Figure 2. Analysis of short-chain prenyl transferase activity by radio-HPLC: (A) using GPP as allylic substrate; (B) using DMAPP as allylic substrate. IPOH, isopentenyl alcohol; DMOH, dimethylallyl alcohol; GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol.

results are expressed as a normalized percentage of FPP formation, using the average of duplicate experiments.

Metal Requirement. Twelve CC—CA pairs of larval V/1 *M. sexta* were dissected dry (i.e., no saline was used) and homogenized in 75 μL of optimum assay buffer devoid of $MgCl_2$. After removal of cellular debris, endogenous metals were removed by several filtrations of the homogenate through a 30 000 MW cutoff Microcon microconcentrator (Amicon). The volume of the resulting solution was adjusted to 60 μL and aliquoted (12.5 μL) into 500 μL Eppendorf tubes. Equal quantities of 2× $MgCl_2$ or $MnCl_2$ assay buffer were added to provide final divalent metal concentrations of 0 and 2.5 mM. Prenyl transferase activity was determined by the addition of GPP and [^{14}C]IPP, as previously described. For accuracy, replicate experiments were performed using separately dissected tissue.

Inhibition of Prenyl Transferase Activity by the Addition of NEM. Corpora cardiaca—corpora allata homogenates were preincubated with *N*-ethylmaleimide (NEM) at increasing concentrations (0, 2, 4, 8 mM) for 10 min on ice. The effect of this material on prenyl transferase activity was determined by observing the decrease in FPP formation using GPP and radioactively labeled IPP as substrates.

RESULTS AND DISCUSSION

While the presence of FPP synthase activity in insect corpora allata can be inferred from whole gland assays using acetate and mevalonate (or their homologues) as precursors, the development of an *in vitro* assay for measuring the coupling of allylic and homoallylic pyrophosphate precursors has previously met with limited success. In a related study on IPP isomerase, trace amounts of FPP were observed from the coupling of IPP with two molecules of DMAPP using CC—CA homogenates of the adult female *M. sexta* (Baker et al., 1981). FPP synthase activity in *Bombyx mori* was observed using DMAPP and GPP as precursors; however, these results are not directly related to JH biosynthesis since whole insect homogenates (minus silk glands and gut) were used (Koyama et al., 1985a,b). Because of the enzyme's central importance in the formation of the JH carbon skeleton, we became interested in developing a simple *in vitro* assay that measured prenyl transferase activity in CC—CA homogenates for use in subsequent enzyme characterization studies.

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Table 2. Corpora Allata Prenyl Transferase Activity Using Different Detergents (at 1% w/v Each)

detergent	FPP formation, % (normalized)
Triton X-100	100
Tween 80	85
CHAPS	17
deoxycholate	9

Effect of Additives. Although significant cytosolic FPP synthase activity can be detected in homogenates of decapitated *M. sexta* larvae whose guts were removed, only small amounts of FPP formation are detected in corpora allata homogenates when conventional assay buffers (20–100 mM Tris-HCl or phosphate buffer, pH 7, 2–5 mM $MgCl_2$, containing 0–10 mM KF to inhibit endogenous phosphatases) are used. Similar low levels of short-chain prenyl transferase activity have been noted in plants and bacteria and have been attributed to either enzyme lability or membrane association (Green et al., 1975; Takahashi and Ogura, 1981; Spurgeon et al., 1984; Sagami and Ogura, 1985; Dudley et al., 1986; Heide and Berger, 1989; Croteau and Purkett, 1989). Because activity in these cases was increased by the addition of glycerol and detergent, we decided to examine the effect of several additives on corpora allata prenyl transferase activity. As seen in Table 1, the addition of Triton X-100 resulted in a substantial increase in enzymatic activity. FPP formation was further enhanced by the addition of both 0.15% BSA and 25% glycerol; however, a combination of the protease inhibitor PMSF and BSA had an inhibitory effect.

Detergent Requirement. An examination of different detergent concentrations shows that prenyl transferase activity increases almost 10-fold with the addition of Triton X-100 to a concentration of 3% (w/v) and remains level up to 6% detergent (Figure 1). Several other detergents were tested for their ability to enhance insect prenyl transferase activity. Tween 80 was nearly as effective in activating the prenyl transferase, while the addition of ionic detergents such as deoxycholate and CHAPS resulted in little or no activity enhancement (Table 2). The latter result suggests that the presence of detergent is important for enzyme activation rather than substrate solubilization, supporting the possibility that corpora allata prenyl transferase activity is membrane associated.

Enzyme Properties. Incubation of GPP and ^{14}C-labeled IPP with larval corpora allata homogenates, followed by enzyme denaturation and radio-HPLC analysis of the corresponding terpenols, provided clean formation of FPP and small amounts of geranylgeranyl pyrophosphate (GGPP, Figure 2A). Similar experiments using DMAPP as the allylic substrate resulted in a mixture of GPP, FPP, and GGPP (Figure 2B). The buildup of the C_{10} intermediate is not commonly found with FPP synthases and may reflect either the presence of a separate GPP synthase activity or inhibition of the second coupling reaction caused by our current assay conditions. Typical conversions were 15–25% for farnesyl pyrophosphate formation using GPP as the allylic substrate and 15–20% GPP with 8–10% FPP when incubations were performed in the presence of DMAPP.

Incubations with GPP and ^{14}C-labeled IPP showed an increase in FPP formation with increasing protein concentration and incubation time. FPP formation increased linearly up to about 3 gland pair equiv/25 µL of assay buffer (Figure 3A) and began to plateau by 6 gland pair equiv (data not shown). Prenyl transferase
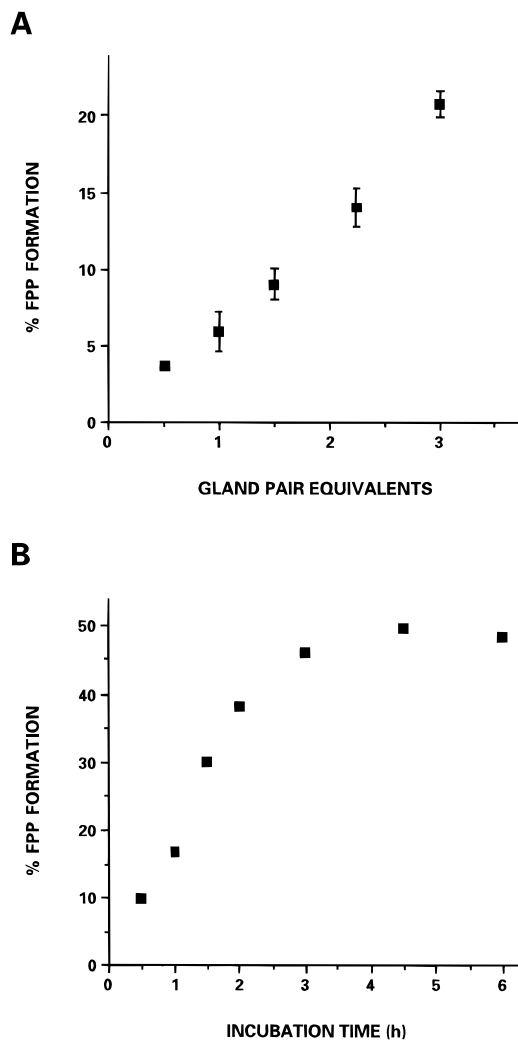

A

B

Figure 3. FPP synthase activity: (A) with increasing enzyme concentration; (B) with increasing incubation time. Graph A represents the average of duplicate experiments; graph B represents single data point experiments but is consistent with replicate studies.

activity remained constant when homogenates were incubated for up to 3 h (Figure 3B). As with all prenyl transferases, the insect enzyme requires either Mg^{2+} or Mn^{2+} for activity. The addition of 2.5 mM Mg^{2+} and Mn^{2+} to prefiltered homogenates resulted in 13- and 18-fold increases in prenyl transferase activity, respectively.

In addition to their role in protein form interconversion, cysteine residues are present within the active site of several prenyl transferases (Yoshida et al., 1989; Koyama et al., 1994). To examine the insect enzyme's similarity to other prenyl transferases, CC–CA homogenates were incubated with the cysteine modifying agent NEM (Lundblad, 1995). At 2 mM concentration, NEM decreased FPP formation by 50%, suggesting the presence of an active site cysteine (Figure 4).

Conclusions. Unlike the enzymes obtained from whole-body preparations, insect prenyl transferase activity in CC–CA homogenates requires the presence of several additives for optimum activity. The coupling of IPP with either DMAPP or GPP is most effectively achieved in the presence of nonionic detergents such as Triton X-100. The strict detergent requirement suggests that the enzyme is membrane associated, in contrast to most FPP synthases. Work is currently underway to better characterize this enzyme system and
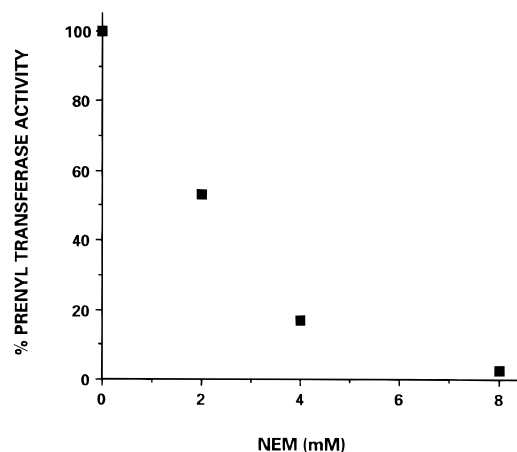
Figure 4. Inhibition of FPP synthase activity by the addition of NEM. Data represent single point experiments.

to determine its role in lepidopteran JH homologue biosynthesis.

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