Farnesol and farnesal dehydrogenase(s) in corpora allata of the tobacco hornworm moth, *Manduca sexta*

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Abstract The metabolism of [^{3}H]farnesol was studied in cell-free preparations of corpora allata from the tobacco hornworm, *Manduca sexta,* to assess the role of this presumed biosynthetic precursor of juvenile hormone (JH) III. A reversed-phase ion-pair liquid chromatographic (RP-IPC) procedure was devised to separate farnesol from several potential intermediates in its presumed metabolism to JH III: farnesal, farnesoic acid, 10,11-epoxyfarnesoic acid, and methyl farnesoate. Following incubation of (2*E*,6*E*)-[1,5,9-^{3}H]farnesol with homogenates of corpora allata from fifth instar larvae or adult female *M. sexta,* and analysis by RP-IPC, the major radiolabeled products corresponded to farnesoic acid, farnesal, and a polar product(s) presumably derived from the tritium on C-1 of farnesol. Inclusion of NAD^+ in the incubations conducted with crude homogenates resulted in enhanced [^{3}H]farnesol metabolism, decreased accumulation of [^{3}H]farnesal, and increased levels of [^{3}H]farnesoic acid. Substitution of $NADP^+$ for NAD^+ was ineffective, suggesting that farnesol and/or farnesal dehydrogenase were NAD^+-dependent enzymes. Pellet fractions obtained by differential centrifugation of crude homogenates exhibited both farnesol and farnesal dehydrogenase activity but only the latter was clearly stimulated by addition of NAD^+. The alcohol/aldehyde dehydrogenase(s) showed some substrate specificity for the 2*E* isomer; nerol and (2*Z*,6*E*)-farnesol were barely metabolized under conditions in which either geraniol or (2*E*,6*E*)-farnesol were rapidly oxidized. The identity of the [^{3}H]farnesal zone obtained from RP-IPC was further established by normal-phase liquid chromatography and by gas–liquid chromatography–mass spectrometry. This is the first demonstration of farnesal production by insect corpora allata and provides compelling evidence for the potential intermediacy of this compound in JH III biosynthesis.—**Baker, F. C., B. Mauchamp, L. W. Tsai, and D. A. Schooley.** Farnesol and farnesal dehydrogenase(s) in corpora allata of the tobacco hornworm moth, *Manduca sexta. J. Lipid Res.* 1983. **24:** 1586–1594.

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Biogenesis of the carbon skeleton of the insect juvenile hormones (JH 0, I, II, III; **Fig. 1**) by the corpora allata (CA) is generally believed to proceed via a typical sesquiterpenoid biosynthetic pathway in the case of JH III, or with some modifications in the case of the ethyl-branched hormones (JH 0, JH I, and JH II) (see e.g. 1, 2). There is a considerable body of evidence for the intermediacy of propionate in the generation of the unusual ethyl side branches of the higher homologs via homomevalonate (1–3). Following formation of farnesyl pyrophosphate (FPP, or its homologs), it is believed that a pyrophosphatase or phosphatase of unknown specificity releases the free alcohol which is in turn oxidized via the aldehyde to the corresponding acid. The final two biosynthetic modifications involve epoxidation of the C-10,11 double bond, probably via a cytochrome P-450 linked enzyme (4, 5), and methylation of the acid by an *O*-methyl transferase utilizing *S*-adenosyl methionine as cofactor (5, 6); the sequence in which these two reactions take place is thought to vary according to the insect order studied (7). Although the latter two steps have been studied in detail in some species, the conversion of the pyrophosphate ester intermediate to the corresponding carboxylic acid has received scant attention. Reibstein et al. (7) showed conversion of [^{3}H]farnesyl pyrophosphate to farnesoic acid, epoxyfarnesoic acid, methyl farnesoate, and JH III by adult female *M. sexta* CA homogenates but the levels of products formed were minute (picomoles or less) considering the incubation period (20 hr). Madhavan et al. (8) were able to show by histochemistry that CA from *Drosophila melanogaster* contained an alcohol and aldehyde dehydrogenase that could oxidize farnesol and farnesal. In addition, using agar-gel electrophoresis and in situ enzyme assay, they showed that homogenates of whole flies contained separable "octanol" and alcohol dehy-

Abbreviations: RP-IPC, reversed-phase ion-pair chromatography; JH, juvenile hormone; CA, corpora allata; FPP, farnesyl pyrophosphate; LC, liquid chromatography; THF, tetrahydrofuran; LSC, liquid scintillation counting; TLC, thin-layer chromatography; CC-CA, corpora cardiaca-corpora allata complex plus attached aorta fragments; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; GLC–MS, gas–liquid chromatography–mass spectrometry; THF, tetrahydrofuran; DiBAL, diisobutylaluminum hydride.

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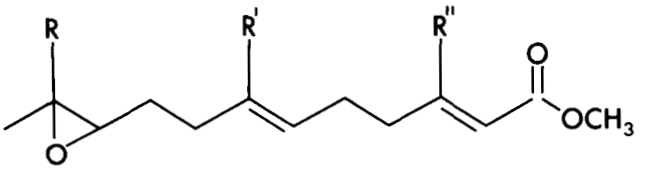

1 $R = R' = R'' = C_2H_5$ (JHO)
2 $R = R' = C_2H_5, R'' = CH_3$ (JHI)
3 $R = C_2H_5, R' = R'' = CH_3$ (JHII)
4 $R = R' = R'' = CH_3$ (JHIII)

Fig. 1. Structures of JH 0, I, II, III.

drogenase enzymes that could also oxidize farnesol and farnesal, although these studies were not directed at detailed identification of products, cofactors, etc. Emmerich et al. (9) showed that homogenates of *Tenebrio molitor* larvae were able to convert farnesol to farnesoic acid via farnesal; the homogenate oxidized farnesol at a greater rate than either ethanol or glycerol. Recent studies by Feyereisen, Koener, and Tobe (10) using intact *Diploptera punctata* CA and by Kramer and Kalish[2] using fifth instar *M. sexta* CA, showed that farnesol can stimulate JH III biosynthesis appreciably. The latter results suggest the presence of an active farnesol dehydrogenase in these glands and prompted us to study metabolism of farnesol by corpus allatum enzymes. For this purpose we developed an analytical system to monitor the various intermediates formed from farnesol, and attempted to determine if farnesal was indeed an accumulative or transient intermediate during conversion of farnesol to farnesoic acid, and hence JH III.

MATERIALS AND METHODS

Materials

[1,5,9-^{3}H]Farnesyl pyrophosphate (FPP, specific activity 12.3 Ci/mmol) was purchased from the New England Nuclear Corporation, Boston, MA. Farnesol (~96% 2*E*,6*E* isomer), 10,11-epoxyfarnesoic acid (containing ~30% of the 3-*exo*-methylene isomer) and methyl farnesoate (~99% 2*E*,6*E* isomer) were generous gifts of Dr. Richard Anderson (Zoecon Corporation Chemistry Department). Nerol (ICN) and geraniol (Kodak) were purified by preparative LC on a 16 × 250 mm LiChrosorb SI-100 column (7 μm) using 12% ether in pentane (50% water-saturated). (2*Z*,6*E*)-Farnesol (prepared by DiBAL reduction of the corresponding isomer of methyl farnesoate) was purified by reversed-phase LC on an 8 × 250 mm LiChroprep RP-8 column with acetonitrile–water 60:40 solvent and using an LDC 214 nm fixed wavelength detector for monitoring purposes. Farnesoic acid (containing >99% of the 2*E*,6*E* isomer) was prepared from the methyl ester by transesterification to the 2-(trimethylsilyloxy)ethyl ester followed by hydrolysis of the latter with tetrabutylammonium fluoride trihydrate in THF (11), a scheme devised by Drs. I. Cloudsdale and R. Anderson (Zoecon Corp.) to avoid the usual formation of *exo*-methylene isomers catalyzed by base hydrolysis. JH III was obtained from stock, prepared as described previously (12). Farnesal (~90% 2*E*,6*E* isomer) was prepared from the corresponding alcohol essentially according to the method of Corey and Schmidt (13).

[1,5,9-^{3}H]FPP was hydrolyzed to the alcohol by incubation with alkaline phosphatase (calf intestine, type 1, Sigma) in 0.1 M glycine/NaOH, pH 9.5, containing 10 mM $MgCl_2$. Unlabeled farnesol was added and the aqueous solution was extracted with ethyl chloride (0–4°C). The product was purified by TLC on a prewashed (CH_3OH) 5 × 20 × 0.05 cm silica GF plate (Analtech) using ether–hexane 1:1 as solvent. After elution of the radiolabeled zone with ether containing ~200 μl DMSO, the ether was gently evaporated and the DMSO solution was stored at −20°C when not in use. The specific activity of the product was estimated from the UV absorption of an aliquot subjected to LC and its associated ^{3}H content. Specific activity was 37 mCi/mmol and 30 mCi/mmol in two separate batches of product prepared. β-NAD^+ and bovine serum albumin (BSA) were obtained from Sigma; β-$NADP^+$ was from ICN.

Methods

Manduca sexta were reared as described previously (14). Generally, CA were removed as complexes along with corpora cardiaca plus attached aorta fragments (CC-CA) (14) from 0–48-hr-old adult females, but in one experiment the various component tissues were separated for individual assay. Glands were dissected in saline containing 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$, and 3 mM $CaCl_2$ (a modification of the *Manduca* saline buffer devised by Cherbas, ref. 15), drop-rinsed in 0.1 M Tris-HCl buffer, pH 7.4, containing 1% BSA, and then homogenized in a micro glass tissue grinder containing the latter buffer. Buffer washings of the tissue grinder were combined with homogenate such that the final solution generally contained one CC-CA pair equivalent per 20 μl. Typically, 50-μl volumes of homogenates or fractions deriving from differential centrifugation (in a Beckman L3-40 ultracentrifuge) were incubated in capped 1-ml shell vials containing [^{3}H]farnesol in DMSO, with or without added cofactors. Final concentration of farnesol was 15 μM and

[2] Kramer, S. J., and F. Kalish. Personal communication.

DMSO ~1%. Incubation was at 28 ± 1°C in a water bath. At desired intervals, 20-μl volumes were removed from the incubations to plastic micro centrifuge tubes containing 1 μg each of farnesoic acid, farnesol, and farnesal in 30 μl of CH_3CN. Precipitated protein was sedimented by centrifugation and the supernatant was reserved for analysis.

Analytical methods

Analyses of enzymatic incubations were by reversed-phase ion-pair chromatography (RP-IPC) on a 4.6 × 250 mm RP-8 column using 0.01 M tetrabutylammonium phosphate (TBAP) in 60% CH_3CN. The solvent mixture was generated by a Spectra Physics 8700 solvent delivery system from three reservoirs containing 0.1 M TBAP in water, CH_3CN (Burdick and Jackson, glass-distilled), and glass-distilled water; in some instances (requiring a very stable baseline at 214 nm), the 0.01 M TBAP was prepared directly in 60% CH_3CN; flow rate was 2 ml/min. UV absorption of eluting compounds was monitored with a fixed wavelength (214 nm) LDC UV III Monitor or a Spectra Physics 8440 variable wavelength detector plus a Spectra Physics 8300 fixed wavelength (254 nm) detector in series. One-minute or 30-sec fractions were collected for quantification of ^3H by liquid scintillation counting (LSC) in Packard 2425 or 460C Tri-Carb liquid scintillation spectrometers. Normal-phase liquid chromatography was conducted on a Zorbax-SIL column (4.6 × 220 mm) using a modular instrument as described previously (14). Gas–liquid chromatography–mass spectrometry (GLC–MS) was conducted on either a 2 m 3% OV-17/0.4% Carbowax 20 M column at 150° → 300°C, 5°C per min, or a 2 m 3% OV-101/Ultrabond II column at 170° → 260°C, 5°C per min, using a Hewlett Packard 5985A system in the electron impact mode.

RESULTS

Preliminary studies were aimed at developing a useful chromatographic system for separation of farnesoic acid, farnesol, and FPP. Reversed-phase LC per se was unsatisfactory; analysis on an RP-8 column using acetonitrile water mixtures under both isocratic and gradient conditions resulted in poor peak shape and peak tailing when either farnesoic acid or farnesyl pyrophosphate was injected. Inclusion of 0.01 M potassium phosphate buffer, pH 7.0, slightly improved peak shape of the latter components; use of triethylammonium phosphate buffer (pH 7.0 or 7.5) resulted in improved peak shape, but decreased the retention of farnesoic acid and FPP. Best results were obtained by using 0.01 M tetrabutylammonium phosphate, pH 7.3, in 60% acetonitrile. Under these conditions good separation of farnesoic acid from farnesol was obtained with acceptable peak shape of components; separation of FPP from farnesoic acid was only marginal. However, for the study of [^3H]farnesol metabolism the latter chromatographic system was deemed suitable, since it was anticipated that farnesol would not be phosphorylated.

In an early time-course experiment, [^3H]farnesol was incubated with a 5,000 *g* (10 min) supernatant from *M. sexta* CC-CA homogenates in the absence of added cofactors; RP-IPC analysis indicated formation of several ^3H-labeled products. Most prominent were a material less polar than farnesol and a polar product that eluted close to the solvent front; a lower level of labeled product was found to co-elute with farnesoic acid. Co-injection of (2*E*,6*E*)-methyl farnesoate, 10,11-epoxyfarnesoic acid, and JH III confirmed that the two predominant unknown ^3H zones did not correspond to these standards. The identity of the radiolabel eluting near the solvent front can almost certainly be attributed to products formed directly or indirectly from transfer of ^3H from C-1 of farnesol; evidence for this is presented later. It was deduced that farnesal might account for the radiolabeled product ($k' = 6.2$) eluting after farnesol ($k' = 4.5$). This was corroborated by synthesis of (2*E*,6*E*)-farnesal and analysis of the product by RP-IPC. **Fig. 2** shows the LC profile for various standards and the ^3H label distribution of products obtained following a 1-hr incubation of [^3H]farnesol with a 5,000 *g* (10 min) supernatant prepared from a CC-CA homogenate without additional cofactors.

Further identification of radiolabeled products

The identity of the [^3H]farnesoic acid zone from RP-IPC was studied further following extraction into hexane and methylation with diazomethane. The radiolabeled product coeluted with (2*E*,6*E*)-methyl farnesoate on a normal-phase LC system (Zorbax-SIL; 4% ether in pentane, 50% water-saturated).

The labeled zone corresponding to farnesal on RP-IPC (see above) was partitioned into ether–pentane 6:4, and the extract was evaporated and analyzed by normal-phase LC on a Zorbax-SIL column (solvent: 6% ether in pentane; 50% water-saturated). The radiolabel of farnesal co-eluted with the UV absorbing peak. Unequivocal evidence for the identity of farnesal was obtained from GLC–MS analysis. A 1-hr incubation conducted with homogenate equivalent to ten CC-CA pairs and 6 nmol of [^3H]farnesol was quenched with CH_3CN and centrifuged, and the supernatant was subjected to RP-IPC analysis (but without addition of unlabeled standards). The farnesal zone, detectable by UV at 254 nm and also as radiolabel by LSC, was collected and recovered by brine–pentane partitioning. The concentrated pentane extracts were estimated (by LSC) to contain >400 ng of product. GLC–MS analysis clearly indicated the presence of two

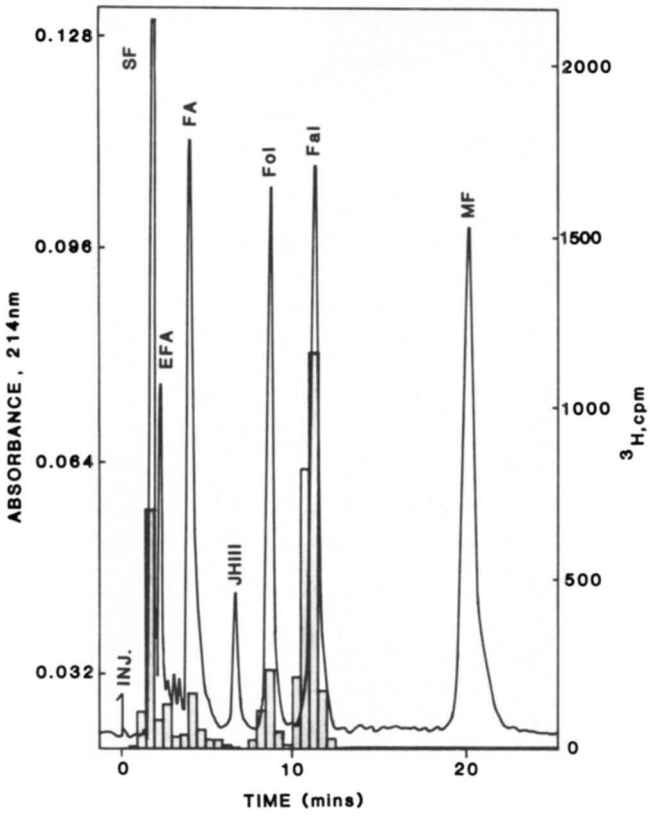

Fig. 2. Separation of a mixture containing epoxyfarnesoic acid (EFA), farnesoic acid (FA), JH III, farnesol (Fol), farnesal (Fal), methyl farnesoate (MF), and radiolabeled products from a 1-hr incubation of [1,5,9-^{3}H]farnesol with a 5,000 *g* (10 min) supernatant prepared from adult female *M. sexta* CC-CA without additional cofactors. Column: RP-8, 4.6 × 250 mm. Solvent: 0.01 M tetrabutylammonium phosphate, pH 7.3, in 60% CH_3CN; flow rate was 2 ml/min. SF, solvent front. Shaded histogram = ^{3}H, cpm.

isomers of farnesal (presumably 2*E*,6*E* and 2*Z*,6*E*) in a ratio of ~2:1. Relative abundances of the major ions from the 2*E*,6*E* isomer were: m/z 69 (100%), 84 (79%), 81 (20%), 41 (10%), 93 (10%), 95 (8%), 220 (1%); those from the isomer presumed to be 2*Z*,6*E* were: m/z 69 (100%), 81 (62%), 84 (60%), 95 (29%), 109 (21%), 93 (18%), 107 (18%), 220 (1%).

Localization of farnesol/farnesal dehydrogenase activity

To simplify the dissection procedure, CA from adult female *M. sexta* were generally removed as a complex containing corpora cardiaca and aorta fragments, and homogenates of this complex were used as a source of enzyme. Previously it has been shown that only the CA exhibits JH biosynthetic activity in vitro (14); however, we felt that it was necessary to determine if the observed farnesol/farnesal dehydrogenase activity also derived only from the CA portion of the gland complex. Separation of the complexes into their individual components followed by homogenization and assay indicated that only the preparations from CA showed activity (~420 pmol of farnesol oxidized per gland pair per hr, **Table 1**); furthermore, no activity had leaked out of the various tissues during the "dissection" procedures as medium used in these surgical manipulations was also shown to be devoid of activity.

Only a cursory examination was made of the cellular distribution of farnesol/farnesal dehydrogenase activity following homogenization of CC-CA. Assay (in the presence of 0.5 mM NAD^+) of both supernatant and pellet fractions, following low speed centrifugation (1000 *g*, 10 min) to remove larger debris and cell nuclei, showed that the supernatant contained 75% of the enzyme activity. In another experiment an homogenate was subjected to centrifugation at 5000 *g* for 10 min; the resulting supernatant was then centrifuged for a further 60 min at 150,000 *g*. The 5000 *g* pellet (presumably containing cellular debris, nuclei, and mitochondria), microsomal fraction, and soluble fraction contained 52%, 15%, and 33% of the enzyme activity, respectively. The distribution profile of labeled products, following assay of the various fractions by RP-IPC, indicated that both farnesol and farnesal dehydrogenase were present in each fraction.

Farnesol/farnesal dehydrogenase activity in CA from other stages of *M. sexta*

In order to determine if there were any correlation between in vivo JH titer and farnesol dehydrogenase levels, enzyme activity was determined also in CA removed from 0–18-hr or 72–96-hr-old (wandering stage) *M. sexta* fifth instar larvae. It is known that JH titers are very low in wandering stage animals while they are quite high in early fifth instar larvae (16, Bergot et al.[3]). Our results demonstrated enzymatic activity (Table 1) in both preparations from larvae; the activity in gland homogenates from wandering stage animals was lowest and both larval preparations had lower activity than that typically obtained in homogenates from adult glands.

Cofactor requirements

Since many alcohol/aldehyde dehydrogenases have a cofactor requirement for NAD^+, we investigated the effect of this compound at several concentrations on farnesol metabolism by CC-CA homogenates. It was clear that inclusion of NAD^+ drastically altered the ^{3}H product profile. In the absence of added cofactor, [^{3}H]farnesal accumulated (Fig. 2); but in the presence of NAD^+, farnesal levels were low while [^{3}H]farnesoic acid and ^{3}H polar products were greatly enhanced (**Fig. 3**). (Changes of concentration of NAD^+ within the range 0.5–10 mM had

[3] Bergot, B. J., et al. Unpublished studies.

TABLE 1. Metabolism of [1,5,9-^{3}H]farnesol by homogenates of corpora allata from *M. sexta* larvae or adults, and by corpora cardiaca, aorta fragments, or cardiaca-allata-aorta complexes of adults

Source of Homogenate	Farnesol Metabolized[a]	Farnesal Produced[a]	Farnesoic Acid Produced[a]
Adult female, 0–48 hr old, separated CA	211	43	130
Adult female, 0–48 hr old, separated CC	7	1	5
Adult female, 0–48 hr old, separated aorta fragment	29	1	0
Dissection medium from above	29	0	0
Adult female, 0–48 hr old, CC-CA-aorta complexes	202[b]	55	136
0–24 hr Vth instar larvae, CA	117	73	7
96–120 hr Vth instar larvae, CA	91	57	36

Homogenates of appropriate glands were prepared in 0.1 M Tris-HCl buffer, pH 7.4, containing 1% BSA, such that 20 μl of buffer contained one gland-pair equivalent. Fifty-μl incubations containing 15 μM [^{3}H]farnesol and 2 mM NAD^+ were conducted at ~28°C. Twenty-μl aliquots were removed at timed intervals and quenched with 30 μl of CH_3CN containing 1 μg each of farnesoic acid, farnesal, and farnesol. Following sedimentation of precipitated protein, samples were analyzed by RP-IPC as described in Methods; pmol values were calculated from ^{3}H dpm in corresponding fractions and known specific activity of products (assuming no dilution by endogenous unlabeled products).

[a] pmol/gland pair equivalent after 30-min incubation.

[b] Mean of three experiments; others were single determinations.

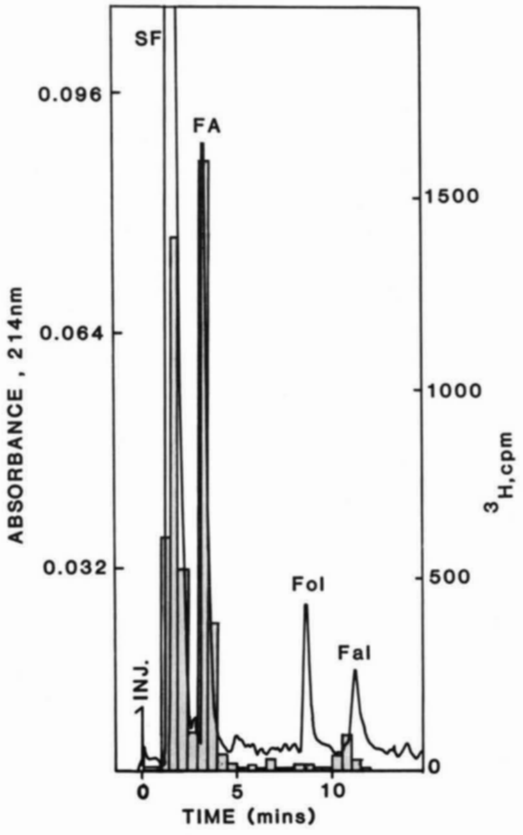

Fig. 3. Distribution of radiolabeled products formed from [1,5,9-^{3}H]farnesol incubated with adult female *M. sexta* CC-CA homogenates in the presence of 2 mM NAD^+. SF, solvent front; FA, farnesoic acid; Fol, farnesol; Fal, farnesal. Column: RP-8, 4.6 × 250 mm. Solvent: 0.01 M tetrabutylammonium phosphate, pH 7.3, in 60% CH_3CN; flow rate was 2 ml/min. Shaded histogram = ^{3}H, cpm.

little effect on ^{3}H profiles.) The time course of events associated with [^{3}H]farnesol metabolism by *M. sexta* CC-CA homogenates plus or minus NAD^+ is shown in **Fig. 4.** It appeared that addition of NAD^+ stimulated the oxidation of farnesal to farnesoic acid. This was substantiated in a separate experiment in which radiolabeled farnesal (generated by incubation of homogenates with [^{3}H]farnesol in the absence of NAD^+) was isolated by RP-IPC, partitioned, then re-incubated with enzyme preparation either in the absence or presence of 2 mM NAD^+. Following RP-IPC analysis of aliquots from these incubations, it was evident that high levels of [^{3}H]farnesoic acid had been formed only in the presence of NAD^+. NAD^+ may also be a cofactor for conversion of farnesol to farnesal, although the evidence for this is more indirect. A kinetic study conducted with a homogenate preparation without added NAD^+ showed a more or less linear loss of farnesol with time and concomitant appearance of farnesal, farnesoic acid, and labeled polar material. Upon addition of NAD^+ (2 mM) to this preparation, the rate of farnesol disappearance increased. Surprisingly, addition of 2 mM NAD^+ had little effect on the rate of disappearance of farnesol catalyzed by a resuspended high speed pellet (150,000 *g*, 60 min) fraction, whereas the generation of farnesoic acid by the same pellet fraction was enhanced by addition of NAD^+. $NADP^+$ (2 mM) was also tested as cofactor for the alcohol and aldehyde dehydrogenases; however, distribution profiles of labeled products generated by these homogenates were exactly the same as those when no cofactor was added.

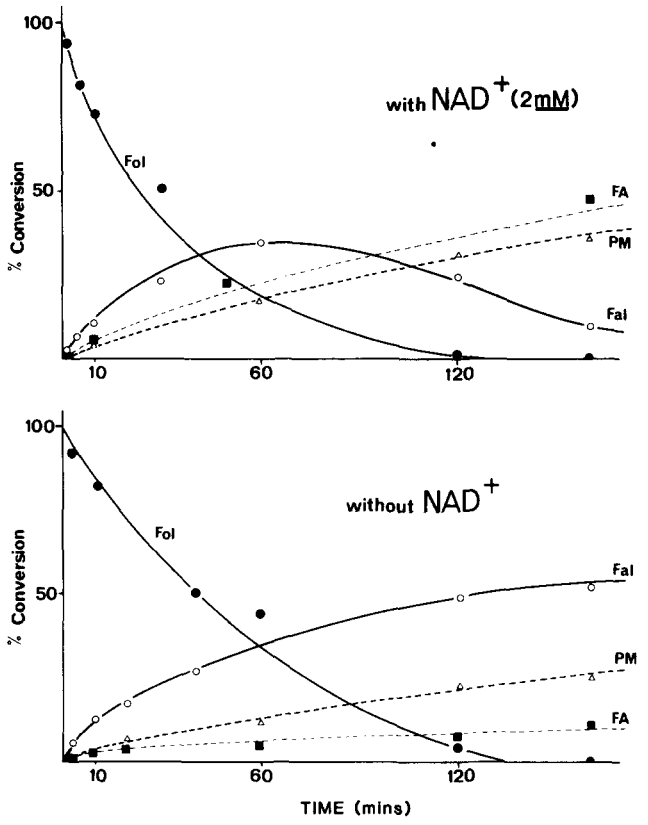

Fig. 4. Time course of the distribution of radiolabeled products formed from [1,5,9-^{3}H]farnesol incubated with an homogenate of adult female *M. sexta* CC-CA with or without 2 mM NAD^+. ●, farnesol; ○, farnesal; ■, farnesoic acid; △, polar material.

Polar [^{3}H]-labeled metabolites

The radiolabeled zone which eluted close to, or at, the solvent front of the RP-IPC system following active metabolism of [^{3}H]farnesol can at least in part be explained as originating from the C-1 methylene ^{3}H atoms of the [1,5,9-^{3}H]farnesol. Radiolabel in this polar zone was not extractable into hexane, ether, or ethyl acetate under either basic or acidic conditions. Theoretically, reduced cofactor (presumably NAD^3H) would be expected as the initial radiolabeled product following transfer of ^{3}H from both farnesol and farnesal. We conducted a limited study to determine if this transfer could be demonstrated in our crude enzyme preparation. A chromatographic system for separation of NAD^+, NADH, $NADP^+$, and NADPH was developed based on the ion-pair solvent used in the routine RP-IPC analyses. A linear gradient of 0.01 M TBAP, pH 7.3, in 100% water programmed to 50% CH_3CN containing 0.01 M TBAP, pH 7.3, gave excellent separation of all four cofactors (**Fig. 5**). However, when labeled polar products derived from routine RP-IPC of a typical incubation were collected and reanalyzed on this gradient system, essentially no radiolabel was found to co-elute with NADH or NADPH. The result is perhaps not too surprising since in the crude preparation many competing reactions could potentially utilize NADH or NADPH, including mitochondrial electron transport, resulting in the generation of miscellaneous ^{3}H-labeled products.

Alternative substrates

The levels of farnesol and farnesal dehydrogenase activity in the adult female *M. sexta* CC-CA homogenates allowed us to investigate alternative substrates semi-quantitatively without necessitating a radiolabeled sample of each. Homogenates were incubated with nonlabeled samples of (2*E*,6*E*)-, (2*Z*,6*E*)-farnesol (96%, 99% isomeric purity, respectively), geraniol (>99% 2*E*), or nerol (>99% 2*Z*), ~15 μM each; 40-μl aliquots were removed, quenched with acetonitrile, centrifuged to remove precipitated proteins, and then the supernatant was analyzed by RP-IPC. Geraniol disappeared at a rate comparable to that of (2*E*,6*E*)-farnesol while (2*Z*,6*E*)-farnesol and nerol metabolism was minimal. In addition, UV-absorbing peaks corresponding to farnesoic acid and farnesal were observed as products from (2*E*,6*E*)-farnesol while corresponding UV-absorbing products from (2*Z*,6*E*)-farnesol were barely detectable. A product from geraniol oxidation showed chromatographic and spectral (215 and 254 nm) properties expected for geranial; the corresponding aldehydic product of nerol was generated to a much lesser extent.

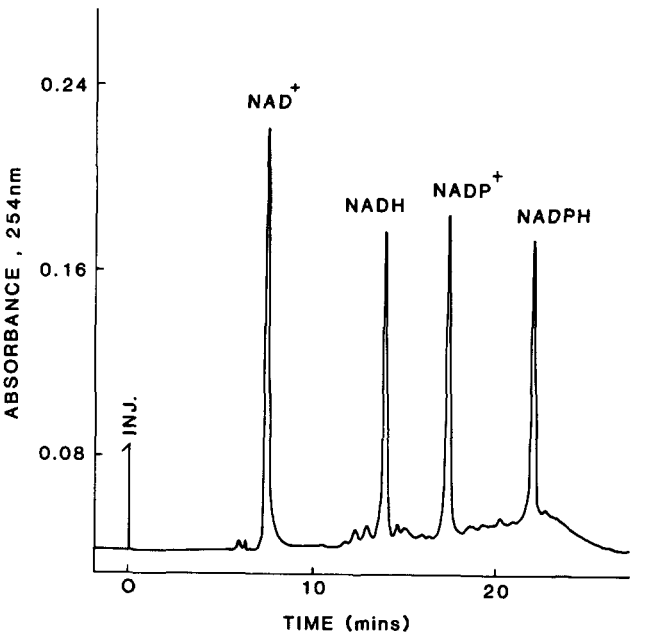

Fig. 5. Separation of a mixture containing NAD^+, NADH, $NADP^+$, and NADPH (~5 μg each). Column: RP-8, 4.6 × 250 mm. Solvent: a linear gradient of 0.01 M tetrabutylammonium phosphate, pH 7.3, in 100% H_2O going to 0.01 M tetrabutylammonium phosphate, pH 7.3, in 50% CH_3CN during 20 min; flow rate was 2 ml/min.

DISCUSSION

Reversed-phase LC analysis of biosynthetic products has an advantage over normal phase LC in that a preliminary extraction of desired components from aqueous medium into solvent is unnecessary; furthermore, normal-phase LC is often unsuitable for analysis of polar metabolites. Even on reversed-phase LC many polar compounds (e.g., carboxylic acids) show unsatisfactory chromatographic properties unless their ionization is suitably suppressed (e.g., by inclusion of dilute acid to effect protonation of the carboxylate anion). In the present study, we wished to separate several biosynthetic precursors of JH III; several of these precursors, although lipophilic, also contained ionizable functional groups. Because both JH III and its epoxy acid precursor contain an acid-labile 10,11-epoxy group, the use of an acidic solvent system was untenable. Previously we have experienced considerable success in analyzing ionizable compounds of varying hydrophobicity by using reversed-phase ion-pair liquid chromatography (RP-IPC, 17, 18). Here we found that application of similar solvent conditions results in complete separation of JH III and five of its immediate biosynthetic precursors. However, we could not obtain satisfactory peak-shape characteristics with FPP under the same solvent conditions, possibly due to incomplete ionization of the pyrophosphate moiety at the pH used (the silica-based RP-8 column is not recommended for use above pH 8).

In preliminary experiments, [1,5,9-^{3}H]farnesol at 15 μM seemed to have poor solubility in the buffer utilized even in the presence of 1% DMSO used to disperse it. The poor solubility was manifest as highly variable counts in several aliquots of the same farnesol solution. The solubility problem was circumvented by including 1% BSA in the buffer. Previously, Christophe and Popják (19) had prepared 0.1 M farnesol and geraniol in 2% BSA to overcome solubility problems with these alcohols. Feyereisen et al. (10) studied effects of farnesol on stimulation of JH III synthesis in *Diploptera* CA incubated in TC-199 medium containing Ficoll, but no proteins; it is likely that they had a nonhomogenous solution at the very high concentrations utilized (up to 320 μM, typically 200 μM), despite the presence of 0.002% Tween 80 detergent in normal TC-199 medium.

The RP-IPC analytical procedure was instrumental in the detection of farnesal as an intermediate formed during oxidation of farnesol to farnesoic acid by *M. sexta* CC-CA homogenates. Although farnesal has long been regarded as a potential precursor of JH III (see e.g., 7, 10), this is the first demonstration of its formation by CA. From GLC–MS analysis of biosynthesized farnesal isolated via RP-IPC it appears that both 2*E* and 2*Z* isomers were formed. The mass spectrum of the biosynthetic (2*E*,6*E*)-farnesal was essentially identical to that of the 2*E*,6*E* synthetic standard, but slightly different from the biosynthetic 2*Z*,6*E* isomer. Major ions observed from mass spectra of both isomers compared favorably with previously reported values (20, 21). It is known that in some plants, isomerization of (2*E*,6*E*)-farnesol to the corresponding 2*Z*,6*E* isomer occurs via the corresponding aldehyde intermediates (22, 23). Similar isomerization has been observed using liver alcohol dehydrogenase (24, 25); interestingly, both (2*E*,6*E*)- and (2*Z*,6*E*)-farnesal have been detected in the defense secretion of soldier termites, *Curvitermes strictinasus* (21). Capellini et al. (25) also showed that isomerization of farnesal could occur nonenzymatically in the presence of albumin, H^+, OH^-, and RS^-. Although neutral conditions were utilized during the isolation of farnesal in the present study, chemically mediated isomerization cannot be ruled out.

One of the prime functions of the CA (a pair of neurohemal organs located close to the insect brain) is the generation of the juvenile hormones (JH) which play an important function during development of juvenile stadia as well as serving a gonadotropic function in the adult. The localization of farnesol/farnesal dehydrogenase activity in the CA (but not the closely associated corpora cardiaca and aorta) is strongly suggestive that farnesol and farnesal are true intermediates of JH biosynthesis. The relatively high activity of the dehydrogenase enzymes compared to the levels of JH biosynthesis normally observed in *M. sexta* CA in vitro is in line with our observations that farnesol stimulates JH III biosynthesis by intact CC-CA from adult *M. sexta*[4] or CA from fifth instar *M. sexta*.[2]

Although it is known that the JH titer in fifth instar d3 *M. sexta* (wandering stage) is very low or nil (16, Bergot et al.[3]), it is interesting that considerable farnesol/farnesal dehydrogenase activity was still measurable in gland homogenates from animals of that age. However, recent in vitro studies have indeed shown that isolated CA are capable of biosynthesizing the JH acid skeleton at this stage (26; Kramer et al.[5]); the in vivo titer of JH may then be maintained at a low level due to high activity of JH-specific esterase(s) (27). The reason why the JH acid skeleton should be biosynthesized at a time when titers are low or nil is presently unknown; in this regard, Granger et al. (26) have suggested a hormonal function for JH III acid, although there is little (if any) evidence for this. The activity observed for farnesol dehydrogenase of ~200 pmol per gland pair per hr is almost two orders of magnitude higher than the rate of in vitro JH biosynthesis by cultured intact CA under similar conditions. Perhaps this enzyme does not play a regulatory role and

[4] Baker, F. C., and L. W. Tsai. Unpublished studies.
[5] Kramer, S. J., et al. Unpublished studies.

is present in excess capacity, which may be consistent with relatively slight changes in enzyme level during development.

The metabolism of farnesol by *M. sexta* CC-CA homogenates has many similarities to the oxidation of farnesol by a mammalian liver preparation. Thus, Christophe and Popják (19) found that (2*E*,6*E*)-farnesol was oxidized to the corresponding (2*E*,6*E*)-farnesoic acid. In addition, as we observed in our insect preparation, the 2*Z*,6*E* isomer of farnesol did not function as a substrate while geraniol was readily oxidized to (2*E*)-geranoic acid. Although farnesal was not observed as an intermediate from farnesol in the liver preparation, (2*E*,6*E*)-farnesal (but not the 2*Z*,6*E* isomer) could be oxidized to the corresponding acid (19). In our system as well, only (2*E*,6*E*)-farnesoic acid was produced by farnesal dehydrogenase. As the GLC–mass spectral evidence indicates that the farnesal produced is a mixture of 2*E*,6*E* and 2*Z*,6*E* isomers, it appears that (2*Z*,6*E*)-farnesal is not a substrate for the farnesal dehydrogenase enzyme. We cannot rule out formation of (2*Z*,6*E*)-farnesal as a strictly chemical reaction (perhaps even an artifact of work-up); any nonutilizable (2*Z*,6*E*)-farnesal isomer produced in vivo might be expected to re-equilibrate with 2*E*,6*E* isomer rather than accumulate as a dead-end metabolite.

In agreement with many alcohol/aldehyde dehydrogenase enzyme studies conducted previously, (including those of Emmerich et al. (9) on farnesol oxidation in homogenates of *Tenebrio* larvae) NAD^+ appeared to be the preferred cofactor in the insect CA preparation. In a rat liver cytosolic preparation it appeared that NAD^+ was tightly bound to the enzyme since even treatment with a Dowex-1 ion exchange resin only partially reduced the enzyme activity (19). We found that resuspended pellets of CC-CA homogenates following high speed centrifugation showed considerable farnesol dehydrogenase activity without addition of NAD^+, hinting that a bound cofactor may also be operational in the insect system. When using a high speed pellet fraction without added NAD^+, very little farnesoic acid was produced from [^{3}H]farnesol despite the accumulation of considerable [^{3}H]farnesal; however, on addition of 2 mM NAD^+ to the same preparation, the [^{3}H]farnesoic acid increased dramatically. Farnesal dehydrogenase may therefore contain little or no bound cofactor itself but instead may require free NAD^+ as a direct electron acceptor. Enzyme purification will be necessary in order to determine whether the latter interpretations are indeed correct.

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