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NADPH DEPENDENT EPOXIDATION OF METHYL FARNESOATE TO JUVENILE HORMONE IN THE COCKROACH *Blaberus giganteus* L.

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*SUMMARY

Corpora allata and corpora allata homogenates from the cockroach *Blaberus giganteus* are able to convert tritium labeled *trans*, *trans*-methyl farnesoate into insect juvenile hormone III. The epoxidation appears to be enzymatic occurring largely in the 100,000 g precipitate or microsomal fraction of corpora allata homogenates. The epoxidation is NADPH dependent, requires molecular oxygen and is inhibited by carbon monoxide, methylene blue, SKF 525A, and piperonyl butoxide.

The juvenile hormone (JH) of insects seems to regulate larval development as well as adult reproduction and maturation in some cases (1), and the juvenile hormones and their mimics (juvenoids) offer some promise as insect pest control agents by prolonging larval stages or blocking reproduction. The biosynthesis of the juvenile hormones by epithelial endocrine glands, the corpora allata (CA), has been previously investigated *in vivo* (2,3) and *in vitro* (4-10). An inhibitor of JH biosynthesis might cause precocious development or decreased reproductive potential of a pest insect which could be a useful pest control tool, especially if the larval stage were destructive. As a possible step in the biosynthesis of JH III (methyl-10,11-epoxy-3,7,11-trimethyl-*trans*,*trans*-2,6-dodecadienoate) (4), the epoxidation of the model substrate methyl farnesoate (I) to JH (III) was chosen for study (Fig. 1).

Materials and Methods

Lightly chilled female *Blaberus giganteus* L. were immersed in chilled sodium phosphate buffer (0.1 M, pH 7.4) and the CA removed through the neck membrane. Extraneous tissue was removed from the CA followed by washing and homogenization in cold Grace's medium (Pacific Biologicals, Berkeley, Calif.) or phosphate buffer, both containing 1% bovine serum albumin (BSA). The homogenate was centrifuged at 12,000 g (10 min) to precipitate the mitochondrial, nuclear, and debris fraction, then at 100,000 g (90 min) to sediment the microsomal fraction. Precipitates were resuspended in enough buffer to yield two CA equivalents per 100 µl unless specified otherwise. As controls, homogenates and tissue fractions of *B. giganteus* fat body (0.3 mg/ml protein in the crude homogenate) and haemolymph (diluted 100:1) were run simultaneously with the CA. The metabolism of methyl farnesoate was also examined in rat liver microsomes prepared as above in phosphate buffer containing $1 \cdot 10^{-5}$M $MgCl_2$ without BSA (1 ml incubations, 37°C, 15-30 min).

Enzyme incubations were carried out in carbowax treated (11) 1 dram shell vials in 100 µl of medium containing 1-4 gland equivalents at 30°C in a shaking incubator for 1 to 4 hours. Methyl farnesoate (I) ($6 \cdot 10^{-8}$M final concen-

tration) was added in 1 µl of 50% aqueous ethanol while cofactors (NAD, NADH, NADP or NADPH $1 \cdot 10^{-5}$M) and water soluble inhibitors (methylene blue $1 \cdot 10^{-6}$M and SKF 525A $5 \cdot 10^{-3}$M; provided by Dr. C. E. Berkoff of Smith, Klein and French Laboratories, Philadelphia, Penn.) were added in 10 µl of distilled water, and the organosoluble inhibitors (piperonyl butoxide $5 \cdot 10^{-5}$M and diisopropyl paraoxon $1 \cdot 10^{-4}$M) were added in 0.5 µl of 20% aqueous ethanol. Occasionally incubations were carried out under atmospheres of carbon monoxide, nitrogen or oxygen. Intact CA (1-4 glands/100 µl) were similarly incubated in Grace's medium (4), usually under an oxygen enriched atmosphere.

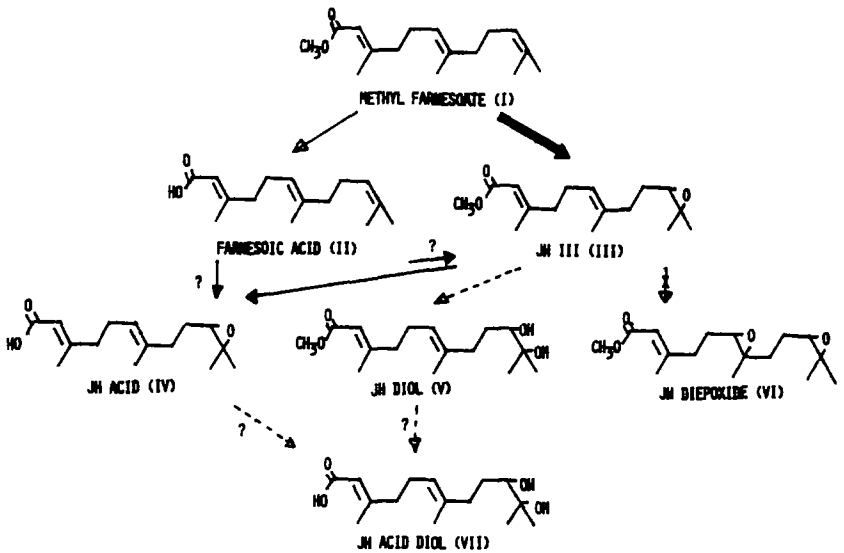

FIG. 1

Metabolic pathways of methyl farnesoate in B. giganteus corpora allata homogenates. A heavy line indicates a major pathway, while dotted lines indicate minor pathways. A question mark or an X indicates a pathway not independently established or a pathway not found, respectively.

The reaction was halted by the addition of freshly distilled ethyl acetate (3 vol, 2x) containing a trace of pyridine and in some cases the antioxidant 2,6-di-t-butyl-p-cresol and 20 µg each of the appropriate standards. The organic extract contained over 97% of the tritium and was dried (Na_2SO_4), concentrated (N_2), spotted on thin layer chromatoplates (silica gel, F254, Brinkman, Westbury, N. Y.) and developed in the first dimension with hexane: ether 6:1 followed by development with benzene: n-propanol 10:1 containing 0.5% acetic acid in the second dimension or in 1 dimension with hexane: ethyl acetate 3:2. Other solvent systems were used to help verify metabolite structures by repeated co-chromatography. Reference standards were observed by their quenching

of fluorescence when viewed under short-wavelength uv light (254 nm). Plates were scanned (Varian tlc scanner, Walnut Creek, Calif.) or exposed to X-ray plates (12) (to locate radioactive compounds) and the appropriate spots scraped for analysis by liquid scintillation counting (lsc). In some cases the metabolites were extracted from the silica gel with ethyl acetate and exposed to previously described microchemical tests for further structural information (13, 14). Authentic standards were synthesized as described earlier (14-16) and their structures verified spectrally.

Farnesoic acid (II) (8.4 Ci/mmol, ^{3}H labeled) (provided by W. Hafferl, Radiochemist, Palo Alto, Calif.) was methylated with diazomethane and the resulting methyl ester purified by tlc in hexane: ether 6:1 followed by high pressure liquid chromatography (20μ LiChrosorb SI 60 column 1m x 22mm eluted with half water saturated 1% ether in freshly distilled pentane and monitored by its uv absorbance at 254 nm on a LDC 1200 uv monitor, Riviera Beach, Fla.) to obtain the pure trans, trans-isomer. Studies on farnesoic acid labeled with deuterium oxide using Hafferl's catalytic exchange procedure indicated that the labeling occurred at the carbon 2 olefinic proton and on the carbons alpha to the 2,3-double bond.

Results and Discussion

There is little metabolism of methyl farnesoate by intact CA under the conditions examined (Table 1). The extent of metabolism is quite erratic, varying from under 0.1 to 6% epoxide formation and 1 to 24% ester cleavage. The erratic metabolism is probably caused by variability in the staging of the B. gigenteus in their reproductive cycle and the small number of CA per incubation. Incubation under oxygen seemed to increase epoxidation, but variability precluded definite conclusions. Tobe and Pratt (10) found no increase in epoxidation of farnesoic acid by the CA of Schistocerca gregaria at oxygen tensions higher than atmospheric. Homogenization of the CA may allow more methyl farnesoate and/or NADPH to reach enzyme sites, thus increasing epoxidation in cell free systems. The presence of an antioxidant in the extracting solvent did not decrease the amounts of epoxidized products (III, IV) found.

The production of JH from methyl farnesoate increases with increased CA equivalents in the homogenate or increased incubation time, but does not occur after heat or pronase treatment of the CA homogenates, indicating that the reaction is enzymatic. The majority of the epoxidase activity appears to be membrane bound with essentially no epoxidase in the 100,000 g supernatant (Table 1). The 12,000 g precipitate shows high epoxidase and esterase activity and probably contains poorly homogenized tissue, due to difficulties encountered because of the very small amount of tissue being used.

With the microsomal fraction, NAD, NADP, and NADH only slightly increase epoxidation of methyl farnesoate while NADPH causes a significant increase in epoxidation as does addition of the soluble fraction. In phosphate buffer the addition of NADPH to the crude homogenate has no noticeable effect on the metabolism of methyl farnesoate, indicating that there are adequate co-factors or co-factor generating systems for the metabolism of the low level of substrate. In Grace's medium the addition of NADPH greatly increases the epoxidation of methyl farnesoate, indicating the destruction of endogenous co-factor or the inhibition of NADPH generating systems.

Methylene blue, SKF 525A, and piperonyl butoxide are common inhibitors of the microsomal mixed-function oxidases in both mammals and insects. The weak inhibition of the CA epoxidase by SKF 525A and piperonyl butoxide and the strong inhibition by methylene blue are thus not surprising. Nitrogen seems to retard epoxidation as found for S. gregaria CA (10) while epoxidation is en-

TABLE 1

Metabolism of Methyl Farnesoate (I) by Blaberus giganteus Corpora Allata and Enzyme Systems

Conditions				Percentage recovered tritium co-chromatographing with major metabolite standards			
Enzyme	NADPH	Buffer	Inhibitor	I (Ester)	II (Acid)	III (JH)	IV (JH Acid)
CA	-	G	O	69	21	2	3
-	±	G or P	± all	>96	<1	<1	<1
H	-	G	-	67	22	2	3
H	+	G	-	33	11	12	42
H	-	P	-	42	3	27	26
H	+	P	-	42	3	28	26
S1	+	G	-	18	2	61	15
P1	+	G	-	43	4	15	34
S2	+	G	-	95	2	1	1
P2	+	G	-	18	3	63	11
P2	-	G	-	90	3	1	1
H	+	P	CO	71	2	13	13
H	+	P	N	56	3	19	18
H	+	P	O	37	2	30	27
H	+	P	P	63	5	15	16
H	+	P	S	98	<1	<1	<1
H	+	P	M	93	5	<1	<1
H	+	P	I	44	<1	41	13
B	±	G	-	3	96	<1	<1
F	±	G	-	20	79	<1	<1

All experiments shown were performed at least 3 times on at least 2 separate enzyme preparations with appropriate controls. All percentages were generally $\leq\pm5\%$ with the same enzyme preparation and separate preparations were $<\pm10\%$ with regard to epoxidase activity except for intact corpora allata which gave highly variable results. The amount of esterase activity also varied greatly between enzyme preparations. Abbreviations are as follows: Enzymes: CA = corpora allata, H = crude CA homogenate, S1 = 12,000 g soluble fraction, P1 = 12,000 g resuspended pellet, S2 = 100,000 g soluble fraction, P2 = 100,000 g resuspended pellet, B = haemolymph, and F = fat body homogenate; Buffers: G = Grace's medium and P = 0.1M pH 7.4 phosphate buffer, both containing 1% BSA; Inhibitors: CO = carbon monoxide, N = nitrogen, O = oxygen, P = piperonyl butoxide ($5\cdot10^{-5}$M), S = SKF 525A ($1\cdot10^{-3}$M), M = methylene blue ($1\cdot10^{-6}$M), and I = isopropyl paraoxon ($1\cdot10^{-4}$M). All incubations were at 30°C for 4 hrs.

hanced by oxygen, indicating that molecular oxygen is probably necessary for the reaction. The inhibition of epoxidation by carbon monoxide may indicate the presence of a haeme protein necessary for the reaction. Diisopropyl paraoxon causes little change in total epoxidation while reducing ester cleavage.

It is interesting that much more JH acid (IV) than farnesoic acid (II) is found. This observation probably indicates that there is more esterase activity on JH III than methyl farnesoate or that farnesoic acid is very rapidly epoxidized. The lack of reduction of total epoxidase activity (III & IV) by diisopropyl paraoxon supports the hypothesis that the epoxidase is more active on methyl farnesoate than farnesoic acid and thus that there is higher esterase activity on JH III than methyl farnesoate. Although the above evidence tentatively supports the preference of methyl farnesoate over farnesoic acid for epoxidation by corpora allata homogenates, higher concentrations of paraoxon do decrease total epoxide formation (III & IV) and methylation of JH acid (IV) or farnesoic acid by S adenosyl methionine in the crude homogenate cannot be ruled out (6, 9).

The specificities of the various enzymes in CA homogenates need to be examined using a variety of substrates and inhibitors in B. giganteus in order to determine the likely biosynthetic route to JH (III). Much of the esterase activity is soluble at 100,000 g while the epoxidase activity is sedimented. It is not certain that the esterase activity is intrinsic to the CA as there is relatively high esterase activity in the haemolymph and fat body (Table 1) which may contaminate even carefully dissected and washed CA.

Most of the metabolism of methyl farnesoate by CA homogenates can be explained by epoxidation and ester cleavage. Only trace amounts of JH diol (V) and JH acid diol (VII) are found, and as reported earlier for Schistocerca gregaria no cis farnesoic acid is found (8). The lack of JH diepoxide (VI) formation suggests specificity of the epoxidase for the 10,11 double bond although this could be attributed to the ease of chemical and biological oxidation of the 10,11 compared to other double bonds.

The structure of JH III was substantiated by several methods in addition to co-chromatography with an authentic standard in five tlc solvent systems. The metabolically formed radioactive JH was diluted with cold standard (very small scale acid catalyzed epoxide hydration often gives anomalous results) and converted to the JH diol by acid catalysis with 87% of the extracted tritium co-chromatographing with the diol by tlc. The diol was then converted to its boronic acid diester (93%) with n-butyl boronic acid or to its corresponding aldehyde with lead tetraacetate (85%). The epoxide was also converted to two major products, presumably tetrahydrofuran diols by treatment with osmium tetroxide. The metabolically formed JH acid was converted to a compound co-chromatographing with authentic JH III (79%) by treatment with excess etheral diazomethane. Of the remaining radioactivity only 3% co-chromatographed with methyl farnesoate while 13% remained at the origin.

As shown in Table 1, B. giganteus fat body homogenates and haemolymph convert methyl farnesoate to farnesoic acid. No other metabolites are detected with or without NADPH and under 0.1% of the tritium from the above incubations co-chromatographed with JH. Mammalian microsomes without NADPH only catalyze ester cleavage to give farnesoic acid, while with NADPH many metabolites are produced, including the JH diol (V), JH acid diol (VII) and cyclic tetrahydrofuran products (not shown in Fig. 1. See references 14 and 16). Other unidentified products are formed but under 0.1% of the tritium co-chromatographed with JH or JH diepoxide (VI), indicating rapid epoxide hydration to the corresponding diols or cyclic products as observed for similar compounds (17).

The presence of a JH epoxidase in CA is predicted by earlier studies using whole CA in vitro (4, 6-10), and the JH epoxidase from B. giganteus CA behaves similarly to microsomal mixed-function oxidases from mammals and insects. It is distinguished by the fact that rapid hydration of the epoxide to the diol does not occur in CA homogenates as it seems to in unhindered epoxides in homogenates of whole insects or mammalian tissues (17-19). A similar NADPH dependent epoxidase acting on farnesoic acid has been recently found in the CA of a holometabolous insect, Manduca sexta, (9), and we have also found it in the CA of other hemimetabolous insects. Thus a CA NADPH dependent epoxidase appears to be an enzyme involved in the biosynthesis of JH, although the exact structure of the natural substrate of this epoxidase awaits further study.

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