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Biosynthesis of the Juvenile Hormones of *Manduca sexta*: Labeling Pattern from Mevalonate, Propionate, and Acetate

(organ culture/corpora allata/high-resolution liquid chromatography/homomevalonate/homoterpenoid)

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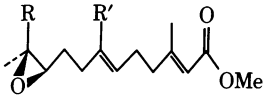
ABSTRACT **Using organ culture, high-resolution liquid chromatography, and microchemical techniques, we demonstrated the efficient incorporation *in vitro* of several radiolabeled precursors into the two juvenile hormones of *Manduca sexta*. JH II, a homosesquiterpene hormone, reported from *M. sexta* as well as several other insects, incorporates radiolabel from acetate, mevalonate, and propionate. JH III, a sesquiterpene hormone recently reported as a natural product of *M. sexta*, incorporates label from acetate and mevalonate, but not from propionate. Based on the position of the labeled atoms in the precursors and upon the position of incorporation obtained from label-distribution data, a scheme for juvenile hormone biosynthesis is advanced.**

Identification of the juvenile hormones **1** and **2** (JH I and JH II) of *Hyalophora cecropia* as novel homoterpenoids (1, 2) aroused considerable interest in their mode of biosynthesis. These same hormones have been reported from two other species of Saturnid moths, *H. gloveri* (3) and *Samia cynthia* (4), and are also produced *in vitro* by the corpora allata of *H. cecropia* (5). Since these hormones are the first known occurrence of ethyl-branched (homo-) terpenes in animals and since considerable practical effort is being directed to development of synthetic analogs of juvenile hormones as pesticides, efforts have been underway in several laboratories to determine their biosynthetic origin. We recently reported (6) the finding of a third natural juvenile hormone **3** [JH III,† methyl (2*E*,6*E*)-(10*R*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate]. This new terpene hormone, co-occurring with the homoterpenoid JH II (but without any detectable JH I), was



1	R = R′ = Et	(JH I)
2	R = Et, R′ = Me	(JH II)
3	R = R′ = Me	(JH III)

produced *in vitro* by gland cultures from the Sphingid moth, *Manduca sexta*. The unique advantages of the *in vitro* technique coupled with the microchemical methods previously described prompted us to use them to explore the biosynthesis of these two hormones.

Positive conclusions regarding juvenile hormone biosynthesis are few. The methyl of the methoxycarbonyl moiety of both JH I and JH II is derived from the *S*-methyl of methionine (7). In addition, good incorporation of the epoxy acid derived from JH I, has been witnessed in *H. cecropia*, although it may not be an obligatory intermediate (8). However, no information has been available on biosynthesis of the carbon skeletons of the juvenile hormones, except for a slight incorporation of [2-^{14}C]acetate (7), and a complete lack of incorporation of methionine in the chain of homoterpenoids JH I (7) and JH II (6, 7).

We now report that juvenile hormone biosynthesis proceeds by a terpenoid pathway with isoprenoid skeletal units arising from mevalonate, and homoisoprenoid units arising from one propionate and two acetates, presumably through homomevalonate (Fig. 1). At present the only perceivable difference from the classical terpenoid biosynthetic scheme is the ability of the insects' corpora allata to produce a homoisoprenoid precursor.

MATERIALS AND METHODS

Animals, Preparation of Cultures, and Extraction Procedures were the same as described (6).

Biosynthetic Precursors. [2-^{14}C]Acetic acid, potassium salt (ICN, specific activity 56 Ci/mol), [2-^{14}C]propionic acid, sodium salt, (Amersham–Searle, specific activity 15.6 Ci/mol), [1-^{14}C]propionic acid, sodium salt (Amersham–Searle, specific activity 48 Ci/mol), and DL-[2-^{14}C]mevalonic acid lactone (Amersham–Searle, specific activity 17.5 Ci/mol) were used as received. DL-[2-^{14}C]Mevalonic acid dibenzylethylene diamine salt (Amersham–Searle, specific activity as mevalonate 4.4 Ci/mol) was converted to the potassium salt by passage through a potassium-equilibrated Amberlite IR-120 column, followed by evaporation of the aqueous effluent under reduced pressure at room temperature. All precursors were reported to be greater than 98–99% radiochemically pure. Precursors were separately made up to final concentrations of 5 or 9 μCi/ml in Grace's Insect T.C. Medium (GIBCO), supplemented with 1% (w/v) bovine-plasma albumin (fraction V, Metrix).

Chromatography. High-resolution liquid chromatography was performed on an apparatus similar to that described (6)

Abbreviation: JH, juvenile hormone.

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† We propose (at the suggestion of Prof. K. Dahm) the adoption of "JH III" as a standard abbreviation for the new hormone, in preference to "C_{16}JH," "$C_{16}H_{26}O_3$ hormone," or "Manduca JH 1."

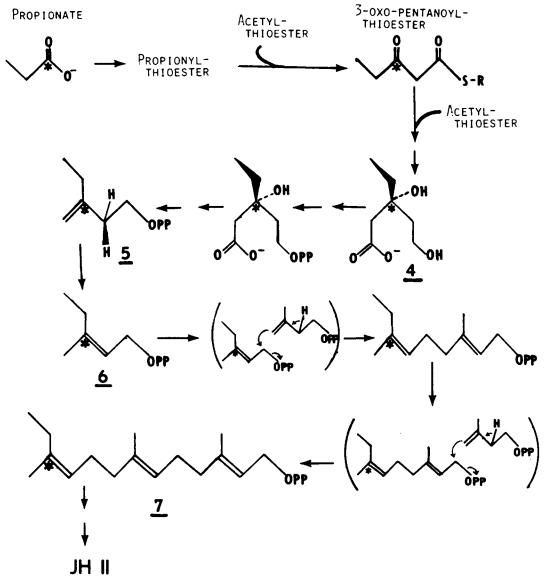

FIG. 1. Hypothetical biosynthesis of the carbon skeleton of JH II from two isoprenoid units and one homoisoprenoid unit. Biosynthesis of the unusual homoisoprenoid precursor is patterned on what is known of the biosynthesis of standard isoprenoid precursors. Atoms marked with an *asterisk* would contain label from [*1*-^{14}C]propionate. Intermediate thioesters could be formed from either coenzyme A or an acyl-carrier protein.

except that a 0.5 m × 2.4 mm inner diameter column of LiChrosorb SI60-10 silica (E. M. Labs, available as MicroPak column from Varian Associates) was found to give better resolution of JH I, II, and III than the 3-m column previously used. Also substitution of a Chromatronix model 230 ultraviolet absorbance detector gave about 3- to 5-times greater sensitivity than the type used previously. Preparative thin-layer chromatography of degradation products was performed on Analtech precoated 5 × 20 × 0.05-cm Silica Gel GF plates.

Radioactivity was measured on Packard Instruments model 2425 or 3380 liquid scintillation counters, with a solution of toluene–2-methoxyethanol 2:1 containing 0.55% PPO (2,5-diphenyloxazole; for routine work) and toluene containing 1.5% PPO and 0.1% bis-MSB *p*-bis(*o*-methylstyryl) benzene (for up to 0.3- to 0.5-mg samples of dinitrophenylhydrazones). Absolute activity was determined by the automatic external standardization method, with occasional double checking by internal standardization.

Mass of Derivatives Was Determined (unless otherwise noted) by ultraviolet spectroscopy (Hitachi-Perkin Elmer model 124). Concentrations were determined by measuring extinctions at λ_{max}, with comparison to standard solutions. Standards for ultraviolet spectra were prepared gravimetrically from unlabeled, recrystallized samples of each derivative. Specific activity was determined by radioassay of the same solution used for extinction measurements.

Reagents and Reference Standards. 3,5-Dinitrobenzoyl chloride was recrystallized from ether–hexane before use. All other reagents were of at least "analytical reagent" purity and were used without further purification. The 2,4-dinitrophenylhydrazine reagent was a 0.25 M solution in 60:40 95% ethanol–85% phosphoric acid. Reference samples of derivatives **9a**, **9b**, **12**, and **13** were prepared by standard methods and were carefully purified by recrystallization. Their melting points (Kofler hot stage, uncorrected) were: butanone 2,4-dinitrophenylhydrazone (**9a**), 110–112°; acetone 2,4-dinitrophenylhydrazone (**9b**), 123–125°; levulinyl 3,5-dinitrobenzoate (**12**), 71–72°; and levulinic acid *p*-phenylphenacyl ester (**13**), 95–97°.

EXPERIMENTAL

Microchemical experimental conditions (where applicable) are summarized below. In the following sequences, JH II or III from incubation with mevalonolactone was combined with that from mevalonate cultures.

Epoxide Hydration. Conditions were the same as those used previously (6). Anomalous reaction products were occasionally encountered if the tetrahydrofuran was not freshly distilled from lithium aluminum hydride. After further dilution with synthetic carrier diol (3–7.5 mg, carefully purified by preparative thin-layer chromatography), specific activity of the diol **8a** (or **8b**) was determined.

Sodium Periodate Cleavage. Diol **8a** or **8b** (10–25 μmol, specific activity about 350–2400 dpm/μmol) was oxidized with sodium periodate in aqueous methanol in a specially designed flask (a custom-fabricated micro-kjeldahl flask fitted with a bonded stainless steel side-arm) connected in series to two 1-ml traps (Kontes Microflex tubes) containing 2,4-dinitrophenylhydrazine solution. After addition of sodium thiosulfate, a stream of nitrogen was bubbled through the reaction to carry acetone (or butanone) into the trapping vials where their crystalline 2,4-dinitrophenylhydrazones formed. These derivatives were extracted and purified by thin-layer chromatography; their specific activity could be determined readily, despite their notorious quenching properties, since the mass of added authentic diol carrier was so low. The flask residue, containing aldehydo-ester **10**, was treated with sodium borohydride, and the resulting hydroxy-ester was isolated by extraction.

Formation of 3,5-Dinitrobenzoate ***11***. The hydroxy-ester (purified by thin-layer chromatography) from the previous step was treated with 3,5-dinitrobenzoyl chloride in dry pyridine. The mass of the oily 3,5-dinitrobenzoate ester **11** was determined gravimetrically in order to establish its specific activity.

Catalytic Ruthenium Tetroxide Cleavage of ***11***. A modification of the procedure of Piatak *et al.* (9) was used, with excess sodium periodate and periodic acid in aqueous *tert*-butyl alcohol with a catalytic amount of ruthenium dioxide (54% ruthenium, Engelhard Industries). After addition of 2-propanol to the buffered reaction, neutral compounds were extracted and purified by thin-layer chromatography to give pure, crystalline levulinyl 3,5-dinitrobenzoate **12**, whose specific activity was determined. Acidic extracts of the reaction mixture were treated with *p*-phenylphenacyl bromide and sodium bicarbonate in dimethylformamide. Purification of these reaction products by thin-layer chromatography (and in several cases, high-resolution liquid chromatography) gave crystalline levulinic acid *p*-phenylphenacyl ester **13**. No other *labeled* acidic derivative other than that from levulinic acid could be isolated by these methods, although many unlabeled by-products were observed.

TABLE 1. *Incorporation of radiolabeled precursors into JH II and III*

Precursor	[2-^{14}C] Acetate	[2-^{14}C] Mevalonate	[2-^{14}C] Mevalonolactone	[2-^{14}C] Propionate	[1-^{14}C] Propionate
Specific activity					
Ci/mol	56	4.4	17.5	15.6	48
dpm/μmol($\times 10^{-6}$)	124	9.77	38.9	34.6	107
Concentration in culture medium					
dpm/ml ($\times 10^{-6}$)	11.1	20.0	11.1	11.1	11.1
Total no. of gland pairs incubated*	75	61	125	60	20
Total dpm recovered from TLC "JH-zone" ($\times 10^{-6}$)	0.31	0.071	0.087	0.67	0.059
Total dpm recovered from HRLC ($\times 10^{-6}$)					
JH II	0.138	0.016	0.046	0.428	0.057
JH III	0.050	0.050	0.032	0.072	0

TLC, thin-layer chromatography; *HRLC*, high-resolution liquid chromatography.

* Variations in culture incubation times preclude any direct correlation between the number of gland pairs incubated and the total dpm of recovered hormones.

RESULTS

Gland cultures were prepared and incubated with the labeled precursors for 3–21 days. Extracts of the culture media were purified by thin-layer chromatography (6) and then stored until sufficient quantities were in hand to warrant separation by high-resolution liquid chromatography. At that time the radiolabeled, natural hormone mixtures were diluted with about 20 μg each of synthetic JH I, II, and III (10). Aliquots of each batch were then subjected to high-resolution liquid chromatography, demonstrating coincidence of labeled hormone with its respective carrier (Table 1). After separation by preparative liquid chromatography, JH II and III from each precursor were converted to their respective 10,11 diols **8a** and **8b** by acidic hydration for greater storage stability. Pools of diols from each precursor were stored separately until sufficient material was available for degradation studies.

As additional proof of identity of each labeled hormone, the diol derivatives **8a** and **8b** were subjected to high-resolution liquid chromatography; complete coincidence of radiolabel and synthetic carrier was observed for each diol from each precursor. In addition, conversion of each diol to its 10-(+)-α-methoxy-α-trifluoromethylphenylacetate ester (6) gave two separable diastereomeric esters from each racemic synthetic diol. For this derivative of both hormones from all precursors, high-resolution liquid chromatography showed coincidence of radiolabel with only the faster eluting diastereomer, thus confirming the optical activity (and 10*R* absolute configuration) (6) of both natural hormones from each precursor.

Since radiolabeled JH III was formed only indirectly from [2-^{14}C]propionate and not from [1-^{14}C]propionate (Table 1), we did not attempt rigorous identification by derivative formation of JH III derived from [2-^{14}C]propionate. After separation by high-resolution liquid chromatography of extracts from incubation with either [1-^{14}C]- or [2-^{14}C]propionate, a small amount of labeled material was recovered with a retention volume identical to that of JH I. This material has not been subjected to further identification.

The fact that acetate, propionate, and mevalonate were apparently efficiently incorporated into JH II, and acetate and mevalonate into JH III, lends support to our basic hypothesis concerning the mode of biosynthesis of these hormones.

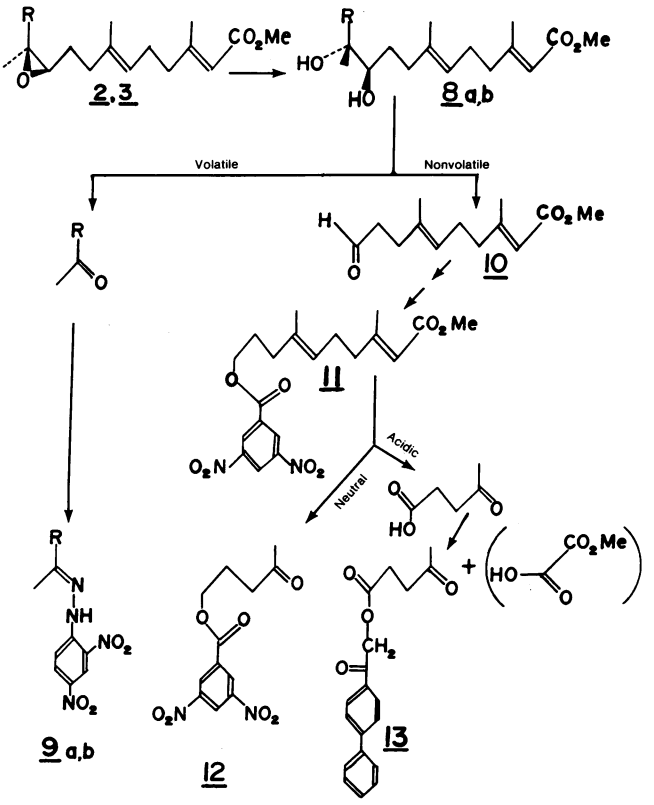

FIG. 2. Degradative scheme for JH II and JH III. Derivatives **8a** and **9a** are formed from JH II (R = Et), while compounds **8b** and **9b** are derived from JH III (R = Me). All other derivatives (**10, 11, 12,** and **13**) do not differ between the two hormones.

In order to estimate better the location of the labeled atoms, a series of degradative reactions was designed to cleave either JH II or III into four fragments, three of which could be recovered as crystalline derivatives. The basic scheme is shown in Fig. 2, the details are outlined in *Experimental*, and the results of specific activity measurements are presented in Table 2.

DISCUSSION

In our previous investigation (6) we demonstrated that corpora allata from adult females of *M. sexta* were able to

TABLE 2. *Degradation of JH II and JH III*

Precursor and hormone	Measured specific activity of compounds in degradative schemes (dpm/μmol)						
	8a	**8b**	**9a**	**9b**	**11**	**12**	**13**
[2-^{14}C]Acetate							
JH II	1905		231		1715	747	683
JH III		850		182	—	290	305
[2-^{14}C]Mevalonate							
JH II	334		≤ 3		382	162	175
JH III		690		262	494	241	237
[2-^{14}C]Propionate							
JH II	2360		2320		301	111	117
JH III		384		69	266	102	102
[1-^{14}C]Propionate							
JH II	275		248		≤ 3	—	—
JH III		0		—	—	—	—

TABLE 3. *Comparison of the predicted number of labeled atoms in each derivative (from Fig. 3) with the observed number of labeled atoms calculated from the specific activity ratios of diols 8 to their degradation products (from Table 2)*

Hormone and precursor	No. of labeled atoms assumed in **8**	No. of labeled atoms found in derivatives (predicted no. given in parentheses) **9a** or **9b**	**12**	**13**	**11**	Nonisolable 2-carbon fragment
JH II from						
[*2*-^{14}C]Acetate	8.00	0.97 (1)	3.14 (3)	2.87 (3)	7.20 (7)	1.02 (1)
[*2*-^{14}C]Mevalonate	2.00	$\leq$0.02 (0)	0.97 (1)	1.05 (1)	2.29 (2)	−0.04 (0)
[*2*-^{14}C]Propionate	1.00	0.98 (1)	0.05 (0)	0.05 (0)	0.13 (0)	−0.08 (0)
[*1*-^{14}C]Propionate	1.00	0.90 (1)	— (0)	— (0)	$\leq$0.01 (0)	0.10 (0)
JH III from						
[*2*-^{14}C]Acetate	9.00	1.93 (2)	3.07 (3)	3.23 (3)	—	0.77 (1)
[*2*-^{14}C]Mevalonate	3.00	1.14 (1)	1.05 (1)	1.03 (1)	2.15 (2)	−0.22 (0)

Data were normalized on the assumption that the number of labeled atoms found and predicted for the parent diols **8** was the same. *Dash* (—), data not measured. Data for the nonisolable two-carbon fragment was calculated from the observed values: [**8** − (**9** + **12** + **13**)].

survive *in vitro* and produce detectable levels of JH II and III for at least several weeks. Addition of labeled [*methyl*-^{14}C]-methionine to the culture medium led to synthesis of both juvenile hormones labeled in the ester methyl carbon. The specific activity of the hormones was within 10% of that of the precursor (i.e., a dilution of 1.1-fold), indicating a strong dependence of the biosynthesis of juvenile hormone on methionine (most likely through *S*-adenosylmethionine) for formation of the methyl ester moiety.

In the present study, specifically labeled precursors were selected to test our hypothesis concerning the biosynthesis of juvenile hormone. The minute quantities of hormones collected precluded accurate mass determination, and therefore we were unable to measure the specific activity of the labeled hormones. Consequently, the effect of the various precursors on the overall rate of juvenile hormone production or on the ratio of JH II to III cannot be assessed.

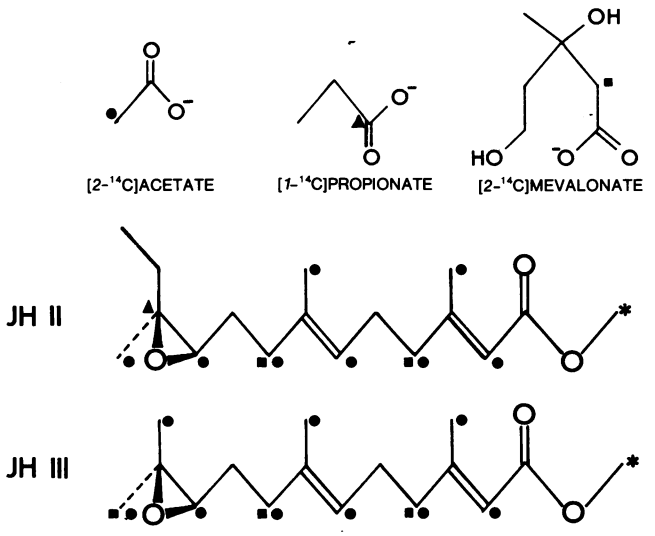

FIG. 3. Labeling pattern anticipated in JH II and JH III (according to the hypothesis in Fig. 1) from [*2*-^{14}C]acetate (●), [*1*-^{14}C]propionate (▲), and [*2*-^{14}C]mevalonate (■). Label from the [*methyl*-^{14}C] of methionine incorporates into the hormones as indicated by *asterisks* (6). Position of label from [*2*-^{14}C]propionate is not indicated here, since some apparent metabolism of this precursor led to equivocal results (see *Discussion*).

Our primary concern in this work was to demonstrate that the selected precursors were incorporated into juvenile hormones and that the distribution of the labeled carbon atoms was that predicted by our hypothesis. To verify the distribution, a procedure was developed for partial degradation that was relatively simple, gave high yields, and was suitable for use on a micromolar scale. It allows cleavage of the juvenile hormone skeletons and isolation of three distinct crystalline substances; the fourth fragment (oxalic acid or its unstable methyl half-ester) is nonisolable, presumably due to oxidative instability. Before degradations were performed, each hormone was rigorously identified as the proper optically active, biosynthetic product by described (6) criteria; in addition, purification of the crystalline derivatives by thin-layer or high-resolution liquid chromatography gave sufficiently high purity to render recrystallization unnecessary. As additional evidence in support of the hypothesis, we isolated the oily intermediate dinitrobenzoate **11** for specific activity determination.

The crucial test of our hypothesis (outlined in Fig. 1) lies in the data presented in Table 3. The agreement between observed and predicted (Fig. 3) numbers of labeled atoms for each of the various derivatives of a given substrate is extremely good, except for the hormones derived from [*2*-^{14}C]-propionate. It is clear that the sesquiterpene JH III is derived from 3 mol of mevalonate, or the equivalent 9 mol of acetate. Thus, formation of the carbon skeleton of this hormone follows, in gross details, the familiar terpene pathway. It is equally clear that JH II incorporates only 2 mol of mevalonate, and, since it was separately found to incorporate 8 mol of acetate (six of which would form the 2 mol of mevalonate) and 1 mol of propionate, the homoisoprenoid segment of the skeleton must arise from the remaining two acetate units and the single propionate unit. We believe the most logical way for this to occur is as shown in Fig. 1. The proposed order of incorporation of propionate into the homoisoprenoid precursor should generate the *cis* (*E*) double-bond geometry which we believe is required for *cis* epoxide formation.

The proposed scheme demands that propionate be entirely excluded from JH III or from derivatives **11**, **12**, or **13** of JH II. However, gland cultures containing [*2*-^{14}C]propionate incorporated radiolabel into JH II and JH III in a 6:1 ratio, and the radioactivity in degradation products **11**, **12**, and **13**

of JH II were significantly greater than zero. On the other hand, substitution of [*1*-^{14}C]propionate showed no detectable incorporation into JH III or into derivatives **11, 12,** and **13** from JH II. We interpret these data to indicate that either a labeled impurity (such as acetate) was present in the [*2*-^{14}C]-propionate, or more likely, that *in vitro* metabolism of this precursor generated other precursors. Although the degradation data for JH III from [*2*-^{14}C]propionate (Table 2) cannot be fully explained by assuming metabolic conversion of the precursor to [*1*-^{14}C]acetate (or by assuming presence of [*2*-^{14}C]-acetate as an impurity), the number of metabolic pathways involving propionate is sufficiently great to preclude any simple resolution of this difficulty. However, the data based on [*1*-^{14}C]-propionate are unambiguous.

Other information supporting the hypothesis in Fig. 1 comes from the recent findings of two groups of workers (11, 12) investigating the enzyme prenyltransferase (farnesyl pyrophosphate synthetase, EC 2.5.1.1), responsible for formation of farnesylpyrophosphate from one equivalent of 3,3-dimethylallyl (3-methylbut-2-enyl) pyrophosphate and two equivalents of isopentenyl pyrophosphate. This enzyme is rather nonspecific for analogs of 3-methylbut-2-enyl pyrophosphate homologous at either terminal methyl group (11, 12). By incubating various homologous artificial substrates with prenyltransferase, these workers have shown that even butyl-branched dimethylallyl pyrophosphate analogs (i.e., *E* and *Z* 3-methylhept-2-enyl pyrophosphate) are converted by the enzyme into trishomofarnesols. Also, the rate of incorporation of the homolog (*Z*)-3-methyl-pent-2-enyl pyrophosphate (**6,** Fig. 1) is only about one-third that of the natural 3-methylbut-2-enyl pyrophosphate (12), resulting in a homofarnesyl pyrophosphate with the same carbon skeleton as JH II.

Corresponding work on homologous artificial substrates of isopentenyl pyrophosphate (3-methyl-but-3-enyl pyrophosphate) has been less intense. However, a recent communication (12) reports that of four homologous substrates examined, only 3-ethylbut-3-enyl pyrophosphate (**5,** Fig. 1) can condense with 3-methylbut-2-enyl pyrophosphate in the presence of prenyltransferase (giving a homofarnesylpyrophosphate). Further details on the apparently greater specificity of this enzyme for its nonallylic pyrophosphate (as opposed to allylic pyrophosphate) substrate will be of interest.

The results of these studies clearly indicate that prenyltransferase enzyme, from sources as unrelated to insects as pig and rat liver or pumpkin fruit, is readily capable of synthesizing the homosesquiterpenoid skeleton of JH II‡. Thus, the novel aspects of the biosynthesis of JH I and JH II must lie in the insects' ability to produce a homoisoprenoid precursor, such as homomevalonate.

While we have not formally clarified the mode of JH I biosynthesis, it can be inferred from the preceding discussion that it probably arises from coupling of two homoisoprenoid precursors such as **5** and **6** and 1 equivalent of normal isoprenoid precursor from mevalonate. Brief speculation (13, 14) that JH I and JH II arise biogenetically from a homolog of mevalonate is therefore probably correct. Nonincorporation of propionate and mevalonate into the JH of *H. cecropia* adult moths *in vivo* was reported (7), however, a reinvestigation (Profs. K. H. Dahm and H. Röller, personal communication) has shown that [*1*-^{14}C]propionate incorporates into the carbon chains of JH I and JH II. The nonincorporation of methionine into the chains of JH I (7) and JH II (6, 7), *together* with our present positive incorporation data, clearly rules out a hypothesis for JH I biosynthesis based on alkylation of double-bond isomers of methyl farnesoate by *S*-adenosylmethionine (16). Also, a one-carbon homologation of JH III by C-1 of propionate to produce JH II is inconsistent with the observed acetate labeling pattern of JH II. An alternative hypothesis compatible with observed labeling patterns would call for replacement of C-12 of JH III by a two-carbon unit somehow derived from C-1 (and presumably C-2) of propionate to produce JH II, but this is extremely unlikely. Finally, a recent suggestion, based solely on failure to detect incorporation of certain precursors, that JH II biosynthesis is "significantly different" from terpenoid pathways is in error (17).

We thank Dr. Richard C. Jennings for discussions and comments on the manuscript.

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‡ A very recent report of the Tohoku University group (12, 13) demonstrates that incubation of **5, 6,** and isopentenyl pyrophosphate with prenyltransferase leads to formation of a bishomofarnesyl pyrophosphate, with identical carbon skeleton to JH I, and a homologous trishomofarnesyl pyrophosphate, with three "ethyl branches", [Koyama, T., Ogura, K. & Seto, S. (1973) *Chem. Lett.* **1973,** 401–404].