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# The Structure of Dolichols Isolated from *Manduca sexta* Larvae

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**We isolated and analyzed dolichols from fifth stadium larvae of *Manduca sexta*, in an effort to determine if *M. sexta* produces ethyl-branched dolichols. Like other lepidopterans, this animal is known to produce the ethyl-branched, homoisoprenoid juvenile hormones. However, we were unable to detect any ethyl-branched dolichol homologues. *Manduca sexta* larvae at this stage do contain the common eukaryotic dolichols, principally dolichols 17, 18, 19 and 20 in an ~3:7:5:2 ratio. Analysis of the yeast-containing artificial diet showed the presence of dolichols 13, 14, 15, 16, and 17 in an ~2:6:8:6:3 ratio. We attempted to determine whether *M. sexta* synthesized dolichols *de novo*; preliminary experiments using [5-$^3$H]mevalonate incubated with two *M. sexta* tissues *in vitro* were unsuccessful. In light of earlier studies on dolichol identity in two fly species, we conclude that insects can synthesize dolichols, even though they are known to be incapable to synthesizing sterols from acetate.**

Mevalonate Homomevalonate Juvenile hormone Isoprenoid Mass spectrometry RPLC

## INTRODUCTION

Dolichols are long-chain 2,3-dihydro-poly-*cis*-isoprenols containing a saturated isoprene residue $\alpha$ to the hydroxyl group [Fig. 1(A)]. They were first identified by Pennock *et al.* (1960), and are present in all eukaryotes (Morris and Pullarkat, 1987; Hemming, 1992). Naturally occurring dolichol homologues contain 13–24 isoprene units (Hemming, 1992). Although the physiological role of dolichols has not yet been clearly defined, it is known that the phosphorylated form, dolichyl phosphate, shuttles polysaccharides onto asparagine residues of proteins during glycoprotein synthesis (Kornfeld and Kornfeld, 1985). Dolichols are derived biosynthetically from farnesyl pyrophosphate (FPP), onto which more isoprene units are added by *cis*-prenyl transferase (Ericsson *et al.*, 1991). Farnesol and isoprene units are synthesized from mevalonate [Fig. 2(B)], which also serves as the precursor for a wide range of steroids and other isoprenoids (Beytía and Porter, 1976), including the side chain of ubiquinone (Pennock and Threlfall, 1983), as well as the farnesyl and geranylgeranyl groups attached to certain cellular proteins (Clarke, 1992).

One essential group of isoprenoid compounds found only in insects are the sesquiterpenoid juvenile hormones (JH) (Fig. 3). The most common form of JH is JH III [Fig. 3(C)], which is found in at least one stage of development of most insects (Trautmann *et al.*, 1974; Schooley *et al.*, 1984; Baker, 1990). The less common forms, e.g. JH 0, JH I, JH II, and 4-MeJHI [Fig. 3(A), (B), (D) and (E)] are believed to be found only in Lepidoptera (Bergot *et al.*, 1980; Schooley *et al.*, 1984; Baker, 1990), and differ from JH III by having one or more ethyl branches at $C_3$, $C_7$ or $C_{11}$, and 4-MeJHI having an extra methyl group at $C_4$. Propionate has long been known to serve as the biogenetic source of these ethyl side branches (Schooley *et al.*, 1973). Subsequent studies have shown that corpus allatum enzymes of *M. sexta* can condense propionyl-CoA with acetyl-CoA to give 3-hydroxy-3-ethylglutaryl-CoA (Baker and Schooley, 1978), which can then be reduced to homomevalonate (Lee *et al.*, 1978). Homomevalonate [Fig. 2(A)] incorporates specifically into the ethyl-branched JH (Jennings *et al.*, 1975). More recently, isoleucine and valine were found to be the sole metabolic progenitors of propionate in *M. sexta* corpora allata (Brindle *et al.*, 1987). Comparative metabolism studies have shown that propionate and acetate units, arising from the metabolism of [U-$^{14}$C]isoleucine incorporate into JH of lepidopteran insects, but they do not incorporate into JH of non-lepidopteran insects (Brindle *et al.*, 1988). Furthermore, organic acid metabolites of [U-$^{14}$C]isoleucine, including [$^{14}$C]propionate and [$^{14}$C]acetate, are secreted from the intact corpus allatum of lepidopteran insects, but not from the corpora allata of non-lepidopteran insects (Brindle *et al.*, 1988, 1992). From these data, it is tempting to speculate that the ability of lepidopterans

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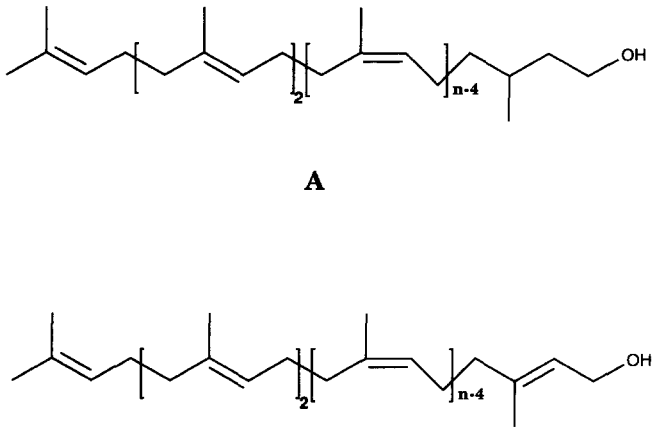


FIGURE 1. Dolichol (A) and polyprenol (B) structures, "n" indicates the number of total isoprene units present in each homologue. By convention, a particular homologue is called "dolichol n" or "polyprenol n."

to metabolize branched-chain amino acids to propionate may correlate with their ability to produce homoisoprenoid structures, including, but not limited to, ethyl-branched JH homologues. Recently we have shown that other lepidopteran tissues, especially the fat body and Malpighian tubules, readily metabolize [U-$^{14}$C]isoleucine to organic acid metabolites (Halarnkar and Schooley, 1995). While insects are known to lack the ability to biosynthesize cholesterol from acetate (Rees, 1985), they are able to synthesize other isoprenoids (Beedle *et al.*, 1975).

Therefore we have isolated dolichols from *M. sexta* to see whether they might include homologues containing ethyl side branches. Based on molecular weight analysis of *M. sexta* dolichols by chemical ionization mass spectrometry, we found only the typical eukaryotic dolichols derived from mevalonate to be present in fifth instar larvae. No indications of homomevalonate derived ethyl-branched dolichols were found.

## MATERIALS AND METHODS

### *Chemicals*

Bovine heart dolichol standards, pyrogallol, alumina powder for column chromatography (super I WB-5 basic) and KCl were purchased from Sigma Chemical Co. Ethylene chloride was from Baxter, and other solvents were obtained from Fisher Scientific. Diethyl ether was purified grade, and all other solvents were HPLC grade. RS-[5-$^3$H(N)]mevalonolactone (35 Ci/mmol) was purchased from DuPont NEN.

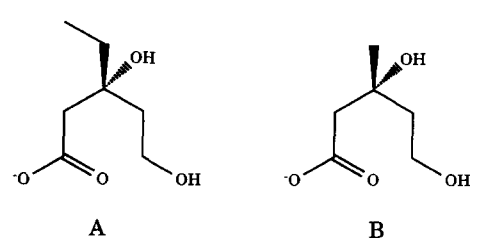


FIGURE 2. The structures of homomevalonate (A) and mevalonate (B).

### *Insects*

The *M. sexta* colony was reared essentially as described by Yamamoto (1969). Larvae were frozen in liquid nitrogen and stored at −80°C. Fifth instar animals, ranging from day 1 to the prewandering period, were used because of their large size, abundance of fat body, and evidence that dolichol content is elevated in older stages of insects (Morris and Pullarkat, 1991). Ethyl-branched JH (JH I and JH II) are the predominant homologues present at this stage of *M. sexta* development (Baker *et al.*, 1987).

### *Saponification and subsequent isolation of neutral lipids*

The saponification and extraction procedures were modified after the methods described by Burgos *et al.* (1963) and Adair and Keller (1985). 1 kg of larval tissue was thawed at room temperature and soaked in 1.5 l. of hexane for 10 min to remove cuticle lipids, and the insect bodies were transferred to a commercial blender (Waring CB-6). 3 l. of methanol, 600 g of KOH, and 7.5 g of pyrogallol were added, and the mixture was homogenized at top speed for 1 min. The homogenate was then transferred into Kimax glass tubes (2.5 × 15 cm) with Teflon-lined screw caps. The lipids were saponified in an 80°C water bath for 1 h, then cooled to room temperature.

The contents of the tubes were pooled into an Erlenmeyer flask, and 3 l. of freshly distilled ether was added. After thorough mixing, the phases were allowed to separate, and the upper ether phase was collected. The ether extraction procedure was repeated three times, and the combined ether phases were then washed three times with 20% KCl solution to remove polar components. Non-saponifiable lipids were obtained after evaporation of ether with a rotary evaporator (Büchi Rotavapor-R). The lipids were redissolved in 3 l. of heptane and stored at 4°C overnight to precipitate sterols. The removal of the precipitate was achieved by filtration via glass wool fiber anchored in a glass Pasteur pipette. The filtrate was then rotary evaporated, and lipids were redissolved in 70 ml hexane.

### *Alumina matrix absorption–desorption of neutral lipids*

Alumina matrix absorption–desorption of neutral lipids is much faster for purifying large quantities of lipids than column chromatography. 70 g of heat activated (450°C, 12 h) alumina was placed into the neutral lipid solution in 70 ml of hexane. This suspension was mixed well by stirring for 5 min, and hexane was decanted off. This hexane wash step was repeated twice. The alumina matrix was then washed sequentially three times each with 140 ml hexane containing 2, 15, and 30%

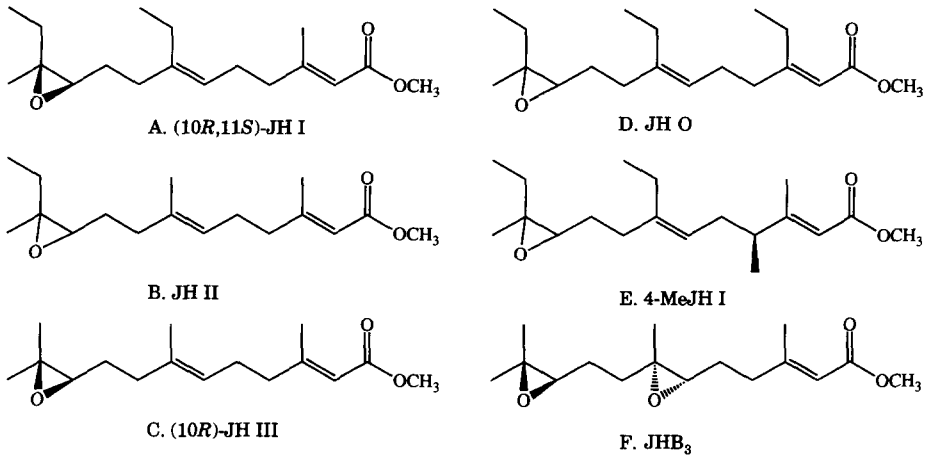


FIGURE 3. The structures of the known naturally occurring juvenile hormones.

ether, respectively. The three washes of each ether concentration were pooled and dried in the rotary evaporator, and the lipids were redissolved in 0.5 ml of chloroform:methanol (2:1 by volume). An aliquot of each fraction was assayed on analytical thin layer chromatography (TLC) plates to determine dolichol purity as described below. The 15% ether in hexane wash yielded the highest amount of dolichols.

*Thin layer chromatography (TLC)*

Preparative TLC was carried out to fractionate the 15% ether extract on a Whatman silica gel plate (20 × 20 cm, 1000 $\mu$m). An aliquot representing one-quarter of the lipids was loaded onto one lane of a plate via a 20 $\mu$l disposable micropipet (Fisher Scientific), with dolichol standard loaded in a separate lane. After all the solvent had evaporated, the plate was developed in hexane:ethyl acetate (3:1 by volume). After air drying, the plate was covered carefully with aluminum foil, except for the lane occupied by dolichol standards, and the plate was placed in an iodine chamber for 1 h. The dolichol standard band was stained a brownish color. A band spanning 0.5 cm above to 0.5 cm below the authentic dolichol standard was scraped off the plate, and the lipids were extracted three times with 200 ml ether. Then the ether was removed in the rotary evaporator and the residue redissolved in 2 ml of 10% isopropanol in methanol.

Analytical TLC was carried out on silical gel GF plates (Analtech) to identify the dolichol-containing fractions eluted from alumina and RPLC steps described below. Only about 5% of each fraction was loaded on the plate. The developing solvent and plate visualizing procedures were the same as described for preparative TLC.

*Reversed phase liquid chromatography (RPLC)*

Two RPLC separations, adapted from the method described by Crick and Carroll (1987), were used to further purify dolichols obtained from preparative TLC. The liquid chromatograph was a Perkin–Elmer series 410 BIO with a Perkin–Elmer LC 235 Diode Array detector. For the first purification step, a 100 × 4.6 mm RP-18 column (Applied Biosystems) was used with a solvent gradient from 100% methanol to methanol:isopropanol (1:9) in 60 min at 2 ml/min, and then the gradient returned to the initial condition in 10 min. Ultraviolet absorbing (210 nm) fractions were analyzed by analytical TLC, and the fractions that co-migrated with dolichol standards were pooled and evaporated under $N_2$. The residue was redissolved in 0.5 ml of 10% isopropanol in methanol for further RPLC purification.

The second RPLC purification was carried out on a Vydac $C_{18}$ narrow bore column (2.1 × 150 mm). The lipids suspended in 0.5 ml of 10% isopropanol in methanol were diluted with 2 ml of methanol and applied to the column. The solvent gradient was from 100% methanol to methanol:isopropanol (1:9) in 60 min, held for 10 min, then returned to the initial condition in 10 min. The rate of solvent delivery was 200 $\mu$l/min. Other conditions were the same as in the first RPLC purification. The fractions that absorbed at 210 nm were collected individually, dried under $N_2$, and redissolved in 20 $\mu$l of methylene chloride for mass spectrometric analysis.

*Mass spectrometry*

Chemical ionization, fast atom bombardment, and electron impact ionization mass spectra were obtained on a Finnigan MAT SSQ 710 mass spectrometer. Chemical ionization (CI) using methane reagent gas proved to be the most sensitive of these methods for detecting molecular ions of dolichols. The dolichol fractions isolated from analytical RPLC were deposited onto a solid probe tip, and the solvent was allowed to evaporate. Normally, CI reagent gas pressure was about 8000 mTorr inside the ion source. We usually scanned from $m/z$ 900 to 1600 in 1.5 s. The probe temperature was set from 100 to 350°C with a 200°C/min increment. Several scans were averaged to improve the signal-to-noise ratio.

*Analysis of dolichols isolated from M. sexta artificial diet*

The dolichol homologue profile of 0.5 kg of *M. sexta* diet was also analyzed by the same procedures as described for the insect extracts, except that the initial hexane treatment to remove cuticular lipids was omitted.

*Computer software for data processing*

The mass spectral data were saved as text files; the actual $m/z$ values determined usually differed by non-integral units. Accordingly, we rounded the experimental $m/z$ values to integers. Plotting of the mass spectra was accomplished by importing the data from text files using DeltaGraph® Professional. Calculation of theoretical isotope distribution of dolichol molecules was done with Isotope™ software (v1.6.4.). For ease of comparison, the isotope distribution was plotted with DeltaGraph®.

## RESULTS

*Extraction, alumina matrix absorption–desorption and TLC*

About 1.65 g of non-saponifiable yellowish lipids were obtained from 1 kg of *M. sexta* larvae after total neutral lipid extraction and precipitate removal via filtration.

*Manduca sexta* dolichols were desorbed from the alumina matrix with 15% ether in hexane. The resulting extract was fortunately rather low in contaminating lipids. None of the other solvent compositions tested desorbed appreciable amounts of dolichols from the alumina matrix, as determined by RPLC.

The $R_f$ value for the dolichol standard was 0.52 on analytical TLC, and was 0.60 on preparative TLC. On a preparative TLC pilot test of *M. sexta* lipids, there were four iodine stained bands with $R_f$ 0.44, 0.64, 0.82, and 0.89. The silica coating from 0.5 cm above and below the band with $R_f$ of 0.64 was scraped off the plate, and the lipids were recovered with ether.

*RPLC purification*

Two successive RPLC separations were needed for dolichol purification. The chromatogram for the first RPLC indicated the presence of many contaminating lipids [Fig. 4(A)]. The dolichols eluted near the end of the gradient with retention times of 30–50 min. Significant amounts of hydrocarbons also eluted after 30 min. The chromatogram from the second RPLC step demonstrates the enrichment and separation of different dolichol homologues [Fig. 4(B)]. The retention time of dolichols on this smaller bore (2.1 mm Vydac $C_{18}$) column was from 50 to 70 min, and from 30 to 50 min on the 4.6 mm column (Applied Biosystems RP-18). It is possible that the doublet peaks of each dolichol homologue observed in this chromatogram are due to the presence of either geometrical isomers or polyprenols, the α-unsaturated analogues of dolichols [Fig. 1(B)]. However, the mass of dolichols isolated was insufficient for investigation of these possibilities by NMR.

*Chemical ionization mass spectrometry (CI/MS) analysis of dolichols*

Chemical ionization mass spectrometry (CI/MS) proved to be the most sensitive mass spectral technique tested for the analysis of dolichols. Though methane provides more fragmentation than many CI/MS reagent gases, the spectra still contained very few fragment ions. Because of the high molecular weight of these materials (> 1000 Da), and because proton transfer is the predominant ionization mechanism, the molecular ions observed represent an envelope of protonated isotopic species (Fig. 5). We have shown only the molecular ion envelope, due to the following considerations: first, the significance of the envelope profiles; second, the lack of significant fragmentation of dolichols produced by chemical ionization; third, the presence of hydrocarbon peaks in the spectra.

The dolichol standard obtained from Sigma was extracted from bovine heart. When 5 μg of the standard mixture (unknown homologue percentage) was analyzed by CI/MS, the signal-to-noise ratio was about 5 for dolichol 17, 20 for dolichol 18, 30 for dolichol 19, and 25 for dolichol 20. The isotopic envelope of the protonated molecular ions of the standard had a relative isotope ratio close to the theoretical value, as calculated by Isotope™ software, with the exception of having one extra peak, which was 1 a.m.u. less than the protonated monoisotopic peak (Fig. 5, A2 and A3; B2 and B3; C2 and C3; D2 and D3; see "Discussion"). For example, the isotope ratio in the mass spectrum of protonated dolichol 18 standard ($C_{90}H_{149}O$; Fig. 5, B2), was very close to the theoretically calculated ratio; $(MH^+):(MH^+ + 1):(MH^+ + 2):(MH^+ + 3):(MH^+ + 4)$ being 96.8:100:51.3:17.4:4.4 ($m/z$ 1245:1246:1247:

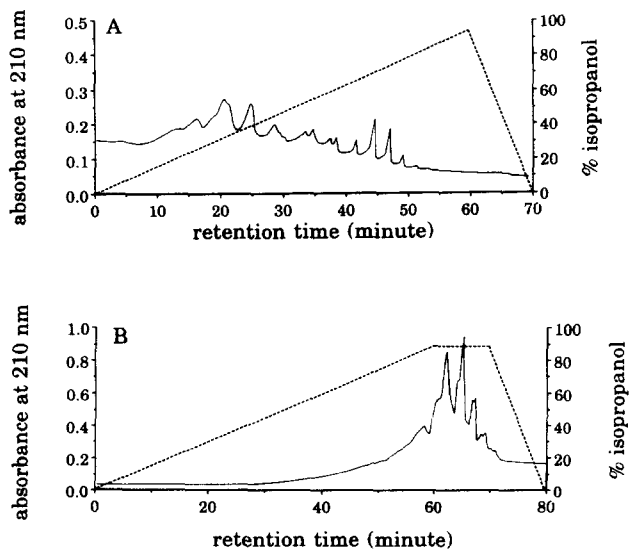


FIGURE 4. RPLC purification of *Manduca* dolichols. (A) The first RPLC step was performed on a 4.6 mm ABI RP-18 column. Dolichols eluted between 30 and 50 min after the beginning of the gradient. (B) The second RPLC step was performed on a 2.1 mm Vydac $C_{18}$ column. Dolichols eluted between 50 and 70 min after the beginning of the gradient. The dotted lines indicate the solvent gradient.

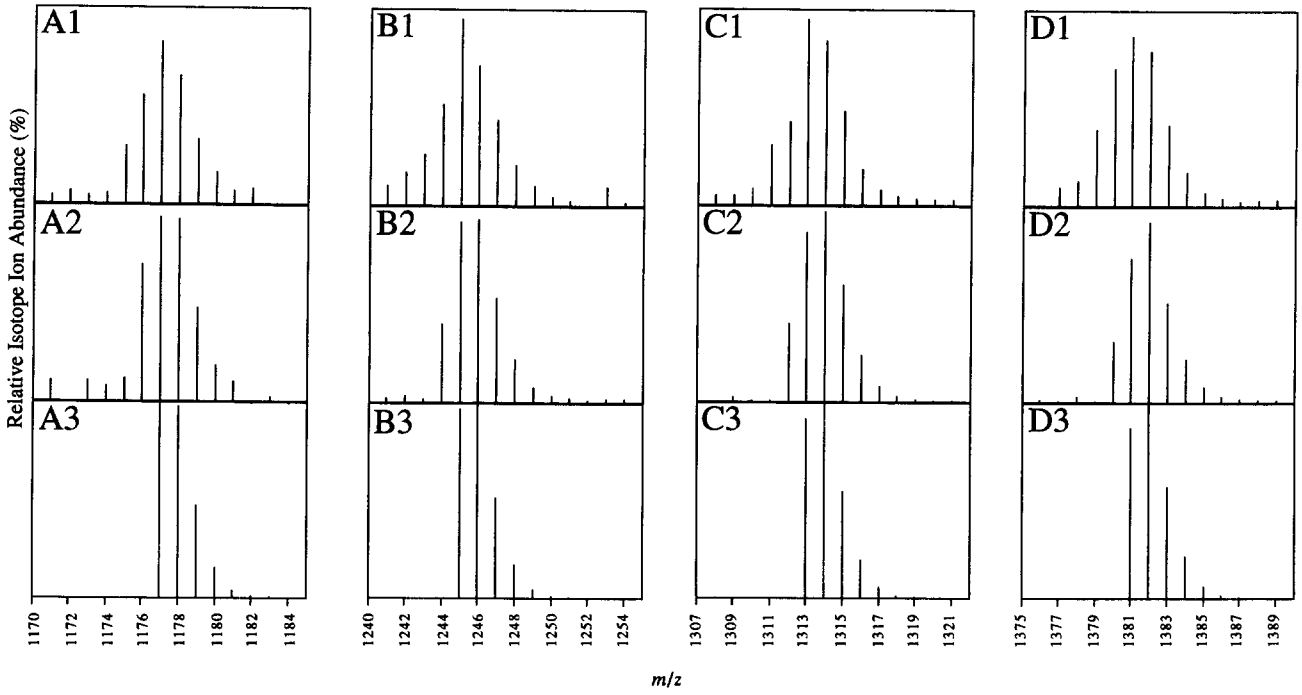


FIGURE 5. The chemical ionization mass spectra of *M. sexta* dolichols, bovine dolichol standard and the calculated isotope distribution (Isotope[tm] software) for dolichol homologues 17–20. Column A: dolichol 17; B: dolichol 18; C: dolichol 19; D: dolichol 20. Row 1: *M. sexta* dolichol; 2: bovine dolichol standard; 3: theoretical protonated molecular ion distribution.

1248:1249) (Fig. 5, B2 and B3), except for having a $M^+$ peak ($m/z$ 1244) that is about 40% the height of peak $m/z$ 1246, the latter being the base peak.

The molecular ion envelopes for *M. sexta* dolichols 17–20 were strong, whereas for dolichols 15 and 16, the molecular ion signals were much weaker. Only the spectra for dolichols 17 to 20 are presented because of their relatively higher signal-to-noise ratio (Fig. 5, A1, B1, C1 and D1). The signal-to-noise ratio was approx. 10 for dolichol 17, 25 for dolichol 18, 15 for dolichol 19, and 10 for dolichol 20. Curiously, the envelope of peaks does not correspond precisely to the spectra obtained for dolichol standards with the same method (Fig. 5, A1 and A2; B1 and B2; C1 and C2; D1 and D2). The most abundant isotope peak observed in each *M. sexta* dolichol homologue was always 1 a.m.u. less than the corresponding peak in the standard (except for dolichol 17). For instance, $m/z$ 1245 was the most abundant ion in *M. sexta* dolichol 18, whereas 1246 was most abundant in bovine dolichol 18 (Fig. 5, B1 and B2). Also, *M. sexta* dolichols had at least one significant peak, that was not seen in the standard, but which was 1 a.m.u. less than the peak with the lowest mass unit of the corresponding dolichol standard. For example, $m/z$ 1311 was found in the *M. sexta* dolichol 19 spectrum with about 30% of the intensity of the base peak, $m/z$ 1313 (Fig. 5, C1). No appreciable $m/z$ 1311 was present in the dolichol 19 standard spectrum (Fig. 5, C2). Mass spectral analysis indicated that both of the doublet peaks from the second RPLC run [Fig. 4(B)] represent the same dolichol homologue. Possible explanations for these curious results will be addressed in the "Discussion" section.

The major interfering substances present were hydrocarbons, the majority being quite large, with 60–75 carbons (data not shown). Their presence was clearly identifiable by the characteristics of hydrocarbon mass spectra. They were volatilized from the mass spectral probe tip at up to 300°C, whereas dolichols showed up at about 330°C. Thus, their presence did not interfere with our analyses of dolichols to a significant degree.

*Dolichol homologues in M. sexta tissue and diet*

A comparison of the relative ion abundance indicated that dolichols 17, 18 and 19 are the predominant homologues in *M. sexta* tissue (Table 1). There were no indications of ethyl branch containing dolichols, with usual dolichol molecular mass plus one or two multiples of 14 a.m.u. It is possible that some other homologues could be present but not detectable because of a low amount present, since there are several small peaks on the chromatogram which did not yield any mass spectral information (Fig. 4).

The dolichols found in the artificial diet are shorter than the ones found in *M. sexta*, with a homologue profile ranging from dolichols 13 to 17, with dolichols 14, 15 and 16 as the predominant homologues (Table 2).

## DISCUSSION

The ubiquitous isoprenoid biosynthetic pathway involves the incorporation of three acetate molecules into one mevalonate. The enzyme responsible for the last step in this process is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the point of regulation for

TABLE 1. Relative abundance of major dolichol homologues in *M. sexta* larvae[a,b]

| Isoprene unit number per dolichol molecule | Relative abundance of each homologue to total dolichols (%) |
|---|---|
| 17 | 19 |
| 18 | 39 |
| 19 | 30 |
| 20 | 12 |

[a] Relative abundance was determined by the ratio of the total molecular ion intensity of a particular homologue to the sum of the total molecular ion intensity of all the dolichol homologues.

[b] Dolichols 15 and 16 are not presented here because they were present in a very low amount in our analysis and did not show significant u.v. absorbance on the chromatogram. They were only identified by mass spectra, with very small quantities of molecular ions.

isoprenoid and cholesterol biosynthesis in vertebrate liver (Beytía and Porter, 1976). In Lepidoptera, two acetate molecules and one propionate molecule are involved in the synthesis of homomevalonate, which is a required precursor for the synthesis of ethyl-branched JH. Although the biosynthesis of JH is confined to the corpora allata, the secretion of propionate and other labeled isoleucine metabolites out of cells is found in several other lepidopteran tissues, including fat body and Malpighian tubule (Halarnkar and Schooley, 1995). In that study, four insect species representing four non-lepidopteran insect orders were found to not secrete isoleucine metabolites. Taken together, these results suggest that there could be other ethyl-branched isoprenoid products made in other lepidopteran tissues.

The goal of these experiments was to determine whether ethyl side branches are present in isoprenoids other than JH. Within the limitations of our research methodology, no novel ethyl-branched dolichols were found in larval *M. sexta*. These results do not exclude the possibility that very small quantities of ethyl-branched dolichols were present below our limits of detection.

Insects do not synthesize sterols from small organic precursors, as they lack the enzyme that converts farnesyl pyrophosphate to squalene (Goodfellow *et al.* 1973; Sagami and Lennarz, 1987). They consequently require a dietary supply of sterols. In order to see whether there were any differences between *Manduca* dolichols and the dolichols present in the artificial diet, we also analyzed the dolichol homologue profile of the diet. The *M. sexta* larval dolichol profile is quite different from the dolichol homologue distribution of the artificial diet. *Manduca sexta* larvae contain dolichols 17, 18, 19 and 20 as the major homologues in an approximate 3:7:5:2 ratio, with smaller amounts of dolichols 15 and 16. The ratio of dolichols in *M. sexta* is similar to that found in other insects. *Calliphora erythrocephala* larvae contain predominantly dolichols 18 and 19, with a smaller amount of dolichol 17 (Beedle *et al.*, 1975). Throughout all of the developmental stages of *Drosophila melanogaster*, the major dolichol homologues are dolichols 17 and 18, with a smaller amount of dolichol 16 (Morris and Pullarkat, 1991). In our colony, the major dietary dolichols are dolichols 13–17, with dolichols 14, 15, and 16 as the predominant homologues. This is the same as the dolichol homologue profile of baker's yeast (Dunphy *et al.*, 1967; Tarvares *et al.*, 1977); and yeast is a significant component of our *M. sexta* diet (Yamamoto, 1969). Although insects do not synthesize sterols, they do appear to synthesize their own dolichols. This process could be accomplished by simple elongation of dietary dolichols, or by *de novo* biosynthesis from acetate.

TABLE 2. Relative abundance of major dolichol homologues in *M. sexta* artificial diet[a]

| Isoprene unit number per dolichol molecule | Relative percentage of each homologue to total dolichols (%) |
|---|---|
| 13 | 8 |
| 14 | 24 |
| 15 | 33 |
| 16 | 22 |
| 17 | 13 |

[a] Relative abundance was determined by the ratio of the molecular ion intensity of a particular homologue to the sum of the molecular ion intensity of all the dolichol homologues.

We also studied *in vitro* metabolism of mevalonate by *M. sexta* tissues (fat body and corpora allata). Very little [5-$^3$H]mevalonate seemed to enter the fat body, as evidenced by a lack of detectable non-polar metabolites (data not shown). Following incubation of corpora allata with [5-$^3$H]mevalonate, radio-TLC analysis indicated the sole labeled metabolite corresponded to JH, and no incorporation into a zone corresponding to dolichol was observed. Beedle *et al.* (1975) have shown incorporation of [2-$^{14}$C]mevalonate into ubiquinones, dolichols, and farnesol in axenically grown larvae of *C. erythrocephala*. A number of earlier reports have shown high levels of the mevalonate shunt pathway in insect tissues (Kaplanis *et al.*, 1961; Nes *et al.*, 1982). This pathway yields many labeled products, resulting from degradation of mevalonate to acetoacetyl- and acetyl-CoA, which complicates detection of genuine isoprenoid metabolites.

Silberkang *et al.* (1983) were able to show the utilization of the mevalonate pathway for the synthesis of dolichol, farnesol and ubiquinone by *Drosophila* $K_c$ cells. They also found that homomevalonate was incorporated into $K_c$ cell lipids about one-tenth as efficiently as mevalonate (Watson *et al.*, 1985). This is interesting as *Drosophila* is not able to synthesize ethyl-branched JH (Sliter *et al.*, 1987; Baker, 1990). Incubating the corpora allata of *M. sexta in vitro* with [5-$^3$H]homomevalonate only slightly enhances their production of JH I and JH II (Jennings *et al.*, 1975; Schooley *et al.*, 1976), apparently due to the poor uptake of this intermediate by the corpus allatum. It may be that the result with *Drosophila* $K_c$ cells is due to more efficient transport of homomevalonate into the cells. A rather loose substrate specificity of the enzymes leading to mevalonate/homomevalonate

formation has been demonstrated in insect and rat tissues (Baker and Schooley, 1981). Also, subsequent enzymes of the isoprenoid biosynthesis pathway have been demonstrated to have a low degree of substrate specificity as well (reviewed by Schooley and Baker, 1985). All of these factors suggest that data on incorporation of homomevalonate are not a reliable indicator of the ability of a species to synthesize homoisoprenoids *in vivo*.

Dolichols were not present in *M. sexta* in large quantity. This necessitated the sensitivity of mass spectrometry for detection and identification. We found that fast atom bombardment and electron impact protocols (Hermansson *et al.*, 1992) were inferior to chemical ionization in terms of sensitivity for the production of molecular ions.

It is rather unusual to obtain the protonated molecular ion peak for long-chain alcohols, like dolichols, using CI/MS. Normally, in higher alcohols the protonated molecular ion is formed, which rapidly eliminates $H_2O$ (Harrison, 1983). We did not observe significant $M + H^+$-18 peaks in our spectra, either of the standard or insect samples. With our conditions, little fragmentation occurred, and many low mass ions were obscured by ions generated from hydrocarbon contaminants (*M. sexta* cuticle and fat body are rich in fats). Therefore, we have displayed in Fig. 5 only the molecular ion envelopes with which the reader may discern the pattern of isotopes.

The spectra of dolichol standards indicated the presence of one extra peak not predicted by the theoretical calculated isotope distribution based on a CI proton transfer mechanism; this peak was 1 a.m.u. less than the protonated monoisotopic peak (Fig. 5, A2 and A3, B2 and B3, C2 and C3, D2 and D3). One possible explanation for this observation is the involvement of a charge exchange ionization mechanism (producing $M^+$ ions), in addition to a proton transfer mechanism (producing $MH^+$ ions). Secondly, incomplete proton transfer might have occurred under our experimental conditions. Thirdly, electron impact ionization might be present in addition to CI. If mixed ionization mechanisms are involved, it is unclear why the rest of the spectra matched the theoretical calculation based solely on CI proton transfer mechanism nearly perfectly, except for the extra peak mentioned above. Protonated and non-protonated ions should have similar profiles differing by 1 a.m.u.; however, the summed profiles would not match the results predicted by Isotope$^{tm}$. Regardless of what mechanism really occurred with the standards, similar processes could have also happened in our *M. sexta* dolichol analysis.

Our mass spectra of dolichols isolated from *M. sexta* are similarly intriguing, because the most abundant isotope peak observed in each *M. sexta* dolichol homologue was 1 a.m.u. less than the corresponding peak in the standard, except for dolichol 17. This might be due to the fact that very low amounts of insect dolichols were obtained for CI/MS analysis, while more than 10 times as much (5 $\mu$g) standard was used. The standards and natural dolichols were also run at different times, with possible differences in instrumentation response. Consequently, an exact comparison of their mass spectra profiles was difficult.

It is not clear whether insects have polyprenols in addition to dolichols. The phenomenon of doublet peaks observed in the HPLC chromatogram [Fig. 4(B)] has also been reported in a study of polyisoprenoid alcohols (including polyprenols and dolichols) in seeds (Ravi *et al.*, 1984). Dolichols are present in all eukaryotes, whereas polyprenols are found mainly in bacteria and plant tissues (Hemming, 1992). However, polyprenols have also been found in bovine pituitary gland (Radomińska-Pyrek *et al.*, 1979) and hen oviduct (Hayes and Lucas, 1980). It might be possible that we have isolated a mixture of polyprenols and dolichols from *M. sexta*, and the mass spectra we obtained were the molecular weight information of that mixture instead of dolichols only. Polyprenols have two less protons than the corresponding dolichols, due to their $\alpha$-desaturation. However, *only* additional $M^+$-1 peaks were clearly found in comparing *M. sexta* dolichol spectra with the spectra of the standard (Fig. 5, A1, B1, C1 and D1). We emphasize that the mass spectra of *M. sexta* dolichols did not provide solid evidence for the presence of polyprenols.

Further studies are needed to determine whether other isoprenoid compounds, such as ubiquinones, can contain ethyl-branched isoprene units. Thus, although the ability of the corpus allatum in *M. sexta* to produce ethyl-branched JH structures is definitive, whether it can produce other ethyl-branched homoisoprenoid compounds remains a mystery. There may be a unique ability of the cells of the corpus allatum to supply high levels of propionyl-CoA units to HMG-CoA synthase.

## REFERENCES


Adair W. L. and Keller R. K. (1985) Isolation and assay of dolichol and dolichyl phosphate. *Meth. Enzymol.* **111**, 201–215.

Baker F. C. and Schooley D. A. (1978) Juvenile hormone biosynthesis: identification of 3-hydroxy-3-ethylglutarate and 3-hydroxy-3-methylglutarate in cell-free extracts from *Manduca sexta* incubated with propionyl- and acetyl-CoA. *J. Chem. Soc., Chem. Commun.* 292–293.

Baker F. C. and Schooley D. A. (1981) Biosynthesis of 3-hydroxy-3-methylglutaryl-CoA, 3-hydroxy-3-ethylglutaryl-CoA, mevalonate, and homomevalonate by insect corpus allatum and mammalian hepatic tissues. *Biochim. Biophys. Acta* **664**, 356–372.

Baker F. C., Tsai L. W., Reuter C. C. and Schooley D. A. (1987) *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem.* **17**, 989–996.

Baker F. C. (1990) Techniques for identification and quantification of juvenile hormones and related compounds in arthropods. In *Morphogenetic Hormones of Arthropods* (Edited by Gupta A. P.), pp. 389–453. Rutgers Univ. Press, New Brunswick.

Beedle A. S., Walton M. J. and Goodwin T. W. (1975) Isoprenoid biosynthesis in aseptic larvae of *Calliphora erythrocephala*. *Insect Biochem.* **5**, 465–472.

Bergot B. J., Jamieson G. C., Ratcliff M. A. and Schooley D. A. (1980) JH zero: new naturally occurring insect juvenile hormone from

developing embryos of the tobacco hornworm. *Science* **210,** 336–338.

Beytía E. D. and Porter J. W. (1976) Biochemistry of polyisoprenoid biosynthesis. *A. Rev. Biochem.* **45,** 113–142.

Brindle P. A., Baker F. C., Tsai L. W., Reuter C. C. and Schooley D. A. (1987) Sources of propionate for the biogenesis of ethyl-branched insect juvenile hormones: role of isoleucine and valine. *Proc. Natl Acad. Sci. U.S.A.* **84,** 7906–7910.

Brindle P. A., Baker F. C., Tsai L. W. and Schooley D. A. (1992) Comparative metabolism of isoleucine by corpora allata of non-lepidopteran insects versus lepidopteran insects, in relation to juvenile hormone biosynthesis. *Archs Insect Biochem. Physiol.* **19,** 1–15.

Brindle P. A., Schooley D. A., Tsai L. W. and Baker F. C. (1988) Comparative metabolism of branched-chain amino acids to precursors of juvenile hormone biogenesis in corpora allata of lepidopterous *vs.* nonlepidopterous insects. *J. Biol. Chem.* **263,** 10,653–10,657.

Burgos J., Hemming F. W., Pennock J. F. and Morton R. A. (1963) Dolichol: a naturally-occurring $C_{100}$ isoprenoid alcohol. *Biochem. J.* **88,** 470–482.

Clarke S. (1992) Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *A. Rev. Biochem.* **61,** 355–386.

Crick D. C. and Carroll K. K. (1987) Extraction and quantitation of total cholesterol, dolichol and dolichyl phosphate from mammalian liver. *Lipids* **22,** 1045–1048.

Dunphy P. J., Kerr J. D., Pennock J. F. and Whittle K. J. (1967) The plurality of long chain isoprenoid alcohols (polyprenols) from natural sources. *Biochim. Biophys. Acta* **136,** 136–147.

Ericsson J., Thelin A., Chojnacki T. and Dallner G. (1991) Characterization and distribution of *cis*-prenyl transferase participating in liver microsomal polyisoprenoid biosynthesis. *Eur. J. Biochem.* **202,** 789–796.

Goodfellow R. D., Radtke H. E., Huang Y. S. and Liu G. C. K. (1973) Studies on squalene biosynthesis in *Sarcophaga bullata larvae. Insect Biochem.* **3,** 61–65.

Halarnkar P. P. and Schooley D. A. (1995) A comparative catabolism study of isoleucine by insect and mammalian tissues. *Comp. Biochem. Physiol.* **110b,** 357–365.

Harrison A. G. (1983) *Chemical Ionization Mass Spectrometry*, pp. 97–99. CRC Press, Boca Raton.

Hayes G. R. and Lucas J. J. (1980) A novel N-acetylglucosaminyl polyprene in hen oviduct. *J. Biol. Chem.* **255,** 7536–7539.

Hemming F. W. (1992) Dolichol: a curriculum cognitionis. *Biochem. Cell. Biol.* **70,** 377–381.

Hermansson K., Jansson P., Löw P., Dallner G., Swiezewska E. and Chojnacki T. (1992) Analysis of long-chain polyisoprenoids by fast atom bombardment mass spectrometry. *Biol. Mass Spectrum.* **21,** 548–553.

Jennings R. C., Judy K. J. and Schooley D. A. (1975) Biosynthesis of the homosesquiterpenoid juvenile hormone JH II [methyl (2E,6E,10Z)-10,11-epoxy-3,7,11-trimethyltridecadienoate] from [5-$^3$H]homomevalonate in *Manduca sexta*. *J. Chem. Soc., Chem. Commun.* 21–22.

Kaplanis J. N., Dutky R. C. and Robbins W. E. (1961) The incorporation of 2-$^{14}$C-mevalonate into housefly lipids. *A. Entomol. Soc. Am.* **54,** 114–116.

Kornfeld R. and Kornfeld S. (1985) Assembly of asparagine-linked oligosaccharides. *A. Rev. Biochem.* **54,** 631–664.

Lee E., Schooley D. A., Hall M. S. and Judy K. J. (1978) Juvenile hormone biosynthesis: homomevalonate and mevalonate synthesis by insect corpus allatum enzymes. *J. Chem. Soc., Chem. Commun.* 290–292.

Morris G. N. and Pullarkat R. K. (1987) A micromethod for the estimation of blood dolichol. *Lipids* **22,** 58–60.

Morris G. N. and Pullarkat R. K. (1991) Dolichol levels during development and ageing of *Drosophila melanogaster*. *Comp. Biochem. Physiol.* **98b,** 267–269.

Nes W. D., Campbell B. C., Stafford A. E., Haddon W. f. and Benson M. (1982) Metabolism of mevalonic acid to long chain fatty alcohols in an insect. *Biochem. Biophys. Res. Commun.* **108,** 1258–1263.

Pennock J. F., Hemming F. W. and Morton R. A. (1960) Dolichol: a naturally occurring isoprenoid alcohol. *Nature* **186,** 470–472.

Pennock J. F. and Threlfall D. R. (1983) Biosynthesis of ubiquinone and related compounds. In *Biosynthesis of Isoprenoid Compounds* (Edited by Porter J. W. and Spurgeon S. L.), pp. 191–303. Wiley, New York.

Radomińska-Pyrek A., Chojnacki T. and St. Pyrek J. (1979) Fully unsaturated decaprenol from bovine pituitary glands. *Biochem. Biophys. Res. Commun.* **86,** 395–401.

Ravi K., Rip J. W. and Carroll K. K. (1984) Differences in polyisoprenoid alcohols of mono- and dicotyledonous seeds. *Lipids* **19,** 401–404.

Rees H. H. (1985) Biosynthesis of ecdysone. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by Kerkut G. A. and Gilbert L. I.), Vol. 7, pp. 249–293. Pergamon Press, Oxford.

Sagami H. and Lennarz, W. J. (1987) Glycoprotein synthesis in *Drosophila* $K_c$ cells. *J. Biol. Chem.* **262,** 15,610–15,617.

Schooley D. A. and Baker F. C. (1985) Juvenile hormone biosynthesis. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by Kerkut G. A. and Gilbert L. I.), Vol. 7, pp. 363–389. Pergamon Press, Oxford.

Schooley D. A., Baker F. C., Tsai L. W., Miller C. A. and Jamieson G. C. (1984) Juvenile hormones O, I, and II exist only in lepidoptera. In *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones* (Edited by Hoffmann J. A. and Porchet M.), pp. 373–383. Springer, Heidelberg.

Schooley D. A., Judy K. J., Bergot B. J., Hall M. S. and Jennings R. C. (1976) Determination of the physiological levels of juvenile hormones in several insects and biosynthesis of the carbon skeletons of the juvenile hormones. In *The Juvenile Hormones* (Edited by Gilbert L. I.), pp. 101–117. Plenum Press, New York.

Schooley D. A., Judy K. J., Bergot B. J., Hall M. S. and Siddall J. B. (1973) Biosynthesis of the juvenile hormones of *Manduca sexta*: labeling pattern from mevalonate, propionate, and acetate. *Proc. Natl Acad. Sci. U.S.A.* **70,** 2921–2925.

Silberkang M., Havel C. M., Friend D. S., McCarthy B. J. and Watson J. A. (1983) Isoprene synthesis in isolated embryonic *Drosophila* cells—I. Sterol-deficient eukaryotic cells. *J. Biol. Chem.* **258,** 8503–8511.

Sliter T. J., Sedlak B. J., Baker F. C. and Schooley D. A. (1987) Juvenile hormone in *Drosophila melanogaster*: identification and titer determination during development. *Insect Biochem.* **17,** 161–165.

Tarvares I. A., Johnson N. J. and Hemming F. W. (1977) A sensitive quantitative assay method for dolichols, cholesterol and ubiquinone using high-pressure liquid chromatography. *Biochem. Trans.* **5,** 1771–1773.

Trautmann K. H., Masner P., Schuler A., Suchý M. and Wipf H. K. (1974) Evidence of the juvenile hormone methyl (2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH-3) in insects of four orders. *Z. Naturf.* **29c,** 757–759.

Watson J. A., Havel C. M., Lobos D. V., Baker F. C. and Morrow C. J. (1985) Isoprenoid synthesis in isolated embryonic *Drosophila cells*. *J. Biol. Chem.* **260,** 14,083–14,091.

Yamamoto R. T. (1969) Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *J. Econ. Entomol.* **62,** 1427–1431.

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