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The Structure of Ubiquinones Isolated from Developing Embryos of *Manduca sexta*

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Like many other lepidoptera, *Manduca sexta* synthesizes juvenile hormones (JH) that differ from classic isoprenoids by having one or more ethyl, instead of methyl, branches attached to the hydrocarbon chain. This difference originates from the utilization of homomevalonate in the place of mevalonate for synthesis of the different forms of JH. No other ethyl-branched homoisoprenoid has been found in lepidopteran insects. We reported earlier that only methyl-branched dolichols were detected in *M. sexta* larvae. We have now isolated ubiquinones from the embryos of *M. sexta* and then analyzed them by chemical ionization mass spectrometry. No ubiquinone homologue with an ethyl-branched side chain was detected. The predominant homologue found is ubiquinone Q-9 entirely with methyl-branches, with only a trace amount of ubiquinone Q-10. These results suggest that JH are synthesized by specialized enzymatic mechanisms in the corpora allata, while other major isoprenoids are synthesized by the more common enzymes present in eukaryotic systems. © **1998 Elsevier Science Ltd. All rights reserved**

Isoprenoid Juvenile hormone Ubiquinol Coenzyme Q Mass spectrometry

INTRODUCTION

Ubiquinones are a group of quinone homologues that exist in all eukaryotic organisms, gram-negative bacteria, and some photosynthetic bacteria (Fig. 1); they contain a 2,3-dimethoxy-5-methylbenzoquinone nucleus, with an all-Z geometry polyprenyl side chain in the 6-position. Q-1 through Q-13 (where the number refers to how many isoprene units are contained in the prenyl chain) have been isolated from various organisms. In most organisms one homologue (usually Q-10, Q-9, or Q-8) represents the great majority of forms present (Ramasarma, 1985), although there are exceptions in micro-organisms, which may contain a range of ubiquinones including traces of Q-1 to Q-4 (Daves, *et al*., 1967). Ubiquinones are found in the inner mitochondrial membrane, where they play a crucial role in the respiratory electron transport system. More recently, the reduced form of ubiquinone (ubiquinol) has been implicated as a potential antioxidant for preventing oxidative damage (Beyer, 1992; Ernster and Dallner, 1995).

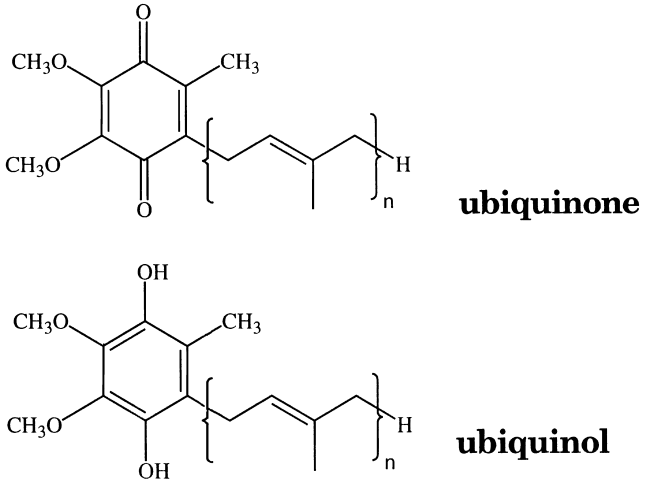

FIGURE 1. The structure of ubiquinones.

Ubiquinones are derived biosynthetically from 4-hydroxybenzoate plus a polyprenyl side chain (Olson and Rudney, 1983). The latter is derived from farnesyl pyrophosphate (FPP) produced *via* the isoprenoid pathway, onto which more isopentenyl pyrophosphate units are added by *trans*-prenyl transferase (Pennock and Threlfall, 1983; Teclebrhan *et al*., 1993). Mevalonate has long been regarded as the sole precursor for a wide range of steroids and other isoprenoids (Beytıa and Porter, 1976), including dolichols, terpenes and carotenoids, as well as farnesyl and geranylgeranyl groups attached to some important cellular proteins (Clarke, 1992). However,

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recently a non-mevalonate pathway to isopentenyl pyrophosphate from glyceraldehyde phosphate has been described in bacteria (Rohmer *et al*., 1993) and chloroplasts of plants (Lichtenthaler *et al*., 1997).

Manduca sexta and other lepidoptera synthesize juvenile hormones (JH) that differ from classic isoprenoids by having one or more ethyl, instead of methyl, branches attached to the hydrocarbon backbone (Schooley *et al*., 1984). The ethyl-branched homologue of isoprene units in these JH (JH 0, JH I, JH II and 4-MeJH I) is derived from homomevalonate, presumably due to lax enzyme specificities (Baker and Schooley, 1981; Jennings *et al*., 1975). Corpus allatum (CA) enzymes of *M. sexta* can condense one propionyl-CoA with two acetyl-CoA to give hydroxyethylglutaryl-CoA (Baker and Schooley, 1978), which is then reduced to homomevalonate (Lee *et al*., 1978). Isoleucine and valine have been found to be the sole metabolic progenitors of propionate and certain other organic acids in *M. sexta* CA (Brindle *et al*., 1987); propionate and other acidic metabolites of Ile and Val are secreted by lepidopteran but not by non-lepidopteran CA (Brindle *et al*., 1992, 1988). The metabolism of [U-^{14}C]isoleucine to propionate and other organic acid metabolites has been demonstrated in other lepidopteran tissues, especially the fat body and Malpighian tubules (Halarnkar and Schooley, 1995). The processes of isoleucine and valine metabolism to propionate, homomevalonate, and eventually ethyl-branched JH appear to be lepidopteran-specific (Schooley *et al*., 1984). However, the fate of these metabolites in tissues other than CA is unclear. Thus it is possible that lepidopterans may be able to metabolize isoleucine and valine *via* propionate to homoisoprenoid structures other than JH. We previously isolated long-chain isoprenoid alcohols, dolichols, from whole bodies of *M. sexta* larvae, and detected no ethyl-branched dolichols (Li *et al*., 1995). Because embryos of *M. sexta* are known to produce a high titer of ethyl-branched JH (JH 0 and 4-MeJH I) (Bergot *et al*., 1980), which are derived entirely from homomevalonate rather than mevalonate, whereas larval JH are derived from about half mevalonate and half homomevalonate (Baker *et al*., 1987), we decided to determine whether embryos make ubiquinones with ethyl-branched side chains.

Based on chemical ionization mass spectrometry analysis of ubiquinones purified by HPLC, we found that predominately Q-9, plus a trace amount of Q-10, is present in *M. sexta* embryos. No ubiquinone with an ethyl-branched side chain was detected, based on mass spectral information.

MATERIALS AND METHODS

Chemicals

All LC grade solvents were purchased from Fisher Scientific. The Q-6 and Q-10 standards were obtained from Sigma Chemical Company.

Insects

The insects were reared as described earlier (Yamamoto, 1969). Eggs from *M. sexta* moths were collected each day, then allowed to mature for two days in the incubator at 27°C (larva typically emerge from eggs after 4 days under these conditions). Embryos were stored at −20°C.

Lipid extraction

M. sexta embryos (200 g) were homogenized in 800 ml chilled H_2O and 1400 ml hexane/ethanol (5/2) with a Polytron homogenizer (Brinkmann Instruments). Solvent mixtures are v/v unless stated otherwise. The organic phase was filtered through a Buchner funnel (Whatman No. 3 filter paper), and dried with a rotary evaporator (Buchi Rotavapor-R). The residues were dissolved in 50 ml hexane. Insoluble material was removed by filtering through glass wool fiber anchored in a glass Pasteur pipette. The lipids were stored at −20°C.

Column chromatography

The column was prepared with a slurry of 300 g of silica gel (Sigma 68–200 μm, 60 Å pores) suspended in 1 l hexane. The solvent was drained to 1 cm above the silica bed before sample was added. The crude lipid extract was placed on top of the silica bed; care was taken not to disturb the bed. The eluent was collected in 30 ml fractions. The following solvents were used sequentially for elution: (1) 2 l hexane; (2) 2 l hexane/ether (H/E,14/1); (3) 2 l H/E (9/1); (4) 2 l H/E (4/1).

Thin layer chromatography (TLC)

Samples collected from column chromatography and later from LC were tested on analytical TLC plates (Analtech, silica gel GF, 250 μm, 10 × 20 cm), developed with H/E (4/1). Aliquots of 20 μl were taken from each fraction for TLC analyses. Unsaturated lipids were visualized by iodine vapor. Column chromatography fractions that produced bands with an R_f value on the TLC separation between the values for ubiquinone 6 and 10 standards were regarded as containing ubiquinones, and were used for further analyses. Fractions from bands with the proper R_f were pooled and dried. The residues were redissolved in 1 ml ethyl acetate for separation by preparative TLC.

Preparative TLC plates (Universal Scientific Inc, silica gel G, 1000 μm, 60 Å pore, 20 × 20 cm) were prewashed with methanol, and allowed to air dry overnight. The lipids were applied onto the plate with a TLC streaker (Kontes) about 1 cm above the bottom of the plate. Twenty μl each of ubiquinone 30 and 50 standards (1.2 μg/μl) were also placed on one end of the plate. The plates were developed in H/E (4/1). After air drying, the plate was covered with aluminum foil, except for the lanes occupied by the standards. The ubiquinone standards were visualized in the iodine chamber, and the silica powder within the unexposed region of the plate only (that between the R_f values of Q-6 and Q-10) was scraped

off with a razor blade and collected in a 20 ml centrifuge tube. The lipids were extracted from the silica gel with 10 ml of absolute ether. After 5 min centrifugation (Dynac BD, 3400 rpm), the supernatants were collected and the residues were re-extracted two times. The combined supernatants were dried and redissolved in 10 ml methanol.

Reversed-phase liquid chromatography (RPLC)

The liquid chromatograph included a Perkin Elmer series 410 LC pump, a Perkin Elmer LC 235 Diode Array detector and a strip chart recorder (Kipp and Zonen BD 41). UV absorbance was monitored at both 275 and 290 nm (the maximum absorbing wavelengths for oxidized and reduced ubiquinones, respectively). The preparative TLC-purified ubiquinones were further purified by two successive RPLC steps. The first system consisted of a Vydac C_{18} column (4.6 × 150 mm), eluted with 100% methanol at 1 ml/min. The second system consisted of an Applied Biosystems Brownlee RP-18, 100 × 4.6 mm, eluted with a solvent gradient from methanol/H_2O (9/1) to 100% methanol in 10 min, and then to methanol/dichloromethane (1/1) in 40 min, and back to 100% methanol in 5 min, at a flow rate of 2 ml/min. The detector was set at 275 nm and 290 nm to monitor oxidized and reduced ubiquinones, respectively. After a confirmatory analysis by TLC, fractions were dried under N_2 and were redissolved in 0.5 ml of pentane for a normal-phase liquid chromatography purification.

Normal-phase liquid chromatography

The liquid chromatograph consisted of a Haskel pneumatic amplifier pump and a Spectra Physics SP8440 UV/Vis detector set at 275 nm. Samples loaded onto a Brownlee silica column (220 × 4.6 mm) were eluted with isocratic pentane/ether (95/5) at a flow rate of 1.5 ml/min. The detector was set at 275 nm to monitor oxidized ubiquinones. UV absorbing fractions were dried and redissolved in 20 μl methylene chloride for mass spectrometric analysis.

Mass spectrometry

Chemical ionization (CI), fast atom bombardment (FAB), and electron impact (EI) ionization mass spectra were obtained on a Finnigan MAT SSQ 710 mass spectrometer. CI with methane as the reagent gas proved the most sensitive method tested for obtaining spectra of ubiquinones. The isolated ubiquinones were deposited onto a solid probe tip, and the solvent was allowed to evaporate. CI reagent gas pressure was about 8000 mTorr inside the ion source. The probe temperature was set from 100 to 350°C with a 200°C/min increment. The scan range was from m/z 100 to 1000. Several scans were averaged to improve the signal-to-noise ratio.

RESULTS

We were able to isolate relatively small amounts (~2.4 to 4 μg) of ubiquinones from 200 g of eggs. They were eluted from the silica column chromatography with hexane/ether (9/1). No other fractions contained a significant amount of ubiquinones, as determined by analytical TLC. The R_f values for ubiquinone 50 and 30 standards were 0.30 and 0.26 on the analytical TLC plate, and was 0.38 and 0.30 on the preparative TLC plate. The silica coating from the preparative TLC plate within the range of R_f 0.3 to 0.4 was scraped off and the lipids eluted with ether.

Two successive RPLC steps and one normal phase LC step were needed to adequately purify ubiquinones for mass spectral analysis. Analyses of samples less vigorously purified suggested the presence of fatty acid derivatives. The retention times for Q-9 and Q-10 were 26.2 and 28.4 min on the second RPLC chromatogram, respectively; the ratio of Q-9 to Q-10 was greater than 99:1, based on the integrated peak areas from the last RPLC. The ubiquinone-containing fractions were vacuum-dried and the residues were redissolved in 0.5 ml of hexane. A final LC purification of Q-9 was performed before mass spectral analysis, giving two major peaks on the chromatogram with retention times of 16.4 and 20.4 min. The peaks contained ubiquinone and ubiquinol, the oxidized and reduced forms, respectively (see below). Curiously, the reduced form (ubiquinol) predominated in this purification. Reanalysis of the purified, oxidized Q-9 with another normal phase separation using the same LC conditions showed the presence of a mixture of similar amounts of reduced and oxidized Q-9, suggesting the occurrence of reduction during analysis or storage. In this context it is interesting to note that in human plasma, a well oxygenated environment, the reduced forms of ubiquinones commonly predominate over the oxidized forms (Finckh *et al*., 1995; Yamamoto and Yamashita, 1997).

Successful identification of Q-9 and Q-10 was accomplished by chemical ionization mass spectrometry (CI/MS), with the observation of *m/z* 794.1 and 796.0 as the M^+ ions of oxidized and reduced Q-9 and 862.0 as the M^+ ion of oxidized Q-10, respectively (Fig. 2). The mass spectra of Q-9 and Q-10 contained strong ions at *m/z* 197 (Fig. 2), which are the characteristic hydroquinone benzelium ions derived from ubiquinones (Daves, *et al*., 1966; Karr *et al*., 1982). However, we did not generally observe a significant *m/z* 235 ion, which is commonly seen in EI spectra of ubiquinones (Daves, *et al*., 1966).

DISCUSSION

We found no evidence of ethyl-branched ubiquinones in embryonic *M. sexta*. Our results are consistent with the available data on ubiquinone homologue distribution in other insect species, including several lepidopteran species (Chefurka, 1965). Q-9 is the only ubiquinone homologue found in adults of *Musca domestica*, *Pieris rapae*, *Sarcophaga bullata* and *Antheraea pernyi* (Goodfellow *et al*., 1972; Lester and Crane, 1959; Szar-

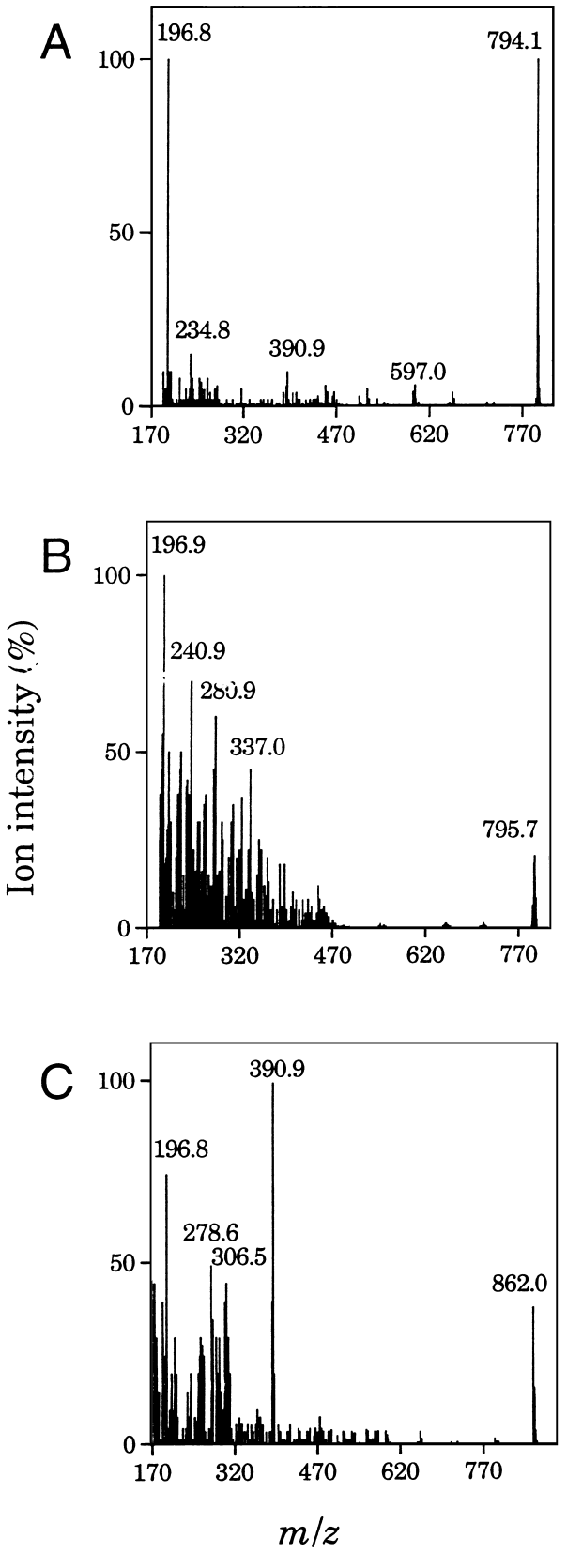

FIGURE 2. Mass spectra of ubiquinones isolated from *M. sexta*. (A) oxidized Q-9; (B) reduced Q-9 and (C) oxidized Q-10

kowska and Michalek, 1960). Q-10 is the only homologue found in pupa of *Celerio euphorbiae, Sphinx pinastri,* and adults of *Anatis ocellata* (Heller *et al*., 1960). Q-8 to Q-10 were identified in larvae of *Calliphora erythrocephala* (Laidman and Morton, 1962). *Drosophila* embryonic cells (K_c) could incorporate [5-^{3}H]mevalonate into Q-9 as determined by radio-TLC (Havel *et al*., 1986). In contrast, Q-10 is the most common homologue found in vertebrates, and Q-6 and Q-7 are the ubiquinones found in yeasts (Olson and Rudney, 1983). None of these species contain ethyl-branched ubiquinones.

In a prior study we found that *M. sexta* larval dolichols are derived only from the five carbon isoprene unit (Li *et al*., 1995), but larval JH are synthesized from similar amounts of isoprenoid and homoisoprenoid units. In contrast, in embryos the JH produced are almost entirely homoisoprenoid in origin (JH 0, 4-MeJH I) (Bergot *et al*., 1980), yet embryonic *M. sexta* ubiquinones are also derived from the five carbon isoprene unit. What specialization allows the CA to biosynthesize JH, while regular isoprenoids are synthesized elsewhere in the insect? Does the embryo itself synthesize its own ubiquinones and other essential isoprenoids, or are they derived from adult female?

As all insect cells should be able to synthesize ubiquinones when cells enlarge or divide, we decided to test the ability of corpora allata to synthesize ubiquinones. In a preliminary experiment, only labeled JHs were unambiguously identified. It is possible that the metabolic pathway in CA can somehow channel most isoprene/homoisoprene units into JHs, but not into other isoprenoids.

In summary, we successfully isolated Q-9 and Q-10 from embryos of *M. sexta*. Q-9 is the predominant homologue. No novel ubiquinones derived from homomevalonate were found with our current methods. Further investigation is needed to determine the regulation of isoprenoid metabolism in lepidoptera insects.

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