More importantly, in terms of the possible mode of substrate-enzyme binding, in folic acid the C(4) oxygen and N(10) hydrogen are on the same side of the pteridine ring, hydrogen-bonded through a water molecule, whereas in the enzyme-inhibitor complex the methotrexate C(4) amino group and N(10) methyl are on opposite sides of the ring. Thus the orientation of the pteridine ring in the methotrexate-enzyme complex differs from the orientation of the same ring in folic acid by an ~180° rotation about the C(6)-C(9) bond. This rotational difference is illustrated in Fig. 4. A number of spectroscopic observations have indicated that there are differences in the way methotrexate and folate bind to DHFR. Matthews *et al.* (*3*) found from model-fitting experiments that the DHFR active site could accommodate a molecule with the orientation of the pteridine ring opposite to that found for methotrexate; strong evidence for such an altered orientation for folate has recently been provided by Charlton *et al.* (*4*). The conformation of folic acid in the crystal adds further support for this concept and provides a model at the atomic resolution level for what that orientation may be.

The intermolecular hydrophobic interaction between the pteridine and phenyl rings in the folic acid crystal structure (Fig. 3) is also suggestive of conformational arrangements that could occur in the enzyme-folate-NADPH complex. The phenyl ring of methotrexate participates in hydrophobic interactions in both DHFR complexes: in *Escherichia coli* with an isoleucine and in the *Lactobacillus casei* complex through a parallel stacking arrangement with the aromatic ring of a phenylalanine. It is likely that the folate *p*-aminobenzoyl ring binds in a similar manner. In the *L. casei* ternary complex the methotrexate pteridine ring is inclined at about 45° to the plane of the NADPH nicotinamide. It may be that folate-DHFR productive binding and reduction of folate by hydride transfer from NADPH in the ternary complex are facilitated by a parallel association of the pteridine and nicotinamide rings similar to the stacking interaction observed in the folic acid crystal.

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JH Zero: New Naturally Occurring Insect Juvenile Hormone from Developing Embryos of the Tobacco Hornworm

Abstract. *A new insect juvenile hormone was isolated from developing embryos of the tobacco hornworm moth,* Manduca sexta. *The new hormone was found with juvenile hormone I and is a 1-carbon homolog of this substance. The assigned structure is methyl (2*E*,6*E*,10-*cis*)-10,11-epoxy-3,7-diethyl-11-methyl-2,6-tridecadienoate, which constitutes a trishomosesquiterpenoid skeleton. This is the first chemical identification of any juvenile hormone from insect eggs.*

Three insect juvenile hormones (JH) have been isolated and characterized from various species representing several insect orders (*1*, *2*). Biosynthetically the most "conventional" (*3*) of the JH's, JH III (structure **4** in Fig. 1) is found in at least one stage of development in nearly all insects surveyed to date (*1*). The homosesquiterpenoid and bishomosesquiterpenoid, JH II (**3**) and JH I (**2**), occur mainly in Lepidoptera, although both have been reported in one species of cockroach (*4*). The latter JH's, arising biosynthetically from propionate and acetate precursors (*3*), are produced by pathways that now appear to be peculiar to insects. Theoretically, organisms that produce JH I could have the biosynthetic capability of elaborating even higher sesquiterpenoid homologs to serve potentially in a hormonal capacity. We now report the identification of JH 0 (**1**), isolated together with its biosynthetic cognate JH I from eggs of the tobacco hornworm *Manduca sexta*. Comparison of mass spectral and chromatographic data from JH 0 and two of its derivatives with synthetic standards confirmed the assignment of structure as a homolog of JH I, bearing an ethyl group at C-3. Approximately 200 ng of natural material was available for all of the structure elucidation work performed.

We recently have studied JH titers throughout the life cycle of *M. sexta* (*5*) using coupled gas chromatography (GC)–mass spectroscopy (MS). By means of a multiple peak-scanning technique, selected ion monitoring, we use the mass spectrometer as a sensitive and selective GC detector, monitoring one (or more) prominent ions produced by electron impact fragmentation of a JH derivative resulting from d_3-methanolysis (**1a** to **4a** in Fig. 1). Such analysis of a purified and appropriately derivatized extract allows quantitative and qualitative analysis of insect JH titers (*6*).

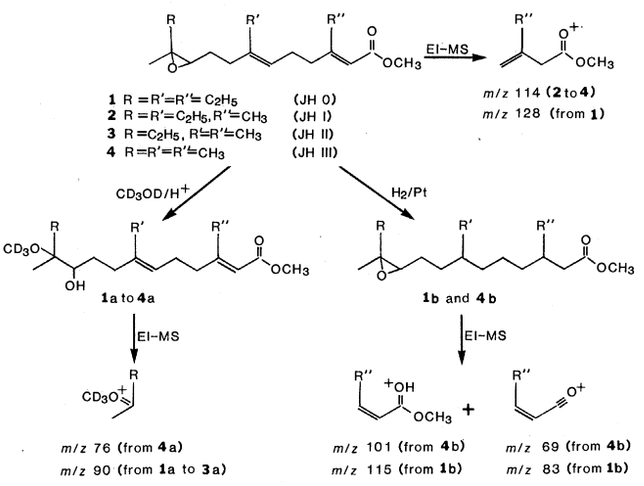

Fig. 1. Important mass spectral fragment ions of the juvenile hormones (**1** to **4**), their methoxy-d_3-hydrin derivatives (**1a** to **4a**), and their tetrahydro derivatives (**1b** and **4b**); *EI*, electron impact.

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During the titer studies, extracts from *Manduca* eggs of various ages were analyzed. When we examined samples of eggs 6 to 29 hours before hatching (48 to 72 hours after oviposition), we noticed—in addition to JH I—a second peak of slightly longer retention time than that of the C_{18} hormone (Fig. 2). Titers of 1.5 to 5 ng/g (fresh weight) for JH I and 2 to 3 ng/g for the unknown were sufficiently high to allow determination of total electron impact and methane chemical ionization mass spectra of the respective d_3-methoxyhydrin derivatives. The mass spectra of the presumed JH I derivative were identical in all respects to synthetic JH I d_3-methoxyhydrin, whereas the unknown gave electron impact and chemical ionization spectra similar to those of JH I with the simple addition of one methylene unit to certain diagnostic ions. Examination of the d_3-methoxyhydrin (**1a**) derived from authentic (*7*) methyl (2*E*,6*E*,10-*cis*)-10,11-epoxy-3,7-diethyl-11-methyl-2,6-tridecadienoate (**1**, JH 0) showed complete identity of electron impact and chemical ionization mass spectra and GC retention behavior with the unknown.

Further data were provided by GC-MS analysis of an egg extract containing JH as the underivatized epoxide (60 to 100 ng), rigorously purified by three sequential liquid chromatography purifications. Identity of retention time and a virtually superimposable electron impact spectrum were found for the unknown when compared with authentic JH 0 standard. Significantly, the appearance of an ion at a mass-to-charge ratio (*m/z*) of 128 verifies that the "extra" methylene unit is located between C-2 and C-4 (Fig. 1); JH I, JH II, and JH III all give a distinctive fragment at *m/z* 114, characterized (*8*) as originating from allylic cleavage between C-4 and C-5 with hydrogen migration. No ion at *m/z* 114 was seen for JH 0. Biosynthetic considerations, furthermore, would suggest either a 3-ethyl or, less likely, a 4-methyl substituent [for example, faranal (*9*)].

In the structure elucidation of cecropia JH, Röller *et al.* (*10*) employed MS analysis of the deoxohexahydro derivative (formed from palladium-catalyzed hydrogenation-hydrogenolysis of JH I) to locate branch points on the chain (Fig. 1). Accordingly, we hydrogenated approximately 100 ng of purified JH 0, isolated from ~ 50 g of eggs, under controlled conditions, using PtO_2 suspended in absolute ethanol. Studies with synthetic JH III showed that under these conditions no hydrogenolysis of the oxirane moiety occurs, and smooth hydrogenation to the tetrahydro derivative (**4b**) is accomplished. The electron impact mass spectrum of the hydrogenated natural material was indistinguishable from that of authentic tetrahydro-JH 0 (**1b**). In particular, the base peak at *m/z* 115 and the corresponding fragment at *m/z* 83 confirm the assignment of the extra methylene group as a 3-ethyl substituent (Fig. 1).

Because there was so little material, we were not able to determine the stereochemistry of natural JH 0 by other spectral means. However, studies by glass capillary GC with flame ionization detection were instructive. We achieved complete baseline resolution of all eight possible geometric isomers (*11*) of JH I (25-m SE-52 column, 85,000 effective plates). In addition, we observed completely coincident retention times of natural and synthetic JH 0, and successfully resolved 2*E*,6*E*,10-*cis*-JH 0 from its synthetic 2*E*,6*E*,10-*trans* isomer (*7*). By extension of the results of separation of all JH I isomers, our capillary column would have resolved the other geometric isomers of JH 0. Taken together these data suggest that the configuration for *Manduca* JH 0 is 2*E*,6*E*,10-*cis*.

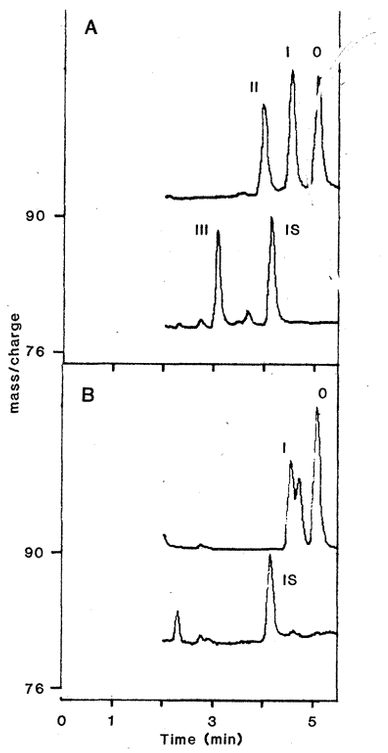

Fig. 2. Selected-ion chromatograms from gas chromatography-mass spectroscopic analysis. (A) JH methoxy-d_3-hydrin reference standards: upper trace, *m/z* 90 (JH II, JH I, and JH 0); lower trace, *m/z* 76 [JH III and IS (internal standard)]. Conditions: 2 m by 2 mm, 3 percent OV-17 on a Carbowax-modified support, programmed from 225° to 260°C at 5°C per minute, helium carrier at 40 ml/min. (B) Purified and derivatized JH extract from day 2 eggs of *M. sexta*. Analytical conditions as above. The peak eluting between JH I and JH 0 is an unknown whose identity is currently under investigation.

A biochemical precedent for the carbon skeleton of JH 0 was established by Koyama *et al.* (*12*), who showed that prenyltransferase (farnesylpyrophosphate synthetase) isolated from pig liver could synthesize trishomofarnesyl pyrophosphate (the presumed biosynthetic precursor of JH 0 in insects) when supplied with the appropriate homoisoprenoid substrates. Moreover, trishomofarnesene (*13*), bishomofarnesene (*14*), homofarnesene (*14*), and faranal (*9*), a bishomosesquiterpenoid, have been reported to be constituents of ant trail pheromones. Together with JH 0, JH I, and JH II, these are the only naturally occurring homoterpenoids documented to date, and it is curious that all appear to have physiological significance as chemical messengers in the Insecta.

The structural analogy of JH 0 to JH I is not sufficient evidence of itself to claim that JH 0 is also a hormone. However, Staal (*15*), using a substitution assay in allatectomized *M. sexta* larvae, compared the biological potency of the then-known natural hormones (JH I, JH II, and JH III) with JH 0 and two other JH analogs. He found that JH I and JH II have quantitatively indistinguishable biological activity, with JH 0 having a similar potency at higher levels, but lower potency at lower levels; JH III was almost 100-fold less active at all levels than JH I, JH II, or JH 0. Thus, JH 0 has high biological activity in larvae of the organism from whose embryos it can be isolated. Moreover, studies of the temporal fluctuation of these hormones in *M. sexta* eggs (*5*) reveal that neither JH I nor JH 0 is present in newly laid eggs, but a high titer of each is observed after 2 to 3 days of development, with a precipitous drop at eclosion (3.5 to 4 days from oviposition). Thus, JH 0 appears to satisfy criteria permitting its classification as a hormone.

This report constitutes the first chemical identification of any juvenile hormone in insect embryos, although the existence of biological activity in egg extracts is well known (*16*) and predates the first published structure of any JH (*10*). The actual role of endogenous JH in the developing embryo remains speculative. Several researchers (*17, 18*) have observed distinct activity periods of differentiated endocrine glands within embryos of several insects. Dorn (*17*) observed a correspondence of the activity

phases of the prothoracic glands and corpora allata to epidermal secretions during embryonic molts in *Oncopeltus*. These activity phases were marked by glandular volume expansion (secretion), followed by contraction (quiescence), phenomena typical of glandular activity in postembryonic development (*18*). Using chromatographic and radioimmunoassay methods, Lageux *et al.* (*19*) detected well-defined peaks of ecdysone titer in *Locusta migratoria* embryos that correlated with cuticular apolysis, an indication of hormonal regulation of embryonic molting. Because of the mutual interaction between prothoracic gland and corpora allata functions during insect larva development (*18*), JH may well assume a parallel indispensability during embryonic ecdysis.

Kaplanis *et al.* (*20*) found that *M. sexta* eggs 0 to 24 hours old were devoid of molting hormone, whereas older eggs yielded relatively large quantities of 26-hydroxy-α-ecdysone. These data and ours reveal an apparent correlation between the appearance of molting hormone and juvenile hormone in *M. sexta* embryos (*5, 20*). The major embryonic molting hormone differs chemically from that found in larvae (*21*) and pupae (*22*); we see an analogous situation with JH, where the major constituents of embryonic JH's (0 and I) are distinct from those of larval (*5*) (JH II, lesser amount of JH I) and adult female (*5*) (JH II and JH III) JH in *M. sexta*. It is noteworthy that JH 0, biochemically the most unusual of the JH's, occurs in *M. sexta* only in the ontogenetically primitive stages.

It appears that embryonic development in insects requires a functioning endocrine system, similar to that demanded for postembryonic development. However, in embryos of *M. sexta*, structural alteration in the respective hormones could imply that biosynthetic enzyme systems of differing precursor specificities are present. Whether the structural differences are in fact obligatory during embryogenesis or simply reflect a fortuitous biosynthetic occurrence has yet to be resolved.

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Swelling of Nerve Fibers Associated with Action Potentials

Abstract. *Swelling of nerve fibers during the action potential was demonstrated by three different methods. Generation of a propagated nerve impulse in a crab nerve produced an outward movement of 50 to 100 angstroms of the nerve surface and a rise in swelling pressure on the order of 5 dynes per square centimeter. In squid giant axons, the amplitude of the observed outward movement of the surface was small.*

We have demonstrated a small movement of the nerve surface associated with the propagation of an action potential. In spite of reports by previous investigators (*1*) on this subject, to our knowledge the existence of such changes has never been substantiated. In particular, there has been no unequivocal demonstration whether it is a swelling or a shrinkage. Obviously, extremely small, transient movements of soft tissues (*2*) have escaped reliable measurements. Using three independent methods, two optical and one mechanoelectrical, however, we have succeeded in obtaining evidence for the occurrence of a rapid and transient swelling of the crab nerve accompanied by excitation. A preliminary experiment on squid giant axons indicated the existence of a similar mechanical change at the surface.

One of the methods used was to modulate the intensity of the light transmitted from a source to a photodetector by the movement of a small object placed on the nerve (Fig. 1A). The light source was a 100-W quartz-iodine lamp (Osram). The light from the lamp was led to the surface of a nerve by means of a bundle of four plastic fiber optics (each 0.12 μm in diameter). In most cases, claw nerves of the crab *Callinectes sapidus* were used. The chamber in which optical measurements were carried out was so designed that a small tension could be applied to the nerve. A small (about 3 mg)

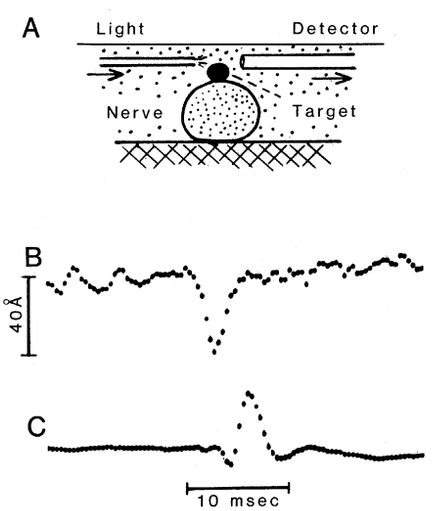

Fig. 1. (A) The method of detecting a small movement of the light-obstructing target on a crab nerve associated with production of action potentials. (B) Transient decrease in the light intensity (an upward movement of the target) produced by nerve stimulation. (C) Extracellularly recorded action potential. Brief stimulating pulses were delivered at the time marked by the beginning of the time marker. The temperature of the preparation was 21°C.

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