ENZYMATIC SYNTHESIS OF INSECT JUVENILE HORMONES[1]

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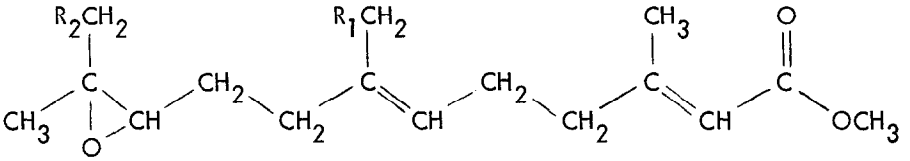
Summary: Cell-free extracts of the corpus allatum-corpus cardiacum complex from the tobacco hornworm, Manduca sexta Johannson, catalyze the formation of labeled juvenile hormones from [methyl-^{3}H]-S-adenosyl-L-methionine. Enzymatic activity is confined to the 12,000 x g supernatant. The radioactive products were identified by their chromatographic properties and chemical derivatizations. Radioactivity was confined exclusively to the methyl ester function.

Insect juvenile hormones, control agents that inhibit development during metamorphosis (1),are synthesized by the corpus allatum (2,3), a small gland behind the brain. The chemical structures of three of these hormones have been determined (3-5). The investigation of their biosynthetic pathway has been stimulated by the possibility that a disruption of the pathway can be used as a method of insect control.



	C_{16}JH	C_{17}JH	C_{18}JH
R_1 =	H	H	CH_3
R_2 =	H	CH_3	CH_3

Methionine was found to be the source of the ester methyl group of JH[3] in adults of the silk moth Hyalophora cecropia (6,7) and in excised corpora allata from adults of the

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[3] Abbreviations: JH: juvenile hormone; GLC: gas-liquid chromatography; TLC: thin layer chromatography; SAM: S-adenosylmethionine.

tobacco hornworm, Manduca sexta (3). Homogenization of the excised glands, however, destroyed their ability to incorporate methionine into JH.

In bacteria, fatty acid methyl esters are formed by an enzymatic alkylation using S-adenosylmethionine (8). If a similar pathway exists in insects, then the failure of homogenized corpora allata to utilize methionine lies in the absence of the methionine activating enzyme in these extracts. We have used gland homogenates to investigate the role of SAM as the methyl donor in JH biosynthesis.

EXPERIMENTAL

Plastic-backed silica gel TLC sheets were the product of Brinkmann Instruments. GLC was performed at 151° with a Hewlett-Packard Model 402 using a 2m x 3mm inner diameter glass column packed with 3% OV-17 on Gas-Chrom Q, 100-200 mesh (Applied Science Laboratories). Half-minute fractions were collected in small Teflon tubes attached to the exit port. The tubes were rinsed with ether and the extract was radioassayed. Collection efficiency was 50%. Radioactivity was measured with a Nuclear Chicago Isocap 300 scintillation counter using a scintillation solution of 4 grams 2,5-diphenyloxazole and 5 mg p-bis-[2-(5-phenyloxazolyl)]-benzene (both scintillation grade from Research Products International) per liter toluene, and counting efficiency was determined by the sample channels ratio method, checked by internal and external standards.

[methyl-^{3}H]-S-adenosyl-L-methionine (4.54. Ci/mmole, 1.10 x 10^{-4}M) was obtained from New England Nuclear. Isomeric unlabeled C_{18}JH (17% active trans,trans,cis)was the gift of Dr. A.J. Manson of Ayerst Laboratories, Montreal. This material gave four major peaks on GLC. Isomerically pure cecropia C_{18}JH and C_{16}JH were purchased from Eco-Control. Each of these gave a single GLC peak. Farnesenic acid-10,11 diol (purity >95% by NMR and GLC) was the gift of Mr. Stephen B. Bowlus and Dr. John Katzenellenbogen of the University of Illinois, and was converted to the methyl ester by treatment with diazomethane in ether. Chemical conversion of JH to the diol, cleavage of the diol, and reduction were done by the method of Judy et.al. (3).

M. sexta eggs were the gift of Dr. R.A. Bell. Larvae were reared on an artificial diet (9), as modified by R.A. Bell, at 27° with a 15-hour photoperiod. Using the technique of Judy et.al. (3), the corpus allatum-corpus cardiacum complex was dissected

from female adults 0- to 3-days after emergence, separated from most of the aorta, and rinsed in saline. We used the entire gland complex rather than the corpus allatum alone because it is more convenient to isolate and because the corpus cardiacum has been shown (2,3) to have no effect on JH synthesis in vitro.

Incubations: Initially, we used Grace's medium, which includes 5% (v/v) heat-treated insect hemolymph (10). Five µl of [methyl-^{3}H]-SAM (0.55 nmole, 5.5×10^6 DPM) was transferred to the mortar of a glass microhomogenizer made by the method of Hall et.al. (11). Five µl of 0.1 M phosphate buffer, pH 7.5, was added to neutralize the acid in which the SAM was supplied. The liquid was evaporated under a stream of nitrogen and 10 µl of medium was added. The saline-washed glands were added to the mortar and ground to a fine suspension. After incubation at 25° in a sterile desiccator saturated with water vapor, a 1 µl aliquot was withdrawn and applied to a TLC sheet. Carrier JH was added, and after development in hexane/ethyl acetate, 7/3, iodine vapor was used to locate the position of the hormone. The sheet was cut into centimeter pieces and each segment was radioassayed.

Following publication of the conditions used by Judy et.al. (3) with intact glands, we substituted 1% (w/v) bovine serum albumin for insect hemolymph in the medium, and the excised glands were washed twice in medium saturated with phenylthiourea, then once in medium, before transfer to the mortar. After 12-17 hours' incubation with labeled SAM, as in the first method, the entire mixture was applied to a TLC sheet. The mortar was rinsed with benzene, and the rinse was applied to the same spot. Unlabeled JH was applied in an adjacent channel and the sheet was developed in anhydrous ethyl ether. The hormone region was eluted with one ml of methanol, giving quantitative recovery of JH. The methanol was evaporated and the labeled product was stored in benzene at -20°.

Centrifugal Fractionation: Ten µl of homogenate, prepared by the second method but without SAM, was placed in a 0.3 ml Pierce Reacti-Vial. The vial was inserted into a Sorvall rubber adaptor and the suspension was centrifuged at 12,000 x g for 5 minutes. The supernatant was drawn off with a small capillary tube and the pellet was washed three times and resuspended in medium. Supernatant and pellet were each incubated with the usual amount of labeled SAM, then extracted and assayed by the second method.

TABLE 1

Incorporation of Labeled Methyl Group of S-adenosylmethionine into Juvenile Hormones[a]

	No. of Complexes	Hours of Incubation	TLC:DPM in JH zone[b]	GLC:[c] %C_{16}	%C_{17}	%C_{18}
First Incubation Method						
No glands + SAM	---	40	0	--	--	--
Whole glands + [methyl-^{3}H]-L-methionine[d]	2	64	1130	--	--	--
Whole glands + SAM	20	24	0	--	--	--
Ground glands + SAM:						
(exp.1)	5	18	14410	--	--	--
(exp.2)	5	19	9760	--	--	--
(exp.3)	5	18	13400	33	5	11
Ground glands + SAM (exp.4)	3	0	0	--	--	--
		16	3550	--	--	--
		40	7700	--	--	--
Second Incubation Method						
Ground glands + SAM	3	12	47600	--	--	--
Ground glands + SAM	7	17	65400	33	8	11
Supernatant + SAM	3	12	33600	12.5	8.5	32.5
Pellet + SAM	3	12	0	--	--	--

[a] Incubations contained, except where noted, homogenates of corpora allata-corpora cardiaca complexes from 0- to 3-day old adult female Manduca sexta; 5.5×10^{-5} M [methyl-^{3}H]-S-adenosyl-L-methionine (5.5×10^6 DPM); in 10 µl Grace's medium containing 30 µg/ml penicillin-G, 100 µg/ml streptomycin sulfate, and either 4.8% (v/v) heat-treated hemolymph from chilled diapausing Antheraea polyphemus pupae (first method) or 1% (w/v) bovine serum albumin Fraction V (second method).

[b] In method 1, 1 µl of the 10 µl incubation was spotted on TLC; total DPM are the DPM found x 10. In method 2, total incubation mixture was spotted and DPM determined directly.

[c] Figures given are the per cent of total recovered radioactivity eluted with the respective JH peaks.

[d] 3.4×10^{-4} M methionine, final specific activity 294 mCi/mmole.

RESULTS AND DISCUSSION (TABLE 1)

Using our first technique we repeated the finding (3) that intact glands incorporate the methyl group of methionine into compounds that migrate with JH on TLC. When we substituted SAM for methionine no incorporation occurred. Homogenized glands, however, incorporated labeled SAM at a much greater rate (1-1.4 pmole in 18 hours) than did intact glands using methionine (0.03 pmole in 64 hours). Incorporation was linear with time for 40 hours. This rate was even greater with the second method (phenylthiourea and bovine serum albumin); homogenates of three gland complexes incorporated, in 12 hours, 3-5 pmole (0.6 to 0.9% of added substrate) into material in the JH zone of TLC.

A large amount of radioactivity was located at the origin of the TLC sheet, where SAM remains, and smaller amounts were uniformly distributed between the origin and the JH zone, but none migrated further than JH. A possible precursor of JH, methyl farnesenate, was not significantly labeled. This compound was shown to have a R_f on TLC greater than that of JH (0.72 vs 0.65 with hexane/ethyl acetate, 7/3). This fact, together with the evidence from GLC (see below), demonstrates that, if methyl farnesenate and its C_{17} and C_{18} homologues are intermediates in JH biosynthesis, their formation cannot be rate-limiting.

The major products of the incubation were shown to be authentic hormones by chemical conversion to derivatives (3). The labeled products extracted from TLC were mixed with 150 µg of carrier C_{18} JH (Ayerst). After conversion to the diol with perchloric acid, the products were separated by TLC in hexane/ethyl acetate, 7/3. The major TLC band corresponding to authentic diol ($R_f = 0.22$) contained 50% of the radioactivity. This figure accords well with the results obtained by GLC (see below). The labeled diol was eluted and treated with periodate, and the resulting aldehyde was reduced to the alcohol with sodium borohydride. The radioactive product cochromatographed with the converted carrier on TLC ($R_f = 0.29$), with recovery of 83% of the radioactivity originally present in the diol. Upon transesterification of the labeled JH with sodium ethoxide, all the radioactivity was found in the methanol derived from the methyl ester function, as had been found with JH produced by intact glands using methionine (3); none was recovered in the nonvolatile residue.

Several TLC extracts, mixed with C_{16} and C_{18} JH carriers, were separately analyzed by GLC. In each case about 50% of the recovered label originally present in the TLC extract cochromatographed with the JH peaks (C_{17} JH was assumed to fall between the C_{16} and C_{18} peaks). In addition to C_{16} JH (the major product) and C_{17} JH, both of which are produced by the intact glands of M. sexta (3), GLC indicated that C_{18} JH was also labeled by these homogenates. One other major peak, with a retention time less than that of the JH's but much greater than that of methyl farnesenate, contained 13-35% of the recovered label. The identity of this compound has not yet been determined.

Centrifugation of the homogenate produced a clear supernatant that contained all the enzymatic activity. Again about 50% of the labeled product of this incubation co-chromatographed with the hormones on GLC. The major product of the supernatant, however, was C_{18} JH.

Although the presence of C_{16} JH in Manduca hemolymph has been verified, the search for C_{17} and C_{18} JH was inconclusive (3). Our results indicate that all three of the hormones thus far identified from several insects are produced by M. sexta gland homogenates, but in variable ratios. This variation may reflect differences in the availability of endogenous precursors.

The experiments reported here show that SAM is an intermediate in the transfer of a methyl group from methionine to JH. An enzyme from the corpus allatum can use SAM for the alkylation of a JH precursor to form the methyl ester function.

ACKNOWLEDGEMENT

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perature circulator (Model FT) with control accuracy of ± 0.002°C. A Beckman thermometer and a calibrated thermometer (accuracy ± 0.05°C) were used to measure the temperature of the bath. In the data reported here, a heating rate of 6-8°C hour and natural cooling rate of about 5° per hour were employed.

RESULTS AND DISCUSSION

In the presence of excess water, the lipid bilayers formed from DMPC, DPPC and DSPC, undergo transitions from the "gel phase" to the lamellar smectic liquid crystal phase. Transition temperatures determined using calorimetry are 23.70, 41.75 and 54.24, respectively.[7,8] Figure 1A shows typical current-voltage curves of a DPPC membrane at three different temperatures. Figures 1B and 1C show the temperature dependencies of our U-tube impregnated with DPPC and DSPC. There are distinct breaks in slope at the transition temperature of these lipids. Transition temperatures determined in this fashion for DMPC, DPPC and DSPC are 23.6, 41.5 and 54.2, respectively. The observed conductivities of the U-tube set-up are consistent with the idea that all, or much of the current passes through the bilayers. However, accurate determination of transition temperatures could nevertheless be possible even if much of the current did not pass through the bilayers, since the geometry of the lipid-glass matrix might change at the transition temperature.

Figure 2A shows that there are two discernable breaks in the temperature dependence of the electrical conductivity of a 50:50 mixture of DMPC and DPPC. The two temperatures 33.7 and 28.7 correspond closely to temperatures 33.9 and 28.8 known from spin-label studies to represent the onset and completion of lateral phase separations in this mixture of lipids.[9]

Figure 2B and 2C show the conductivities of DMPC-DPPC and DPPC-DSPC mixtures, this time in the presence of valinomycin. Again, breaks in slope of conductivity vs. temperature are seen at temperatures corresponding to the beginning and end of lateral phase separations, but there is no marked effect of valinomycin on the size of these rather small changes.

Figure 3 gives the results of three experiments involving pure DPPC in the presence of valinomycin. Here there are marked enhancements in