STUDIES OF PREVENTION OF METAMORPHOSIS BY JUVENILE HORMONE IN ONCOPELTUS FASCIATUS (DALLAS)

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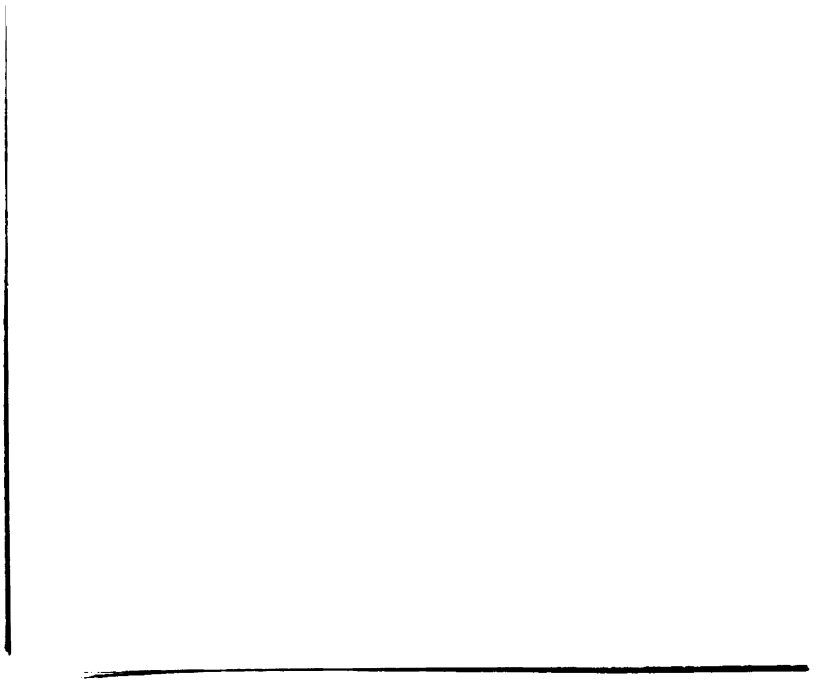

ABSTRACT

STUDIES OF PREVENTION OF METAMORPHOSIS

BY JUVENILE HORMONE IN ONCOPELTUS FASCIATUS (DALLAS)

By

Thomas M. Brown

Application of insect juvenile hormone or its analogues to the newly-ecdysed last-instar larva prevents metamorphosis and causes a supernumerary larva in species of Hemiptera. This phenomenon was investigated in O.fasciatus with regard to the effect on lipid metabolism and to the possible interaction of the moulting hormone, ecdysterone. Topical application of deuterated cecropia juvenile hormone, injection of glycerol-U-^{14}C and lipid analysis by column and thin layer chromatography and liquid scintillation spectrometry indicated depression of neutral lipid synthesis and of phospholipid turnover 115 hours after treatment. Topically applied ethyl trimethyl dodecadienoate produced juvenile hormone effects but was not synergized by simultaneous ecdysterone injection as reported in Tenebrio molitor.

STUDIES OF PREVENTION OF METAMORPHOSIS BY JUVENILE HORMONE IN ONCOPELTUS FASCIATUS (DALLAS)

By

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Submitted to

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PART I

EFFECT OF JUVENILE HORMONE ON THE INCORPORATION OF GLYCEROL-U-^{14}C INTO LIPIDS OF *ONCOPELTUS FASCIATUS* (DALLAS)

INTRODUCTION

Insect juvenile hormone has been known to be essential for oögenesis in many adult female insects since the pioneering studies of Wigglesworth (1936). More recent work with female cockroaches has shown that juvenile hormone exerts a control over lipid synthesis, turnover and transport in connection with egg production (Vroman, Kaplanis and Robbins, 1965; Gilbert, 1967).

The lack of juvenile hormone is essential for metamorphosis of an insect to the imago (Schneiderman and Gilbert, 1964). Stephen and Gilbert (1970) have demonstrated an inverse correlation of juvenile hormone titre and lipid synthesis in the metamorphosis of the cecropia silkmoth, Hyalophora cecropia. Morohoshi and Kiguchi (1969) also showed that juvenile hormone promotes lipid consumption in the developing silkworms, Bombyx mori.

The application of an appropriately timed dose of juvenile hormone to the milkweed bug, Oncopeltus fasciatus, inhibits metamorphosis and promotes a supernumerary larval instar. This study was initiated to determine the effect of a morphogenetically active dose of synthetic C-18 cecropia juvenile hormone on the lipid dynamics of the milkweek bug fifth-instar larva.

MATERIALS AND METHODS

Experimental Animals

The large milkweed bug, Oncopeltus fasciatus (Dallas) was reared in glass jars on milkweed seed. Water was provided in cotton-plugged vials. The culture was maintained at 28±1° C. and 50 percent R.H. on a 14:10 (day-night) photoperiod. This insect was chosen for its definitive response to juvenile hormone analogues (Bowers, 1968; Brown and Monroe, 1972), its large size, and its ability to withstand injection with no adverse effects.

Experimental Chemicals

The synthetic juvenile hormone (SJH) used in these studies was methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyl-trideca-2,6-dienoate as prepared by Bieber, Sweeley, Faulkner and Petersen (1972) and was a gift of Dr. Charles C. Sweeley, Michigan State University, East Lansing, Michigan.

Since it was imperative than an effective but sublethal dose of SJH be employed, bioassay by topical application in acetone to newly-ecdysed fifth-instar larvae was completed and scored according to a simplified system (Brieger, 1971).

Glycerol-U-^{14}C was obtained from Amersham/Searle Corp., Arlington Heights, Illinois in ampules of 50 µCi and the radiochemical purity was found to be 98 percent by thin layer chromatography. Solutions of SJH in acetone and glycerol-U-^{14}C in water were prepared immediately before treatment. All solvents used in this study were glass distilled.

Experimental Design

Eggs less than one day old were collected and reared until ecdysis to the fifth instar was observed. Fifth-instar larvae less than 13 hours old were selected for uniform size and 15 bugs were placed in each of 10 jars or treatment groups.

Treatment order was randomized and each group of 15 larvae received topical application of either 1.0 µg SJH in acetone or acetone as a control. Bugs were then given food and water, aged for five time intervals, and injected with .05 µCi glycerol-U-^{14}C in 1 µl water. Bugs were anesthesized on ice for 2-4 minutes prior to injection with a 30 gauge needle inserted through the intersegmental membrane between the 7th and 8th abdominal segments. Seven hours after injection, the larvae were cold anesthesized, weighed, and frozen under nitrogen at -32° C. until analysis.

Topical applications and injections were done with a calibrated microapplicator (Biotronics, Brookings, South

Dakota) fitted with a 250 µl Hamilton syringe. The above procedures were completed in triplicate.

Analytical Techniques

The larvae were homogenized in water, refluxed for 90 minutes in acetone-ethanol, 1:1 (v/v) at four times the aqueous volume, and vacuum filtered (Kaplanis, Robbins and Tabor, 1960). Residues were dried, weighed and an aliquot combusted to $^{14}CO_2$ and water in a Nuclear Chicago combustion apparatus. The $^{14}CO_2$ was trapped in 10 ml monoethanolamine-methyl cellosolve, 1:2 (v/v), and radioassayed with a Nuclear Chicago Unilux I (model 6850) liquid scintillation spectrometer. A toluene-methyl cellosolve fluor mixture was used in all radioassays which were done in triplicate.

Lipids were extracted three times with ethyl ether, backwashed with water four times, dried over sodium sulfate and radioassayed. Lipids were added in chloroform to a 1:1 x 6 cm Unisil 200-235 mesh adsorption column (Clarkson Chemical Co., Williamsport, Pa.) packed in chloroform. Neutral lipids were eluted with 100 ml chloroform and then 100 ml methanol eluted the polar lipids. Neutral lipids were radioassayed and duplicate aliquots dried by vacuum oven and weighed. Polar lipids were radioassayed and phosphate determined in duplicate by the method of Bartlett (1959).

Thin layer chromatography (TLC) was done on 6.5 x 20 cm glass plates coated with Silica Gel G (Brinkmann

Instruments, Inc., Westbury, N. Y.). Neutral lipid classes were separated by developing with hexane-ethyl ether-glacial acetic acid, 70:30:1, (v/v/v). Plates were visualized with iodine vapor and zones were marked as in Figure 1. After the iodine had dissapated, zones were scraped, sonicated for 30 seconds in the scintillation mixture, and radioassayed.

Polar lipid classes were separated by developing with chloroform-methal-water, 65:35:5 (v/v/v). Zones were visualized (as in Figure 1), scraped, and radioassayed as above. Zones of phospholipid were determined from Rf standards provided by Dr. Loran L. Bieber and by spraying sample plates with ninhydrin reagent to detect amines and Dragendorf's reagent to detect quaternary amines.

Figure 1. Zonal separation of lipids by thin layer chromatography on Silica Gel G.
a. Polar lipids as developed in chloroform-methanol-water, 65:35:5 (v/v/v).
b. Neutral lipids as developed in hexane-ethyl ether-glacial acetic acid, 70:30:1 (v/v/v).
PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; LPE, lysophosphatidyl ethanolamine; SP, sphingomyelin; LPC, lysophosphatidyl choline; SE, sterol esters; TG, triglyceride; FA, free fatty acids; DG, diglyceride; FS, free sterol; F, solvent front; U, unknown.

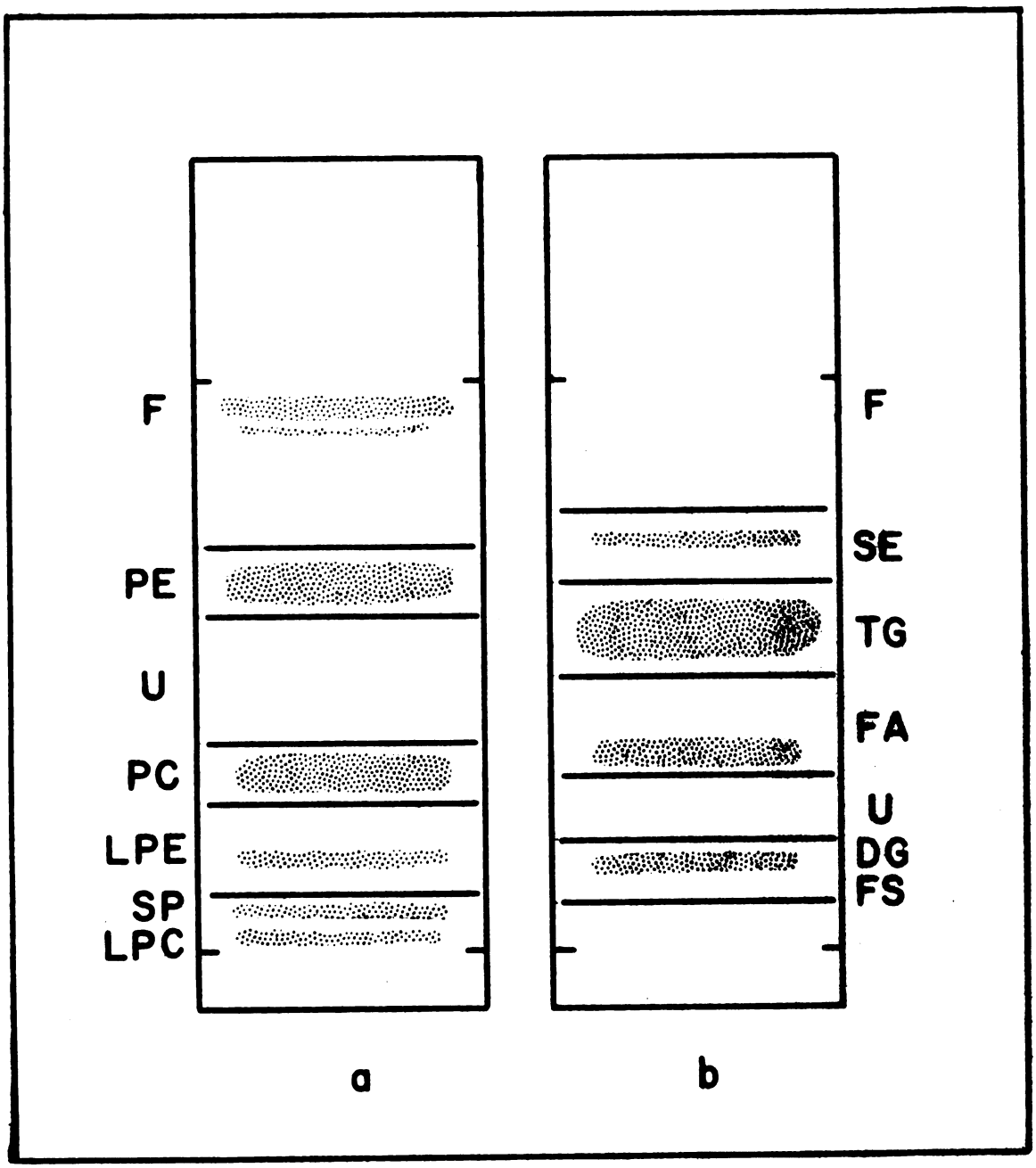

Figure 1

RESULTS

Figure 2 described the morphogenetic effects of SJH on fifth-instar milkweed bugs. From these data the 1 µg per bug dose was chosen as the standard for investigation of effects on lipids. This dose was about 51 percent effective while causing no pre-ecdysis mortality.

Table I consists of growth data for control and SJH-treated larvae during the first five days of the fifth instar. Weights were taken after the 7 hours of exposure to injected glycerol-U-^{14}C. The lean dry weight was the weight of the air dried residue from which total lipids and aqueous had been extracted. The mean lean dry weight ranged from 12.6 percent to 16.0 percent of the mean dry weight. SJH had little effect on weight through the first 4 days, but the fifth day showed a noticeable depression in both wet and lean dry weight in the SJH-treated larvae.

Table II gives recovery of the injected radioactivity in lipid aqueous and residue. The lipids showed no SJH effect except for the depression in the 120 hour group. Age had little effect on lipid recovery except for the 24 hour group which was significantly low. Aqueous recovery demonstrated a continuous increase over the first

Figure 2. Bioassay of methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyltrideca-2,6-dienoate (SJH) in milkweed bug fifth-instar larvae. Scored as 1, adult with curled wings; 2, larval-adult intermediate; 3, perfect supernumerary larva. Each point represents the mean score of 25 insects.

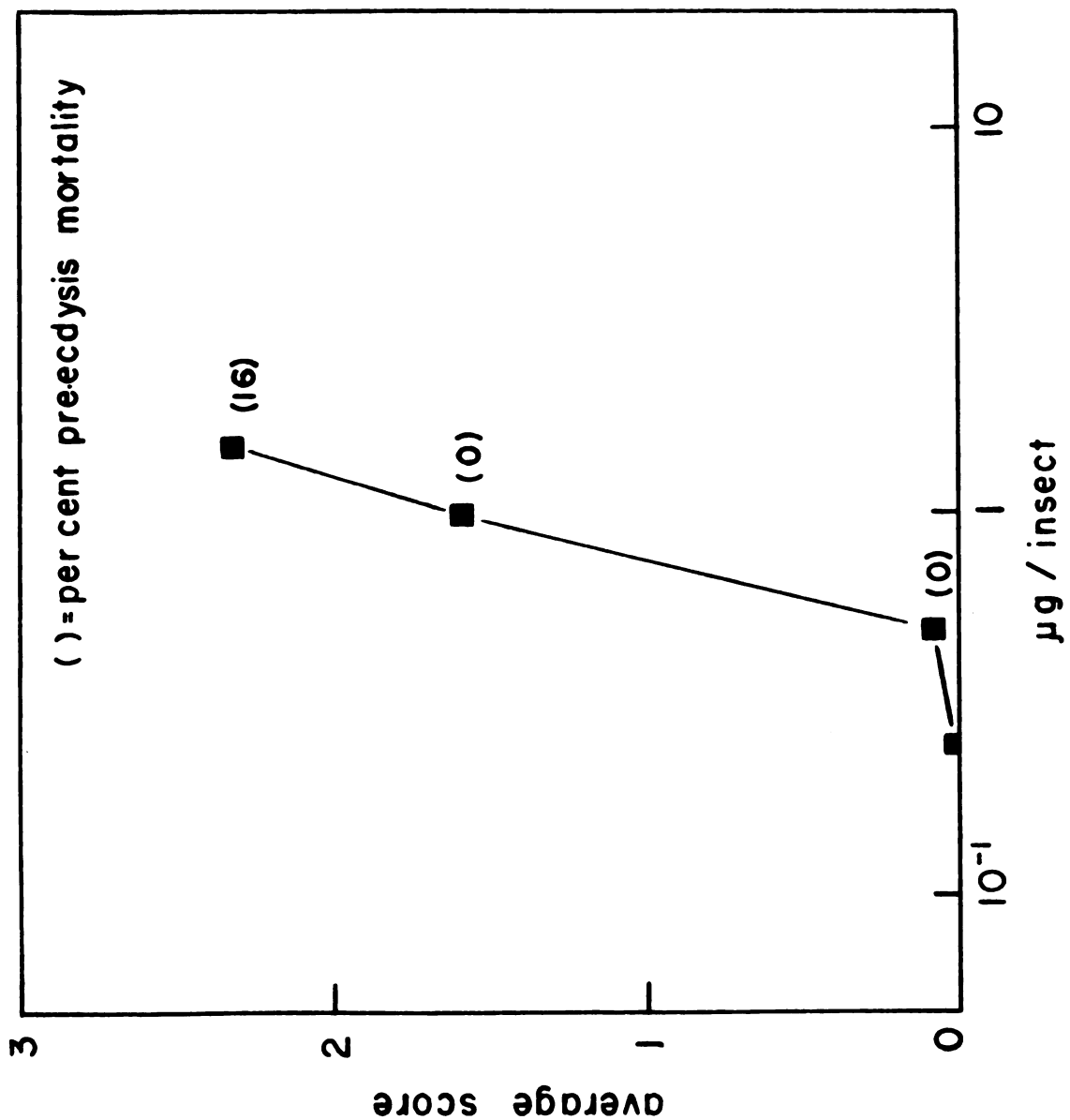
( ) = per cent pre-ecdysis mortality
(16)
(0)
(0)
average score
0
1
2
3
10^{-1}
1
10
µg / insect

Figure 2

Table I. Wet Weight and Lean Dry Weight of Milkweed Bug Fifth-instar Larvae after Synthetic Juvenile Hormone (SJH)* Treatment and Injection of Glycerol-U-^{14}C.

Age Injected† (hours)	Wet weight 15 larvae (mg)		Lean dry weight 15 larvae (mg)	
	Control	SJH	Control	SJH
24	453±13	446±20	61± 2	56± 8
48	686±40	650±34	113± 5	103± 2
72	867±46	875±27	135± 5	140± 9
96	997±15	993±30	148± 4	149±16
120	1012±65	934±40	150±16	139±12

*Topically treated with methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyltrideca-2,6-dienoate.

†Age after ecdysis to fifth-instar.

Table II. Recovery of Injected Radioactivity* in the Lipids, Aqueous, and Residue of Milkweed Bug Fifth-instar Larvae Treated with Synthetic Juvenile Hormone (SJH).†

Age Injected‡ (hours)	Percentage of Injected					
	Lipids		Aqueous		Residue	
	Control	SJH	Control	SJH	Control	SJH
24	13.7±0.5**	13.9±1.6	17.6±3.1	17.3±2.6	20.1±6.2	23.1±4.2
48	24.4±1.1	25.1±2.4	20.2±2.1	21.3±1.2	18.5±2.4	17.7±2.0
72	22.5±3.6	24.2±1.8	28.6±0.7	30.1±2.4	15.5±2.2	13.2±1.8
96	26.7±2.6	25.5±5.8	36.5±3.6	38.5±2.0	15.3±1.9	16.4±2.2
120	25.3±2.7	19.2±2.3	36.2±4.6	34.4±3.6	18.1±2.1	18.4±4.2

*Recovery after 7 hours of exposure to glycerol-U-^{14}C.

†Topically treated with methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyltrideca-2,6-dienoate.

‡Age after ecdysis to fifth-instar.

**Mean of 3 observations (15 larvae per observation) ± standard deviation.

4 days but showed no effect of SJH. Recovery in residue was strikingly highest in the 24 hour larvae, declined through the 72 hour group and increased to the 120 hour larvae. SJH did not influence this pattern in the residue.

In Table III is represented the ng-equivalents of glycerol-U-^{14}C (1 ng = 470.4 dpm) in the respective areas from TLC separation of neutral lipids. Other than the general depression of incorporation by SJH in 120 hour bugs, the hormone treatment showed no consistent effect. Triglyceride incorporated the greatest part of the label going from about 78 percent of the neutral lipid on the first day to about 82 percent on day 2, to 84 percent on day 3 and declining to about 70 percent on days 4 and 5.

The incorporation of label into the diglyceride and sterol area showed a striking increase in the 96 hour group and became about 20 percent of the neutral lipid radioactivity on day 5.

Free fatty acids were extremely variable in incorporation of glycerol-U-^{14}C while sterol esters showed a pattern of incorporation which became about 5 percent of the neutral lipid labelled on day 2 and declined to less than 1 percent on day 5. The remaining radio-label in the neutral lipids is represented in the origin, front and unknown (the area between the free fatty acids and the diglycerides).

The variability of the fatty acid component might have been due to the number of metabolic steps involved

Table III. Incorporation* of Injected Glycerol-U-^{14}C into Neutral Lipids of Milkweed Bug Fifth-instar Larvae Treated with Synthetic Juvenile Hormone (SJH).†

Age Injected ‡ (hours)	Total ng-equivalents in 15 larvae									
	Triglyceride		Diglyceride + Sterol		Free fatty acids		Sterol esters		Origin + front + unknown	
	Control	SJH	Control	SJH	Control	SJH	Control	SJH	Control	SJH
24	344± 28**	340± 67	30.6±11.3	32.4± 7.0	19.3± 6.2	22.8±10.6	20.8±5.9	18.7± 8.0	26.4± 9.6	26.9± 7.0
48	643± 68	653± 44	41.5±10.0	43.0±17.4	35.9±29.9	38.4±25.2	42.9±9.7	34.1±13.1	23.2± 7.7	40.6±28.5
72	574± 65	603± 45	40.8±10.6	46.3±18.6	27.0±14.2	27.0±14.3	29.4±3.8	30.3± 8.5	20.1±11.8	15.5± 9.2
96	543±112	534±119	142.1±15.5	121.6±58.7	42.3±37.0	33.8±38.6	17.9±7.9	11.2± 2.3	34.2±31.9	29.4±25.4
120	486±171	355± 28	144.9±24.9	102.2±26.8	31.5±20.2	24.6±21.3	3.7±3.3	3.8± 0.3	31.3±28.6	23.6±26.7

*Incorporation after 7 hours of exposure.

†Topically treated with methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyltrideca-2,6-dienoate.

‡Age after ecdysis to fifth-instar

**Mean of 3 observations ± standard deviation.

from glycerol-U-^{14}C to fatty acid via acetate as compared to glycerol-U-^{14}C to glycerides via α-glycerol phosphate. Saponification of a neutral lipid sample resulted in recovery of 82.8 percent of the radioactivity in glycerol, 9.9 percent in fatty acid, and 7.3 percent as unsaponifiable lipid.

Table IV represents the radioactivity recovered in the various polar lipid components separated by TLC. SJH had no significant effect on absolute incorporation of label into phospholipids. A continuous geometric increase was observed over the initial 4 days, but there was at most a slight gain from day 4 to day 5.

Figure 3 depicts changes in neutral lipid weight through the period studied. Both control and hormone curves did plateau at day 3 but the treated bugs contained less neutral lipid throughout the 5 days. While weight of neutral lipid rapidly increased to day 3, specific activity declined as shown in Figure 4. This probably reflects both decreased synthesis and the increased glycerol pool due to feeding. After day 3, specific activity of the treated bugs declines more rapidly than the control larvae. The separation of specific activities on day 5 indicates a depression of neutral lipid synthesis as also seen in Table III.

Figure 5 describes the amount of lipid phosphorous in the polar lipid fraction. Except for the final day, the hormone again showed a general depression of growth

Table IV. Incorporation* of Injected Glycerol-U-^{14}C into Phospholipids of Milkweed Bug Fifth-instar Larvae Treated With Synthetic Juvenile Hormone (SJH).†

Age Injected ‡ (hours)	ng-equivalents in 15 larvae									
	Phosphatidyl choline		Phosphatidyl ethanolamine		Lysophosphatidyl choline + sphingomyelin		Lysophosphatidyl ethanolamine		Front + 2 unknowns	
	Control	SJH	Control	SJH	Control	SJH	Control	SJH	Control	SJH
24	5.5±1.1**	4.2±0.6	10.5±3.5	7.2± 1.8	8.2± 1.5	7.8± 2.2	5.7± 4.5	5.2±2.7	11.1±8.5	9.6±4.9
48	10.8±3.6	8.7±1.7	15.1±4.8	10.4± 2.9	21.0±10.0	18.0± 7.3	6.1± 5.1	7.6±5.1	11.7±4.5	7.7±1.5
72	18.4±2.6	22.1±9.7	19.1±2.4	23.9± 9.9	34.6±14.4	46.3±21.7	9.4± 3.3	8.9±4.0	8.6±3.0	11.5±6.4
96	34.6±4.9	35.6±7.0	38.3±0.9	38.2±12.4	48.3± 6.1	39.4±15.3	13.2±10.0	13.3±8.4	23.6±9.9	19.5±9.7
120	40.7±4.3	36.6±6.4	49.2±5.7	44.4± 8.7	47.3±23.6	36.8±22.8	11.9± 5.8	11.3±6.2	19.8±9.2	16.4±6.3

*Incorporation after 7 hours of exposure.

†Topically treated with methyl-$\underline{d}_3$-10,11-epoxy-7-ethyl-3,11-dimethyltrideca-2,6-dienoate.

‡Age after ecdysis to fifth-instar.

**Mean of 3 observations ± standard deviation.

Figure 3. Effect of Synthetic Juvenile Hormone (SJH) on the weight of neutral lipid in the milkweed bug fifth-instar larvae. (Each point represents the total weight in 15 larvae. SJH applied at 12 hours.)

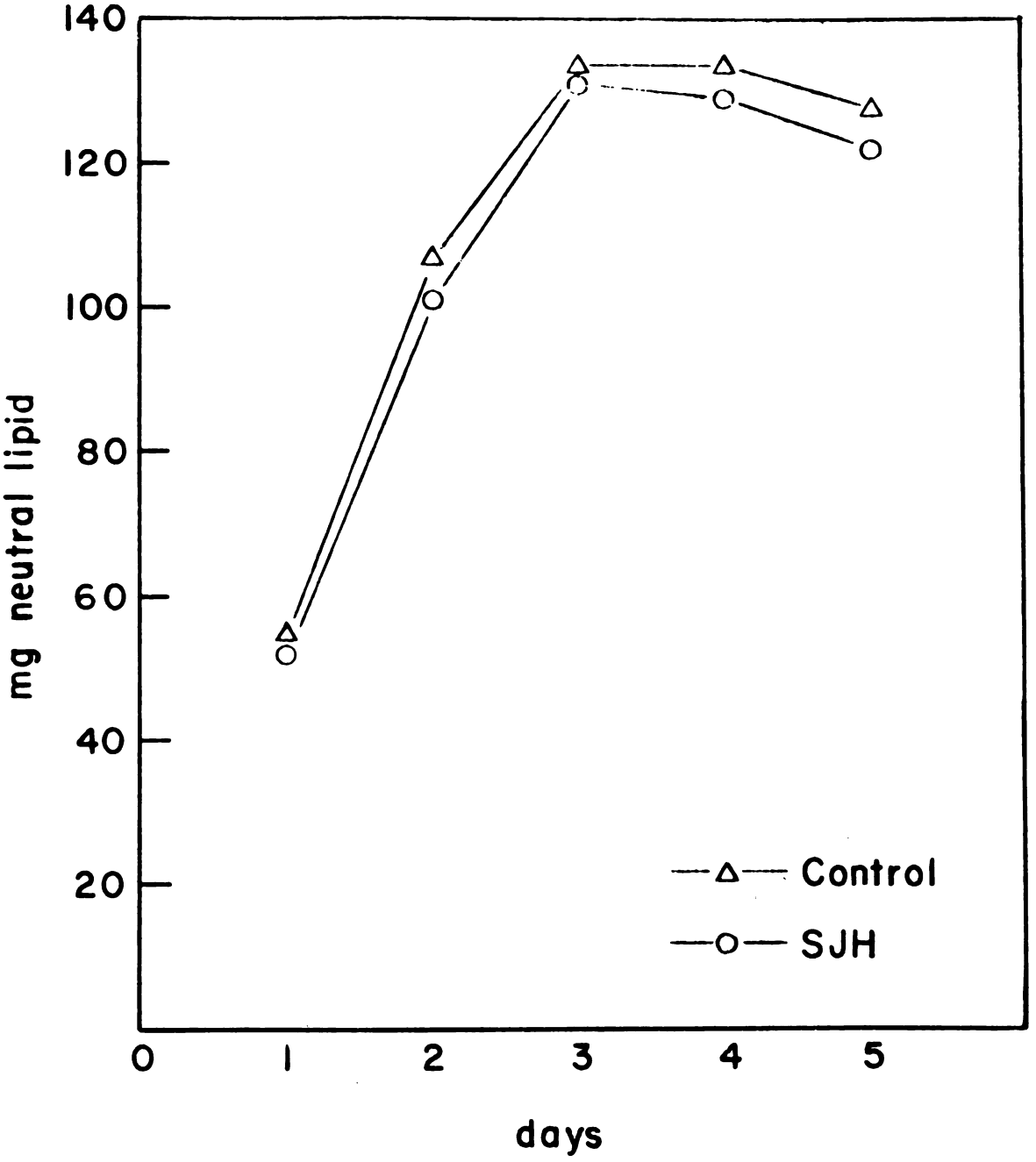
140
120
100
80
60
40
20
mg neutral lipid
0
1
2
3
4
5
days
Control
SJH

Figure 3

Figure 4. Effect of Synthetic Juvenile Hormone (SJH) on the specific activity of the neutral lipids of milkweed bug fifth-instar larvae. (SJH applied at 12 hours.)

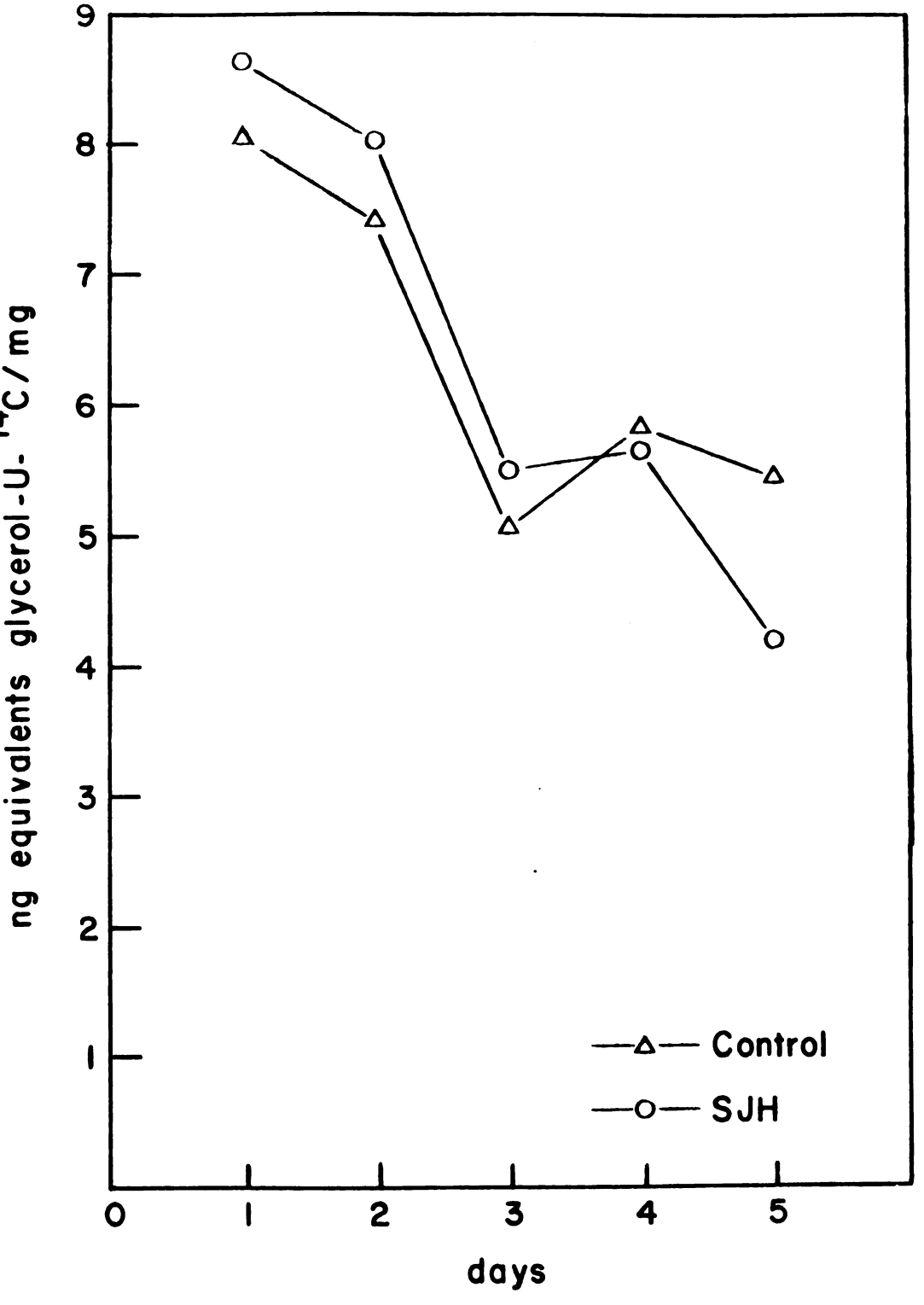
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
days
ng equivalents glycerol-U-^{14}C/mg
Control
SJH

Figure 4

Figure 5. Effect of Synthetic Juvenile Hormone (SJH) on the polar lipid phosphorus of milkweed bug fifth-instar larvae. (Each point represents the total in 15 larvae. SJH applied at 12 hours.)

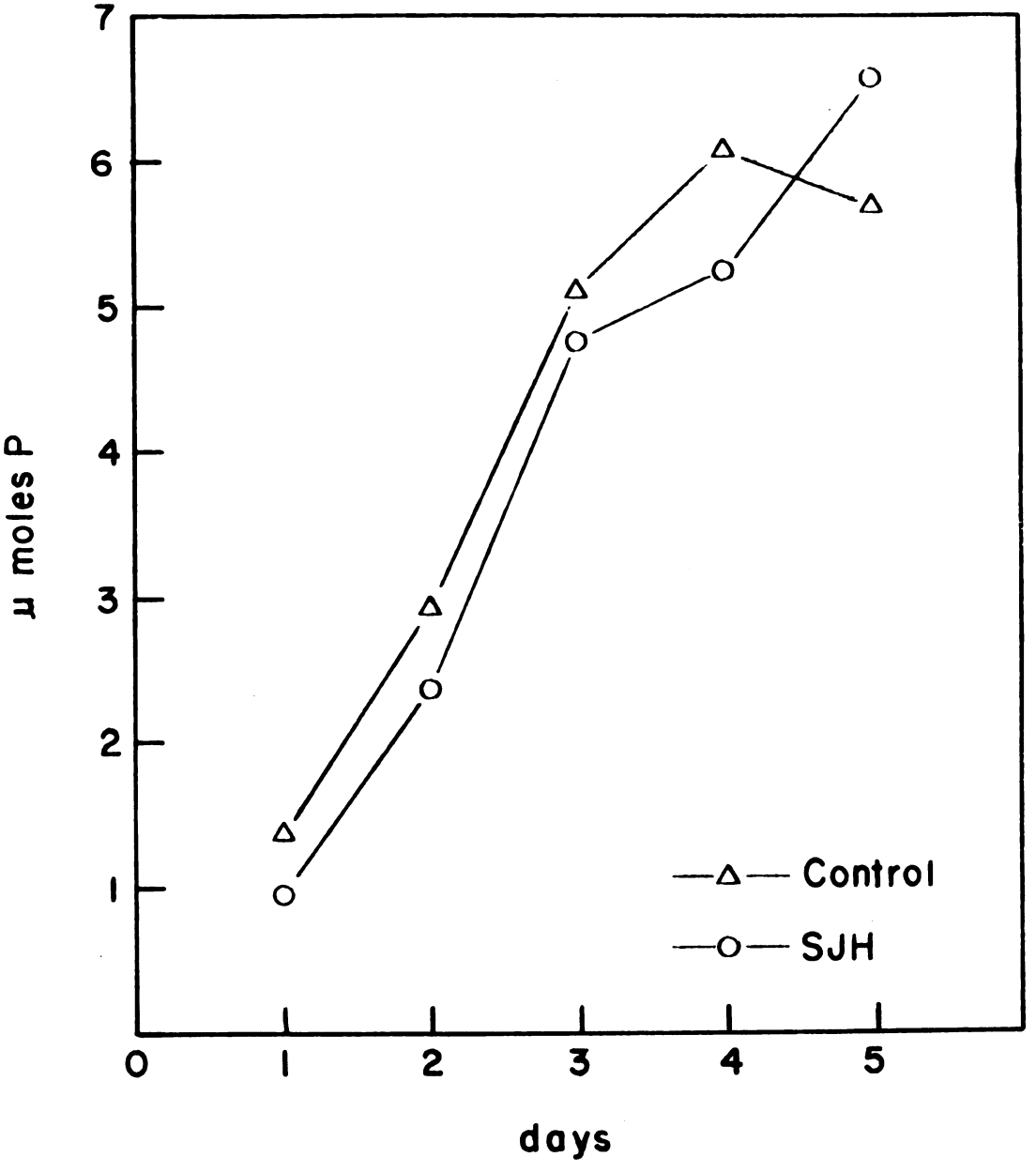

Figure 5

from the control. Figure 6 shows that specific activity of the polar lipids followed a pattern similar to the neutral lipids. The sharp rise in the control specific activity from day 3 compared to the small increase in lipid phosphorous may indicate turnover in the phospholipids at that time. It is interesting that the SJH-treated bugs showed nearly the opposite pattern.

There was no statistically significant SJH effect found when the data of Figures 3, 4, 5 and 6 were examined by analysis of variance.

Figure 6. Effect of Synthetic Juvenile Hormone (SJH) on the specific activity of the polar lipids of milkweed bug fifth-instar larvae. (SJH applied at 12 hours.)

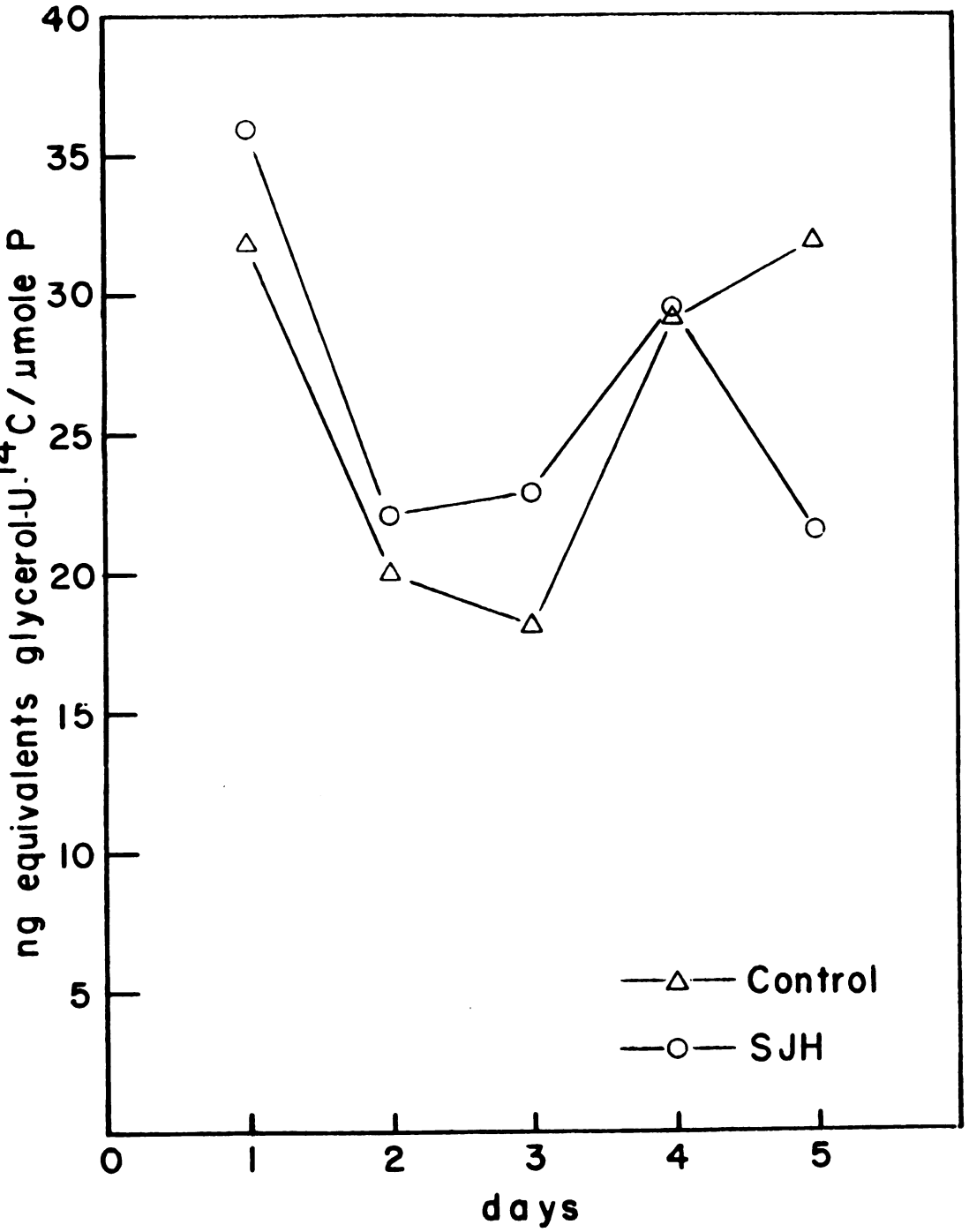
40
35
30
25
20
15
10
5
0
1
2
3
4
5
days
ng equivalents glycerol-U.^{14}C / µmole P
Control
SJH

Figure 6

DISCUSSION

For the purpose of this study, it was desirous to employ the actual juvenile hormone rather than an analogue. After unsuccessful attempts to purchase the C-18 cecropia juvenile hormone, the deuterated compound was accepted as a substitute. The bioassay data (Figure 2) were comparable to those for the C-18 compound in O. fasciatus (Bagley and Bauernfeind, 1972).

The experiment was terminated on day 5 because SJH-treated larvae began ecdysis shortly thereafter. The normal length of the fifth instar was 140 hours.

The 1.5 µg dose of SJH (as well as higher doses of analogues) produced some larvae which failed to feed or grow at all. This may have been a toxic effect of hormone imbalance. It was avoided in this study by using the 1 µg dose as seen in the weight data for days 3 and 4 which showed no weight loss in SJH treated bugs. The weight loss in day 5 SJH larvae reflected a delayed response to the hormone rather than general physiological toxicity.

The recovery of glycerol-U-^{14}C through the instar followed different patterns in lipids, aqueous and residue (Table II). The fact that residue incorporated the most label in the 24 hour group was probably due to cuticle

synthesis which proceeds at a rapid rate at that time. Endocuticlar chitin might have incorporated label from glucose-U-^{14}C via gluconeogenesis to N-acety-D-glucosamine.

The rise in recovery in the aqueous was probably due to accumulation of unconverted glycerol-U-^{14}C and labelled carbohydrates and amino acids. Pyrrochoris apterus, another seed-feeding Hemipteran with a 7 day fifth instar, was shown to have a steadily declining respiratory rate through the 5th day (Novak and Slama, 1962). The low respiratory rate and the cessation of feeding after 96 hours as observed in O. fasciatus by Beck, Edwards, and Medler (1958) were characteristic of the metamorphosing Hemipterans during the last part of the fifth instar. This would account for accumulation of injected glycerol-U-^{14}C due to decreased catabolism.

The previous studies of lipid metabolism in O. fasciatus did not include the dynamics of the fifth-instar larva. Kinsella (1966) compared the lipid composition of the egg, the nymph, and the adult by appearance of thin layer chromatograms, but quantitated only the relative proportion of fatty acids by gas-liquid chromatography. Yurkiewicz and Whelchel (1968) quantitatively compared the various lipid classes of the normal adult with a melamic mutant. The relative proportions of neutral lipids which they found compare very well to the data obtained here. However, they reported lesser proportions of lysophosphatidyl choline + sphingomyelin and lysophosphatidyl ethanolamine by

phosphorus analysis than were indicated by the radioactivity incorporated in this study.

The dramatic increase in label of the diglyceride + sterol fraction was attributable to diglyceride mobilization for energy utilization in metamorphosis as was also observed with acetate-1-^{14}C incorporation in Heliothis zea (Lambremont and Graves, 1968). Sterol biosynthesis has not been observed in insects (Robbins et al., 1971).

The effect of SJH on the neutral lipid was to increase specific activity during the rapid growth phase and decrease specific activity during metamorphosis when neutral lipid weight was declining (Figures 3 and 4). This effect was also noted in phospholipid dynamics (Figures 5 and 6) where the specific activity followed a U-shaped curve in the control. While the turnover of phospholipid during metamorphosis was indicated in the control, SJH inhibited this turnover which might indicate an inhibition of differentiation.

Bassi and Feir (1971 a,b) found that an injected JH analogue caused increased synthesis of haemolymph acid phosphatase during the last half of the fifth instar. They speculated that this phosphatase might have been involved with phospholipids and membrane permeability. The possibility that phospholipid synthesis for differentiating membranes is inhibited by juvenile hormone through the action of a phosphatase arises as a question to be investigated.

SUMMARY

1. Synthetic C-18 cecropia juvenile hormone (SJH) was bioassayed in the milkweed bug and 1 µg per bug was found to be an effective but sublethal dose.

2. Fifth-instar larvae less than 12 hours old were treated with 1 µg SJH and injected with glycerol-U-^{14}C each day through day 5. Incorporation of label into lipid components was compared to control larvae.

3. SJH had no significant effect on larval weight or on absolute incorporation of label into lipids through day 4. Larval weight and incorporation of label into di- and triglycerides were depressed by SJH on day 5.

4. Incorporation of label into triglycerides declined after day 2, while labelled diglyceride increased three fold on day 4 in both control and treated larvae.

5. Phosphatidyl choline and phosphatidyl ethanolamine incorporated label geometrically through day 4 in both control and treated larvae.

6. Specific activities of the neutral lipid and polar lipid were dynamic and are discussed with relation to differentiation although there were no statistically significant differences due to SJH treatment.

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PART II

EFFECT OF ECDYSTERONE ON ETHYL TRIMETHYL DODECADIENOATE ACTION IN *ONCOPELTUS FASCIATUS* (DALLAS)

INTRODUCTION

Several studies have been made on synergists of juvenile hormone activity in various insects (Corey, et al., 1971; Cruickshank and Palmere, 1971; Wheeler and Thebault, 1971; Redfern et al., 1972). Reddy and Krishnakumaran (1972) found a high synergistic effect of ecdysterone (the moulting hormone) on several juvenile hormone analogues in Tenebrio molitor pupae. Of the analogues tested the greatest degree of synergism was obtained with ethyl trimethyl dodecadienoate which was synergized 14-fold by ecdysterone. This study was initiated to determine whether ecdysterone could synergize ethyl trimethyl dodecadienoate when administered jointly to large milkweed bug larvae.

MATERIALS AND METHODS

Experimental Insects

The insect used in this study was the large milkweed bug, Oncopeltus fasciatus (Dallas). Previous experiments have shown it to be useful in assaying juvenile hormone activity (Bowers, 1968). The insects were reared on milkweed seed sterilized at 15 lb pressure for 20 minutes. Eggs less than 24 hours old were collected for all experiments and, after surface sterilization in 0.1% hypochlorite solution (Chlorox$^{(R)}$) for 15 minutes, were placed in 1 qt rearing jars with sterilized seed and water (this procedure prevented bugs from becoming diseased while in culture). The culture was maintained at 29°C and the first fifth instar larvae produced were removed and discarded. Twenty-four hours later the newly moulted fifth instar larvae were collected, giving a culture of relatively uniform age.

Experimental Chemicals

The juvenile hormone analogue used was ethyl-3,7,11-trimethyl-2,4-dodecadienoate (ETD) as reported by Staal et al. (1971), and was purified from an experimental emulsifiable concentrate obtained from Zoecon Corp., Palo Alto, Calif. The compound was purified by a distilled water-hexane partition followed by adsorption liquid

chromatography employing a 1 x 5 cm colum of 5 g of 200-235 mesh Unisil silicic acid (Clarkson Chemical Co., Inc., Williamsport, Pa.). The column was eluted with 100 ml fractions of ethyl ether, benzene and toluene with the ETD eluting in the latter fraction. The ETD thus obtained was then tested for purity using thin layer chromatography, gas-liquid chromatography (Research Specialties Corp. 600 series), and gas chromatography-mass spectrometer combination. Results indicated the presence of two geometric isomers composed of 90+% of the total mixture. Mass spectral analysis gave a parent molecular ion at m/e 266. A base peak at m/e 142 and a prominent peak at P-45 (loss of $CH_3CH_2O\cdot$) were consistent with theoretical fragmentation patterns.

Ecdysterone (β-ecdysone) was purchased from Mann Research Laboratories, N. Y. Two types of solvents were used for injection of the ecdysterone: peanut oil or insect Ringer solution (Ephrussi and Beadle, 1936) containing 10% ethanol. All solutions were freshly prepared prior to treatment.

Treatment Techniques

All treatments were made using a calibrated micro-applicator (Biotronics, Brookings, South Dakota) with a 250 µl Hamilton syringe fitted with a 30 gauge needle. The milkweed bug fifth-instar larvae were anesthetized prior to treatment by placing them on ice for 2-4 minutes.

Injections were made using 2 and 3 µg ecdysterone in 1 µl peanut oil. A 10% Chlorox(R) solution was utilized to sterilize the needle after every injection to prevent infection. After injections were completed, 1.6 µg ETD was applied topically in 1 µl acetone to the abdomen of the milkweed bug fifth instar larvae. This concentration which was intermediate in juvenile hormone action (little activity to production of perfect supernumerary larvae) was purposely chosen so that any synergistic effect from ecdysterone could be easily determined. Control insects were injected with insect Ringer solution with 10% ethanol and a topical application of ETD, injected with an ecdysterone solution and a topical application of acetone, or a topical application of acetone. Each test group consisted of 20 insects.

The test insects were evaluated after ecdysis according to a scoring system (presented in Results) based on retention of larval characteristics.

RESULTS

Table V summarizes the results of these tests. ETD and ecdysterone-treated insects showed no significant increase in juvenile hormone activity when compared to insects treated with ETD only. In fact, those insects treated with ETD alone showed a higher score than those treated with both compounds or with ecdysterone alone. Juvenile hormone-like activity of the ecdysterone alone was very low in these studies. The most definitive indicator of juvenile hormone activity, the supernumerary (sixth-instar) larva, was produced in the control (1.6 µg ETD), but was not produced in any other treatment. The solvent used with ecdysterone or ETD appeared to have no effect on juvenile hormone activity.

Table V. Injection of Ecdysterone, Followed by Topical Application of 1.6 µg Ethyl Trimethyl Dodecadienoate (ETD) on Milkweed Bug Fifth-Instar Larvae.

Test Group	No. of tests	Average* score
Control (acetone)†	3	0
Control (1.6 µg ETD)‡	5	2.2
Control (1 µg ecdysterone in Ringer solution)	1	0.5
Control (3 µg ecdysterone in Ringer solution)	1	0.5
Control (4 µg ecdysterone in Ringer solution)	1	1.0
ETD + 2 µg ecdysterone in Ringer solution	3	1.7
ETD + 3 µg ecdysterone in Ringer solution	3	1.6
ETD + 1 µg ecdysterone in peanut oil	1	1.8
ETD + 1.5 µg ecdysterone in peanut oil	1	1.3
ETD + 2 µg ecdysterone in peanut oil	1	1.6
ETD + 3 µg ecdysterone in peanut oil	1	1.7

*Scoring was based on the following: 0, normal adults and fifth-instar larvae; 1, abnormal adults with normal wing length, black scutellum and larval abdominal cuticle, or fifth-instar larvae dead in preparation for ecdysis; 2, black scutellum, shortened curled wings, incomplete ecdysis, wing pad rupture often present; 3, yellow scutellum, short curled wings, incomplete ecdysis; 4, yellow scutellum, wings intermediate length with curled ends; and 5, perfect supernumerary larvae. ETD treatment at 1.6 µg produced 5% supernumerary larvae.

†All test solutions were 1 µl total volume.

‡Purposely chosen concentration so that synergistic effects could be more easily determined.

DISCUSSION

The dosage of ethyl trimethyl dodecadienoate (ETD) used in these studies was established at 1.6 µg per test insect in order to allow for higher scores if synergism occurred. In preliminary tests, this dosage produced 5% supernumerary larvae. A higher dose tested, 3.2 µg ETD, gave 10-15% supernumerary larvae and a mean score of 2.9, while 6.4 µg ETD produced 20-30% supernumerary larvae and a mean score of 3.8. Very little response to ETD was observed at the level of 0.4 µg per insect, with a mean score of 0.1 for this concentration.

In other preliminary tests, ecdysterone was found to be highly toxic at dosages above 4 µg per insect and elicited a very low response when treated below 1 µg per insect. Schneiderman (1972) also noted the toxicity of ecdysones to the cynthia silkworm.

Utilization of a different carrier solvent such as peanut oil rather than insect Ringer solution gave no significant change in the scores. It was felt that perhaps the peanut oil would give a different rate of release for the ecdysterone, and therefore, an increase or decrease in any synergistic effect upon ETD action. Whether or not such a change in the release rate for ecdysterone

occurred is not known, although the data tends to indicate that if it did occur there was no effect, especially no synergistic effect.

The results indicate that ecdysterone does not act as a synergist of ETD juvenile hormone activity in the large milkweed bug. In all instances the score of the ETD control was higher than the groups where joint treatments of ecdysterone and ETD were conducted. This may indicate an inhibition of juvenile hormone activity by the ecdysterone. Congote (1969) found that a simultaneous treatment of juvenile hormone and ecdysone in a blowfly resulted in an inhibition of RNA synthesis in fat body nuclei normally present when either juvenile hormone or ecdysone were introduced. Patel and Madhaven (1969) observed that an application of ecdysterone and juvenile hormone did not trigger any RNA or protein in the ricini silkworm. This antagonistic action of ecdysterone and juvenile hormone might be reflected in the slight inhibition observed in these studies.

A possible explanation for the lack of any synergistic effect could be that this occurrence is specific for only certain insects. Thus the synergistic effect that Reddy and Krishnakumaran (1972) found with T. molitor may be order specific or even species specific and would not be demonstrated in O. fasciatus. The degree of specificity of this effect can be determined only by assay of these compounds in insects representing other orders.

Mechanism involved in either synergism or inhibition of juvenile hormone analogues have not been defined, but possibilities have been discussed. The role of the mixed-function oxidase system of the microsomes was discussed by Reddy and Krishnakumaran (1972). This enzyme system was found to be stimulated by ecdysterone and by Law's mixture (a juvenile hormone analogue) in a strain of Musca domestica in studies by Yu and Terriere (1971). In recent work with ETD, Terriere and Yu (1973) found that in M. domestica microsomal preparations, B-esterase was more responsible for metabolism than were the microsomal oxidases. However, ETD metabolism by microsomal oxidases was induced to higher levels with juvenile hormone, phenobarbital, and dieldrin, while B-esterase metabolism was not. This induction is similar to the induction of mammalian liver microsomal oxidases by drugs and steroids (Orrenius et al., 1968). Since ecdysterone is a steroid, it is possible that it would act to induce microsomal oxidases to metabolize the ETD and inhibit its effect. Although this deduction would explain our results with O. fasciatus, it does not agree with the synergistic effects obtained in T. molitor by Reddy and Krishnakumaran (1972).

Further studies must be done in vivo and in vitro to to determine the nature of the interaction of ETD and ecdysterone.

SUMMARY

Ethyl trimethyl dodecadienoate exhibited a strong juvenile hormone action in large milkweed bug larvae, Oncopeltus fasciatus, while ecdysterone caused minimal juvenilizing effects. Topically applied ethyl trimethyl dodecadienoate was not synergized by simultaneously injected ecdysterone.

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APPENDIX

LITERATURE REVIEW

The need for a safe and specific insecticide has stimulated research in insect juvenile hormones. A tremendous potential has been forecast for the use of juvenile hormone analogues to upset the delicate balance of the insect hormonal system controlling the insect pest while having no effect on non-target organisms.

The majority of research to this time has centered on the production of juvenilizing effects in many different insects by a myriad of synthetic compounds (see reviews below). More work must now be directed in determining the precise biochemical action of these compounds as well as the mode of action, and biodeactivation of the natural juvenile hormone.

Comprehensive reviews of the history of juvenile hormone research have been published. The biology of the hormone and its function in relation to other insect endocrines was extensively discussed by Schneiderman and Gilbert (1964). The chemical aspects of juvenile hormone as isolated from the silk moth, Hyalophora cecropia, were reviewed by Roller and Dahm (1968). Williams (1967) coined the term "third-generation pesticides" to describe juvenile hormone and its analogues in his examination of the

insecticidal potentialities of these agents. Excellent general summaries of juvenile hormone research included an address by Berkoff (1970), an article by El-Ibrashy (1970), and a book by Wigglesworth (1970). More recent reviews included those by Bowers (1971) and Slama (1971) as well as the book by Menn and Beroza (1972).

Chemistry

Roller et al. (1967) published the structure of juvenile hormone isolated from the cecropia silk moth as determined by mass spectrometry, nuclear magnetic resonance spectrometry, gas-liquid chromatography, and identification of reaction products. Synthesis confirming the structure to be methyl trans, trans, cis-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate was later described (Roller and Dahm, 1968; Trost, 1970). Meyer et al. (1970) showed that there were actually two cecropia juvenile hormones, one being the tridecadienoate molecule identified by Roller and Dahm, and the other being a dodecadienoate molecule otherwise identical. Dahm and Roller (1970) then found these two hormones to be present in the giant silk moth, Hyalophora gloveri. Meyer and Hanzmann (1970) measured optical rotation of the compounds finding that they are not racemic.

Bieber et al. (1972) used accelerating voltage alternation mass spectrometry of a deuterated carrier compound to quantitate the C-18 juvenile hormone in H. cecropia. They found 1.8 μg in the adult male and 0.6 μg in the adult female.

Control of Metamorphosis

Juvenile hormone is secreted from the corpora allata into the haemolymph in which it is carried to target tissues where it effects a response. Although for many years the juvenile hormone has been claimed to be secreted from the corpora allata, two minute glands posterad of the insect brain, it was not until recently that Roller and Dahm (1970) actually isolated the hormone as produced by corpora allata *in vitro*.

Several functions have been claimed for juvenile hormone, but the most investigated property is its morphogenetic activity. Various viewpoints have been taken as to how growth and differentiation are affected by the juvenile hormone. Williams (1967) suggested that the moulting hormone, ecdysone, activity promotes growth and differentiation toward maturity while juvenile hormone functions as a "brake" or restraint on this development. Roller and Dahm (1968) concurred with this view stating that ecdysone induces metamorphosis and initiates moulting while the juvenile hormone modifies the expression of the moult in such a way as to favor development of larval structures. Wigglesworth (1970) saw a slightly more active role for juvenile hormone by maintaining that it is not only arrests differentiation, but that it has a qualitative effect on growth, possibly by activation of the larval genome. In any case, it has been established that larval moults are accompanied by a high juvenile hormone titer;

that this titer declines to allow a larval-pupal moult, while juvenile hormone must be absent during the pupal-adult moult for normal metamorphosis. Wigglesworth (1970) cited that the epidermal cells involved in certain moults could also be induced to form "younger" cuticle when juvenile hormone was present in greater concentrations than normal. This reversal in "differentiation" supports the hypothesis of an active role for juvenile hormone. Williams and Kafatos (1971) stated a theoretical model of action in which master genes (larval, pupal and adult) were controlled by juvenile hormone-dependent repressors; successive master genes were derepressed as juvenile hormone titre declined.

Functions in the Adult

In the adult insect, the juvenile hormone has been shown to produce gonadotropic effects by Roller and Dahm (1968). They used the natural juvenile hormone of cecropia to induce yolk deposition in allatectomized cockroaches, Periplaneta americana and Leucophaea maderae.

Adams and Nelson (1969) discussed the relationship of the corpus allatum to reproduction in the housefly, Musca domestica. They hypothesized that juvenile hormone stimulates vitellogenesis and that an oöstatic hormone can inhibit the juvenile hormone when necessary in the reproductive cycle. Adams (1970) found that injection of extracts containing the oöstatic hormone caused

ovariectomized female houseflies to store juvenile hormone as determined by observation of corpus allatum size.

Sahota et al. (1970) reported that the Douglas-fir beetle, Dendroctonus pseudotsugae, might postpone ovarial development when not on host logs through the withholding of its juvenile hormone.

In similar studies, juvenile hormone has also been implicated as a regulatory factor in diapause. Wigglesworth (1970) examined this role which has been established through experiments in which brainless, diapausing pupae of various insects have been caused to terminate diapause by injection of hormone analogues. This experimental phenomenon has been termed the prothoracatropic effect by some authors. To investigate this possible regulatory mechanism in nature, de Wilde (1969) used pure juvenile hormone to calibrate his bio-assay technique, and then determined the hormone titer of the haemolymph of the Colorado potato beetle, Leptinotarsa decemlineata, through adult life. He found a definite correlation between the juvenile titer and behavior leading to diapause.

Metabolic Effects

The effects of juvenile hormone on various metabolic processes in the adult insect have been studied, usually with some attempt to relate the results to either reproduction or diapause. Slama (1964) employed the Warburg respirometer to study metabolism in allatectomized female

adult bugs, Pyrrhocoris apterus. He concluded that juvenile hormone regulated reproductive respiratory metabolism. However, in studies of the African migratory locust, Locusta migratoria, Minks (1967) measured respiration and oxidative phosphorylation in isolated flight muscle mitochondria with α-glycerophosphate and pyruvate/malate substrates, then added homogenized corpora allata. No effect on oxygen consumption was seen with either substrate, but stimulation of oxidative phosphorylation in vitro was observed with α-glycerophosphate.

Zalokar (1968), working with the German cockroach, Blattela germanica, demonstrated with radioactive precursors that activation of corpora allata (by oötheca removal) stimulated incorporation of uridine-^{3}H into RNA followed by increased protein synthesis.

Induction of yolk protein synthesis by topical application of a juvenile hormone analogue was observed by Brookes (1969) who used the amputated abdomena of adult female cockroaches, L. maderae. Engelmann (1971), using the same insect, found that synthesis of a female specific protein in allatectomized females was induced by synthetic C-18 cecropia juvenile hormone. He used immunological technique for isolation of the protein which was labelled by injection of leucine-^{14}C.

Lipid metabolism has also been affected by juvenile hormone. Vroman et al. (1965) found that the triglycerides of P. americana females were synthesized at twice the normal

rate with allatectomy. Studies in vitro with adult female L. maderae by Gilbert (1967) showed that lipid synthesis in ovaries was enhanced during oögenesis, while being simultaneously depressed in fat body.

In a study of adult Drosophila melanogaster, Butterworth and Bodenstein (1969) found female corpora allata and synthetic juvenile hormone to stimulate adipose tissue in the male fly. A controversial result of this study was that simultaneous implantation of both male and female corpora allata did not give the stimulatory reaction.

Shaaya and Sekeris (1970) investigated the control of oötheca formation in P. americana finding that juvenile hormone regulated the synthesis of protocatechuic acid.

Metabolic effects of juvenile hormone in developing insect larvae and pupae have also been investigated. Sehnal (1966), who performed implantation of glands with larvae and pupae of the wax moth, Galleria mellonella, concluded that the additional juvenile hormone elicited increased oxygen consumption only when induced morphological changes were taking place. This was in concordance with the concept of indirect metabolic action of the hormone as discussed by Wigglesworth (1970).

Similar results were observed regarding the effect of juvenile hormone on the haemolymph protein of the silk moth, Antheraea polyphemus, by Blumenfeld and Schneiderman (1968). The accumulation of protein in the blood on injection of juvenile hormone extract into female pupae was

caused, not by regulation of protein synthesis, but by failure of affected oocytes to utilize this accumulating protein.

Larvae of Bombyx mori were higher in lipid content when allatectomized than the control as shown by Morohoshi and Kiguchi (1969). This depression of lipid content by the hormone was also observed in H. cecropia by Steven and Gilbert (1970) who found that a high titer was accompanied by a low synthesis of fatty acids. When the corpora allata were inactive in last instar larvae, the lipid was rapidly accumulated. Increasing juvenile hormone titer in pupae corresponded with a decreasing rate of lipid synthesis. They also mentioned that this correlation was found in studies in vitro.

Mode of Action

It is clearly remarkable that this compound of great biological activity, eliciting spectacular changes in insects, can be so well known in its identity and effects and yet can be completely mysterious in its mode of action. The solving of this mystery awaits further research along the lines of the following pioneering experiments.

Beerman and Clever (1964) noted that injections of ecdysone into the midge, Chironomus tentans, brought about explosions of "puffs" of the giant polytene chromosomes of the salivary glands. These puffs were thought to be related to increased gene activity which resulted in

increased RNA synthesis. Lezzi and Gilbert (1969), again working with C. tentans, reported that ecdysone also affected the Balbiani ring 1 which is a giant puff of a tissue-specific nature. They further showed that juvenile hormone decreased Balbiani ring 1, but that it induced a puff in another region. From these and additional data, they suggested that juvenile hormone may be an antagonist to ecdysone. Laufer and Holt (1970) working with the midge, Chironomus thummi, and using a mixture of juvenile hormone analogues, also found effects on a Balbiani ring. However, they found no antagonism in relation to the ecdysone puffs in this species.

Although these reports showed that ecdysone and juvenile hormone treatments induced chromosome puffs in vivo, it is not known if this activation was a direct or an indirect result of the hormone. Treatment in vitro to isolated chromosomes in a strictly defined medium must be done to answer this problem.

Chromosome puffs are also caused by changes in metallic ion concentration and the possibility exists that hormones may act indirectly on gene activity through the regulation of cellular ions as discussed by Lezzi and Gilbert (1970). There may even be a longer chain of events initiated by the interaction of the hormone with a lipid membrane as Baumann (1968) has shown juvenile hormone to depolarize salivary gland cell membrane in G. mellonella.

Metabolism

Very little work has been reported to date on the biological metabolism of juvenile hormone. However, it seems certain that the major deactivation steps are ester hydrolysis and epoxide hydration as was found in Schistocerca gregaria and R. prolixus by White (1972) using tritiated methyl farnesoate. The same inactivation steps were observed by Slade and Zibitt (1972) with synthetic C-18 juvenile hormone labelled with ^{14}C in the 2 position. They found ester hydrolysis occurred first in Manduca sexta, while either reaction could occur initially in H. cecropia.

Insect Growth Regulators

Control of insects through the disruption of the hormonal system is becoming a possible alternative to the use of persistent pesticides. A very active area of research involves the induction of juvenile hormone effects by synthetic analogues of the hormone now described as growth regulators.

Two of the most tested compounds are methyl 7,11-dichloro-3,7,11-trimethyl-2-dodecenoate and 10,11-epoxy farnesenic acid methyl ester. The former was contained in the mixed farnesenic acid derivatives prepared according to Law et al. (1966). Insecticidal potentiality of this compound has been demonstrated by Spielman and Skaff (1967) with the mosquito, Aedes aegypti, Vinson and

Williams (1967) with the body louse, Pediculus humanus, Thomas and Bhatnagar-Thomas (1968) with pests of stored grain, White (1968) with the cabbage aphid, Brevicoryne brassicae, and Hintze (1969) with Cerura vinula, a moth. This analogue exhibited ovicidal properties on the spruce budworm, Choristoneura fumiferana, in tests by Retnakaran (1970) and on the bugs, P. apterus and Oncopeltus fasciatus, and the silk moth, H. cecropia, in experiments by Riddiford (1970a, 1970b).

Bowers et al. (1965) synthesized 10,11-epoxy farnesenic acid methyl ester finding it to be active in the milkweed bug, O. fasciatus, and in the yellow mealworm, Tenebrio molitor. This analogue also produced juvenilizing effects in the flesh fly, Sarcophaga bullata, as shown by Srivastava and Gilbert (1969) and in M. domestica as demonstrated by Herzog and Monroe (1971).

Hundreds of other natural and synthetic compounds have been found to have juvenilizing activity in various insects. Levinson (1966) found farnesol and nerolidol to be active in T. molitor. Activity of p-1,5-dimethylhexyl benzoic acid derivatives was noted by Suchy et al. (1968) with bugs of the genus Dysdercus. Bowers (1968) found activity with several well-known insecticide synergists. He also (1969) increased the potency of certain synergists by altering side chains. Wigglesworth (1969) tested forty-two compounds on Rhodnius prolixus. Fifteen analogues were assayed on G. mellonella by Jarolim et al. (1969).

Combinations of several functional groups and terpenoid skeletons were made and tested on T. molitor by Swartz et al. (1970). Several commercial compounds were assayed in the stable fly, Stomoxys calcitrans, by Wright (1970) and in the greasy cutworm, Agrotis ypsilon, by El-Ibrashy and Mansour (1970). Riddiford et al. (1971) synthesized imino analogues which were observed to exert a synergistic effect when tested with cecropia juvenile hormone on A. polyphemus and P. apterus.

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