Propionate as a Precursor of Juvenile Hormone in the Cecropia Moth

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Adult 3–4 day old males of the giant silk moth *Hyalophora cecropia* incorporate the label of [1-^{14}C]propionate and [1-^{14}C]butyrate in JH-I and JH-II. [1-^{14}C]propionate is used for the biosynthesis of the hormones as an intact C_3-unit. Three possible intermediates in the biosynthesis of JH-I — (E,E,Z)-7-ethyl-3,11-dimethyl-2,6,10-tridecatrienoic acid, its methyl ester, and the corresponding alcohol — were converted in low yields to the hormone. Methyl 7-ethyl-3,11-dimethyltridecanoate was not incorporated. Mevalonate applied to larvae before pupation was not used for the synthesis of a hypothetical JH-I precursor.

From several species of insects and from cultures of corpora allata *in vitro* three compounds have been isolated which may be called "juvenile hormones". Juvenile hormone I (JH-I, **1**) is the major [1] hormonally active compound in extracts of adult male *Hyalophora cecropia* and is accompanied by 10–30% of the lower homologue (JH-II, **2**) [2]. The cecropia moth does not accumulate the hormone after removal of the corpora allata during the pupal stage [3]. We have been able to maintain corpora allata of this species *in vitro* and to isolate JH-I from the culture medium [4]. These two results show clearly that the hormone identified as JH-I is produced by the corpora allata, a gland which was thought to be the physiological source of the juvenile hormone since 1936 [5]. During previous studies on the biosynthesis *in vivo* we had found that methionine is an efficient donor of C_1-units for the ester methyl group of JH-I and JH-II [6]. Judy *et al.*, culturing corpora allata of *Manduca sexta in vitro*, consequently added labelled methionine to the medium in order to facilitate the isolation of the expected JH-I. Surprisingly, they found the biological activity produced in the cultures not associated with JH-I but with JH-II and a further lower homologue, JH-III (**3**) [7]. At this time, the juvenile hormones I, II and III either individually or in combination have been discovered in species of lepidoptera, coleoptera and orthoptera [2, 4, 7–15]. They have been isolated from extracts either of whole animals or from cultures of corpora allata. It is not known whether in each case the compound identified indeed serves as "the juvenile hormone" of the respective species.

A major question in the biosynthesis of JH-I is the origin of the ethyl chains at C(7) and C(11). Additional C-atoms might be added to an intermediate like farnesol through transfer of C_1-units. On the other hand, the biosynthesis may follow the established mevalonate-sesquiterpene pathway with the exception that the biochemical equivalent of propionyl acetate instead of acetyl acetate is formed and utilized in the initial steps. A crucial intermediate in this sequence is homomevalonate, two molecules of which plus one molecule of mevalonate would serve as precursors for JH-I (Fig. 1). In the organ culture system of *Manduca sexta* corpora allata, the labels of methionine, acetate, and mevalonate were readily assimilated in JH-II and JH-III. Propionate was incorporated in JH-II. The isolated hormones were cleaved in four fragments, and the

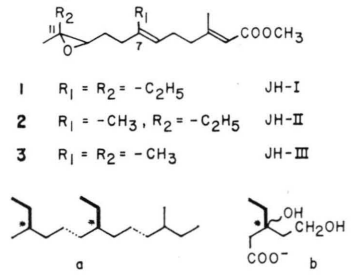

Fig. 1. Juvenile hormones. Scheme of propionate incorporation in JH-I. The carbon skeleton **a** consists of one isoprene and two homoisoprene units which are derived from homomevalonate **b**. The propionate residue appears in heavy print; C(1) is marked with an asterisk.

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respective labelling patterns were consistent with the assumption that the ethyl group at C(11) of JH-II is derived through incorporation of propionate by way of homomevalonate [16].

In contrast to these *in vitro* studies, *H. cecropia in vivo* [6] does not efficiently utilize administered mevalonate for the biosynthesis of JH-I. After injection of ^{3}H-labelled mevalonate, the specific activity of JH-I was at least five orders of magnitude smaller than that of farnesol isolated from the same moths. Acetate, on the other hand, was incorporated in a low yield, but equally well in JH-I and farnesol. The most likely explanation for this discrepancy is biosynthesis of JH in a compartment not accessible to exogenous mevalonate. The processes maintaining this compartment *in vivo* break down upon explantation of the gland. Since acetate can be assimilated *in vivo* and *in vitro*, one may postulate that propionate should behave similarly. In our original experiments with [1-^{14}C]propionate, the applied dose – 5.5×10^6 dpm/moth – might have been too small to allow with certainty detection of incorporation. The recovery of ^{14}C in JH-I after administration of the same dose of [1-^{14}C]acetate was also close to the limit of detectability (totally recovered 50 dpm; 1.4 μg JH/moth). It is impossible to predict an incorporation ratio for propionate relative to acetate as long as neither the size nor the turnover rate of the propionate and acetate pools are known. We therefore repeated the incorporation experiments with larger doses of propionate. In control experiments [1-^{14}C]butyrate was injected into moths of the same batch; *trans,trans*-farnesol and the fatty-acid fraction were isolated as internal reference compounds.

A second explanation for the discrepancy between the *in vivo* and *in vitro* experiments with regard to the incorporation of mevalonate may be found in the assumption that in *H. cecropia* the carbon skeleton of JH is synthesized from mevalonate not in the adult, but in some previous stage. The adult moth of this species does not feed and all its constituents are thus derived from food ingested by the larvae. In the adult the stored intermediate would then be converted to JH by relatively minor chemical modifications which, on the basis of our labelling experiments with methionine, would include the introduction of the ester methyl group. A likely time for the biosynthesis of the hypothetical precursor is the period when the animal undergoes pupation. We therefore injected labelled mevalonate, and for control purposes acetate, in the 5th instar larvae just before pupation and isolated JH, farnesol, and some fatty acids from the adult moth.

Adult cecropia moths will convert exogenous JH-I acid (**4**) to JH-I in high yield [17]. Corpora allata from adult female *Schistocerca gregaria* when incubated with *trans,trans*-farnesoic acid produced and released into the medium JH-III. From the glands themselves the unepoxidized methyl ester was isolated. Incubation with the trienoic acid (like **7**, differently labelled) produced JN-I and the trienoic methyl ester [12]. In order to probe the final phases of JH-I biosynthesis in cecropia we synthesized from the chloro derivative **5** the tritium labelled trienoic ester **6**, the trienoic acid **7**, the alcohol **8**, and the saturated ester **9** (Fig. 2). These compounds were injected into young adult moths and the recovery of label was determined after isolation of JH-I and JH-II.

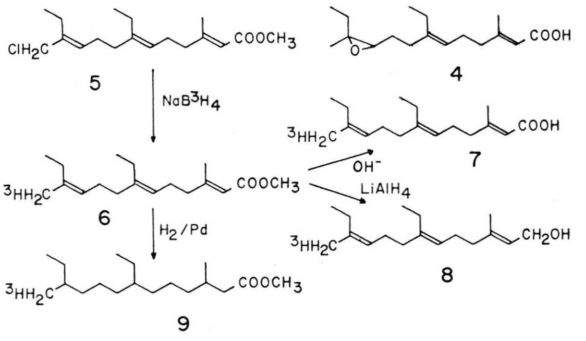

Fig. 2. Synthesis of compounds with the carbon skeleton of JH-I.

Experimental

Adult Moths

H. cecropia were bought as diapausing pupae from Richter Butterfly Farm, N.Y., and were kept at 4 °C. Adult development was initiated by exposure to 25 °C. 2 – 3 day old male adults were injected between the 6th and 7th abdominal segment with the labelled precursor dissolved in either 20 – 50 μl insect-Ringer solution or, if insoluble in this medium (**6**, **8**, **9**), in 2 – 5 μl olive oil. After an incubation period of 15 – 16 hours, the animals were sacrificed.

Larvae

Eggs of *H. cecropia* obtained from the above mentioned adults were allowed to hatch and the larvae

were reared on an artificial diet [18]. After a final defecation and commencing of spinning, 5th instar larvae were injected with 10 μCi (±) [5-^{14}C]mevalonate or 50 μCi [^{14}C]acetate in 100 μl insect-Ringer solution. The larvae were punctured with the needle in an anterior direction lateral to the second proleg. The animals went through diapause; the male adults that successfully emerged were sacrificed when 4 days old.

Radiochemicals

Sodium[^{3}H]borohydride (2.17 Ci/mmol) and sodium[1-^{14}C]propionate (31.2 μCi/μmol) were purchased from ICN Chemical and Radioisotope Division, Irvine, California – DL[5-^{14}C]mevalonic acid (11.8 μCi/μmol) and sodium[2-^{14}C]acetate (50 μCi/μmol) were purchased from Schwartz/Mann, Orangeburg, N.Y. – sodium[1-^{14}C]butyrate (24 μCi/μmol) was purchased from Amersham Searle Corp., Des Plaines, Ill.

Methyl-(2E,6E,10Z)-7-ethyl-3-methyl-11-(^{3}H-methyl)-2,6,10-tridecatrienoate (**6**)

25 μmol methyl (2E,6E,10E)-11-chloromethyl-7-ethyl-3-methyl-2,6,10-tridecatrienoate (**5**), 23 μmol sodium[^{3}H]borohydride and 46 μmol 1,5-hexadiyne in 220 μl dimethyl sulfoxide were sealed in a vial under nitrogen and kept for 4 hours at 50 °C. The reaction mixture was extracted with pentane, the extract washed with water, dried over sodium sulfate, and resolved by TLC on silica-gel H (E. Merck, Darmstadt; benzene/5% ethyl acetate). Yield 40% by GLC. Spec. act. 1.2×10^{9} dpm/μmol. Before injection, this material was diluted with the unlabelled ester to a specific activity of 43×10^{7} dpm/μmol and purified by GLC on OV-1.

Trienoic acid **7**, *trienic alcohol* **8**, *and saturated ester* **9**

After dilution with unlabelled **6**, **7** was prepared from **6** by saponification with methanolic/aqueous KOH. The acid was purified by TLC on silicagel in hexane/ethyl acetate/acetic acid 17:2:1 ($R_F \cong 0.5$). The alcohol was obtained through reduction of **6** with $LiAlH_4$ and purified by TLC on silicagel in benzene/15% ethyl acetate ($R_F \cong 0.2$), and by GLC on EGSS-X. After hydrogenation of **6** on Pd-black, the saturated ester **9** was purified by GLC on OV-1.

Isolation of JH-I, JH-II, farnesol, and fatty acid methyl esters

All components were isolated as previously described [6]. They were further purified by GLC to constant specific activity. The data of the propionate incorporation experiment in the following described are representative. 1.11×10^{7} dpm [1-^{14}C]propionate (6.93×10^{7} dpm/μmol) dissolved in 400 μl insect-Ringer solution were injected into ten 2–3 day old male cecropia moths. After an incubation period of 16 hours, the animals were divided into two groups of five which were worked up separately (data for the second group in parentheses). Extraction with ether gave 1.46 g crude oil with a ^{14}C-activity of 5.07×10^{6} dpm (0.75 g, 4.95×10^{6} dpm). Cold precipitation left 130 mg (not measured) in the filtrate which was resolved by column chromatography on LH-20 and two TLC separations on silicagel. The amount of the hormones after the second TLC separation was determined by analytical GLC on XE-60 and Carbowax 20M: 8.8 μg JH-I and 1.8 μg JH-II (11.0 μg JH-I and 2.5 μg JH-II). The hormone fraction was diluted with 17.6 μg unlabelled JH-I and 14.7 μg JH-II and resolved by preparative GLC on XE-60. The individual hormones were further purified by successive GLC separations. After each separation the recovery (%) was determined by analytical GLC with methyl stearate as the standard, while another aliquot was used for measurement of the ^{14}C-activity. The specific activities are calculated for the amounts of JH-I and JH-II present in the TLC-fraction. GLC-XE-60: 90% JH-I, 82% JH-II (94% JH-I, 89% JH-II). GLC-Carbowax 20M: 76% JH-I, 194×10^{3} dpm/μmol; 65% JH-II, 236×10^{3} dpm/μmol (74% JH-I, 195×10^{3} dpm/μmol; 64% JH-II, 134×10^{3} dpm/μmol). The JH-I preparations were combined, also the JH-II preparations, and collected from OV-1: 85% JH-I, 202×10^{3} dpm/μmol; 90% JH-II, 165×10^{3} dpm/μmol. GLC-EGSS-X: 86% JH-I, 197×10^{3} dpm/μmol; 82% JH-II, 164×10^{3} dpm/μmol. After the OV-1 separation aliquots of JH-I and JH-II were hydrogenated to the saturated ester (12 μg JH-I, yield 90%; 4.3 μg JH-II, yield ~20%). GLC-XE-60: 74% JH-I-derivative, 166×10^{3} dpm/μmol; ~100% JH-II derivative, 350×10^{3} dpm/μmol.

Gas Chromatography

Hewlett Packard Series 5750 instrument alternatively equipped with a thermal conductivity or a flame ionization detector. Carrier gas: helium, 60 ml/min; operating temperatures: injection port 200–230 °C; TC-detector 210–220 °C, FID 270 °C. 180 cm glass columns, 6 mm diameter: 3% XE-60 on 100/120 mesh Gas-Chrom Q; 3% OV-1 on 60/80 mesh Gas-Chrom Q; 3% EGSS-X on 100/120 mesh Gas-Chrom Q and 3% Carbowax 20 M on 80/100 mesh Gas-Chrom Q (all packings from Applied Science Laboratories, State College, Pa.).

Radioassay

Radioactivity was measured by liquid scientillation counting with a Tri-Carb 3375 (Packard Instrument Co., Downers Grove, Ill.). Scintillation-solutions were dioxane/10% naphthalene/0.7% PPO or toluene/0.5% PPO/0.01% dimethyl-POPOP. The counting efficiency was determined by the automatic external standardization method.

Results and Discussion

I. Propionate in the adult male

After injection of similar doses of [1-^{14}C]acetate [6], [1-^{14}C]propionate, and [1-^{14}C]butyrate in male cecropia moths, the label of all precursors was incorporated in JH-I to a similar extent (Table I). The incorporation ratios of about 10^{-3}% are low. The juvenile hormones were isolated as previously described by extraction with ether, low temperature precipitation, column chromatography in benzene/acetone 1 : 1 on Sephadex LH-20, and two thin layer separations on silicagel in the solvent systems chloroform/ethyl acetate 2:1 and benzene/ethyl acetate 19:1 [6]. Following the second TLC-separation the amounts of JH-I and JH-II in the hormone fraction were determined by gas chromatography. Unlabelled JH-I and JH-II were added and the hormones isolated individually by preparative GLC. The quantity of JH recovered in each case was measured by analytical GLC against methyl stearate as the standard. Liquid scintillation counting of another aliquot determined the radioactivity and allowed calculation of the specific activities. A series of preparative separations, followed by determinations of specific activities, established the purity of the hormone preparations. In the propionate experiments, for instance, the hormones were purified successively by GLC on XE-60, Carbowax 20M, OV-1, and EGSS-X. Also, after the OV-1 separations, an aliquot was hydrogenated to the saturated ester [8] which was repurified on XE-60. Throughout these procedures the radiolabel remained with JH-I, JH-II, or their derivatives, respectively. The hormones of the butyrate experiment were purified with the same result on XE-60, OV-1, and EGSS-X; the aliquot for hydrogenation was taken after the OV-1 separation. Consequently, in spite of the small incorporation ratio, there is no doubt that the isolated radiolabel is contained indeed in the hormones and not in associated impurities.

The most important question to decide at this point was whether propionate and butyrate are incorporated directly or only after degradation to acetate. Because of the small amounts of labelled JH-I in our hands the obvious solution, namely mapping of the ^{14}C-distribution in the molecule, was not possible. Before procuring more of the labelled hormone we decided therefore to use farnesol as an internal reference compound. We had already shown that farnesol is synthesized from acetate in the adult moth. According to the biosynthesis scheme for terpenes neither propionate nor butyrate can be incorporated in farnesol unless they are first degraded to acetate or, in the case of butyrate, at least to acetoacetate.

Farnesol was isolated as reported earlier [6]. After determination of the yield by gas chromatography, the samples were diluted with unlabelled farnesol and repurified by successive GLC-separations on XE-60 and Carbowax 20M: their specific activities remained unchanged. Butyrate was incorporated in JH-I and farnesol; the specific activity of each compound was approximately one half of that found after acetate incorporation (Table I). This result makes a specific role of butyrate as a precursor for JH-I unlikely, even though it does not prove it conclusively. [1-^{14}C]propionate, in contrast, was incorporated in JH-I but not in farnesol. The latter result shows that the label at C(1) of propionate does not reappear in the metabolism as acetate. In

Table I. Incorporation of acetate, propionate, and butyrate in JH-I and farnesol. Three to five adult male cecropia moths were used for each experiment. [a] Amounts in the hormone fraction of the second TLC separation.

Precursor	Spec. act. [dpm/μmol]	Dose/moth [dpm]	Juvenile hormone Isolated per moth [a]		Spec. act. [dpm/μmol]	*trans,trans*-Farnesol Isolated per moth [a]		Spec. act. [dpm/μmol]
			[μg]	[dpm]		[μg]	[dpm]	
[2-^{14}C]acetate	11.2×10^7	9.8×10^7	1.1	1,300	350×10^3	0.15	230	340×10^3
[1-^{14}C]propionate	6.93×10^7	11.1×10^7	1.8	1,150	190×10^3	0.08	0	0
[1-^{14}C]propionate	6.93×10^7	11.1×10^7	2.2	1,450	200×10^3	0.08	0	0
[1-^{14}C]butyrate	5.3×10^7	11.1×10^7	1.3	610	140×10^3	0.10	85	190×10^3

view of the previous experiments with methionine, it seems highly unlikely that the label of propionate is transferred to JH as a C_1-unit. Consequently, [1-^{14}C]propionate is incorporated in JH-I as an intact C_3-unit. This interpretation of our results with regard to [1-^{14}C]propionate metabolism finds support in that the label is not recovered in the fatty acid fraction either. Aliquots of extracts from each experiment were transesterified with methanol/BF_3; the methyl esters of saturated, monounsaturated, and diunsaturated fatty acids were separated by TLC on silicagel, TLC on silicagel impregnated with silver nitrate, and GLC on EGSS-X. After administration of [1-^{14}C]butyrate, 5.2 μg methyl stearate contained 1080 dpm ^{14}C, while in the propionate experiment no activity was detectable in 25 μg of the same compound. Since actetate in the experimental animal is readily incorporated in the saturated and monounsaturated fatty acids [6], it can be concluded that degradation of [1-^{14}C]propionate to [^{14}C]acetate is insignificant, if occurring at all.

JH-II was always isolated and purified through the same sequence as JH-I, including hydrogenation. The propionate experiments yielded 0.4 and 0.5 μg JH-II/moth with specific activities of 240×10^3 and 130×10^3 dpm/μmol, respectively. From the butyrate experiment 0.3 μg JH-II/moth with a specific activity of 300×10^3 dpm/mol was isolated. Because of the smaller amounts of JH-II, the mass measurements and consequently the data for specific activities are less reliable than those for JH-I. In addition, later experiments have shown (unpublished data) that the relative rates of JH-I/JH-II biosynthesis change drastically during the life of the moth. Therefore, from the specific activities no conclusion can be drawn with regard to the theory, which requires two moles of propionate for the biosynthesis of JH-I and one mole of propionate for JH-II.

II. Mevalonate in larvae

In order to search for a JH precursor which might be synthesized during a period preceding adult life, we injected mevalonate in ten 5th instar larvae of cecropia shortly before pupation. The animals went into diapause. Four males emerged successfully eleven months later and were sacrificed when four days old. From the control experiment where [2-^{14}C]acetate had been injected, we recovered of ten larvae only three healthy males. JH-I, JH-II, farnesol and fatty acids were isolated and purified as described above. If during pupation mevalonate is used for the biosynthesis of a JH-precursor which is subsequently stored until adult emergence, a high radioactivity in JH is to be expected in spite of the long incubation period. Otherwise, through randomization, a general low labelling in all compounds similar to that after acetate injection was anticipated. No incorporation of mevalonate in JH or farnesol was detectable (Table II). The experiment does not disprove the hypothesis since the timing of the injection may be crucial and the experiment allows wide variations in this respect. It may be interesting to note, however, that after injection of acetate the specific activity of methyl palmitate approached that observed in the experiment with adult moths (27×10^3 dpm/μmol), while the specific activity of JH was two orders of magnitude lower (1.3×10^3 vs. 240×10^3 dpm/μmol in the adult [6]). This may be taken as additional evidence for the complete synthesis of JH-I from acetate – and propionate – in the adult moth.

Table II. Incorporation of label after injection of precursor in larvae. [a] In the acetate experiment three and in the mevalonate experiment four adult males emerged and were used.

Precursor	Spec. act. [dpm/μmol]	Dose/larva [dpm]	Juvenile hormone		
			Isolated per moth [a]		Spec. act.
			[μg]	[dpm]	[dpm/μmol]
[2-^{14}C]acetate	11×10^7	11×10^7	2.9	29	1.3×10^3
[5-^{14}C]mevalonate	2.6×10^7	2.2×10^7	0.7	0 ± 18	~0

	trans,trans-Farnesol			Methyl palmitate		
	Isolated per moth		Spec. act.	Amount isolated		Spec. act.
	[μg]	[dpm]	[dpm/μmol]	[μg]	[dpm]	[dpm/μmol]
	0.1	0 ± 13	~0	36	1020	7.7×10^3
	0.1	0 ± 18	~0	48	0 ± 18	~0

Table III. Precursors with JH-I skeleton. Five to seven adult males were used in each experiment. The data for the epoxy acid **4** and [2-^{14}C]acetate had been published [6, 17].

Precursor	Spec. act. [dpm/μmol]	Dose/moth [dpm]	Juvenile hormone Isolated per moth [μg]	[dpm]	Spec. act. [dpm/μmol]	Incorporation [%]
epoxy acid **4**	5.5×10^7	3.5×10^5	1.8	20,200	3.3×10^6	5.6
trienoic ester **6**	43×10^7	2.6×10^5	1.3	130	29×10^3	50×10^{-3}
trienoic acid **7**	43×10^7	4.0×10^5	2.1	130	19×10^3	33×10^{-3}
trienic alcohol **8**	43×10^7	4.0×10^5	2.3	77	10×10^3	19×10^{-3}
saturated ester **9**	12×10^7	4.0×10^5	2.2	~0	~0	~0
[2-^{14}C]acetate	11×10^7	98×10^6	1.1	1,300	350×10^3	1.3×10^{-3}

III. Precursors with JH-I skeleton

In contrast to the epoxy acid **4** [17], other compounds with the carbon skeleton of JH-I (**6** – **9**) are not efficiently converted to the hormone (Table III). It is not surprising that the moth cannot produce the necessary double bonds in **9** but one might have expected it capable of introducing the oxirane ring as well as the methyl ester group. The incorporation of the trienoic ester **6**, the trienoic acid **7**, and the trienic alcohol **8** is still higher than degradation to acetate and *de novo* synthesis of JH-I would allow. Degradation to acetate prior to incorporation of label is also excluded by the fact that in all of these experiments JH-II showed no detectable activity. Since exogenous mevalonate *in vivo* is not available for the biosynthesis of JH in the corpora allata, the same difficulty may exist for larger precursors. In this case the methylation of the epoxy acid in the adult male may not be under the control of the corpora allata but could be a reaction in other tissues, not specific for JH. Also, the conversions of **6**, **7** and **8** to the hormone involving oxidations and methylation are chemically of such a general nature that the observed incorporation in JH-I may not be related to the biosynthesis of the natural hormone. We are presently studying this problem with the use of allatectomized animals and with corpora allata *in vitro*.

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