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Dimethyl sulphoxide inhibits *in vitro* synthesis of juvenile hormone I and II and stimulates juvenile hormone III by corpora allata of insects†

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Dimethyl sulphoxide (DMSO) specifically inhibits the rates of biosynthesis of juvenile hormone I (JH I) and juvenile hormone II (JH II) in corpora allata (CA) of 0 day last instar larvae of *Manduca sexta* maintained *in vitro*. Addition of mevalonate (Mev), propionate and homomevalonate (HMeV) to the medium increased the rate of biosynthesis of both JH II and JH III in normal CA. Even with added Mev, DMSO drastically reduced the rate of synthesis of both the higher homologues, but enhanced the rate of JH III synthesis even more with DMSO alone. The mated adult female CA which secretes JH II and JH III, and almost no JH I, also showed similar pattern of response to DMSO in larval CA. Allatotropin-stimulated JH II synthesis was also blocked by DMSO. DMSO inhibited JH II synthesis in *Galleria mellonella*, but the rate of JH III synthesis showed no increase. Likewise, in the CA of

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adult male, *Hyalophora cecropia* which secretes only JH I and JH II acids, DMSO suppressed the synthesis of both acids, but there was no elevation in the rate of JH III acid synthesis. DMSO stimulated the rate of JH III synthesis, the only homologue secreted by the adult female CA of cockroach, *Blaberus discoidalis*. Further *in vivo* studies showed that DMSO enhanced the growth of the silkworm larvae and silk fibre production.

Keywords: Dimethyl sulphoxide, juvenile hormones, lepdopteran insects, silk fibre.

MORPHOGENESIS and reproduction in insects are regulated by juvenile hormone (JH), a sesquiterpene hormone, secreted by the corpora allata (CA). *In vitro* CA of all lepidopteran species examined to date synthesize three structurally related JHs; JH I and JH II with ethyl side chains and JH III with only methyl side chains. All other insects secrete only JH III[1,2]. Studies to elucidate the biochemical basis of the unique capacity of lepidopteran CA to produce the higher homologues have demonstrated one difference between lepidopterans and non-lepidopterans. The ethyl branches of the higher homologues are derived from propionyl-CoA formed by transamination of branched-chain amino acids, isoleucine and valine; this pathway is formed in CA of only lepidopterans[3]. Many of the enzymes involved in JH biosynthesis have been characterized and their genes cloned[4], but we have yet to identify the other special features of the lepidopteran CA that enable them to synthesize the higher homologues. Recent studies have focused on the enzyme farnesyl diphosphate synthase (FPPS), which catalyses the coupling of isoprenoid and/or homoisoprenoid units to form the carbon skeleton of JHs[5–10]. Three forms of the enzymes with differing substrate preference and subcellular localization have been identified in lepidopterans, but their specific role in JH homologue synthesis is still uncertain. The isoforms of the enzymes are homodimeric, but recent observations indicate the possibility that heterodimers may be more active in synthesizing the higher homologues[8].

During the course of our studies on the regulation of JH biosynthesis in *Manduca sexta*[11,12], we used dimethyl sulphoxide (DMSO) as an agent to increase the permeability of CA *in vitro* to precursors such as mevalonate (Mev) and homomevalonate (HMev). Surprisingly, we found a drastic reduction in the rate of JH I and JH II synthesis and a concomitant enhancement of JH III synthesis. In this communication we report the inhibitory effect of DMSO on higher homologue synthesis in CA of three species of lepidopterans. Increase in JH synthesis was observed only in CA of species that normally make this homologue, but not in CA which either do not produce or make only trace quantities of JH III. Further, we have shown that DMSO increases the rate of JH III synthesis in CA that produce only JH III as in the cockroach, *Blaberus discoidalis*.

The larvae of *M. sexta* were reared and staged as described previously[13,14]. Silkworms (*Anthearaea assama*) were reared at the campus laboratory (North East Institute of Science and Technology (NEIST), Jorhat) as described previously[15].

The CA were dissected out in Graces medium and one pair of CA with attached corpora cardiaca was transferred into 0.1 ml medium-199 with Hank's salt and glutamine (GIBCO), 20 mM Hepes (pH 6.5), and 2% Ficoll (Sigma, USA) containing L-[^{3}H-methyl]methionine. The CA were incubated for 3 h in the dark at 30°C. The effects of DMSO (20% (v/v) prepared in acetone) on JH biosynthesis were tested *in vitro* under the same experimental condition. The incubation was terminated by the addition of 100 µl methanol containing 2.5 µg each of JH I and JH II as carriers and JH III as internal standard, and 100 µg trioctanoin to prevent the loss of JH during evaporation. A small column of 50 mg Licroprep (RP-8, 25–45 µm, E Merck, Germany) in a Pasteur pipette plugged with glasswool was prewashed with methanol. Medium plus methanol were filtered through the column; the column was washed with 1 ml or more of 50% methanol and emptied with gentle air pressure.

The JHs were eluted with 1 ml hexane; the elute was evaporated to dryness in a stream of dry nitrogen. The JHs recovered from Licroprep were resolved by isocratic HPLC on µPorasil with 3% ether in hexane, half saturated with water, at a flow rate of 1 ml/min. The JHs were eluted within 30 min. The recovery calculated from the UV traces (248 nm) was usually between 70 and 100%. Then 1 mm fractions were diluted with 4 ml scintillation cocktail and analysed for ^{3}H. The amount of JHs produced in the experiment was then calculated from the specific activity of the L-methionine in the medium, with the assumption of a specific incorporation ratio of 1 (no isotopic dilution).

Newly ecdysed fifth instar larvae of *A. assama* were collected from the stock reared on som (*Machilus bombycina*) at the NEIST campus. DMSO (100 mM) dissolved in acetone (HPLC-grade, E Merck) was topically applied at the neck region with the help of Hamilton microsyringe. The treated, untreated and control (treated with acetone only) were allowed to feed separately under the same environmental condition.

Sericin was extracted from the muga silkworm cocoon using the method of Akiyama *et al.*[16]. Silk fibre analysis was carried out according to Krishnaswami *et al.*[17].

In vitro CA of *M. sexta* larvae secrete primarily JH I and JH II[18]. During initial studies on the regulation of JH synthesis by neurohormones, we added precursors such as propionate, Mev and HMev to enhance the rate of JH production. However, these precursors were not effective, perhaps because of poor permeability into the CA[2]. Since DMSO is known to increase the entry of various substrates into the cells[19], in some preliminary experiments we tested the effect of 10 and 20% (v/v) of DMSO in the

Table 1. Effect of dimethyl sulphoxide (DMSO) on juvenile hormone (JH) biosynthesis by larval corpora allata (CA) of *Manduca sexta**

Treatment	*N*	JH I	JH II	JH III	JH total ± SEM
0 d V	20	0.21 ± 0.05	0.37 ± 0.07	0.06 ± 0.01	0.64 ± 0.08
0 d V DMSO	21	0.02 ± 0.01	0.19 ± 0.06	0.48 ± 0.05	0.69 ± 0.07

*0 d larval CA were incubated in control medium and medium with 20% DMSO.
Rate of JH biosynthesis: (pmol/h/CA pair ± SE).

Table 2. Effect of DMSO on JH biosynthesis by larval CA of *M. sexta* with added precursors*

Treatment	*N*	JH I	JH II	JH III	JH total ± SEM
0 V	7	0.29 ± 0.06	0.50 ± 0.11	0.04 ± 0.01	0.83 ± 0.16
0 V DMSO	7	0.01 ± 0.01	0.20 ± 0.06	0.52 ± 0.11	0.73 ± 0.16
0 V + Prop	5	0.31 ± 0.06	0.60 ± 0.16	0.06 ± 0.02	0.97 ± 0.20
0 V + Prop + DMSO	8	0.07 ± 0.02	0.15 ± 0.03	0.04 ± 0.01	0.26 ± 0.05
0 V+Mev	5	0.13 ± 0.01	0.64 ± 0.08	0.22 ± 0.02	0.99 ± 0.04
0 V + Mev + DMSO	6	<0.01	0.11 ± 0.01	0.59 ± 0.07	0.71 ± 0.08

*0 d larval CA were incubated in control medium, medium with 20% DMSO and medium with 0.1 mg/ml Mev and Prop.
Rate of JH biosynthesis: (pmol/h/CA pair ± SE).

medium on JH synthesis. Surprisingly, DMSO inhibited JH I and JH II synthesis, but stimulated JH III synthesis. A more detailed study was then conducted with fifth instar day-0 CA. Table 1 shows that JH I production was almost totally abolished, JH II synthesis was reduced to 50% or less, and JH III increased about eight-fold in CA treated with DMSO. Total JH production was therefore nearly the same as in the control glands.

To test whether DMSO blocks the metabolism of isoleucine and valine to propionyl-CoA, a pathway specific for lepidopteran CA[20], we added unlabelled propionate to the medium at 0.1 mg/ml of the medium containing DMSO. Although in the control incubated propionate alone caused only a small increase in JH I, JH II and JH III production, DMSO blocked the synthesis of the higher homologues even with added propionate. Further, propionate almost completely inhibited the increase in JH III synthesis caused by DMSO and hence total JH was less than 30% that of the control glands (Table 2). This result implies that DMSO inhibits a later step in the JH synthesis, perhaps in the formation of HMev.

We then examined the effect of Mev and HMev, either alone or in combination, on JH synthesis in the presence of DMSO. When Mev alone (0.1 mg/ml) was added to the medium the rate of JH I synthesis decreased, that of JH II increased slightly and of JH III several fold higher than in the control CA (Table 2). DMSO plus Mev showed the same pattern of inhibition as with DMSO alone. JH III production was almost the same as with DMSO alone (Table 2), suggesting lack of additive effect with Mev for JH III production. HMev alone (0.5 mg/ml) had no effect on the rate of synthesis of JH I, but enhanced JH II synthesis (two assays; av-pmol/h/CA pair; JH I, 0.17; JH II, 0.54; JH III, 0.05; total JH, 0.76). DMSO blocked JH I and JH II synthesis even with added HMev, but unlike with DMSO alone, there was no increase in JH III production. HMev plus Mev increased the synthesis of the higher homologues. In the presence of DMSO, production of both JH I and JH II decreased sharply, and the increase in JH III was not to the same extent as with DMSO alone.

Unlike larval CA which secrete predominantly JH I and JH II and only a small amount of JH III, adult female CA produce high amounts of JH II and JH III and trace levels of JH I. When mated, 3-day-old female CA was tested with DMSO, we found that DMSO inhibited JH II synthesis and increased JH III synthesis (Table 3) as in larval CA. Inhibition of JH II synthesis was more pronounced, but stimulation of JH III synthesis was relatively lower than in larval CA. A *Manduca* allatotropin (ATANA) analogue stimulates the production of all the three homologues, more so JH II than the other two[11]. In presence of DMSO, the ATANA-stimulated increase in JH II synthesis was severely curtailed and was nearly the same as in the control glands. JH III synthesis increased to almost double that with ATANA alone, thus demonstrating an additive effect (Table 3).

Larval CA of *Galleria mellonella* secrete mainly JH II and only trace amounts of JH I and JH III. DMSO almost totally inhibited JH II synthesis, but did not stimulate JH III production in contrast to *M. sexta* CA, and hence total JH production was reduced to almost undetectable levels (Table 4). CA of adult male *H. cecropia* secrete only JH I and JH II acids and virtually no JH III acid[21]. DMSO inhibited the synthesis of the acids of the higher homologues but did not cause a compensatory increase in JH III acid unlike in *M. sexta*, thus resembling the pattern in *G. mellonella*.

Table 3. JH biosynthesis by adult female CA of *M. sexta* in the presence of DMSO and ATH*

Treatment	*N*	JH I	JH II	JH III	JH total ± SEM
3 d mated–fed	10	0.06 ± 0.01	1.10 ± 0.22	1.21 ± 0.21	2.37 ± 0.31
3 d mated–fed + DMSO	6	0.05 ± 0.01	0.24 ± 0.06	1.91 ± 0.44	2.20 ± 0.48
3 d mated–fed + ATH	6	0.13 ± 0.03	5.53 ± 1.50	3.48 ± 1.20	9.14 ± 2.50
3 d mated–fed + ATH + DMSO	7	0.05 ± 0.01	0.98 ± 0.18	5.30 ± 0.70	6.33 ± 0.86

*3-day-old CA of mated female were incubated in control medium, medium with 20% DMSO, medium with 20 nM ATH and medium with 20 nM ATH, and 20% DMSO. Rate of JH biosynthesis: (pmol/h/CA pair ± SE).

Table 4. Response of larval CA of *Galleria mellonella* to DMSO*

Treatment	*N*	JH I	JH II	JH III	JH total ± SEM
0 VIII	6	<1.0	24.0 ± 3.60	1.00 ± 0.02	25.3 ± 3.87
0 VIII + DMSO	6	<1.0	<1.0	<1.0	<3.0

*0 d VIII larval CA were incubated in control medium and medium with 20% DMSO.
Rate of JH biosynthesis: (pmol/h/CA pair ± SE).

Table 5. Response of adult female CA of *Blaberus discoidalis* to DMSO*

Treatment	*N*	JH III
6–10 virgin females	8	2.85 ± 0.87
6–10 virgin females + DMSO	5	10.30 ± 2.47
6–10 virgin females + Mev	4	9.94 ± 0.93
6–10 virgin females + Mev + DMSO	4	8.30 ± 1.70

*6–10 d adult virgin female CA were incubated in control medium, medium with 20% DMSO, medium with 0.1 mg/ml Mev and medium with 0.1 mg/ml Mev and 20% DMSO.
Rate of JH biosynthesis: (pmol/h/CA pair ± SE).

Next we tested the effect of DMSO on CA which produce only JH III (all non-lepidopteran CA). *In vitro* these glands cannot be forced to synthesize higher homologues even with added precursor such as propionate and HMev[2,21]. CA of 6–10-day-old virgin females of the cockroach *B. discoidalis* were incubated in the medium containing DMSO. There was a four-fold increase in the rate of JH III synthesis. Mev alone had a similar effect, but Mev plus DMSO did not cause any additional increase (Table 5).

The shell weight of the cocoons formed from the DMSO-treated larvae (topically applied) was higher than that of the control (Table 6). The cocoons were used for fibre estimation and there was considerable increase in the fibre content. Moreover, the fibres were longer and had higher non-breakable filament length compared to the control.

In vitro CA from postembryonic stages of lepidopteran insects secrete JH I, JH II and JH III; the homologue ratio depends on the stage and age of the insects. Thus larval CA secrete JH I and JH II, but little JH III, whereas young adult female CA produce more JH II and JH III and CA of old female more JH III, less JH II and virtually no JH I[2,11,12,22]. The factors that regulate homologue ratios in different stages are not known. At the biochemical level we know that the acetate–Mev pathway provides methyl branches of JH III, whereas the propionate–HMev pathway leads to the construction of ethyl branches of the higher homologues[2]. Branched-chain amino acids, isoleucine and valine, have been shown to form propionyl-CoA, a necessary precursor for the higher homologues[3,23]. CA of other species of insects which make only JH III lack this pathway[20]. Additionally, they appear to be isoforms of prenyltransferase with differing substrate preference for isoprenoid and homoisoprenoid units[5,8,24]. It was, therefore, of interest when we accidentally discovered that DMSO inhibited specifically JH I and JH II synthesis, and increased JH III synthesis by CA of larval *M. sexta*. Further, we found that DMSO inhibited the production of the higher homologues in adult female CA of same insect with concomitant increase in JH III. This inhibitory effect is more pronounced on JH I compared to JH II. Since JH I requires two homoisoprenoid units and JH II only one, it may seem that the synthesis of homoisoprenoid units is limited by DMSO. The reduced synthesis of JH I and JH II would lead to an increase in isoprenoid units available for JH III synthesis and may explain the compensatory increase of this homologue. The increase in JH III almost equals the decrease of the other two and consequently, total JH production is essentially the same as in the control glands. This pattern is common to both larval and adult CA (Tables 1 and 3). However, this simple explanation does not account for all the observed effects. In adult female CA stimulated by ATANA in the presence of DMSO, the increase in JH III is appreciably less than the corresponding decrease in JH II. Both *G. mellonella* larval CA and *H. cecropia* adult male CA do not show compensatory increase in JH III and JH III acids respectively. Importantly, *B. discoidalis* CA which make only JH III, also increased JH III production in response to DMSO (Table 5). This suggests that DMSO

Table 6. Response of larvae of non-mulberry silkworm, *Antheraea assama* to DMSO*

Treatment	*N*	Weight of cocoons (g)	Weight of silk fibre (g)	Weight of sericin (g)
0 d V + DMSO	10	4.150 ± 0.34	2.314 ± 0.27	1.578 ± 0.24
Control (untreated)	10	3.400 ± 0.14	1.343 ± 0.24	2.701 ± 0.19
0 d V + acetone	10	3.995 ± 0 0.34	1.294 ± 0.27	1.521 ± 0.24

*Values are mean of three replicates of ten cocoons each ± SE.
Values are statistically significant over the control ($P < 0.05$).

directly affects some step(s) in the Mev pathway leading to an increase in the JH III synthesis.

The DMSO effect on JH I and JH II synthesis does not appear to be due to interference with the formation of propionyl-CoA from isoleucine and valine, since neither propionate nor HMev restored a normal production of the higher homologues in the presence of DMSO. So the blocking must be at some point beyond the HMev formation. It is noteworthy that both propionate and HMev prevented the DMSO-stimulated increase in JH III synthesis. Although the sample size is small, our experiments suggest that the upstream intermediates of the HMev pathway decrease Mev formation, since the combination of HMev and Mev with DMSO resulted in an increase in JH III production. HMev may also affect some step(s) downstream from Mev. Watson *et al.*[25] reported that HMev reduced incorporation of Mev into the lipids. HMev also inhibited JH III synthesis by adult female CA, but significantly enhanced JH I synthesis in addition to JH II (unpublished observation). Since adult females of all ages make only low amounts of JH I, the pronounced increase (nearly ten-fold) in JH I with HMev indicates that the glands have the potential for high rates of JH I synthesis. In contrast, ATANA has only a marginal effect on JH I synthesis (Table 3), suggesting that this allatotropin primarily stimulates some step(s) beyond the Mev–HMev formation.

DMSO has been used extensively both *in vivo* and *in vitro* as an agent to increase membrane permeability and as a cryoprotectant[19]. Yet, the exact mechanism of action of DMSO is not known. It is possible that it induces transient enzyme modification (phosphorylation, etc.) leading to changes in affinity or substrate specificity. DMSO did not appear to cause irreversible changes in JH biosynthesis, since CA exposed to DMSO first and then transferred to normal medium regained the pattern of synthesis of normal CA. Likewise, CA maintained in DMSO when transplanted into allatectomized fourth instar larvae induced larval molt, implying that they secreted the higher homologues[26]. In the present context it should be noted that heteromeric association of subunits of the isozymes of FPPS appear to change the substrate preference[27]. Perhaps DMSO may depress heteromer formation, thus favouring JH III synthesis.

Application of JH III on silkworm larvae generally increases silk production. This has been proved in large scale with mulberry and non-mulberry silkworms. Since the incorporation of DMSO *in vitro* enhances the synthesis of JH III in comparison to JH I and JH II, incorporation of DMSO *in vivo* probably enhances the JH III biosynthesis and ultimately increases the silk fibre production, as observed in DMSO-treated non-mulberry silkworms. Treatment with DMSO by topical application on larvae enhanced the growth of silkworms to produce more silk fibre of good quality. Considering the above facts the present study may prove helpful to the sericulture farmers of North East India.

Although the present work with DMSO and early precursor provides only glimpses of the complexities of JH biosynthetic patterns in Lepidoptera, more such physiological studies would complement the recent molecular approaches to elucidate the biosynthetic pathways for the higher homologues and in the regulation of homologue ratios during the development of lepidopterous insects.

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Role of thidiazuron in *in vitro* induction of embryogenesis in nucellar tissue of *Mangifera indica* L. var. Dashehari, leading to plantlets

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High frequency differentiation of embryos took place in 100% explants of predisposed nucellar tissue of *Mangifera indica* L. var. Dashehari, proliferated using 2,4-dichlorophenoxyacetic acid for a short duration of only 7 days initially, particularly under the influence of thidiazuron. The nucellus contained in ovular halves of 25- to 30-day-old young fruits proliferated into dark-brown compact tissue, over which differentiation of discrete sectors of whitish, pale and granular pro-embryogenic tissue was induced. Enormous number of embryos differentiated in the proembryogenic tissue during its subsequent subculture in the optimum treatment containing 0.15 mg l^{-1} each of 6-benzylaminopurine and 2-isopentenyladenine, along with 0.5 mg l^{-1} indole-3-acetic acid. Amongst the embryos of five developmental stages cultured in the liquid-processing medium, the best development was evinced by embryos with av 1.51 cm length. However, amongst the processed embryos, 90% matured and 80% germinated, while 60% did actually convert into plantlets. The properly acclimatized and *in vitro*-raised plantlets showed more than 80% transplant success, and during 4 months in the potted pure Soilrite under *ex vitro* conditions displayed an increase in shoot length and produced new leaves as well.

Keywords: Convertibility, embryo proliferation, monoembryonic, nucellar embryogenesis, pulse treatment.

NUCELLAR embryogenesis is the sole option for clonal propagation of *Mangifera indica* L. (mango), as regenera-

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