Molecular and Cellular Endocrinology, 77 (1991) 159–166

MOLCEL 02491

Evidence for regulation of juvenile hormone biosynthesis operating before mevalonate in locust corpora allata

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(Received 13 November 1990; accepted 15 January 1991)

Key words: Insect; *Locusta migratoria*; Corpora allata; Mevalonate; Mevalonolactone; Juvenile hormone; Mevinolin; Regulation

Summary

Exogenous mevalonate poorly stimulated juvenile hormone III (JH-3) biosynthesis by corpora allata from *Locusta migratoria*. However, mevalonolactone strongly stimulated glands from different physiological states and fully restored JH synthesis in mevinolin-treated glands. Asymmetry in spontaneous rate of JH release was abolished by exogenous mevalonolactone. After transection of nervus corporis allati 1 (NCA-1), the rate of mevalonolactone-stimulated JH synthesis was maintained at the preoperative levels although the spontaneous rate of JH biosynthesis decreased rapidly. These results suggest that the spontaneous asymmetry of JH biosynthesis and the low rate of JH biosynthesis by denervated corpora allata both result from non-stimulation or inhibition acting on the JH pathway before the utilisation of mevalonate.

Introduction

The mevalonate pathway in insects produces juvenile hormones (JH) which play a crucial role in insect physiology by controlling, for instance, metamorphosis and reproduction (see Feyereisen, 1985; Schooley and Baker, 1985). JH are biosynthesized in small endocrine glands, the corpora allata, under the control of the insect brain. The JH biosynthetic pathway is postulated to be controlled essentially by two mechanisms (Tobe and Pratt, 1976; Feyereisen, 1985): first, the activity of one or few rate-limiting enzymes in the biosynthetic pathway may be regulated; second, the concentration of enzymes involved in the pathway may change under the influence of regulatory factors.

This paper is part of an extensive investigation to localize the rate-limiting enzymes of the JH biosynthesis pathway in the corpora allata of the African locust and examines enzymatic steps subsequent to mevalonate formation. We have taken advantage of two characteristics of JH biosynthesis by locust corpora allata:

First is the large asymmetry in the spontaneous rates of JH biosynthesis by paired locust corpora allata (Couillaud and Girardie, 1987). It has been proposed that this asymmetry of JH production results from an autonomic regulation of each gland involving enzymatic targets (Couillaud and Girardie, 1987). The in vitro stimulation of JH production by exogenous precursors such as farnesol and farnesoic acid suppressed asymmetry.

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This suggested that rate-limiting enzymes are located prior to farnesol.

Second is the fact that locust corpora allata isolated from the brain-corpora cardiaca complex exhibit a very low rate of JH biosynthesis (Couillaud and Girardie, 1985). Such induced-low rate of JR production can be stimulated within 3 h when corpora allata are co-incubated with brain extract (Couillaud and Girardie, 1990). This suggests that enzymes involved in JH synthesis are still present in disconnected glands and that the regulation of JH biosynthesis by the factor present in the brain extract is probably acting on the activity of one or few rate-limiting enzymes. Restoration of JH production by exogenous farnesoic acid or farnesol supported a regulation prior to farnesol (Couillaud et al., 1988).

JH-III, the single JH produced by locust corpora allata (Pratt and Weaver, 1978; Mauchamp et al., 1985), is a sesquiterpenoid derived from mevalonate (Schooley and Baker, 1985). Exogenous mevalonate has been shown to serve as JH-III precursor in the corpora allata of several species (Feyereisen, 1985; Schooley and Baker, 1985; Feyereisen and Farnsworth, 1987). The present paper reports the stimulation of JH biosynthesis in unoperated, disconnected and mevinolin-treated corpora allata by mevalonate and mevalonolactone. Our physiological experiments demonstrate that rate-limiting enzymes in this locust are located prior to mevalonate.

Materials and methods

Insects

Locusta migratoria migratorioides were reared at 30°C under crowded conditions. They were fed every morning on fresh wheat and bran. All experimental females were isolated on the first day of adult life. Nervus corporis allati 1 (NCA-1) transection was performed on day 8, using Pascheff Wolff scissors on CO_2-anaesthetized females.

Chemicals

Racemic mevalonolactone (mevalonic acid lactone) from Sigma (Taufkirchen, F.R.G.) was converted to mevalonate by titration with 1 N NaOH. Mevinolin (a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase) was a gift from Dr. A.W. Alberts (Merck Institute for Therapeutic Research, Rahway, NJ, U.S.A.). JH-III was purchased from Calbiochem (San Diego, CA, U.S.A.).

Corpora allata incubations

Glands were incubated at 28°C in TC 199 (Flow Laboratories, Irvine, U.K.) supplemented with Ficoll (20 mg/ml, Sigma, Taufkirchen, F.R.G.) and [^{14}C-*methyl*]methionine (final specific activity 1.5 GBq/mmol, Amersham, Buckinghamshire, U.K.). Racemic mevalonate and mevalonolactone in aqueous solutions were added directly in labelled culture medium. Mevinolin, dissolved in dimethyl sulphoxide (DMSO) was added in incubation medium (final concentration 10 μM, 1% DMSO). Standard incubation time was 3 h. For consecutive incubations, corpora allata were first incubated 30 min with labelled methionine for equilibration and then culture medium, supplemented or not, was changed every hour. Linearity in the spontaneous rate of JH biosynthesis has been published previously (Girardie et al., 1981).

JH biosynthesis

JH biosynthesis was determined using a radiochemical assay according to Pratt and Tobe (1974). The validity of this assay in *Locusta migratoria* has been described extensively (Girardie et al., 1981; Mauchamp et al., 1985; Couillaud et al., 1987). JH-III was separated by silica thin-layer chromatography (TLC) (Merck, Ref. 5735, Darmstadt, F.R.G.) in a xylene/ethyl acetate (4:1) solvent system. Radioactivity of fractions sliced from the TLC plastic plates was determined by liquid scintillation counting with a Beckmann LS 2800 spectrometer (Palo Alto, CA, U.S.A.).

Osmotic pressure measurement

Osmotic pressure was determined at the freezing point using a Knauer osmometer (Berlin, F.R.G.). Changes in osmotic pressure were induced by NaCl addition in incubation medium.

Results

Effect of mevalonate and mevalonolactone on JH biosynthesis

When present in the incubation medium at 120 mM (optimal concentration, result not shown),

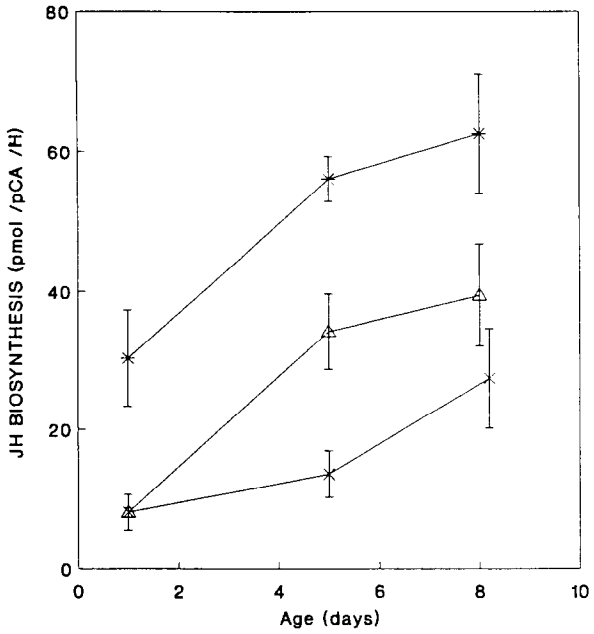

Fig. 1. Spontaneous (×), 120 mM (3*RS*)-mevalonate-stimulated (△) and 120 mM (3*RS*)-mevalonolactone-stimulated (*) rate of JH biosynthesis by corpora allata from 1-, 5- and 8-day-old adult females. Each point (±SEM) represents the mean of 6–10 incubation with single pairs of corpora allata from adult females.

racemic mevalonate stimulated the rate of JH-III biosynthesis by corpora allata from 5-day-old adult females (Fig. 1), but did not stimulate corpora allata from day-1 and day-8 females. However, mevalonic acid lactone added in the incubation medium at the same dose strongly stimulated JH synthesis in corpora allata from the three ages. Mevalonic acid lactone stimulated the rate of JH synthesis 3.7, 4.3 and 2.3 times on days 1, 5 and 8 respectively. On day 5, the mevalonic acid lactone-stimulated rate of JH biosynthesis was 1.6 times higher than mevalonate-stimulated rate.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase from *Diploptera* has been shown to produce only (3*R*)-mevalonate (Feyereisen and Farnsworth, 1987) and mevalonate kinase from *Sarcophaga* was found to phosphorylate only one isomer (presumably 3*R*) of a (3*RS*)-mevalonate mixture (Goodfellow et al., 1972). Thus, in the locust, effective concentrations of mevalonate are probably half of the concentrations of racemic mevalonate reported in the present paper.

Changes in pH and osmotic pressure and their effect on JH biosynthesis

During the 3 h incubation, mevalonic acid lactone slowly acidified the medium as revealed by colour changes of phenol red and pH monitoring (Fig. 2). Mevalonate did not modify the pH of the incubation medium. In order to discriminate between the effects of mevalonic acid lactone and of pH, the rate of JH biosynthesis was determined at different pH. Fig. 3 clearly illustrates that a decreasing pH resulted in a significant decrease in JH biosynthesis. Thus, the stimulation of JH biosynthesis by exogenous mevalonic acid lactone cannot be assigned to the acidification of culture medium. In spite of the pH decrease in incubation medium, the mevalonic acid lactone exhibited a strong stimulating effect on JH biosynthesis in vitro.

We also checked changes in osmotic pressure resulting from such high a concentration of mevalonate or mevalonic acid lactone. The typical osmotic pressure for TC 199, as prepared for JH radiochemical assay (see Materials and Methods) is 270 mOsm. Osmotic pressure in mevalonic acid lactone-supplemented medium was 275, 310 and

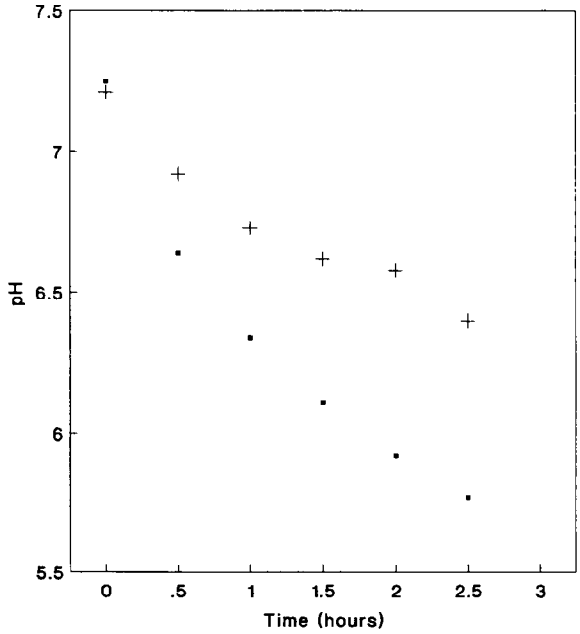

Fig. 2. pH monitoring of incubation medium containing 120 mM (+) or 160 mM (■) mevalonolactone at 30°C during the 3 h incubation time.

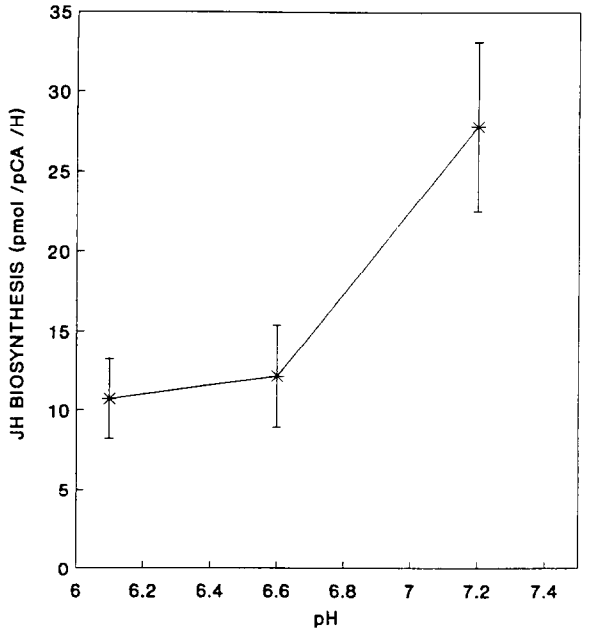

Fig. 3. Influence of the pH of the incubation medium on the rate of JH biosynthesis. Each point (±SEM) represents the mean of 13 incubations (3 h) with single pairs of corpora allata from 8-day-old adult females.

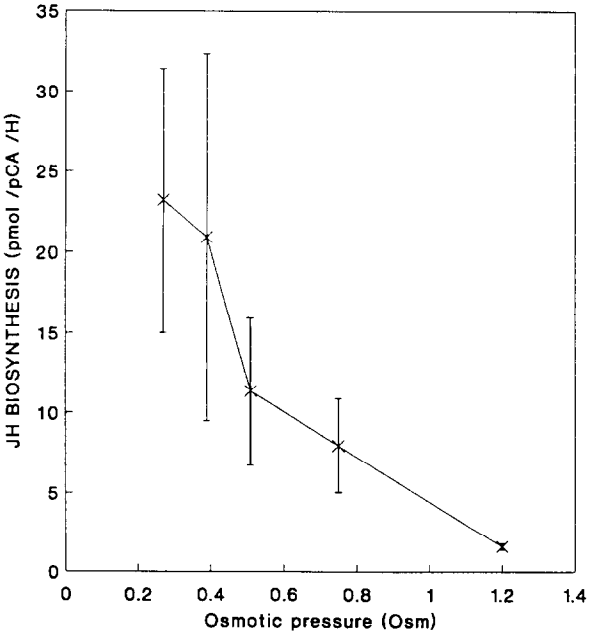

Fig. 4. Influence of changes in osmotic pressure of the incubation medium on the rate of JH biosynthesis. Changes in osmotic pressure were induced by NaCl addition in incubation medium. Each point (±SEM) represents the mean of ten incubations with single pairs of corpora allata from 8-day-old adult females.

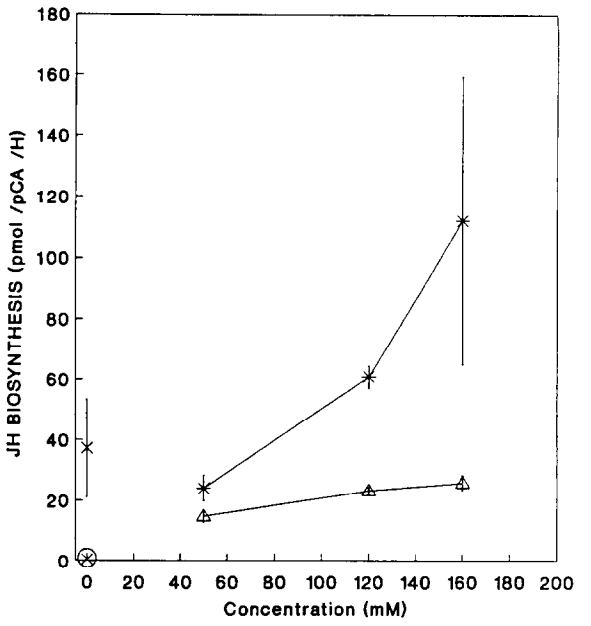

Fig. 5. Stimulation of JH synthesis by mevalonate (△) and mevalonolactone (*) on corpora allata incubated with mevinolin (⊗). Spontaneous activity of untreated gland is shown for comparison (×). Each point (±SEM) represents the mean of 6–10 incubations with single pairs of corpora allata from 8-day-old adult females.

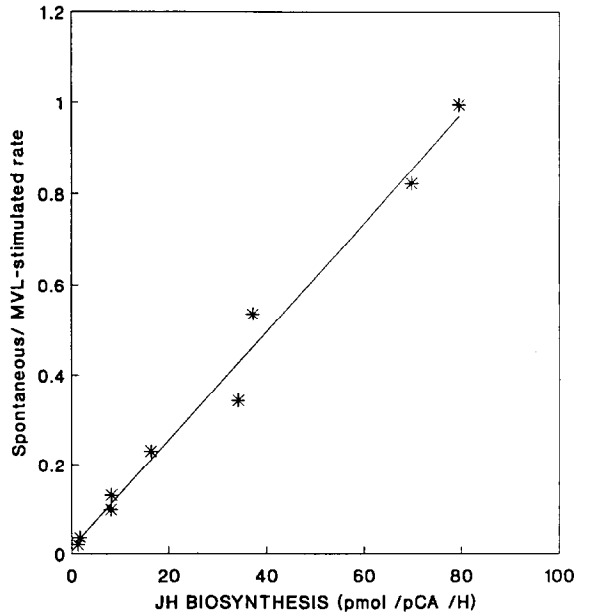

Fig. 6. Relation between the spontaneous rate and the ratio between spontaneous and 120 mM (3*RS*)-mevalonic acid lactone-stimulated rate of JH biosynthesis by individual pairs of corpora allata from 8-day-old adult females.

325 mOsm for respectively 50, 120 and 160 mM of racemic mevalonic acid lactone. Higher osmotic pressure was measured in mevalonate-supplemented medium with 308, 380 and 430 mOsm for respectively 50, 120 and 160 mM of racemic mevalonate. Fig. 4 illustrates the effect of osmotic pressure on the rate of JH biosynthesis. An increase in osmotic pressure resulted in a decline in the rate of JH biosynthesis. However, the higher osmotic pressure observed with mevalonate compared with mevalonic acid lactone cannot be the sole cause of the poor stimulating effect of mevalonate.

Mevalonate and mevalonic acid lactone on mevinolin-treated corpora allata

Because mevinolin is a potent inhibitor of HMG-CoA reductase and JH biosynthesis in locust corpora allata (B. Mauchamp and F. Couillaud, unpublished results), it was expected that mevinolin would suppress the level of endogenous mevalonate. Mevinolin (10 μM) suppressed JH production by corpora allata from 8-day-old adult females (Fig. 5). When mevalonic acid lactone was added to the incubation medium containing 10 μM mevinolin, JH production was restored at the mevalonic acid lactone-stimulated rate of JH biosynthesis, i.e. 2 times higher than the spontaneous rate (compare Fig. 5 and Fig. 1). Thus, the missing endogenous mevalonate is completely replaced by mevalonate from exogenous mevalonic acid lactone. However, mevalonate added in the incubation medium did not restore the spontaneous rate of JH biosynthesis in mevinolin-treated glands. Exogenous racemic mevalonate, even at 120 mM, did not replace the endogenous mevalonate (Fig. 5). The results suggested a poor penetration of mevalonate when compared to mevalonic acid lactone.

Ratio between spontaneous rate and mevalonic acid lactone-stimulated rate of JH biosynthesis

To characterize the stimulation of JH biosynthesis by exogenous mevalonic acid lactone, the ratio between spontaneous and mevalonic acid lactone-stimulated rate of JH biosynthesis (called FEAR, Tobe and Pratt, 1976) has been determined by successive 2 h incubations. For each individual pair of corpora allata, the ratio was

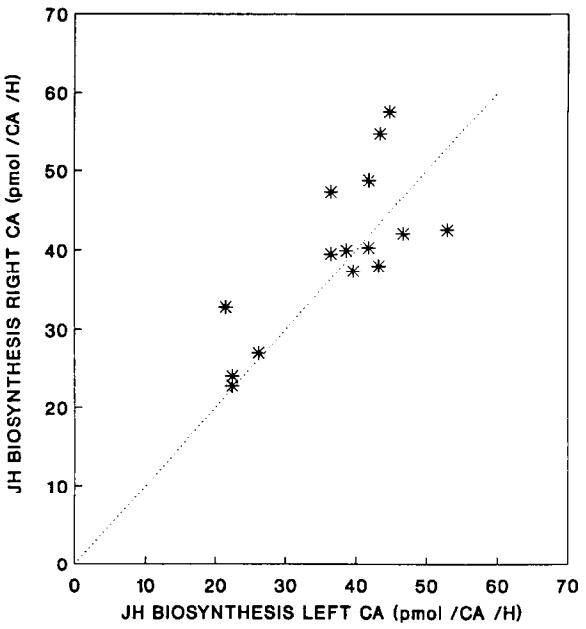

Fig. 7. Rate of JH biosynthesis by left and right corpora allata from 8-day-old adult females when stimulated with 120 mM (3*RS*)-mevalonolactone.

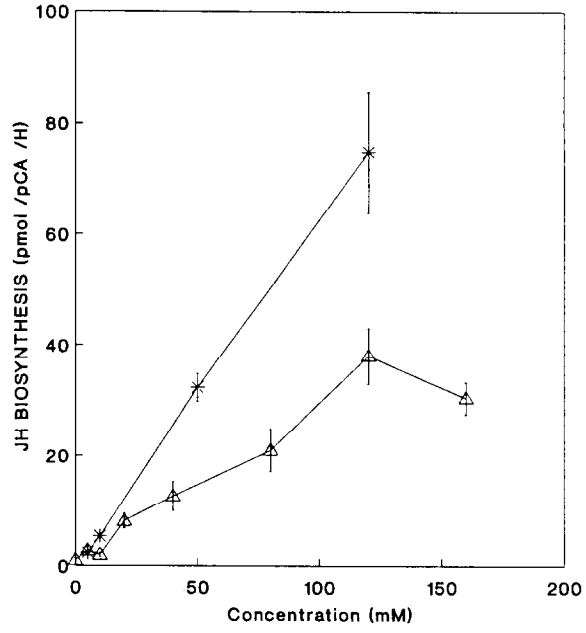

Fig. 8. Influence of mevalonate (△) and mevalonolactone (*) concentrations on the rate of JH biosynthesis by corpora allata from 11-day-old adult females with NCA-1 transected on day 8. Each point (±SEM) represents the mean of 6–10 incubations with single pairs of corpora allata.

always less than 1 (Fig. 6). Glands with low spontaneous activity exhibited low ratio values, while glands with high spontaneous activity exhibited high ratio values ($r = 0.99$; $n = 9$). This suggests that the spontaneous rate of JH synthesis did not reflect any differences in the capacity of the enzymatic steps posterior to mevalonate formation.

Asymmetry of JH biosynthesis and mevalonic acid lactone stimulation

Because left and right corpora allata in the locust present a large asymmetry in the spontaneous rate of JH biosynthesis and not in farnesoic acid or farnesol-stimulated rate (Couillaud and Girardie, 1987), we investigated asymmetry in mevalonic acid lactone-stimulated rate of JH production (Fig. 7). The mevalonic acid lactone-stimulated rate of JH biosynthesis was similar for the left and the right gland of a pair. Thus, the asymmetry in JH biosynthesis did not involve limiting activities of enzymes posterior to mevalonate formation.

Mevalonate and mevalonic acid lactone stimulation of disconnected corpora allata

Both mevalonate and mevalonic acid lactone were added in incubation medium in an attempt to restore JH biosynthesis by 11-day-old corpora allata that were isolated from the brain-corpora cardiaca complex by in situ transection of NCA-1 on day 8 (Fig. 8). Three days after the operation, racemic mevalonic acid lactone at 120 mM entirely restored the mevalonic acid lactone-stimulated rate of JH production typical of day-8 corpora allata. This suggests that enzymes acting after the mevalonate formation are still present in the corpora allata and thus are not responsible for the low rate of JH biosynthesis by disconnected corpora allata. Mevalonate also stimulated JH production by disconnected glands at a level close to the spontaneous rate of JH biosynthesis.

Discussion

The stimulation of JH-III biosynthesis by exogenous precursors such as farnesol and farnesoic acid gives useful insights into the regulation of the corpora allata because it is indicative of the degree of saturation of the enzyme situated beyond the entry of those precursors (Feyereisen, 1985; Schooley and Baker, 1985). Exogenous mevalonate has been shown to serve as JH-III precursor in the corpora allata of several insect species (see Schooley and Baker, 1985; Feyereisen and Farnsworth, 1987). However, the use of exogenous mevalonate as a probe of the physiology of the corpora allata has been restricted to the viviparous cockroach *Diploptera punctata*. Problems were encountered because of the limited penetration of mevalonate into the gland cells (Feyereisen and Farnsworth, 1987). For instance, mevalonate was not able to restore JH-III synthesis when endogenous mevalonate synthesis was blocked by the HMG-CoA reductase inhibitor mevinolin (Feyereisen and Farnsworth, 1987). Results presented in this paper largely support those conclusions as mevalonate was not able to restore JH synthesis in mevinolin-treated glands in the African locust.

However, mevalonolactone does restore JH synthesis in mevinolin-treated glands, and the mevalonolactone-stimulated rate of JH synthesis in mevinolin-treated glands is comparable to the mevalonolactone-stimulated rate of non-treated glands. In the locust, mevalonolactone stimulated the rate of JH biosynthesis by corpora allata of different ages (days 1, 5 and 8) and the stimulation appeared to be dependent on the spontaneous JH-III release rate. In *Locusta*, as in *Diploptera* (Feyereisen et al., 1981), low activity glands showed low ratio values (ratio between spontaneous and mevalonic acid lactone-stimulated rate of JH biosynthesis), whereas high activity glands exhibited high ratio values when stimulated with mevalonolactone. The present data show that the mevalonolactone-stimulated rate of JH biosynthesis always exceeded the spontaneous rate. Thus, under spontaneous synthesis conditions, enzymatic steps of JH biosynthesis subsequent to mevalonate formation are not rate limiting in corpora allata of adult female locusts.

Efficient concentrations for mevalonolactone stimulation of JH synthesis are very high and result in a progressive acidification of incubation medium. Decreasing the pH of the incubation medium reduces JH production by the corpora allata (Tobe and Pratt, 1974; this work Fig. 3). These harmful circumstances of incubation may

be responsible for the relatively low mevalonolactone-stimulated rates of JH synthesis (60 pmol/pair of corpora allata/h) when compared with farnesol or farnesoic acid-stimulated rate (around 250 pmol/pCA/h) (Couillaud et al., 1988). The slight stimulation may be also attributable to cell membrane permeability problems (Feyereisen et al., 1981). Thus, the activities measured in the presence of mevalonolactone are lower than the maximal velocities of enzymatic steps posterior to mevalonate formation (at least for the alcohol and aldehyde dehydrogenase, methyl transferase and epoxidase) and therefore probably do not reflect the total amount of enzyme present in the corpora allata. At least, the slight stimulation may indicate that the concentration of enzymes involved in synthesis from mevalonate to farnesol is lower than the concentration of enzymes converting farnesol into JH-III.

Mevalonolactone has been used to investigate the asymmetry in spontaneous rate of JH biosynthesis by locust corpora allata. When stimulated by mevalonolactone, left and right glands exhibited very similar rates of JH biosynthesis as had been previously shown with farnesoic acid (Couillaud and Girardie, 1987). Thus, enzymatic equipment for JH biosynthesis from mevalonate does not differ between left and right glands and asymmetry in spontaneous rate of JH biosynthesis may involve some kind of individual regulation of each gland acting prior to mevalonate. Morphological and experimental evidence suggest that this individual regulation comes from allatostimulating factors of the lateral neurosecretory cells (Couillaud and Girardie, 1987). Some tracts of the lateral neurosecretory cells extend to the corpora cardiaca, cross them and enter the NCA-1 to reach the corpora allata (Cassier and Fain-Maurel, 1970). This organisation provides opportunities for local action directly on allatal cells. Furthermore, deprivation of lateral factors by destruction of the lateral neurosecretory cells, or NCA-1 transection result in a decline of JH biosynthesis (no stimulation) (Couillaud and Girardie, 1985). Disconnected corpora allata exhibit then a constant low rate of JH biosynthesis. Severance of the left NCA-1 results in a decrease in the rate of JH biosynthesis only by the disconnected gland, suggesting that the stimulating factor of the right size cannot act on the left size through the hemolymph (Couillaud and Girardie, 1987).

We show in this paper that mevalonolactone is able to restore JH biosynthesis in disconnected corpora allata (NCA-1 transection 3 days before). Enzymatic steps subsequent to mevalonate formation are not responsible for the low rate of JH biosynthesis by disconnected glands and thus, the allatostimulating factor coming through the NCA-1 acts on JH biosynthesis prior to mevalonate. Three days after the NCA-1 transection, enzymes subsequent to mevalonate formation are still present. This is consistent with the fact that brain extract can stimulate JH biosynthesis by disconnected corpora allata during the 3 h time of an in vitro incubation (Couillaud and Girardie, 1990) and supports the idea of 'a rate limiting step' prior to mevalonate kinase (i.e. acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase).

Whether the allatostimulating factor coming through the NCA-1 is also responsible for the asymmetry in spontaneous rate of JH biosynthesis remains to be clearly established but the present data show that both asymmetry and stimulation by the NCA-1 factor involve early steps in the JH pathway prior to mevalonate.

Acknowledgments

I thank F. Rossignol for her technical assistance and R. Feyereisen for reading of the manuscript.

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