The Influence of Substrate Concentrations on the Rate of Insect Juvenile Hormone Biosynthesis by Corpora Allata of the Desert Locust *in vitro*

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The rate at which isolated corpora allata of adult female *Schistocerca gregaria* incorporate [^{3}H]farnesenic acid and [^{14}C]methionine into C_{16} juvenile hormone *in vitro* was examined at different concentrations of farnesenic acid, methionine, O_2 and H^+ ions. Maximum juvenile hormone biosynthesis is obtained at a farnesenic acid concentration of $20\,\mu$M. The range of optimum L-methionine concentrations (0.1–0.4mM) encompasses the physiological concentration of this substrate in the haemolymph. Hormone biosynthesis is dependent on O_2, but is not stimulated by hyperbaric oxygen tension. The glands had a maximum synthetic activity at pH8.0, but their activity was more reproducible in the the physiological range pH7.0–7.5. At pH6.5 and less, the synthetic ability was considerably decreased. The relative incorporations of the labelled substrates into methyl farnesoate and C_{16} juvenile hormone indicate that [^{3}H]farnesenic acid comes into isotopic equilibrium within the gland more rapidly than [^{14}C]methionine. The incorporations into methyl farnesoate become stoicheiometric after 20min incubation and into C_{16} juvenile hormone after a further 10min. Labelled juvenile hormone is detectable after 10min incubation and the rate of incorporation is constant for up to 4h. It is proposed that the described method may be usefully employed to assess the physiological changes in the enzymic competence of the glands to effect the last two stages in C_{16} juvenile hormone biosynthesis.

Juvenile hormone is known to be a regulator of metamorphosis in all insect species investigated (Wigglesworth, 1970); it also regulates various other physiological phenomena in different insects including the stimulation of sexual development in adult insects (Engelmann, 1970) and the determination of phase polymorphism in swarming locusts (Joly & Meyer, 1970). Two juvenile hormone-active compounds were first isolated from the abdomens of adult males of the silk moth *Hyalophora cecropia* (Röller *et al.*, 1967) and were found to be the methyl esters of 10,11-epoxy-7-ethyl-3,11-dimethyl-*trans, trans,cis*-2,6-tridecadienoic acid and 10,11-epoxy-3,7,11-trimethyl-*trans,trans,cis*-tridecadienoic acid (these compounds are derivatives of side-chain homologues of farnesenic acid 3,7,11-trimethyl-*trans,trans*-2,6,10-dodecatrienoic acid). Röller & Dahm (1970) employed long-term organ cultures of isolated corpora allata and brain complexes to prove finally that the corpus allatum, a glandular part of the retrocerebral complex in insects, is the site of synthesis and release of these hormones. Similar techniques have been used to demonstrate and identify the natural juvenile hormones of the tobacco hornworm *Manduca sexta* (Judy *et al.*, 1973b), and the

grasshopper *Schistocerca vaga* (Judy *et al.*, 1973a). In these two cases, the principle hormone was identified as the lower homologue methyl 10,11-epoxy-3,7,11-trimethyl-*trans-trans*-2,6-dodecadienoate (C_{16} juvenile hormone). The carboxylic acid ester group, which appears to be unique among known animal hormones, probably depends solely on the *S*-methyl group of exogenous L-methionine as its methyl source (Pratt & Tobe, 1974a,b), possibly through the formation of *S*-adenosylmethionine (Reibstein & Law, 1973).

The competence of the glands to effect the last two stages in juvenile hormone biosynthesis from added farnesenic acid, namely esterification of the acid followed by 10,11 epoxidation of the olefinic ester, is much higher than their competence to fabricate the entire molecule *de novo* (Pratt & Tobe, 1974a) suggesting that it may be possible to exert direct experimental control over the rate of juvenile hormone biosynthesis in active corpora allata by adding various concentrations of the required substrates to the incubation medium. In the present paper we have developed an assay system *in vitro* for measuring the overall enzymic competence of the glands to effect the two last stages in hormone synthesis.

Experimental

Animals and dissection

Animals were reared and dissected as previously described (Pratt & Tobe, 1974a). Adult females were killed 15–19 days after adult emergence, which corresponds to a period of usefully high activities of the glands *in vitro* (S. S. Tobe & G. E. Pratt, unpublished work). Within each experiment the pairs of glands from each animal were separated and randomized throughout the series.

Materials

L-[*Me*-^{14}C]Methionine (56–58 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *trans-trans*-[2-^{3}H]Farnesenic acid (25 mCi/mmol) was prepared from the methyl ester and stored under the same conditions as previously described (Pratt & Tobe, 1974a). Ficoll was from Pharmacia (G.B.) Ltd., London W.5, U.K. Tissue-culture medium TC 199 (Hank's salts, plus glutamine) was obtained from Grand Island Biological Co., New York, N.Y., U.S.A. All other reagents were AR grade. Glass-distilled water was used for preparing aqueous solutions, and all media were filtered through Millipore filters (0.22 μm) immediately before addition of labelled substrates.

Media

In those experiments where the concentration of methionine was held constant, we used TC 199 medium fortified with Ficoll (20 mg/ml); L-[*Me*-^{14}C]-methionine was added to a final concentration of 0.29 mM (final sp. radioactivity, 36.5 mCi/mmol). In other experiments, we used a Ringer solution (Pratt & Tobe, 1974a) to which was added various concentrations of L-methionine. L-[*Me*-^{14}C]Methionine was incorporated into media by adding appropriate volumes of altered media to tubes containing freeze-dried portions of a standard aqueous solution of the labelled methionine. *trans,trans*-[2-^{3}H]Farnesenic acid was incorporated into media by adding appropriate volumes of filtered media to tubes containing evaporated portions of a standard iso-octane solution of the labelled farnesenic acid.

Incubations

Corpora allata (4) were incubated in 0.1 ml of medium contained in capped conical-bottomed glass vials (8 mm internal diameter). The vials were gently agitated on a wrist-action shaker (approx. 150 cycles/min) under air unless otherwise stated, in a light-proof incubator kept at 30°C. The reactions were stopped and extracted as described by Pratt & Tobe (1974a). In those cases where the contents of the glands were

analysed separately from the medium, medium was aspirated at the end of the incubation, the glands were washed for 15 s in non-radioactive medium, and the combined medium and wash were extracted separately from the glands.

Chromatography

T.l.c. of the extracts was performed on 0.25 mm plastic-backed Silica F$_{254}$ plates (E. Merck A.-G., Darmstadt, Germany) with either ethyl acetate–light petroleum (b.p. 40–60°C) (1:3, v/v) or ethyl acetate–benzene (1:3, v/v). The labelled products were detected by a combination of fluorescence-quenching densitometry (Vitatron TLD) and gas-flow radioactivity-detection (Berthold Scanner, 2 mm window) of the developed chromatograms.

Scintillation spectrometry

Standard solutions and sections of t.l.c. were counted in 10 ml of 1% 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 10% ethanol, 5 mM-acetic acid in toluene, in a Packard 3375 liquid-scintillation spectrometer with perforated tape output. Standard [1,2-^{3}H]- and [1-^{14}C]-hexadecane (The Radiochemical Centre) were used to provide data for a 5-point curve-fitting Fortran IV program for solving double-label quench-correction equations.

Results

Effect of farnesenic acid concentration

Fig. 1 shows the effect of various concentrations of added farnesenic acid on the total production of C$_{16}$ juvenile hormone measured after an incubation period of 3 h. The results of two separate experiments carried out in the TC 199-based medium are shown, in which the entire contents of the incubation tubes (i.e. glands plus medium) were extracted together for analysis. The quantity of hormone biosynthesized was calculated from the dual-label radioactivities, on the basis of both the ^{3}H incorporation from farnesenic acid and from the ^{14}C incorporation from methionine. Both calculations yield the same value within experimental limits since, with the exception of hormone synthesized under conditions of very low exogenous methionine concentration, the molar incorporation ratios of farnesenic acid to methionine are close to unity (Pratt & Tobe, 1974b). The obvious difference between the two curves derives from the natural difference in synthetic activity of corpora allata taken from adult females of different ages. In the experiments described in the present paper the rate of hormone synthesis in the absence of added farnesenic acid was barely detectable, leading to an almost complete dependence of hormone synthesis
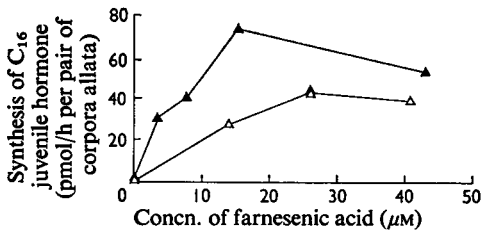
Fig. 1. *Influence of [^3H] farnesenic acid concentration on the rate of C_{16} juvenile hormone synthesis*

In each experiment two pairs of corpora allata from *S. gregaria* were incubated at 30°C under air for 3 h in 0.1 ml of TC 199 medium containing [^{14}C]methionine (final concentration 0.29 mM, final sp. radioactivity 36.5 mCi/ mmol) and appropriate concentrations of [^{3}H]farnesenic acid (25 mCi/mmol). Reactions were terminated by addition of 0.2 ml of ethanol, and chloroform extracts were submitted to t.l.c. Incorporation of both isotopes into C_{16} juvenile hormone was measured by liquid-scintillation spectrometry of the appropriate sections of the chromatograms. Each point represents an individual determination. Different symbols indicate two entirely separate experiments.
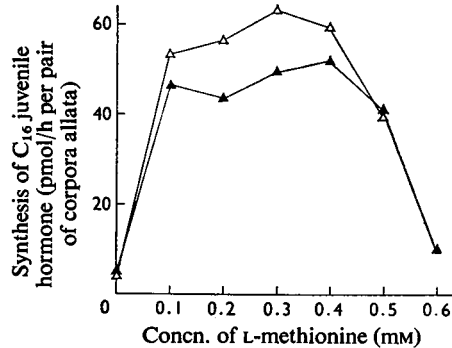

Fig. 2. *Influence of [^{14}C]methionine concentration on the rate of synthesis of C_{16} juvenile hormone*

In each experiment two pairs of corpora allata from *S. gregaria* were incubated at 30°C under air for 3 h in 0.1 ml of Ringer solution (see the Experimental section) containing 20 μM-[^{3}H]farnesenic acid (25 mCi/mmol) and appropriate concentrations of L-methionine. The reactions were terminated and the contents of the tubes analysed as described in Fig. 1. Each point represents an individual determination. Different symbols indicate two entirely separate experiments.

on added precursor acid. In other experiments where there was an appreciable rate of synthesis *de novo* of hormone (no precursor acid present in the medium), the addition of farnesenic acid to the medium brought about further increases in the rate of biosynthesis. It can be seen that the rate of hormone biosynthesis increases with increasing concentration of added farnesenic acid. The optimum concentration of farnesenic acid appears to vary somewhat between different experiments and to lie within the range 16 to 28 μM. Concentrations in excess of 40 μM are markedly inhibitory, possibly because of a non-specific cytotoxic effect. In the experiments described below we used farnesenic acid at a concentration of 20 μM.

Effect of methionine concentration

In these experiments we used only a single label ([^{3}H]farnesenic acid) and varied the concentration of added L-methionine in the Ringer medium. Incubations were carried out for 3 h, and the entire contents of the tubes were analysed. The results of two typical experiments are shown in Fig. 2. When no methionine is present in the medium the rate of hormone synthesis is very low, but this strong dependence on exogenous methionine is apparently satisfied at low concentrations, and high rates of synthesis are obtained at concentrations ranging from 0.1 to 0.4 mM. The apparent inhibitory effect of methionine concentrations in excess of 0.5 mM was not further investigated.

We have employed as a routine a methionine concentration of 0.29 mM in those experiments requiring an optimum concentration of the amino acid.

Effect of O_2 supply

The effect of oxygen tension on the rate of hormone synthesis was investigated by incubating glands for 3 h in media containing optimum concentrations of [^{14}C]methionine and [^{3}H]farnesenic acid under different gases. The incubation media were appropriately gassed immediately before addition to the glands. These experiments showed that increasing the oxygen tension above that of atmospheric tension did not stimulate either esterification or epoxidation of the farnesenic acid. On the other hand, anaerobic conditions (pure N_2) completely suppressed the formation of epoxy ester C_{16} juvenile hormone and resulted in a 90% inhibition of total esterification of the farnesenic acid.

Kinetics of incorporation

To investigate the linearity of juvenile hormone biosynthesis and release during the course of incubations lasting several hours, we terminated incubations at various intervals up to 4 h and analysed the contents of glands and medium separately. We have previously shown that the unepoxidized olefinic ester is not released from the glands and that, at least in the case of corpora allata taken from reproductively active
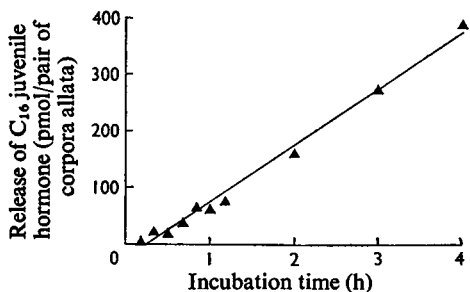
Fig. 3. *Kinetics of release of newly synthesized radiolabelled* C_{16} *juvenile hormone*

Two pairs of corpora allata from *S. gregaria* were incubated under air at 30°C in 0.1 ml of TC 199 medium containing 0.29 mM-[¹⁴C]methionine (sp. radioactivity 36.5 mCi/mmol) and 20 μM-[³H]farnesenic acid (25 mCi/mmol), for appropriate periods of time. Medium was separated from the glands by aspiration, and the glands were washed briefly with 0.1 ml of non-radioactive medium; reactions were immediately stopped by addition of 0.2 ml of ethanol to the glands and to the combined medium and washings. Labelled products were separated and measured as described in Fig. 1. The amount of C_{16} juvenile hormone synthesized was calculated from the amount of [¹⁴C]methionine incorporated. Each point represents an individual determination.

Fig. 4. *Molar incorporation ratios observed during the first* 60 min *incubation with* [¹⁴C]*methionine and* [³H]*farnesenic acid*

Incubation conditions, separation of glands from medium and analysis of labelled products were as described in Fig. 3. The incorporation of radioactivity from both labelled substrates into methyl farnesoate and C_{16} juvenile hormone was used to calculate the apparent rate of molar incorporation from their known specific radioactivities in the medium, and the results are expressed as a molar incorporation ratio. ▲, Methyl farnesoate; △, C_{16} juvenile hormone within the glands; ○, C_{16} juvenile hormone released into the medium. Each point represents an individual determination.

female locusts, the C_{16} juvenile hormone is rapidly released from the glands once its synthesis is complete (Pratt & Tobe, 1974a). Fig. 3 shows the rate of release of C_{16} juvenile hormone from the glands (based on the incorporation of [¹⁴C]methionine), which indicates that it is linear over a period of 4 h and that the establishment of this constant rate of release occurs after a lag period not exceeding 15 min. Significant quantities of labelled juvenile hormone are detectable in the medium after 10 min incubation. The progress curve for total C_{16} juvenile hormone synthesis (omitted from Fig. 3 for the sake of clarity) describes an essentially similar relationship, owing to the fact that the hormone is not stored to any appreciable extent within the glands. Only at incubation times of less than 20 min is the radioactivity of hormone within the glands equal to or greater than that present in the medium. Shortly thereafter the radioactive hormone within the glands assumes a steady-state value corresponding to between 10 and 20 pmol of C_{16} juvenile hormone/pair of glands.

We have also examined the relative rates of incorporation of [¹⁴C]methionine and [³H]farnesenic acid into both C_{16} juvenile hormone and methyl farnesoate. The molar incorporation ratios into these compounds were calculated from the measured radioactivity on the basis of the known final sp. radioactivity of the respective radioactive precursors in the incubation medium. It has already been
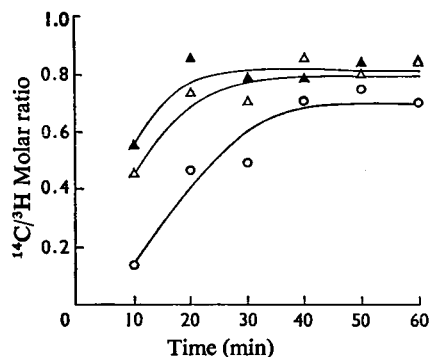
reported that the isotope incorporation ratios measured on the product resulting from incubations lasting 3 h approximate to unity (Pratt & Tobe, 1974b). Any divergence from this value during the initial stages of the incubation presumably indicates a difference in the relative rates of penetration and isotopic equilibration of the two labelled substrates into those precursor pools which provide the immediate substrates for the esterification reaction. Fig. 4 shows that the ¹⁴C/³H ratios are significantly less than one during the initial stages of incorporation into the methyl farnesoate, the C_{16} juvenile hormone within the gland and the C_{16} juvenile hormone released into the medium. The molar incorporation ratios stabilize at values approximating to unity, in the following predicted sequence: methyl farnesoate by 20 min, C_{16} juvenile hormone within the gland by 30 min, C_{16} juvenile hormone released into the medium by 40 min (asymptotic). These results indicate that exogenous labelled farnesenic acid is incorporated into methyl farnesoate earlier than exogenous labelled methionine, but that this apparent difference in equilibration time is no longer manifested in the product of the esterification reaction by 20 min. It appears to take a further period of approx. 10 min for this disproportionately labelled material to pass through the pool of C_{16} juvenile hormone within the gland.
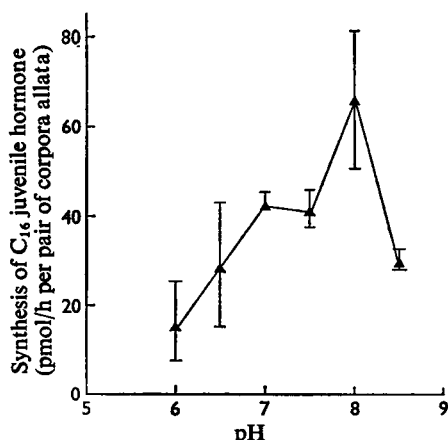
Fig. 5. *Influence of H^+ concentration on the rate of synthesis of C_{16} juvenile hormone*

In each experiment two pairs of corpora allata from *S. gregaria* were incubated at 30°C under air for 3 h in 0.1 ml of Ringer solution (see the Experimental section) containing 0.29mM-[^{14}C]methionine (sp. radioactivity 36.5mCi/mmol) and 20μM-[^{3}H]farnesenic acid (25mCi/mmol). The pH of the Ringer solution was adjusted before addition of the labelled substrates with either 0.1M-NaOH or 0.1M-HCl. The reactions were terminated and the contents of the tubes were analysed as described in Fig. 1. Each point represents the mean from four incubations and the vertical bars indicate the total observed range.

Effect of pH on incorporation of [^{3}H] farnesenic acid

The influence of extra-glandular pH on the rate of incorporation of [^{3}H]farnesenic acid into C_{16} juvenile hormone was investigated by using Ringer solution containing optimum L-methionine concentration. The pH was adjusted with either 0.1M-NaOH or 0.1M-HCl before sterile filtration, which also served to remove the precipitate of calcium phosphate which formed at pH values greater than 7.5. The relationship shown in Fig. 5 is the arithmetic mean of four separate experiments, each of which have the same characteristic curve. It can be seen that the relationship is complex and appears to consist of several components. The synthetic activity of the glands is similar at pH7.0 and 7.5; moreover, there is a low inherent variability in this pH range. Below this pH, there is a progressive decrease in the synthetic activity of the glands and the overall reproducibility decreases markedly. There is a similar variability at pH8.0, although in all cases the rate of synthesis of hormone by the glands is maximum at this pH. At pH8.5, the highest value tested, the synthetic activity of the glands again declines.

These results indicate that the synthetic activity of the glands may be determined most reliably over the pH range 7.0 to 7.5, although the values obtained are less than those realised at pH8.0. Incubation of the glands at pH6.5 or lower is clearly disadvantageous since it results in rates of hormone biosynthesis which are both low and irreproducible.

Discussion

This is the first reported assessment of the influence of culture conditions on the rate of juvenile hormone synthesis by corpora allata of any insect, and represents a first step in an on-going investigation of the endocrine functions of the corpora allata of adult female *S. gregaria*. In the present paper we concern ourselves solely with the ability of the glands to effect the last two stages in the biosynthesis of juvenile hormone, namely the formation of the olefinic ester, methyl farnesoate, and its epoxidation at the C_{10}–C_{11} double bond to yield C_{16} juvenile hormone. The remarkably high rate of C_{16} juvenile hormone biosynthesis from added [^{3}H]farnesenic acid by isolated corpora allata of adult female *S. gregaria* (Pratt & Tobe, 1974a) makes these glands a particularly convenient tool for examining the influence of experimental conditions on the activity of the last two enzymic stages and facilitates a precise kinetic analysis of the incorporation.

Influence of substrate concentrations

During the course of sexual maturation in female *S. gregaria*, there are changes in the ability of the corpora allata to synthesize C_{16} juvenile hormone either *de novo* or from [^{3}H]farnesenic acid (S. S. Tobe & G. E. Pratt, unpublished work). Our results indicate that glands having different capacities for synthesizing juvenile hormone from [^{3}H]farnesenic acid require similar concentrations of the acid for optimum response. On this basis we recommend the use of [^{3}H]farnesenic acid at concentrations between 15 and 25μM for the routine measurement of the overall enzymic competence of corpora allata to effect the last two stages in C_{16} juvenile hormone biosynthesis.

The extremely low rates of C_{16} juvenile hormone biosynthesis exhibited by glands incubated in media containing no L-methionine indicate that the glands contain no appreciable stores of this or any alternative methyl donor. The glands are capable of high synthetic activity over a finite range of L-methionine concentrations (0.1–0.4mM), and appear to have an optimum response at 0.3–0.4mM. This is in good agreement with our previous results (Pratt & Tobe, 1974a) from experiments utilizing casein hydrolysate to adjust *inter alia* the concentration of L-methionine in the medium. This is consistent with the suggestion that L-methionine is the most important source of C_1 units for esterification, which derives from the finding

that stoicheiometric amounts of labelled methionine and farnesenic acid are incorporated into the hormone product (Pratt & Tobe, 1974a). We report here a similar stoicheiometry from incubations in TC 199 medium, indicating that the glands do not utilize any of the potential C_1 donors present in this complete medium to a significant extent. The reported concentration of L-methionine in the haemolymph of adult female *S. gregaria* lies within the range in which the glands have high synthetic activity *in vitro* (Benassi *et al.*, 1961). We can offer no explanation for the overt inhibition of C_{16} juvenile hormone synthesis at concentrations in excess of 0.4mM-L-methionine.

It is hardly surprising that O_2 is an essential substrate for the epoxidation of methyl farnesoate to C_{16} juvenile hormone. In addition the pronounced inhibition of esterification of [^{3}H]farnesenic acid occurring under anaerobic conditions indicates a general metabolic dependence by the glands on aerobic respiration.

Influence of pH

Isolated corpora allata are capable of synthesizing C_{16} juvenile hormones from [^{3}H]farnesenic acid over the entire pH range 6.0–8.5. However, both the rate of synthesis and its reproducibility varies markedly within this range. Consistently high (although not maximal) rates of synthesis were obtained at pH 7.0 and 7.5, whereas outside this range rates of synthesis were much more variable. It is noteworthy that at pH values of 6.5 or lower, the rate of synthesis of juvenile hormone is both unpredictable and markedly suppressed. Thus the use of media of low pH such as that of Grace (1962), which has been used by other workers to investigate the synthesis of juvenile hormones by corpora allata of a closely related species (*S. vaga*)(Judy *et al.*, 1973a), is not to be recommended on the basis of our more detailed investigations.

Kinetics of incorporation

Measurements of the incorporation of both [^{3}H]farnesenic acid and [^{14}C]methionine into methyl farnesoate and the final product, C_{16} juvenile hormone (Fig. 4) show a marked disparity between the incorporations of these two compounds during the first 30min of incubation. The possible causes of these early differential incorporations have not been investigated.

The earlier attainment of stoicheiometric incorporation in methyl farnesoate than in C_{16} juvenile hormone provides additional kinetic evidence for the suggestion that methyl farnesoate is an obligatory intermediate in the biosynthesis of C_{16} juvenile hormone (Pratt & Tobe, 1974a). Similar considerations of the pattern of incorporation into C_{16} juvenile hormone present within the gland indicate

that this component is also turning over rapidly, and this is confirmed by the fact that the separate radioactivities in this component reach a constant steady-state value after 30min incubation. As is to be expected in a terminal accumulation pool, the molar incorporation ratios in C_{16} juvenile hormone released from the gland increase to a value of one asymptotically, and are experimentally distinguishable from one until 2h after the start of the incubation.

The very high rate at which peripheral tissues such as fat-body, mid-gut and Malpighian tubules convert [^{3}H]farnesenic acid into water-soluble derivatives *in vitro* (G. E. Pratt, unpublished work) make it very unlikely that exogenous farnesenic acid is available to the corpora allata *in vivo*. The addition of farnesenic acid to the incubation medium apparently by-passes earlier steps in juvenile hormone biosynthesis, which appear to be rate-limiting under our rearing conditions, and allows the direct measurement of the ability of the glands to carry out the final two stages in juvenile hormone biosynthesis. The method we have described appears to be satisfactory in that, apart from early transient differences in the rates of incorporation of [^{14}C]-methionine and [^{3}H]farnesenic acid, the rates of synthesis and release of hormone are constant for at least 4h. The ability to quantify independently both the rate of hormone synthesis and its rate of release from the corpora allata should provide a basis for monitoring the endocrine activity of the glands during development and maturation, and in particular should facilitate the elucidation of those stages in the synthesis *de novo* of juvenile hormone which are subject to physiological control under different environmental conditions.

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