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Long-term in vitro incubation of the locust corpus allatum

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Abstract

Corpora allata (CA) of sexually mature female locusts (*Locusta migratoria*) were incubated in a new agar-based medium up to 30 days at 33°C and tested periodically by short-term radiochemical assay for juvenile hormone-III (JH-III) production. After keeping the CA for 24-48 h under conditions of long-term incubation, a marked increase in activity was observed initially weakly active glands, but not in initially highly active ones. Subsequently, the activity declined, but on average it remained higher for up to 26 days than glands with low initial levels. The degree of activation of the CA at 24-48 hours and the duration of their viability in the conditions of long-term incubation differed among different sets of experiments. Variations in the activity of different glands tested on the same day and those of the same glands tested on different days were considerable, as high as the variations found in freshly excised glands. We conclude that: (1) mature adult CA, that are already active, show an endogenous JH-biosynthetic activity in vitro for several weeks without the involvement of insect-derived components outside of the CA, and (2) mature adult CA that exhibit low initial rates of JH-production, are subject to some degree of inhibition in vivo which seems to be relieved during long-term incubation and/or to some degree of (non-specific?) activation in vitro. However, long-term incubation does not activate the CA to the full capacity of their epoxidase and methyl transferase enzyme systems because farnesoic acid (FA)-stimulated rates of JH-III production were always higher than basal rates.

Key Word Index: Corpus allatum, juvenile hormone-III production, long-term incubation, short-term radiochemical assay, in vitro activation of low activity corpus allatum, *Locusta migratoria.*

Introduction

Long-term *in vitro* incubation of insect corpora allata (CA) had been reported about two decades ago (Marks, 1970; Seshan and Levi-Montalcini, 1971), mostly as a convenient method to yield sufficient quantitites of juvenile hormone (JH) for characterization of the hormone(s) produced by the CA (Röller and Dahm, 1970; Judy *et al.*, 1973a, 1973b, 1975, Müller *et al.*, 1974, Jennings *et al.*, 1975). However, only Judy *et al.* (1973a) provided definitive proof that the incubated CA indeed produced JH in vitro for up to 15 days. More recently Wilhelm *et al.* (1987) investigated JH production by *in vitro* incubated CA, but they tested the glands for only 4 days and added haemolymph to the medium. In the mid-1970's, research interest shifted from long-term incubation of the CA to a short-term radiochemical assay developed by Pratt and Tobe (1974) first for the CA of the desert locust (*Schistocerca gregaria*). The radiochemical assay has been improved and adapted successfully to measure the activity of the CA of many other insects in various developmental stages, with the assumption that JH production measured in vitro with this assay reliably reflects in vivo JH secretory activity of the CA (review by Tobe and Stay, 1985).

We report here on long-term incubation of CA taken from sexually mature female locusts and cultured in vitro in a new agar-based medium, for up to 30 days, without any insect derived component in the medium except the CA. We assessed by the short-term radiochemical assay (see above) JH production by individual glands before and periodically during long-term incubation. This combination of long-term incubation of the CA and periodical assessment of their secretory activity may be useful for testing on the CA effects of long-term regulatory factors, such as allatotropic or allatostatic neuropeptides (review by Tobe and Stay, 1985; recent summarizing articles by Applebaum *et al.*, 1991, Stay *et al.*, 1991, Pratt *et al.*, 1991, Kataoka *et al.*, 1989, Bhashkaran *et al.*, 1990).

Materials and methods

Crowded locusts, *Locusta migratoria migratorioides* (R.& F.), from the Jerusalem stock colony (Lazaroviçi and Pener, 1977) were maintained in 60-litre locust cages (Hunter-Jones, 1961), under continuous illumination and high but not constant temperatures (32-39°C) with some daily fluctuations. The locusts were fed daily on graminae, usually Kikuyu grass (*Pennisetum clandestinum*), and flaked oats, *ad lib.*

Corpora allata were excised, under sterile conditions, from 20-30-day-old sexually mature adult females. Glands originating from locusts kept in the same cage and excised on the same day were considered as a set of experiments. Each gland was cleaned of adherent tissues and nerves and placed with the aid of a steel loop into minimum essential medium (MEM). The rates of JH-III production of such freshly excised glands (also termed as day 0 glands) were measured individually, under sterile conditions, for 2-3 h with the radiochemical assay of Pratt

and Tobe (1974), as modified by Gadot and Applebaum (1985). In some instances, after assessment of the basal rate of JH-III production, the glands were submitted immediately to a new short-term (2-3 h) radiochemical assay with 100 μmolar farnesoic acid (FA, 70% pure, Sandoz Crop Protection, Palo Alto, California) in the medium. Farnesoic acid is transformed to JH-III in the CA by epoxidase and methyl transferase and these two enzymes are not rate limiting. Therefore, presence of FA in the medium bypasses earlier rate limiting step(s) in JH-III biosynthesis and so stimulates JH-III production (Tobe and Pratt, 1976, reviews: Feyereisen, 1985, Tobe and Stay, 1985).

Following the short-term radiochemical assay, individual glands were transferred to long-term incubation. The jelly-like "long-term medium" was freshly prepared by mixing 100 fl of LB-15 medium (Munderloh and Kurtti, 1989; with minor modifications), fortified with glucose, tryptose phosphate broth (TPB, Difco), fetal calf serum (FCS) and gentamicine, with 200 fl of modified MEM containing 1% agar (kept at 40-50°C to prevent solidification) in a 2-cm-diameter watch glass. The final concentration of the gentamicine was 50 μg/ml and that of the TPB and FCS was 6.7% each. A more detailed description of this long-term medium is provided elsewhere (Gadot, Pener and Schal, submitted). With the aid of a steel loop, a single gland was placed onto the medium (which had meanwhile cooled and solidified) in each watch glass. The watch glass was covered immediately with a glass cover and sealed with hot paraffin to prevent evaporation. The CA in the watch glasses were incubated at 33°C. Periodically, the CA were removed from the long-term medium and their basal, and sometimes also FA-stimulated, rates of JH-III production were assessed by the short-term radiochemical assay. The glands were then returned to fresh long-term medium which was thus changed every few days. All procedures were performed under sterile conditions.

Results

Corpora allata (CA) kept in the long-term medium and submitted periodically to short-term radiochemical assays continued to produce JH-III in vitro up to 30 days (Fig. 1A). However, glands from the same set of experiments showed large variations in the rates of JH-III production and the variations were considerable also between different sets of experiments (compare Fig. 1A to 1B). Data presented for individual glands clearly show that these variations were high both for different glands tested on the same day and for the same gland tested on different days (Fig. 2).

Fig. 1A and 1B indicate low JH-III production by freshly excised (day 0) glands and an increase in CA activity on day 2. Fig. 2 shows a similar trend, except for one gland whose activity decreased from day 0 through days 1 and 2. Glands, showing a decrease in activity during the first 1-2 days of long-term incubation were less frequent than glands exhibiting an increase in activity during this period. Nevertheless, in a few sets of experiments the

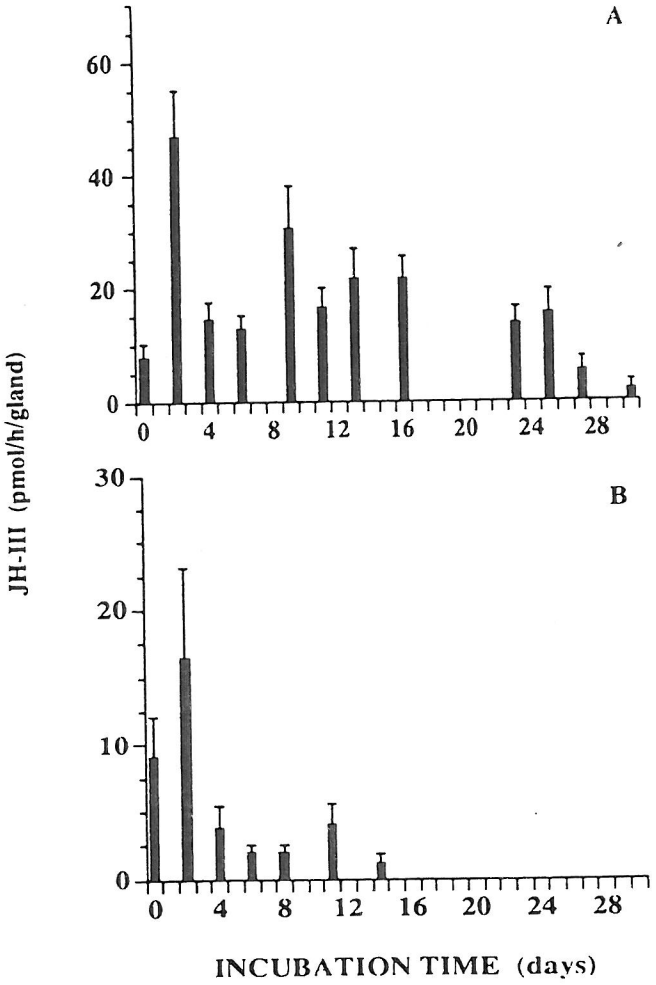

Fig. 1. Rates of JH-III production (pmol/h/gland) by CA kept under long-term incubation (days), as assessed periodically by short-term radiochemical assay; average values (columns) and SE (bars on top of columns) are shown. A and B present two independent sets of experiments, each based on 8 glands (n=8), with relatively high and low rates of JH-III production, respectively. Note difference in scale of ordinate between A and B. Glands of set B were not assayed beyond day 14.

proportion of the former was higher than usual (in set A, Table 1, even higher than 50%). Detailed inspection of the data revealed that such a decrease in activity was correlated with a high initial (day 0) rate of JH-III production. Table 1 clearly demonstrates this point: the activity of initially (day 0) weakly active glands increased on day 1 or 2, whereas that of initially highly active glands decreased. The differences between glands exhibiting high and low initial activity disappeared later (see day 10 and 11 in Table 1).

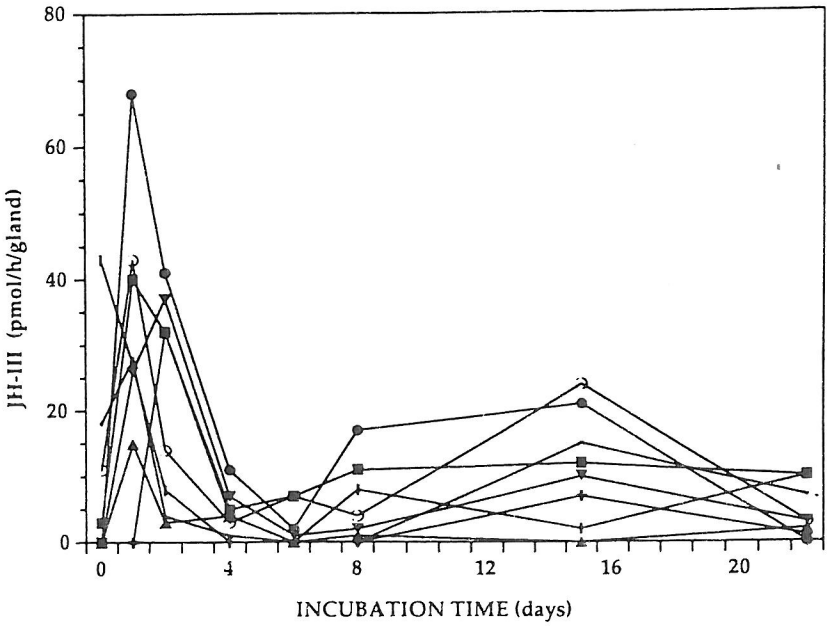

Fig.2. Rates of JH-III production (pmol/h/gland) by CA kept under long-term incubation (days), as assessed periodically by short-term radiochemical assay. Results are presented for 8 individual glands (each marked by a different symbol) in one set of experiments (different from those shown in Fig. 1).

Table 1. Rates of JH-III production in pmol/h/gland, average ± SE (in parentheses, n), by CA showing initially (day 0) low (<20 pmol/h/gland) or high (>20 pmol/h/gland) activity in two independent sets of long-term incubation experiments.*

Set+	day of incubation	Initial activity of CA	
		Low	High
A	0°	7.9±1.7 (8)	85.6±12.0 (10)
	1	20.0±3.4 (8)	37.4±7.3 (10)
	3	3.1±0.5 (8)	4.0±0.5 (10)
	10	10.2±4.5 (3)	11.3±5.1 (6)
B	0°	1.6±1.6 (5)	92.7±18.3 (3)
	1	16.0±7.0 (5)	34.0±7.5 (3)
	2	6.4±1.1 (5)	3.3±1.4 (3)
	11	5.4±1.5 (5)	4.3±2.3 (3)

*CA were kept under long-term incubation and assessed periodically by short-term radiochemical assay.
+Sets are selected for high proportion of initially (day 0) highly active glands; these sets are different from those presented in Fig. 1 and 2.
°Freshly excised glands.

Addition of FA to the medium of the short-term radiochemical assay always stimulated JH-III production. However, on the first 3 days of the long-term incubation, both highly active and relatively inactive CA exhibited similar FA-stimulated rates (Table 2). Consequently, the ratio FA-stimulated rate/basal rate was much higher for glands showing low basal rates than for those showing high basal rates. FA-stimulated rates of JH-III production declined considerably during the first few days of long-term incubation (Table 2), and remained relatively unchanged for two-three weeks, before declining again (Table 3).

Table 2. Basal and farnesoic acid (FA)-stimulated rates of JH-III production in pmol/h/gland, average ± SE (in parentheses, n), by CA showing initially (day 0) low (<20 pmol/h/gland) or high (>20 pmol/h/gland) activity during the first few days of long term incubation.*,+

Day of incubation	Initial activity of CA			
	Low		High	
	Basal rate	FA-Stimulated rate	Basal rate	FA-stimulated rate
0°	10.5±1.4 (4)	169.0±4.7 (5)	93.0±23.4 (4)	185.0±22.1 (4)
1	20.0±3.4 (8)	67.9±5.8 (8)	37.4±7.3 (10)	75.7±11.9 (10)
3	3.1±0.5 (8)	46.5±8.0 (8)	4.0±0.5 (10)	49.2±7.6 (10)

* and ° As in Table 1
+ Same set of experiments as A in Table 1

Table 3. Basal and farnesoic acid (FA)-stimulated rates of JH-III production in pmol/h/gland, average ±SE (in parenthesis, n), by CA kept for longer periods under long-term incubation.*

Set+	Day of incubation	Basal rate	FA-stimulated rate
I	11	6.2±2.1 (8)	39.8±5.9 (8)
	13	8.4±1.5 (8)	42.3±6.4 (8)
II	15	11.4±3.0 (8)	55.4±7.8 (8)
	22	4.5±1.4 (8)	21.8±5.2 (8)

* As in Table 1
+ These sets of experiments are different from those shown in Table 1.

Discussion

The present study demonstrates that CA originating from sexually mature adult females of *Locusta migratoria* can be cultured in a medium without any insect-derived component, except those in the CA, and the glands continue to produce JH-III in vitro for several weeks. We do not claim that our long-term medium is optimal. In fact, the decrease of FA-stimulated rates of JH-III production within the first few days of the long-term incubation (Table 2) and later (Table 3) indicates suboptimal conditions. Nevertheless, our findings allow some conclusions to be drawn.

Assessed by the short-term radiochemical assay, freshly excised CA of *L. migratoria* show very large variations in the rates of JH-III production (Girardie et al., 1981, Pratt and Pener, 1983, Couillaud and Girardie, 1985, Gadot and Applebaum, 1985). These variations are usually explained by brief periods of maximum glandular activity, i.e. the glands are assumed to produce JH in "pulses". The present results show that the variations in the rates of JH-III production of CA kept in long-term incubation are as high as those observed in freshly excised glands (compare SE for day 0 to those for later days in Figs. 1A and 1B). Moreover, the same individual gland may show large variations in the rates of JH-III production on different days of long-term incubation (see Fig. 2). These findings suggest that the "pulsatile" JH-III producing activity is an endogenous characteristic of the CA which may operate also in vivo without specific neural or neurosecretory signals. Alternatively, though less probably, the "pulsatile" activity of the long-term-incubated CA may be an experimental artefact, but then the similar large variations in JH-III production shown by freshly excised glands (such glands are presumed to reflect in vivo activity, see Introduction) and by glands kept in long-term incubation must be just a coincidence.

The phenomenon of temporarily increasing activity in initially (day 0) weakly active glands and decreasing activity in initially highly active CA within the same sets of experiments (Table 1) is surprising because a standardized long-term medium would not be expected to induce such contrasting heterogenous effects. CA showing high initial activity are rather infrequent; Table 1 presents selected sets of experiments with a relatively high proportion of such glands. Fig. 2 (one gland out of 8 showing high initial activity and subsequent decrease) and Fig. 1 (low average activity of the glands on day 0 and an increase by day 2) present more typical results. The presumably pulsatile activity of the CA (see above) may well account for the low proportion of glands with high inital activity and the decrease in their JH-production may also be explained by pulsatile activity and/or by relatively rapid depletion in the glands of some rate limiting factor or substance which is then replaced only slowly and/or in low quantities from the medium.

However, pulsatile activity alone seems to be insufficient to explain the high proportion of glands showing a rather high increase in activity after a day or two of long-term incubation. Pulsatile activity allows for large differences in average activity of CA on different days, but because it also leads to large variations among different glands tested on the same day, the averages obtained for different days should not differ significantly. However, in the set of experiments shown in Fig. 1A, the difference in the rates of JH-III production between day 0 and day 2 was statistically highly significant ($P<0.01$, non-parametric randomization test for matched pairs, see Siegel, 1956); glands kept in long-term incubation for two days produced about 6-fold more JH-III than freshly excised glands. Data on which Fig. 1B and Fig. 2 are based reveal a similar trend, though with smaller differences. The results presented in Table 1 are not representative in this respect because the sets are selected to show a high proportion of initially highly active glands whose activity then declines.

It may be concluded, therefore, that pulsatile activity cannot be the sole factor to account for the increase in the average rate of JH-III production from day 0 to day 1 or 2 (Figs. 1 and 2). If so, one must infer that a day or two of long-term incubation relieves some in vivo inhibition presumably still operating in most freshly excised glands, or that glands that exhibit low initial rates of JH-III production are subject to some in vitro activation.

Dale and Tobe (1988) reported that CA of adult females of *L. migratoria* exhibit a heterogenous response to high concentration of potassium; glands showing low initial rates of JH production were stimulated strongly by potassium, while those showing high initial rates of JH production much less so. Although these authors used repeated short-term radiochemical assays and so followed the rate of JH-III production only up to a maximum of 450 min, the heterogenous response of the glands to potassium found by Dale and Tobe (1988), and the heterogenous response to one or two days of incubation in the long-term medium found in the present study, are surprisingly similar. However, the mechanism(s) responsible for such heterogenous response of the CA is (are) not yet known in either case.

Production of JH-III by CA kept for several weeks under long-term incubation, the possibility of pulsatile JH-III production by denervated CA kept in vitro without insect-derived components (except the gland itself), and the heterogenous response of the glands in the first day or two of long-term incubation, strongly suggest some "autoregulation" of the CA. Therefore, the glands may be less dependent than generally presumed on immediate neural or neurosecretory signals and these signals may only tune or modulate the activity of the glands. However, it must be stressed that such "autoregulation" seems to be limited to already active CA. We repeatedly observed, in several independent sets of experiments (Gadot, Pener and Schal, unpublished), that inactive CA, originating from newly fledged females, do not become active under the present conditions of long-term incubation.

The limited "autoregulation" of already active CA does not invalidate possible utility of our long-term incubation system for studying effects of neurohormonal regulatory factors on

the CA (see INTRODUCTION, also for references). However, the existence and possible effects of some limited "autoregulation" should be taken into account in such studies.

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References

Applebum S.W., Gadot M., Hirsch J. and Abd El-Hadi F. (1991) Allatal stimulation and inhibition in locusts. In Insect Neuropeptides (Edited by Menn J.J., Kelly T.J. and Masler E.P.), pp. 152-163. ACS Symposium Series No. 453, American Chemical Society, Washington D.C.

Bhaskaran G., Dahm K.H., Barrera P., Pacheco J.L., Peck K.E. and Muszynska-Pytel M. (1990) Allatinhibin, a neurohormonal inhibitor of juvenile hormone biosynthesis in *Manduca sexta*. *Gen. comp. Endocr.* **78**, 123-136.

Couillaud F. and Girardie A. (1985) Control of C-16 juvenile hormone biosynthesis in active corpora allata of female African locusts. *Int. J. Invertebr. Reprod. Dev.* **8**, 303-315.

Dale J.F. and Tobe S.S. (1988) Effect of high potassium concentrations on juvenile hormone release in vitro from corpora allata of *Locusta migratoria*. *Physiol. Ent.* **13**, 21-27.

Feyereisen R. (1985) Regulation of juvenile hormone titer: Synthesis. In Comprehensive Insect Physiology Biochemistry and Pharmacology, Vol. 7, Endocrinology I (Edited by Kerkut G.A. and Gilbert L.I.), pp. 391-429. Pergamon Press, Oxford.

Gadot M. and Applebaum S.W. (1985) Rapid in vitro activation of corpora allata by extracted locust brain allatotropic factor. *Arch. Insect Biochem. Physiol.* **2**, 117-129.

Girardie J., Tobe S.S. and Girardie A. (1981) Biosynthèse de l'hormone juvenile C*16* (JH-III) et maturation ovarienne chez le criquet migrateur. *C. r. hebd. Seanc. Acad. Sci.* Paris Série III, **293**, 443-446.

Hunter-Jones P. (1961) Rearing and Breeding Locusts in the Laboratory. Anti-Locust Research Centre, London.

Jennings R.C., Judy K.J., Schooley D.A., Hall M.S. and Siddall J.B. (1975) The identification and biosynthesis of two juvenile hormones from the tobacco budworm moth (*Heliothis virescens*). *Life Sci.* **16**, 1033-1039.

Judy K.J., Schooley D.A., Dunham L.L., Hall M.S., Bergot B.J. and Siddall J.B. (1973a) Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. Natl. Acad. Sci.* U.S.A. **70**, 1509-1513.

Judy K.J., Schooley D.A., Hall M.S., Bergot B.J. and Siddall J.B. (1973b) Chemical structure and absolute configuration of a juvenile hormone from grasshopper corpora allata *in vitro*. *Life Sci.* **13**, 1511-1516.

Judy K.J., Schooley D.A., Troetschler R.G., Jennings R.C., Bergot B.J. and Hall M.S. (1975) Juvenile hormone production by corpora allata of *Tenebrio molitor in vitro*. *Life Sci.* **16**, 1059-1066.

Kataoka H., Toschi A., Li J.P., Carney R.L., Schooley D.A. and Kramer S.J. (1989) Identification of an allatotropin from adult *Manduca sexta*. *Science* **243**, 1481-1483.

Lazarovici P. and Pener M.P. (1977) Juvenile hormones (JHs) and completion of oocyte development in the African migratory locust: A comparative and quantitative study. *Gen. comp. Endocr.* **33**, 434-452.

Marks E.P. (1970) The action of hormones in insect cell and organ cultures. *Gen comp. Endocr.* **15**, 289-302.

Müller P.J., Masner P., Trautmann K.H., Suchý M. and Wipf H.-K. (1974) The isolation and identification of juvenile hormone from cockroach corpora allata *in vitro. Life Sci.* **15**, 915-921.

Munderloh U.G. and Kurtti T.J. (1989) Formulation of medium for tick cell culture. *Exp. appl. Acarology* **7**, 219-229.

Pratt G.E. and Pener M.P. (1983) Precocene sensitivity of corpora allata in adult female *Locusta migratoria* after electrocoagulation of the pars intercerebralis neurosecretory cells. *J. Insect Physiol.* **29**, 33-39.

Pratt G.E. and Tobe S.S. (1974) Juvenile hormones radiobiosynthesised by corpora allata of adult female locusts *in vitro. Life Sci.* **14**, 575-586.

Pratt G.E., Farnsworth D.E., Siegel N.R., Fok K.F. and Feyereisen R. (1991) Two types of allatostatic peptides from brains of the cockroach Diploptera punctata. In Insect Neuropeptides (Edited by Menn J.J., Kelly T.J. and Masler E.P.), pp. 177-192. ACS Symposium Series No. 453, American Chemical Society, Washington D.C.

Röller H. and Dahm K.H. (1970) The identity of juvenile hormone produced by corpora allata *in vitro. Naturwissenschaften* **57**, 454-455.

Seshan K.R. and Levi-Montalcini R. (1971) In vitro analysis of corpora cardiaca and corpora allata from nymphal and adult specimens of *Periplaneta americana. Arch. ital. Biol.* **109**, 81-109.

Siegel S. (1956) Nonparametric Statistics for the Behavioral Sciences. McGraw Hill, Kogakusha, Tokyo.

Stay B., Woodhead A.P., Joshi S. and Tobe S.S. (1991) Allatostatins; Neuropeptide inhibitors of juvenile hormone synthesis in brain and corpora allata of the cockroach *Diploptera punctata.* In Insect Neuropeptides (Edited by Menn J.J., Kelly T.J. and Masler E.P.), pp. 164-176. ACS Symposium Series No. 453, American Chemical Society, Washington D.C.

Tobe S.S. and Pratt G.E. (1976) Farnesenic acid stimulation of juvenile hormone biosynthesis as an experimental probe in corpus allatum physiology. In The Juvenile Hormones (Edited by Gilbert L.I.), pp. 147-163. Plenum Press, New York and London.

Tobe S.S. and Stay B. (1985) Structure and regulation of the corpus allatum. *Adv. Insect Physiol.* **18**, 305-432.

Wilhelm R., Riechsteiner R. and Lanzrein B. (1987) On the competence of corpora allata to synthesise juvenile hormone and the dependence of their activity on haemolymph factors in the cockroach, *Nauphoeta cinerea. Insect Biochem.* **17**, 971-975.