Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects

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Summary. The prothoracic glands of a variety of insects were tested for their ability to synthesize ecdysteroids in vitro. More specifically, they were evaluated for their ability to produce 3-dehydroecdysone and ecdysone using both radioimmunoassay and reverse phase high performance liquid chromatography. Three categories of insect prothoracic glands were noted: a) those producing much more 3-dehydroecdysone than ecdysone; b) glands synthesizing almost equivalent amounts of each of these two ecdysteroids; c) prothoracic glands that yielded more ecdysone than 3-dehydroecdysone. In addition, the 3-oxoecdysteroid 3β-reductase activity of the hemolymph of these insects was evaluated for its ability to convert 3-dehydroecdysone to ecdysone. The lepidopteran species tested yielded the most potent enzyme activity, although activity was demonstrated in members of other orders. These data indicate that the dehydroecdysone-ecdysone axis is not restricted to moths and butterflies.
Key words. Molting hormone; 3-dehydroecdysone; 3-oxoecdysteroid 3β-reductase; ecdysteroid; prothoracic glands.

The hemolymph ecdysteroid titer determines many aspects of insect development and molting and is itself regulated in large measure by the synthetic activity of the prothoracic glands[1]. Although it was originally believed that ecdysone was the sole ecdysteroid secreted by the prothoracic glands[2,3], recent studies have shown that 3-dehydroecdysone is the major ecdysteroid secreted by the prothoracic glands of *Manduca sexta*, that it is rapidly converted to ecdysone by a hemolymph 3-oxoecdysteroid 3β-reductase (3β-reductase), and that ecdysone is then hydroxylated to the more active molting hormone, 20-hydroxyecdysone, by peripheral tissues[1,4,5].

Since the original studies only utilized the prothoracic glands of *Manduca* and the 3β-reductase of the hemolymph of 3 species of moths, it was important to determine if the dehydroecdysone-ecdysone axis was a phenomenon characteristic of the Lepidoptera, was a new central dogma relevant to all insects, or was a rather specific occurrence in one or a few species of moths. Such studies cannot be conducted by simply monitoring the hemolymph ecdysteroid titer with a radioimmunoassay (RIA) using a single antibody preparation even if coupled to high performance liquid chromatography (HPLC)[6,7], both because of the inability of most antisera now utilized to quantify both side chain and A ring substituted ecdysteroids, and because 3-dehydroecdysone is rapidly converted to ecdysone in the hemolymph. We have therefore used in vitro procedures and several ecdysteroid antisera to investigate the prevalence of the dehydroecdysone-ecdysone axis in insects representing several orders.

Materials and methods

Larvae of the silkworm, *Bombyx mori*, (Gunpo-Shuhgyoku), were reared on an artificial diet under a 12L:12D photoperiod at 25 ± 1 °C[8], while *Manduca sexta* were raised on an artificial diet under a 18L:6D photoperiod at 26 ± 1 °C.

Sarcophaga peregrina, Calliphora vicinia, Tenebrio molitor and *Leucenia separata* were from stocks raised in our laboratories under standard conditions. Representatives of other species were collected near Kanazawa University (*Pieris rapae, Papilio xuthus, P. protenor*) and raised on their food plant in the laboratory, or were graciously supplied by our colleagues: *Leucophaea maderae* (J. Koeppe, U.N.C.); *Aedes aegypti* (L. Whisenton, U.N.C.); *Periplaneta americana* (M. Miura, NIH, Japan); *Galleria mellonella* (N. Granger, U.N.C.); *Precis coenia* (H.F. Nijhout, Duke Univ.); *Heliothis virescens, H. zea, Trichoplusia ni, Epilachna varivestis* and *Leptinotarsa decemlineata* (N.C. State University); *Locusta migratoria* (K. Yamazaki, Tokyo Metropolitan Univ.). Lyophilized hemolymph of diapausing *H. zea* was from R. Meola (Texas A&M Univ.) while lyophilized hemolymph of adult locust was a gift from S. Applebaum (Hebrew Univ.).

Ecdysone and 20-hydroxyecdysone were purchased from Sigma, Simes and Rhoto, while ponasterone A was a gift from Takeda Pharmaceutical Ltd. and 3-dehydroecdysone was a gift from M. Thompson (USDA). [1,2-^{3}H]Cholesterol (56 Ci/mmol) and [23,24-^{3}H]ecdysone (60 Ci/mmol) were obtained from New England Nuclear Corp. [^{3}H]Dehydroecdysone was recovered from the medium in which *Manduca* prothoracic glands were incubated with [1,2-^{3}H]cholesterol and was purified by J. Warren[4]. All labelled ecdysteroids and precursors were purified by RP-HPLC before use[4,6].

Prothoracic glands and ring glands were dissected out as described previously[4,5,8,9] and incubated in 50 or 100 µl Grace's medium (GIBCO) for 2–6 h at 25 °C. For incubation of the prothoracic glands with protease, one pair of glands was incubated in 100 µl Grace's medium containing 0.02 % protease (type XXV, Sigma)

for 4 h. Ecdysteroids were extracted, separated and identified basically by RIA and RP-HPLC[4,5,7]. For RP-HPLC, a C_{18} μBondasphere column (Waters, 3.9 mm × 15 cm) or Hibar LiChrosorb column (Merck, RP-18, 3.9 mm × 30 cm was employed. RP-HPLC used a 5–30% acetronitrile/water gradient, a 50-min run, and fractions were collected every 0.5 min. Although the retention times of reference compounds varied, ecdysone and 3-dehydroecdysone were easily identified by differential RIA[7]. Three different antisera were used for the ecdysteroid RIA[7], H-22 with high affinity for ecdysone and 20-hydroecdysone, the H-2 with high affinity for 3-dehydroecdysone[4], and the S-3 with high affinity for ecdysone and dehydroecdysone[5] (table 1). The basic procedure has been detailed[7], and in the present experiments either protein A or ammonium sulfate was used to separate bound from unbound ligand.

3β-Reductase activity of the various hemolymph samples was assayed in one of three ways. The first used the procedure already described in which hemolymph was passed through a Sephadex G-15 column and appropriate fractions diluted 1:1 with culture medium and monitored by noting the conversion of [^{3}H]dehydroecdysone to [^{3}H]ecdysone in the presence of NADPH[1,4]. In the second method, aliquots of medium were assayed with the H-22 antiserum before and after the addition of 3β-reductase + NADPH and the amount of ecdysone present quantified. The third procedure utilized the same fractions but these were diluted with 50 mM phosphate buffer (pH 6.8) containing 1 mM EDTA, 1 mM dithiothreitol and a few crystals of phenylthiourea. In the latter case, 20 μl of the diluted hemolymph fraction plus 180 μl phosphate buffer were incubated at 25 °C in the presence of 3-dehydroecdysone (final concentration 0.43 μM) and NADPH (final concentration 0.5 mM). After termination of the reaction, the ecdysteroid content was measured by RIA using the H-22 antiserum.

Results

Ecdysteroids secreted by prothoracic glands of various species. Although the use of differential RIA using the H-2 and H-22 antisera for the identification and quantification of ecdysteroids has been discussed previously[6,7], cross-reactivity studies have not been reported for our S-3 antiserum used in the current study. Table 1 reveals that the S-3 antiserum showed very high affinity to both ecdysone and dehydroecdysone while the H-22 antiserum had negligible reactivity with 3-dehydroecdysone (< 5% of S-3); both antisera showed moderate to high affinity for 20-hydroecdysone and ponasterone A. By utilizing the H-22 and S-3 antisera therefore, the amount of ecdysone and 20-hydroxyecdysone in a sample could be determined with reasonable accuracy and reproducibility.

Table 1. Cross-reactivity of ecdysteroids to the H-22 and S-3 antisera

Ecdysteroids	H-22	S-3
Ecdysone	1.0	1.0
3-Dehydroecdysone	23	1.0
20-Hydroxyecdysone	2.0	2.3
Ponasterone A	3.0	3.4

Table 2. The ratio of dehydroecdysone:ecdysone produced by the prothoracic glands of 11 species of Lepidoptera in vitro.

Species	Stage	Ratio of 3-dehydro-ecdysone:ecdysone
Bombyx mori	Wandering larva	0.03
Galleria mellonella	Wandering larva	2.57
Heliothis virescens	Day-1-2 pupa	3.05
Heliothis zea	Day-1-2 pupa	3.24
Leucania separata	Wandering larva	0.97
Manduca sexta	Day-1 pupa	7.00
Papilio protenor	Wandering larva	10.64
Papilio xuthus		
1st generation	Wandering larva	0.77
2nd generation	Wandering larva	12.63
4th generation	Wandering larva	35.93
Pieris rapae	Spinning larva	12.00
Precis coenia	Day-1 pupa	23.09
Trichoplusia ni	Day-1-2 pupa	8.70

The first series of species distribution experiments consisted of the 2-h incubation of prothoracic glands of 11 species of Lepidoptera, as well as a beetle, and determination by the H-22 antibody procedure of the ratio of dehydroecdysone:ecdysone. As table 2 reveals, the lepidopteran larvae and pupae produced significant quantities of 3-dehydroecdysone, ranging from 36 times the amount of ecdysone for the glands from *Papilio xuthus* (fourth generation) larvae to 7-fold for *Manduca* pupae to about 2.5 for *Galleria* larvae and less than 0.1 for *Bombyx* larvae. No 3-dehydroecdysone was detected in incubation medium that contained the prothoracic glands of feeding, last instar *Tenebrio molitor* larvae. Similar studies were conducted with the ovaries of the mosquito *Aedes aegypti* and the cockroach *Leucophaea maderae* since both were reported to synthesize ecdysteroids[10], but neither synthesized 3-dehydroecdysone in vitro.

These data indicated that the synthesis and secretion of 3-dehydroecdysone was a general feature of the prothoracic glands of Lepidoptera, but perhaps was not a feature of insects in general. Further studies were then conducted on more lepidopterans, flies and another cockroach. The prothoracic glands of *Papilio protenor* larvae immediately after gut purge and those from *Pieris rapae* larvae at the time of spinning synthesized in vitro about 90% 3-dehydroecdysone and 10% ecdysone (fig. 1 and table 2). For *Leucenia separata,* about 50% of the ecdysteroids synthesized was 3-dehydroecdysone and 50% ecdysone, whereas the prothoracic glands of *Bombyx mori* larvae 1 day after the onset of spinning synthesized ecdysone almost exclusively. The ring glands of *Sarcophaga peregrina* also produced essentially no 3-dehydroecdysone, but surprisingly the prothoracic glands of

Periplaneta americana synthesized a 1:1 ratio of dehydroecdysone: ecdysone (fig. 1).

Since *P. xuthus* is multivoltine (4–5 generations per year) in the environs of Kanazawa, prothoracic glands from last instar larvae representing several generations were extirpated within 3 h of gut purge and incubated for 3 h.

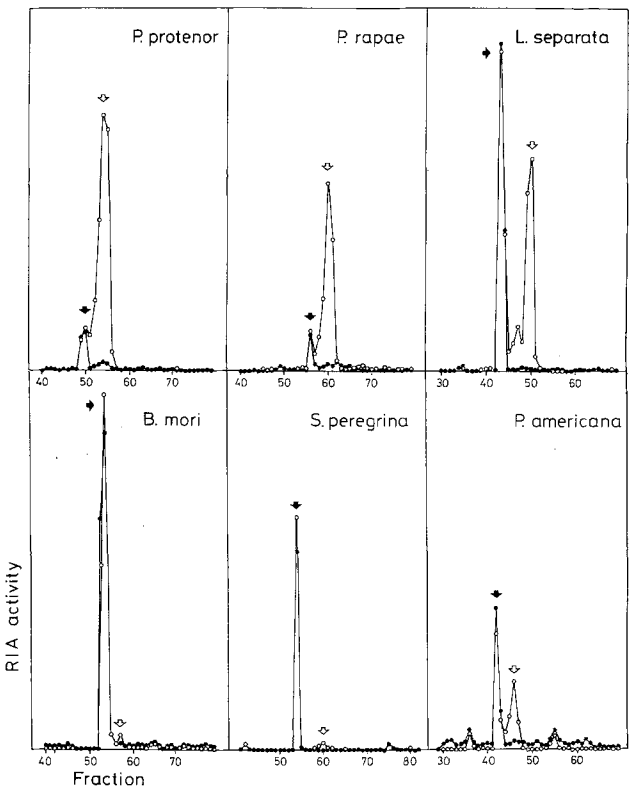

Figure 1. Reverse-phase HPLC of ecdysteroids produced in vitro by prothoracic glands of six species of insects. Fractions were quantified by RIA using the H-22 antiserum (filled circles) and S-3 antiserum (open circles). Filled arrows denote ecdysone while open arrows denote 3-dehydroecdysone.

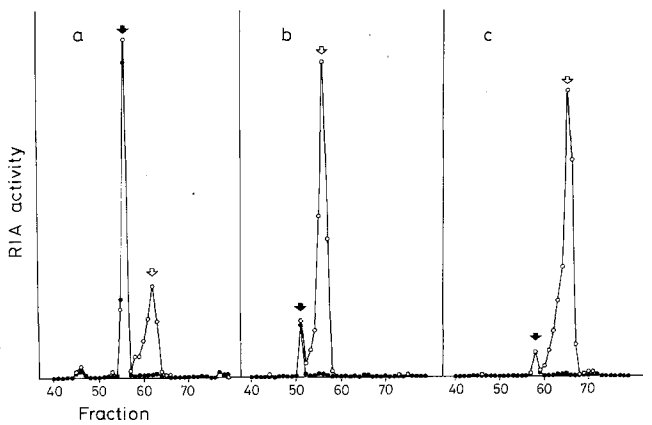

Figure 2. Reverse-phase HPLC of ecdysteroids produced in vitro by the prothoracic glands of the multivoltine *Papilio xuthus*. Fractions were quantified by RIA using the H-22 antiserum (filled circles) and S-3 antiserum (open circles). Filled arrows denote ecdysone while open arrows denote 3-dehydroecdysone. Prothoracic glands were extirpated from larvae of the first generation (a), second generation (b) and fourth generation (c). See text for details.

The resulting data revealed seasonal variations in the quantitative nature of the ecdysteroids synthesized and secreted by the prothoracic glands with glands from first generation larvae synthesizing almost equal quantities of ecdysone and 3-dehydroecdysone whereas 3-dehydroecdysone was the predominant product of glands from second and fourth generation animals (fig. 2 and table 2).

Origin of 3-dehydroecdysone. Of the data generated, the most perplexing was the finding that the prothoracic glands of *Bombyx* secreted only trace amounts of dehydroecdysone, in contrast to the glands of other lepidopterans tested. One possibility was that *Bombyx* prothoracic glands produced 3-dehydroecdysone that is reduced to ecdysone before being liberated from the glands. This could be a consequence of 3β-reductase activity associated with the especially thick basal lamina of the *Bombyx* prothoracic glands due to the very high enzyme activity associated with *Bombyx* hemolymph (see below). To test this possibility, very active glands, day 3 of the fourth instar[11], were incubated in medium containing protease that would presumably inactivate any 3β-reductase associated with the basal lamina. The resulting data revealed no difference between protease treated and control *Bombyx* prothoracic glands in their capacity to synthesize and secrete ecdysteroids (fig. 3). RP-HPLC analysis showed that prothoracic glands secreted neither 20-hydroxyecdysone (fractions 34–35, fig. 3a) nor 3-dehydro-20-hydroxyecdysone (fractions 37–38). Two small peaks were observed but neither corresponded to 3-epiecdysone since they were only detected by the H-22 antiserum which does not exhibit cross-reactivity with 3-epiecdysone[6]. In the presence of protease, 35.8 ng ecdysone per gland pair were produced (fractions 42–44, fig. 3a) and 37.0 ng in the control gland pair (fractions 43–44, fig. 3b). The quantity of 3-dehydroecdysone synthesized and released was 6.1 ng in the presence of protease (fractions 47–49, fig. 3a) and 7.4 ng in the absence of the enzyme (fractions 47–49, fig. 3b).

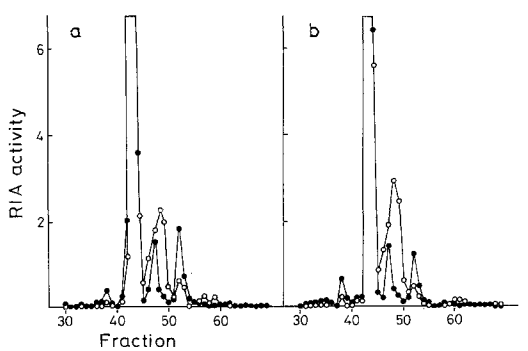

Figure 3. Effect of protease on ecdysteroid synthesis by the prothoracic glands of *Bombyx mori*. Prothoracic glands were incubated in the presence (a) or absence (b) of protease for 4 h. Ecdysteroids were separated by RP-HPLC and fractions quantified by RIA using the H-22 (filled circles) and S-3 antiserum (open circles).

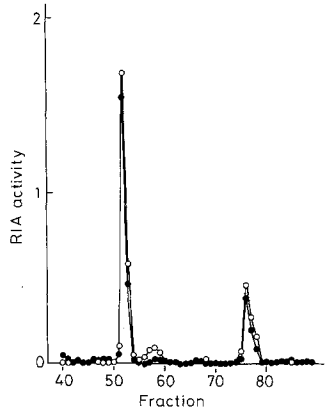

Figure 4. The endogenous ecdysteroids of the prothoracic glands of *Bombyx* larvae. 50 pairs of day-8 (2 days after the onset of spinning) larval prothoracic glands were extracted, subjected to RP-HPLC and quantified by RIA using the H-22 antiserum (filled circles) and S-3 antiserum (open circles).

Thus, a small but significant amount of 3-dehydroecdysone appeared to be synthesized by *Bombyx* glands and to determine whether it was an oxidative product of ecdysone in the medium or was indeed present in the glands, gland homogenates were analyzed by RP-HPLC. Figure 4 reveals that these homogenates did yield 3-dehydroecdysone and demonstrated that *Bombyx* glands do produce a predominance of ecdysone and a small but significant quantity of 3-dehydroecdysone. Although these data tend to alleviate the possibility that the basal lamina of the *Bombyx* prothoracic glands is responsible for the preponderance of ecdysone released into the incubation medium, we cannot be sure that protease treatment effectively reduced the putative 3β-reductase activity of the basal lamina.

Manduca glands also produced both ecdysone and 3-dehydroecdysone in vitro. It was possible that hemolymph 3β-reductase clinging to the outer boundary of the basal lamina was responsible as was hypothesized for the *Bombyx* prothoracic glands. To test this hypothesis, *Manduca* prothoracic glands were rinsed extensively in several changes of Grace's medium or lepidopteran saline before being placed in culture. The detergents Triton X-100 (0.01 %) and Noniodet P-40 (0.01 %) were also used as washing solutions. The production of both ecdysone and dehydroecdysone was monitored for 2 h in the washed glands, while the unwashed contralateral glands served as controls. In no case did the difference in the 3-dehydroecdysone: ecdysone ratio change by more than 10 % after washing. Oxidized cytochrome was added to the culture medium to a concentration of 25 µM in an attempt to dislodge $NADPH^{12}$ from any free reductase clinging to the outside of the prothoracic glands. After a 2 h incubation in the cytochrome C culture medium no appreciable change in the 3-dehydroecdysone: ecdysone ratio was observed. These results indicate that both ecdysone and 3-dehydroecdysone are intrinsic products of the gland.

3-Oxoecdysteroid 3β-reductase activity in hemolymph of various species. Although a small amount of 3β-reductase seemed to be irreversibly bound to, or inherently associated with, the prothoracic gland, much greater concentrations of the enzyme were found in the hemolymph of both *Manduca* and *A. polyphemus*[4,5]. In order to deter-

Table 3. 3β-Reductase activity in the hemolymph of various insect species

Species		Stage	Conversion (percent)	Conversion (nmol/ml/min)	Method*
Blattaria	*Leucophaea maderae*	Last nymphal stage	< 1%	–	1
		Adult female mated + 14 days	< 1%	–	1
	Periplaneta americana	Last instar larva	–	0.04	3
Orthoptera	*Locusta migratoria*	Adult, day 1–3	< 2 %	–	1
		Last instar larva, day 5		0.03	3
Lepidoptera	*Antheraea polyphemus*	Chilled pupa	> 99 %	–	2
	Bombyx mori	Last instar larva, wandering	–	3.02	3
	Heliothis zea	Pupa, day 1,3	92%	–	1
	Hyalophora cecropia	Diapausing pupa	99 %	–	2
	Leucania separata	Last instar larva, wandering	–	0.78	3
	Manduca sexta	Last instar larva, day 6	> 99 %	–	2
		Diapausing pupa, day 21	> 99 %	–	2
	Papilio xuthus	Last instar larva, spinning	–	1.34	3
	Pieris rapae	Last instar larva, spinning	–	3.71	3
	Precis coenia	Pupa, day 1	87 %	–	1
	Trichoplusia ni	Pupa, days 1–2	96 %	–	1
Coleoptera	*Epilachna varivestis*	Pupa	4 %	–	1
	Leptinotarsa decemlineata	Last instar larva, wandering	< 1 %	–	1
	Tenebrio molitor	Pupa, day 1	< 2 %	–	1
Diptera	*Aedes aegypti*	Adult female, fed + 22 h	< 1 %	–	1
	Calliphora vicinia	White puparium	< 2 %	–	1
	Sarcophaga peregrina	Last instar larva, mature	–	< 0.01	3

* Enzyme activity was determined (1) by method 1, (2) method 2, or (3) method 3 in 'Materials and methods'.

mine the taxonomic distribution of the 3β-reductase, hemolymph from several other species of insects representing 5 orders were tested for enzyme activity. Since endogenous ecdysteroids could have potentially obscured the results gathered by RIA, and since removal of these hemolymph ecdysteroids would be difficult when very small volumes of hemolymph were to be assayed, 3β-reductase activity was monitored by quantifying by HPTLC the conversion of [^{3}H]dehydroecdysone to [^{3}H]ecdysone in whole hemolymph samples during 1 h at room temperature. In the absence of hemolymph, all of the radiolabel migrated with the 3-dehydroecdysone marker. This assay relies on the observation that 3-oxo-ecdysteroid 3α-reductase, which further reduces 3-dehydroecdysone to 3-epiecdysone, is not a component of the hemolymph samples studied. Using this protocol, some of the data in table 3 were generated. All of the lepidopteran species tested showed high 3β-reductase levels ($> 85\%$ conversion, or > 38 ng 3-dehydroecdysone reduced/100 µl hemolymph). The lowest activity in the Lepidoptera was that in the hemolymph of *L. separata,* but even in this case where the activity is 0.78 nmol/ ml/ min, 100 µl hemolymph has the capacity to convert 36 ng 3-dehydroecdysone to ecdysone in 1 min. All of the non-lepidopteran species tested had negligible reductase activity in their hemolymph.

Discussion

The data demonstrate that the prothoracic glands of a variety of insects synthesize and release both 3-dehydroecdysone and ecdysone in vitro, and that the hemolymph 3β-reductase responsible for mediating the conversion of 3-dehydroecdysone to ecdysone is for the most part relegated to the Lepidoptera. Within the Lepidoptera, there are dramatic differences in the ratios of dehydroecdysone: ecdysone produced by the prothoracic glands, with *Bombyx* glands synthesizing and secreting a preponderance of ecdysone while the glands of *Manduca* and *Leucania* synthesize mainly 3-dehydroecdysone. The physiological significance of these variations in ecdysteroid ratios is not known, but one could speculate that they have developmental importance. For example, the data presented here showed changing ratios among the various generations of the multivoltine insect *P. xuthus* even when the prothoracic glands were from the exact same development stage. Further, the ratio of 3-dehydroecdysone: ecdysone in *Manduca* varies with developmental state (Sakurai and Gilbert, unpublished information). That the synthesis and secretion of 3-dehydroecdysone is not limited to *Manduca,* and indeed not to the Lepidoptera, is an important finding that requires investigation of more species representing other orders before it can be considered a general feature of the endocrine mediated molting axis.

Our previous studies revealed that the 3-dehydroecdysone secreted by the prothoracic glands of *Manduca* was converted to ecdysone by a 3β-reductase in the hemolymph[4,5]. When *Manduca* glands were incubated in vitro for 47 h, the amount of ecdysone increased in time concomitant with a decrease in 3-dehydroecdysone while the total amount of ecdysteroids (ecdysone + 3-dehydroecdysone) did not increase after 5 h of incubation[4]. We first assumed, therefore, that 3β-reductase associated with the glands reduced the 3-dehydroecdysone to ecdysone ratio after the former is secreted in the case of those insects in which the prothoracic glands secrete a great deal of ecdysone in vitro, e.g. *Bombyx*, and even for those where 3-dehydroecdysone predominates but a significant quantity of ecdysone is secreted, e.g. *Manduca.* Since the 3β-reductase was found at high levels in the hemolymph, residual hemolymph clinging to the outside of the glands could have been responsible for the conversion. Attempts to remove 3β-reductase by rinsing the glands in many changes of saline or protease, or gentle washing with detergents failed to alter the ratio of 3-dehydroecdysone: ecdysone produced by the prothoracic glands in vitro. Since simple rinsing of the prothoracic glands did not remove 3β-reductase activity, we attempted to remove the NADPH cofactor from the enzyme by using cytochrome C as an NADPH scavenger[12]. Since intracellular NADPH would be replenished via biochemical redox reactions, but not be available to extracellular enzyme, a change in the ratio of dehydroecdysone: ecdysone would have been expected if the extracellular enzyme was responsible for the conversion of dehydroecdysone to ecdysone. However, no change was noted in that ratio when the glands were monitored in vitro. The failure of these methods to alter the ratio of 3-dehydroecdysone: ecdysone suggests that both 3-dehydroecdysone and ecdysone are intrinsic products of the prothoracic gland cells.

Although the finding that 3-dehydroecdysone is the major product of the prothoracic glands of most Lepidoptera is a recent event[4,5], it had been identified as a metabolite of ecdysone almost twenty years ago, the oxidation of ecdysone being mediated by ecdysone oxidase[13,14]. The oxidase is present in several insect tissues[15], but is in very low titer in the prothoracic glands of *Manduca*[1,4], indicating that the dehydroecdysone secreted by the prothoracic glands in vitro is not a breakdown product of ecdysone, but rather a prohormone, or hormone in its own right.

All species and stages of Lepidoptera examined here possessed potent 3β-reductase activity regardless of the assay employed. Although *Bombyx* prothoracic glands secrete very little dehydroecdysone in vitro, *Bombyx* hemolymph yielded relatively high enzyme activty as did hemolymph from diapausing pupae of *H. cecropia* whose glands are essentially inactive. Further, the amount of enzyme activity in *Manduca* hemolymph at any stage of development is much greater than needed to mediate the reduction of all the dehydroecdysone secreted by the prothoracic glands[5]. Thus, although it would have been gratifying to demonstrate that fluctuations in 3β-reductase activity

were important regulatory components in the maintenance of the ecdysteroid titer, we have not yet been successful in showing such regulatory control.
Clearly the discovery of the prothoracic gland secretory product 3-dehydroecdysone raises more questions than it answers. The knowledge that prothoracic glands synthesize 3-dehydroecydsone from 7-dehydrocholesterol[4,5] is a first step in elucidating the ecdysone biosynthetic pathway. The putative roles of dehydroecdysone in the morphogenetic changes associated with metamorphic commitment and molting are undefined, but are deserving of future study.

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Neuropeptide Y inhibits corticosterone secretion by isolated rat adrenocortical cells[1]

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Summary. Studies with isolated rat adrenocortical cells have shown that neuropeptide Y (NPY) inhibits both basal and ACTH-stimulated corticosterone secretion. These results suggest the regulatory role of NPY in corticosterone secretion from the adrenal gland, especially during stress.
Key words. Neuropeptide Y; adrenal cortex; isolated adrenocortical cells; corticosterone secretion; inhibitory effect; rat.

Neuropeptide Y is widely distributed in neurons of the central and peripheral nervous system. NPY is contained in adrenal chromaffin cells and in nerve fibers passing through the adrenal cortex and medulla of various animal species, and it is co-released with catecholamines during sympathetic activation[2-4]. Evidence is available for the participation of this peptide in the systemic and/or paracrine adrenomedullary mechanism regulating aldosterone secretion, whereas the action of NPY on corticosterone secretion remains an open question[5,6]. Therefore we examined the effect of NPY on basal and ACTH-stimulated corticosterone secretion by isolated rat adrenocortical cells.

Materials and methods

Isolated rat adrenal cells were prepared by the method of Sayers et al.[7] with modifications described previously[8]. Adrenal glands from adult female Wistar rats (140–160 g) were removed, decapsulated, dissected into small pieces and preincubated for 5 min in Krebs-Ringer bicarbonate buffer (pH 7.3) enriched with 0.3% glucose (KRBG). The medium was discarded and adrenal tissue digested by 0.5% collagenase (type I, Sigma) in KRBG. The collagenase digestion was carried out by gentle stirring with a magnetic stirrer for 15 min at 37 °C and was repeated. Collected medium was centrifuged at 300 × g for 10 min and the sediment resuspended in KRBG containing 0.4% bovine serum albumin (Cohn's V fraction, Sigma) KRBGA). Cell viability was tested with the trypan blue test (0.5% solution) and the percentage of stained cells was always less than 5–6%. Adrenocortical cells were counted in a Burker chamber.
Cells were incubated for 60 min at 37 °C in a Dubnoff incubator (shaking at 50 rpm). Depending upon the experiment the number of cells used varied from 90,000 to 150,000 per ml. NPY (porcine, Sigma) was added in 0.9% NaCl and ACTH (1–24 ACTH, Sigma) in