the vaginal closure membrane. Perforation of the membrane is an estrogen-dependent event[9] and thus TAM acted as an agonist in this target tissue. The dose of TAM used in this study was four times higher (on a body weight basis) than the effective dose in rats[10] but did not prolong luteal function in the guinea pig. TAM is metabolized to 4-monohydroxytamoxifen in vivo and others have shown that this form is more potent[1,10]; however, TAM is also effective when this metabolic conversion is blocked[11]. It is unknown whether TAM or its metabolite was the active agent in the present study.

ENC exerted luteolytic effects whether administered early in the luteal phase or beginning at midcycle. However, ENC was not as potent as E2, requiring approximately ten times the amount of E2 to be effective. In most of the systems studied, ENC (the trans isomer of clomiphene) is a potent estrogen antagonist[12] and clomiphene (a mixture of cis and trans isomers) blocks the luteolytic effect of E2 in primates[5,13]. Agonistic effects of ENC have also been reported[14].

The uterus appears to mediate the luteolytic effect of both ENC and E2, as demonstrated by the absence of luteolysis in hysterectomized guinea pigs treated with either factor (this study and reference 2). Because E2 increases uterine PGF production in guinea pigs, it seems likely that ENC would do the same, but uterine PGF production in response to ENC will have to be measured to verify this. TAM decreases guinea pig uterine PGF production in vitro[15], but did not affect luteal activity in this study. Enclomiphene also affects gonadotropin secretion[16] and luteal steroidogenesis[5], but these potential mechanisms apparently do not play a significant role in the guinea pig because of the absence of any effect of ENC in hysterectomized animals.

In summary, this investigation has demonstrated 1) the luteolytic dose of E2 yields blood levels similar to those measured in the utero-ovarian vein prior to the onset of spontaneous luteolysis, 2) ENC induces premature luteolysis in the guinea pig, and 3) the luteolytic effect of ENC is mediated by the uterus, probably by increasing uterine PGF production.

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Ecdysteroid receptors located in the central nervous system of an insect

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Summary. Using thaw-mount autoradiography for steroid hormones, we obtained direct evidence for a nuclear localization of ecdysteroid binding sites in target organs of blowfly (*Calliphora vicina*) larvae. The binding sites revealed properties of ecdysteroid receptors. Endocrine cells of the ring gland were found to be target tissues of ecdysteroids. This observation provides morphological evidence for a network of complex interendocrine regulation. In the central nervous system receptor-containing neurons were identified which include many, if not all, neurosecretory cells of the brain. A map of ecdysteroid sensitive cells of the larval brain is presented.
Key words. Ecdysteroid; steroid hormone receptor; central nervous system; interendocrine regulation; autoradiography; fly.

Ecdysteroids serve as the sole steroid hormone system in arthropods. They elicit a wide variety of effects which range from control of moulting to the induction of vitellogenins[2]. These effects are mediated by hormone receptors. So far there has only been indirect evidence suggesting the localization of ecdysteroid receptors within target cells[3].

Blowfly larvae contain ecdysteroid-binding molecules that fulfill all criteria of steroid hormone receptors: they exhibit an affinity for DNA as well as tight hormone binding (K_d = 30 nM with 20-hydroxyecdysone), show analogue specificity, and a low steroid-binding capacity[4]. The radiolabelled hormone analogue ponasterone A (PoA = 25-deoxy-20-hydroxyecdysone) reveals an increased affinity for the receptor (K_d = 1 nM)[4].

Results and discussion. When tissues, dissected from late third instar larvae (L 3 d 7) of the blowfly (*Calliphora vicina*), were incubated with a low concentration of [^{3}H]PoA the ecdysteroid was taken up and reached a plateau after about 1 h. The final amount of PoA resorbed depended on the age and physiological stage of the larvae from which the tissues were prepared. Higher rates of uptake were obtained with larvae arrested in their development by an additional 7 days

at 4° C (L 3 d 14). These larvae did not pupariate. This treatment prevented a rise in ecdysteroid titre as was determined by radioimmunoassay.

Washing of the tissues led to the release of radioactivity. An initial rapid loss (for about 30 min) apparently resulted from nonspecificially bound PoA as determined with a 100-fold excess of unlabelled PoA in a separate experiment (fig. 1). Competition of [^{3}H]PoA binding with other ecdysteroids (data not shown) revealed the same ligand specificity (PoA > muristerone A > 20-hydroxyecdysone > makisterone A > ecdysone), as was detected by biochemical analysis of the ecdysteroid receptors[4]. The results of these experiments indicated that salivary glands and the central nervous system (CNS) contained ecdysteroid receptors and also provided information to minimize nonspecific binding in autoradiographic experiments.

To localize the ecdysteroid receptors we used thaw-mount autoradiography for steroid hormones, as developed by Stumpf and Sar[5]. Salivary glands and the CNS were dissected from blowfly larvae (L 3 d 14) and washed in blowfly Ringer to remove endogenous ecdysteroids. The tissues were then incubated in vitro with the radiolabelled hormone analogue PoA (4 nM), washed again and finally frozen in liquid nitrogen. Preparation of frozen tissue sections and their exposure to photographic emulsion followed in detail the protocol for quantitative autoradiography[6]. After appropriate exposure times the slides were photographically processed and the sections stained.

The distribution of radioactivity, as indicated by silver grains, demonstrated a distinct nuclear localization of ecdysteroid-binding sites (fig. 2). Autoradiographic experiments were always accompanied by two types of controls: in the presence of a 100-fold excess of unlabelled PoA the localization of radioactivity in nuclei was absent (fig. 2G); the other type of control excluded positive and negative chemography[5,6].

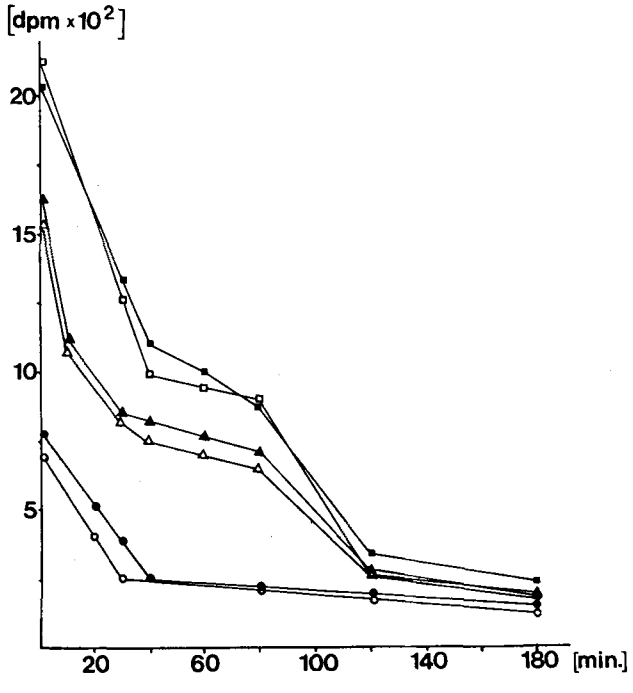

Figure 1. Release of radioactivity from brain-ring gland complexes (closed symbols) and salivary glands (open symbols) following an in vitro incubation with [^{3}H]PoA (4 nM; 60 min). Tissues from normal blowfly larvae (L 3 d 7; triangles) accumulated less radioactivity than tissues from larvae kept under cold conditions (4 °C) for an additional seven days (L 3 d 14; squares). However, the kinetics of release were similar. The rapid decrease of radioactivity in the control experiments (circles; brain-ring gland complexes from L 3 d 14 incubated with an 100-fold excess of unlabelled PoA) indicated that washing for 40 min was sufficient to remove radiolabel bound nonspecificially. Detailed technical information (insect culture, radiotracer, chemicals, methods) is found in reference Lehmann and Koolman[4].

The existence of ecdysteroid receptors was first demonstrated in a cell line of *Drosophila*[7]. Ecdysteroid binding moiety was found in cytosol and cell nucleus. More recent work especially with imaginal discs from *Drosophila* suggests, although indirectly, a nuclear localization of ecdysteroid receptors[3]. The preferential nuclear localization of unoccupied high-affinity binding-sites observed in this study directly suggests that ecdysteroid receptors reside in the nuclear compartment where they bind the hormone and initiate the hormonal effects on transcription. This observation is in accordance with the 'nuclear' model[8] favoured for most steroid hormone systems[9].

Localization of ecdysteroid receptors in salivary glands (fig. 2F) served as a positive control, because these glands are a known target of ecdysteroids[10,11]. They contain ecdysteroid-binding sites as suggested by autoradiography with ecdysone[12] and 20-hydroxyecdysone[13] as well as by indirect immunofluorescence[14].

Adjacent to the brain of blowfly larvae, as of other dipterans, is a ring-like structure (fig. 3) with the function of a composite endocrine gland[15]. This ring gland is the source of several hormones: a) ecdysone[16] secreted by lateral cells which are equivalent to prothoracic glands (PG) of other insect species, b) juvenile hormone(s) secreted by corpora allata (CA) equivalents, and c) a variety of neuropeptide hormones produced, stored, and secreted by corpora cardiaca (CC) cells. All three cell types of the gland showed nuclear concentration of label (fig. 2A, B, and C). Apparently various functions of these endocrine glands are under the control of ecdysteroids. This appears to reflect the 'interendocrine regulation' of insect development which may be elucidated by in vitro experiments[17].

Within the brain, nuclei of two groups of neurosecretory cells (NSC) in the pars intercerebralis were strongly radiolabelled (fig. 2D). These are NSC group 1 according to the nomenclature of Vijverberg[18]. They innervate the CC and are potential sources of the hyperglycaemic hormone[19]. Less label was detected in some NSC from groups 3 and 4 of the periphery of pars intercerebralis (fig. 2H), which innervate the CC and CA via nervi corporis cardiaci I and c. allati. Cells of these two groups control in lepidopteran species, such as *Manduca sexta*, the function of CA by an allatotropin and allatostatin[20]. Also the cells of the dorso-lateral NSC group 11 were labelled. They innervate the PG and CA equivalent of the ring gland via nervus corporis cardiaci II and nervus corporis allati. In lepidopteran species these cells are the source of the large form of the prothoracicotropic hormone[21,22].

Secretion of ecdysone by brain-ring gland complexes in vitro can be inhibited by addition of ecdysteroids[23] suggesting a feed-back loop by the hormone produced. Indications for a feed-back loop controlling the haemolymph titre of ecdysteroids has also been obtained in other insect species[24–27]. Our observation that ecdysone synthesizing cells (PG equivalents) as well as the presumed prothoracicotropes (NSC group 11) and their neurohaemal organ (CA or CC) contain ecdysteroid receptors, suggests a multiple feed-back mechanism: directly on ecdysone synthesis and indirectly on PTTH synthesis and release. In contrast to most other insect tissues PGs are unable to convert their secretory product ecdysone to 20-hydroxyecdysone[28], the active form of the hormone. This difference may become understandable in the light of the finding that PGs are a target tissue of the moulting hormone.

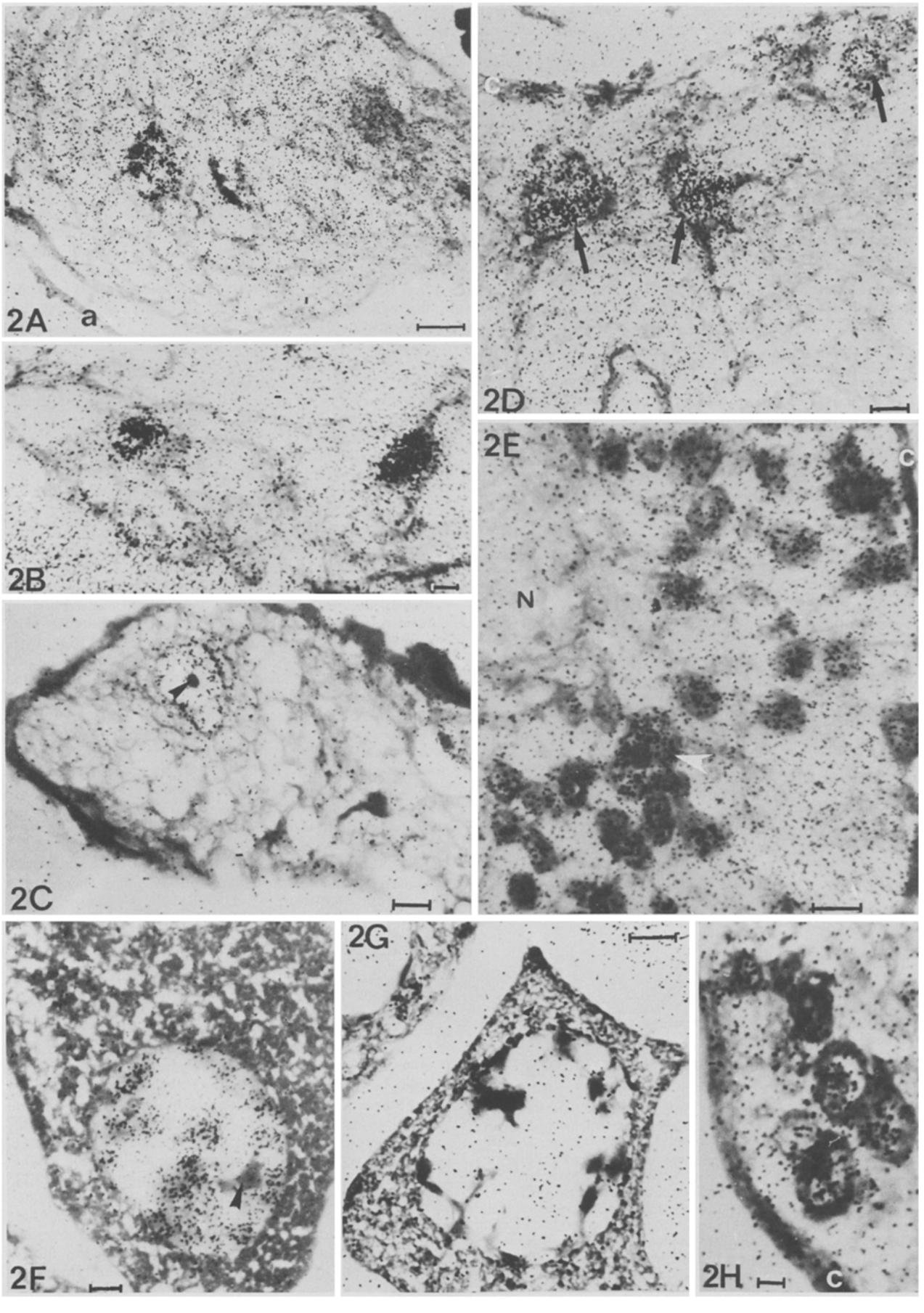
2A
a
2B
2C
2D
2E
N
C
2F
2G
2H
C

Figure 2. Autoradiograms of endocrine glands and CNS of 3rd instar blowfly larvae after incubation with the ecdysteroid analogue [^{3}H]ponasterone A.
Radioactivity is concentrated in nuclei of cells in corpus allatum (*A*) and corpus cardiacum (*B*), prothoracic gland cells (*C*) of the ring gland, in nuclei of the median NCS group 2↑, and of few small cells ↑ in the pars intercerebralis (*D*), of M-NCS group 3 and 4 (*H*) in the protocerebrum of the NCS-group 7 (white arrow), and of many other small cells of the suboesophageal ganglion (*E*). *F* and *G*: Accumulation of radioactivity in salivary gland cell nuclei after incubation in 4 nM [^{3}H]ponasterone A (*F*) and random distribution of radioactivity after incubation with an additional 100-fold excess of unlabeled ponasterone A as a control (*G*). Nucleoli ▲ in *C* and *F* show no concentration of silver grains. Aorta wall, a; connective tissue, c; neurophil, N; bar: 10 µm.
Tissues were taken from third-instar larvae (L 3 d 14) of the blowfly, *Calliphora vicina*, washed for 2 h in Calliphora Ringer and then incubated in radiolabelled PoA (4 nM; spec. radioact. 180 Ci/mmol; 22 °C for 1 h), followed by an additional washing for 40 min; all incubations at 22 °C. Stained with methyl green-pyronine; 136 days (fig. 2 *F*: 76 days) exposure to Kodak NTB-3; 4-µm sections.

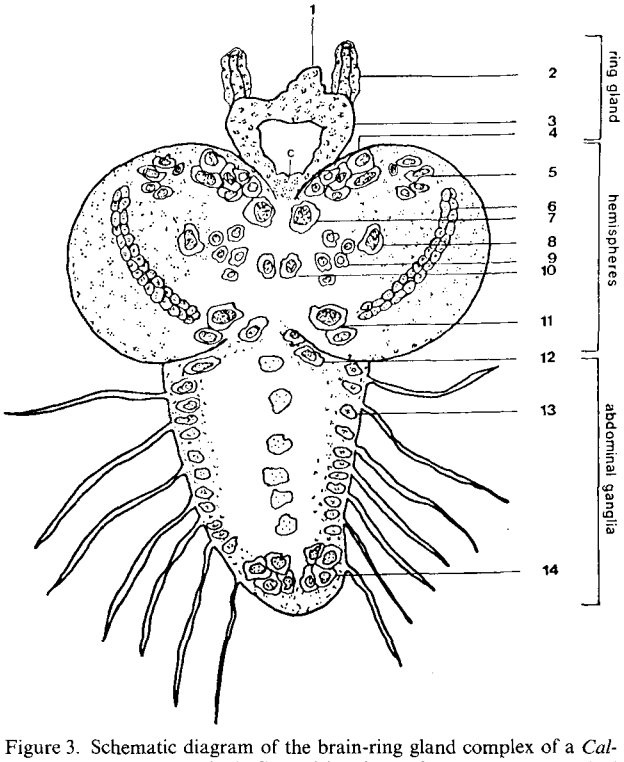

Figure 3. Schematic diagram of the brain-ring gland complex of a *Calliphora vicina* larva (L 3 d 14). Cells with ecdysteroid receptors are marked with dots.
1, Corpus allatum; 2, endodermis around the trachea; 3, prothoracic gland; c, corpus cardiacum; 4, median NSC group 3 and 4; 5, lateral NSC group 11; 6, cells of the optic lobes; 7, median NSC group 2; 8, NSC group 5; 9, small non-neurosecretory cells around group 5; 10, median NSC group 1; 11, NSC group 6; 12, NSC group 9 + 10 in the dorsal median suboesophageal ganglion and in the abdominal ganglion; 13, NSC group 7; 14, fused NSC group 7 + 8 in the last abdominal ganglion. Nomenclature of the NSC groups in the larval brain of *C. vicina* according to Vijverberg[18]. Cell group 11 has been newly defined.

Neurones capable of binding ecdysteroids were also found in the optic lobes of the brain hemispheres, the suboesophageal ganglion (fig. 2 E) and the attached and fused abdominal ganglion. Figure 3 gives the location of all neurones of the larval CNS in which we observed radiolabel to be concentrated in nuclei. Although the functional relevance of these ecdysteroid receptors (with the exception of the feed-back loop) is as yet obscure, the results of the present studies indicate that many neurones are under the control of ecdysteroids.

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