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Effects of the pyrimidine-containing cytochrome P-450 inhibitor, fenarimol, on the formation of 20-OH ecdysone in flies

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Summary. The effect of fenarimol, a pyrimidine-containing cytochrome P-450 inhibitor, was tested in vitro on brain-ring gland complexes of *Calliphora vicina* (Dipt., Calliphoridae), and on microsomes prepared from the fat body of 0-h wandering stage larvae of *Neobellieria bullata* (Dipt., Sacrophagidae). Fenarimol had no influence on the formation of ecdysone, but it was an effective inhibitor of cytochrome P-450-dependent ecdysone 20-monooxygenase.
Key words. Calliphora vicina; *Neobellieria bullata*; fenarimol; synthesis of 20-hydroxy ecdysone; cytochrome P-450 inhibitor; microsomal ecdysone 20-monooxygenase.

20-hydroxy ecdysone, the active metabolite of the insect steroid hormone, ecdysone, has multiple functions. It influences not only the regulation of molts, but also other physiological processes during insect development [2]. The precursors of this hormone are usually dietary sterols which are converted into ecdysone via cholesterol [3]. In immature stages the main site of transformation of cholesterol to ecdysone is in the prothoracic glands (in Dipteran species, the ring gland), although other organs can also carry out this conversion which includes several hydroxylation steps. Then, in peripheral tissues, such as the fat body, Malphigian tubules, gut, etc., the secreted ecdysone is converted (hydroxylated) by the cytochrome P-450-dependent ecdysone 20-monooxygenase enzyme system into 20-hydroxy ecdysone.

Injection of triarimol [4], a pyrimidine-containing fungicide (inhibitor of ergosterol biosynthesis [5]), into larvae of *N. bullata* at the wandering stage, caused a delay in the pupariation process. This effect was reversed by 20-hydroxy ecdysone. Later dietary experiments with triarimol [6] and fenarimol [7] showed molting disturbances of *N. bullata*; 0.2% fenarimol added to the diet inhibited the molt to the next stage, causing permanent 1st instar larvae (for about 4–6 days)[8], while at 0.1%, it induced precocious pupariation [10, 11]. Compounds containing nitrogen heterocycles (pyridine, pyrimidine, imidazole or triazole rings) are well known as inhibitors of cytochrome P-450-dependent monooxygenases [12] via reversible ligand formation with heme. Effects caused by fenarimol in vivo suggested that this compound probably acts on the biosynthesis of 20-hydroxy ecdysone.

Materials and methods

Effects of fenarimol (fig. 1) on the biosynthesis of ecdysone were analyzed with undisrupted tissue (i.e. intact cells), while the effects on 20-hydroxylation were measured with a crude enzyme preparation. Larvae of

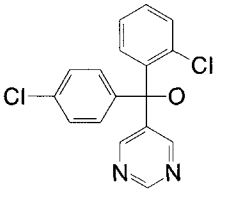

Figure 1. The chemical structure of fenarimol.

the blowfly, *Calliphora vicina* Robineau-Desvoidy (Dipt., Calliphoridae) were reared on beef muscle at 23 °C and 50% relative humidity with light from 07.00 to 19.00 h. Brain-ring gland complexes (BRCs) were dissected from mature 3rd instar larvae (6th day after hatching). BRCs were washed with Calliphora-Ringer[13] and incubated individually in 500 µl Calliphora-Ringer solution for 6 h at 37 °C. After removing the BRCs, the secreted ecdysone was determined by using solid phase RIA[13]. Fenarimol was added in acetone solution (1 µl) to give a final concentration of 1 mM. In a control experiment, the effect of the same amount of acetone was also tested. Experiments were conducted in triplicate.

Fat bodies were dissected from 0-h wandering stage larvae of the fleshfly, *Neobellieria bullata* Parker (Dipt., Sarcophagidae). On the fifth day after hatching, wandering stage larvae were treated with Ohtaki-type wet synchronization[9] for 48 h before dissection. Fat bodies were homogenized in isoosmotic Neobellieria-Hepes buffer (20 mM HEPES, 20 mM KF, 260 mM sucrose, pH 7.2, 340 mOsm)[10], and the homogenate centrifuged at 600 × g three times for 5 min, then at 15,000 × g for 10 min, and finally at 200,000 × g for 75 min, to isolate the microsomal fraction. This fraction was resuspended in hypotonic Neobellieria-Hepes buffer (120 mOsm). Microsomal protein equivalent to 2.5 larval fat bodies was preincubated with the inhibitor (from 10^{-4} M to 10^{-7} M) for 10 min, then an NADPH generating system (3.4 mM NADPH, 2.6 mM glucose-6-phosphate, 0.6 IU glucose-6-phosphate dehydrogenase) and 4.22 pmol of [^{3}H] ecdysone (0.25 µCi/mmol, NEN) were added, and the mixture was further incubated for 30 min at 30 °C[10]. The reaction was stopped by addition of ethanol, the supernatant was evaporated to dryness under vacuum and the samples dissolved in ethanol were separated by TLC, developing twice with chloroform-ethanol (4:1, v/v)[14]. The conversion of ecdysone into 20-hydroxy ecdysone was measured quantitatively by scraping off the radioactive spots for assay in an LKB Wallac 1219 Rackbeta LSC. The activity of the microsomal fraction was assayed in duplicate. Inhibition of ecdysone 20-monooxygenase was expressed as an IC_{50} value and calculated by probit analysis.

Results and discussion

After incubation of brain-ring gland complexes with 1 mM fenarimol, there was no indication of interference with ecdysone biosynthesis or release (table), despite the fact that various hydroxylations on nucleus and side-chain are catalyzed by certain cytochrome P-450-dependent monooxygenases[15,16]. We concluded that the symptoms observed in vivo[5-10] could not arise because of the lack of ecdysone. This is reminiscent of the observation that 10 mM triarimol did not inhibit the fucosterol-24(28)epoxide cleavage enzyme (which may not be a cytochrome P-450 isozyme) involved in sitosterol dealkylation of phytophagous insects[17].

	Ecdysone released
Control	4.72 ng (± 0.44)
Treated with 1 mM fenarimol	4.50 ng (± 0.66)

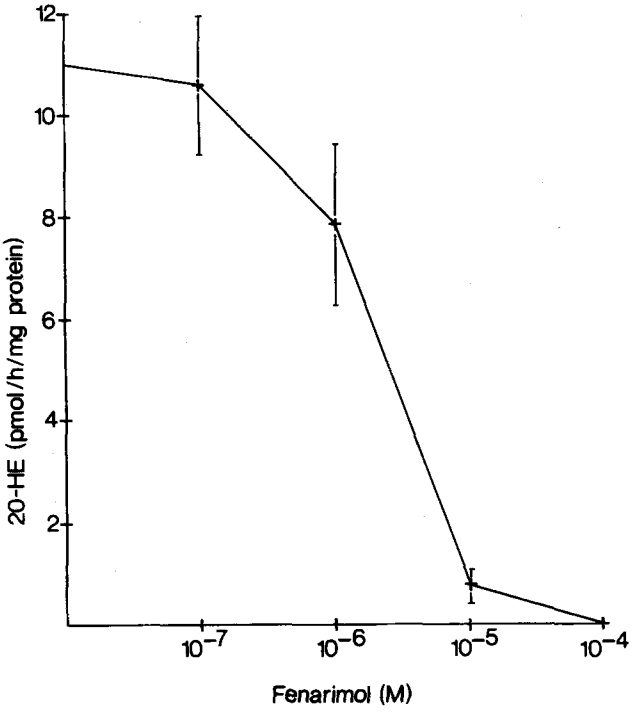

Figure 2. Inhibitory effect of fenarimol on microsomal ecdysone 20-monooxygenase.

Nevertheless, fenarimol is a very potent inhibitor of the cytochrome P-450-dependent microsomal ecdysone 20-monooxygenase. The IC_{50} value of fenarimol is 0.66 µM (0.39–1.12 µM) on microsomal ecdysone 20-monooxygenase of fat bodies of *N. bullata* (fig. 2).

The cytochrome P-450 isozyme system is a widely distributed enzyme superfamily which performs oxidative functions in different organisms[18]. Differences in the structure of the apoprotein, of the substrate channel, and of the substrate binding site have been demonstrated in various cytochrome P-450 isozymes. Thus, different cytochrome P-450 isozymes may have different sensitivities to inhibitors. During 20-hydroxy ecdysone formation, fenarimol seems to be a partially selective inhibitor of microsomal ecdysone 20-monooxygenase, while the other insect steroidogenic hydroxylases, the mitochondrial deoxyecdysone 2-monooxygenase[19] and the C-14, C-25, and C-22 hydroxylases, involved in ecdysone synthesis, may be far less sensitive to it.

It is interesting that fenarimol shows this apparent selectivity for the cytochrome P-450 enzyme catalyzing the formation of 20-hydroxy ecdysone, although it is also known to inhibit the production of methyl-6,7; 10,11-bisepoxy-3,7,11-trimethyl-(2*E*)-dodecenoate (JHB_3) by larval ring glands of *Drosophila melanogaster* in vitro[20],

and it has unspecific, toxic effects on *Pseudaulacaspis pentagona* (Hom., Diaspididae) and its parasitoid, *Prospaltella berlesei* (Hymen., Aphelinidae)[21]; this suggests that it may have inhibitory effects on other polysubstrate monooxygenases involved in xenobiotic metabolism in insects.

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Tamoxifen 'sex reverses' alligator embryos at male producing temperature, but is an antiestrogen in female hatchlings

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Summary. Tamoxifen is an anticancer drug widely used in the treatment of estrogen-dependent breast cancer. In hatchling alligators it acts as a pure antiestrogen in that it completely blocks the effect of estradiol-induced oviductal hypertrophy and completely blocks the estradiol-induced hepatic vitellogenin secretion. Paradoxically, when injected into alligator eggs incubated at 33 °C, a temperature which would normally result in 100% male hatchlings, tamoxifen 'sex reverses' the embryos into apparently normal female hatchlings.
Key words. Tamoxifen; sex determination; alligators.

In some teleost and amphibian species functionally reproductive adults of either sex can be produced by treating the larvae with androgens or estrogens, though there are a number of paradoxical exceptions in which androgens feminize and estrogens masculinize[1,2]. In amniote vertebrates it has not been possible to 'sex reverse' embryos in the male direction with androgens, but estrogens will sex reverse reptile embryos into apparently normal females[3–5], and will produce feminized but sterile birds[1]. Tamoxifen has been reported to block the feminizing action of estradiol in chick embryos[6] and of DES in quail embryos[7]. Treatment of bird embryos with tamoxifen alone has given conflicting results. In one study no effect was noted[6], but in two other studies a partial 'masculinizing' of the left ovary and partial development of the normally regressed right gonad were reported[8,9]. Despite this apparent masculinizing action, the Mullerian duct did not appear to be affected. Similar 'masculinizing' effects have been noted in turtle embryos treated with tamoxifen[4].

The sex of alligators is determined by the temperature at which the eggs are incubated. Eggs incubated at 33 °C produce 100% male hatchlings, and eggs incubated at 30 °C produce 100% female hatchlings[10–12]. Injection of estrogen into eggs incubated at 33 °C will 'sex reverse' the embryos and produce female hatchlings[3], but the converse is not true. It has not been possible to produce male hatchlings from eggs incubated at 30 °C by injecting androgens[13]. If, as has been suggested, ovarian development is dependent upon synthesis of estrogen in the developing gonad[4,5,14], then it may be possible to 'sex reverse' embryos at female producing temperatures by