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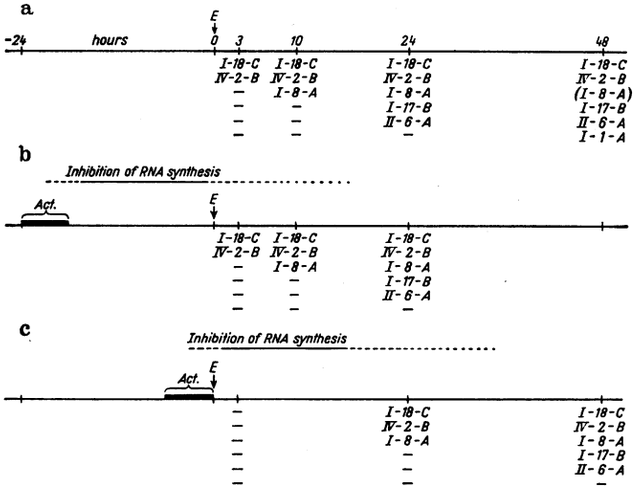
Actinomycin and Puromycin: Effects on Sequential Gene Activation by Ecdysone

Abstract. *A temporary inhibition of RNA synthesis leads to a corresponding delay in the formation of all puffs which are stimulated by ecdysone in the salivary gland chromosomes of* Chironomus tentans. *Inhibition of protein synthesis does not influence the induction of those puffs which appear shortly after injection of ecdysone. Puffs which develop after a longer period are delayed in appearance. It is concluded that early reacting genes are involved in those processes leading to the sequential activation of the puffs which appear later.*

Molting in insects is induced by the hormone ecdysone from the prothoracic glands (*1*). An early effect of this hormone is to change the activity of specific gene loci. This has been found in studies on the puffing phenomenon in salivary gland chromosomes of *Chironomus tentans* (*2*). After injection of ecdysone into last-instar larvae, two puffs (I-18-C and IV-2-B) appear within the next 15 to 30 and 30 to 60 minutes, respectively. In normal, as well as in experimentally induced metamorphosis, the appearance of these puffs is followed by a sequential activation of other loci (*2, 3*). The order in which puffs appear at some of these loci after an injection of ecdysone is shown in Fig. 1*a*. Changes in the puffing pattern during metamorphosis, comparable to those found in *C. tentans*, occur in other insects as well. That they are induced by or depend on the presence of ecdysone has been shown in *Acricotopus* and in *Drosophila* (*4*). In *Chironomus tentans*, the size of the two early induced puffs is controlled by the concentration of ecdysone. They differ, however, in their reaction thresholds and in their reactivities against different hormone concentrations (*3*). Puffing of some of the later-reacting loci is also dependent on the presence of ecdysone. These loci, however, react only 2 or 3 days after ecdysone injections, even when very large amounts are injected; thus they cannot be influenced by changes of hormone concentrations (*2, 3*). These results suggest that the early puffs may be controlled more directly by ecdysone than are the later ones. To determine whether early-reacting genes participate in the chain of processes which lead to an activation of the puffs which appear later, we temporarily inhibited RNA and protein synthesis.

The synthesis of RNA was inhibited by incubating *C. tentans* larvae in a culture medium containing 0.2 μg of actinomycin C_1 per milliliter (*5*). After 6 hours they were transferred to a medium lacking actinomycin. At various intervals, some larvae were killed, and the salivary glands were explanted and incubated for 30 minutes in a sucrose medium containing tritiated uridine (1.29 c/m*M*; 1.0 μc/ml) (*6*). The glands then were squashed and tested for RNA synthesis by means of autoradiography (*7*).

After 4 to 6 hours of treatment with actinomycin, incorporation of H^3-uridine had strikingly decreased. Seven to eight hours after beginning the actinomycin treatment (that is, 1 to 2 hours after the transfer of the larvae into a fresh medium) RNA synthesis could no longer be detected by our method (Fig. 2). Fifteen to twenty hours after trans-



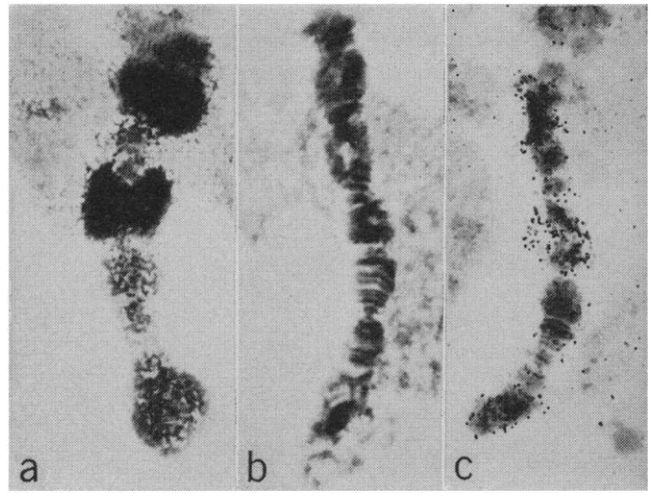


Fig. 1 (left). Puff-formation at representative loci of salivary gland chromosomes of *Chironomus tentans* 3, 10, 24, and 48 hours after injections of ecdysone (*E*). In *b* and *c* larvae were pretreated with actinomycin for 6 hours (*Act.*). The symbols indicate the chromosome regions in which the puffs are located. Their exact locations and details of their behavior in normal and experimentally induced metamorphosis have been published (*2, 3*). The number of animals used in *b* and *c* were 28 and 25, respectively. Fig. 2 (right). Autoradiographs of the IVth chromosome of *C. tentans* after incubation of the salivary glands in a medium containing H^3-uridine for 30 minutes. For *b* and *c*, the larvae were treated for 6 hours with actinomycin 10 hours and 20 hours, respectively, before the salivary glands were incubated with H^3-uridine.

fer—with a relatively high degree of variability—RNA synthesis began to recover (Fig. 2*c*). For some time, however, incorporation of H^3-uridine remained lower than it was in the untreated controls. Parallel with the inhibition of RNA synthesis, puffs and Balbiani rings disappeared. They gradually reappeared after RNA synthesis was resumed.

To study the interference of actinomycin with the processes of hormone action, we undertook two series of experiments. In the first series we injected the hormone immediately after larvae had been treated with actinomycin for 6 hours. As shown by the autoradiographic experiments, no, or almost no, RNA synthesis occurred under these conditions for the 15 to 20 hours following the hormone injection. New puffs could not be induced by ecdysone during this period (*8*). In larvae fixed 24 and 48 hours after being treated with actinomycin and injected with ecdysone, that is after RNA synthesis was resumed, puffs had formed as shown in Fig. 1*c*. A comparison of Figs. 1*a* and 1*c* shows that the stage reached in these experiments 24 hours after the injection of ecdysone corresponds to the stage reached normally after about 5 to 10 hours. Larvae fixed 48 hours after the injection showed puffing patterns which are normally attained after 24 hours. Thus the appearance of all puffs in these experiments was delayed for approximately the same period of time that RNA synthesis was inhibited. The data given in Fig. 1*c* were obtained with the most advanced larvae of our experiments (about half the larvae injected). In other larvae only puffs I-18-C and IV-2-B had formed after 24 hours, or only I-18-C, IV-2-B, and I-8-A after 48 hours. This variability corresponds to the variability in the recovery of RNA synthesis.

In a second series of experiments we injected ecdysone 16 and 24 hours after transferring the larvae to actinomycin-free culture medium, that is shortly after RNA synthesis had resumed. The larvae were fixed 3, 9, and 24 hours later. Figure 1*b* shows which puffs had developed in these larvae; again, the most advanced stages of puff-formation were selected. A comparison with Fig. 1*a* shows that, at any one time, the puffing pattern was the same as in the larvae not treated with actinomycin. Although the variability was larger than in the untreated larvae, between 60 and 90 percent of the larvae had

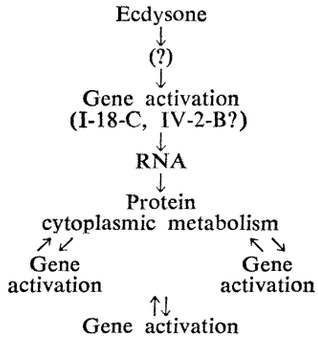


Fig. 3. Diagram of the first steps in the action of ecdysone.

reached the normal stage. It follows from these results that 15 to 20 hours after actinomycin treatment—that is, as soon as RNA synthesis had resumed—the cell was able to respond normally to the hormone. Obviously, the nonappearance of the typical puffs 24 hours after injection of ecdysone (and actinomycin treatment) in the first series of experiments cannot be due to general damage of cell metabolism. The results therefore show that it is a necessary condition for the initiation of all subsequent molting processes that some early genes are induced and deliver informational RNA. It follows that some early inducible genes must participate in the processes which lead from the primary action of ecdysone to the activation of the later-appearing puffs; or, to put it another way, some gene activations are closer to the primary action of the hormone than are others (*9*).

Protein synthesis was inhibited with the antibiotic puromycin (*10*). Its effect on protein synthesis in the salivary gland cells was examined autoradiographically after injections of tritiated leucine (10 c/m*M*; injected 1 μc in 1 μl). After an injection of 2.5 μg puromycin (this leads to a concentration of about 100 to 120 μg/ml in the larva) protein synthesis was almost completely inhibited for the next 4 to 5 hours.

In one experiment, ecdysone was injected into larvae 1 hour after the injection of puromycin. The larvae were fixed 3 hours later. Thus ecdysone was present only during the period of inhibition of protein synthesis. Puffs I-18-C and IV-2-B were induced in all these larvae. The mechanism by which ecdysone induces these puffs, therefore, does not seem to include the synthesis of new proteins. The larvae in another group were fixed 24 hours after each received a combined injection of ecdysone and puromycin. In most of these larvae only those puffs were present which normally appear earlier than 15 hours after the injection of ecdysone, while those puffs which normally appear after 15 to 20 hours were missing. Puffing of these loci, therefore, seems to be delayed, corresponding to the duration of time in which protein synthesis is inhibited. Thus, in contrast to the processes leading to the induction of puffs I-18-C and IV-2-B, those which lead to the induction of the puffs which appear later apparently do involve protein synthesis.

Our results show that ecdysone very specifically activates only a few gene loci. Through the RNA messages produced by these genes, and the proteins determined by them, some processes in the cytoplasm are set in motion which lead to the activation of other genes. Thus, the first steps in the action of ecdysone can now be described as shown in Fig. 3. The mechanism by which ecdysone influences the activity of the primary genes (the question mark in Fig. 3) is not yet known.

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