Inhibitory effect of β-ecdysone on protein synthesis by blowfly fat body in vitro

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Summary. Synthesis of blow fly calliphorin and other blood proteins by larval fat body in organ culture is inhibited by β-ecdysone. The findings suggest a novel function for the hormone.

Insect fat body, like vertebrate liver, is the major source of plasma proteins[3,4]. In the blowfly *Calliphora,* the rapid synthesis of plasma proteins during the actively feeding phase of the 3rd larval instar falls off sharply as the fully grown larva leaves its food and commences to wander prior to pupariation[5]. This decline is well illustrated by biosynthesis of the major larval blood protein calliphorin[6] (or protein C[7]); during larval growth its concentration in the blood increases dramatically to about 15% (w/v) in *C. stygia* at the cessation of feeding[7]. Subsequently, during the wandering stage, fat body synthesis of calliphorin and of most other plasma proteins either ceases, or declines to a low rate[3]. This termination of protein synthesis is associated with profound cytological changes in the fat body cells involving nucleolar degradation[8], loss of ribosomes[9], and subsequent accumulation of protein granules[4]. The biological signal(s) initiating wandering and associated physiological changes such as those described is still unknown, but a barely detectable increase in ecdysone level[10], subsequently confirmed as a small peak of ecdysteroids by RIA (J. Koolman, personal communication) occurs in the early 3rd instar larva. To test whether this might be one of the signals involved we examined whether ecdysone could inhibit protein synthesis by the isolated fat body in organ culture. Our findings now indicate that ecdysone does inhibit protein synthesis in such a system. The procedure was basically as follows.

C. vicina R.D. (*C. erythrocephala* Meig.) was reared aseptically on a semidefined diet[11]. Fat body explants prepared from 3rd instar larvae were placed in organ culture in a manner similar to that described for pineal glands[12]. The explants were incubated in the absence (control) or the presence of varying concentrations of β-ecdysone, and the incorporation of L-[^{35}S]-methionine from the medium into secreted protein and/or calliphorin was measured. In all gel experiments calliphorin includes minor contamination by the related protein II[6].

The developmental age of the donor larvae was first examined for 2 reasons. First, to test our experimental system by demonstrating a decrease in the rate of protein synthesis associated with larval age as found in vivo[4,5] and in vitro[6]; second, the best chances of observing an ecdysone effect would be with fat body having maximal rates of endogenous synthesis. Accordingly, fat bodies from larvae aged 4, 5, 6, 7 and 8 days after egg laying were examined for rates of labelled methionine incorporation into both retained and secreted calliphorin and into corresponding bulk protein. As expected, these rates expressed on a 'per mg fat body protein' basis progressively declined between the 4th and 7th days. However, the fat body protein content increases exponentially from days 4 to 6 to attain a maximum on the 8th day (figure 1), and thus a more meaningful portrayal is bulk protein and calliphorin production by the entire organ calculated from the corresponding explant data (figure 1). The curves indicate maximal protein synthesis between the 5th and 6th days and hence 4-day larvae fat bodies were employed for subsequent experiments, these becoming equivalent to 5-day fat bodies after 20 h pre-incubation (vide infra).

To verify that incorporation of the labelled precursor, L-[^{35}S]-methionine, into secreted protein was a measure of true protein synthesis, the effects of 3 known inhibitors of protein synthesis were next examined. Fat body explants were pre-incubated for 15 min with either cycloheximide (10^{-4} M), emetine-2HCl (10^{-4} M) or puromycin (2×10^{-5} M) in modified Grace's medium prior to incubation for 1 h in fresh medium containing the indicated concentration of inhibitor plus 1 μCi of [^{35}S]-methionine. In each case radioactivity in the secreted proteins was negligible compared to controls lacking inhibitor.

Addition of up to 10^{-4} M β-ecdysone to the incubation medium showed only minor and inconsistent effects on the incorporation of isotope into secreted proteins during the first 4 h of incubation. However, the natural termination of protein synthesis requires about 2 days in vivo (figure 1). If the putative action of ecdysone should be by inhibition at the transcriptional level, then possibly calliphorin mRNA[13], like some other insect mRNA's[14], may have a half-life of many hours. Accordingly, fat bodies were incubated in the presence of hormone for 20 h, at which time isotope was added and incorporation into protein was measured during the subsequent 2 h of incubation. Under these conditions an inhibitory effect of β-ecdysone on protein synthesis was clearly demonstrable as judged by intensity of labelling of the separated, secreted protein bands on fluorograms. A typical fluorogram, depicted in the inset in figure 2, shows the visible reduction in band labelling intensity following incubation with 10^{-4} and 10^{-6} M β-ecdysone and a somewhat less pronounced reduction with 10^{-7} M ecdysone.

For a more quantitative analysis, 2 approaches have been employed. 1. The conspicuous calliphorin bands were excised from dried gels following fluorography and counted. Despite the rather wide scatter of values derived from replicate experiments, the data (figure 2) substantiate the visual findings: [^{35}S]-methionine incorporation into calliphorin was inhibited by about 35% at 10^{-8} M ecdysone, and by about 65% at 10^{-7} M or higher concentrations of hormone. Particularly noteworthy is the disproportionate inhibition of calliphorin secretion relative to the bulk protein, indicative of some degree of specificity as opposed to a general inhibition. 2. Pure calliphorin was used to prepare rabbit anti-serum according to experiments to be published elsewhere. This anti-serum was then utilized to selectively immunoprecipitate[15] radioactive calliphorin secreted into the medium in the presence of varying concentrations of ecdysone. The results of 2 such experiments indicated that after incubation with 10^{-12}, 10^{-6} and 10^{-4} M ecdysone, the radioactivity in calliphorin was 109, 50 and 44% respectively of controls lacking ecdysone. Confirming our conclusion that ecdysone inhibits fat body protein synthesis we have shown, albeit less satisfactorily than with isotope, that the intensity of corresponding Coomassie Brilliant Blue-stained bands after SDS-gel electrophoresis of secreted proteins was less after incubation with 10^{-6} M ecdysone than in its absence (not illustrated), which supports the fidelity of the radio-tracer experiments.

To assess whether our findings could be ascribed to a nonspecific effect of any polyhydroxylated steroid, we substituted varying concentrations of 22-iso-α-ecdysone – a biologically inactive epimer[15] kindly donated by Dr Clive A. Henrick – in place of β-ecdysone. We estimate from the resulting fluorograms that the 22-iso-ecdysone was only

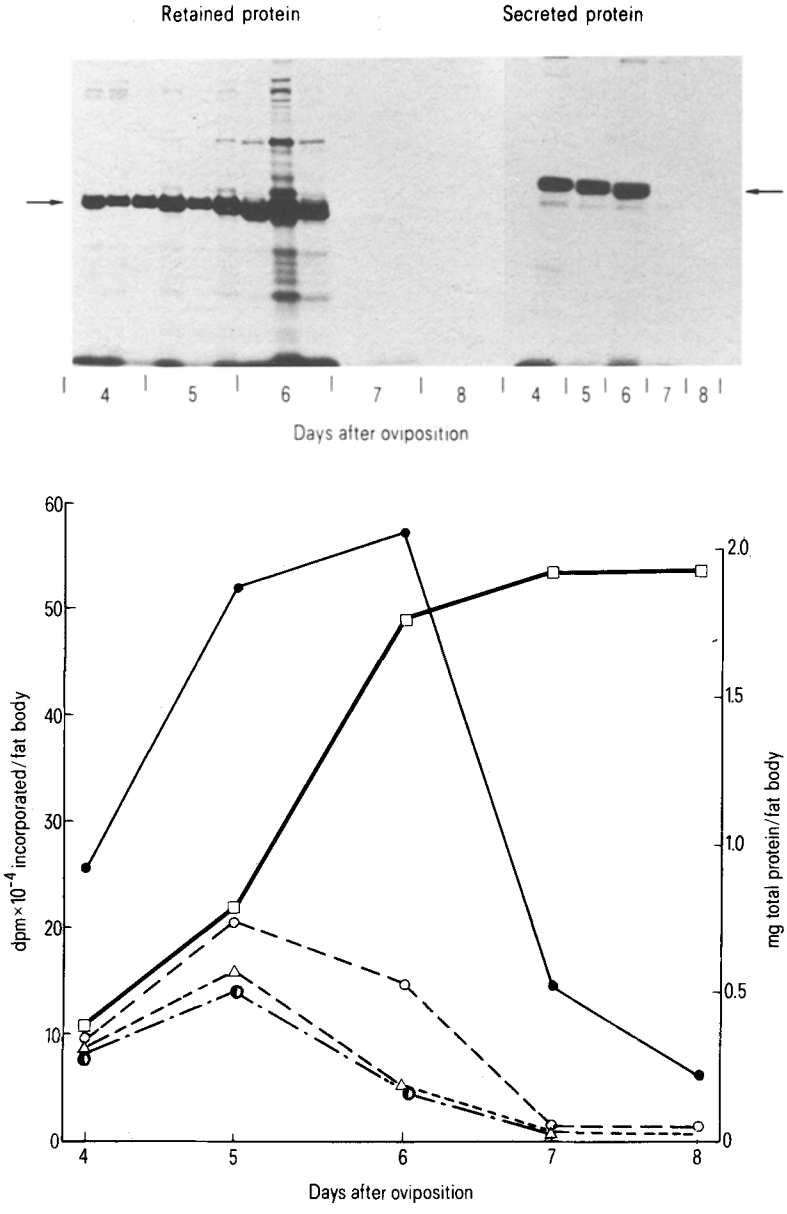
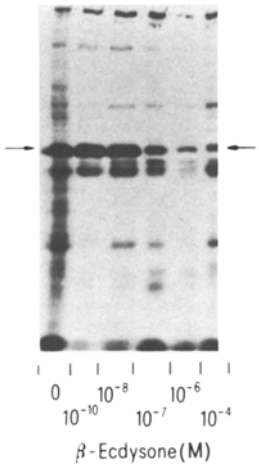


Fig. 1. Incorporation of [^{35}S]-methionine into retained and secreted proteins by *C. vicina* fat body in relation to larval age. Fat bodies were either carefully dissected out in toto for whole organ protein estimation, or were aseptically removed from sterile larvae of the desired age and placed on 4–5 mm^2 of 400-mesh nylon screening[12] on the bottom of 16 mm wells of a Costar tissue culture cluster (Microbiological Associates, Bethesda, MD) containing 200 µl of medium. The explants were teased to form as flat a sheet of fat body as possible. The modified Grace's[21] medium consisted of Grace's medium diluted with 20% (v/v) of sterile water; the methionine content was reduced to 1.3×10^{-3} mM, and 20 units of penicillin and 20 µl of streptomycin ml^{-1} were added. The culture dish was placed in a chamber on a rocking platform (9 cycles min^{-1}) at 25–28 °C in a flowing atmosphere of H_2O-saturated 95% O_2-5% CO_2. After 1 h the explants were transferred to 200 µl fresh medium containing 2 µCi L-[^{35}S]-methionine (Amersham, 400–900 Ci/mM). At the end of 2 h the explants were removed, frozen and thawed, and sonicated in 200 µl of water. Protein concentrations using pure calliphorin standards were measured by the method of Goa[20] after prior delipidation in methanol-chloroform (2:1) for whole fat bodies, and by modified Lowry[23] for the explants. For electrophoretic analysis 25 µl aliquots of sonicate or incubation medium were mixed with an equal volume of a solution containing 2% (w/v) sodium dodecyl sulphate (SDS), 0.02% (v/v) β-mercaptoethanol, 0.2% (v/v) glycerol, and 0.002% phenol red in 0.1 M Tris-HCl buffer pH 6.8 and heated for 1 min at 100 °C. SDS slab-gels of 7.5% polyacrylamide were run as described[24] and subjected to fluorography[25] either directly, or after staining with Coomassie Brilliant Blue R250. Calliphorin bands (indicated by arrows) were cut out of the dried gels using developed fluorograms as templates, dissolved in 30% H_2O_2 containing 4% (v/v) NH_4OH at 50 °C overnight, neutralized with acetic acid and counted in 10 ml Aquasol (New England Nuclear) at 85% efficiency. The appropriate correction for recovery of radioactivity from such gel slices was derived from purified, labelled calliphorin of known specific activity subjected to identical treatment. The recovery was found to be 51%.

To measure [^{35}S]-methionine incorporation into total protein, 40 µl aliquots were heated for 15 min at 90 °C with 10% (w/v) TCA containing 5 mM L-methionine and were filtered on Whatman GF/A discs. The precipitated protein was washed twice with 5 ml of the TCA solution and then twice with diethyl ether prior to drying at 50 °C. The dry protein was digested with NCS solubilizer (Amersham/Searle) for 2 h at 50 °C, neutralized with acetic acid and counted as above. Above left: fluorograms of retained proteins depicting 3 separate experiments for each of the days indicated to show the variation between individual explants; above right: fluorogram of secreted proteins. Below: protein content □; synthesis of ● retained protein; ○ retained calliphorin; △ secreted protein; ◐ secreted calliphorin.



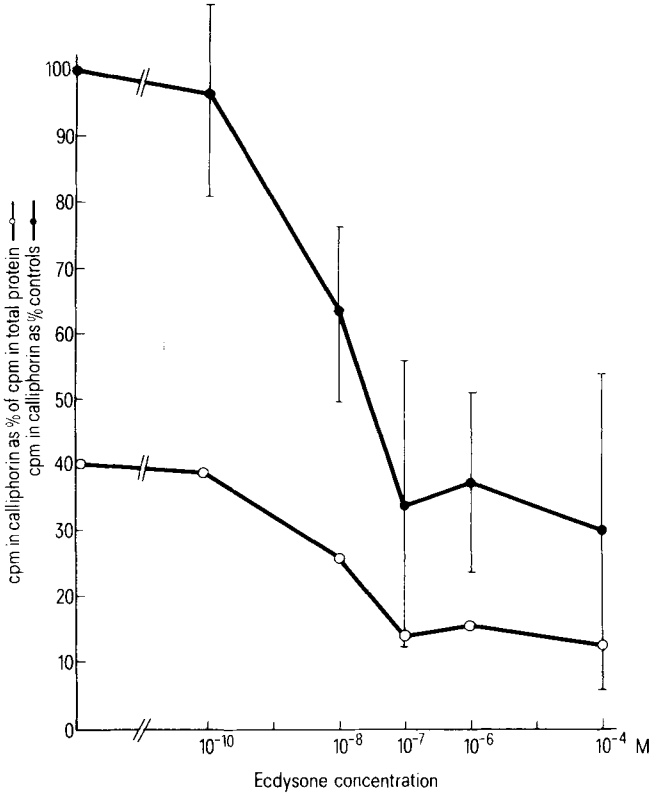


Fig. 2. Effect of β-ecdysone on incorporation of [^{35}S]-L-methionine into calliphorin by *C. vicina* fat body explants. Incubation conditions as in legend to figure 1 except for the following: 1. β-ecdysone (Sigma) was dissolved in water and standardized by densitometry at 240 nm[26] prior to addition to the medium at the indicated concentrations; 2. explants were incubated for 10 h in the absence of isotope but in the presence of hormone, and for a further 10 h in fresh medium. A 2nd change was then made to fresh medium containing 2 μCi of [^{35}S]-methionine and incorporation measured over the ensuing 2 h as described in figure 1. The calliphorin + protein II bands on the dried fluorograms were excised, and the remainder of the electrophoretic tracks were cut into equal pieces which were individually counted as in the legend to figure 1. Counts in calliphorin determined in the presence of ecdysone are expressed as a percentage of controls lacking hormone. For each concentration of ecdysone the total counts per track were normalized to 100% and counts in the calliphorin bands also expressed as a percentage of this total. The quantitative data are the means of 5 sets of experiments, the bars indicating the numerical range of the data.

1–10% as active as the natural hormone, and conceivably this slight activity could be due to traces of biologically active contaminants. A synthetic juvenile hormone analogue, ZR 515 (Zoecon Corp.) had no effect at concentrations up to 10^{-4} M.

Our finding that ecdysone inhibits blowfly fat body protein synthesis in vitro is contrary to reports of variable, but overall stimulatory effects on such synthesis primarily in vivo[17–19]. These studies, however, were performed at significantly higher ecdysone concentrations employing physiologically older larvae, and the experiments were of shorter duration. No experiments directly comparable to ours have been reported, although the results of long-term exposure of a *Drosophila* cell line to β-ecdysone[20] were similar. Two additional aspects of our experiments should be noted. First, although the modified Grace's medium we have employed was the most satisfactory of several media tested, nevertheless the rate of protein synthesis after 22 h of incubation had declined to about a third of the initial rate. Thus, the response to ecdysone might in fact be greater than that observed. Second, maximal ecdysone inhibition of [^{35}S]-methionine incorporation into secreted fat body proteins was observed only at low methionine concentration in the medium. Thus, the inhibition with 3.3×10 mM methionine (as in undiluted Grace's medium) was neither as consistent nor as marked as that described above. The physiological significance of this dependency upon methionine concentration remains to be determined.

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