

**Effect of 20-hydroxyecdysone on synthesis and uptake of arylphorin
by the larval fat body of *Calliphora vicina* (Diptera: Calliphoridae)**

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***Calliphora vicina*, arylphorin, ecdysteroids, protein synthesis, receptor mediated endocytosis**

Abstract. During the final larval instar of the blowfly *Calliphora vicina* the storage protein arylphorin is stage-specifically produced and re-absorbed by the fat body cells. In this study the hormonal regulation of arylphorin biosynthesis and endocytosis has been investigated in vitro and in vivo. Application of 10 to 50 ng 20-hydroxyecdysone (20E) in vivo or of 10^{-7} to 10^{-6} M 20E in vitro specifically inhibits arylphorin translation without altering the arylphorin mRNA level. After termination of arylphorin synthesis in vivo, it can be artificially re-induced by neck-ligation, an effect which can be reversed by application of 50 ng 20E. This suggests that the small rise in titre of 20E at the end of feeding stage is responsible for the inhibition of arylphorin-mRNA translation in vivo. 20E stage-specifically stimulates arylphorin uptake by the larval fat body in vitro, as well as in vivo (Burmester & Scheller, in prep.). We assume two defined but different post-translational effects of 20E on arylphorin metabolism.

INTRODUCTION

During larval development insects synthesize large amounts of characteristic storage proteins or hexamerins with a molecular mass in the 5×10^5 Da range (Telfer & Kunkel, 1991). Those which are characterized by a high content of aromatic amino acids (aryl groups) are generally termed arylphorins. In the blowfly *Calliphora vicina* the synthesis of arylphorin is restricted to the fat body of the final larval instar (for review, see Scheller et al., 1990). During the feeding period the fat body cells secrete arylphorin into haemolymph, where it accumulates and eventually makes up 80% of total haemolymph proteins (Sekeris & Scheller, 1977).

Arylphorins act as a source for aromatic amino acids and energy for protein synthesis during metamorphosis (Levenbook & Bauer, 1984), serve as an ecdysteroid carrier in the haemolymph (Enderle et al., 1983) and as a component of the sclerotizing system of the cuticle (for review, see Peter & Scheller, 1991). Arylphorin of *Calliphora vicina* is composed of six polypeptides, each of 759 amino acids (Naumann & Scheller, 1991). At the end of the final larval instar arylphorin is selectively taken up by the fat body cells (Burmester & Scheller, 1992).

It is generally assumed that insect larval development, moulting, puparium formation and the onset of metamorphosis are controlled mainly by juvenile hormone and ecdysteroids. The influence of both hormones on biosynthesis and utilization of arylphorin was the subject of several studies. It has been demonstrated that juvenile hormone represses the

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gene expression of some larval storage proteins (Jones et al., 1988; Memmel & Kumaran, 1988). Several papers reported that ecdysteroids accomplish a stimulating effect on the transcription of LSP-1 β and LSP-2 genes in *Drosophila melanogaster* (Jowett & Postlethwait, 1981; Lepesant et al., 1982; Powell et al., 1984), while another study suggested that 20-hydroxyecdysone (20E) inhibits transcription of the same gene (i.e. LSP-2: Richards, 1981). Several studies found no influence of ecdysteroids on larval serum proteins (i.e. Caglayan & Gilbert, 1987). By contrast, an inhibitory effect of 20E on arylphorin synthesis was postulated by Pau et al. (1979) for *Calliphora vicina*, by Ray et al. (1987) for *Galleria mellonella* and by Webb & Riddiford (1988) for *Manduca sexta*. Schenkel et al. (1983) reported that translatable arylphorin mRNA is present in the form of mRNPs after cessation of arylphorin biosynthesis, and they proposed that a small rise in titre of 20E might be responsible for the cessation of arylphorin translation in vivo.

The results referring to the influence of ecdysteroids on the utilization of arylphorin are less contradictory. Collins (1969) was the first who demonstrated hormonal control of the formation of storage granules by executing ligation experiments and Lepesant et al. (1978) showed that in a *Drosophila* ecdysteroid-deficient mutant the fat body failed to accumulate larval serum proteins. Elevation of arylphorin uptake by larval fat body induced by experimental changes in the ecdysteroid level were demonstrated repeatedly (Tojo et al., 1981; Ueno & Natori, 1982; Ismail & Gupta, 1990). Detailed biochemical studies in *Sarcophaga peregrina* (Ueno et al., 1983; Ueno & Natori, 1984) revealed that the sequestration of storage protein is mediated by a specific receptor which is activated by 20E.

In our studies on *Calliphora vicina* we showed that arylphorin is selectively taken up from the haemolymph by the fat body cells at the end of the last instar, probably by the mechanism of receptor mediated endocytosis (Burmester & Scheller, 1992). We discovered three arylphorin binding proteins (ABP 130, ABP 96, ABP 65; named according to their molecular mass in SDS-PAGE) in the fat body cell membrane fraction; these were later proved to be derived from a single protein, the proposed arylphorin receptor precursor (Burmester & Scheller, in prep.). It could be demonstrated in vivo that 20E is responsible for the initiation of the stage-specific sequestration of arylphorin. By the action of 20E ABP 96 is cleaved into ABP 65 and a 30 kDa protein, which does not bind arylphorin. Recently we determined the complete cDNA sequence and protein structure of the arylphorin receptor precursor (Burmester & Scheller, in prep.).

In summary, 20E was proposed to be involved in the control of both arylphorin synthesis and utilization but this is unlikely because the two processes occur at different times of larval development. We decided to examine both possible effects and to develop a model of the action of 20E in arylphorin metabolism.

MATERIALS AND METHODS

ABBREVIATIONS USED: 4dL, (5dL...) – four (five...) days old larvae; 20E – 20-hydroxyecdysone; ABP – arylphorin binding protein; ArR – arylphorin receptor; PAGE – polyacrylamide gel electrophoresis; SDS – sodium dodecyl sulphate; SEM – standard error of the means.

ANIMALS. *Calliphora vicina* Robineau-Desvoidy larvae were reared on bovine meat at 23°C and 65% relative humidity under a long day photoperiod (16 h light : 8 h dark). The egg deposition was restricted to 30 min. Under these conditions, the 3rd larval instar starts 3 days after egg deposition, feeding period lasts to day 6 and is followed by the wandering stage. Puparia are formed on day 8, and adult eclosion follows after 10 additional days.

PROTEIN EXTRACTION FROM THE HAEMOLYMPH AND LARVAL FAT BODY. Fat bodies were excised on ice under a binocular microscope and transferred to the desired medium. Haemolymph was collected from decapitated larvae and diluted in 140 mM NaCl, 10 mM MgCl₂, 0.01% phenylthiourea, 15 mM KH₂PO₄/Na₂PO₄, pH 6.3. The protein concentrations were determined according to the method of Bradford (1976).

IN VIVO-LABELLING OF FAT BODY AND HAEMOLYMPH PROTEINS. Larvae of desired age received injections of 10 µCi ³⁵S-L-methionine dissolved in sterile insect saline. To obtain ecdysone-deficient insects, the larvae were neck-ligated on day 6 and used for protein labelling on day 7. Varying amounts of 20E (Calbiochem) dissolved in 10% ethanol were injected. After 1 h incubation at room temperature the larvae were decapitated, haemolymph collected and fat bodies dissected. The proteins were analysed by SDS-PAGE.

IN VITRO-LABELLING OF SECRETED FAT BODY PROTEINS. Three fat bodies of 4dL per assay were dissected, washed twice with 1 ml of Grace's insect medium (without methionine; GIBCO-BRL) and incubated in 200 µl of the same medium with 10 µCi ³⁵S-L-methionine and the desired concentration of 20E. After 2 h the fat bodies were removed, the incubation medium was centrifuged 10 min at 10,000 × g, and the proteins in the supernatant were precipitated with five volumes of acetone (-20°C). The resulting pellet was dissolved in sample buffer and subjected to SDS-PAGE.

POLYACRYLAMIDE GEL ELECTROPHORESIS AND FLUOROGRAPHY. One-dimensional polyacrylamide gel electrophoresis was performed under denaturing conditions by the method of Laemmli (1970) using 6% stacking and 10% separating gels. Proteins were stained with Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid, destained and photographed. Subsequently the gels were soaked in "Amplify" (Amersham) and submitted to fluorography on X-ray films.

ARYLPHORIN LABELLING AND PURIFICATION. In vivo-labelling of arylphorin was performed by injecting 5 µCi ³⁵S-L-methionine (1,200 Ci/mM, NEN-DuPont) diluted in sterile insect saline (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂) into 4dL. Six hours after injection haemolymph was collected and arylphorin purified as described by Burmester & Scheller (1992). The specific activity of labelled arylphorin was about 1,000–2,000 cpm/µg.

IN VITRO UPTAKE OF ARYLPHORIN. Hand-dissected fat bodies from 4dL, 7dL or 7dL neck-ligated on day 6 were washed twice in Schneider's medium and incubated for 2 h with 50 or 100 µg of ³⁵S-arylphorin with or without the desired amount of 20E in 200 µl Schneider's medium (GIBCO-BRL). The incorporated amount of arylphorin was measured as described before (Burmester & Scheller, 1992).

RNA PREPARATION AND NORTHERN BLOTTING. Five 4dL injected with 20E were incubated at room temperature for 2 h. Total RNA was extracted according to Scheller & Karlson (1977) and subjected to electrophoresis in a 0.8% agarose gel containing 1 M formaldehyde. After electrophoresis, the gels were rinsed in 20 × SSC and transferred to nitrocellulose. Northern blot analysis was performed according to standard procedures (Sambrook et al., 1989) using as probe the genomic arylphorin gene fragment pC223 (Naumann & Scheller, 1991), labelled with ³²P-CTP by random priming.

RESULTS

CONTROL OF ARYLPHORIN BIOSYNTHESIS BY 20E IN VIVO. Arylphorin is stage-specifically synthesized during the feeding period of the last larval instar (Scheller et al., 1990). Since the fat body of 3dL is fragile and difficult to be excised, and the mortality of the larvae after injection is rather high (about 30%), while 5dL already contain a high concentration of arylphorin, and a change after 1 h incubation is difficult to measure, we chose 4 dL for our labeling experiments. The larvae have a survival rate of about 90% after injection. In all experiments performed we got specific labelling of fat body proteins.

The in vivo response of the fat body to changing concentration of 20E is dosage-dependent (Fig. 1). While injections of 10 ng 20E into 4dL does not change the level of labelled arylphorin in the fat body or haemolymph, injection of 20 ng 20E clearly reduces radioactivity of the arylphorin band, and 50 ng 20E also causes a slight reduction. Surprisingly, the labelling rises to the normal level after application of 100 or 200 ng 20E.

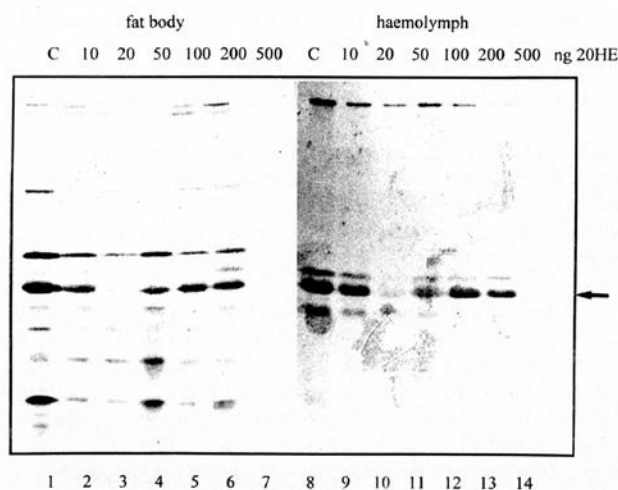


Fig. 1. Effect of 20E on protein synthesis in vivo. 4dL received injections of 20E and were kept for 1 h at room temperature; subsequently the proteins were labelled for 1 h with $10 \mu\text{Ci } ^{35}\text{S}$ -methionine. Proteins of the fat body (left) and the haemolymph (right) of single animals were separated on SDS-PAGE, stained, fixed, dried and subjected to fluorography. The arrow indicates the position of arylphorin. Lane 1, 8 - control; 2, 9 - 10 ng; 3, 10 - 20 ng; 4 + 11 - 50 ng; 5, 12 - 100 ng; 6, 13 - 200 ng; 7, 14 - 500 ng 20E.

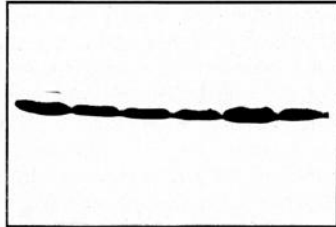
Besides arylphorin, and a protein with a slightly higher molecular mass designated as protein II (Munn & Greville, 1969, Markl et al., 1992), no other protein seems to be affected by the applications of 5 to 200 ng 20E. Injection of 500 ng causes a reduction of overall protein biosynthesis and brings about death of most larvae. It should be mentioned that in some experiments the inhibitory effect of 20E was variable between 5 to 20 ng, indicating a dependence on exact age and size of the larvae.

EFFECT OF 20E ON ARYLPHORIN SYNTHESIS IN VITRO. We incubated fat bodies of 4dL with 5×10^{-8} to 5×10^{-6} M 20E and in the presence of $10 \mu\text{Ci } ^{35}\text{S}$ -methionine. Proteins released into the incubation medium within 2 h were precipitated and analyzed on SDS-PAGE. As depicted in Fig. 2, 10^{-7} to 10^{-6} M 20E reduced the amount of labelled arylphorin in the medium, while total amount of released protein seems to be unaffected (Fig. 2a). Supporting our results obtained in vivo, labelling of arylphorin rises to a normal level when fat bodies are incubated with higher concentration of 20E.

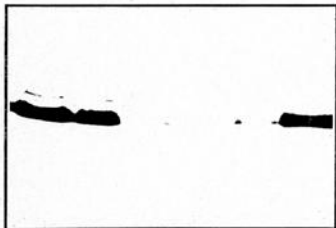
ANALYSIS OF THE EFFECT OF 20E ON THE LEVEL OF ARYLPHORIN mRNA. To examine the influence of 20E on the steady state level of arylphorin mRNA we assessed the actual amount of arylphorin mRNA by Northern blotting. Five 4dL received injections of 10, 20, 50, 100 or 500 ng 20E per larva and were incubated for 2 h. Insect saline without 20E was injected into control larvae. As shown in Fig. 3, up to 500 ng 20E have no detectable effect on the amount of arylphorin mRNA.

EFFECT OF NECK-LIGATION ON ARYLPHORIN BIOSYNTHESIS. The experiments described above demonstrate that 20E can inhibit arylphorin biosynthesis in vivo at the time when this protein is translated. We tested whether arylphorin biosynthesis is re-initiated when ecdysteroids are removed by separating the posterior body part from the site of ecdysteroid synthesis: 6dL were neck-ligated and left for one day at room temperature to allow the endogenous hormone level to decline. Control larvae were left non-ligated. On day 7 one half of the ligated larvae received injections of 50 ng 20E and all larvae were injected with $10 \mu\text{Ci } ^{35}\text{S}$ -methionine. As shown in Fig. 4, arylphorin is not synthesized in control larvae on day 7. After neck-ligation the arylphorin band is labelled, and injection of 50 ng 20E terminates arylphorin biosynthesis.

a. Coomassie staining

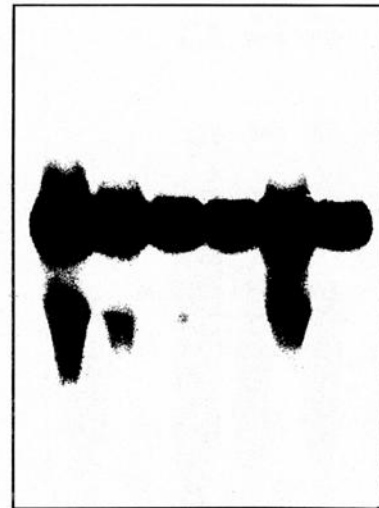


b. fluorography



1 2 3 4 5 6

Fig. 2. Labelling of proteins released by the fat body in vitro after application of 20E. Three fat bodies of 4dL were excised and incubated for 2 h in 200 μ l methionine-free Grace's insect medium with 10 μ Ci 35 S-methionine and the indicated amount of 20E. After 2 h the fat bodies were removed and the proteins released into the medium precipitated with acetone (-20° C) and analyzed on SDS-PAGE. Lane 1 – control; 2 – 5×10^{-8} M; 3 – 10^{-7} M; 4 – 5×10^{-7} M; 5 – 10^{-6} M; 6 – 5×10^{-6} M 20E in medium.



1 2 3 4 5 6

Fig 3. Northern blot analysis of arylphorin mRNA from larvae after treatment with 20E. 20 μ g of total RNA from larvae which received varying amounts of 20E was separated on an agarose gel and probed with 32 P-labelled arylphorin DNA probe as described. Lane 1 – control; 2 – 10 ng; 3 – 20 ng; 4 – 50 ng; 5 – 100 ng; 6 – 500 ng 20E.

ARYLPHORIN UPTAKE IN VITRO. The capability of larval fat body of different developmental stages to sequester arylphorin in vitro was investigated by incubating single fat bodies with 100 μ g 35 S-arylphorin for 2 h in Schneider's insect medium. The incorporation of radioactivity rate is low in 4dL and early 5dL, rises to a small transient peak in late 5dL decreases on day 6 and early day 7, and finally rises to a very high rate at the end of the 3rd instar (Fig. 5). The rate of radiolabelling in 8dL is about eight times as high as in early 4dL.

INDUCTION OF ARYLPHORIN UPTAKE WITH 20E. The capability of 20E to induce the uptake of arylphorin was examined in fat bodies of intact 4dL, and 7dL, and 6dL neck-ligated for 24 hr (Fig. 6). The radiolabelling rate is low in 4dL and high in 7dL fat bodies and 20E has no enhancing effect on the fat bodies at these stages. When 6dL were neck-ligated and the fat bodies of the posterior body part were tested for their ability to take up arylphorin in vitro at day 7, we found that the radiolabelling was low. Application of 5×10^{-7} to 10^{-6} M 20E leads to a significant increase in arylphorin uptake, while higher concentrations (5×10^{-5}

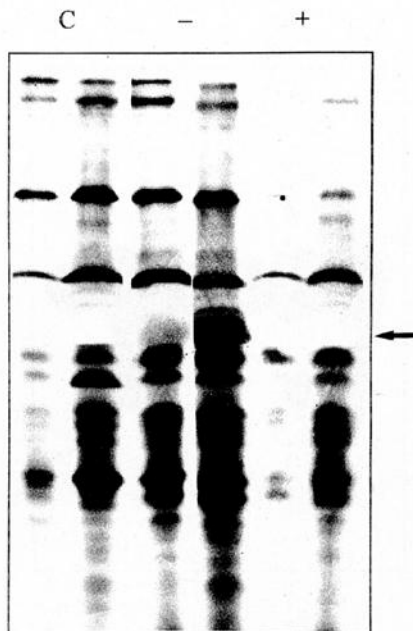


Fig. 4. Induction of arylphorin biosynthesis by neck-ligation. 6dL were neck-ligated and left at room temperature for 24 h. Larvae used as controls were left non-ligated (C). At day 7 some of the ligated larvae received injections of 50 μg 20E (+), the others not (-). The fat body proteins were labelled with ^{35}S -methionine for 1 h and analysed by SDS-PAGE and subsequent fluorography. The arrow indicates the position of arylphorin.

to 10^{-5}) have an inhibitory effect. However, there was a high degree of variability in all experiments and thus a large standard deviation.

CONCLUSIONS AND DISCUSSION

Our studies focus on the stage-specific endocytosis of arylphorin by the larval fat body (Burmester & Scheller, 1992, in prep.). When analyzing the influence of 20E on this process we obtained evidence that this hormone also modulates the biosynthesis of arylphorin. This confirmed our previous assumptions (Schenkel et al., 1983) and led us to carry out this comparative study which concerns both issues.

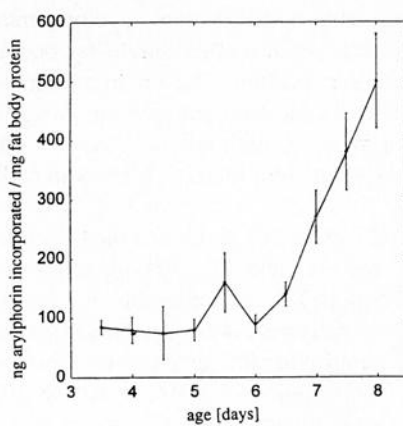


Fig. 5. Arylphorin uptake in vitro. Fat bodies from 4dL to 8dL were excised every 12 h and incubated in 200 μl Schneider's medium with 100 μg ^{35}S -arylphorin. After 2 h incubation at room temperature the amount of arylphorin taken up was calculated. Points (\pm SEM) are averages for four larvae.

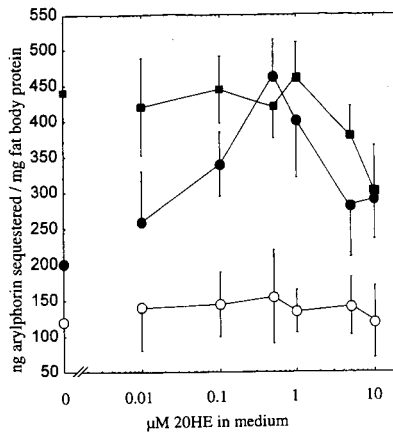


Fig. 6. Effect of 20E on arylphorin uptake in vitro. Fat bodies from 4dL (○), 7dL (■) and 7dL neck-ligated at day 6 (●) were incubated with the indicated amount of 20E as described. After 2h the sequestered arylphorin was determined. Points (\pm SEM) are calculated from four fat bodies.

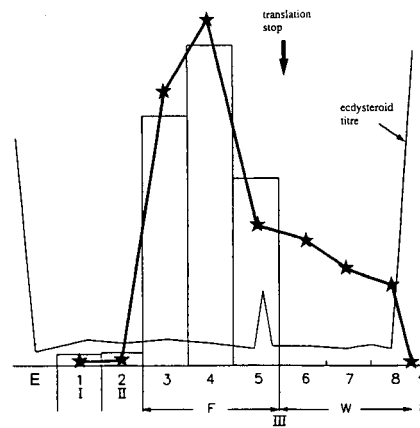


Fig. 7. Transcription and translation of arylphorin throughout the 3rd larval instar (adapted from Schenkel et al., 1983). The relative amount of in vitro-translatable arylphorin-mRNA (★), and the amount of arylphorin actually synthesized in vivo (vertical bars) were depicted. The concentration of ecdysteroids was depicted according to the results of Koolman (1980). The age of the larvae is indicated in days after egg deposition. E – egg; I – 1st larval instar; II – 2nd larval instar; III – 3rd larval instar; F – feeding stage; W – wandering stage; P – puparium formation.

The stage specific biosynthesis of arylphorin during the feeding period of holometabolous larvae was suspected to be regulated by 20E but experimental data were confusing, sometimes reporting a stimulation of arylphorin synthesis (Jowett & Postlethwait, 1981; Lepesant et al., 1982; Powell et al., 1984), while other studies proposed inhibition (Pau et al., 1979; Ray et al., 1987; Webb & Riddiford, 1988). The results obtained in this study demonstrate that biosynthesis of arylphorin is specifically inhibited by a definite concentration of 20E: Applications of either 10 to 50 ng 20E per larva in vivo, or 10^{-7} to 10^{-6} M 20E in vitro, result in a repression of arylphorin synthesis. However, 20E has no influence on the arylphorin export machinery: None of the 20E concentrations tested caused a reduction of arylphorin released in vitro.

20E does not alter the amount of arylphorin mRNA within 2 h after application but a reduction of arylphorin biosynthesis was established after 1 h incubation. Since the amount of arylphorin mRNA remains constant, while the translation of arylphorin decreases, we suggest that arylphorin biosynthesis is not regulated primarily on transcriptional level in wandering larvae and that 20E inhibits arylphorin biosynthesis on the translation level, this is consistent with the effect of neck-ligation; this removal of endogenous 20E causes a renewal of arylphorin biosynthesis. These results agree with the earlier observations which showed that in vitro-translatable mRNA is detectable as mRNPs up to the end of the third larval instar, and that the cessation of biosynthesis of arylphorin is not

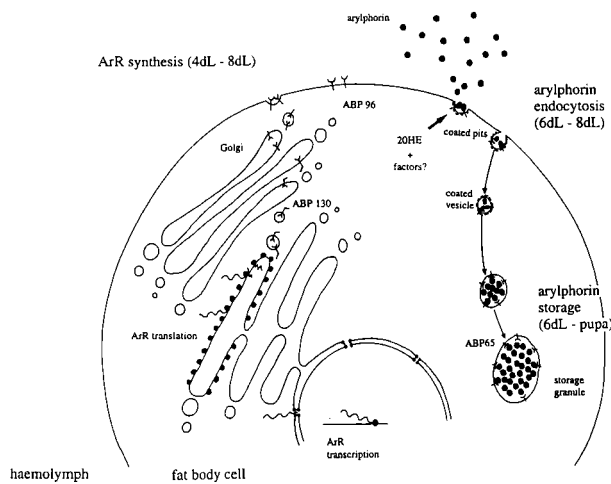


Fig. 8. Model showing the role of 20E in control of arylphorin incorporation. ArR - arylphorin receptor. For further discussion please refer to the text.

due to the absence of arylphorin mRNA (Schenkel & Scheller, 1986). In Fig. 7 we summarize current knowledge about the regulation of arylphorin biosynthesis.

Here it should be mentioned that the data on ecdysteroid titre (Koolman, 1980; Fig. 7) indicate a decline of total ecdysteroids after a small peak on day 5 and a strong rise of titre on day 8. This does not agree with our data about the ecdysteroid-inducible processes. Our results show that ligation of 6dL causes an ecdysone-responsive re-initiation of arylphorin translation, while according to Koolman (1980) nearly no ecdysteroids are present at this stage. A possible explanation is that the established titre does not reflect the actual concentration of 20E in the fat body cells. It was shown that up to 90% 20E is bound to arylphorin in the hemolymph and is, therefore, inactive (Enderle et al., 1983).

Our data give no explanation why the arylphorin biosynthesis rises to the normal level after application of more than the 50 ng 20E *in vivo*, or 10^{-6} M 20E *in vitro*, respectively, whereas general protein biosynthesis is not affected by as much as 200 ng 20E (Fig. 1). There must be a molecular mechanism which specifically regulates arylphorin biosynthesis. Our finding contradicts the reports of variable, but overall stimulatory effects of ecdysteroids on general protein biosynthesis *in vivo* (e.g. Neufeld et al., 1968; Arking et al., 1969). These studies, however, were performed with physiologically older larvae. No experiments directly comparable to ours have been reported, and, as far as we know, there is no result that shows a specific repression of a protein by a distinct hormone concentration. Nevertheless, these data might explain some of the confusions about the influence of 20E on arylphorin, serum protein, or LSP synthesis in Diptera and Lepidoptera.

The fat body of *Calliphora* larvae sequester arylphorin stage-specifically at the end of the 3rd larval instar *in vitro*. These results confirmed our previous studies carried out *in vivo* (Burmester & Scheller, 1992). There is a small rise in arylphorin sequestration in late 5dL which was not observed in the *in vivo* studies. Whether this peak is linked to the proposed small rise in ecdysteroid titre at this stage (Koolman, 1980) could not be determined.

Our present evidence that 20E is an essential factor for arylphorin sequestration, confirms similar results obtained by Ueno & Natori (1982). We could demonstrate that 20E is linked to the sequestration process by cleaving the proposed arylphorin receptor ABP 96 into a 65 and a 30 kDa protein (unpublished). In Fig. 8 we suggest a model of receptor-mediated endocytosis of arylphorin which is based on the present as well as on hitherto unpublished results: We propose that the arylphorin receptor (ArR) is synthesized throughout the last larval instar from day 4 to day 8. The ArR gene is transcribed and the mRNA is translated into a single protein precursor (ABP 130), which undergoes post-translational processing into ABP 96, probably in the Golgi apparatus. The presence of ABP 96 is not sufficient to initiate arylphorin uptake. In the late 3rd instar larva, 20E induces the receptor-mediated endocytosis of arylphorin. Characteristic coated pits and coated vesicles could be observed in this stage (unpublished results; Marx, 1983). During the process of arylphorin uptake ABP 96 is cleaved into ABP 65, and a 30 kDa protein. This is accomplished either by a direct influence of 20E on ABP 96 or by an unidentified mechanism that might also modify ArR in this process. ABP 65 eventually is involved in the storage of arylphorin in the protein granules that remain in the fat body throughout metamorphosis.

Both considered mechanisms are believed to act on post-transcriptional level. However, it was not resolved whether 20E interacts directly with arylphorin mRNA or with arylphorin receptor. Proteins encoded by ecdysteroid-inducible genes may be involved in either of these effects. For instance, 20E might stimulate the transcription of genes encoding proteins involved in the formation of RNPs, which are thought to be involved in the inactivation of arylphorin-mRNA (Schenkel et al., 1983). Furthermore, 20E may induce a specific protease which activates the arylphorin receptor by cleavage at a distinct site. This would explain the need of at least one other factor beside 20E for the initiation of arylphorin uptake.

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