Effects of Ecdysterone and the Juvenile Hormone Analogue Methoprene on Protein, RNA and DNA Synthesis in Wing Discs of *Calliphora vicina*

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The protein, RNA and DNA content of wing discs increase exponentially during the last larval instar. Biosynthesis of protein, RNA and DNA was studied by injecting labelled precursors into larvae or white prepupae and measuring the incorporation in the wing discs dissected out after an appropriate time. Protein synthesis is stimulated by ecdysterone, methoprene has no effect. Biosynthesis of rRNA is increased in wing discs of white prepupae after ecdysterone or methoprene injection. Methoprene inhibits the synthesis of mRNA, while ecdysterone has no clear-cut effect within the limits of our method (gel electrophoretic analysis). Ecdysterone and methoprene have no detectable influence on incorporation of thymidine into DNA, but the incorporation label from uridine into DNA is diminished; this effect may be due to changes in the precursor pool.

Introduction

The postembryonic development of insects is under the control of the prothoracic gland hormone, ecdysone, and the hormone of the corpora allata, the juvenile hormone. According to the presently accepted theory of the action of these hormones, they exert their influence on development at the level of the cell nucleus [1]. It has been shown that new species of messenger RNA and proteins are produced [2], though it is not yet clear if this is due to control of transcription, as originally postulated by Karlson [3], or if post-transcriptional modification of hnRNA is an additional or the main mechanism.

The biochemical action of ecdysteroids and juvenile hormones has been studied either in whole larvae or in certain tissues: the epidermis [4] and the fat body [5, 6]. But the most important developmental changes take place, at least in holometabolous insects, in the imaginal discs. It was therefore of interest to study biochemical changes in these discs during normal development, and under the influence of exogenous hormones.

In the present paper, we wish to report data on the biosynthesis of protein, RNA and DNA in imaginal discs (wing discs) in the larvae of *Calliphora vicina* R.D. from the 4th day of larval development (early III. instar) to the 8th day (white prepupae) and on the influence of exogeneous hormones (ecdysterone and methoprene) on the biosynthesis of these macromolecules.

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Materials and Methods

Calliphora vicina (= *C. erythrocephala* Meig.) were reared on bovine meat at 298 °K and relative humidity of 65%. The larvae form puparia at the end of the seventh day after egg deposition. Blowflies were allowed to deposit eggs only for 45 min daily in order to have larvae of the same developmental stage.

Abbreviations: 4 d-L: four days old larvae; WP: white prepupae.

Chemicals: [^{3}H]uridine (24 Ci/mmol), [^{3}H]thymidine (28 Ci/mmol) and [^{35}S]methionine (700 Ci/mmol) were purchased from Amersham Buchler, ecdysterone from Rohto, Osaka (Japan). Methoprene (trademark Altosid) was a gift of Dr. O. Schooley, Zoecon Corporation, Paolo Alto. Methoprene (Isopropyl(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate) is an effective dipterian larvicide with high juvenile hormone activity [7].

Injection of radioactive precursors and hormones. The chemicals were injected between the 3rd and

4th posterior segment of the larvae with a glass microcapillary. The minimal volume applied was 1 μl, the maximal 5 μl. Hormones were dissolved in ethanol and then diluted with distilled water.

Determination of RNA and DNA. 50 discs were homogenized in 10 ml ice-cold trichloroacetic acid-acetone-water-mixture (50 g : 1 l : 1 l) and centrifuged at 10,000 × *g* for 5 min at 273 °K and then washed three times with 5% TCA. The pellets were worked up according to the procedure of Schmidt and Thannhauser [8].

For measurements of radioactivity, the washing procedure was repeated until the supernatant was free of radioactivity. One series of disc was dissected immediately after injection. The radioactivity in this samples was substracted from all others in order to substract the counts, arising from unspecific absorption.

RNA was determined according to Ceriotti [9] and DNA according to Burton [10]. Standards were RNA from *Calliphora* fat bodies for RNA, calf thymus DNA for DNA and 2-deoxy-D-ribose for deoxy-sugars.

Extraction of proteins from wing discs. Wing discs were dissected and immediately homogenized at 273 °K in a Potter-Elvejhem homogenizer in a 0.02 M phosphate buffer, pH 6.9, containing 0.02% phenylthiurea. The homogenates were centrifuged at 10,000 × *g* for 30 min and the supernatants were used for the electrophoretic characterization of the proteins or the determination of the protein content with bovine serum albumine as a standard by the method of Lowry *et al.* [11].

Gel electrophoresis and fluorographs. SDS acrylamide gel electrophoresis was run according to the method of Laemmli and Faure [12] on 7.5% gels. Fluorography of the dried gel slabs was performed using the technique of Laskey and Mills [13].

Preparation of nucleic acids. Nucleic acids were extracted, purified and electrophoresed as previously described [5]. In a typical preparation 50 discs were ground with 1 ml frozen starting buffer. The homogenate was extracted with a phenol-chloroform-isoamyl alcohol mixture and the interphase reextracted with alkaline buffer. Nucleic acids were precipitated with ethanol from the aqueous phase at a final concentration of 0.24 M ammonium acetate.

Results and Discussion

Protein, RNA and DNA content of imaginal discs. As a prerequisite to the studies on biosynthesis, it was necessary to measure protein, RNA and DNA during the course of normal development of imaginal discs. The results are shown in semilogarithmic plots in Figs 1 – 3. It can be seen that the increase of protein, RNA and DNA follows straight lines from day 4 to the white prepupae where growth stops. Obviously, imaginal discs are a good example of a tissue in exponential growth.

Similar conclusions have been reached by Bougues [14] using dry weight and wing disc volumes as parameters.

Our RNA values determined by the colorimetric method of Ceriotti [9] (which measures the ribose

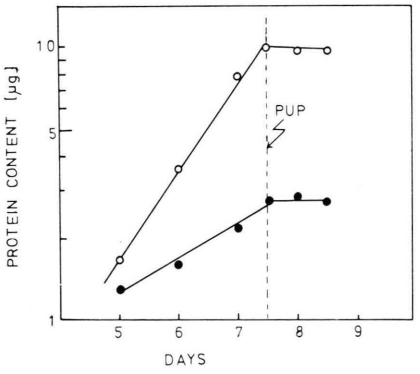

Fig. 1. Eovlution of the protein content of imaginal wing discs ○—○ and leg discs ●—● during the III. instar of *Calliphora*. Age of larvae is given in days after egg deposition. The dashed line (PUP) indicates the beginning of puparium formation (WP). Each point is the average of 50 discs.

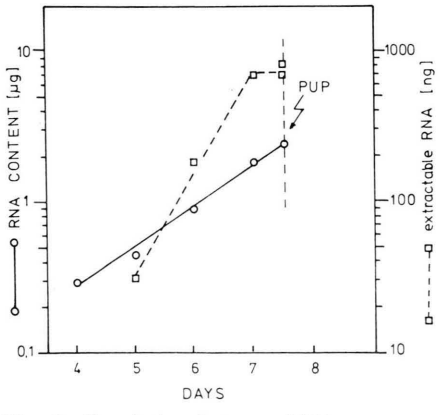

Fig. 2. Correlation between RNA content and extractable RNA from wing discs of III. instar larvae. Each point is the average of 50 discs.

moiety) also agree with the data of Bougues. It may be mentioned, however, that the extraction of native RNA from wing discs, using the method of Scheller and Karlson [5], yields only 10 – 30% of the colorimetrically determined RNA (Fig. 2). This discrepancy is probably due to the fact that some RNA is not extractable but remains bound to structures. Moreover, the colorimetric method may give too high values due to other carbohydrates. However, extractable RNA also increases exponentially.

The DNA content of the discs increases from 4 day old larvae to white prepupae by a factor of 18.6 (Fig. 3). Disc cells are generally believed to be diploid; the increase would therefore correspond to approx. four divisions per cell. We have tried to calculate the number of cells per disc assuming the same gross genome organisation as in *Drosophila*. The somatic nucleus of a *Drosophila* cell contains 0.4 pg DNA [15], the genome size has been estimated to 0.7×10^{11} Daltons. The genome of *Calliphora* is 2.43 times larger (1.7×10^{11} Daltons) [16], the DNA content should therefore be 0.97 pg per nucleus. Using this constant, the number of cells per disc has been calculated from the DNA content (Table I). In the same table, the ratios of RNA/DNA and protein/DNA are listed. Both are higher than those for *Drosophila*, but these data stem from a mass isolation procedure containing all kinds of imaginal discs [17]. The ratios show a falling tendency during the third larval instar, a fact that we cannot interpret at the moment, perhaps indicating that the nucleus of the very small disc cells occupies more and more space within the wing disc cell.

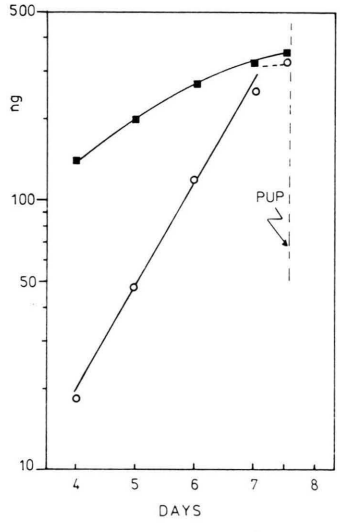

Fig. 3. DNA content and sugar components of DNA in wing discs. ○—○ DNA content in acid precipitable fraction [8]; ■—■ sum of all molecules containing 2-deoxy-D-ribose.

The protein/RNA ratio does not change very much during early III. instar and remains constant from day 6 onwards. This might indicate that transcription, translation and metabolism of proteins and RNA are well balanced; there seems to be a steady state, though we may assume that the nature of the proteins and the RNA may change during this interval. As will be shown below, there is rather active RNA and protein synthesis, obviously balanced by degradation of at least part of these macromolecules, though there is still an over-all increase of protein and nucleic acids per disc.

Protein biosynthesis. [^{35}S]Methionine was used as precursor to measure protein biosynthesis. Fig. 4 shows incorporation of label into salt soluble proteins of wing-discs as a function of time after a single injection of 3.3 μCi [^{35}S]methionine into

Table I. Biochemical composition of wing discs of III. instar *Calliphora*. The numbers in parenthesis show the manifolds in a column compared to the value of 4 dL.

	Cell number per disc $\times 10^{-3}$	Deoxyribose ng/disc	DNA ng/disc	RNA μg/disc	Protein μg/disc	RNA DNA	Protein/ DNA	Protein/ RNA
4 dL	19	140 (1)	18 (1)	0.28 (1)	0.8* (1)	15.6	44.4	2.9
5 dL	49	195 (1.4)	47 (2.6)	0.48 (1.7)	1.7 (7.1)	10.2	36.2	3.6
6 dL	121	265 (1.9)	117 (6.5)	0.85 (3.1)	3.6 (4.5)	7.3	30.8	4.2
7 dL	260	315 (2.3)	250 (13.9)	1.87 (6.7)	7.8 (9.8)	7.3	31.2	4.3
WP	345	345 (2.5)	334 (18.6)	2.2 (7.9)	9.5 (11.8)	6.6	28.4	4.3

* This value is extrapolated from the protein plot (Fig. 1).

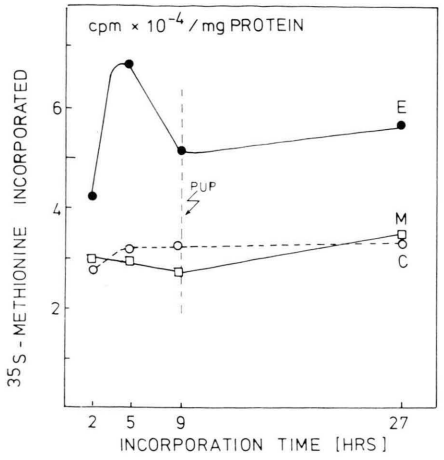

Fig. 4. Effects of ecdysterone (100 ng/larva) and methoprene (1 μg/larva) on the [^{35}S]methionine incorporation into salt soluble proteins of wing discs of 7 dL. Methionine was injected (together with the hormone) at zero time.

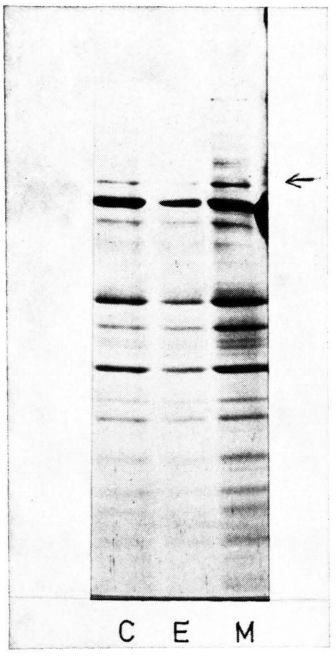

Fig. 5. Analysis of the proteins synthesized in wing disc of 7 dL. Fluorography of SDS-acrylamide gel electrophoresis of salt-soluble protein from wing discs. Larvae were injected with 3.3 μCi [^{35}S]methionine (C = controls) and additional with 100 ng ecdysterone (E) or 1 μg methoprene (M). 4 h after injection the discs were dissected and the proteins extracted as described in materials and methods. The extracts were then subjected to electrophoresis and the slab gels were submitted for fluorography. The arrow indicates the position of Calliphorin.

each 7 day old larvae. Methionine is incorporated within 5 h, afterwards the specific activity of the protein shows very little change. When ecdysterone is injected together with the labelled amino acid, the specific activity of the protein is much higher, and synthesis continues with a higher rate for 5 h. Afterwards the specific activity decreases again; this may be due to a rapid turnover of proteins in wing disc or to a dilution of the methionine pool with non-labelled material. (We have not been able to measure pool size in the small discs.)

A similar effect of ecdysterone on protein synthesis in imaginal discs of *Drosophila* has been described by Kuniyuki and Fristrom [18]. These authors used leucine as precursor; they also provided evidence that it is indeed protein synthesis that is specifically stimulated by ecdysterone.

Considering the fact that ecdysteroids induce striking morphogenetic changes in imaginal discs during and after formation of puparium, it was not surprising to see that ecdysterone stimulates protein biosynthesis in these discs shortly before pupariation. It was of interest to see if the measured incorporation of labelled methionine into protein is due to a more or less general increase in protein synthesis or if it is due to the induction of specific proteins by ecdysterone, as one would predict from its mode of action. We have therefore separated the labelled proteins by slab gel electrophoresis and analysed the gels by fluorography. As can be seen from Fig. 5, no major differences can be observed in the protein bands newly synthesized in the presence of ecdysterone or methoprene compared to the controls. The results agree with the situation in *Drosophila* discs, reported by Fristrom [19]. It should be stressed, however, that the failure to detect differences may be due to limitations of the method used.

It is remarkable that the specific protein Calliphorin which is synthesized in the fat body [20] and is utilized for tanning of the cuticle [21] could be detected as a main band within the electrophoretic pattern by immunological techniques. We are not yet in a position to decide whether this protein is also synthesized by the wing discs or derived from the fat body via hemolymph.

Methoprene, the juvenile hormone analogue, had no significant influence on protein synthesis when injected together with the labelled amino acid. Fristom *et al.* working with *Drosophila* wing discs cultured *in vitro* found an inhibition of leucine incorporation by juvenile hormone preparations

[22], while Patel and Madhavan [23] found an increase of leucine incorporation into discs of the *Ricini* silkworm. But these experiments may not be comparable to our studies.

RNA synthesis. In order to analyze RNA synthesized *in vivo* by wing discs, 50 7dL received injections of 5 μCi [^{3}H]uridine. Immediately after the injection we started dissection of the discs. The last discs of one series were dissected after 45 min, so that we could analyze RNA which is labelled from 5 to 45 min. The analysis of the pulse-labelled RNA on 2.2% polyacrylamide gels yielded a heterodisperse pattern in the high molecular weight-region (Fig. 6). The profile also showed distinct peaks at 4S/5S, 18S and 28S, which can be ascribed to rRNA, and a peak at 32S, which can be ascribed to rRNA precursor molecules. When the RNA was labelled with [^{3}H]methyl-methionine and separated on 7.5% PAA-gels, only the 4S-peak showed radioactivity, *i. e.*: tRNA [24].

The heterodisperse pattern in the higher molecular weight region was severely suppressed when the larvae received α-amanitin (100 μg/larva) simultaneously with the RNA-precursors (not shown). α-amanitin inhibits DNA-dependent RNA polymerase B, responsible for the transcription of structural genes into mRNA precursors. Our results indicate that mRNA- and rRNA-precursors are synthesized in a high rate by the wing discs shortly before evagination. A very rapid processing of the rRNA precursors into the cytoplasmatic 28S- and 18S RNA fraction with a molecular weight of 1.42 $\times 10^6$ resp. 0.7×10^6 could be observed. The molecular weights for the 28S and 18S rRNA molecules agree well with those found in other insects, for *Drosophila* 1.4 resp. 0.73×10^6 D [25]; *Antheraea*, 1.4 resp. 0.65×10^6 D [26]; *Aedes*, 1.5 resp. 0.7 $\times 10^6$ D [27] and *Dysdercus* [28], 1.5 resp. 0.7 $\times 10^6$. The values for *Chironomus tentans* seem to be higher; they reach the size of mammalian with

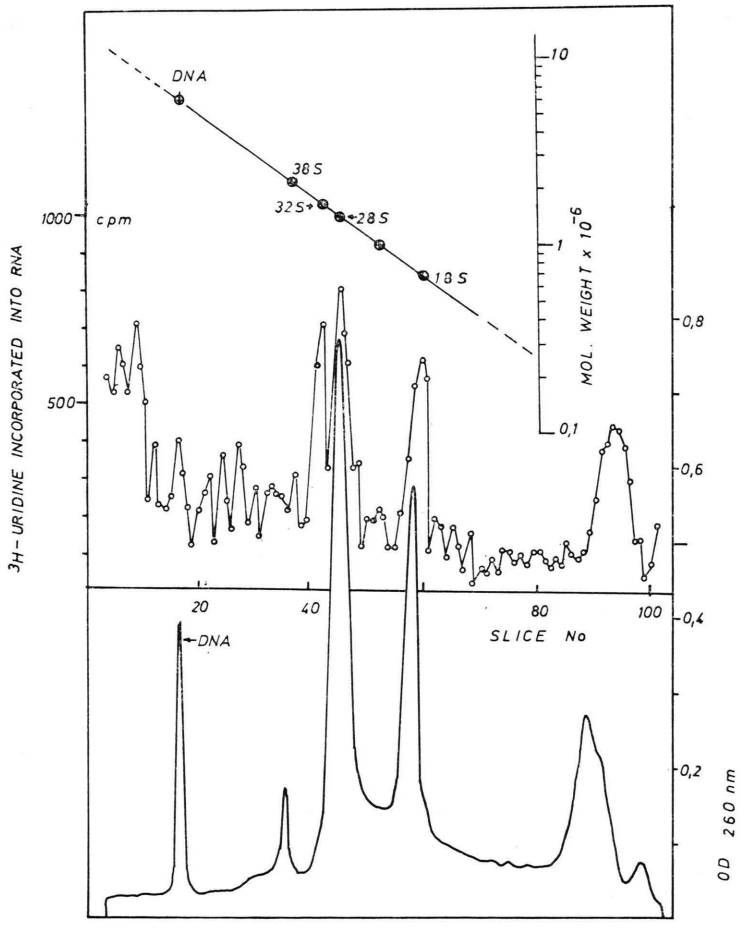

Fig. 6. Pulse-labelling and molecular weigt determination of wing disc RNA from 7 dL, analysed on 2.2% polyacrylamide gel.

1.75 resp. 0.7×10^6 D [29]. Within the animal kingdom only the size for the 28S-component varies, the 18S remains constant throughout all eukaryotes.

We also tried to translate total RNA from wing discs in a wheat germ system into protein, but increasing RNA concentrations caused inhibition of amino acid incorporation. The *in vitro* translation of poly(A)$^+$ RNA from wing discs is under way in our laboratory.

The following experiments were designed to study the quantitative and qualitative effects of ecdysterone and methoprene on transcription. White prepupae received injections of 4 μCi [^{3}H]uridine (control animals) and 4 μCi [^{3}H]uridine + 100 ng ecdysterone or 1 μg methoprene (experimental animals). 4 hours after injection the wing discs were dissected, the RNA extracted and the radioactivity measured. The RNA from the experimental animals displayed a much higher specific radioactivity as the RNA from the controls, indicating a significant increase of uridine incorporation into RNA of wing discs after hormone treatment (Table II).

Table II. Specific radioactivity of wing disc RNA, labelled *in vivo* 4 h with [^{3}H]uridine (4 μCi).

	cpm/μg RNA
WP, controls	1200
WP, ecdysterone treated, 100 ng/larva	2485
WP, methoprene treated, 1 μg/larva	3060

We always observed that the incorporation of [^{3}H]uridine into wing discs was higher than in other tissues like fat body or epidermis.

The situation in wing discs of WP is rather different compared to the fat bodies from the same developmental stage. The fat body tissue of WP does not respond with a significantly higher RNA synthesis after the treatment with ecdysteroids [5, 6]. In contrast, the stimulation of fat body by ecdysone and ecdysterone is high when the insects received the hormones in the middle of the III. instar, *i. e.* at a time when the endogenous titer of ecdysteroids is at a minimum in the whole animal [30]. But we do not know much about ecdysterone concentrations within the *imaginal discs* during different developmental stages.

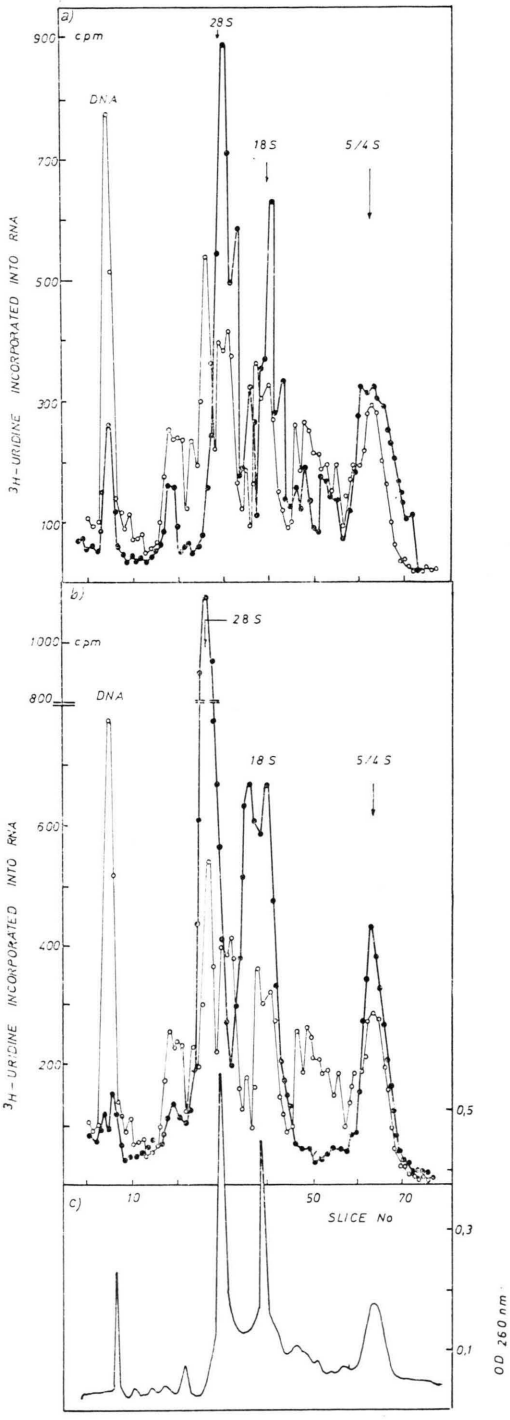

Fig. 7. Effects of ecdysterone and methoprene on RNA biosynthesis in wing discs of white prepupae. 4 μCi [^{3}H]uridine and the hormone was injected 4 h before dissection, the RNA was analyzed on 2.2% polyacrylamide gel.
a) Effect of ecdysterone: ○—○ controls, ●—● ecdysterone (0.1 μg per animal).
b) Effect of methoprene: ○—○ controls, ●—● methoprene (1 μg per animal).
c) Optical density at 260 nm (RNA concentration).

To study the qualitative effects of ecdysterone and methoprene on transcription, 10 μg samples of RNA from the experiment described above were analysed by gel electrophoresis. The results are shown in Fig. 7 a and b. The optical profile (Fig. 7 c) was indistinguishable in samples from control and experimental larvae. The profiles of radioactivity labelling showed distinct peaks at 4/5S, 18S, 28S, 32S and 38S which can be attached to ribosomal RNA and precursors. In the RNA from WP, either treated with ecdysterone or methoprene, the label in the rRNA peaks are doubled. These proportions are similar to those in fat body tissue of 5dL. The bulk of wing disc RNA induced by ecdysterone and methoprene is certainly ribosomal RNA. It is known that the capacity of the nucleoli to synthesize ribosomal precursors is much greater than the actual synthesis, *i. e.* nucleolar RNA synthesis is in a suppressed state. Augmentation of RNA synthesis apparently requires the production of a certain species of mRNA with a rapid turnover which acts either directly or after being translated into protein.

In the profile of the mRNA region, very little and rather irregular differences can be seen between ecdysone-treated WP and controls (Fig. 7). Here our gel electrophoretical analysis is not sensitive enough to tell us whether the synthesis of mRNA is induced by ecdysterone in wing discs of *Calliphora*. A more sensitive method to give an answer to this question will be the *in vitro* translation of mRNA from imaginal discs and hybridization techniques using unique sequence DNA and poly(A)$^+$ RNA (in preparation). For the time being, the absence of demonstrable differences in RNAs synthesized in wing discs should not be taken as evidence indicating that there are no differences in the mRNA species in the presence and the absence of ecdysterone. For fat body cells, the activation of structural genes by ecdysterone could be demonstrated by means of hybridization experiments with unique sequence DNA and poly(A)$^+$ RNA.

Methoprene completely inhibited the incorporation of [^{3}H]uridine into the RNA which sediments between 18S and 5S, the so called messenger region (Fig. 7 b). It could be that methoprene either suppresses the transcription of mRNA precursor molecules or causes a rapid degradation of mRNA, *i. e.* a posttranscriptional process. That might be a hint that ecdysterone and the juvenile hormone analogue methoprene act in an antagonistic manner controlling synthesis and inactivation of mRNA.

Our results concerning RNA synthesis in imaginal discs agree with findings from *in vitro* experiments of other authors for *Drosophila* [19] and *Galleria* [31]. Bougues [14] reported that the period of maximal rRNA synthesis in wing discs of *Calliphora* corresponded to the moment of the highest ecdysteroid titer in the animal.

DNA synthesis. The gel electrophoresis of our nucleic acid preparations from wing discs always revealed a distinct peak near the start (Figs 6 and 7). After labelling *in vivo* with [^{3}H]thymidine, only this peak contained radioactivity (Fig. 8); moreover, this nucleic acid was resistent to RNase but

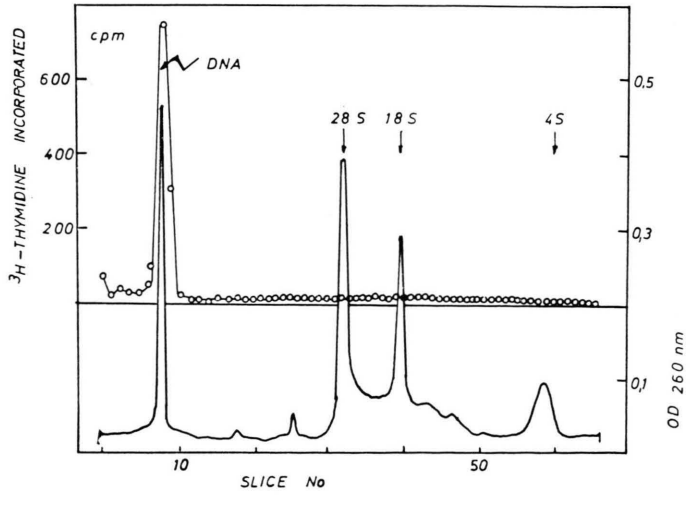

Fig. 8. Gelelectrophoretical analysis of nucleic-acid preparation from 7 dL wing discs, labelled for 4 h with [^{3}H]thymidine.

completely digested by DNase. We therefore concluded that this component represents DNA. Its molecular weight could not be determined because the linear relation between the electrophoretic mobility and the logarithm of the molecular weight does not hold in this gel region near the start, but it must be rather high compared to the RNA.

When 7 dL were treated with [^{3}H]thymidine and ecdysterone or methoprene, only minimal changes in incorporation of the DNA-precursor into the DNA fraction of wing discs are seen. The DNA peak also showed a high level of radioactivity when [^{3}H]uridine was used to label the nucleic acids. In this case, the incorporation of radioactivity into the DNA peak shows dramatic changes under the influence of the hormones. Ecdysterone severely inhibited incorporation (Fig. 7 a) whereas methoprene suppressed incorporation nearly totally (Fig. 7 b).

Since DNA contains thymine instead of uracil present in RNA, [^{3}H]uridine must be converted into [^{3}H]thymidine. The biosynthetic pathway consists of reduction of UDP to dUDP, the following hydrolysis from dUDP to dUMP and methylation of dUMP to dTMP. The last reaction is catalyzed by the enzyme thymidilate synthetase. Our results can be explainined by the assumption that the hormones either alter the activity of the enzyme systems (or the coenzymes) or affect the allosteric control mechanisms in the biosynthesis of deoxyribonucleoside-5-triphosphates, thereby influencing the precursor pool.

Changes in the nucleotide pool can also be inferred from the total content of deoxyribose in the wing disc (Fig. 3). In 4 dL, the amount of total deoxyribose present in form of DNA is about 4% of the total, while in WP it goes up to 27%. The only explanation of these figures is that the nucleotide pool of DNA precursors decreases towards pupariation. Since the larval-pupal transition is under the control of ecdysterone, this might be a side effect of the hormone.

The data presented here show that imaginal discs respond to ecdysteroids and to juvenile hormones *in vivo* with changes in the rate of synthesis of RNA and protein. A more detailed analysis of the RNA and protein species might give some clues to the molecular mechanism of morphogenesis and of action of these morphogenetic hormones in this interesting target tissue.

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