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Original Article

Cuticle secretion in *Drosophila* wing imaginal discs *in vitro*: parameters of exposure to 20-hydroxy ecdysone

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ABSTRACT We have cultured *Drosophila* wing imaginal discs *in vitro* under a variety of hormonal conditions in order to determine whether cuticle secretion is enhanced by a withdrawal of 20-hydroxy ecdysone at one of two points in development, corresponding to the drop in hormone titer during the prepupal period, and to the fall in hormone levels during the later stages of imaginal differentiation. We found that these treatments did not enhance either pupal or adult cuticle secretion.

KEY WORDS: *wing disc, 20-hydroxy ecdysone, tissue culture, Drosophila, metamorphosis*

Introduction

The imaginal discs of the fruit fly *Drosophila* evaginate and differentiate during the prepupal and pupal stages, each disc forming a specific region of the exoskeleton of the adult fly. The first stage of this process, known as evagination, involves the transformation of the folded sheet of epithelial cells into the shape of the appendage it is destined to form - for example, the leg or the wing. Differentiation involves the secretion of two layers of cuticle. The first of these, the thin pupal cuticle, is deposited from 3h to 12h after pupariation (Fristrom *et al.* 1982; Fristrom and Liebrich, 1986), after which it is shed by a process known as apolysis. The second is the adult or imaginal cuticle, which is thicker and incorporates the bristles and trichomes (hairs) which pattern the exoskeleton of the adult fly. This cuticle is secreted from 48 hours after pupariation onwards *in vivo* (Reed *et al.*, 1975). Although both cuticles exhibit the same basic morphology, the structure and secretion of the pupal cuticle has received the most attention in recent years (Fristrom *et al.*, 1982; Doctor *et al.*, 1985; Fristrom and Liebrich, 1986). The outer layer, the epicuticle, is subdivided into a cuticulin layer or outer epicuticle, and an inner epicuticle composed of proteins. The inner layer or procuticle is a much thicker, laminated structure composed of chitin and proteins (Andersen, 1979).

The secretion of the pupal and adult cuticles from the imaginal disc epithelium is under the control of the moulting hormone 20-hydroxy ecdysone (Fristrom *et al.*, 1973; Milner and Sang, 1974; Milner, 1977a). *In vivo* assays of molting hormone in *Drosophila* (Hodgetts *et al.*, 1977; Handler, 1982; Bainbridge and Bownes, 1988) have shown that pupariation occurs at a peak in hormone titer. This is followed by a fall, reaching a minimum 12 hours after pupariation (AP) which coincides with pupation proper; after this a second, higher peak is reached at 35-40 hours AP. The titer then falls off stepwise until the adult fly ecloses at around 100 hours AP.

To what extent is this complex pattern of hormone exposure necessary for the normal development of imaginal discs? Hormonal requirements for the secretion of the pupal cuticle *in vitro* have been examined over the past few years using mass-isolated discs cultured in Robb's medium (Fristrom *et al.*, 1982; Fristrom and Liebrich, 1986). These authors found that procuticle deposition required a 6 hour pulse of 20-hydroxy ecdysone (20HOE) followed by its complete removal, and that this hormonal regimen also facilitated the deposition of cuticulin. A continuous exposure to 0.1 ug/ml of 20HOE permitted the secretion of epicuticle only, and this was often discontinuous (Fristrom *et al.*, 1982). Thus, these authors have concluded that the hormonal requirement for normal pupal cuticle secretion *in vitro* mirrors the titer of 20HOE found during the prepupal and early pupal period. However, Milner and Muir (1987) found that pupal cuticle with a continuous epicuticle and a thick, laminated procuticle was formed on continuous exposure to 0.1 ug/ml of 20HOE. At a later stage of culture, a structured imaginal cuticle was also laid down. Clearly, these results are not in accord with those of Fristrom *et al.* (1982).

Little attention has been paid to the relationship between hormone exposure and the later stages of imaginal cuticle formation. One study of interest is that of Schwartz and Truman (1983), who administered exogenous hormone to pupae of the tobacco hornworm, *Manduca sexta*. This was found to inhibit the later stages of adult development, for example wing pigmentation. Conversely, ligation of the abdomen, which brought about a preco-

Abbreviations used in this paper: AP, after pupariation; 20HOE, 20-hydroxy ecdysone.

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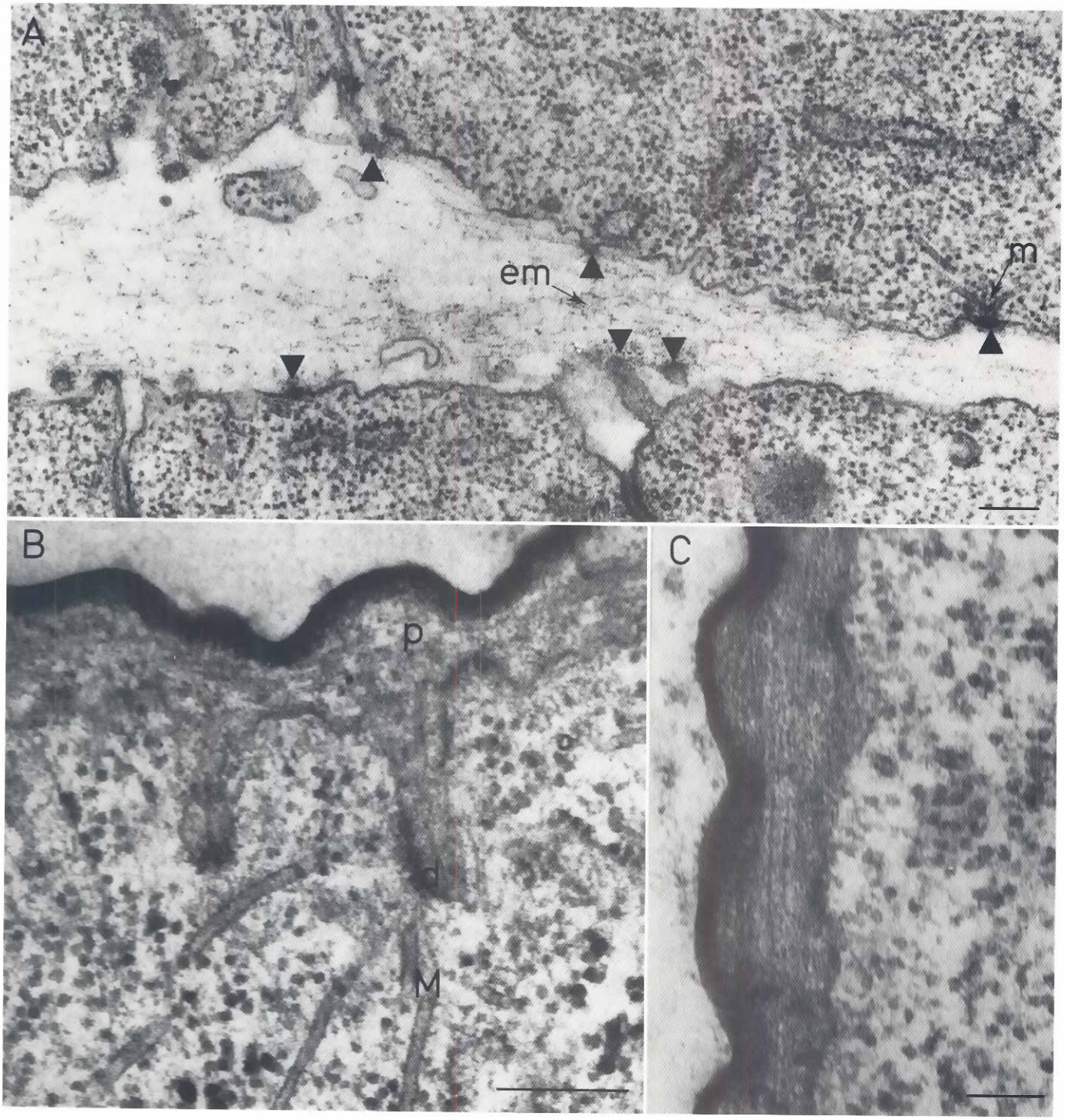


Fig. 1. Control discs either cultured without 20HOE or continuously exposed to 0.1 ug/ml of 20HOE, and fixed after 21 h. (A) *The apical surfaces of epithelial cells in an uneverted disc after culture without hormone (culture 1.1). Microvilli (arrowheads) secrete extracellular matrix material (em) into the lumen of the disc. On occasions the microvilli appear to be closely associated with microtubules (m).* **(B)** *Continuous epicuticle (e) and procuticle (p) formed by a disc continuously exposed to 0.1 ug/ml 20HOE (culture 1.2). Dense projections from the cell surface (d) appear to be associated with microtubules (m).* **(C)** *Pupal cuticle from a disc cultured as in 1B but with a medium change to fresh medium plus 0.1 ug/ml of 20HOE after 6 hours of culture (culture 1.3). Bar represents 0.2 um.*

cious reduction in ecdysteroid titer, accelerated development. We wished to determine whether a similar phenomenon could be observed in *Drosophila, i.e.* whether a drop in ecdysteroid titer would enhance imaginal cuticle secretion or pigmentation in culture.

The present study is an examination of the relationship between hormone titer and the secretion of both pupal and adult cuticles by the wing imaginal disc of *Drosophila* during culture *in vitro*. The cell biology of wing disc metamorphosis in this culture system in response to a continuous exposure of 0.1 ug/ml of 20HOE has already been documented (Milner and Muir, 1987), and the present study compares these findings with cuticle secretion under a variety of different hormonal conditions.

Results

Experiments and controls are grouped into four series, depending on the duration and conditions of culture. Summaries of all cultures may be found in Table 1.

Series 1, 21 hour fixation

The four experiments in this series mimic the hormonal conditions and duration of culture used in Fristrom's experiments (Fristrom *et al.*, 1982) in order to determine whether similar results would be obtained under our conditions of culture.

For the first control culture (1.1), discs were cultured without hormone to assess whether they had been exposed to 20HOE *in vivo* prior to dissection. No cuticle was secreted (Fig. 1A), and very little evagination occurred (Table 1), indicating that this possibility could be discarded. Microvilli are present, which appear to be still secreting the fibrous extracellular matrix material observed in late third instar discs (Fristrom and Rickoll, 1982; Milner *et al.*, 1984). Microvilli are sometimes associated with microtubules (Fig. 1A). In control 1.2, discs were continuously exposed to 0.1 ug/ml of 20HOE with no medium change. A continuous epicuticle and laminated procuticle was formed (Fig. 1B) the mean thickness of which was 0.25 um. Control 1.3 similarly involved a continuous exposure to 0.1 ug/ml of 20HOE, but with a medium change to fresh medium plus hormone after 6 hours of culture (Fig. 1C). Here, the pupal cuticle formed to an average thickness similar to that of control 1.2, but the evagination index score for control 1.3 was found to be significantly lower ($p>0.0001$) than that for control 1.2, demonstrating that the 6 hour medium change disrupted the evagination process. It was, however, not clear whether the reduced level of evagination was due to the removal of conditioning factors or to mechanical damage during the medium change.

The three experimental sets in this series all involved a medium change at 6 hours of culture. In culture 1.4, discs were incubated in 1.0 ug/ml of 20HOE before the change, and then in medium lacking hormone (Fig. 2A, B). In the best examples, the pupal cuticle secretion at 21 hours of culture was thicker (0.4 um) and particularly well formed. For culture 1.5, the hormone level prior to the medium change was reduced to 0.1 ug/ml. This treatment resulted in the formation of epicuticle only, which was in places discontinuous (Fig. 2C, D). Discs in culture 1.6 were incubated in medium containing 1.0 ug/ml of hormone before the medium change, and thereafter in 0.1 ug/ml. Relatively thin cuticle formed with a procuticle which was thin, rather unstructured and occasionally absent (Fig. 2 E, F) indicating that this hormone titer may have been superoptimal.

Series 2, 48 hour fixation

The duration of culture for series 1 was chosen to approximate to the overnight culture used by Fristrom *et al.* (1982). In series 2 we asked whether additional pupal cuticle is laid down if the culture period is extended to 48 hours. *In situ* the ecdysteroid titer falls to a minimum about 10 hours after pupariation, after which it rises again (Handler, 1982; Bainbridge and Bownes, 1988). We attempted to mimic this by changing experimental cultures to medium lacking hormone between 8 and 17 hours, to determine whether this hormonal regime improved cuticle secretion.

Control 2.1. Discs were exposed to 0.1 ug/ml of 20HOE throughout the culture period, with medium changes to fresh medium containing hormone at 8 and 17 hours of culture. The best examples secreted a complete, well-defined pupal cuticle approximately twice as thick (0.5 um) as that in controls 1.1 and 1.2, indicating that pupal cuticle secretion continues during the second day of culture. As one would expect after the additional culture period, the pupal cuticle was apolysed in many areas, and Fig. 3a shows apolysis taking place in control 2.1.

Both experiments 2.2 and 2.3 involved a medium change at 8 hours to medium lacking 20HOE. In experiment 2.2 the discs were returned at 17 hours to medium containing hormone, whereas in 2.3 the medium change was to medium lacking hormone. Thus, experiment 2.2 attempted to conform more closely to the *in situ* parameters of hormone exposure, while 2.3 was an extension of 1.5 to determine whether extra culture time would permit additional

TABLE 1

PARAMETERS OF CULTURE IN SERIES 1 TO 4

culture	no. of discs	fixation time (hours or days)	time of medium change(s) (hours or days	20HOE titer before/after medium change (ug/ml)	mean evagination (E) or differentiation (D) score + standard deviation
1.1	28	21h	none	0.0	E 0.5 + 0.5
1.2	22	21h	none	0.1	E 2.9 + 0.5
1.3	27	21h	6h	0.1/0.1	E 2.1 + 0.6
1.4	25	21h	6h	1.0/0.0	E 2.5 + 0.6
1.5	28	21h	6h	0.1/0.0	E 2.3 + 0.8
1.6	29	21h	6h	1.0/0.1	E 2.1 + 0.4
2.1	28	48h	none	0.1	E 3.7 + 0.5
2.2	16	48h	8h+17h	0.1/0.0/0.1	E 3.4 + 0.5
2.3	17	48h	8h+17h	0.1/0.0/0.0	E 3.4 + 0.6
3.1	21	7d	none	0.1	D 3.5 + 0.4
3.2	23	7d	3d	0.1/0.0	D 3.6 + 0.5
3.3	34	7d	3d	0.1/0.1	D 3.2 + 0.4
3.4	29	7d	4d	0.1/0.0	D 3.6 + 0.4
3.5	21	7d	4d	0.1/0.1	D 3.6 + 0.4
3.6	34	7d	5d	0.1/0.0	D 3.8 + 0.3
3.7	35	7d	5d	0.1/0.1	D 3.6 + 0.4
3.8	13	7d	8h+17h+4d	0.1/0.0/0.1/0.0	D 3.3 + 0.4
3.9	25	7d	8h+17h+4d	0.1/0.1/0.1/0.1	D 3.0 + 0.2
4.1	27	7d	5d	0.1/0.0	D 3.9 + 0.3
4.2	26	7d	5d	0.1/0.1	D 3.9 + 0.3
4.3	24	7d	5d	0.1/0.0	D 3.6 + 0.3
4.4	20	7d	5d	0.1/0.1	D 3.7 + 0.3

cuticle secretion. Experiment 2.2 yielded pupal cuticle which was in the best cases comparable to that formed in 2.1, but tended to be a little thinner (0.4 um). Examples from both ends of the spectrum are shown in Fig. 3B and 3C. The pupal cuticle produced in 2.3 was less substantial than that formed in 2.2, but in good cases a fibrous procuticle was found (Fig. 3D). Poorer examples only secreted epicuticle (Fig. 3E).Thus, cuticle secretion in culture 2.1 was more satisfactory than in 2.2 or 2.3, suggesting a continuing requirement for 20HOE after 8 hours of culture for complete pupal cuticle deposition. Evagination scores did not differ significantly between cultures 2.2 and 2.3, although evagination was significantly better in culture 2.1 than in cultures 2.2 and 2.3 ($p<0.05$). This difference can be ascribed to the disruptive effect of the 6 h medium change. There were also significant differences between series 2 and series 1 cultures ($p<0.0001$) reflecting the fact that evagination is not complete after 21 hours of culture.

Series 3, 7 day fixation

In series 3, the principal consideration was to investigate the effect of a withdrawal of hormone in the later stages of culture. Medium changes were performed after 3, 4 and 5 days (see Table 1). In each case, the experimental set was changed to medium lacking 20HOE, while the controls were changed to medium with 0.1 ug/ml of hormone. The differentiation scores of experimental and control cultures were then compared using an analysis of variance. Mean scores are given in Table 1. The only significant difference was between 3.2 and 3.3 ($p=0.011$), and this seems to be the result of unusually poor differentiation in 3.3, the control culture.

We also attempted to roughly mimic the hormonal parameters found *in vivo* by medium changing discs to no hormone between 8 and 17 hours, raising the hormone level again and dropping it at 4 days (culture 3.8 and control 3.9). Both cultures differentiated poorly compared to cultures 3.1-3.7, and we suspect that this was simply due to additional medium changes being deleterious to the final level of differentiation.

Discs from each culture were examined at the ultrastructural level, but no significant differences in the adult cuticle were found. Some examples are shown in Fig. 4. Considerable variation was observed between discs from the same culture - compare Fig. 4A and 4B, both from culture 3.1. Both well-organized cuticle (Fig. 4B) and cuticle with a disorganized procuticle (Fig. 4C) from culture 3.6 is shown.

Series 4, 7 day fixation, conditioned medium

In this last series of experiments, discs were medium changed after 5 days of culture to medium either with or without 20HOE that had been conditioned by culturing approximately 40 wing discs in 150 ul of medium in a 96 microwell plate for 4 days (cultures 4.1, 4.2) or 6 days (cultures 4.3, 4.4). This was done in order to eliminate any possible loss of medium conditioning at the medium change. Again, no significant differences between experimentals and controls were observed.

The utilization of hormone by discs in culture

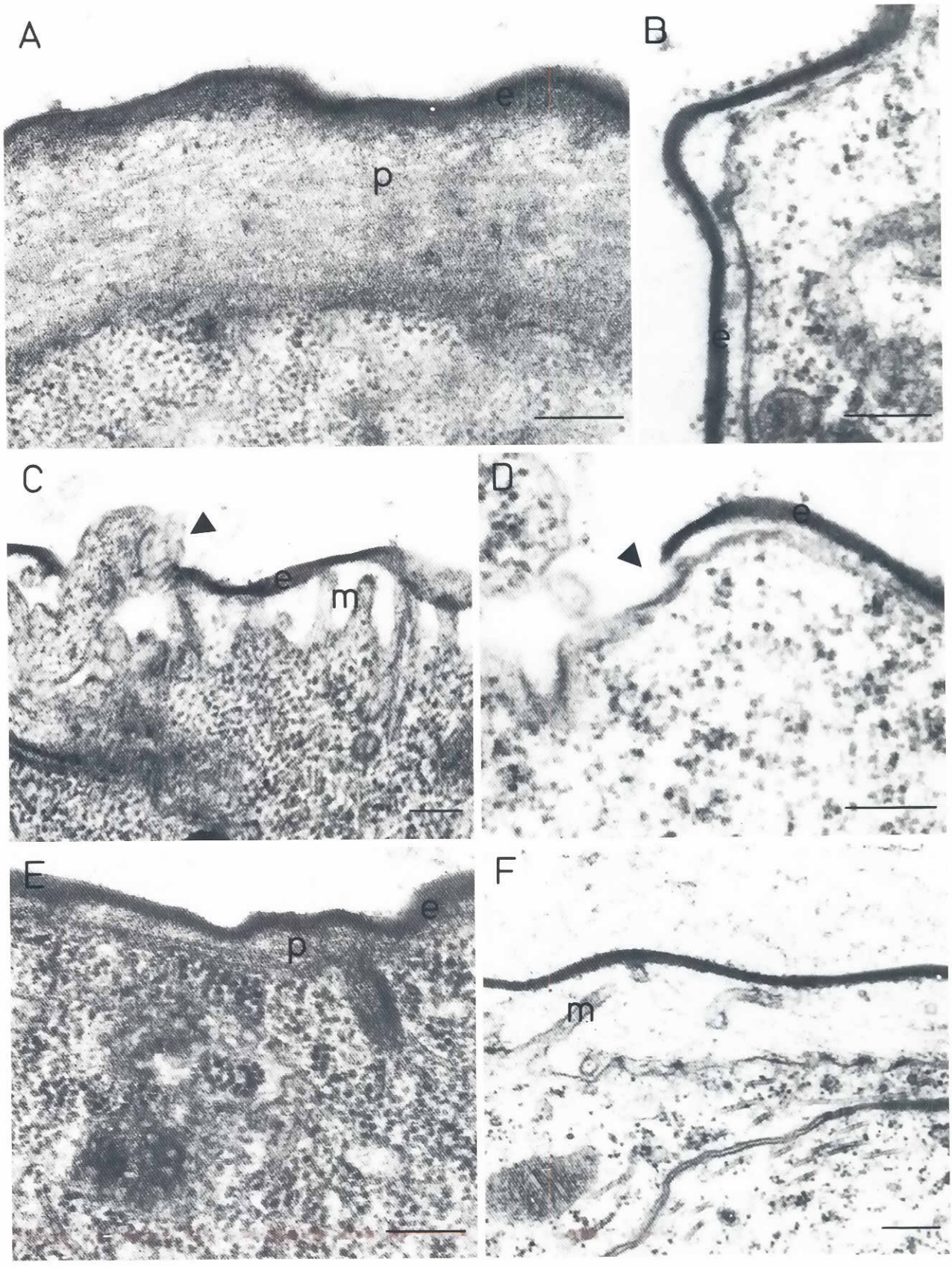
We wished to determine whether discs degrade or sequester 20-hydroxy ecdysone during the early period of culture, in order to eliminate the possibility that fresh hormone added at an early medium change might replenish the supply available to the differentiating disc. To do this, wing discs were cultured in medium containing 0.1 ug/ml of 20-hydroxy ecdysone for either one or two days. The culture slide was then opened, the old discs removed and freshly dissected wing discs added to the medium. The newly added discs were scored for evagination after 1 day and differentiation after 1 week in order to determine whether sufficient hormone remained after 1 or 2 days of culture to support the complete sequence of differentiative events. Control discs were cultured in the same medium throughout, but the culture drops were opened after 1 day of culture for 2 minutes to parallel the treatment of experimental cultures.The mean evagination (E) and differentiation (D) score for the control was E 3.7 and D 3.7 (n=30), for discs in medium pre-used for 1 day E 3.9 and D 3.8 (n=31), and for discs in medium pre-used for 2 days E 3.9 and D 3.6 (n=31). These results indicate that hormone is not degraded or sequestered to any significant degree during the early culture period.

Discussion

The object of the first two sets of experiments was to re-examine the results of Fristrom *et al.* (1982), using our culture medium. A full replication of these experiments was not possible as we do not have a system for mass isolating discs as is done in Fristrom's laboratory. Our fixation time of 21 hours for set 1 was chosen to approximate to their "overnight incubation", and the 48 hour incubation time for set 2 was designed to determine whether pupal cuticle secretion continued on the second day of culture. We found that although a 6 hour pulse with 1.0 ug/ml 20HOE gave the best cuticle secretion, and a continuous exposure to 0.1 ug/ml of 20HOE gave rise to both an inner epicuticle and a laminated procuticle (Fig. 1B, C). This is in contrast to the results of Fristrom and Liebrich (1986) who found that the latter hormonal regimen would only permit cuticulin deposition. The presence of hormone at our optimal concentration of 0.1 ug/ml was required after 6 hours in order to form a satisfactory pupal cuticle complete with procuticle (compare cultures 2.2 and 2.3). We have also observed that cuticle secretion may continue until apolysis occurs at around two days of culture.

The reasons for the differences between the results presented here and those of Fristrom *et al.* (1982) are not easy to assess. They may, however, include the culture medium used and the method of disc isolation. If the conditions of culture are not optimal, then an early pulse with a high concentration of hormone may achieve more rapid and therefore more complete development than a lower titer of hormone. Fristrom *et al.* (1982) suggest that a major difference between mass-isolated discs and hand-dissected discs from late third instar larvae is that the latter have been exposed to the rise

Fig. 2. Discs of series 1 medium changed at 6 hours and fixed at 21 hours of culture. (A) *and* **(B)** *good and poor examples of cuticle secretion in discs cultured in 1.0 ug/ml of 20HOE before, and no hormone after, the medium change (culture 1.4).* **(C)** *and* **(D)** *examples of discs cultured in 0.1 ug/ml of 20HOE before, and no hormone after the medium change (culture 1.5). Epicuticle only was formed, and this was discontinuous in places (arrowheads).* **(E)** *Good and* **(F)** *poor examples of cuticle secretion in discs cultured in 1.0 ug/ml of 20HOE before, and 0.1 ug/ml after, the medium change (culture 1.6). e - epicuticle, p - procuticle, m - microvilli. Bar represents 0.2 um.*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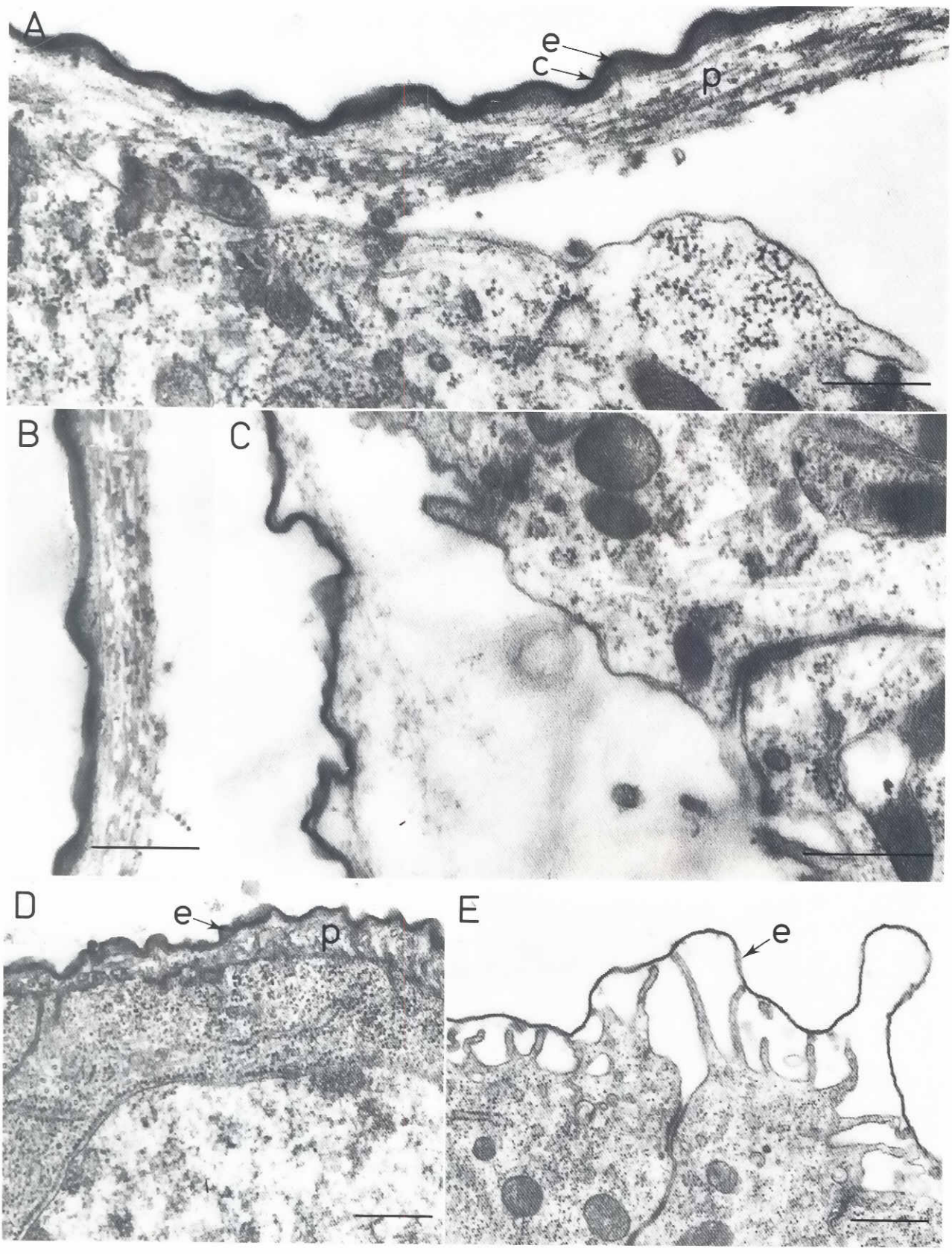
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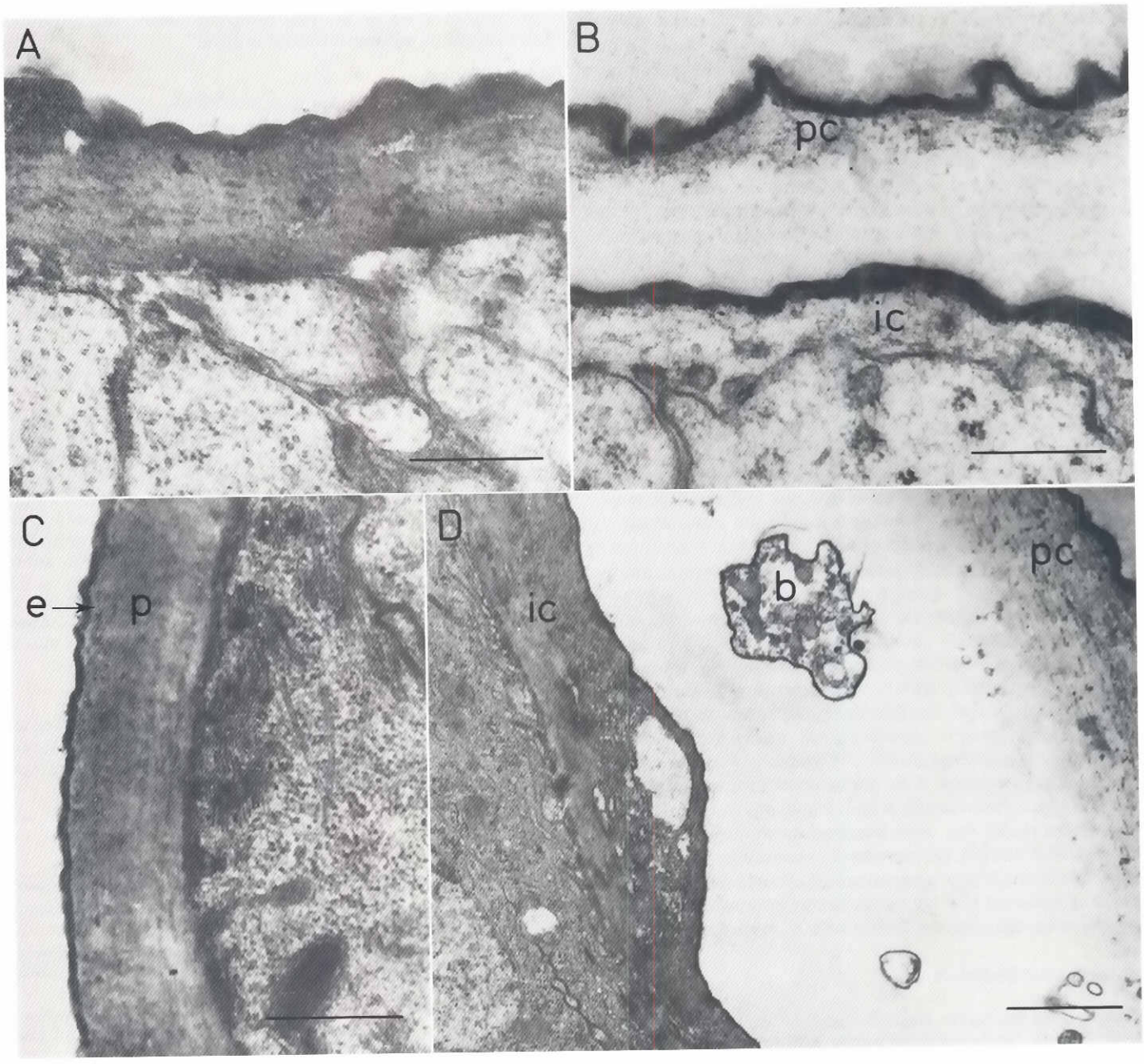

Fig. 4. Imaginal cuticle secretion in discs cultured for 7 days before fixation. (A) *Good and* **(B)** *poor examples of adult cuticle secretion in controls which were not medium changed (culture 3.1). In the latter the procuticle of both the imaginal and overlying apolysed pupal cuticles appears very eroded and floccular.* **(C)** *Good and* **(D)** *less well-structured examples of imaginal cuticle secretion in discs changed to medium containing no hormone after 5 days (culture 3.6). b - bristle, e - epicuticle, p - procuticle, ic - imaginal cuticle, pc - pupal cuticle. Bar represents 0.5 um.*

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Fig. 3. Pupal cuticle secretion examined after 48 hours of culture (cultures 2.1 - 2.3). (A) *Disc exposed to 0.1 ug/ml of 20HOE throughout the culture period, with medium changes at 8 and 17 hours of culture. The cuticle is just undergoing apolysis.* **(B)** *Good and* **(C)** *poor examples of discs medium changed at 8 hours to no hormone and again at 17 hours to medium containing 0.1 ug/ml of 20HOE.* **(D)** *Good and* **(E)** *poor examples of discs changed to no hormone at 8 hours and again to fresh medium lacking hormone at 17 hours. The latter micrograph is of cuticle from the thoracic region of the disc. c - cuticulin, e - epicuticle, p - procuticle. Bar represents 0.5 um.*

in ecdysteroid titer required for procuticle secretion *in vivo* before dissection. However, no cuticle was secreted by discs cultured without 20HOE (culture 1.1), and even after an exposure of 6 hours to 0.1 ug/ml 20HOE, discs still secreted only a discontinuous epicuticle and no procuticle (culture 1.5). We may therefore conclude that the degree of exposure to hormone *in vivo* must be minimal. Another point of variance between different culture systems is differing ratios of tissue:medium:air. Our system utilises a relatively small volume of medium, and this may maximize conditioning of the medium by the discs. It is also worth pointing out that medium changing at 6 hours of culture disrupts evagination, and this may indirectly enhance the degree of cuticle secretion. The disc then tends to remain at a stage termed internal eversion which is a particularly favorable configuration for good differentiation (Milner, 1977b).The fact that discs can undergo complete differentiation in medium in which discs had previously been incubated for one or two days strongly suggests that discs do not degrade or sequester hormone during the early stages of incubation. Thus, the possibility that medium changes replenish the supply of hormones to the disc may be discounted.

Experiments in series 3 and 4 were designed to determine whether the secretion and/or tanning of the imaginal cuticle was enhanced by ecdysteroid withdrawal during the latter stages of culture. No evidence in support of this premise was evident either from the differentiation index scores or from electron microscopy.

Our results do not support the contention that a drop in ecdysteroid titer is necessary for the normal development of the pupal or adult cuticle in *Drosophila* imaginal discs. However, it should be borne in mind that evidence from other insects such as *Tenebrio* (Slama, 1980), *Manduca* (Schwartz and Truman, 1983), and *Sarcophaga* (Zdarek and Denlinger, 1987) has suggested that ecdysteroids can delay or prevent normal development if injected into pupae at an inappropriate time. The tanning of *Manduca* pupal cuticle *in vitro* is inhibited if the period of exposure to hormone is prolonged beyond the optimal period (Mitsui and Riddiford, 1976), and a similar result has been obtained for *Plodia* wing discs by Dutkowski *et al.* (1977). We feel that our results may put a question mark against the developing consensus that the prepupal and pupal drops in ecdysteroid titer are necessary for normal differentiation, and emphasize the need for further work in this area.

Materials and Methods

Wing imaginal discs were obtained from late third instar larvae of the Oregon-S strain of *Drosophila melanogaster*. Eggs were collected over a two hour period, surface-sterilized with sodium hypochlorite, and placed in sterile Petri dishes containing David's medium (David, 1955). Discs were hand-dissected under sterile conditions and cultured in Shields and Sang's medium M3 (Shields and Sang, 1977) with modifications for low serum (Edwards *et al.*, 1978), supplemented with 2% fetal bovine serum. Each culture was set up with two wing discs in a column drop (approx. 1-2 ul of medium) between a glass cavity slide and a siliconized cover slip. The culture was sealed with petroleum jelly and kept at a constant 25 ± 0.5°C. 20HOE (Simes, Milan) was dissolved directly in the culture medium and then diluted to the concentration required. Alterations in the hormone concentration during the culture period were achieved by medium changes. The culture was unsealed and the old medium was carefully removed using a pulled Pasteur pipette. The discs were washed twice in fresh medium containing the new level of hormone before the final aliquot of medium was added and the culture resealed.

At the end of the culture period, discs were scored using an eversion and differentiation index to assess development. Each disc was scored on a scale of 0 to 4, the points being defined as follows, with reference to previously published Figures illustrating them:

Evagination index

0 - no evagination (Milner and Sang 1977 Fig. 1b), 1 - internal evagination (Milner and Sang, 1977, Fig. 3a; Milner *et al.*, 1984, Fig. 4c), 2 - partial evagination (Milner and Sang, 1977, Fig. 3b), 3 - full evagination but small wing blade less than 300 um long (Milner, 1977b, Fig. 2e, f). 4 - full evagination with good extension of the wing blade (Milner and Muir, 1987, Fig. 2a).

Differentiation index

0 - no cuticle, 1 - pupal cuticle secretion and apolysis, no imaginal differentiation (Milner, 1977a, Fig. 1b, c; Milner and Muir, 1987, Fig. 2c), 2 - pupal cuticle apolysis and some imaginal differentiation which remained unpigmented after one week of culture (Milner, 1977a, Fig. 1d), 3 - pupal cuticle apolysis, complete imaginal differentiation but poor cuticle pigmentation (Milner and Muir, 1987, Fig. 7c), 4 - as 3 but good cuticle pigmentation after 7 days (Milner and Muir, 1987, Fig. 5b).

Disc development was assessed by calculating mean scores for each treatment. Differences between experimentals and controls were evaluated using a two-sample t-test with 95% confidence limits (minitab). Following scoring, discs were fixed with glutaraldehyde and osmium tetroxide, dehydrated with alcohol, and embedded in Araldite for thin sectioning as previously described (Tucker *et al.*, 1986; Milner and Muir, 1987). Thin sections (60 nm) were stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined using a Phillips 301 transmission electron microscope. Measurements of cuticle thickness were performed on 5-10 sections from at least 3 discs per treatment. Because of the difficulties of obtaining sections at right angles to the plane of the cuticle, measurements should be regarded as approximations only. Sections were taken from the central region of the wing blade unless otherwise noted in the text.

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