**In vitro** imaginal disc development and moulting hormone

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**SUMMARY**

Imaginal leg and wing discs obtained from late-third-instar *Drosophila* larvae were cultured *in vitro* in various concentrations of ecdysterone ranging from $10^{-10}$ to $10^{-5}$ M in order to test the effect of hormone concentration on evagination and cell differentiation.

At the optimal concentration of $8 \times 10^{-8}$ M discs evaginated normally, secreted the pupal cuticle, underwent apolysis, differentiated imaginal structures and secreted the imaginal cuticle.

At suboptimal concentrations ($10^{-8}$ M and less), evagination was incomplete in a variable proportion of appendages. Morphogenetic movements were limited to the earlier ones; so that appendages did not emerge from the peripodial sac. Subsequent development, whenever it occurred, took place inside the peripodial sac. This particular type of 'endoevagination' was only obtained with sub-optimal hormone concentration.

At supra-optimal concentrations ($10^{-6}$ M and more), evagination was always complete but further differentiation was inhibited.

These results show that endoevagination is strictly related to insufficient supply of hormone and that morphogenesis and cell differentiation in imaginal discs are two independent phenomena, which respond to different levels of hormone stimulation.

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**INTRODUCTION**

The *in vitro* development of imaginal discs of *Drosophila* has been described successively by Sengel & Mandaron (1969), Mandaron (1970), Fristrom (1976), Milner (1977), Mandaron & Guillermet (1978), Edwards, Milner & Chen (1978) and Martin & Schneider (1978). It comprises essentially two phenomena: the morphogenesis of the appendage, which occurs during evagination, and cell differentiation. The evagination process may be subdivided into three phases: (1) segmentation of the appendage inside the peripodial sac; (2) emergence of the appendage outside of the peripodial sac (evagination proper) and (3) elongation of the segments of the evaginated appendage. Toward the end of evagination, epidermal cells reject the pupal cuticle (pupal apolysis) which they have started to secrete at the beginning of metamorphosis. The imaginal cuticle

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is then deposited, while the characteristic imaginal structures (bristles, hairs, sensilla) develop. A particular type of evagination was recently described under the (improper) name of endoevagination (Milner, 1977; Guillermet & Mandaron, 1978). In this case the appendage does not emerge out of the peripodial sac, inside which it performs its whole development. In particular, cell differentiation occurs as if evagination had taken place normally.

What are the causes of endoevagination? Is it an accidental occurrence of \textit{in vitro} cultures, independent of hormone concentration in the medium, as suggested by Milner (1977)? In which case it would correspond to a trivial phenomenon due to imperfect culture conditions. Contrariwise, is endoevagination a particular biological process directly linked to hormonal concentration? In order to answer these questions the influence of ecdysterone concentration on the development of imaginal discs was studied \textit{in vitro}, with particular attention to evagination.

**MATERIAL AND METHODS**

Leg and wing discs were obtained from late-third-instar larvae of an Oregon strain of \textit{Drosophila melanogaster}, some 4 h before pupation (embryos and larvae were raised at 25 °C). At this time in our rearing conditions salivary glands were considerably swollen. This means that discs had probably experienced some hormone \textit{in situ} prior to explantation, but nevertheless remained unable to develop further \textit{in vitro}, without hormone supply.

Discs were cultured \textit{in vitro} at 26 °C in the medium M2 (Mandaron, 1971, modified 1979) in the presence of ecdysterone. The latter was provided by S.I.M.E.S. (Milan/Italy) and is known to contain 1 % makisterone (Lafont, personal communication). This hormone was very stable as shown by its analysis, which did not reveal any alteration nor by-products after one year of conservation in 20 % ethanol solution at 4 °C. Several experimental series were performed in which the hormone concentration in the medium was the only variable. Besides the optimal concentration of \(8 \times 10^{-8} \) M, the following concentrations were tested in eight series of 100 explanted discs: \(2 \times 10^{-10}, 8 \times 10^{-10}, 8 \times 10^{-9}, 2 \times 10^{-8}, 2 \times 10^{-7}, 2 \times 10^{-6}, 2 \times 10^{-5} \) M. The development of the discs was analysed with three different techniques: (1) time-lapse cinematography, at the rate of 1 image/min, (2) light microscopy: discs at various stages of development were embedded in Faure's liquid for study of chitinized structures, (3) transmission electron microscopy. After 1, 2 or 3 days of culture appendages were fixed at 1 h at room temperature in modified Karnovsky's fixative (Poodry & Schneiderman, 1970): 2-5 % glutaraldehyde and 2-5 % formaldehyde in 0-1 M sodium cacodylate buffer at pH 7-4. They were rapidly washed in several changes of buffer before being post-fixed for 1 h in 1 % osmium tetroxide in 0-1 M veronal sodium acetate buffer. Dehydration in a graded series of ethanol lasted for 1 h. The passage through propylene oxide did not exceed 30 min.
Discs were then embedded in Epon. Thin sections were stained for 5 min in uranyl acetate and for 5 min in lead citrate.

**RESULTS**

(A) Microcinematographic observations

(1) Development of discs under optimal hormonal conditions ($8 \times 10^{-8}$ to $2 \times 10^{-7}$)

In the vast majority of cases (95 of 100 explanted discs) evagination occurred in three phases (Mandaron & Guillermot, 1978) and was complete (Fig. 1).

Phase 1. This occurred inside the peripodial sac. As the epidermal folds became progressively unfolded, the distal tip of the future appendage moved towards the peduncle which had joined the disc to the larval epidermis *in situ*.

Phase 2. The distal tip penetrated into the widening lumen of the peduncle. Later the more proximal segments followed and thrust outward through this opening. Thus the appendage became completely everted outside the peripodial sac. At the end of this phase the peripodial membrane was found at the base of the appendage, where it persisted throughout the rest of the *in vitro* development.

Phase 3. As soon as the appendage emerged out of the peripodial sac, the segments elongated very rapidly. The length of the appendage was multiplied by a factor of about two. The change in cell shape (transformation from cuboidal to squamous epithelium), which had started during the preceding phases, came to an end during this phase. Evagination and cell flattening started, in almost all cases, in the distal segments (tarsus and wing), and later propagated towards proximal segments (tibia and femur, wing hinge), and finally extended to the peripodial membrane. The progression was thus disto-proximal.

The subsequent *in vitro* development of discs, namely pupal apolysis and formation of imaginal structures, has been described elsewhere (Sengel & Mandaron, 1969; Mandaron, 1970; Milner, 1977).

(2) Development of discs in the presence of low ecdysterone concentration (less than $8 \times 10^{-8}$ M)

In these suboptimal hormonal conditions the percentage of normally evaginated appendages decreased in inverse proportion to the hormone concentration.

(a) In the presence of $2 \times 10^{-8}$ M ecdysterone, only 50 % of explanted discs evaginated completely in a way similar to that described for control discs cultured in $8 \times 10^{-8}$ M ecdysterone. The remaining 50 % of discs developed in the following manner: phase 1 of evagination was identical to that of controls. Segmentation occurred in the distal part of the disc and this then folded over the presumptive proximal segments of the future appendage. Morphogenetic movements ceased shortly before the control appendages emerged out of the peripodial sac. Phases 2 and 3 were lacking. Therefore the proximal segments (tibia and femur, wing hinge) were not formed or formed incompletely (Figs. 2 and 3).
Furthermore, the elongation of the appendage, which normally occurs after the emergence out of the peripodial sac, did not take place. The appendage remained small, its linear dimensions being about half those of the control appendages (750 μm), although the proportions between the length of the segments remained normal (Figs. 1, 3). Further development of these ‘endoevaginated’ discs (pupal apolysis and formation of imaginal structures) occurred entirely inside the peripodial sac. As in the case of control appendages, cell flattening and pupal apolysis progressed in a disto-proximal direction.

(b) In the presence of $8 \times 10^{-9}$ M ecdysterone, all 100 explanted discs endoevaginated. Their development was strictly comparable to that of endoevaginated discs cultured in $2 \times 10^{-8}$ M hormone.

(c) In the presence of hormone concentration lower than $8 \times 10^{-9}$ M, namely $2 \times 10^{-9}$ and $8 \times 10^{-10}$ M, all discs endoevaginated. At the concentration of $2 \times 10^{-10}$ discs did not develop at all.

(3) Development of discs in the presence of high ecdysterone concentration ($2 \times 10^{-6}$, $2 \times 10^{-5}$ M)

Even with highest concentrations, evagination was complete in all discs (100 discs in each of the three series). However, at the end of evagination, wings appeared to be exceedingly swollen, their epithelial wall being abnormally thin. In none of these cases were pupal apolysis or imaginal differentiations observed.

**Figures 1–6**

Fig. 1. Control appendages 8 days after disc explantation in medium M2 in the presence of $8 \times 10^{-8}$ M of ecdysterone.

Figs. 2–6. Cuticular differentiation on endoevaginated legs and wings 8 days after disc explantation in medium M2 in the presence of $8 \times 10^{-9}$ M of ecdysterone.

Fig. 2. Wing developed inside the peripodial sac. After shedding of the pupal cuticle *(p.c.)* the imaginal cuticle *(i.c.)* has been secreted and bristles have developed. × 125.

Fig. 3. Segmentation of the endoevaginated leg has occurred. The pupal cuticle *(p.c.)* has been secreted on the whole appendage and also on the peripodial membrane *(p.m.)*. Length of the appendage is smaller than that of the appendage in Fig. 1. × 250.

Fig. 4. Specific cuticular differentiations on the tarsus of an endoevaginated leg. × 250.

Fig. 5. Endoevaginated wing. Inside the peripodial sac the different parts of the wing are recognizable, with bristles and hairs. Bristles are also very well developed on the peripodial membrane. × 250.

Fig. 6. After the peripodial sac has been mechanically disrupted cuticular structures on the wing (bristles, hairs) can be clearly seen.

*a*, *Apodema*; *b*, bristle; *c*, coxa; *cl*, claws; *f*, femur; *i.c.*, imaginal cuticle; *p.c.*, pupal cuticle; *p.m.*, peripodial membrane; *s*, sex comb; *t*, tarsus; *ti*, tibia; *th*, thoracic part of the wing; *tri*, triple row of bristles on the anterior margin of the wing; *w*, wing; *w.h.*, wing hinge.
Fig. 7. Influence of the ecdysterone concentration on the evagination and differentiation of wing or leg discs cultured in vitro in the medium M2. Vertical bars indicate the frequency of endoevaginated and evaginated appendages, and the frequency of appendages which underwent complete imaginal cuticular differentiation.

(B) Light-microscope observations

(1) Differentiation of appendages cultured in the range of $8 \times 10^{-8}$ and $2 \times 10^{-7}$ M ecdysterone

From 72 h in culture onward, imaginal structures (imaginal cuticle, bristle, hairs, sensilla, sex comb, claws, pulvilli, etc.), comparable to those formed in situ, differentiated (see Mandaron, 1970; Milner 1977).

(2) Differentiation of appendages cultured in low concentrations of hormones (less than $8 \times 10^{-8}$ M)

(a) At hormonal concentrations of $2 \times 10^{-8}$ and $8 \times 10^{-9}$ M, epidermal cells of 'endoevaginated' discs differentiated normally after 72 h in culture and gave rise to typical imaginal structures (Figs. 2–6). Furthermore, the cells of the presumptive proximal segments, which did not undergo morphogenetic movements during endoevagination, nevertheless formed imaginal structures (Figs. 2, 5, 6). Likewise, the cells of the peripodial membrane itself differentiated and formed various imaginal structures such as cuticle and bristles (Figs. 5–6). Bristles that developed on endoevaginated appendages were usually longer and thicker than those which formed on control appendages.

(b) In hormone concentration less than $8 \times 10^{-9}$ M the proportion of discs performing imaginal differentiations became very low (see Fig. 7).
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(c) At hormonal concentration of 10^{-10} M no imaginal differentiations were observed.

(3) Differentiation of appendages cultured in high concentrations of hormones (8 \times 10^{-7}, 2 \times 10^{-6}, 2 \times 10^{-5} M)

At 8 \times 10^{-7} M only 50\% of the 100 explanted discs differentiated poor imaginal structures and at 10^{-6} and 10^{-5} M differentiations were never observed although discs evaginated quite normally.

(C) Transmission electron microscope observations

(1) Appendages cultured in a concentration of ecdysterone ranging from 8 \times 10^{-8} to 2 \times 10^{-7} M

Besides the observations reported before (Sengel & Mandaron, 1969; Mandaron, 1971, 1976) it is worth mentioning that nerve fibres, tracheol trunks and tracheoles were constantly present.

(2) Appendages cultured in low concentrations of ecdysterone (less than 8 \times 10^{-8} M)

Electron microscopy confirmed the change in cell shape already observed in time-lapse cinematography. Epidermal disc cells which measured from 8-9 \mu m in height and 2-3 \mu m in length at the time of explantation, were only 2-3 \mu m high at the end of evagination; their length, measured along the proximal axis of the appendage, reached 5 \mu m (Fig. 8). The epidermis very infrequently showed a few necrotic cells. The development of nerve fibres (Fig. 9) and tracheae was similar to that of control discs. Among cuticular differentiations, only pupal cuticle was examined in some detail. It was composed of an epicuticle some 20 nm thick and of a stratified procuticle, the thickness of which reached 1-2 \mu m just before apolysis (Fig. 10).

DISCUSSION

It is known that in the absence of moulting hormone a cultured imaginal disc shows no sign of development: it does not evaginate nor differentiate. In the presence of ecdysterone, at concentrations ranging from 8 \times 10^{-8} to 2 \times 10^{-7} M, evagination is complete and epidermis differentiates pupal and imaginal structures similar to those which develop in situ (Sengel & Mandaron, 1969; Mandaron, 1971, 1976; Milner, 1977; Edwards et al. 1978). At concentrations lower than these apparently optimal concentrations, only the first phase of evagination takes place: the appendage does not emerge out of the peripodial sac, inside which it performs its entire development. The percentage of ‘endoevagination’ and further differentiations are inversely related to the hormonal concentration (Fig. 7) (Guillermet & Mandaron, 1978). It appears that suboptimal concentration of hormone is, in our culture conditions, the cause of ‘endoevagination’. Thus ‘endoevagination’ is not a cultivation accident, but rather a biological phenomenon, strictly related to low concentrations of
Figs. 8-10. Electron microscopic observations of differentiations on endoevaginated wing or leg discs cultured in the medium M2 in the presence of $8 \times 10^{-8}$ M of ecdysterone.

Fig. 8. Three days after explantation, the epidermal cells of a leg disc have changed their shape; they have become squamous. ×16,500.

Fig. 9. Nerve fibres differentiated in a wing 3 days after explantation of the disc. ×21,750.

Fig. 10. One day after explantation of a leg disc a very thick and stratified procuticle has been secreted on the appendage. At this time, cells of the peripodial membrane (p.m.) are just beginning to secrete their own cuticle. ×18,750.

epi, Epicuticle; m, microvilli; N, nucleus; pro, procuticle; n, nerve; P→D, proximo-distal axis.

moulting hormone. This conclusion is opposed to that of Milner (1977) for whom 'internal eversion is not an artifact induced by any particular concentration of $\beta$-ecdysone, as it is observed with suboptimal ($2 \times 10^{-8}$ M) and superoptimal ($2 \times 10^{-6}$ M) concentration of $\beta$-ecdysone . . .'. Indeed in the modified medium of Shields and Sang, Milner obtained 50% of endoevaginated legs and 10% of endoevaginated wings at optimal hormone concentration. These
surprising results are probably due to the constitution of the particular medium used by this author. The endoevagination obtained by Milner with $10^{-5}$ M $\beta$-ecdysone probably reflects a developmental block of the disc due not only to hyperecdysonism but also to the constitution of the medium. In our culture conditions, hormonal concentration of $10^{-6}$ M always led to complete evagination of the discs, even though apolysis and imaginal differentiation were precluded. It should be mentioned that optimal hormonal concentrations leading to a normal development of the discs, and which were found to be of $4 \times 10^{-7}$ M in Shields and Sang's medium (Milner, 1977) and $8 \times 10^{-8}$ M in our medium, can vary with the ionic concentration of sodium and potassium in the medium. Indeed a variation of the $\text{Na}^+ / \text{K}^+$ ratio probably changes the permeability of the cell membrane.

In endoevaginations caused by hormonal concentrations of the order of $10^{-10}$ and $10^{-9}$ M, pupal and imaginal structures appear to be qualitatively better than those formed in normally evaginated discs cultured under 'optimal' hormonal conditions. Thus the pupal cuticle, the thickness of which exceeds 1 $\mu$m, is clearly stratified, while that of normally evaginated appendages reaches a thickness of only 0.5 $\mu$m and shows a fewer number of strata. Likewise bristles are longer and thicker than those of control appendages. These results may be due to the establishment of a hormonally more favourable micro-environment inside the peripodial sac than that which exists outside. The present results, obtained in vitro, show that cell differentiation can occur in the absence of evagination. This fact, which was also mentioned by Milner for in vitro culture, had already been described in detail by Hadorn and his group (1966) in the case of discs transplanted into a larva prior to metamorphosis. In these transplantations, discs or disc fragments form a membraneous vesicle inside which pupal and imaginal structures differentiate from the epidermal cells of the disc (Hadorn, 1966; Poodry & Schneiderman, 1970). However, these two types of development—development of transplanted discs and in vitro development of endoevaginated discs—although they lead to comparable structures, result from different mechanisms. Indeed in vivo discs are placed under the optimal physiological conditions of the host. The absence of morphogenesis of transplants can therefore not be explained by inadequate hormonal concentrations. It is possible that handling of the discs during transplantation is responsible for the absence of evagination, as suggested by Mandaron (1971): according to whether discs are explanted with a transplantation spoon or through a glass capillary comparable to those used in in vivo transplantations, discs will or will not evaginate in vitro.

While the characteristic morphogenetic movements occurring during evagination are not a requisite of further cell differentiation, it appears that change in cell shape is a sine qua non condition. In all circumstances, in situ, in vivo or in vitro, during normal evagination or endoevagination, cells must pass from a prismatic to a cuboidal and to a squamous shape before undergoing

In vitro endoevagination reveals that morphogenesis and cell differentiation are two independent processes, which may be initiated by different hormonal concentrations. How does this finding compare with the in situ conditions? Since Shaaya & Karlson’s (1965) report it is well known that hormonal concentration in hemolymph, or in total extracts, varies during the course of metamorphosis. More recently, by using radioimmune assay techniques, several authors have extensively studied the variations of the ecdysone titre during the post-embryonic development. Unfortunately results were somewhat contradictory. According to Borst et al. (1974), for the 12 h period between puparium formation and pupation – that is, during the time of evagination of the discs – ecdysone titre increased about threefold. By contrast, Hodgetts, Sage & O’Connor in Drosophila (1977), in agreement with the results of Shaaya and Karlson in Calliphora, showed that, while the titre was very high at the time of the puparium formation, it decreased dramatically during this 12 h period after puparium formation, and increased again just prior to adult cuticle deposition – that is, at the time of the cell differentiation. This second peak was found to be higher than the first one. Likewise, De Reggi, Hirn & Delaage (1975) showed a very high ecdysone level at the puparium formation (more than twice that found in mature larvae) but did not publish any results regarding stages beyond 6 h after puparium formation. Besides, concerning the ecdysone titre inside the discs, at the time of their evagination, the only report of Borst et al. (1974) showed a very low level compared to that in the hemolymph at the same time. Until new data are available, it seems quite impossible to establish a good correlation between the stages of disc development and the ecdysone titre in the hemolymph. For instance, we do not know if, in situ, evagination requires more ecdysone than differentiation or vice versa. The fact that endoevaginated appendages, despite their normal proportions, are smaller than normally evaginated ones raises a certain number of questions. It is generally admitted that, during in vitro development at hormonal concentration of the order of $10^{-7}$ or $10^{-8}$ M, disc cells do not proliferate or proliferate very little (Mandaron, 1970; Fristrom, Logan & Murphy, 1973; Fristrom, Fristrom, Fekete & Kuniyuki, 1977; Bullmore, 1977). The rare mitotic figures which can be observed, particularly in tormogen or trichogen cells, correspond to differentiation mitoses. The very small number of cell divisions could explain the differences in size between normal in situ appendages and those that develop in vitro. Furthermore, in the case of endoevaginated appendages the second and third phases of normal evagination are omitted, so that the lack of elongation of the appendage could by itself explain their small size. Indeed, length is normally multiplied by a factor 1:5–2 during the third phase of evagination. Surprisingly wings and legs obtained by Milner in modified Shields and Sang’s medium are smaller than those obtained in Mandaron modified medium. According to this author this dif-
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ference appears to be due to the lack of expansion of the segments in the proximo-distal direction in his experiments (compare Milner (1977), p. 108, fig. H, and Mandaron (1972), p. 107, fig. 10).

As cell death is extremely infrequent, one is led to admit that control appendages and endoevaginated ones comprise approximately the same number of cells. The absence of elongation in endoevaginated appendages may result from an incomplete change in cell shape. Transmission electron microscopy revealed that the lengthening of cells along the proximo-distal axis of the appendage was less pronounced in endoevaginated appendages (5 \mu m) than in totally evaginated ones (5–7 \mu m; Mandaron, 1976).

Finally endoevagination reveals that cells of the peripodial membrane, like those of the disc proper, undergo differentiation under the influence of moultng hormone and thus form imaginal structures. Peripodial membrane cells are therefore target cells for the hormone (Mandaron & Guillermet, 1978; Guillermet & Mandaron, 1978). The peripodial membrane is not destroyed during metamorphosis, as it was generally considered before (Fristrom, Raikow, Petri & Stewart, 1969; Poodry & Schneiderman, 1970; Mandaron, Guillermet & Sengel, 1977). Its role could be to provide continuity between the appendages and the thoracic ventral sclerites, as was already suggested by Sprey & Oldenhave (1974) in the case of Calliphora. This unexpected result is also in agreement with observations by Kiss (personal communication), according to which, in the case of the mutant ‘broad’ where disc evagination does not occur, the cells of the peripodial membrane can be seen to secrete a pupal cuticle.

REFERENCES


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