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The eversion and differentiation of *Drosophila melanogaster* leg and wing imaginal discs cultured *in vitro* with an optimal concentration of β-ecdysone

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SUMMARY

The stages of the eversion and differentiation of prothoracic leg and wing discs of *Drosophila melanogaster*, in Shields and Sang's medium 3, are described. The range of specific imaginal structures produced, including patterns of sensilla trichodea and sensilla campaniformia, are noted, and the relationship of these structures to those differentiated *in situ* is discussed.

INTRODUCTION

During recent years, culture *in vitro* has been widely used to examine the development of many insect tissues. Examples from *Drosophila* include the differentiation of larval and imaginal tissue from early embryos (Shields, Dübendorfer & Sang, 1975; Dübendorfer, Shields & Sang, 1975), and the chromosomal puffing sequences of cultured salivary glands (Ashburner 1973, 1974; Richards, 1976). Another such system is that of the eversion and differentiation *in vitro* of imaginal discs, first described by Mandaron (1970, 1971, 1973), and subsequently examined by Fristrom and co-workers (e.g. Chihara, Petri, Fristrom & King, 1972), Oberlander and associates (e.g. Benson, Oberlander, Koreeda & Nakanishi, 1974), and Milner & Sang (1974, 1976*a*). These studies open up opportunities for examining the morphological changes involved in disc development, and for determining the hormonal requirements for eversion and differentiation in a system which minimizes the effect of substances released into the haemolymph by other insect organs, an effect which complicates studies using culture *in vivo*. Also, the biochemical manipulation of disc development may be performed in a system free from the possible degradation of test substances, or their metabolism to compounds with an altered activity, which cannot be excluded from *in vivo* experiments involving the direct injection of compounds into the haemolymph, where they are in contact with all the major organs of the insect.

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Unfortunately, different *in vitro* systems, utilizing different culture media, may yield different results in response to similar experimental manipulations. For example Mandaron (1973) found that β-ecdysone would not induce normal eversion and differentiation in leg discs, whereas Chihara *et al.* (1972) and Milner & Sang (1974) found that β-ecdysone was much more active than α-ecdysone in inducing morphogenesis in *Drosophila* discs. Also, different degrees of morphogenesis are found in different experimental systems. For example, Oberlander, Leach & Tomblin (1973) report only a minor level of morphogenesis and cuticle formation in lepidopteran discs, without the imaginal differentiation observed for *Drosophila* discs by Mandaron (1970, 1971, 1973) and Milner & Sang (1974, 1976*a*).

It would therefore appear to be of importance to catalogue the stages of eversion and differentiation of imaginal discs in an experimental system which supports a repeatably high level of morphogenesis, with comparisons to development *in situ*, so that the usefulness of that particular experimental system for further investigations may be evaluated. This has been partially performed for the leg disc only by Mandaron (1970, 1973), using Mandaron's medium M (1971). However, it has been found that ecdysone-induced differentiation in this medium is very difficult to obtain in a repeatable manner (Milner 1975), as different batches of medium M vary widely in their ability to support development. Shields and Sang's medium 3 (Shields & Sang, 1976), when supplemented with a suitable batch of foetal bovine serum, would appear to satisfy the requirements of both good imaginal differentiation and repeatability of results with different batches of medium (Milner & Sang, 1974). This medium, which was devised for the differentiation of *Drosophila* embryonic tissues, contains the concentrations of amino acids and ions found in *Drosophila* larval haemolymph, and therefore corresponds more closely to the natural situation. β-ecdysone regularly induces disc differentiation when added to this medium, and the present paper outlines a system for describing the eversion stages and differentiation of leg and wing discs with an optimal concentration of β-ecdysone, which may be of use in future studies of imaginal morphogenesis.

MATERIALS AND METHODS

The procedures used for the sterile culture of larvae from an Oregon S stock of *Drosophila melanogaster*, and for the dissection and culture *in vitro* of their imaginal discs, has been previously described (Milner & Sang, 1974). Discs from late third instar larvae were cultured at 25 °C in medium containing 0·2 μg/ml (4×10^{-7} M) of β-ecdysone. Donor larvae had finished feeding, and were at the wandering stage, but had not yet everted their anterior spiracles. This sample of β-ecdysone was isolated from the bark of the tree *Dacrydium intermedium* (Russell, Fenemore, Horn & Middleton, 1972), and contained a minor contaminant of Makisterone A.

RESULTS

The timing of morphogenetic events during culture *in vitro* with an optimal concentration of hormone is necessarily approximate, as the time course of eversion varies from disc to disc according to the exact developmental stage reached by the larva before dissection, and, presumably, according to the degree of damage inflicted upon the disc during handling. The stages of disc eversion were numbered sequentially, and related to the period of time after culture initiation during which the majority of discs had undergone the processes specific to that stage.

The development of the prothoracic leg disc

A pair of prothoracic leg discs from a late third instar larva (stage 0) is shown in Fig. 1A. A concentric, folded organization exists, and it is the unfolding of this pre-existing structure that constitutes eversion. The sequence of morphogenesis in culture is as follows:

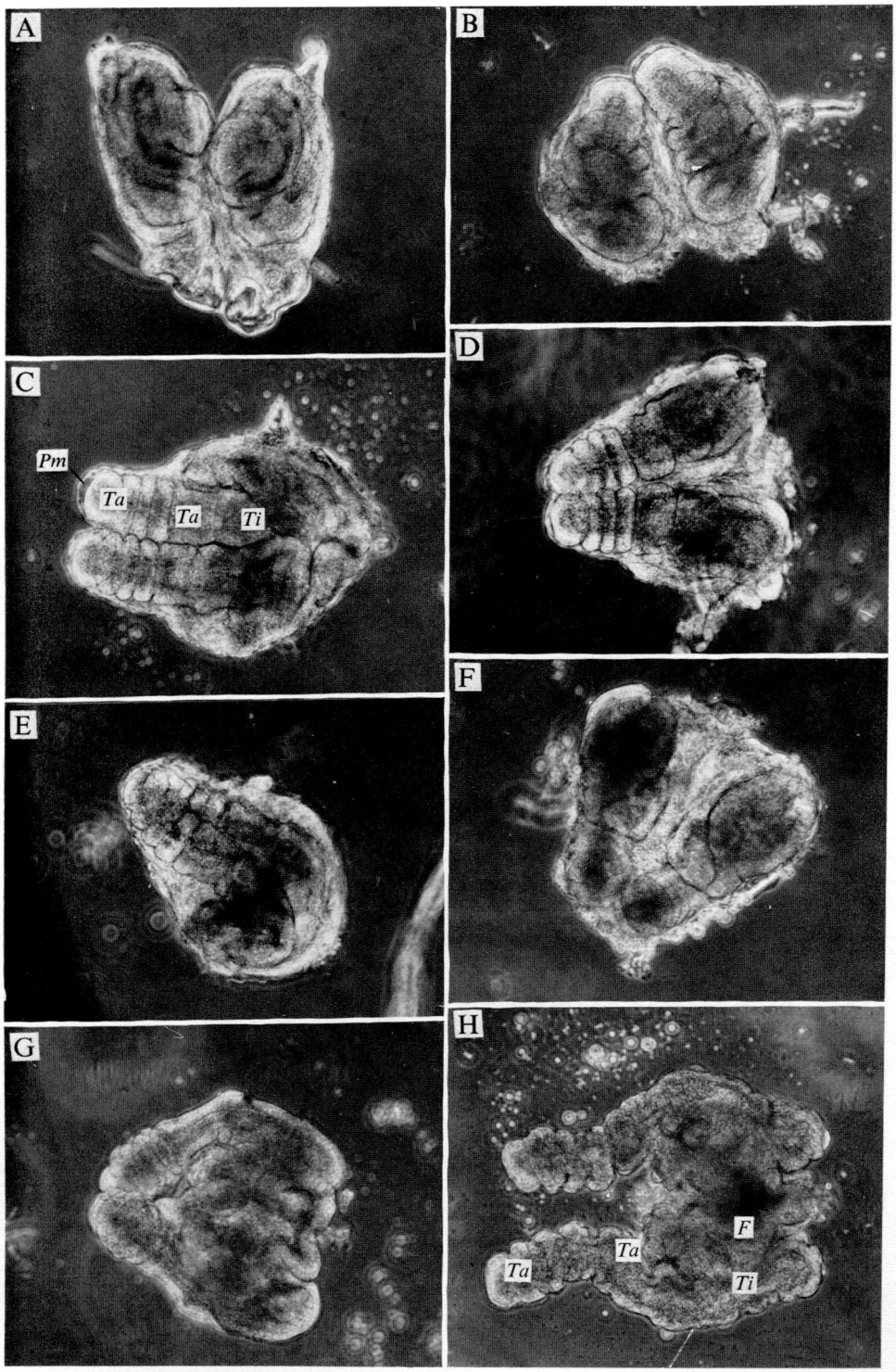
Stage 1 (6–9 h). Extension of tarsal segments begins, pushing the peripodial membrane outwards (Fig. 1B).

Stage 2 (9–11 h). As eversion proceeds, the five tarsal segments and the distal part of the tibia become visible. The peripodial membranes of the two discs are increasingly unified as the tarsi push outwards, the disc pair sharing a common lumen (Fig. 1C). During the early part of this stage it can be seen that tarsal segments 4 and 5 are not clearly separated, whereas towards the end of stage 2 the boundary between these two segments is clearly defined. This separation of tarsal segments 4 and 5 has been observed to occur *in situ* betwen $1\frac{1}{4}$ and 2 h after pupariation (Vogt, 1946).

Stage 3 (11–13 h). Up to this point the pair of legs have everted in parallel. Now, the everted tarsi become angled toward one another (Fig. 1D). This probably results from the forces of eversion being countered by the fully stretched peripodial membrane. Also, the tarsi are now angled downwards relative to the plane of eversion of the tibia (Fig. 1E), presumably due to the same cause.

Stage 4 (13–14 h). The peripodial membrane begins to break down, and cells can be seen rounding off and falling away. These cells appear to break down very rapidly, and in a matter of 15–30 min the membrane is represented only by a cloud of rounded up cells and sub-cellular debris, floating freely in the medium. The tarsi are angled towards the camera in this photograph (Fig. 1F).

Stage 5 (13–14 h). As soon as the constraint of the peripodial membrane is removed, the tarsi open outwards to reveal the femura, which up to this point have remained inside the body of the discs (Fig. 1G). The distal parts of each leg remain bent, and do not lie in the same plane, and the debris of the peripodial membrane can be clearly seen.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Stage 6 (14–24 h). The everted structures elongate slightly, and come to lie in one plane (Fig. 1 H).

Stage 7 (24–48 h). The pupal cuticle, previously secreted by the disc epithelium, is shed by the contraction of the underlying tissue, leaving a gap between the imaginal epithelium and the cuticle layer (see Fig. 2 F). This process is known as apolysis.

Stage 8 (48–72 h). The characteristic imaginal cuticular structures, for example the claws and bristles, differentiate from the imaginal epidermal cells and may be seen using phase contrast optics (for photographs, see Milner & Sang, 1976*b*). Characteristic bristle patterns, such as the tibial transverse rows, and the sex comb of the male differentiate. These structures, and the imaginal cuticle, are not pigmented at this stage. Under poor culture conditions, some cell breakdown may occur, filling the space between the leg epidermis and the shed pupal cuticle, and thus obscuring the underlying differentiation.

Stage 9 (72 h–7 days). During this period, partial pigmentation of the imaginal cuticle, and of the cuticular structures, occurs in some, but not all of the cultures. The differentiation of myogenic elements has been observed, giving rise to leg movements.

No further differentiation is seen, and the imaginal tissue eventually becomes necrotic and dies. It is often difficult to distinguish between necrosis and pigmentation at this stage.

In approximately 50 % of prothoracic leg pairs, an abnormal mode of eversion, called internal eversion, may occur. In these cases, eversion proceeds normally until stage 2, after which the peripodial membrane, instead of breaking down, contracts back to re-form the original shape of the disc pair. During this process, the everted tarsi cross over each other, and are forced back into the lumen of the disc, where they are completely surrounded by the peripodial membrane and tissue which will form femoral, coxal and prothoracic structures. Here, the tarsi apolyse and differentiate normally, forming structures which are usually better pigmented than those differentiated by normally

FIGURE 1

The sequence of eversion of a pair of prothoracic leg discs under optimal hormonal conditions (see text for detailed descriptions).

(A) Stage 0 (immediately after culture initiation).

(B) Stage 1 (6–9 h after culture initiation).

(C) Stage 2 (9–11 h).

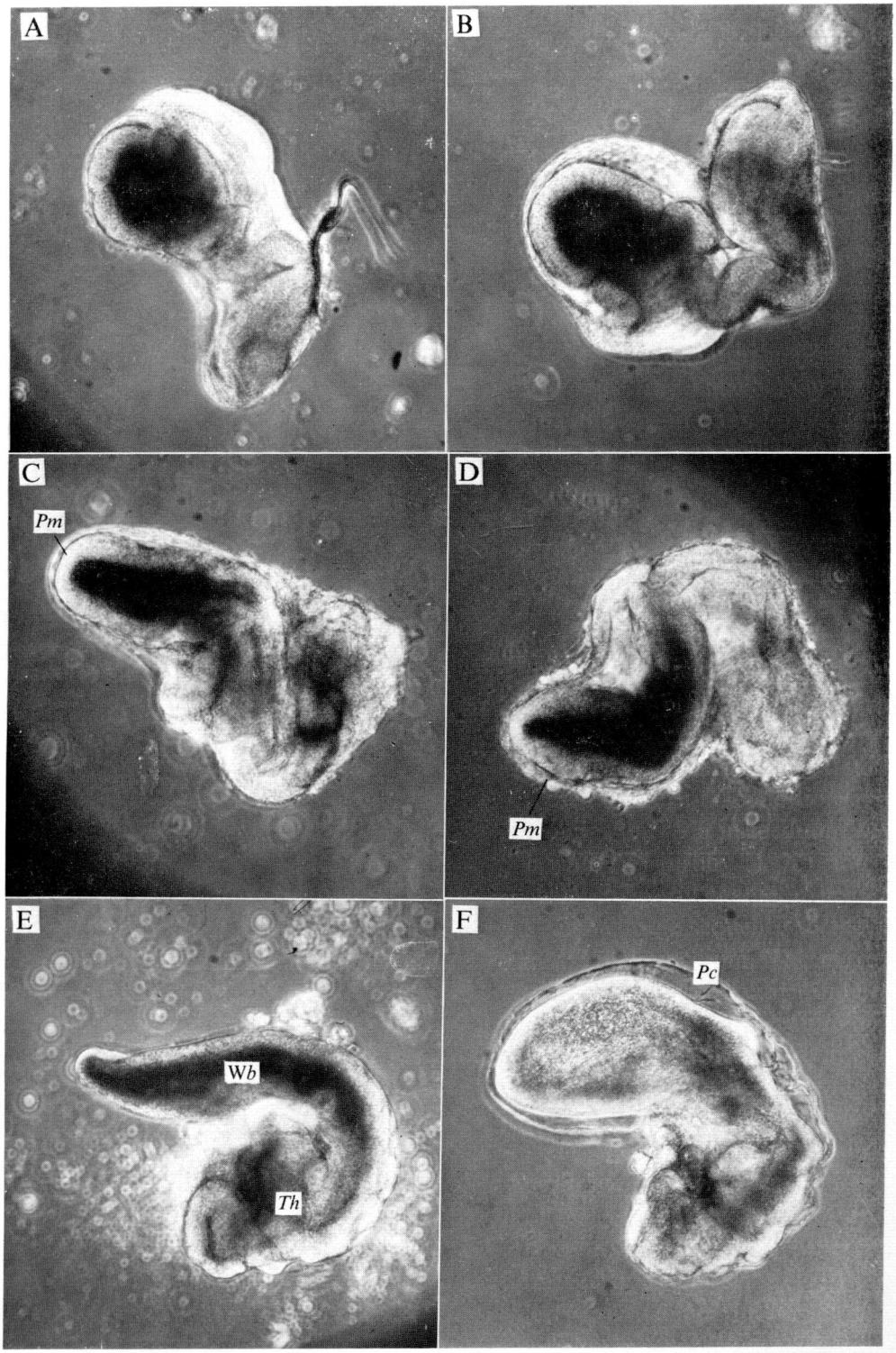
(D) Stage 3 (11–13 h), top view.

(E) Stage 3 (11–13 h), side view.

(F) Stage 4 (13–14 h).

(G) Stage 5 (13–14 h).

(H) Stage 6 (14–24 h). *F*, femur; *Pm*, peripodial membrane; *Ta*, tarsus; *Ti*, tibia. All magnifications × 110.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everted disc pairs. In these, the specific patterns of cuticular differentiation described below can be most clearly seen. This may be due to the fact that differentiation here occurs in a medium which is well conditioned by the surrounding layer of cells, providing an environment which is presumably better for development. Internal eversion is not an artifact induced by one particular concentration of β-ecdysone, as it is observed with suboptimal (0·02 μg/ml) and super-optimal (20 μg/ml) concentrations of β-ecdysone, and also with α-ecdysone, at a range of concentrations.

In squash preparations, particularly of internally everted leg disc pairs at stage 9, the cuticular differentiation present may be seen in greater detail. The unguitractor plate, the pulvillus and the unguis, all parts of the claw organ (Ferris, 1950), and the apodemes, may be seen. The sex comb of male prothoracic legs, the transverse bristle rows of the basitarsus and tibia, and patterns of trichomes specific to prothoracic, coxal and tibial regions differentiate (see Fig. 3A). Also, groups of sensilla trichodea (St) and sensilla companiformia (Sc) are present. These include the St 13 of the prothorax (Fig. 3A), the prothoracic-coxal joint, the St 3, St 4 and St 8 of the coxa, the Sc-8, Sc+5, 2GSt, St 5 and Sc 3 of the trochanter, and the Sc 1 and Sc 11 of the femur (see Schubiger, 1968, for descriptions of these structures). However, in many cases elements of a particular pattern are lost, incomplete, or unrecognizable. Examples of these include a sex comb incorporating a socket which lacked a bristle, and Sc−8 and Sc+5 from the trochanter without the accompanying edge bristle. The bristles of the femur are particularly deficient, and in most cases are either extremely stunted or missing, the characteristic long bristles of the distal femur having never been observed. Also, the degree of pigmentation attained is in all cases less than is found for structures differentiated *in vivo*.

The development of the wing disc

Dorsal mesothoracic discs, like leg discs, are folded structures, which give rise to the wing blade and much of the imaginal mesothorax (Bryant, 1975). Figure 2A shows a side view of a wing disc from a late third instar larva. The wing pouch, which forms the distal part of the wing blade, can be seen protruding

FIGURE 2

The sequence of eversion and differentiation of wing discs under optimal hormonal conditions (see text for detailed descriptions).

(A) Stage 0 (immediately after culture initiation).

(B) Stage 1 (6–9 h after culture initiation).

(C) Stage 2 (9–11 h).

(D) Stage 4 (13–14 h).

(E) Stage 5 (14–24 h).

(F) Stage 7 (24–48 h). *Pc*, pupal cuticle; *Pm*, peripodial membrane; *Th*, thoracic elements; *Wb*, wing blade. All magnifications × 110.

from the plane of the disc, within the peripodial membrane. The sequence of eversion and differentiation under conditions optimal for development *in vitro* (0·2 μg/ml of β-ecdysone) is as follows:

Stage 1 (6–9 h). The wing pouch begins to push outwards (Fig. 2B).

Stage 2 (9–11 h). Extension of the wing blade within the peripodial membrane continues, and the upper and lower surface of the wing blade come together (Fig. 2C).

Stage 3 (11–13 h). The everting wing blade begins to bend, presumably due to the resistance of the fully stretched peripodial membrane (see Fig. 2D). This pressure tends to open up the structure somewhat, showing the union of the dorsal and ventral surfaces of the wing blade, and the tapering of the thick imaginal epithelial cell layer into the thin peripodial membrane.

Stage 4 (13–14 h). The peripodial membrane begins to break down, cells and sub-cellular debris rounding up and falling away (Fig. 2D). As with the leg disc, this process occurs rapidly, within 30 min.

Stage 5 (14–24 h). The released wing blade straightens somewhat, and the thoracic part of the wing disc contracts into a mass at the base of the wing (Fig. 2E). The debris of the peripodial membrane can be seen.

Stage 6 (24–48 h). Apolysis from the pupal cuticle occurs (Fig. 2F).

Stage 7 (48–72 h). Imaginal differentiation occurs, forming the bristles of the costa, triple row and double row, along the anterior margin of the wing (see Milner & Sang, 1976*b*). The double row of hairs along the posterior margin of the wing also differentiates.

Stage 8 (72 h–7 days). As in the leg disc, pigmentation occurs during this latter phase of development, allowing squash preparations to be made. Pulsations of the thoracic region can be observed, indicating the development of myogenic elements.

A high proportion (>90 %) of wing discs evert, and differentiate the bristles and trichomes of the wing blade. Structures specific to the wing hinge and mesothorax may be observed after preparing squashes of stage-8 discs, the terminology used in the following description being that of Bryant (1975). The two scutellar bristles, trichomes of the mesonotum, microchaetes (Fig. 3C), and bristles and sensilla of the tegula may be seen, as well as bristles of the costa, triple row and double row, hairs of the posterior row, and trichomes of the wing blade. Other cuticular structures of the wing hinges, such as the axillary sclerites, the humeral plate, the unnamed plate, and the yellow club may be recognized. Sensilla trichodea of the tegula, and sensilla campaniformia (Sc) of the wing hinge, including Sc 25, Sc 12 and Sc 4d (Fig. 3B), are found. Again, patterns, although recognizable, are in some cases incomplete–for example, the Sc 25 illustrated in Fig. 3B appears to possess only 19 sensilla, whereas the adjacent Sc 12 is complete.

It is of interest to determine the relationship of the dimensions of imaginal structures differentiated *in vitro* to those formed *in situ*. Table 1 shows the dimen-

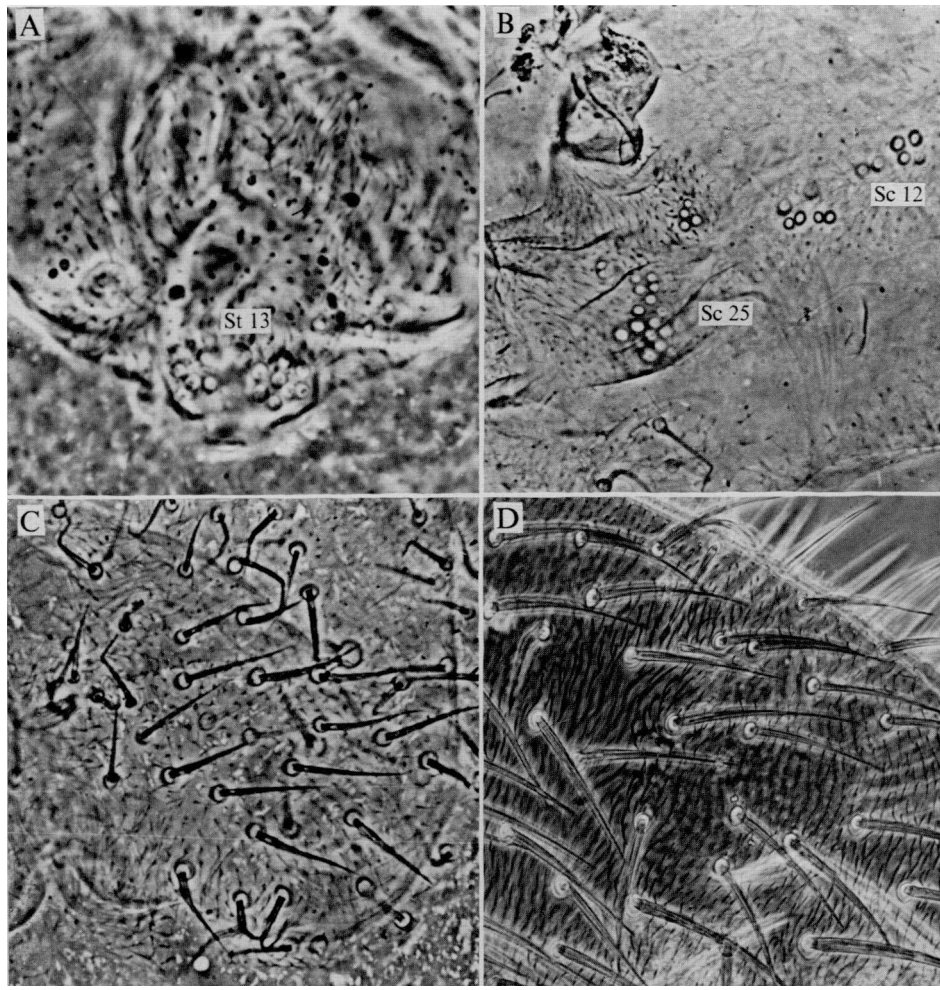

Fig. 3. Imaginal differentiation of leg and wing discs *in vitro* and *in situ*. (A) Sensilla trichodea (St 13) of the prothorax, and prothoracic trichomes, differentiated *in vitro* by a pair of prothoracic leg discs. (B) Sensilla campaniformia (Sc 25 and Sc 12) from the dorsal wing hinge, differentiated *in vitro*. (C) Mesothoracic trichomes and microchaetes of the dorsal mesothorax differentiated *in vitro* from a wing disc. (D) Michochaetes and trichomes from the mesothorax differentiated *in situ*. Magnifications: A × 400; B, C × 475, D × 285.

sions of leg segments and wing blades formed *in vitro*. The length of leg segments are approximately one-third normal, but the widths of differentiated segments in most cases exceeds the value for development *in situ*. Thus, a failure of morphogenesis *in vitro* is a lack of expansion of the segments in the proximo-distal direction. Wing blades, however, compare less favourably to normal development than do leg segments. The wing blade everted *in vitro* had dimensions only one-fifth to one-seventh of those found *in situ* (Table 1). Part of the explanation for this may lie in the folding which is present both in wing blades everted *in*

Table 1. *Overall dimensions of everted structures 48 h after culture initiation with 0·2 μg/ml β-ecdysone*

		Size *in situ* (μm)	Size *in vitro* (μm)
(i) Lengths of leg segments			
Tarsal segment	5	85	34
	4	74	23
	3	85	23
	2	125	23
	1	188	75
Tibia		495	165
Femur		570	174
(ii) Widths of leg segments			
Tarsal segment	5	33	54
	4	34	49
	3	36	50
	2	38	49
	1	55	43
Tibia		93	52
Femur		184	47
(iii) Length of wing blade			
Male		1915	331
Female		2280	
(iv) Width of wing			
Male		896	180
Female		1020	

Numbers represent the average of a minimum of five observations.

Table 2. *Dimensions of cuticular structures formed following differentiation* in vitro *with 0·2 μg/ml of β-ecdysone*

	Differentiated *in situ* (μm)	Differentiated *in vitro* (μm)
Leg		
Claw (unguis)	37–43	14–17
Tarsal bristles	26–74	11–29
Sex comb bristles	34–46	23–26
Tibial bristles (traverse rows)	26–46	14–20
Prothoracic trichomes	9–11	6–9
Wing		
Bristles (anterior margin)	34–46	14–17
Hairs (posterior margin)	34–51	14–23
Wing blade trichomes	11–23	6–8
Thoracic trichomes	9–14	4–7
Thoracic bristles	51–456	17–71

vitro and *in situ*, the final dimensions of the wing blade *in situ* only being attained after emergence by means of hydrostatic pressure (Robertson, 1936; Bodenstein, 1950). This additional extension of the wing blade is of course not available to a cultured disc.

The size of imaginal cuticular structures differentiated by both leg and wing discs is shown in Table 2. Because of the difficulty in recognizing specific bristles in some areas, the range of sizes of bristles is given, and compared to the range found *in situ*. Structures formed *in vitro* are, with a few exceptions, two- to three-fold smaller than their equivalents differentiated *in situ* (compare Fig. 3C and 3D, showing the differentiation of similar areas of the mesothorax *in vitro* and *in situ*).

Thus, although the majority of markers used to recognize particular areas of leg and wing cuticle may be found in preparations differentiated *in vitro*, qualitative and quantitative deficiencies in these patterns exist in comparison to cultures differentiated *in situ*.

DISCUSSION

The time sequence of eversion and differentiation of imaginal discs under the conditions of culture described above may be compared to the sequence both *in situ* and in other culture media. *In situ*, leg and wing discs have completed eversion 6 h after puparium formation (Robertson, 1936), the apolysis of the pupal cuticle occurring at approximately 20 h (Bodenstein, 1950). The differentiation of imaginal cuticular structures occurs at approximately 36 h, but they remain unpigmented until about 70 h after pupariation (Robertson, 1936). Thus, eversion and differentiation occur more slowly *in vitro* than *in situ*, under these experimental conditions. However, Mandaron (1973) reports that eversion requires only 9 h in medium M with 3 μg/ml of α-ecdysone, whereas Fristrom, Logan & Murphy (1973) note that maximal eversion in Robb's medium is achieved approximately 15 h after culture initiation with 0·1 μg/ml of β-ecdysone, a similar time sequence to the 13–14 h reported above for eversion in Shields and Sang's medium. The more rapid development found by Mandaron may well be the result of either different hormonal or medium conditions, or perhaps a slightly higher temperature of incubation.

One of the deficiencies of leg disc development *in vitro* is the failure of the segments to elongate following the eversion of the appendage. This process has been suggested by Fristrom & Fristrom (1975) to be caused by cell rearrangement, and presumably the culture medium or hormone titre used does not sufficiently mimic *in situ* conditions for this process to occur. The internal eversion of half the cultured pairs of prothoracic leg discs also represents a deviation from normal development. However, it is interesting to note that the structure formed by this process closely resembles leg disc development *in vivo*, after injection into a late third instar larval host, where the tarsus is also everted inside a hollow vesicle (Poodry & Schneiderman, 1970).

Further deficiencies are the reduced size, incomplete patterns, and poor pigmentation of imaginal cuticular structures differentiated *in vitro*. However, the above report demonstrates that the majority of the cuticular patterns specific to both leg and wing discs may be found during differentiation *in vitro*. Sensilla and trichomes of the ventral prothorax, and sensilla, trichomes and bristles of the dorsal mesothorax and wing hinge are formed by prothoracic leg and wing discs respectively, in contrast to Mandaron's (1970, 1973) failure to find these structures. Especially important is the observation that leg and wing sensilla develop in culture, as these structures may be used to identify the presence of particular areas of the disc following differentiation *in vitro*.

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