The Differentiation Centre Inducing the Development from Larval to Adult Leg in *Pieris brassicae* (Lepidoptera)

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WITH TWO PLATES

It is generally supposed that the imaginal bud of the leg in Lepidoptera, which Bodenstein (1935, 1937) reported to be visible in the second segment during the last larval instar of *Vanessa urticae*, develops to produce the adult leg by histogenesis.

Bodenstein (1935, 1937) concluded that the material in the second segment of the larval leg normally forms the anlage of the adult leg but that the regions anterior and posterior to this anlage also have the capacity to develop into a leg, though the potentiality existing in the ectoderm of such parts is not usually realized. He therefore interpreted his results from extirpation and transplantation of the legs of *Vanessa* on the hypothesis that the adult leg is derived from an imaginal bud just like the imaginal bud of a wing.

But later (1941) he wrote: 'the labile topographical anlage pattern of the leg field may be roughly as follows: the distal segments of the caterpillar leg represent the anlage of the tarsus, the second segment the anlage of the tibia and the first, the proximal segment, the anlage of the femur'. This idea served to explain the fact that duplicated or triplicated leg formation occurred in *Pyrameis* and *Phryganidia*; but it was inconsistent with his former ideas on the imaginal bud.

In fact, however, Gonin (1894) had studied the relation between the larval leg and the imaginal structure within it in *Pieris brassicae*. He termed the out-growth within the larval leg the 'femoro-tibial bud', but he did not explain how the bud developed to form the femur and tibia. He also pointed out that the trachea was bent along the epidermis of the bud, that the folds at the inside of the leg were deep, whereas those at the other side were not, and that the coxa and trochanter might be derived from the root of the leg bud. But he did not pursue the later development in any detail.

In the present paper, therefore, the post-embryonic development of the leg in *Pieris* is more fully described and some problems concerned with this development are discussed.

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MATERIAL AND METHODS

All experiments were carried out on caterpillars of *Pieris brassicae* which came from the stock culture maintained at the Entomological Field Station, University of Cambridge. The fourth, fifth, prepupal, and pupal stages of *Pieris* kept at 25°C last 2, 3, 2, and 9 days respectively.

For histological work Carnoy's fluid was used as fixative. After fixation of the intact caterpillars for one hour the heads and abdomens were removed and the remaining parts were fixed for a further hour. Sections were cut in paraffin at 6 μ to 14 μ. Hansen's iron trioxyhaematin was mainly used for staining.

For cauterizing or transplanting the distal segments of the leg the animal was narcotized with ether. The motionless caterpillar was then placed under the binocular microscope on a mound of plasticine in which a depression was made that just about fitted the animal's body. A fine pipette, the broad end of which was connected with a rubber tube and whose narrow end exactly fitted the third segment of caterpillar's leg, was used to hold the leg during operation. This is very necessary when handling the tender larvae just after moulting. The end of the rubber tube is held in the mouth and the distal segment of the leg is sucked into the narrow end of the pipette.

This method is particularly useful when inverting the transplant; it is possible to hold the graft in position and thus to prevent the blood from flowing out. For burning restricted parts of the leg an electrically heated needle was used.

The right mesothoracic leg was used for most observations and experiments; the effects on the adult leg were observed in the pupa 2 or 3 days before emergence.

RESULTS

1. Development of the pupal legs

Immediately after the last larval moult the epidermis at the outer side of the third segment of the leg has elongated cells and many intercellular spaces, like the caudate cells described by Lower (1957) in the wing epidermis of Lepidoptera. The cell layer at the outer side of the second segment, on the other hand, is very thin except for a small anterior region between the second and third segments where cell divisions occur actively from the very beginning of the last instar (Text-fig. 1; Plate 1, fig. A). This region can be recognized through the surface of the soft leg after moult because its rich tracheal supply makes it look like a pale spot. In the present paper it is regarded as a 'differentiation centre'.

The area of cellular multiplication spreads gradually from this region throughout the whole leg epidermis, especially toward the base of the second segment, to build the femoro-tibial bud. In *Pieris* the thickened part of the second segment is about 30 μ in thickness, or twice that of the general epidermis, and is conspicuous on the outer side of the second segment about 6 hours after the last
moult at 25°C; and at that time a trachea with many tracheoles is closely applied to the thickened part (Text-fig. 2).

The epidermis, which is attached to the cuticle at the beginning of the last instar, becomes separated from it about 3 hours after the last moult except at the points of attachment of the muscles. The epidermal layer can thus become thickened and folded, the thickened region of the leg agreeing closely with those parts of the leg which are sclerotized. The parts which have many bristles, that is, the inner side of the second and third segments, remain without thickening.

On the second day of the fifth instar the most highly thickened part measures about 60 μ in thickness and the femoro-tibial bud begins to evaginate. The trachea, which runs immediately below the thickened epidermis, follows the evagination, as pointed out by Gonin. At this time two further thickened regions, resulting from the spread of cell divisions through the epidermis, become visible.
One is at the inner side of the articular membrane between the third and fourth segments; it is deeply invaginated, while the outside wall has been folded several times; the other is at the proximal inner side of the second segment. Later a third thickening appears between the second and third segments also.

Some folds which occur in the middle part of the second segment and near the top of the out-growth, as a result of active cell division, cause the evaginated out-growth to enlarge more and more. Gradually many folds appear on the third and fourth segments also and the surface area becomes very extensive (Text-fig. 3).

During the prepupal stage the epidermis retracts to form the exuvial space;
this space contains the moulting fluid, which begins to appear about 3 hours after the last moult. During this period all the folds in the epidermis extend to build the parts of the pupal leg (Plate 2, fig. F).

Ultimately the first segment builds the coxa, the thickened part at the proximal inside end of the second segment grows to form the trochanter, and the outgrowth of the femoro-tibial bud forms the femur and tibia, as was pointed out by Gonin. During the prepupal period a septum appears in this out-growth, and after pupation the femur and tibia are completely separated. The third and fourth segments give rise to the tarsus. The inner part of the second segment may contribute to the side wall of the femur and that of the third segment may become the side wall of the tibia, whereas Gonin described them all as contributing to build the tarsus (Text-fig. 4).

The epidermis begins to lay down pupal cuticle on the second day after prepupation.

2. Development of the adult legs

At 25 °C. the prepupal period lasts 2 days and then pupal ecdysis occurs. A most striking change takes place within the out-growth. Although a septum develops already during the prepupal period, it is not till the first day after pupation that pupal cuticle appears in this septum and extends from the bottom to the top of the out-growth to separate the two parts, femur and tibia (Plate 1, figs. E–H).

On the second pupal day tanning of the pupal cuticle with quinones takes place, the femur and tibia are differentiated, and then the epidermis retracts, acquiring adult characteristics progressively. The imaginal epidermis is formed at about the fourth day of pupal life and scale-forming cells are found.

In the light of these observations it is not surprising that Bodenstein (1937) obtained chimeric legs when two distal segments of the foreleg were transplanted on to corresponding part of the hind leg.

3. Experiment on the differentiation centre

If the second segment is removed in the third instar, as was done by Bodenstein (1933b), a dwarf leg regenerates (Plate 1, fig. B); if removed in the fourth or fifth instar, no leg regenerates. But when the part of the differentiation centre alone is removed or burned at the fourth instar, the corresponding adult leg has a thin tibia, especially in the proximal half, and an incomplete tarsus, though usually with the claw intact. This suggests that the regulative capacity to build the adult leg still remains at the fourth instar.

In general the new formation of the lost parts of an insect leg depends on the amount of material remaining and on the length of time available for regulation to occur. However, if the differentiation centre is removed or burned at the beginning of the fifth instar, no differentiation of the leg parts takes place in spite of the large amount of material remaining, but an out-growth resembling a larval leg with some adult characters, bearing short hairs and claw, develops at the top
of the adult trochanter. The differentiation centre is clearly important for the development of the leg in its natural condition.

When the two and a half distal segments, including the differentiation centre, are rotated through 180° at the beginning of the last instar, to see how the thickened part appears, the distal part fails to continue its development; it degenerates, and then the larval epidermis regenerates from the stump. Finally, a larval leg-like structure develops at the top of the adult trochanter (as happens likewise after removing or burning the differentiation centre at the same stage). When the two distal segments were rotated through 180° close to the differentiation centre within the first hour after the last larval moult, and 2 days later (just before prepupation) the caterpillars were killed and sections cut, no differentiated epidermis could be found in the transplant, the epidermis of which gradually degenerated. However, when the same experiment was done on larvae about 6 hours after moulting, thickened epidermis was developing in the transplant. This was still more evident when the operation was done about 20 hours after moulting (Plate 2, figs. A–B). This result means that the thickened epidermis within the transplant, which is rotated after some degree of determination has taken place (within 12 hours after the last moult, at 25°C.), can develop (provided it has a sufficient oxygen supply, as mentioned below). But the transplant which is moved before determination gradually degenerates. After determination the thickened epidermis develops to form another leg. This result agrees entirely with Bodenstein’s (1937) application of the centre hypothesis of Mangold (1929).

However, duplicated leg formation was scarcely ever obtained in *Pieris* (only two examples among 63 operated caterpillars). That is presumably because there is insufficient time available for regulation, for the last instar in *Pieris* at 25°C. lasts only 3 days, while *Phryganidia* caterpillars (Bodenstein, 1937) required 17–20 days from the time of operation to the time of pupation. Therefore, there may be another factor in addition to the differentiation centre in the production of the adult leg.

When the outer side of the first or second segment (the side on which the differentiation centre lies) is severely cauterized at some stage after the last moult, no leg develops as a rule, except the coxa and sometimes the trochanter. If the membrane between the first and second segments is cauterized in the last instar, when the femoro-tibial bud grows out conspicuously from inside the membranous region, the epidermis of the distal part, which has differentiated already, gradually degenerates (Plate 2, fig. C). This probably results from damage to the tracheae.

If, on the contrary, the outside of the second segment is burned slightly on the first day of the last instar, the adult emerging from such a caterpillar has a rather longer femur and tibia, but the tarsus is not differentiated (Plate 1, fig. C). But if the outside of the membranous segment is burned slightly on the second day, or at the beginning of the third day, the adult has a short femur and tibia and a
relatively complete tarsus (Plate 1, fig. D). The burned epidermis of the second segment is considered to have approximately the same properties as the epidermis within the membranous segment which was burned later, because the femoro-tibial bud grows and evaginates. The development of an undifferentiated tarsus (Plate 1, fig. C) also depends perhaps upon the shortage of oxygen supply to the undetermined part.

When only the middle portion of the outside of the second segment is cut at

![Text-Fig. 5. Diagrammatic outline of experiments and their results. Arrows show cauterizations, broken lines indicate extirpations or rotations, and normal stages are given at the left of the figure.](image-url)

the beginning of the last instar so as to damage the main trachea running into the distal part, and its inside epidermis is left without damage, an incomplete leg arises, presumably dependent on the remaining tracheoles; but an almost complete leg develops when the trachea happens to escape all damage. Naturally, in these cases, leg formation is delayed. In a single example of the last instar, sections of which were cut, the trachea had formed many new branches after wound healing (Plate 2, fig. E). On the other hand, duplicated or triplicated legs frequently develop when rotation of the distal part (comprising as many as three segments) of the limb was effected early in the fourth instar, because the damaged trachea can regenerate and often branch during the two last instars. This fact supports the suggestion that duplicated or triplicated leg formation is dependent upon development of newly formed tracheae. The experiments are summarized in Text-fig. 5.
DISCUSSION

As mentioned above, a trachea with tracheoles is found near the differentiation centre at the beginning of the last instar, and, after the cell divisions in this region, the trachea becomes closely applied to the epidermis and finally bends proximally in the second segment, following the growth of the femoro-tibial bud. The larval leg at the fourth instar has the same trachea, with some tracheoles arising from it in the same position, but no determination in the leg occurs during the fourth instar. However, some further tracheoblasts are found in this same part of the leg during the last moult (Plate 2, fig. D). These apparently lead to an increase in the number of tracheoles during the last instar, and this region, which is the differentiation centre in the natural condition, begins active cell divisions immediately after the last moult, probably because it now gets a sufficient oxygen supply. Consequently thickened regions appear successively in the different parts of the leg. It is at these stages that determination seems to take place.

According to the experimental evidence mentioned above, determination and development of the epidermis depends upon the oxygen supply. Whenever the epidermis outside the differentiation centre can obtain a sufficient oxygen supply during the early stage after the last moult, as the result of the approach of a newly formed trachea, that epidermal part likewise may be able to become a new differentiation centre. All the epidermis in the leg may well have this capacity, but those parts which are remote from the trachea in the natural condition may need some substances or stimuli for their differentiation.

The moulting hormone will induce the epidermis to develop adult characters, but it will not cause the release of the leg-forming potencies in the larval epidermis if the oxygen supply is insufficient. Stimuli of some kind from the differentiation centre, which has been activated by the moulting hormone in the presence of sufficient oxygen, seem necessary in addition and to act through the epidermis. Adult leg formation and the realization of adult characteristics in the caterpillar leg are considered to be different phenomena. The moulting hormone causes the larval epidermis of the leg to produce its imaginal characters (as in pieces of epidermis from a young larva transplanted into a later stage), but this hormone does not induce each part to develop to the corresponding part of the leg without the stimuli coming from the differentiation centre.

Since the differentiation centre may be expected to absorb more hormone than other parts, when it receives a sufficient oxygen supply from an early stage, it will differentiate at the very beginning of the last instar, and stimuli produced in it may induce cell division and self-differentiation in the remainder of the limb, provided this receives an appropriate oxygen supply. It may perhaps do this by activating the respiratory enzymes.

Williams (1951) claimed that the function of the thoracic gland hormone is to preside over the synthesis of cytochrome C, and Wigglesworth (1957) supported
the view that the essential change induced by the moulting hormone is protein synthesis, and that as protein synthesis is an endothermic process for which energy is required, respiratory enzymes will be necessary. He considered two possible ways in which the moulting hormone could exert its action: the hormone might provide some material substance needed by the cells to ensure the activity of the enzymes used for protein synthesis, or act by influencing permeability within the cells, existing enzymes thus gaining access to their potential substrates. Haget (1953) has studied the gradual acquisition of the capacity for self-differentiation by the various regions of the ectoderm, and the associated loss of its ability to regulate. He has shown that the process is dependent on an influence which spreads from the differentiation centre through the sheet of ectoderm in the egg (Waddington, 1957). The observations on post-embryonic development outlined above agree with these results. Wigglesworth (1954) noted that one of the most impressive features of normal growth is synchronization and co-ordination of the process in all parts of the body. In discussing two possible media for maintaining simultaneous development, the nervous system and the epidermis, he writes that synchronous development in different parts of the integument may perhaps be maintained by chemical or other stimuli transmitted by way of the continuous epidermis. The synchronous development in the different parts of the caterpillar's leg would clearly agree with this suggestion.

Thus it would appear that in post-embryonic development the substance from the formative centre, that is, the hormone secreted by the prothoracic gland, reaches the differentiation centre of the leg which is induced to develop and to give rise to stimuli that are transmitted by way of the epidermis; just as, in the egg, embryonic development begins after chemical substances from the formative centre reach the differentiation centre. The principle of egg development, that determination proceeds step by step, agrees with that of post-embryonic development of the larval leg.

Whether the other imaginal organs have similar centres that are activated by the hormone and give rise to stimuli which induce differentiation in the surrounding parts is not known, either because they are often too small or because determination proceeds too quickly for such a centre to be recognized.

**SUMMARY**

1. Post-embryonic development from larval to adult leg in *Pieris brassicae* has been studied histologically.
2. It is suggested that the epidermis between the second and third segments of the caterpillar's leg, where cell divisions occur actively soon after the final moult, may be a differentiation centre which induces development from larval to adult leg, and that stimuli of some kind are transmitted from this centre by way of the continuous epidermis, producing cell divisions and determination.
3. The differentiation centre may be the region which absorbs more hormone than other parts when it receives a sufficient oxygen supply from an early stage.
4. Adult leg formation and the realization of adult characteristics in the caterpillar leg are considered to be different phenomena.

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REFERENCES


EXPLANATION OF PLATES

PLATE 1

FIG. A. Stage about 3 hours after the last moult. tr, trachea; trl, tracheole.

FIG. B. A dwarf leg (b) in caterpillar which was extirpated at the third instar and then regenerated; and its section (B).

FIGS. C–D. Adult legs emerged from the caterpillar, the legs of which were cauterized slightly. C, cauterizing the outer side of the second segment of caterpillar's leg. D, cauterizing the membrane between the first and second segments at a later stage. Arrows show the cauterized areas; the legs on opposite side were left untreated as controls.

FIGS. E–H. Pupal leg just after pupation, showing division into femur and tibia. E, the upper part of the femoro-tibial bud. F, near its middle part. G, its next lower part (femur and tibia are separate). H, its lowest part. ti, tibia; f, femur.
PLATE 2

FIGS. A–B. Caterpillar's legs at the second day after rotation of the distal parts through 180°. A, rotation was made on the second day after last moult. B, rotation was made on the first day (left leg), and normal leg (right leg). Arrows show the amputated parts.

FIG. C. A degenerating larval leg arises after severely cauterizing the membrane between the first and second segments at the second-day stage. Arrow shows the area cauterized. No tracheae are visible.

FIG. D. The new epidermis in the leg during the last moult. Many tracheoblasts (trb) are visible.

FIG. E. Caterpillar's leg showing many tracheal branches newly formed after cutting the trachea together with the outer side of the second segment.

FIG. F. A prepupal leg newly formed in the larval leg.

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