The Post-embryonic Development of the Tracheal System in *Drosophila melanogaster*

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**SUMMARY**

The fate of the tracheal system is traced from the first larval instar to the adult stage. The basic larval pattern conforms to that shown for other Diptera Cyclorrhapha (Whitten, 1955), and is identical in all three instars.

According to previous accounts the adult system directly replaces the larval: the larval system is partly shed, partly histolysed, and the adult system arises from imaginal cell clusters independently of the preceding larval system.

In contrast, it is shown here that in the cephalic, thoracic, and anterior abdominal region there is a definite continuity in the tracheal system, from larval, through pupal to the adult stage, whereas in the posterior abdominal region the larval system is histolysed, and the adult system is independent of it in origin. Moreover, in the pupal stage this region is tracheated by tracheae arising from the anterior abdominal region and belonging to a distinct pupal system.

Moulting of the tracheal linings is complete at the first and second larval ecdyses, but incomplete at the third larval-pupal and pupal-adult ecdyses. In consequence, in both pupal and adult systems there are tracheae which are secreted around pre-existing tracheae, others formed as new 'branch' tracheae, and those which have been carried over from the previous instar. In the adult the newly formed tracheae of the posterior abdominal region fall into a fourth category. Most of the adult thoracic air sacs correspond to new 'branch' tracheae of other instars.

The pre-pupal moult and instar are discussed with reference to the tracheal system and tentative suggestions are made concerning the true nature of the pre-pupal cuticle. There is no pre-pupal tracheal system.

Events traced for *Drosophila* would seem to be general for Cyclorrhapha, both Acalypterae and Calypterae. The separate fates of the anterior and posterior abdominal systems, in contrast with the straightforward development in Diptera Nematocera, would appear to mark a distinct step in the evolution of the system in Diptera.

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INTRODUCTION

ALTHOUGH the tracheal system of insects has been much studied, knowledge of the development of the system is scanty and contains many contradictions. Older workers came to one of two conclusions concerning the fate of the system during metamorphosis in holometabolous insects: either that the tracheal system is entirely destroyed during the pupal stage, so that the adult system is independent of the pre-existing larval system; or else that small parts of the main tracheae of the adult system are formed around stumps of previous larval tracheae (Peréz, 1910), the rest of the larval system being destroyed by 'histolysis', and the remainder of the adult system arising independently of pre-existing larval tracheae. Keister's (1948) recent results on the development of the tracheal system in Sciara coprophila (Diptera Nematocera) favour Palmén's (1877) belief that the same tracheal epithelium persists right through to the adult stage, rather than Peréz's (1910) conception of the origin of the adult system from nests of imaginal cells on the larval tracheal stumps.

The present investigation in Drosophila melanogaster Meigen (Diptera Cyclorrhapha) is an attempt to determine the detailed topography of the tracheal system in each of the stages from the first larval instar to the adult stage, and to investigate the relationship existing between them. The main object has been to determine whether there is a definite continuity in the system from larva to adult, or whether, as concluded by Robertson (1936) and Bodenstein (1950), the adult system directly replaces and is independent of the larval system, in which case there is no distinct functional pupal system.

The development of the tracheal system is a more difficult problem in a member of the Diptera Cyclorrhapha than in the Nematocera, of which Sciara coprophila is a member (Keister, 1948). In the Nematocera there are four larval instars, a pupal instar, and lastly the adult stage. Each instar possesses its own tracheal system, and by a relatively simple process the cuticle and tracheal system of any instar is replaced by that of the subsequent instar, the tracheal system of the former being divided into its component metameres and removed through the respective pairs of spiracles. In this way the fourth larval system is replaced by the pupal, and this in turn by the adult. In the Cyclorrhapha the problem is complicated by the developmental processes occurring between the last larval instar and adult stage. Instead of four, there are three larval instars, and the last (third) larval cuticle is retained as the so-called 'puparium'. Existing evidence of the processes occurring subsequently to this is contradictory. Fraenkel (1938) observed in Calliphora erythrocephala that the abdomen only of the pupa is invested in a thin membrane lying between pupal cuticle and the puparium. Previously to this Snodgrass (1924) noted a thin membrane completely investing the pupa of Rhagoletis, and he suggested that it is a pre-pupal cuticle which corresponds to the cuticle of a suppressed fourth instar larva. This would bring the
development more in line with that found in the Diptera Nematocera, where there are four larval instars. A study of the tracheal system must necessarily involve a consideration of the general cuticle with which it is continuous. Robertson (1936) for Drosophila reports the presence of a pre-pupal cuticle completely investing the larva, but does not discuss it in connexion with the tracheal system.

Details of the moulting of the larger and finer tracheal branches in the different developmental stages of Drosophila melanogaster are also considered. Keister (1948) showed that in Sciara coprophila, whereas shedding of both tracheal and tracheolar intima is complete at each of the inter-larval ecdyses, at the pre-pupal and pre-adult ecdyses certain of the pre-existing ultimate branches are retained and not shed, so that the final adult system possesses a number of pupal and even preceding larval elements. On the other hand, Wigglesworth (1954) has shown for Rhodnius prolixus (Hemiptera) that at each ecdysis the lining of the tracheole is never shed with the cuticle, although the lining of the trachea is removed.

MATERIALS AND METHODS

All stages were studied either in the living state or after injection with cobalt sulphide (Wigglesworth, 1950). After injection, specimens were dissected or sectioned. The living stages were investigated by dissection under a binocular dissecting microscope, while the details of moulting were followed exclusively by phase-contrast microscopy.

Whereas the events taking place in the tracheal system at, before, and after the inter-larval ecdyses can be seen through the transparent body-wall, opacity of the puparium makes such a study of the subsequent stages difficult, and in the later stages almost impossible. Although individual specimens are only 3 mm in length, the sequence of events subsequent to puparium formation is not simultaneously observable in all the tracheae. Therefore, the following account is of a composite picture obtained from several hundred whole and dissected, living and injected specimens.

The work was begun in the Zoological Laboratory, Cambridge, and has been continued at Queen Mary College (University of London).

THE LARVAL SYSTEM

The tracheal system of the larva is essentially similar in all three instars, the chief difference being the presence of an increasing number of smaller tracheae and tracheoles in the successive stages. The pattern formed by the main tracheae is identical with that seen and described by Whitten (1955) in other Diptera Cyclorrhapha. Each of the main tracheae can, therefore, be homologized with those described for other Diptera, and the terms used in the following description correspond to those given in the above paper, rather than those given in the descriptive accounts by Ruhle (1932) and Haskins and Enzmann (1937). The larval system is redescribed in detail as both of the
latter descriptions omit several tracheae which form an essential part of the basic tracheal framework; this must be determined in detail before the subsequent development can be traced.

The first instar system is metapneustic, while the second and third are

![Diagram of larval tracheal system in Drosophila](image)

**Fig. 1A. Drosophila: the larval tracheal system. Dorsal view. Semi-diagrammatic.**

amphipneustic. The spiracular tracheae of the non-functional spiracles two to nine are collapsed to form solid spiracular cords.

In common with other Diptera Cyclorrhapha, there are a pair of dorsal and a pair of lateral longitudinal trunks, joined on each side by eight transverse connectives which give off the spiracular threads of spiracles two to
Connecting the two dorsal trunks are ten dorsal anastomoses arranged as in fig. I, A.

Posterior to the anterior junctions of the lateral and dorsal trunks, a dorsal cervical trachea arises from each dorsal trunk. Shortly after its origin each divides. At the level of the supraoesophageal ganglia each posterior branch gives off a trachea to the ganglion and then unites with its fellow in a short median anastomosis corresponding to the dorsal cervical anastomosis of other Diptera (Whitten, 1955). This is omitted from the description by Haskins and Finzmann (1937).
Each ventral cervical trachea, before passing anteriorly, gives rise to a medianly directed trachea; this is one of the first pair of ventral ganglionic tracheae. It gives off a branch to the central nervous system, and almost immediately anastomoses mid-ventrally with its fellow to form the first ventral anastomosis. Only the first three pairs of ventral ganglionic tracheae form mid-ventral anastomoses. These tracheate areas of the nerve mass corresponding to the pro-, meso-, and meta-thoracic ganglia of the Diptera Nematocera.

The last two pairs of ventral ganglionic tracheae, instead of traversing the abdomen to tracheate the posterior region of the ventral nerve mass, terminate abruptly without reaching the nerve mass. This is obviously a variation from the normal condition seen in the Diptera Cyclorrhapha; possibly other species of *Drosophila* or even different strains of *melanogaster* are normal in this respect. Haskins and Enzmann (1937) described the same condition in their material.

**The Pupal System**

The functional pupal tracheal system differs very greatly from the larval. The metameric tracheal pattern so characteristic of the system in the larva is not immediately evident. However, on closer investigation, such a pattern can be made out in the thoracic and anterior abdominal regions.

The only functional spiracles at this stage are the first thoracic pair which are well developed, biperforate, and lie at the anterior angles of the prothorax.

The head is tracheated by numerous ramifications of two pairs of tracheae, both of which arise from the region of the functional 'prothoracic' spiracles. One pair enters the head ventrally to the other and tracheates the ventral regions of the head, including the developing proboscis. The two dorsal tracheae, almost immediately on entering the head, are connected by a transverse anastomosis. The dorsal tracheae send numerous branches to the developing eyes and to the brain.

The most striking differences from the larval tracheal system are in the abdominal region where, posteriorly, there is no evidence of a metamerically arranged pattern of tracheae connected with either non-functional or functional spiracles. Instead, the whole of this region is tracheated by the numerous ramifications of two pairs of tracheae, arising from the anterior abdominal region.

The thoracic and anterior abdominal tracheae are best considered together. The spiracular trachea opening at the 'prothoracic' spiracle is very broad and continues posteriorly as a longitudinal dorsal tracheal trunk. From it, shortly after its origin from the spiracle, a trachea of much narrower diameter arises, which also passes posteriorly as a lateral longitudinal trunk. Both continue as far as the anterior abdominal region.

The two broad longitudinal trunks are connected dorsally by five dorsal anastomoses. All, except the first, are of very narrow diameter, and obscured beneath opaque pupal cells, being visible only by micro-dissection. The second differs from the three succeeding ones by giving rise to numerous
tracheae and tracheoles which supply the developing thoracic flight muscles. The first of these anastomoses is very much broader in diameter and unites the two dorsal trunks posteriorly to the origin of the lateral trunks. The two pairs of trunks of each side are connected by four tracheae. The

![Diagram of Drosophila tracheal system](image)

**Fig. 2. Drosophila: the pupal tracheal system. Dorsal view. Semi-diagrammatic.**

first arises from the dorsal trunk posteriorly to the origin of the second dorsal anastomosis. The succeeding connectives arise between the origins of successive dorsal anastomoses. This arrangement immediately recalls an identical arrangement between the anterior dorsal anastomoses and the transverse connectives of the larval system.

From each of the lateral trunks, immediately posterior to their origin, a trachea arises which passes ventrally. It first gives off a trachea to the first
pair of legs, then a trachea to the nervous system, and finally unites with its fellow in the mid-ventral line. Posteriorly to the origin of the first ganglionic tracheae each lateral trunk gives rise to a trachea which, passing medio-ventrally, first gives off a trachea to the nervous system and then unites with its fellow in the mid-line. The second pair of leg tracheae arises from the lateral trunk separately and posteriorly to the origin of the second pair of ventrally anastomosing tracheae. A third pair of ventrally anastomosing tracheae and the third pair of leg tracheae arise separately and in a corresponding position to the second pairs, but between the origins of the first and second transverse connectives. The relative positions of these ventral tracheae are clearly identical with those of the first three ventral ganglionic tracheae of the larval system; they similarly unite in the mid-line to form three ventral anastomoses.

A trachea to each wing arises from the lateral longitudinal trunks immediately after their origin anteriorly. Each bifurcates near the wing base, both branches entering the wing. No corresponding posterior wing trachea has been observed either in dissection or after injection. In the case of the haltere, a single trachea arises from the lateral trunk just anteriorly to the origin of the second transverse connective; no corresponding anterior trachea appears to reach or enter the haltere.

The spiracular tracheae associated with the four pairs of transverse connectives appear collapsed and the corresponding spiracles inconspicuous and non-functional.

The two pairs of very much-branched tracheae which ramify throughout the posterior abdominal region arise from the third and fourth transverse connectives at the origin of these from the dorsal trunks. Posteriorly to the origins of the fourth connectives, the broad dorsal trunks end blindly in a characteristic and curious way.

**The Adult System**

The adult tracheal system bears even less resemblance to that of the preceding pupal and larval stages than does the pupal to the larval system. Miller (1950), in describing the adult system, simply remarks on the enormous changes which have taken place in the head and thoracic region, accompanying evolution of air sacs. According to him the basic metameric pattern—implying the serially repeated pattern characteristic of the insect tracheal system in general—is clearly evident only in the mid-abdominal region. He does not attempt to compare the system with that found in the larva, nor could he have compared his description with those of other Diptera, for his is one of the rare accurate descriptions in print of the tracheal system of an adult dipteran (see Whitten, 1953).

For the purpose of tracing the development of the system through to the adult, it is necessary to redescribe the adult system, concentrating on the basic pattern underlying the complex arrangement of air sacs in the head and thorax. Consequently, Miller's descriptive terms for the different air sacs will
not be used consistently, but only where they help in describing the detailed structure overlying the fundamental pattern.

In the adult there are nine pairs of functional spiracles. They occupy positions corresponding to the first nine pairs of larval spiracles, which are, according to Keilin (1944) intersegmental in position.

The head (figs. 3, 9) contains a large number of air sacs which give off numerous tracheae and tracheoles supplying the eyes, brain, and other structures. According to Miller (1950) the distal air sacs all arise from a pair of ventral and a pair of dorsal sacs which have diverged from a single pair of sacs entering the head from the region of the 'pro-thoracic' spiracles. It would, however, appear that the paired dorsal and ventral sacs actually enter the head separately, as two pairs of sacs, those of the two sides being very closely apposed and therefore appearing to be single. Each of the dorsal sacs first gives off a branch to the brain; this is narrow in diameter and tracheate in form. Secondly, a trachea is given off laterally which expands into the postocular air sac of Miller (1950); this passes antero-ventrally and tracheates the eyes. This is followed by a sac-like branch arising medially which anastomoses with its fellow mid-dorsally. Opposite the origin of this, there arises laterally a branch which tracheates the brain. The main dorsal vessels finally expand into the paired dorsal sacs. The paired ventral sacs each give off first a very large frontal sac and then continue as the post-genal air sacs which ultimately pass into a trachea that extends the length of the proboscis, supplying branches to the various structures.

In the abdomen the system is clearly composed of tracheae constituting a definite pattern. Passing from the first pair of spiracles to the posterior end are two pairs of longitudinal trunks. The first pair, situated more dorsally, begins anteriorly as a continuation backwards of the first thoracic spiracular trachea. It is very broad in diameter and generally sac-like until reaching the region of the fifth pair of spiracles. Here it suddenly becomes narrow and continues in its more tracheate form as far as the posterior end of the abdomen. In the thoracic region, tracheae and tracheoles arising from it pass mainly to the dorsal median indirect flight muscles. The more laterally placed trunk is joined to the dorsal trunk immediately behind the first thoracic spiracle. In the thoracic region it is hardly recognizable as a single longitudinal element, being expanded into a series of air sacs. At the level of the third pair of spiracles it becomes more tracheate and extends in this form to the region of the ninth pair of spiracles. In the thoracic region small branches arise from the sac-like dilations of the lateral trunks to tracheate mainly the more superficial tergo-sternal flight muscles.

The dorsal and lateral trunks of each side are connected, besides their junctions anteriorly and posteriorly, in seven places. In the abdominal region they are connected by long transverse connectives; in the region of the second pair of spiracles the connectives are much shorter and stouter.

The two broad dorsal trunks are connected by a regularly placed series of nine dorsal anastomoses. The first, which is much broader and more sac-like
than the rest, and the second, which is very narrow, occur between the region of the first thoracic spiracles and the first pair of connectives, at the level of the second pair of spiracles. The subsequent anastomoses arise between the origins of successive transverse connectives and the ninth pair of spiracles.

The third to ninth pairs of spiracular tracheae arise from the transverse connectives at the ventral end near their junction with the lateral trunks.

Other tracheae in the abdominal region will not be considered here as they hardly constitute part of the fundamental pattern. It is significant that posteriorly a tenth pair of spiracles appears to be absent. In fact, a tenth
The tracheal metamere—as found in the larva—appears to be completely absent, so reducing the total complement of spiracles and dorsal anastomoses from ten to nine.

In the thorax the basic framework is obscured by the many large air sacs (figs. 3, 10). The three leg tracheae arise in positions corresponding exactly to those in the pupa. The second pair arises directly from the lateral trunks anteriorly to the second pair of spiracles. The third pair similarly arises from the lateral trunks anteriorly to the third pair of spiracles. The first pair, however, arises from a pair of branches tracheating the nervous system. These correspond in position and origin to the first ventral ganglionic tracheae of the pupa and larva and likewise form a mid-ventral anastomosis. The second and third pairs of ventral ganglionic tracheae similarly arise directly from the lateral trunks anteriorly to the origins of the respective second and third leg tracheae. The second ganglionic trachea is given off by the expanded portion of the lateral trunk, posteriorly to the first thoracic spiracle. The third anastomosis is distinctly sac-like, but the tracheae arising from it to pass to the nervous system are tracheate.

The wing and haltere tracheae also arise in positions corresponding to those in the pupa. They are narrow in diameter, except that the base of the wing trachea arises from a small air sac. Neither the wing nor haltere tracheae are described by Miller (1950). From Chapman's (1918) review of the wing tracheae in insects, two pairs of both wing and haltere tracheae might have been expected. In *Drosophila* they are very difficult to follow, but certainly neither in the living nor injected adult has a second trachea been found to penetrate either the wing or haltere.

**The First Larval Ecdysis**

The tracheal lining of the second instar larval system is secreted around that of the first, extending even into the finest tracheae and tracheoles. The result is that the larval lining of the first instar is completely replaced by that of the second. In addition, a number of new secondary 'branch' tracheae are formed. Quite a while before ecdysis occurs taenidia appear in the newly formed lining. Immediately before the moult the old lining breaks into its ten tracheal metameres, as demonstrated by Keilin (1944) for dipteran larvae in general. The breaks in the longitudinal trunks occur posteriorly to the origin of the respective transverse connectives and in the mid-line in the dorsal and ventral anastomoses and dorsal cervical anastomosis.

At ecdysis, the tracheal metameres, being attached to the cuticle at their respective spiracles, are withdrawn along with them. In this way the cervical tracheae, first dorsal anastomosis, and part of the longitudinal trunks together with their branches are removed with the first pair of spiracles; similarly, the second dorsal anastomosis, first transverse connective, and relative parts of the trunks and their tracheae are withdrawn with the second pair of spiracles, and so on.

After withdrawal of the old intima, the second instar system rapidly fills
with air and the second to ninth spiracular tracheae collapse, while the spiracular tracheae of the first and last pairs of spiracles remain open. The places through which the old system was removed close up and remain as ecdysial scars, which are inconspicuous even in the larger first and last pairs of spiracles.

THE SECOND LARVAL ECDYSIS

The details of this ecdysis are very similar to the events recorded for the first larval ecdysis. In the cases of the tracheae in which the moulting process has been followed in detail, moulting is complete; even the intima of the tracheoles is withdrawn and replaced by a new third instar tracheal lining. Besides the addition of new 'branch' tracheae the main difference lies in the formation of the more elaborate last pair of spiracles and of the finger-like anterior spiracles. The spiracular tracheae of spiracles two to nine again collapse to form solid cords.

THE LARVAL—PUPAL ECDYSIS

Prior to pupation, the fully grown third instar larva ceases to feed and extends its anterior and posterior spiracles; the larval cuticle gradually becomes hardened to form the characteristic puparium with its operculum demarcated anteriorly. Darkening occurs gradually and for some time the puparium remains sufficiently light in colour for the internal tracheal system to be observed through the puparium wall, at least in the dorsal region of the anterior end. Right from the beginning of puparium formation, changes occurring in the ventral and in the more posterior region are more difficult to see.

Through the puparium wall the pupal first thoracic spiracles can be seen developing median to the base of the larval first thoracic spiracular tracheae (figs. 4, 6). Later, the tracheal lining within the spiracles is secreted and is seen to be continuous with the new pupal lining which is secreted at the same time; this is laid down around the existing larval lining and not independently of it. The new pupal tracheal lining is secreted around the first five dorsal anastomoses, the first four transverse connectives, and the longitudinal trunks only as far as the region immediately posterior to the origin of the fourth pair of transverse connectives. At this point, larval and pupal tracheal linings are confluent. Posteriorly to this, no pupal lining—discernible under an oil immersion objective—is secreted by the tracheal epithelium. This striking state of affairs was re-confirmed many times and is substantiated by subsequent events.

Not all of the tracheae in the anterior region are surrounded by a newly secreted pupal tracheal lining; in this respect the process is essentially different from that occurring at the inter-larval ecdyses where the lining, even in the finest tracheae and tracheoles, appears to be replaced. Examples of tracheae in which the larval intima is retained in the pupal stage include the ganglionic tracheae arising from the ventral anastomoses (fig. 5, D, E), and the tracheae arising from the dorsal anastomoses (fig. 5, A). In contrast, at the inter-larval
ecyyses both the tracheae and their tracheoles are surrounded by the newly secreted intima and moulting is complete.

The processes occurring at this stage differ in another remarkable respect from those taking place at the previous ecdyses. Whereas at the inter-larval ecdyses the production of new 'branch' tracheae and tracheoles is confined to elements of small diameter and of relative unimportance, when the pupal tracheal system is formed a number of large tracheae, giving rise to numerous smaller ramifications, are laid down. However, they are essentially similar to
the smaller ‘branch’ tracheae of previous instars in that their lining appears simultaneously to, and is continuous with, that secreted around the neighbouring pre-existing framework of larval tracheae. The most striking of these exclusively pupal branch-tracheae are (a) tracheae arising from the two branches of the cervical tracheae, which will eventually tracheate the develop-

![Diagram](image)

Fig. 5. Drosophila: individual tracheae of the third instar larva shortly before pupation, showing the positions of the existing larval relative to the recently secreted pupal tracheal linings. A, the second dorsal anastomosis. B, the ventral cervical trachea. C, the dorsal cervical trachea. D, the second ventral anastomosis. E, the third ventral anastomosis.

ing head structures including the eyes (fig. 5, B, c), (b) the numerous fine tracheae arising from the second dorsal anastomosis which will subsequently tracheate the developing thoracic flight muscles (fig. 5, A), and (c) the two pairs of large tracheae arising from the third and fourth transverse connectives immediately after their origin from the dorsal longitudinal trunks (fig. 11, A); these ramifying tracheae will be responsible for the tracheation of the entire posterior abdominal region.

Before withdrawal of the larval tracheal linings, breaks occur in the larval tracheal system dividing it into its ten pairs of tracheal metameres. Withdrawal
of the larval linings starts first in the anterior region, extending subsequently to the abdominal metameres. Each pair of metameres is withdrawn through a pair of ecdysial openings in the pupal cuticle. In the case of the first pair of tracheal metameres (fig. 6) breaks occur mid-dorsally in the dorsal cervical anastomosis and first dorsal anastomosis, and in dorsal and lateral trunks in the region immediately posterior to the first dorsal anastomosis. Simultaneously with the severing and withdrawal of these larval tracheae associated with the first pair of spiracles, internal cell movements are set up, and the head becomes evaginated. The two pairs of posteriorly directed dorsal cervical tracheae and the cervical anastomosis are swept forward in the general stream and come to lie inside the everted head (fig. 8, B). The first dorsal anastomosis, initially superficial, comes to lie in a deeper position, almost completely obscured dorsally by pupal cells. Breaks in the cervical tracheae occur at the junction of larval and pupal linings so that the ultimate tracheae are larval, the main tracheae are pupal formations around pre-existing larval, while the much-divided branches are new pupal structures having no precursors in the preceding larval stage.

As the tracheae attached to the larval first spiracles are withdrawn, they become collapsed, whereas the spiracular trachea itself always remains air-filled and rigid. The 'hole' (fig. 6, B) in the tracheal lining—at the extremity of the spiracular trachea—simply represents, morphologically, the breaking point in the dorsal longitudinal trunk. The portion of the trunk posteriorly to this belongs to the second metamere; the latter, with its associated tracheae, is withdrawn along with the second pair of spiracles.

Withdrawal of the larva away from the puparium wall is followed by contractions of the abdomen and secretion of the thin 'pre-pupal' membrane. Simultaneously with these contractions a large air bubble (fig. 4) lying within the larval abdomen is released, and the air rapidly spreads between the pupal cuticle and the newly secreted 'membrane'. The more
posterior larval tracheal metameres are simultaneously withdrawn and come to lie on the inner surface of the membrane. The spiracular threads of the second to ninth spiracles remain attached to the non-functional spiracles in the wall of the puparium. The threads themselves traverse the space between the puparium and the thin membrane, pass through the latter, and open into the collapsed tracheae which lie on its inner surface.

If a pupa is removed from the puparium immediately after the withdrawal of the larval tracheae, air bubbles are to be seen attached to the abdominal ecdysial openings; this suggests that the bubble of air lying within the larva in some way becomes transferred to the outside of the pupa by way of these ecdysial openings. This may be supplementary to its possible escape more posteriorly during simultaneous removal of the larval proctodaeal lining. As noted by Robertson (1936) the bubble within the larva rapidly decreases in size at the time of, and subsequently to, the appearance and spreading of air between the pupal cuticle and pre-pupal membrane.

A pupa dissected immediately after withdrawal of the larval tracheae shows that the tracheal epithelium of the posterior abdominal region is intact and continuous with that of the more anterior region, but there is no corresponding tracheal lining. The breaking point in the dorsal trunks between the removed sixth pair of larval metameres and the new pupal lining of the fifth tracheal metameres can be clearly seen. The open end of the air-filled tube appears to contract either by its own elasticity or by that of the taenidia in its walls: there is no escape of air from the open end nor entry of fluid from the region.
Whitten—Development of Tracheal System in Drosophila

posterior to it. Dissected pupae of later stages reveal the presence of the more anterior tracheal epithelium only; by this stage the posterior parts of the larval tracheal epithelium have disappeared, and it must be inferred that it has been histolysed. Fig. 7 shows a portion of a trachea of the posterior abdominal region in Calliphora erythrocephala immediately after withdrawal of the larval tracheal lining. Here, seen under the highest magnification available, a distinct break occurs in the larval lining between the trachea and tracheole; the lining of the tracheoles is retained and presumably absorbed along with the cells, while the lining of the trachea is withdrawn, leaving behind only the epithelial cells. As the events taking place in Calliphora have, in other respects, been found to parallel so closely those in Drosophila, the same retention of tracheolar linings probably also occurs in Drosophila.

The larval metameres are removed through the usual number of ten pairs of ecdysial openings in the pupal cuticle. These subsequently close to form minute ecdysial scars. In the anterior region the first pair of spiracles remains functional but the spiracular tracheae of the second to fifth appear collapsed and the spiracles non-functional.

The pupal-adult ecdysis

The basic framework of the adult system is formed by secretion around the linings of the pupal system (figs. 8, 9, 10), whilst most of the larger air sacs of the head and thorax correspond to ‘secondary’ branch tracheae. In some cases this newly secreted adult intima is not significantly greater in diameter than the enclosed pupal intima. However, in the case of the air sacs the epithelium has become withdrawn from the pupal lining to a considerable extent, and the newly secreted adult lining is very much folded and of considerably greater diameter than the enveloped pupal intima (fig. 12). Whatever the peculiar properties of the epithelium which secretes the lining of the more sac-like parts, it is continuous with the lining, giving rise to the more tracheate adult elements. In fact, in the region between first and second pairs of spiracles the tubular longitudinal trunks of the pupa can be seen surrounded by the adult intima, which anteriorly and posteriorly is secreted in sac-like form separated by a distinctly tracheate region (fig. 10).

In many cases, as at the pupal ecdysis, the newly secreted adult intima does not extend into the smaller tracheae and tracheoles, and as a result the pupal tracheae are retained in the adult. This happens in the case of the ganglionic tracheae arising from the thoracic ventral anastomoses, and the wing and haltere tracheae (fig. 8). The air sacs arising from the region of the first and second pairs of ‘thoracic’ spiracles correspond to the ‘branches’ of previous stages (fig. 10). Their intima is secreted simultaneously with, and is continuous with, that of the adult intima secreted around the pre-existing pupal trunks. While still within the puparium they also have a broad, collapsed, much-folded appearance.

In the abdomen (figs. 3, 11) the adult intima is secreted in a tracheate form around the pupal tracheae and in the later stages taenia are clearly seen.
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Fig. 8. Drosophila: the anterior end of the tracheal system in ventral view A, the larva. B, the pupa. C, the late pupa showing the relationship of the pupal to the newly secreted adult linings. D, the adult. Semi-diagrammatic.

Fig. 9. Drosophila: the head of a late pupa in posterior view, showing the relative positions of the pupal and newly secreted adult linings (air sacs shown in expanded air-filled condition).
The tracheal epithelium surrounds the posterior 'broken' end of the pupal trunk, and at the inner posterior angle continues in a medio-posterior direction. It is here much narrower in diameter as also is the adult intima which it secretes: this is probably due to its not surrounding a pre-existing pupal trachea. The main tracheae forming this posterior adult system are the two longitudinal trunks, four dorsal anastomoses, three transverse connectives, and four pairs of spiracles. A tenth tracheal metamere does not appear to be formed.

Fig. 10. *Drosophila*: the thorax of a late pupa in left lateral view, showing the relative positions of pupal and newly secreted adult linings (air sacs shown in expanded air-filled condition).

At ecdysis, breaks occur in the pupal intima, dividing the system into the five pairs of tracheal metameres of which it is composed. The breaks occur in positions corresponding to those in the first five pairs of larval tracheal metameres, i.e. in the mid-dorsal and mid-ventral lines, and along the longitudinal trunks in the region posterior to each respective transverse connective.

Fig. 11, c is of the mid-abdominal region of a mature pupa of *Calliphora erythrocephala* (Cyclorrhapha, Calyptrae). It shows the relationship of the newly secreted adult tracheal lining to the existing functional pupal system. The relationships are almost identical with those of *Drosophila* (fig. 11, b). The only significant difference is that the lining around the dorsal trunk is sac-like in *Calliphora*. This is particularly interesting in that the presence of a pair of large air sacs in the abdomen is fairly general for Calyptrae, whereas they are generally absent in Acalyptrae.

Great changes appear to take place in the tracheal epithelium during the pupal stage. The present investigation has been mainly concerned with the
fate of the tracheal lining at the different ecdyses. Although this is closely linked with the fate of the tracheal epithelium itself, work remains to be done on the fate of the individual cells constituting the epithelial layer to determine the extent of reorganization, replacement, and histolysis which occurs.

Certain general principles have, however, already emerged. In the first place there is a definite continuity in the tracheal epithelium during the pupal stage. The adult tracheal system does not arise independently of the pupal.
FIG. 5

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but the adult tracheal lining is secreted around that of the pupal by the same tracheal epithelial layer—though possibly not by the same tracheal epithelial cells. Secondly, the majority of the adult air sacs are produced as new branches of the pre-existing system similar to the new branches formed at each of the preceding stages. Thirdly, the lining of certain pupal tracheae is not replaced by that of the adult, so that moulting, as in the case of the larval/pupal ecdysis, is incomplete. Lastly, the origin of the adult system posterior to the fifth pair of spiracles is completely different from that anterior to it. There are no tracheal metameres in this region in the pupal stage. The pupal trunks and their epithelium are seen to end blindly at the level of the fifth pair of spiracles. However, there are two remarkable features about the newly formed posterior adult system. First, the general pattern formed is identical with that of the preceding larval stage, except for the absence of a tenth tracheal metamere, and secondly, there is no distinction or break between the pattern in the functional anterior and posterior regions, and continuity in the tracheal trunks is maintained. However, from fig. 11, B it can be seen that the longitudinal axis of the dorsal trunk posteriorly is not continuous with the trunk more anteriorly, but comes off instead more medially and at an angle. It is also significantly narrower in diameter, a characteristic remarked on but not explained by Miller (1950). It is clearly due to the fact that posteriorly it is a new development, but anteriorly is the final product of successive secretions of new tracheal tubes outside the preceding tracheal tubes.

DISCUSSION

The nature and origin of the tracheae in the different instars

The form of the tracheal system at the various stages from first larval instar to adult is explained by the developmental processes involved: the peculiarities of each particular stage are due to the combined effect of the method and extent of secretion of the new tracheal linings, the method and extent of the separation and withdrawal of the old linings, together with the general fate of the tracheal epithelial cells.

In the case of the three larval instars, the same tracheal epithelium is responsible for the successive secretion of the tracheal linings at all three stages, and moulting is complete. Thus the larval tracheae are divisible into two categories only: (a) those constituting the basic framework, and (b) new 'branch' tracheae.

In the case of the pupal system, instead of moulting being complete at the previous ecdysis, the linings of certain of the larval tracheae and tracheoles are retained in the pupa. As a result, the tracheal parts are divisible into three categories: (a) the basic framework; (b) branch formations; and (c) those carried over from the third larval stage. It follows that the number and form of the shed larval tracheal linings, which lie within and are attached to the piparium at the functional and non-functional larval spiracles, can be interpreted from a study of the processes involved in their removal. For instance, a
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mechanical explanation is provided for the 'hole' in the tracheal linings of the first pair of spiracles. The physiological significance of these holes is discussed by Snodgrass (1924) for Rhagoletis, and by Robertson (1936) for Drosophila.

In the same way, the pupal-adult moult is incomplete and the same three categories of tracheae exist in the adult as in the pupa: (a) those forming the basic pattern, (b) branch tracheae, and (c) tracheae carried over from the preceding pupal, and in some cases larval stages. However, unlike the pupal tracheae, the form of (a) and (b) may be either 'tracheate' or expanded and sac-like. The tracheae of the posterior abdominal region fall into a fourth category. These are tracheae of which the epithelium appears for the first time during the pupal stage, replacing, functionally, the larval epithelium which was histolyed earlier in the pupal stage. This adult tracheal epithelium and the linings are continuous with those of the tracheal metameres of the more anterior region; they may even be classified as an exaggerated example of branch tracheae (category (b)).

The fate of the tracheal system during development

Since Robertson's (1936) results on the development of this system are quoted by Bodenstein (1950) in the most recent and comprehensive work on all aspects of Drosophila morphology, anatomy, and development, it is important to compare the results given by these workers with those obtained in the present investigation. The following tabulation of events taking place in the tracheal system at 25°C—arrived at from the study of serial sections of accurately timed stages—is selectively quoted from Bodenstein after Robertson. The numbers are inserted by the present author for subsequent comparison.

1. At 97 hours. The imaginal lateral spiracles, originating as outgrowths from imaginal cells on the great lateral trunks have connected with the hypoderm.

2. At 98 hours. The imaginal spiracles form a tube filled with newly secreted chitin.

3. At 99 hours. The larva contracts away from the anterior end of the puparium and partly withdraws the anterior tracheal linings.

4. At 100 hours. The pre-pupal moult occurs; a lumen appears in the imaginal abdominal tracheal tubes, which connects the tracheal tracts with a pore in the pupal membrane. The stigmata are probably not functional.

5. At 108 hours. Pupation occurs. The anterior and posterior tracheal linings are shed. The newly formed tracheal tube linings of the pre-pupal stage are also withdrawn (a). The newly formed system in the head is everted (b). The newly formed imaginal pro-thoracic spiracles connect with the lateral larval trunks (c).

6. At 110 hours. The abdominal tracheae are still larval.

7. At 114 hours. The pupal intima of the abdominal longitudinal trunks is broken off near the posterior end.

8. At 120–156 hours. The posterior tracheal trunks are histolyed. The
chitinous intima shrinks into the anterior tracheal trunks which form the large thoracic air sacs.

9. At 158 hours. The closing device of the imaginal stigmata is established.
10. At 192 hours. Emergence occurs. The chitinous intima is shed through the pro-thoracic spiracles.

Although the results of the present work indicate the same time sequence, the interpretations of the fate of the tracheal system are quite different from those outlined above. The above interpretation of the facts available is consistent with the fragmentary evidence and with the generally accepted theory that the imaginal tracheal system is an entirely new formation directly replacing the larval, part of which is then histolysed and part withdrawn with the cuticle. The results of the present investigation would suggest the following drastic modification of the interpretations of the sequence of events given in Bodenstein (1950).

1. 97 hours. The so-called ‘imaginal spiracles’ are in fact the pupal spiracles (see p. 134); they develop mesad to the larval spiracles and communicate with the future pupal tracheal system.
2. 98 hours. The pupal spiracles do indeed form a tube which is the pupal intima enclosing the fluid-filled lumen. It bears a similar relationship to the larval spiracle as did the third instar spiracle to the second, that is, after ecdysis the opening in the pupal cuticle through which the attached trachea is removed closes and an ecdysial scar remains. Although not conspicuous in Drosophila, this can be seen clearly in Calliphora.
3. 99 hours. The tracheal linings are not partly withdrawn at this stage, if ‘withdrawn’ suggests a partial break in the system and actual removal of the tracheae. The pro-thoracic spiracular tracheae are indeed stretched almost to what would appear to be their maximum degree of taughtness, but origins of the first dorsal anastomosis and ventral and dorsal cervical tracheae and lateral and dorsal trunks are still clearly seen. The dorsal and lateral trunks are partly outside the pupal cuticle and have been drawn through the pupal ecdysial tube, but no break has yet occurred in the larval system of this region.
4. 100 hours. The statement concerning a ‘pore’ is clearly confused, as also is the statement that the spiracles are probably not functional, since the term ‘functional’ here conveys the meaning that they play no part in the elimination of the larval system. In fact, the larval system breaks up into ten pairs of tracheal metameres which are withdrawn through the ten pairs of ecdysial openings associated with ten pairs of pupal spiracles; the cast tracheal linings can be seen lying closely apposed to the inner surface of the so-called pre-pupal cuticle.
5. 108 hours. (a) It is difficult to interpret quite what is involved in the statement concerning the shedding of the anterior and posterior tracheal linings together with the newly formed tracheal tube linings of the pre-pupal stage. The latter are referred to previously to this—in 4—as the imaginal abdominal tracheal tubes. (b) The newly formed tracheal system refers to the newly developed adult system of the head. In actual fact, they are the functional
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head tracheae of the pupal stage. (c) It is not the imaginal pro-thoracic but the pupal thoracic spiracles which connect with the lateral and dorsal pupal trunks, and not with the lateral larval trunks.

6. *310 hours.* The intima of the larval abdominal trunks have already been removed by way of the abdominal spiracles, but the tracheal epithelium remains and is subsequently histolysed.

7, 8, and 9. Even without contradictory evidence it would be very peculiar if the intima of the abdominal region really shrank into the anterior tracheal trunks. It is even more unlikely that this intima should subsequently be shed through the first thoracic spiracles. The first thoracic spiracles of which stage is not stated; previously to this, only adult spiracles have been mentioned. Robertson (1936) also states that the pupal spiracles appear to be retained in the adult.

Post-embryonic development of the system in other Diptera

The discrepancies noted above are of particular importance because it appears that this sequence of events for *Drosophila*—a member of the Diptera Cyclorrhapha, Acalypterae—is true also for *Calliphora*—a member of the Diptera Cyclorrhapha, Calypterae. The sequence in both differs considerably from that seen in the 'lower' Diptera Nematocera, as illustrated by *Sciara coprophila* (Keister, 1948). In the latter case, the same tracheal epithelium appears to persist through to the adult stage, secreting the successive tracheal linings of the four larval, the pupal, and the adult instars. The presence, in the Diptera Cyclorrhapha, of separate fates for the tracheal epithelium in the anterior and posterior regions marks a distinct step in the evolutionary development within the Diptera.

Moulting of the tracheal and tracheolar linings

The sequence of events taking place in the anterior tracheal metameres of *Drosophila* is essentially similar to that occurring throughout the body of *Sciara* (Keister, 1948). In both of these Diptera, moulting is complete at the inter-larval ecdyses and incomplete at the larval-pupal and pupal-adult ecdyses. These Diptera differ from *Rhodnius* (Hemiptera) (Wigglesworth, 1954) in which the tracheal linings of the tracheoles are never shed; here, at all ecdyses, the new lining of the trachea is continuous with the old lining of the tracheole and only the old lining of the trachea is shed at ecdysis. Wigglesworth (1954) suggests that a possible distinction between tracheoles and tracheae may be that the intima of the former is never shed, whereas the tracheal linings of the latter are, and that in *Sciara* true tracheoles appear only at the third larval instar. However, if this were the case, such relatively large tracheae in *Drosophila* as those arising from the main cervical tracheae in the pupal head, or the adult wing and haltere tracheae, or the ventral ganglionic tracheae of the pupa and adult would all come under this definition of tracheoles, since they are not shed at the appropriate ecdysis.
The fate of the tracheal epithelium during development

In *Drosophila* and also in *Calliphora*, the larval tracheal epithelium closely surrounds the tracheal lining and is composed of large nuclei occurring at scattered intervals in the enclosing strand of cytoplasm. The cell outlines are not distinct and the epithelium resembles the tracheal epithelium of *Sciara* (Keister, 1948) in appearing to be a syncytium containing large scattered nuclei. The spiral thickenings of the new intima become evident some time before ecdysis of the old lining.

The fate of the individual tracheal epithelial cells is not finally settled. All the evidence presented here suggests that in the head, thorax, and anterior abdominal region the same tracheal epithelium persists into the adult stage. However, in the pupal stage changes certainly seem to take place within the epithelium itself. For instance, at this stage the nuclei of the epithelium are more frequent and smaller than in the larval stages. Also, the surface area of the epithelium is greatly increased—particularly in areas which will secrete the sac-like folded linings of the future adult air sacs—and the epithelium has to separate quite a way from the pupal tracheal lining before secreting this adult lining.

The origin of secondary 'branch' tracheae

It remains to be determined whether the secondary tracheae of earlier instars arise in a way similar to that shown for *Rhodnius prolixus* (Wigglesworth, 1954). In the latter they arise by division of cells of the existing epithelium, followed by migration of these cells outwards as branches; the cells subsequently secrete the tracheal lining of the new 'branch' tracheae. It seems likely from a study of the simultaneous and continuous secretion of the new tracheal lining in the 'branch' tracheae and main tracheae that a similar process operates in *Drosophila*. Also the same process appears to occur in the formation of branch tracheae of the adult stage regardless of whether they are tracheate or sac-like, since both types are frequently secreted by adjacent regions of the tracheal epithelium.

Finally, whereas the larval tracheal epithelium in the posterior abdominal region is undoubtedly histolysed, the method of origin and reorganization of the replacing adult tracheal epithelium remains to be determined. Three possibilities present themselves: first, that these last four tracheal metameres are regenerated from 'imaginal clusters'—if this is so little of the classical theory (Peréz, 1910) of the origin of the adult system remains; secondly, that there is a backward migration of tracheal epithelial cells from the tracheal epithelium surrounding the blind ends of the pupal dorsal and lateral longitudinal trunks; thirdly, that the adult system of this region arises by a combination of these two processes.

The tracheal system and the pre-pupal stage

The present work on the tracheal system cannot ignore the question of the 'pre-pupal' instar. It has shown that there is certainly no functional pre-pupal
tracheal system interposed between the third instar larva and the pupa. However, although no positive evidence has emerged on the nature of the secreted membrane, a certain amount of negative evidence throws doubt on its reputed identity as the cuticle of a distinct 'pre-pupal' instar (Snodgrass, 1924), corresponding to the fourth larval instar of Diptera Nematocera.

Invariably, when the operculum of the puparium is removed, the object within the anterior end of the puparium is either the headless pupa or the evaginated pupal head. In the former case removal of the operculum often brings about artificial eversion of the head, accompanied by partial or complete removal of the larval spiracular tracheae, through the ecdysial openings in the new pupal cuticle. There is no suggestion—under microscopic examination—of a membrane corresponding to the so-called pre-pupal cuticle surrounding the pupal head; at no time is there any separate cuticle attached to the operculum; if present this is very closely apposed to the inner layer of the puparium. On the other hand, the membrane lies loose in the abdominal region and can, with care, be isolated. Likewise, dissection of the corresponding stage in Calliphora has never revealed a separable cuticle in the head region, whereas, contrary to Fraenkel's (1938) observation, the pre-pupal cuticle is evident as a separate membrane as far as the level of the pupal first thoracic spiracles. Removal of the operculum reveals the membrane with a 'torn' edge which suggests that it has been torn from the innermost layer of the operculum, with which it appears to be continuous. When pupae of subsequent stages are removed from the puparium, the 'pre-pupal' cuticle, with the larval tracheae lying on its inner surface, is frequently found lying apposed to the pupal cuticle. In such cases the spiracular threads or the posterior spiracular tracheae have been severed from their connexion with the spiracles on the puparial wall during removal from the puparium.

Simultaneously with the withdrawal of the tracheae of the first pair of tracheal metameres and eversion of the pupal head, the larval mouth armature and stomodaeum are ejected. Similarly, the larval proctodaeum is removed along with the posterior tracheae of the last pair of tracheal metameres. According to Robertson (1936) the 'pre-pupal' cuticle extends into the stomodaeum and proctodaeum and also into the spiracles. However, the whole conception of this membrane as a distinct pre-pupal cuticle is difficult to accept after consideration of the development of the tracheal system, since the larval tracheal system is replaced directly by the pupal system which is secreted around it—except in the posterior abdominal region where no pupal system is found. Also retention of the larval tracheae, larval mouth armature, and larval stomodaeum and proctodaeum until the pre-pupal moult, appears to be anomalous, although it may be a mechanical necessity.

There is no definite evidence from any of the Cyclorrhapha of the chemical constitution of this membrane or pre-pupal cuticle (Wolfe, 1954, a, b, 1955). Clearly the problem posed is whether the membrane is epi-cuticular or endocuticular in nature. If epi-cuticular it may be defined as a distinct cuticle and the anomaly with respect to the tracheal system remains. If, however,
the cuticle proves to be endo-cuticular, its relation to the tracheal system and to the larval stomodaeum and proctodaeum would be more easily understood. Possibly, owing to the peculiar pupation within the last larval skin, it is mechanically essential for the larval epithelium to separate from the larval cuticle, particularly posteriorly. If the epithelium is still secreting endo-cuticle, as it has been doing continuously throughout the third larval instar (Wolfe, 1955), the separate membrane or 'pre-pupal' cuticle would result. Since the epithelium of the spiracles, stomodaeum, and proctodaeum is continuous with the general hypodermis, it would not be surprising if withdrawal of the epithelium, accompanied by continued secretion of the endo-cuticle, resulted in this separable membrane extending into these invaginations also.

There is an additional reason for questioning the true identity of this 'pre-pupal' cuticle. Snodgrass (1924), Fraenkel (1938), and Robertson (1936) liken the pre-pupal instar to the suppressed fourth larval instar of the Diptera Nematocera. Yet it has been shown here that, in Drosophila, moulting of the tracheal system is incomplete at the third larval-pupal moult, although complete at the two inter-larval ecdyses. In Sciara (Nematocera) (Keister, 1948) moulting is also incomplete at the last larval-pupal ecdysis, but complete at the three preceding inter-larval ecdyses. In both, the tracheal system of the larval instar prior to the pupal instar shows incomplete moulting. If a suppressed fourth instar—or 'pre-pupa'—intervenes in the Cyclorrhapha, the tracheal system of this instar has disappeared. Also, the distinctive characteristics of the fourth larval-pupal ecdysis in the Nematocera have been taken over by the third larval-pupal ecdysis in the Cyclorrhapha. (Subsequent work on the pre-pupal cuticle forms the basis of a further publication.)

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Fig. 1

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