Weismann's ring and the control of tyrosinase activity in the larva of *Calliphora erythrocephala*

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The blood and tissues of third instar *Calliphora* larvae contain a glucose dehydrogenase system which shows maximum activity at the crop-full stage and declines as pupation is approached. This decline is prevented by destruction of Weismann's ring at 3 days. It is suggested that dehydrogenase activity is the cause of the unstable reducing power of the blood and consequent inactivation of tyrosinase. The liberation of the 'pupation hormone' coincides with the complete and abrupt termination of dehydrogenase activity which leads to tyrosinase activity and hardening of the larval cuticle to form the puparium. Weismann's ring is therefore not merely active immediately before puparium formation, but exercises a regulatory influence on developmental processes during the life of the larva. Although no direct evidence is available of the existence in blowfly larvae of a juvenile hormone such as that found in *Rhodnius* by Wigglesworth, the results obtained seem consistent with the presence and later elimination of such a hormone.

1. **Introduction**

In a previous paper (Dennell 1947) an account was given of the formation of the puparium in *Sarcophaga falculata*. The dihydroxyphenol responsible for hardening the cuticle of the third larval instar to form the puparium arises from the oxidation of tyrosine in the blood. As pupation is approached the blood tyrosine increases in amount, and concurrently the enzyme tyrosinase makes its appearance and increases in abundance during the latter part of larval life. In spite of the presence in the blood of both enzyme and substrate, however, no oxidation of tyrosine takes place until the pupation hormone is liberated. Then the pupal contraction occurs and the cuticle becomes hard and dark. The existence in the blood of other insects of some factor inhibiting tyrosinase activity had been noted by previous workers, notably Graubard (1933), and was fully evident in *Sarcophaga*, where it was observed.
that the increase in abundance of tyrosine and tyrosinase is accompanied by a progressive fall in the oxidation-reduction potential of the blood. It was therefore suggested (Dennell 1947) that the inhibition of tyrosinase activity before pupation is due to a fall in potential below the level at which the oxidation of tyrosine is possible. Various treatments result in the release of tyrosinase activity before the normal time of pupation, the release being accompanied by a rise in potential. The possible causes of the low potential of the blood were discussed, and since the treatments resulting in rise of potential and release of tyrosinase activity bear a strong resemblance to those which in general inhibit the activity of dehydrogenases, it was speculated that it might be due to the presence in the blood of an enzyme system of this type.

This view is confirmed by the evidence presented in this paper. Not only is a dehydrogenase system truly present, but its activity is controlled by Weismann's ring which has been shown by a number of workers to exert a profound influence on the course of pupation and metamorphosis.

The work has been carried out on the third instar larva of *Calliphora erythrocephala* since considerable relevant information, which will be discussed in the text, was already available for this species and for *C. vomitoria*. The culture methods adopted were similar to those used for *Sarcophaga* (Dennell 1947). All ages quoted in the following account are those of larvae reared at 25° C.

2. THE DEMONSTRATION AND CHARACTERIZATION OF DEHYDROGENASE IN THE LARVA

Although the reduction of methylene blue and other dyes by the blood of *Sarcophaga* larvae under experimental conditions (Dennell 1947) may be due to their utilization as hydrogen acceptors by an enzyme of the dehydrogenase type, it is clear that in the intact larva some other acceptor must be utilized if an enzyme of this type is truly present. It was therefore desirable at the outset to study the oxygen uptake of the larval blood and tissues under various conditions. Using the Barcroft differential respirometer this has given very significant results.

It may be stated at once that the chopped tissues of larvae which have ceased to feed show a significant oxygen uptake in the presence of cyanide. Ten larvae were washed in Ringer solution, chopped and suspended in the respirometer flask in 5 ml. Ringer solution to which KCN solution had been added to give a final concentration of 0.1 M. The thermostat was maintained at 20° C. Under these conditions larvae of different ages showed oxygen uptakes varying from 6 to 36 cu. mm. in 30 min., being greatest in larvae 4 days old. That this uptake is due to enzymic oxidation carried out by the tissues is shown by the fact that it is completely inhibited by boiling the larvae for a few minutes. Since dehydrogenases are typically not inactivated by cyanide the observed oxygen uptakes may be ascribed to the activity of an enzyme of this type, and since oxygen is directly utilized as the hydrogen acceptor we may refer to the enzyme in question as an aerobic dehydrogenase.

Further evidence that the oxygen uptake of the chopped larvae is due to a dehydrogenase system is given by treating the tissues, prepared and suspended
as before, with methylene blue and phenylurethane. The addition of methylene blue in a final concentration of 0.01% results in an initial acceleration of uptake, but this increase is not maintained, an inhibiting effect being clearly shown after 30 min. (figure 1c). The addition of phenylurethane to saturate the suspension medium causes marked depression of the uptake, corresponding to approximately 60% after 1 hr. (figure 1d). In view of the inhibiting effects of methylene blue and narcotics on dehydrogenases generally these observations constitute confirmation of the opinion that the recorded uptakes are indeed due to the activity of such an enzyme.

In searching for the possible substrate of the enzyme the general occurrence of glucose in insect blood was borne in mind. Frew (1929) demonstrated its presence and fluctuation in amount in the blood of Calliphora larvae, and it seemed natural to look to it, in the first instance, as possibly constituting the naturally occurring substrate. The result, however, of adding glucose to the simple tissue preparations was disappointing. On adding glucose to a final concentration of 1% no increased uptake is to be observed, nor is the uptake maintained for a longer period than without added glucose. Indeed, the observations point rather to a depression of the activity of the preparation (figure 1b). In view of the work of Harrison (1931), who found that a glucose dehydrogenase preparation from mammalian liver showed a marked optimum in its activity towards glucose in varying concentrations, the possibility that glucose formed the substrate sought was not immediately rejected. It seemed possible that, since the preparation consisted not only of the tissues but also of the haemolymph with its contained glucose, addition of further glucose might result in a total concentration greater than the optimum and so reduce the
oxygen uptake. It was therefore decided to attempt the preparation of a dehydrogenase extract freed from glucose by washing. Fifty 4-day larvae were washed clean, chopped, and ground with sand in cold distilled water. After several washings the ground tissues were suspended in \( \frac{m}{15} \text{K}_2\text{HPO}_4 \) and shaken gently for some time. The tissue residue was removed by centrifuging, and 2.5 ml. of the extract were added to 2.5 ml. phosphate solution in the right-hand flask of the Barcroft apparatus. KCN was added to give a final concentration of 0.1 M. The low oxygen uptake of a tissue extract prepared in this manner and shaken for 45 min. is shown in figure 2a. On the addition of glucose to a concentration of 1% to a further portion of the same extract the uptake was greatly increased (figure 2b). It appeared probable that the slight uptake shown at a might be ascribed to the utilization of residual glucose in the ground tissues, and to test this preparations were made by shaking in phosphate for longer and shorter periods. The uptake shown by an extract shaken for 25 min. is seen from figure 2c to be considerably higher than that of the extract shaken for 45 min. Conversely the prolongation of the period of shaking to 1 hr. resulted in complete elimination of uptake. The effect of addition of glucose to the extract shaken for 25 min. is shown in figure 2d. From these experiments it may reasonably be concluded that not only is glucose the substrate oxidized, but that the dehydrogenase involved in its oxidation is located in the tissues and not primarily in the haemolymph which is not included in the extract.

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Further evidence of the occurrence of the enzyme in the tissues and only to a slight extent in the blood is given by experiments in which the oxygen uptake of chopped and washed larvae is compared with that of larvae which are chopped but not washed, so that the blood is included in the preparation. The latter preparations

![Figure 2](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/)

***Figure 2.*** The cyanide insensitive respiration of extracts of 50 4-day larvae. Preparations a and b were extracted for 45 minutes, and c and d for 25 minutes. Preparations a and c without glucose. Glucose to a final concentration of 1% was added to preparations b and d.
consistently show a slightly higher uptake than that of the washed larvae. Additionally larvae were cut under Ringer solution and gently squeezed so that the blood was expelled. The larvae were then removed, and the blood showed only a slight uptake in the presence of cyanide. The significance of these and the preceding observations will be discussed in reviewing the work of Graubard (1933).

In view of the observations of Levenbook (1947) on fructose and the reducing value of insect blood it was of interest to repeat the observations made on the oxygen uptake of phosphate extracts, adding fructose instead of glucose. In no experiment was the addition of fructose observed to modify the residual uptake of the extract, so that the possibility that fructose may serve as substrate for the dehydrogenase studied may be excluded. This, indeed, is to be expected from the differing molecular configuration of glucose and fructose. Levenbook noted that no great amount of fructose is present in the blood of the late third instar larva of Calliphora erythrocephala, although it is abundant in the larva of Gastrophilus intestinalis.

No attempt has been made in this work to identify the oxidation product of glucose, but it is noteworthy that in experiments on phosphate extracts of larval tissues to which cyanide was added similar oxygen uptakes were recorded with and without KOH papers in the flasks of the Barcroft manometer. The course of oxidation does not therefore involve the production of CO₂. Harrison (1931) found in his study of the glucose dehydrogenase from mammalian liver that the oxidation product was gluconic acid, but his dehydrogenase showed different characteristics from that discussed here, and it does not necessarily follow that this is true of Calliphora.

The interpretation to be placed on these experiments is then that an aerobic glucose dehydrogenase occurs in the larva of Calliphora and is located principally in the tissues. Hewitt (1937) has shown that well-marked reducing conditions may be established in bacterial cultures, even when aerated, apparently as the result of dehydrogenase activity. The presence in blowfly larvae of an aerobic dehydrogenase may well therefore account for the falling oxidation-reduction potential of the blood and consequent inactivation of tyrosinase until the time of puparium formation. If this explanation is valid it is probable that fluctuations in dehydrogenase activity, related to the appearance of tyrosinase in the blood and the liberation of the pupation hormone, are to be demonstrated in growing larvae. It will be shown in the remainder of this paper that such changes not only do occur but bear an intimate relation to the activity of Weismann’s ring, the gland responsible for the secretion of the pupation hormone.

3. Changes in dehydrogenase activity during growth of the larva

As previously stated, it was found that the oxygen uptake in the presence of cyanide was found to be greatest with larvae 4 days old, an observation amply confirmed in a study of the cyanide insensitive respiration of growing larvae up to the time of pupation.

Ten larvae were taken daily from the same batch and chopped in Ringer solution as previously described. Cyanide was added and the oxygen uptake of the
preparation observed. These experiments were repeated on numerous batches of larvae, and the results of a considerable number of determinations are expressed in figure 3b. It will be seen that dehydrogenase activity is first apparent at about 2 days, and rises to a maximum at 4 days. Thereafter a steady decline takes place up to the time of pupation. The maximum at 4 days, although occurring when the crop is full, cannot be due to bacterial activity in the crop contents, for it is equally apparent when observations are made on larvae from which the crop is removed, or which have been well washed after chopping. During the early part of larval life, up to about 3½ days, the larvae are still growing, and it may be objected that this alone is sufficient to account for the increase in dehydrogenase activity during this period. But on the other hand, it is clear that the fall in oxygen uptake noted after 4 days must represent a real decline in dehydrogenase activity since no change in size of the larva takes place.

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To meet this objection, daily observations were made not only of the cyanide insensitive respiration but also of the total respiration of the chopped larvae. Parallel with the rise in dehydrogenase activity the total respiration reaches a maximum at 4 days and declines towards pupation (figure 3a). The rise in total respiration between 2 and 4 days is accounted for by the increase in size of the larvae, and the fall after 4 days is presumably related to the more sluggish habits of the larvae after the cessation of feeding. It is now possible however to express dehydrogenase activity as percentage of the total respiration and so eliminate the effect of growth and variation in size of larvae from different batches. When this is done it is seen (figure 4) that dehydrogenase activity rises to approximately 30% of the total respiration at 4 days, and falls to approximately 12% not long before pupation. A true increase in dehydrogenase activity, followed by a marked decline, therefore takes place.
The figures given by Frew (1929) for the fluctuation in the glucose content of the larval blood are of immediate interest here, and are indicated by the broken curve in figure 4. At 4 days, when dehydrogenase activity and therefore glucose oxidation reach a maximum, Frew found the glucose content of the blood to be at a minimum. It is perhaps unfortunate that Frew does not expressly state the temperature at which his larvae were reared, but it is implied (p. 215) that it was 21° C and not 25° C as employed in the present work. At first sight this discrepancy appears to detract somewhat from the close correspondence between the figures for glucose content and dehydrogenase activity, but it may be pointed out that in the present work observations were made at precisely daily intervals only, as those of Frew appear to have been. The maxima and minima referred to do not therefore necessarily occur precisely at 4 days, and it seems not unlikely that if observations were made at more frequent intervals of both glucose content and dehydrogenase activity in larvae reared at the same temperature close correspondence would be demonstrated. Frew remarks that 'It is probable that changes in glucose concentration may have some connection with pupation, though the connection may not be one of cause and effect'.

That the changes in dehydrogenase activity and glucose content of the larval blood are indeed intimately related to the formation of the puparium is indicated by observations on critical stage larvae in which the pupation hormone (Fraenkel 1935) has been liberated, and on white pupae. In these stages tyrosinase is active (Dennell 1947), and no dehydrogenase activity is to be expected if this is indeed the cause of tyrosinase inactivation. It is therefore important that no cyanide insensitive
respiration has been detected in larvae which have entered the critical period (as indicated by ligaturing others of the same batch, see Fraenkel 1935), nor in slightly older larvae which showed some contraction of the posterior three segments which are dragged in movement, a sign that pupation is imminent. Similarly, no uptake was observed in white pupae, but in the light brown pupae, and more markedly in older pupae, measurable uptakes were consistently recorded (figure 4). It is significant that this rise in dehydrogenase activity in the early stages of pupation is matched by a fall in the glucose content of the blood (figure 4). In contrast to these results, larvae a little before the critical period show a cyanide insensitive respiration amounting to about 12% of the total respiration. On no occasion has an intermediate figure been recorded for larvae between the critical period and the pupal contraction.

It may therefore be postulated that when the pupation hormone is liberated at the critical period one result is the inhibition of dehydrogenase activity (figure 4). At the same time the electrode potential of the blood rises abruptly (Dennell 1947), so permitting the oxidation of tyrosine with the formation of the polyphenol responsible for the hardening of the puparium. Strong evidence of the part played by Weismann’s ring in controlling the enzyme systems involved in the formation of the puparium will be given in the next sections of this paper.

4. WEISMANN’S RING AND THE CONTROL OF DEHYDR OGENASE ACTIVITY

Previous authors have shown clearly that Weismann’s ring is intimately concerned in the hormonal control of pupation in ecylorrhaphous larvae. The existence of a hormone responsible for the onset of pupation in Calliphora erythrocephala was demonstrated by Fraenkel (1935), and Burtt (1937) suggested that Weismann’s ring, a gland dorsal to the central nerve ganglia, is the source of this hormone. Burtt expressed the view that the ring constituted the homologue of the corpora allata suspected to be responsible for hormone production in other insects. Later (1938) he demonstrated decisively by experimental methods that Weismann’s ring is intimately concerned with pupation in C. vomitoria. Non-feeding larvae 2 to 3 days before pupation from which the ring was removed, or in which it was destroyed by cauterizing, failed to pupate although they survived for a considerable time. But Burtt did more than demonstrate the part played by the ring in older larvae. Turning his attention to younger larvae which had not ceased to feed, that is, aged about 3 days at 25°C, he showed that the effect of removal or destruction of the ring was not only to inhibit pupation but to cause a great extension of the feeding period. After operation the larvae continued to feed, in one instance for as long as 39 days, and died without emptying the crop preparatory to pupating.

Burtt’s experiments point emphatically to the activity of Weismann’s ring at two distinct periods in the life of the third instar larva, and cannot be neglected here. In the present work it has been shown that at corresponding periods marked physiological changes occur in relation to the formation of the puparium, and it was therefore of critical interest to repeat the operations carried out by Burtt and to determine their effect on the dehydrogenase activity of the larvae.
Since it was considered inadvisable for the present purpose to open the larvae they were cauterized as described by Burtt (1938) except that they were not anaesthetized before the operation. The effects of the operation on the behaviour of the larvae were precisely as described by Burtt, but further than this, study of the oxygen uptake of preparations of the operated larvae in the presence and absence of cyanide revealed profound modifications of the fluctuations in dehydrogenase activity shown by normal larvae. Initially the respiratory characteristics of unoperated larvae were observed, and a considerable number of larvae were then cauterized for later observation. Larvae cauterized 24 hours before pupation (figure 5′c), that is, before the critical period at which the pupation hormone is liberated, show no sudden inhibition of dehydrogenase activity such as occurs in normal larvae at the critical period (figure 4). In the operated larvae dehydrogenase activity continues unchecked for some time after the normal period of pupation. A somewhat similar result is given when Weismann's ring is cauterized in larvae 3½ days old (figure 5′a). Dehydrogenase activity once more remains approximately constant, continuing at the level characteristic of larvae of this age until well after the normal time of pupation. Larvae cauterized at 4½ days show a different result (figure 5′b). Dehydrogenase activity falls with increasing age of the larvae which, unlike those cauterized at 3½ days, cease to feed, but again does not show inhibition at the critical period. Different results are therefore produced by cauterization immediately before and immediately after the period when dehydrogenase activity of the normal larva reaches its height.

![Figure 5](http://rspb.royalsocietypublishing.org/)

**Figure 5.** The effects on respiration of cauterizing Weismann's ring at different times. a, b and c, total respiration; a′, b′, and c′, cyanide insensitive respiration. The time of pupation of normal larvae is indicated at 7½ days.

The effect of cauterization on total respiration is illustrated in figure 5a, b and c. A progressive fall takes place similar to that occurring in normal larvae. As a result the dehydrogenase activity of larvae cauterized at 3½ days, expressed as percentage of total respiration, shows a marked increase from 28 to 66% in the experiment.
illustrated. This is in marked contrast to the fall occurring in normal larvae (figure 4), but it should be pointed out that the operated larvae are often more sluggish than the normal so that the fall in total respiration is probably exaggerated.

The simplest explanation of these results would be that not only is Weismann's ring active at two distinct periods in larval life, as Burtt (1938) found, but at these periods it exercises control over the glucose dehydrogenase system described. Observations on normal and cauterized larvae indicate that this control is not exercised in the same way at the critical periods of 4 and 7 days. It seems that the hormone which may be presumed to be liberated at 4 days checks the production of further dehydrogenase by the tissues, with the result that a progressive decline in activity of this enzyme follows. At the critical period of 7 days the pupation hormone completely inhibits residual dehydrogenase activity, so setting in train the events leading to the hardening of the larval cuticle as the puparium. A less simple explanation, however, arises from the work of Wigglesworth (1934, 1936, 1940, 1947, 1948) and will be discussed later.

5. WEISMANN'S RING AND THE CONTROL OF TYROSINASE PRODUCTION

It has been shown (Dennell 1947) that in Sarcophaga the blood of larvae from the crop-full stage onwards darkens on exposure to the air for some minutes, indicating the presence of both tyrosine and tyrosinase. The delay in darkening seems to be due to the progressive disappearance of the inhibiting conditions which prevent darkening of the blood in intact larvae. Further, and separately from this observation, tyrosine and the oenocytoid-like cells suspected to secrete tyrosinase were shown to increase in the blood from the crop-full stage onwards. Since in the present work Weismann's ring has been shown to control dehydrogenase activity and therefore tyrosinase activity, it was necessary to discover whether it exerts a direct effect on tyrosinase production.

Experiments have therefore been carried out on the darkening of exposed blood of Calliphora larvae cauterized before and after the crop-full stage. The blood of larvae cauterized at 3 days is found to darken on exposure on filter paper more strongly than that of unoperated larvae when examined at 6½ days, that is, before the time of the critical period. No doubt is left of the greater potency of the tyrosinase system in the operated larvae, in spite of the greater dehydrogenase activity already referred to. A similar though less marked difference between the blood of operated and control larvae is obtained by cauterizing at 4½ days, and a still smaller difference when older larvae are cauterized. It is to be noted that the darkening of the blood of the operated larvae, although more intense than that of control larvae, takes place more slowly, as might be expected from the fact that operation at about 3 days results in greater dehydrogenase activity. This darkening of the blood of operated larvae is inhibited by cyanide, as is that of control larvae when examined before the critical period. It is therefore not due to the non-enzymic oxidation of a phenol already present in the blood.

These results indicate that while activity of Weismann's ring at 4 days does not appear to initiate tyrosinase production, the ring exerts a controlling
influence from this time onwards, and may be regarded as maintaining the balance between potential tyrosinase activity and the inhibiting dehydrogenase system.

6. Discussion

The view that tyrosinase activity in the blood of the larval blowfly is held in check by the low oxidation-reduction potential produced by the activity of a dehydrogenase system finds support from a number of sources. The occurrence of such a system is entirely adequate to explain the low potential of the blood found before pupation, and it is to be particularly emphasized that the disappearance of dehydrogenase activity shortly before pupation, at the critical period when the pupation hormone is liberated, is accompanied by a rapid rise in potential (Dennell 1947). The reduction of dyes by the blood may be ascribed to the action of a dehydrogenase, and the darkening of exposed blood only after an interval to the fact that not until the reducing power of the blood is eliminated, either as a result of destruction of dehydrogenase or disappearance of its substrate by oxidation, is oxidation of tyrosine possible. The effect of dilution of the blood in accelerating darkening may perhaps be due to the reduction of glucose concentration below the optimum for dehydrogenase activity, although the possibility of a direct effect on the rate of destruction of the enzyme cannot be excluded. And further the treatments effective in inducing the premature darkening, though not contraction, of intact larvae due to tyrosinase activity show a striking correspondence with those which in general inhibit dehydrogenases.

The results obtained by Graubard (1933) are in agreement with the conclusions reached during this work. Graubard found that some inhibiting factor or substance is present in *Drosophila* larvae, and is in some way less accessibly situated than is tyrosinase. Extracts made by grinding the larvae with sand showed greater inhibition of tyrosinase than those prepared without sand, a result completely in accordance with the observation here that dehydrogenase is present to only a slight extent in the blood, being mainly found in the tissues. The further observation of Graubard that extraction with chloroform water results in a high yield of tyrosinase agrees with the fact that chloroform is an effective inhibitor of dehydrogenases. The work of Kuwana (1937) is also significant here. In this work it was found that the reducing power of silkworm blood shows considerable diminution, though not complete disappearance, on exposure. This diminution is accompanied by melanosis, and Kuwana was of the opinion that the unstable reducing power of the blood might be due to the presence of a phenol which on exposure becomes oxidized to a coloured compound. But in the light of the present work it is understandable that on exposure dehydrogenase activity of the blood may diminish, so causing a loss of reducing power. Similarly the observations of Levenbook (1947) are important in indicating the instability of insect blood. The blood of the third instar larva of *Gastrophilus* contains a considerable amount of fructose which diminishes when the blood is allowed to stand, suggesting that here perhaps a fructose dehydrogenase may be operative. It may well be, indeed it is probable, that in different insects different dehydrogenase systems may serve as inhibitors of tyrosinase.
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activity, just as within the general plan of the mechanism of phenolic hardening of the cuticle different oxidation products of tyrosine appear to be involved (Pryor, Russell & Todd 1947).

It may be concluded, then, that in blowfly larvae tyrosinase activity is inhibited by the agency of a dehydrogenase system as was suggested as a result of work on Sarcophaga (Dennell 1947). Neither the presence of ascorbic acid as in plants, nor of a phenol as suggested in the silkworm by Kuwana (1937), is adequate to explain the observed inhibition of tyrosinase. The possibility that the apparent liberation of tyrosinase activity in blowfly larvae at the time of puparium formation is actually the result of the production of tyrosinase from its precursor protyrosinase under the action of a lipidic activator, as occurs in the eggs of Melanoplus (Bodine 1945), has not been confirmed. It should be emphasized, however, that while it is convenient to speak of tyrosinase inhibition this is not strictly correct. Tyrosinase is not actually inactivated, and the appearance of inactivation arises from the fact that at the low potential obtaining in the blood the oxidation of tyrosine is not possible.

The significance of the control of dehydrogenase activity by Weismann's ring must now be considered. During the past 15 years it has become increasingly apparent that moulting and metamorphosis in insects takes place under the hormonal control exercised by the components of a neuroglandular complex situated in the head. The components of this system are the corpora cardiaca and allata, and the pars intercerebralis of the brain. Weismann's ring of the cyclorrhaphous larva of Diptera contains the histological elements of the corpus cardiacum and corpus allatum and is responsible for the control of growth, moulting, and pupation (see Scharrer & Scharrer 1944). Following the work of Fraenkel (1935) considerable evidence has accumulated pointing to the production by Weismann's ring of a hormone specifically causing pupation, and Becker & Plagge (1939) have claimed to have isolated this hormone and have enumerated some of its properties. Becker & Plagge showed that older larvae are more susceptible than younger ones to the action of the hormone. They ligatured larvae of Calliphora before the critical period so that the posterior portions were unable to receive the hormone and therefore did not pupate. Injection of the blood of critical stage larvae, however, in which the pupation hormone was present, into these posterior portions caused pupation. Receptors in which the anterior end had pupated 14 to 24 hours after ligaturing were more sensitive to the action of the hormone than those in which pupation of the anterior end had occurred 72 to 96 hours after ligaturing. Fraenkel (1935) had previously found similar differences in larvae ligatured at different periods before pupation. These results are not in conflict with the observations on the fluctuations in dehydrogenase activity made in the present work. Reference to figure 4 of this paper shows that the larvae ligatured by Becker & Plagge a little before the critical period would show less dehydrogenase activity than those ligatured earlier. Inhibition of dehydrogenase by the pupation hormone may therefore be expected to be most complete in these larvae, with the result that they form the most sensitive receptors. But it must be remembered that the blood of critical stage larvae used as donors contains not only the hormone but also some
polyphenol arising from its action, so that it is difficult in these experiments to
distinguish between the effects of the hormone and the direct action of the poly­
phenol. It is to be noted that later Becker (1941) adopted the view that the material
previously isolated (Becker & Plagge 1939) was not the hormone but some chemical
to which the integument would respond after activation by the hormone.

So far it has been presumed that the hormone liberated at the critical period is
indeed specifically a pupation or metamorphosis-inducing hormone, but it seems
possible that it is no more than a general developmental and therefore moult-
inducing hormone which evokes pupation and metamorphosis only in virtue of the
conditions obtaining at the time it is liberated. This view arises from a considera-
tion of the theory of metamorphosis put forward by Wigglesworth (1934, 1936, 1940,
1947, 1948) as the outcome of his work on Rhodnius, and supported by embryo-
logical and histological findings (Henson 1946). In Rhodnius two hormones are
responsible for control of the course of development. One is secreted in the dorsum
of the brain and causes growth and development with the production of imaginal
characters. It is referred to by Henson (1946) as a general developmental hormone,
not merely as a moulting hormone as Wigglesworth described it, and this procedure
will be followed here. In the nymphal instars the activity of this hormone is
modified by a second, the juvenile, hormone secreted by the corpus allatum, so that
the adult form is not assumed. The assumption of the imaginal condition is therefore
due to the developmental hormone exerting its full effect in the 5th instar nymph
in the absence of the juvenile hormone. Wigglesworth (1947, 1948) gives reasons for
believing that in the 5th nymph the corpus allatum not only does not secrete
juvenile hormone, but actively removes from the blood any traces that remain. He
goes on to suggest that it may be the elimination of juvenile hormone in Diptera
which gives the impression of a 'metamorphosis-promoting hormone'.

Although there is as yet no direct evidence of the production and elimination of
a juvenile hormone in cyclorrhaphous Diptera Wigglesworth's view has much to
recommend it, and the evidence in its favour may be reviewed here.

Burtt (1938) studied the effects of removal or destruction of Weismann's ring in
young third instar larvae of Calliphora, and found that removal of the ring at 3 days
causes arrest of development. The imaginal buds cease to grow, and the larva
continues to feed. These results may be interpreted by supposing that during the
first 3 or 4 days of larval life juvenile hormone is present, but is later eliminated by
the ring gland. Exirpation of the ring at 3 days therefore prevents the disappearance
of the hormone, so that the imaginal buds remain in a retarded or juvenile condition.
Further, in the present work it has been found that up to 4 days dehydrogenase
activity of the larva rises and thereafter falls, and that cautering the ring at 3 days
prevents this fall. If we are justified in regarding the production of dehydrogenase
as a juvenile character, since it inhibits tyrosinase activity and therefore opposes
the production of the puparium, then in accordance with the interpretation
placed on Burtt's results it is found that conditions promoting the juvenile state
prevail in the early part of larval life. Perhaps the production of dehydrogenase by
the tissues is stimulated by juvenile hormone, and the decline in dehydrogenase
activity which occurs after 4 days follows the disappearance of this hormone.
In *Rhodnius* the corpus allatum appears to produce the juvenile hormone when exposed to the stimulus provided by the secretion of the developmental hormone, so that the juvenile hormone eliminated by the corpus allatum in the fifth stage nymph is that remaining from the previous moult. In the fifth stage nymph the developmental hormone, secreted on the stimulus of feeding, results in the absence of juvenile hormone in mitosis of the epidermal cells in 5 days, the appearance of new cuticle in 18 days, and moulting after 28 days (Wigglesworth 1940). Although the imaginal cuticle is still not fully formed it is hardened immediately after ecdysis, and further production of endocuticle takes place for at least 3 weeks (Wigglesworth & Gillett 1936). In *Calliphora* the developmental hormone responsible for the initiation of the production of the cuticle of the third instar larva is liberated at the end of second instar life, and as in *Rhodnius* cuticle formation is incomplete when ecdysis takes place. Growth of endocuticle takes place after moultiing, and may even continue after the pupal contraction as in *Sarcophaga* (Dennell 1947). But hardening of the cuticle does not take place on ecdysis, as it does in *Rhodnius*, it is delayed until the end of the third larval stadium when puparium formation takes place. The developmental hormone liberated at the end of this stadium is in no way related to the secretion of the third instar cuticle or to the possible early presence of juvenile hormone, but is that which initiates the production of the prepupal cuticle. If juvenile hormone is indeed present in *Calliphora* at the beginning of the third instar it must, if Wigglesworth’s views are applicable, have been secreted by the ring gland in response to the stimulus provided by the liberation of the developmental hormone of the second instar. Secretion of the cuticle of the early third instar larva therefore proceeds in the presence of juvenile hormone and therefore conforms to the larval pattern.

From this comparison emerges the essential difference in the time of hardening of the cuticle in the adult *Rhodnius* and the puparium of *Calliphora*. In *Rhodnius* hardening occurs at the beginning of the stadium, but in *Calliphora* the hardening of the third instar cuticle to form the puparium is delayed so that the process of puparium formation is overlapped by the developmental cycle which involves the production of the cuticle of the next instar. To this may be ascribed the function of the developmental hormone liberated at the end of third instar life in inducing hardening of the puparium. Whereas in *Rhodnius* the developmental hormone does not, apparently, provide the stimulus to hardening of the cuticle, in *Calliphora* the hormone is liberated at precisely the time when the components of the enzyme mechanism responsible for hardening the cuticle have been assembled. It appears that by inhibiting dehydrogenase and so releasing tyrosinase activity the developmental hormone has assumed the additional function of stimulating puparium formation, whereas in insects generally the stimulus causing hardening of the cuticle remains obscure (Wigglesworth 1939).

If we adopt the view held by Wigglesworth (1947, 1948) and Henson (1946) the critical feature of metamorphosis is the withdrawal of the modifying influence of the juvenile hormone so that growth and differentiation are directed towards the development of imaginal characteristics. The decline in dehydrogenase activity after 4 days noted in the present work, and the observation of Burtt (1938) that the
destruction of Weismann’s ring at 3 days arrests the growth of imaginal buds, suggests that the elimination of juvenile hormone may begin in Calliphora at about 3 days before pupation. It is noteworthy that after this time the crop begins to empty, a process accompanied in Sarcophaga by the appearance of tyrosine and tyrosinase in the blood and the production of polyphenol oxidase by the epidermal cells (Dennell 1947). These are, of course, preparations for puparium formation and it is evident that the crop-full condition is a decisive period in the developmental sequence. Additional evidence of this is given by the observations of Drummond (1939), who ligatured Sarcophaga larvae of different ages for a short period and then freed them. Larvae subjected to temporary ligature before the critical period underwent pupation in both halves, indicating, as Fraenkel (1935) had pointed out for Calliphora, that the stimulus to pupation is not nervous since the effect of the temporary ligature is to interrupt connexion between the central and peripheral parts of the nervous system. But when the operation was repeated on younger larvae many remained alive without pupating after the normal larvae from the same batch had pupated. Drummond therefore suggested that since the ring gland was present in these larvae which failed to pupate the stimulus causing liberation of the pupation hormone cannot have been received. As a result of a number of experiments on larvae of different ages he concluded that in Sarcophaga the interval between stimulus and pupation is about three days. It is clear, however, that Drummond’s results may equally well be explained by the suggestion that at about 3 days before pupation stimulation of the ring gland takes place and is followed by the elimination of juvenile hormone. If the stimulus is not received juvenile hormone persists and dehydrogenase activity does not decline, so that the developmental hormone liberated later is unable to effect complete inhibition of this enzyme and puparium formation therefore does not take place. In connexion with Drummond’s observations it should be noted that Burtt (1938) found that severing the nerves between Weismann’s ring and the brain in Calliphora larvae 4 days before pupation had the same effect as removal or destruction of the ring.

The work of Bodenstein (1943, 1944) on Drosophila seems also to point to the existence of a decisive phase in larval life. Bodenstein showed that not only growth but differentiation is controlled by the ring gland, and came to the conclusion that the same hormone is involved in both these processes. He states (1944) that whether growth or differentiation takes place in organ disks depends on a definite relationship between hormone level and organ competence. Young organ disks of third instar larvae are able to respond to hormone stimulation with growth, but as they become older this capacity decreases, while their capacity to respond with differentiation increases. Although Bodenstein explained his results by reference to hormone level and tissue competence, they may possibly be better explained in terms of the elimination of juvenile hormone.

Throughout this paper, as a matter of convenience, the terms ‘puparium formation’ and ‘pupation’ have been used as synonymous. It is evident however that the term pupation should be restricted to the production of the pupa, whereas puparium formation is the earlier hardening of the larval cuticle. Both puparium formation and pupation result from the onset of metamorphosis, which on the
views suggested here must be regarded as initiated in the larva as early as the end of the feeding period. The term ‘pupation hormone’ applied to the hormone liberated at the critical period at the end of the third instar therefore appears to be misleading. In conclusion, it seems that Weismann’s ring is to be regarded as not merely active immediately prior to pupation, but as influencing the whole course of third instar larval life.

I am grateful to Dr V. B. Wigglesworth for reading the manuscript of this paper, and to Dr H. Henson for discussion of the views presented in his review.

Appendix

After the completion of this paper my attention was drawn by Dr V. B. Wigglesworth to the paper by Danneel (1946) recording the presence of a dehydrase in insects. Since this paper is as yet inaccessible to me I am unable to comment on its significance in the light of the present work.

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