CYTOCHROME OXIDASE ACTIVITY DURING DIAPAUSE AND
METAMORPHOSIS OF THE JAPANESE BEETLE (POPILLIA
JAPONICA NEWMAN)*

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Bodine (1934) showed that the respiration of the pre- and postdiapause egg
of the grasshopper *Melanoplus differentialis*, is markedly inhibited by cyanide,
whereas that of the diapause egg is cyanide-insensitive. Allen (1940) studied
cytochrome oxidase during the embryonic development of this insect and
found that the concentration of this enzyme remains low throughout diapause
and the first part of postdiapause. Williams (1946, 1947, 1948 a) described the
diapause condition in the pupa of the moth *Platysamia cecropia*, and the role
of the brain and prothoracic glands in its regulation. In 1948 b he postulated a
mechanism whereby these glands may function in the termination of the dia-
pause condition. As in the egg of the grasshopper, the respiration of the dia-
pause pupa is insensitive to cyanide. Williams ascribed this insensitivity to a
disruption of the cytochrome system. Later in the pupal period when the brain
becomes active, there is an enormous increase in cytochrome oxidase which
persists as long as the brain is active and then decreases; the stimulation of the
prothoracic glands by the brain hormone is followed by the synthesis of cyto-
chrome *c*. In this way, the cytochrome system is restored and adult develop-
ment is able to proceed. Subsequently, Sanborn and Williams (1950) found
that the cytochrome system of the larva (cytochrome *a*) disappears before the
time of pupation. Diapause in this insect is associated with an almost complete
absence of cytochrome *c* and cytochrome oxidase; and its termination, with
the restoration of the cytochrome system under the influence of hormones.

Ludwig (1928, 1932) described a larval diapause in the Japanese beetle,
*Popillia japonica*. Its position in development is conditioned by nutrition and
temperature, occurring in the last or third larval instar at 25°C. The present
experiments were undertaken to determine whether this larval diapause is
associated with a defect in the cytochrome system as postulated by Williams
(1948 b) for the pupal diapause of the *Cecropia* moth.

Williams (1950), Sacktor (1951 a), and Bodenstein and Sacktor (1952),
found that the activity of cytochrome oxidase during the pupal stage follows

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the characteristic U-shaped curve associated with oxygen consumption. It thus appears that most of the oxidative metabolism of metamorphosing insects is mediated through the cytochrome system. Since studies have already been made on the changes in oxygen consumption during the metamorphosis of the Japanese beetle (Ludwig, 1931), it was decided to extend these experiments to include a study of the activity of cytochrome oxidase throughout the period of metamorphosis.

Material and Methods

The larvae used in the diapause experiments were obtained from eggs collected in the laboratory; and the metamorphosing individuals, from larvae collected in the field. The larvae of both groups were kept individually in 1 ounce metal salve boxes containing moist soil to which several grains of wheat were added to serve as food. They were kept at a constant temperature of 25°C. until used in the experiment. Each larva was examined every 4 or 5 days, and food or water added as needed. On the approach of a molt, the insect was examined daily until it had molted and the date of its molt was recorded. In this way, an accurate record of the history of each individual was obtained.

The activity of cytochrome oxidase was determined on individual insects by the method of Cooperstein and Lazarow (1951), using a Beckman DU spectrophotometer. The insect was weighed, inactivated by placing it in an ice bath, then thoroughly homogenized in a glass homogenizer containing enough ice cold phosphate buffer (0.03 molar adjusted to pH 7.4) to give a 10 per cent homogenate. During this process, the homogenizer was held in a bath of ice water. The homogenate was then diluted with enough of the cold phosphate buffer to make a dilution of 1:200 and passed through a cloth filter. It was then kept in an ice bath until used.

The cytochrome c was prepared in a phosphate buffer at pH 7.4 by adding 5 mg. to each 10 ml. of solution. It was then reduced by the addition of a few crystals of sodium hydrosulfite, and the excess reducing agent was removed by bubbling air through the solution for about 15 minutes. In making a determination on the activity of cytochrome oxidase, 2.5 ml. of reduced cytochrome c was mixed, by inversion, with 0.5 ml. of the diluted tissue homogenate in a Beckman cuvette. The first reading on the density of the mixture was made within 1 minute after the addition of the homogenate to the cytochrome c, and readings were made at intervals of 1 minute for a period of 5 minutes. At the end of that time, 0.1 ml. of 1/10 potassium ferricyanide was added to the cuvette, mixed by inversion, and a reading made of the optical density of the oxidized cytochrome c. A correction was made in the final calculation for the change in density resulting from the addition of the ferricyanide. All readings were made at a wave length of 550 mμ. Calculations of cytochrome oxidase activity were made using the formula given by Cooperstein and Lazarow (1951).

1 The cytochrome c was obtained from the Sigma Chemical Company. The writer is indebted to Dr. Bertram Sacktor, Army Chemical Center, for suggestions regarding the preparation of different solutions used in these experiments.
RESULTS

The change in activity of cytochrome oxidase during the second and third larval instars, as well as in the prepupal and pupal stages is shown in Fig. 1. The values for cytochrome oxidase activity are expressed as $\Delta \log [\text{Cy Fe}^{+++}]$ per minute for homogenates in the dilution of 1:1,000. There was a slight increase in activity from 0.022 to 0.025 during the second instar. The newly molted third instar larva had an activity of 0.027. At the end of the 1st week of the third instar, there was a rapid increase, the values reaching a level of between 0.074 and 0.083 during the diapause stage. Since each point plotted for diapause larvae represents an average of at least four determinations, a total of more than 30 readings was made on diapause larvae. Under the conditions of this experiment, diapause occurs in the full grown larva and lasts approximately 50 days. This stage usually begins approximately 5 weeks after the second molt and continues until approximately the 12th week. The larva
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then feeds for a short time and soon enters the prepupal stage. The transformation of the larva to the prepupa was accompanied by a rapid decrease in the activity of cytochrome oxidase which continued into the early pupal period, reaching a low value of 0.019 in insects 2 days after pupation. Enzyme activity remained low until 4 days after pupation and then increased rapidly throughout the remainder of the pupal period, reaching a high value of 0.095 just before adult emergence.

Determinations on the activity of cytochrome oxidase were also made on newly emerged adults, using a dilution of 1:10,000. For purposes of comparison, the values given are calculated on the basis of a 1:1,000 dilution. The average value obtained for 12 adult female beetles was 0.25 ± 0.012; and for 9 males it was 0.40 ± 0.028. These means are significantly different since their difference is more than five times its probable error.

Readings were made on the activity of cytochrome oxidase in larvae, prepupae, and pupae, using homogenates prepared in a phosphate buffer containing NaCN. In every case, the enzyme was completely inhibited by cyanide in a concentration of 1/1,000.

DISCUSSION

The high activity of cytochrome oxidase recorded for the diapause larva indicates that this condition may be physiologically different from diapause in the egg or pupal stages. Eggs and pupae are closed systems in that there is no exchange of materials with the environment except gases and water. On the other hand, the diapause larva does take in food occasionally, although to a very limited extent compared with the feeding of growing larvae. Larval diapause appears not to be associated with a defect in the cytochrome system as indicated by the work of Bodine (1934) for the egg of the grasshopper, and as postulated by Williams (1948 b) for the pupa of the Cecropia moth. However, in the present experiments, no readings were made on the presence of cytochrome c but only on the activity of cytochrome oxidase. It is possible for the oxidase to exist without its substrate. This condition was reported by Stotz (1939) for embryonic and tumor tissues of the rat, and by Williams (1948 b, 1951) for Cecropia pupae. Williams found that cytochrome oxidase increases at the time the brain hormone becomes active; whereas the synthesis of cytochrome c depends on the activity of the prothoracic glands. After the brain hormone disappears cytochrome oxidase decreases and remains low until the 2nd day after the initiation of adult development, when it again increases. The onset of adult development shows a positive correlation with the behavior of cytochrome c but a negative correlation with changes in cytochrome oxidase. A comparable relationship between the presence of hormones and the activity of cytochrome oxidase could not be demonstrated experimentally by Bodenstein and Sacktor (1952) in adults of Drosophila virilis.

The observations of Bodine (1934), Bodine and Boell (1934), and Williams
that the respiration of diapause insects is largely insensitive to cyanide and carbon monoxide, may be considered evidence that the cytochrome system is not important in their respiration. It has generally been assumed that respiration during diapause is largely mediated by the flavoprotein system as the terminal oxidase. However, the recent observations of Bodine, Lu, and West (1952) that the addition of sodium succinate to a homogenate of grasshopper eggs greatly speeds up the rate of oxygen consumption during diapause, but not during pre- and postdiapause, indicate that the succinoxidase system (which is believed to include cytochrome c and cytochrome oxidase) may be present although not active in the normal diapause egg. The fact that the induced respiration is inhibited by cyanide and other inhibitors of the oxidase adds support to this idea. Levenbook (1951) reported that the respiration of the diapause larva of the horse bot fly, *Gastrophilus intestinalis*, goes through a cyanide- and carbon monoxide-sensitive heavy metal protein, probably cytochrome oxidase. Furthermore, MacDonald and Brown (1953) found that the diapause of the larch sawfly, *Pristiphora erichsonii*, which occurs in the pre-pupal or eonymph stage, shows no decrease in cytochrome oxidase and no change in cyanide sensitivity. It must therefore be concluded that in certain insects there is a high activity of cytochrome oxidase during diapause. In these forms, diapause may be controlled by some mechanism other than the cytochrome system.

The activity of cytochrome oxidase obtained for metamorphosing Japanese beetles is very similar to that reported by Sacktor (1951 a) using the house fly, *Musca domestica*; and by Bodenstein and Sacktor (1952) with the fly *Drosophila virilis*. It follows the characteristic U-shaped curve obtained by many workers for oxygen consumption during metamorphosis. Ludwig (1931) found that the oxygen consumption of the Japanese beetle decreased during the first 2 or 3 days of the pupal stage and then increased. Fig. 1 shows that the activity of cytochrome oxidase also reached a low value in the 2nd and 3rd days of pupal life and then increased. These observations add evidence to the theory advanced by Wolsky (1938) that the oxygen consumption observed during metamorphosis may be attributed to the quantity or activity of the cytochrome system. Sacktor (1951 a) found that the cyanide-insensitive respiration of house fly pupae remained relatively constant throughout metamorphosis. Hence, cyanide is much more effective at the beginning and end of metamorphosis when the oxygen consumption and cytochrome oxidase activity are high, than when they are low. He attributes most of the cyanide-sensitive respiration to cytochrome oxidase and suggests that the remainder may be associated with another enzyme, probably tyrosinase.

The very high activity of cytochrome oxidase observed in the adult Japanese beetle is in agreement with the work of Sacktor (1951 a) for the adult house fly. The average standard activity value (calculated by using the formula of Cooperstein and Lazarow, 1951) for the adult Japanese beetle is 3.14, and the
value obtained by Sacktor is 3.4. The higher rate of enzyme activity in males as compared with females is also in agreement with the results of other workers. Sacktor (1951) found that, on a weight basis, the male house fly has a higher rate of cytochrome oxidase activity than the female. Barron and Tahmisian (1948) studied the oxidative metabolism of the skeletal muscles of the cockroach, *Periplaneta americana*, and found the $Q_{O2}$ for the male to be 5.0, and for the female, 2.6. Sacktor and Bodenstein (1952) determined the cytochrome oxidase activity of the muscles of the same species of cockroach and obtained a standard activity of 2.93 for males and 1.60 for females. However, they found the cytochrome oxidase activity of some other tissues, such as the brain, nerve cord, and fore gut, to be higher in the female than in the male.

**SUMMARY**

1. Determinations were made on the activity of cytochrome oxidase of individual Japanese beetles during growth, diapause, and metamorphosis. All readings were made on homogenates at a dilution of 1:1,000, except for adult beetles, when the final dilution was 1:10,000.
2. The activity of the enzyme increased during larval growth from a low value of 0.022 in the second instar, to high values ranging from 0.074 to 0.083 in diapause third instar larvae.
3. The high activity of cytochrome oxidase during larval diapause indicates that this condition may be physiologically different from that occurring in the egg or pupal stages of most other insects.
4. During metamorphosis, the activity of cytochrome oxidase follows the characteristic U-shaped curve associated with respiratory metabolism. It thus appears that most of the oxidation occurring in metamorphosing individuals is mediated through the cytochrome system.
5. The activity of cytochrome oxidase is significantly higher in the adult male than it is in the adult female; the values (calculated on the basis of a 1:1,000 dilution) were 0.40 ± 0.028 and 0.25 ± 0.012, respectively.

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