Some Quantitative Changes Observed in *Philosamia ricini* Pupal Haemolymph during Metamorphosis

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1. The quantitative variations of the concentrations of uric acid, citrate, nucleic acids and total and acid-soluble phosphorus in the pupal haemolymph of *Philosamia ricini* during metamorphosis have been studied. 2. The mean value for total nitrogen in the haemolymph and total loss in weight of the insects during metamorphosis have also been recorded.

During the larva-pupa-adult transformation of holometabolous insects, a number of remarkable changes take place in the quiescent pupa in respiratory rate and in the occurrence and metabolism of protein, carbohydrates, lipids and nucleic acids (Gilbert & Schneiderman, 1961; Harvey, 1962). Patterson (1957) made some observations, both qualitative and quantitative, on the free α -amino nitrogen fraction of *Tenebrio molitor* pupal tissues during metamorphosis and correlated them with the changes observed in other non-protein tissue fractions. The absence of similar published data on the biochemistry of *Philosamia ricini* pupal haemolymph and the quantitative chemical changes occurring in certain metabolic processes during metamorphosis prompted us to undertake the present study.

MATERIALS AND METHODS

Insects. Larvae of *Philosamia ricini* were reared at 27° in the laboratory in glass dishes kept in a wooden box (18 in. $\times$ 18 in. $\times$ 18 in.) with wire-netted sides and top. The larvae were fed on fresh castor leaves. Humidity was controlled by keeping a damp pad of cotton wool in the box. After 28 days of larval period in five instar stages, they attached themselves to suitable places in the box and spun cocoons. These were detached and stored at room temperature in glass dishes. This has been termed by us 'day 0' and the 17 days taken by a day-0 pupa to be completely transformed into an adult moth as the 'period of metamorphosis'.

Haemolymph. All estimations were carried out on haemolymph pooled from 12 chilled pupae at each stage by making an incision on the posterior end of the dorsal side of the pupa with sharp scissors and draining the pale-straw-coloured fluid into a Pyrex dish immersed in salt-ice mixture.

Total nitrogen. This was estimated by digesting 0.5 ml. of pupal haemolymph with 2 ml. of conc. H_2SO_4 (sp. gr. 1.84) and 0.1 g. of the copper catalyst of Chibnall, Rees & Williams (1943) in a graduated 10 ml. micro-Kjeldahl flask. Distillation was carried out in a semi-micro Parnas apparatus

and nitrogen was titrated as ammonia with 14.3 mN- H_2SO_4 , with Tashiro's indicator.

Uric acid. This was estimated by the method of Brown (1945) as modified by Dresel & Moyle (1950). To 0.5 ml. of haemolymph diluted to 5 ml. with water were added 1 ml. each of 10% (w/v) sodium tungstate and 0.67N- H_2SO_4 . The mixture was shaken well, diluted to 10 ml. with water and centrifuged. To 5 ml. of this protein-free supernatant were added 2 ml. of 12% (w/v) NaCN and 2 ml. of 50% (w/v) urea followed by 1 ml. of the uric acid reagent. After incubation at 38° for 1 hr., the volume was made up to 25 ml. and the colour density measured in a Gallenkamp colorimeter with an Ilford 608 (red) filter.

Nucleic acids. These were precipitated from 0.5 ml. of haemolymph by the addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid. After thorough mixing, the precipitate was isolated by centrifugation and was extracted with 7 ml. of 5% (w/v) trichloroacetic acid at 90° for 20 min. RNA was estimated by colorimetric method of Meijbaum (1939) in samples of this 5% trichloroacetic acid extract. To 1 ml. of the extract was added 1 ml. of conc. HCl (sp. gr. 1.19) containing $FeCl_3$ (0.1%, w/v) and orcinol (0.1%, w/v) and the mixture was heated in a boiling-water bath for 45 min. according to the procedure of Albaum & Umbreit (1949). After cooling, the extinction was measured at 670 m μ in a Unicam SP.600 spectrophotometer. A calibration curve was prepared from a standard solution of RNA. RNA was also determined by the method of Euler & Hahn (1947) and estimations by these two methods agreed fairly well.

Citrate. The method used for the determination of citrate was essentially the micro-colorimetric method of Weil-Malherbe & Bone (1949) in which vanadate was used as a specific oxidizing agent. The pentabromoacetone formed by subsequent bromination was extracted from light petroleum with thiourea buffered with borate as described by Natelson, Pincus & Lugovoy (1948). Haemolymph (0.2 ml.) was diluted to 3.0 ml. with distilled water, 1 ml. of 20% (w/v) trichloroacetic acid was added and after 1 min. the mixture was centrifuged. The supernatant (1.5 ml.) was diluted to 5 ml. with N- H_2SO_4 in a stoppered 25 ml. conical flask. To this 5 ml. of 27N- H_2SO_4 was added and while it was still warm 5 ml. of bromide-bromate-vanadate oxidizing agent was added. After mixing, the flask was stoppered and

20–25 min. was allowed for the formation of pentabromoacetone. A freshly prepared solution (2 ml.) of 22% (w/v) FeSO_4 was added to this. The solutions were mixed and the flask was left unstoppered for about 10 min. When no smell of bromine was detected 7 ml. of light petroleum (b.p. 80–100°) was added and the flask was well shaken for 3 min. The ethereal layer was removed and transferred to a stoppered tube containing approx. 1 g. of anhydrous Na_2SO_4 . After being shaken and kept standing for 5 min. a 4 ml. sample was added to 4 ml. of buffered thiourea, pH 9.2. This mixture was thoroughly shaken for 10–15 min. and the aqueous layer was filtered through Whatman no. 1 filter paper. The extinction was measured at 445 m μ in a Unicam SP. 600 spectrophotometer within 15–30 min. Standard curves were not always reproducible by this method, but on any given occasion the curve was linear over the concentration range 50–200 μg . of citric acid. Citrate estimations were made in the range 100–200 μg . and for this reason stated suitable standards were always set up.

Total phosphorus. This was estimated by Allen's (1940) method by digesting 0.5 ml. of pupal haemolymph with HClO_4 . Oxidation was completed by addition of 1 or 2 drops of H_2O_2 . The phosphorus content was determined as described by Pant & Agrawal (1963).

Acid-soluble phosphorus. For the determination of acid-soluble phosphorus 0.5 ml. of haemolymph was deproteinized with 4.5 ml. of 5% trichloroacetic acid, and after being kept at 0° for 5 min. the mixture was centrifuged. The precipitate was re-extracted with 5 ml. of 5% trichloroacetic acid twice and the supernatants were combined. Phosphorus was estimated as in the above after diluting the washings to a known volume.

RESULTS AND DISCUSSION

During metamorphosis the energy required by the pupae is derived from endogenous sources. This energy is stored in the pupal tissues in the form of glycogen and fat derived from the food that the insect gorges during its larval stages. The pupae therefore continuously lose weight, and, since the rate of utilization of this stored energy is not a constant factor, the loss in weight correspondingly varies.

The average loss in weight of 12 pupae with their

Fig. 1. Variation in weight of *Philosamia ricini* pupae during metamorphosis. Values represent the means of 12 pupae.

cocoons intact was recorded daily during metamorphosis; the loss was rapid during the first 5 days but later became more gradual, and at about the ecdysis period practically no loss occurred (Fig. 1). The average total loss in weight was 0.88 g./pupa.

Since uric acid is the major end product of nitrogenous metabolism in insects (Baldwin, 1940; Florkin, 1949) and is stored in their tissues during metamorphosis, the rate of its accumulation in the tissues could be considered as an index of deaminative activity at various developmental stages of metamorphosis. Previous investigations (Heller & Moklowska, 1930; Evans, 1932, 1934) on the concentration of uric acid in blood revealed that during metamorphosis this value varied only a little and generally approximated to that in a saturated aqueous solution (6.5 mg./100 ml.). The variation of uric acid concentration in the pupal haemolymph of *Philosamia ricini* during metamorphosis is recorded in Fig. 2. The maximum concentration (16.4 mg./100 ml.) is on day 15 at about ecdysis and the lowest (3.4 mg./100 ml.) on day 2. During the first phase (days 0–7) the values show the familiar U-shaped variation with an approximately constant concentration during days 2–6, suggestive of minimal deamination rate. Corresponding to the high concentrations on days 7, 9 and 15, sharp falls were observed on days 2, 8, 10, 12 and 16. These periods in all probability depict periods of high and low deaminative activity respectively. The rises and falls of concentrations of uric acid could also be attributed to uricase, the presence of which has been reported in the haemolymph of some insects during certain developmental stages (Brown, 1938; Razet, 1954; Ross, 1959).

Fig. 2. Variation of uric acid concentration in *Philosamia ricini* pupal haemolymph during metamorphosis. Values are the means of three to six estimations from the pooled haemolymph of 12 pupae.

The presence of a high concentration of citrate in an insect was reported by Tsuji (1909), who estimated it in the larval haemolymph of *Bombyx mori*. Since then the only citrate analysis of insect haemolymph has been by Levenbook (1950) and Levenbook & Hollis (1961), who investigated it in the haemolymph of *Gastrophilus intestinalis* and in other insects belonging to six different Orders examined at various stages of development. The values obtained were higher than those found for the blood of higher organisms. This led Levenbook & Hollis (1961) to regard this phenomenon as a systematic 'biochemical characteristic of insects'. Patterson's (1956, 1957) observations on the blood of *Rhodnius prolixus* and the tissue homogenate of *Tenebrio molitor* supported this view.

Patterson (1956) observed that the total body citrate in *Tenebrio molitor* varied reciprocally with the respiration, a typical feature of insect adult development. He postulated that citrate accumulation in this insect is inversely proportional to the activity of the tricarboxylic acid cycle. However, Levenbook & Hollis (1961) have not been able to confirm this in two other species, *Phormia regina* and *Prodenia eridania*. They observed no rise or fall in the concentration of citrate during metamorphosis in either of the species tested. On the contrary, a marked utilization of citrate was recorded during the first third of the pupal period, the quantity utilized being much more in *Phormia regina* than in *Prodenia eridania*. In addition, after attaining the minimum the concentration remained constant for the rest of the adult development period. The results of the present investigation (Fig. 3), however, do not agree entirely either with the findings of Patterson (1956, 1957) or with those of Levenbook & Hollis (1961). This difference may be due to the fact that, unlike the present authors, Levenbook & Hollis (1961) and Patterson (1956) employed tissue

homogenates instead of haemolymph. The haemolymph citrate content shows marked variations throughout, with several rises and falls, the minimum (166mg./100ml.) being on day 3, when the developing pupa shows advanced histolysis. On day 11 the concentration rises to the maximum (362mg./100ml.), during which period (days 9–11) the proventriculus, ventriculus and rectum become well formed, reproductive organs become distinct and wing buds appear. Another high concentration (355mg./100ml.) observed is on day 6, when (days 4–6) the complete formation of pupa takes place. Simultaneously, the histolysis of fore and midgut muscles is completed, the latter shortens, contents of the malpighian tubules disappear and appreciable quantities of fat bodies are deposited. There is also a marked fall on day 16, just before ecdysis. There is a typical U-shaped variation in the citrate concentration between days 8 and 14, when most of the tissue for the preparative adult is formed.

In the absence of any correlation between dietary and haemolymph citrate concentrations, Levenbook & Hollis (1961) concluded that citrate in insects was of endogenous origin. Consequently, the recurring rise and fall in its concentration during the various developmental stages indicate the rates of citrate synthesis and utilization. Nevertheless, it is very difficult to suggest a satisfactory explanation for the different variations in concentration afforded by these four insects, two of them belonging to the same Order.

During metamorphosis, measurements of RNA might indicate periods of intensive protein-synthetic activity, since there is a correlation between the protein-synthetic activity and the relative accumulation of RNA. Agrell (1953) studied the changes in the RNA/DNA ratio in *Calliphora* during metamorphosis and the variation presented a U-shaped curve. Patterson (1957) studied these changes during metamorphosis of *Tenebrio* pupae and his results agreed more or less with those of Agrell (1953). Several authors have pointed that each cell nucleus contains a relatively constant amount of DNA (Vendrey & Vendrey, 1948; Mirsky & Ris, 1949; Leuchtenberger & Vendrey, 1952); others have maintained that the amount of DNA varies widely from tissue to tissue and even within the nuclei of one cell type (Brachet, 1947; Moricard, 1949; Schrader & Leuchtenberger, 1949).

In the present work, although RNA was found in the haemolymph of *Philosamia ricini*, DNA could not be detected at any of the developmental stages. Fig. 4 shows that, except for the prepupal period (0–3 or 4 days), when the RNA content increased and reached the maximum (97.76mg./100ml. of haemolymph), from the beginning of the pupal life there is a sudden fall (28.05mg./100ml.) and from day 4 to day 10 or 11 the concentration remains

Fig. 3. Variation of citrate concentration in *Philosamia ricini* pupal haemolymph during metamorphosis. Values are the means of duplicate determinations from the pooled haemolymph of 12 pupae.

more or less constant, after which it rises again on day 12 to 84.76 mg./100 ml. of haemolymph. Thus this U-shaped variation of RNA during days 4–12 may indicate that at the beginning of pupal period as well as on days 11 and 12 protein-synthetic activity is more rapid than at any stage during metamorphosis, whereas the minimal synthesis occurs during days 4–9 or 10. Identical results were obtained at all stages of development (days 0–17) when RNA was determined by the methods of Mejbaum (1939) and Euler & Hahn (1947). The variation between individual specimens of haemolymph taken at any particular stage was over 25% in only one instance.

The chemical changes occurring in the phosphorus compounds during metamorphosis of insects are of obvious significance. Tsuji (1909) found about 20% of phosphates in the ash of the blood of silkworms, and analyses of various insect haemolymphs have revealed that the total phosphate content is about 20–80 mM (Wyatt, 1961; Altman & Dittmer, 1961). Of the total phosphorus 'pool' only a small fraction is inorganic orthophosphate, and this has been confirmed for a number of species of insects (Buck, 1953). Most of the organic phosphorus is acid-soluble and only a small fraction is due to lipid or phosphoprotein (Wyatt, Loughheed & Wyatt, 1956; Karpiak, 1957; Ito, Shigematsu & Horie, 1958). A number of organic phosphorus compounds have been isolated and identified from the haemolymph of several species of Lepidoptera by employing chromatographic, chemical and enzymic methods (Wyatt, Kropf & Carey, 1963).

Levenbook (1953) studied the variation of phosphorus compounds during metamorphosis of the

blow-fly *Calliphora erythrocephala* Meig, and concluded that, though no change occurred in the amounts of acid-soluble lipid or nucleic acid phosphorus, that of inorganic phosphorus increased rapidly during pupation, attaining the maximum about half-way in adult development, and then declined. The content of easily hydrolysable phosphorus was high at the beginning and the end of pupation and the lowest after about one-third of the pupal period. However, no evidence for the presence of protein or inorganic (pyro- or meta-) phosphate was found. Most of the previous workers have reported the total phosphorus concentrations in insect tissues to be more or less constant during metamorphosis, though the concentrations of acid-soluble phosphorus varied. However, our results for haemolymph show variations in concentration of both types of phosphorus compound investigated.

During metamorphosis, two concurrent processes take place: the formation of a number of new organs and characteristic structures of the individual adult accompanied by the progressive disintegration of the old larval tissues by histolysis and phagocytosis. Since the circulating haemolymph supplies the reserves for histogenesis and collects breakdown products, the variation of concentrations of total and acid-soluble phosphorus is understandable. Fig. 5 shows that, after the prepupal period, when the larva changes to pupa on day 3 or 4, up to day 12 the pattern for the total phosphorus concentration is the usual U-shaped pattern. The lowest concentration of phosphorus (53.52 mg./100 ml.) is on day 16 and the maximum (160 mg./100 ml.) on day 17,

Fig. 4. Variation in RNA content in *Philosamia ricini* pupal haemolymph during metamorphosis. Each point is the mean of six determinations performed on the haemolymph of individual pupae. The vertical lines denote the upper and lower limits of the S.E.M. of the estimations.

Fig. 5. Variation of total phosphorus concentration in *Philosamia ricini* pupal haemolymph during metamorphosis. Values are the means of duplicate determinations from the pooled haemolymph of 12 pupae.

Fig. 6. Variation of acid-soluble phosphorus in *Philosamia ricini* pupal haemolymph during metamorphosis. Values are the means of duplicate determinations from the pooled haemolymph of 12 pupae.

Fig. 7. Variation of total nitrogen content in *Philosamia ricini* pupal haemolymph during metamorphosis. Values are the means of duplicate determinations from the pooled haemolymph of 12 pupae.

just at the time of ecdysis. On the other hand, the concentrations of acid-soluble phosphorus vary as shown in Fig. 6. The concentration of phosphorus increases at the beginning of the pupal period, reaches the maximum on day 6 and then declines.

Evans (1932, 1934) and Agrell (1951) studied the variation of total tissue protein nitrogen during the metamorphosis of *Lucilla*, *Tenebrio molitor* and *Calliphora* respectively. Their investigations on soluble and insoluble protein fractions indicate that as the metamorphosis proceeds more insoluble proteins are formed at the expense of the soluble ones, evincing histogenesis. Patterson (1957) recalculated the total nitrogen values for the nitrogen content of individual pupa (excluding the cuticle) and obtained an approximately constant value of 2.64 mg. of total nitrogen/pupa.

Total nitrogen content of *Philosamia ricini* pupal haemolymph at different stages of development during metamorphosis is recorded in Fig. 7. The mean value is 3.62 g./100 ml. of haemolymph.

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