BLOOD PROTEINS OF THE SILKWORM
BOMBYX MORI L.

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INTRODUCTION

Though metamorphosis in holometabolous insects is an example of the integration and regulation of numerous systems and homeostasis, the associated changes in blood and tissue composition calls for a thorough study. As early as 1924, Heller (1924) observed fluctuations in blood protein concentrations during metamorphosis. The potential value of the electrophoretic analysis of blood proteins of insects in entomological systematics was clearly demonstrated by Zweig and Crenshaw (1957) and Stephen (1958). Moreover, their investigations led to the important conclusion that fluctuations in the blood protein patterns during insect life are of little value unless the age of the insect is known exactly. Some information has been gathered on the blood protein patterns, of the Bombyx and other insect species during metamorphosis, but the data available are somewhat confusing and are at the same time not quantitative (Aizazawa and Murai, 1957; Oda et al., 1956; Kobayashi and Komatsu, 1956; Denuee, 1958; Steinhauer and Stephen, 1959; Groulade et al., 1961). In fact the only detailed report so far made was by Laufer (1960) who traced the variations in the blood proteins of the Cynthia and Cecropia silkworms during their life-cycle.

In the present investigation an attempt has been made to study in detail the electrophoretic behaviour of blood proteins of the silkworm Bombyx mori L. throughout its life-cycle. The changes which occur in the total and nonprotein nitrogen contents during different stages of growth are also indicated.

MATERIALS AND METHODS

Disease-free silkworms of pure Mysore variety at appropriate stages were selected from a large stock. Since up to third moult blood could not

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be obtained in sufficient quantities, a known weight of the larvae was homogenised, centrifuged at high speed and the clear supernatant obtained therefrom was used in all determinations. Eggs were also treated similarly. From the third moult onwards blood was extracted from a large number of worms through a puncture on the third or fourth segment and was pooled (usually only 0.1 to 0.2 ml. of blood could be obtained from a fully grown larva). For the analysis of individual samples, the blood drop was directly taken on to a micropipette. A known amount (15 to 20 μl.) of the blood or protein extract (30 to 40 μl.) was spotted on a thin strip of Whatman No. 1 filter-paper, air dried and was then used for the electrophoretic run. Since variations have been observed between individual and pooled samples (Stephen, 1961), usually five replicates were run for each sample; two of them from individual worms and the other three from pooled samples.

The electrophorograms were prepared according to Giri (1956). The agar solution for spreading on plates was prepared by mixing equal volumes of a 1% agar in water (autoclaved at 20lb. for 20 minutes) and veronal-acetate buffer of pH 8.6 and ionic strength 0.1 μ. The electrophorograms were run for about 6 hours at a constant current flow of 4 ma. per plate and at a voltage gradient of 300 V for the first two hours and 250 V for the remaining period.

After the electrophoretic run the electrophorograms were dried overnight in a current of air and stained with amido-black for all the proteins. The stain used for lipoproteins was sudan black and Basic Fuchsin was used for glycoproteins. For quantitative estimations the optical densities of the stained zones were measured, in the densitometer (Photovolt Corp. Model 50·1 A, New York) and area measured by a planimeter. Total protein was determined by nesslerization after Kjeldahl digestion of the extract or whole blood (Johnson, 1941). Nonprotein nitrogen was determined similarly from trichloracetic acid nonprecipitable supernatant.

**RESULTS**

Figure I shows the general blood protein pattern of fully-grown larva compared with that of normal human serum. The protein bands have been numbered 1 to 6 starting from the one with the least anodic migration. Henceforth, protein zones 1, 2, etc., will be referred to as protein 1, protein 2, etc., Proteins 1, 2 and 3 exhibit apparent cathodic mobility similar to serum γ-globulin. Protein 2 seems to correspond to the fibrinogen. Protein 5 corresponds to β2-globulin and 6 lies in between α2 and β1 components. Protein 1 does not have any corresponding member in normal serum and there is no
Blood Proteins of the Silkworm Bombyx mori L.

Protein in the silkworm blood that corresponds to serum albumin. Proteins 1 and 2 which are faint in Fig. I can be clearly seen in Fig. III.

Figures II b, c and a show the electrophorograms obtained from larvae first stage through the pupal stage to the third egg stage. The slight differences observed in the distances moved by the same protein zone during the larval stage was presumably due to slight variations in the running time and temperature. Likewise, larvae at the first and second molts gave very diffused patterns due to the very low concentration of the proteins. However, plates run with extracts of larvae in second molt clearly showed two bands on the cathode side. Three bands became clear in the larvae from the third molt. Thereafter, the three bands drifted apart, the mobility towards the anode decreasing considerably. During the metamorphosis of the larvae into pupae, proteins 2 and 5 disappeared altogether within three days, and none of these proteins were present in ten-day old pupae when two new protein [Fig. II c, 12 and 13] zones appeared. The eggs soon after laying contained all the six protein zones that were observed in mature larvae with almost similar mobilities [Fig. II a (1)]. However, these zones disappeared again after ten days when the eggs hatched [Fig. II a (3)].

Figure III shows the blood protein patterns at the final larval stage of worms infected by flacherie and grasserie. It could be seen that worms affected by the former did not show any change in the picture whereas in the latter no band could be discerned distinctly. Figure IV shows the protein pattern of the blood from mature silkworms just two days prior to spinning, but whose silk glands were removed at the commencement of fifth instar compared with that of normal worms. It could be seen that protein 5 was missing from the former.

Figures V and VI show the concentration of total nitrogen and non-protein nitrogen at various stages of growth. Both increased steadily during larval life but suffered a sudden decrease during spinning and maintained an almost steady value during pupal life. There was a considerable increase in both the forms of nitrogen in the newly deposited eggs; however, it suffered a decrease during egg development.

Table I shows the percentage composition of the different protein fractions at different stages of growth. The fact that the percentage of a specific protein at different stages has been included under protein of a particular number does not imply either that the proteins are the same or that they have the same mobility. This is particularly true of proteins 1 and 2 during larval life and all the components during pupal life. The percentages of
**Fig. V. Total Nitrogen during the Life-cycle of Silkworm**

- $L_1$, $L_2$, etc., indicate larval moults
- $P_1$, $P_5$ and $P_9$ indicate 1, 5 and 9-day old pupae
- $E_1$, $E_5$ and $E_9$ indicate 1, 5 and 9-day old eggs

**Fig. VI. Non-Protein Nitrogen during the Life-cycle of Silkworm**

- $L_1$, $L_2$, etc., indicate larval moults
- $P_1$, $P_5$ and $P_9$ indicate 1, 5 and 9-day old pupae
- $E_1$, $E_5$ and $E_9$ indicate 1, 5 and 9-day old eggs
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TABLE I

Per cent. composition of proteins at different stages of growth of the silkworm

<table>
<thead>
<tr>
<th>Protein band No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg 1</td>
<td>..</td>
<td>1.7</td>
<td>1.7</td>
<td>21.8</td>
<td>21.2</td>
<td>30.7</td>
</tr>
<tr>
<td>Egg 2</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>18.5</td>
<td>40.3</td>
<td>20.4</td>
</tr>
<tr>
<td>Egg 3</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>Larvae I</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>Larvae II</td>
<td>..</td>
<td>..</td>
<td>2.9</td>
<td>4.6</td>
<td>38.6</td>
<td>10.5</td>
</tr>
<tr>
<td>Larvae III</td>
<td>..</td>
<td>3.1</td>
<td>3.1</td>
<td>5.5</td>
<td>35.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Larvae IV</td>
<td>..</td>
<td>2.2</td>
<td>3.0</td>
<td>8.7</td>
<td>31.0</td>
<td>18.3</td>
</tr>
<tr>
<td>Larvae V a</td>
<td>..</td>
<td>0.9</td>
<td>1.8</td>
<td>16.6</td>
<td>29.3</td>
<td>22.2</td>
</tr>
<tr>
<td>Larvae V b</td>
<td>..</td>
<td>0.5</td>
<td>0.7</td>
<td>21.0</td>
<td>22.1</td>
<td>30.0</td>
</tr>
<tr>
<td>Pupae 1</td>
<td>..</td>
<td>1.4</td>
<td>..</td>
<td>23.9</td>
<td>55.3</td>
<td>..</td>
</tr>
<tr>
<td>Pupae 2</td>
<td>..</td>
<td>1.5</td>
<td>..</td>
<td>38.8</td>
<td>59.7</td>
<td>..</td>
</tr>
<tr>
<td>Pupae 3</td>
<td>..</td>
<td>..</td>
<td>26.4</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>Moths</td>
<td>..</td>
<td>..</td>
<td>23.5</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
</tbody>
</table>

Proteins 3 and 5 increased from larval stage to pupation; proteins 5 and 6 disappeared completely within 5 days of pupation, the former disappearing earlier than the latter. Proteins 4 and 6 decreased from larvae I to mature larvae only to increase again during early pupal life. The percentage of proteins at egg I stage corresponded closely with that of mature larvae. Electrophorograms suitable for reading in the densitometer could not be obtained at the third egg and larvae I stages and therefore, the percentages at these two stages are not indicated.

The electrophorograms of the blood of mature larvae did not take up any lipoprotein stain in spite of spotting high concentrations of blood; whereas the Fuchsin stain gave only a uniform staining throughout the plates,
Kobayashi and Komatsu (1956) have reported that protein 6 corresponds to \( \alpha_{\epsilon} \)-globulin in this insect. In the present study it can be clearly seen to lie between \( \alpha_{s} \)- and \( \beta_{s} \)-globulins. Further, no new component in the blood of 5th instar silkworm, which they designate as \( M \), could be discerned. However, protein 5 corresponding to \( \beta_{\epsilon} \)-globulin seems to correspond to their protein \( M \). This observation is in agreement with that of Groulade et al. (1961), who were also unable to observe protein \( M \) at that particular stage. The absence of lipoprotein stain uptake may be due to the extremely small concentration of lipids present in silkworm blood or even due to the limitations of the technique. There was no protein zone corresponding to albumin as claimed by Shigematsu (1958).

Stephen (1961), while studying roaches obtained 5 bands in all the cases and Laufer (1960) studying other species of silkworms found 4 and 5 bands in \textit{Cecropia} and \textit{Cynthia} respectively. We have found six zones consistently throughout the larval stage and this is in contradiction to the four zones reported by Kobayashi and Komatsu (1956), three zones by Aiazawa and Murai (1947) and a number of bands by Denuce (1958). However, the fact that there was a difference in the number of protein zones observed in different species of silkworms indicated that the blood protein pattern was perhaps species specific and therefore could be with advantage used as a tool in identifying different species as suggested by Stephen (1961).

Figures II a, b and c reveal many interesting features. Considerable changes in the number and concentrations of blood protein components during development is obvious. The change in the number of components during larval development is slight since the differentiation is negligible during this period. The absence of protein 1 at the larva 1 stage may be due to its presence at the limit of sensitivity of the protein staining procedure. Laufer (1960) has reported the maximum uptake of stain in \textit{Cecropia} blood during the pupal stage. Such an increase in staining could not be observed in this case; in fact there was a reduction in the intensity of stain uptake in different components from the pupal 1 stage to moth stage. No differences were observed between male and female pupae (9 days old) in both of which only two components got separated into bands. This observation is in sharp contrast with four to five bands reported by Laufer (1960) in \textit{Cecropia} blood. The most interesting observation was however the presence of all the 6 zones at the egg 1 stage. Laufer (1960) has indeed found direct incorporation of certain blood proteins directly into the egg, but not the \textit{en masse} incorporation of all
the proteins as in this case. The gradual disappearance of these proteins during egg development indicates their utilisation for building up of insoluble cuticular proteins.

Whether the change in the migration of proteins 1 and 2 during larval development is due to new proteins per se or due to changes in their mobility itself is not definite. The pattern of protein zones in five-day old and ten-day old pupae are so different that it would be necessary to assume that they are different proteins. If this assumption is correct then the conclusion can be drawn that during pupal development the blood proteins are in a dynamic state, some undergoing degradation and some being newly synthesised.

The differences observed in Figs. II a, b and c become more apparent from Figs. V and VI. The gradual increase in the protein and nonprotein nitrogen contents during larval life is due to the larval development. The considerable increase in the nonprotein nitrogen during the fifth instar is due to the presence of considerable quantities of free amino-acids in blood (Fukuda et al., 1955). There is a 30-35 per cent. decrease in the total nitrogen content of blood within a day of spinning. It is known that during formation of silk 70 per cent. of the protein is preformed protein and only about 30 per cent. is formed by indirect means (Fukuda et al., 1958). The reduction in the nitrogen content of the blood (approximately to the same extent) with the simultaneous disappearance of proteins 3 and 5 can be taken as an indication of blood nitrogen getting transformed into silk during spinning. However, the gradual disappearance of some proteins and appearance of new proteins during pupal development is indicative of the dynamic state of the blood proteins compared to the static nature of the total and nonprotein nitrogen, but their exact status remains to be explained.

Unlike the report of Sauerlander and Ehrhardt (1961) who found the absence of three proteins and a diminution of other proteins in the case of P. americana infected with P. aeruginosa and S. marcescens, no differences could be observed in the blood protein patterns between the normal and flacherie attacked worms, except for a reduction of total nitrogen value itself. Since the blood available from the worms attacked by grasserie was very small, the total nitrogen could not be estimated. However, the complete absence of almost all the globular-like proteins in this case is noteworthy and indicates some sort of precipitin reactions.

From Fig. IV some indications as to the origin of protein 5 can be obtained. The fact that this protein is absent in worms whose silk glands were
removed when considered along with the disappearance of the same protein within a day of spinning may very well be due to such a protein being synthesized as well as degraded by the silk glands. Since silk represents almost all the protein that is synthesized during the fifth instar by the silk glands, protein 5 may be considered to act as a reservoir of the protein synthesized by the silk glands or as a means of transport of the blood amino-acids to the silk glands. Attempts to determine the origin of other proteins, by running electrophorograms of extracts of silk glands, intestines, and the fat body did not yield any results. However, Shigematsu (1958) has shown the fat body to be the site of synthesis of protein 3.

In conclusion, it can be said that the silkworm has its own characteristic protein pattern at different stages of growth though it is difficult to interpret with any exactitude its significance in insect physiology.

SUMMARY

The qualitative and quantitative aspects of the proteins of the silkworm blood were studied by the technique of agarophoresis. The blood of larvae at the final stage revealed the presence of six different protein zones. Considerable differences in the patterns were observed at different stages of growth. There was an increase in the total nitrogen of the blood up to the 5th instar and then came a sudden decrease in the one-day old pupae. Nitrogen concentration was at its highest in egg 1 stage and the electrophoretic pattern closely corresponded to the final larval pattern. Results indicate to the involvement of silk glands in the synthesis and breakdown of a protein designated as protein 5.

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REFERENCES


FIG. I. Electrophoretic pattern of normal human serum compared with silkworm blood.

FIG. II(a). Electrophoretic patterns of silkworm blood at various stages of growth.
Fig. 11(b). Electrophoretic patterns of silkworm blood at various stages of growth.
Fig. II (c). Electrophoretic patterns of silkworm blood at various stages of growth.
FIG. III. Electrophoretic patterns of normal silkworm blood and of those attacked by flacherie and grasserie.

FIG. IV. Electrophorograms of blood of normal and silk gland removed silkworms.
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