Studies on the Haemolymph Proteins during the Metamorphosis of the Pine Moth, *Dendrolimus spectabilis* Butler

Chong Myung Yoo and Kyung Ro Lee*

(Biology Lab., Soong Jun Univ., *Dept. of Biology, Kon Kuk Univ.)

솔나방의 變態에 따른 血蛋白質의 變化

柳 鍾 鳴・李 敬 魯*
(崇田大 生物學敎室・*建國大 生物學科)

摘　　要

山林害虫인 솔나방(*Dendrolimus spectabilis* Butler)의 變態에 따른 血蛋白質의 變化를 調査하기 위하여 acrylamide gel 電氣泳動法을 이용하여 測定한 結果는 다음과 같다.

1. 血蛋白質의 band는 移動度에 따라 22개(1—22)의 종류를 나타냈다.

2. 血蛋白質의 濃度는 band 數, 染色强度, 移動度를 가지고 비교할 때 stage에 따라서 定量的 差異를 나타내며, 幼虫이 성장함에 따라 增加하였고, 終齡幼虫에서 가장 높은 濃度를 나타냈다.

3. 血蛋白質의 band는 終齡幼虫을 지나 幼虫器官의 解消가 일어나는 蛹前期에서 減少하고, 成虫器官의 新生이 시작되는 蛹後期에서 다시 增加하였다.

4. PAS 反應, toluidine, Sudan black을 이용한 조직화학적 반응결과 終齡幼虫에서 가장 많은 glycoprotein, mucopolysaccharide, lipoprotein 등이 검출되었다.

5. 脂肪體와 腸에 있어서도 血蛋白質의 band 數와 비슷한 特性을 나타내었고 濃度의 變化도 유사하였다.

INTRODUCTION

The haemolymph of inscts is in direct contact with body tissues and thus its variations in composition provide adequate criteria to judge the metabolic activity associated with the differentiation process. The haemolymph proteins have been

studied by a large number of workers from various viewpoints, especially since
the advent of such techniqes as starch and polyacrylamide gel electrophoresis
which give a high resolution of the protein fractions. The results of earier studies
have been reviewed by Buck (1953) and more recently by Wyatt (1961) and Chen
(1966). A number of studies (Telfer and Williams, 1959a; Whittaker, 1959a ;
Hudson 1966; Chen and Levenbook, 1966 ; Zamburlini and Danieli, 1970 ; Pasteur
and Kastritsis, 1971 ; Pentz and Kling, 1972) shown that haemolymph proteins
change during development and metamorphosis of the insect.

   Gilbert and Schneiderman (1961) reported that the protein content of the haem-
olymph is increased during the successive larval stage, but is decreased in the adult.
Duke and Pantelouris (1963) studied the protein patterns of *Drospphila* larva by
means of starch gel electrophoresis and found that the soluble proteins increased
from 1 band in the Ist instar larvae to a total of 11 in the late 3rd instar. Chen
and Levenbook (1966) observed that the total protein concentration rises rapidly
in the growing larva of blowfly, goes down in the early pupa and during
adult differentiation, and falls off sharply at the time of the emergence of the
adult. They have identified a total of 19 haemolymph proteins in the developing
Phormia larvae. Van Der Geest and Borgsteede (1969) investigated the protein
changes in the haemolymph of *Pieris brassicae* during the last larval instar and
the beginning of the pupal stage, and found that a number of high molecular
weight proteins were increased in concentration during the development, and in
addition, they identified the presence of the enzymes, such as esterases, leuci-
aminopeptidase, alkaline and acid phosphatases.

   Zamburlini and Danieli (1970) separated the soluble proteins of the larvae and
the haemolymph of *Drosophila hydei* at different stages. They reported that the
larval age could be recognized from the electrophoretic pattern of the larval
proteins, and identified 20 discreate bands at the end of development.

   Pasteur and Kastritsis (1971) studied the changes in protein patterns of whole
fly homogenates, haemolymph, fat body, and salivary glands, and found the exi-
stence of both qualitative and quantitative changes in them. The present study
was undertaken to determine the changes of pattern of soluble haemolymph
proteins during the development and metamorphosis of pine moth, *Dendrolimus
spectabilis* Butler and to identify these proteins by use of specific staining methods.
The study on the tissue patterns was also accompained.

MATERIALS AND METHODS

Test Insect
The overwintering larvae of pine moth were collected frcm the vicinity of

Taejon city and reared at 26°C in a rearing box containing pine needles. The pine needles were changed daily, and at the desired age the samples were obtained from the box. The experiments were carried out at the following stages; larval, prepupal, pupal, adult, and egg stages.

Collection of Haemolymph

The animals were rinsed in distilled water and dried with absorbent paper. The haemolymph was aspirated into a micropipette and collected in a glass cooled in an ice-bath. Haemocytes were removed by centrifugation. In general, the largest haemolymph volumes were obtained from the 8th instar larval stages. In all cases a few crystal of phenylthiourea (PTU) were added to the pooled haemolymph in order to inhibit tyrosinase action. Until further experiments the haemolymph was stored at−20°C.

Protein Determination

Protein concentration was measured by the biuret reaction according to the method of Gornal et al. (1949).

The proteins were first precipitated with 5% trichloroacetic acid, and the absolute values were calculated from a standard curve prepared from pure serum albumin.

Electrophoresis

Polyacrylamide gels for electrophoresis were prepared with Canalco's premixed solutions according to the technique of Ornstein and Davis (1964). The electrophoretic compartment was made at the Zoological Institute, Cologne University and the power supply was obtained directly from Heizinger Co., München, Germany. In this experiment, the writers used a 7.5% gel of polyacrylamide and Tris glycine buffer (pH 8.3). And 5μl haemolymph was applied to each gel tube for obtaining an acceptable electrophoresis, which was stopped when the tracking dye (BPB) reached 1 cm from the bottom of the tube. After the migration was complete, the gels were removed from the glass tube. The electrophoresis runs for each stage were repeated 5 times.

Stains

General proteins were stained in a saturated solution of Amido Black 10B in 7% glacial acetic acid for 24 hours. Destaining was achieved in 24 hours by diffusing out in a 7% glacial acetic solution, and the gels were preserved in 7% aectic acid.

Glycoproteins were identified by the method of Caldwell and Pigman (1967). Gels were incubated for 1 hour at room temperature in 7.5% acetic acid and then for 1 hour in 0.2% periodic acid at 4°C.

They were subsequently transferred to Schiff's reagent after being rinsed in 15% acetic acid. The glycoproteins show up as purple bands. For the detection of lipoproteins, the method of Chippendale and Beck (1966) was used. Excess stain

was removed after 24 hours in 70% ethanol. Acidic mucopolysaccharides was
stained in 1% solution of toluidine-blue in 3.5% acetic acid followed by destaining
in 3.5% acetic acid (Rennert, 1967).

RESULTS

1. Protein concentration

Table 1 shows the concentration of haemolymph proteins during development
and metamorphosis of pine moth, *Dendrolimus spectabilis* Butler. As can be seen
from the data summarized in Table 1, the protein concentration increased grad-
ually with the growth of larva. Particulary impressive is the rapid increase in
the concentration of haemolymph protein in the mature larva shortly before
puparium formation. The haemolymph protein concentration was 87mg/ml in the
early 8th instar larva, but it had increased to 102mg/ml in the late 8th instar.
In the prepupa and pupa, the protein concentration decreased markedly, showing
a minimum value in the adult.

Table 1. Change in haemolymph protein concentration during the
metamorphosis of the pine moth, *Dendrolimus spectabilis* Butler

Developmental stages	Protein concentration (mg/ml)
Overwintering larva	47
4th instar larva	50
5th instar larva	62
6th instar larva	68
7th instar larva	73
8th instar larva I (early stage)	87
8th instar larva II (middle stage)	92
8th instar larva III (late stage)	102
Prepupa I, 1 day	84
Prepupa II, 2 days	66
Pupa, 1 day	64
Pupa, 3 days	61
Pupa, 7 days	55
Pupa, 10 days	56
Pupa, 14 days	55
Adult, 1 day (female)	21

From Table 1, it is clear that there are two stages which show a distinct drop
in the protein concentration during the transformation of the larva into the
prepupa, and at the time of adult emergence.

2. Ontogenetic patterns

Haemolymph protein patterns of the pine moth, *Dendrolimus spectabilis* Butler
at different stages from the overwintering larva to the adult were examined by

means of acrylamide gel disc electrophoresis. Figs. 1, 2, 3, and 4 show a series of protein patterns in the haemolymph of pine moth of varying intensities which appear to change in number as in concentration, or intensity, throughout the life cycle. For convenience the protein bands were numbered from 1 to 22, starting with the most rapidly migrating component. By this experiment, a total of 22 protein bands were obtained. However, these bands were not identified commonly at all stages. The drawings were made by gel-to-gel comparisons out the drawings for the slow moving bands were difficult because they were very close one another. As represented in Figs. 1, 2, 3 and 4, each developmental stage of pine moth exhibits both qualitative and quantitative differences in their protein patterns and, in general, the number of protein bands increases with the development of larvae, whereas the increase in conentration of several bands occurs, too. Band 18 is the predominant one in all stages. This band increases rapidly in its concentration so that the distinction among bands, 18, 19, 20 and 21 is no longer apparent in the 8th inster larval and prepupal stages. But in the pupal, adult, and egg stages, this band appears as two bands. And 10 bands, 1, 2, 3, 5, 7, 10, 11, 13, 16 and 17 occur only in specific developmental stages. Of them, band 1 appears only in the stages from the 7th instar larva to the prepupa, and band 10, in the late pupa and adult stages, respectively. The protein pattern of the egg has a different picture, and the number of bands was reduced signficantly, comparing with those of larval, pupal, and adult stages. Fig. 5 shows the changes in protein patterns of haemolymph, fat body and intestine. Each tissue exhibits its specific pattern. Band 18 in the haemolymph is present as several thin bands in fat body and intestine. And bands, 1, 3, 6, 8, 9, 13, 15, 16, 17 and 18 are the proteins which are common to three tissues at this stage. Histochemical studies of general proteins were performed in the manner described above. The results of experiment are shown in Figs. 6, 7 and 8. Several proteins were stained with Schiff's reagent and Sudan black, These proteins are glyco- and lipoproteins, and present mainly in the slow moving fraction. Three acidic mucopolysaccharides were also found.

DISCUSSION

The change in protein concentration in the haemolymph of the pine moth, *Dendrolimus spectabilis* is essentially similar to those already found for *Deilephila euphorbiae* (Heller and Moklowska, 1930), *Hyalophora cecropia* (Chefurka, 1953), *Popillia Japonica* (Ludwig, 1954), *Bombyx mori* (Wyatt et al., 1956), *Samia cynthia* (Laufer, 1960), *Phormia regina* (Chen and Levenbook, 1966), *Pieris brassicae* (Van Der Geest, 1969), and *Ephestia kühniella* (Cölln, 1969). In general, the concentration of protein in insects rises in the late larval stage, decreases in the pupa and

during adult development, and falls sharply at the time of adult emergence. The concentration in the present study is increased with the development of larva and showed a maximum in the mature larva, and decreased thereafter.

In the newly emerged adult the protein concentration falls to a value as low as 21mg/ml. In particular, a marked increase of protein concentration in pine moth occurred in the 8th instar larva III stage. Wyatt et al. (1956) reported that the haemolymph protein in *Bombyx mori* rose from 1.2% in the early 3rd stage to 5.3% in the late 5th stage. Apparently the same is true for *Samia cynthia* whose protein concentration increases rapidly from the 3rd instar to a maximum in the spinning 5th instar (Laufer, 1960). Chen and Levenbook (1966) reported that the average haemolymph protein content was found to be nearly 20% in the fully-grown larva of *Phormia regina*. The concentration changes of proteins in insects are either indirectly the result of hormone action or are the endocrine secretions themselves (Laufer, 1960). These facts were also clearly demonstrated by the implantation experiment of corpora allata for the 5th instar larva of *Pyrrhocoris apterus* (Brettschneiderova and Novak, 1965).

The changes in protein concentration are also obvious from the electrophoretic patterns of haemolymph. The protein patterns present an interesting changes. They change significantly throughout the larval-pupal-adult transformations. Similar changes in protein pattern were observed by Telfer (1953) who examined cecropia haemolymph with immunochemical procedures. The over-all increase of protein concentration in bands occurs rapidly from the 5th instar larva, and the number of protein bands also increases with the larval development, the maximum number being at the last instar larval stage.

That is; the soluble proteins of haemolymph increased from 16 bands in the 4th instar larva to a total of 22 in the last instar larva. Bands 1, 2, 3, 10, and 12 began to appear from the 7th, 6th, 5th, and 7th instar larval stages, respectively. However, after the larval stages, the appearance and disappearance of these bands and others are clearly discernible.

The fact that each protein component appears at a definite stage and exhibits its own ontogenetic pattern of variation implicates a genic control of synthesis (Chen and Levenbook, 1966). Band 5 appears only in larval stages, hence this band seems "larval protein." The patterns of several glyco- and lipoproteins were found to change during the metamorphosis. These proteins increased rapidly in concentration in the 8th instar larva III, but during the prepupal and pupal stages, a decrease of these high molecular weight proteins was noted. Particularly, the change of glycoprotein is in accord with the findings of Van Der Geest and Borgsteede (1969) in *Pieris brassicae*. Carbohydrates in the haemolymph of insect are reported to present in the form of glycoproteins (Wigglesworth, 1965). It is

assumed that they are mainly reserves which are being used during pupation (Van Der Geest and Borgsteede, 1969). Some possible functions of haemolymph proteins have been suggested by several investigators. Laufer (1960, 1961, 1964) demonstrated that nearly all blood proteins of *Hyalophora cecropia* and *Sama cyinthia* act as enzymes. Subsquently, Siakotos (1960a, b) reported that blood proteins of cockroach *Periplaneta* may be involved in the transport of nutrients such as lipids and carbohydrates which are joined to the proteins as conjugated groups.

SUMMARY

The blood proteins of pine moth, *Dendrolimus spectabilis* Butler of different developmental stages were investigated by disc electrophoresis in acrylamide gels. Blood protein concentration was also determined during the metamorphosis. Protein concentration increased gradually with the growth of larva, reaching a maximum in themature larva, and the increase of protein bands also was accompanied. As the larva transforms into the prepupa the number of protein bands as well as the protein concentration dropped. A total of 22 bands were identified throughout the stages. Histochemical staining of the acrylamide gels by the PAS method, Toluidine blue O, and Sudan black demonstrated that the carbohydrate, mucopolysaccharide, and lipid were associated with certain blood proteins.

REFERENCES

Brettschneiderova, Z. and V.J.A. Novak, 1965. The effect of the juvenile hormone on the protein level in the haemolymph of the bug *Pyrrhocoris apterus* during metamorphosis. *Zool. Jb. Physiol. Bd.* **71** : 665—668.

Buck, J. B., 1953. Physical properties and chemical composition of insect blood; *In* Roeder: *Insect Physiology*, pp. 147—190 Wiley, New York.

Caldwill, R.C. and W. Pigman, 1965. Disc electrophoresis of human saliva in polyacrylamide gels. *Archs Biochem. Biophys.* **110** : 91—96.

Chefurka, W., 1953. Biochemical studies on the blood of the giant silkworm, *Platysamia cecropia*. Thesis, Harvard University, Cambrige, Massachusetts.

Chen, P.S., 1966. Amino acid and protein metabolism in insect development. *Adv. Insect Physiol.* **3** : 53—132.

Chen, P.S. and L. Levenbook, 1966. Studies on the haemolymph proteins of the blow fly, *Phormia regina* 1. Changes in ontogenetic patterns. *J. Insect Physiol.* **12** : 1595—1605.

Chippendale, G.N. and S.d. Beck, 1966. Haemolymph proteins of *Ostrinia nubilalis* during diapause and prepupal differentiation. *J. Insect Physiol.* **12** : 1629—1638.

Cölln, K., 1969. Unterschungen zur Ontogenese der Haemolymphe Protein von *Ephestia kühniella. Z. Verh. Zool. Ges.* **29** : 121—127.

Davis, B.J., 1964. Disc electrophoresis-11. Method and application to human serum prote-
    ins. *Ann. N.Y. Acad. Sci.* **121** : 404—427.

Duke, E.J. and E.M. Pantelouris, 1963. Ontogenesis of lymph proteins in *Drosophila mel-
    anogaster*. *Comp. Biochem. Physiol.* **10** : 351-355.

Gilbert, L. I. and H.A. Schneiderman, 1961. Some biochemical aspects of insect metamo-
    rphosis. *Amer. Zool.* **1** : 11—51.

Gornall, A.G.,C.J. Bardawill and M.M. David, 1949. Determination of serum proteins by
    means of the biuret reaction. *J. Biol. Chem.* **177** : 751—777.

Heller, J. and A. Moklowska, 1930. Ueber die Zusammensetzung des Raupenblutes bei
    Deilephila euphorbiae und deren Veranderungen im Verlaufe der Metamorphose. VII.
    Chemisch Untersungen uber die Metamorphose der Insecten. *Biochem. Z.* **219** : 473—489.

Hudson, A., 1966. Proteins in the haemolymph and other tissues of the developing tomato
    hornworm, *Protoparce quinquemaculata* Haworth. *Can. J. Zool.* **44** : 541—555.

Laufer, H., 1959a. Identification and characterization of some blood proteins in the deve-
    lopment of giant silk moths. *Anat. Record.* **134** : 597.

Laufer, H., 1960. Blood proteins in insect development. *Ann. N.Y. Acad. Sci.* **89** : 490—515.

Laufer, H., 1961. Forms of enzymes in insect development. *Ann. N. Y. Acad. Sci.* **94** :
    825—835.

Laufer, H., 1964. Macromolecular patterns in development and evolution. *In*: Taxonomic
    Biochemistry and Serology (Leone C.A., Ed.), pp. 171—189.

Ludwig, D., 1954. Changes in distribution of nitrogen in blood of Japanese beetle, *Popilla
    japonica* Newman, during growth and metamorphosis. *Physiol. Zool.* **27** : 325—334.

Ornstein, L.A. 1964. Disc electrophoresis-1. Background and theory. *Ann. N.Y. Acad. Sci.*
    121 : 312—349.

Pasteur, N. and C.D. Kastritsis, 1971. Developmental studies in *Drosophila*. 1. Acid pho-
    sphatases, esterases, and other proteins in organs and whole-fly homogenates during
    development of *D. pseudoobscura*. *Develop. Biol.* **26** : 525—536.

Pentz, S. and H. Kling, 1972. Protein pattern in the haemolymph of the pharate pupa of
    Plodia interpunctella. J. Insect Physiol. **18** : 2277—2283.

Rennert, O.M., 1967. Disc electrophoresis of acid mucopolysaccharides. *Nature. Lond.*
    213 : 1133.

Siakotos, A.N., 1960a. The conjugated plasma proteins of the American cockroach. 1. The
    normal state. *J. Gen. Physiol.* **43** : 999—1013.

Siakotos, A.N., 1960b. The conjugated plasma proteins of the American cockroach. 11.
    Changes during the molting and clotting process. *J. Gen. Physiol.* **43** : 1015—1030.

Telfer, W.H. and C.M. Williams, 1953. Immunological studies of insect metamorphosis.
    1. Qualitative and quantitative description of the blood antigens of the cecropia silk-
    worm. *J. Gen. Physiol.* **36** : 389—413.

Van Der Geest and F.H.M. Borgsteeds, 1969. Protein changes in the haemolymph of *Pieris
    brassicae* during the last larval instars and the beginning of the pupal stage. *J.
    Insect Physiol.* **15** : 1687—1693.

Whittaker, J.R., 1959. A starch gel electrophoretic study of insect haemolymph proteins. M. Sc. Thesis, Queen's University, Kingston, Ontario.

Wigglesworth, V.B., 1965. The Principles of Insect Physiology. Methun. London.

Wyatt, G.R., T.C. Lougheed and S.S. Wyatt. 1956. The chemistry of insect haemolymph of the silkworm, *Bombyx mori*, and two other species. *J. Gen. Physiol.* **39** : 853—868.

Zamburlini, P. and G.A. Danieli, 1970. Acrylamide gel electrophoresis of *D. hydei* proteins at different stages of larval development. *DIS* **45** : 85—86.

EXPLANATION OF FIGURES
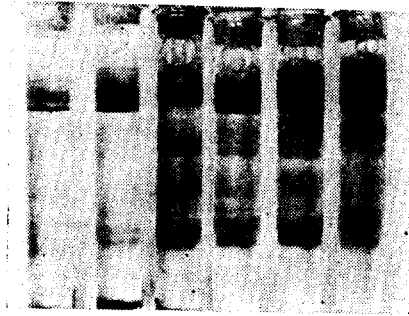
Fig. 1. Photograph of haemolymph protein patterns in *Dendrolimus spectabilis* Butler; Left to right, A: overwintering larva; B: 4th instar larva; C: 5th instar larva; D: 6th instar larva; E: 7th instar larva; F: 8th instar larva I (early stage); TD: trcking dye.
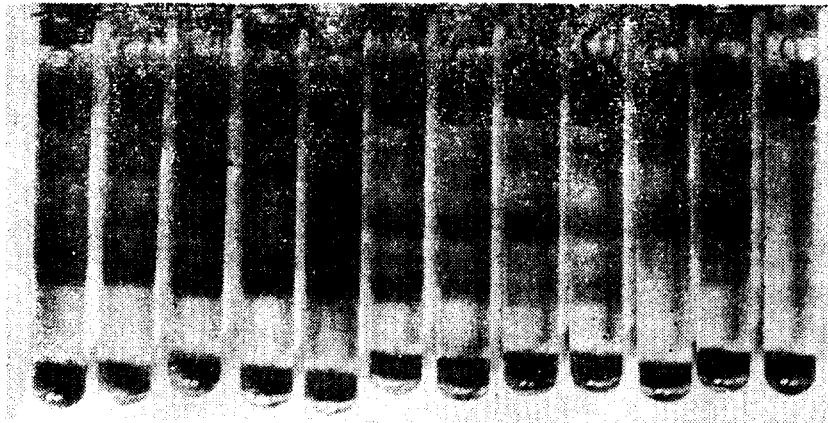
Fig. 2. Photograph of haemolymph protein patterns in *Dendrolimus spectabilis* Butler. Left to right, F: 8th instar larva I; G: 8th instar larva II; H: 8th instar larva III; I: prepupa I; J: prepupa II; K: pupa, I day; L: pupa, 3 days; M: pupa, 7 days; N: pupa, 10 days; O: pupa, 14 days; P: adult, I day (female); Q: egg stage.
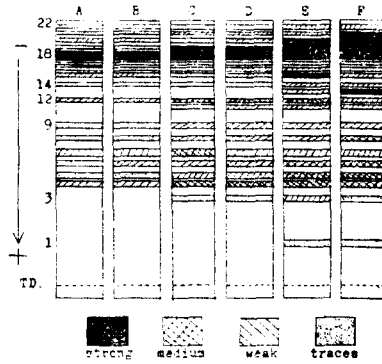
Fig. 3. Diagrammatic representation of haemolymph protein pattern in *Dendrolimus spectabilis* A: Overwintering larva; B: 4th instar larva; C: 5th instar larva; D: 6th instar larva; E: 7th instar larva; F: 8th instar larva; TD: tracking dye.
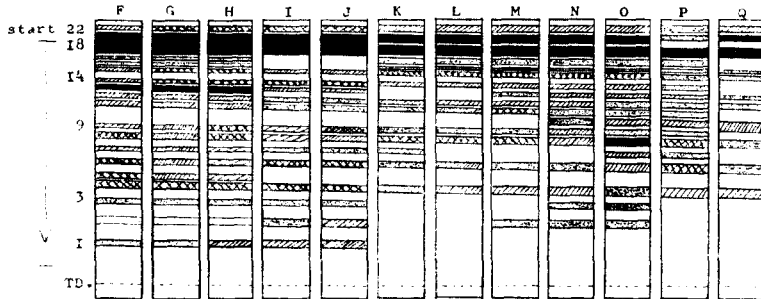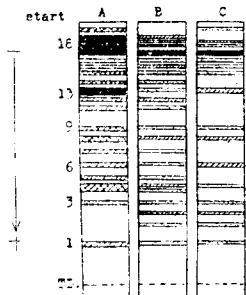

Fig. 4. Diagrammatic representation of haemolymph protein pattern in *Dendrolimus spectabilis* Butler.
F: 8th instar larva I; G: 8th instar larva II; H: 8th instar larva III; I: prepupa I; J: prepupa II; K: pupa, I day; L: pupa, 3 days; M: pupa, 7 days, N: pupa, 10 days; O: pupa, 14 days; P: adult, I day (female); Q: egg stage; TD: tracking dye.

Fig. 5. Changes in electrophoretic pat-
terns of protein in several tissues of the
8th instar larva 111.
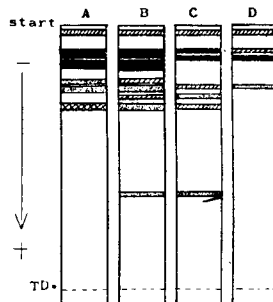A: haemolymph;   B: fat body;
C: intestine

Fig. 6. Changes in glycoprotein patte-
rns of haemolymph during the met-
amorphosis of *Dendrolimus spectabilis*
Butler.
A: 8th instar larva II; B: 8th instar
larva III (spinning stage); C: prepupa
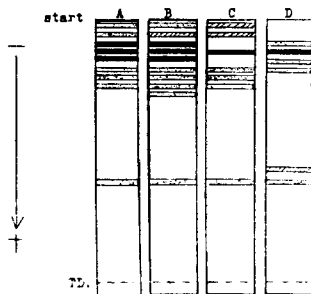I; D: pupa, 14 days; TD: tracking dye.

Fig. 7. Changes in lipoprotein patterns
of haemolymph during the metam-
orphosis of *Dendrolimus spectabilis*
Butler.
A: 8th instar larva II; B: 8th instar
larva (spinning stage); C: prepupa 1;
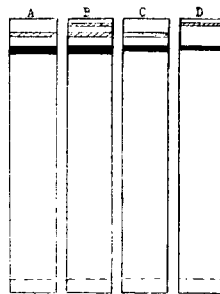D: pupa, 14 days; TD: tracking dye.

Fig. 8. Changes in acidic mucopolysa-
ccharide patterns of haemolymph during
the metamorphosis of *Dendrolimus
spectabilis* Butler.
A: 8th instar larva II; B: 8th instar
larva (spinning stage); C: prepupa 1;
D: pupa, 14 days.