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Changes of haemolymph proteins in *Pieris rapae* L. during the cuticle formation and hardening process

Hak Ryul Kim and Eul Won Seo
(Department of Biology, Korea University)

배추흰나비의 큐티클 形成과 硬化에 따른 혈림프 단백질의 變化

金 學 烈·徐 乙 源
(高麗大 生物學科)
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摘 要

큐티클 形成및 硬化過程中 血蛋白質의 變化와 起源을 규명하고자 acrylamide gel electrophoresis와 immunodiffusion 方法을 使用하였다.

Acrylamide gel electrophoresis에서 적어도 19개의 protein band가 혈림프에서 發見되었으며 脂肪體에서는 13개의 band가 確認되었는데 이들은 대체적으로 일정한 pattern을 유지하였다. 또한 혈림프와 脂肪體의 一般的인 protein band의 pattern은 3~4개의 강하게 染色된 band와 몇개의 가는 band가 gel의 상단에 存在하는 것이 특징이었고 적어도 5개 이상의 haemolymph protein band가 蛹期初에 걸쳐 일정하게 나타났다.

Immunodiffusion test에서는 8~9개의 血蛋白質이 蛹期初에서 나타났는데 그중 2개의 血蛋白質은 蛹期前에 脂肪體에서 나타났으며 다른 두 血蛋白質도 蛹化직 후 脂肪體에서 나타남으로써 脂肪體가 이들 血蛋白質의 起源임을 암시하였다.

INTRODUCTION

Changes in the protein pattern of the haemolymph during the metamorphosis involving drastic morphological changes such as histolysis and histogenesis indicate different functional conditions in the metabolism.

Laufer (1960, 1963) has demonstrated specific changes in the haemolymph protein composition related to development, hormonal activity, and ovarian growth in insects. Chen and Levenbook (1966) also proved characteristic changes of protein pattern in the haemolymph during both pupal and adult stages. Pentz and Kling

(1972), on the while, found remarkable changes in the protein pattern at the critical point after ecdyson secretion. In relation with cuticle formation, Tobe and Loughton (1969) have shown that blood proteins are not degraded to amino acids before incorporation into cuticle and Fox *et al.* (1972) also suggested that a number of haemolymph proteins are present at all stages and some of these proteins are routinely absorbed from the blood into the cuticle relatively unchanged during cuticle formation and hardening process.

Present work concerns protein changes and their possible origin in the haemolymph especially during the period of cuticle formation and hardening process.

MATERIALS AND METHODS

Cabbage worms (*Pieris rapae* L.) were reared in the vinyl house and the last inster larvae were transferred to the laboratory room and grown on the cabbage leaves in rearing box until moulting to prepupa. Haemolymph and fat body samples were taken from the pupae at the intervals of 0, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7, and 8 hour after ecdysis. For convenience' sake, these intervals were described as fallows; S1 for newly ecdysed, S2 for 15 min after pupation, S3 for 30 min after pupation, and S4~S11 for 1~8 hr after pupation respectively.

Collection of haemolymph and fat body

Haemolymph was aspirated into micropipette from pupa rinsed in the distilled water and stored in the test tube cooled in an ice-bath. As a moderate amount of haemolymph was collected, haemocytes and tissue fragments were removed from the haemolymph by centrifugation at 3,000 rpm for 15 min and a few crystals of phenylthiourea were added to the haemolymph samples in order to prevent blackening of the haemolymph with melanin, formed as the result of tyrosinase activity. It was found that phenylthiourea is a tyrosinase inhibitor and has no effect on the general protein pattern of the haemolymph (Geest and Borgsteede, 1969). The haemolymph samples were then stored at −20° C. The fat body was isolated from the pupa in a cold Ringer solution and as a moderate amount of fat body was collected, they were homogenized and centrifuged at the same conditions as described in the above. The supernatant was then carefully pipetted out and used as samples in all experiments.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in 5% acrylamide gel and Tris-borate buffer (Tris 9.825 g, Boric acid 0.765 g, EDTA 0.444 g, Calcium lactate 0.384 g in 1,000 ml) at pH 8.9. Ten μl of either haemolymph or fat body samples were applied to each gel tube. The experiment was repeated at least five times at each stage. Gels were stained in Coomassie Brilliant blue (0.25%) for 1 hr,

destained in 7% glacial acetic acid, stored in 2% acetic acid, and scanned with TG 2970 densitometer.

Immunodiffusion

Antiserum was prepared by injecting rabbits with 0.8 ml Freund's complete adjuvant and 0.2 ml of pooled haemolymph from pupa 30 min after ecdysis as previously described by Terando and Feir (1966). The adjuvant is necessary in order to obtain a high titer of antibodies against haemolymph antigens (Walz, 1963). The injections were given at the intervals of two days during the first week. The fourth injection was given 1 week after the third. Two weeks after the fourth injection, the fifth one was given. Forty-eight hours later blood was taken from the marginal ear vein. The antiserum was stored at −20° C. Immunodiffusion was performed in agar as described by Ouchterlony (1958). Haemolymph and fat body samples were placed in Ouchterlony plates where they were allowed to react with antiserum to pupal haemolymph. They were stained with Amido Black 10 B. For comparison with human serum, the human serum was also allowed to react with antibody to pupal haemolymph.

RESULTS

Acrylamide gel electrophoresis

Haemolymph samples from the early pupal stage show little qualitative difference in their protein components (Fig. 1 and 3). At least 19 haemolymph protein bands are separated, though not all of them occur at any one single stage. For purposes of comparison, the protein bands were numbered by 1 through 19 according to their mobilities (Fig. 1). Band 10 which is most intensely stained throughout all stages was considered as the main haemolymph protein (Fig. 3). Other 7 bands (2, 4, 5, 7, 8, 13, 14) are also present at all stages, though their intensity are somewhat lighter than that of band 10, whereas 11 bands (1, 3, 6, 9, 11, 12, 15, 16, 17, 18, 19) occur only at the specific developmental stages. The haemolymph of these stages is characterized by the presence of 3 to 5 very thin bands as well as 3 to 4 densely stained bands near the top of the gel. Also, as shown in Fig. 3, intensities of bands 4, 5, 6, 7, 8 and 9 in the middle region of the gel drastically drop to low level from immediately after pupation to 30 min after ecdysis, and thereafter maintain a low level consistently throughout that period.

There are three relatively fast migrating anodic bands (1, 2, 3) over all stages, of which upper fraction (band 3) is mostly present after stage S7 (4 hrs after pupation) with the exception of the presence at stages S3 and S4 (30 min and 1 hr after pupation), and the lower one appears intermittently only within 3 hrs after pupa-

tion, whereas middle rather thick fraction occurs at all stages. Several thin bands (16, 17, 18, 19) in S3 (30 min after pupation) increase rapidly in their concentrations so that the distinctions between them are no longer apparent in haemolymph one hour after pupation (S4). These similar cases occur between stages S6 to S7 and stages S8 to S9 respectively. There are two bands between bands 10 and 13 at stages S4, S8 while one band between them at stages S7, S9, S10, and S11. Band 9 is also present between bands 8 and 10 at stages S1, S4, S5, S6, S8, S10, and S11 while band 6 appears consistently up to stage S9.

One notable thing is that band 15 which is alway densely stained, is gradually narrowed from stages S1 (newly ecdysed pupa) to S4 (1 hr after pupation) but drastically broadened until stage S6 (3 hrs after pupation), then again gradually narrowed to disappearance at stage S10 (Fig. 1).

Compared with rather diverse protein pattern in haemolymph, protein pattern of fat body has somewhat different picture. It shows a relative similarity between all developmental stages (Fig. 2). Total 13 bands are obtained from the fat body at all stages. Bands 8 and 10 are intensely stained throughout all stages but band 10 is broader than band 8 and divided into two bands between stages S4 and S9. Other 6 bands (1, 2, 4, 6, 7, 13) appear over the whole period but 4 bands (3, 5, 9, 12) are present only at the particular stages. That is, band 12 is present at stages S2 and S9 but band 9 between stages S1 to S2 and S8 to S11 and band 5 appears only at stages S1, S4 and between stages S9 and S11 while band 3 occurs at stages S1, S2, S4, S5 and between stages S7 and S11. A transverse relationship between haemolymph proteins and fat body proteins could not be determined mainly because they are runned at different season. To solve a part of this problem, immunodiffusion was applied.

Immunodiffusion

In relationship between haemolymph proteins and fat body proteins, the immunodiffusion tests with haemolymph show three precipitin lines at prepupal stage and eight to nine precipitin lines during up to 8 hr pupal stage (Fig. 4a, b). In any case human serum did not react with antiserum against insect haemolymph. All three lines of the prepupal stage are continuous with neighbouring pupal precipitin lines and eight to nine lines of the early pupal stage are also continuous each other between different haemolymph samples. These data indicate that at least three of newly pupal haemolymph proteins are already present in haemolymph of prepupa and all newly pupal haemolymph proteins occur constantly without qualitative changes during the early pupal stage. In relation with the extracts of the fat body (Fig. 4c), on the while, two precipitin lines were found at the prepupal stage while three to four lines are present during up to 8 hr pupal stage. All corresponding lines are continuous between different fat body samples, showing that at

least two pupal haemolymph proteins are already present in fat body of prepupal stage while four proteins including above two prepupal proteins are constantly present in fat body during the early pupal stage. In the reaction with the extracts of the fat body and the haemolymph on the same plate (Fig. 4d), there appear also at least two precipitin lines continuous each other between fat body and haemolymph of prepupal stage while four lines between fat body and haemolymph during up to 8 hr pupae.

DISCUSSION

Several authors have found fifteen to over twenty different proteins in insect haemolymph (Chen and Levenbook, 1966; Chippendale and Beck, 1966; Wang and Patton, 1968). However, there was no intensive study on changes of haemolymph proteins especially during the cuticle formation and hardening process. The present work showed that in *Pieris rapae* 19 protein bands are present in the haemolymph while 13 fractions in the fat body and they remain at a rather constant level during this period.

These findings well support the report by Chen and Levenbook (1966) that haemolymph proteins remain at a rather constant level during pupal stage and prove more substancially that this situation could be applied to the period of cuticle formation immediately after ecdysis. Geest and Borgsteede (1969) found in *Pieris brassicae* that some quantitative changes occur during larval and pupal development; that is, the intensity of a number of protein bands increases rapidly toward the end of the larval life with especial concentration increase in the slow-moving fractions but decrease during the pharate pupal and pupal stages.

Present electropherograms also show a relatively similar picture to those of the early pupal stages of *Pieris brassicae.*

Loughton and West (1965), on the other hand, found the progressive formation of new haemolymph protein fractions and the disappearance of others during the ontogeny of *Malacosoma americanum, Rothschilotia orizaba,* and *Vespula arenaria,* and McCormick and Scott (1966) indicated that among a total of 19 protein fractions separated in the haemolymph of the developing *Locusta migratoria migratorioides,* one band appears shortly before the moult, reaches a maximum at the time of moulting, decreases thereafter, and disappears entirely after 24 hrs. The present study also shows that band 15 which is densely stained gradually decreases until 1 hr after ecdysis (Fig. 1) and also bands 4, 5, 6, 7, 8, and 9 strikingly decrease during first half an hour after pupation (Fig. 3). Considering that most of the cuticle protein is synthesized within 1 hr after ecdysis in *Pieris rapae* (Kim *et al.*, 1979) and some haemolymph proteins are absorbed directly into the cuticle relativ-

ely unchanged during cuticle formation (Fox *et al.*, 1972), this gradual decrease could be considered in relation with formation of cuticle protein. Also, temporal absence of certain bands (for examples bands 1, 3, 9, 11, 12) shown in Fig. 1 might be interpreted to be due to concentrations of these proteins below the detectable level rather than to their total absence.

On the origin of haemolymph proteins, Laufer (1960) found, by using starch-gel electrophoresis, three proteins in the fat bodies of female giant silk moth with similar mobilities to those of three components of the haemolymph, and considered that the fat body was strongly implicated as the source of those haemolymph proteins. Price (1973) also reported that the fat body synthesizes many of the serum proteins in a variety of insect.

Present data also clearly prove that some haemolymph proteins are antigenically very close with proteins in fat body before and after pupation, suggesting the fat body as the possible source of some haemolymph proteins during this period. Boavida and Roberts (1975) indicated that most of the serum proteins could have been synthesized in the fat body while remainder in the haemocytes, malpighian tubules, and salivary glands. Results presented here also suggest that at prepupal stage two of three haemolymph proteins are found in the fat body and during the early pupal stage only three to four of more than 8 haemolymph proteins are closely related with the fat body, suggesting presence of other organs as haemolymph protein origin.

SUMMARY

Changes and possible origin of haemolymph proteins during the cuticle formation and hardening are determined by means of acrylamide gel electrophoresis and immunodiffusion. The results by acrylamide gel electrophoresis showed at least 19 protein bands in the haemolymph and 13 fractions in the fat body with relatively constant pattern during the period of cuticle formation and hardening. Both haemolymph and fat body proteins are generally characterized by the presence of three to four heavy stained bands and several thin bands near the top region of the gel. At least over five haemolymph proteins are constantly present during this period. Immunodiffusion tests show that of total eight to nine pupal haemolymph proteins two proteins were already detected in the fat body before pupation and other two proteins were also found in the fat body immediately after pupation, suggesting fat body as possible source of these two haemolymph proteins.

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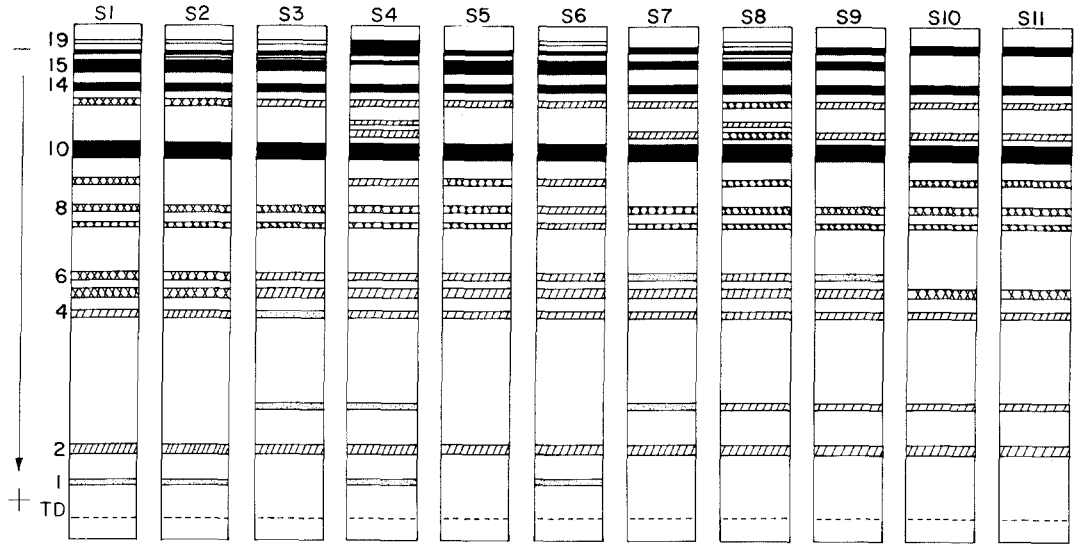

Fig. 1. The electrophoretic pattern of the haemolymph proteins at different times during the cuticle formation and hardening process.
S1: newly ecdysed pupa. S2: 15 min pupa. S3: 30 min pupa.
S4–11: 1–8 hr pupae. (▬ strong; ▩ medium; ▨ weak; ▤ trace.)

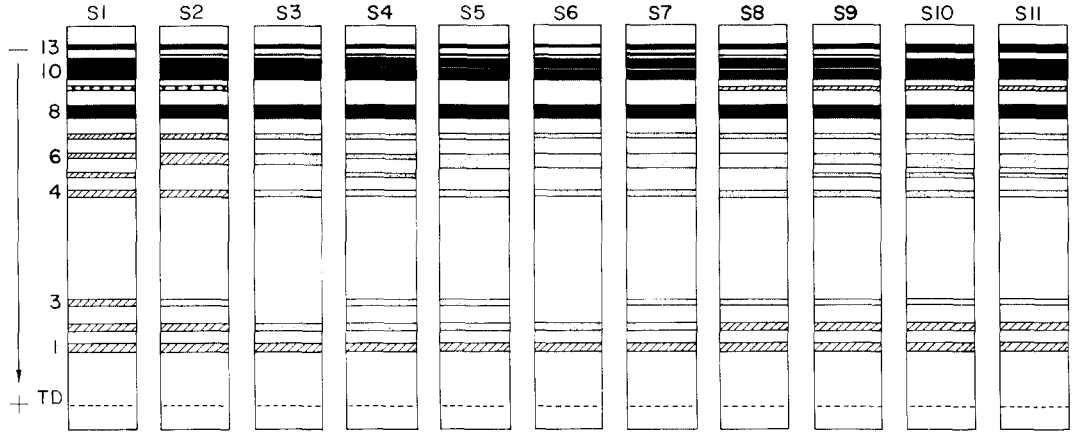

Fig. 2. The electrophoretic pattern of the fat body proteins at different times during the cuticle formation and hardening process.
S1; newly ecdysed pupa. S2; 15 min pupa. S3; 30 min pupa.
S4–11; 1–8 hr pupae.

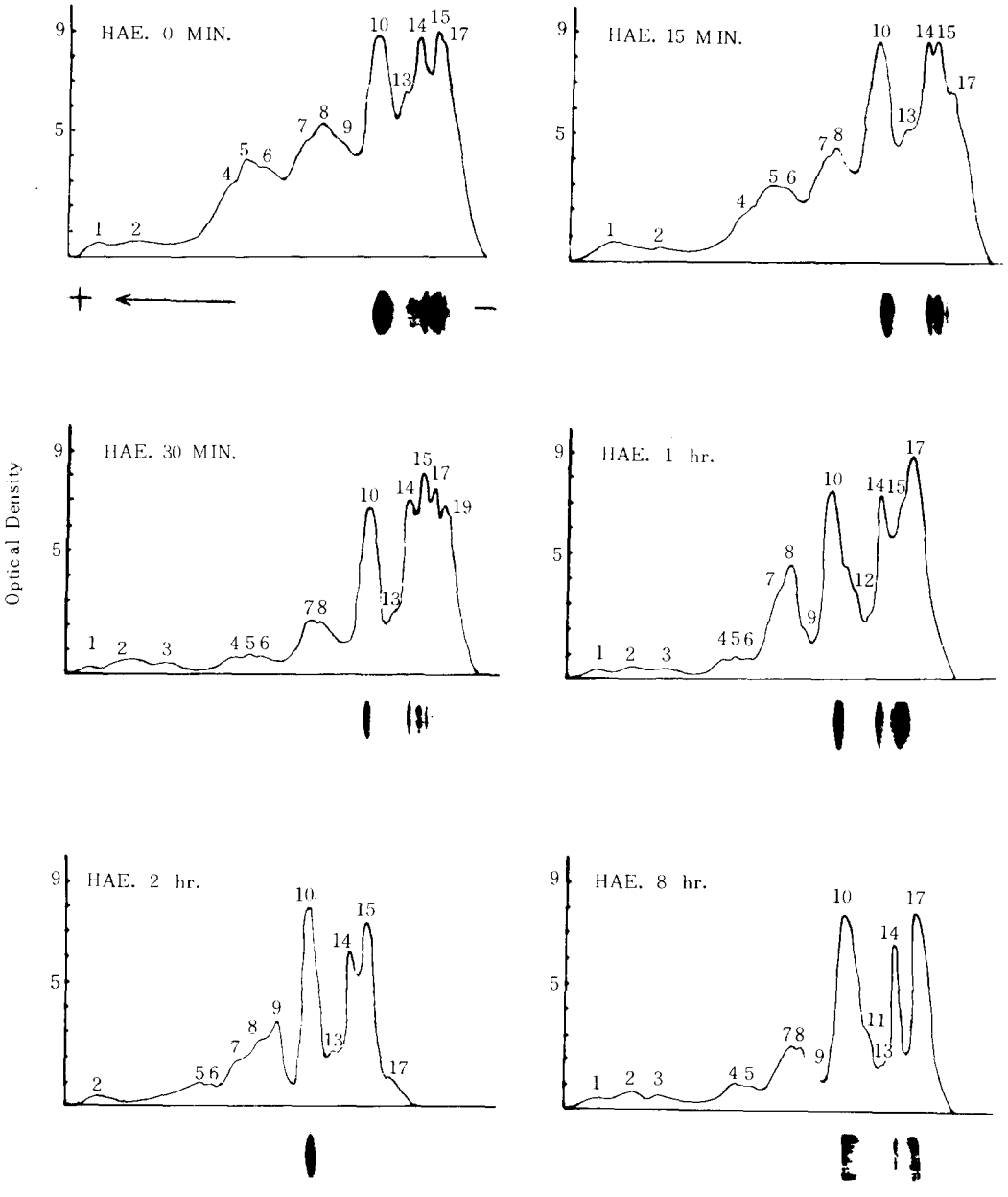

Fig. 3. Electrophoretic patterns of the haemolymph proteins of *Pieris rapae* during 8 hrs after pupation.

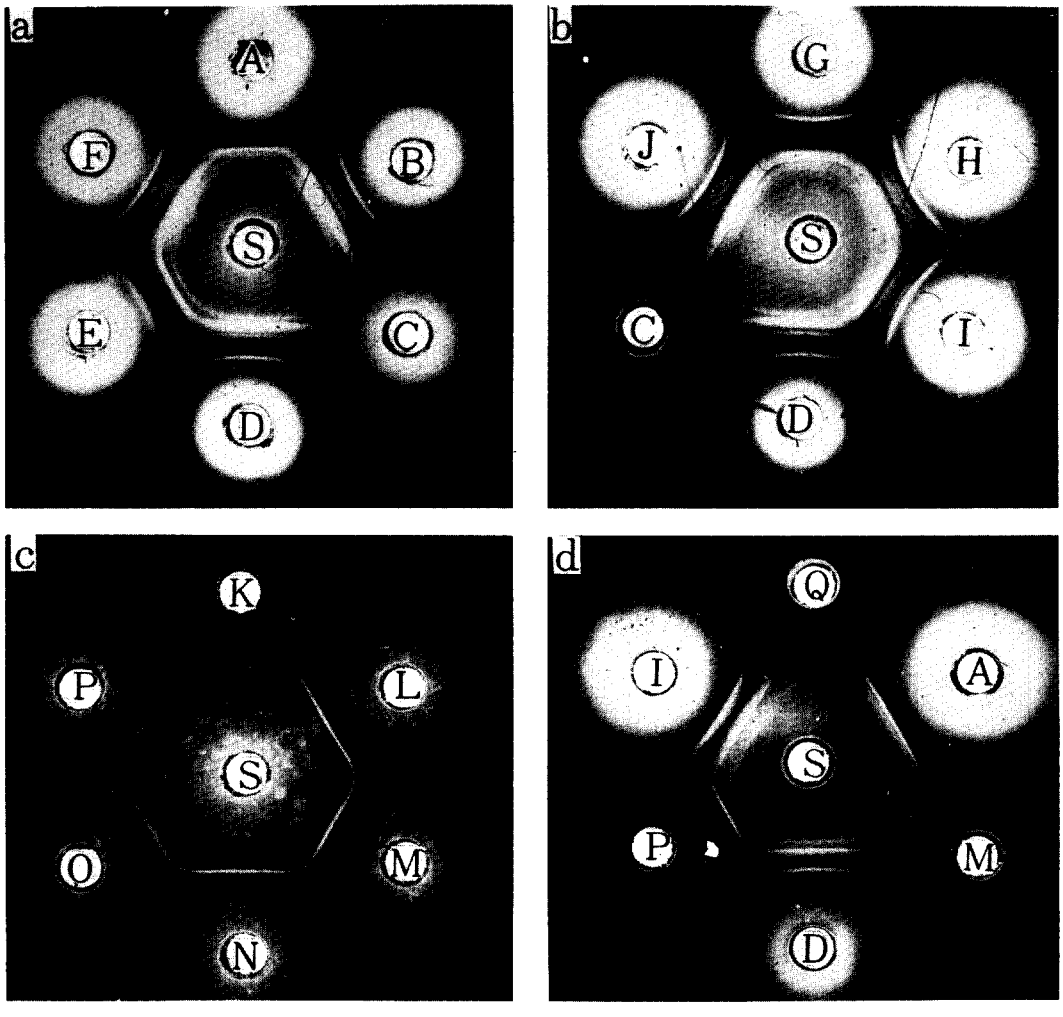

Fig. 4. Immunodiffusion patterns given by haemolymph and fat body of *Pieris rapae at* different times with antiserum against haemolymph from *Pieris rapae* 30 min after pupation; A: prepupal haemolymph, B: newly ecdysed pupal haemolymph, C: human serum, D: 30 min pupal haemolymph, E: 1 hr pupal haemolymph, F: 3hr pupal haemolymph, G: 5 hr pupal haemolymph, H: 6 hr pupal haemolymph, I: 8 hr pupal haemolymph, J: 1 day pupal haemolymph, K: newly ecdysed pupal fat body, L: 15 min pupal fat body, M: 30 min pupal fat body, N: 3 hr pupal fat body, O: 5 hr pupal fat body, P: 8hr pupal fat body, Q: prepupal fat body, S: antiserum.