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Distribution and Quantitative Change of Vitellogenin During Egg Formation of *Bombyx mori* L.

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누에 (*Bombyx mori*)의 卵形成過程에 따른 Vitellogenin의 分布 및 量的 變化

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摘 要

누에의 卵形成時期동안 자성 혈림프에서 DEAE-cellullose column을 使用하여 vitellogenin을 순수하게 分離해 낸 후, 電氣泳動法과 免疫學的 方法을 使用하여 각 器官에 따른 vitellogenin의 分布와 이의 量的 變化를 調査하였다.

누에의 vitellogenin은 난성숙시기에 걸쳐 혈림프, 脂肪體 및 卵巢에 모두 分布하고 있으며, 특히 蛹時期동안 vitellogenin이 脂肪體에서 放出되는 量과 卵母細胞에서 흡수되는 量은 서로 거의 비슷하게 일어나고 있으며 혈림프에서는 羽化直前까지 거의 一定하게 維持되고 있다. 免疫學的으로 볼 때, 脂肪體내의 vitellogenin은 蛹化後 7日까지만 확인되었으며 그 이후 급격한 量的 감소를 나타내었으며, 또한 血蛋白質은 卵巢蛋白質과 最小한 3개의 同質인 蛋白質을 갖고 있는 것으로 보아 卵形成에 vitellogenin 이외의 다른 血蛋白質도 관여되는 것 같다.

INTRODUCTION

It is generally known in insects that female-specific protein, vitellogenin is synthesized in extraovarian tissues, taken up selectively by follicle cells, and deposited in egg yolk. Pan *et al.* (1969) reported that female protein is synthesized *in vitro* in the fat body of *H. cecropia*, suggesting that vitellogenin synthesized in the fat body is released into haemolymph and then transported to the developing oöcytes.

The existence of immunologically identical proteins in the haemolymph to those in the oöcyte has been demonstrated in *Nauphoeta* (Buhlmann, 1976), *Leucophaea* (Dejmal and

Brookes, 1972) and *Periplaneta* (Bell, 1969), and vitellogenin has also been immunohistochemically detected in the ovary and fat body of *Blattela germanica* (Tanaka and Ishiaki, 1974).

As to the silkworm, *Bombyx mori*, Ono *et al.* (1975) confirmed that female-specific protein is present in the haemolymph of female pupae and egg yolk but not in the haemolymph of male pupae and larva of either sex, while egg-specific protein only in the egg yolk, and Irie and Yamashita (1980) reported that vitellin and other yolk proteins undergo quantitative changes during embryo development. However, little has been studied about the detailed transporting process of vitellogenin through haemolymph.

Present work is aimed at the quantitative changes of vitellogenin in haemolymph, fat body, and ovary during oöcyte development of *Bombyx mori*.

MATERIALS AND METHODS

The fifth larval silkworms were obtained from National Sericultural Laboratory at Soo Won and segregated sexually, then reared on fresh mulberry leaves until pupated. Haemolymph, fat body, and ovary were collected at two-day intervals during oöcyte development just after pupation.

Collection of haemolymph, fat body, and ovary

Haemolymph was collected into cold test tube containing small amounts of phenylthiourea by puncturing the abdomen with sharp needle and centrifuged at 10,000 rpm for 10 min to remove haemocytes and tissue fragments and stored at -20°C until used. Upon the collection of haemolymph, the pupae were rinsed two to three times in distilled water and then fat body and ovary were disssected out in cold Ringer solution and weighed, homogenized and then, centrifuged as above and only supernatant was used as protein sample.

Measurement of protein concentrations in organs

Protein concentration was determined by the method of Biuret (Gornall *et al.*, 1949) and bovine serum albumin was used as standard.

Electrophoresis

Electrophoresis was conducted in 5% polyacrylamide and Tris-glycine buffer (pH, 8.3) and after electrophoresis gel was stained with 0.25% Coomassie brilliant blue R 250 and then destained in 7% glacial acetic aced. TG 2970 densitometer was used to measure relative concentration of each fraction and relative concentration was expressed as area for each peak (cm^2/peak).

Immunodiffusion test

Ovary containing mature egg was dissected out just after emergence and protein sample of which was used as antigen, and antiserum was made accodring to the method described previously (Terando and Feir, 1966). Immunodiffusion was carried out by Ouchterlony method (1949), using 1% agarose buffered with 0.01 M phosphate buffer (pH, 7.0)

containing 0.1% sodium azide and 0.15 M NaCl. Diffusion was conducted at room temperature for 3 days and then rinsed to remove unreacted antibody and antigen, and stained with 1% amido black 10 B to visualize precipitin line.

Purification of vitellogenin

Vitellogenin present in the haemolymph of female pupae was obtained according to the method developed by Chen *et al.* (1977) with slight modifications and concentrated in a freeze dryer.

RESULTS

As shown in Fig. 1, vitellogenin was purified from the haemolymph of female pupae. For DEAE-cellulose chromatography, haemolymph was saturated to 75% with ammonium sulfate and vitellogenin was further precipitated in a low ionic strength solution. The precipitate obtained in this way was applied to DEAE-cellulose. The first ten fractions were eluted with 0.04 M phosphate buffer at the speed of 9 ml/hr, and the next 10 fractions (11th to 20th fraction) were eluted with 0.04 M phosphate buffer containing 0.25 M KCl. Each fraction was measured at 280 nm and only fractions 14, 15, 16, were collected according to the values of O.D. Vitellogenin obtained in this way was completely concentrated in the freeze dryer and its purity was examined by 5% acrylamide gel electrophoresis and immunodiffusion test. As shown in Fig. 2a, when the haemolymph proteins of male and female pupae were separated on electrophoresis, one protein fraction with the same electrophoretic mobility as that of vitellogenin was confirmed in female haemolymph, fat body, and ovary but not in male haemolymph

In the immunodiffusion test, a single precipitin line formed as vitellogenin was reacted with antiserum against ovary extract, and this line was continuous to one of precipitin

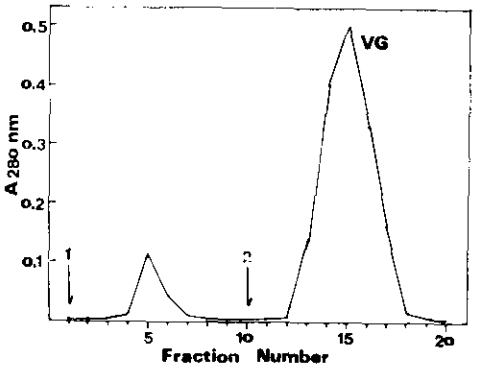

Fig. 1. Separation of silkworm vitellogenin (VG) by DEAE cellulose chromatography. Eluents: (1) 0.04 M phosphate buffer (pH 6.0); (2) 0.25 M~0.04 M phosphate buffer. Fraction volume: 3 ml.

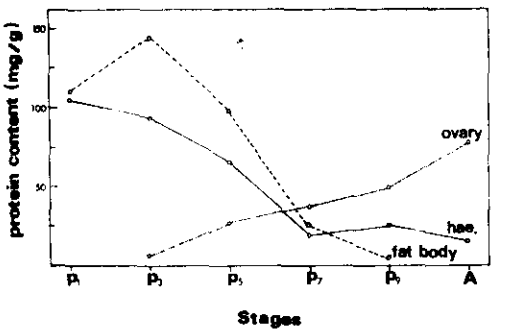

Fig. 3. Changes of protein concentration among the haemolymph, fat body, and ovary during egg maturation. Letter symbols indicate; p_1—newly ecdysed pupa, p_3—3 day pupa, p_5—5 day pupa, p_7—7 day pupa, p_9—9 day pupa, A—newly emerged adult.

lines of haemolymph, fat body, and ovary, but not to any one of precipitin lines of male haemolymph (Fig. 2b), indicating that this female-specific protein is a purified vitellogenin of silkworm. Therefore, this marker protein was used to determine the distribution and quantitative change of vitellogenin during egg maturation.

The concentration of haemolymph protein reaches the peak immediately after pupation but shows continual decrease towards the egg maturation. The concentration of fat body protein has the peak 3 days after pupation but continuously decrease thereafter. On the contrary, oöcyte development was first detected 3 days after pupation and protein concentration of ovary increases with oöcyte growth, reaching the peak of 74 mg/g just after emergence. Thus, during the oocyte growth protein concentrations of haemolymph and fat body decrease but those of ovary continuously increase (Fig. 3), as shorwn by the close similarity in electrophoretic pattern.

Protein patterns of haemolymph, fat body, and ovary, and the homogeneity of these proteins were confirmed by electrophoresis and immunodiffusion, and quantitative analysis of vitellogenin was done by densitometric scanning. As shown in Fig. 4a. the intensity of protein bands of haemolymph decreases with oöcyte growth and just after emergence some protein bands disappear. Immunologically one of haemolymph precipitin lines is continuous to vitellogenin, indicating the presence of the same protein in haemolymph. An immumological reaction with anti-ovary extract shows a constant pattern over the egg maturation. Interaction of anti-ovary extract with haemolymph protein shows at least 3 identical proteins in haemolymph (Fig. 5a). The quantitative ratio of vitellogenin to total haemolymph protein and its concentration change were measured, based on the haemolymph protein pattern. The relative percentage of vitellogenin maintains constant level over the period of just after pupation up to 5-day old pupae but increases to high value of about 23% 7 days after pupation and decrease thereafter, and the intensity of vitellogenin reaches the peak just after pupation, and then maintains for a while, but decreases at the end of egg

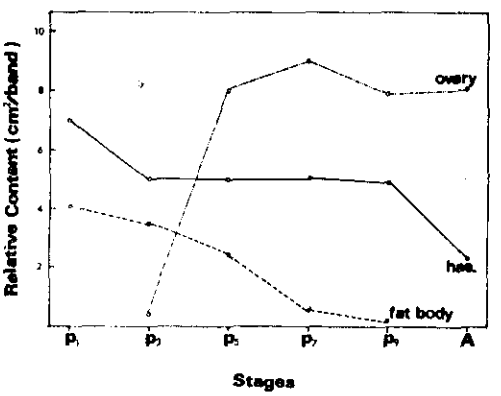

Fig. 6. Changes of relative content of vitellogenin in haemolymph, fat body, and ovary during the egg maturation.

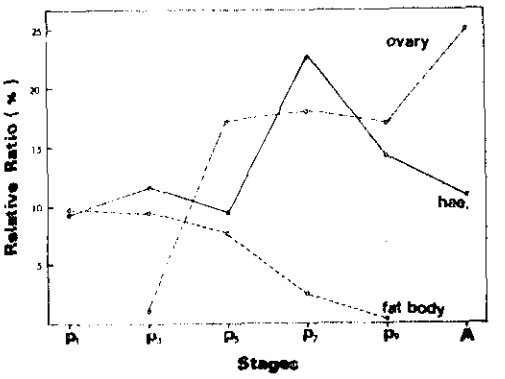

Fig. 7. Changes in relative ratio of vitellogenin to the total haemolymph, fat body, and ovary protein during the egg maturation.

maturation.

Protein pattern of fat body represents the similarity to that of haemolymph protein, showing that there are concentration decrease and qualitative change over the egg maturation (Fig. 4b). Immunologically vitellogenin of fat body was detected until 7 days after pupation, over this period oöcyte growth takes actually place but not thereafer (Fig. 5b). That is, it is considered that most of vitellogenin in fat body is released into haemolymph over the period of oöcyte growth. The ratio and concentration of vitellogenin in fat body decrease with egg maturation.

Electrophoresis of ovarial protein shows almost constant pattern over the period of 5 days after pupation starting oöcyte growth to just after emergence completing egg maturation but represents a little qualitative change at the distal end of gel (Fig. 4c). Immunologically ovary has a strong precipitin line identical to vitellogenin and also at least 4 other precipitin lines (Fig. 5c). The intensity of vitellogenin maintains relatively high level up to egg maturation and the relative ratio to total ovary protein increase to about 25% just after emergence.

According to above results, the intensity of vitellogenin maintains relatively constant level in ovary but decreases in fat body with oöcyte growth. These facts indicate that vitellogenin in fat body is released into the haemolymph at the same amount as it is taken up by ovary (Fig. 6). Also, relative ratio of vitellogenin continues to decrease in fat body but reaches the peak 7 days after purpation in haemolymph, even it shows a great increase just after emergence in ovary (Fig. 7).

DISCUSSION

Vitellogenin and vitellin in different insects have been confirmed by electrophoretic mobility and immunological reaction. In the present work, purified vitellogenin was confirmed as female-specific protein on electrophoresis. This protein has the same electrophoretic mobility as each one of female haemolymph, fat body, and ovary proteins but none of male haemolymph. Immunologically the interaction of antiserum against ovary extract with vitellogenin showed a unique line consistent with each one of female haemolymph, fat body, and ovary precipitin lines but not with male haemolymph, supporting above results and also suggesting that vitellogenin is taken up intact by ovary. Also, at least three haemolymph proteins showed the homogeneity with ovary proteins, indicating that other haemolymph proteins in addition to vitellogenin are involved in egg formation. Izumi and Tomino (1980) reported that as purified vitellogenin and vitellin were cross-reacted, these two proteins were immunologically homogeneous, suggesting that major protein moiety of haemolymph vitellogenin is transferred intact to developing oöcytes. Vitellogenin was also reported to be selectively absorbed (Kunkel and Pan, 1976; Ferenz, 1978) or taken up through micropinocytosis (Telfer and Melius, 1963; Bassemir, 1977; Goltzene, 1977). Especially Ferenz and Lubzens (1981) reported that during the release of vitellogenin into

ovary through micropinocytosis, other proteins present in external fluid are also absorbed non-specifically, these proteins absorbed accounting for about 5% of total vitellogenin quantity taken up by oöcytes.

Concerning the change of protein concentrations of various organs, Izumi and Tomino (1976) reported that protein concentration of haemolymph of female silkworm gradually decreases over the period of larval-pupal transformation but vitellogenin in haemolymph increases, suggesting that vitellogenin is synthesized in the fat body during larval-pupal ecdysis. Scheurer and Leuthold (1969) also reported that total haemolymph protein concentration decreases during oöcyte maturation, showing the good agreement with our result. In present work protein concentrations gradually decrease during egg maturation in haemolymph and fat body but increase up to 74 mg/g in ovary.

In the 5% acrylamide gel electrophoresis, protein patterns showed the quantitative as well as qualitative changes in fat body and haemolymph but only slight qualitative change in ovary. However, Scheurer (1969) reported that in cockroach, *Leucophaea maderae* more protein fractions were confirmed in ovary than in haemolymph, and also more distinct qualitative and quantitative changes take place in ovary during egg maturation, showing a little discrepancy with our result.

As to the time of synthesis of vitellogenin in fat body, Ono *et al.* (1975) reported that female specific protein is synthesized in fat body one or two days before larval-pupal ecdysis in silkworm, and Telfer (1954) also reported that female protein is synthesized before metamorphosis in *Cecropia*.

In the present experiment, quantitative change of vitellogenin was analyzed using densitometer. Vitellogenin decreases in fat body but maintains constant level for a while in haemolymph and also relatively high level in ovary, indicating that vitellogenin is released into haemolymph at the same amount as it is taken up by ovary. Especially these results are in good accord with immunolgical data. Haemolymph and ovary showed almost constant immunological reaction during egg maturation (especially with stronger reaction in ovary) while fat body showed no antigenity such as vitellogenin 7 days after pupation but drastic quantitative decrease in general. Also, relative ratio of vitellogenin in ovary accounts for about 25% of total protein just after emergence. According to Irie and Yamashita (1980). vitellin was known to occupy about 40% of soluble protein of newly laid egg in silkworm. Therefore, it is considered that vitellogenin of silkworm is continuously deposited into the oöcyte throughout the period of just after emergence to oviposition time.

SUMMARY

Vitellogenin was purified from the haemolymph of female pupae during egg formation of *Bombyx mori* using DEAE-cellulose column and its quantitative change in various organs with age was examined by electrophoresis and immunodiffusion.

Vitellogenin is distributed in the haemolymph, fat body, and over the egg maturation and especially maintains a constant level in the haemolymph until just before emergence,

indicating that vitellogenin is released into the haemolymph at the same amount as it is taken up by oöcytes during pupal period. Immunologically vitellogenin was confirmed to be in the fat body until 7 days after pupation and to undergo a drastic decline thereafter. Also, the interaction of anti-ovary proteins with haemolymph proteins showed at least 3 homogeneous proteins, indicating that other proteins as well as vitellogenin are involved in egg formation.

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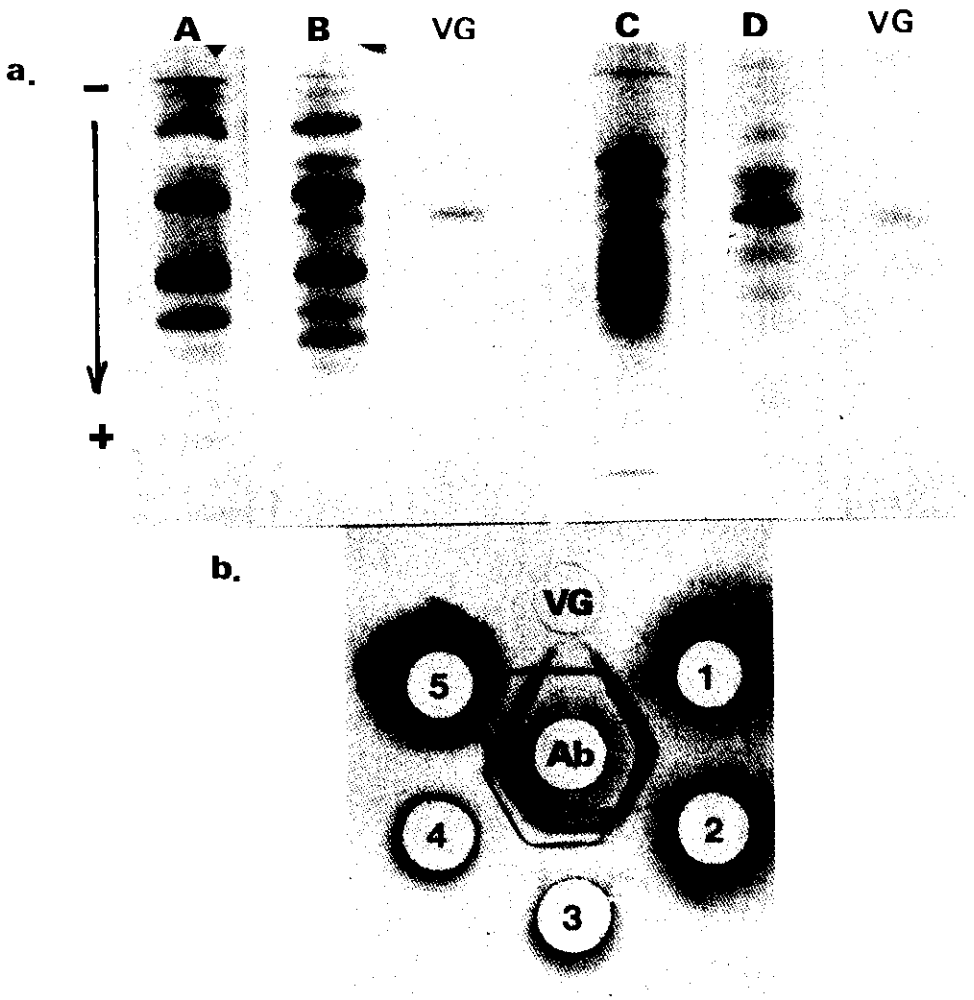

Fig. 2. Identification of purified vitellogenin by electrophoresis and immunodiffusion. a) Comparison of protein bands prepared from (A) male haemolymph, (B) female haemolymph, (C) fat body, (D) ovary, and (VG) purified vitellogenin. b) Immunodiffusion pattern against ovary extracts; 1, 2; female haemolymph: 3, 4; ovary: 5; male haemolymph: Ab; antibody.

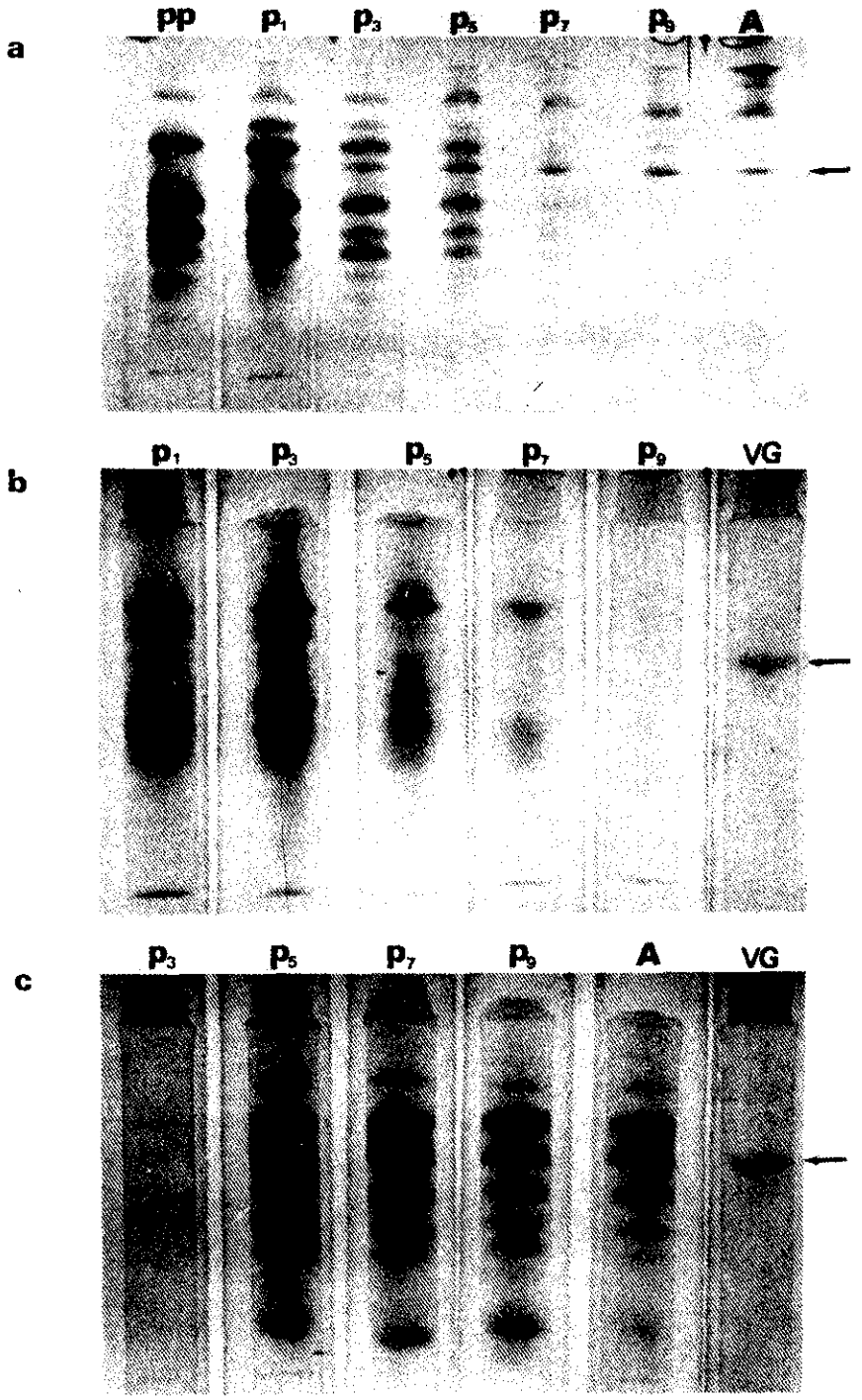

Fig. 4. Changes of electrphoretic patterns during the egg maturation. a) haemolymph protein, b) fat body protein, c) ovary protein.

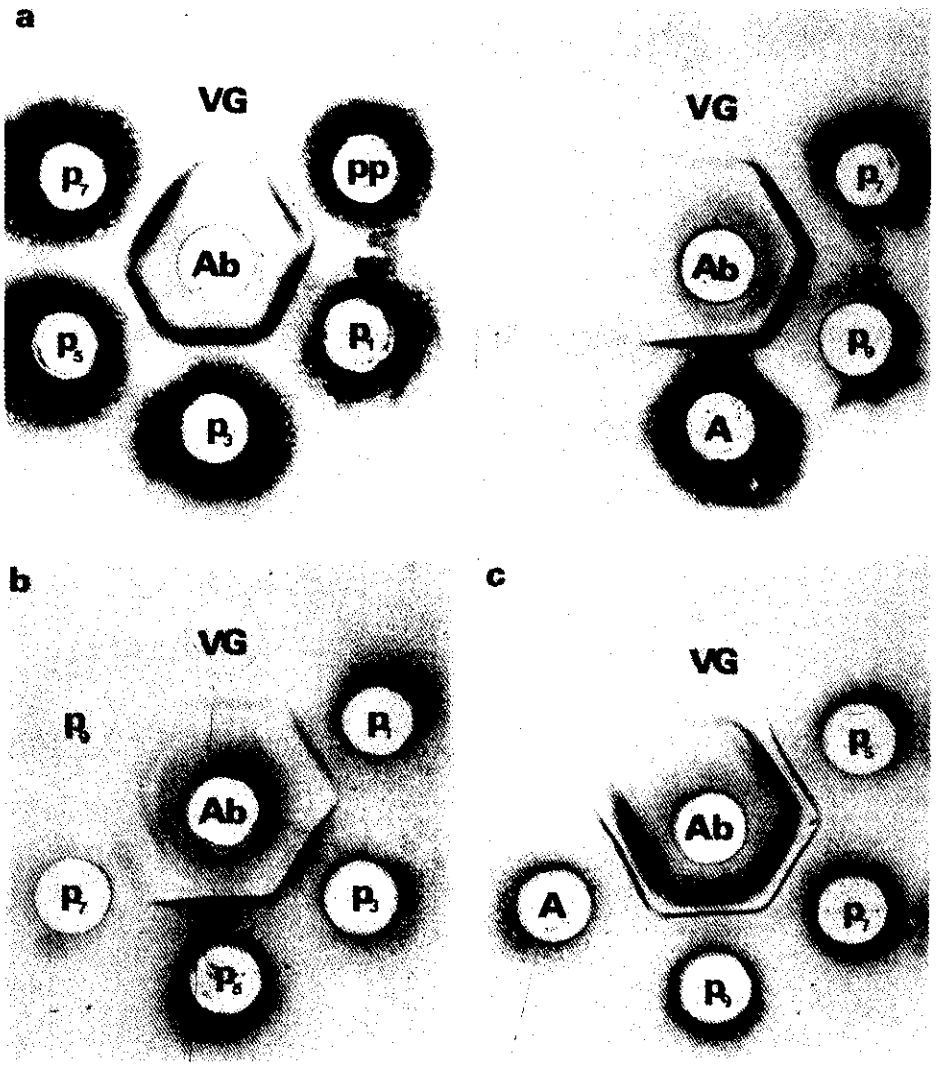

Fig. 5. Double diffusion precipitation patterns against ovary protein during the egg maturation. a) haemolymph protein, b) fat body protein, c) ovary protein.