Tissue Culture of the Rice Stem Borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae)

II Morphology and *in Vitro* Cultivation of Hemocytes

Jun Mitsuhashi

Division of Entomology, National Institute of Agricultural Sciences, Nishigahara, Kita-ku, Tokyo, Japan

(Received November 1, 1965)

The blood of *Chilo suppressalis* larvae was found to contain 7 types of hemocytes, namely, prohemocytes, plasmatocytes, granular hemocytes, spherule cells, oenocytoids, podocytes, and vermiform cells. Primary cultures of the hemocytes were successfully maintained for over 12 months by changing the medium once a week. Prohemocytes and plasmatocytes multiplied by mitoses, whereas other types of hemocytes did not survive for long in the primary cultures. Subculturing of cells was tried from primary cultures by taking parts of the cell colonies 7 months after the culture was set up. Subcultured cells multiplied by mitoses and survived for over 5 months. The plasmatocytes usually formed cell sheets. The prohemocytes were often found on the plasmatocyte cell sheets. The prohemocytes aggregated to form cell masses or they suspended themselves freely in the medium. During the cultivation, morphological changes of the cells, which suggested the transformation of prohemocytes into plasmatocytes, were observed.

INTRODUCTION

Insect hemocytes have been known since the time of Swammerdam (1637–1680). But despite the great number of investigations on insect blood, there are still many questions, especially about function, which remain unsolved. Insect hemocytes consist of several types of cells which seem to vary in different species. Many workers have classified insect hemocytes, but their classifications are not unified in detail. The classification of hemocytes has been reviewed by Munson (1953), Wigglesworth (1959), Jones (1962, 1964), etc.

The first attempt to cultivate insect hemocytes was made by Glaser (1917). He cultivated hemocytes of several lepidopterous insects, and kept those of *Lymauntria dispar* alive in hanging drops of blood for as long as 17 weeks. Since that time, hemocytes of various other insects have been cultivated, all in short term cultures. (Taylor, 1935; Wermel, 1938 a, b; Fischer and Gottschewski, 1939; Millara, 1946; Arvy and Gabe, 1946; Horikawa and Kuroda, 1959; Martignoni and Scallion, 1961; Marks and Reinecke, 1965). Cultivations for long periods or subcultures of insect hemocytes have not as yet succeeded.

In the present study, various types of hemocytes in larvae of the rice stem borer, *Chilo suppressalis* Walker, were examined and were cultivated for long periods in synthetic media.
MATERIALS AND METHODS

*C. suppressalis* larvae used in this study were aseptically reared in the same manner as described in the previous paper (MITSUHASHI, 1965 b). Diapausing larvae were found to be most suitable.

Morphological studies were made on living hemocytes with a phase contrast microscope, and also on fixed and stained hemocytes with an ordinary microscope. Staining techniques used were DELAFIELD’s hematoxylin–eosin after fixation with BOUIN’s fixative, MALLORY’s triple stain after fixation with SUSA-picric fixative, combination of DELAFIELD’s hematoxylin–eosin stain and MALLORY’s triple stain after fixation with SUSA-picric fixative, CLARK’s paraaldehyde fuchsin stain (CLARK, 1955) after fixation with SUSA-picric fixative, and Sudan black B–neutral red stain after fixation with 10 per cent formalin.

The basic culture medium and the culture vessels used in the cultivation of the hemocytes were the same as those used in the cultivation of various tissues of *C. suppressalis* larvae (MITSUHASHI, 1955 b). The following procedures were employed for setting up the culture. Larvae were held up side down in one hand and the anterior and posterior halves bent back. A proleg was then punctured with a sterilized needle, and the blood which oozed out was allowed to drop onto a modified RINGER-TYRODE’s salt solution (CARLSON, 1946) placed on the bottom of a culture vessel. Most of the hemocytes sank and attached themselves to the glass surface within a few minutes. Some hemocytes aggregated to form masses while others became flat by sending out pseudopod-like cytoplasmic processes. Certain types of hemocytes seldom attached themselves to the glass surface. After most of the hemocytes settled, they were washed by changing the salt solution several times. This washing removed any hemocytes which had not yet settled, and hemolymph which caused darkening of the solution. After washing, the RINGER-TYRODE’s solution was replaced with culture medium, which was done by changing the medium five times.

Cultivation was carried out at 25°C under natural light. The culture medium was changed once a week. Observations were made with an inverted phase contrast microscope as described in the previous paper (MITSUHASHI, 1965 b).

RESULTS

*Morphology of Hemocytes*

The blood of *C. suppressalis* contained 7 types of hemocytes which could be distinguished from each other by their size, shape, staining property and behavior.

1) *Prohemocytes* were small cells and were spherical when they were in hemolymph (Fig. 1). The diameters of the cell and the nucleus were about 10 μ and 3 μ respectively. Prohemocytes rarely became flat, but aggregated to form cell masses on the glass surface. The cytoplasm of the cell contained fine granules. The granules stained black with MALLORY’s triple stain. With other staining methods, the granules could not be distinguished from other parts of the cytoplasm.
Figs. 1-9. Bright phase contrast appearance of various types of hemocytes in diapausing larvae of *Chilo suppressalis* (×1,200). 1: Prohemocyte. 2: Plasmatocyte. 3: Flattened plasmatocyte (one hour after the blood was drawn out). 4: Granular hemocyte. 5: Flattened granular hemocyte (one hour after the blood was drawn out). 6: Spherule cell. 7: Oenocyte. 8: Podocyte. 9: Vermiform cell.

2) *Plasmatocytes* were spherical or elliptical and sent out so many slender cytoplasmic processes that they looked like chestnut-bur when they were in hemolymph (Fig. 2). The diameters of the cell and the nucleus were about 10 μ
and 3 \( \mu \) respectively. Plasmatocytes easily adhered to the glass surface and became very flat by spreading their cytoplasm like pseudopodia soon after they were brought into the culture vessels. The flattened plasmatocytes took on irregular forms and varied in size (Fig. 3). The sizes of the flattened cells ranged from 20 \( \mu \) to 40 \( \mu \) and those of the nuclei from 7 \( \mu \) to 15 \( \mu \). The rim of the flattened cells was often obscure at the parts farthest from the nuclei. The cytoplasm of the cells usually had several vacuoles. The cytoplasm stained very faintly with all the staining methods used.

3) **Granular hemocytes** were spherical or elliptical when they were in hemolymph (Fig. 4). Granular hemocytes had processes on their surface, but the processes were so short and fine that they were difficult to recognize. When hemolymph was spread on glass, the granular hemocytes soon attached themselves to the glass surface, and gradually became flat by spreading their cytoplasm outward (Fig. 5). The cytoplasm of the cells contained many granules of various sizes. The large granules in the flatted cells were about 2 \( \mu \) in diameter. The diameters of the cell and the nucleus were about 10 \( \mu \) and 3 \( \mu \) respectively when the cell was freely suspended in hemolymph, whereas the diameter of the cell and the nucleus became about 25 \( \mu \) and 5 \( \mu \) respectively when the cell flattened on the glass surface. The cytoplasm stained faintly purple with DELAFIELD's hematoxylin-eosin, but was scarcely stained in other staining techniques. Most of the granules were found to be eosinophilic. However, even in the same cell, individual granules gave different colors with MALLORY's triple stain or CLARK's paraldehyde fuchsin stain, suggesting that they were of different chemical composition. The granules did not appear to be lipids, since they were not stained with Sudan black B.

4) **Spherule cells** were round hemocytes with an irregular outline like mulberry fruit (Fig. 6). Their irregular outline was due to the presence of very large inclusions. The diameters of the cell and the nucleus were about 10 \( \mu \) and 3 \( \mu \) respectively. The nuclei often seemed to be depressed by inclusions. The spherule cells seldom attached themselves to the glass surface, when they were brought into the culture vessels. The cytoplasm of the spherule cells was difficult to recognize, because the inclusions filled the whole area of the cells except for the nuclei. The inclusions were spherical and their diameter was about 3 \( \mu \). The inclusions showed affinity to aniline blue in MALLORY's triple stain, and this was a prominent characteristic of spherule cells. The same staining properties of inclusions have been reported in the spherule cells of *Bombyx mori* (NITTONO, 1960). The inclusions were not stained with Sudan black B.

5) **Oenocytes** were elliptical or ovoid cells with smooth outline (Fig. 7). The major diameter of the cells was about 20 \( \mu \). The nucleus was round and about 10 \( \mu \) in diameter. Oenocytes rarely adhered to the glass surface when they were brought into the culture vessels. The cytoplasm of the oenocytes in *C. suppressalis* was intensely acidophilic, although in many other insects it has been reported to be basophilic (JONES, 1962).

6) **Podocytes** were the only cells which had a very flat form in hemolymph, although the cells of some other types became flat when the hemolymph was drawn out onto the glass surface. The podocytes were stellate (Fig. 8), and the diameters of the cell and the nucleus were about 20 \( \mu \) and 8 \( \mu \) respectively. The cells did not alter their shapes after being taken out of the insect hemocoel, and they
never attached themselves to the glass surface. These characteristics of podocytes agree with the observations of Jones (1959) on podocytes of Prodenia eridania. The cytoplasm of the podocytes was scarcely stained in the staining techniques used. Podocytes have been reported from only one species of lepidopterous insect and from only one species of dipterous insect (Jones, 1962).

7) Vermiform cells were very slender cells with tapered ends (Fig. 9). The length of the cells varied from 20 μ to 150 μ and the diameter of the nucleus was about 2 μ. The veriform cells never attached themselves to the glass surface. Vermiform cells have been reported from only one species of lepidopterous insect and from only one species of coleopterous insect (Jones, 1962).

The staining properties of the various hemocytes are summarized in Table 1.

Differential hemocyte counts were not made in this study, but the spherule cells predominated. Prohemocytes, plasmatocytes and granular hemocytes were next in abundance and almost equal in number. There were few oenocytes, and podocytes and veriform cells were rare. The ratio of these hemocytes in hemolymph seemed to vary in different individuals.

Table 1. Staining Properties of Hemocytes in Diapausing Larvae of Chilo suppressalis

<table>
<thead>
<tr>
<th>Type of hemocytes</th>
<th>Parts of cell</th>
<th>Staining</th>
<th>Combination of Delafield’s hematoxylin-eosin stain and Mallory’s triple stain</th>
<th>Clark’s paraaldehyde fuchsin stain</th>
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<tbody>
<tr>
<td>Prohemocytes</td>
<td>cytoplasm</td>
<td>reddish purple</td>
<td>dark red</td>
<td>brown</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>dark red</td>
<td>brown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>deep purple</td>
</tr>
<tr>
<td>Plasmatocytes</td>
<td>cytoplasm</td>
<td>purple</td>
<td>reddish purple gray</td>
<td>dark green</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>orange yellow</td>
<td>dark red</td>
</tr>
<tr>
<td>Granular hemocytes</td>
<td>cytoplasm</td>
<td>pale purple</td>
<td>yellow</td>
<td>blue</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>yellow, red, blue or gray</td>
<td>dark red or green</td>
</tr>
<tr>
<td></td>
<td>granules</td>
<td>pink</td>
<td>dark greenish</td>
<td>orange yellow, red, green, brown, or blue</td>
</tr>
<tr>
<td>Spherule cells</td>
<td>cytoplasm</td>
<td>purple</td>
<td>red</td>
<td>bluish green</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus inclusions</td>
<td></td>
<td></td>
<td>deep purple</td>
</tr>
<tr>
<td>Oenocytes</td>
<td>cytoplasm</td>
<td>reddish purple</td>
<td>light blue</td>
<td>blue</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>deep red</td>
<td>dark brown</td>
</tr>
<tr>
<td>Podocytes</td>
<td>cytoplasm</td>
<td>pale gray</td>
<td>pale blue</td>
<td>bluish gray</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>orange</td>
<td>dark green</td>
</tr>
<tr>
<td>Vermiform cells</td>
<td>cytoplasm</td>
<td>reddish purple</td>
<td>bluish gray</td>
<td>pale green</td>
</tr>
<tr>
<td></td>
<td>chromatin in nucleus</td>
<td>purple</td>
<td>purple</td>
<td>dark green</td>
</tr>
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<td></td>
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<td>dark red</td>
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</table>
Cultivation of Hemocytes

1. Primary cultures

Some types of hemocytes showed marked morphological changes when they were brought into the culture vessels. The plasmocytes and granular hemocytes soon attached themselves to the glass surface and became flat by spreading their cytoplasm. The prohemocytes aggregated to form cell masses and some...

Fig. 10. Plasmocyte cell sheet. Bright phase contrast (×200). Fig. 11. Prohemocyte masses. Bright phase contrast (×200).
times other types of hemocytes were taken into the prohemocyte aggregates. The spherule cells, oenoctoids, podocytes and vermiform cells did not attach themselves to the glass surface, most of them being removed from the culture vessels when the hemocytes were washed with RINGER-TYRDE's solution.

Of the 7 types of hemocytes, the plasmatocytes and prohemocytes survived and multiplied by mitoses for long periods, the former forming cell sheets and the latter forming cell masses (Figs. 10 and 11). The granular hemocytes usually did not survive for more than 2 days. When the granular hemocytes began to degenerate, the cells usually released some granules into the medium surrounding them, and the cytoplasm gradually became hyaline. These changes were followed by death of the cells. In the degenerated granular hemocytes, the cytoplasm seemed to lose its viscosity, and active Brownian movement of the granules was seen in the cytoplasm. The nuclear boundary was very distinct in the degenerated granular hemocytes. The spherule cells, oenoctoids, podocytes and vermiform cells did not show marked morphological changes, but they gradually degenerated.

The plasmatocytes initiated multiplication by mitoses usually within a week after the culture was set up. At this time, however, mitoses were seen only occasionally. They often formed cell sheets (Fig. 10). Blister formation of the plasmatocytes became active usually after 2 to 3 weeks. First small vacuoles appeared in the cytoplasm (Fig. 12), then the vacuoles swelled up and became blisters (Fig. 13). This blister formation seemed to be the result of active pinocytosis of the cells. Active blister formation of the plasmatocytes lasted for 1 to 2 months and the blisters coalesced and covered the whole area of the glass surface (Fig. 14). After this stage the blisters gradually disappeared. Hollow spherical vesicles were sometimes formed on the plasmatocyte cell sheets. The vesicles consisted of a mono-layer of cells, mostly plasmatocytes, and were filled with liquid, probably from the medium that surrounded them. The vesicles were similar to those obtained in the cultivation of leafhopper tissues (MITSUHASHI and MARAMOROSCH, 1964; MITSUHASHI 1965a) and in cockroach tissues (LARSEN, 1964; MARKS and REINECKE, 1964). Vesicle formation was considered to be one of the ways in which cells multiplied without keeping contact with the glass surface. From the 6th month, the frequency of mitoses in plasmatocytes increased to some extent. At the interphase of mitoses, the nuclei of the cultivated plasmatocytes contained granular chromatin, which looked like chromosomes. It was, therefore, difficult to distinguish the nuclei of mitotic prophase from those of interphase. But metaphase and the succeeding stages of mitoses were easily recognized (Fig. 15). Sometimes, multipolar mitoses, most often tripolar mitoses, were seen in the plasmatocytes (Fig. 16). With increasing time of cultivation, very large plasmatocytes, probably polyploid, appeared. Binucleate cells were common in large plasmatocytes (Fig. 17). When the plasmatocytes began to degenerate, some droplets appeared in the cytoplasm around the nuclei (Fig. 18). The droplets were identified as lipid by Sudan black B staining.

The prohemocytes started multiplication by mitoses within a week after the culture was set up. They were often seen on the plasmatocyte cell sheets. During cultivation prohemocytes rarely became flat by attaching themselves to the glass surface, and retained their spherical shape. In the early periods of cultivation, most of the prohemocytes were not fixed to any surrounding materials, and they
Fig. 12. Vacuoles appearing in plasmacyte. N, nucleus; V, vacuole. Bright phase contrast (×1,400). Fig. 13. Blisters formed on plasmacyte. B, blister; N, nucleus; V, vacuole. Bright phase contrast (×1,400). Fig. 14. Network of blisters formed on plasmacyte cell sheet. Bright phase contrast (×200).
Fig. 15. Mitotic anaphase of plasmacocyte. Chromosomes are moving to poles. Bright phase contrast (× 1,500). Fig. 16. Tripolar mitoses in plasmacocyte at metaphase. Bright phase contrast (× 1,500). Fig. 17. Binucleate plasmacocyte. Bright phase contrast (× 1,200). Fig. 18. Plasmacocyte containing droplets. Droplets are seen mostly around the nucleus. Bright phase contrast (× 1,200).
often showed small vibrating movement like Brownian movement. The frequency of mitoses in the prohemocytes increased synchronously with that in the plasmatocytes. The prohemocytes did not form cell sheets, but they formed tissue-like cell masses or sometimes networks. During cultivation, some prohemocytes showed various shapes and sizes. The largest prohemocyte measured was 50 μ in diameter. Some prohemocytes sent out cytoplasmic processes, and some others took on an elongated form (Fig. 19). Since the prohemocytes were not flat, detail of the nuclei was difficult to see with the phase contrast microscope. Mitoses were only recognized after metaphase (Fig. 20). When the prohemocytes started to degenerate, the cytoplasm became granular. 

The primary culture of the plasmatocytes and prohemocytes was maintained for more than 12 months and the cells are still multiplying by mitoses, the medium being changed once a week.

2. Subculturing

Seven months after the culture was set up, subculturing was tried 20 times from a primary culture at irregular intervals. In the 7 month old primary culture, only the prohemocytes and plasmatocytes survived. The plasmatocytes formed cell sheets and the prohemocytes built up tissue-like cell masses, which were so thick that the site of multiplication could easily be seen with the naked eye.

The procedure for subculturing was as follows: Small portions of the plasmatocyte cell sheets and prohemocyte masses were scratched with sterile needles and the cells which were isolated from the glass surface were transferred into a new culture vessel by means of a pipette. Trypsinization was also employed for detaching cells from the glass surface. The treatment with 0.1 per cent trypsin for 10 minutes at 25°C freed the cells from the glass surface without deleterious effects. After the transfer of the cells was completed, a small amount of the culture medium which was previously conditioned with cultivated hemocytes, was added to the subcultured cells. Sometimes fresh culture medium was used instead of the conditioned medium. The fresh medium proved to be harmless for the subcultured cells.

Soon after the subculturing of cells was completed, some plasmatocytes migrated from the explanted cell masses, and became flat on the glass surface (Fig. 21). But the prohemocytes scarcely ever attached themselves to the glass surface. Mitoses became active usually 2 days after subculturing. Multipolar mitoses were also occasionally observed.

Cell growth after subculturing varied. In some cases, the plasmatocytes grew much faster than the prohemocytes, while, later, the prohemocytes multiplied gradually on the newly formed plasmatocyte cell sheets. In other cases, however, the prohemocytes multiplied much more rapidly than the plasmatocytes. In these cases, most of the prohemocytes multiplied by mitoses without attaching themselves to the glass surface.

During cultivation, some cells showed morphological changes. The prohemocytes were usually small, round cells, but sometimes they became pyri-form or elongate. Especially when they attached themselves to the glass surface, they sent out cytoplasmic processes and took on a similar form to the plasmatocytes. The prohemocytes with cytoplasmic processes sometimes formed networks (Fig. 22).
Fig. 19. Elongated prohemocyte. Bright phase contrast (× 1,200). Fig. 20. Mitotic telophase of prohemocyte. Bright phase contrast (× 1,500). Fig. 21. Cell migration from the subcultured cell mass (CM). Bright phase contrast (× 300). Fig. 22. Network of elongated prohemocytes. Bright phase contrast (× 300).

Some of the prohemocytes markedly increased in size. The larger prohemocytes reached 50 μ in diameter (Fig. 23), although the original prohemocytes were only about 10 μ in diameter. It is possible that the very large prohemocytes were polyploid, but the detail of their nuclei could not be seen on account of their thickness. In the plasmatocytes very large cells, most likely polyploid, became
common during cultivation. Binucleate cells were also seen frequently in the plasmatocytes. The nuclei of the plasmatocytes were usually round, but sometimes, in giant cells, they were irregular (Fig. 24). Such plasmatocytes with irregular shaped nuclei very much resembled the large epithelial cells obtained in the cultivation of testis tissues of the same species (Mitsuhashi, 1965 b).

In the primary culture from which the cells were subcultured, the parts of the glass surface exposed by removal of the cells were soon covered with cells, mostly plasmatocytes, migrated from the surrounding area. Mitoses were much more frequently observed in these areas than in those areas on which were not scratched for subculturing. It is, therefore, probable that the removal of the cells from the cell masses stimulated the multiplication of the remaining cells. In this manner, the primary culture soon built up its cell population after the removal of a considerable number of cells for subculturing, and this made it possible to subculture the cells repeatedly from the same primary culture.

Serial subculturing was also tried. A part of the primary culture was subcultured, and after the cells multiplied to cover the whole area of the glass surface, a part of the secondary culture was again subcultured. In this way, cells were successively subcultured for 8 generations. Cells did not lose their multiplying ability by repeated subculturing.

3. Effects of medium modifications

The culture medium was modified in order to test the effect of certain substances on cell growth.

An increase in concentration of fetal bovine serum up to 20 per cent promoted cell multiplication and the frequency of mitoses was markedly increased. On the other hand, complete absence of fetal bovine serum in the culture medium prevented cell multiplication, but did not kill the cells.

Addition of C. suppressalis hemolymph (obtained from diapausing larvae and heated at 75°C for 10 minutes, frozen at -5°C, and centrifuged) to the culture medium at a final concentration of 5 per cent did not promote cell multiplication. In this medium mitoses were often observed but their frequency was not higher than that in the medium containing 20 per cent fetal bovine serum. However, in this medium cells from degenerating cell masses were able to recover. When culture conditions became unfavorable for cells, by sudden change of the component of the medium for instance, the cells began to degenerate. The prohemocytes freely suspended in the medium aggregated and the plasmatocyte cell sheets shrank and then cells of both types formed rough networks (Fig. 25). At this stage of the degeneration, individual cells were difficult to recognize. But if the medium containing C. suppressalis hemolymph was supplied at this stage, the formed networks were broken down and cell migration from the cell masses resumed (Fig. 26). On the other hand, when the degeneration of cells proceeded further, substitution of the medium was not effective and lipid droplets appeared in the cytoplasm of the cells, and finally the cells detached themselves from the glass. In some cases, numerous blisters were formed on the cells during their degeneration.

Addition of Bombyx mori hemolymph1 to the culture medium showed about

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1 The blood of B. mori was obtained from matured larvae, prepupae and pupae respectively. The hemolymph was prepared in the same manner as that of C. suppressalis.
Fig. 23. Very large prohemocyte (cf. Fig. 1). Bright phase contrast (×600). Fig. 24. Irregular shaped nucleus in plasmocyte. N, nucleus. Bright phase contrast (×300).
Fig. 25. Aggregate of degenerating cells. Individual cells can not be detected. Bright phase contrast (×200). Fig. 26. Cell migration from the aggregate of degenerating cells. Mitoses (arrow) can be seen. Bright phase contrast (×200).
the same effects on cell growth as that of *C. suppressalis*. No difference was found in the growth promoting effects of the hemolymph prepared from blood of *B. mori* at different stages of growth.

When the concentration of TC-199 in the culture medium was increased two fold, cell growth was impaired. Granules appeared in the cytoplasm and cells degenerated.

**DISCUSSION**

The blood cells of lepidopterous insects have been studied in detail on several species (Yeager, 1945; Arnold, 1952; Clark and Chadbourne, 1955; Jones, 1959; Rosenberger and Jones, 1960; Nittono, 1960). The hemocyte picture is different in different species even in the same order, and unification of hemocyte types is difficult. Even in the same species, for instance in *B. mori*, the hemocyte picture varies in different strains or races (Nittono, 1960). In *C. suppressalis* larvae, 7 types of hemocytes were recognized and the hemocytes were similar in most respects to those of other lepidopterous insects but were different in detail. The ratio of each hemocyte type seemed to vary in different individuals, although differential hemocyte counts were not made. This variation may be due to differences in physiological conditions. There are many reports on the variation in hemocyte counts in relation to physiological conditions (Jones, 1962).

The hemocytes of *C. suppressalis* aggregated to form cell masses when they were drawn out. Similar clumping of hemocytes has been reported in *Prodenia eridania* larvae (Rosenberger and Jones, 1959). Some hemocytes of *C. suppressalis* adhered to the glass soon after they were brought into the culture vessels, and became flat and formed cell sheets. Similar cell adhesion and formation of cell sheets or syncytia have also been reported in several insect species when their hemocytes were cultivated in vitro (Glaser, 1917; Arvy and Gabe, 1946; Rosenberger and Jones, 1959; Clark and Harvey, 1965).

Mitoses were often observed in plasmacytes as well as in prohemocytes in the present study. Arvy and Gabe (1946) reported that in the cultivation of *Forficula auricularia* hemocytes, mitoses could be observed in the hemocyte cell sheets formed on the glass surface, Lüscher (1948) observed cell division of hemocytes in *in vivo* cultivation of *Rhodnius prolixus* tissue.

Previously there have been no reports of successful attempts at subculturing insect hemocytes. In the present study two different techniques were applied in order to detach the cells from the glass surface; mechanical and chemical treatments. When the cells were harvested from the glass surface by scratching, they aggregated to form cell masses, and the cell masses were mechanically dissociated by pipetting in and out in the new culture vessels. In vertebrate cell cultures trypsinization has been widely used for collecting cells from the glass surface. But some insect cells have been reported to be sensitive to this treatment (Martignoni, Ziec and Wagner, 1958; Aizawa and Vago, 1959). In the previous paper (Mitsuhashi, 1965 b), most tissues of *C. suppressalis* were found to be quite tolerant to trypsinization, and in the present study the hemocytes of this species also proved to be tolerant to treatment with trypsin. This finding facilitated the subculturing of the hemocytes.
In general, fresh medium has been considered to be rather toxic to cultivated cells. For this reason, many culturists have used the medium conditioned by the cells concerned, especially in subculturing. But, in the case of *C. suppressalis* hemocytes, fresh medium was used without deleterious effects.

The success in subculturing *C. suppressalis* hemocytes suggests the possibility of establishing an insect hemocyte cell-line. The hemocytes survived long enough and the mitotic activity of cells remained almost constant over the entire period of cultivation. If the multiplication of hemocytes can be accelerated by improving culture conditions, it should be possible to establish a cell-line. For this purpose, attention must be given, not only to the improvement of the culture medium but also to the use of different culture methods. For example, HORIKAWA and KURUDA (1959), in the culture of the hemocytes of *Drosophila melanogaster*, obtained good cell multiplication by rotation culture.

During cultivation, cells with various forms intermediate between prohemocytes and plasmatocytes appeared, suggesting the transformation of prohemocytes into plasmatocytes. Transformation of hemocytes has been studied experimentally in *Drosophila* (RIZKI, 1962), and it is hoped that transformation of hemocytes in *C. suppressalis* will be clarified when a clone of prohemocytes is established.

REFERENCES


