THE PROLONGED GROWTH AND SURVIVAL OF OVARIAN TISSUE OF THE PROMETHEA MOTH (CALLOSAMIA PROMETHEA) IN VITRO*

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ABSTRACT

1. The ovarian tissues from diapausmg pupae of the promethea moth (Callosamia promethea) have survived and grown for 186 days under in vitro conditions. There was continual cell migration and multiplication for a period of 53 days, followed by a period of 47 days during which no cells migrated from the tissues. Between the 100th and 105th days after setting up the cultures, cell migration was resumed, and by the 111th day 250 cells were present in the medium. A few cell divisions were observed between the 126th and 136th days. After the tissues were subcultured on the 140th day, the explant culture continued to survive, but the cell culture died 3 days later.

2. The tissues were subcultured a total of 6 times during the 186 days. By the introduction of a piece of live tissue into the cell cultures, the growth and survival of the cells were increased from 8 days to about 20 days.

3. It is possible that the tissues had become adapted to the medium during their long survival, as the cells which migrated from them after 100 days showed considerably longer survival than those in earlier cultures.

In recent years it has become increasingly apparent that many problems in many branches of entomology will not be adequately solved until the technique of growing insect tissues in vitro for long periods becomes available. This is particularly true in studies of the transmission of plant and animal viruses by insects. In fact, many of the recent attempts to culture insect tissues have been made for the purpose of studying methods of transmission of viruses by insects (Wyatt, 1956; Grace, in press). The technique will also serve as an extremely useful tool in problems of insect biochemistry, physiology, genetics, morphology, and pathology.

Attempts to grow insect tissues in vitro were first made by Goldschmidt in 1916, but it is only in the past 2 or 3 years that significant progress has been made. With the exception of the experiments carried out by Trager (1935), in which he grew the ovarian tissue of the commercial silkworm

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(Bombyx mori L.) for 2 weeks in hanging drop cultures, the attempts have resulted in very little, if any, growth and survival for short periods. Improved results were obtained by Wyatt (1956) and Grace (in press), in which the ovarian tissue of the larval silkworm (B. mori) grew for up to 3 weeks and survived for 4 weeks in roller tubes and hanging drop-cultures. Loeb and Schneiderman (1956) were able to maintain the epidermis from the pupal wing and antenna of Samia walkeri (Drury) and Antheraea polyphemus (Cram.) alive in vitro for as long as 6 weeks, but no growth was obtained.

Only two attempts have been made to subculture the tissues (Trager, 1935; Grace, in press). In neither case was it possible to carry the tissues through more than two subcultures. This paper describes in detail the growth and survival in vitro for 186 days of the ovarian tissue of diapausing pupae of the promethea moth (Callosamia promethea) in a partially synthetic medium formulated by Wyatt (1956), and modified by Grace (in press). This is the longest period in which insect tissues have been grown in vitro, and represents a big step towards the achievement of continuous growth of insect tissues comparable to that obtained in plant and vertebrate tissue culture.

Materials and Methods

The experiments were performed on ovarian tissue obtained from diapausing pupae of the promethea moth (Callosamia promethea). The ovaries in the diapausing pupae are enclosed within the ovarian sac and lie in the abdominal cavity embedded in the fat body. To remove them, the 5th and 6th abdominal tergites were removed and the ovaries pulled free. They were then transferred to a drop of culture medium, freed of any attached non-ovarian tissue and cut into pieces about 1 mm. in size.

The basic medium, consisting of salts, amino acids, organic acids, and sugars, was made up as described by Wyatt (1956). Since the members of the vitamin B complex were found to have a beneficial effect on the cultures (Grace, in press), each of them was added to the medium at a concentration of 0.01 µg./ml. Cholesterol, although it appeared to have no effect on the survival or growth of the cells in culture (Grace, in press), has been shown to be essential for normal growth of insects, and was added at a concentration of 0.03 mg./ml. The medium contained 3 per cent plasma obtained from diapausing pupae of the promethea moth. The pupae were bled from the wing, and the blood was collected in sterile centrifuge tubes packed in ice. Immediately after collection, it was heated at 60°C. for 5 minutes, and then deep-frozen for 24 hours to inhibit the action of the enzyme tyrosinase. After centrifugation to remove the blood cells and precipitated proteins, the clear yellow supernatant was added to the medium.

To inhibit the growth of bacteria, penicillin and streptomycin were added to the medium at a concentration of 0.03 mg./ml. and 0.1 mg./ml., respectively.

The principal culture method used was the hanging drop in slides with a central...
depression of about 0.8 mm., or Maximov slides. The cover glass containing the tissues in about 0.005 ml. of medium was sealed to the slide with a mixture (1:1) of paraffin wax (m.p. 54°C.) and vaseline. Cultures were also grown for short periods in roller tubes and shaking flasks. The medium in the cultures was renewed once every 5 days and all cultures were incubated at 25°C.

Just prior to dissection the pupae were surface-sterilized with 70 per cent alcohol. All glassware was sterilized by dry heat (160°C. for 1½ hours). The media were sterilized by passage through sintered glass (u. f.) filters.

The criteria used to adjudge growth in the cultures were the presence of mitoses in the migrated cells and an increase in the cell population. Those cells were considered healthy in which the cytoplasm remained clear and movements took place. When fat-like, highly refractive droplets appeared in the cytoplasm, the cells ceased to move, rounded up, and after 1 or 2 days began to degenerate.

**Subculturing**

As the cells which grow from the explants do not remain attached to them, but either float freely in the liquid medium or become attached to the cover glass, the most practical method of subculturing was the following. When the cell population in the cultures had become dense, the tissue was removed, special care being taken to remove as few free cells as possible. After washing the tissue twice to remove any adherent cells, it was set up in a fresh culture. The other culture which now contained only cells was replenished with fresh medium and resealed.

**Experiments and Results**

Six hanging-drop cultures were set up with 1 explant in each culture. After 48 hours many cells had moved from the explant, the majority remained floating in the medium, while very few became attached to the cover glass. The cells floating in the medium remained round in shape and did not move actively, whereas those attached to the cover glass became spindle-shaped or otherwise irregular in outline and moved quite actively on the glass. Numerous mitotic divisions were observed after 72 hours. The time from metaphase to complete cell division was 15 to 20 minutes at 25°C.

After 6 days in culture, during which time the number of cells had increased markedly (Fig. 1) and mitoses were very numerous, the 6 explants with the medium and as many cells as possible were transferred to a 25 ml. Erlenmeyer flask. Six explants from freshly dissected ovaries were also placed in the flask. The volume of the medium was made up to 1.0 ml., and the flask placed on a shaking table and shaken at 80 revolutions per minute for 4 days. The shaking motion was not violent; it merely swirled the medium in a circular motion over the tissues. During the shaking, 10 of the 12 explants became attached to the wall of the flask and, although it was not possible to study the culture closely, a small area of cells could be seen surrounding each attached explant.

At the end of 4 days, the 12 explants, as many free cells as possible, and the
medium were removed from the flask and transferred to a roller tube. To ensure that the explants became firmly attached to the walls of the tube, the medium was added 1/2 hour after the explants were introduced. The volume of medium was made up to 1.0 ml., and the tube rotated at 1 revolution per minute.

After 24 hours cells had begun to migrate from the explants, and the number continued to increase during the next 5 days. The cells became attached to the wall of the tube and formed a "halo" around each explant. Due to the continual rotation of the medium, the cells became spindle-shaped. Mitoses could not be observed because of the rounded surface of the tube, but the cells remained healthy and free of granules. After 6 days' rotation, the explants, but not the cells, were removed from the tube, washed in a drop of medium to remove any attached cells, and set up in 6 hanging drop cultures (each culture containing 2 explants). The medium in the roller tube was renewed. This was the first time the tissues were subcultured.

Sixteen days after the cultures had been initiated, the muscle sheaths around the ovarioles began to contract. No movement of the explants was noticed prior to this. In the intact promethea pupae contractions were not noticed in the ovaries during diapause, but set in 2 to 3 days after the onset of adult development. Similarly, the ovarian tissues of late 5th instar silkworm (B. mori) larvae showed contractions after the tissues had been in culture about 9 days, but none were noticed in the insect until the 2nd or 3rd day after pupation (Grace, in press).

Cells began to migrate from the subcultured tissues 48 hours after they had been set up, and the number gradually increased both by migration from the explant and by cell division. During the first 3 days the cells remained in open growth and then began to clump together (see Fig. 2). Mitoses were very numerous both in the free floating cells and in the cells at the edges of the groups. The period from metaphase to complete cell division was the same as in the original cultures, i.e., 15 to 20 minutes. In the roller tube culture which contained only cells, granules appeared in the cells, both in the cytoplasm and the nucleus, 3 days after subcultures had been made. By the 8th day practically every cell was granulated and many were degenerating. This culture was discarded.

The hanging drop cultures were again subcultured 10 days after the first subculture. Since the cell population in the first subcultures was smaller than in the original hanging drop cultures, on subculturing, 11 explants were divided between 2 hanging drops so that these contained 5 and 6 explants, respectively. The 12th explant was placed in a culture containing all the cells from the previous cultures because the presence of a piece of tissue had been shown to increase growth and prolong survival in cultures containing only free cells of the silkworm ovary (Grace, in press).

As before, cells began to migrate from the explants (which continued to
contract very actively) after 48 hours and continued to increase in number. Although some cells tended to form groups, the majority remained separate (Fig. 3).

In the cell culture there was a slow, but noticeable increase in the number of cells over a period of 16 days. Whereas in the previous cell culture granulation became noticeable after about 3 days, in this culture very few cells showed granulation up to 13 days. The explant continued to move actively, and small groups of cells migrated from it. By the 17th day many of the cells at the edge of the drop had flattened out on the cover glass, their cytoplasm becoming very tenuous, thread-like, and granulated. Gradually the cells closer to the explant became granulated, until by the 20th day only a few cells close to the explant were still healthy. No mitoses were observed after the 18th day, but the explant remained quite active.

Because of the large number of explants in each culture, the size of the cell population after 7 days was large enough to subculture a third time. The vast majority of cells in these cultures were very healthy, but a few cells at the periphery of migration showed granulation. It was also observed that the contractions of the explants became slower and more irregular after the 3rd day. This was probably due to the depletion of the oxygen supply in the chambers by the tissues, and to the quick accumulation of toxic metabolites in the medium because of the large number of explants. To improve conditions on subculturing, 4 cultures were set up, 3 of which contained 3 explants each, while the remaining culture comprised 2 explants and, in addition, all the cells from the cultures.

In the cultures containing only explants, the migration of cells and the growth of the cell population were slow for the first 3 days. Several mitoses were observed in cells after 48 hours. During the next 4 days the population increased slowly, the greatest population being attained in culture 2, which numbered about 600 cells.

There was a slow, but noticeable increase in the cell population of the "cell" culture, due in part, no doubt, to migration of cells from the explant. The cells remained healthy in all parts of the culture, and mitoses were fairly numerous for 12 days. By the 19th day after subculturing, the majority of cells were dead.

The cultures were divided a fourth time 13 days after the third subculture into 3 cultures each with 3 explants, and the cells and the explant from the second subculture in a fourth culture. Two days later, 2 of the cultures containing 3 explants became contaminated with fungi and were discarded. The remaining cultures showed very poor growth. After 7 days there were not more than 200 cells in each explant culture, and most of the cells in the cell culture had died. As very little growth occurred, the 3 remaining explants and the 2 explants from the third subculture were placed in 1 culture, and the cells and 1 explant in another culture.

Although the explants continued to contract, not one cell was observed to
migrate from them over a period of 47 days. During this time the 5 explants slowly grew into one mass. The medium was routinely changed every 5 days, and occasionally the tissue was torn to encourage cell migration. The cell culture continued to grow for 8 days, after which time the cells gradually died. The explant remained active and was transferred to the explant culture 14 days after subculturing.

Between 100 and 105 days after the cultures were first set up, cells began to migrate again from the tissue mass. The migration continued, and by the 111th day 250 cells were present (Fig. 4). No mitoses were recorded during this time. On the 126th day, 4 mitoses were observed, and further divisions were found on each of the 127th, 135th, and 136th days. Some cells by the 136th day had become granulated, especially those furthest from the tissue mass.

On the 140th day the culture was subcultured as previously. No cells appeared in the explant culture until 11 days later (151st day), when 2 cells were present. The cells in the cell culture remained healthy, and 2 mitoses were observed on the 143rd day; but by the 151st day the majority had died.

The cell population in the explant slowly increased, although some cells died. By the 161st day 20 cells were counted and 1 division was recorded. No divisions have been noticed subsequent to this date.

Although after 186 days in culture the migration of cells from the tissues is slow and intermittent, the tissue mass continues to contract very strongly, and there is every reason to suppose that it will continue to survive.²

**DISCUSSION**

The *in vitro* culture of insect tissues has for many years lagged behind plant and vertebrate tissue culture, but the experimental results reported above clearly indicate that the long term culture of insect tissues is now possible.

It is of interest that the tissues from the diapausing pupae have continued to grow and survive in a medium lacking the growth or prothoracic gland hormone during the entire culture period. In previous studies using ovarian tissue of the silkworm *B. mori*, superior growth and slightly longer survival were obtained when hormone extracts were added to the medium (Grace, in press). Schmidt and Williams (1953) showed that the spermatocytes of the cecropia silkworm required the presence of the growth hormone for their development, and no development took place when the spermatocytes were cultured in a medium containing blood from diapausing individuals. Perhaps the ovarian tissues become hormone-independent when grown *in vitro*.

During the 6 months in which the cultures have grown it has been possible to subculture the tissues 6 times, but it is still not possible to obtain the con-

² *Note Added in Proof.*—The tissue mass has continued to survive without growth and is now 336 days old (48 weeks) and still showing activity.
tinuous growth of cells. The addition of a living explant to the cell cultures had a decidedly beneficial effect on the growth of the cells. Whether this effect was due to the secretion by the tissue of some factor into the medium or to the removal of substances by the tissue from the medium has not yet been determined, but is currently being studied.

Although the cells ceased to migrate from the culture about 53 days after they were set up, growth did not stop altogether, as evidenced by the growing together of the 6 explants into one mass. The most outstanding feature of the experiment was the rather sudden resumption of cell migration from the 100-day-old tissues.

It is probable that some of the cells which had migrated from the tissues at 100 days survived for between 36 and 51 days, as granulation was first observed about the 136th day and the majority of cells were dead by the 151st day. This is a considerably longer survival than has been obtained previously, and is explained on the assumption that the tissues have become adapted to the medium.

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REFERENCES

EXPLANATION OF PLATE 7

Fig. 1. Cells from a 6 day old culture in hanging drop. × 97. (Photograph by J. A. Carlile.)

Fig. 2. Clumping of cells in the first subculture. 7 days. × 97. (Photograph by J. A. Carlile.)

Fig. 3. Cells migrating from ovarian tissue, second subculture. 5 days. × 213. (Photograph by J. A. Carlile.)

Fig. 4. Migrated cells from the ovarian tissue 111 days after the cultures were first set up. × 97. (Photograph by J. A. Carlile.)
(Grace: Growth and survival of ovarian tissue)