EFFECTS OF VARIOUS SUBSTANCES ON GROWTH OF SILKWORM TISSUES *IN VITRO*

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Summary

Cells from the ovaries of late final instar silkworms have undergone mitosis in hanging-drop preparations for 19 days and survived for 29 days in a medium consisting of Wyatt's solution plus 10 components of the vitamin B complex, cholesterol, and extracts of endocrine and ovarian tissues. Attempts to subculture the cells met with only partial success.

The addition to Wyatt's medium of 10 members of the vitamin B complex and *meso*inositol improved the appearance of the cells but did not affect their growth or survival. Neither cholesterol nor nucleic acids had any effect on the growth or survival of the tissues.

The replacement of the sugars of Wyatt's medium by trehalose had a deleterious effect on growth and survival. When either glucose and fructose or sucrose were replaced by trehalose growth and survival were not affected. The addition to the medium of extracts of endocrine organs or of ovaries increased markedly the number of mitoses in the cultures.

Only ovaries from larvae which had reached the fourth instar produced cells which grew in culture. The only cells of the silkworm which grew in culture were those of the ovariole covering. Cell density within the cultures had an effect on their growth and survival.

I. Introduction

In recent years several attempts have been made to grow insect tissues *in vitro* for long periods. The majority of these investigations have been concerned with developing the technique for use in the study of problems associated with the transmission of plant and animal virus diseases by insects (Trager 1935; Maramorosch 1956; Wyatt 1956).

The major difficulty has been the lack of balanced physiological solutions formulated from adequate knowledge of the composition of insect haemolymph. Although there are a number of "Ringer" solutions (see Roeder 1953, p. 161) for a variety of insects, these have been found in most cases to bear little relation to the composition of the haemolymph of the insects for which they were designed. Most of these solutions were developed for the purpose of maintaining tissues for short periods only.

In 1956, Wyatt, Loughheed, and Wyatt made chemical analyses of the haemolymph of the silkworm *Bombyx mori* (L.). From this information Wyatt (1956) formulated a medium in which she was able to obtain growth of ovarian tissue of

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the silkworm for 2 weeks in roller tube cultures. No attempts were made to subculture the tissues, and continuous growth of any insect tissue in culture has still not been achieved.

In the present investigations, attempts have been made to improve the growth of the cultures by studying (1) the effect of adding to Wyatt's medium various substances which may promote growth of the tissues, and (2) the effect of age and other characteristics of the tissues.

II. Material and Methods

(*a*) *Insect Culture*

The silkworms (*B. mori*) were reared in shallow metal trays and fed on mulberry leaves. During the winter months they were kept in incubators at a temperature of $24 \pm 2°C$. During the warmer months the trays were placed on benches in the laboratory.

As it is not possible to obtain freshly picked leaves during the winter, the silkworms were fed during these months on leaves stored in plastic bags and kept in a refrigerator at about 4°C. Under these conditions the leaves kept remarkably fresh for approximately 3 months, provided no water was present on the surface of the leaves.

(*b*) *Materials Used*

(i) *Ovaries.*—In the majority of experiments the tissues used were from last stage (5th instar) larvae which had ceased feeding and in which the rosy coloration of the prepupae had appeared. Tissues from younger larvae were also cultured. No trouble was experienced with fungal, viral, or bacterial diseases of the silkworms. The larvae were surface-sterilized by washing twice with 70 per cent. alcohol, the second wash being a few minutes prior to dissection. No contamination was experienced using this technique. The larvae were starved for 24 hr to ensure an empty gut at the time of dissection.

Ovaries were obtained by removing the tergite from the 5th abdominal segment. Usually the ovaries remained in the larvae but were occasionally removed with the tergite. They were placed in a drop of medium and freed from any adhering fat-body or other tissue. They were then cut into pieces about 1 mm^3. One ovary produced three to six pieces of tissue, depending on its size.

The ovariole sheaths were removed as follows: The ovarioles were separated from the ovarian sac; an ovariole was held by the oviduct with a pair of fine forceps; another pair of forceps was placed so that they straddled the ovariole. By gently pulling on the oviduct, the sheath was slipped off unbroken. It was picked up in a wide-mouthed pipette and cultured in a drop of medium.

(ii) *Haemolymph.*—Haemolymph was obtained by snipping off the caudal horn on the 8th abdominal segment and collecting it in chilled, sterile centrifuge tubes. It was essential to collect the haemolymph in chilled tubes to prevent the formation of melanin-like pigments which render it toxic to the tissues. The haemolymph was either centrifuged immediately or deep-frozen until required. Usually it was obtained from late 4th instar larvae, but occasionally from prepupae.

(iii) *Haemocytes.*—To obtain haemocytes for culturing, 5th instar larvae were bled from the caudal horn, the haemolymph being collected in a sterile centrifuge tube packed in ice. It was necessary to bleed about five larvae to accumulate enough cells for each culture. As soon as possible after bleeding, the haemolymph was lightly centrifuged to collect the cells into a firm pellet which could be cut into pieces about 1 mm^3.

(iv) *Embryos.*—Embryos at two stages of development were studied. The youngest embryos were those in which segmentation was just completed but the appendages were still small outgrowths. The older embryos were nearly fully developed, the mandibles and eyes were pigmented, and the heart and gut showed movements.

By careful manipulation it was possible to remove the embryos from the eggs without breaking them, and without carrying over much yolk. Before being set up in culture the embryos were washed three times in the medium. The hypodermis of the older embryos were torn into several pieces to ensure that all the tissues were bathed by the medium. Although it was very easy to transfer the older embryos to a hanging drop without breaking them, the younger embryos invariably broke apart when being lifted through the surface film of the medium.

(*c*) *Basic Medium*

In all the experiments described in this paper Wyatt's (1956) physiological solution containing 2 per cent. heat-treated haemolymph (60°C for 5 min) was used as the basic medium. Media were sterilized by passage through a Seitz or sintered-glass filter. Penicillin and streptomycin were routinely added to the media at concentrations of 0·03 and 0·1 mg/ml respectively.

(*d*) *Tissue Extracts*

(i) *Endocrine Organs.*—Ring glands were taken from 200 larvae of *Lucilia cuprina* (Wied.) in the middle of the last instar, and the prothoracic glands from 20 last-stage nymphs of *Periplaneta americana* (L.). Adhering tissues were removed, and the glands transferred to 1·0 ml of medium (which did not contain any haemolymph) in a sterile centrifuge tube and macerated. The resulting suspension was stirred thoroughly and allowed to stand for 1 hr. It was then centrifuged for 5 min at 2200 *g*. The supernatant was filtered through a sintered-glass filter and a solution containing 2 per cent. heat-treated haemolymph added.

(ii) *Silkworm Embryo.*—Embryo extract was obtained from eggs in which diapause had been broken by immersing them (24 hr after laying) in concentrated (35 per cent.) hydrochloric acid for 3 min. The eggs were washed several times in tap water and dried on filter paper. After the acid treatment the eggs were incubated for 6 days at 25°C, then surface-sterilized in 70 per cent. alcohol, and finally ground in a mortar with a little medium to which a small amount of sterile sand had been added. The suspension was then centrifuged at 90 *g* for 5 min to remove the tissue debris and sand. The supernatant was transferred to a fresh sterile tube and centrifuged at 9000 *g* for 60 min. The final supernatant which was still slightly

cloudy was then transferred to a sterile tube, stoppered, and kept in a deep-freeze unit until required. (It was not possible to clarify the extract by centrifugation at this speed.)

By placing the extract in boiling water for 2 min a large amount of protein was precipitated. Centrifuging now produced a clear supernatant.

(e) *Techniques*

Glassware was cleaned by boiling with soft soap for 10 min, rinsing in tap water, and re-boiling for 10 min in distilled water. It was then rinsed once in distilled water, dried, and sterilized in an oven at 160°C for 1½ hr.

The principal culture method used was the hanging drop in small depression slides. Single explants were placed in approximately 0·005 ml of medium. Cultures were also set up in Carrel flasks, roller tubes, hanging drops containing perforated "Cellophane", and solid clots of fowl plasma.

The criteria used to estimate growth and survival were respectively the presence of mitoses in cells which had migrated from the tissues 3–4 days after setting up the cultures, and lack of granules, fatty droplets, or other abnormalities of the cytoplasm.

III. Experimental

(a) *Cell Growth in the Basic Medium*

In previous experiments carried out over a period of 2½ years all the substances (vitamins, cholesterol, nucleic acids, hormone, and tissue extracts) studied had been tested for their effect on growth of silkworm larval tissues in Trager's (1935) medium. However, little or no effect was noticed when these substances were present, perhaps because the medium did not resemble sufficiently the haemolymph of the silkworm. Eventually, in Trager's medium to which had been added B vitamins, cholesterol, and 10 per cent. silkworm plasma it proved possible to keep the cells active for 5–6 days, but very few mitoses occurred after 3 days and the tissues never survived for longer than 9–10 days.

In all experiments with Wyatt's medium it was noted that, during the first 24 hr after setting up the cultures, very few cells migrated from the tissues, and those that did stayed close to them. During the next 24 hr the number of cells which moved away from the tissues increased considerably. The majority of cells adhered to the cover-glass or the surface of the drop although some remained suspended in the medium. The cells which adhered to the surfaces became either irregular in outline or spindle-shaped and moved in an amoeboid manner, whereas those which were in suspension remained spherical. After 72 hr many cells had migrated well away from the explant. Although they remained active, very few cells migrated further so that, after a few days, a number of cells formed a "ring" around the explant. The cells within the ring continued to move in all directions but, as the culture aged, the number which came to lie near the explant increased. It was also observed many times that the number of mitotic divisions increased after the first 48–72 hr, continued at a constant level for a few days, and then gradually dropped as more and more cells became granulated. Eventually the

mitoses ceased even though there were a large number of active, transparent cells near the explant. Finally, granulation and subsequent degeneration began in the cells furthest from the tissue, and gradually moved towards the explant until all cells had become degenerate. Frequent renewal of the medium did not prevent degeneration of the cells.

(*b*) *Growth in Modifications of the Basic Medium*

The basic medium has been modified by addition of a number of metabolites or extracts. These together with their effects are listed in Table 1.

(i) *Vitamins.*—The members of the vitamin B complex are necessary for normal growth of insects (see Trager 1953). The following members of the B group were added: thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, folic acid, *p*-aminobenzoic acid, choline, and *meso*inositol. At a concentration of 10 μg/ml

TABLE 1

EFFECT ON TISSUES OF ADDITIONS TO WYATT'S MEDIUM

+ = better growth or survival; − = harmful; 0 = no effect

Modification	Effect	Modification	Effect
B vitamins (10 μg/ml)	−	Trehalose replacing either hexoses or sucrose	0
B vitamins (0·01 μg/ml)	0	Endocrine extract	+
*meso*Inositol	0	Embryo extract	−
Cholesterol	0	Treated embryo extract	0
Ribonucleic acid, thymus nucleic acid (separately or together)	0	Ovary extract	+
Trehalose replacing all sugars	−	Endocrine extract plus trehalose replacing all sugars	+ +

(except choline at 100 μg/ml, and *meso*inositol at 2 μg/ml) heavy granulation of the cells occurred after 4 days, the cytoplasm became threadlike, and the cells did not survive longer than 1 week. When only *meso*inositol at 2 μg/ml or all vitamins at 0·01 μg/ml were added, there was a definite improvement in the appearance of the cultures. Many more cells migrated from the tissues but there was no increase in the number of mitoses or survival of the tissues.

(ii) *Cholesterol.*—This growth factor (essential for normal growth in the Diptera, Lepidoptera, and Coleoptera) was added to the basic medium at a concentration of 0·03 mg/ml to produce a saturated solution. Its presence did not appear to have any effect on the survival or growth of the tissues.

(iii) *Yeast Nucleic Acid and Thymus Nucleic Acid.*—These nucleic acids were added separately or together to the medium in several concentrations (10, 20, and 30 μg/ml). In no instance did the addition of either or both of these acids have any effect.

(iv) *Trehalose.*—Wyatt and Kalf (1956) have recently shown that trehalose is present in silkworm blood at the surprisingly high concentration of 500 mg/100 ml.

Three sugars (glucose, fructose, and sucrose) were included in Wyatt's medium. In media containing trehalose as the only sugar at the same concentration (150 mg/100 ml) as the sugars in the basic medium, or at 500 mg/100 ml, there was very little migration of cells from the tissues even after 1 week in culture.

When either the glucose and fructose or the sucrose were replaced by trehalose the number of cells migrating from the tissues was about the same as in the original medium. However, when trehalose was used in the medium containing extracts of endocrine organs (see below) good cell growth and multiplication was obtained.

(v) *Endocrine Organ Extracts.*—The number of cells which migrated from the tissues in the hormone-containing medium (prepared as described in Section II) was the greatest ever obtained (Plate 1, Fig. 1). Mitoses were first noticed 8 days after establishing the cultures, and cells in all parts of the medium underwent mitosis, whereas previously the mitoses were found to be more numerous in cells near the tissues. The period from metaphase (the first easily recognizable stage of mitosis) until complete cell division was 15–20 min. During the next 5 days numerous mitotic figures were noticed both in cells near the explant and at the periphery of migration. The number sharply decreased until, by the 17th day, only one or two near the explant could be found. No granulation or decreased mobility of the cells was observed in any of the cultures for about 14–16 days. As found by other workers, the first signs of granulation were in the cells furthest from the tissue. By 21 days the majority of cells had become round and granular but a few cells close to the explant were still transparent and active.

(vi) *Embryo Extracts.*—The supernatant from ground, centrifuged eggs, when added to the medium at concentrations of 2–5 per cent. caused heavy granulation of the cells within 4 days.

In media containing 5 or 10 per cent. of the treated embryo extract (see Section II), large numbers of cells migrated from the tissues. All the cells were still transparent after 9 days and active except for a few at the margin of migration. Granulation of the cells developed subsequently until, by 14 days after the cultures had been prepared, only a few transparent cells were present.

(vii) *Ovarian Extracts.*—The fact that the cells nearest the tissue seemed to survive longer than those further away suggested a possible effect by the tissue on the medium. Twelve ovaries from late, last-instar larvae were ground very thoroughly in 1·0 ml of haemolymph-free medium. The solution was filtered and 2 per cent. of heat-treated blood (60°C for 5 min) was added.

Tissues set up in this medium showed very marked migration of the cells with numerous mitoses after 48 hr, comparable with that obtained using the medium containing hormone extract. Granulation of the cells began to appear about the 14th day, and by the end of 21 days the majority of the cells were degenerating. Apparently the ovarian extract had some beneficial effect for, prior to the use of this medium and that containing hormone extracts, mitoses were very few and only rarely did cells survive longer than about 2 weeks.

As the presence of either endocrine organ or ovarian extracts in the medium had a very beneficial effect on growth, a medium was prepared in which both extracts

were present in the same amounts as when they were added singly. Migration and multiplication of the cells was profuse. Mitoses were first noticed 48 hr after establishing the cultures and were numerous for 12–14 days. During this time no granulation occurred and the cells were active. After the 14th day the number of mitoses decreased and about the 16th day some of the outermost cells contained granules. Mitoses could still be found near the explant after 18 days but they were scarce. Granulation of the cells spread progressively inwards and by the 20th day no mitoses were found and the majority of the cells were degenerating.

An attempt was made to test further the effect of the endocrine organ and ovarian extracts on growth. In a previous experiment in which sucrose, fructose, and glucose were replaced by trehalose, the growth and multiplication of the cultures was very poor. This experiment was repeated, with the addition of endocrine organ extract to the medium. After about 3 days migration was marked. On the 8th day ovarian extract was added when changing the medium. Quite a large number of mitoses were observed 2 days later and one or two could still be found after 19 days. A number of cells in these cultures had quite obviously undergone mitosis to the stage where cell division was almost but not quite complete (Plate 1, Fig. 2). By the end of 21 days granulation was far advanced. The results of this experiment demonstrate that the endocrine organ and ovarian extracts have a beneficial effect on the survival and growth of the tissues.

(*c*) *The Effect of "Conditioned" Medium*

To determine whether conditioning of the medium had any effect on growth of insect tissue, six pieces of ovary tissue were placed in 0·1 ml of medium and incubated for 48 hr at 26°C. The medium was then used in hanging-drop cultures each containing one explant in 0·005 ml of medium. The migration of cells was poor and many became granulated after 4 days. It is possible that the practice of changing the medium in hanging-drop cultures every 48 hr may have an adverse effect on growth. In some cultures the medium was, therefore, changed only every 4th day and in others once weekly. In neither series of cultures was survival or growth increased.

(*d*) *Modification of the Vapour Phase*

In an attempt to study the effects of modification of the gas phase either CO_2 or O_2 was blown under the coverslip for 30 sec prior to sealing it to the slide. Each time the medium was changed the gassing procedure was repeated. Control cultures which were not treated in any way were set up at the same time. The migration of cells from the tissues and the length of survival were the same as in the control cultures when either CO_2 or O_2 was used. The pH of the medium dropped from 6·4 to 6·2 after 48 hr in those cultures gassed with CO_2.

To decrease the CO_2 tension in normal cultures, two very fine capillaries were sealed with paraffin on to a depression slide so that there could be a flow of gas in and out of the chamber. Drying out of the cultures did not occur. These treatments had no effect on the growth or survival of the tissues.

(e) *Modifications of the Tissue Explants*

In all the studies on insect tissue culture in which the silkworm has been used, cells from the ovaries are the only ones which have grown in culture (Trager 1935; Grace 1954; Wyatt 1956). The fat-body, gut, salivary glands, muscles, testes, nerve, blood cells, and hypodermis have all failed to grow.

In this section experiments will be described in which the effects on growth have been studied of (i) the age of the ovaries used, (ii) culturing the ovariole sheath removed from the ovariole, (iii) increasing the cell concentration in the cultures, (iv) other tissues, particularly embryonic tissues and haemocytes, (v) subculturing.

(i) *Age of the Ovaries.*—The ovaries of 2nd, 3rd, 4th, and 5th instar larvae (early and later in the instar) were cultured in Wyatt's basic medium to which was added B vitamins, cholesterol, and 10 per cent. silkworm larval plasma.

TABLE 2

EFFECT OF AGE OF THE OVARIES ON GROWTH AND SURVIVAL

Instar from which Ovaries Taken	No. of Explants per Culture	Growth and Survival
2nd or 3rd instar	2 ovaries	No growth. Survived for 6 days. No cell migration
4th instar	2 ovaries	No growth. Survived for 10 days. A few cells migrated into medium
5th instar		
(*a*) Early	2 explants	No growth Survived for 16 days. Fair cell migration
(*b*) Late	1 explant	Limited growth. Survived for 16 days. Good migration of cells into medium

The ovaries of the early instar larvae were too small to cut into pieces so the ovarian sac was opened and the ovarioles were teased out. Usually (because of their small size) two ovaries were placed in each hanging-drop culture. The ovaries of the 5th instar larvae were cut into three to five pieces depending on their size and one explant placed in each culture.

The results as set out in Table 2 show that, at least on this rather unsatisfactory medium, no growth and very short survival was obtained in the cultures of 2nd, 3rd, and 4th instar ovaries. In cultures of the ovaries from 5th instar larvae no difference was observed in the length of survival between those taken early or late in the instar, but the amount of cell migration and the number of mitoses observed were much greater in cultures of the late 5th instar ovaries. After about 5 days in culture some of the ovarioles of the late instar ovaries showed movements, which were due to the contractions of the muscle sheath around the ovariole. Muscular contractions of the tissues have not been observed in any larvae but only in pupae at least 3 days after pupation.

(ii) *Cultures of the Ovariole Sheath.*—In order to determine whether the only cells which grow in culture are those of the lining of the ovariole, the sheaths were

removed from the ovarioles of the prepupae (as described above) and cultured in hanging drops. The naked ovarioles were also cultured. Large numbers of cells migrated from the sheaths and mitoses were observed over a period of about 5 days. After 10 days in culture many of the cells were granulated and the cultures were discarded after 2 weeks. Not one cell migrated from the naked ovarioles.

(iii) *Size of Explant.*—It had been observed that, in cultures containing either the endocrine organ or the ovarian extract or both, cell migration and multiplication were very marked. However, although the period of survival was somewhat increased, the tissues were still not capable of surviving and multiplying for long periods. It is possible that the better growth in the cultures is not only due to the presence of extracts but also to a second factor, namely the size of the population of cells in the medium. To test this, a series of cultures was set up in which an explant was placed in a drop of medium containing hormone extract. After 9 days, when the cell population was dense and a few mitoses could be found near the tissue, an explant from another culture was added. Twenty-four hours later mitoses were very numerous. Dividing cells were found near the explant, and there were also many at the periphery. The majority of cells rounded up and the cytoplasm became dense prior to division, but mitoses were observed in some cells (especially those far from the explant) in which cytoplasm remained transparent and spread in a thin sheet across the coverslip. Five days after adding the second explant the mitoses became scarcer and by the 7th day all divisions had stopped. Although a very large number of cells were still healthy, many of the outer ones had become granulated. After 25 days granulation of the cells had increased and the cultures had degenerated by the 29th day.

The control cultures, containing only one piece of tissue, showed a few mitoses until they were 14 days old, after which the cells began to degenerate.

(iv) *Culture of Tissues from Embryos.*—A few attempts were made to grow embryonic tissues but have so far met with little success. The younger embryos failed to survive in culture longer than 10 days. A large number of cells containing what appeared to be fat droplets were liberated into the medium, but no mitoses were observed. The gut and heart of the older embryos continued to contract for up to 14 days but there was no growth of cells into the medium and no mitoses were observed.

(v) *Haemocyte Cultures.*—Haemocytes were placed in hanging-drop cultures in Wyatt's medium containing 10 members of the vitamin B complex, endocrine organ extract, and 2 per cent. heat-treated haemolymph. Even when a crystal of phenylthiourea was added, all cells had turned black within 24 hr and were degenerating after 3 days.

(vi) *Attempts to Subculture.*—During the early stages of the work several attempts were made to subculture the tissues. The method consisted of removing the explants from the culture, care being taken to leave behind as many free cells as possible. After renewing the medium, the tissues were implanted in one hanging drop and the cells in another.

It was not until tissue extracts were added to the medium and the consequent increased population of cells was obtained that it was possible to obtain a successful

subculture. After 14 hr the subculturing cells had begun to migrate from the tissues and migration and multiplication continued for about 3 days, but the cell population in the medium never reached the size of the parent cultures.

In several cultures, when the cells were quite numerous and mitoses were still evident, a second subculture was made, separating the cells from the tissues. Again cells were present after about 24 hr and one or two mitoses could be found. The number of cells increased very slowly over the next 2–3 days and by the 5th day there would usually be about 300 cells in the medium. The number of cells did not increase any further and granulation started about the 6th day, the cultures not surviving for more than 8 days. It was never possible to re-implant the inoculum more than twice or to subculture the cells more than once.

IV. Discussion

In the experiments outlined above three facts stand out. First, it is obvious that, although Wyatt's medium is superior for the *in vitro* culture of silkworm ovaries to any other medium which has previously been used, it does not contain all the factors necessary for the continued growth of the tissues.

Second, after the cells have been in culture for about 16 days, the number of mitoses decreases sharply and many of the cells begin to degenerate. Only in a few cultures have the cells stayed healthy for as long as 3–4 weeks.

Third, with the addition of the endocrine organ and ovarian extracts the number of mitoses and the number of cells present in the medium were greatly increased, but the length of survival and the periods during which mitoses are present have not been increased much beyond that obtained by Wyatt (1956).

The partial success obtained when the tissues were subcultured indicated that the medium contained nearly all the factors needed to prolong growth but that further detailed studies of the growth requirements of the tissues need to be carried out.

The presence of silkworm embryo extract had less effect than was anticipated on the basis of work with vertebrate cultures. Whole extract had a deleterious effect which could be ascribed to the large amount of yolk and protein material present. Gaulden and Kokomoor (1955) reported that "the frequency of mid mitotic neuroblasts in grasshopper embryos in hanging-drop cultures increases as the quantity of yolk in the cultures was increased up to quantity equivalent to one-quarter that in the egg". Although no yolk *per se* was present, the extract was made from 2000 eggs in 1·0 ml of medium. After boiling the extract, when much of the protein was removed, the tissues grew better. A similar result was obtained in a comparison between whole plasma and plasma in which much of the protein had been removed by heating. Apparently insect tissues (at least the ovaries) under culture conditions, do not tolerate a large amount of protein. In this they differ markedly from vertebrate tissues for which it is important to include proteins in the medium to obtain growth.

The improvement in growth resulting from the addition of cells to the medium is evidence that the larger the size of the inoculum (up to a limit) the more quickly

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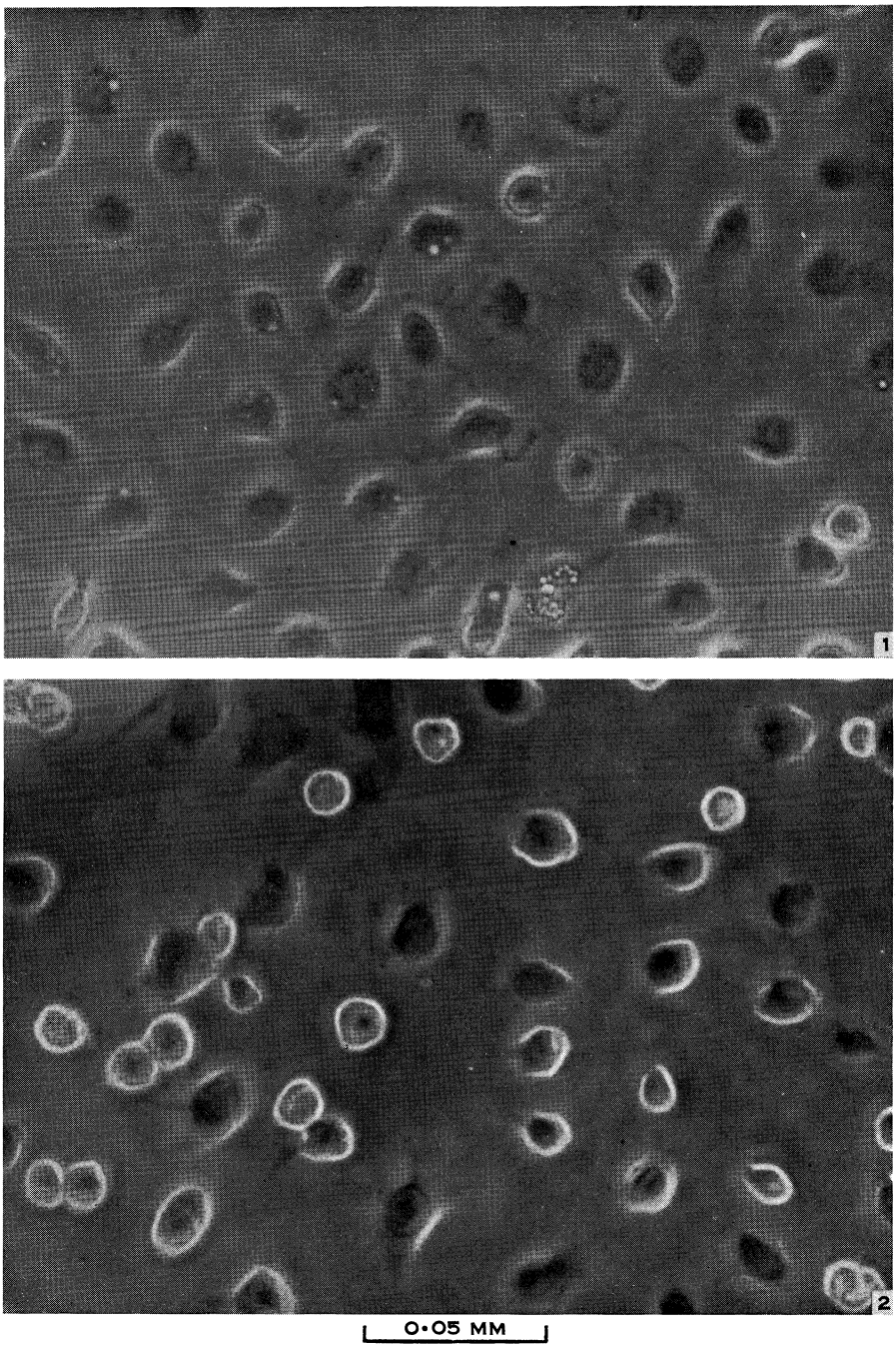

Fig. 1.—Cells from the lining of the ovarioles of 5th instar larvae of the silkworm cultured for 8 days in Wyatt's medium containing hormone extract. × 380. Phase contrast.

Fig. 2.—Numerous cells in telophase in Wyatt's medium containing hormone extract, ovary extract, and trehalose, after 20 days. × 380. Phase contrast.

will growth proceed. In growth studies of bacteria and vertebrate tissues the size of the inoculum (either separate cells or explants) has been shown to determine to a large degree whether the cells will grow or not. Experiments such as these are very similar to those in which conditioned media were found to increase the ability of the cells to grow in culture. It is probable that the greater the size of the inoculum the less time is needed for the medium to become optimal for growth.

The results of the subculturing experiments are interesting in that they show (1) that only in those cultures in which the cell population was high was it possible to obtain growth after subculturing; and (2) that the presence of the explant has a growth-promoting effect on the cells. In experiments using the ovaries of diapausing pupal *Callosamia promethea* (Drury) it has been possible to carry the cells through a second subculture only if a piece of surviving ovary was present (Grace, unpublished data). The results are encouraging when it is recognized that it is difficult to maintain growth in first subcultures of some vertebrate tissues, but after they have grown in the first subculture it becomes relatively easy to make subsequent subcultures. Until it is possible to carry out repeated subcultures, the continuous growth of insect tissues cannot be said to have been achieved.

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