CULTURE IN VITRO OF TISSUE FROM THE SILKWORM, BOMBYX MORI L.*

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PLATES 1 TO 3

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An investigation of insect tissue culture was undertaken in the hope that this technique might be developed into a useful tool for the study of insect virus multiplication and for virus titrations. That this should be practicable was indicated by the work of Trager (1935), who obtained development of polyhedral virus inclusion bodies in cells of the silkworm, *Bombyx mori* L., *in vitro*. Initially, the intention was to corroborate his work and adapt it to routine use. The project developed into an investigation of improved methods for insect tissue culture.

There have been a number of attempts to grow insect cells *in vitro*, with some degree of success reported (see Bibliography of tissue culture, also, Gavrilov and Cowez, 1941). Observations have been made on organ growth, cell survival, and parabiotic development, which are reviewed by Schmidt and Williams (1953). Two recent notes (Goodchild, 1954; Grace, 1954), however, report largely negative results and point out that as yet no one has been able to maintain insect tissue cultures for an extended period of time by subculturing, and that even maintenance of insect cells *in vitro* for more than short periods is uncertain. In the present work, after variable results were achieved with Trager's method, a new culture medium was developed based on the composition of silkworm hemolymph. Results with this were promising, and indicated some questions requiring further investigation.

Materials and Methods

The experimental insects were chiefly silkworms (*Bombyx mori* L.) reared in the laboratory on mulberry leaves. No single strain was available at all times so several strains including both white and yellow-blooded, mostly the latter, were used. Some of the earlier winter work was accomplished with pupae of *Samia walkeri* F. and F. (supplied through the kindness of Dr. T. N. Tahmisian, Argonne National Laboratory, Chicago) and larvae of the forest tent catepillar, *Malacosoma disstria* Hbn. (thanks to Mr. W. L. Sippell, Forest Insect Laboratory, Sault Ste. Marie, Ontario).

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By using greenhouse-grown mulberry foliage it was eventually possible to rear silkworms throughout the year.

Experimental insects were surface-sterilized by immersion for 1 minute in 0.1 per cent hyamine (Angus, 1952), then washed in three changes of sterile tap water, and dried in sterile Petri dishes containing filter paper. Blood was drawn by clipping the side of an abdominal leg, and collected in a 6 × 50 mm. tube, in which it could be centrifuged. The ovaries were dissected out as described by Trager (1935), cleaned of adhering fat body, and cut into pieces of about 1 mm.[3] One ovary produced 3 to 8 such explants, depending on the size of the larva.

All glassware was cleaned with Ivory soap, the detergent 7×, or acid (equal parts concentrated nitric and sulfuric), then thoroughly rinsed in tap water and double distilled water and finally sterilized. Media were sterilized by autoclaving (stock solutions of salts for Trager's medium) or filtration through UF sintered glass.

When virus was used it was purified according to Bergold (1947, 1953) but employing aseptic technique. To the final preparation were added streptomycin and penicillin at the concentrations of 0.5 mg. per ml. and 0.03 mg. per ml., respectively.

Two principal culture methods were employed. Hanging-drop cultures were prepared with a single explant in 0.005 ml. medium spread on a glass coverslip, inverted over a 0.8 mm. depth depression slide, and sealed with vaseline. In the later phases of the work, 12 × 50 mm. culture tubes were used. containing 4 to 6 explants and 0.15 ml. of medium and closed with rubber stoppers (Central Scientific Co., No. 44181, serum bottle stopper inserted upside down). These tube cultures were placed in a roller rotating at 6 R.P.H., or occasionally held stationary. Incubation was at 28 to 29°C.

The method of judging cultures was necessarily rather subjective. The number, appearance, and pattern formed by the cells were noted. At the beginning, counts were made of cells distinct from the explant, but as the work progressed this became impossible and comparative values indicated by plusses were resorted to. Cells that were transparent with few granules and no large fat globules were considered healthy. Movement of the cells would continue, however, for some time after they had lost their transparency.

EXPERIMENTAL

Use of Trager's Method.—Hanging-drop cultures were prepared with ovarian explants from late 5th instar silkworm larvae in Trager's physiological solution (Table I) containing 10 per cent centrifuged silkworm hemolymph. After 1 day of incubation these cultures generally showed, on the glass surface, the drop surface, or both, a small area containing irregular round to spindle-shaped cells in open growth (Fig. 1). The number of cells thus distinct from the explant increased for 5 to 6 days. Although many of these had undoubtedly migrated from the explant, some of the increase was due to mitosis, as metaphase and the succeeding phases of division were occasionally observed. During the same period, however, there occurred a change from transparent to granular appearance (Fig. 2), progressively from the peripheral cells, inwards, which was not prevented by renewing the medium. In addition to such "aging," the cultures were unsatisfactory because of great individual variation and the very limited amount of growth.

Some such cultures were inoculated after 48 hours' incubation with silkworm polyhedral virus, and formation of polyhedral bodies was subsequently observed. The infection was very uneven, however, and in many cells the pathological process would stop in the stage of the "ring zone" or incipient polyhedra (Bergold, 1943). The number of polyhedra per cell was also less than in blood cells drawn from infected larvae.

In an attempt to improve the cultures, peptic digests of egg albumin (as used by Trager), of bovine fibrin, and of bovine serum albumin were added to the physiological solution. These did improve the number and transparency of the cells. Use of digests was not continued, however, because different preparations varied considerably in effect, and because it was hoped that for virus work a chemically better defined medium or natural fluids, might be used.

TABLE I

Composition of Trager's Physiological Solution

	Concentration, millimolar
NaCl	15
$MgCl_2 \cdot 6H_2O$	1
$CaCl_2$	1
$NaH_2PO_4 \cdot H_2O$	1.5
K_2HPO_4	1.5
Maltose	60
Total	80
Freezing point depression	0.285°C.
pH	6.7

Tissues other than ovarian were tried as explants. With muscle, nerve, epidermis, or midgut, no cells developed separately from the explant. Some cells appeared from imaginal discs—which themselves increased in size—and from abdominal ganglia and testes. At all times the best results were in cultures from ovary.

As these cultures were not satisfactory, more profound modifications of the methods were tried, as described in the following sections.

Inhibition of Tyrosinase Activity.—The activity of a tyrosinase or phenol oxidase in insect hemolymph, producing melanin *via* intermediary quinones, has long been recognized (Sussman, 1949). At the outset of this work the tyrosinase activity was not counteracted in any way save by dilution (5 to 25 per cent) with physiological solution. After several days incubation the medium had darkened visibly. Dr. C. M. Williams (personal communication) suggested the use of phenylthiourea as a tyrosinase inhibitor. By preventing the production of probably toxic quinones this might allow the use of a higher percentage of hemolymph in the medium and thereby improve the cultures. When this was tried (recrystallized sample of phenylthiourea kindly provided by Dr. Williams), there was some decrease in granularity of the cells, but their appear-

ance was still not satisfactory, and it seemed likely that the phenylthiourea, or an impurity in it, was toxic. Therefore, other possible inhibitors were tested by mixing with cell-free hemolymph in small test tubes and observing the darkening visually. Each was tested at several dilutions, of which the figures in Table II are in each case the strongest since even these did not give permanent inhibition.

These inhibitors were then tested at the required concentrations (with respect to the blood content of the medium) in hanging-drop cultures of ovarian plants in Trager's medium. Glutathione proved superior, in both number and appearance of the cells, to any of the other inhibitors, and it was used as routine for several months. As it did not afford permanent inhibition, however, further experiments were later carried out with heat treatment. Destruction of silkworm blood tyrosinase by heat was first recorded by Ducceschi (1902), and Levenbook (1950) was able to prevent darkening of *Gastrophilus* hemo-

TABLE II

Chemical Inhibition of Tyrosinase in Silkworm Hemolymph in Test Tubes at Room Temperature

	Concentration in hemolymph	Period before development of visible darkening
Phenylthiourea	Saturated	Indefinite
p-Aminobenzoic acid	Saturated	1 wk.
Ascorbic acid	0.1 M	4 days
Glutathione	0.03 M	4 days
Disodium ethylene diamine Tetraacetate	0.03 M	12 hrs.

lymph without coagulation by heating at 60°C. for 5 minutes. In my experiments with silkworm hemolymph, coagulation started at 52°C., but the heated blood still darkened on standing. It was then found that the enzyme is precipitated though not completely inactivated at 60°, and that the supernatant solution from blood heated at 60° for 5 minutes, then chilled and centrifuged, remained clear indefinitely. Hemolymph thus treated proved superior in cultures to that inhibited with glutathione. Glutathione was then tested in dilutions for its effect on cultures with heat-treated hemolymph, and it was found to be detrimental at final concentrations of 10^{-4}M or greater, and to have no marked effect at 10^{-5}M.

Modification of the Physiological Solution.—Since insects have an open circulatory system in which the organs are continuously bathed in the hemolymph, it seemed reasonable that a culture medium should resemble the hemolymph in its composition. Since Trager's solution differed in osmotic pressure and proportions of the cations (values published since Trager's work) from silkworm hemolymph, experiments were done to find out whether correction of these differences would improve the cultures.

It was known (Bialaszewicz and Landau, 1938; Tobias, 1948) that silkworm hemolymph is high in potassium rather than sodium. Equating or reversing the proportions of these metals relative to Trager's solution, however, did not cause significant differences in the cultures. A solution was also prepared with sodium, potassium, calcium, and magnesium in proportions corresponding to the analysis of Bialaszewicz and Landau (1938) and including sufficient EDTA (ethylene diamine tetraacetic acid) to prevent precipitation of calcium and magnesium salts. The cells in this medium were more numerous than in Trager's but slightly more granular, although the usual peripheral zone of degenerating cells was not apparent.

It was also on record (*cf.* Buck, 1953) that insect hemolymph commonly has a high content of free amino acids, but at the outset of this work a quantitative analysis of these in the silkworm was not available. When this information became available (Sarlet, Duchateau, and Florkin, 1952; Wyatt, Loughheed, and Wyatt, 1956) a solution was prepared containing amino acids as well as cations in their natural proportions, except for calcium which was reduced by 20 per cent to allow for protein binding, by analogy with mammalian serum. The amino acids had sufficient chelating power to prevent precipitation of calcium and magnesium salts, so EDTA was no longer used. Although apparently absent from the hemolymph, tryptophan, cystine, and cysteine were included because the two former had been shown by Fischer (1948) to be essential for growth of chick fibroblasts, and when added to Trager's medium they were found to have a stimulatory effect on insect tissue culture. The three sugars found in the blood analyses (Wyatt, Loughheed, and Wyatt, 1956) were included, but to increase reserve energy their total was raised to a level comparable with that usual in vertebrate tissue culture media. Except for the lack of the organic acids and the presence of aspartic and glutamic acids and their amides in the proportions found in hemolymph (Wyatt, Loughheed, and Wyatt, 1956; see also below), this solution resembled that described in Table III.

When this physiological solution, with the addition of heat-treated hemolymph, was tested in cultures, it was at once noted that they were greatly superior to any previously obtained (Fig. 5). The cells were more numerous and retained their transparency and activity for a longer period than cells in Trager's medium, this difference being confirmed in repeated experiments. In the improved cultures obtained with the new medium it was generally possible to recognize differences when single factors were varied. For example, when media adjusted to different pH levels were tested, better cultures were obtained at pH 6.35 than at 6.16, or 6.5 or higher. Testing changes in osmotic pressure, it was found that the solution as formulated, or diluted by one-third, was better than when stronger or weaker than this range.

It will be noted (Table III) that the medium differs from hemolymph (Wyatt,

Loughheed, and Wyatt, 1956) in the ratio of aspartic and glutamic acids to their amides. This change was made with the idea that increased aspartate and glutamate might be helpful by combining with ammonia formed in the medium.

TABLE III

Composition of Physiological Solution for Bombyx mori

	Mg. per 100 ml.	mM		Mg. per 100 ml.	mM
1. Inorganic Salts			*4. Amino Acids*		
NaH_2PO_4	110	8	L-Arginine HCl	70	3.3
$MgCl_2 \cdot 6H_2O$	304	15	DL-Lysine HCl	125	6.9
$MgSO_4 \cdot 7H_2O$	370	15	L-Histidine	250	15.7
KCl	298	40	L-Aspartic acid	35	2.63
$CaCl_2$	81	7.2	L-Asparagine	35	2.65
			L-Glutamic acid	60	4.08
2. Sugars			L-Glutamine	60	4.11
Glucose	70	3.9	Glycine	65	8.66
Fructose	40	2.2	DL-Serine	110	10.5
Sucrose	40	1.1	DL-Alanine	45	5.05
			β-Alanine	20	2.25
3. Organic Acids			L-Proline	35	3.2
Malic	67	5	L-Tyrosine	5	0.27
α-Ketoglutaric	37	2.5	DL-Threonine	35	2.94
Succinic	6	0.5	DL-Methionine	10	0.67
Fumaric	5.5	0.5	L-Phenylalanine	15	0.9
			DL-Valine	20	0.7
			DL-Isoleucine	10	0.77
			DL-Leucine	15	1.14
			L-Tryptophan	10	0.61
			L-Cystine	2.5	0.1
			Cysteine HCl	8	0.5

Total concentrations of inorganic components (mM): Na, 8; K, 40; Mg, 30; Ca, 12; Cl, 107; sulfate, 15; phosphate, 8.

Freezing point depression: 0.53°C.

Preparation: Inorganic salts, excepting $CaCl_2$, were dissolved in 30 ml. of H_2O; the $CaCl_2$ in 7.2 ml.; sugars in 10 ml.; organic acids in 5 ml., neutralized with KOH; and amino acids in 40 ml. The solutions were mixed, the $CaCl_2$ being added last. The pH was adjusted to 6.35 and finally water was added to a total volume of 100 ml.

Culture medium: Above solution plus heat-treated hemolymph.

When cultures in media containing the natural and increased ratios of acids to amides were compared, the cells in the latter were slightly more transparent after a week's incubation.

It was known (Levenbook, 1950) that the hemolymph of *Gastrophilus* contains a number of organic acids in sufficient total quantity to make up approximately one-fifth of the anion balance. The only analysis of these reported for

silkworm blood is the early work of Tsuji (1909), whose values (as cited by Yamafuji, 1937) are so high as to appear of doubtful accuracy. When a medium was prepared containing citrate, malate, lactate, and succinate in amounts corresponding to Tsuji's analysis, it gave cultures with very little development. A number of organic acids (neutralized with KOH) were then tested biologically by adding them at various dilutions to culture media, and the following were the highest concentrations found to give no visible detrimental effect: malate, 10 mM; α-ketoglutarate, 5 mM; fumarate, 1 mM; succinate, 1 mM; and citrate, 0.1 mM. Lactate had detrimental effects at 0.1 mM, the lowest concentration tested (the possibility of impurities must be considered as technical grade was used). Tested individually, only malate had a slightly stimulatory effect. The first four above were tested in combination in the concentrations shown in Table III, and it was found that addition of this group produced significant stimulation in the cultures.

In case the presence of protein might be beneficial, crystalline bovine serum albumin was tested dissolved in the medium at 2 mg. per ml. and 10 mg. per ml. in a single experiment near the end of this investigation and did produce some improvement over the controls.

As it was of interest to compare the requirements of tissues from different types of animals, Dr. J. M. Morgan kindly supplied a sample of his synthetic medium 150, identical with medium 199 used for maintenance of vertebrate tissue cultures (Morgan, Morton, and Parker, 1950) except for being adjusted to pH 6.47. When silkworm ovarian explants were placed in this medium, with and without addition of heat-treated hemolymph, it was found to be quite unsuited for maintenance of silkworm cells as these degenerated completely within 2 days.

Effect of Hemolymph and Tissue Extracts.—In the above experiments, the culture medium consisted of synthetic physiological solution with a certain proportion of hemolymph added. When hemolymph inhibited with glutathione was tested in different percentages with Trager's solution, 50 per cent blood gave the best cultures. With the new physiological solution and heat-treated hemolymph, good cultures could be obtained with from 5 to 50 per cent of hemolymph. In most of the experiments with this solution, 10 per cent was used. Undiluted heat-treated hemolymph gave poorer cultures with granular cells. On the other hand, when the synthetic solution was tested without any addition of blood, the number of cells developing was very low, but these did retain normal appearance.

To test the effect of the hemolymph as a source of hormones, cultures were made from insects of different ages. Early experiments using *Malacosoma disstria* larvae, Trager's medium, and 10 per cent hemolymph without any tyrosinase inhibitor, indicated that larvae taken just after the last larval molt or just before pupation produced two or three times as many cells in culture

as did those taken in the middle of the instar. On the basis of this result and because of the convenience of working with large insects, silkworms were regularly taken for culturing after the termination of feeding and when the rosy coloration of the prepupae had started to appear. The only experiment on the question of age with silkworms using heat-treated blood and the new solution showed that larvae during the period from cessation of feeding to prepupae are better sources of both blood and tissue than pupae 3 to 4 days old. Further investigation of this question is desirable.

In the hope of providing stimulatory substances not present in the hemolymph, an embryo extract was prepared from silkworm eggs. Newly laid eggs were acid-treated to prevent diapause (Koch, 1951), and after 4 to 5 days' development were surface-sterilized, washed, ground in an equal volume of physiological solution, heated to 60°C. for 5 minutes, and then centrifuged. A small amount (less than 1 per cent) added to the medium improved the cultures. As this extract was not satisfactorily clarified by centrifuging, cultures containing a larger proportion of it also contained a coagulum. Chick embryo extract (commercial lyophilized product reconstituted) was also tested; it appeared to have a stimulatory action when present as a trace only (about 0.2 per cent), but in larger quantities produced cellular abnormalities such as binucleates.

Description of Cultures.—In hanging-drop cultures prepared with the physiological solution and heat-treated hemolymph as described, cells were observed in moderately dense development (Fig. 5) and increased in number for 5 to 7 days until they finally covered an area about 3 to 5 times the diameter of the explant. They ordinarily resembled fibroblasts in open growth, but sometimes in compact brush formations (Figs. 4 and 6) very similar to, but less rapid in growth than, vertebrate fibroblast cultures. In roller tubes the cells were similar (Fig. 8) but during the first day formed a "tail" behind the explant around the tube in the direction of rotation. Frequently in about 4 days the tube was covered fairly evenly, and the tails as such were no longer evident. At this stage the cells often appeared less elongate (Fig. 9), particularly when mitoses were numerous.

In the improved medium the cells degenerated less rapidly than in any of the media previously used (compare Figs. 2 and 5), and even at the first signs of outgrowth were freer of granules and much more transparent in both bright field and phase contrast microscopy. Degeneration was diagnosed by increase in granularity and the appearance of small fat droplets in the cytoplasm. Usually after the cytoplasm had become granular, the cells became rounded and less securely affixed to the glass. Sometimes cells were seen to become very flattened, and since the cytoplasm was still free of large numbers of granules, it was then difficult to define. These were designated "veil" cells and their significance is not known.

Whereas mitosis in Trager's medium took about 50 minutes from metaphase to the completion of telophase, in the new medium this took only 20 to 25 minutes (Fig. 7 *a* — *e*).

In some roller tubes the medium was changed twice weekly with physiological solution and heat-treated hemolymph stored at 4°C., or sometimes with treated hemolymph from freshly bled larvae. Such tubes showed gradually decreasing cell activity and the cells would become either round and granular with droplets or flattened, in a period varying from 10 days to 3 weeks, leaving only a few transparent and still active cells. Normal and rapid mitoses were, however, observed in tubes that were 2 weeks old.

Occasionally, in roller tube cultures a continuous sheet of cells would develop (Fig. 3) like the beginning of epithelial development. These sheets were observed in Trager's medium as well as in the new medium. The area of such a sheet would increase even after the fibroblasts were degenerating. Because of the optical properties of these sheets in roller tubes, observation of mitosis in them was not possible.

DISCUSSION

Previous attempts at culturing insect tissue have been made using variously composed salt solutions and a variety of natural fluids. The salt solutions have resembled vertebrate physiological solutions or have been the result of trial and error. A solution based on blood analyses has been designed for physiological studies with *Gastrophilus* but has not been used in tissue culture (Levenbook, 1950). Fluids of insect origin, such as hemolymph (Glaser, 1917; Goldschmidt, 1916; Trager, 1935; etc.), grasshopper broth (Frew, 1928), egg yolk (Carlson, 1946), contents of lysed pupae (Stern, 1940), and homogenized larvae (Gavrilov and Cowez, 1941), have been used, as well as fowl plasma, chick embryo juice, bacteriological broth, and peptic digests.

During the early part of the present work, discouraging results forced the author, finally, to undertake formulation of a new medium of composition based on that of the experimental insect's hemolymph. This seemed logical because in vertebrate culture media use is made of solutions resembling serum in salt content, and further, because in insects the circulatory system is only partially closed so that many cell types are bathed directly in hemolymph. This new medium, containing high potassium and magnesium, low sodium, and abundant amino acids in the proportions found in hemolymph, produced cultures greatly superior in number and appearance of cells to any other tried. It is of interest to note the recent paper by Eagles (1955) reporting that the optimal concentrations of amino acids for the growth of carcinoma cells (strain HeLa) are closely correlated with their levels in human serum.

The results with respect to cations may appear inconsistent with those recently published by Barsa (1954). Using *Samia walkeri*, an insect with blood

cation ratios similar to those in *Bombyx*, and isotonic salt solutions, she obtained optimal maintenance of heart beat with a lower potassium:sodium ratio than is in the blood and with no magnesium. However, the significance of this result is that the biological effects of the metals must be different in simple chloride solutions than in the presence of other hemolymph components. As Barsa points out, magnesium is bound by organic constituents, including amino acids. Also, requirements for cell multiplication may differ from those for maintenance of heart beat.

Although the medium designed to resemble hemolymph proved better than any yet tried, it probably can be simplified and improved. For example, in view of complex formation between amino acids and the divalent cations it seems possible that their simultaneous reduction would not be detrimental. Also, it is unlikely that the three sugars used in imitation of hemolymph are necessary, and probably glucose alone would serve equally well.

On the other hand, further additions to the medium would probably be beneficial. Considering the composition of hemolymph, the effect of sugar phosphates might be of interest—in a preliminary experiment glucose-1-phosphate appeared to give slight stimulation. Since the blood of late fifth instar silkworm larvae contains about 11 volumes per cent CO_2 (Florkin, 1937), the use of bicarbonate in the medium and a controlled atmosphere in the culture tubes might be favorable.

The use of silkworm egg extract, though it produced noticeable improvement in cultures, was not made routine because of the difficulty of getting sufficient eggs and of clarifying small quantities of the extract. Some of the stimulus derived may be due to the presence of normally intracellular substances, as has been indicated in vertebrate culturing. The superior quality of ovarian tissue over all others in insect culturing may possibly be because this organ, even in the larva, is able to synthesize or store some of the growth-stimulating substances of the embryo. The cultures used for testing the egg extract were made from larvae about to pupate and therefore already containing increased amounts of the growth and differentiation hormone (Bounhiol, 1938; Williams, 1952). If the stimulants in egg extract and pupal hemolymph are the same, it would be understandable that the former might prove the better source for culturing, since it does not contain the products of histolysis which a culture may be less capable of coping with than is the whole animal.

Long term experiments with subculturing of either suspended fibroblasts or epithelium-like sheets are certainly indicated. As some vertebrate tissues are slow in adjusting to growth *in vitro* and therefore develop increasingly well after subculturing, so it might be with insect cells. Unfortunately, the author's work had to be concluded with many such questions still not investigated and before virus multiplication could be observed in the new medium. The first amino acid and inorganic salt medium was in fact tested only 3 months before the work was stopped.

SUMMARY

1. Ovarian tissue from *Bombyx mori* L. larvae about to pupate was cultured in Trager's (1935) salt solution and 10 per cent hemolymph, with indifferent results. Improvement of cultures was sought by modifying the culture medium.

2. To reduce the activity of the tyrosinase, hemolymph for culture medium was heated for 5 minutes at 60°C., and the coagulated protein removed.

3. A physiological solution was formulated containing cations and amino acids as they occur normally in silkworm hemolymph. In both hanging-drop and small tube cultures use of this medium brought about increased cell number, improved cell appearance, more rapid mitoses, and longer life of cultures.

4. To the solution formulated from analyses, tryptophan, cystine, cysteine, malate, fumarate, succinate, and α-ketoglutarate were added after testing individually, resulting in improved growth in cultures.

5. Use of a silkworm egg extract prepared 4 to 5 days after acid treatment produced an increase in cell number.

6. In small roller tube cultures, when the new medium was changed twice a week, the cells spread over the walls of the tube in 4 or 5 days (Figs. 8 and 9), rapid mitoses were observed after 2 weeks, and transparent active cells were present at 3 weeks. Subculturing was not attempted.

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EXPLANATION OF PLATES

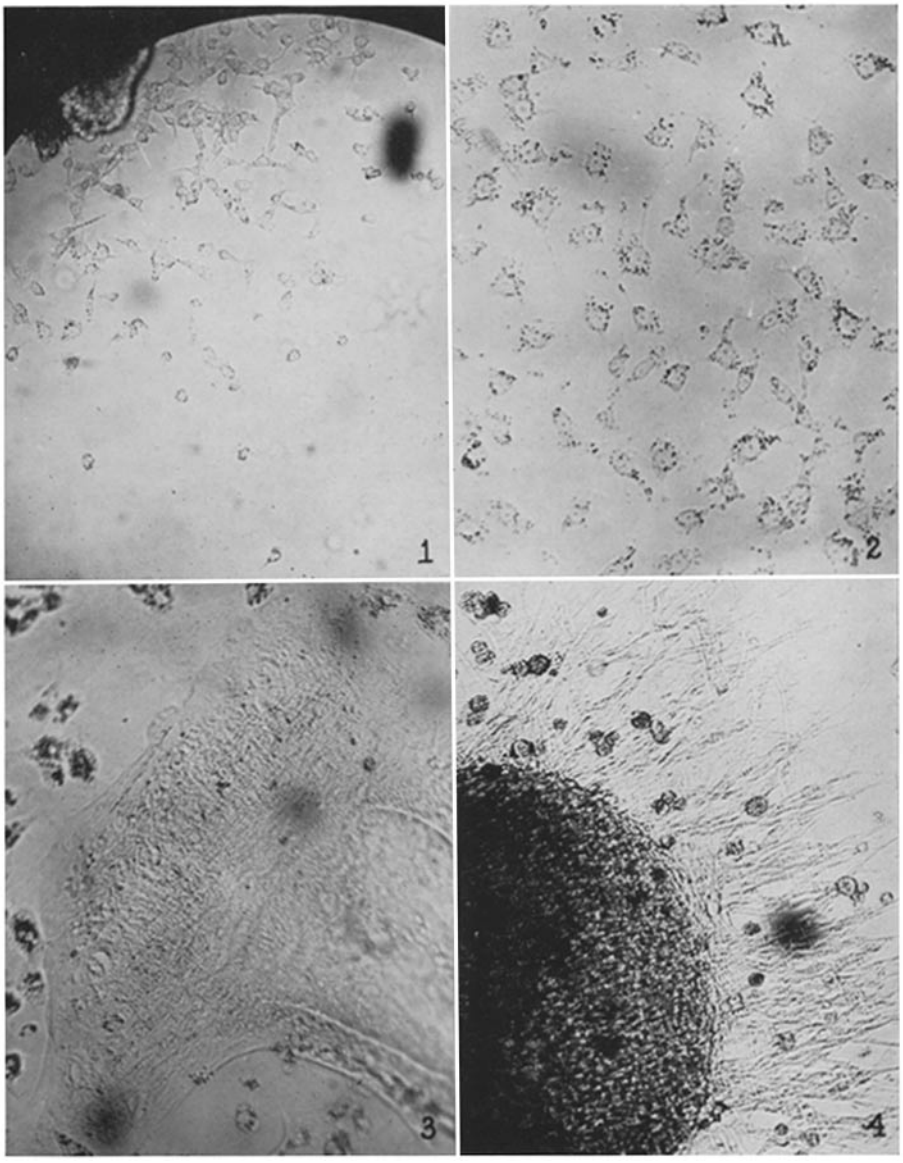
PLATE 1

FIG. 1. Cells developing from ovarian tissue in hanging-drop of Trager's medium, 18 hours. × 165.

FIG. 2. As Fig. 1, 24 hours. × 360.

FIG. 3. Epithelium-like development in roller tube in Trager's medium, 2 weeks. × 135.

FIG. 4. Brush formation of fibroblasts in hanging-drop of medium with composition as in Table III plus glucose-6-phosphate, 10 days. × 100.



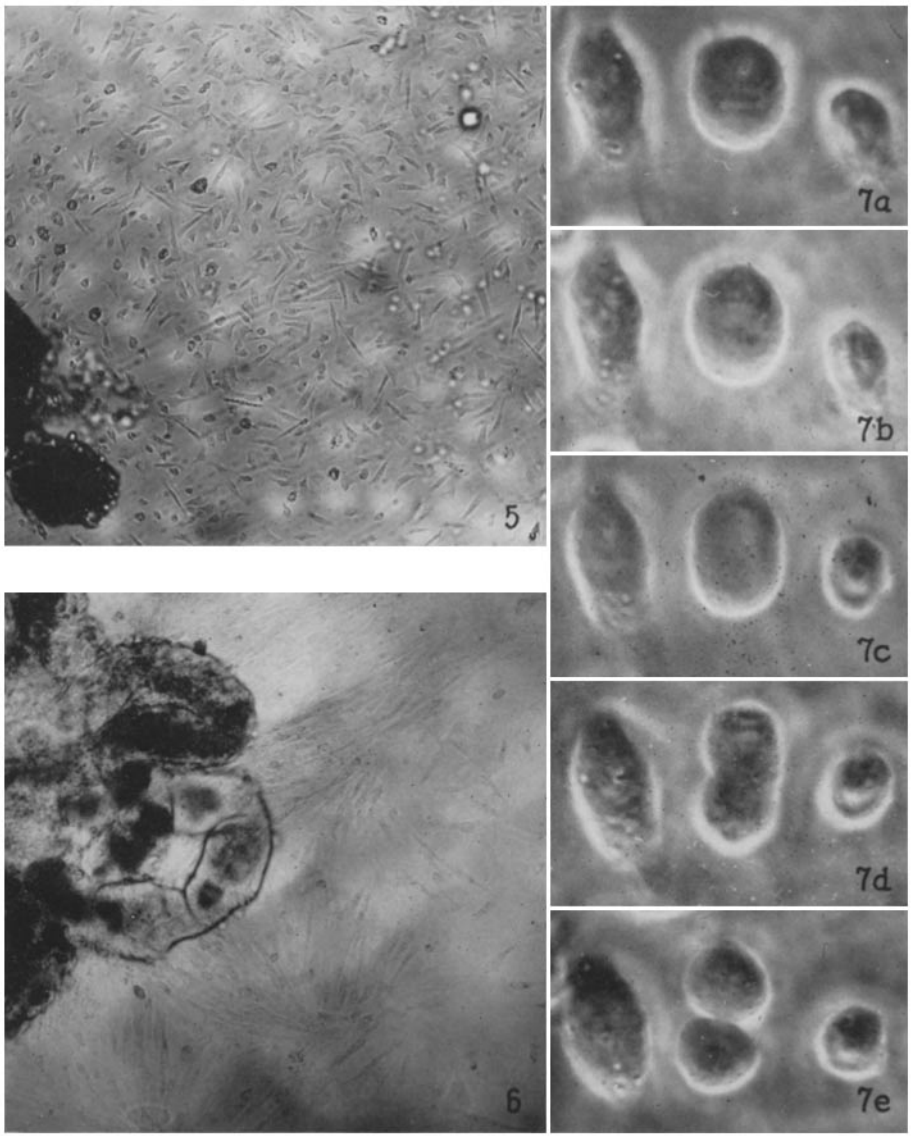
(Wyatt: *In vitro* culture of silkworm tissue)

PLATE 2

FIG. 5. Cells developing from ovarian tissue in hanging-drop of medium with composition as in Table III, 4 days, phase contrast. × 72.

FIG. 6. Brush formation of fibroblasts in hanging-drop of medium without organic acids but with aspartic and glutamic acids and their respective amides in the quantities found normally in the hemolymph, 5 days. × 86.

FIG. 7. Mitosis in hanging-drop of medium with composition as in Table III, 4 days, phase contrast. × 800. *a*, 0 minutes; *b*, 2 minutes; *c*, 4½ minutes; *d*, 9 minutes; *e*, 20 minutes.

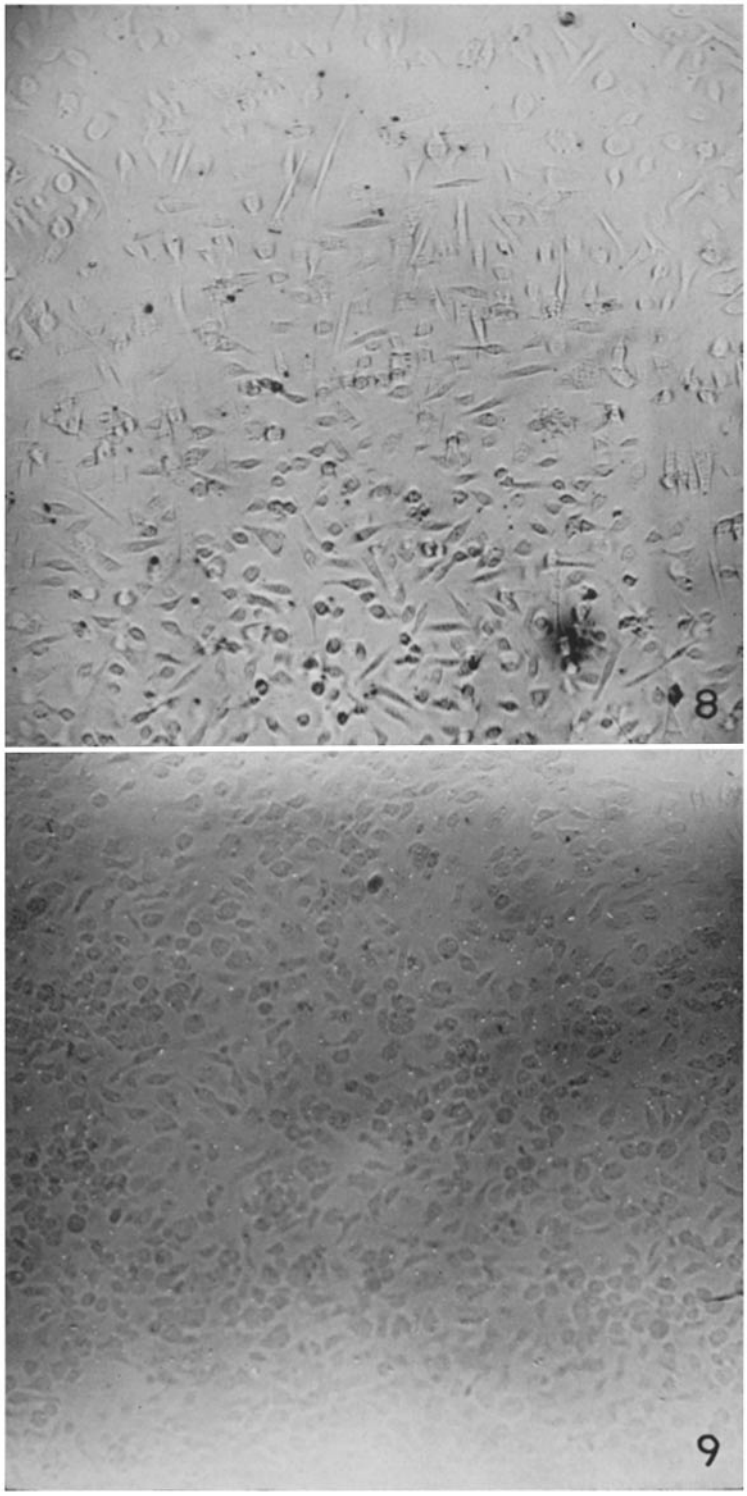


(Wyatt: *In vitro* culture of silkworm tissue)

PLATE 3

FIG. 8. Fibroblasts in roller tube in medium, as in Table III, 5 days. × 130.

FIG. 9. Fibroblasts in roller tube in medium without organic acids but with aspartic and glutamic acids and their respective amide forms in the quantity found normally in hemolymph, 6 days. × 130.



(Wyatt: *In vitro* culture of silkworm tissue)