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THE GROWTH OF INSECT BLOOD CELLS IN VITRO.[1]

By R. W. Glaser.

In order to obtain an insight into various pathological changes taking place in diseased insect tissue, I undertook a series of experiments dealing with the cultivation of such tissue in vitro. The degenerative changes occurring in normal and in pathological blood cells were especially studied for the reason that the blood is frequently used in diagnosing the health of a particular insect. In the polyhedral diseases of insects a general picture of the progress during the later stages of the disease can be obtained by examination of the blood. This type of disease is recognized by the fact that nucleoprotein reaction bodies, termed polyhedra, are formed within the nuclei of the blood and certain other tissue cells. It was also of considerable interest to ascertain whether slides with growing insect tissue could be infected with the polyhedral disease virus and whether polyhedra would form within the nuclei of cells thus infected. By way of comparison, it was also of interest to see through what changes normal cells pass when permitted to degenerate naturally.

Incidentally, a number of observations were made in regard to the morphology and behavior of growing insect blood cells and I will present the observations in the hope of stimulating work along these lines. Although the cultivation of insect tissue is not new, this method of studying various embryological, morphological, and physiological questions pertaining to entomology has been almost entirely neglected and I am convinced that the cultivation of tissues will greatly simplify the solution to many difficult problems.

Goldschmidt in 1915, by means of the tissue culture method,

[1] Contribution from the Entomological Laboratory of the Bussey Institution in coöperation with the U. S. Bureau of Entomology. (Bussey Institution No. 125.)

studied the spermatogenesis of *Samia cecropia* L. Spermatogonia or young spermatocytes were kept alive for about three weeks, and the follicle membrane for some weeks more. This piece of work and that of Lewis and Robertson, 1916, on the male germ cells of *Chorthippus curtipennis* Scudd (*Stenobothrus curtipennis* Harris) seem to be the only examples of insect tissue cultivation found in the literature.

For the experiments here described the larvæ of *Malacosoma americanum*, *Cirphis unipuncta*, *Laphygma frugiperda* and *Porthetria dispar* were used. My method did not differ materially from those of Harrison, Carrel, Goldschmidt, etc. However, since most of my experiments dealt with the cultivation of insect blood I will briefly outline the method of procedure. The larvæ to be operated upon are held upside down in one hand and the anterior and posterior halves bent back. A proleg is then thoroughly washed with 80 to 95 per cent. alcohol after which it is clipped with very fine aseptic scissors. The drop of blood which oozes out is caught on a sterile cover slip which is then placed on a sterile depression slide and the edges sealed up with sterile vaseline. A great many slides were prepared in this manner, *i.e.*, the blood corpuscles were simply mounted in their own plasma. In other cases Locke's solution,[1] or a mixture of Locke's solution and plasma was found satisfactory. In general Locke's solution is isotonic with insect tissue and can be very freely used for cultivation and for the washing out of old cultures in order to free them of harmful by-products. Locke's solution has no particular advantage over the plasma, except that the preparations are a bit more transparent, owing to the fact that large amounts of fibrin have been eliminated.

Blood was obtained from healthy *Malacosoma americanum* larvæ and six slides prepared. In a few days some of the blood cells disintegrated, but the majority lived and multiplied. In ten days beautiful syncytia had formed (Pl. I, fig. 1). At the end of this time three of the slides were inoculated with some polyhedral material which had been passed through Berkefeld Grade "N" candles. The other three slides were kept as checks. All slides were observed for forty days. After this the cells in both experiments and checks ceased growing and disintegrated normally.

[1] Locke's solution consists of NaCl 0.9 per cent., $CaCl_2$ 0.025 per cent., KCl 0.042 per cent., $NaHCO_3$ 0.02 per cent., Dextrose 0.25 per cent., Peptone 0.2 per cent.

The inoculated slides showed no indications of the formation of polyhedral bodies within the nuclei of the blood cells. This experiment was repeated twice more and with the same result; no difference between the experiments and checks was observed.

Twelve healthy *M. americanum* larvæ were fed with polyhedral virus passed through Berkefeld Grade "N" filters. As checks the same number of larvæ were infected with the virus sterilized by autoclaving. At the end of ten days tissue culture preparations were made with blood taken from the experimental animals and from the checks. The slides were studied at once and it was found that two thirds of them, representing blood taken from animals fed with the unsterilized virus, showed infection. The early stages of polyhedra were discernible within the nuclei of many of the blood cells. Other cells still seemed to be in a normal condition. The slides representing blood taken from the checks appeared perfectly normal. The next day all of the slides were again examined, but no change was noticed with the exception that some of the cells had divided. In one day more nearly all of the experimental blood cell nuclei were beset with large and small polyhedra. In six to seven days the blood cells from the experimental animals began to disintegrate with the liberation of small and large, well formed, typical polyhedra. The cells on the check slides disintegrated normally five days later.

A large number of the blood cells of *M. americanum* are of the mulberry corpuscle type (Pl. I, fig. 2). These are not so well adapted to cultivation as the ordinary blood cells (Pl. I, figs. 3 and 4). For this reason the experiments were repeated with the blood of *Porthetria dispar* in which the mulberry cells are in the minority. In these experiments it was likewise impossible to infect growing blood cells with the polyhedral virus, but if animals were first infected the formation of the polyhedra could be traced very nicely by taking the blood from the infected animals in about ten or twelve days and studying by means of the tissue culture method.

What do these experiments signify? Several possibilities at once suggest themselves, but I will merely outline the two most probable. First of all let us suppose that I have actually cultivated the polyhedral virus on the tissue culture slides. Then why is it impossible to infect such tissue directly with the virus? Why is it necessary to give the virus "a start" within the insect itself? Per-

haps the early stages of the virus require some particular organ or tissue or some particular condition. The insect itself fulfills the required condition, but the blood cells growing in vitro do not. The later stages of the virus, however, find the conditions suitable on the tissue culture slides.

Then again I may not have cultivated the virus at all. The caterpillars were infected with the polyhedral virus which may have a strong affinity for some particular tissue other than the blood. Toxins may be elaborated and getting into the blood may start the degenerative changes which culminate in the formation of polyhedral bodies. These degenerative changes, after beginning within the animal, may later proceed outside of it on the tissue culture slides in the absence of the virus. I think that a series of passage infections would clear up the whole matter. This I have not yet attempted. A series of animals should be infected with fresh virus. In ten or twelve days tissue culture slides should be prepared from the blood. When polyhedral bodies begin to form, another series of animals should be infected from the slides. In ten or twelve days the blood should be taken from these animals and kept on slides and if polyhedra form, a fresh series of animals should be again infected and so on. Such a series must, of course, be accompanied by suitable checks. If the animals in the later experimental series die typically and if there is no increase in the period from infection to death (about twenty days) it would be fairly certain that the virus has been cultivated and that one is not dealing with a partial recovery of the amount of the virus originally used.

In cultivating insect tissue it is always well to prepare a great many slides. A few become contaminated with bacteria, but many disintegrate normally without showing the least inclination towards growth. The ability of the tissue to grow well also seems to a slight degree to vary according to the species of insect. The tent-caterpillar blood, for instance, does not grow as readily as the blood taken from the true army or fall-army worm. The blood from these two species does not grow nearly so well as the blood of the gipsy-moth caterpillar. One should never discard slides for at least a week or more. Very frequently nearly all of the cells will disintegrate during the first five or six days. A few, however, live and these later increase and multiply forming beautiful

syncytia. I have frequently given up slides as hopeless on account of what seemed to me to be complete disintegration, yet on reëxamination in about two weeks, I was astonished to find clusters of healthy looking, growing cells.

I have kept true army and fall-army worm blood preparations alive for one month without washing out the cultures or transferring them to a fresh medium. Gipsy-moth blood cultures have been kept alive for as long as seventeen weeks without washing or transferring. It is true, the cells were no longer vigorous and showed signs of beginning degeneracy, but they were alive. After washing out these old cultures with sterile Locke's solution and filter paper, as is usually done, and transferring to a fresh medium like Locke's solution the cells grew and multiplied as before.

In so far as the morphological elements contained in insect blood are concerned, the ordinary amœbocytes (Pl. I, figs. 3 and 4) are the only ones which multiply in tissue cultures. The minute amœbocytes (Pl. I, fig. 5), the mulberry corpuscles (Pl. I, fig. 2), and the cytoplasmic free cells (Pl. I, fig. 6) described by me in 1915[1] always disintegrate. A difference of opinion seems to exist in the literature as to the origin of the blood corpuscles of larval and adult insects. From my studies it appears that the blood cells, after their differentiation from the mesoderm during embryological development, simply maintain their numerical equilibrium in larvæ and adults by dividing mitotically at certain intervals. I cannot find any so-called blood corpuscle forming tissue at least in sections of caterpillars.

Some of the visible changes observed on the culture slides in normal degenerating blood cells have proved instructive and have further helped to strengthen my views (published elsewhere) in regard to the nature of the polyhedral bodies found in the nuclei of certain pathological cells. Normal disintegration of insect blood cells is always accompanied by the formation of protein crystals within their cytoplasm. Crystalline disintegration accompanied by granular disintegration seems to be the rule in normal disintegrating insect tissue. Granular disintegration alone seems to be exceptional in blood cells at least. In the polyhedral diseases of insects protein crystals are likewise formed within the

[1] Wilt of gipsy-moth caterpillars. Journal of Agricultural Research. Vol. IV, No. 2, May, 1915, p. 113.

degenerating cells, but here the crystals are formed within the nuclei instead of within the cytoplasm.

Normal blood cells in the early stages of disintegration show distinct granulations and also small highly refractive greenish crystals within the cytoplasm (Pl. I, fig. 7). The nucleus does not show any changes till rather late. In a few days the cytoplasmic crystals become more and more numerous and likewise grow in size (Pl. I, fig. 8). Still later they reach the size of 5 and 6μ and assume a shape very closely simulating polyhedra. In a few days more the cells disintegrate, completely liberating granules and crystals (Pl. I, fig. 9). Some of these liberated crystals measure 15μ or more in diameter (Pl. I, fig. 10). Millon's reagent demonstrates their protein nature and I believe that they are similar in many ways to the polyhedra. Of course, the composition of the polyhedra is different, since they are formed within the nuclei under pathological conditions, but what I wish to emphasize is that insect tissue has a normal tendency towards crystalline disintegration. Is it, therefore, so surprising to find crystals (polyhedra) within the degenerating nuclei in a series of insect diseases?

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EXPLANATION OF PLATE.

Fig. 1. Syncytium of growing amœbocytes.
Fig. 2. A type of mulberry corpuscle.
Fig. 3. Amœboid amœbocyte.
Fig. 4. Round amœbocyte.
Fig. 5. Minute amœbocyte.
Fig. 6. Cytoplasmic free cell.
Fig. 7. Degenerating amœbocyte with a few small, refractive crystals in cytoplasm.
Fig. 8. Degenerating amœboycte with numerous large, refractive crystals in cytoplasm.
Fig. 9. Disintegrated amœbocyte showing liberated granules and crystals.
Fig. 10. Double crystal formed by a normally disintegrating amœbocyte.

NOTES ON REARING INSECTS FOR EXPERIMENTAL PURPOSES AND LIFE-HISTORY WORK.[1]

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INTRODUCTION.

During the past two years I have been rearing insects for experimental and life-history studies. While engaged in this work it became necessary to develop new rearing methods and to modify some of the old ones.

The rearing of insects from egg to adult is not always an easy task. Unforeseen difficulties arise anew with every species, in consequence of which I am offering these notes with the hope that entomologists will find them serviceable.

During the life history of insects reared artificially, the following must be observed:

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[1] Contribution from the entomological laboratory of the Bussey Institution in coöperation with the U. S. Bureau of Entomology. (Bussey Institution, No. 121.)

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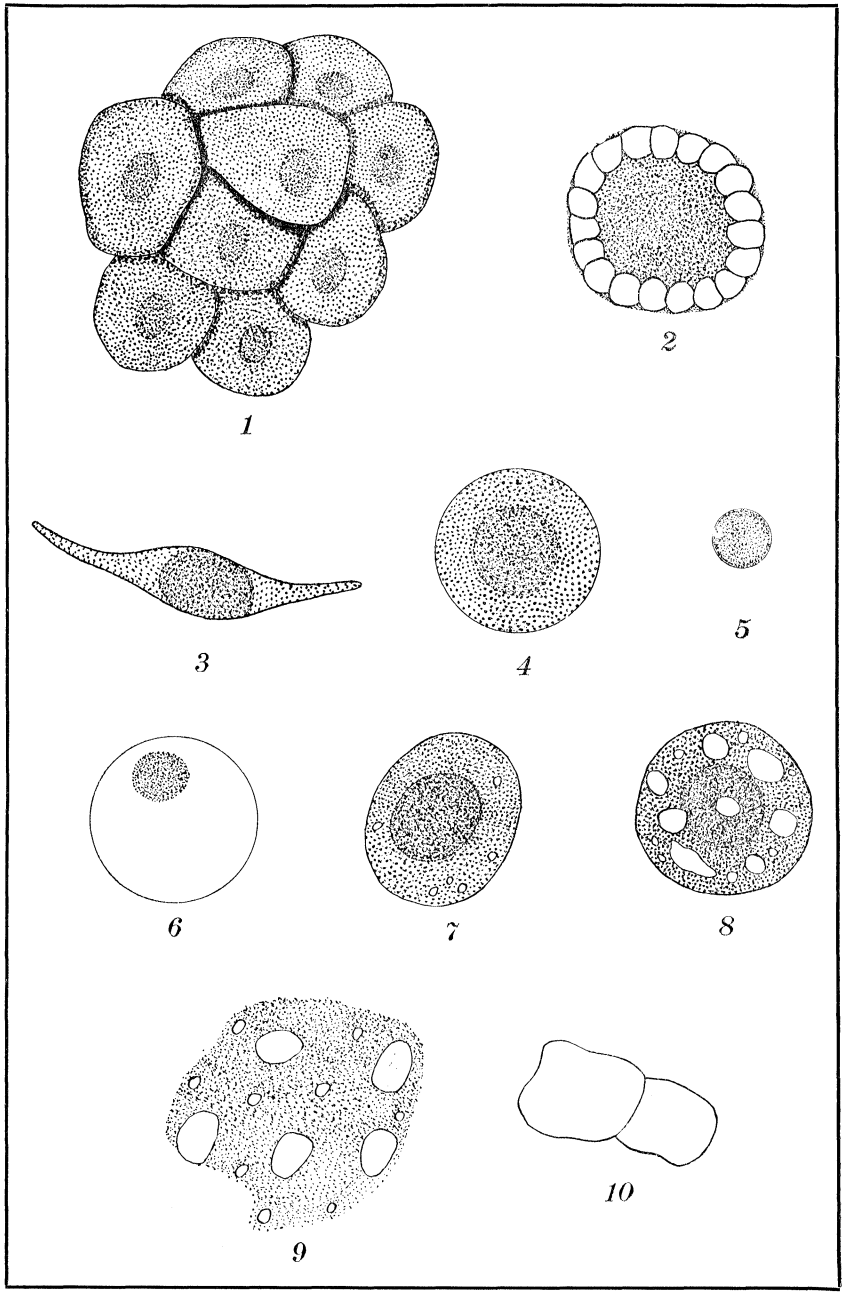

GLASER—*Growth of Insect Blood Cells in Vitro.*