

CULTIVATION OF THE VIRUS OF GRASSERIE IN SILK-WORM TISSUE CULTURES

BY WILLIAM TRAGER,* PH.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Adequate evidence has been presented by Glaser and Chapman, 1913, Paillot, 1924, Glaser, 1927, and others (Glaser in Rivers, 1928) to show that the polyhedral diseases of caterpillars are due to specific filterable viruses. Since many filterable viruses of vertebrates have been propagated in tissue cultures (Carrel in Rivers, 1928; also, vaccinia (Li and Rivers, 1930, Rivers and Ward, 1933); vesicular stomatitis (Carrel, Olitsky, and Long, 1928); fowl pox (Findlay, 1928); Virus III (Andrewes, 1929, *a*, *b*); herpes (Andrewes, 1930); foot-and-mouth disease (Hecke, 1930, Maitland and Maitland, 1931); common cold (Dochez, Mills, and Kneeland, 1931); pseudorabies (Traub, 1933); yellow fever (Haagen, 1934), as well as two plant viruses in growing excised root tips (White, 1934)), it seemed worth determining whether a virus disease of an invertebrate could be similarly maintained, and whether it would behave in culture in a manner like that of virus diseases of vertebrates. Moreover, cultures of a polyhedral virus might be expected to be of special value in studies on the nature of inclusion bodies. It is well known that many filterable viruses bring about the formation, within certain cells of the host organism, of nuclear or cytoplasmic inclusion bodies, all of highly characteristic appearance, but none so definite and easily observed without fixation or staining as the polyhedral nuclear inclusions which occur in silkworms affected by grasserie.

The culture of silkworm tissue, the propagation in these cultures of the virus of grasserie, and some observations on the formation of

* Fellow, Medical Board National Research Council, 1933-34.

polyhedra in cells living *in vitro* will all be detailed in the succeeding portions of this paper.

General Methods

A few general remarks must first be made. The silkworms were raised from healthy stock obtained from Dr. Glaser and maintained free of virus for several years. They were kept in a room carefully guarded from the introduction of infective material. During the entire breeding season no cases of grasserie occurred among the stock of silkworms. Indeed, very few stock silkworms were sick from any cause whatever, except during the late fall, when the food was of inferior quality and a number of deaths from bacterial disease occurred. Experimental silkworms were kept singly in half pint bottles and were generally treated in the manner described by Glaser and Lacaillade (1934). Material which was to be tested for infectivity was diluted with water to a known extent and 0.01 to 0.02 cc. of it fed to caterpillars previously starved for 1 day, using usually 5 caterpillars for each dilution. In this way rough titrations of the virus could be carried out.

Cultivation of Silkworm Tissue

All reported attempts at the cultivation *in vitro* of insect tissue have met with relatively slight success.

Goldschmidt (1915) obtained growths of the follicle cells of male gonads removed from pupae of *Samia cecropia*. These growths occurred in hanging drops of hemolymph. No mitoses could be found. Glaser (1917) observed the formation of syncytia by the amebocytes in hanging drops of silkworm blood. Lazarenko (1925) saw similar amebocyte syncytia in hanging drops of blood of the larva of the beetle, *Oryctes nasicornis*, and the writer has noted such syncytia in hanging drops of blood of the cockroach, *Periplaneta americana*, of silkworm larvae, and of pupae of *Samia cecropia*. Lazarenko found that the syncytia were formed by an extreme flattening, spreading out, and anastomosing of the amebocytes, and that no multiplication of these cells occurred. Our own observations support this view. Frew (1928), using the imaginal discs of blow-fly larvae in larval or pupal body fluid sterilized by Berkefeld filtration, was unable to get multiplication of the cells. However, entire imaginal discs of the legs, when placed in pupal body fluid, evaginated and underwent a partial development into segmented limbs. Lewis and Robertson (1916) developed a balanced salt solution containing glucose and grasshopper bouillon or peptone, in which the testis cells of *Chorthippus curtipennis* lived for some time and underwent normal mitotic division. Belař (1929) used a similar modified Ringer's solution, but without peptone or bouillon, to observe mitosis in spermatocytes of the grasshopper, *Chorthippus lineatus*. Neither of the latter two workers noted division in any cells other than the germ cells.

In the present investigation the effects were tried of a number of balanced salt solutions on the tissues of full grown larvae of *Bombyx mori*. All the solutions had a concentration approximately equivalent to a 0.05 M NaCl solution, as this was at the center of the favorable osmotic pressure range, and a pH of 6.7, since this is the pH of silkworm hemolymph (Glaser, 1925, Demyanovskii *et al.*, 1932). A medium was finally found in which good initial cultures could be

TABLE I
Composition of the Culture Media

	Solution A		Solution B	
	<i>cc. per 100 cc.</i>	<i>moles per liter</i>	<i>cc. per 100 cc.</i>	<i>moles per liter</i>
Maltose, 0.3 M.....	20.0	0.06	20.0	0.06
NaCl, 0.3 M.....	5.0	0.015	0.4	0.0013
MgCl ₂ ·6H ₂ O, 0.2 M.....	0.5	0.001	0.5	0.001
CaCl ₂ , 0.2 M.....	0.5	0.001	0.5	0.001
NaH ₂ PO ₄ ·H ₂ O, 0.2 M.....	0.75	0.0015	0.75	0.0015
K ₂ HPO ₄ , 0.2 M.....	0.75	0.0015	0.75	0.0015
Egg albumin digest, 0.137 M*.....	0	0	10.0	0.0137
Distilled water.....	72.5		67.1	
Freezing point depression, °C.†.....	0.285		0.290	
Nitrogen per cc., mg.†.....	0		1.19	

The media were prepared by mixing the indicated amounts of sterile 0.3 or 0.2 M solutions with sterile distilled water. The stock solutions were sterilized by autoclaving, except the maltose and the digest, which were filtered through a Berkefeld N.

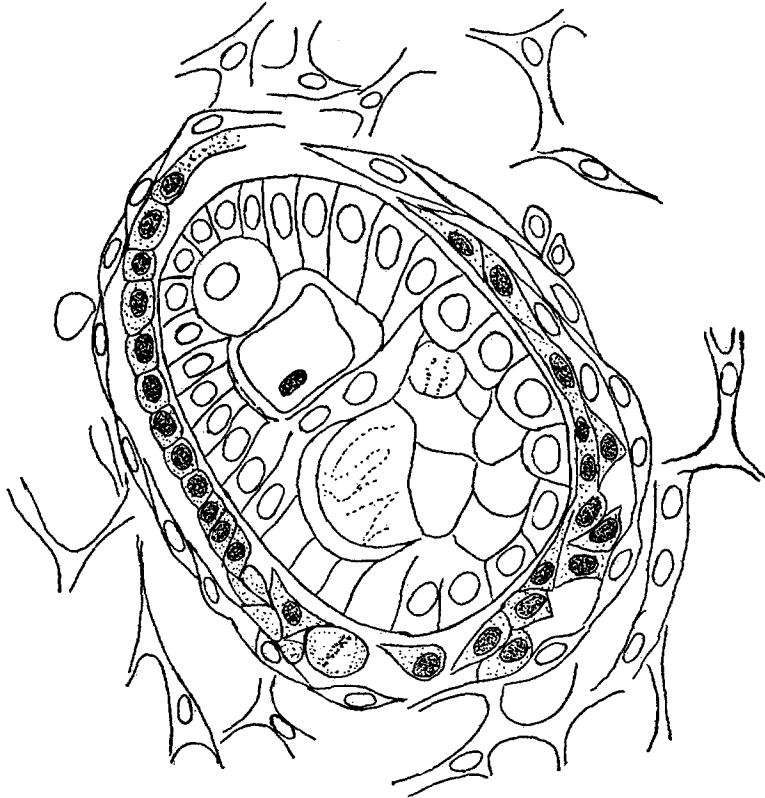
* The molarity of the digest was calculated from the amount of NaOH needed to neutralize it and from its final volume after heating. That the figure obtained is sufficiently accurate is shown by the fact that the freezing point depression of Solution B is only slightly higher than that of A.

† These determinations were made by Dr. William F. Bruce.

obtained of a certain silkworm tissue derived from the female gonads. The compositions of this medium (A) and of a modification of it (B) are given in Table I.

Note that the media contain maltose. Neither sucrose nor glucose in equimolecular amounts gave growths as good as those obtained with maltose. Medium B differs from A in that it contains an egg albumin digest prepared in the manner of Baker (1929). Growth was usually greater in Medium B than in A,

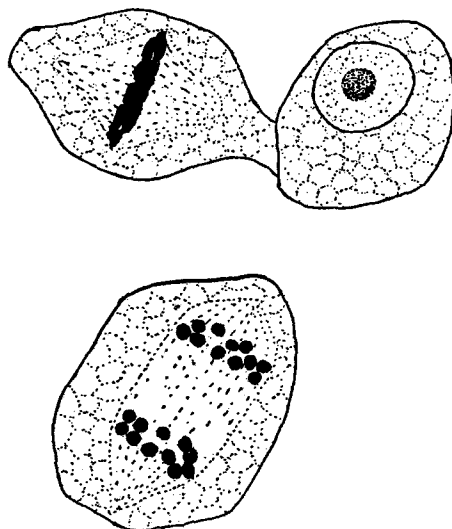
especially if the media were used without the addition of silkworm serum. The best growths were obtained when the medium contained from 5 to 25 per cent (usually 10 per cent) silkworm serum. The latter was secured by bleeding several large silkworms aseptically from the proleg, pooling the blood, and centrifuging out the blood cells.



TEXT-FIG. 1. Section through ovarian tube of 5th instar silkworm. The stippled cells represent the layer which grew in culture.

The tissue cultures were prepared in the following manner. Full grown female silkworms were immersed in 1:1000 mercuric chloride solution for 30 to 45 minutes. This treatment did not injure the caterpillars as, if they were subsequently washed and allowed to dry out, they recovered completely. It did, however, anesthetize them, rendering them temporarily motionless and very easy to handle. After immersion in mercuric chloride each larva was washed in two Petri dishes of sterile water and was then bled from a proleg into a small Petri dish. It was next

placed in another small Petri, and with fine sharp scissors the entire dorsal wall of the 8th segment was cut off (care being taken not to cut into the alimentary canal) and placed in the blood previously obtained. Usually the imaginal buds of the ovaries came out along with the dorsal body wall of the 8th segment. Sometimes they did not, and then it was necessary to dissect them out with glass needles and place them in the dish with the blood. In the former case the gonads were dissected away from the body wall, also with glass needles. All the instruments used were sterile, and aseptic precautions were observed throughout. Each gonad (which contains four coiled ovarian tubes) was divided into 3 to 4 pieces. Each



TEXT-FIG. 2. Mitotic figures from a 3 day old culture stained with crystal violet.

fragment of tissue so obtained (about 0.5 to 1 mm. in diameter) was set up in a hanging drop (0.005 cc.) of culture fluid on a sterile cover-slip vased on to a microculture slide. The cultures were kept in an incubator at 27.5–28.5°C. By these methods very few cultures were ever contaminated by bacteria. Nevertheless, rarely would more than 75 per cent of the cultures prepared at one time be successful. Sometimes the lack of success of a culture could be attributed to an insufficient number of cells of the right type (see below) in the explant, but for other cases there was no obvious explanation. It was noted that tissues from caterpillars with colorless blood generally gave better growths than those from caterpillars with yellow blood.

In successful cultures small numbers of wandering connective-tissue-like cells appear after 1 or 2 days incubation. These cells come

from the tissue forming the lining of the ovarian tubes (Text-fig. 1). The cells forming the loose stroma of the larval gonad (Machida, 1926) do not grow in culture. By the 3rd day, the lining cells are very numerous, and they become more so on the 4th and 5th days. Usually the cells then remain in good condition for another week or two. They have numerous fine pseudopodia, and exhibit slow ameboid movement. The growth takes place in 2 layers, one against the cover-slip and one on the surface of the hanging drop. Fig. 1 is a photograph of a small portion of a 6 day old growth, with only the layer on the cover-slip in focus. Several cultures which were fixed and stained showed mitotic figures (Text-fig. 2). Cells growing in media containing silkworm serum have more cytoplasmic granules than those in serum-free media.

The cultures could not be kept going indefinitely, although a few first subcultures were successful. The initial cultures were, however, perfectly suitable for the cultivation of the polyhedral virus.

No tissue cultures were obtained when pieces of silkworm nerve, muscle, fat, silk-gland, leg or wing bud, or male gonad were explanted under the conditions suitable for growth of the lining cells of the ovarian tubes.

Cultivation of the Virus

Initial cultures of the silkworm polyhedral virus have been repeatedly obtained simply by explanting the gonad tissue into culture medium containing the virus. One example will suffice.

Blood from a typical case of grasserie was drawn aseptically and diluted 1 in 25 with Medium A. The mixture was centrifuged and the clear supernatant liquid stored 3 days in the ice box while its sterility was being tested in a broth tube inoculated from it. 1 part of this liquid was then further diluted with 3 parts of Solution B to give a total virus concentration of 1 in 100. Another part of the liquid was diluted with 3 parts of a mixture of Solution B and silkworm serum, giving a virus concentration of 1 in 100 and a serum concentration of 25 per cent. Proper control mixtures were made of Solutions A and B, and A and B with serum. Each of the 4 mixtures was used as the medium for 3 hanging drop cultures of female gonad tissue. The drops, in this as in all the other experiments, were measured out with a fine pipette to be 0.005 cc. in volume.

After 1 day of incubation, all the cultures were about the same and showed a few outwandering cells. On the 2nd day, growth had begun in all but one control

culture, but there was no growth in cultures containing the virus. On the 3rd day, intranuclear polyhedra began to appear in some of the ovarian tube lining cells in cultures infected with virus both with and without serum. Subsequently, all but one of the controls gave typical excellent cultures. In the infected cultures more polyhedra appeared, and in those containing serum a fatty degeneration of the cells soon set in.

On the day when the cultures were set up, a portion of the original virus mixture was diluted with water to give virus concentrations of 1:10,000, 1:100,000, and 1:1,000,000, and each dilution was fed to 5 silkworms. None of the 15 worms thus fed contracted the disease. After 4 days' incubation, an infected culture in the medium with serum was teased up and diluted with water to give virus concentrations (in terms of the original virus) of 1:100,000, 1:1,000,000, and 1:5,000,000. Several of the worms fed the first two dilutions came down with grasserie, showing that the virus had multiplied more than 1000 times. After 9 days' incubation an infected culture in the medium without serum was similarly diluted to give virus concentrations of 1:1,000,000 and 1:10,000,000. 2 of the 5 worms fed the first, and 1 of the 5 fed the second dilution contracted typical polyhedral infections, showing that in this culture the virus had multiplied over 10,000 times.

The strain of virus thus obtained in culture was subcultured by transferring a bit of infected tissue to a healthy tissue culture, in which a large outgrowth of cells had already occurred. The strain, started July 28th, was subcultured 9 times, the last subculture being performed October 19th. It regularly brought about the formation of typical polyhedra, usually within 2 days after inoculation. Its activity was further tested by feeding 3rd, 4th, and 5th passage cultures (diluted with water) to silkworms, and it was found to be capable of producing the disease.

In this strain the subcultures were not made in a quantitative way, so that no estimate of the extent of multiplication is possible. Another strain, however, was subcultured in the following manner.

After the polyhedra were well formed in any given culture, and the cells were beginning to die (usually 1 week after inoculation, although longer intervals were occasionally permitted between subcultures) the culture was teased up in 0.25 cc. of Solution A or B. 0.005 cc. of this dilution was then added to a healthy culture from 3 to 10 days old, usually 4, effecting a further dilution of 1 in 2, so that at each subculture the virus was diluted 1:50 except in the 1st subculture when it was diluted 1:100. This strain of virus was carried through 8 passages (7 subcultures) from Aug. 27th to Oct. 26th. The 2nd and 7th passage cultures were each stored 2 weeks in the refrigerator before being subcultured. In the 7th subculture the concentration of the original virus was 1:156,250,000,000,000, yet polyhedral bodies formed in the usual manner.

When this strain was started the virus used was titrated in silkworms at 1:1000, 1:10,000, and 1:100,000. None of these dilutions produced the disease. Third passage culture virus was titrated in silkworms and gave two cases at a dilution of 1:1,250,000,000 of the original virus (the highest dilution used). There can thus be no doubt that the polyhedral virus of silkworms can multiply and form polyhedral bodies in ovarian tube lining cells living *in vitro*.

In an attempt to discover whether the virus could multiply in other silkworm tissues, the following experiment was performed.

One of the 3rd passage cultures (the same as used for the worm titration above) of the strain last described was diluted in the usual way with 0.25 cc. of culture medium. 0.005 cc. was then added to each of two 4 day old ovarian tube lining cell cultures and also to similar cultures containing explants of fat, silk-gland, and muscle tissue, also 4 days old. In the ovarian tube lining cell cultures, polyhedra appeared on the 2nd day.

After 6 days' incubation, 1 of each of the 4 types of cultures was titrated in silkworms, using, for the lining cell cultures, dilutions of 1:6,250,000,000 and of 1:62,500,000,000, for the fat, silk-gland, and muscle cultures only a dilution of 1:6,250,000,000. The infected lining cell cultures, even at the higher dilution, produced the disease in caterpillars, but no cases appeared among the worms fed from cultures of the other three tissues. That the virus did not multiply in silk-gland and muscle tissue is perhaps not surprising, since polyhedra do not form in these tissue *in vivo*. But in the fat-body polyhedra do form *in vivo*. That they could not be detected and that the virus did not multiply in fat-body *in vitro* may be due to the fact that the fat cells do not grow as do the lining cells, and indeed may survive only a short time. In this connection the following observations of Andrewes (1929, *b*) are of interest. He found that while Virus III multiplied and formed inclusions in cultures of rabbit testis, no inclusions were formed in cultures of liver, spleen, kidney, or bone marrow, even though Virus III *in vivo* can infect many different tissues. These latter tissues, however, did not survive as well under the culture conditions as did the testis tissue.

Two strains of polyhedral virus were started with blood from infected silkworms diluted with culture medium and passed through a

Berkefeld N filter. These were not kept going. Subcultures of the other strains had to be discontinued in the late fall when silkworms were no longer available.

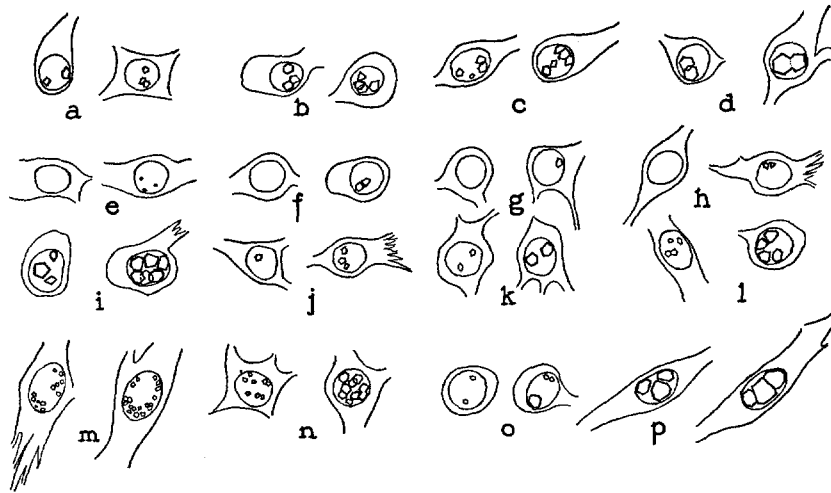
Observations on the Formation of Polyhedra in Tissue Culture

Although cytoplasmic or nuclear inclusion bodies are characteristic of many filterable virus diseases (Cowdry in Rivers, 1928), relatively few investigators have concerned themselves with the behavior of these bodies in tissue cultures of viruses. Rivers, Haagen, and Muckenfuss (1929) first reported the appearance of Guarnieri bodies in cultures of adult rabbit cornea infected with vaccinia. At the same time Andrewes (1929, *a, b*) described the formation of typical intranuclear inclusions in the interstitial cells of rabbit testis cultures infected with Virus III, and also in tissue cultures of herpes virus (1930). Haagen (1934) observed nuclear inclusions in rabbit cornea and testis cultures infected with yellow fever virus, while Traub (1933) found the inclusions characteristic of pseudorabies virus to occur in only about 60 per cent of cultures in which the virus had multiplied, as judged from titration experiments.

In the writer's work with silkworm grasserie virus, typical intranuclear polyhedra were always found in at least some of the cells of an infected tissue culture. The number of cells showing well formed polyhedra varied, not with the source of virus used for infection, but with the condition of the tissue culture at the time of infection and during the next few days. Cultures in which the outgoing cells were healthy at the time of infection, as judged from their activity and the appearance of the nucleus and cytoplasm, gave the best and most rapid polyhedral body formation. In such cultures, polyhedra frequently begin to appear within 24 hours after infection, and are present in most of the cells within 48 hours. During several succeeding days the polyhedra increase in size and number. Most of the infected cells meanwhile remain superficially in good condition; *i.e.* the cytoplasm looks like that of uninfected cells and the cells show ameboid movement. Fig. 2 is a photograph, taken 5 days after infection, of a 4th subculture of the strain of virus first discussed in the preceding section. Compare this with Fig. 1, the photograph of an uninfected tissue culture. Note that the infected cells have hypertrophied and

that practically all contain polyhedra. The number and size of the polyhedra in individual cells, however, vary enormously.

About a week after infection of a culture, the cells begin to die; *i.e.*, the cytoplasm becomes dense and granular and movement ceases. Some of the dead cells burst and liberate the contained polyhedra. In some cultures, this process continues until the culture has degenerated into a mass of tissue debris and large numbers of free polyhedra (Fig. 3). In other cultures most of the polyhedra are retained within the dead cells.



TEXT-FIG. 3. Stages in the formation of polyhedra in tissue culture. Each pair of drawings represents the same cell at intervals of: for *a* to *e*, 3 hours; for *f* to *h*, 8 hours; for *i* to *p*, 12 hours.

When tissue cultures which contained degenerating cells were infected with polyhedral virus, the appearance of the first polyhedra was delayed, and might take as long as 4 days. No polyhedra ever formed in cells already degenerate, while the polyhedra that formed in other wandering cells of such cultures remained small and few in number. In some very poor cultures, in which the outgoing cells were few and already degenerate at the time of inoculation, polyhedra appeared only in some of the ovarian tube lining cells which had remained within the explant. Such cells might be expected to have a better chance of survival under adverse conditions than cells which

have wandered out into the medium. Thus it appears that more and larger polyhedral bodies are formed in active healthy cells than in less active, less healthy cells. This may be taken to indicate that polyhedra are a product of cell activity under the influence of the virus. It is interesting to note that Andrewes (1929, *b*) observed that the inclusions of Virus III were subsequently formed if the tissue cultures were incubated for 48 hours or less before addition of the virus, but they were not formed if the tissues were incubated for 72 hours or more before addition of the virus. In the latter case the cells were probably in poor condition.

Individual cells of infected cultures, at the early stages, were kept under observation for varying periods of time, and all changes in their appearance recorded by means of sketches. Some of these drawings are given in Text-fig. 3. They show that the infected cells changed in shape, that new polyhedra appeared, and that the existing polyhedra increased in size. Within a period of 3 hours a new polyhedral body could become large enough to be visible with the magnification of $\times 1800$. Note in the drawings of Text-fig. 3 that the larger inclusion bodies are of definitely polyhedral shape even when they are not crowded together. Hence the polyhedral form of the bodies cannot be the result of mechanical pressure, as happens when many bodies ordinarily spherical are crowded together in a confined space.

DISCUSSION

The virus of silkworm grasserie apparently behaves in culture much as do the viruses of vertebrates. It multiplies only in the presence of active living cells, it brings about the formation of typical inclusion bodies, and it does not, with successive tissue culture passage, lose any of its characteristic properties.

One other point remains to be discussed. The ovarian tube lining cells of silkworms multiply extensively during the pupal stage to keep pace with the growth of the ovarian tubes, and eventually they form a tissue of the adult moth. Now adults of *Bombyx mori* are completely refractory to the polyhedral disease. Even older pupae, in which the adult tissues are already formed cannot be infected with the virus, although very young pupae are susceptible (Glaser in

Rivers, 1928). Since at least one tissue of the adult has now been shown to be susceptible when obtained before the adult is formed and grown *in vitro*, one may suppose tentatively that the immunity of the adult is a humoral and not a tissue immunity.

SUMMARY

A medium has been developed in which certain cells from the gonads of female silkworms multiply and live for periods of 2 to 3 weeks.

In such tissue cultures, strains of silkworm grasserie virus were maintained in successive passages up to the number of ten. The virus multiplied greatly and typical polyhedral bodies formed in the cells of infected cultures.

I take pleasure in acknowledging the help and advice of Dr. R. W. Glaser.

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EXPLANATION OF PLATES

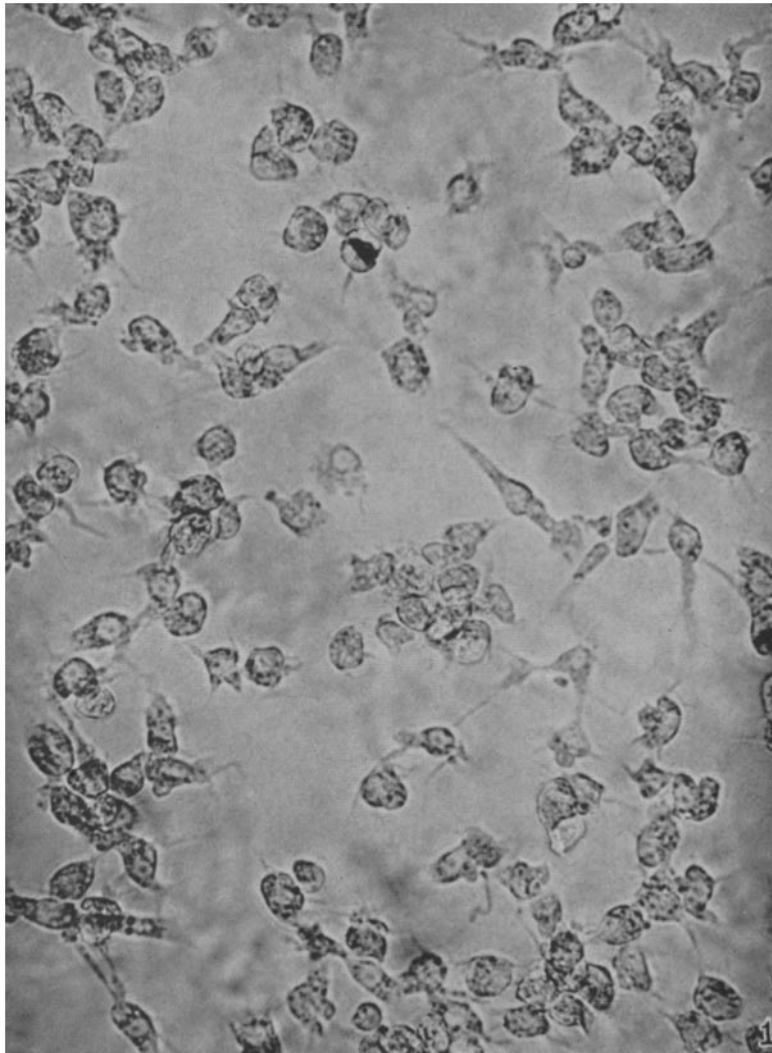
PLATE 21

FIG. 1. Photograph of normal 6 day old culture. ×445.

PLATE 22

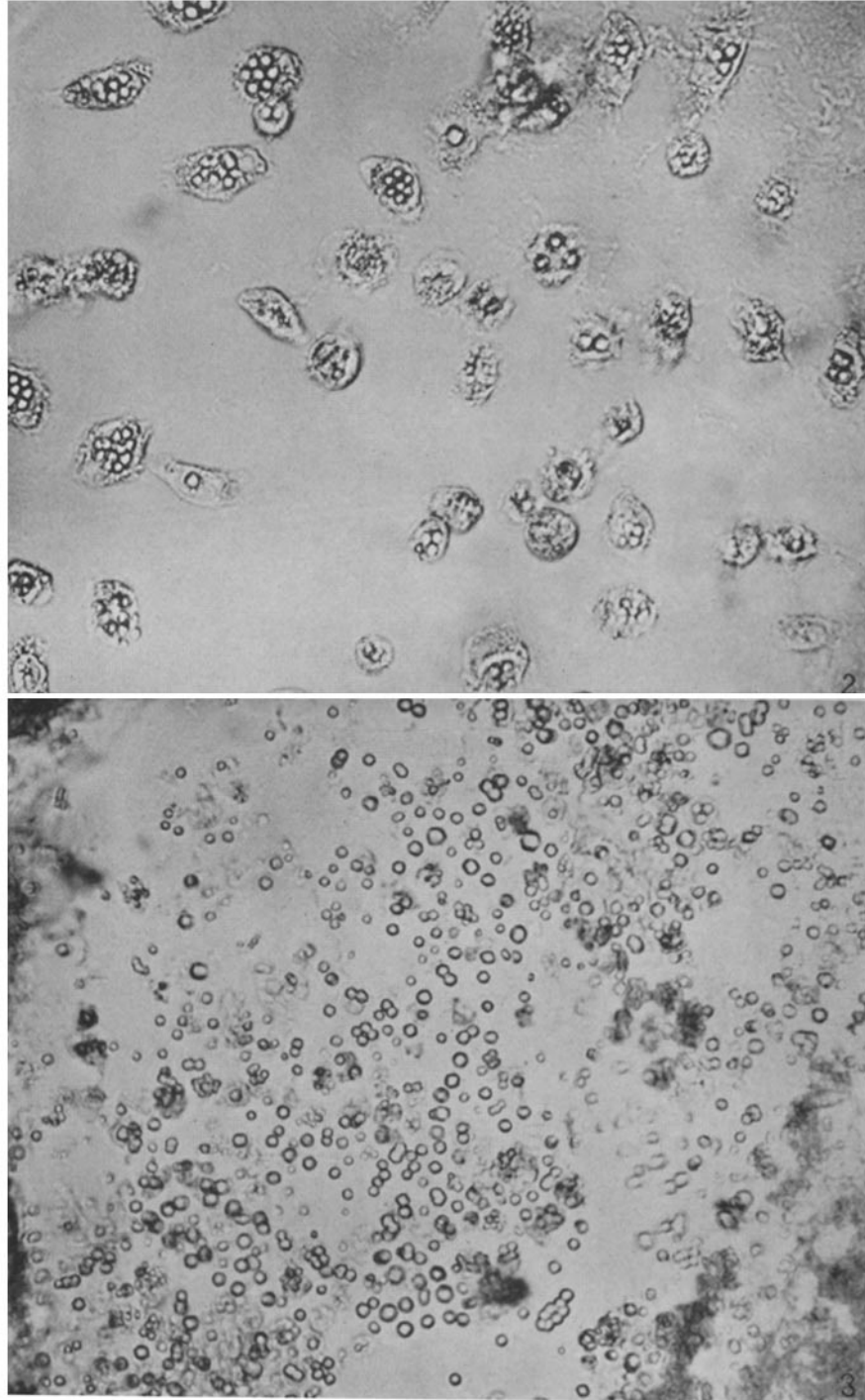
FIG. 2. A culture 5 days after infection with polyhedral virus. ×445.

FIG. 3. A culture 8 days after infection with polyhedral virus. ×445.



Photographed by J. A. Carlile

(Trager: Grasserie virus in silkworm tissue cultures)



Photographed by J. A. Carlile

(Trager: Grasserie virus in silkworm tissue cultures)