age utilization of dry matter among the diets differed significantly ($p < .01$). Although the percentage utilization of the three diets had not been determined previously, the values we obtained are acceptable. Our utilization value of 41 percent is similar to the value of 48 percent determined by the gravimetric method when fresh sprouts were fed to fifth-instar larvae of the cutworm (*8*). A further indication of the reliability of the method is that the percentage utilization of the sprout : cellulose diet (50 : 50 wt/wt) was about half of that found for the sprout diet. Such a result would be expected if it is assumed that little or no cellulose is digested by cutworm larvae. The percentage utilization of pith determined by the Cr_2O_3 procedure was low and is compatible with the slower rate of larval growth (Table 1).

The simplicity of the Cr_2O_3 procedure indicates that it should be useful for studies of food utilization by other insect species with biting and chewing mouth parts provided they will feed on artificial diets in which Cr_2O_3 has been incorporated. Because utilization values determined by the Cr_2O_3 method are computed from the concentrations of the indicator compound in food and excreta, quantitative separation and recovery of all of the excreta is not necessary, whereas the gravimetric method requires quantitative measurement of all food consumed and excreta produced. Although the Cr_2O_3 procedure was used here to determine dry matter utilization it can be extended to measure utilization of other dietary components. The gravimetric and Cr_2O_3 procedures are being compared with both the pale western cutworm and a grasshopper, *Melanoplus bivittatus* Say, and preliminary results support the usefulness of the indicator method for studies of food utilization.

A. J. McGinnis
R. Kasting

Canada Agriculture Research Station, Lethbridge, Alberta

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14 April 1964

Insect Tissue Culture: Use of Blastokinetic Stage of Leafhopper Embryo

Abstract. *To find the proper material for cultivation in vitro of cells of leafhopper vectors of plant viruses, embryonic tissues of the six-spotted aster leafhopper* (Macrosteles fascifrons) *were tested during early developmental stages, during blastokinetic movement, and in late developmental stages. Growing cells were only obtained from the stage of blastokinetic movement. This stage can be determined visually in leafhopper eggs.*

Several species of leafhoppers (Homoptera, Cicadellidae) are capable of harboring and transmitting pathogenic plant viruses that multiply in both plants and insect vectors (*1*). Repeated attempts to cultivate leafhopper tissues in vitro have been made, since tissue cultures would provide a useful tool for the study of the interactions of plant viruses and insect cells. Although various organs of nymphs and of adult leafhoppers had been maintained in vitro for several weeks (*2*), no growing cells were obtained in these earlier experiments. Until now, successful cultivations of arthropod cells had been carried out mainly with ovarian tissues of Lepidoptera (*3*), larval and pupal tissues of Diptera (*4*), nymphal tissues of Ixodina (*5*), and embryonic tissues of Orthoptera (*6*).

Tissues of the regenerating cockroach leg have been used successfully by Marks and Reinecke for tissue culture purposes (*7*). Cells and tissues of holometabolous insects are grown fairly easily in vitro, but those of paurometabolous insects can be cultivated only with considerable difficulty. These difficulties have been partly overcome in the cultivation of embryonic tissues of the six-spotted aster leafhopper, *Macrosteles fascifrons* Stål (*8*). After the initial success in growing such cells, it was expected that any undifferentiated embryonic cells obtained from leafhopper eggs would prove suitable for cultivation. However, this expectation was not fulfilled. It seemed that the stage of embryonic development was of critical importance for the attempted tissue culture. Experiments were therefore conducted to find the developmental stage of the leafhopper embryo which would grow in vitro.

For oviposition, 200 adult six-spotted leafhoppers were caged on young rye plants (*Secale cereale* L.) grown in 10-cm pots. Insects were transferred to groups of fresh plants every day. Rye plants with deposited eggs were maintained at a constant temperature of 25°C; standard fluorescent tubes provided 6600 lu/m^2 of light during a 16-hour day. Under these controlled conditions, first-instar nymphs hatched 11 days after oviposition. Daily transfers of insects to fresh plants facilitated the collection for each test of large numbers of embryos at the same stage of embryonic development. On the 1st, 3rd, 5th, 7th, 8th, 10th, and 11th day

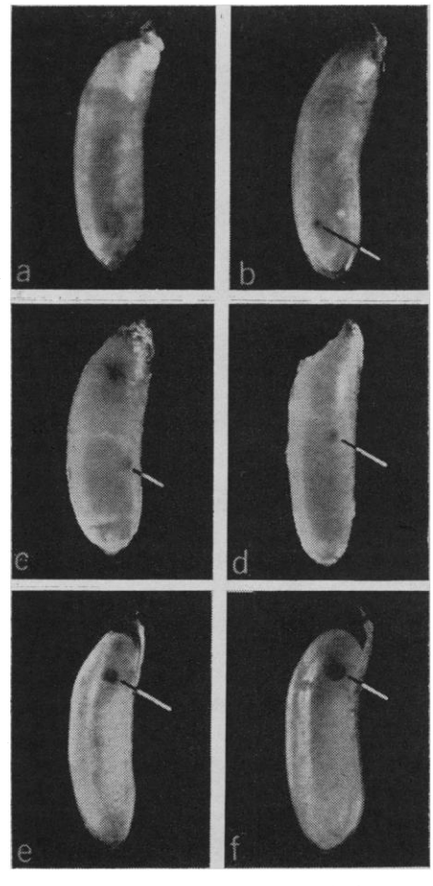

Fig. 1. Developmental stages of the embryo of the six-spotted aster leafhopper (× 30). *A*, One of the early development stages, 5-day-old egg. The pigmentation of eye discs is not yet visible. *B–D*, The eggs in the stage of blastokinetic movement during the 7th and 8th day. Slightly pigmented eye discs arise in the posterior position of the egg (*B*), then gradually move to the anterior position (*C* and *D*). Eggs in late development stages: *E*, 10-day-old egg; *F*, 11-day-old egg, just before hatching.

after oviposition eggs were excised from the rye plant leaves with metal needles and placed in a small petri dish with 5 ml of a culture medium containing 20 ml of modified Vago's *Bombyx mori* medium No. 22 (*8*), 20 ml of Morgan's synthetic medium TC 199 (*9*), 6 ml of serum from a bovine fetus (*9*), and 100 units per milliliter of penicillin and streptomycin. The *p*H was adjusted to 6.4 with a 7.5 percent sodium bicarbonate solution. After all eggs were excised and immersed in this medium, batches of eight to ten eggs were collected at a time for surface sterilization. The removal of eggs from the culture medium was facilitated by means of a micropipette with an orifice slightly narrower than the diameter of a leafhopper egg. By gentle suction, up to ten eggs were gathered at the tip of a pipette, removed from the medium, washed in Earle's balanced salt solution (*9*), then placed for 60 seconds in 70 percent ethyl alcohol. Afterwards the eggs were gathered in the same manner with a micropipette, washed again in fresh Earle's balanced salt solution, and immersed for 10 minutes in 0.1 percent Hyamine 2389 (*9, 10*). After sterilization of the surface, the eggs were washed in Earle's balanced salt solution and passed three times through fresh culture medium on Maximow slides. Under a dissecting microscope the posterior end of an egg was slightly pressed down with the point of a fine dissecting knife, and the egg shell at the anterior end was cut with a second sterile knife. With the pointed blade of this knife the embryo was then gently moved into the culture medium. Dissected embryos were washed by passing them through fresh culture medium three times; they were then treated with an 0.02 percent trypsin solution (*8*) and resuspended in 2 ml of culture medium. "Sitting-drop" cultures were prepared by placing 0.2 ml of medium with suspended trypsinized tissue fragments on the cover glass of each V-H tissue culture flask (*11*). Ovarian tissue fragments were also set up as sitting-drop cultures. Other details of the culture technique have been described (*8*). Usually 25 eggs were dissected on one Maximow slide, and a minimum of 100 eggs in the same developmental stage was used for each test. Three replications were made for each developmental stage.

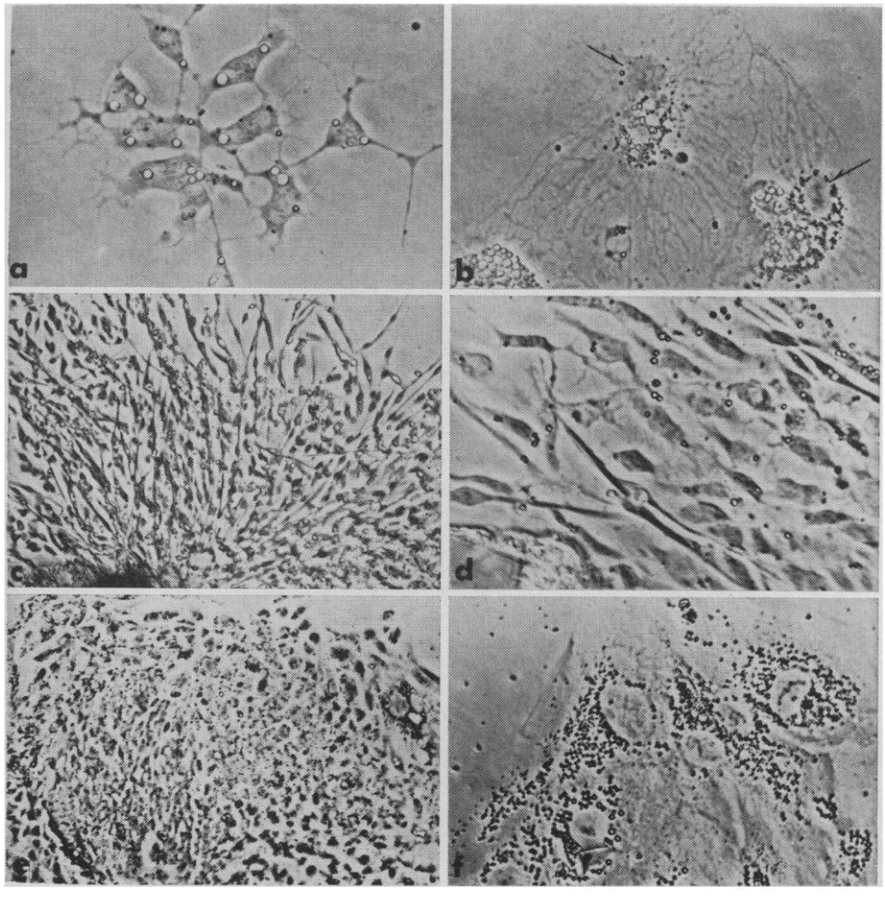

Fig. 2. Cells of the six-spotted aster leafhopper embryo in the stage of blastokinetic movement, growing in vitro as seen under phase contrast. *A*, Growth from an individual cell on the 6th day. During the initial 2 days of cultivation a cell multiplies about tenfold, then by the 6th day large oil droplets appear in the cytoplasm (× 570). *B*, Large phagocyte-like cells of a 10-day-old culture. Arrows point to groups of engulfed bacteria (× 520). *C*, Networks of fibroblast-like cells from a larger tissue fragment with contractile movements, on the 19th day (× 130). *D*, A portion of the same fibroblast network, under higher magnification (× 570). *E*, Outgrowth of epithelial cells from a larger contracting tissue fragment, on the 19th day (× 120). *F*, A portion of the same outgrowth (× 570). Note mitotic figures and well-spread cytoplasmic membranes with a number of large granules in the cytoplasm.

Embryonic tissues during early developmental stages, on the 1st, 3rd, and 5th day after oviposition, and in late developmental stages (that is, 10th and 11th day), as well as ovarian tissues of fifth-instar nymphs and of adults, yielded no growing cells. Trager (*12*) has emphasized that in other insect groups, as well as in other stages—larval or pupal—the finding of the proper developmental stage could be of critical importance for the success of cultivation in vitro. However, it may not always be possible to determine visually the right moment for dissection. Fortunately, the developmental stages of the embryo of the six-spotted aster leafhopper can be recognized visually with ease. The successive developmental stages of the embryo are represented in Fig. 1 (*A–F*). The eye, as can be seen from these photographs, moves from the posterior to the anterior end of the egg. The stage that yielded growing cells, and thus the only one suitable for cultivation in vitro, is shown in Fig. 1, *B*, *C*, and *D*. This stage, obtained on the 7th and 8th day after oviposition, was identified as the stage of blastokinetic movement.

When embryonic tissue fragments in this stage of blastokinetic movement were placed in V-H culture flasks, a number of single cells became attached to the surface of the cover glass during the initial 2 hours of cultivation. The number of cells increased about tenfold during the next 2 days (Fig. 2*A*). However, these cells did not survive for more than 8 days. Another type of cell migrated from the explants, by ameboid movements, 24 hours after the start of cultivation.

Cytoplasmic membranes of these large cells spread widely, showing an intricate fine texture (Fig. 2*B*). After 3 to 4 days there appeared in the Golgi region granules which gradually increased in size and number. After 6 days, bacteria, probably symbionts of the embryo, were seen engulfed in the cytoplasm. No cell divisions were observed. Although the origin and nature of the large cells remain obscure, they resemble phagocytes obtained from the cultured circulatory organ of Lepidoptera (*13*). Fibroblast-like cells and epithelial cells of both smaller and larger tissue fragments in the stage of blastokinetic movement also became attached to the glass surface and grew in vitro. After 24 hours of initial cultivation, fibroblast-like cells appeared and continued to grow, forming networks (Fig. 2, *C* and *D*). After 48 hours of cultivation, epithelial cells began to grow, forming a fairly compact cell sheet (Fig. 2, *E* and *F*). Cells derived from smaller tissue fragments began to degenerate and eventually became detached from the glass surface on the 15th day. Cells derived from larger tissue fragments, which had contractile movements for more than 15 days, continued to grow for more than 40 days.

Whether the need for fairly large numbers (100 embryos) for each successful cultivation test reflected a statistical chance to find in this number an adequate supply of tissues at the proper stage of development, or whether there was a definite need for some growth factors supplied in adequate amounts only by larger tissue masses, could not be ascertained. The latter seems more plausible at the moment. It also would explain the better growth consistently obtained from larger fragments of tissues compared to poor growth from smaller fragments. So far, it has not been possible to determine whether cells that became attached to the cover glass within the initial 2 hours of cultivation, and that degenerated usually after 8 days, were derived from the same tissues as cells that eventually grew well, or whether these earliest growing cells were hemocytes of the embryo.

The medium for cultivation cannot be considered as an "insect tissue culture medium," since differences between groups of arthropods are often more pronounced than differences between mammals on one side, and birds or reptiles on the other. Several attempts have been made to improve the composition of the culture medium. Among others, hemolymph of Crustacea was tried as an additive to our medium, but it failed to enhance the growth of cells in vitro.

HIROYUKI HIRUMI*
KARL MARAMOROSCH
Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

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* On leave from the Department of Anatomy, Wakayama Medical College, Wakayama-shi, Japan.

27 March 1964

Carotenoids of Cavernicolous Crayfish

Abstract. *Small amounts of β-carotene and lutein were found in* Orconectes pellucidus pellucidus. Cambarus bartonii tenebrosus *from the same cave contained much less carotenoid than surface crayfish. Astaxanthin, the principal carotenoid of most Crustacea, was absent from* O. p. pellucidus, *but accounted for 83 percent of the carotenoid of* C. b. tenebrosus. *These findings support other observations that pigmentation is dependent on the amount of carotenoid in the diet rather than on the presence of light. Furthermore, they suggest that* O. p. pellucidus *has lost or has never developed the ability to oxidize dietary carotenoids.*

Crustacea contain large amounts of carotenoid, most of it in the form of astaxanthin, which usually occurs conjugated with protein in the integumentary pigment of the animals. This is true of surface forms as well as bathypelagic and benthic members of this class. Astaxanthin is formed by the oxidation of dietary carotenoids (*1*). Intermediate products of oxidation have been demonstrated in *Artemia salina* (*2*) and *Carcinas maenas* (*3*). Vitamin A, also an oxidation product of dietary carotenoids, is involved in the visual process of many species of Crustacea including the crayfish *Orconectes virilis* and *Procambarus clarkii* (*4*). Vitamin A has not been detected, however, in many other crustacean species (*5*).

Cavernicolous Crustacea exhibit very little external pigmentation and their eyes are degenerate or absent. No carotenoid was found in the isopod *Aselus aquaticus cavernicolous* (*6*) or in cavernicolous amphipods of the genus *Niphargus* (*7*). Beatty (*8*) examined cave detritus and found substantial amounts of carotenoid in flood debris consisting chiefly of old leaves and pieces of wood. He concluded that the absence of pigment might result from the absence of light (*7*). The validity of this hypothesis was questioned by Maguire (*9*), who showed that color development in the crayfish *Procambarus simulans simulans* was independent of light and dependent upon nutrition. The presence of carotenoids in benthic marine Crustacea, living in the perpetual absence of light, tends to confirm that carotenoid pigmentation is not light induced.

We had the opportunity to compare

Table 1. Carotenoid content of *Orconectes pellucidus pellucidus* and *Cambarus bartonii tenebrosus*.

Species	Weight (g)		Total carotenoid (μg)
	Wet	Oil	
O. p. pellucidus	65.6	0.774	15.7*
C. b. tenebrosus	127	1.27	722

* Since the spectrum obtained for the total extract exhibited much nonspecific absorption (Fig. 1), this value represents the sum of the fractionated carotenoids.