Pure cultures of *Chlorella pyrenoidosa,* Van Niel's strain Z 2.2.1, were cultured autotrophically and synchronously in Schmidt's medium (*2*). In some cases, cultures were exposed to P^{32}-phosphate, S^{35}-sulfate, or C^{14}-carbon dioxide for one life cycle of growth.

Samples of 100 g (wet weight) of *Chlorella* cells were stirred in 300 ml of dilute KOH (*p*H 11, at 25°C for 1 hour). An equal volume of 88 percent phenol was then added, and the mixture was shaken thoroughly. The phases were separated by centrifugation, and the upper, aqueous phase, which contained the glycopeptide, was treated two more times with phenol to free it completely of protein. The residual phenol was extracted with ether, the glycopeptide remaining in the aqueous fraction. Sodium acetate (2 percent dry weight) and three volumes of cold ethanol were added. The resultant precipitate was separated by centrifugation and dissolved in 0.01*M* $MgCl_2$. The sample was then treated with snake-venom phosphodiesterase (*p*H 8.8 at 35°C for 24 hours) to hydrolyze the bulk of the RNA present. The hydrolysate was eluted from a diethylaminoethylcellulose (DEAE) column with a gradient of sodium chloride. The glycopeptide, which eluted between 1.0 and 1.5*M*, was then hydrolyzed with 0.5*N* KOH for 18 hours at 35°C and rechromatographed on a DEAE column.

After this purification, the glycopeptide had no absorption in the visible or ultraviolet regions, and only one fraction, which was a symmetrical peak, was eluted from the DEAE column. Sialic acid was assayed in both the phenol-water extract and the final preparation by the thiobarbituric acid method (*3*). The yield, 10 μmole of sialic acid, amounted to 80 percent of the sialic acid found in the phenol-water extract. That no thiobarbituric acid reaction occurred unless the sample was first treated with 0.1*N* HCl at 80°C for 1 hour indicated a ketosidic linkage. The spectrum of the chromogen is identical with that produced in the reaction when known *N*-acetyl neuraminic acid (one of the sialic acids) is used. Further evidence that the unknown is indeed a sialic acid included a positive Partridge reaction for amino sugars (*4*) and a positive HCl test (compound turns dark in 0.1*N* HCl at 100°C but not at 80°C).

The glycopeptide gave an intense metachromatic reaction with toluidine blue at *p*H 7 but gave no uronic acid reaction (*5*). No incorporation in vivo of radioactive sulfate or phosphate into the glycopeptide was detected. Thus, the metachromasy and strong interaction with DEAE indicate that the carboxyl function of the sialic acid units are free in the polymer. The material was nondialyzable and was excluded by G-25 Sephadex (cross-linked dextran gel) which indicates a molecular weight of over 10,000.

The polymer contained 9.5 μmole of glucose equivalent of nonamino sugars per micromole of sialic acid as determined by the anthrone reaction (*6*), 1 μmole of reducing sugar before any acid treatment (*7*), and 875 μg of protein (*8*). After 0.1*N* HCl hydrolysis for 1 hour at 80°C, reducing functions were increased to 2.75 μmole.

A C^{14}-labeled sample having 15,000 count/min per micromole of sialic acid was isolated. This material was chromatographed on Whatman No. 1 paper by the descending method with ethyl acetate, water, and pyridine (5:7:2) (*9*). Spots were detected by autoradiography, also by spraying with silver nitrate then with alcoholic sodium hydroxide. The untreated polymer remained at the origin.

After mild acid hydrolysis another spot appeared at $R_{glucose}$ (movement relative to that of glucose) of 0.13, identical to the location of *N*-acetyl neuraminic acid. The material at this location reduced silver ion and gave positive Partridge (*4*) and ninhydrin tests. These are all expected properties of sialic acids. After more severe acid hydrolysis the sialic acid spot was no longer found, but spots at $R_{glucose}$ of 0.65 and 1.86 appeared. The materials at both of these locations were able to reduce silver ion. These mobilities coincide with those of glucosamine and L-rhamnose, respectively.

Three types of polymers containing sialic acid have been isolated: (i) glycoproteins—such as that from the ovine submaxillary gland—from which the moiety containing sialic acid is released by mild alkali (*10*); (ii) glycoproteins—such as that from equine erythrocytes—in which the protein-carbohydrate linkage is stable to mild alkali (*11*); (iii) colominic acid—obtained from *Escherichia coli*—a polymer which contains only sialic acid, none of which is released by mild alkali (*12*). Very mild acid hydrolysis cleaves the ketosidic linkage of the sialic acid in all three types of polymer.

The glycopeptide isolated from *Chlorella* has properties most closely resembling the second type of polymer. It is stable to mild alkali and contains both amino acids and carbohydrates.

More thorough identification of the components of the glycopeptide from *Chlorella* will require larger amounts of material or the application of more sensitive methods. However, it seems important to note that this glycopeptide, which contains sialic acid, does occur in *Chlorella.*

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25 June 1964

Tobacco Mosaic Virus: Cytological Evidence of the Synthesis in the Nucleus

Abstract. *Living tomato hair cells were directly injected with tobacco mosaic virus or tobacco mosaic virus RNA with a fine glass needle. Use of a fluorescent tobacco mosaic virus antibody showed the presence of the virus protein in the nucleus 6 to 18 hours after the injection. As time of infection progressed, staining of the cytoplasm became more prominent.*

The nuclei of plant cells may play an important role in the formation of tobacco mosaic virus (TMV) (*1*). However, these observations have all been made on cells that became infected with TMV indirectly. This report shows that the TMV infective process may be induced by direct injection of TMV or TMV-RNA into living tomato hair cells. From observations on the course of the infection both by phase microscope and by the use

of fluorescent antibody specific for TMV protein, the nuclei of hair cells appear to be the first organelles to show the presence of newly synthesized TMV protein.

A common strain of TMV found in Japan was purified by differential centrifugation. The TMV-RNA was isolated and purified by the phenol method (*2*). A tomato stem about 1 cm in diameter was cut off near the base of the plant. Strips of epidermal tissue (5 by 5 mm) located about 5 cm from the base were peeled off. The hair cells on the surface of the strips were dissected away except for one large trichome attached to the epidermis by a cluster of small cells. The large cell at the base just above the cluster of cells was used for injection. The strip containing this base cell was fastened with adhesive tape to the glass stage of a dissecting microscope. A fine glass needle, 3 to 5 μ in diameter at one end, was fastened to a mechanical stage of another microscope. While the cell was being observed with the microscope, the needle was inserted into the base cell. One drop of TMV (3.0 mg/ml in 0.05*M* phosphate buffer, *p*H 7) or TMV-RNA (0.7 mg/ml in the same buffer) was placed on the needle. The needle was then carefully withdrawn from the cell, this act sufficing to permit entry of the virus into the cell. Infection by this procedure occurred with 30 to 80 percent success. The inoculated hair cells were placed on a wet filter paper contained in a petri dish which was incubated at 25°C under continuous illumination from fluorescent lamps. After definite periods of inoculation, hair cells were cut through the cluster of cells away from the epidermis, mounted in water, and observed under the phase microscope. Only living hair cells, as determined by their protoplasmic streaming, were treated with fluorescent antibody.

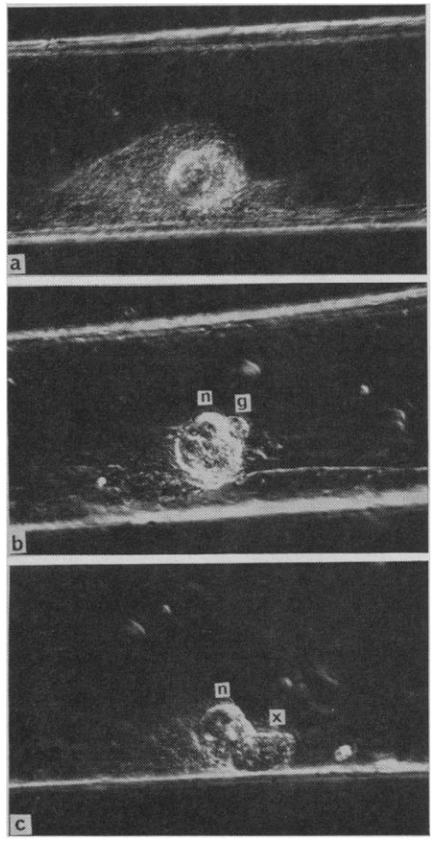

Fig. 1. *A*, Conglomeration of cytoplasmic vesicles around the nucleus, 6 hours after injection of TMV into the tomato hair cell. *B*, A small granular appearing substance (*g*) adhering to the nucleus (*n*) 18 hours after injection of TMV. *C*, X-body formation (*x*) adhering to the nucleus (*n*) 24 hours after injection of TMV. Observed under phase microscope (× 500).

The γ-globulin fraction of antibody (1:512 titer to TMV) was conjugated with fluorescein isothiocyanate. The titer of fluorescent antibody was 1:64 as determined by precipitin test. The base cells were fixed in cold acetone for 5 minutes and then washed twice with phosphate-buffered saline. The base cell was then cut at the end distal to the cluster of cells in order to produce a tubelike structure with an opening at one end. The open end of the cell was then dipped into a fluorescent antibody solution for 2 to 3 hours at 37°C. The cells were then washed for 1 day with several changes of phosphate-buffered saline. The presence of antibody was detected by fluorescence microscopy.

When observed by phase microscopy, the first evidence of TMV infection was the migration of the nucleus towards the point of entry of the needle and virus into the base cell. The migration of the nucleus was followed by an acceleration of protoplasmic streaming, and in about 6 hours there was a noticeable conglomeration of cytoplasmic network vesicles and contained organelles such as sphaerosomes and mitochondria clustering around the nucleus. Sometimes a small granular appearing substance could be seen adhering to the nucleus (Fig. 1). Tobacco mosaic virus crystals often, but not always, appeared at a still later stage, ordinarily 30 hours after injection of the virus.

Soon after injection of tobacco mosaic virus, a specific fluorescence characteristic of the reaction of the antibody with TMV protein was sometimes observed near the position where the virus was injected into the base cells. However, this fluorescence reaction disappeared within 1 to 3 hours after injection. Six hours after injection, a new fluorescent antibody reaction could be detected in about 50 percent of the cells injected with TMV. The antibody reaction was mainly confined to the nucleus and was less frequently found in both the nucleus and the cytoplasm adhering to the nucleus. As the time of infection increased, the percentage of cells showing the TMV antibody reaction increased. However, the reaction was less apparent in the nuclei and

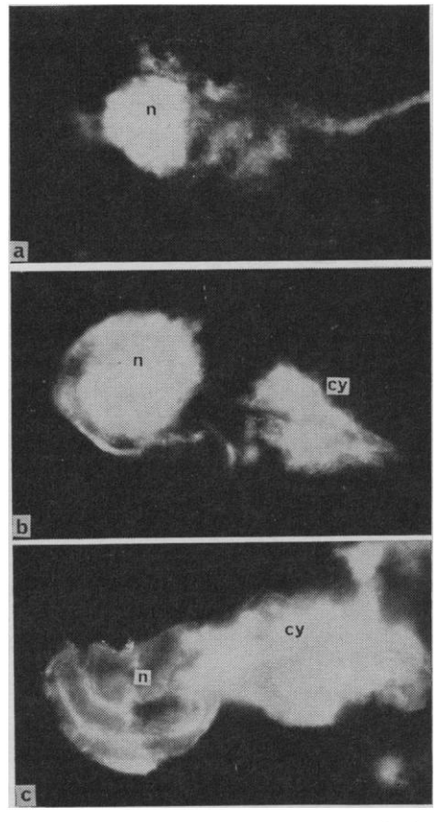

Fig. 2. *A*, Fluorescent TMV-antibody stain of the tomato hair cell at 6 hours after the injection of TMV-RNA. Note the nucleus (*n*) alone shows a positive reaction, (× 630). *B*, The nucleus (*n*) as well as the cytoplasm (*cy*), located near the nucleus, exhibit the specific fluorescence at 18 hours after the injection of TMV (× 900). *C*, The nucleus (*n*) remains rather unstained, whereas the cytoplasm (*cy*), producing from the nucleus, is extensively stained at 24 hours after the injection of TMV (× 900). Observed under fluorescent microscope.

more prominent in the cytoplasm immediately surrounding the nuclei (Fig. 2).

Hair cells which were not injected, or injected with water, did not remain stained by fluorescent TMV-antibody after the cells were washed with phosphate-buffered saline. The validity of the fluorescent antibody identification of TMV was verified by first treating a base cell injected with TMV with TMV-antibody that was not conjugated with dye. These cells were then treated with fluorescent antibody. No definite specific fluorescence was recognized. Further evidence of the specificity of the fluorescent antibody reaction was found by the use of TMV inactivated by irradiation with ultraviolet light. By this means, TMV was inactivated so that it no longer produced lesions on pinto bean leaves but still retained its serological properties. The inactivated TMV was injected into hair cells. Within a few hours after injection, the nuclei sometimes showed a reaction to fluorescent antibody. However, the reaction disappeared 6 hours after injection, and no further antibody stain either on the nuclei or other cell components was seen at later times of observations.

When TMV-RNA was injected, no reaction of the type found with TMV was evident immediately after injection. However, 6 hours after injection a fluorescent reaction typical of TMV protein was detected in the nucleus and less frequently in the cytoplasm surrounding the nucleus. With longer periods after injection, the fluorescent antibody reaction had the same character found with infections induced by TMV.

Previous observations of cells already infected with TMV have all suggested that the nucleus takes part in the TMV infectious process. The results of this investigation with cells that were directly infected with either TMV or TMV-RNA confirm previous observations and provide rigorous new evidence to show that the nucleus is the first organelle in the injected cell to respond to the presence of virus. Furthermore, the nucleus is the first organelle in which TMV protein, as identified by a specific fluorescent antibody, can be detected. From results of biochemical studies, Reddi (*3*) stated that TMV may be synthesized in the nucleus. Only after the virus protein has been found in the nucleus can its presence be identified in the cytoplasm, and then only in areas that are closely adhering to the nucleus. These results are inconsistent with the observation by Schramm and Röttger (*4*), who demonstrated that the cytoplasm alone around the nucleus exhibited the specific fluorescence. Of particular interest is the observation that uninfectious TMV injected into hair cells still finds its way to the nucleus before it eventually disappears as a reactant to fluorescent antibody. Apparently, there is a highly specific interaction between TMV and the nucleus which signals the beginning of the TMV infectious process leading ultimately to the formation of new TMV nucleoprotein rods in the injected cell.

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Circulatory Adaptation to Diving in the Freshwater Turtle

Abstract. *The heart of the freshwater turtle has a functional ventricular septal defect. In ambient air there is some shunting of blood from the left to the right ventricle through the defect. During prolonged diving or N_2 inhalation, the shunt is reversed and blood from the tissues bypasses the lung and enters the aorta directly. This adaptation appears to be based on the exclusive use of anaerobic glycolysis as an energy source during prolonged diving.*

The circulation and external ventilation of vertebrates must be geared to intracellular metabolic requirements. The freshwater turtle is able to dive for extended periods in the absence of molecular oxygen (*1*). The fundamental adaptation which permits this is the animal's ability to exist for prolonged periods on energy derived solely from anaerobic glycolysis (*1*, *2*). In this report we describe an unusual circulatory adaptation which allows a more effective supply of substrates for anaerobic metabolism of the tissues.

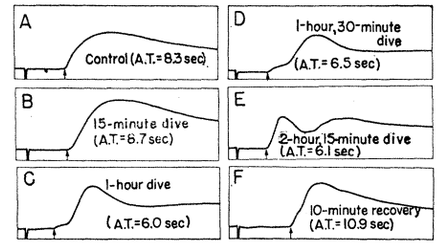

Fig. 1. Cardiac shunt progression. The effect of diving on dye-indicator curves obtained from the freshwater turtle. The time between injection of the dye and its appearance in the arterial system (↑) is indicated by A.T. (appearance time).

Freshwater turtles, *Pseudemys scripta elegans* and *floridana*, weighing between 1 and 2 kg, were used for our studies. A total of 40 experiments were conducted. Preliminary investigations of the anatomical features of turtle heart, aortae, and pulmonary arteries were made by direct inspection after appropriate dissection.

The pathways of blood flow were determined by use of dye indicator techniques (*3*). The Gilford continuous, automatic densitometer (*4*), commonly used for cardiac output measurements in man, was modified so that dye indicator studies could be performed in the turtle. The indicator dye used was indocyanine green. Peripheral arteries and veins in the neck were isolated and intubated with polyethylene catheters (PE 50). For the documentation of right to left cardiac shunts the bolus of dye was injected into a peripheral vein and the appearance times and contours of the time-concentration curves were monitored in arterial blood. For the documentation of left to right cardiac shunts the dye bolus was injected directly into the left ventricle after removal of the plastron, and indicator-dye concentrations were monitored in arterial blood. Heart rates were obtained from arterial pressure tracings. Experiments were performed in ambient air, during diving, during the inhalation of 100 percent N_2, and after the intravenous injection of 20 mg of NaCN.

The heart of the turtle has four chambers. As has been reported for all reptiles (except Crocodilia) the intra-