tion of several fractions. Use of fluorochromasia from FDA as the sorting parameter in this cell sorter may have other applications in selecting functionally different groups of cells. The possibility of substituting other fluorogenic substrates for FDA should be considered as well as the use of other fluorescent dyes and of fluorescent antibody techniques.

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Chloroplast Replication and Growth in Tobacco

Abstract. *Tobacco etioplasts replicate at least twice during light-induced development. Their size is doubled over the same period. The two processes do not show the same kinetics. 5-Fluorodeoxyuridine inhibits chlorophyll synthesis during the normal course of "greening" by inhibiting chloroplast growth. This compound also inhibits cytokinin-induced chloroplast replication.*

Chloroplasts in higher plants and algae contain their own DNA (*1*) and complete protein-synthesizing machinery (*2*), but very little is known about the function of this DNA in chloroplast development. To study the effect of inhibitors of DNA synthesis on the development of etioplasts into chloroplasts, we used the inhibitor 5-fluorodeoxyuridine (FUDR), which inhibits DNA synthesis in bacteria and tobacco (*3, 4*) primarily by inhibiting thymidylate synthetase (*5*). The effect is reversible with thymidine.

Etiolated leaves of *Nicotiana tabacum* L. var. Maryland Mammoth were obtained by cutting stems of mature, greenhouse-grown plants into 20-cm sections which were rinsed in 4.0 percent hypochlorite (commercial bleach with detergent) solution and placed in the dark in flasks containing a nutrient solution. Etiolated leaves of lateral shoots were harvested after 2 weeks, soaked in water containing a trace of laboratory detergent for 15 to 20 minutes, agitated in a 2.0 percent hypochlorite solution for 2 minutes, rinsed in sterile water, and placed in petri dishes on a layer of sterile filter paper. Disks (2 mm in diameter) from several leaves were placed together in each dish to randomize variation. All manipulations were performed under a dim green light. The dishes were kept in the dark for 2 hours before exposure to light; longer periods of preliminary treatment in the dark did not affect the result. The dishes were kept at 25°C in a light intensity of 30 lu/mm^2 provided by Sylvania cool white VHO fluorescent tubes with neutral density filters.

The standard culture medium (S+S) contained Murashige and Skoog salts (*6*), 0.8 percent agar, and 0.5 percent sucrose. Other components were added as indicated. Thymidine, uridine, and FUDR were sterilized by filtration before addition to the medium.

Chlorophyll was determined according to the method of Arnon (*7*). Chloroplasts and etioplasts in cells were counted and measured in sections from fixed tissue. The leaf disks were embedded in paraffin by standard methods, sectioned at 40 μm, and stained in safranin and then fast green. Plastids were counted by moving the plane of focus through whole cells. Chloroplast size was determined by measuring the greatest dimension with an ocular scale. The number of cells per leaf disk was determined by hemacytometer counts of cells separated in 5 percent chromic acid, and cell sizes were measured on photographs of the microscopic slides. Observations were made with a Zeiss photomicroscope equipped with a ×100 phase-contrast objective.

Disks were exposed to ^{3}H-thymidine (250 μc/ml, specific activity 11.7 c/mmole) for 2 hours, fixed by freezing, and embedded in paraffin (*8*). Sections were cut at 6 μm, treated with either deoxyribonuclease (Worthington, ribonuclease-free; 1 mg/ml in 0.1M acetate buffer, pH 5, and 0.005M $MgSO_4$) or with plain buffer, covered with Ilford L4 emulsion by the liquid-emulsion method (*9*), exposed for 8 to 10 days, and developed with Kodak D19 developer.

Material was fixed for electron microscopy in glutaraldehyde and OsO_4 as described (*10*). The tissue was embedded in Epon (*11*) and sectioned with a diamond knife. Sections were mounted on uncoated grids, then stained with lead citrate (*12*), and viewed in a Zeiss 9A electron microscope.

The etiolated tobacco leaves are not completely uniform, so the absolute values for chlorophyll content vary between experiments although the pattern remains the same. The graphs and tables, therefore, give representative data rather than average values.

Chlorophyll synthesis in the tobacco leaf disks is inhibited about 50 to 60 percent by FUDR ($10^{-4}M$) after 4 days in the light. This is reversed by thymidine at the same concentration but not by uridine (Table 1). 5-Fluorodeoxyuridine has little effect on fresh weight and none on cell size and cell number per leaf disk. The course of chlorophyll synthesis (Fig. 1) in the control shows the normal pattern of a lag phase followed by a rapid increase in chlorophyll content (*13*). During the lag phase there is no visible effect of FUDR, but the following rapid synthesis of chlorophyll is largely inhibited by FUDR. A reduction in chlorophyll content of the whole tissue could be the result of abnormal chloroplasts, fewer chloroplasts, or smaller chloroplasts.

Since chloroplasts from FUDR-treated

Table 1. Inhibition of chlorophyll synthesis by $10^{-4}M$ FUDR in etiolated leaf disks exposed to light for 4 days and reversal of this inhibition.

Additions to S+S medium	Chlorophyll (μg/g, fresh weight)
None	375.4
FUDR	220.5
Thymidine	414.4
Uridine	446.0
FUDR and thymidine	366.6
FUDR and uridine	228.6

Table 2. Effect of FUDR on kinetin-induced chloroplast replication. Etiolated disks cultured on S+S and kinetin and S+S, kinetin, and FUDR in the dark for 7 days and exposed to light for 7 days.

Additions to S+S medium	Chloroplasts (No./cell)	Chloroplast size (μm)
Kinetin	135	4.6
FUDR and kinetin (0.5 mg/liter)	50	2.5

material have a normal ultrastructure (Fig. 2), we studied chloroplast number per cell during light-induced development. There is little cell division during this period. The chloroplast number per cell increases over time, but FUDR does not inhibit this replication (Fig. 3). The increase in number is equivalent to two to three division cycles in the plastid population. Chloroplast number per cell in palisade cells and spongy mesophyll cells does not differ.

5-Fluorodeoxyuridine inhibits chloroplast growth (Fig. 4). The sudden increase in chloroplast size about 24 hours after illumination, which is inhibited by FUDR, also coincides in time with the start of rapid chlorophyll synthesis which is inhibited by FUDR (Fig. 1). We conclude that the reduction in chlorophyll content by FUDR is caused by a reduction in chloroplast size.

We tested the viability of the disks and the reversibility of FUDR inhibition by culturing leaf disks in the light for 4 days on S+S medium containing FUDR and then transferring them to S+S medium containing thymidine. There is an initial lag period followed by a rapid chlorophyll synthesis, but the recovery was not complete during the test period (Fig. 5). When the disks were transferred to S+S alone, they did not recover as fast, but their recovery was still significant. Chloroplast size also increases during recovery. When FUDR-treated leaf disks were transferred to a medium containing both FUDR and thymidine, their recovery was as good as when they were transferred to thymidine alone. Disks were exposed to FUDR for 4 days and then to ^{3}H-thymidine for 2 hours to determine whether this recovery involved DNA synthesis and, if so, where this occurred. Autoradiography showed labeling in the cytoplasm in all cells, and in the nuclei of a few cells. Most of the label is removed with deoxyribonuclease.

While FUDR does not inhibit chloroplast replication in the leaf disks cultured as described above, it will inhibit kinetin (6-furfurylaminopurine)-induced chloroplast replication (Table 2). 5-Fluorodeoxyuridine almost completely inhibits chlorophyll synthesis in the disks treated with kinetin, and there is no lag phase in this inhibition. The plastids in this tissue have a very abnormal ultrastructure. It is not known if there is DNA synthesis during the more than 100 percent increase in chloroplast numbers per cell in the tissue treated with kinetin.

Results with the tissue on S+S provide information about the normal be-

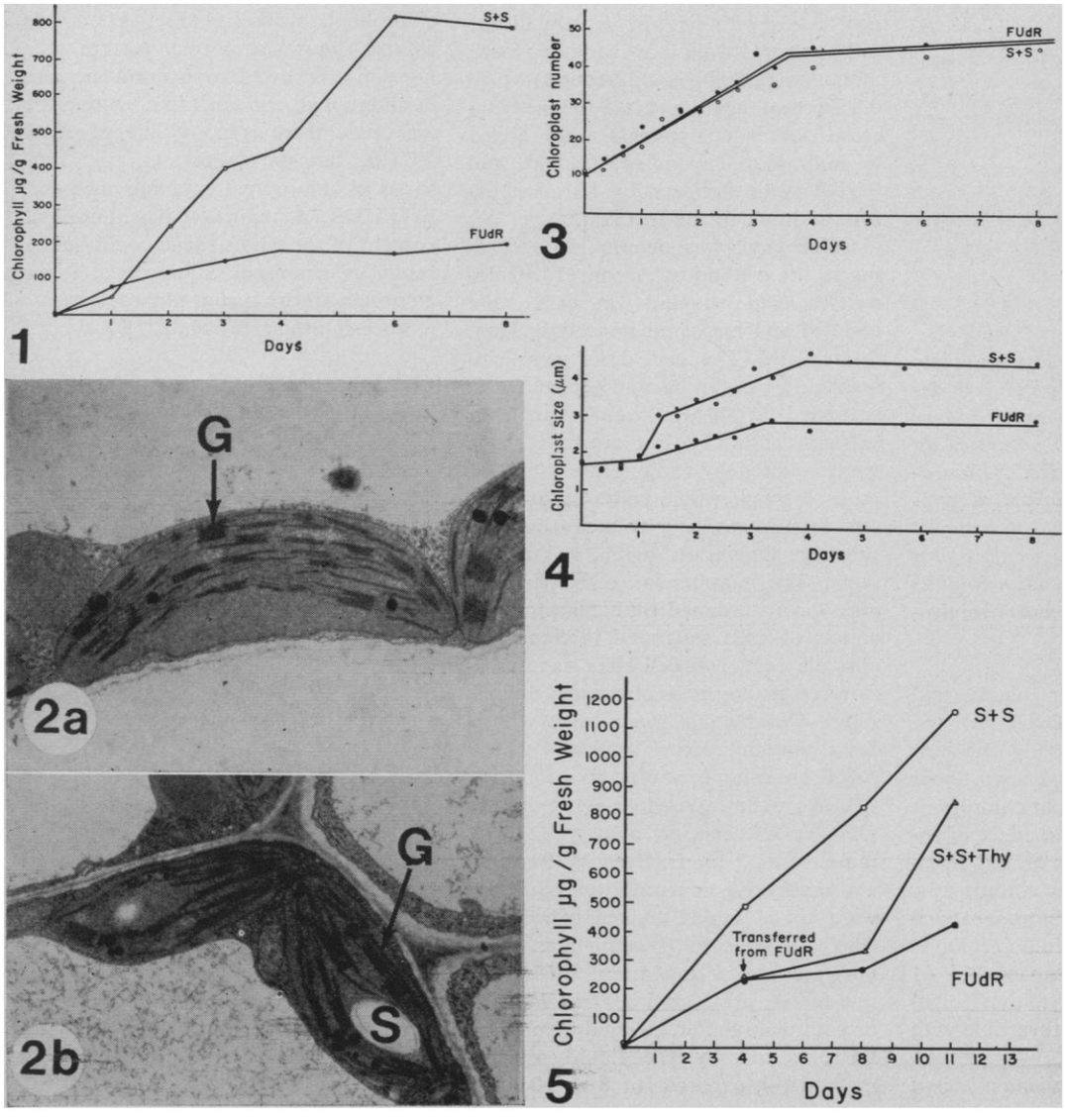

Fig. 1. Chlorophyll synthesis in etiolated tobacco leaf disks when incubated on surcrose and salts (S+S) and sucrose, salts, and FUDR media.

Fig. 2. Effect of FUDR treatment on chloroplast ultrastructure. (a) Chloroplast from tissue exposed to light for 4 days on S+S (*G*, grana). (b) Chloroplast from tissue exposed to light for 4 days on S+S and FUDR (*G*, grana; *S*, starch).

Fig. 3. Chloroplast replication during light-induced development. Etiolated leaf disks were incubated in the light on sucrose and salts (S+S) and sucrose, salts, and FUDR media.

Fig. 4. Chloroplast growth during light-induced development. Etiolated leaf disks were incubated in the light on sucrose and salts (S+S) and on sucrose, salts, and FUDR media.

Fig. 5. Recovery of FUDR-treated disks on S+S and thymidine. Disks were cultured on S+S and FUDR for 4 days in the light before being transferred to S+S and thymidine (arrow).

havior of the chloroplast population during light-induced development. There are some observations that young chloroplasts can divide (*14*) and a few studies of chloroplast numbers in mature cells (*15*). Fasse-Franzisket (*16*) showed that in normal development of the light-grown leaf of *Agapanthus umbellatus* the plastids replicated several times. Plastids in etiolated leaves multiplied very slowly, but, if the etiolated leaves were exposed to light, plastids divided very rapidly until the normal number was reached. Glydenholm (*17*) demonstrated that there is no increase of chloroplast numbers in 16-day-old etiolated bean leaves during "greening." In our own material the plastids replicate at least twice. Replication starts immediately, and the shape of the curve indicates that this division is regular and asynchronous, whereas growth lags by about 24 hours and does not progress linearly.

We now have a method of specifically inhibiting chloroplast growth without permanently damaging the plastids. We can separate chloroplast growth from replication and thus explore the regulation of chloroplast development. In order to understand the action of FUDR on chloroplast growth, it must be shown that FUDR acts on DNA synthesis. There is a report (*3*) that it also affects RNA synthesis and that the effects are reversible with uridine. The effect of FUDR on higher plants has always been reversible with thymidine (*4*, *18*) and RNA synthesis in tobacco callus was not affected by FUDR (*4*).

An important question is whether DNA synthesis during chloroplast growth occurs in the nucleus or in the plastid. Theoretically, it would be enough to show that chloroplasts treated with FUDR contain less DNA than those in the control, but since differences would not be more than 25 to 50 percent, it would be virtually impossible to purify the plastids enough to produce significant results. The autoradiographic data show that DNA synthesis occurs during plastid recovery from FUDR inhibition. Most of it is localized in the cytoplasm, so it seems likely that it is indeed this DNA synthesis that is involved in plastid growth.

The number of plastids increases considerably during greening, so it is not surprising that inhibiting DNA synthesis should influence chloroplast development. It is, however, most surprising that this inhibition has no effect on normal chloroplast replication. One explanation for this phenomenon is that the plastids contain more than one copy of their DNA, so that they can divide a few times even without additional DNA synthesis, but that DNA redundancy is necessary for the chloroplasts to attain their normal size. This hypothesis demands that plastid replication would come to a halt after a few division cycles, and the kinetin data show that this is so. 5-Fluorodeoxyuridine did inhibit kinetin-induced plastid division. Another consequence is that FUDR would have no influence on plants in which no plastid replication occurs.

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Calibrated Membranes with Coated Pore Walls

Abstract. *Extremely uniform small-radius pores, formed in thin mica membranes, have been coated with mono- and multimolecular layers of fatty acids. Stearate monolayers orient themselves on the pore walls in layers 25 to 28 angstroms thick. The resulting membranes, with radii of the order 100 angstroms or less, may have applications in simulating certain features of biological membranes and in fabricating highly selective membranes for differential dialysis or ultrafiltration.*

Price and Walker (*1*) described a technique for producing uniform holes of molecular dimensions in certain nonmetallic materials. Holes are produced by exposing the material to massive, high energy particles which traverse the solid and leave tracks along which the material undergoes radiation damage. The damaged material along the tracks can be removed in a chemical etching solution which creates fine, hollow channels while the rest of the solid remains unaltered. By controlling the radiation exposure and etching time, holes smaller than 50 Å in diameter and hole densities up to 10^{11} holes per square centimeter have been produced (*2*).

Starting with thin mica sheets containing holes formed by this irradiation-etching technique, we coated the channel walls with stearate multilayers (*3*). Stearate deposited onto the surface of the sheet migrates into the pores, forming oriented layers on the pore wall. We summarize here our procedure for preparing coated holes in mica membranes and for measuring the thickness of the built-up layers.

Our membranes were made from clear muscovite (*4*), a natural mica, cleaved to thicknesses of 7 to 8 μm and cut into circular disks approximately 5 cm in diameter. The mica was irradiated by exposure to fission fragments resulting from the neutron-induced fission of U^{235}. Both the mica sheets and the uranium source were mounted in an evacuated collimating device placed in the thermal column of a nuclear reactor (*5*). After irradiation, holes were etched in a 20 percent HF solution (*1*).

Recrystallized, radioactive stearic acid (*6*) was used for the depositions by the Langmuir-Blodgett technique