10-ml portions were placed in 120-ml, flat-sided, glass prescription bottles. The bottles were incubated on their sides for 90 minutes at 37°C. The lymphocytes and any nonadherent polymorphs were gently decanted, centrifuged, washed twice in medium, and cultured as described. This procedure effectively reduced the number of contaminating polymorphs and provided a reasonably pure culture inoculum of small lymphocytes.

However, the presence of polymorphonuclear leukocytes in the cultures had no obvious stimulatory effect on cellular transformation, since their removal did not reduce the percentage of blastoid cells after 5 days of culture. Indeed, rather than provoke stimulation of blastoids, the presence of granulocytes may inhibit the growth and differentiation of lymphocytes in vitro (*3*).

Spontaneous transformation to blastoid cells was also studied in cultures of guinea pig peripheral lymphocytes. Erythrocytes from nonimmunized guinea pigs were sedimented by the addition of an equal volume of 3 percent gelatin to heparinized blood, and the cells were cultured in 20 percent rabbit or fetal calf serum. By the 5th day of culture, between 58 and 72 percent of the cells were blastoid in appearance, whereas only 0.8 to 2.5 percent were blastoid before culture.

The potential for spontaneous transformation was not confined to circulating lymphoid cells; a similar change was also observed in 5-day cultures of splenic lymphocytes of guinea pig. The splenic tissue was teased apart in minimal essential medium, filtered through mesh, washed three times with medium, and then cultured for 5 days at 37°C in minimal essential medium supplemented with fetal calf serum (20 percent). By contrast with the peripheral lymphocytes, the splenic cells were more heterogeneous in appearance at the beginning of culture. Many of the splenic lymphocytes were larger than the circulating lymphocytes; most of them had a dense, deeply basophilic, nuclear chromatin, while approximately 10 to 16 percent had reticulated nuclei similar to those of the blastoid cells. By the 3rd day of culture with PHA present, 75 to 85 percent of the splenic cells were blastoid in appearance, and by the 5th day 55 to 75 percent of the cells grown without PHA had transformed spontaneously into blastoid cells. Both the PHA-promoted and spontaneously transformed splenic and peripheral blastoid cells were capable of synthesis of RNA and DNA, as evidenced by labeling with tritiated cytidine and tritiated thymidine, respectively.

The stimulus for transformation to blastoids is not known. The immature cells that developed spontaneously or with PHA present tend to appear in aggregates. It has been suggested that the blastogenic properties of PHA may be related to cell-membrane contacts caused by agglutination of leukocytes. Thus one may speculate that spontaneous transformation to blastoids is also related to the lymphocytic aggregation that always occurs to some extent, even when cells are cultured in the absence of PHA. Possibly the transformation reflects a response by the small lymphocytes to antigen or antigens in the culture media. I tried to eliminate certain obvious antigens, such as foreign proteins and penicillin, but cell-bound antigens or antigens resulting from cell degeneration conceivably may have elicited such response.

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Kinetin-Induced Chloroplast Maturation in Cultures of Tobacco Tissue

Abstract. *Cultured tobacco tissue possesses proplastids capable of differentiating into mature chloroplasts. Kinetin (6-furfurylaminopurine) is a specific requirement for this differentiation; the absence of this compound results in a blockage of the formation of grana. The possibility that kinetin exerts a direct effect upon chloroplast differentiation is considered.*

We describe here the effect of the plant growth regulator, kinetin (6-furfurylaminopurine), on chloroplast maturation and chlorophyll synthesis in cultured tobacco tissue. The function and fine structure of developing chloroplasts in this system has already been reported (*1*). Kinetin has been implicated in many aspects of plant metabolism, and it is required for the growth of many cultured tissues (*2*).

The tissue used in these experiments was originally isolated from pith of *Nicotiana tabacum* L. var. Maryland Mammoth, and it had been subcultured for a period of 2 years. This strain of tobacco tissue will grow in the absence of kinetin, but complete chloroplast development occurs only in the presence of this compound. A modified Murashige and Skoog medium (*3*), which was used as the basic culture medium, contains the auxin, naphthalene acetic acid, at a concentration of 0.5 mg/liter. Explants from tissue grown without light (dark-grown) were grown in continuous light (light-grown) on media with or without kinetin. Light was supplied by a bank of warm-white fluorescent tubes providing an intensity of 500 ft-c (45 lu/m^2) at the level of the cultures. Both light- and dark-grown tissues were cultured at 25°C. Tissue samples were taken from both light- and dark-grown cultures at either 28 or 45 days for chlorophyll determinations (*4*) and for fine-structure examination by electron microscopy. The tissue was fixed in 1.5 percent $KMnO_4$, dehydrated in a graded water-acetone series, and stained in 1.0 percent uranyl nitrate. The fixed and stained material was embedded in epoxy resin (*5*) and sectioned with a diamond knife. Sections were mounted on uncoated grids and viewed in the Siemens Elmiskop I.

The proplastid illustrated in Fig. 1*A* is typical of those found in tissue grown in the dark for 28 days on a medium containing kinetin (0.5 mg/liter). Lamellar formation appears to occur by vesiculation and elaboration of the inner proplastid membrane. Lamellar sheets, as seen by serial sections, are formed in the proplastids, but there is no fusion of lamellae to form grana. Prolamellar bodies have not been observed in the proplastids of dark-grown cultured tissue. Cultured tissue growing in the light in

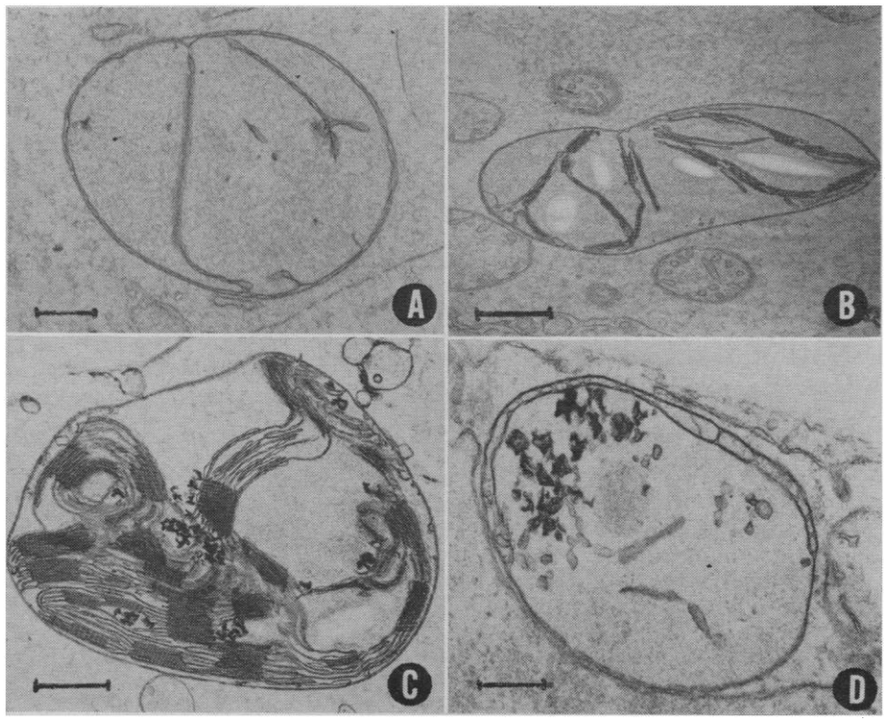

Fig. 1. Electron micrographs of plastids from cultured tobacco tissue. Tissues were fixed in $KMnO_4$ and then stained with uranyl nitrate. (*A*) Proplastid from dark-grown tissue. Scale, 0.1 μ. (*B*) Chloroplast from tissue cultured in the light for 28 days in the presence of kinetin. Extensive grana development is lacking and starch grains are abundant. Scale, 1.0 μ. (*C*) Chloroplast from tissue cultured in the light for 45 days in the presence of kinetin. Scale, 1.0 μ. (*D*) Plastid from tissue cultured in the light for 28 days in the absence of kinetin. Scale, 1.0 μ.

the presence of kinetin (0.5 mg/liter) possessed partially differentiated chloroplasts (Fig. 1*B*). A typical value for the chlorophyll content of tissue with chloroplasts at this stage of development is 2.7×10^{-4} mg of chlorophyll per milligram of dried tissue. The chlorophyll content of such tissue grown on media containing less than 0.5 kg of kinetin per liter falls sharply and approaches zero between 0.05 and 0.01 mg per liter. The type of chloroplast illustrated in Fig. 1*B* has abundant starch grains and grana composed of three to five closely approximated lamellae. The inner plastid membrane appears to be involved in lamellar formation in a way similar to that observed in the proplastids of dark-grown tissue. We have observed prolamellar bodies in developing chloroplasts of young tobacco leaves, but such structures have never been observed in partially differentiated chloroplasts from light-grown, cultured tissue. In contrast to these partially differentiated chloroplasts is the fully matured chloroplast shown in Fig. 1*C*. This chloroplast is from 45-day-old, light-grown, cultured tissue past the log phase of growth. The average chlorophyll content of such tissue is 1.5×10^{-3} mg of chlorophyll per milligram of dried tissue, which is close to the value for mature tobacco leaves (6.3×10^{-3} mg of chlorophyll per milligram of dried tissue). In addition, the chloroplast shown in Fig. 1*C* is similar in size and structure to chloroplasts from mature tobacco leaves and demonstrates that complete development of this organelle occurs in cultured tissue. The plastid shown in Fig. 1*D* manifests the effect produced by the absence of kinetin in the medium and is from light-grown tissue of the same age as tissue containing chloroplasts shown in Fig. 1*B*. The size of the undifferentiated plastid is similar to a normal chloroplast, and lamellar formation is a result of vesiculation of the inner limiting membrane. The orderly differentiation of lamellae into grana is blocked, and aggregations of densely stained vesicles appear instead. The structure of this organelle resembles that of the proplastids of albino mutants of barley after exposure to the light (*6*).

The necessity of kinetin for chloroplast maturation in this cultured tissue is, as far as we know, the first clear correlation between a known plant growth regulator and the differentiation of a specific organelle. We have also found that another cytokinin, 6-benzylaminopurine, stimulates chlorophyll synthesis at lower concentrations than kinetin does. With the use of other growth regulators such as auxin and gibberellin we have not been able to duplicate the kinetin effect on chloroplast development. In fact, gibberellic acid inhibits chloroplast maturation in tobacco tissue even though it stimulates tissue growth. The stimulation of growth is probably the explanation for its failure to induce chloroplast maturation, since chloroplast maturation and growth rate of this cultured tissue are inversely related (*1*). The kinetin requirement for chloroplast maturation is not directly related to its requirement for tissue growth, since the tissue grows well at concentrations of kinetin too low for chlorophyll synthesis. Conversely, the optimum kinetin concentration for chlorophyll synthesis (1.0 mg/liter) inhibits growth. Kinetin is an effective inhibitor of chlorophyll degradation in senescing plant tissues (*7*), and it increases chlorophyll levels in tobacco leaves (*8*). The implication of kinetin in both protein and nucleic acid metabolism (*9*), coupled with the demonstration of nucleic acids in chloroplasts (*10*) and the apparent ability of isolated tobacco chloroplasts to synthesize proteins (*11*), suggests the possibility that kinetin exerts a direct effect upon chloroplast differentiation.

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