division, followed in time by cytodifferentiation of tubular gland cells, a process that culminates in the appearance of a specific cell product, lysozyme. To determine at which point in time progesterone antagonizes the action of estrogen, administrations of progesterone were begun from 1 to 4 days after the onset of estrogen administration. After 5 days of total treatment, the magnum portion of the oviduct was examined for total content and concentration of DNA, RNA, protein, and lysozyme. Even a 1-day delay in the onset of progesterone treatment resulted in a significant ($P = .01$) increase in the content of DNA by day 5 (compare E1 EP4 with EP5) (Table 1). As the onset of administration of progesterone was delayed for longer periods, the increase in the content of total DNA, as well as in the contents of RNA and protein, was proportionately greater. In addition, the amount of lysozyme also correlated well with the length of the delay in starting the administration of progesterone (Table 1). Thus, even a 1-day delay allowed for the appearance of a detectable amount of lysozyme. A correlation between cell proliferation and subsequent functional differentiation was also evidenced by histological examination of oviduct from chicks treated with estrogen for 2 days and then with combined treatment for 3 days. Nests of normally appearing tubular gland cells with cytoplasmic granules were present among the stromal cells (Fig. 2d). This histological picture, then, is a combination of those patterns characteristic of the magnum after 5 days of either estrogen or combined treatment (Fig. 2, b and c).

The exact site of action of progesterone in antagonizing estrogen-induced cell proliferation and cytodifferentiation of tubular gland cells is unknown. Our results suggest that estrogen-induced proliferation of potential tubular gland cells commences within 24 hours, but that subsequent cytodifferentiation of such cells culminates in the appearance of lysozyme only after 72 hours (Fig. 1). Progesterone prevents occurrence of these processes when given continuously with estrogen, but does not prevent limited cell proliferation, lysozyme synthesis, and formation of tubular gland cells when administrations are begun within 24 hours after the initial estrogen. We therefore suggest as a tentative hypothesis that progesterone acts by inhibiting initial proliferation of potential tubular gland cells, but once this has occurred, cytodifferentiation and lysozyme synthesis are not inhibited.

The progesterone antagonism in this system is relatively tissue-specific, since the estrogen-induced increases in concentrations of calcium, lipid, and phosphoprotein in blood are not affected (*6, 7, 16*). Furthermore, the antagonism appears to be noncompetitive, since the inhibitory effect of progesterone cannot be reversed by increasing the dose of estrogen (*6, 16*). One of the early effects of estrogen in rat uterus is an increased uptake of amino acids and water (*17, 18*). We demonstrated this effect in chick oviduct and found that it is not inhibited by the concomitant administration of progesterone (*16*).

We anticipate that the phenomenon of progesterone inhibition of estrogen-induced tubular gland proliferation may be useful in understanding hormonal regulation of the development and function of this specific cell type.

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19. Supported by research grant P427 from the American Cancer Society.

9 September 1968

Haploid Plants from Pollen Grains

Abstract. *A method is presented by which hundreds of haploid plants of various species of* Nicotiana *can be raised from pollen grains. Stamens should be excised when pollen grains have been individualized, but are still uninucleate and free of starch. When grown in vitro on a relatively simple medium, some pollen grains proliferate into embryo-like structures which develop in stages similar to those of zygotic embryos. The plantlets mature and flower profusely, but do not set seed.*

Haploid organisms are desirable for at least two reasons: mutations induced in them are readily visible, and doubling their chromosomes, with colchicine for example, leads directly to homozygous individuals. This method for obtaining haploid plants in large numbers is based on the stimulation of cell division in immature pollen grains which leads to the production of a plant from the male prothallus alone (androgenesis).

Three to four weeks after stamens of the proper developmental stage have been planted on a suitable medium, embryos and plantlets can be seen emerging from some anthers (Fig. 1). These plantlets can be transplanted to individual tubes on a simplified medium. Once they have formed an adequate root system, they may be transplanted to pots and raised to mature plants which flower profusely. Chromosome counts made on preparations from root tips excised either from plantlets in test tubes or from adult plants have shown the plants to be haploid. A visible sign of the haploid condition was the fact that the flowers did not set seed. In general, haploid plants and flowers were smaller than diploids by about one-third. Large numbers of haploid plants have been raised in this manner from the following species: *Nicotiana tabacum* var. "Wisconsin 38," var. "Red flowered, large leaved" (a mutant originated at Debreczen, Hungary), var. "Corolle double X" (a mutant originated at Bergerac, France), var. "Coulo" (a white-flowered mutant), var. "Maryland Mammoth," and var. "White Burley," *N. sylvestris, N. affinis* (a white-flowered variety), and *N. rustica* (a white-seeded mutant of var. "Zlag").

Procedure is as follows: (i) Select flower buds at stage 2 in the case of *N. tabacum* and stage 3 with *N. sylvestris* (Fig. 2). (ii) Dip the cut end of the pedicel into molten paraffin, then disinfect the whole bud by dipping into 70 percent ethanol and immersing

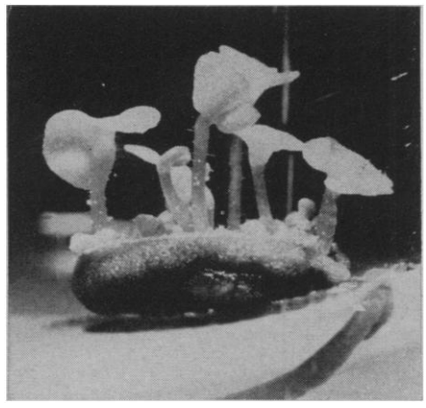

Fig. 1. Plantlets emerging from an anther of *Nicotiana tabacum* var. "Corolle double X" after 1 month of culture in vitro (× 5).

for about 3 minutes in a filtered suspension of calcium hypochlorite (7 percent). (iii) Remove the stamens aseptically and plant them on the nutrient medium (*1*). (iv) Grow the cultures at 28°C (day) and 22°C (night) under fluorescent tubes supplemented with incandescent light giving about 5500 lu/m^2 outside the culture tubes. After 4 to 6 weeks, the plantlets may be transfered onto "Medium T" which is devoid of indole-3-acetic acid (IAA) and contains only 1 percent sucrose. (v) After the plantlets have developed a sufficient root system, transplant them with the rest of the agar medium into a mixture of peat and vermiculite (1 : 1 by volume) and water them with a nutrient solution such as Hoagland's. For about 1 week after the transfer to pots, the plants should be covered with a polyethylene bag to prevent desiccation. The haploid plants originate from haploid cells, namely, the pollen grains. Certain pollen grains increase in size and form a round mass of cells. The integuments of the pollen grain break and liberate what may represent the globular stage of normal embryogenesis. Indeed the following steps copy exactly those which normal embryos undergo, namely, the "heart," "torpedo," and "cotyledonary" stages (Fig. 3). Later the embryos germinate, that is, they start to respond to geotropic stimuli, their cotyledons turn green, and their radicles develop.

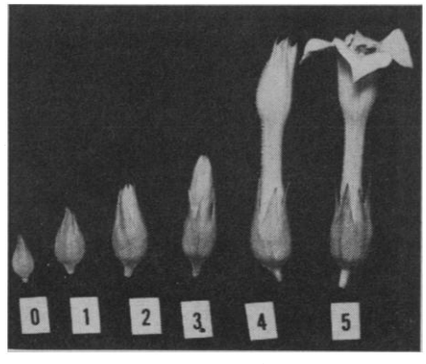

Fig. 2. Stages of flower development in *Nicotiana tabacum* var. "Wisconsin 38."

Some abnormalities have been observed in the number and shape of cotyledons, including the fusion of the cotyledons in a peltate structure.

Initially (*2*), a small percentage of stamens produced embryos and plantlets. This was due in part to inadequacy of the medium, but mostly to selecting an improper stage of pollen development at planting. Neither anthers excised at the tetrad stage nor anthers excised shortly before pollen was shed gave rise to any plantlets. Mature pollen grains, sown aseptically in vitro, produced normal pollen tubes, but no plantlets. Formation of plantlets occurred when pollen grains were fully individualized, uninucleate, and devoid of starch. This corresponds roughly to stage 2 of *N. tabacum*, characterized by the tip of the petals reaching the tip of the sepals (Fig. 2). The percentage of success obtained with flower buds at various stages is shown in Table 1; stamens excised at stage 2 produced the greatest number of plantlets. Addition of gibberellic acid (GA) to the medium did not alter the pattern.

Many constituents of the medium have been tested for their ability to promote the formation of haploid plantlets. These include NH_4^+ ions, sucrose, growth regulators, certain amino acids, and nucleic acid constituents.

Although they seem important for the formation of vegetative embryos in the wild carrot (*3*), ammonium ions ($2 \times 10^{-2} M$) only hastened somewhat the production of visible plantlets 4 weeks after the beginning of the culture but, compared to nitrate ions, they did not increase the percentage of stamens producing plantlets.

Without the presence of a sugar in the medium, no plantlets were produced in experiments done with *N. tabacum* var. "Wisconsin 38" and var. "Maryland Mammoth." Good production occurred when the sucrose concentration ranged from 0.5 to 3 percent, the optimum being about 2 percent.

Two auxins, IAA and 2,4-dichlorophenoxyacetic acid (2,4-D), were tried at various concentrations. In general, they enhanced the formation of plantlets, the optimal concentration of IAA being 0.1 mg/liter in the case of *N. tabacum* var. "Wisconsin 38" and *N. sylvestris*. Relatively high concentrations of auxin caused malformations, especially in the cotyledons.

Table 1. Effect of the stage of flower bud development upon the production of haploid plantlets. Stamens of *Nicotiana tabacum* var. "White Burley" planted on medium H plus kinetin (0.2 mg/liter), indoleacetic acid (0.1 mg/liter) and 2 percent sucrose. In each case, 48 stamens were planted.

Floral stage	Stamens producing plantlets (%)	
	After 3 weeks	After 4 weeks
1	0	21
2	13	45
3	2	2
4	0	0
1*	0	20
2*	23	45
3*	0	0
4*	0	0

* Gibberellic acid (1 mg/liter sterilized by filtration) added in this series.

Kinetin was also tried, alone and in combination with IAA. The medium initially used (*2*) contained 0.2 mg/liter of kinetin, but it was found that kinetin at this concentration generally reduced the percentage of stamens producing plantlets. This depression was not relieved by the addition of adenine (40 mg/liter).

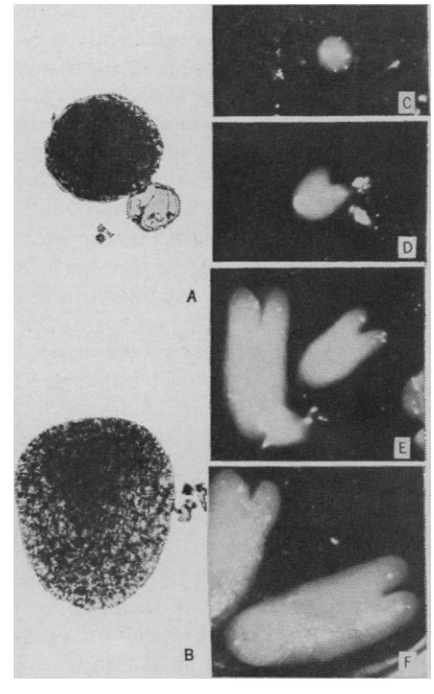

Fig. 3. Stages of embryonic development from the pollen grain. (A) Spherical mass of cells along the empty integuments of the pollen grain (× 198); (B) establishment of polarity (× 198); (C) globular stage (× 66); (D) "heart stage" (× 66); (E) early "torpedo" stage (× 66); and (F) "torpedo" stage (× 66).

At 1 mg/liter GA slightly enhanced the speed with which plantlets appeared, but did not improve the final percentage of stamens producing embryos. It did not allow younger stages (such as stage 1) to produce more embryos (Table 1). At 1 mg/liter, GA caused an abnormal elongation of the hypocotyl and production of spindly, chlorotic plantlets.

Abscisic acid (ABA) from 10^{-7} to $10^{-5}M$ did not reduce the percentage of stamens producing plantlets, but it delayed their development markedly. In the presence of $10^{-6}M$ or more of ABA, the embryos formed were shorter and thicker than the controls and remained in an ungerminated condition for at least 2 months (see above the three criteria for germination) by which time the controls had developed into plantlets with green leaves, reaching 5 cm in height.

In some experiments, addition of L-glutamine or of L-asparagine at 1 to 3×10^{-3} M stimulated the production of plantlets from excised stamens. L-arginine, at the same concentrations, was completely inhibitory. The purine and pyrimidine constituents of nucleic acids gave variable results, adenine (at 1 to $3 \times 10^{-4}M$) being generally inhibitory. Addition of all the bases together ($10^{-4}M$ of each) did not improve the percentage of stamens producing plantlets.

The fact that the male prothallus can proliferate and form embryos explains certain abnormalities reported in the literature. Thus haploid plants which had only the characters of the male parent have been obtained in crossing *N. digluta* by *N. tabacum* (*4*) or *N. tabacum macrophylla* by *N. langsdorffii* (*5*). In these cases, androgenesis probably occurred in the embryo sac. This natural tendency may be increased to such a degree by the present method that it is a practical way to produce haploid tobacco plants at will (*6*).

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References and Notes

1. Medium H, consisting of: (i) Mineral salts (mg/liter)—KNO_3, (950), NH_4NO_3, (720); $MgSO_4 \cdot 7\ H_2O$ (185); $CaCl_2$ (166); KH_2PO_4 (68); $MnSO_4 \cdot 4\ H_2O$ (25); H_3BO_3 (10); $ZnSO_4 \cdot 7\ H_2O$ (10); $Na_2MoO_4 \cdot 2\ H_2O$ (0.25); $CuSO_4 \cdot 5\ H_2O$ (0.025). In addition, 5 ml/liter of a solution of 7.45 g of disodium ethylenediaminetetraacetate and .557 g of $FeSO_4 \cdot 7\ H_2O$ in 1 liter of distilled water was added. (ii) Organic addenda (mg/liter)—*myo*-inositol (100); glycine (2); nicotinic acid (5); pyridoxine HCl (0.5); thiamine HCl (0.5); folic acid (0.5); and biotin (0.05). (iii) Sucrose (20 g/liter). (iv) Difco Bacto-agar (8 g/liter). Indole-3-acetic acid (IAA) at 0.1 mg/liter is beneficial but not indispensable. The *p*H is adjusted to 5.5 with HCl or NaOH before the agar is added. The medium was sterilized in an autoclave for 15 minutes at 20 pounds per square inch.
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6. While this paper was being processed for publication, it came to our attention that K. Nakata and M. Tanaka [*Jap. J. Genet.* **43**, 65 (1968)] had also obtained embryos from cultured anthers of *N. tabacum*. Their highest reported yield of 6 percent falls short of the yield of embryo-producing anthers (45 percent) reported here.
7. The authors are indebted to the Station de Recherches pour l'Amélioration des Planets de Grande Culture, Gembloux, Belgium, and to the Institut Expérimental du Tabac, Bergerac, France, for seeds. We also thank M. Mousseron for a sample of *cis*-abscisic acid. Photographs taken by B. Norreel; the microscopic preparations of Fig. 3 made by S. Hamon.

20 September 1968

Algae: Amounts of DNA and Organic Carbon in Single Cells

Abstract. *An analysis of ten different unicellular algae, varying in size and containing from 10 to 6000 picograms of carbon per cell, indicates that the amount of DNA per cell is in direct proportion to cell size. The content of DNA is equal to approximately 1 to 3 percent of the cellular organic carbon.*

There has been speculation on the evolutionary significance of the amount of DNA in diverse plant and animal cells (*1–3*). Most data on DNA as a function of cell size deal with bacteria or with vertebrates or higher plants. Commoner (*4*) has postulated that DNA plays important physiological roles in addition to its role as the template for genetic information, and that DNA content of a cell should be proportional to cell size. To test the hypothesis that DNA is directly proportional to cell size, it is essential to use cells which are closely related phylogenetically and which are similar in physiology and nutrition. These criteria are met by eukaryotic, unicellular algae that are growing photoautotrophically. The smallest cells used in this investigation (*Monochrysis lutheri* and *Navicula pelliculosa*) contained approximately 10 pg of organic carbon and 0.1 pg of DNA per cell, and the largest cells (*Gonyaulax polyedra*) contained 6000 pg of carbon and 200 pg of DNA per cell. The DNA content per cell therefore is nearly directly proportional to cell size as determined by total organic carbon content. Because these cells could be expected to require about the same amount of DNA-template information, the large variations in DNA per cell (up to 2000 times) indicate that DNA does much more than merely convey genetic information in the cell.

The ten species of unicellular algae used were unialgal, bacteria-free cultures grown as described (*5, 6*). Samples of the algal suspensions were filtered through HA (0.45-μ pore size) Millipore filters, and the cellular contents of DNA were determined by a fluorometric measurement with 3,5-diaminobenzoic acid dihydrochloride. This procedure is based on that of Kissane and Robbins (*7*), with modifications for laboratory cultures of phytoplankton (*8*). Samples of algal suspensions were also filtered through glass fiber filters and the total cellular organic carbon was determined by measurement of CO_2 by infrared gas analysis after complete combustion of the sample by wet oxidation (*9*). Cell counts were determined with a Coulter model A particle counter.

There is nearly a direct proportionality between cell size and content of DNA in these species (Fig. 1). These values represent total cellular DNA and thus include nuclear DNA as well as any extranuclear DNA. It is unlikely, however, that the observed correlation between DNA content and cell size can be attributed solely to extranuclear DNA. The amount of DNA in mitochondria and chloroplasts generally accounts for only a few percent of the total cellular DNA. In *Euglena gracilis*, for example, the DNA of the mitochondria and the plastids together amounts to less than 5 percent of the nuclear DNA, as judged by microdensitometer readings of ultraviolet-absorption photographs of DNA separated on cesium chloride density gradients (*10, 11*). Studies with chloroplast-containing flagellates and with their colorless counterparts also show that the amount of DNA contained in the chloroplasts is minor compared to the amount in the nucleus (*10*). Therefore the correlation between cell size and DNA (Fig. 1) is caused predominately by varying amounts of nuclear DNA. Microscopic examination of