Table 1. Effects of neonatal testosterone propionate (TP) treatment, blinding, and the pineal gland on the body weights and the size of the testes and of accessory organs (seminal vesicles and coagulating glands) in male albino rats.

Animals (No.)	Body weight (g)*	Testicular weight (mg)	Accessory organ weight (mg)
	Group 1: Untreated		
8	318 ± 11	3110 ± 69	389 ± 31
	Group 2: Blinded, sham-pinealectomized		
8	254 ± 8†	2508 ± 94†	238 ± 27†
	Group 3: Blinded, pinealectomized		
8	310 ± 10	3060 ± 103	368 ± 34
	Group 4: TP-treated, sham pinealectomized		
10	301 ± 14	2589 ± 78†	291 ± 19†
	Group 5: TP-treated, pinealectomized		
9	292 ± 9	2480 ± 96†	278 ± 26†
	Group 6: TP-treated, blinded, sham pinealectomized		
9	217 ± 11†	906 ± 161‡	44 ± 12‡
	Group 7: TP-treated, blinded, pinealectomized		
8	246 ± 5†	2155 ± 79†	255 ± 23†

*Mean body and organ weights ± standard errors. †Significantly different ($P<.05$) from similar weights of untreated controls (group 1). ‡Significantly different ($P<.001$) from similar weights of untreated controls (group 1).

blinded, TP-treated animals) were, for the most part, normal in appearance. The small testes of blinded, androgen-treated rats with intact pineals exhibited a marked diminution in the number of spermatids.

The hypothalamic sites at which androgen acts to modify the cyclic release of luteinizing hormone appear to be the anterior and preoptic areas (*6*). Our results show that TP-treatment or blinding alone does not greatly retard gonadal development, although the testes are smaller than normal under both conditions. However, when the treatments are combined, the overall effect is an apparent restriction of folliculotropin synthesis or release (*4*). Possibly, the onset of gonadotropin synthesis or release is delayed or decreased, and, if animals had been maintained for longer periods of time, there would have been normal (or at least increased) spermatogenic activity and gonadal size. The specific site at which the pineal principles act is still undefined (*7*).

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References and Notes

1. J. C. Hoffmann, *Neuroendocrinology* **2**, 1 (1967).
2. R. J. Reiter, "The pineal gland: A report of some recent physiological studies," *Edgewood Arsenal Technical Report 4110* (Edgewood Arsenal, Maryland, 1967).
3. A. Moszkowska, *Progr. Brain Res.* **10**, 564 (1965); R. J. Reiter and R. J. Hester, *Endocrinology* **79**, 1168 (1966).
4. C. Kordon and J. C. Hoffmann, *Compt. Rend. Soc. Biol.* (Paris), **161**, 1262 (1968); J. C. Hoffmann, C. Kordon, J. Benoit, *Gen. Comp. Endocrinol.* **10**, 109 (1968).
5. R. A. Hoffman, and R. J. Reiter, *Science* **148**, 1609 (1965); R. J. Reiter, R. A. Hoffman, R. J. Hester, "The role of the pineal gland and of environmental lighting in the regulation of the endocrine and reproductive systems of rodents," *Edgewood Arsenal Technical Report 4032* (Edgewood Arsenal, Maryland, 1966); R. J. Reiter, *Neuroendocrinology* **2**, 138 (1967).
6. G. W. Harris, *Endocrinology* **75**, 627 (1964); R. A. Gorski, *J. Reprod. Fertil.* (Suppl.) **1**, 67 (1966); F. Neuman and W. Elger, *Endokrinologie* **50**, 209 (1966).
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Starch Accumulation in Shoot-Forming Tobacco Callus Cultures

Abstract. *Microscopic histochemical examinations of cultured tobacco callus disclosed a strong correlation between starch accumulation and shoot initiation. The accumulation started before any observable organized development and was heaviest in cells of loci which ultimately gave rise to organ primordia. Treatment of tissue cultures with gibberellin prevented starch accumulation and organ formation.*

Cultures of plant callus are useful in the exploration of factors regulating the initiation of plant organs (*1*). In contrast to the more conventional methods, in which stem cuttings are used, callus cultures offer better control of nutritional and environmental factors and permit one to distinguish between initiation of organs and their outgrowth from preexisting primordia. It has been shown with callus cultures that the basic regulatory mechanism underlying plant organ initiation involves a balance between auxin and cytokinin (*2*). Unfortunately, this mechanism cannot be demonstrated among a wide range of plant species, and attempts to induce organ initiation through treatments with auxin, cytokinin, and other morphogenic substances have been generally unsuccessful. Possibly, the elucidation of the regulatory mechanism can be more effectively accomplished if information concerning the internal physiology of the cells which participate in organogenesis becomes available.

Treatment of tobacco callus cultures with various amounts of indoleacetic acid and kinetin apparently results in correlated changes in the composition of scopoletin and its glycoside, scopolin in the tissue and in the nutrient medium (*3*). The significance of this finding remains unknown. Lee (*4*) suggested that the action of phenolic compounds, including L-tyrosine, in stimulating or inhibiting shoot initiation in tobacco callus culture lay in their ability to regulate the activity of indoleacetic acid oxidase and thus the effective concentration of indoleacetic acid in the tissue. Lee further suggested that, since tyrosine is also a precursor of lignin, some lignification of cells may be a prerequisite of organ initiation; thus, he supported the concept of Steward *et al.* (*5*). However, Dougall (*6*) showed that tyrosine was more rapidly incorporated into protein than into lignin.

We conducted a microscopic histochemical study on freeze-substituted samples of cultured tobacco (*Nicotiana tabacum* L., var. Wisconsin 38) callus. The procedure of tissue culture was a modification of one previously described (*7*). The cultures were maintained in constant darkness in one of two nutrient media, one containing 2 mg each of indoleacetic acid and kinetin per liter, and another further supplemented with these substances (in milligrams per liter): L-tyrosine, 100; adenine sulfate, 160; and NaH_2PO_4, 340. The first medium caused the development of only unorganized callus, whereas the latter promoted the initiation of large numbers of shoots. The cultures were sampled frequently during development and compared histologically and histochemically. The metabolism with respect to carbohydrates, nucleic acids, and proteins was investigated. The most dramatic difference was that of starch accumulation. The periodic acid-Schiff, aniline blue-black staining procedure was used; the presence of starch was confirmed by the use of the IKI and zinc-chlor-iodide reagents (*8*).

The callus initially showed little or no stored starch and no discernible cell division. The cells were highly vacuolated and contained inconspicuous

nuclei and cytoplasm. Vascular elements were also evident in the tissue in the form of highly lignified xylem. After 8 days, the tissue which was destined to produce shoots had an abundance of starch granules of various sizes. In contrast, the tissue not forming organs had accumulated considerably less starch. This was randomly distributed, whereas starch granules in the organ-forming tissue appeared more densely accumulated in specific loci, from which organ primordia ultimately arose. By the 13th day cells of tissues not forming organs were still highly vacuolated, parenchymatous, and conspicuously lacking in starch granules (Fig. 1). Nodule-like nests of cells, or Torrey's meristemoids (*9*), could be seen in organ-initiating cultures (Figs. 2 and 3). These cells could be distinguished from the ordinary parenchyma callus cells because they were smaller, isodiametric, and thinner walled; furthermore, they lacked apparent vacuoles, had densely staining cytoplasm and prominent nuclei, and were densely filled with starch grains. On further culture the meristemoids developed into well-defined shoot primordia and eventually gave rise to readily discernible shoots. Since the cultures were grown in complete darkness, the shoots remained etiolated. As the primordia emerged as shoots, stored starch decreased in cells of the no longer meristematic regions of the newly formed organs; cells of the meristematic regions, for example, initial cells of leaf and stem in the shoot apex continued to store starch, although somewhat less.

We suggest that the accumulation of starch functions in the initiation of organized structures in plants. We do not think that it is a coincidental product of the process of organ initiation. First, the accumulation preceded any observable organized development and, in the organ-forming callus, the development of the meristemoids was confined to regions containing heavy deposits of starch. Second, this conclusion was supported by a supplementary experiment with gibberellin. Addition of gibberellin to the culture medium represses organ formation in tobacco callus (*10*). Moreover, gibberellin induces the *de novo* synthesis of α-amylase in barley endosperm (*11*), and thereby causes the disappearance of starch. We followed histochemically the accumulation of starch granules in tobacco callus treated with gibberellin (GA_3). The culture conditions were the same as before, but the organ-forming medium was supplemented with $10^{-6}M$ GA_3. Shoot initiation was absent in treated tissues, and starch accumulation was markedly decreased. Furthermore, the starch granules were smaller in size and randomly distributed in the tissue (Fig. 4). Third, in other organogenetic processes, starch accumulation has been a conspicuous feature, for example, the initiation of cotton embryos and the induction of floral parts in cauliflower (*12*).

The physiological significance of starch accumulation in organ initiation requires elucidation. Perhaps it reflects the high energy requirement of the organogenetic process. As a source of energy, starch possesses a distinct advantage. Its degradation results in high yields of glucose-1-phosphate, the subsequent catabolism of which through glycolysis produces substrate adenosine triphosphate, without the expenditure of existing high-energy phosphates.

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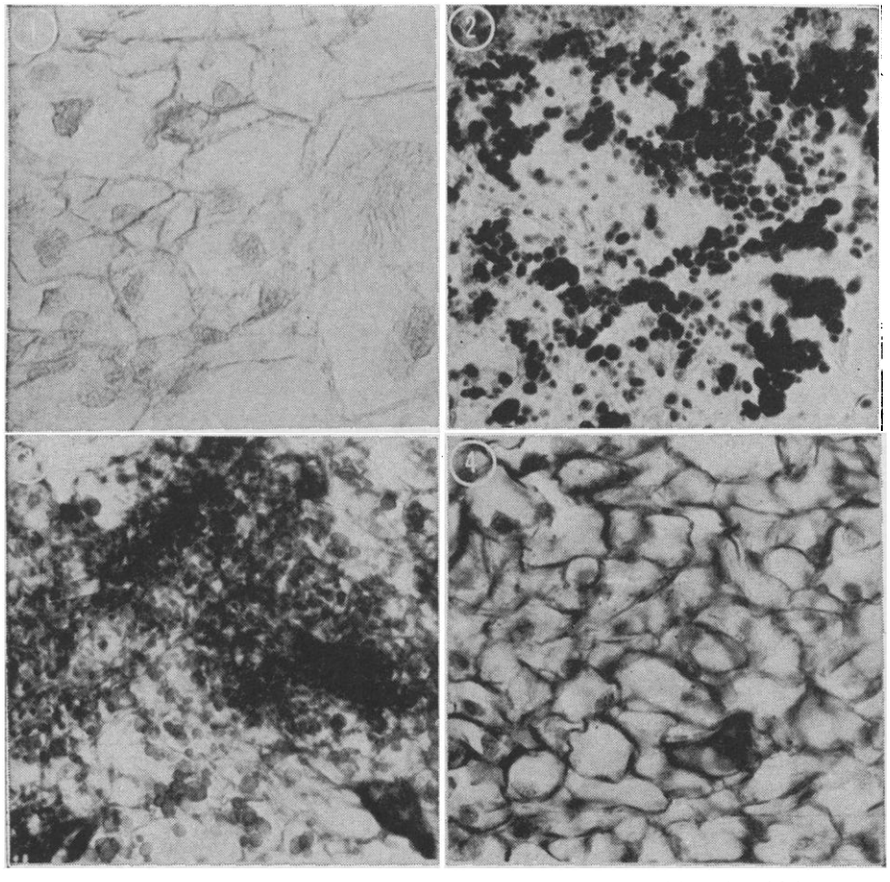

Fig. 1. Section through tobacco callus cultured 13 days under conditions in which organs are not formed. Note absence of starch. Fig. 2. Section through tobacco callus cultured 13 days under shoot-forming conditions. Note heavy deposits of starch granules. Fig. 3. A more advanced stage of shoot initiation showing accumulation of starch granules in association with meristemoid cells. Fig. 4. Section through 16-day-cultured tobacco callus showing reductions in starch accumulation and shoot initiation, resulting from addition of gibberellin to nutrient agar. All figures × 235; IKI stain in Figs. 1 and 2; periodic acid-Schiff, aniline blue-black stain in Figs. 3 and 4.

References and Notes

1. F. Skoog, *Amer. J. Bot.* **13**, 19 (1944).
2. ——— and C. O. Miller, *Symp. Soc. Exp. Biol.* **11**, 118 (1957).
3. J. A. Sargent and F. Skoog, *Plant Physiol.* **35**, 934 (1960); F. Skoog and E. Montaldi, *Proc. Nat. Acad. Sci. U.S.* **47**, 36 (1961).
4. T. T. Lee, thesis, University of Wisconsin (1962).
5. F. C. Steward, M. O. Mapes, K. Mears, *Amer. J. Bot.* **45**, 705 (1958).
6. D. K. Dougall, *Australian J. Biol. Sci.* **15**, 619 (1962).
7. T. Murashige and F. Skoog, *Physiol. Plant* **15**, 473 (1962); T. Murashige and R. T. Nakano, in preparation.
8. W. A. Jensen and D. B. Fisher, *Planta* **78**, 158 (1968); W. A. Jensen, *Botanical Histochemistry* (Freeman, San Francisco, 1962), pp. 198–202.
9. J. G. Torrey, *Advance Morphogenesis* **5**, 39 (1966).
10. T. Murashige, *Science* **134**, 280 (1961); *Physiol. Plantarum* **17**, 636 (1964).
11. J. E. Varner and G. Ram Chandra, *Proc. Nat. Acad. Sci. U.S.* **52**, 100 (1964).
12. W. A. Jensen, *Brookhaven Symp. Biol.* **16**, 179 (1963); S. Sadik and J. L. Ozbun, *Can. J. Bot.* **45**, 955 (1967).
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