*ACTIVATION OF METABOLIC SYSTEMS DURING TUMOR-CELL FORMATION**

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Braun[1-4] has clearly demonstrated that the transformation of normal plant cells into crown-gall tumor cells includes the progressive activation of enzyme systems which, once in operation, become a permanent part of the metabolic machinery of the altered cells. The work in our laboratory has paralleled that in Dr. Braun's with surprisingly similar results. We have, however, utilized a different technique, permitting us, in some particulars, to garner information not readily obtainable with his system and to determine some of the interrelations between the etiological agents acting on the cells and the activation of specific metabolic systems.

A quantitative bioassay has been developed for these studies which utilizes the cambial-adjacent phloem tissues of carrot roots.[5] Tumor tissues formed in the surface of these disks can be weighed, giving a direct measure of the rates of duplication of tumor cells. We have delimited with some precision the temporal relations of the processes leading to tumor-cell formation in carrots, as well as the nature of the etiological agents (Fig. 1).

Transformation of carrot phloem cells into tumor cells occurs in three phases, designated "conditioning," "induction," and "promotion." Optimal conditioning takes 16–20 hours and requires the action of several causal agents normally present in wound juice.[2, 6, 7] The bacteria are unnecessary at this time. By pre-assay, carrot roots are obtained which are deficient in one of these agents,[7] and these roots possess only limited tumor-forming capacity. Conditioning may also be limited by restricting the time of action of these agents, by interfering with their action with antimetabolites of the conditioning agents, or by slowing down the process with cold treatment.

Induction takes 60–70 hours for completion and requires the action of a tumor-inducing principle (T-iP) which may be a DNA synthesized by genetically competent crown-gall bacteria in the presence of a T-iP synthesis factor also present in the wound juice.[8] Induction may be limited at will by using strains of crown-gall bacteria synthesizing a less effective T-iP or by holding the conditioned and inoculated disks for given periods of time during the induction phase at elevated temperatures[9] where T-iP is presumably not synthesized by crown-gall bacteria.

Incipient tumor cells formed by the action of conditioning and induction agents are promoted into primary tumor cells by auxins.[10, 11] Promotion requires 28–30 hours in carrot. This phase has been inhibited by presenting an antiauxin to the tissues during the critical time. Tumors are macroscopically evident within 12 hours after the end of transformation, and their rates of duplication are linear for 10–15 days.

For optimal transformation, the amounts, composition, and presentation times of all causal agents must be precisely regulated. Any deviation from the optimum results in the formation of slow-growing tumors. The exogenous stimulation of slow-growing tumors resulting from incomplete conditioning, induction, or promotion by specific growth factors may be taken as presumptive evidence that the

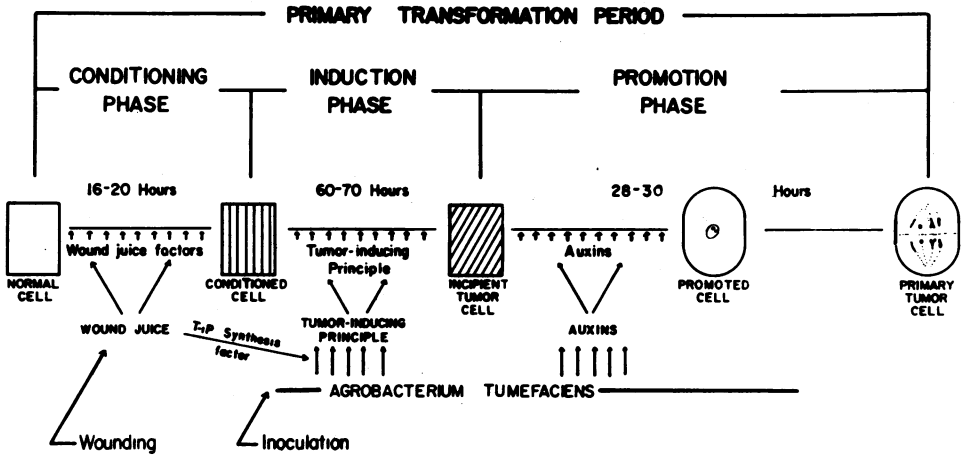

FIG. 1.—Diagrammatic representation of the transformation of normal plant cells into primary crown-gall tumor cells.

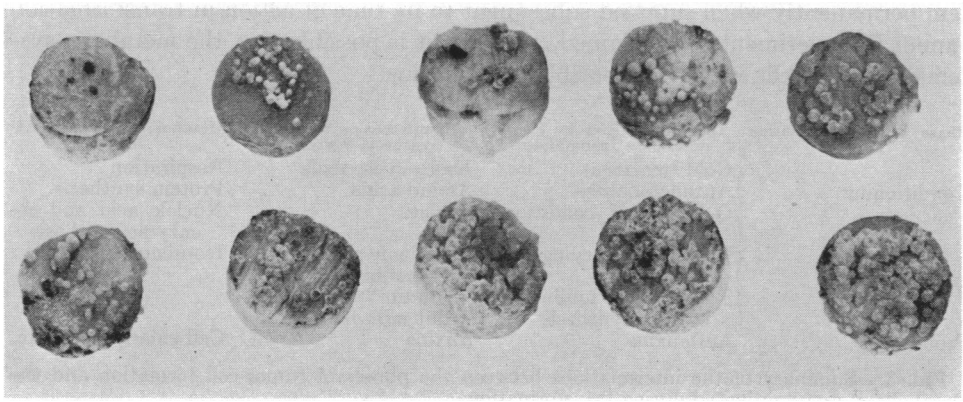

FIG. 2.—Distribution of tumor sizes following endogenous limitation of wound-juice factors required for conditioning.

metabolic system synthesizing that factor was not fully activated by the causal agents operative during the modified step. Thus analyses of the subcellular consequences of transformation are feasible.

The procedure used was as follows. Disks of cambial-adjacent carrot phloem were exposed to the agents acting in the three steps of transformation, and one of these steps was modified. At the end of the transformation period, the disks were transferred to Petri dishes containing water agar plus the test compound, and, after a period of growth, the tumors were weighed. Figure 2 shows a series of tumors resulting from endogenous limitations of one of the conditioning agents. There is a complete spectrum of tumorous growth capacities ranging from virtually no duplication to relatively massive and confluent tumorous development. Similar pictures could be presented following incomplete induction or promotion.

The significant findings to date are presented in Figure 3. We have found that, following incomplete transformation, three biochemical categories of slow-growing

tumors are produced, each characterized by its own set of nutritional deficiencies.[12] Slow-growing or weak tumors, formed following incomplete conditioning, can be stimulated only by purine ribosides, several amino acids, and one or more of the intermediates of the Krebs cycle. Weak tumors formed following incomplete induction require mevalonic acid, gibberellic acid, kinetin, and coconut milk for maximum growth. Preliminary data indicate that the kinetin response may be due to its mimicking a natural cell-division factor found in coconut milk and in crown-gall tumor tissues. This cell-division factor is not any of the known fractions of coconut milk. The slow-growing tumors resulting from incomplete promotion are stimulated only by indoleacetic acid or its biological equivalent. It is important to note that compounds that stimulate weak tumors of one category are without positive effect on tumors from another etiological group. Finally, extracts of crown-gall tissues contain all the substances which positively affect the growth of slow-growing tumors of any category. The positive effects of the required growth factors are observed only with incompletely activated tumors, and the increased growth is apparent only as long as the required factor is available. The stimulation is temporary, not permanent. The failure of these substances to activate the system permanently when supplied subsequent to its time of action in transformation cannot be experimentally explained, although it is possible that the metabolic systems of tumor cells are not susceptible to activation.

Phase of Transformation Period	Technique Used to Render Phase Incomplete	Incompletely Activated Synthesis Systems	Metabolic Systems Affected
Conditioning	Cold treatment	Krebs cycle acids	Respiration
	Antimetabolites	Amino acids	Protein synthesis
	Quantity of conditioning agents	Purines	Nucleic acid and coenzyme synthesis
Induction	Bacteria of varying virulence	Mevalonic acid Gibberellins	Isoprenoid synthesis
	Temperature inhibition of T-iP synthesis	"Kinetin" and "Coconut milk factor"	
Promotion	Antiauxins	Auxins	Cell enlargement, etc.

FIG. 3.—Summary of the interrelations between the phases of tumor-cell formation and the metabolic systems activated during transformation.

Even within the relatively short time of induction, there is evidence that the activation of different metabolic systems occurs independently and in a given order. Thus mevalonic acid prototrophy is found within 24–48 hours, while kinetin and gibberellic acid independence requires 48–72 hours. Exact correspondence of data between two different plant systems cannot be expected. Pyrimidines and vitamins were not required by slow-growing carrot tumors of any category but were required by *Vinca*,[4] and *Vinca* tumors do not seem to require mevalonic acid or organic acids. Apparently, different metabolic systems are either already developed in healthy cells of various plants or are not involved in the growth of the tumors derived from these cells.

The interrelation between the chemical nature of the etiological agent and that of the growth factor synthesized by the activated metabolic system is quite intriguing. A purine and possibly amino acids are required for conditioning, and we may assume that purine- and amino acid–synthesizing systems are developed. An identical situation holds for auxins in promotion. In crown gall, etiological agents apparently activate systems permanently regulating the synthesis of compounds

chemically related to the agents, a finding best explained by invoking a feedback mechanism.

The stability of the acquired synthetic systems is one of some practical importance in oncology. Since growth of plant tumors is dependent upon the activation of metabolic systems, their selective destruction should stop growth. We have found that the systems activated in carrot during conditioning, particularly those synthesizing amino acids and purines, are temperature-labile,[13] that visible radiation inhibits the gibberellic acid system activated during induction, and that ionizing radiation will suppress the auxin-regulating systems.[14] Further work on this concept may prove useful in the control of animal tumors.

It must be emphasized that the ability of plant tumor cells to synthesize all required growth factors is not the entire "cause" of crown gall. Morphogenic (graft) autonomy of tissues is observed following transformation periods shorter than those required for full activation of all metabolic systems,[15] and there is no evidence that certain respiratory and energy metabolism characteristics are related to growth-factor synthesis.[16]

The acquisition of the ability to synthesize any given growth factor can be treated formally and is of general biological importance.[16] Enzyme synthesis may be completely or partially prevented by a series of factors, not the least being the inherent genetic capacity of the cell. For a cell to be even potentially capable of synthesis, there must be an adequate genetic potential which can be modified only by mutation. For adequate enzymatic potential—and crown gall is a case in point—etiological agents must act on enzyme-forming sites to permit the development of the metabolic system concerned.

Finally, the synthesis of the factor requires precursors, which are made available by other metabolic systems. When all necessary conditions are fulfilled, the cell is prototrophic for the growth factor being studied. There are sufficient data in the literature to suggest that morphogenic alterations in the behavior of many cells—both plant and animal—may be so analyzed. It is for this reason that crown gall may be a useful model for growth and differentiation in cells.

Viewed historically, the crown-gall problem has proceeded in a series of decade-long steps. The first decade was concerned with Smith's struggle to convince the doubters that bacteria could indeed be the cause of plant disease. The second ended with a relatively precise structural analysis of the crown-gall syndrome, and the third found our knowledge of the chemical structure of the tissue on a firm foundation. The fourth decade was the period in which the biology and chemistry of the causal agents began to assume its present form, and the fifth, now ended, has taken the problem to the intracellular level discussed today. Research on crown gall has, of necessity, been on the frontiers of current knowledge in cellular biology and has been both a recipient and a donor of knowledge on this frontier. This situation will, happily, continue.

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STIMULATION OR INHIBITION OF VIRUS OF INFECTED AND INSECT-GALL TISSUES AND SINGLE-CELL CLONES

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Plant tissue culture has provided an important method for clarifying normal and diseased growth. The method has been used in the present studies with tissues from a variety of plant species to study stimulation and inhibition of diseased growth of crown gall, virus, and insect-gall origins and the normal homologous tissues from the same species. Recent success in culturing for unlimited periods clones of tissue established from hand-picked single cells of the diseased and normal tissue masses[1] provided another means for elucidation of similarities and differences among the cells in the tissues.

The early independent works of Kotte, of Robbins, and of White showed the possibility of the in vitro growth of isolated root tips.[2] Their successes indicated the need for the proper balance of inorganic salts, carbohydrates, and growth substances to culture isolated plant tissues successfully. These early studies led to the development of true tissue or cell cultures, respectively, by White, by Gautheret, and by Nobecourt.[2] Certain of these tissues and others from roots, stems, and leaves have been described in this symposium as tools for studying the specialized problems of differences between normal and diseased growths.

In order to understand these diseased conditions better, many other studies have been made with isolated tissues that grew in vitro. These came from crown galls, virus-infected tissue, and insect galls. The results of some of these studies are described below.

Tissues of Crown Gall and Normal Origins.—The tissue for these studies came from cultures established from 5 to 16 years previously. They were transferred aseptically to fresh medium[3] at regular intervals as needed. All experimental cultures were started by transferring good stock tissue to a sterile Petri plate, cutting it with a sterile scalpel into seed pieces 20 mg. in mass and by transferring four good pieces with a sterile wire loop to each experimental culture bottle. Replications were usually six culture bottles for each experimental condition.

With these tissue cultures, it was possible further to clarify the balance of inorganic salts, sugars, alcohols, organic acids, and various nitrogen compounds re-