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*STUDIES ON THE METABOLISM OF PLANT NEOPLASMS, V. AUXIN AS A PROMOTING AGENT IN THE TRANSFORMATION OF NORMAL TO CROWN-GALL TUMOR CELLS**

BY RICHARD M. KLEIN AND GEORGE K. K. LINK

DEPARTMENT OF BOTANY, UNIVERSITY OF CHICAGO

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The cellular alterations involved in the formation of crown-gall tumors in plants have recently aroused renewed interest in associated processes Because this, it is important to distinguish between those processes involved in the change of a normal into a tumor cell and those concerned with the subsequent behavior of such a cell. A conceptual and semantic scheme is proposed to serve as a working hypothesis for planning experiments and for relating experimental findings to the general problem of crown-gall genesis.

The techniques and conclusions of Braun and coworkers,[1–3] as well as work under way in the Chicago laboratory, permit an experimental delimitation of several of the processes involved in tumorization (in the limited sense of change of normal cells into primary tumor cells). Braun and Laskaris[1] discovered that an attenuated strain of *Agrobacterium tumefaciens*, the incitant of crown-gall, was incapable of inducing typical tumors in susceptible plants unless the affected tissues received an external supply of either natural (native) or synthetic auxin. They concluded that it was likely that at least two substances were involved in alteration and stimulation of tumor cells, only one of these possibly being auxin. Even earlier, Link, *et al.*,[4] concluded that auxin could be only one entity in a complex of causal factors, all of which were related to tumor formation, and Link and Eggers[5] conclusively demonstrated hyperauxiny in crown-gall tumors and in the contiguous parts of tumor-bearing tomato plants. The subsequent discovery and partial characterization of the action and nature of a "tumor-inducing principle" by Braun[6, 7] demonstrated that at least one of the non-auxinic substances appeared to be a complex molecule of limited heat stability. It is very important for the evaluation of the results of the present study to note that this substance virtually completes its specific action in approximately 36 to 48 hours after introduction of the bacterial

thalli via a wound into the host tissues. On the basis of these reports, it now appears that attenuated strains of crown-gall bacteria are still capable of producing or stimulating the production of Braun's tumor-inducing principle but either lack the ability to produce sufficient, or the right kind of, auxin in the milieu of the plant or to stimulate auxin production by the plant tissues themselves.

With this information at hand, experimental procedures were developed to determine the temporal interrelations between Braun's tumor-inducing principle and auxin, each of which appears to be a necessary, but not a sufficient, causal factor in the genesis of crown-gall tumors. One of the purposes of this paper is to demonstrate that auxin is a promoting substance or cocarcinogen,[8] which acts on the cell while it is in the process of being transformed into a primary tumor cell. (Without auxin a cell which has been altered into an incipient tumor cell through the action of tumor-inducing (incepting) substances, will not be completely transformed into a typical primary tumor cell.) Experiments have also been performed to determine how long incipient tumor cells may remain positively disposed to the promoting action of auxin.

Methods.—A single synthetic auxin, γ-indole-*n*-butyric acid, was used, the choice of this promoting agent being made because of its rapid action.[1] Tomato plants (var. Bonny Best) were grown in 4-inch pots of garden loam in a greenhouse room. When the plants were 30 cm. tall the topmost internode was severed below its node leaving the second apical internode as a stump. All test plants were inoculated with a culture of a single colony isolate of an attenuated strain of crown-gall bacteria (A66) by a needle puncture about 1 cm. below the cut surface. Control plants were similarly bisected but received a sterile puncture in the same site. Only one inoculation or puncture was made in a plant. The inoculated plants and their controls were each divided into two lots. Inoculated and control plants forming the first lot were immediately treated on the cut surface with 0.01 ml. of a 2.5% paste of indole butyric acid in lanolin prepared by the method of Michel.[9] At various time intervals from 0 time to 240 hours after inoculation the auxin paste was removed from the plant by slicing off a 0.2 mm. disk of tissue from the top of the stump. The control and test plants of the second lot received auxin paste at time intervals after inoculation ranging from 0 time to 480 hours. Ten test and ten control plants were used for each time period. As soon as they became visible, axillary buds were removed to minimize the native auxin supply in the tissues. The experiments were terminated 30 days after inoculation.

Experimental Results.—It is evident from figure 1 that the removal of auxin prior to at least 36 to 48 hours after inoculation prevents the appearance of tumors. From 36 hours to 60 hours after inoculation and application of auxin, the eventual size of the tumors appears to be a func-

tion of the length of the auxin presentation time. Under experimental conditions it is difficult to determine exactly when the auxin began its specific promoting action. It is certainly inoperable prior to *at least* 36 hours. However, evidence for the presence of auxin within the tissues after the removal of the auxin-containing paste can be seen in figure 1 where limited auxin tumors formed on treated stumps which had their auxin source removed 24 hours after application. It appears, therefore, that auxin already moved into the tissues may induce some tumor-promoting action.

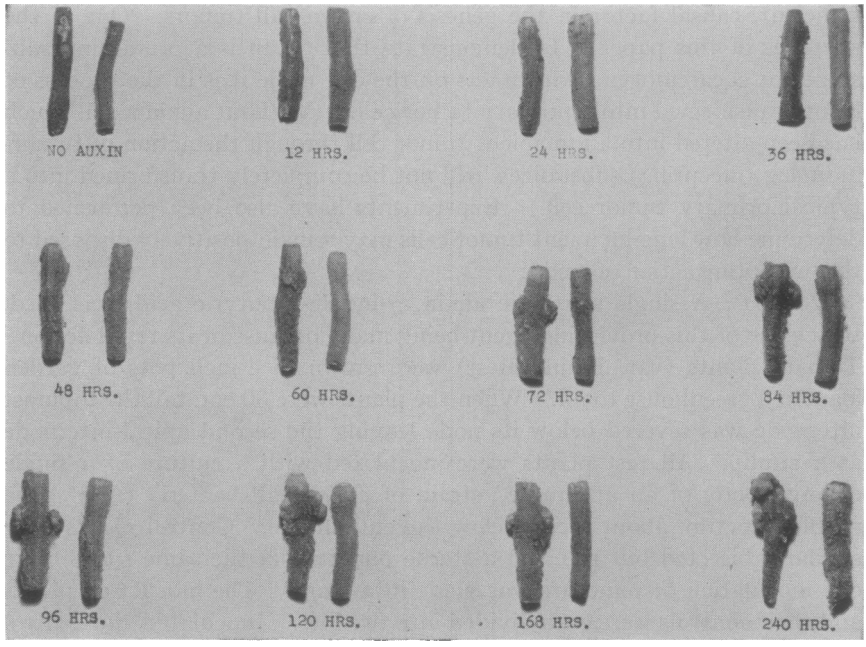

FIGURE 1

Sections of stems from decapitated tomato plants inoculated with the attenuated A66 culture of crown-gall bacterial and treated with auxin at time of inoculation. Auxin-containing paste removed at times indicated under the sections. Inoculated sections at left, control sections at right.

Subsequent to the "60-hour removal" of auxin, there is no further need for supplementary growth substance for there were no significant size differences among tumors formed under conditions of extended auxin presentation time. These findings indicate that a relatively high auxin titer and/or a definite presentation time are required for complete promotion of those cells rendered positively predisposed to promotion by the action of tumor-inducing substance and other, yet unknown, factors.[10]

The incipient tumor cells, once formed, remain positively predisposed

to auxin promotion for some time (figure 2). When auxin paste was applied as late as 10 days after inoculation with attenuated bacteria, the affected cells were transformed into rapidly growing tumor cells. However, when 15 or more days elapsed between inoculation and promotion, the resulting tumors contained root primordia. This probably indicates that the host plant had, to some extent, gained control over the development of the neoplastic tissues. It is of interest that root development from tomato crown-gall tissues has not been observed previously and that this root development did not occur in auxin-treated control material in this study.

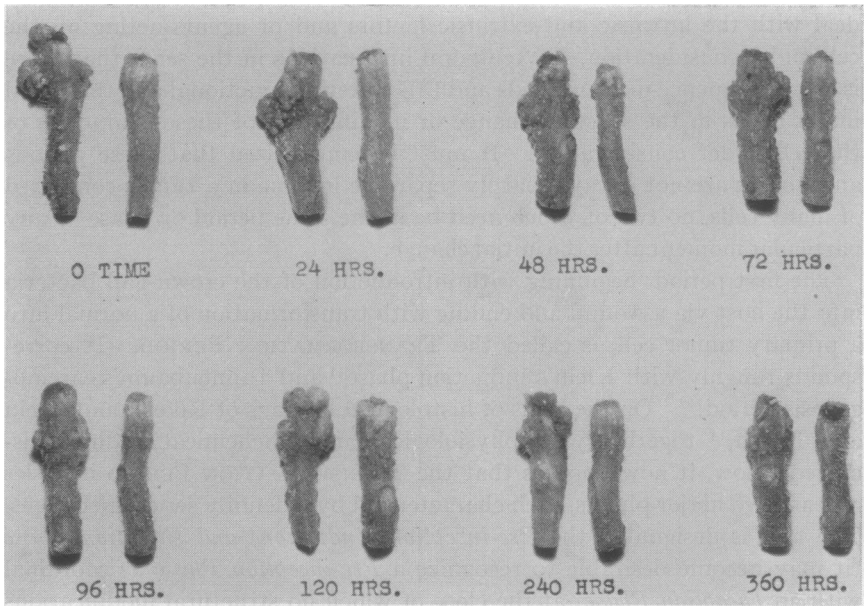

FIGURE 2

Sections of stems from decapitated tomato plants inoculated with attenuated A66 culture of crown-gall bacteria. Auxin-containing paste applied to stumps at times indicated on figure. Inoculated sections at left, control sections at right.

Discussion and Conclusions.—During the last fifty years of research on crown-gall, several attempts have been made to delimit morphological, physiological (including biochemical) and etiological stages in the formation of tumors.[1, 11, 12] Klein,[11] for example, identified three histologically recognizable phases: (1) initiation, ending with the onset of tumor cell multiplication; (2) growth, the phase of rapid tumorous cell proliferation; and (3) maturation, the phase of differentiation of tumor cells into sclerids, etc. Several biochemical changes and activities were associated with these

phases. Consideration of this and previous schemes, together with more recent findings, has necessitated revision and extension of current concepts and semantic schema to deal with the genesis of crown-gall. While this paper deals mainly with the first period of crown-gall formation, the subsequent periods are discussed to show their relation to one another and to the first period.

Genesis of the new entity—a crown gall tumor—from a normal cell or cells can be divided into three periods of cellular change. These periods, when necessary, are subdivided into phases which in turn may be divided into subphases. The terms used here to designate the various periods and phases of tumor cell genesis have (1) etiological implications in that they deal with the intrinsic and extrinsic factors and/or agents acting on the cell under consideration, (2) temporal implications in the sense that there is a time sequence in the periods and (3) material, functional and relational implications in the sense of change or modification of these characters of the cell under consideration. It must be emphasized that these periods and phases are not always sharply separable in time in a tumor composed of many cells, no two of which need be in the same period or phase at any particular moment after the initial change.

The first period, beginning with introduction of the crown-gall bacteria into the host via a wound and ending with transformation of a normal into a primary tumor cell, is called the TRANSFORMATION PERIOD. It corresponds roughly with Klein's induction phase[11] and Tannenbaum's carcinogenesis period.[12] On the basis of histological findings of Riker[13] and Klein and Rasch,[14] together with physiological and biochemical findings discussed below, it now appears that the TRANSFORMATION PERIOD includes at least two major phases, each characterized by a definite series of changes. The first is designated the *Pre-inception (Induction) and Inception Phase* (it may become desirable to recognize a *Pre-inception Phase* as coordinal with an *Inception Phase*) at the close of which no structural modifications are detectable in the prospective tumor cell but during which extensive biochemical changes do occur. These include changes in respiratory levels and pattern, changes in the levels of metabolic intermediates and modifications in the activities of cytoplasmic and nuclear components.[11] These facts leave little doubt that profound changes occur in the affected cells prior to observable structural alterations. Temporally and etiologically, this is the phase during which Braun's tumor-inducing principle is active[6] and during which the cell acquires the potentiality for autonomous behavior but not the capacity for rapid duplication.[10] A cell so incepted, yet histologically and possibly cytologically[15] indistinguishable from non-affected cells, is designated as an *incipient tumor cell.* The incipient tumor cell has become positively predisposed to the action of those other etiological factors and agents which are essential to complete its change into a primary tumor cell.

The second major part of the TRANSFORMATION PERIOD is designated the *Promotion and Completion Phase* (there are indications on etiological grounds that each of these may also have to be recognized as a distinct phase). The etiological factors, processes, and changes occurring during this phase involve a series of cytological, biochemical and physiological changes which are, as yet, incompletely studied. These include cell volume increase, heightened activity of cytoplasmic and nuclear material as judged by changes in staining reactions and biochemical analyses, and a number of altered metabolic activities.[11, 14] It is during this phase that auxin is required as a promoting agent or "cocarcinogen," one of the factors needed to promote, and possibly complete, the change from an incipient to a primary tumor cell. The experiments reported here indicate that these substances are inactive prior to the end of the *Inception Phase*, since the pre-incipient cell is not positively predisposed to the specific promoting action of auxin until it is incepted. There is little doubt that auxin is not the only entity active during the *Promotion and Completion Phase.*

The experimental prolongation of promotion and completion by withholding auxin not only indicates that this substance is specifically required to complete tumorization but also demonstrates that an incipient tumor cell is capable of retaining its positive predisposition to promotion for a considerable length of time. This suggests, but does not prove, that fundamental and presumably permanent alterations were engendered in the incipient tumor cell during the *Pre-induction and Inception Phase.* The application of anti-auxin subsequent to auxin-induced promotion has been shown by de Ropp[17] to be ineffective in preventing tumorization. Klein and Klein,[18] however, did not observe inhibition of tumor formation in tomato plants sprayed with a low concentration of maleic hydrazide, an antiauxin, prior to or concurrently with inoculation by a virulent strain of *A. tumefaciens.* Since there was no way of determining the relative concentrations of auxin and antiauxin at the site of cellular transformation and calculations showed that the antiauxin was in very low concentration within the plant, these latter findings do not invalidate the thesis presented in this report. Attempts to introduce sufficiently large concentrations of maleic hydrazide into tissues being acted upon by attenuated bacteria and auxin were unsuccessful. Further study on this problem is now under way.

Once transformation to an autonomous primary tumor cell has been completed, the cell is ready for the second period of change and behavior, the DUPLICATION PERIOD. During this period a primary tumor cell, as well as its progeny, begins to divide in a rapid but uncoordinated manner. Secondary transformation processes (appositional growth[16]) may also occur during this period when normal cells abutting tumor cells are transformed into tumor cells by agents contributed by primary tumor cells and/or

their progenies. Since Link and Eggers demonstrated hyperauxiny in tissues contiguous to tumors, it may be that these elevated auxin levels play a causal role in appositional tumor genesis. These secondary transformation processes may be different than those involved in primary transformation because the bacteria, and presumably tumor-inducing principle, are no longer required after the primary transformation.[2] The DUPLICATION PERIOD is the time of main increase in the size of the tumorous mass.

With the onset of differentiation of tumor cells into mature elements, tumor sclerids and cells simulating vascular tissue, and with organization of the entire mass of tumor tissue and occluded non-tumor cells into a definite structural, functional, and relational entity, the DIFFERENTIATION AND ORGANIZATION PERIOD begins. Even during this period primary and secondary transformation processes may be in progress and the resultant cells add to the mass of the neoplasm.

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