Nucleic Acids and Tumor Genesis in Broad Bean*

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In an earlier communication, marked changes were reported in the level of nucleic acids during structurally well defined stages in the genesis of crown gall tumors in tomato (9). In view of the distinctive pattern of these changes, it seemed advisable to extend these studies to other plant tumors to ascertain whether nucleic acid changes were, in fact, concurrent with the process of tumorigenesis and to determine whether they were related to the histological and cytochemical changes that occur in tumor genesis. Furthermore, it was important to determine whether these changes were restricted to tumors or were characteristic of plant neoplastic growth in general.

Broad bean (*Vicia faba*, var. English Windsor) was chosen for this study, to compare earlier studies on *Agrobacterium tumefaciens* tumors in tomato with a different host and pathogen. The bean is not susceptible to *A. tumefaciens*, but when inoculated with *A. rubi* does form tumors which are grossly distinct from those formed by tomato and many other hosts (4). Furthermore, bean tissues possess large cells and nuclei which can be studied histochemically.

MATERIALS AND METHODS

Bean seeds were treated with 0.1 per cent Semesan, soaked overnight, and planted in loam soil. The pots, each containing three plants of uniform size, were placed at random on a greenhouse bench. Normal photoperiod was supplemented with 350–400 foot candles of light (pot level), supplied by 200-watt incandescent bulbs. Twenty-one days after planting, the fifth internode was inoculated with *Agrobacterium rubi* (Hildebrand) Starr and Weiss (obtained from Dr. L. C. Coleman), by a single puncture with a trident previously dipped into the culture; control plants were punctured with a sterile trident. Stem segments were collected at various time intervals after treatment and sliced into thin discs about 0.3 mm. thick. All collections were made at noon on the appropriate day, the first 1 hour after treatment. Slices included only the puncture and were put in water, blotted, and weighed before chemical examination. For cytological examination, slices were immediately put into 3:1 absolute alcohol: acetic acid, or into 10 per cent neutral formalin for fixation. All dry weight values were obtained after 24 hours at 95° C.

Desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) were estimated by the method of Ogur and Rosen (13), with 200–300 mg. fresh weight of tissues for each analysis. The DNAP extraction temperature was reduced to 70° C.; the number of cold and hot extractions was increased by one each. Percentage standard errors of aliquot portions of a homogenate of *Vicia faba* stems were 3 per cent for RNAP and 2.5 per cent for DNAP. Percentage standard errors for sextuplicate analyses of individually weighed samples were 9 per cent for RNAP and 4 per cent for DNAP. In these studies, three weighed samples were analyzed.

For the study of auxin-induced neoplasms of bean, the same test procedures were followed. Three per cent indoleacetic acid (IAA) in lanolin (12) was applied in a 1-cm. ring to the middle of the fifth internode, making available a large presentation area for penetration of the auxin (10). Control plants were not treated; lanolin alone had no effect. The lanolin paste was removed prior to slicing. Samples were taken only from the treated areas.

Fixed tissues were prepared in the usual manner for histological study. Relative amounts of DNA in individual nuclei were estimated by microphotometric determinations of Feulgen-stained sections (DNA-Feulgen), as previously described (17). Sections of both alcohol-acetic and formalin-fixed tissues were stained for 1 hour with Feulgen reagent after hydrolysis for 12 minutes at 60° C. in 1 N HCl. Longitudinal sections were found most satisfactory to assure measurement only of whole, spherical nuclei. Because bean nuclei were too dark to measure accurately at 560 mμ (absorption peak of the Feulgen-DNA complex), measurements were made off the peak at 600 mμ.

RESULTS

Biochemical.—The DNAP of prospective bean tumor tissues rose sharply following inoculation, reaching a peak at 2 days (Chart 1). Subsequently, there was a decrease in the DNAP level to a point below that found at the time of inoculation. The lowest level of DNAP in tumorous tissue was reached at 5 days, followed by a secondary rise to a plateau from 9 days until the close of the study. In control material, a steadily decreasing level of DNAP was observed. That this peak in DNAP is not due to an increment of bacterial DNA inci-

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dent to multiplication of the bacteria in the tissues was determined by inoculating susceptible plants with a completely avirulent strain of *Agrobacterium*.[1] Although these bacteria multiplied normally in the inoculated tissues (8), the DNAP curve for the critical period was identical with that of punctured control tissues.

Changes in RNAP were less striking (Chart 2). Immediately following inoculation, there was a small, possibly significant, rise in RNAP. During the next 4 days, RNAP in control and inoculated tissues showed a parallel decrease, followed by a secondary rise from the 9- to 20-day period. Except for the small initial rise in RNAP of prospective tumorous tissues at the 1- to 2-day period, no significant differences between control and tumorous tissues were noted.

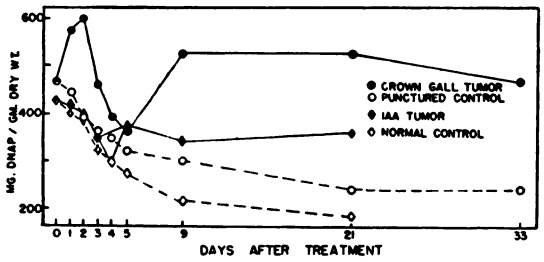

CHART 1.—Levels of desoxyribonucleic acid phosphorus during the genesis of tumors and auxin-induced neoplasms of broad bean.

No differences were found in DNAP levels of either auxin-treated plants or untreated controls during the first 4 days (Chart 1). When the auxin-neoplasm became macroscopically evident (5 days after IAA treatment), there was a secondary rise in DNAP to a level slightly below that found at zero time. This level remained constant throughout the period of study. RNAP levels of IAA-treated tissues were strikingly similar to those DNAP changes observed for the early period of crown gall development (Chart 2). There was an initial rise, reaching a peak 2 days after application, followed by a fall to the level of untreated control tissues within 9 days. No further changes were noted.

Histological.—Initiation of tumor genesis was histologically detected within 2 days after inoculation by increased cambial activity, and within 3 days by enlargement of cortical, endodermal, and vascular parenchyma cells. Tumorous tissues showed extensive cell division within 5 days (Fig. 2) in comparison to control stems (Fig. 1). Complete disorganization of normal stem architecture occurred within 20 days following rapid proliferation of all parenchymatous tissue elements as well as cell enlargement, de-differentiation, and division of perimedullary tissue, pericyclic fibers, and cortical fiber bundle cells.

The high degree of normal stem architecture maintained in IAA-induced proliferations, as reported by Palser (14), is in striking contrast to the disorganization found in the autonomous growth of bean tumors. Cell enlargement and initiation of cell division were reported by Palser to occur within 30 hours, while divisions in most parenchymatous tissues were found at 48 hours after treatment of decapitated bean stems.

Cytochemical and cytological.—The amount of DNA-Feulgen complex per nucleus was determined in over 800 nuclei of tumor and control tissues. Values from normal tissues were those expected from previous findings (17, 18). Cambial telophase nuclei contained the diploid or 2C amount of DNA and prophases, twice the diploid or 4C amount (dotted lines of Chart 3); interphase values spread between these limits. It thus appears that DNA synthesis here occurs in interphase, as found in many other plant and animal tissues (see 18 for review). In spite of the marked DNAP increase shown biochemically in 2-day tumor tissues, no significant differences in DNA-Feulgen values over the control were found. It is unlikely that, if such an increase included Feulgen-positive material, it would be unnoticed in measurements on individual nuclei. The increase apparently cannot be associated primarily with cell division, since tumor proliferation did not reach a maximum until 5 days or later after inoculation. By this time, the DNA found biochemically dropped sharply. Also, at the time of the DNA decline there was no evidence of cell death, except in the wound pseudocicatrice. This degeneration was essentially the same in treated and control sections (Figs. 1 and 2). No Feulgen-positive material was seen in the cytoplasm of control or inoculated tissues.

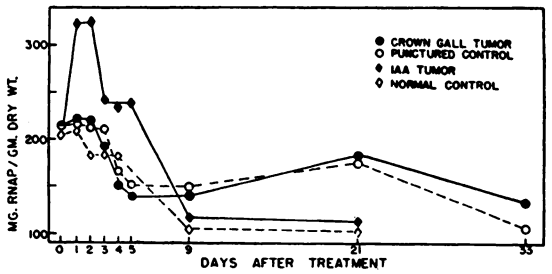

CHART 2.—Levels of ribonucleic acid phosphorus during the genesis of tumors and auxin-induced neoplasms of broad bean.

[1] (Klein, unpublished data, 1953).

Determinations of DNA-Feulgen per nucleus in mature tumorous tissue 33 days after inoculation, and in wound periderm of 33-day-old control tissues, gave values grouping well into polyploid classes: 2C, 4C, 8C, and 16C. Division of polyploid nuclei occurred in the maturing tumor 20 and 33 days after inoculation. No polyploid divisions were found in wound periderm of 20- and 33-day control stems. Details of the histology and nucleic acid changes accompanying tumor development will be published elsewhere.

Contrary to previous reports of aberrant nuclear division as characteristic of tumor tissues of plants (16, 19), only two instances of micronuclei adjacent to reconstruction nuclei, and only one case of a bi-nucleate cell were observed. Similar abnormalities may be seen in normal tissues. As suggested by Levine (11) aberrant divisions, therefore, are not necessarily characteristic of plant tumor cells.

DISCUSSION

Several critical points in the relation of nucleic acids to tumor genesis have been examined. Primary among these was the demonstration that an increase in DNA 1–2 days after inoculation of virulent bacteria occurred in the genesis of crown gall tumors (9) but not in auxin-induced neoplasms.

The distinctive pattern of DNAP changes in tumor genesis, as determined biochemically, shows little direct correlation at present with either histological or cytochemical observations. Satisfactory technics, as have been applied in the case of animal tissue studies (15), do not exist for direct cell number estimation in plant tissues. Total nitrogen as a base for cell number has also been shown to be unsatisfactory (9, 12). Consequently, no exact correlations between biochemically observed levels of DNAP and relative amounts of DNA-Feulgen per nucleus have been attempted here. Since the 1- to 2-day peak in DNAP was not reflected in Feulgen determinations, this increase seems best interpreted as either a result of specific action of prospective tumor cells, or a product of virulent, tumor-inducing bacteria. Avirulent bacteria which do not induce tumor formation nor evoke the DNAP peak[1] can be altered into tumor-inducing forms which do evoke this peak by treatment with the nucleic acid of virulent forms. Strains and species of Agrobacterium, including *A. rubi*, possessing one host range, can acquire new host ranges by these technics (8). It may be concluded that the DNA peak represents synthesis of a specific nucleic acid by virulent bacteria, this nucleic acid apparently playing an etiological role in the transformation of normal cells to tumor cells. In later stages of tumor growth, elevated DNAP levels may be due to larger numbers of cells per unit weight of tissue, and to the increased proportion of polyploid cells in the crown-gall tissue mass (Rasch, unpublished data, 1952).

In contrast to the unusual nucleic acid pattern during the early phase of tumor genesis, DNAP and RNAP levels during the development of auxin-induced proliferations present no findings which differ from those observed in studies on normal cell growth. Heightened RNAP levels found 2 days after application of IAA may be correlated with histological findings of enlargement of affected cells at that time (14) and possibly with protein synthesis. High rates of protein synthesis have been found to accompany auxin-induced cell enlargement in pea stems by Christiansen and Thimann (3). Stimulation of mitosis in auxin-induced neoplasms during the initial response to treatment (14) was reflected biochemically by a steady decline in DNAP levels during the critical 1- to 3-day period. Although timing of histological responses to treatment in auxin neoplasms may be more rapid than that found here for the tumors, changes of DNAP levels in the two cases cannot be directly related to cell numbers or cell division.

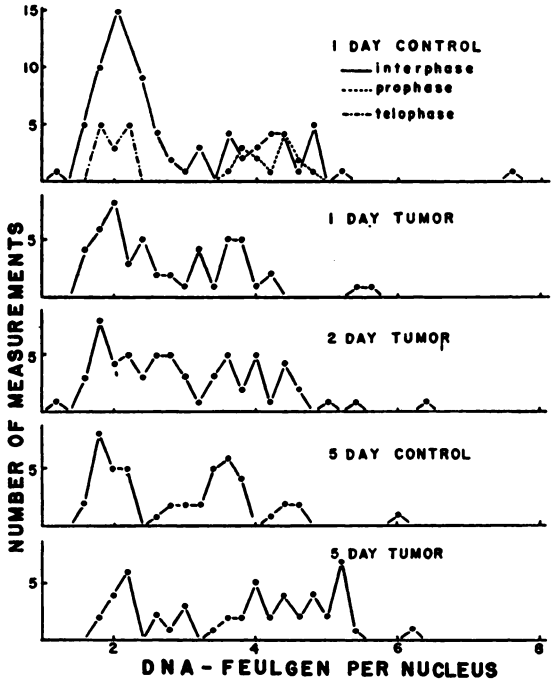

CHART 3.—Amounts of DNA-Feulgen, in arbitrary units, in individual nuclei from control and tumorous stem tissues of broad bean.

This evidence would further indicate that the DNAP peak of the prospective tumorous tissues is not characteristic of normal growth responses.

From Coleman's previous study on mitotic figures from IAA-induced divisions of cortical parenchyma (5), and the present cytochemical study of interphase nuclei of several stem tissues, it can be concluded that polyploidy alone is not sufficient to characterize tumor tissue. Similar findings for several mammalian tumors have been recently presented (1). Divisions of highly polyploid nuclei, however, may be characteristic of tumor development, for only diploid division figures were found in the older control tissues after completion of the mitotic response to wounding. Polyploid divisions, moreover, are known to be extremely rare in normal tissues (6, 7).

SUMMARY

Levels of desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) were determined at various times during the genesis of tumors and auxin-induced proliferation on stem tissues of broad bean. Prospective tumorous tissues showed a sharp peak of DNAP levels 2 days after inoculation, a maximum depression at 5 days, and a gradual increase to a plateau by 9 days. No marked changes in RNAP were noted. Auxin-induced neoplasms showed a sharp initial rise in RNAP at 2 days after application of IAA, but only slightly elevated DNAP levels.

Initiation of tumor genesis was histologically detected within 2 days after inoculation by increased cambial activity and at 3 days by enlargement of stelar parenchyma. Tumorous tissues showed extensive cell division within 5 days. Complete disorientation of normal stem architecture occurred within 20 days.

Photometric determinations of over 800 Feulgen-stained nuclei of inoculated and control tissues showed no significant increase in relative amounts of DNA per nucleus in tumor tissues during the first 5 days. No evidence for cytoplasmic localization of DNA was found. Although the number of and division of polyploid nuclei may be characteristic of tumorous growth, both wounded and inoculated tissues showed a pattern of DNA values essentially similar to that found in areas of meristematic growth, as compared to mature, nondividing tissues.

Comparisons of histological and cytological findings with biochemically observed changes in nucleic acid levels accompanying crown gall tumor genesis have indicated that the 1- to 2-day peak of DNAP may be a result of specific action of prospective tumor cells, or a product of virulent tumor-inducing bacteria.

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Figs. 1 and 2.—Broad bean stem x-sections. Fig. 1.—control 5 days after wounding; Fig. 2.—tumor 5 days after inoculation. ×110.