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NECESSITY OF INDOLEACETIC ACID FOR THE DUPLICATION OF CROWN-GALL TUMOR CELLS[1,2]

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The presence of a crown-gall tumor in vivo is frequently associated with numerous modifications of the growth behavior of the host. Included among these are severe epinasty, suppression of the development of lateral shoots, delay in petiolar abscission, and increased cambial activity. Many investigators found that indoleacetic acid (IAA) could induce almost identical abnormalities independent of the presence of a tumor (1, 14). It was postulated by Link and Eggers (18) that crown-gall tumors contained high concentrations of IAA that were carried throughout the host plant and were responsible for the observed changes. Indeed, bioassays of tomato plants demonstrated that there was much more auxin in tumor tissues than in corresponding normal stem tissues (18). This report could not at the time be confirmed (24), but the present evidence supports the findings of Link and Eggers. Although it was not then known whether the crown-gall bacteria synthesized the IAA present in tumor tissues or whether it was formed in the tumor cells, DeRopp (3) later concluded that the tumor cells themselves were the primary source of the auxin. Both Kulescha (15) and Henderson and Bonner (10) demonstrated that there was more IAA in the tissues of sterile tumors in vitro as compared with in vitro cultures of normal tissues and that IAA was synthesized by tumor cells.

Attempts to prove that these high endogenous levels of IAA are necessary for the duplication of tumor cells have failed since additions of IAA to crown-gall tissue in vivo or in vitro were generally inhibitory or without significant effect on tumor-cell duplication (3, 11, 15). The simplest way to demonstrate a positive role for IAA in the growth of crown-gall tissues would be to reduce the biologically effective level of IAA with concomitant reduction in growth. Control rates of growth should be restored by addition of this growth substance.

Two lines of attack on this problem have appeared. The first of these is the radiation technique discovered by Skoog (28) and developed by Gordon (6, 7, 35). Here, controlled doses of ionizing radiation reduce the endogenous level of IAA, presumably by blocking its synthesis. The doses of radiation used are not permanently inhibitory to other cellular processes, although higher doses have profound effects on chromosomes, nucleic acid biosynthesis, and other processes (22, 23) which are not reversible with IAA. Levin and Levine (19) and Rivera (25) found that hard x-rays would prevent the growth of tumors on Ricinus as judged by increases in tumor size or by microscopic examination of the affected cells. Stapp and Bortels (29), however, were unable to observe any reduction in tumor growth on tomato plants following x-irradiation. Recently, Waggoner and Dimond (33) demonstrated that ionizing radiations delayed the appearance of

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tumors in tomato and suppressed the growth of previously formed tumors. None of these workers attempted to reverse the radiation-induced inhibitions of tumor-cell growth with exogenous supplies of IAA.

The second technique now available for reducing the biologically effective level of IAA in tissue requires a specific antimetabolite. Experiments of this type, to be of positive value, must result in inhibitions of growth by the presumed antimetabolite which may be reversed by appropriate concentrations of the metabolite, in this instance, IAA. Interpretation of the results may be complicated by the fact that exogenous supplies of IAA may be inhibitory when supplied to tumor tissues which contain endogenous IAA. Thus, both antimetabolite and metabolite may be independently inhibitory, and inhibitions induced by either compound should be reversible by the other (5). The reports of McRae and Bonner (21) and Gentile and Klein (5) suggested that 2,4,6-trichlorophenoxyacetic acid (TCPA) might be a specific antimetabolite for IAA.

The experiments reported at this time, using both radiation and antimetabolite approaches, give presumptive evidence that IAA is, in fact, required for the duplication of the crown-gall tumor cells which synthesize it.

Materials and Methods

Radiation Studies: Tomato plants (var. Bonny Best) were grown from seed in flats of garden loam. Plants were selected for uniformity at the three leaf stage and repotted in glass shell vials of one inch diameter. After allowing several days for recovery from transplanting injuries, test plants were inoculated with *Agrobacterium tumefaciens* (strain S 5-6) by a single puncture through the second apical internode with a needle dipped into an 18-hour broth culture of the bacteria. Control plants received sterile punctures. All plants were kept on a greenhouse bench for 108 hours to permit complete transformation of normal cells into primary tumor cells (14). They were then irradiated with x-rays, gamma-rays, or fast neutrons at the dose levels given in table I. X-rays were supplied by a General Electric Maximar x-ray unit at 250 KV and 15 milliampere with a 1-mm copper filter. Dose rate was controlled by altering the distance between the source and the zone of puncture. Exposures to the other ionizing radiations were carried out in a gamma-neutron radiation chamber (31) at Argonne National Laboratory's CP-3′ reactor. Fast neutrons were produced through fission, by allowing slow neutrons from the thermal column of the reactor to impinge upon a uranium plate. Appropriate shielding devices of boron carbide prevented slow neutrons from reaching the plants. The gamma contamination of the fission neutrons was approximately 7 % of the total physical dose. Pure gamma radiation was obtained from 18 sources of cobalt^{60} at the opposite end of the chamber from the uranium converter plate.

Neutron dose was measured in roentgen equivalents physical (rep) with Victoreen condenser r-chambers which had been calibrated against a tissue equivalent chamber of 10.1 % hydrogen composition (27) and a Hurst-Ritchie proportional counter (12). For gamma rays, the same condenser chambers were calibrated by the U. S. National Bureau of Standards.

Table I

Inhibition of the Duplication of Crown-gall Tumor Cells in Vivo by Ionizing Radiation and Reversal of Inhibition with Indoleacetic Acid *

Radiation	Dose r or rep/min	Total dose	Av. tumor diameter as % of control ***	
			Lanolin	Lanolin ** IAA
Fast neutrons	3.4	340 rep	95	103
	3.4	550 rep	28	100
	4.6	950 rep	7	90
X-rays	10.6	500 r	99	100
	10.2	1000 r	76	98
	12.2	1500 r	8	80
	47.5	5000 r	7	7
Gamma rays	12.2	1500 r	106	106
	12.5	2100 r	58	101
	12.2	2850 r	7	90

* Tomato stems inoculated 108 hr prior to irradiation.

** Lanolin pastes applied to surface of bisected internode bearing this tumor.

*** Tumor diameter measured 18 days after irradiation and treatment. Control = tumors on untreated plants.

The plants were irradiated in a single vertical plane in a Lucite exposure box, 16 inches high, 16 inches wide, and two inches thick. Approximately 30 plants were irradiated simultaneously, half the vials arranged in the bottom of the box, the other half placed in a shelf eight inches above. The intensity of the radiation field was carefully mapped for all exposures with a Victoreen rate-meter. Total doses received at the zone of puncture on the stems of the tomato plants varied $\pm$ 5 % from the values listed in table I. Eight to 10 plants were used for each variable in all experiments.

Within one hour after irradiation, all plants were decapitated just below the apical node, leaving intact the second internode bearing the inoculation site. Lanolin or lanolin-IAA pastes (1 % IAA) were placed on the cut surfaces with a warmed syringe; each plant received 0.01 ml. The plants were transferred to pots of loam and kept on a greenhouse bench for 18 days. Axillary shoots formed during this period were removed to minimize growth responses to any other source of auxin. Tumor-cell duplication was judged by determination of the diameters of stem and tumor with a micrometer gauge. During the period of study, increases in tumor size was due almost entirely to the duplication of tumor cells (14).

Tissue cultures of a secondary crown-gall tumor of sunflower (36) were grown on White's medium for 20 days at 24 $\pm$ 2° C. The tissues were irradiated with

x-rays in the culture tubes, were weighed, transferred to fresh tubes of White's medium or White's medium containing 5 mg/l IAA, and were incubated for 30 days at 24° C. Final fresh weight values were determined and the "percentage growth" was calculated (final weight − initial weight/initial weight). Increase in fresh weight is here used as a measure of the growth by cell division since cell enlargement occurs infrequently under the conditions used (14).

ANTIMETABOLITE STUDIES: Sunflower tissue cultures were grown on White's medium; all media used for normal tissues was supplemented with naphthaleneacetic acid (NAA) at 10^{-7} gm/l. The following tissues were used: normal, habituated (9), secondary petiolar crown-gall (36), and primary stem crown-gall (2). In each case, several morphological types of tissue were noted in the cultures as received in this laboratory and, before being used, they were selected to consist of a single type. Indoleacetic acid and TCPA were each added to White's medium to final concentrations of 1 to 50×10^{-5} and 0.1 to 10×10^{-5} M, respectively. Mixtures of these compounds were made in all combinations with the concentrations used. Fragments of tissue were grown on control media for two weeks before being weighed to within 2 mg and were transferred to test media where they grew for 30 days at 27° C. Percentage growth values were determined and the data are reported as percentages of the percentage growth of tissue grown without supplements. Five replicates of each variable were set up; the standard deviation approximated 12 %.

RESULTS

RADIATION STUDIES: Fast neutrons were the most effective in suppressing tumor-cell duplication, 550 roentgen equivalents physical (rep) suppressing about 75 % of tumor growth as compared with non-irradiated control (table I). X-rays and gamma-rays were less effective than fast neutrons. Complete inhibition of tumor growth was achieved with 950 rep of fast neutrons, 1500 roentgens (r) of x-irradiation, or 2850 r of gamma-irradiation. With these doses, the addition of IAA reversed the inhibitions although 1500 r of x-irradiation affected metabolic activities not reversible with IAA since complete restoration of tumor growth did not occur. It is of interest that following 5000 r x-irradiation, the inhibition of tumor growth was not at all reversible with IAA. In contrast to the findings of Waggoner and Dimond (33), no spontaneous recrudescence of tumor growth was observed following any radiation treatment. It is noteworthy that suppression of growth was achieved with dose levels considerably below those used by Waggoner and Dimond.

Suppression of tumor-cell duplication by ionizing radiation and restoration of growth by IAA was also demonstrated with tissue cultures of crown-gall origin (table II). Five mg/l IAA (5.7×10^{-5} M) in the medium independently reduced growth to its half maximum level, essentially the same growth inhibition reported by Hildebrandt and Riker (11). X-irradiation to 1000 r total dose independently suppressed growth 68 %. When irradiated tissues were grown on media containing that level of IAA which reduced growth to half the control value, the growth was significantly superior to that obtained following irradiation alone or growth in the presence of IAA. Here, as with tumor tissues in vivo, IAA was capable of reversing the inhibitory effects of radiation and IAA appears to be necessary for growth of the tumor. On the basis of these experiments, and the results of Weber and Gordon (35), it appears that IAA is required for the duplication of tumor cells when they are rendered deficient in auxin by a radiation-induced blocking of IAA synthesis. These data indicate that IAA is required for the duplication of crown-gall tumor cells.

TABLE II

EFFECT OF IONIZING RADIATION AND INDOLEACETIC ACID ON GROWTH OF TISSUE CULTURES OF SECONDARY CROWN-GALL

TREATMENT	NO. TISSUES	MG FRESH WT		% GROWTH	STD DEVIATION	% CONTROL
		INITIAL	FINAL			
Control	10	242	569	130	16 %	100
Control + IAA *	10	267	442	66	13 %	51
Irradiated ** ...	15	263	372	42	18 %	32
Irradiated + IAA	15	265	545	106	14 %	82

* IAA = 5 mg/l.
** 1000 r total dose of x-rays given 10 r/min.

ANTIMETABOLITE STUDIES: Since this hypothesis would be strengthened if confirmed by an independent method, we turned to the antimetabolite technique for reducing the biologically effective level of IAA in the cells. The study was enlarged to include normal tissue cultures known to require an exogenous supply of IAA, or its equivalent, for growth, and habituated tissue which is presumed to be autotrophic for IAA. The sunflower tissues used differed in their growth rates; the 30-day increase in weight for normal tissues was 200 %; habituated tissues, 250 %; primary crown-gall tissue, 190 %; and secondary crown-gall tissue, 100 %. Thus, crown-gall tumor tissues do not necessarily grow at faster rates than do corresponding normal tissues.

For all tested tissues, both IAA and TCPA were independently inhibitory. To be considered as significant, stimulation, inhibition, or reversal of growth percentages of any variable must deviate from the appropriate control by at least 15 to 20 %. Table III gives the concentration of IAA and of TCPA necessary to reduce the percentage growth to 50 % of the control value. These data suggest that secondary petiolar crown-gall tissue was the least able to adjust to changes in the biologically effective concentration of IAA on either side of its endogenous level (which may be presumed to be near optimal). Primary tumor tissue was the most adaptable. Normal tissues

TABLE III

CONCENTRATIONS OF INDOLEACETIC ACID OF TRICHLOROPHENOXYACETIC ACID NECESSARY TO REDUCE THE GROWTH OF SUNFLOWER TISSUE CULTURES TO THE HALF MAXIMAL LEVEL

SUNFLOWER TISSUE CULTURE	IAA ($M \times 10^{-5}$)	TCPA ($M \times 10^{-5}$)
Normal	5.0	0.13
Habituated	1.0	1.0
Primary tumor	30.0	4.0
Secondary tumor	4.0	0.05

of sunflower, as might be expected, apparently can tolerate increased concentrations of IAA better than they can adjust to any reduction in IAA levels. Habituated tissues had a relatively narrow zone of tolerance.

Growth of the tested sunflower tissue in the presence of different combinations of IAA and TCPA showed a number of interesting correlations. For normal tissue (table IV), 1 and 5×10^{-5} M IAA significantly reversed the inhibitions of growth induced by 0.1×10^{-5} M TCPA and all tested concentrations of IAA reversed the inhibitions caused by 0.5×10^{-5} M TCPA. In one instance, the reversal of the inhibitions of each compound alone (double inhibition) was complete. TCPA at 1.0×10^{-5} M almost completely prevented the growth of the tissue. For normal tissues of sunflower, IAA and TCPA acted as an anti-metabolite-metabolite system and IAA apparently is required for growth. This was expected since auxin must be supplied to this tissue for any growth.

For both primary and secondary crown-gall tissues which do not require an exogenous supply of auxin, similar patterns of inhibition and reversal were obtained. These data, too, are presumptive evidence that IAA is required for the duplication of these two types of crown gall tumor tissue.

Although both IAA and TCPA were independently inhibitory for habituated tissue, neither substance significantly reversed the inhibitions caused by the other. There was, however, evidence that they did interact since the addition of 50×10^{-5} M IAA to tissues grown with TCPA resulted in synergeistic inhibitions. Habituated tissue appears to differ qualitatively from normal and tumor tissues from the same species.

DISCUSSION

There is little doubt that IAA or its biological equivalent is required for the growth of most plant tissues. The previously noted failure of workers to demonstrate its necessity for crown-gall tumor tissues is now clear. Most tumor tissues possess the ability to maintain endogenously a level of IAA necessary for optimal growth. When an additional amount of IAA is supplied to them, the total level becomes supra-optimal and inhibition or "toxicity" is observed. Mature tissues in vivo and tissue cultures of normal cells, which usually do not synthesize sufficient IAA for optimal growth, may be stimulated by the addition of IAA in proper amounts. This is not to imply that the auxin-utilizing systems of crown-gall cells are qualitatively unique; they probably are quantitatively different from the cells from which they are derived. Although it would simplify the situation were high endogenous levels of IAA solely responsible for the observed growth behavior and metabolic patterns characteristic of crown-gall cells (14), this does not appear to be true. Many of the biochemical activities and morphogenic characteristics of tumor cells are quite different from normal cells or from neoplastic cells activated by auxins. One must conclude that the ability of crown-gall tumor cells to synthesize and to use the high levels of IAA found in them are only two of the activities which differentiate them from normal cells.

Examination of the data obtained by two techniques indicates that IAA is required for the multiplication of crown-gall tumor cells. Previous work showed that auxin is one of the agents necessary for the complete transformation of normal cells into primary tumor cells (14). Other work (30) suggests that IAA may be involved in the differentiation of mature tumor cells. It would appear that one substance—IAA—plays three major etiological roles in the life history of a crown-gall tumor cell.

It might be expected that anti-auxins would sup-

TABLE IV

INHIBITION OF THE GROWTH OF SUNFLOWER TISSUES IN VITRO BY EITHER INDOLEACETIC ACID OR 2,4,6-TRICHLOROPHENOXYACETIC ACID AND REVERSAL OF THESE INHIBITIONS BY THE OTHER COMPOUND. RESULTS EXPRESSED AS PERCENT OF THE PERCENTAGE GROWTH OF CONTROL CULTURES

TCPA ($M \times 10^{-5}$)	IAA ($M \times 10^{-5}$)					
	0.0	0.1	1.0	5.0	10.0	50.0
	Sunflower—Normal					
0.0	100	87	72	49	24	..
0.1	44	59	69	100 *	..	..
0.5	36	64	69	77 *	..	..
1.0	23	0	0	0	0	..
	Sunflower—Habituated					
0.0	100	..	58	..	55	34
0.1	96	..	86	..	68	16
1.0	49	..	39	..	52	18
5.0	25	..	22	..	19	8
10.0	20	..	11	..	17	12
	Sunflower—Primary crown gall					
0.0	100	..	113	..	74	39
1.0	75	..	78	..	90 *	29
5.0	40	..	52	..	25	17
10.0	28	..	47	..	22	11
	Sunflower—Secondary crown gall					
0.0	100	..	69	..	26	11
1.0	24	..	57	..	93 *	30
5.0	8	..	22	..	31	80 *
10.0	1	..	3	..	10	80 *

* Variables showing significant reversals of the double inhibitions.

press tumor growth in vivo. Roberts (26) reported that a naturally-occurring "anti-auxin" will reduce tumor growth on tomato plants and would also decrease the severity of the accompanying epinasty and adventitious root formation characteristic of the response of this host to IAA diffusing from the tumor. Waggoner and Dimond (34) reported that maleic hydrazide, a reputed anti-auxin, acted in a similar fashion but Klein and Klein (13) and Manil and Straszewska (20) had previously obtained negative results with this compound. The failure of maleic hydrazide to inhibit tumor-cell multiplication in vivo is in contrast to its effects in vitro where it severely reduced tumor growth (16), but it remains to be determined whether maleic hydrazide is an anti-auxin. The data presented here does show that TCPA can be used as an anti-auxin in vitro.

It is of interest that the data on TCPA reported here and those reported by Gentile and Klein (5) on Diplodia do not fit the kinetic theory developed by McRae and Bonner (21). The latter authors based their theory on the TCPA-IAA interactions on growth by cell elongation. Both Gentile and Klein and the present authors measured growth by increases in the weight of cell masses. In the Diplodia studies, as well as in the tissue cultures, growth is primarily by cell division. These differences in technique and results may indicate that IAA plays different roles in cell enlargement and in cell division (4).

The radiation data reported here are of interest independent of their relation to the IAA problem. Fission neutrons and gamma rays dissipate their energy in matter in distinctly different ways. Ions from gamma irradiation are more uniformly scattered throughout tissue, whereas fission neutrons, through proton recoil, cause more concentrated aggregates of ion pairs (17). This physical difference in specific ionization and linear energy transfer appears to be of significance in the relative biological effectiveness of these radiations. Although the data presented in table I are insufficient to justify setting a definite figure for the relative biological effectiveness, it is clear that fission neutrons are somewhat more effective and cobalt^{60} gamma rays less effective than 250 KVP x-rays for this biological criterion. These results confirm similar experiments in which a variety of organisms (mice, chicks, fruit flies, grasshoppers, kidney bean seeds, etc.) have been exposed in a gamma-neutron chamber (32).

Summary

The reduction of the endogenous or biologically effective level of IAA in crown-gall tumor tissues was accomplished in vivo and in vitro by the use of ionizing radiations (x-rays, gamma rays, and fast neutrons) and by the use of a specific anti-auxin. In all cases, the primary effects were a reduction in the growth of the tissues. These inhibitions of growth could be reversed wholly or in part by supplying the tumor cells with appropriate concentrations of IAA. These results give presumptive evidence that IAA may be specifically required for the duplication of crown-gall tumor cells which presumably synthesize optimal amounts of auxin.

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EFFECT OF MANGANESE AND CERTAIN OTHER METAL CATIONS ON ISOCITRIC DEHYDROGENASE AND MALIC ENZYME ACTIVITIES IN PHASEOLUS VULGARIS[1,2]

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It is well established that many of the enzymes in plants require metal ions for activity. An examination of the effect of variable levels of mineral ions in culture media on enzyme activities of tissues offers possibilities of explaining the roles and interactions of metals in metabolism. Brown and Steinberg (2) have reported that leaves from tobacco plants grown in Fe-deficient media were low in peroxidase activity and that leaves from Cu-deficient plants were characteristically low in ascorbic acid oxidase activity. Another example of reduced activity of a metalloenzyme caused by a metal deficiency is the observation of Nicholas, Nason and McElroy (13) that nitrate reductase of Neurospora was markedly reduced when the fungus was grown in media containing insufficient Mo. Other metal deficiencies had little effect on nitrate reductase activity.

Decreased metalloenzyme activities of tissues of plants grown in media deficient in the metal associated with the enzyme are expected. The influence of a metal deficiency on the activity of enzymes not known to be directly associated with the particular metal in question is more difficult to interpret. The findings by Nason, Kaplan, and Oldewurtel (12) that Zn deficiency in Neurospora resulted in a striking increase in diphosphopyridine nucleotidase is of this type. It has also been shown (11) that Cu-deficient tomato leaves exhibited abnormally high isocitric dehydrogenase activity. This was interpreted in terms of a specific effect of Cu deficiency on protein synthesis and not on the basis of any direct relationship between Cu and the enzyme.

It has been established in numerous cases that high concentrations of certain metals apparently cause induced deficiencies of other metal cations. An example of such an interrelationship was shown by the work of

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