NITROGEN AND PHOSPHORUS FRACTIONS, RESPIRATION, AND STRUCTURE OF NORMAL AND CROWN GALL TISSUES OF TOMATO

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(WITH ELEVEN FIGURES)

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Introduction

The development of masses of tumorous tissue following implantation of *Agrobacterium tumefaciens* into susceptible plants has been of considerable interest to biologists for half a century. Among the many studies on these crown galls, few, however, have attempted to compare and correlate the structure and vital activities of normal and tumorous tissues from the time of inoculation through the various phases of development of the normal control plant and of the tumorous plant. For the tomato plant, RIKER (**24, 25**) has made sequential histological and cytological examinations of these tissues; RIKER *et al.* (**26**) and LINK and EGGERS (**15**) have studied the auxin levels; and LINK and GODDARD (**16**) have made a preliminary investigation of the respiration of the control and tumorous hypocotyl tissues throughout the period of tumor development.

The work reported in this paper was undertaken to compare some of the biochemical properties of normal and tumorous tissue and correlate these findings with the modifications in structure that occur during development. Nitrogen and phosphorus fractions and respiratory intensities were selected for this study because of their well-known relationships to energy and growth metabolism.

Materials and methods

Approximately 6,000 tomato seeds (*Lycopersicon esculentum* Mill. var. Bonny Best) were sown into 10 flats of soil on June 6, 1950. When the seedlings were 22 to 24 days old they were selected for uniformity and transplanted into flats (100 plants/flat) or into five-inch pots (one plant/pot). The 4000 plants so selected were divided into two lots, each occupying half of an east-west facing greenhouse room. On July 5, 1950, 24 days after germination, the plants of one lot were inoculated in the middle of the aboveground portion of the hypocotyl with a 24 hour old nutrient-dextrose broth culture of *Agrobacterium tumefaciens* (Univ. of Chicago strain S 5–6). The composition of the medium and the method of inoculation was that of BRAUN (**2**) and all of the inoculated plants developed tumors. The plants of the control lot were similarly wounded in the middle of the hypocotyl with a sterile needle. The hypocotyl was selected for this study because it

is the first axis segment to complete elongation and because of its relatively uniform diameter and stage of development under uniform cultural conditions (**15**).

Two days before inoculation and at time intervals of 2 to 44 days after inoculation, samples were collected for analysis. After the 44th day the plants began to flower and, as previously noted by STAPP and BORTELS (**31**), the tumors no longer grew after the plants were in bloom. All collections were made at 9:00 A.M. on the designated days. The plants in flats were used only for the first two collections when very large numbers of plants were required; thereafter the potted plants were used exclusively. For the first two collections after inoculations only the water-soaked tissues surrounding the punctures were used since this zone apparently delimits the prospective tumorous area (**23**). When the tumors were macroscopically evident only the enlarged portion of the hypocotyledonary cylinder was examined. Control segments routinely included the middle third of the hypocotyl. The selected hypocotyl segments were removed with a razor blade and rinsed with running tap water. During the earlier collection periods tissues from at least 150 control or tumorous plants were used and a minimum of 75 plants were sampled in all later collections. Tissue from the same plant was used for all of the studies reported here.

NITROGEN ANALYSES

Four to six gram samples of fresh tumor or control hypocotyl tissues were placed in weighing bottles, and, after determining their initial weight, were heated at 110° C for two hours. The tissues were then dried to constant weight in a vacuum oven at 60° C (**17**), and the percentage dry weight was calculated from these data. The dry samples were ground in a Wiley mill to pass a 60 mesh screen and stored in the dry state until analyzed. Total nitrogen was determined by the semi-micro Kjeldahl method using selenium as the digestion catalyst. Soluble nitrogen was extracted from the dry tissue for 18 hours at room temperature by 55% ethyl alcohol containing 5% trichloroacetic acid and four subsequent extractions were made at 80° C for 20 minutes each. Aliquot portions of the pooled soluble fraction were analyzed as for total nitrogen. The insoluble residue contained no alcohol-soluble nitrogen after the final extraction. Protein nitrogen was determined by difference and all data were referred to both wet and dry weight bases. The percentage standard deviations averaged 3%.

PHOSPHORUS ANALYSES

Several modifications were introduced into the phosphorus fractionation scheme develop by JUNI *et al.* (**10**) and the revised procedure is given here in detail. This method has the advantage of requiring a short time for a complete analysis and may prove useful in general surveys of the phosphorus metabolism of higher plants. Total phosphorus was measured after digestion of the plant tissue at 140° C for 18 hours in open tubes with 10 *N*

H_2SO_4 and concentrated HNO_3 and aliquot portions of the wet ash were analyzed by a modification of the FISKE and SUBBAROW method (**9**). The reduced phosphomolybdate color was fully developed within five minutes by including ethanol to a final concentration of 10% by volume (**28**) and was read in a Klett photoelectric colorimeter with the red filter.

Weighed samples of fresh tissue (1 to 1.5 grams) were placed together with fine sand in small glass mortars containing a frozen paste of 10% trichloroacetic acid and thoroughly ground. The mortars were kept at 4° C for two hours with occasional regrinding following which the samples were quantitatively transferred to centrifuge tubes using small volumes of cold 5% trichloroacetic acid as rinse fluid. After cold centrifugation the residues were re-extracted four times with cold 5% trichloroacetic acid, the pooled extracts made alkaline (pH 8.2) with NH_4OH, $MgCl_2$ added to a final concentration of 4% and the extracts stored overnight at 4° C. No additional acid-soluble phosphorus was extractable upon further treatment with cold trichloroacetic acid. The inorganic phosphorus in the extracts had precipitated by the next morning and was separated from the acid-soluble organic compounds by centrifugation. Preliminary trials showed that no phosphorus-containing esters were precipitated by this treatment. The precipitates were washed four times with small portions of dilute (1:10) NH_4OH, and the washings were added to the acid-soluble organic fraction. Aliquot portions of the pooled acid-soluble organic fraction were wet ashed for total phosphorus. This fraction was not further characterized. The inorganic phosphorus was analyzed by the methods outlined by JUNI *et al.* (**10**), metaphosphate being precipitated at pH 4.5 to ensure the maximum yield.

The residue remaining after the extraction of the acid-soluble fractions was dried at 65° C, transferred to soxhlet extraction thimbles, and extracted for two hours with ethyl ether (25 to 30 complete extraction cycles). The ether extract was analyzed for lipid phosphorus. The fat-free residues were then extracted four times with 5% trichloroacetic acid for 10 minutes at 90° C and the extracts pooled to form the nucleic acid fraction. This procedure (**29**) apparently removed only nucleic acids since no other phosphorus-containing compound was detected. Desoxypentose was determined using diphenylamine reagent (**8**) with the resulting color checked for absorbtion maximum, and the desoxyribonucleic acid (DNA) was calculated as a tetranucleotide from standard curves prepared by the method of SCHNEIDER (**29**). Since, as reported by OGUR and ROSEN (**21**), hot trichloroacetic acid treatments will hydrolyze pentosans, the standard pentose tests are unreliable as measures of ribonucleic acid in plant tissues. For this reason ribonucleic acid phosphorus and ribonucleic acid tetranucleotide (RNA) were calculated as the difference between the total nucleic acid phosphorus and the DNA phosphorus. Inasmuch as any determinate errors in the diphenylamine test would affect the observed concentrations of both nucleic acids, this method may not be completely accurate. However, this modification of the Schneider procedure was subsequently compared with the OGUR and ROSEN

(**21**) method with excellent agreement between the two techniques with regard to separation and analysis of the nucleic acids of tomato stem tissues.

The fat-free and nucleic acid-free residues were wet-ashed and analyzed for phosphorus. This fraction contained only protein-bound phosphorus.

In preliminary trials during the development of this phosphorus fractionation scheme, the average percentage standard deviation was 2.5%. Recoveries of added phosphorus compounds to plant tissues was 96% to 103% with an average recovery of $\pm$ 3% from theory. In the studies reported at this time it was impossible to run a sufficiently large number of replicate analyses at any collection period to calculate standard errors. In an effort to ensure reasonable dependability of the results, tissue fragments from at least 75 plants were pooled at each collection and all fractionations were run in duplicate. In no case did the duplicates vary more than 5% from their mean. The sum of the phosphorus in each fraction was usually 3% to 5% below the total phosphorus values and in view of the number of transfers and dilutions, this does not seem to be excessive.

Manometric methods

Tumorous and control tissues were cut free-hand into thin (0.5 to 0.8 mm.) slices with a razor blade and transferred to water at room temperature. After all the tissue had been sliced the water was filtered off through a porcelain filter. This rinsing of the tissue removed most of the bacteria (**20, 27**) and also served to randomize the slices from a number of plants. The buffer consisted of half strength Kreb's Ringer solution (to supply ionic cofactors) + 0.01 M potassium phosphates adjusted to pH 5.3 for respiratory study and pH 6.0 for the study of fermentation. Hydrogen or nitrogen gas, previously passed over heated copper filings to remove oxygen, was bubbled through the fermentation buffer for 10 minutes before it was used. Approximately 600 mg. of fresh tissue and 3.0 ml. of the appropriate buffer solution were placed in the main compartments of 25 ml. Warburg vessels. Respiratory gas exchange was measured by a one-vessel modification of the Warburg direct method (**13**) with air as the gas phase. Fermentation (anaerobic respiration) was measured in oxygen-free nitrogen or hydrogen and any bound CO_2 was released by acid tipped in at the end of the run. All runs were made in duplicate at 28° C. Respiration and fermentation were followed for 120 and 90 minutes respectively. All tissues were dried at 110° C to constant weight. The duplicate vessels agreed to within 10% on a dry weight basis.

Results

Histological findings

Free-hand sections of normal and tumorous hypocotyl tissues made at each collection showed the same sequence of histological changes noted by Riker (**24**). Only rare cell divisions were noted in control or prospective

tumorous tissues during the first four to five days after puncture or inoculation. At the end of this time cells about the point of the wound were beginning to divide. This period is designated as the induction phase (IP) and is so indicated on all figures. The control plant sections showed that the number of new cells formed in the puncture zone subsequent to the induction phase was small and they were restricted to the periphery of the wound. Cell division stopped about eight days after wounding. These new cells simulated a wound phellem in that the cells were tabularly arranged but they did not show extensive cell wall suberization. In the inoculated plants, however, cell division continued and did not slow down until about 20 days after inoculation. This period of rapid tumorous cell proliferation is designated as the growth phase (GP). These tumorous cells do not constitute a typical callus because the cell divisions, which take place in all planes, were not preceded by extensive cell enlargement. During the growth phase the only new cells formed in the control hypocotyl tissues were those cut off by normal cambial activity, and the maturation of the control tissues proceeded in normal fashion.

Fourteen to 20 days after inoculation the tumor consisted almost entirely of small meristem-like cells, and it appeared that by this time the maximum number of tumorous cells per unit weight or volume of tissue had been formed. The third phase of tumor development was initiated at this time and is designated as the maturation phase (MP). During this period tumorous cell division decreased rather sharply and had almost ceased by the end of the study. Large numbers of tumorous cells, instead of dividing, were enlarging and maturing. The walls were undergoing secondary thickenings, dead elements (sclerids, wound tracheids, etc.) were seen, and necrobiotic changes were evident in the protoplasts. Nevertheless, the proportion of young cells was still considerably higher in the tumorous tissue than in the control tissue. In the control tissue, the number of new cambial derivatives decreased and maturation of the several tissues continued up to the end of the study.

Weight changes

Nagy *et al.* (**19**) and Link and Eggers (**15**) did not find any significant differences in the percentage dry weight between tumor and control at the time of their collections of material. In the experiments reported at this time, there was a distinct difference between these tissues after the fourteenth day of tumor development (fig. 1). Although the percentage dry weight of the tumorous tissues remained constant after this time, that of the control increased steadily until the close of the study. These differences in dry weight have been reported for old tumors and their controls by Klein and Keyssner (**11**) and would be expected if, as noted above, the tumor consisted of young cells while the control tissues showed considerable secondary thickening. It should be remembered, however, that these differences might not be observed if the control tissues remained quite succulent

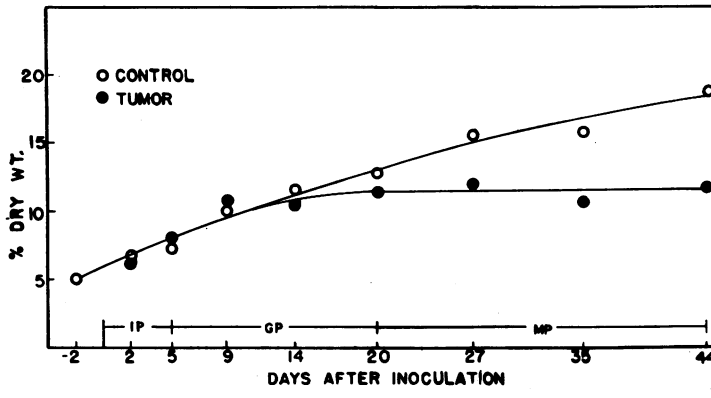

FIG. 1. Changes in the percentage dry weight of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

throughout the period of study. The relation of the dry weight to the calculations of gas exchange rates and to the levels of tissue constituents will be discussed later in this paper.

NITROGEN CHANGES

The relation of soluble and protein nitrogen to the time after wounding or inoculation is illustrated in figure 2. If there were any nitrogen changes during the induction phase in response to the wounding of the control plants, the changes were either too small to be detected by the analytical methods used, or the samples were too large to uncover purely local changes. The shapes of the curves for the protein and soluble nitrogen of the control tissues are typical of the changes in concentration to be found in normal tissues with increasing age when they are calculated to a dry weight base. When plotted on a fresh weight base, the curves showed a slowly rising level of nitrogen (or other) fraction with time.

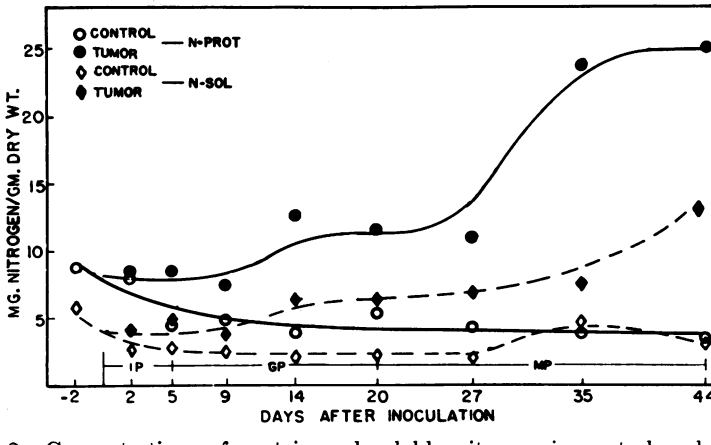

FIG. 2. Concentrations of protein and soluble nitrogen in control and tumorous tomato hypocotyl tissues during tumor ontogeny.

The major increase in protein nitrogen of the tumorous tissue occurred relatively late in development and appeared to be initiated at the time when the tumor changes from primarily numerical cell increase to cell volume increase. The soluble nitrogen showed the same pattern but the rate of accumulation was smaller. Prior to the present study, only the 35 to 44 day stages have been used for the reported differences in the nitrogen content of normal and tumorous tomato tissues (**11, 19**).

The relative proportion of protein to soluble nitrogen constituents during development is of considerable interest. KLEIN and KEYSSNER (**11**) reported a predominance of protein nitrogen in tomato tumors five to six weeks after inoculation while the opposite was true for control tissues. This appears to be true only during the period of tumor maturation since protein was not being preferentially synthesized during the main period of tumor growth. Since soluble nitrogen also increased in the tumor during the maturation phase, albeit at a slower rate than protein nitrogen, the synthesis of protein did not occur entirely at the expense of the available amino acids and amides. The stunted and nitrogen-deficient appearance of the tumorous plants would lead one to postulate that the nitrogen of the whole plant was being mobilized into the tumor.

NUCLEIC ACID CHANGES

The variations in the level of DNA in tumorous tissue during the period of study were quite striking (fig. 3). Two days after inoculation there was a peak in the level of DNA with no evidence of cell division about the point of inoculation. The recent work of SWIFT (**32**) suggested the possibility that there may be a duplication of chromatin in these pre-tumorous cells since in normal plant tissues the DNA per nucleus doubles preceding cell division. As no corresponding change was noted in the wounded control tissues, this increase in DNA may be the first indication of the subsequent burst of cell division noted during the early growth phase. However, the subsequent drop in the concentration of DNA is not consistent with this duplication hypothesis since, unless DNA was being destroyed, the curve should either form a plateau at this level or increase until the second peak was reached. It is possible that this initial, high DNA level represents some yet unknown activity of the pre-tumorous cells which may be directly related to the processes of tumor induction. The second peak in DNA level of the tumorous tissue is almost certainly due to the large numbers of cells per unit weight of tissue. Measurements of the relative amounts of DNA per normal and tumorous cell nucleus by the methods of SWIFT (**32**) at the time of the second peak (14 days after inoculation) did not show any significantly higher concentration of DNA in the tumorous cell nucleus as compared to the control (H. H. Swift, unpublished). The histologically observed enlargement and maturation of the tumorous cells subsequent to the growth phase would explain the drop in the DNA level since the number of cells per unit weight of tissue appeared to have decreased after the 14 to 20 day peak.

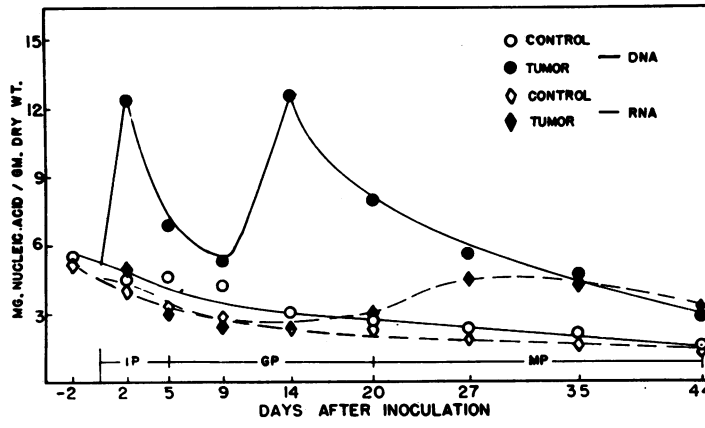

FIG. 3. Concentrations of desoxy- and ribonucleic acids in control and tumorous tomato hypocotyl tissues during tumor ontogeny.

Since the concentration of RNA in the tumorous tissues followed that of the control tissues during the induction and growth phases in spite of the obvious increase in cell numbers, it would appear that the cellular concentration of this nucleic acid does not remain constant during the period of tumorous cell multiplication (fig. 3). The rise in the RNA concentration in the tumorous tissue 27 to 35 days after inoculation occurs at the time protein accumulation is greatest and cell division had largely been supplanted by enlargement and maturation. This association of changes in the concentration of RNA and protein has been postulated as a causal relation by CASPERSSON (**6**) but has not been reported in higher plants. This problem will be discussed in another section of this paper.

The ratio DNA/RNA of the control tissue was approximately 1.0 to 1.2 throughout the mature life of the plant (fig. 4). Another indication of the constancy of a ratio of this type can be noted in the nucleo-cytoplasmic ratio of normal tissue as reported by RIKER (**26**) although the specificity of DNA and RNA in plant nuclei and cytoplasm respectively has not been established. In the tumor the ratio varied, reaching a peak of 4.8 at the end of the growth phase. The fall in the nucleic acid ratio during the mat-

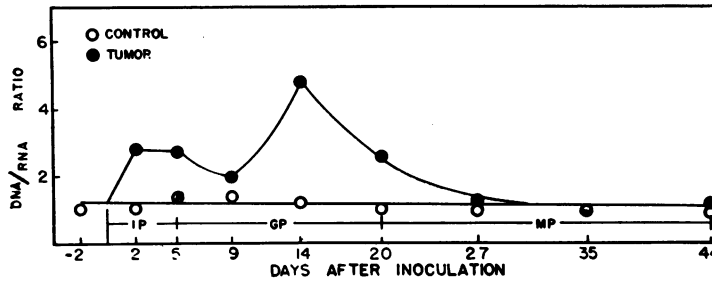

FIG. 4. Changes in the ratio: DNA/RNA of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

uration phase to that found in the control is, to some extent, a biochemical confirmation of the histologically observed maturation of the tissues. It is doubtful, however, whether this normal ratio indicates that the tumor can now be compared to controls of the same chronological age.

Phosphorus changes

The peak in the accumulation of total phosphorus in the tumorous tissue was observed during the late growth phase (fig. 5). This piling up of total phosphorus in crown gall tissues has been studied recently by Tsao and Whaley (**33**) using phosphorus 32 accumulation in Bryophyllum tumors. These authors also reported higher concentrations of total phosphorus in

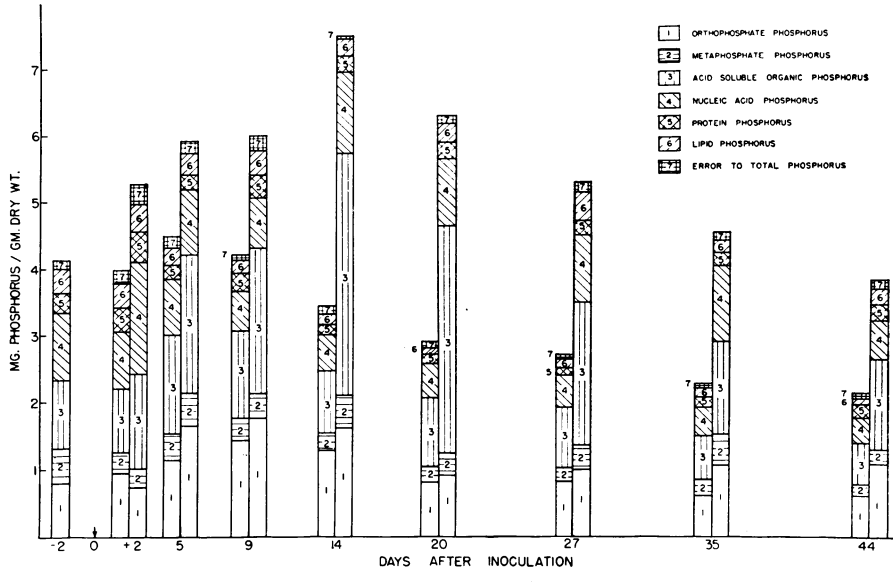

Fig. 5. Concentrations of various phosphorus-containing tissue fractions of control and tumorous tomato hypocotyl tissues during tumor ontogeny. Single bar at 2 days before inoculation (−2 days) represents normal hypocotyl tissues only. Left bar at all collection times represents fractions in control tissues; right bar represents tumorous tissues.

tomato stem tumor tissues as compared to normal tissues, their values closely approximating those reported here. When the total phosphorus values reported in the present study were calculated on a DNA base rather than per unit tissue weight in an effort to determine the relative concentrations of phosphorus per cell, the differences in the levels of phosphorus in control and tumorous tissue disappeared and the time course curves were practically superimposable.

The orthophosphate phosphorus content of the control tissue comprised about 25% of the total tissue phosphorus throughout the period of study. In the tumor, however, during the late induction and early growth phases,

there was some indication that the orthophosphate phosphorus became a somewhat greater fraction of total phosphorus than was true of the corresponding control. The orthophosphate contribution to the total phosphorus of the tumor then decreased to about 20% during the growth phase, and the proportion again rose to the control value towards the end of the maturation phase.

The presence of metaphosphate has been reported in fungi (*Aspergillus, Neurospora,* and yeasts) but has not previously been noted in the tissues of higher plants. The compound (or polymer) has not yet been assigned any specific role in metabolic processes. In the hypocotyl tissues of the controls, metaphosphate phosphorus remained a fairly constant fraction of the total phosphorus throughout the period of study. In the tumorous tissues, its relative importance decreased markedly as the total phosphorus content of the tissues rose. Whether this indicates that this little-studied fraction is of slight importance in the phosphorus metabolism of proliferating plant tissues or that it serves as a stable pool for other phosphorus-containing compounds must await further study. In view of the increase in cell numbers in the tumorous tissues, it is certain that metaphosphate did not remain constant in amount in tumor cells as compared to control levels.

The remarkable increase in the components of the acid-soluble, organic phosphorus fraction of the tumorous tissue is of considerable interest. During the late growth phase of tumor development this fraction, containing phosphorus esters, acid-soluble nucleotides, etc., comprised more than half of the total tissue phosphorus. When calculated to a DNA base instead of weight, there is good reason to infer that these components increase in amount per cell. It is doubtful whether this increase is due entirely to a block in the Meyerhof-Embden pathway since no decrease in the rates of tumorous tissue fermentation was observed. During the late maturation phase of tumor development the relative role of these compounds decreased until, at the end of the study, they formed approximately the same fraction of total phosphorus as was found for the controls. The detailed fractionation scheme developed for the analysis of these components by LEPAGE and UMBREIT (**14**) would be most useful in an analysis of this problem but could not be used in the present investigation.

Although the nucleic acid phosphorus and lipid phosphorus fractions remained reasonably constant as percentages of total phosphorus in both tumorous and control tissues during the period of study, there were small increases in the percentages of tumorous tissue protein phosphorus during the growth phase of development. Here the time dependent changes in the level of this component appeared to be more closely related to DNA (and hence cell numbers) than to the weight of the tissue. The rise in protein phosphorus of the tumor during the early induction phase, two days after inoculation, which is unrelated in increases in cell numbers, may be significant since BRAUN (**3**) has postulated that the tumor inducing principle may be protein in nature.

Gas exchange

The relative constancy of the respiratory quotient (R.Q.) of control hypocotyl tissues close to unity would indicate that throughout most of the mature life of this organ the primary respiratory substrate is carbohydrate (fig. 6). This corroborates the finding of Caldwell and Meiklejohn (**5**). In the present study the R.Q. was 0.92, 27 days after planting (22 days after seed germination) and then the R.Q. levels off at 0.97 to 0.99 when the plants were in the four to six leaf stage, 28 days after germination. The progressive modifications of the R.Q. of the slices of tumorous tissue suggests that the substrate for respiration was changing. The initial drop to 0.85 immediately after inoculation might indicate that protein was being oxidized but no data are available at this time to evaluate this finding. It is also possible that this R.Q. change is more closely related to an incomplete oxidation of carbohydrate with an accumulation of its end products (organic acids, etc.).

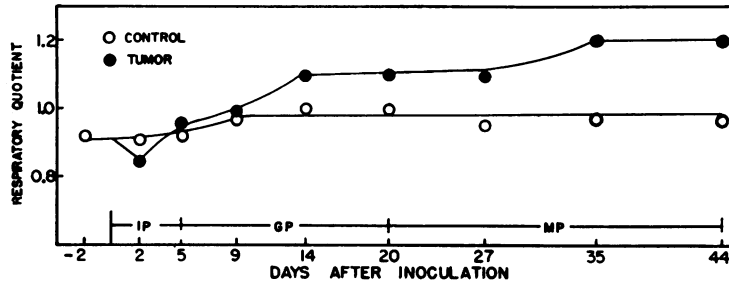

Fig. 6. Respiratory quotient of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

The subsequent rise in the R.Q. to 1.2 during the maturation phase would suggest the utilization of compounds in a higher state of oxidation than carbohydrate. Although the nature of this substrate is unknown, it is of interest that Klein and Keyssner (**12**) reported that five to six week old tomato tumors were more alkaline than control tissues and contained more titratable acidity. This suggests that there might be an accumulation of organic acids in the tumorous tissues which might be available for respiratory utilization. It should be remembered, however, that this high R.Q. may reflect a concomitant aerobic glycolysis of the tumorous tissues; but again, no convincing evidence is available.

The rates of gas exchange of control and tumorous tissue slices were plotted on weight, nitrogen, phosphorus, and nucleic acid bases in order to compare these standards as to significance and to gain an insight into the types of response of these tissues during their various phases of development. It must be emphasized that the gas exchange rates of isolated tissues cannot be assumed to represent the activities of these tissues in the environment of the intact plant.

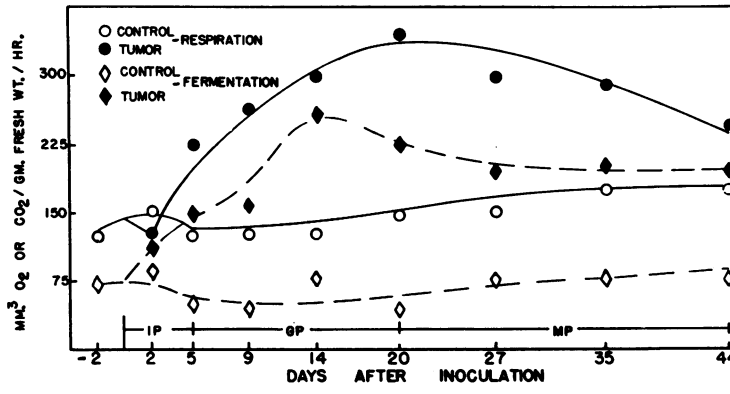

FIG. 7. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a fresh weight basis.

The curves for oxygen uptake (respiration) and anaerobic CO_2 production (fermentation) on fresh and dry weight bases (figs. 7 and 8) show that the gas exchange rates of tumorous tissues were considerably higher than those of the controls with the peak in respiration occurring at about the end of the growth phase. On both these bases there were small but significant decreases in tumor respiration immediately after inoculation and a slight increase in fermentation at the same time. The curves for oxygen uptake on a fresh weight base are quite similar to those reported by LINK and GODDARD (**16**). However, these authors did not find any decrease in oxygen uptake upon tumor initiation when they calculated rates on a fresh weight base. The steady decreases in gas exchange rates of control tissue slices calculated on dry weight standards is primarily a function of the increments of non-respiring elements (vessels, fibers, cell walls, etc.) which make up

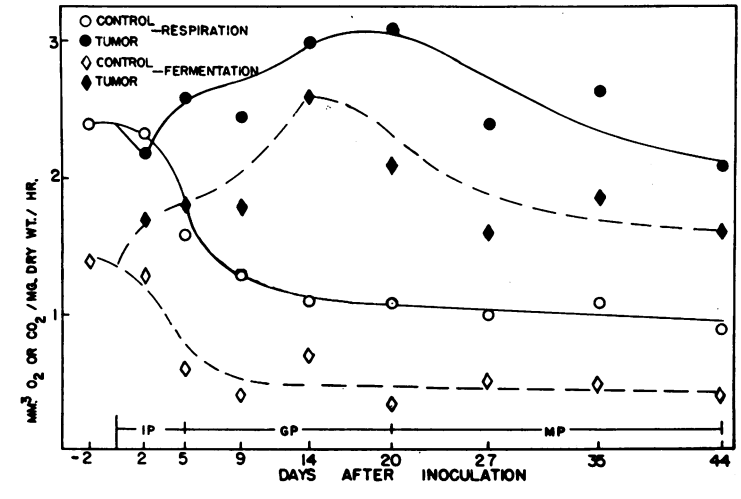

FIG. 8. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a dry weight basis.

increasingly greater fractions of the dry weight with increasing age of the tissues. When fresh weight was used as the reference standard, the ascending control curves during the period of study are related more to the changing number of cells per unit volume of tissue since the dead material, although increasing in amount, comprises a smaller fraction of the fresh weight than of the dry weight.

Nitrogen bases have been suggested and used for the calculation of the gas exchange rates of plant tissues because of the ease of determining this cell fraction and because of the assumption that nitrogen will represent living material. These views have been discussed by BURRIS and WILSON (4). The gas exchange rates of control and tumorous tissue slices have been plotted for total nitrogen (fig. 9), soluble nitrogen, and protein nitrogen. There was good agreement in the shapes of the curves when total and protein nitrogen were used as standards for calculation. The respiration of the

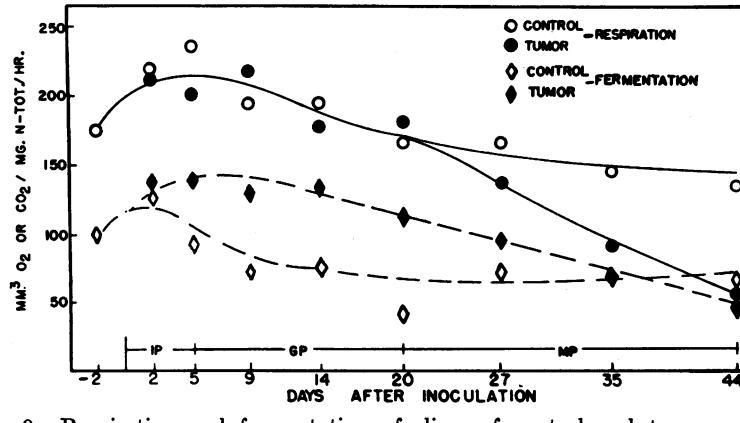

FIG. 9. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a total nitrogen basis.

tumorous tissue was identical to that of the control during the induction and growth phases after which the respiration of the tumorous tissue fell off sharply. It will be remembered that this was the time that the tumor was maturing and nitrogen, particularly protein nitrogen, had started to accumulate. On the other hand, the rates of fermentation remained higher in the tumor throughout most of the period of examination, descending to the control level only at the end of the study. If these were the only data considered, it would appear that the fermentation of tumorous tissue was accelerated while respiration was unaffected during the induction and growth phases, falling off during the maturation phase. When soluble nitrogen was the standard, the picture was more like that seen on weight bases.

Various phosphorus fractions were also used for determining gas exchange rates. When the rates of control and tumorous tissues were plotted to a total phosphorus base (fig. 10) it can be seen that both the respiratory and fermentative capacities of the tumorous tissue parallel those of the con-

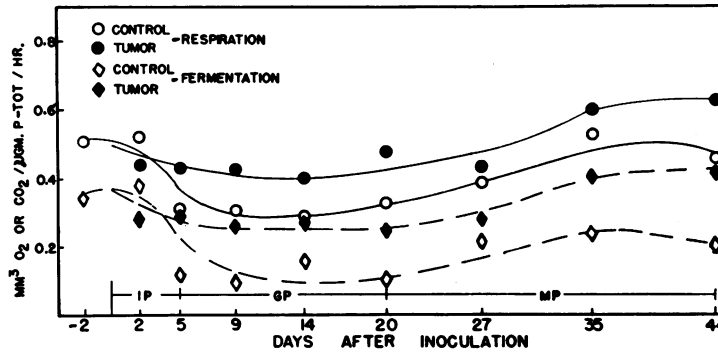

FIG. 10. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a total phosphorus basis.

trol tissues but are at somewhat higher levels. The striking similarity of the total phosphorus based curves to those employing a DNA standard (fig. 11) is particularly important. Since it was reported that DNA is reasonably constant per tomato cell nucleus, this nucleic acid should be an accurate measure of the number of living cells in any particular tissue sample. Therefore, these latter curves should compare the gas exchange rates of control and tumorous tissue on a more nearly cellular level than would be possible using any other biochemical characteristic of the tissue. Under the conditions of these studies (excluding the early induction phase values which are unrelated to cell numbers) the rates of gas exchange of tumorous tissue (tumorous cells) were parallel to but were greater than those of the control tissues (control cells) throughout most of the period of study. This elevated rate of gas exchange of the tumorous cell was initiated during the late induction phase, was maintained during the growth phase, and showed a further increase during the maturation phase. This secondary increase in gas exchange during the late maturation phase was also noted when total phosphorus was used as the reference standard. When protein phosphorus was used as the base, the gas exchange rates of the tumorous tissue were less

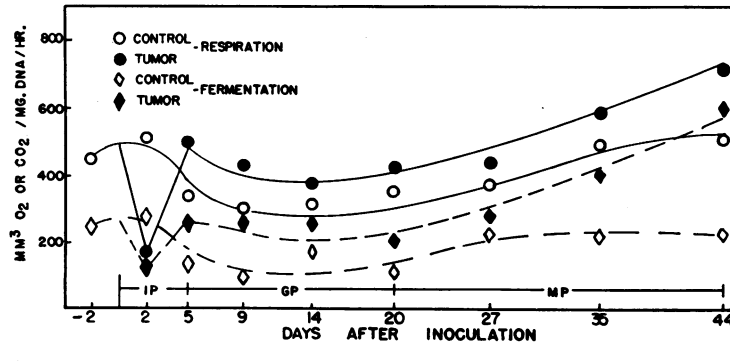

FIG. 11. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a desoxyribonucleic acid basis.

constant than those calculated to the other bases but the previously noted acceleration of the rates during the late maturation phase was evident.

Discussion

The patterns of the curves for gas exchange rates of the slices of control and tumorous hypocotyl tissues during the study varied considerably depending on the base used for the calculations. The problem remains as to the most adequate base for expressing gas exchange rates to reflect the metabolism of tissues which, in addition to being modified by normal aging processes, differ as a result of experimental procedure. If the concentration of DNA remains fairly constant per nucleus during most of the life of a cell and this nucleic acid is restricted to the nucleus, DNA would be the standard of choice. The use of nucleic acid standards for determining metabolic activities of small amounts of surviving tissue was suggested by BERENBLUM *et al.* (**1**) and used by DAVIDSON and LESLIE (**7**). The inadequacy of weight or nitrogen bases for this type of calculation is quite evident in a study of this kind in which there were definite weight differences during development of the control and tumorous tissues and nitrogen, particularly protein nitrogen, accumulated during the terminal stages of tumor development. Further, this accumulation of nitrogen, occurring at the time that DNA was decreasing in amount per unit weight of tissue, *i.e.*, the number of cells per unit weight were decreasing, indicates that the concentration of nitrogenous compounds per cell did not remain constant during tumor ontogeny.

Whether the striking correspondence in the sequential gas exchange rates of normal and tumorous tissues when based on total phosphorus and DNA constitutes evidence that total phosphorus was in the same concentration in control and tumorous cells must await further study. If this suggestion is borne out in future research, it may be possible to refer gas exchange (and possibly other metabolic activities) preferentially to a total phosphorus base, *i.e.*, $Q_{O_2}(P)$. This cell constituent is easily measured and determinations have the same accuracy as do those for nitrogen.

Gas exchange rates and nitrogen and phosphorus fractions, calculated to the various bases used in this study, were converted to percentages of the control as advocated by MITCHELL *et al* (**18**). Although the scatter of points was somewhat reduced, the results of these calculations are not reported at this time since the method of presentation does not permit rapid comparison with the results of other workers. Without exception the conclusions drawn from plots of these calculations were the same as those obtained by the more usual methods.

It is important to determine whether there were any specific and characteristic changes in gas exchange upon tumor initiation. The sharp rise in the DNA level at this time, which is unrelated to cell numbers, prevented comparisons on this basis. When weight, nitrogen, and total phosphorus data were considered, the respiration of the incipient tumorous tissue was somewhat depressed and fermentation was increased. Since it will be re-

membered that in this study the percentage dry weight and stages of development of control and incipient tumorous tissue were the same at this time, the conclusion that fermentation was increased and respiration decreased upon the initiation of the neoplastic state appears to be valid. As noted in the section on the R.Q., there was an even more pronounced depression in the rate of aerobic CO_2 production.

The biochemical behavior of the tumor during the induction phase is of particular importance since, as demonstrated by BRAUN (**2**), normal host cells have received maximum tumor-inducing stimulus during this period, some stimulation occurring even in 36 to 48 hours. Both protein and soluble nitrogen levels were elevated in this time and total phosphorus had also increased above the control level. Especially noteworthy were the increases in DNA and protein phosphorus of the incipient tumor. All of these modifications in the composition of the stimulated tissues occurring before cell divisions were evident. The relation of these changes and the modifications of gas exchange rates to the processes leading to the establishment of the tumorous state are, at present, unknown. In view of the high energy of inactivation of BRAUN'S tumor inducing principle (**3**), the increases in protein phosphorus, protein nitrogen, and DNA are suggestive.

The characterization of the tumor during the growth phase is most difficult. The respiration of the tumorous tissue (tumor cells) during this period is at a higher level than that of comparable control tissues (control cells) and the modifications in the R.Q. suggests that the primary respiratory substrate has become different than that of the control. The rates of tumor fermentation also were above control levels. The proportion of protein nitrogen to soluble nitrogen suggests that the dividing tumorous cell did not accumulate protein. No special changes were noted in the relative concentration of most of the phosphorus fractions although the production and/or accumulation of esters and acid soluble nucleotides was greatly accelerated. In attempting to evaluate the relations among protein nitrogen, RNA, and cell development, one is confronted by the lack of any direct experimental evidence for any more than a coincidental association of these factors in the tissues of higher plants. In a critical discussion of these phenomena, SPIEGELMAN and KAMEN (**30**) state: "While the experiments . . . show that protein synthesis or modification is associated with marked changes in nucleotide synthesis, none can be interpreted to exhibit a rigorous causal relation between the two processes." Thus it can only be stated that the association of these cellular components during the maturation phase of crown gall development parallels that found in other organisms. PETRIE (**22**) has reviewed the attempts to correlate protein synthesis in plants with changes in respiratory activity and concluded that, to date, these studies have not been marked with any great degree of success. Examinations of the curves relating to the changes in protein nitrogen of the tumorous tissue (fig. 2) and those concerned with the time-course of gas exchange calculated to total phosphorus (fig. 10) or DNA (fig. 11) standards show that at the time of

maturation of the tumor there were almost simultaneous increases in respiration and protein accumulation. This association of processes, too, cannot be definitely stated to be directed but is suggestive of more than a casual coincidence of a number of vital activities.

The significance of the sequential changes noted among the various factors and processes studied during the development of the control and tumorous tissues with regard to cause and effect are obscured by the lack of adequate information on their exact roles in the economy of the constituent tissues. Furthermore, the characterization of any of these developmental stages must be based on numerous structural, biochemical, and biophysical diagnostic aids since no one feature is capable of delimiting the facets of a growing, changing system or cell. Differences in structure, biochemical activity, and/or cell constituents are valid insofar as they are specifically related to a particular phase in the development of normal or pathological tissues. Therefore, the level or activity of any cellular character will be a function of the chonological and physiological age of the tissue and its stage of development in addition to the conditions imposed by the experiment.

Summary

1. In an effort to compare some of the biochemical properties of normal and tumorous tissues of the tomato hypocotyl and to correlate these findings with the modifications in structure that occur during the development of the tissues, a study has been made of the nitrogen and phosphorus compounds, the structure, and the respiratory intensities of these tissues from the time of the induction of the crown gall until the flowering of the plants when the tumors stopped growing.

2. During the first five days after inoculation or wounding of the tissues, *i.e.*, the induction phase of tumor development, only rare cell divisions were noted in either control or incipient tumorous tissues. No differences in the percentage dry weight were observed. By the end of the induction period, there were significant increases in both protein and soluble nitrogen fractions of the tumorous tissue but no modification of the ratio between these constituents. Immediately after inoculation, there were increases in the concentrations of desoxyribonucleic acid, and protein phosphorus in the inoculated tissues, and less marked increases in the concentration of the acid-soluble organic phosphorus compounds. In the wounded control plants, all nitrogen and phosphorus fractions examined showed the expected decrease in concentration when calculated on dry weight bases. Immediately after inoculation there was a slight depression in the rate of oxygen uptake of the pre-tumorous tissues as compared to the controls and a drop in the respiratory quotient from 0.92 to 0.85. There was, however, a pronounced increase in the rate of fermentation of the incipient tumorous tissues.

3. The period from the end of the induction phase until approximately 20 days after inoculation was marked structurally by rapid cell divisions in the tumorous tissue. By the end of this growth phase of tumor develop-

ment, there appeared to be the maximum number of tumorous cells per unit weight or volume of tissue. Cell divisions about the point of wounding of the controls had ceased a week after inoculation. The percentage dry weight of the control tissues continued to increase during this and the subsequent period of development but that of the tumorous tissues became established at a constant value by the end of the growth phase. There was some evidence of the accumulation of nitrogen in the tumorous tissue, although the ratio between protein and soluble fractions maintained itself at close to the pre-induction value. A peak in the concentration of DNA per unit weight of tissue was observed by the end of the growth phase but histochemical measurements indicated that the amount of this nucleic acid per tumorous cell nucleus was the same as that of the control. The production and/or accumulation of phosphorus esters and acid-soluble nucleotides was accelerated in the tumor during this period. The respiration rate of the tumorous cells paralleled that of the control cells and was at a higher level. The respiratory quotient of the control tissues was approximately 1.0 but that of the tumor rose to 1.2. The rates of fermentation of the tumorous cells paralleled those of the control and were also elevated.

4. The maturation phase of tumor development was structurally marked by tumorous cell enlargement and the formation of mature and dead elements. The drop in the ratio between the nucleic acids during this period to that found in the control is, to some extent, a biochemical confirmation of the histologically observed maturation of the tumorous tissues. There was an association among protein synthesis, ribonucleic acid, gas exchange, and cell maturation in that the concentrations and/or rates of activity of all of them increased at the same time.

This report is a continuation and extension of the studies on the metabolism of neoplastic plant tissues begun by Professor G. K. K. Link and is the third in a series of papers.

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