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*STUDIES ON THE REGULATION OF CERTAIN ESSENTIAL BIOSYNTHETIC SYSTEMS IN NORMAL AND CROWN-GALL TUMOR CELLS**

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A tumor cell that has acquired a capacity for autonomous growth is generally considered to be a permanently altered cell that reproduces true to type and against the growth of which there is no adequate control mechanism in a host. The question as to what makes such tumor cell types deficient in internal or unresponsive to the external control mechanisms that govern so precisely the growth of all normal cells within an organism is fundamental and constitutes the ultimate basis of the tumor problem generally. This problem appears now to have been largely resolved in the case of the plant tumor cell. Experimental findings reported in the plant field clearly demonstrate that as a result of the transformation of a normal cell to a tumor cell, a radical reorientation in synthetic activities occurs. This metabolic reorientation progresses from the precisely regulated metabolism concerned with differentiated function to one involving a permanently increased synthesis of the

nucleic acids, mitotic proteins, and other substances concerned specifically with cell growth and division.[1, 2] This shift in metabolism is triggered by irritation accompanying a wound. It may be permanently fixed by any one of several distinct types of tumorigenic agencies and it is maintained in the plant tumor cell by virtue of the fact that all of those agencies permanently activate a series of biosynthetic systems, the products of which are concerned specifically with cell growth and division. Biosynthetic activation in a plant tumor cell has been most thoroughly investigated in the non-self-limiting neoplastic disease known as crown gall. The results of those studies demonstrate that the transition from a normal plant cell to a fully autonomous crown-gall tumor cell is a gradual and progressive process.[3-5] By interrupting the transformation process at intervals by thermal treatment, it was possible to obtain tissues that show varying grades of neoplastic change ranging from slowly growing benign to rapidly growing fully autonomous tumor cell types. A physiological and biochemical study of such tissues has demonstrated that, as a result of the transformation of a normal plant cell to a fully autonomous tumor cell, a series of quite distinct but well defined biosynthetic systems become progressively and permanently activated.[6] It was suggested, moreover, that the degree of activation of those systems within a tumor cell determines the rate at which that cell type grows. An attempt was made in the present study to characterize, in part at least, the mechanism by which the diverse biosynthetic systems, which represent the entire area of metabolism concerned with cell growth and division, become progressively and permanently activated. It is with that problem that the present study is concerned.

Materials and Methods.—Normal cells, as well as the partially altered moderately fast growing and the fully transformed rapidly growing crown-gall tumor cells of *Vinca rosea* L. used in this investigation, have been maintained as stock cultures in this laboratory for more than five years. The three tissues were originally isolated according to methods previously described.[5, 6] The two types of tumor cells were routinely grown on White's basic culture medium[7] containing 1% agar and 2% sucrose. The normal cells were maintained on that medium supplemented with naphthalene acetic acid, 6-furfurylaminopurine, glutamine, asparagine, inositol, and cytidylic and guanylic acids.

In the experiments reported below, White's basic culture medium containing 1% thoroughly cleaned Difco bacto agar and 2% sucrose was used. Organic compounds and inorganic salts were added to the basic medium where desired at the following concentrations: meso inositol 100 mg/l, glutamine 200 mg/l, asparagine 200 mg/l, cytidylic acid 100 mg/l, guanylic acid 100 mg/l, naphthalene acetic acid 1 mg/l, 6-furfurylaminopurine 0.5 mg/l, KCl 845 mg/l, $(NH_4)_2SO_4$ 790 mg/l, $NaNO_3$ 1,800 mg/l, $MgSO_4 \cdot 7H_2O$ 1,000.0 mg/l, and $NaH_2PO_4 \cdot H_2O$ 300 mg/l. Solutions of glutamine, asparagine, cytidylic and guanylic acids, inositol, and α-methyl glutamic acid were sterilized by filtration and added aseptically to the culture medium. All other compounds were sterilized by autoclaving at 15 lb. pressure for 15 min. The pH of all solutions was adjusted to 5.5 with NaOH.

The normal and tumor tissues used in these studies were cut to a standard size, each piece a cube with sides approximately 4 mm in length. The experiments were carried out in 50-ml Erlenmeyer flasks, each of which contained 20 ml of the desired medium. All treatments were carried out in duplicate. The experimental

flasks were incubated at 25°C for 7 to 8 weeks and growth was measured on a wet-weight basis.

Experimental Results.—An attempt was made in this investigation to study the effects of mineral salts on the activation of biosynthetic systems in normal and two types of crown-gall tumor cells isolated from *Vinca rosea*. In these studies, two media were compared. The first of these was White's basic medium; the second, White's medium in which three salts, KCl, $NaNO_3$, and NaH_2PO_4, were added at levels indicated in the section above.

The fully transformed crown-gall tumor cell can utilize the mineral salts and sucrose in White's basic medium for its continued rapid growth. This cell type grown on White's basic medium was used as the standard in these studies. The partially transformed tumor cell, like the fully altered cell type, can also utilize the mineral salts and sucrose in the basic culture medium for its continued growth. However, the growth rate of such cells is only about one-half that of the fully transformed tumor cell type. The implication of this would appear to be that such cell types can synthesize all of the factors required for growth and division from mineral salts and sucrose in the basic medium but that one or more of those factors is limiting for rapid growth. It was found[6] that when White's basic culture medium was supplemented with an auxin (naphthalene acetic acid), meso inositol, and glutamine, growth of the partially altered tumor cell approached that of the fully transformed tumor cell type. These metabolites were found to be synthesized by both the fully transformed and the partially altered tumor cells when such cells were grown on White's medium. Inositol was found to be present in both types of tumor tissue following hydrolysis of those tissues with 6 *N* HCl and assaying the hydrolysate, the pH of which was adjusted to 5.5, with the inositol-requiring yeast *Kloeckera apiculata* according to the procedures of Ridgway and Douglas.[8] Inositol was found to be present at a level of 7 mg/100 gm wet weight in fully transformed tumor tissue and 4 mg/100 gm in partially transformed tumor tissue. The requirement of glutamine for growth of both the partially and the fully transformed tumor cell types was demonstrated with the use of α-methyl glutamic acid, a known competitive inhibitor of glutamine. Growth of both tissues was inhibited by α-methyl glutamic acid. The fully transformed tumor cell, however, was forty times more sensitive to that inhibitor than was the partially altered cell type. This inhibition was in both instances completely reversed by supplying glutamine to the α-methyl glutamic acid–containing culture medium.

That a kinin is synthesized by both types of tumor cells was demonstrated by isolation of that substance from such tissues and by its partial characterization. This will be the subject of a later communication. It is now well established that auxin is synthesized by crown-gall tumor cells, and, judging from the degree of hydration of the two tissues, the fully transformed tumor cells synthesize more auxin than do the partially altered cells.

Normal cells of the type from which the tumor cells were derived do not appear to synthesize any of those substances when planted on White's basic medium. Only when that culture medium was supplemented with an auxin, a kinin, meso inositol, glutamine, asparagine, and cytidylic and guanylic acids did the normal cells proliferate as rapidly as did the fully transformed tumor cells grown on White's basic medium. The relationship of growth of the three tissues on White's medium

and on that medium containing various organic supplements is shown in Figure 1.

When the partially transformed tumor cells and the normal cells were planted on a modified White's medium to which three salts, KCl, $NaNO_3$, and NaH_2PO_4 were added, a strikingly different picture was obtained, as shown in Figures 2 and 3. Growth of partially transformed tumor cells on that medium supplemented only with an auxin (Fig. 2) was significantly better than was the growth of such cell types on White's basic medium supplemented with an auxin, glutamine, and inositol (Fig. 1). It would appear, therefore, that the partially altered tumor cell types lost their exogenous requirement for rapid growth for both inositol and glutamine as a result of modifying White's basic medium by the addition of three salts. Yet, as can be seen in Figure 2, both inositol and glutamine remained limiting, since the addition of either of those substances to the medium increased somewhat the rate of growth of the tissues.

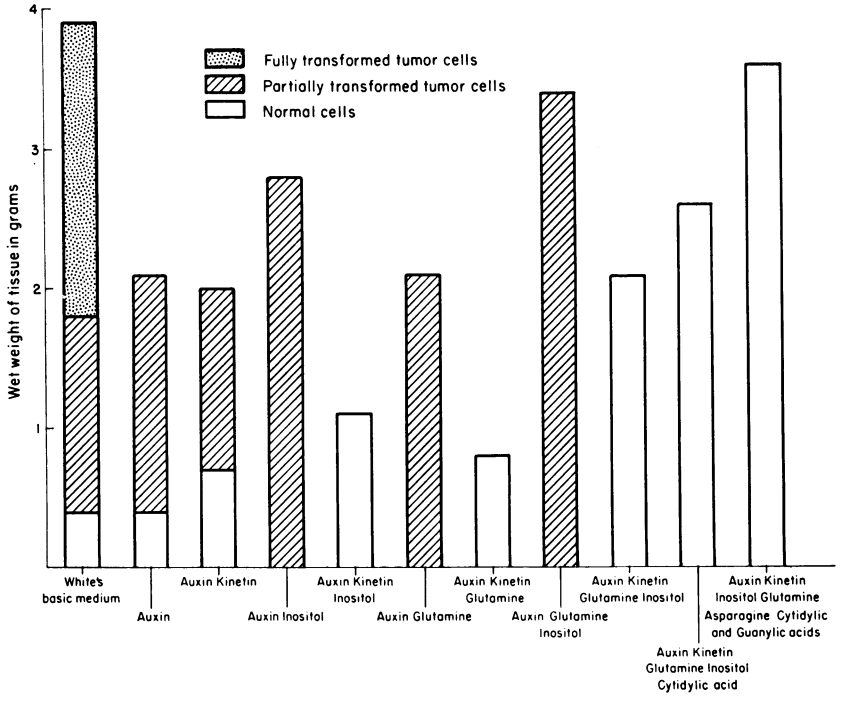

FIG. 1.—Fully transformed, partially transformed tumor tissue, and normal tissue grown on White's basic medium supplemented as indicated above.

The effect of modified White's medium on the growth of normal cells was even more striking. On that medium, the normal cells required only an exogenous source of an auxin, kinetin, and inositol to achieve a growth rate comparable to that obtained on White's basic medium supplemented with the seven substances found necessary for the rapid growth of such cell types. Thus, four of the seven exogenous organic requirements found necessary for rapid growth were eliminated by fortifying the basic medium with inorganic salts. This is shown in Figures 1 and 3.

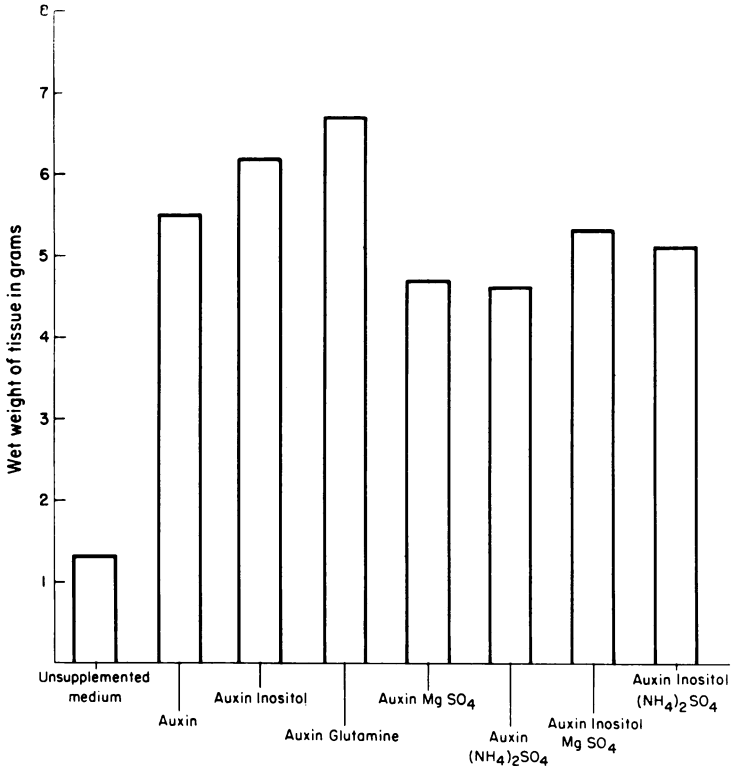

FIG. 2.—Partially altered tumor cells grown on White's basic medium fortified with KCl, $NaNO_3$, and NaH_2PO_4 and supplemented as indicated above.

Of particular interest in these studies was the finding that meso inositol played an important role in facilitating the uptake and/or utilization of ions by the normal cells. The increased salt level present in modified White's medium was largely without effect on the growth of normal cells unless inositol was present in the culture medium in addition to an auxin and kinetin. While it is true that some growth of the normal cells does occur on that medium with only auxin and kinetin supplements, this limited growth can perhaps be accounted for on the assumption that a small amount of inositol is synthesized by the normal cell types under those conditions or that a sufficient number of ions penetrate, by virtue of their increased concentration in the medium, to permit some growth of the normal cells to occur. That there is a relationship between the concentration of certain ions in the culture media and the amount of growth of the normal cells resulting from the addition of only an auxin and kinetin to the media is evident from the data shown in Figures 1 and 3.

The role of inositol in facilitating the uptake and/or utilization of ions was most dramatically illustrated when ammonium sulfate was applied to modified White's medium on which the normal cells were grown. In the presence of an auxin and kinetin, that salt had no effect or perhaps a slight inhibitory effect on the growth of the normal cells. Under similar cultural conditions but with added inositol, a very rapid proliferation of such cell types occurred. Growth on modified White's

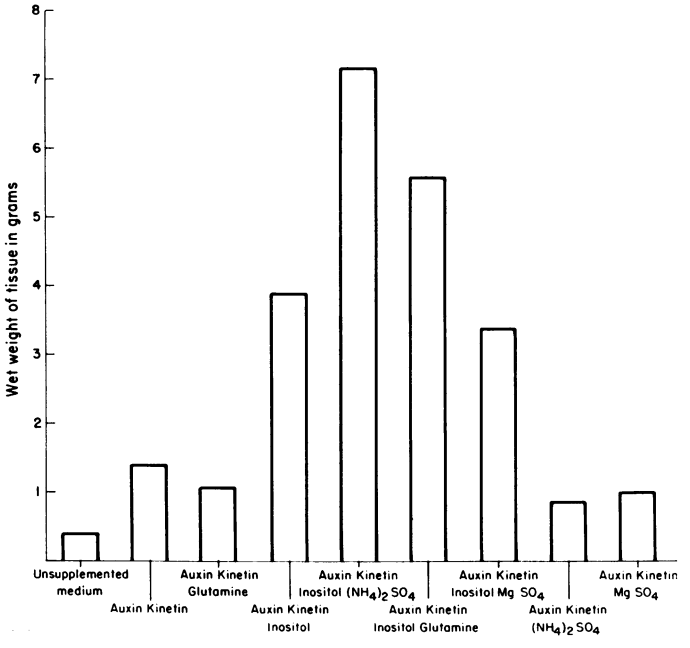

FIG. 3.—Normal tissue grown on White's basic medium fortified with KCl, $NaNO_3$, and NaH_2PO_4 and supplemented as indicated above.

medium in the presence of ammonium sulfate, an auxin, kinetin, and inositol reached almost twice the level of tissue grown under similar conditions but in the absence of that salt. That the ammonium ion rather than the sulfate ion was limiting for rapid growth in this instance is suggested by the fact that when $MgSO_4$ was substituted for $(NH_4)_2SO_4$, no significant effect on growth of the normal cell was observed over that found in comparable controls. Similar although somewhat less dramatic results were obtained when glutamine, another source of reduced nitrogen, was substituted for ammonium sulfate. The results obtained in these studies are summarized in Figure 3.

In contrast to the stimulatory effects that ammonium sulfate exerted on the growth of the normal cells, no such effect was observed on the growth of either the partially transformed or the fully transformed tumor cell types. The reason for this is not yet clear. The answer does not, however, appear to lie in fundamental differences in the ability of the normal cells and the two types of tumor cells to reduce nitrate nitrogen. The fully transformed tumor cell can very effectively reduce the nitrate nitrogen present in White's basic medium since such cell types grow and divide actively on that medium. The normal cells can apparently also do this but to a much more limited extent if the basic medium is supplemented with an auxin and kinetin or with those two substances and inositol. However, when White's medium is supplemented with auxin, kinetin, and inositol and fortified with KCl, $NaNO_3$, and NaH_2PO_4, the normal cells grow at a rate comparable to that of the fully transformed tumor cells on White's basic medium (Figs. 1 and 3). Since both tissues grow at approximately equal rates and since there is no reduced nitrogen in either medium, it appears that both types of tissue are capable of reduc-

ing nitrate nitrogen at essentially equal rates under their respective conditions of culture. The difference in the two tissues appears to lie in their respective abilities to transport rather than to utilize the nitrate ion.

Discussion and Conclusions.—An attempt was made in this investigation to characterize the mechanism by which certain biosynthetic systems shown to be essential for cell growth and division are regulated in normal and crown-gall tumor cells of *Vinca rosea*. The results obtained in these studies indicate that a number of the biosynthetic systems shown to be permanently unblocked in the plant tumor cell are ion-activated systems. Of particular interest in these studies was the finding that ion uptake and/or utilization was greatly facilitated by and probably dependent upon the availability of meso inositol. These findings suggest a physiological function for that compound in the experimental test system used here.

Fully transformed tumor cells appear to have a very effective ion-transport system. This is evidenced by the fact that such cell types can utilize ions efficiently when they are present at the concentration found in White's basic culture medium. Normal cells and to a lesser extent the partially transformed tumor cells require significantly higher levels of nitrate, phosphate, and potassium ions than are present in White's basic medium to synthesize certain essential metabolites in amounts required for their rapid growth. This suggests that those cell types have a less efficient ion-transport system than does the fully transformed tumor cell and that greater concentrations of certain essential ions are necessary to penetrate to the proper locus in such cell types. This information indicates that, as a result of the transition from a normal plant cell to a tumor cell, changes in permeability or ion-transport mechanisms occur. As a result of such changes, essential ions penetrate to the proper locus in a tumor cell but are unable to do so in a normal cell unless the concentration of such ions is raised significantly in the medium.

Reduced nitrogen in the form of ammonium sulfate greatly stimulated the growth of normal cells but not the two types of tumor cells used in this study. The reason for this is not yet clear. The answer does not, however, appear to rest on any fundamental difference in the ability of the normal cells and the two types of tumor cells to reduce nitrate nitrogen. In this instance, too, meso inositol greatly facilitated the uptake and/or possibly the utilization of the ammonium ion.

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