predict that fused, intact fragments of differing tissues will, by a spreading process, assume an organization identical with that achieved through autonomous segregation by intermixed cells of the same tissues. This prediction is substantiated. When viewed in the light of previous work by others, the results demonstrate that the mutual anatomical relationships of tissues in the organism must be determined in appreciable measure by the quantitative adhesive differences deduced from these observations, operating to minimize the surface free energy of the system.

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*ON THE ACTIVATION OF CERTAIN ESSENTIAL BIOSYNTHETIC SYSTEMS IN CELLS OF VINCA ROSEA L.**

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It has been found that as a result of the transition from a normal plant cell to a fully autonomous rapidly growing crown-gall tumor cell, a series of quite distinct but well defined biosynthetic systems, which represent the entire area of metabolism concerned with cell growth and division, become progressively and permanently activated.[1] This leads to the permanently increased synthesis by such tumor cell types of the nucleic acids, mitotic proteins, and other substances concerned specifically with growth accompanied by cell division.[2, 3] Included among the biosynthetic systems shown to be activated in the plant tumor cell are those involved in the synthesis of two growth-regulating substances one of which, an auxin, is concerned with cell enlargement while the other, a kinin, is mitogenic and acts synergistically with the first to promote growth accompanied by cell division.[4]

In addition, the systems concerned with the synthesis of the vitamin myo-inositol, glutamine, asparagine or aspartic acid as well as with purines and pyrimidines are permanently activated in the tumor cell types. The degree of activation of these biosynthetic systems appears, moreover, to determine the rate of growth of the plant tumor cells. These systems are precisely regulated in normal cell types.

By comparing fully autonomous tumor cells with normal cells of the type from which the tumor cells were derived, it was recently concluded that four of the seven biosynthetic systems shown to be permanently unblocked in the plant tumor cell are ion-activatable systems.[5] This, in turn, suggests that changes in membrane permeability or in ion-transport systems accompany the cellular transformation. As a result of such changes, essential ions penetrate to proper loci in tumor cells but are apparently unable to do so in normal cell types. Fully autonomous tumor cells utilize ions very efficiently when grown on White's basic medium; the normal cells do not.

An attempt was made in this study to characterize the nature of the mechanism involved in the permanent activation of the remaining three essential metabolic systems which are concerned, respectively, with the synthesis of auxin, myo-inositol, and kinin in the plant tumor cell.

Experimental Methods.—Normal cells of *Vinca rosea* L. were used in this study. These cells have been maintained in culture on White's basic medium[6] fortified with 1 per cent Difco agar, 2 per cent sucrose, and supplemented with naphthalene acetic acid 1 mg/l, kinetin (6-furfurylaminopurine) 0.5 mg/l, myo-inositol 100 mg/l, glutamine 200 mg/l, asparagine 200 mg/l, cytidylic acid 100 mg/l, and guanylic acid 100 mg/l. With the exception of naphthalene acetic acid and 6-furfurylaminopurine, which were incorporated directly into the medium and sterilized in an autoclave at 15 lb pressure for 15 min, organic substances were sterilized by filtration and added aseptically to the sterile culture medium.

In the experiments reported below, White's basic culture medium containing 2 per cent sucrose and 1 per cent Difco bacto-agar that had been cleaned by washing thoroughly with distilled water, 95 per cent alcohol, and acetone, was used. The organic supplements, where used, were added at the concentrations indicated above. Inorganic salts were added where desired at the following concentrations: KCl 845 mg/l, $NaNO_3$ 1,800 mg/l, $NaH_2PO_4 \cdot H_2O$ 300 mg/l, and $(NH_4)_2SO_4$ 790 mg/l. The pH of all solutions was adjusted to 5.5 with NaOH.

The experiments were carried out in 50-ml Erlenmeyer flasks, each of which contained 20 ml of the desired medium. The normal *Vinca* tissues used in these studies were cut to a standard size, each piece a cube with sides approximately 4 mm in length. Treatments were generally carried out in triplicate and the experiments were repeated at least twice. The experimental flasks were incubated at 25°C in diffuse light for a period of about 7 weeks. Growth was measured on a wet-weight basis.

Experimental Results.—Activation of the auxin system: It has long been recognized that auxin, the plant hormone concerned with cell enlargement, plays a central role in the establishment of a capacity for autonomous growth of the plant tumor cell. It is generally believed, moreover, that most normal cell types require an exogenous source of auxin for growth. Fully autonomous crown-gall tumor tissue, on the other hand, grows profusely and synthesizes not only large amounts of auxin but also all other factors required for growth and cell division when planted on

White's basic culture medium. It was of interest to learn, therefore, how the biosynthetic system responsible for the production of that essential growth hormone is permanently activated in the plant tumor cell. In studying that question an attempt was made to determine the method by which the auxin synthesizing system may be activated in normal cells of the type from which the tumor cells were derived. This was done by comparing the growth of normal *Vinca* cells on White's basic medium and on that medium containing various combinations of essential organic substances and inorganic salts. The results of certain pertinent aspects of those studies are shown in Figures 1 and 2.

These experiments clearly demonstrate that normal *Vinca* cells grow profusely without an exogenous source of auxin if White's basic medium is fortified with four salts, KCl, $NaNO_3$, NaH_2PO_4, and $(NH_4)_2SO_4$ as well as with kinetin and inositol (Fig. 2, *D*). Growth of such tissues was poor, on the other hand, when the basic medium itself was supplemented with kinetin, kinetin and inositol, or with those substances and $(NH_4)_2SO_4$ (Fig. 1, *C*, *D*, and *E*).

The beneficial effect of ions in excess of those found in White's basic medium on the growth of normal *Vinca* cells is evident from the pictures shown in Figures 1 and 2. Nevertheless, some growth of the normal cells did occur on the basic medium supplemented with kinetin or with kinetin and inositol. This suggests either that such tissues synthesized very small amounts of auxin under those conditions of culture, or that sufficient amounts of auxin were carried over with the inoculum to permit a limited amount of growth to occur.

Since normal *Vinca* cells grow profusely on an appropriate culture medium in the absence of an exogenous source of auxin, an attempt was made to determine whether such tissues could be carried through a number of successive transfers on an auxin-free medium without a reduction in the growth rate of the tissues. Such tissues have now been carried through ten successive passages at 1-month intervals on White's medium supplemented with kinetin, inositol, KCl, $NaNO_3$, NaH_2PO_4, and $(NH_4)_2SO_4$ without any indication that a reduction in the growth rate of such tissues had occurred. It can, therefore, be assumed that those tissues do not require an exogenous source of auxin for their continued rapid growth on an auxin-free culture medium.

The question as to whether such tissues actively synthesized an auxin under those conditions of culture was examined. In those studies tobacco pith parenchyma tissue was used as the test object. The cells of such tissue possess an absolute exogenous requirement for auxin for cell enlargement, and for an auxin and a kinin for growth accompanied by cell division. Fragments of tobacco pith tissue approximately 1 cm long and 5 mm wide and high were isolated from the middle third of large tobacco plants and planted with their long axis in contact with White's basic medium fortified with 1 per cent agar and 2 per cent sucrose. Normal *Vinca* tissue that had grown on the above auxin-free medium for four passages was cut to about the same size as the pith tissue fragment and was placed on the upper surface of the pith tissue. The normal tissue was fed by placing a slice of White's agar medium supplemented with kinetin, inositol, KCl, $NaNO_3$, NaH_2PO_4, and $(NH_4)_2SO_4$ on the upper surface of the tissue. About 3 weeks after the experiment was initiated, a pronounced callusing response was observed at the physiological base of the pith tissue fragments. In those studies two sets of controls were used.

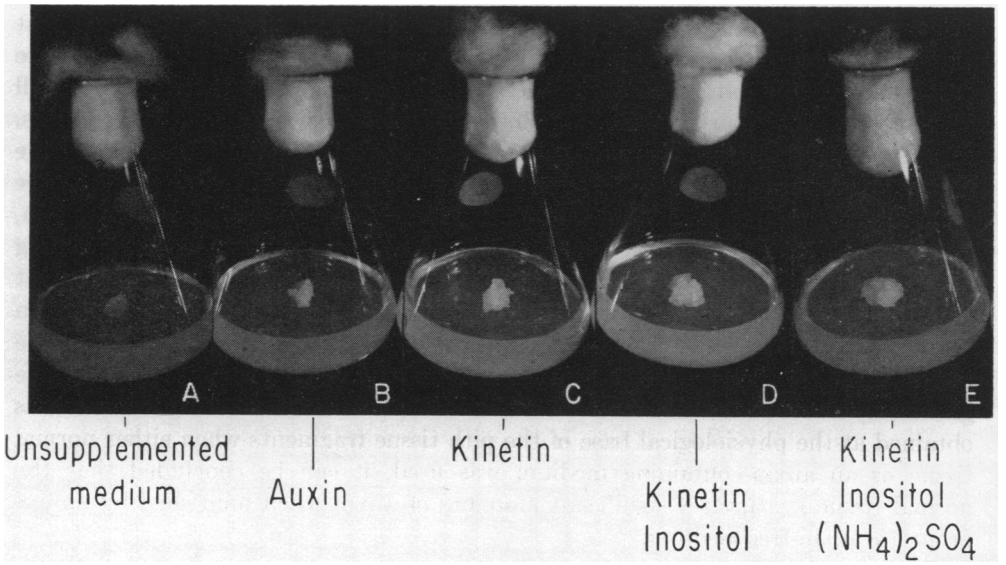

FIG. 1.—Normal *Vinca rosea* tissue grown on White's basic medium supplemented as indicated above.

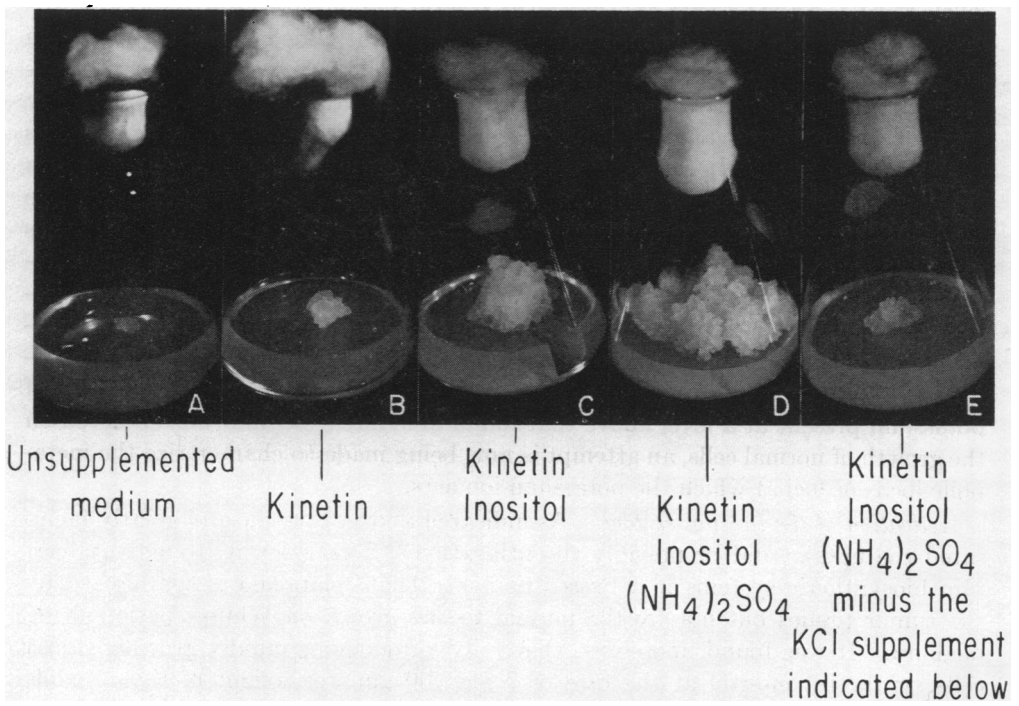

FIG. 2.—Normal *Vinca rosea* tissue grown on White's basic medium fortified with KCl, $NaNO_3$, and NaH_2PO_4 and supplemented as indicated above.

In both sets pith tissue was isolated as indicated above and planted on White's medium. In place of the normal *Vinca* tissue used in the studies above, a slice of White's agar medium was used. On the upper surface of the agar in the first set

of controls was placed a slice of the supplemented White's agar of the type that was used to feed the normal tissue. In that series no callusing was observed at the basal end of the pith fragment. Microscopic examination revealed that cell divisions had not occurred there. Such tissue turned brown and died in 2–3 weeks. The second set of controls was set up in a manner similar to that described for the first set of controls except that the upper layer of agar contained an auxin in the form of naphthalene acetic acid at a concentration of 1 mg/l in addition to kinetin, inositol, KCl, NaH_2PO_4, $NaNO_3$, and $(NH_4)_2SO_4$. The top layer of agar containing the growth substances was renewed every 4–5 days in all instances. In the last set of controls callus responses similar to, but not so pronounced as, those found in the series of tests in which the normal *Vinca* tissue was used, were observed at the physiologically basal end of the pith tissue fragment. Since tobacco pith tissue grows only in the presence of an auxin and since a pronounced callusing response was obtained at the physiological base of the pith tissue fragments when either normal tissue or an auxin-containing medium was used, it can be concluded that the normal tissue synthesizes significant amounts of auxin when nourished by an appropriate auxin-free medium.

The beneficial effect that the ammonium ion exerted on the growth of normal cells was striking. Upon addition of that ion to the basic medium containing elevated levels of KCl, $NaNO_3$, NaH_2PO_4, as well as kinetin and inositol, growth was approximately twice that found on a similar medium without added $(NH_4)_2SO_4$. It was pointed out previously[5] that the uptake and/or possibly the utilization of the ammonium ion by the normal cell types was greatly facilitated by and was probably dependent upon the availability to the cells of myo-inositol. The present studies have demonstrated that the potassium ion also plays an essential role in nitrogen metabolism in this system. This is illustrated in Figure 2, *D* and *E*. As can be seen from the legend accompanying Figure 2, the media used in those two instances were the same except that the KCl supplement was eliminated in *E*. Sodium chloride at the same molar concentration was ineffective in replacing the KCl requirement. It should be recalled that the fully autonomous tumor tissue grows profusely on White's basic culture medium and thus utilizes very efficiently the potassium and other ions present in that medium. Since such a striking effect of potassium present at a level above that found in White's medium was observed on the growth of normal cells, an attempt is now being made to characterize the metabolic locus or loci at which the potassium ion acts.

Activation of the inositol system: As indicated above, myo-inositol greatly facilitated the uptake and/or possibly the utilization of ions essential to activate certain biosynthetic systems in *V. rosea* tissues. That compound is synthesized by the tumor tissues but not by the normal tissues grown on White's basic culture medium. It was found, moreover, that fully autonomous rapidly growing tumor cells synthesize inositol at the rate of 7 mg/100 gm wet weight of tissue, while partially transformed moderately fast growing tumor cells synthesize about 4 mg/100 gm wet weight of tissue.[5] It was further observed in those studies that normal *Vinca* cells grow on White's medium supplemented with auxin, kinetin, KCl, $NaNO_3$, and NaH_2PO_4. The growth of the cells on that medium, although slow, was about three times as great as the growth of similar cells on White's basic medium supplemented only with an auxin and kinetin. Since the normal tissues

used in those studies were routinely cultured on an inositol-containing medium, the amount of growth observed in the above instances may have resulted from residual inositol that was carried over in the tissue fragments used in the experimental studies. An attempt was, therefore, made to learn whether the normal cells synthesize inositol in the presence of auxin, kinetin, and elevated salt levels or whether perhaps the inositol requirement was circumvented by raising the level of three salts in the basic culture medium. In order to study that question normal *Vinca* tissue was carried through ten passages at 1-month intervals on White's basic medium containing naphthalene acetic acid, kinetin, and increased levels of KCl, $NaNO_3$, and NaH_2PO_4. The rate of growth of the tissues at the end of ten passages was somewhat better than that found in the first passage. Thus, no reduction in growth rate was observed. Assays for inositol, using the method of Ridgway and Douglas[7] and the inositol-requiring yeast *Kloeckera apiculata* as the test organism, showed that the amount of inositol present in the tissues after the second passage on an inositol-free medium and after the sixth passage was the same. In both instances, approximately 2 mg of inositol per 100 gm wet weight of the tissues were obtained. It can be concluded, therefore, that myo-inositol is synthesized by the normal tissues under appropriate conditions of culture but not in amounts required for optimal growth. When the culture medium used above was supplemented with myo-inositol, excellent growth of the tissues occurred. These studies suggest that increasing the levels of certain ions in the basic culture medium favors an increased synthesis of myo-inositol by the normal cells. However, unlike the other biosynthetic systems shown to be activated by ions, it has not been possible to achieve an optimal synthesis of inositol by manipulating either the type of ion or the concentration of ions in the culture medium. It, nevertheless, appears likely that the inositol synthesizing system is also, either directly or indirectly, an ion-activatable system. The evidence reported earlier[5] demonstrated that myo-inositol was somehow rather specifically concerned with ion-uptake and/or possibly utilization in this system. Those studies also suggested that ion-uptake was quite inefficient in the presence of suboptimal levels of available inositol. Since the rate of the ion-uptake appears, within limits, to be a function of the concentration of available inositol, and since the normal cells synthesize suboptimal amounts of that substance under the conditions of culture used, it appears likely that not enough ions penetrate to the proper locus in the normal cell to activate fully the inositol synthesizing system. Normal *Vinca* cells appear to take up ions poorly and only by increasing significantly the level of certain ions in the culture medium was it possible to partially activate the inositol synthesizing system. This would appear to be a relatively inefficient method of making ions available to a cell when compared with the highly efficient capacity of the fully transformed tumor cells to utilize ions present at a concentration found in White's basic culture medium.

The kinin synthesizing system: Normal *Vinca* cells appear to possess an absolute exogenous requirement for kinetin or a naturally occurring kinin for growth. It has thus far not been possible to activate the kinin synthesizing system in normal cell types by manipulating either the type of ion or the concentration of ions in the culture medium. Since that system appears to be solidly blocked in normal *Vinca* cells, it may perhaps be the biosynthetic system that is specifically activated by the tumor-inducing principle responsible for the transformation of normal plant cells

to crown-gall tumor cells. If that is true, then either the activation of the kinin synthesizing system also accomplishes the fundamental changes observed in the capacity of the cells to take up and/or possibly utilize ions as described above, or the tumor-inducing principle exerts a dual function and accomplishes both types of cellular change independently of each other. On the other hand, the kinin synthesizing system, like the other biosynthetic systems described above, may be an ion-activatable system but the methods used in this study were not adequate to demonstrate that possibility. It should be recalled that the properties of the membranes found in the tumor cell appear to be quite different from those found in normal cell types.

Discussion and Conclusions.—The results of studies reported here together with those published earlier[5] may be interpreted to indicate that six of the seven essential biosynthetic systems shown to be permanently unblocked in the crown-gall tumor cell are, either directly or indirectly, ion-activatable systems. Only the activation of the metabolic system concerned with the synthesis of the mitogenic hormone, kinin, cannot as yet be accounted for on that basis.

These studies indicate, then, that changes in membrane permeability or in ion-transport systems accompany the cellular transformation. Such changes would appear to represent a most fundamental difference between a normal plant cell and a crown-gall tumor cell, since they permit the activation by ions of a large segment of the metabolism concerned specifically with cell growth and division. These studies may also give insight into the mechanism by which such biosynthetic systems are regulated in cells of normal plants. It is interesting to speculate, on the basis of work reported above, that the mechanism involved in the activation of the auxin synthesizing system by normal cell types in culture may be similar to that concerned with the synthesis of large amounts of auxin by cells in the meristematic regions of actively growing roots and shoots in an intact plant.

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