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A plausible model for reversal of neoplastic transformations in plants based on multiple steady states

(phenotypic reaction switch)

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ABSTRACT **We offer a plausible interpretation of some experiments on the reversal of neoplastic transformations in plants. We suggest that normal cells and tumorous cells represent multiple stable-steady states corresponding to a reaction feedback mechanism. The (autocatalytic) feedback loop is constructed from observations on the role played by *myo*-inositol: it increases the permeability of ions through the membrane and the biosynthetic pathway to *myo*-inositol is activated by ions. Provided that the permeabilities of nutrients (sugars and salts) are a product-enhanced function of *myo*-inositol, then we have a (oversimplified) model that can exhibit multiple stationary stable states, one or two depending on the exogenous nutrients and *myo*-inositol concentrations, and reversible and irreversible transitions from one of these states to the other are possible. From this model, straightforward simple experiments are suggested. We also propose that recent models dealing with the intracellular calcium regulation by hormones, where one key step requires the hydrolysis of inositol phospholipids, take into account free *myo*-inositol and endogeneous hormone concentrations (e.g., auxins).**

Hairy root disease and crown gall tumors are neoplastic growth on plants incited by virulent strains of the soil bacteria *Agrobacterium rhyzogenes* and *Agrobacterium tumefaciens*, respectively. These processes are associated with the incorporation in the plant host cells of large plasmids (Ri and Ti plasmids, respectively) harbored by these bacteria. In both cases these plasmids contain two regions necessary for tumorigenesis. They include the transferred DNA (T-DNA) region and the *vir* (virulence) region. The T-DNA contain genes that (*i*) specify the oncogenic phenotype (through hormone biosyntheses) and (*ii*) direct the production and secretion of low molecular weight, tumor-specific compounds called opines (e.g., octopine and nopaline). As a result, the transformed plant cells can grow in sterile cultures without addition of the phytohormones auxins and cytokinins (1, 2).

Most examples of reversal of the crown gall tumorous states studied (3–9), rarely capable of rooting and sensitive to surinfection by *Agrobacterium*, have turned out to be explained by either deletion (10–13) or methylation of part or the totality of T-DNA sequences (14, 15). Hairy root tumors induced by *Agrobacterium* strains carrying mannopine Ri plasmids can, however, spontaneously regenerate plants, albeit with altered morphology, that still contain and express T-DNA genes (16–19).

Striking formal similarities exist between crown gall transformation and habituation. The term habituation refers to the loss of a requirement for externally supplied phytohormones by plant tissues and cells in culture (20). Habituation is a form of neoplastic transformation involving heritable, progressive changes in cell phenotype that can result in autonomous growth. However, habituation occurs in the absence of a recognizable infectious agent and appears to have an epigenetic basis; it is reversible at rates much faster than expected for back mutations (21, 22).

From these observations, a fundamental question arises: How can cells that appear to have the same genetic constitution (host genome plus plasmid) exhibit different physiological and metabolic states (different phenotypes)? It was already proposed (23–28) that behaviors of this type may find their basis in the intrinsic properties of interdependent cellular regulatory mechanisms, with some kind of feedback loop; as a result, the mechanism may exist in alternative steady states (bistability). These loops may occur in homogeneous (bio) catalysis when a reaction intermediate triggers its own synthesis (product activation) or inhibits its own degradation (substrate inhibition). The coupling of a reaction mechanism occurring within a membrane to the concentration-dependent permeabilities of the substrates can also generate a feedback loop (29–31). Some documented cases of epigenetic differences have been found in microorganisms (24, 32–34) that result in a divergence from a common initial situation and persist indefinitely in numerous states of regime. An analysis of a Monod–Jacob model for induction and repression (enzyme β-galactosidase) has shown that for a wide variety of enzymic systems with several types of "cross-feedback" it is possible to find alternative stable states, each corresponding to functioning of one of the pathways and inhibition of the others. Thus if the system finds itself in a situation where one of the pathways has a head start, due to temporarily metabolic advantages, all other pathways are inhibited permanently (35).

The fact that habituation mimics the malignant transformation processes and has an epigenetic basis leads to the proposal that tumorigenesis is a form of nonappropriate differentiation involving cellular mechanisms encountered in the normal development (36). Meins and Binns (37) have proposed a heuristic model incorporating (autocatalytic) regulatory processes for the case of cytokinin-habituated explants of tobacco pith parenchyma (Havana 425). The habituated state is maintained by a positive feedback loop in which cytokinins induce their own synthesis or inhibit their degradation. In this hypothesis, the primary explant requires the presence of exogeneous cytokinins for growth; when the external concentration exceeds a threshold value, the cell turns to the habituated state and remains in that state as long as the feedback mechanism is not interrupted. It is thus suggested that the habituated phenotype is maintained by regulatory metabolic systems able to generate alternative steady states. The predictions of such an hypothesis were confirmed unequivocally by experiments: (*i*) the treatment of competent tissues with cytokinins above a critical threshold concentration (1 nM) induces cytokinin habituation (38); (*ii*) cells depleted of cytokinins by blocking cytokinin production momentarily, either by incubating cloned, cold-sensitive

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tissue at 16°C or by subculturing tissues on medium containing low concentrations of auxin, can return to the nonhabituated state. Tissues incubated on high-cytokinin medium under nonpermissive conditions remained habituated when assayed under permissive conditions, whereas tissues incubated on low-cytokinin medium shifted to the nonhabituated state. Moreover, tissues in this state regained their habituated character when incubated on high-cytokinin medium under permissive conditions (39).

Evidence also exist to show that in plant cells certain regulatory molecules induce their own production—e.g., ethylene by flower tissue of morning glory (40), cyclic AMP by aggregating cells of the cellular slime mold *Dictyostelium discoideum* (41), and auxin by cultivated explants of *Nicotiana glauca* × *langsdorffii* hybrid (42); also the flowering stimulus in certain photoperiodic plants has self-perpetuating properties (43).

In complementary studies on normal and crown-gall tumor cells of *Vincea rosea*, Braun and co-workers (44–46) made an attempt 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. The fully transformed tumor cells can utilize the mineral salts and sucrose in a basic culture medium for its continued rapid growth. The partially transformed cells can also utilize mineral salts and sucrose for continuous growth but with a rate only about one-half that of the fully transformed cells. When the basic culture medium is supplemented with an auxin, inositol, and glutamine, the growth of these latter cells approaches that of the fully transformed 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 the basic medium. Only when that culture medium is supplemented with an auxin, a kinin, inositol, glutamine, asparagine, and cytidylic and guanilic acids do the normal cells proliferate as rapidly as do the fully transformed tumor cells grown on the basic medium. On a basic medium modified by addition of three salts—i.e., KCl, $NaNO_3$, and NaH_2PO_4—the normal cells require only an exogeneous source of auxin, inositol, and kinin to achieve a growth rate comparable to that obtained on the basic medium supplemented with the seven organic substances found necessary for the rapid growth of such cells. Thus, four of the seven exogeneous organic requirements (glutamine, asparagine, cytidylic, and guanilic acids) found necessary for rapid growth were eliminated by fortifying the basic medium with organic salts. Of particular interest was the finding that inositol plays an important role in facilitating the uptake and/or utilization of ions by the normal cell: the increased salt level present in the modified medium is largely without any effect on the growth of normal cells unless inositol is present in the modified medium in addition to auxin and kinin. Taken together, these observations suggest that changes in membrane permeability or in ion transport systems accompany the cellular transformation (47). Fully autonomous tumor cells utilize ions very efficiently; the normal cells do not. In normal cells the synthesis of inositol is at a very low level but can be increased with low efficiency by increasing the mineral salt concentration. Such changes 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.

Recent studies on the anabolism and catabolism of phytohormones indicate sophisticated control and regulation processes occurring in the cell (48). As a simplification we focus on the role of inositol. The experimental results on crown gall tumor and observations on inositol lead us to suggest the abstraction of a particular, but obviously not solitary, feedback loop: the rate of production of inositol increases with internal mineral salt ion concentrations, and the permeability of these ions in turn is an increasing function of inositol concentration. If we write the mechanism in the simplest possible way, that is

$$\text{S (sugars and salts)} \rightarrow \text{P (inositol)},$$

and require the permeability of S, h_S, to be a product-enhanced function of P, then we have a model system that has been described in detail (ref. 27; see also Fig. 1 *A* and *B* and its caption). Multiple stationary stable-steady states exist, one or two depending upon the exogeneous concentrations S_0 and P_0, and reversible and irreversible transitions from one of these states to the other are possible. At low values of S_0 and P_0 there exists only one branch of stable states, which we label 1*, and which we assume to correspond to the least proliferating cells, the normal cells. For these cells, the concentration of inositol inside the cell, P, is low. If P_0 is held fixed at its low value and S_0 is increased (Fig. 1*C*), there is some increase in P, but then there occurs a point at which a transition from one branch of steady states (1*) to another (3*) becomes possible, and P increases sharply. This branch of stable states (3*) has a high concentration of P and can therefore proliferate rapidly. We identify this branch with the most proliferating, autonomous cancer cells. The model further predicts that (*i*) at fixed P_0, exceeding a critical value, there exists only one branch of stable states (3*) (Fig. 1*C*, upper curve); (*ii*) at low value of S_0 (held fixed) there are two branches (1* and 3*) available with reversible transition between them achieved by increasing P_0 (1* → 3*) (Fig. 1*D*, case 1); (*iii*) at high value of fixed S_0 there exists only the branch (3*) (Fig. 1*D*, case 3); (*iv*) at intermediate value of fixed S_0 (Fig. 1*D*, case 2) there are two stable branches, but only the irreversible transition (1* → 3*) occurs on increasing P_0: once (3*) is attained, then on decreasing P_0 the system remains on (3*).

These predictions suggest a number of straightforward experiments on normal and cancerous cells. Can a transition from cancerous to normal cells be achieved by decreasing salt and sugar concentrations (S_0)? Can a transition from normal to cancerous cells be achieved by increasing S_0? What changes, if any, are attained by changes in the external inositol concentration (P_0)?

This proposed positive feedback loop model centered around the *myo*-inositol moiety, and resting upon global experimental observations, is an extreme simplification of a likely much more complex mechanism. The *myo*-inositol moiety is a key molecule involved in an increasingly complex network of metabolic interconversions (49). Essentially, there are three potential sources of cellular inositol: uptake from the medium, *de novo* synthesis, and recycling of inositol-containing compounds such as inositol phosphates and inositol phospholipids. Most cells appear to transport inositol via a very-low-affinity, passive mechanism, whose precise characteristics and specificity have not been fully defined as yet. Inositol is synthesized from glucose 6-phosphate by the way of inositol 1-phosphate (inositol-1-phosphate synthase, NAD^+ dependent), which is further dephosphorylated by a specific monophosphatase. This pathway is the sole synthetic route that links carbohydrate metabolism to cyclitol formation in the plant kingdom. Inositol is the precursor of all other naturally occurring inositols. D-Glucuronic acid is formed from inositol. The UDP derivatives of glucuronic acid may be further converted to UDP-galacturonic acid and hence to pectins (oxidative pathway). Methylated inositols are thought to be precursors of methylated sugars in plant cell walls and involved in their modification. The complex galactinol is formed by combination of inositol with galactose. Surprisingly, inositol also forms enzymatically reversible

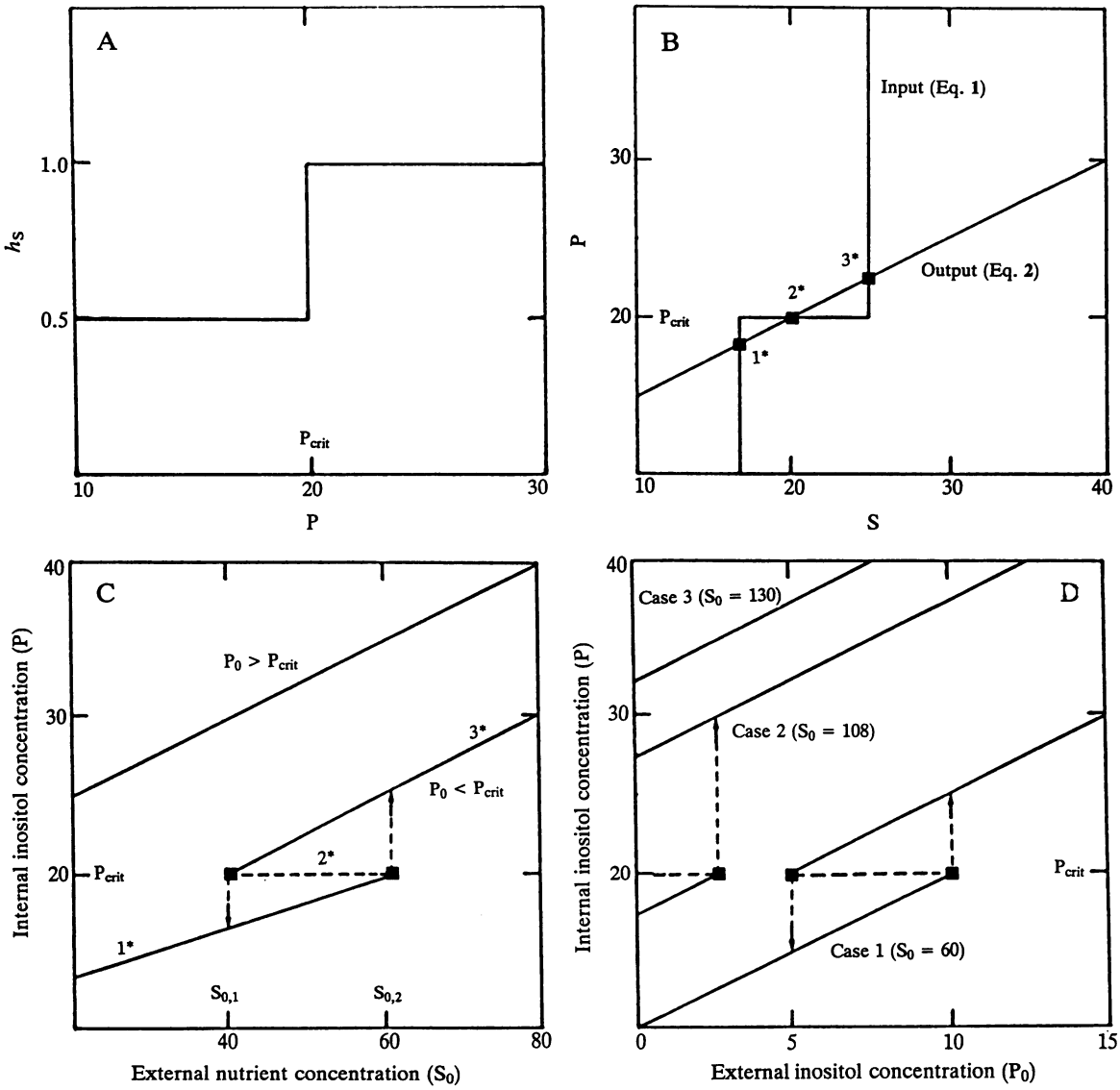

FIG. 1. The model system consists of two species, S and P, with an irreversible reaction, S → P, and a net reaction rate, W, such that $W = kS$. The system is open to mass transfers by diffusion, and the rates of change of the two species are

$$\frac{dS}{dt} = -W + h_S(S_0 - S) \text{ and } \frac{dP}{dt} = W + h_P(P_0 - P), \quad [1]$$

where S_0 and P_0 stand for the (constant) external concentrations. The permeabilities for S and P are denoted h_S and h_P, respectively. We assume for all steady states that $S_0 > S$ and $P_0 < P$. Let the permeability of S be a function of concentration of P, $h_S = h_S(P)$ and take h_P to be constant. One example of cross-correlation dependence of the permeability of S on P may have the functional form

$$h_S(P) = h_S(\infty) + \frac{h_S(0) - h_S(\infty)}{1 + (P/P_{crit})^\gamma}, \quad [2]$$

with $h_S(0) = 0.5$, $h_S(\infty) = 1.0$, $P_{crit} = 20$, and $\gamma = \infty$ (see *A*). With the form of $h_S(P)$ as shown in *A*, we plot the input–output lines in concentration phase space (steady-state conditions obtained from Eq. 1 are shown in *B*, with $k = 1$, $S_0 = 50$, $P_0 = 10$, and h_P = constant = 2). Three steady states are possible for this system (■). The existence of multiple steady states results in typical hysteresis, as shown in *C* in its dependence on S_0 and P_0: if the system is prepared in the region $S_0 < S_{0,1}$ ($S_0 > S_{0,2}$) and S_0 is slowly increased (decreased), then the system makes a discontinuous transition 1* → 3* (3* → 1*) when S_0 passes beyond an upper (lower) critical value $S_{0,2}$ ($S_{0,1}$). The functional dependence of the steady state on the external concentration P_0 demonstrates three general types of behavior, including the important case of irreversible transitions (*D*): Case 1 (S_0 = 60 and all other parameters as in *B*), there is hysteresis behavior analogous to the dependence of P on S_0 (*C*). Case 2 (S_0 = 108), as P_0 is increased from zero, a transition from the lowest to the uppermost branch occurs at a given value; if, however, the system is prepared in a steady state at P_0 exceeding that value, and then P_0 is decreased, the reverse transition does not occur. Case 3 (S_0 = 130), there is only one branch of steady state available to the system.

esters with auxin (indoleacetic acid) (50) and is the precursor of the complex glycolipids—i.e., inositol phospholipids—such as the second messengers inositol 1,4,5-triphosphate and diacylglycerol involved in the hormonal-mediated Ca^{2+} mobilization (51, 52).

Thus, *myo*-inositol appear to be directly and indirectly involved in reaction mechanisms of cellular processes being altered during malignancy. From this, we suggest that during experiments dealing with the measurements and physiological effects of (phyto)hormone balances (mainly auxins and

cytokinins), inositol phospholipid breakdowns and the related Ca^{2+} movements, glycolytic fluxes, and the variations in the *myo*-inositol concentrations should be determined. Recently, a molecular model accounting for the cytosolic Ca^{2+} bistability and spiking was described (53). It may be of interest to include explicitly the concentration of *myo*-inositol in the inositol 1,4,5-triphosphate/phosphatidylinositol 4,5-bisphosphate cycle and reconsider the dynamic potentialities of the system. Then, the effect(s) of varying auxin concentrations (autocrine production and indoleacetic acid–inositol complexation) can be evaluated. This latter aspect may in turn help in answering the issue about the metabolic and physiological implications due to the simultaneous and "additive" autocrine production and receptor-mediated hormonal actions.

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