second, upper peak represents those nuclei in G_2 and M, with a spectrum of S-phase nuclei between these two peaks.

The nuclear DNA contents of a variety of monocotyledonous and dicotyledonous plants are presented in Table 1. These data are in broad agreement with the results of studies in which Feulgen staining was used. Exact correspondence would not be expected, since considerable variability can be observed in the nuclear DNA values obtained with different lines or cultivars of the same species by identical Feulgen staining. In contrast, the data obtained for haploid and diploid *Nicotiana* species with flow cytometry show the predicted doubling of the nuclear DNA content in G_1. Furthermore, in a single species the G_2 peak always appears at exactly twice the DNA content of the appropriate G_1 peak, and the coefficients of variation for the 2C DNA peaks of the various species (Table 1) compare favorably with those obtained with animal cell lines (*13*).

These observations, coupled with a complete absence of artifactual nuclear adhesion (peaks corresponding to the adhesion of three or more nuclei were never observed), lead us to conclude that mechanical chopping does not result in significant DNA hydrolysis or in nuclear degradation or adhesion of intact nuclei liberated by this procedure. The reproducibility of measurements for different individuals of a single species was extensively investigated in *Nicotiana*. The standard deviations of these determinations fall in the range of 4 to 6 percent. This compares to a range of 6.2 to 39.8 percent attainable by microspectrophotometry with Feulgen staining (*2*). Figure 2 shows the binding characteristics of the mithramycin-DNA complex and their analysis by double-reciprocal plotting. The data indicate a single class of binding sites, half-saturated at a mithramycin concentration of 32 μg/ml. Fluorescence was abolished by treatment with deoxyribonuclease (1 mg/ml) for 30 minutes at 37°C, but was unaffected by ribonuclease under the same conditions.

The power of the technique is further illustrated in Fig. 3. The nuclear DNA status of 11 sequential leaves (counting from the apex) of a single *Nicotiana tabacum* was analyzed. The complete analysis, from greenhouse to histograms, required less than 2 hours. The results clearly show the occurrence of a prolonged period of DNA synthesis during leaf maturation after leaf expansion has become complete. Since neither mitotic figures nor binucleate cells were observed in leaf sections that were fixed and observed by conventional microscopy (*2*), we conclude that the process of leaf maturation includes the development of a significant population of cells arrested in G_2.

This method is directly applicable to (i) measurements of nuclear DNA degradation during leaf senescence as a function of leaf age, (ii) measurements of DNA synthesis in tissues in which infiltration with [^{3}H]thymidine is inappropriate or impossible, (iii) measurements of possible positions of cell cycle arrest in response to micronutrient or phytohormone deprivation, (iv) measurements of endopolyploidy in storage tissues, (v) correlations of ploidy level with cell cycle status, photosynthetic efficiency, and plant yield in isogeneic polyploid series, (vi) measurements of the distribution of evolutionary polyploid series in ecology and taxonomy (*7, 14*), and (vii) a systematic, accurate determination of 2C nuclear DNA levels in many, if not all, mono- and dicotyledonous plant species.

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10. The minimal convenient sample size was 12 mg chopped in a volume of 0.2 ml of buffer. The coefficients of variation for the G_1 peaks of nuclei prepared in this manner were similar to those obtained with larger tissue samples.
11. For measurement of nuclear DNA contents, chicken red blood cells were used as internal standards. Normally, the amplification and high voltages of the flow cytometer photomultipliers were adjusted for each species such that the G_0/G_1 peaks were located wherever possible, toward the upper ends of the 256-channel histograms. This maximizes the accuracy of measurement of the position of the fluorescence peaks. The absolute DNA contents of the chicken red blood cells were determined by the method of K. Burton [*Biochem. J.* **62**, 315 (1956)] in which calf thymus DNA is used as the standard.
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15 November 1982; revised 14 January 1983

Desuppression of Leaf Primordia of *Plagiochila arctica* (Hepaticae) by Ethylene Antagonists

Abstract. *Silver nitrate, α-aminooxyacetic acid, and aminoethoxyvinylglycine, three potent inhibitors of ethylene synthesis and action, induced the same kind of phenovariation in the liverwort* Plagiochila arctica *Bryhn and Kaal (Hepaticae) as do antagonists of the synthesis of hydroxyproline-containing protein. This finding (i) supports the hypothesis that hydroxyproline-protein has a role in ethylene-mediated suppression, (ii) provides evidence that the role of ethylene in the correlative development of leafy liverwort gametophytes may be similar to its role in flowering plants, and (iii) contributes to the characterization of a morphoregulatory system that mediates cellular suppression in leafy liverworts and possibly all land plants.*

A series of experiments with leafy liverworts (*1–10*) led to a new hypothesis concerning the morphoregulatory role of a cell-surface protein sensitive to antagonists of the synthesis of hydroxyproline-containing protein (hyp-protein). According to this hypothesis, the cell-surface protein plays a pivotal role in suppressing cellular development in local populations of cells such as leaf and branch primordia. Furthermore, changes in the time or location (or both) of the synthesis of this protein during ontogeny (heterochronous changes) could account for some of the phylogenetically significant differences in leaf and branch morphology of leafy liverworts. Some idea of the different positions of the cell populations (primordia) that may be influenced in liverworts can be seen in Fig. 1. We refer to the hypothesis concerning both morphogenetic action and phylogenetic implications as the suppression hypothesis (*7–10*).

Suppression of cellular development is commonly associated with the phytohormone ethylene. Ridge and Osborne (*11, 12*) suggested that the inhibitory action of ethylene involved hyp-protein. They had found that ethylene-induced sup-

pression of internode development in the pea was correlated with a precocious increase of hydroxyproline in walls of the suppressed cells. We have now found a way of similarly correlating the action of ethylene and hyp-proteins in suppressing leaf and branch primordia of leafy liverworts.

Potent inhibitors of the biosynthesis and action of ethylene have been discovered. We tried three of these—silver nitrate (*13*), α-aminooxyacetic acid (*14, 15*), and aminoethoxyvinylglycine (*15*)—in an initial test of the Ridge-Osborne hypothesis. The plant used in our experiment was *Plagiochila arctica* Bryhn and Kaal. A series of concentrations of each antagonist was tried, and the most effective was used in two subsequent experiments. In both experiments, each of the above-mentioned compounds was injected through the stoppers (*16*) in separate sets of six replicate cultures. Aside from the compounds used, all experimental procedures and culture conditions were the same as those described for experiments with *P. arctica* (*7*) (see legends to Figs. 1 and 2 for concentrations of antagonists found effective).

Although the influence of a single treatment with any of the ethylene antagonists was not as persistent as that observed with hydroxyproline (*7*), all three antagonists consistently induced the same kind of changes in the pattern of morphogenesis. That is, leaves developed from all three ranks of merophytes and leaf primordia instead of just from two of the three. Branch types that may develop in the position of leaves in some taxa (*3, 6*) (Fig. 1), but normally do not in *P. arctica*, also developed. Figure 2 provides a basis for comparing the influence of ethylene antagonists and hyp-protein antagonists with respect to desuppressing leaf primordia. Comparable effects on branching and histological organization at the shoot apex will be published. These examples should suffice to show that ethylene antagonists and hyp-protein antagonists (*8, 17–19*) have a common effect—suppressed primordia are desuppressed.

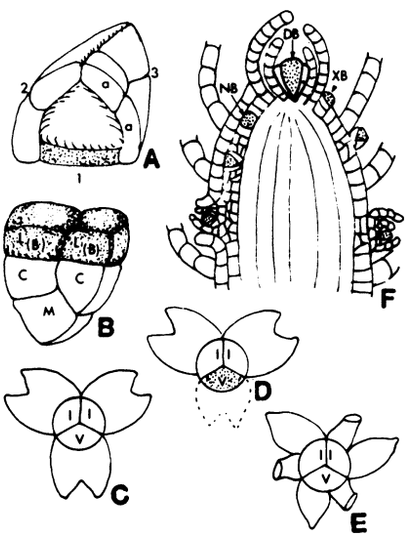

Fig. 1. Diagrams showing aspects of the initiation and distribution of primordia (stippled) influenced by antagonists of ethylene or hyp-protein (or both). (A) Apical cell with three segments (merophytes) "cut off" in sequence. In plants with suberect to creeping growth habits, the merophytes toward the substratum are termed ventral (*v*), the others lateral (*l*). Each merophyte divides to form two (A2), then three cells (A1→A3)). The two anterior cells (*a*) may divide again. (B) Five-celled merophyte resulting from divisions just described with the developmental fates of each cell indicated: medulla (*M*) and cortical (*C*) initials of stem tissues, paired leaf (*L*) initials, and terminal branch (*B*) initial that may differentiate from either of the two leaf initials. (C) Symmetry resulting if all three merophytes contribute equally to stem and leaf development. (D) Symmetry resulting if ventral leaf primordia are completely (no leaf) or partially (reduced leaf in dashed outline) suppressed. (E) Relative positions of branches that may develop from half a leaf primordium but that are normally suppressed in *P. arctica*. (F) Branching not initiated from leaf primordia: dichotomous branching (*DB*) initiated from apical cell, endogenous branches (*NB*) from internal cortical cell, and exogenous branches (*XB*) from superficial stem cells.

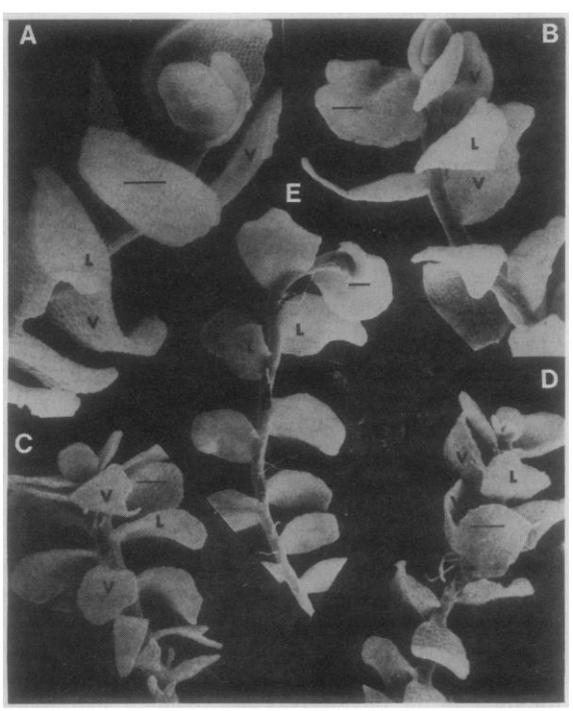

Fig. 2. Photomicrographs of phenovariant and normal plants of *Plagiochila arctica* Bryhn and Kaal. (A) Phenovariant induced by $AgNO_3$ (10 μg/ml), lateral view. (B) Phenovariant induced by α-aminooxyacetic acid (15 μg/ml), lateral view. (C) Phenovariant induced by aminoethoxyvinylglycine (3.5 μg/ml), ventral view. (D) Phenovariant induced by hydroxyproline (5 μg/ml), lateral view. (E) Normal plant, ventral view. Often, the only way to distinguish lateral leaves (*L*) from ventral leaves (*V*) in phenovariants is the occasional occurrence of rhizoids (*r*) in proximity to, or papillae (*p*) in the position of leaves (see Fig. 1, C and D). These two structures normally only develop in the ventral position in *P. arctica*. The only way to know which antagonist was used to induce any of the phenovariants is by knowledge of the experimental conditions. Scale bars on leaves each represent ~ 0.2 mm.

We have isolated an antigen (*10*) that may be useful in immunohistochemical procedures for measuring changes in hyp-protein in response to ethylene antagonism in the minute branch and leaf primordia of leafy liverworts.

There are three important implications of these results. First, they add to the still meager evidence (*20, 21*) that ethylene plays a role in the correlative development of liverworts similar to its role in the development of flowering plants. Second, they support the original proposal by Ridge and Osborne that hyp-proteins may be involved in ethylene-mediated cellular suppression. Third, they provide further evidence that developmental capacities manifest in some members of a taxon but not normally expressed in others may not be "lacking" or "lost," but merely suppressed.

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6 October 1982; revised 20 December 1982

A Parathyroid Hormone Inhibitor in vivo: Design and Biological Evaluation of a Hormone Analog

Abstract. *A synthetic analog of bovine parathyroid hormone (bPTH), [tyrosine-34]bPTH-(7–34)NH_2, was found to inhibit parathyroid hormone action in vivo. When the analog and parathyroid hormone were infused simultaneously to rats at a molar ratio of 200 to 1, the analog inhibited the excretion of urinary phosphate and adenosine 3′,5′-monophosphate. When infused alone at the same dose rate, the analog was devoid of agonist activity. The compound was prepared by following design principles developed for inhibitors of parathyroid hormone, and is believed to be the first antagonist of parathyroid hormone that is effective in vivo.*

Parathyroid hormone (PTH) is a single-chain polypeptide of 84 amino acids which, through its action on kidney and bone, serves a critical role in mineral ion homeostasis. Elucidation of the complex regulatory events involved in mineral ion homeostasis and the physiological role of PTH would be facilitated by availability of an inhibitor of PTH effective in vivo. Hypersecretion of PTH by the parathyroid glands occurs commonly in humans (*1*); the complex diagnostic and therapeutic issues that this disorder poses provide considerable impetus for the development of antagonists of PTH action in vivo.

All of the structural determinants necessary for full biological activity of PTH reside within the NH_2-terminal one-third (residues 1–34) of the native hormone, as has been determined in vitro and in vivo in multiple assays of PTH-stimulated effects on concentrations of 3′,5′-adenosine monophosphate (cyclic AMP) and fluxes in calcium and phosphate (*2, 3*).

Over the last decade, extensive studies of the structure and activity of PTH have delineated within the hormone molecule separable domains responsible for receptor binding and activation (*3–6*). There is an address core, or domain, responsible for receptor binding (that is, the 3–34 sequence) and a small but critical message region (positions 1 and 2) necessary for hormone action. These studies led to the design and synthesis of bovine parathyroid hormone (bPTH) analogs that are potent antagonists in multiple assay systems in vitro (*3*). An analog of the 3–34 sequence, with norleucine (Nle) and tyrosine (Tyr) as substitutions, [Nle^8 , Nle^{18} , Tyr^{34}]bPTH - (3–34)NH_2, proved to be the most potent in vitro antagonist of PTH yet designed (*5*). In vitro, this compound is a true competitive inhibitor of PTH that possesses an avidity for PTH receptors comparable to that of PTH and displays an inhibitory constant (K_i) approximately equal to the affinity constant (K_m) for PTH (*5, 6*). In vitro, this analog is devoid of PTH-like agonist activity (*3, 5, 7*). However, when tested in vivo (*8, 9*), [Nle^8, Nle^{18}, Tyr^{34}]bPTH-(3–34)NH_2 failed to display inhibitory properties; in other investigations in vivo, the analog demonstrated weak, but definite, PTH-like agonist properties in the dog (*10*), the rat (*11*), and in a canine isolated perfused bone system (*12*). Taken together, these studies revealed this 3–34 analog to be an agonist for several parameters of PTH action, including stimulation of hypercalcemia, urinary phosphate and cyclic AMP excretion, and production of 1α,25-dihydroxyvitamin D_3 (*3*). The biological response to this analog in these several systems was less than 1 percent of the response to PTH (on a molar basis).

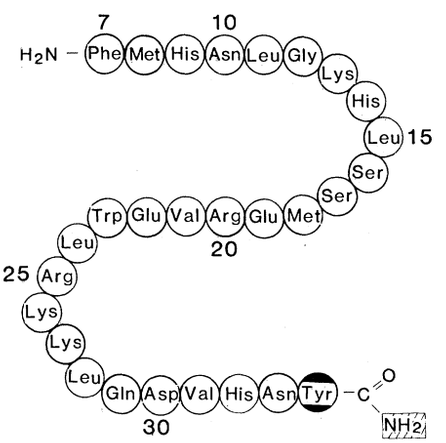

Fig. 1. The amino acid sequence of the analog [Tyr^{34}]bPTH-(7–34)NH_2. Tyrosine at position 34 replaces the phenylalanine of the native sequence and the carboxyl terminus is modified from a carboxylic acid to a carboxamide.

Our approach to designing an inhibitor for PTH in vivo was to modify the NH_2-terminus, either through substitution (*13*) or further NH_2-terminal truncation, in an attempt to eliminate the last traces of agonist activity from the 3–34 sequence without losing completely the avidity for PTH receptors. Substitutions at positions 3 and 4 failed to remove weak agonist activity (*14*).

A series of fragments of PTH containing NH_2-terminal deletions were therefore synthesized and evaluated in a renal radioactive-ligand–receptor binding assay for PTH (*15*). Stepwise deletions at the NH_2-terminus resulted in a progressive decline in receptor avidity, and identified the region 25–34, most distal from the NH_2-terminus, as the principal binding domain of the hormone (*6*). The intermediate sequence 7–34 still bound strongly to PTH receptors and inhibited completely the specific binding of a radioactive-ligand analog of PTH that was biologically active in vitro, although binding avidity was diminished from one-tenth to one-hundredth of that of the 3–34 sequence (*15*). Substitution of a tyrosine amide enhanced the activity of both agonists and antagonists in vitro (*3*). However, other studies revealed that substitution of norleucine for methionine diminished the activity in vitro and was very poorly tolerated in vivo in terms of agonist activity: a 15-fold decline in potency was observed in agonist analogs containing the 1–34 sequence and incorporating this substitution (*16*).

It thus appeared necessary to adopt the following strategy in designing an inhibitor that would be effective in vivo: (i) The amino terminus would have to be truncated beyond position 3 to remove PTH-like agonist properties. (ii) Because this structural manipulation alone would not be sufficient to remove the agonist properties, since the receptor-binding avidity of analogs truncated at the amino terminus is too weak, activity-enhancing modifications would have to be incorporated. (iii) Consideration would have to be given to the fact that alterations of the