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Plant Hormones and Regulators

Gibberellins, cytokinins, and auxins may regulate plant growth via nucleic acid and enzyme synthesis.

J. van Overbeek

Just about 40 years ago, in opposite parts of the world, proof was given of the existence of substances which promote growth of plants. In 1926 Went (*1*), in Holland, provided convincing proof of a diffusible substance obtained from oat seedlings which promoted growth of these seedlings. This was the beginning of auxin research.

Kurosawa in Japan, in the same year (*2*), gave proof of a substance in cell-free fungus filtrate which promoted growth of rice seedlings. This was the beginning of gibberellin research, although the Western world did not take notice until the early 1950's. Auxins and gibberellins are now recognized to be two separate classes of chemicals that cause distinct growth patterns in plants.

Auxins

It is now reasonably certain that the native auxin is indole-3-acetic acid (IAA, Fig. 1) (*3*). Indole-3-acetic acid occurs in minute quantities in growing tissue. Thus, in the shoot of the pineapple plant, only 6 micrograms of auxin are found per kilogram of plant material (*4*). J. P. Nitsch (*5*) calculated that this is like the weight of a needle in a 22-ton truckload of hay. One reason that this concentration is so low is that IAA is constantly being destroyed by indole-3-acetic acid oxidase (*6*). This enzyme system definitely occurs in intact plants (*7*). Indole-3-acetic acid oxidation is usually activated by monophenols and inhibited by orthodiphenols (*8*). Recognition of this fact has clarified the growth-promoting activity of diphenols such as caffeic acid. Previously, they were thought to be auxins; now it is recognized that, by inhibiting the IAA oxidase, these compounds raise the level of native IAA considerably (*9*). This is a form of synergism.

Many synthetic auxins (*10*) have been found. Some of these have a biological activity more potent than that of IAA, probably because they are more persistent in the plant than this native auxin is. The best known of these synthetics is 2,4-dichlorophenoxyacetic acid, the herbicide 2,4-D (*11*). In the United States alone, this chemical is now produced at a rate of over 100 million pounds (45 million kilograms) per year (*12*).

Although synthetic auxins are more stable in plants than the native auxin is, synergism is still found among them. Thus, Veldstra (*13*) reports that the activity of 10^{-6} mole of naphthaleneacetic acid could be increased 40-fold by supplementation with 2×10^{-5} mole of decahydronaphthaleneacetic acid, which is inactive by itself.

Auxins are required for cell elongation as well as for cell proliferation, but they have a multitude of additional effects. In tissue cultures, the native IAA is often replaced by 2,4-D, as 2,4-D is more stable and less likely to undergo biological degradation (*14*).

The author is chief plant physiologist, Agricultural Research Division, Shell Development Company, Modesto, California.

Horticulturists use indolebutyric acid to promote root formation on cuttings because it is less likely to undergo biological oxidation than the native IAA is. Auxins can have striking effects on differentiation. It is well known, for instance, that naphthaleneacetic acid and 2,4-D can cause the vegetative pineapple plant to flower. Recently, Lang and his associates (*15*) removed buds from young cucumber plants and cultured them in vitro. If these buds had been left on the plant, they would have become male flowers, but on the medium they developed into females. This occurred only in the presence of IAA—a hormone which thus seems to be able to affect sex when applied directly to the floral bud.

Gibberellins

Although gibberellins were originally discovered as products of a fungus which parasitizes a higher plant, it has turned out that gibberellins can be considered normal constituents of green plants (*16*). This was first shown in 1956 by Radley (*17*), who made dwarf peas grow tall by giving them extract from normal tall peas. In plants, gibberellin, like auxin, is found in minute quantities: 100 buds of sunflower seedlings contain only 0.001 microgram of gibberellin (*18*).

Best known of the gibberellins, and commercially produced by fermentation from fungal cultures, is gibberellic acid (GA_3, Fig. 1). Several other gibberellins, from GA_1 through GA_{13}, are known (*16*). These differ only slightly in chemical structure from GA_3, but they differ vastly from GA_3 and from one another in biological activity (*19*). Thus, GA_7 is often more active than GA_3, although it differs from it structurally only by the lack of an OH group on carbon number 7. Gibberellins A_7 and A_1 are the only ones known to cause flower formation in forget-me-not; GA_3, GA_4, GA_5, GA_6, and GA_9 do not do so (*20*). Gibberellin A_7, at concentrations as low as 5×10^{-10} gram per milliliter [0.5 part per billion (*21*)], causes the formation of antheridia (the male sex organs) on fern gametophytes. Gibberellin A_5 appears to be responsible, in a rather indirect manner, for the reduced growth rate of dwarf peas. In the dark, seedlings of the dwarf pea and of the normal variety grow equally tall. Only when the seedlings are grown in the light will dwarfs show up (*22*). Both varieties contain GA_1 and GA_5 in about equal proportions, but in the light the tissues of the dwarf are rendered less responsive to GA_5 rather specifically (*23*). The response to GA_1—and to GA_3 too, for that matter—is not much reduced by light. Gibberellin A_5 has a double bond between carbons 2 and 3, while GA_1 has none. In addition, GA_5 lacks the OH group at carbon 2 which GA_1 possesses. These seemingly small differences have a profound effect on the physiology of the plant.

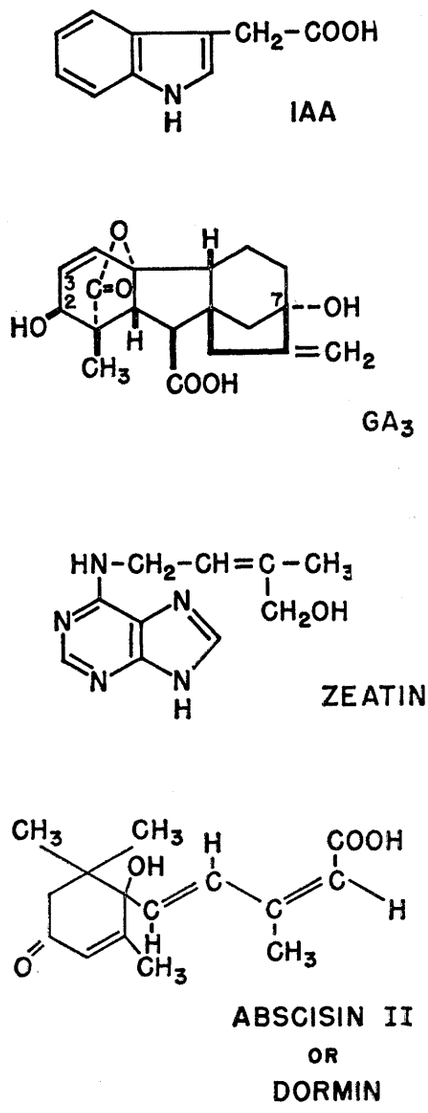


Fig. 1. Structures of naturally occurring plant hormones: IAA, or indoleacetic acid, (*94*), an auxin; GA_3, or gibberellic acid (*3*), a gibberellin; zeatin (*35*), a cytokinin; abscisin II or dormin (*47*, *48*).

While, on the one hand, small changes in molecular structure of gibberellins have far-reaching physiological effects, on the other hand, molecules differing rather notably in structure from GA_3 possess a considerable amount of gibberellin activity. One of these is vitamin E, α-tocopherol (*24*); another is helminthosporol (*25*). Steviol (*26*) and a number of kaurene derivatives (*27*) show gibberellin activity, and these could conceivably act as gibberellic acid precursors. No synthetic gibberellins have yet been made; they are all obtained from natural sources. Gibberellins are linked to diterpene metabolism, just as IAA can be linked with tryptophan metabolism (*28*).

Cytokinins

In 1941 it became clear to me (*29*) that coconut milk contained a potent growth factor, different from anything that was known at that time. This discovery had two effects: tissue and organ cultures could be made with more success than had heretofore been possible, and the search for the new growth factor began. Skoog and his associates at the University of Wisconsin purified the factor, so that its activity increased 4000-fold (*30*). Coconut milk is a natural nutrient and thus is full of sugars and other constituents which make it a very unattractive source from which to isolate growth factors. Therefore, these workers explored other sources of the growth factor which stimulated their tobacco tissue cultures. These attempts met with success in 1955, when an adenine derivative, named kinetin, was isolated from herring sperm DNA (*31*). Other adenine derivatives similar in structure to kinetin were synthesized, and a number of active materials were thus found which were originally named kinins by Skoog, but which recently have been renamed cytokinins (*32*). The discovery of these adenine materials focused attention on nucleic acid.

Cytokinin activity is found in green plants, in their seeds (*33*), in coconut milk, and in the sap stream (*34*). Because cytokinins occur in nature in such small quantities—concentrations of 50 to 100 parts per billion are found in the bleeding sap of the grapevine (*34*)—it was nearly 10 years before the chemical composition of a natural cytokinin was first announced. The announcement was made by Letham and his associates (*35*) in 1964, and their results were supported by those of Miller (*36*). These workers identified the structure of a cytokinin which occurs in young maize seeds. The structure is shown in Fig. 1. Zeatin, as this material was named, is,

like kinetin, an adenine derivative. Zeatin is a hydroxy derivative of the 6-*N*-dimethylallyl adenine which was described by the University of Wisconsin workers (*37*) and which is an isomer of the alkaloid triacanthine. Perhaps the best known synthetic cytokinin is benzyladenine. Recently, cytokinin activity has been described in urea derivatives (*38*), compounds chemically not related to adenines.

In addition to being necessary for cell growth and differentiation, cytokinins have some other interesting physiological roles. One of these is inhibition of senescence. Detached leaves treated with cytokinin stay green longer because they retain their proteins (*39*). Another most interesting characteristic of cytokinins is their capacity to direct the flow of chemicals through the plant (*40*). Mothes applied a drop of radioactive DL-aminoisobutyric acid on the left side of a detached tobacco leaf and a drop of cytokinin on the right side. The cytokinin did not move appreciably, but he found that the amino acid moved from the left to the right. Since this particular amino acid is not synthesized into protein, the phenomenon is not due simply to a source-and-sink relationship (*41*).

Inhibitors

There is a growing conviction among plant physiologists that dormancy is regulated by an interaction of endogenous inhibitors and gibberellins (*42*). Hemberg has long suggested that an inhibitor is involved in the dormancy of the potato tuber (*43*), where it inhibits α-amylase activity (*44*). Wareing has isolated inhibitors from leaves of woody plants kept under short-day conditions (*45*). These inhibitors, appropriately named "dormins," cause a vegetative bud to change into a winter bud by turning the developing leaf primordia into bud scales. One inhibitor in particular is of high potency, being far more active than coumarin. Its inhibitory effects can be overcome by gibberellin. This dormin is a carboxylic acid having a hydroxyl group and an unsaturated ketone function, and it appears to be identical with abscisin II (*46*). Abscisin II was isolated by Addicott's group from young cotton fruits, and its structure was revealed (*47*). Shortly thereafter the material was synthesized by Cornforth (*48*). The inhibitor is an isoprenoid, related to vitamin A (Fig 1). Analogs of abscisin II were made by Ohkuma, but all were considerably less active than the parent compound.

Dormin appears to be involved in complicated interactions with auxins and gibberellins (*49*). It was reported from Wareing's laboratory that dormin inhibits α-amylase activity induced by gibberellin in barley endosperm. This strongly suggests that the inhibitor may function as a gibberellin antagonist in vivo. The inhibitor seems to have little effect on corn dwarfs, but it reduces the growth of normal corn plants, and this inhibition can be overcome by gibberellin. When IAA (1 milligram per liter) was applied to coleoptile sections, it caused optimum growth. Dormin was found to inhibit this growth. Curiously, the original growth rate could be restored by applying gibberellin, but not by applying IAA. Coleoptiles did not respond to gibberellin in the absence of dormin. This new evidence lends additional support to the idea that both gibberellins and auxins are normally involved in plant growth (*50*).

While the search for the chemical identity of natural inhibitors went on, several synthetic growth retardants turned up. Mitchell and his associates were the first to report such a retardant; they found amo 1618 (*51*). This retardant seems to inhibit the biosynthesis of gibberellins (*52*) and not the action of gibberellin once it has been formed (*53*). Several other growth retardants are now known, but they are not necessarily chemically related (*54*). By retarding vegetative growth, these chemicals often promote flower and fruit production. This effect appears to have economic possibilities.

The list of growth-regulating chemicals is by no mean exhausted with mention of auxins, gibberellins, cytokinins, and growth inhibitors. The late P. W. Zimmerman and his associates long counted ethylene among the plant hormones (*55*). This was a reasonable view as this gas is naturally produced by plants and is highly active biologically. Ethylene is probably an important factor in fruit ripening, and its mode of action has been traced to changes in the permeability of lipoprotein membranes. A dramatic increase in the rate of ion leakage out of banana tissue is found when the fruit turns from green to yellow, a process regulated by ethylene (*56*). Ethylene has also been linked to leaf abscission (*57*) and to auxin action (*58*).

Much of the argument as to whether or not a chemical is a hormone is merely a question of semantics. A hormone is principally a chemical signal and, in the words of Heslop-Harrison (*59*), "the essence of a hormone as a chemical signal is that it evokes a response of a kind that is in some way beneficial to the individual or to the species. . . . It is quite essential that the term should be restricted to natural substances which participate in the regulation of life processes." Synthetic chemicals with hormone-like action are referred to as regulators (*60*).

Although, in our desire to classify, we do not by tradition include organic nutrients and inorganic ions among plant hormones or regulators, such distinctions are not found in nature. Sugar may well control growth of fruit in its final ripening stages (*61*). Sugar, together with auxin, is critical in the induction and differentiation of vascular tissue in plants. This combination is so effective that, in fern prothalli, which ordinarily have no vascular tissue, it induces strands of vascular bundles. This induction was achieved in all the prothalli studied (*62*). As discussed below, the mechanism by which gibberellin produces its growth-promoting effect may ultimately be found to include making sugar available.

The mysterious agent which causes pollen tubes to grow down the style toward the ovule, thus bringing about fertilization, turned out to be nothing more complex than a calcium gradient (*63*).

Germination

A seed may be looked upon as a resting bud. Its bursting into activity is the result of a marvelous interplay of hormones. Even in the dormant state there is activity. There may be little or no cell enlargement, but meristematic activity may be high (*64*). Dormancy is a phase of development, a phase of differentiation. The chilling of winter cereals is an example. Here, flower formation is induced during periods of low winter temperatures (*65*). Another example is provided by tulip and hyacinth bulbs, where the physiological preparations for flowering in spring take place in a series of distinct stages when the bulbs are dormant, even when they are stored on the shelves of a warehouse (*66*).

As in winter buds of trees, one finds in seeds a balance between inhibitors and promoters (*67*). Seeds of desert

plants germinate only after heavy rains (*68*), when the water has washed out the inhibitor. The dormant seeds of the wild oat (*Avena fatua*) germinate only when they are in contact with liquid water (*69*).

Recent evidence suggests that gibberellin is the promoter part of the inhibitor-promoter complex. Gibberellin thus may provide a natural mechanism in the breaking of seed dormancy (*70*). This would be in agreement with the situation found in winter buds of trees, as discussed above (*45*).

The most obvious change associated with the initiation of germination is rapid uptake of water. In maize, during the first few days of germination, most of the water is held by the embryo (*71*). The water uptake activates the embryo. This is well illustrated by the germination of lettuce seed in response to light. Red light promotes germination of these seeds. However, the seeds are sensitive to illumination only after they have absorbed sufficient moisture. When they have done so, exposure for 1 minute to 60 foot-candles (660 lumens per square meter) of red light is sufficient to cause 100 percent germination (*72*). The part of the seed that is sensitive to light is probably located in the tip of the hypocotyl of the embryo (*73*), and the light-absorbing pigment is phytochrome, a bluish protein (*74*). Light absorption may set in motion a mechanism which causes the formation or activation of hydrolytic enzymes. These weaken the seed coats. This then allows the young root (radicle) to break through, and the process of germination is on its way (*72*). In addition it has been suggested (*75*) that red light sets in motion a mechanism which enables the embryo to push harder against the seed covers and thus overcome their resistance.

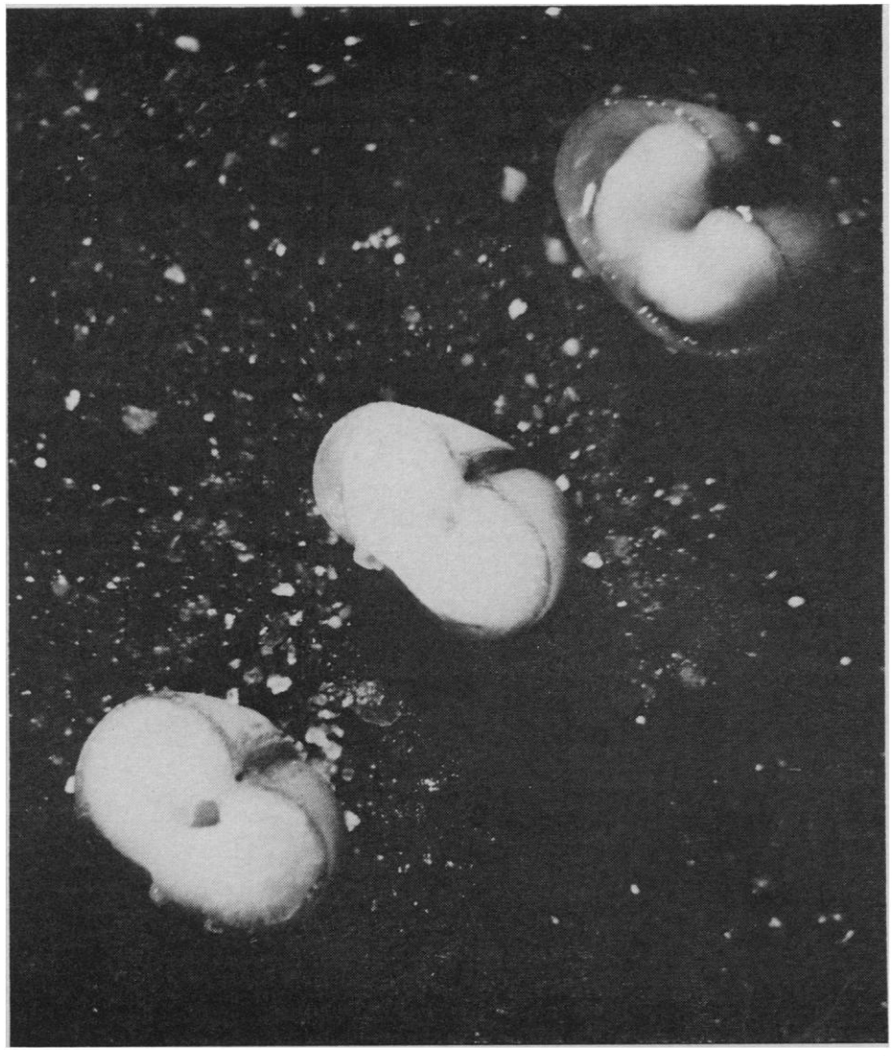


Fig. 2. Three sterile halves of barley seed without embryo. To the open surface of each was added either 0.5 microliter of water, gibberellic acid at a concentration of 1 part per billion, or gibberellic acid at a concentration of 100 parts per billion. The photograph, taken 48 hours later, shows that digestion of the starch-filled storage tissue is already taking place. The hormone gibberellin promotes production and secretion of the enzymes that cause hydrolysis of the storage material. [Photograph courtesy of J. E. Varner]

Gibberellin and Germination

The weakening of the seed coats can also be brought about in darkness, provided the seed is soaked in a gibberellic acid solution (*76*). Whether or not gibberellin is involved in the chain of events which starts with the absorption of red light by phytochrome is not yet clear. However, it seems obvious now that gibberellin has a function in the process of seed germination. The study of the role of gibberellins in seed germination has revealed an amazing story which is truly the high point of modern plant hormone research. This appears to be a case where hormone activity has been revealed close to the molecular level.

Seeds of cereals have two major parts: the germ (embryo) and the food reserves (the endosperm tissue). The cells of the embryo, of course, are very much alive, as out of them will develop the entire plant, by cell division and by cell enlargement. The storage tissue of the endosperm, by contrast, is considered dead, as its cells no longer respire. These dead storage cells, however, are surrounded by a coat of live cells, consisting of three layers of cells in barley. This coat is called the aleurone layer, and although its cells are alive, they do not divide.

In the course of normal germination the starch of the storage cells is hydrolyzed—it liquefies. Haberlandt (*77*) in 1890 recognized that the aleurone layer is a gland which secretes the hydrolytic enzyme (diastase, now called α-amylase) responsible for liquifying the reserve starch. The presence of the embryo is required before the aleurone layer will secrete its hydrolytic enzyme. Thus, when a cereal grain is cut in half along its shortest axis and the half with the embryo is thrown away, the starch in the other half will not liquify, provided of course that microorganisms are kept away.

Since 1940 (*78*) it has been known in Japan that gibberellin hastens germination of barley and rice. But it was

not until 1960 that Yomo in Japan (*79*) and Paleg in Australia (*80*) began to realize that gibberellin is the chemical signal, the hormone secreted by the embryo, that activates the cells of the aleurone layer into secreting hydrolytic enzyme. Concentrations as low as 2×10^{-11} mole of gibberellic acid per liter will initiate starch digestion in the embryo-less halves of barley endosperm (*81*). This was illustrated in experiments by J. E. Varner (see Fig. 2), whose recent work has shown that the secretion of α-amylase by aleurone cells results in *de novo* synthesis of that enzyme protein (*82*). The effect of gibberellin on endosperm with and without the aleurone layer is shown in Fig. 3, drawn from data of MacLeod and Millar (*83*). Gibberellins do occur naturally in germinating barley, as first shown by Radley (*84*). It is further known that, in germinating wheat, new gibberellin is formed in the embryo (*85*).

In addition to inducing formation of α-amylase in the aleurone cells, gibberellin activates enzymes that promote cell-wall degradation (*83*). Not only does this action aid digestion of the endosperm cells, but the cytolytic enzymes also weaken the seed coats and allow the growing germ to burst through.

Gibberellin and Auxin Production

Growth of the embryo, in terms of increase in size, is the result of cell enlargement. Cell division, as such, does not contribute to increase in size. On the contrary, the cotton embryo shrinks after the first divisions, and it is not until the embryo contains approximately 75 cells that it becomes larger than the original zygote cell (*86*).

The growth of cereal seedlings has, by tradition, been associated with the presence of auxins, specifically IAA. Much evidence suggesting that the growth of the coleoptile is indeed con-

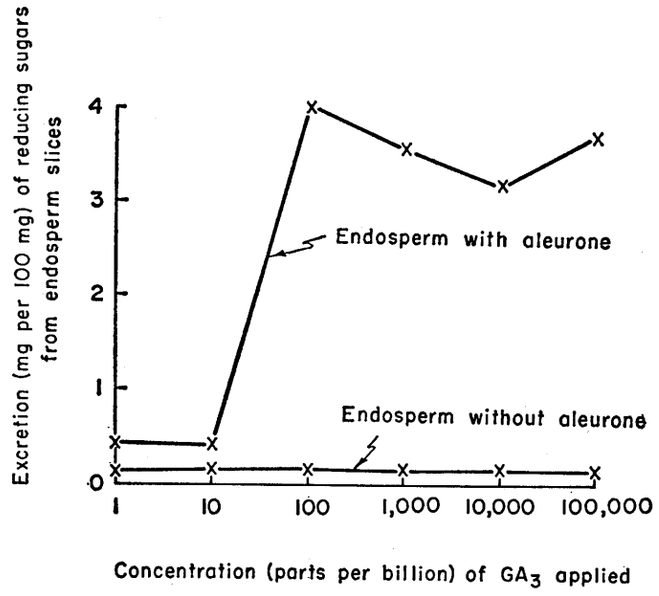
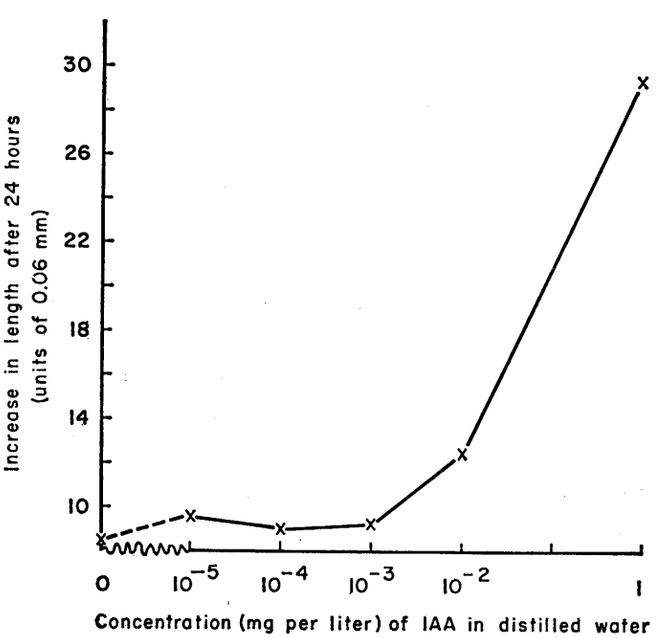
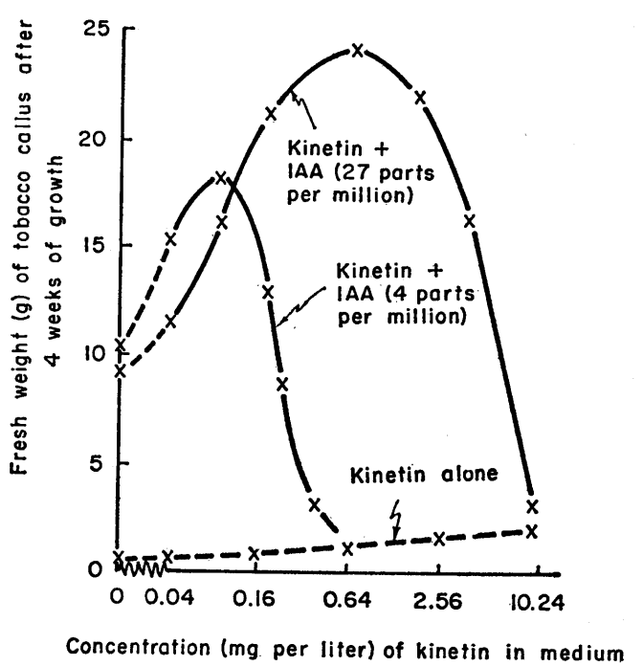


Fig. 3 (top left). Induction of α-amylase by gibberellic acid in barley endosperm tissue. The presence of aleurone cells is a requirement, as these secrete the enzymes that hydrolyze the starch to reducing sugar. [From data of MacLeod and Millar (*83*)]

Fig. 4 (bottom left). Growth response of isolated 3-millimeter sections of oat coleoptiles floating on solutions of IAA in distilled water. [From data of G. Blaauw-Jansen (*136*)]

Fig. 5 (bottom right). Graph showing modifying effect of kinetin on the growth-promoting activity of IAA on tobacco callus growing in culture flasks. [From Murashige and Skoog (*105*)]





trolled by IAA has been accumulated (*87*). The coleoptile is a modified leaf, rolled up like an onion leaf, inside of which are the true leaves of the seedling, nicely protected from the rough soil.

Coleoptile tissue is exceedingly responsive to IAA, as the dosage-response curve of Fig. 4 shows. The data of Fig. 4 were obtained by floating the hollow sections cut from the apical region of oat coleoptiles on the surface of solutions of IAA in distilled water. The duration of the experiment was 24 hours. It is clear that the coleoptile tissue responds with cell elongation to auxin concentrations as low as 10 parts per billion. The coleoptile sections do not elongate under the influence of gibberellin.

It seems likely that, in the seedling, the IAA needed to promote the growth of the coleoptile originates from the reserve proteins in the endosperm. In addition to inducing formation of α-amylase in the endosperm storage cells, gibberellin activates protease from ungerminated barley endosperm (*83, 88*). As a result of protein hydrolysis, tryptophan must occur among the amino acids liberated. Skoog's work (*89*) indicated many years ago that tryptophan serves as a precursor to IAA. The subject has been reviewed more recently, and there is a reasonable amount of direct and indirect evidence that tends to substantiate the concept that IAA is normally formed from tryptophan (*90*). In the seedling, it would seem, tryptophan is converted to IAA in the tip of the coleoptile.

It appears, therefore, that in cereal grains the following picture of interaction of gibberellin and auxin emerges. The embryo, after it is activated by imbibition with water, produces gibberellin. This hormone now moves to the aleurone cells, where it induces synthesis or activation (or both) of cytolytic and hydrolytic enzymes, including protease, which releases tryptophan from the reserve protein of the endosperm. Tryptophan now moves inside the young shoot, and when it arrives at the coleoptile tip it is activated to form IAA. It is well known that IAA moves polarly from the coleoptile tip to the coleoptile base. While moving from tip to base, the IAA sets in motion a mechanism that weakens the walls of the coleoptile cells (*91*). The weakening increases the suction of these cells. Thus, water uptake is increased and cell elongation results. All of this finally is observable as growth of the coleoptile (*92*).

Gibberellin and Roots

The young root emerges from the germinating seed before the coleoptile does, and the reader may well ask what causes this young root to grow. Earlier, Richardson showed that elongation of the radicles emerging from germinating Douglas fir seed is stimulated by gibberellic acid (*93*), and I had come to the conclusion that it is more likely that gibberellin promotes root growth than that auxin does (*94*). More evidence supporting this view is found in the work of Paleg *et al.* (*95*), where it was shown that radicle growth of lettuce is promoted by gibberellins. Especially active is GA_6, which, at a concentration of 100 parts per billion, causes a 40-percent increase in root growth. Except under very special conditions, auxins decrease root growth (*94*), although they are known to promote root *formation* on cuttings. In embryo cultures, gibberellin was more effective than auxin, promoting elongation of the root (*96*). The root has now also been recognized as a producer of gibberellin (*97*), although the shoot tip of seedlings produces gibberellin too (*98*).

If, indeed, gibberellin, rather than auxin, is the principal promoter of growth of the early root, this might explain why the root rather than the coleoptile is first to emerge from the germinating seed: gibberellin is formed a phase ahead of auxin in the differentiation of the seedling.

Even if gibberellin controls root elongation, this does not mean that auxin has no function in the root. Auxin still functions in guiding the root down into the soil in response to gravity. Thimann and his associates, using radioactive IAA, have in recent years substantiated in elegant fashion the hypothesis that the direction-seeking of coleoptiles in response to gravity (geotropism) is caused by a unilateral distribution of IAA in the extreme tip (*99*). When a coleoptile is laid on its side, 60 percent of the auxin goes to the lower side and 40 percent to the upper. The resulting difference in rate of elongation causes the coleoptile to straighten up, away from the pull of gravity. Stems of broad-leaved plants appear to respond to gravity with the same mechanism of unilateral auxin distribution observed for grass seedlings (*100*). In roots, a similar redistribution of auxin has been noted, but because auxins generally have an inhibiting effect on root growth, the root tip grows downward.

Auxin and Cytokinin

I might note one more example of the remarkable coordination found in plant growth. When a shoot of cereal seedlings grows in the dark, both the coleoptile and the leaves inside it grow. Physiologists have noticed that the tips of the leaves stay just a little behind the coleoptile tip. It is not until the coleoptile ceases to grow that the leaves accelerate their growth rate and break through the side of the coleoptile tip. What holds these leaves in check while the coleoptile is growing? When growth of the leaf inside the coleoptile is studied, it is noted that the leaf responds to gibberellin and not to auxin. When auxin is added to the gibberellin, it is found that the auxin prevents the gibberellin from promoting the growth of the leaf (*101*). It would seem, therefore, that, while the coleoptile is growing, some of the auxin serves to hold the gibberellin-controlled growth of the leaf in check. Not until auxin production ceases and the growth of the coleoptile has been completed does the plant shift to another gibberellin-dominated phase of differentiation—leaf development.

In the growth of the seedling, cytokinins also must have their role. Cytokinins occur in seeds, and they have a role in early stages of embryo growth (*102*) and germination (*103*). They cause lignification, and they appear to normalize and stabilize growth (*104*). Cytokinins have a striking capacity to modify the action of other hormones. This is illustrated by data (*105*) taken from the work of Skoog *et al.* and reproduced in Fig. 5. These data pertain to the growth, in vitro, of tobacco callus tissue on a suitable medium containing kinetin alone and in combination with IAA. The cytokinin alone has little effect. The auxin (IAA) alone causes the callus culture to grow to 10 grams, regardless of the concentration used. The presence of kinetin in the medium greatly influences the

response to auxins, boosting the growth to an optimum over a relatively narrow range of cytokinin concentrations —a range which is different for each auxin concentration. At cytokinin concentrations beyond the optimum, growth drops and finally ceases.

Not only do auxin-cytokinin combinations affect growth quantitatively, as indicated in Fig. 5, but there are also striking qualitative effects. Skoog *et al.* (*32*) demonstrated that the differentiation of undifferentiated tissue into roots or shoots is controlled by auxin-cytokinin ratios. Even complete tobacco plants regenerated from undifferentiated cell masses under the unique control of cytokinin (Fig. 6).

Since the pioneering work of the Wisconsin workers (*106*), it has become recognized that cytokinin is a distinct and constant requirement for rapid growth of normal tissues (*107*). In tumorous plant tissue, such as crown galls, cells have ceased to be dependent upon external sources of cytokinins and auxins. Tumor cells produce their own hormones (*108*) and thus grow independently of the organism in which they occur. In other words, the host plant has lost its hormonal control over these cells.

The stabilizing effect of cytokinins extends also to external influences. Thus, treatment with kinetin protected bean leaves from the growth-retarding effects of gamma radiation (*109*). Kinetin applied to the lower surface of petunia leaf tissue reduced the number of lesions on the upper surface caused by tomato spotted wilt virus (*110*). Prior treatment of the shoots of black currant with kinetin (50 parts per million) provided complete protection against the mutagenic effects of a colchicine treatment without influencing the growth of the seedlings (*111*). It is evident that the functions of cytokinins are just beginning to be recognized.

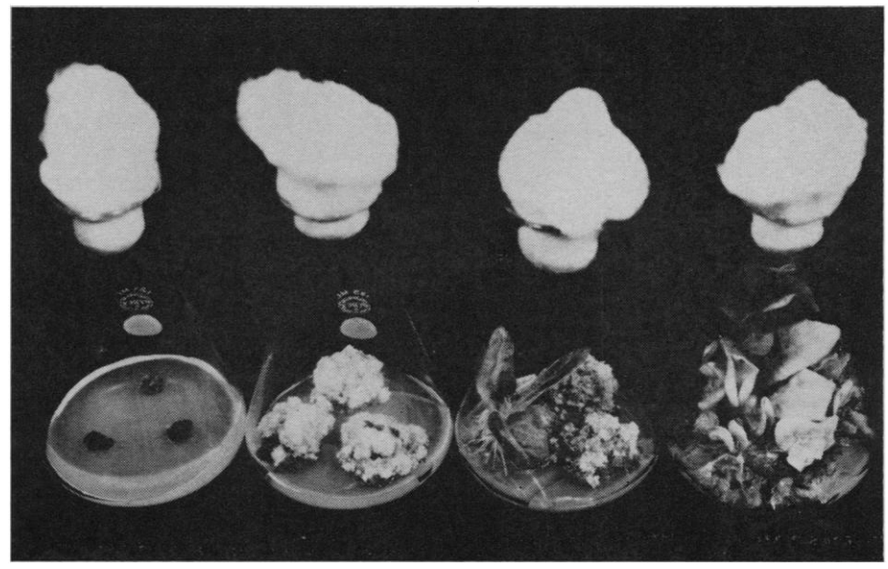


Fig. 6. Regeneration, under the influence of cytokinin, of complete tobacco plants from undifferentiated cell masses. All the flasks contained the basic mineral ingredients used by Linsmaier and Skoog (*107*) plus thiamine (0.4 mg/liter), myoinositol (100 mg/liter), IAA (0.5 mg/liter), sucrose (30 g/liter), and the cytokinin 6-(γ, γ-dimethylallylamino)-purine at concentrations (from left to right) of 0, 0.1, 0.5, and 2.5 micromoles per liter (2.5 micromoles per liter corresponds to 0.5 part per million). This cytokinin is closely related to zeatin. [Skoog, unpublished data]

Gibberellin and Enzymes

In the 1940's, in attempts to find the primary reaction of auxins, a search was made for an enzyme system that would be activated by auxin. The thought was that auxin would bind to a protein as a prosthetic group, thereby conferring enzyme activity upon it. This proved to be a fruitless search. Present indications are that auxins and plant hormones in general are indeed bound to enzymes, but only in a figurative sense. More and more physiologists and biochemists have come to believe that plant hormones act on the nucleic acid system—somewhere between DNA and messenger RNA. This influence, then, would control enzyme formation, and thus the biochemistry and physiology of the plant. It is an attractive concept, but in the first flush of enthusiasm there is danger of overgeneralizing.

In 1954 Skoog (*112*) concluded from his studies on tissue culture that an intimate relationship exists between (i) the effects of auxin on nucleic acids and (ii) growth. Thus, to my knowledge, Skoog was the first to see clearly that the primary effect of plant hormones was on nucleic acid metabolism. This conclusion was further strengthened by the discovery that kinetin, a breakdown product of DNA, was required for growth, by cell division, of tobacco callus (*106*).

The most convincing evidence of the involvement of a plant hormone in nucleic acid metabolism is provided by the work of Varner and Chandra on α-amylase formation under the influence of gibberellic acid (*82*). First, it was demonstrated by incubating the endosperm halves of barley seed (see Fig. 2) with phenylalanine-C^{14} that new amylase protein was formed. Chromatography revealed a clear major peak of amylase in the presence of gibberellic acid and none when gibberellic acid was not present. Later, the aleurone layer, isolated from the storage cells, was used for similar tests. These simple dissection experiments have shown that only aleurone-layer cells are capable of respiration and enzyme synthesis. Aside from the possibility that a layer of living cells surrounding the dead starchy endosperm may provide protection against attack by microorganisms, the only obvious function of the aleurone cells is that of producing and secreting hydrolytic enzymes for digesting the reserves of these dead starchy endosperm cells. It is a delightful nicety that the key to these reserves—gibberellin—is kept by the embryo, the only tissue capable of growth (*82*).

Further direct proof that the entire newly formed α-amylase molecule was identical in composition to the native enzyme was obtained by analyzing its amino acid composition. After being labeled with threonine-C^{14}, the protein was digested with trypsin, and the hydrolysis products were subjected to chromatography. Further separation by electrophoresis yielded 20 labeled peptides and 31 spots giving a positive ninhydrin reaction typical for α-amylase (*82*).

Gibberellin and Nucleic Acids

As might have been expected, it is found that inhibitors of protein synthesis—such as dinitrophenol, puromycin, chloramphenicol, *p*-fluorophenylalanine, and cycloheximide—prevent the gibberellin-induced formation of α-amylase. Of greater significance is the total inhibition of the synthesis of α-amylase by actinomycin D (*82*). Actinomycins are peptide-containing antibiotics and were among the first to be isolated from actinomycetes by Waksman. They are potent antitumor substances which complex with DNA, but not with RNA. The complexing is with the amino group of the base guanine. In this complexing, actinomycin displaces RNA polymerase, which normally hooks on to the DNA guanine (*113*). The result is that actinomycin D inhibits such RNA synthesis as is dependent upon DNA, but not RNA synthesis dependent upon RNA. DNA synthesis itself is not affected by low concentrations of actinomycin D. Since actinomycin D prevents gibberellin action, it follows that the effect of gibberellic acid is upon the expression of the genetic information contained in the DNA, which ultimately controls the production of α-amylase.

$$\text{Action of GA} \downarrow \quad \text{DNA} \longrightarrow \text{mRNA} \longrightarrow \alpha\text{-amylase}$$

The site of action of the plant hormone is thus very close to the genes.

So it appears that the action of gibberellin involves synthesis of messenger RNA, but it remains to be shown whether this is the messenger RNA specific for α-amylase.

It is now generally understood that all live cells contain the complete complement of DNA that is characteristic for the individual. All the genes are present, but only a fraction of them are active at a given time. Genes are thus turned off or on. Such variations in genetic activity result in differences among cells that have the same set of genes (*114*). The turning on and off of genes may be the result of covering and uncovering of DNA by nucleohistones (*115*).

It may thus be that the first act of the hormone gibberellin in the seed leads to the uncovering of some DNA. The now activated DNA would allow production of specific messenger RNA, resulting in the formation of specific enzymes. This would result in changed metabolism, a new phase in differentiation, and the beginning of a new growth cycle.

It is now clear that, in germination, gibberellin also induces formation of several cytolytic enzymes other than α-amylase. It is further clear that gibberellin induces enzyme formation during phases other than germination. In tissues of the carrot and the Jerusalem artichoke, invertase is formed under the influence of gibberellin at a concentration of 10 parts per billion (*116*). As in barley seeds, gibberellin stimulates formation of reducing sugars in dormant potatoes, and release of α-amylase appears to be involved (*117*).

Gibberellin and Elongation

Gibberellins, as is well known, have striking growth-promoting effects. They speed elongation of dwarf varieties to the point where the dwarfs resemble normal tall varieties (*118*). Gibberellins promote growth of fruit (*119*), and in some respects elongation caused by gibberellin resembles that caused by auxin (*120*). One cannot but wonder whether it is the enzyme-inducing effects of gibberellin which cause all these growth phenomena. Cell elongation is an osmotic process, a process of water uptake by the cells. Two major features affect this water uptake: the concentration of osmotically active material inside the vacuole and the resistance of the cell wall to stretching. In coleoptiles, during elongation under the influence of auxins, the concentration of osmotically active material does not increase. On the other hand, the cell wall of coleoptile tissue is markedly weakened by an indirect action of the auxin (*91*). Now it has been shown that gibberellins induce the formation of enzymes that weaken cell walls (*83*). Therefore, one might expect to find that gibberellins do indeed cause elongation by this route.

Gibberellins also induce formation of proteolytic enzymes, and, as was explained earlier, one would expect to find that this process releases tryptophan, which serves as a precursor to IAA. Treatment with gibberellin indeed leads to rapid increases in auxin concentration (*121*).

There is still a third mechanism by which gibberellins may promote cell elongation. The hydrolysis of starch caused by the induction of α-amylase synthesis by gibberellin would increase sugar concentration. This would increase the osmotically active material in the cell sap and would also explain why osmotic concentration is maintained during elongation. Starch hydrolysis during cell expansion has indeed been observed. Horie (*122*), studying the flowers of *Tradescantia*, observed that, in the buds, the epidermis of the petals contained large quantities of starch. This disappeared almost entirely after the flowers had opened. The rapid extension of the petals seems to be connected with the hydrolysis of the stored starch. It is tempting to theorize that gibberellin maintains the osmotic potential of the cells during elongation. Recently, Nanda and Purohit (*123*) have arrived at the somewhat similar conclusion that the enhanced extension growth of tree seedlings caused by gibberellin is probably a consequence of mobilization of stored food.

From these considerations, then, it follows that auxin and gibberellin could act synergistically. One example of this is cited in the interesting recent review of hormone interaction by Daphne Osborne (*124*). Green stem sections of pea seedlings, when floated on IAA in distilled water (10 parts per million), will elongate 85 percent in 24 hours. A similar concentration of GA_3 has no more effect than distilled water alone. However, when the IAA and the GA_3 are combined, elongation becomes 115 percent. This IAA-GA_3 synergism has even been found in the auxin-induced curvatures of *Avena* coleoptiles. Gibberellin A_3 applied by itself, unilaterally, does not cause a curvature. However, when applied together with IAA, it causes a curvature greater than that caused by the same concentration of IAA alone (*125*). Similarly, in *Avena* coleoptiles, gibberellin (1 part per million) increased the geotropic curvature (*126*). Earlier I mentioned the work of Wareing and his associates (*49*), which showed that only in the presence of dormin does gibberellin increase auxin-induced coleoptile growth.

Clarification was provided by the work of Yanagishima and Masuda (*127*). Initially they discovered that normal yeast cells become elongated by auxin only when the medium also contains gibberellin. It was not necessary for auxin and gibberellin to be present at the same time; prior treatment of the cells with gibberellin proved sufficient. They went even further and ex-

tracted RNA from gibberellin-treated cells. Now, this RNA proved to be a suitable cofactor for auxin-induced growth, not only in yeast but also in Jerusalem artichoke tissue cultures.

It is well known that *Avena* coleoptiles do not require exogenous gibberellin in order to respond to auxins. Thinking that perhaps a cofactor was being made under the influence of natural gibberellin, Yanagishima and Masuda extracted RNA from *Avena* coleoptiles. When this extract was added to yeast cells, these were found to respond to auxin much as they responded when gibberellin was added to the culture. To me it seems likely that gibberellin induces the formation of stable messenger RNA, and that this in turn causes the formation of enzymes needed to support auxin-induced growth. Hydrolytic enzymes could well be among those formed.

Hormones and RNA

Hormone action at the nucleic acid level has so far been conclusively demonstrated only with gibberellin, and only with barley endosperm—a nongrowing tissue. What, then, is the action of auxin when it induces cell elongation in *Avena* coleoptiles, the classical example of hormone action?

In work with specific inhibitors of protein synthesis, Noodén and Thimann (*128*) concluded that their experimental evidence supports the hypothesis that IAA activates the formation of one or more new enzymes which act on cell walls to increase plasticity. Cleland (*129*) also found that actinomycin D drastically inhibits RNA synthesis and IAA-induced cell elongation, and Key (*130*) concluded that the action of auxin in regulating cell elongation is more closely related to RNA synthesis than to protein synthesis. Thus, there can be little doubt that RNA synthesis is required for some aspects of auxin-induced cell elongation.

What about the primary action of auxin? Cleland reasoned that, since auxin controls elongation by reducing the rigidity of the cell walls, RNA synthesis should be required for this auxin action if, indeed, primary auxin action is by way of synthesis of new RNA. But, when he put the idea to the test, he did not find this. Treatment of coleoptile tissue with actinomycin D did not impair the auxin-induced extensibility of the coleoptile walls—as measured with the Instron stress-strain fiber analyzer, which was first used for this purpose by Bonner (*91*). Suppose that RNA synthesis is required, instead, to build and maintain the structural components of the plasma membrane, as suggested by the destructive effect of ribonuclease on isolated protoplasts of *Avena* coleptile cells (*131*). Surely, auxin could not promote cell elongation if the plasma membrane were not intact! This argument alone would be enough to explain the need for RNA synthesis in cell enlargement. This also would explain the need for RNA synthesis in the auxin-induced uptake of water by potato tuber slices (*132*). It may not be necessary to assume that the role of RNA in the auxin action is a direct one. Moreover, we should be aware of still other alternatives, and it may be well to reconsider the concept of primary hormone action. As one hears it presented, and as I understand it, this concept envisions *one* primary act, by the hormone, from which all other reactions follow in sequence. At the symposia on steroid hormones held at the AAAS meeting in Berkeley last December (*133*) this concept of *the one exclusive* primary act was questioned as being too simplistic.

It is true that there is abundant evidence that several hormones set off RNA synthesis (*134*). Yet this is often not the first reaction. When estrogen is injected into a rat, the uterus will experience an increased flow of blood within half a minute. Only an hour later there is evidence of new RNA synthesis, and 2 hours later the first detectable new protein synthesis begins. When glucocorticoids are injected into the rat, then within half a minute blood flow in the liver is markedly increased, but it is a matter of hours before massive RNA synthesis (of all kinds) is noted. When androgen is injected there is a "sharpening' of respiration (the concentration of NAD doubles) within half an hour, and this response is not dependent upon new RNA synthesis.

These results with androgen are reminiscent of Cleland's conclusion with auxin: "apparently aerobic metabolism, but not RNA synthesis, is needed for this action of auxin." No one doubts that the action of these steroid hormones involves at some place in the mechanism the need for RNA and protein synthesis. However, one can detect hormone action long before one can show a nucleic acid response. Is it not possible that there are several sites of primary action? One might be an immediate antihistamine-like action on the sphincters upstream of the blood capillaries, another might be action at the site of RNA polymerase action—like one key opening several doors.

And now what about the cytokinins? Earlier I stated that Skoog had come to the conclusion that both IAA and kinetin are involved in nucleic acid synthesis. The two hormones, used together, promoted growth of callus tissue in vitro. After studying the action of inhibitors of protein synthesis on kinetin-induced inhibition of leaf senescence, Osborne (*124*) proposed that the action of this cytokinin in preventing senescence may be directed through an effect on DNA-controlled RNA synthesis, and suggested that the regulatory action of kinetin may take place at the stage of messenger-RNA synthesis. Senescence in the leaf could then be the result of a progressive turning off of the genetic information in the nucleus. Kinetin also stimulates the formation of tyramine methylpherase, an enzyme required in the synthesis of hordenine in the roots of germinating barley (*135*). The state of knowledge about the mode of action of auxins and cytokinins is therefore very much like the state of knowledge about action of the steroid hormones. Hydrocortisone induces synthesis of enzymes (tryptophan pyrrolase and tyrosine transaminase, for instance), which follows the increase in synthesis of the three major types of RNA. However, it remains to be seen whether indeed the hormone-mediated induction reflects a hormonal influence on the mechanisms by which the information contained in the genetic code of DNA is used in the synthesis of RNA templates.

What We Have Learned

With 40 years of plant hormone research behind us, what have we learned?

We have learned the identity of auxins, gibberellins, and cytokinins, and recently even the identity of a natural inhibitor. Curiously, knowledge of the chemical structure of these compounds has given us little direct help in understanding their functions. On the other hand, it has helped greatly as a research tool, making pure chemicals, synthetic

or naturally occurring, available for physiological experimentation.

We have learned that the hormones have a broad function. Auxins do more than produce elongation; cytokinins do more than promote cell division. Auxins cause cell division (think of the cambium) as well as elongation. Cytokinins cause elongation (in leaf tissue) as well as cell proliferation. The functions of hormones overlap. Auxins are known to arise in seeds of developing fruit, and fruit that lacks seed normally ceases to grow. Yet, as Crane has demonstrated, development of a seedless peach can be induced with the aid of gibberellin. This fruit grows and ripens in a manner externally indistinguishable from the growth and ripening of a normal peach. Certainly the seed auxins were not present in these fruits because there were no seeds. In this parthenocarpic fruit, gibberellin has taken on what was formerly considered to be a function exclusively of auxin.

We have learned that plant hormones act in sequence. Cytokinins and gibberellins appear to dominate the early phase of development, auxins becoming dominant later. The concentration of a specific hormone in the plant is by no means constant. On the contrary, the normal pattern appears to be a quick rise to a peak, followed by a quick decline. When the hormone concentration is kept high artificially for long periods, its normal rise and fall is interfered with and the normal interaction between hormones is disturbed. That is why the auxin 2,4-D, with its highly stable molecules, can be used as a weed killer.

We have learned that auxins, gibberellins, and cytokinins interact with one another. A rise or fall in concentration of one affects the response caused by the others.

We are finally beginning to learn that the site of action of hormones—at least of some plant and animal hormones, it appears—is close to the gene. In some cases, possibly by promoting syntheses of messenger-RNA molecules, hormones give rise to new syntheses of specific enzymes. These enzymes, in turn, control the biochemistry and thereby the physiology of the organism. However, one wonders if the concept of *one* site of primary action of a hormone is not too simplistic. Sweeney demonstrated over 25 years ago that protoplasmic streaming in coleoptile cells accelerates within seconds as a response to tiny quantities of auxin (0.01 to 1 milligram of IAA per liter). Such a fast response could not possibly be the result of a DNA-RNA-enzyme process. Why couldn't there be several sites of primary hormone action, just as there are several doors that can be opened by one key? One also wonders whether some of the effects involving RNA suggested by some authors to be primary hormone responses might not simply be RNA required to support the structures of growth (such as the plasma membrane) without which the hormone action cannot express itself.

What we perceive at the frontiers of knowledge is still enveloped in haze, but what shines through is exciting.

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