The Cytokinins

Synthetic and naturally occurring N^6-substituted adenine derivatives profoundly affect plant growth.

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The modern era of research on the cytokinins began in 1955 with the isolation of kinetin, a highly potent growth factor, by Miller *et al.* (*1*). This group, working in the laboratories of Skoog and Strong at the University of Wisconsin, named their product kinetin because of its promotion of cytokinesis (cell division) in tobacco callus tissue, and characterized it as 6-furfurylaminopurine (*2*) (see Fig. 1). As 6-benzylaminopurine and many other N^6-substituted adenines also produce this result, a class name, cytokinin, was proposed and applied to the group as a whole (*3*). In the 13 years since the isolation of kinetin, there have been many reports of its diverse effects on plants. Evidence has accumulated for its role in the following: (i) induction, promotion, or regulation of DNA, RNA, protein, and thiamine biosynthesis; (ii) regulation of organ formation, apical dominance, and branching; (iii) enhancement of flowering and of seed germination; (iv) regulation of phloem transport and mobilization of metabolites; and (v) preservation of flowers, fruits, vegetables, and leaves through prevention of senescence (*4*). Thus, it is now apparent that the cytokinins, acting together with other plant growth substances, play a major role in ensuring orderly growth and development of plants (*5*).

Occurrence of Cytokinins in Nature

The isolation of a pure compound which in very low concentrations could stimulate cell division was the culmination of research begun many years ago. As early as 1892, Wiesner (*6*) suggested that there was a specific substance which stimulated cell division, and Haberlandt and his students in the early 1900's supplied experimental evidence for such a factor (*7*). Later, extracts of many plant materials were shown to permit continued growth of embryos and prolific cell divisions of tissue cultures. For example, van Overbeek *et al.* (*8*) successfully grew datura embryos on a medium containing coconut milk, and Caplin and Steward (*9*) grew carrot tissue cultures on a medium containing coconut milk or extracts from young corn seeds. The Wisconsin workers isolated their active factor by turning from coconut meat, from which they thought they had isolated a purine, to herring sperm DNA.

Kinetin was an artifact, at least in terms of the high concentrations present in the material from which it was isolated. Only old or heated DNA preparations yielded substantial quantities of the active material. Later, Hall and deRopp showed that kinetin could be produced by autoclaving adenine and furfuryl alcohol together (*10*). Still, the high biological activity of kinetin and its analogs was very real. In addition, this activity was fairly restricted to N^6-substituted adenines, suggesting that something closely resembling kinetin was present in plants. In 1964 Letham and his associates characterized such an active factor from young sweetcorn kernels and named it zeatin [from the generic name for corn, *Zea mays* (*11*)]. This material turned out to be identical with the substance isolated but not fully characterized by Miller in 1961 (*12*). Chemically, zeatin is 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino) purine (see Fig. 1). It has since been found in RNA hydrolysates from corn, peas, and spinach and in a fungal growth medium (*13*). The zeatin analog, dihydrozeatin, in which the side chain is saturated, has been found in lupine seeds (*14*). Recently, a close relative of zeatin, 6-(γ,γ-dimethylallylamino)purine (see Fig. 1), has been isolated as the ribonucleoside (2iPA) from serine (*15*) and tyrosine transfer RNA's (tRNA) (*16*) from yeast and from unfractionated soluble RNA (sRNA) hydrolysates from yeast, calf liver, human liver, chick liver, peas, and spinach (*13, 17*).

6-(γ,γ-Dimethylallylamino)purine also has been found as the free base (2iP) in culture filtrates of *Corynebacterium fascians* (*18*). This compound had been synthesized (*19*) prior to its isolation from natural sources and found to be about ten times as active as kinetin in the tobacco callus test (*20–22*). Zeatin appears to be slightly more active than 2iP and is the most active of compounds which have been tested to date (*23*).

Biological Effects of Cytokinins

The discovery of kinetin naturally stimulated efforts to examine the range of its biological activity. Biologists were presented with a new plant growth substance and many, of course, tried it in their own experimental systems. It took only 3 or 4 years for the essential outline of the biological activity of cytokinins to be constructed. An array of results was obtained ranging from initiation of growth [germination of lettuce seeds (*24*)] to retardation of senescence [maintenance of chlorophyll in excised leaves (*25*)]. It soon became obvious that kinetin and its analogs have a great number of diverse biological effects. These effects are also obtained with naturally occurring cytokinins. Although the naturally occurring compounds are often quantitatively more active than kinetin, there is no evidence for qualitative differences in activity between the natural and synthetic cytokinins.

The initial biological test for kinetin was based on ability to promote cell division in tobacco callus tissues. In the absence of kinetin or some other cytokinin very little growth of this tissue occurs. However, given an adequate supply of a cytokinin, rapid cell division takes place.

Commonly, the central cylinder of tissue (pith) of a tobacco plant is isolated aseptically with a cork borer and slabs of this tissue are placed on the

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medium. Subsequently, subcultures are made from this isolate and stocks of tissue may be maintained indefinitely by periodic transfer to fresh media.

Refinements of the growth medium for these tissues (*26*) have resulted in yields of better than 20 g (fresh weight) of tissue from an original 100 to 150 mg planted on 50 ml of medium containing $10^{-7}M$ kinetin and grown for 4 to 5 weeks. Figure 2 illustrates the growth of this tissue on a medium containing the synthetic cytokinin, 6-benzylaminopurine (BAP). It is interesting to note that the growth of this tissue is logarithmic for a considerable period of time and that the growth rate varies with cytokinin concentration rather than proceeding at a single rate and slowing at different times upon exhaustion of the medium.

Among other kinds of tissue cultures utilized for testing cytokinins, probably the soybean callus system as developed by Miller and his associates is the most useful. According to Miller (*27*), this assay gives a somewhat greater linear range of response of total fresh weight to cytokinin concentration than does the tobacco system.

In addition to their promoting cell division, it was soon found that cytokinins influenced the morphology of the cultures (*28*). At a given level of auxin in the medium, usually 2 mg of indole-3-acetic acid (IAA) per liter, loose, friable tissues are obtained with relatively low concentrations of kinetin or other highly active cytokinins (10^{-3} to 10^{-1} μM). Occasionally roots are initiated in older cultures. If the cytokinin concentration is somewhat higher (1 μM), flattened hemispheres of tissue are obtained. At still higher cytokinin concentrations (3 to 10 μM) vigorous shoot formation is induced (*23, 26*). Typical results of this type are shown in Fig. 3. An increase in the auxin level tends to increase the level of cytokinin required to achieve a particular result (*23, 26*). These effects of cytokinins demonstrate the experimental control of leaf and shoot production on a completely defined medium and are strongly suggestive of an important role for cytokinins in the general control of plant form as well as of cell division.

Evidence from studies with intact plants soon indicated that there was an interaction of auxin and kinetin in control of apical dominance. With many plants the side buds are held in check by a substance, presumably IAA, which is made in and transported from the apical bud. In this way the plant controls its form, growing taller at the apex without random, uncontrolled branching of lower regions. If the apex is removed, the side buds grow out. If, however, IAA is applied to the cut stump, the side buds remain inhibited. Application of kinetin will overcome this bud inhibition due to auxin (see *29*).

A similar disturbance of apical dominance in plants is caused by *Corynebacterium fascians*. Samuels (*30*) was able to simulate these symptoms with applications of kinetin. The detection of cytokinins produced by this bacterium (*31*) led to the isolation and characterization of 6-(γ,γ-dimethylallylamino)purine from culture filtrates of *Corynebacterium fascians* (*18*). Thus, it appears that a cytokinin which is elaborated by the bacteria causes the disease symptoms.

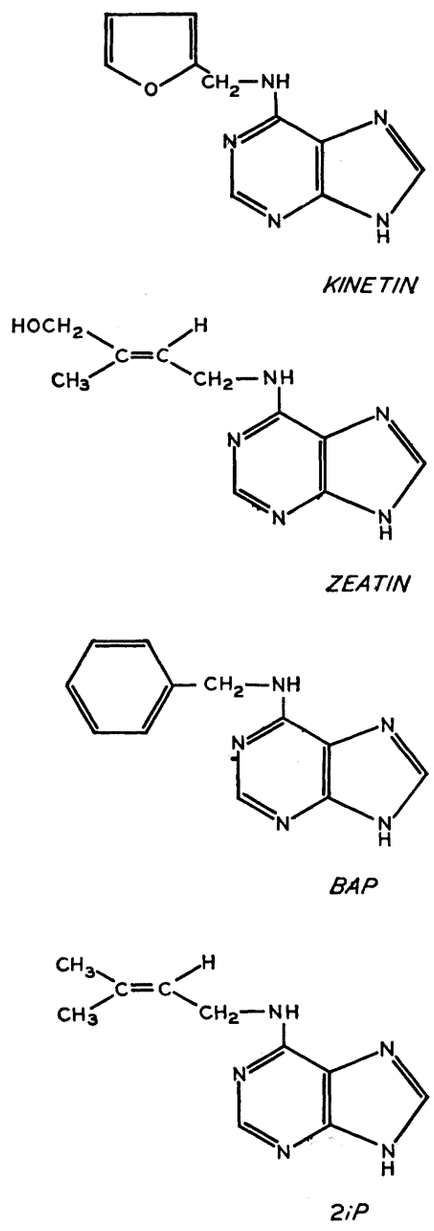

Fig. 1. Structures of four cytokinins. Zeatin and 6-(γ,γ-dimethylallylamino)-purine (2iP) are known to be present in plants. Kinetin and 6-benzylaminopurine (BAP) are commonly used synthetic cytokinins.

Another effect of cytokinins, superficially somewhat different from promotion of cell division or control of morphological form, was reported by Richmond and Lang (*25*). Leaves of the cocklebur plant lose their chlorophyll and turn yellow if floated on plain water for 10 days. If, however, kinetin (1 or 5 mg/liter) was present in the water, much of the chlorophyll and fresh appearance was maintained. Mothes and his associates have confirmed and expanded the initial observations by spotting leaves with kinetin-containing solutions. In addition, they have shown that transport of nutrients is directed toward the cytokinin-treated leaf areas (*32*). Osborne and McCalla (*33*) demonstrated that cytokinin concentrations could be measured by this technique. Other leaf tissues, for example, radish (*22*), can also be used in this test. This general antisenescence effect of cytokinins could be explained on the basis of a maintenance of protein and nucleic acid synthesis in the detached tissue (*34*).

Cytokinins have even been implicated in some germination phenomena and in cell enlargement. Early in the studies on the biological effects of kinetin, Miller (*24*) noted that seeds of certain lettuce varieties which require light for germination will germinate even in the dark if preincubated with kinetin solutions. Skinner, Shive, and their associates (*35*) used this system to test various kinetin analogs. Haber and Luippold (*36*) showed that this promotion of germination was maintained even if cell division was prevented by massive doses of radiation. They concluded that cell enlargement rather than cell division was being stimulated. Results from the leaf expansion test used by Kuraishi, Okumura, and their associates (*37*) would support this conclusion. They showed that a number of cytokinins would, in fact, stimulate the expansion of radish leaf discs. These and other results implicate cyto-

kinins as important controls of cell enlargement as well as cell division.

The metabolic effects of cytokinins are at least as varied as those on other aspects of plant growth. Thus, for example, Letham lists promotion of protein, RNA, lipid, and starch synthesis among the long list of other more specific effects (*4*). Cytokinins have been reported to stimulate production of tyramine methyltransferase in barley roots (*38*), to inhibit certain glycolytic kinases (*39*), and to be substrates for xanthine oxidase (*40*). Apparent decreases in enzyme activity in the hexose monophosphate shunt without concomitant decreases in the activity of glycolytic enzymes have been noted with high ($10^{-5}M$) concentrations of kinetin (*41*). The multiplicity of the above effects, together with results reported elsewhere (*4*), indicates the failure, to date, to specifically link cytokinins to any particular biochemical reaction. Considering the low concentrations of cytokinins which give visible biological effects (10^{-10} to $10^{-11}M$), this is not at all surprising.

An important question which was asked soon after the isolation of kinetin was just how specific are the chemical structure requirements for cytokinin activity? Early work, summarized by Strong (*2*), indicated that high activity was restricted to N^6-substituted adenines. In general, other workers have confirmed these findings (*35, 37, 42*). However, the differing specificities of various tests have prevented full agreement.

In addition, reports of activity of 1-substituted (*22*) and 3-substituted adenines (*20*) appeared in the literature. In both of these cases it was possible to show that neither of these compounds was active. However, some treatments, including heating, convert them into active compounds (*21, 43*). A recent study (*23*) combining the highly specific tobacco callus bioassay with a systematic program of synthesis of adenine derivatives has confirmed the earlier reports of Strong (*2*). In brief, the findings of this study were:

1) Only N^6-substituted adenines definitely possessed cytokinin activity. However, the activity of these compounds varied considerably with the length, degree of unsaturation, and substitutions on the side chain.

2) Modification of the adenine moiety, for example, substitution of *N* for *C* at the 8-position, reduced activity often by 95 percent.

3) A second substituent at the 1- or 3-position of the purine ring nearly eliminates cytokinin activity. However, a second substituent at the N^6-, N^7-, or N^9-position has much less of a depressing effect on activity.

Interestingly enough the N^6-substituted adenosines possess high cytokinin activity. In some cases they are only slightly less active than their corresponding adenines.

Although the most active cytokinins are of the N^6-substituted adenine type, other, quite different compounds possess cytokinin activity. Steward and his associate reported that 1,3-diphenyl urea was an active ingredient in coconut milk (*44*).

Recently, many substituted ureas have been reported to possess low, but reproducible, activity (*45*). Thus, although the most active of the ureas is still considerably less potent than kinetin, they may be classified as cytokinins. The reason for their low activity and their relationship (if any) to the adenine-type cytokinins remain obscure.

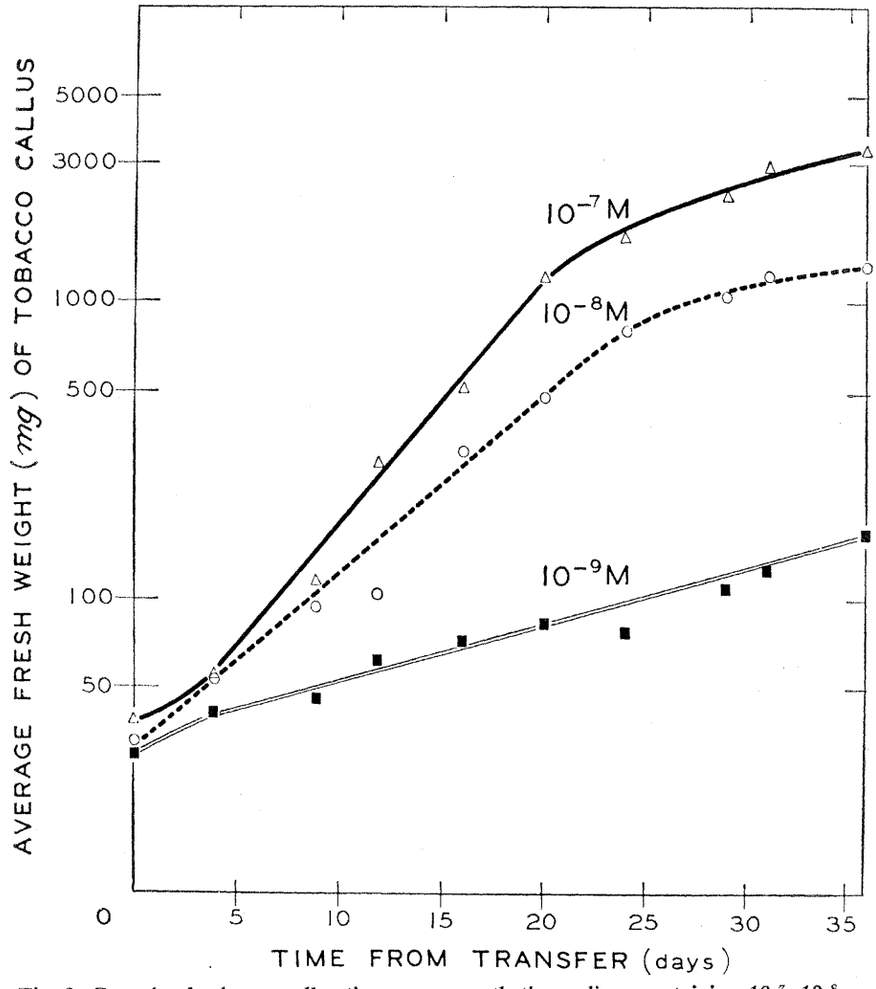

Fig. 2. Growth of tobacco callus tissue on a synthetic medium containing 10^{-7}, 10^{-8}, or $10^{-9}M$ 6-benzylaminopurine. (Helgeson, unpublished results)

Chemistry of Cytokinins

To a large extent it was the advance of knowledge in purine chemistry which permitted the characterization of the naturally occurring cytokinins. As the amounts of natural material are never very large [for example, Hall and his associates (*13*) isolated only 1.9 mg of zeatin riboside from 230 kg of corn] the characterizations have relied very heavily on physical methods, particularly, mass spectrometry and ultraviolet spectroscopy. As the usefulness of these methods is enhanced by extensive lists of compounds for comparison, it is fortunate that the interest in purine derivatives as cancer antimetabolites, as minor constituents of nucleic acids, as interesting natural products, and as cy-

tokinins has greatly increased the number of known purines.

The use of the mass spectrometer has been particularly important in the characterization of naturally occurring cytokinins. In fact, it has been a major basis for most of the identifications made to date (*11, 13, 15, 17, 18*).

Chemical molecules fragment in a predictable fashion when hit by a high-energy electron beam. In usual practice, the positively charged ions produced by the irradiation are spread according to their ratio of mass to charge (*m/e*) on a detector. By observing these fragments one can get some idea of how the molecule is put together. In addition, as a signal resulting from ionization of the whole molecule is often received, a precise determination of its molecular weight is possible.

The technique of inserting the sample directly into the electron beam has permitted analysis of relatively nonvolatile materials such as purines and various nucleoside derivatives (*46*). Within the adenine series a particularly strong peak at *m/e* 135 occurs which is due to the adenine ion ($C_5H_5N_5+$). The difference between the molecular ion (often the peak with the highest *m/e* ratio) and *m/e* 135 will then give a clear indication of the combined atomic weights of the substituents on the adenine ring.

The usefulness of this method may be seen by examining the mass spectra of the isomeric cytokinins, 6-(γ,γ-dimethylallylamino)purine (2iP) and 6-(3-methyl-3-butenylamino)purine (3iP), shown in Fig. 4 (*47*). The adenine ion (*m/e* 135) as well as two other fragments (*m/e* 119, purine ion, and *m/e* 108, adenine minus HCN) which are typical of many adenine derivatives are clearly seen. However, although these compounds differ only in the position of the double bond in the side chain, the fragmentation patterns differ greatly. For example, the signals at *m/e* 188 and *m/e* 160, so prominent in the spectrum of 2iP, are very weak in the spectrum of 3iP. Instead, a very strong peak corresponding to the loss of all but one carbon and two hydrogens of the side chain (*m/e* 148, $C_6H_6N_5+$) is predominant. Thus the molecular weight (203), the presence of the adenine moiety, the combined atomic weights of the side chain (69), and the position of the double bond in the side chain can be deduced from the spectral characteristics of the natural product

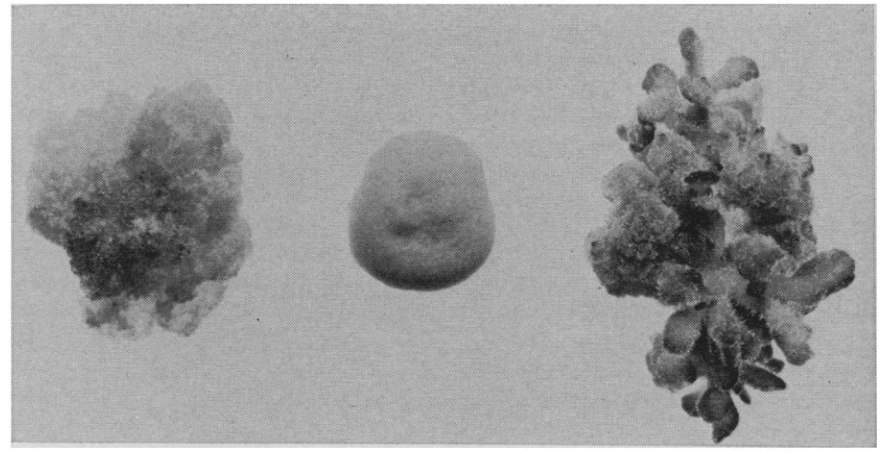

Fig. 3. Growth and differentiation of tobacco callus tissue on medium containing, left to right, 2 mg of indoleacetic acid (IAA) per liter and $10^{-7}M$ kinetin; 2 mg of IAA per liter and $10^{-6}M$ kinetin; 1 mg of IAA per liter and $5 \times 10^{-5}M$ 6-(γ,γ-dimethylallylamino)purine (2iP). All tissues derived from the same stock tissue and all are 4 weeks old. (Helgeson, unpublished results)

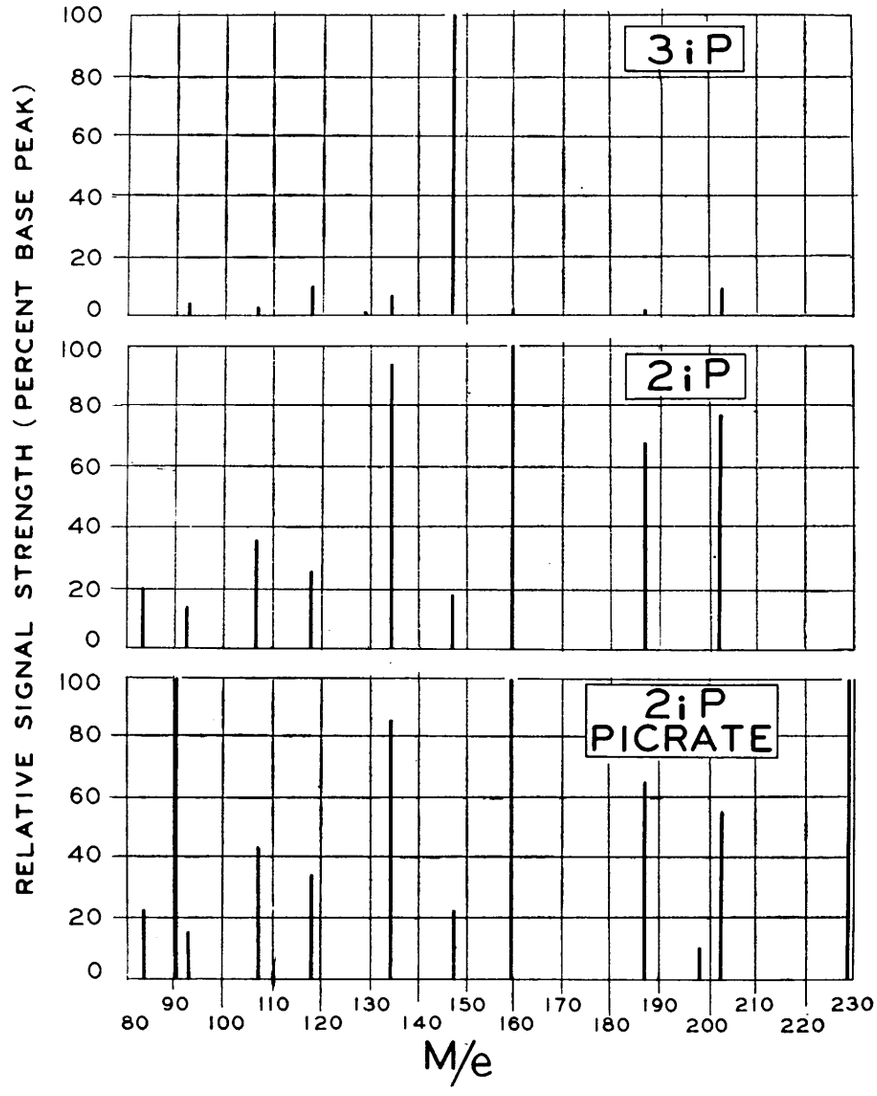

Fig. 4. Simplified mass spectra of 6-(3-methyl-3-butenylamino)purine (3iP), 6-(γ,γ-dimethylallylamino)purine (2iP) and 2iP-picrate. Only abundant ions above *m/e* = 80 shown. [Data taken from Leonard *et al.* (*47*) and Helgeson and Leonard (see *18*)]

as compared with synthetic materials of known structure.

The mass spectrum of 2iP-picrate in Fig. 4 illustrates another fortunate circumstance. In our comparisons of picrates of purines and other substances we noticed that, in every case, the molecular ions of picric acid (*m/e* 229) and its associated base (in the illustrated case, *m/e* 203) were clearly visible (*18*). Furthermore, signals from large fragments of the base (above *m/e* 91) were not obscured by fragments from picric acid, and were identical with signals received from the pure free bases. This circumstance makes the mass spectra of cytokinin picrates almost as useful as those from the free bases. As the picrates of these substances are often more easily crystalized than the free bases and the final product is increased by the weight of picric acid, this method may prove quite useful in the future.

Once the presence of the adenine ring system is detected and the size and fragmentation pattern of the substituent has been obtained, another problem, that of the position of substituent, must be faced. Within the adenine series, ultraviolet spectral analysis has been particularly useful in this regard. Purines and pyrimidines absorb ultraviolet light quite strongly in the region from 250 to 280 mμ. With the cytokinins, as little as 10 to 20 μg in a milliliter of solvent will give useful spectra. During the last few years, synthetic methods and spectral data for many singly or multiply substituted compounds have been reported. Correlations of the ultraviolet spectra of these compounds have shown that the substituted purines can be differentiated on the basis of their ultraviolet spectra (*48, 49*). Thus, for example, the ultraviolet spectra of zeatin shown in Fig. 5 mark it indelibly as an N^6-substituted adenine and the relatively short wavelengths of the maxima indicate that it is probably mono-substituted, that is, all the contained atomic weight of its substituent is included in a single group attached by one bond to the N^6-nitrogen. It is usually best to examine the shape of the spectrum rather than just to note the maxima and minima. The latter notation would, for example, ignore the diagnostically important shoulder on the 1-substituted adenines which extends all the way to about 330 mμ. Absolute ethanol made to 95 percent with water, aqueous base, or acid is a useful solvent

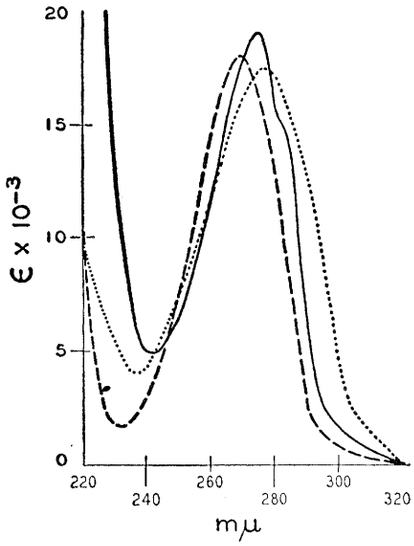

Fig. 5. Ultraviolet spectra of zeatin in 95 percent ethanol. The spectrum in neutral ethanol is denoted by a dashed line (– – –); the spectrum in acidic ethanol is denoted by a dotted line (. . .); the spectrum in basic ethanol is denoted by a solid line (——). (Helgeson and Leonard, unpublished results)

both for giving maximum structure to the spectrum and for solubilizing many adenine derivatives. Furthermore, spectra in acidic, neutral, and basic solutions are desirable for making definitive comparisons. Otherwise, for example, the identical spectra in basic and neutral solution, characteristic of a nucleoside, may not be seen.

Comparison of spectra should be made with the same solvent system. If one compares the published data for zeatin (*11*) with the spectrum shown in Fig. 5, one will note that while the data for basic and neutral maxima are identical, the acid maxima differ slightly with the solvent (275 mμ for aqueous acid, 278 mμ for ethanolic acid). A similar situation exists for 6-(γ,γ-dimethylallylamino)purine (*17, 18*).

Information on the structure of the side chain obtained from mass spectrometry and on the position of the substitution obtained from ultraviolet spectroscopy may be supported by results of nuclear magnetic resonance (NMR) spectroscopy. Although cytokinin derivatives may not be very soluble in some common NMR solvents (H_2O, D_2O, $CDCl_3$, and others) they will often dissolve in deuterated dimethyl sulfoxide (DMSO-d_6). Clear signals from the 2 and 8 ring hydrogens are usually seen if, of course, their positions are not substituted. Furthermore, the separation and relative order (2 versus 8) can be correlated with the position of substitution, thus providing collaborating evidence for the assignment from ultraviolet data (*48, 50*). The paucity of the hydrogens in the purine ring system and the rather low field signals for 2 and 8 hydrogens (far away from methyl hydrogen signals of the internal standard) provide a clear look at the hydrogens of any substituent. As reporting of NMR data has become standard in descriptions of synthetic products (*11, 17, 23*), much information allowing reliable assignment of structures to signals received is available today.

In addition, NMR spectroscopy is useful for comparing materials before and after a particular diagnostic reaction. Thus, for example, Leonard and Deyrup were able to deduce the structure of the γ,γ-dimethylallyl side chain of triacanthine, the 3-substituted isomer of 2iP, from NMR studies before and after hydrogenation of the double bond (*50*). However, the usefulness of the method for natural products is limited by the sample size required (several milligrams) unless special procedures (micro sample technique or repetitive scan) are employed.

Although infrared spectra of cytokinins are not particularly useful, the amphoteric nature of the adenine ring system may be used to good advantage. The acid dissociation constants (pK_a) of mono-substituted adenines vary with the position of the substituent (*50*), thus providing another check on a structural assignment. Furthermore, this capability for ionization permits use of cation or anion exchange resin for purifications. This property, coupled with the strong ultraviolet absorptions of these derivatives, makes purifications utilizing resins and paper chromatography quite practical, if somewhat laborious. A useful general procedure for "minor nucleoside" isolation has been published by Hall (*51*).

Chemical and Biological Synthesis

Of course, an important final step in the identification of the natural product has to be the synthesis of the compound with the proposed structure and direct comparison with the natural product. A number of good synthetic methods involving cyclization of pyrimidine and imidazole derivatives (*52*),

exchange amination (*53*), displacement of chlorine (*54*) or an alkyl-thio group (*55*) at the 6-position of the purine ring or direct alkylation (*56*) are available today. Of most general usefulness for cytokinin syntheses are the displacement reactions for preparations of N^6-substituted adenines and adenosines and the alkylation reactions for preparation of adenosine and adenosine-5′-phosphate derivatives.

Kinetin was first efficiently synthesized by the reaction of furfurylamine and 6-methylmercaptopurine (*1*). Although this remains a convenient method today, the reaction with 6-chloropurine and the appropriate amine is probably even more useful. Zeatin and zeatin riboside (*57*) or 2iP (*58*) may be prepared via this method, for example. The ready availability of 6-chloropurine and a number of amines, together with simplicity of the reaction (no sealed tube or extremely high temperatures are required), make this method especially attractive. A typical preparation is illustrated by the upper row in Fig. 6. This general procedure has been found useful for the preparation of cytokinin ribosides (*17*).

An alternative synthesis of cytokinin ribosides and ribotides involves the direct alkylation of adenosine or adenosine-5′-monophosphate (AMP). This reaction takes advantage of the directing influence of a substituent at the 9-position of the purine ring. Leonard and Fujii (*56*) noticed that such a substituent directed alkylation to the 1-position and thereby produced another good synthesis for 1-substituted adenines. Furthermore, the 1-substituted compounds easily rearrange to N^6-substituted (*43*). Recently Grimm and Leonard prepared 6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosyl purine-5′-phosphate by alkylation of the sodium salt of AMP and subsequent rearrangement of the 1-isomer to the N^6-compound (*59*) (Fig. 6).

Although a number of other procedures can be used, particularly when variously substituted compounds are needed to determine the structure-activity relationship of cytokinins, the methods outlined above appear generally useful for preparing highly active cytokinins.

How nature synthesizes these compounds is another matter. The isoprenoid nature of the substituent of the N^6-position of the naturally occurring cytokinins suggests that the biosynthesis of these compounds may parallel, to some extent, the biosynthesis of sterols and other terpenoid compounds. A central compound in this pathway is mevalonic acid (*60*). Recently it has been shown that mevalonic acid is indeed a likely precursor for the γ,γ-dimethylallyl side chain. Peterkofsky (*61*), utilizing a mevalonate-requiring strain of *Lactobacillus acidophilus*, has demonstrated incorporation of radioactive carbon from mevalonic acid-2-C^{14} into transfer RNA and, through hydrolysis and chromatography, has isolated the substance which contains most of this label. Chemical and chromatographic comparisons indicate that this compound is probably identical with 6-(γ,γ-dimethylallyl)adenosine (2iPA). Similar results have been obtained by Hall and his co-workers (*62*).

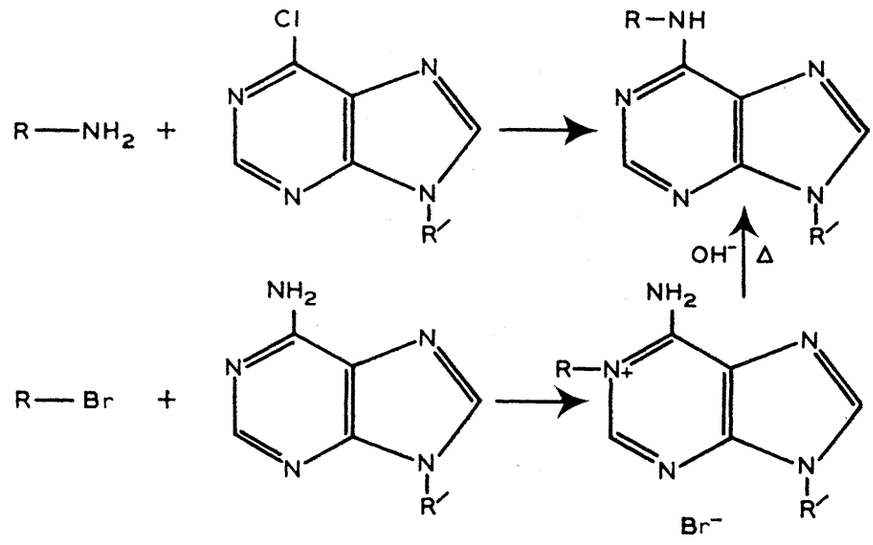

Fig. 6. Two common synthetic methods for cytokinins. The reaction with an amine and 6-chloropurine (upper row) may proceed with R′ equal to hydrogen or some other substituent. The alkylation reaction (lower row) requires that R′ be other than hydrogen (commonly ribose or the sodium salt of ribose-5′-phosphate) if alkylation is to be directed to the 1-position.

Thus we have the interesting finding that three different types of growth substances—the gibberellins, abscisic acid (an inhibitor), and the cytokinins—all start from a common precursor, mevalonic acid.

Cytokinins and Nucleic Acids

Unique among the plant growth substances is the well-documented localization of cytokinins in nucleic acids. Different laboratories with very different directions of research came upon this phenomenon.

Since the isolation of kinetin from DNA preparations, there has been a suspicion that cytokinins might in some way be involved with the nucleic acids. Thus, in the exploratory work with kinetin it was shown that DNA synthesis was stimulated in dividing tobacco tissues when kinetin was present in the medium (*63*). Osborne showed that, during prevention of senescence in xanthium leaves by 6-benzylaminopurine, the synthesis of RNA as well as protein was maintained above control levels (*34*). Although there was considerable breakdown of the cytokinin, little was detected in nucleic acid fractions (*64*). In January 1966 Fox reported incorporation of radioactive N^6-benzyladenine into RNA of tobacco and soybean tissues (*65*). Although there was extensive breakdown of the compound, a small but reproducible amount of intact cytokinin was incorporated into soluble, but not ribosomal, RNA.

During the determination of the base sequences of serine tRNA species, Zachau and his co-workers (*15*) noticed an "odd" base immediately adjacent to the anticodon (the sequence of bases which carries the matching code for the messenger RNA which in turn specifies the proper place for the amino acid in the new protein). In collaboration with Biemann and his associates, this base was identified as 6-(γ-γ-dimethylallylamino)purine, one of the most highly active cytokinins known (*66*). Subsequently, Hall and his associates isolated this compound from RNA hydrolysates from yeast, calf liver, human liver, peas,

and spinach (*13, 17*). This same base has been found adjacent to the anticodon of a tyrosine tRNA by Madison and his associates (*16*). Skoog *et al.* (*67*) detected cytokinin activity in tRNA from yeast, liver, and *Escherichia coli* RNA. Significantly, preparations of arginine, glycine, phenylalanine, valine, and alanine tRNA did not contain cytokinin activity according to tests with the tobacco callus bioassay. The lack of cytokinin activity in alanine tRNA agrees with the absence of such a compound in the base sequence of alanine tRNA as determined by Holley *et al.* (*68*). Thus cytokinins have been found to be present in at least two types of tRNA's and absent from five others. It appears that cytokinins are not generally present in ribosomal RNA (*13, 17, 65*).

Estimates of the total amount of cytokinin present in RNA fractions have been made by several groups. Hall and his associates (*17*) estimated that from 0.05 to 0.1 percent of the bases might be cytokinins. Assuming about 80 bases in each tRNA species and incorporation of only 1 molecule per tRNA molecule, this would amount to from 0.8 to 1.6 molecules of cytokinin per 20 molecules of tRNA. Skoog *et al.* (*67*) estimated about 1 cytokinin in each 20 tRNA molecules. Slightly higher levels (1 to 10) have been estimated by Peterkofsky (*61*) on the basis of incorporation of radioactively labeled mevalonic acid. Data on the incorporation of intact bases tends to suggest the same general level of cytokinin content (*69*). The above considerations, together with the absence of cytokinin from some tRNA species, would suggest that the number of tRNA molecules containing cytokinins must be rather small.

Whether or not the presence of cytokinin in tRNA has any relation to its biological activity is another question. Although it might seem logical that the action of cytokinins can be through tRNA, the fact remains that the essential experimental connection has not been made. However, there is some evidence which is quite suggestive of such a connection. The presence of the cytokinin molecule immediately adjacent to the anticodon in serine and tyrosine tRNA's would suggest that the molecule is there for a specific purpose—perhaps to maintain the proper spacial arrangement for the anticodon. Models of the tRNA molecules would indicate that this is possible. Chemical modifications of cytokinin molecules *in situ* may well provide much information. Iodination of serine tRNA apparently decreases its binding affinity for the messenger-ribosomal RNA complex without affecting the acceptor activity for serine (*70*). After this treatment, no 6-(γ,γ-dimethylallyl)adenosine was detected in the tRNA. Treatments of this type are known to cause a 1 to 6 cyclization of 6-(γ,γ-dimethylallylamino)purine and a subsequent *complete* loss of cytokinin activity (*23, 43, 70, 71*). Thus it would appear that a change in a cytokinin in the tRNA can modify, in some way, the biological activity of the tRNA.

It may not be strictly correct to speak of *a* tRNA for *an* amino acid. Considerable evidence has been accumulated to indicate that several amino acids have multiple acceptor tRNA's (*72*). Serine tRNA, for example, may exist in at least four species. Although Zachau and his co-workers have demonstrated the presence of a cytokinin in two of these species, it is not known whether cytokinins are adjacent to the anticodon in all seryl-tRNA's. Obviously, such a demonstration (or negative conclusion) would have a considerable bearing on any proposed mechanisms of action of cytokinins via nucleic acids. Today several groups are actively working on the problem of the distribution of cytokinins within the total population of tRNA species.

Problems and Prospects

An intriguing feature of the biological effects of cytokinins is the diversity of effects obtained with different concentrations. Cytokinins not only permit (or promote) growth in terms of a "stop and go signal" but they also profoundly affect the growth habit of plants. Thus not only a quantitative change, in terms of duplication of existing cells, but also a qualitative change, production of new types of cells, occurs. It should be emphasized that these cytokinin effects take place within the complex protoplasm of cells and in most of these changes there is at least some evidence of an interaction of cytokinins with other growth substances. For example, an auxin such as IAA is required in tobacco tissue culture media for many responses to cytokinins. Furthermore, changing the level of IAA requires changing the level of cytokinin if a particular biological response is to be obtained (*26*).

In this same tobacco system, the addition of gibberellic acid to a medium containing optimum concentrations of IAA and cytokinins will further increase growth yields. In short, the presence of all three factors is required for maximum growth.

As there is still much to be learned about the cytokinins, perhaps it is too early to speculate on a detailed scheme for their mode of action. If cytokinins act via tRNA's it is possible that either the transfer of information from the gene or the utilization of this information could be affected. As tRNA's are the vehicles for delivering amino acids to the protein synthesis system it is quite possible to invoke a protein synthesis control capability for those transfer RNA molecules which contain cytokinins. On the other hand, there are known cases of apparent control at the information exchange level, for example, suppressor activity of serine tRNA (*73*). More data on the localization of cytokinins within specific tRNA fractions and the biological activity of these specific fractions is greatly needed.

There are other areas of cytokinin research which also need effort in the future. For example, the biosynthesis and degradation of these compounds is poorly understood. Furthermore, the control of biosynthesis and degradation, perhaps even more important than the pathways themselves, remains to be investigated. There is evidence for incorporation of intact cytokinins *and* for alkylation of existing bases already in the tRNA and no clear way of deciding between the alternatives is apparent yet. Perhaps both events occur.

Regardless of their mode of action, the cytokinins and other naturally occurring growth substances have provided useful model systems for studying the biochemistry of plant differentiation and the intermediary metabolism of plants. For example, it is now possible to produce, at will, buds and shoots from tissue cultures grown on completely defined media which contain only those substances known to exist in plants. By utilizing still other combinations of these completely defined materials it is possible to obtain experimental material of precisely known, yet experimentally variable, growth rates. Considerable effort on these defined systems would seem very much in order.

Thirteen years have passed since the isolation of the first crude batch of

kinetin. In retrospect, the progress made in these years has been very substantial. Literally hundreds of synthetic compounds have been prepared and many of them have been biologically active. Similarly, reports in the literature have indicated diverse biological effects in almost all plant types ranging from bacteria to trees. Today, events are moving rapidly in the field of cytokinins research. Perhaps 13 years from now all the pieces of the cytokinin puzzle will be in their proper places. Although this may be too much to ask, one *can* say that if progress is maintained at the present rate, we should understand much more about the intricate control of plant growth and differentiation.

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