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THE CHEMISTRY AND BIOCHEMISTRY OF PLANT HORMONES

Recent Advances in Phytochemistry
Volume 7

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ACADEMIC PRESS New York and London 1974

A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 77-182656

PRINTED IN THE UNITED STATES OF AMERICA

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PREFACE

At first glance one might suspect that our understanding of the chemistry and biochemistry of plant hormones should be further advanced than is the case with animal regulator substances. There are only five generally recognized groups of higher plant hormones: the auxins, the gibberellins, the cytokinins, abscisic acid, and ethylene. On the other hand, animal hormones are more diverse and are often more complex in structure. For example, proteins, peptides, steroids, and phenolic amines, which have major roles as animal hormones, are not presently known to be represented among the regulators of higher plants. Structure proofs for the proteins, and for many of the peptides and steroids, certainly represent more of a challenge than those for the hormones thus far encountered in plants. With regard to the biochemistry of hormones, one might also expect greater progress with plants. The latter have neither a nervous nor a muscular system and therefore require fewer control mechanisms than do their animal counterparts.

Yet it must be admitted at the outset that progress in the understanding of the chemistry, and particularly the biochemistry, of plant hormonal regulation lags behind the understanding of regulation in the animal kingdom. There is no counterpart in plant biochemistry to the elegant work that has been done on the influence of estrogens on uterine processes. We know nothing about carrier proteins involved in the transport of plant hormones, a subject in which considerable progress has been made with animal hormones. The role of cyclic adenosine monophosphate as a second messenger in animal metabolic regulation has been elucidated. Such a role for this compound in higher plants is still in doubt.

We mention these contrasts not in any spirit of discouragement, but rather to point out what a truly challenging research area is represented by the chemical and biochemical aspects of plant hormones. Furthermore, it is our hope that the present volume will be of aid to continued research efforts in this field.

The authors attempted to bring the reader up to date in an area in which the literature is often highly dispersed among physiologically and chemically oriented journals; they have critically evaluated their respective fields and have identified those topics where application of the discussed techniques and experiments can be expected to lead to a deeper understanding of the mode of action of plant hormones.

While there is no complete separation in the treatment of the hormones between chemistry and metabolism, the first three chapters display an orientation toward the chemical approach. In the review on gibberellins, modern isolation and characterization techniques are stressed. After reading this contribution, one can readily appreciate the powers of combining gas–liquid chromatography with mass spectral analysis in the solution of problems that have proven refractory to the more classical approaches The present status of cytokinin chemistry is described with emphasis on methods of structure elucidation, synthesis, and structure–activity relations. This chapter illustrates very well the synergistic effects possible when workers from different areas are able to collaborate. The review of the chemistry of abscisic acid, the most recently discovered hormone discussed in this volume, indicates that with modern techniques, progress in isolation, quantitative determination, characterization, synthesis, etc., can be extremely rapid. It is most remarkable that our knowledge concerning the chemical aspects of this compound is more or less at the same level as that of the other hormones, even though some of these were described almost 40 years ago.

Elucidation of the role of auxins at the molecular level has proven a tremendous challenge to the plant biochemist, and perusal of the chapter by Ray shows that even sophisticated experiments have not yet provided any generally accepted explanations concerning the mode of action of indoleacetic acid. Here, clearly, is an area that will be a challenge to any investigator who wants to take up the gauntlet. In the fifth chapter, evidence is presented that gibberellic acid has an accelerating effect on the synthesis of rough endoplasmic reticulum in barley half-seeds. Since gibberellic acid is also known to enhance the synthesis of various hydrolases in this system, it is of special significance that the membrane effects are observed before *de novo* enzyme synthesis is detectable. The suggested paths for the biosynthesis of ethylene are critically reviewed in the final chapter. At present, the best evidence indicates that methionine is the precursor in higher plants, and glutamic acid in fungi. This work demonstrates the difficulties in determining the biogenesis of simple molecules which arise from common intermediary metabolites. This knowledge of the biosynthesis should facilitate work on the regulation of ethylene biosynthesis.

The work summarized and discussed in the present volume shows the vast scope and multifaceted aspects of modern chemical and biochemical investigations of plant hormones. While progress may be a little slower in some areas than one had anticipated in the first flushes of discovery, the overall outlook is encouraging and will, we hope, lead additional investigators to pursue research in this fascinating and significant field.

We wish to thank the University-Wide Programs, State University of New York, Albany, New York, for its financial support through the Conversations in the Disciplines program. Acknowledgment is also made of the help provided by the SUNY College of Environmental Science and Forestry for hosting the Twelfth Annual Symposium of the Phytochemical Society of North America, under whose auspices this meeting was held. The Editor-in-Chief particularly wishes to thank the contributors to the volume for their cooperation, and also Ms. Diane Green, who achieved miracles in interpreting editorial changes and preparing the final manuscript copy.

V. C. Runeckles
D. C. Walton

Ernest Sondheimer

1923–1973

ERNEST SONDHEIMER

The organizer of the symposium on which this book is based, Dr. Ernest Sondheimer, died during the preparation of this volume.

Ernest Sondheimer was a long-time member of PSNA. Trained as an organic chemist, he became deeply interested in the biochemical and physiological aspects of seed dormancy. During the past nine years, he had been using his training and considerable talents in this area, bringing to the work a fresh viewpoint. His untimely death occurred when his studies on seed dormancy were making rapid progress.

Ernest was the rare combination of dedicated researcher and highly effective teacher, enjoying both activities. Those of us who were his colleagues, students, and friends will miss his interest in and enthusiasm about both his work and ours. Phytochemistry has lost one of its most able and ardent practitioners.

RECENT ASPECTS OF THE CHEMISTRY AND BIOSYNTHESIS OF THE GIBBERELLINS

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School of Chemistry, The University, Bristol, England

Introduction

The gibberellins comprise a large family of diterpenoid acids which were initially discovered as metabolites of *Fusarium moniliforme* (*Gibberella fujikuroi*), the causative fungus of the Bakanae disease of rice. The story of their discovery and emergence as an important group of ubiquitous plant hormones is a fascinating one which has been described in several reviews (e.g., Stodola, 1958; Stowe and Tamaki, 1957; Brian *et al.*, 1960). The gibberellins fulfill the following reasonable criteria for endogenous plant hormones: (*a*) when applied exogenously in extremely small amounts they induce a wide range of plant growth responses; (*b*) they are present in most, and probably all, higher plants; and (*c*) there is circumstantial

evidence that they move within the plant from site(s) of biosynthesis to site(s) of action.

The gibberellins are unique among plant growth hormones in their ability to stimulate growth in many *intact* plants. These growth effects, which are well documented, include: (*a*) phenotypic reversal of dwarfism in plants such as *Pisum* and single gene mutants of *Zea*; (*b*) induction of stem growth in rosette plants which otherwise require long-day photoperiods or exposure to low temperature; (*c*) stimulation of flowering under noninductive conditions in long-day rosette and caulescent plants and in some plants the modification of flower sex expression; and (*d*) breaking of dormancy in shoots of deciduous woody plants, tubers, and seed. In addition to these gross effects on whole plants, gibberellins have been shown to stimulate the *de novo* synthesis and/or excretion of many enzymes, for example, α-amylase, endo-β-glucanase, and endopentosanase during the germination of cereal grain. Some of these effects, especially those related to dormancy, can also be obtained with cytokinins and ethylene.

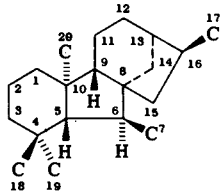
As a consequence of these varied growth responses, the gibberellins have considerable practical potential and many applications have been found for GA_3 and GA_4/GA_7 mixtures which are commercially available in quantity from fermentation of the fungus, *F. moniliforme*. The most important pertains to certain varieties of grapes where judicious spraying eliminates the need for girdling or thinning and produces berries that are larger and more attractive. Other applications include induction of male flowers in cucumbers for seed production, promotion of artichoke bud formation, stimulation of petiole elongation in celery, inhibition of surface defects in oranges, termination of dormancy in potato tubers, and acceleration of germination of barley, thus reducing malting times and increasing the amount of fermentable carbohydrates.

It is not surprising, therefore, that the gibberellins have been the subject of intensive chemical and biochemical study over the past few decades and many thousands of original publications have appeared during this time. Thus the scope of this article is limited to some of the more recent developments. Attention is focused on two important aspects, essential for a rational study of the role of the gibberellins in the hormonal control of plant growth. The first concerns the chemical structures of the gibberellins which are discussed briefly with an emphasis on the modern methods of structure determination. The second aspect concerns the correlation of gibberellin levels with particular growth responses. Here the importance of the dynamic turnover of the hormones is stressed, and methods for the identification and estimation of microgram quantities of the hormones are discussed together with their biosynthetic and catabolic metabolism.

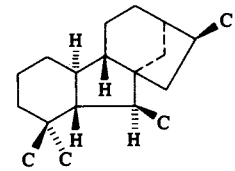
Chemical Structures

The gibberellins are diterpenoid acids which can be subdivided into two groups—the C_{20}-gibberellins containing all twenty carbon atoms of the parent *ent*-gibberellane (**1**) and the C_{19}-gibberellins that have lost carbon-20 and are derivatives of *ent*-20-norgibberellane (**2**). The parent system (**1**) is numbered (Rowe, 1968) in the same was as *ent*-kaurane (**3**) from which the gibberellins are biogenetically derived. For convenience the gibberellins are designated GA_1, GA_2, . . ., GA_n (MacMillan and Takashasi, 1968). The present author has listed structures of GA_1 to GA_{29} (MacMillan, 1971) and of GA_{30} to GA_{36} (MacMillan, 1972b). Structures **4** and **5** show the two most recent discoveries, GA_{37} and GA_{38}; these gibberellins were reported to occur as the D-glucosyl esters in mature seed of *Phaseolus vulgaris* by Hiraga *et al.* (1972).

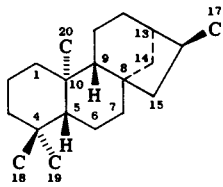
The C_{19}-gibberellins are characterized by the presence of a 19→10-lactone bridge, e.g., GA_3 (**6**), with the sole exception of GA_{11} which contains a 19→2-lactone. Otherwise they differ only in their oxidation state. So far double bonds are found at Δ-1, Δ-2, and Δ-16, and hydroxylation occurs at all carbon atoms with the exception of C-5, C-9, and C-14. Further oxidation to the 2- and 12-carbonyl functions occurs in GA_{26} and GA_{33}. The C_{20}-gibberellins (e.g., compounds **4** and **5**) are characterized by the presence of C-20 which may be present as CH_3, CH_2OH, CHO, or CO_2H functions. Carbons-7 and -19 are always present as CO_2H groups and hy-



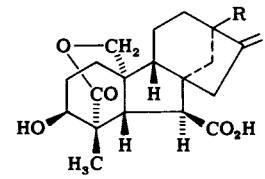
ent-Gibberellane
(C_{20}-Gibberellins)
1



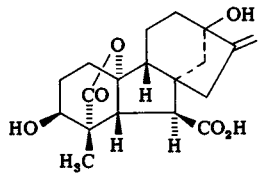
ent-20-Norgibberellane
(C_{19}-Gibberellins)
2



ent-Kauane
3

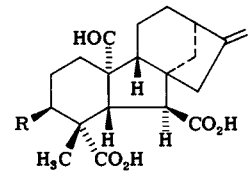


4 R = H, GA_{37}
5 R = OH, GA_{38}



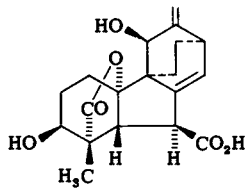
GA_3

6



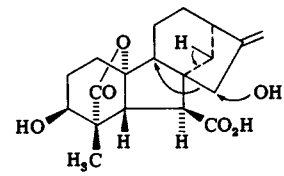
9 R = OH, GA_{36}

10 R = H, GA_{24}



A_{An}

7



GA_4

8

droxylation occurs at C-2, C-3, and C-13. So far only a Δ-16 double bond has been found in the C_{20}-gibberellins. The known gibberellins far from exhaust all permutations of the possible oxidation levels of *ent*-gibberellane (**1**) and *ent*-20-norgibberellane (**2**).

In addition to the thirty-eight known gibberellins, the functional derivatives, listed in Table 1, have been isolated. Of particular interest is the

TABLE 1

NATURALLY OCCURRING GIBBERELLIN DERIVATIVES

Derivative	Source	Reference
3-*O*-Acetyl GA_3	*Gibberella fujikuroi*	Schreiber *et al.* (1966)
Tetrahydro GA_3	*Sonneratia apetala*	Gaskin *et al.* (1972)
3-*O*-β-D-Glucosyl GA_3	*Pharbitis nil*	Yokota *et al.* (1969)
2-*O*-β-D-Glucosyl GA_8	*Phaseolus coccineus*	Schreiber *et al.* (1970)
2-*O*-β-D-Glucosyl GA_{26}	*Pharbitis nil*	Yokota *et al.* (1969)
2-*O*-β-D-Glucosyl GA_{27}	*Pharbitis nil*	Yokota *et al.* (1969)
2-*O*-β-D-Glucosyl GA_{29}	*Pharbitis nil*	Yokota *et al.* (1970)
11-*O*-β-D-Glucosyl GA_{35}	*Cytisus scoparius*	Yamane *et al.* (1971)
GA_4 D-Glucosyl ester	*Phaseolus vulgaris*	Hiraga *et al.* (1972)
GA_{37} D-Glucosyl ester	*Phaseolus vulgaris*	Hiraga *et al.* (1972)
GA_{38} D-Glucosyl ester	*Phaseolus vulgaris*	Hiraga *et al.* (1972)

recent isolation (Hiraga *et al.*, 1972) of the D-glucosyl esters of GA_4, GA_{37}, and GA_{38} which provide the first characterized examples of neutral gibberellins, originally detected by Hashimoto and Rappaport (1966). A notable achievement has been the recent elucidation of the structure (**7**) for the antheridium-inducing factor (A_{An}) from the fern, *Anemia phyllitidis* (Nakanishi *et al.*, 1971). The structure corresponds to a rearranged gibberellin, formally derivable from GA_4, as shown in structure **8**, and helps explain the earlier observations of the gibberellin-like properties of antheridiogens and the antheridium-inducing properties of gibberellins.

Structure Determination

The methods used to determine the structures of the gibberellins have become progressively more sophisticated, paralleling the development of physical methods, in particular nuclear magnetic resonance (NMR) and mass spectrometry (MS), and their application in organic natural product chemistry. Thus the first structure to be established, that of GA_3 (**6**), was determined mainly by classic chemical methods, assisted by infrared and ultraviolet spectroscopy. The chemical evidence for structure **6** has been reviewed (Brian *et al.*, 1960; Grove, 1961). Nuclear magnetic resonance, which was then in its infancy, provided support for the ring A structure of GA_3 toward the end of the structure determination and MS was not employed. Structure **6** and its stereochemistry were subsequently confirmed by X-ray analyses.

Such a wealth of data has now accrued correlating gibberellin structures with their NMR and MS spectra that it is possible to establish the structures of new gibberellins by these spectroscopic methods using 1 mg or less of compound. Occasionally the structure is deducible from MS alone on submicrogram quantities, although it is always prudent in these cases to seek confirmation by NMR or by a partial synthesis. The application of these methods, which are summarized by MacMillan and Pryce (1973), is well illustrated by the detection and structure determination of GA_{36} (Bearder and MacMillan, 1972).

In a search for new diterpenoid metabolites from a mutant of *Fusarium moniliforme* the material remaining after the isolation of GA_3 was methylated and trimethylsilylated, then examined by combined gas chromatography (GC–MS). The total ion current (TIC) trace is shown in Fig. 1 where all the identified peaks are labeled. The peak corresponding to the new gibberellin (GA_{36}) was immediately recognizable from the GC–MS scan as a dimethyl ester monotrimethylsilyl ether, of a C_{20}-gibberellin with a molecular weight of 362 by the characteristic losses of $32(CH_3OH)$ and

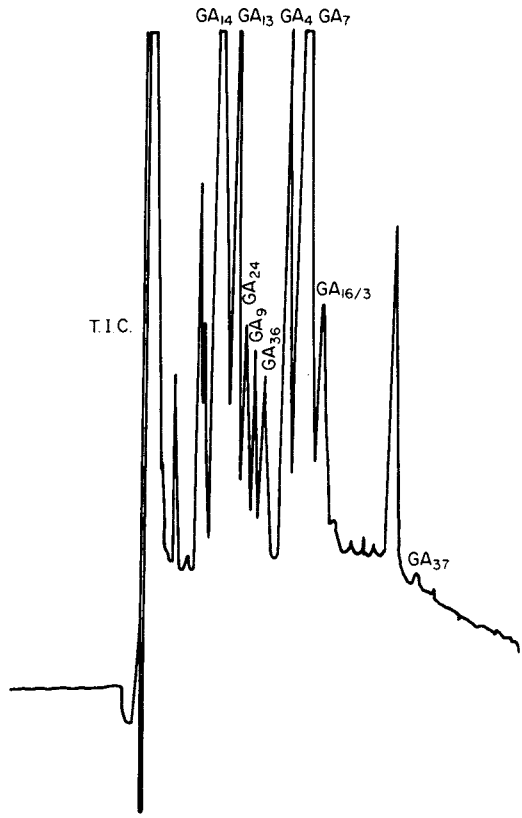


FIG. 1. Total ion current (TIC) trace of MeTMSi derivatized GA_3 mother liquors on 2 percent QF-1, temperature programmed 200°–260°C at 2°/minute.

60(CH_3COOH) amu which occurred successively, and in combination, from the molecular ion. The loss of 28 amu from the molecular ion in combination with the losses of 32 and 60 amu suggested the presence of an aldehyde group, and the intense (base peak) ion at *m/e* 129, characteristic of the trimethylsilyl ether of a 3-hydroxy gibberellin, was present. Thus, from the GC–MS, structure **9** could be deduced for GA_{36} which was then isolated by column and thin-layer chromatography (TLC). Structure **9** was then confirmed by high-resolution MS which defined the molecular formula as $C_{20}H_{26}O_2$ and by the NMR data shown in Table 2.

The NMR data in Table 2 illustrate a number of general correlations. First, the signals for two methoxyl groups, the exocyclic methylene protons, the 4-methyl group, and the aldehyde function (low-field 20-proton)

show the presence of *five* extracyclic carbon atoms. This evidence excludes an *ent*-kaurane derivative that possesses only *four* extracyclic carbon atoms and indicates an *ent*-gibberellane. Second, the AM double doublet of the 5- and 6-protons, with J 13 Hz, is typical of an *ent*-gibberellane. Third, the insignificant change in the chemical shift of the 17-protons on changing the solvent from $CDCl_3$ to C_5D_5N indicates the absence of a 13-hydroxy group; in those cases where a 13-hydroxyl group is present, this change of solvent causes one of the 17-proton signals to move downfield by 0.5 to 0.6 ppm (Hanson, 1965). Fourth, the low-field signal of the 6-proton, compared with GA_{37} methyl ester, for example, shows the presence of carbonyl substituents at C-4 and C-10 (in this case —CHO and $—CO_2H$) (Galt, 1965). Finally, the lower chemical shifts of the 5-proton and of the 4-methyl group, compared with those in the dimethyl ether of GA_{24} (**10**) in the same solvent system shows the presence of a 3β-hydroxyl group (Hanson, 1965).

Concurrently, structure **4** was deduced from similar spectroscopic evidence for the closely related GA_{37} which was obtained by hydrolysis of the naturally occurring glucosyl ester (Hiraga *et al.*, 1972). Particular points of interest in the NMR spectrum of GA_{37} methyl ester are (*a*) the AB-quartet of the 20-protons; (*b*) the higher chemical shift of the 6-proton in the absence of a carbonyl function at C-10; and (*c*) the coincidence of the chemical shifts of the 5- and 6-protons which is a feature of other *ent*-gibberellane 19→20-lactones.

Gibberellins A_{36} and A_{37} were interrelated by sodium borohydride reduction of the former (**9**) to the latter (**4**), and both structures were finally confirmed by the partial synthesis (Fig. 2) of GA_{37} from GA_{13} (**11**) (Bowen

TABLE 2

NUCLEAR MAGNETIC RESONANCE DATA FOR THE METHYL ESTERS OF GA_{36}, GA_{24} AND GA_{37}

Me ester	Solvent	3-H	5-H	6-H	17-H (br)	20-H	4-Me	O-Me
GA_{36}	$CDCl_3$	5.90 (*t*, J 3 Hz)	7.26 (*d*, J 13 Hz)	6.10 (*d*, J 13 Hz)	5.08 5.16	0.32*s*	8.78*s*	6.28*s* 6.37*s*
GA_{36}	C_5D_5N	5.52 br	6.66 (*d*, J 13 Hz)	5.71 (*d*, J 13 Hz)	5.06 5.14	0.04*s*	8.41*s*	6.29*s* 6.37*s*
GA_{24}	$CDCl_3$	—	7.81 (*d*, J 13 Hz)	6.19 (*d*, J 13 Hz)	5.16 5.24	0.38*s*	8.89*s*	6.34*s* 6.43*s*
GA_{37}	$CDCl_3$	6.26 (*t*, J 3 Hz)	7.26*s*	7.26*s*	5.10 5.22	5.58*d* 5.93*d*	8.81*s*	6.35*s*

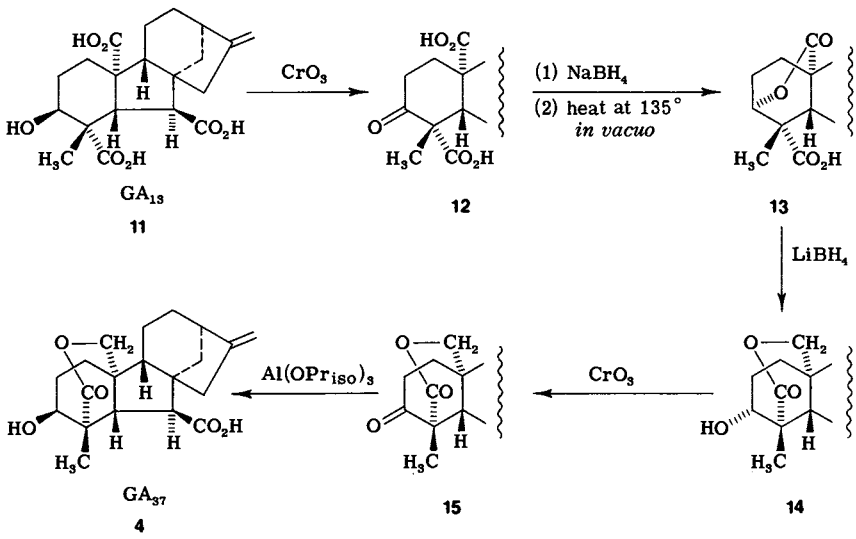


FIG. 2. Partial synthesis of GA_{37} from GA_{13}.

et al., 1972a). In this synthesis, the difficult step was the selective reduction of the most hindered 10-carboxyl group of **11** in the presence of the other two, more reactive carboxyl groups. This selectivity was achieved through the 20, 3-lactone (**13**), prepared from the 3-keto derivative (**12**) of GA_{13} by borohydride reduction followed by heating at 135°. Reduction of **13** with lithium borohydride gave the 3-epimer (**14**) of GA_{37} which was converted into GA_{37} (**4**) by oxidation to the 3-ketone (**15**), followed by reduction with aluminum isopropoxide in isopropanol.

As a likely biosynthetic intermediate between GA_{14} and GA_{36} (see p. 14), GA_{37} was expected to occur in the fungus. This expectation was realized by a careful reexamination, by GC–MS, of the GA_3 mother liquors (Fig. 1) (J. R. Bearder, unpublished data).

The detection of GA_{36} and GA_{37} by GC–MS, the structure determination of GA_{36} and GA_{37} by spectroscopic methods, the chemical interrelating of GA_{36} and GA_{37}, and finally the partial synthesis of GA_{37} from GA_{13}, provide an excellent example of present-day methodology in the structure determination of gibberellins.

Identification in Plants

In order to study the hormonal role of gibberellins in plant growth processes, methods of detection and identification of the endogenous hor-

mones in extremely small amounts are essential. To detail the methods which have been used would require a review of the development in chromatographic methods over the past two decades. It is perhaps sufficient to note that chromatographic methods, even when combined with bioassay, provide circumstantial, not conclusive, identification. The ideal method would require minimal purification of the plant extract and maximal sensitivity. It would also provide conclusive identification without the need for reference samples since these are not generally available. At present the method that best meets these requirements is GC–MS (MacMillan, 1972a). With the availability of reference mass spectra of suitable derivatives (Binks *et al.*, 1969; P. Gaskin and J. MacMillan, unpublished) this method is definitive without the need for reference compounds. It is also sensitive and requires no purification when the concentration of endogenous gibberellins in the extract is greater than 1 percent (Durley *et al.*, 1971). With moderate concentrations, minimal purification (for example, the use of TLC) is required; however, with very low concentrations (parts per million), extensive purification is usually necessary.

A recent example (D. H. Bowen, A. Crozier, J. MacMillan, and D. M. Reid, unpublished) of a very unfavorable situation concerns the identification of GA_1, GA_4, GA_5, and GA_{20} in light-grown seedlings of *Phaseolus coccineus*. Before GC–MS identification of these gibberellins was possible, it was necessary to purify the extract in the following way: (*a*) treatment with polyvinylpyrrolidone (PVP); (*b*) countercurrent distribution; (*c*) successive column chromatography on Sephadex G-10, PVP, charcoal–celite, silica gel, and charcoal–celite; (*d*) preparative TLC; and (*e*) preparative GLC of the methyl esters. These procedures gave partially purified fractions containing ca. 1 percent of each of the gibberellins. In a previous investigation (Crozier *et al.*, 1971) of the gibberellins present in dark-grown seedlings of *P. coccineus*, only GA_4 could be positively identified by GC–MS; two other gibberellins present in trace quantities await conclusive identification but are thought to be GA_1 and GA_{19} or GA_{20}. These results may be compared to those obtained for the gibberellins in the seed of the same variety of *P. coccineus* (Durley *et al.*, 1971).

Many other recent examples could be given to illustrate the power of GC–MS in the analysis of endogenous gibberellin levels. However, in attempting to correlate physiological function with endogenous levels, it is important to remember that the detected gibberellins may not be involved in the growth process; the physiologically active hormones may be turning over at a rate that precludes their detection. In any case, the detected levels only reflect the relative rates of biosynthetic and catabolic metabolism. For these reasons it is essential to investigate the biosynthetic and

catabolic metabolism of the gibberellins and factors that affect the rates of such metabolism. In this way an overall picture of the kinetic changes of the endogenous hormones may be obtained and related to growth processes.

Biosynthetic Metabolism

The outline of the generally accepted pathway of biosynthesis of the gibberellins is shown in Figs. 3 and 4. The evidence has been reviewed (Cross, 1968; Lang, 1970; MacMillan, 1971) up to the beginning of 1970; it is derived from radiolabeling studies, partly with cell-free enzyme systems from *Fusarium moniliforme* and higher plants, but mainly using cultures of *F. moniliforme* that biosynthesize the gibberellins in high yield. The present discussion will be confined to some notable advances that have been made since the last review.

Sequence a (Fig. 3) is well established for both higher plants and for the fungus, and some of the enzymes involved in higher plants have been studied (for leading references, see West and Fall, 1972). However, the demonstration, in higher plant systems, of the ring contraction of the *ent*-kaurane to the *ent*-gibberellane ring system (Fig. 4, sequence b) has presented a barrier for many years. One recent and significant advance, therefore, has been the conversion of mevalonic acid (MVA) into GA_{12}–aldehyde (**22**, Fig. 4) in a cell-free system from immature seed of *Cucurbita maxima* (Graebe *et al.*, 1972). This is the first report of the presence of GA_{12}–aldehyde (**22**) in a higher plant. It is also the first demonstration of sequence b in higher plants. In addition, the *C. maxima* system converts MVA into *ent*-kaur-16-ene (**16**), *ent*-kaur-16-en-19-oic acid (**19**), and *ent*-7α-hydroxykaur-16-en-19-oic acid (**20**) (Fig. 3) (Graebe, 1969; Graebe *et al.*, 1972). All these metabolites were identified by GC–MS. A notable feature of this work was the simultaneous determination, by GC–MS, of the specific radioactivity of these biosynthetic intermediates (Bowen *et al.*, 1972b). Complete characterization was achieved on the microgram scale. Since MVA is converted into these intermediates without dilution of the ^{14}C-label, this enzyme system offers the interesting possibility of producing gibberellins with very high specific radioactivity.

Another notable development concerns the mechanism of ring contraction (sequence b, Fig. 4). It is known from the first tracer studies by Birch *et al.* (1959) that carbon-7 of the *ent*-kaurene precursors is extruded in this ring contraction. Recently Hanson *et al.* (1972) have presented evidence (Fig. 5) that ring contraction involves transfer of hydrogen from the 6- to the 7- position. These authors do not discuss possible mechanisms but, from

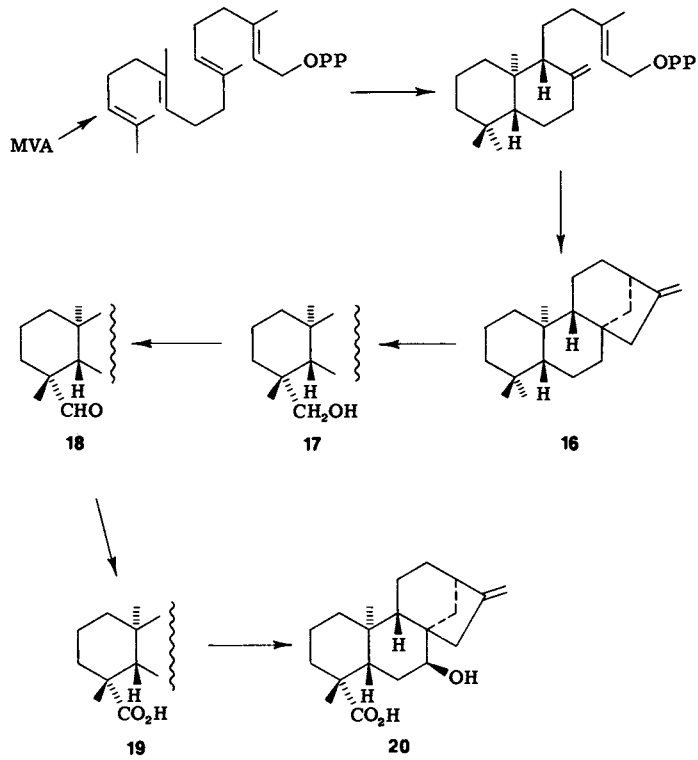


FIG. 3. Gibberellin biosynthesis: Sequence a. MVA—mevalonic acid; PP-pyrophosphate.

previous evidence of their own and others, at least two can be envisaged. They are shown in Fig. 6, depicting the fate of the 6β-H as a ^{3}H-label. Mechanism A involves an initial oxidation step and leads directly to the GA_{12}-aldehyde (**24**). In contrast, mechanism B is nonoxidative and leads to the GA_{12}-monoalcohol (**25**) as the primary product of ring contraction.

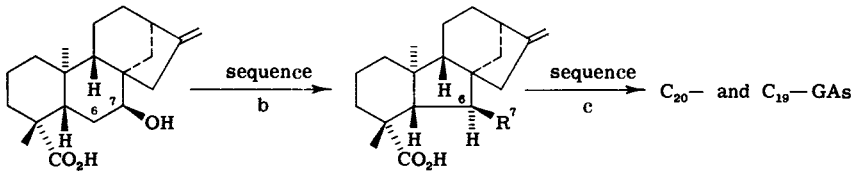


FIG. 4. Gibberellin biosynthesis: Sequences b and c.

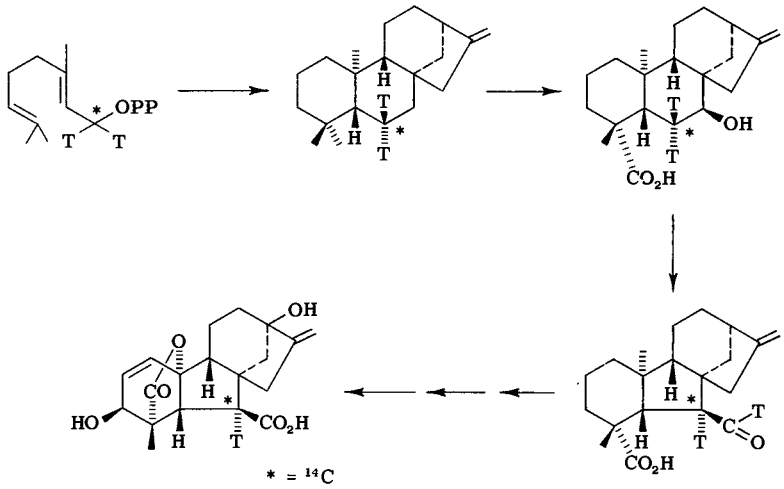


FIG. 5. Incorporation of 1,1-ditritiogeranylpyrophosphate into GA_{12}-aldehyde and GA_3 (data from Hanson *et al.*, 1972); PP—pyrophosphate.

Since GA_{12}-aldehyde was obtained (Hanson *et al.*, 1972) with retention of the initial 6β-^{3}H-label in the aldehydic group, a stereospecific oxidation of **25** to the GA_{12}-aldehyde (**24**) would be required for mechanism B. So far there is no direct evidence that the GA_{12}-alcohol is an obligatory intermediate in gibberellin biosynthesis although it is converted into gibberellins by *F. moniliforme* in very high yield (see p. 15).

Sequence c (Fig. 4) has been the subject of much speculation. In the case of the fungal gibberellins, the direct pathway to GA_3 is not known with certainty. Indeed, as indicated in the foregoing, there is still some doubt as to whether the initial *ent*-gibberellane is GA_{12}-aldehyde (**22**) or its monoalcohol (**23**). Whereas there is little doubt that C_{20}-gibberellins are precursors of C_{19}-gibberellins, the detailed pathways and, in particular, the mechanism of the loss of carbon-20, are not known. Some recent and unpublished work (J. R. Bearder, P. Hedden, J. MacMillan, and B. O. Phinney) promises to answer some of these questions. The approach is of some interest since it makes use of mutants of the fungus in which gibberellin synthesis is blocked (Phinney and Fukiyama, 1969). So far most work has been conducted with a mutant, B1-41a, which is blocked between *ent*-kaur-16-en-19-al (**18**) and *ent*-kaur-16-en-19-oic acid (**19**) (Fig. 3). Compound **18** occurs in the mutant but is not converted into gibberellins; compound **19** is absent but is converted. The metabolism of intermediates and conversion of gibberellins beyond the block has been studied as follows.

The mutant is grown on the ammonium nitrate medium described by Borrow *et al.* (1955) except that a lower initial nitrogen concentration is used. When pigmentation is well-developed, the mycelium is resuspended in the same medium, without ammonium nitrate, but containing the presumptive intermediate; it is buffered to a selected pH value. The resuspended mycelium is cultured for a selected time, then the medium is extracted with ethyl acetate. The extract is derivatized and examined by GLC, GC–MS, and GC–radioactivity counting (RC). The method is illustrated (Fig. 7) for the conversion products of GA_{12}–aldehyde (**22**) at pH 3.5 after 20 hours. The peaks were identified as indicated by GC–MS. The flame ionization detector (FID) trace contains only the background (B/G) peaks indicated so that much useful work can be obtained with nonlabeled substrates. However, the radioactivity (RA) of the conversion products from labeled substrates can be conveniently determined by GC–RC using the method of Belham and Neal (1972) as illustrated in Fig. 7.

With these methods the metabolism of many substrates has been ex-

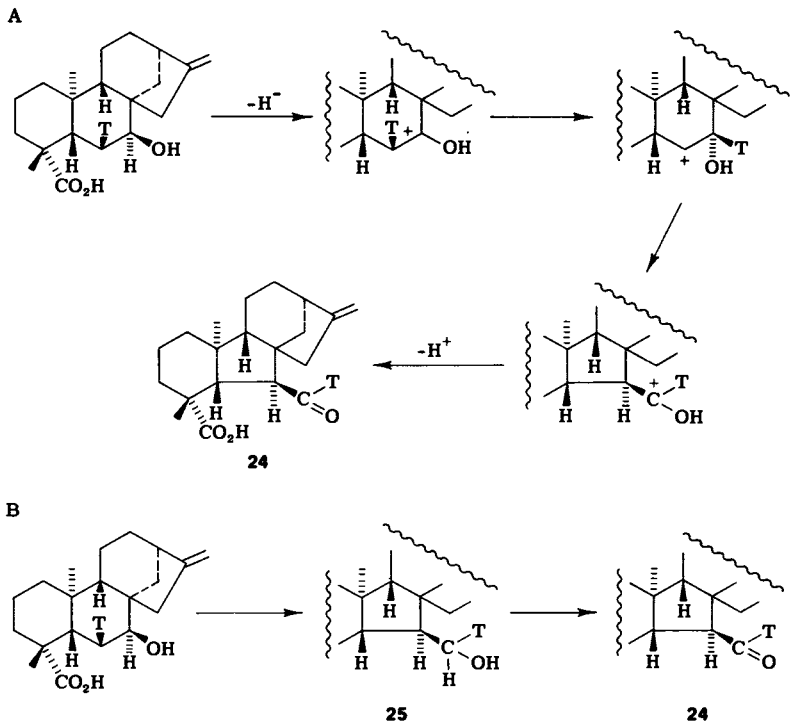


FIG. 6. Two possible mechanisms (A and B) of ring contraction.

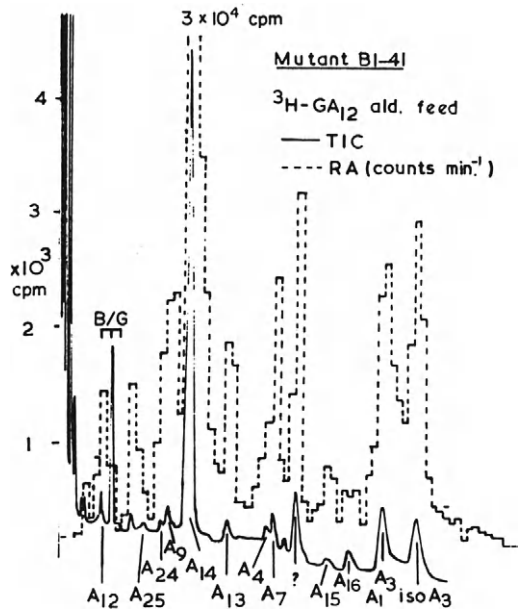


FIG. 7. GA_{12}-aldehyde-6-^3H conversion in *Fusarium moniliforme* mutant B1-41; gas–liquid chromatography (solid lines) and gas chromatography–radioactivity counting (broken lines) in counts per minute of methylated products. Gas chromatography conditions: 3 percent QF-1 column; 180°C for 25 minutes and then 2 percent per minute to 203°C. Unlabeled peaks are unidentified.

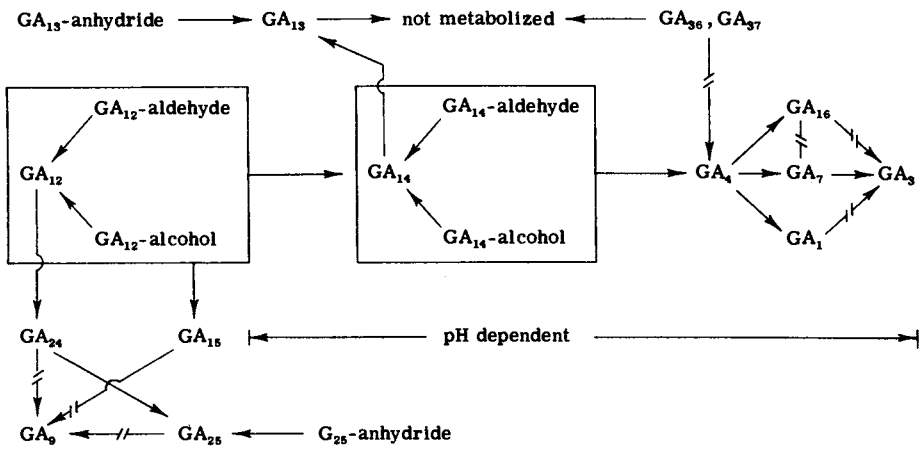


FIG. 8. Conversion products of gibberellins in *Fusarium moniliforme* mutant B1-41a.

amined. Some of the results are summarized in Fig. 8 where all the compounds shown have been fed (except for GA_3) and their conversion products have been identified by GC–MS. Only a few points can be singled out for special comment. Both GA_{12}–aldehyde (**22**) and GA_{12}–monoalcohol (**23**) are rapidly metabolized to GA_{14}, GA_4, GA_7, and GA_3. The results illustrated for GA_{12}–aldehyde in Fig. 7 represent an intermediate stage after 20 hours; after 48 hours, conversion into GA_3 is virtually complete. In contrast, GA_{12} is poorly metabolized to GA_{14}, GA_4, GA_7, and GA_3. Similarly, the 3-hydroxylated analogs, GA_{14}–aldehyde and GA_{14}–monoalcohol are rapidly metabolized to GA_4, GA_7, and GA_3, but GA_{14} is metabolized more slowly; the conversion of GA_{14}–alcohol into GA_3 is illustrated in Fig. 9. The GA_{14}–aldehyde has been shown to be produced from GA_{12}–aldehyde-6-^{3}H by isotope dilution. Since it both occurs in the fungus and is rapidly incorporated into GA_3, the GA_{14}–aldehyde qualifies as the next intermediate after GA_{12}–aldehyde (P. Hedden, unpublished results).

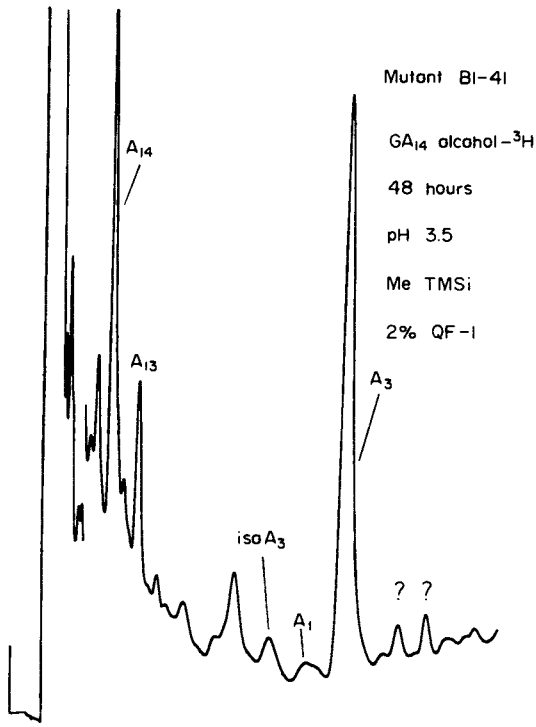


FIG. 9. Total ion current trace of metabolites from GA_{14}–monoalcohol-6-^{3}H in cultures of *Fusarium moniliforme* mutant B1-41a.

The GA_{12} also yields GA_{24} which is converted into GA_{25}. Although it has been suggested that carbon-20 of the C_{20}-gibberellins is lost at the aldehyde level of oxidation, GA_{24} was not converted into GA_9. It is also disappointing that GA_{36} (7), the corresponding 3-hydroxy aldehyde and possible intermediate between GA_{14} and GA_4, was not metabolized. Within the limits of detection, GA_{13}-anhydride and GA_{25}-anhydride did not yield GA_3 and GA_9, respectively, but were completely converted into the corresponding acids, GA_{13} and GA_{25}, which were not metabolized. These results contrast with the recent report (Hanson and Hawker, 1972) that GA_{13}–anhydride-^{14}C is specifically incorporated into GA_3 (0.14 percent) and $GA_{4/7}$ (0.07 percent). The GA_1 is not converted into GA_3 by mutant B1-41; neither is this conversion observed (J. R. Bearder, unpublished results) in mutant R-9 (Spector and Phinney, 1968) in which the conversion of $GA_{4/7}$ into GA_1/GA_3 is blocked. These results supplement those of Pitel *et al.* (1971).

ent-Kaurenoic acid (**19**) and *ent*-7α-hydroxykaurenoic acid (**20**) are also converted into gibberellins in this system. The steps from GA_{12} intermediates to GA_3 are inhibited by high pH, and those from the GA_{14} intermediates may be inhibited by ammonium nitrate although it has still to be firmly established that this is not also a pH effect.

Catabolic Metabolism

Catabolic metabolism has received much less attention than biosynthetic metabolism due in large part to the inaccessibility of gibberellins with sufficiently high specific radioactivity. Most of the reported studies concern the fate of gibberellins in developing seed where hydroxylation and glucosidation occur on maturation. Thus it is reported that GA_3, GA_6, and GA_8 are converted into GA_8-2-*O*-β-D-glucopyranosides (for leading references, see Sembdner *et al.*, 1972) during the ripening of the fruit of *Phaseolus coccineus*. Evidence has been presented (reviewed by Lang, 1970) for the view that the glucosides are biologically inactive storage forms from which the free gibberellins are regenerated during subsequent seed germination. Two recent studies are of special interest since they implicate abscisic acid (ABA) in gibberellin catabolism. Nadeau and Rappapport (1972) have shown that ABA enhances the formation of GA_8–glucoside from GA_1 in germinating seed of *Phaseolus vulgaris*; GA_1, which is a native gibberellin of such seed, was added in physiological concentration as the 1,2-$^3H_2$-labeled compound with a specific activity of 5 Ci/mmole. A similar effect was obtained in aleurone layers in which ABA substantially increased the uptake of GA_1-1,2-$^3H_2$ and its metabolism to four products. Two of these

metabolites were tentatively identified as GA_1– and GA_8–glucosides and one was established to be GA_8. From these results it was postulated that ABA inhibits the responses of the gibberellins by mediating their catabolic metabolism to inactive forms.

However, it is often difficult to decide where biosynthetic metabolism ends and catabolic metabolism begins. Thus, further hydroxylation does not necessarily reduce biological activity; for example, GA_{32}, the 12α, 16β-dihydroxy derivative of GA_3, is at least as active as GA_3 (Coombe, 1971).

Much remains to be done and the increasing availability of gibberellins with high specific radioactivity together with the use of GC–MS to identify and determine specific activity on the microgram scale should stimulate further studies. In this connection it is interesting to note that there is a potential route to a variety of gibberellins of very high specific activity in which MVA can be converted without radioactive dilution to GA_{12}–aldehyde in the *Cucurbita maxima* system; the latter can then be converted into gibberellins again without dilution using the *Fusarium moniliforme* mutant B1-41a.

Concluding Remarks

In this highly selective review, an attempt has been made to provide a perspective for those aspects of the chemistry of the gibberellins which are directly concerned with their function as plant hormones. Many other interesting aspects have had to be omitted, particularly recent progress toward total synthesis. Here the challenge has stimulated many interesting

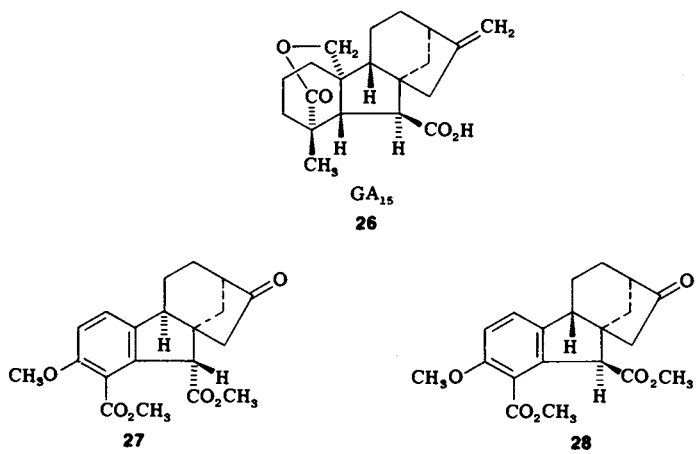


FIG. 10. Total syntheses.

synthetic approaches and several elegant routes to particular features, for example, the ring C/D of GA_3 and ring A of GA_3 and GA_7, have been devised. Of the many recent accomplishments, two may be mentioned (Fig. 10). First, the total synthesis of GA_{15} (**26**) by Nagata *et al.* (1971) and, second, the synthesis of the intermediates **27** (Loewenthal and Schatzmiller, 1972) and **28** (Baker and Goudie, 1972) as their acetals, both of which contain all the necessary features for the synthesis of GA_4 and GA_7.

Acknowledgment

I would like to thank Dr. J. R. Hanson for the friendly exchange of results prior to publication.

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CHEMISTRY OF THE CYTOKININS

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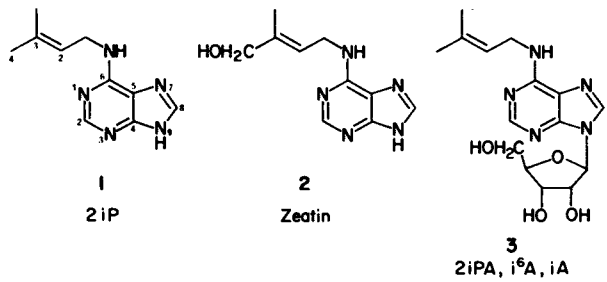
Introduction

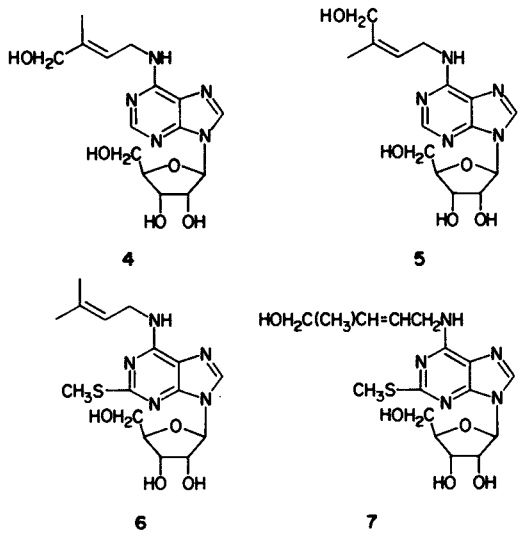
Cytokinins are plant growth substances that play a major role in cell division and cell differentiation. The measurement of cytokinin activity may be based on the growth of tobacco callus tissue, soybean callus tissue, or carrot tissue, the germination of seeds, or the retardation of leaf senescence. In the tobacco bioassay, callus originally derived from the pith of tobacco plants (*Nicotiana tabacum* var. Wisconsin No. 38) is grown on agar using a synthetic mineral nutrient medium containing indole-3-acetic

acid, thiamine hydrochloride, and myoinositol (Linsmaier and Skoog, 1965). Four replicate cultures, with three pieces of callus each, are kept at about 28°C in continuous, weak, diffuse light with varying concentrations of cytokinin present. After a 5-week growth period the total fresh and dry weights yields are determined. The range of concentrations over which the fresh weight increases nearly linearly with the logarithm of the cytokinin concentration (Leonard *et al.*, 1969) is reproducible for a single compound and is, therefore, useful in direct comparison with the linear ranges for other compounds. After a period of undifferentiated growth in the presence of added cytokinin, tobacco callus tissue differentiates to form plantlets which can be transplanted and will generally produce normal plants with viable seed.

Cytokinins have been the subject of numerous reviews (Skoog and Armstrong, 1970; Kende, 1971; Hall, 1970; Letham, 1969; Steward and Krikorian, 1971; Helgeson, 1968; Gaspar and Xhaufflaire, 1968). Accordingly, the present discussion is limited to recent work, with particular emphasis on the researches that have been carried out in a close collaboration between our laboratory at the University of Illinois and that of Professor Skoog at the University of Wisconsin. Together, we have been able to delineate the structural requirements for cytokinin activity, to elucidate the structure and stereochemistry of a number of naturally occurring cytokinins, and to develop the first potent antagonists to cytokinins. Interest in the chemistry of cytokinins stems largely from the observed variations in biological activity that accompany chemical and biochemical conversions and alterations in structure.

6-(3-Methyl-2-butenylamino)purine or "2iP" (or i^6Ade) for N^6-(Δ^2-isopentenyl)adenine (**1**), first obtained synthetically and shown to possess a high degree of cytokinin activity as indicated by detectable growth response at a minimum concentration of $<1 \times 10^{-10}$ M in the tobacco bioassay, was later isolated from cultures of the plant pathogen *Corynebacterium fascians*. The closely related hydroxy compound, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine or zeatin (**2**), first iso-





lated from the milky stage endosperm of sweet corn and later synthesized, shows detectable growth response at even lower concentration. Both of these adenine derivatives show greater activity than their corresponding ribosyl derivatives, compounds **3** and **4**, both of which have been found in nature. Also obtained from natural sources were the cis isomer of compound **4**, namely 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribo-

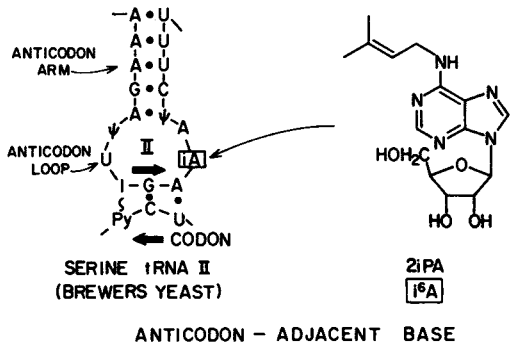


FIG. 1. Anticodon section of serine transfer ribonucleic acid (tRNA) from brewer's yeast. From Zachau *et al.* (1966). 2iPA—6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

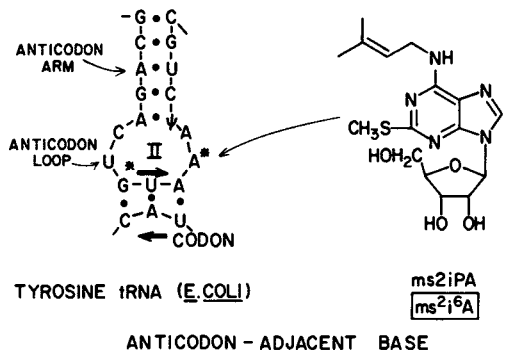


FIG. 2. Anticodon section of tyrosine transfer ribonucleic acid (tRNA) from *Escherichia coli* (from Harada *et al.*, 1968). ms2iPA—6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

furanosylpurine (**5**), 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**6**), and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**7**). It was the occurrence of the cytokinin-active ribonucleosides, **3** and **5**, in certain transfer ribonucleic acid (tRNA) species that translate codons starting with uridine and the location of compounds **3** and **5** adjacent to the 3′-end of tRNA anticodons (Figs. 1 and 2) (Zachau *et al.*, 1966; Harada *et al.*, 1968) that aroused particular interest because plant hormonal activity was found in a structural moiety in a specific site and at a strategic locus in a molecule of known function. These findings also stimulated a search for other cytokinin-active tRNA components and investigation of the metabolic fates of both the exogenous free-base cytokinins and the tRNA-contained modified adenosines.

Chemistry of the Cytokinins: Elucidation of Cytokinin Structures

Generally the meager quantities of natural cytokinin-active material have limited the methodology available for structure establishment to relative chromatographic mobilities, ultraviolet absorption spectra, and low- and high-resolution mass spectrometry (MS). Where separation and identification of known cytokinins are desired, the relative mobilities of the cytokinins chromatographed on Sephadex LH-20 columns in 35 percent ethanol serve as a reliable guide (Armstrong *et al.*, 1969). The relative retention times of the trimethylsilylated cytokinin bases and ribonucleosides in a variety of gas chromatographic systems also serve to identify a

known compound (Most *et al.*, 1968; Upper *et al.*, 1970; Babcock and Morris, 1970). The combination of gas chromatography and MS can be standardized to identify known cytokinins and to detect new ones. Final positive identification of cytokinin structure rests upon quantitative biological activity, mass spectral analysis, and total synthesis (Burrows *et al.*, 1970). The application of MS may be illustrated with the compound found within the second chromatographic zone of cytokinin activity released from the enzymatic digest of *Escherichia coli* tRNA (Burrows *et al.*, 1968, 1969) and also within the fourth and final zone of activity released from wheat germ tRNA (Burrows *et al.*, 1970). The fragmentation pattern was parallel to that of 2iPA or i^6A (**3**) but consistently 46 mass units greater (Fig. 3), and the ultraviolet maximum was at 280 nm rather than at 260 nm. These features suggested the presence of a CH_3S substituent that was not in an N^6 side chain or in the 9-ribosyl moiety but rather on the ring and in the 2-position, and, therefore, structure **6**. The low-resolution mass spectrum had peaks at *m/e* values of 381, molecular ion; 366, corresponding to the loss of CH_3 with a metastable peak confirming this

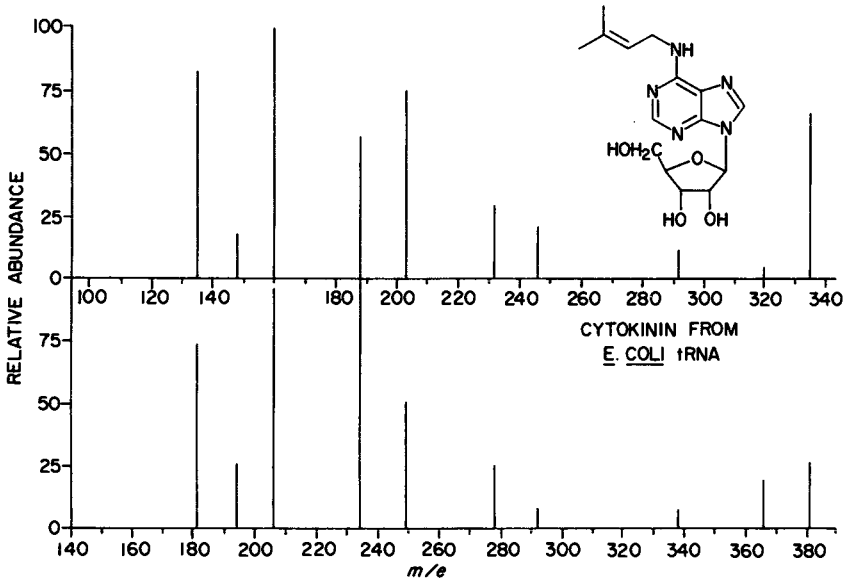
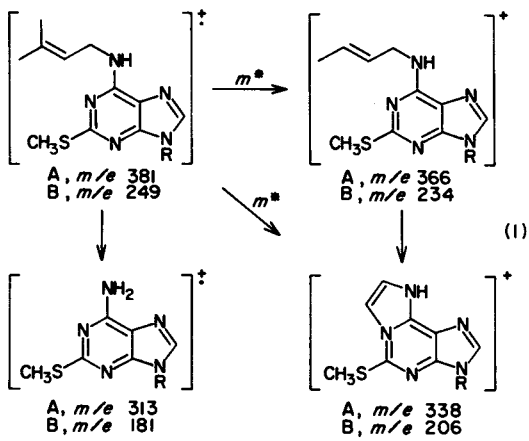


FIG. 3. Mass spectral fragmentation patterns of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**) and a second cytokinin isolated from *Escherichia coli* transfer ribonucleic acid (tRNA) (**6**), with *m/e* scale offset for the latter by 46 mass units. From Burrows *et al.* (1968, 1969).



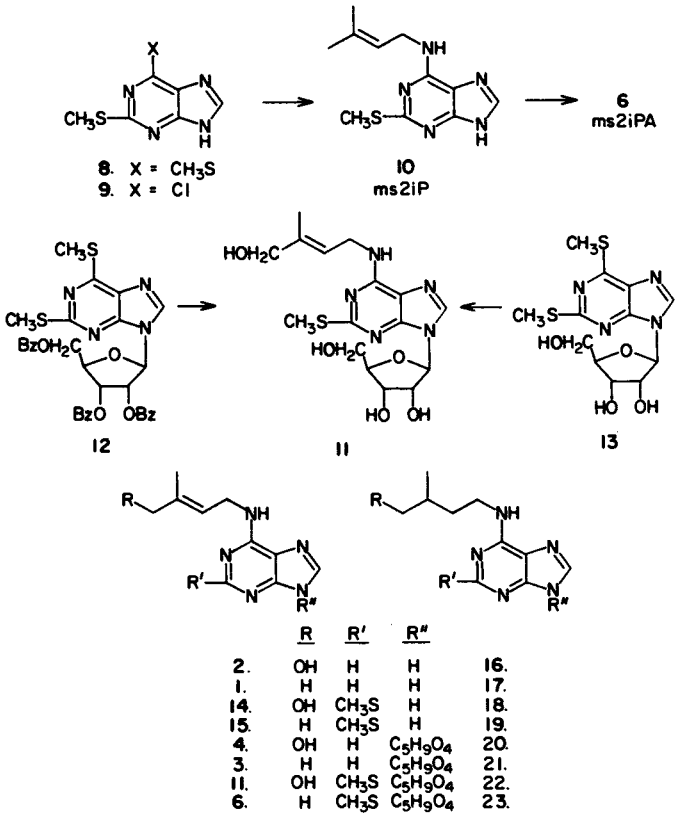
(1)

event; 338, corresponding to $(M\text{-}C_3H_7)^+$ with a metastable peak; and 313, corresponding to $(M\text{-}C_5H_8)^+$. A parallel set of *m/e* values was observed for the deribosyl or B + 1 unit, namely 249, 234, 206, and 181. The partial fragmentation patterns are shown in Eq. (1). The compositions of these and other major peaks were identified by high-resolution MS. Similarly, mass-spectrometric data were used to identify 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ribosyl-2-methylthiozeatin) (**7**) from wheat germ tRNA, which had a fragmentation pattern parallel to that of ribosylzeatin (**4** or **5**) but 46 mass units greater at each stage. The compositions of all the fragments were confirmed using high-resolution MS. Synthesis of the assigned structures, e.g., **6** and **7**, and comparison of the compounds derived from natural and synthetic sources on the basis of their chromatographic properties, biological activity, and ultraviolet and mass spectra generally completed the goal of identification.

Synthesis and Biological Activity of Cytokinins

General Methodology

Structure confirmation by synthesis was achieved usually by standard methods, illustrated by the reaction of either 2,6-bismethylthiopurine or 6-chloro-2-methylthiopurine with excess 3-methyl-2-butenylamine (**8** or **9** → **10**) followed by ribosidation (→ **6**). An alternative sequence of preparation is illustrated by the synthesis of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**11**), which was carried out by two methods. The first involved condensation of 2,6-bismethylthio-9-(2′,3′,5′-tri-*O*-benzoyl)-β-D-ribofuranosylpurine (**12**)



with 4-hydroxy-3-methyl-*trans*-2-butenylamine, followed by debenzoylation in methanolic ammonia and chromatographic purification. The second method relied upon condensation of the unprotected ribofuranoside, 2,6-bismethylthio-9-β-D-ribofuranosylpurine (**13**) with the same hydroxyamine and chromatographic purification. The syntheses of other N^6-substituted adenines and adenosines were unexceptional, being patterned on the displacement of a 6-chloro, 6-methylthio, or 6-methylsulfonyl group with the appropriate amine at reflux in ethanol or 1-butanol. When the amine was more readily preserved or available as the hydrochloride salt, triethylamine was added to the reaction mixture. The methodology lends itself to the synthesis of 2- and/or 8-substituted N^6-substituted compounds as well, in that a good leaving group such as chloro or methylsulfonyl at the 6-position is much more reactive than the same or a poorer leaving group at either the 2- or the 8-position.

Actually, the first synthesis of 2iPA (**3**) involved the alkylation of

adenosine on the 1-position with 1-bromo-3-methyl-2-butene, followed by rearrangement of the 1-substituted compound with base (Leonard, 1965). This method can be generalized for the preparation of N^6-substituted adenosines and, if a hydrolysis step is included, of N^6-substituted adenines (Fleysher *et al.*, 1969).

Whereas N^6-substituted adenines have the highest activity among the presently known cytokinins, other classes of compounds are also active. Thus, the isolation of N,N'-diphenylurea, $C_6H_5NHCONHC_6H_5$, from coconut milk and its cytokinin activity were reported first by Shantz and Steward (1955), and subsequently the relation between structure and cytokinin activity of a series of substituted diphenylurea derivatives was investigated (Bruce *et al.*, 1965; Kefford *et al.*, 1965, 1966). Syntheses in this series depended on the well-known combination of isocyanates with amines. Similar urea syntheses were used for the preparation of substituted 6-phenylureidopurines (Jones and Warren, 1970; Chheda and Hong, 1971; McDonald *et al.*, 1971), which were intermediate in activity between 6-benzylaminopurine and diphenylurea as determined by the tobacco bioassay.

Relation of Structure to Activity

All the naturally occurring cytokinins that are adenine derivatives (e.g., **1**–**7**) have been found to contain an isopent(en)yl side chain, which may be further substituted with a 4-hydroxyl group. The purine ring may be modified with 2-methylthio and 9-β-D-ribofuranosyl groups. Since the compounds presently known to occur naturally did not include all possible combinations of the four modifications (4-OH, side-chain saturation, 2-SCH_3, 9-$C_5H_9O_4$), it seemed reasonable to expect that some of these unknown types might yet be found in nature. Therefore, we accumulated the set of sixteen compounds that encompasses all of the possible modifications and determined the relative activities of these compounds in the tobacco bioassay (Schmitz *et al.*, 1972a). In those cases in which there could be more than one configurational isomer, we arbitrarily tested the trans isomer or the racemic modification, wherever applicable, although we were well aware that differences in configuration may substantially affect cytokinin activity (Hecht *et al.*, 1970b; Leonard *et al.*, 1971; Koshimizu *et al.*, 1968).

The structural features affecting biological activity were, with regard to the side chain, the presence or absence of a double bond in the 2-position and of a hydroxyl group on C-4, and, with regard to the purine nucleus, the substitution of a methylthio group on the 2-position and a 9-β-D-ribofuranosyl group on the 9-position. The activities of these bases and ribonucleosides are shown in Fig. 4. Zeatin (**2**), the most active of the

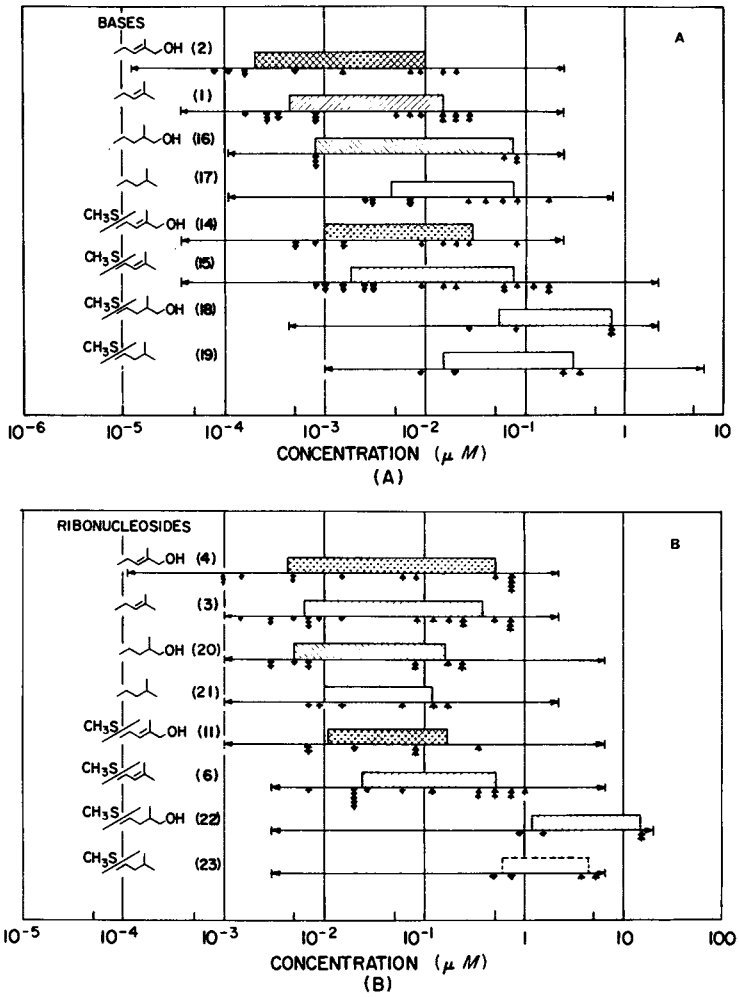


FIG. 4. Relative cytokinin activities of N^6-isopent(en)yl adenine (diagram A) and adenosine (diagram B) derivatives. The compounds are numbered as in the text. For easy reference the substituents on the N^6-position and the methylthio group on the 2-position are indicated in the margin. The base lines represent the tested concentration ranges, and the arrows under the base lines represent the start and end points (in individual experiments) of the concentration range over which growth increases as a nearly linear function of the log of concentration of added cytokinin. Bars represent the average range of the linear growth response. A bar outlined by a dashed line indicates that the compound became toxic at a concentration which was too low to support maximal callus growth. Side-chain structure is indicated by shading of the bars as follows: ▨ isopentenyl group; ▩ 4-OH-substituted isopentenyl group; □ isopentyl group; ▧ 4-OH-substituted isopentyl group. Schmitz *et al.* (1972a). Reprinted with the permission of the publisher of *Phytochemistry*.

naturally occurring cytokinins in the tobacco callus bioassay, was used as a model. The extent to which the modifications, alone or in combination, depress cytokinin activity can be estimated by noting the concentration range over which the linear relationship between the logarithm of cytokinin concentration and growth response is valid. It can be seen that the relative activities follow certain petterns according to the nature and combination of the modifications.

The enhancement of activity by the formal addition of a hydroxyl group to the 4-position of the isopent(en)yl side chain, the markedly lower activity of compounds with a saturated side chain, the consistently lower activity of the ribonucleosides as compared with the corresponding purine bases (Skoog *et al.*, 1967; Leonard *et al.*, 1969), and the reduction in activity accompanying methylthiation of the 2-position of the purine nucleus (Hecht *et al.*, 1970a) were all consistent with earlier findings. Of particular interest are the effects that deviate from the expected pattern. Any single modification of compound **2** except ribosidation causes less than a tenfold reduction in activity. The markedly larger size of the ribonucleosides, as compared with the purines, may produce secondary effects, such as a decrease in permeability, which in themselves could obscure their effectiveness as cytokinins.

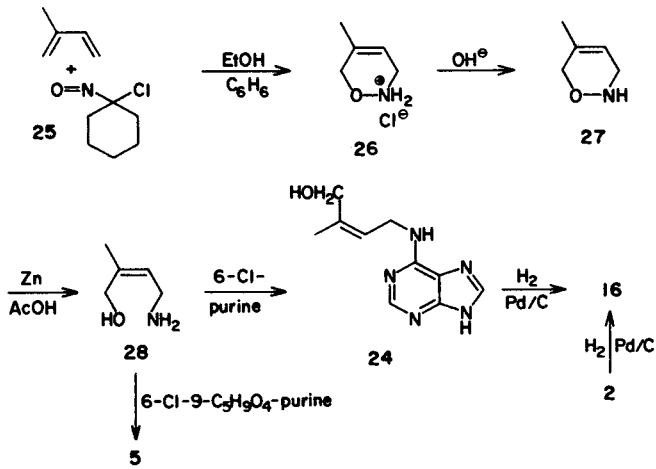
When we brought about two or more modifications simultaneously, the decrease in activity tended to be additive. The combination of two factors which caused by far the greatest loss in activity was double-bond saturation and methylthiation. When combined with a third (loss of the 4-hydroxyl group), however, activity appeared to be partially restored in terms of minimum concentration of cytokinin required to effect a linear growth response (compounds **19** and **23**), but neither of these two compounds was capable of eliciting maximal callus growth over the range of active concentrations. Whether the effectiveness of these compounds as cytokinins is directly related to their presence and role in the tRNA molecule has not been demonstrated. In contrast to its reported effect on ribosomal binding (Gefter and Russell, 1969), the methylthio group in the tobacco bioassay tended to decrease cytokinin activity (cf. especially compounds **6** vs. **3**). The magnitude of this effect was small, however, and this comparison alone does not provide sufficient evidence for or against the possibility that the site of action of the cytokinins is in fact at the tRNA level. Parallel activity studies of substituted purines and the correspondingly substituted 8-aza-9-deaza compounds indicated that the ribosyl moiety is not necessary for the promotion of cell division and growth by exogenously supplied cytokinins (Hecht *et al.*, 1971c).

To summarize, formal modifications of zeatin (**2**) as an exogenously supplied cytokinin by deoxidation, hydrogenation, and methylthiation,

and especially by ribosidation, lead to rather systematic decrements in biological activity. The set of sixteen compounds examined provides an additional basis for predicting activity as a function of structural modification. In addition, characterization of the previously unknown members by physical means and by relative activities is useful for identification of any further cytokinins that may be isolated from natural sources.

Stereoselective Synthesis

The determination of the cytokinin activities of the various structural permutations described in the foregoing left open the question of the effect of cis–trans stereochemistry in the side chain since only the trans isomers (e.g., **2**, **4**, **11**, **14**) were compared. Previous attempts to obtain the pure cis isomer of zeatin (**2**) were unsuccessful mainly because of cis–trans isomerization encountered with the types of intermediates employed. Interest in the synthesis of *cis*-zeatin (**24**) stems from the isolation of a cytokinin assigned the structure 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine or ribosyl-*cis*-zeatin (**5**) from the tRNA of certain plant tissue, e.g., peas, spinach, and corn (Hall *et al.*, 1967; Hall and Srivastava, 1968; Babcock and Morris, 1970) and from the finding that cytokinin activity is influenced by spatial factors including side-chain geometry (Hecht *et al.*, 1970b). We were able to provide a successful synthetic route to *cis*-zeatin (**24**) which utilized cyclic intermediates to ensure correct stereochemistry and was based on the generation of the CH_2OH and CH_2NH_2 groups on the same side of the double bond by a method that would not lead to isomerization at an intermediate



stage (Leonard *et al.*, 1971). The cyclic *O*,*N*-substituted hydroxylamine derivative (**27**) was a desirable precursor of the intermediate, 4-hydroxy-3-methyl-*cis*-2-butenylamine (**28**), which was required for condensation with 6-chloropurine. Compound **27** was obtainable via a Diels–Alder reaction. The reaction of 1-chloro-1-nitrosocyclohexane (**25**) with isoprene was brought about in benzene–ethanol. The crude 5-methyl-3,6-dihydro-1,2-oxazine hydrochloride (**26**) was converted to the free base (**27**), and reductive ring opening of the 5-methyl-3,6-dihydro-1,2-oxazine (**27**) with zinc and acetic acid at room temperature yielded the desired 4-hydroxy-3-methyl-*cis*-2-butenylamine (**28**). The amino alcohol (**28**) was converted to 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine (**24**) by reaction with 6-chloropurine in refluxing *n*-butyl alcohol. The question as to which positional isomer was in hand, that is, which way the isoprene unit was facing in the Diels–Alder condensation, was held in abeyance until the final chromatographic purification of product **24** and determination of the nuclear magnetic resonance (NMR) spectrum. It was found that the two zeatins could be separated in 9:1 chloroform–methanol by thin-layer chromatography (TLC) on silica: trans (**2**), R_f 0.25; cis (**24**), R_f 0.32. Final proof that the synthesis had been stereoselective was achieved by hydrogenation of **24** over 5 percent palladium on charcoal to give (±)-dihydrozeatin (**16**), identified by direct comparison with the product of catalytic hydrogenation of zeatin (**2**). The difference in biological activity between *cis*- and *trans*-zeatin was striking. In the standard tobacco callus bioassay for cytokinin activity, the trans isomer was at least 50 times more active than *cis*-zeatin (Fig. 5).

The route that was successful for the stereoselective synthesis of *cis*-zeatin was adapted to the synthesis of ribosyl-*cis*-zeatin (**5**) by condensation of 6-chloro-9-β-D-ribofuranosylpurine with 4-hydroxy-3-methyl-*cis*-2-butenylamine (**28**) (Playtis and Leonard, 1971). The product was characterized by microanalysis, melting point (202°–205° vs. 206°C for the modified ribonucleoside from tRNA), and mass spectrum [practically identical with that of its isomer (**4**)]. The relationship between synthetic cis and synthetic trans (Shaw *et al.*, 1966) ribonucleosides as geometrical isomers was established by their NMR spectra. Samples of the two isomers were spotted separately and together in TLC on silica gel with a fluorescent indicator and developed with chloroform–methanol (9:1). The R_f values obtained, 0.20 for cis (**5**) and 0.15 for trans (**4**), give a good indication of the relative mobilities of the two isomers in this system. In general, a mixture of **4** and **5** must contain about 5 percent of the minor isomer for it to be detectable by UV visualization on TLC under the conditions specified.

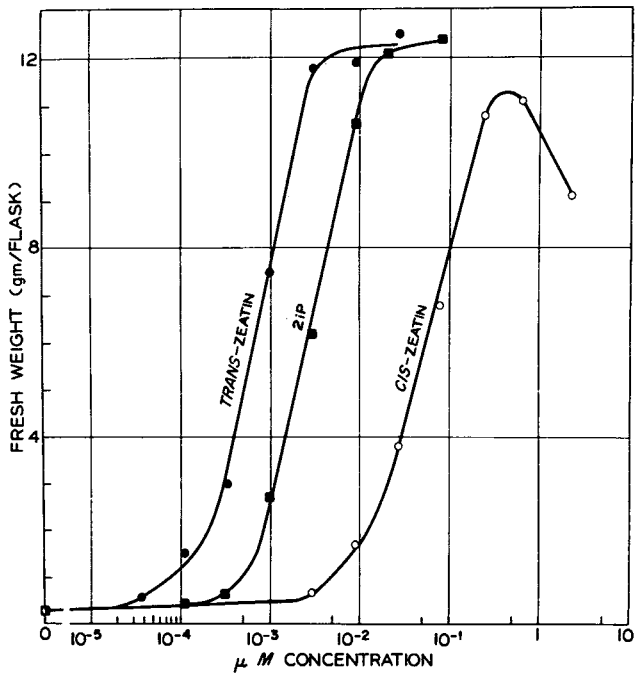


FIG. 5. Comparison of cytokinin activities of *trans*- and *cis*-zeatin and of 6-(3-methyl-2-butenylamino)purine (2iP) in the tobacco bioassay. The curves represent mean values from three experiments. Growth period 35 days within the dates June 10–September 4, 1970. Leonard *et al.* (1971). Reprinted with the permission of the American Chemical Society.

IDENTIFICATION OF RIBOSYL–*cis*- AND *trans*-ZEATIN TYPES IN NATURE

By using both synthetic ribosyl-*cis*-zeatin (**5**) and ribosyl-*trans*-zeatin (**4**) as standards or using a single standard, we were able to identify the stereochemistry of the ribosylzeatins isolated from natural sources. For example, our earlier identification of ribosylzeatin (Burrows *et al.*, 1970) as a component of wheat germ tRNA did not include assignment of side-chain stereochemistry. Chromatography established clearly that this sample was the cis isomer. No trans isomer was detectable on the plate under UV light. The sample of ribosylzeatin which was isolated previously from tobacco callus tRNA (Burrows *et al.*, 1971) was also identified as ribosyl-*cis*-zeatin (**5**). By contrast, the tRNA isolated from young pea shoots (Vreman *et al.*, 1972) yielded a mixture of both cis and trans isomers (**4** and **5**).

The isolation of five cytokinin-active ribonucleosides from the tRNA of 7-day-old green pea shoots (*Pisum sativum* L. var. Alaska) serves to illustrate the general methodology involved. The ethyl acetate-soluble ribonucleosides from the tRNA hydrolyzate were fractionated on a Sephadex LH-20 column with 33 percent (v/v) ethanol and the fractions were monitored by ultraviolet (UV) absorbance at 260 nm and by cytokinin activity, as shown in Fig. 6. Fractions 5, 6, and 7 corresponded to the elution volume of ribosylzeatin (cis and/or trans isomers). Fractions 10 and 12 corresponded to the elution volumes of 2iPA (**3**) and ms-ribosylzeatin (**7**), respectively. Fraction 19, which had barely detectable cytokinin activity, represented the elution volume of ms-2iPA (**6**). No other fractions had detectable cytokinin activity. Fractions 5, 6, and 7 (Fig. 6) were combined, lyophilized, dissolved in distilled water, and fractionated on a Sephadex G-10 column with water as the solvent. Cytokinin activity was detected in fractions 7′, 8′, 9′, and 10′ (Fig. 7), and the elution volume corresponded to that of ribosylzeatin (cis and/or trans isomers). After further purification by ascending paper chromatography with 19 percent

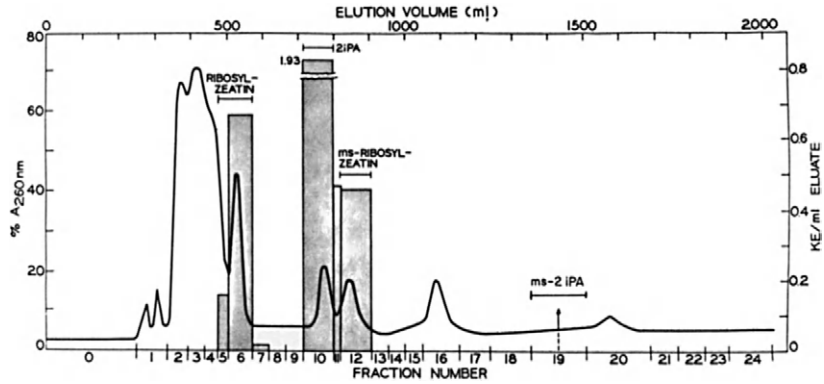


FIG. 6. Preliminary separation of ribonucleosides. Distribution of ultraviolet absorption and cytokinin activity of the ethyl acetate-soluble ribonucleosides of pea transfer ribonucleic acid hydrolyzate in the elution profile of the Sephadex LH-20 column. The dashed arrow in fraction 19 indicates the relatively low, but definite activity in this fraction, corresponding to the elution volume of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ms2iPA). The Sephadex column was equilibrated with eluant before the sample, dissolved in approximately 2 ml of the same, was introduced. Exp. H/16 (10/14/70–12/11/70). LH-20, 139 gm, 61 = 3.7 cm. Eluant, 33.3% EtOH. Flow rate, 33 ml/hr. Volumes collected, 9.5 ml. ———, % A_{260nm}. [ ], Cytokinin activity. KE—Kinetin equivalents. Vreman *et al.* (1972). Reprinted with the permission of the publishers of *Plant Physiology*.

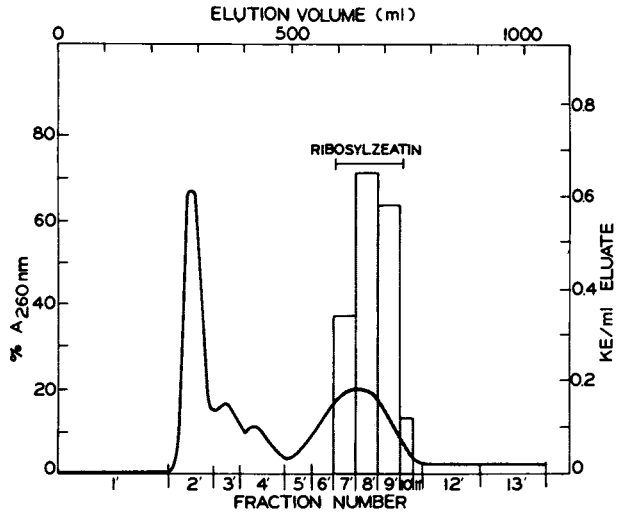


FIG. 7. Purification of ribosylzeatin. Distribution of the cytokinin activity in the elution profile of the solid material from pooled fractions 5, 6, and 7 (Fig. 6). The Sephadex column was equilibrated with eluant before the sample, dissolved in approximately 2 ml of the same, was introduced. Exp. H/16 (12/4/70–2/2/71). G-10, 150 gm, 55 × 3.7 cm. Eluant, dist. H_2O. Flow rate, 150 ml/hr. Volume collected, 9.5 ml. ———, % A_{260nm}. ▭ Cytokinin activity. KE—Kinetin equivalents. Vreman *et al.* (1972). Reprinted with the permission of the publishers of *Plant Physiology*.

(v/v) ethanol as solvent, positive identification was made by comparisons of UV absorption and mass spectra with those of synthetic material. Separation and identification of both cis and trans isomers as being present were then achieved by TLC on silica gel and inspection of the plates under UV light. Both spots were about equally active in the tobacco bioassay. Whether or not the presence of ribosyl-*trans*-zeatin (4) in the tRNA from *Pisum* shoots is related to the abundance of chloroplasts in the starting material has not been determined, but it is of interest that both isomers were isolated from the vegetative green shoots, whereas only the cis isomer was reported from the tRNA of *Pisum* roots (Babcock and Morris, 1970).

For the positive identification of the other cytokinin-active ribonucleosides, fractions 10, 11, and 12 (Fig. 6) were pooled, lyophilized, and fractionated with distilled water on a Sephadex LH-20 column (Fig. 8). Cytokinin activity was found in fractions 3″ and 5″, corresponding to the elution volumes of 2iPA (3) and ms-ribosylzeatin (7), respectively. After further purification of the separate fractions by ascending paper chroma-

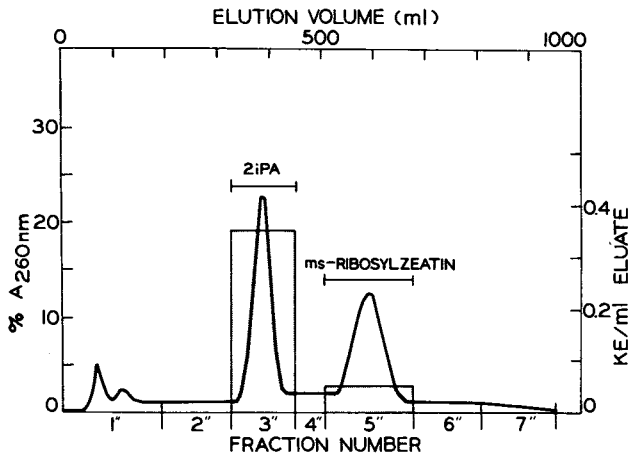
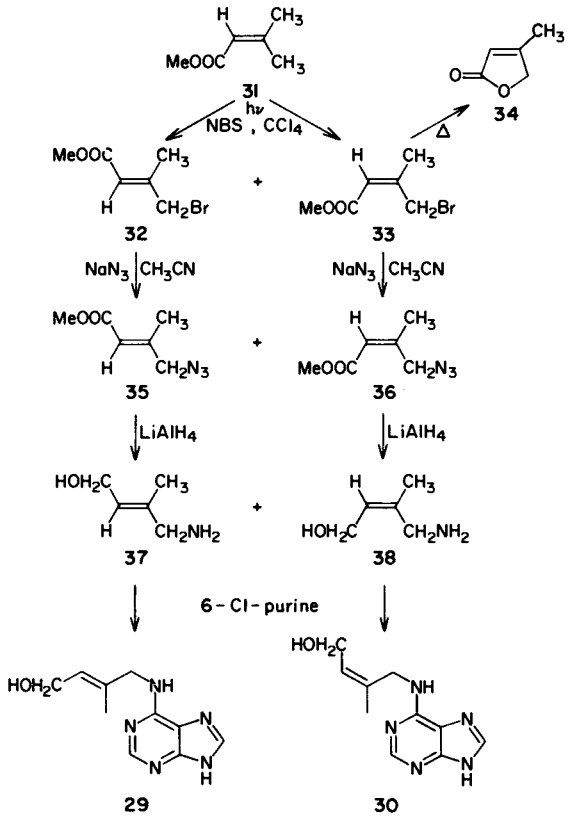


FIG. 8. Separation of ribosyl-2-methylthiozeatin (ms-ribosylzeatin) and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranyoslypurine (2iPA). Cytokinin activity in the elution profile of the solid material from the pooled fractions 10, 11, and 12 (Fig. 6). The Sephadex column was equilibrated with eluant before the sample, dissolved in approximately 1 ml of the same, was introduced. Exp. H/16 (12/12/70–2/2/71). LH-20, 30 gm, 22 × 2.6 cm. Eluant, dist. H_2O. Flow rate, 150 ml/hr. Volumes collected, 9.5 ml. ———, % A_{260nm}. [ ], Cytokinin activity. KE—Kinetin equivalents. Vreman *et al.* (1972). Reprinted with the permission of the publishers of *Plant Physiology*.

tography using dilute ethanol, mass-spectrometric determinations completed the identification of **3** and **7**. The question of the stereochemistry of the side chain in the 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**7**) remained open until the cis isomer was made unequivocally by the condensation of compound **13** with 4-hydroxy-3-methyl-*cis*-2-butenylamine (**28**) (Playtis, 1972) and until a method had been developed that could separate a mixture of the two isomers. The cis isomer of **7** and the trans isomer of **7** (**11**) could be differentiated by TLC on silica gel using chloroform–acetic acid (8:2) or chloroform–methanol (9:1) as the elution solvent, as could the *cis*- and *trans*-zeatins and the ribosyl-*cis*- and *trans*-zeatins, with the cis isomer moving faster. Careful examination of the ms-ribosylzeatin from the tRNA of the green pea shoots has now indicated the presence of both the cis and trans forms (work in progress at the Universities of Wisconsin and Illinois).

GEOMETRIC AND POSITION ISOMERS OF ZEATIN

Because of the variety of modifications of terpenoid structures in nature and because of the influence of side-chain geometry on biological activity,



we decided to make and compare 6-(4-hydroxy-2-methyl-*trans*-2-butenylamino)purine (*trans*-isozeatin) (**29**) and 6-(4-hydroxy-2-methyl-*cis*-2-butenylamino)purine (*cis*-isozeatin) (**30**) (Leonard and Playtis, 1972). The first of these compounds (**29**) had been made earlier by Letham *et al.* (1969) in a survey of synthetic regulators of cell division, and we followed the same synthetic route, which involved γ-bromination of methyl β-methylcrotonate (**31**) to a mixture of methyl *trans*-4-bromo-3-methylcrotonate (**32**) and methyl *cis*-4-bromo-3-methylcrotonate (**33**). During fractional distillation, the *cis*-bromo ester was effectively removed by cyclization to the 3-methylbutenolide (**34**), and the *trans*-bromo ester (**32**) was treated with sodium azide to give methyl *trans*-4-azido-3-methylcrotonate (**35**), which was reduced with lithium aluminum hydride to 4-hydroxy-2-methyl-*trans*-2-butenylamine (**37**), which was then condensed with 6-chloropurine to give *trans*-isozeatin (**29**). We were able to obtain *cis*-isozeatin (**30**) by accepting the original mixture of *cis*- and *trans*-

bromo esters (**33**, **32**), omitting distillation at any stage in the synthetic sequence, postponing the problem of separation until the final stage (**33** + **32** → **36** + **35** → **38** + **37** → **30** + **29**), and monitoring the sequence by NMR spectra and by TLC. The latter method, employing silica gel and chloroform–methanol (9:1) as eluant, was useful for differentiating between *cis*-isozeatin, R_f 0.33, and *trans*-isozeatin, R_f 0.24. Applied to a chromatographic column of silica, the mixture of compounds **30** and **29** was readily separable by elution with the same solvent. Catalytic hydrogenation of **29** produced 6-(4-hydroxy-2-methylbutylamino)purine (dihydroisozeatin, having properties different from those of the isomeric dihydrozeatin that had previously been found in nature and also synthesized (Koshimizu *et al.*, 1967a,b). Comparison of dihydrozeatin (**16**) with dihydroisozeatin in the tobacco callus bioassay showed that a shift of the methyl group from the 3- to the 2-position resulted in a seventyfold decrease in activity. This decrease compares closely with that resulting from shifting the terminal methyl group in zeatin (**2**) to the 2-position in *trans*-isozeatin (**29**) (Schmitz *et al.*, 1972b).

The ribosyl derivatives of the *cis*- and *trans*-isozeatins were obtained just as the *cis* and *trans*-isozeatins themselves, that is, by using 6-chloro-9-β-D-ribofuranosylpurine in the final condensation stage. The *cis*- and *trans*-isozeatin and their ribonucleosides were then compared with the corresponding zeatin derivatives in the tobacco callus bioassay with 2iP (**1**) and 2iPA (**3**) as standards. All the compounds were capable of giving a full growth response when supplied in concentrations up to 20 μM, and they gave roughly parallel curves when yields were plotted against the logarithm of the concentration. For comparison of activities based on all the available data, the ranges in which growth was a nearly linear function of the log of concentration are shown in Fig. 9. It may be seen that no derivative was more active than zeatin (**2**) itself. There was a much greater difference in activity between the *trans*- and *cis*-zeatins than between the *trans*- and *cis*-isozeatins (**29** and **30**), in which the methyl group is in the 2- instead of the 3-position of the side chain but the relative geometries are preserved. The activities of *cis*-zeatin (**24**) and of *trans*-isozeatin (**29**) are actually in the same range, and that of *cis*-isozeatin (**30**) is measurably lower than both. Letham (1972) has recently reported that, on the basis of a different bioassay involving the promotion of expansion of excised radish cotyledons, 6-(4-hydroxy-2-methyl-*trans*-2-butenylamino)purine, *trans*-isozeatin (**29**), has about half the activity of zeatin (**2**) at 10 μM.

The 9-ribosyl derivatives of the *trans*-zeatin, *cis*-zeatin, *trans*-isozeatin, and *cis*-isozeatin series followed an order of decreasing activity in the

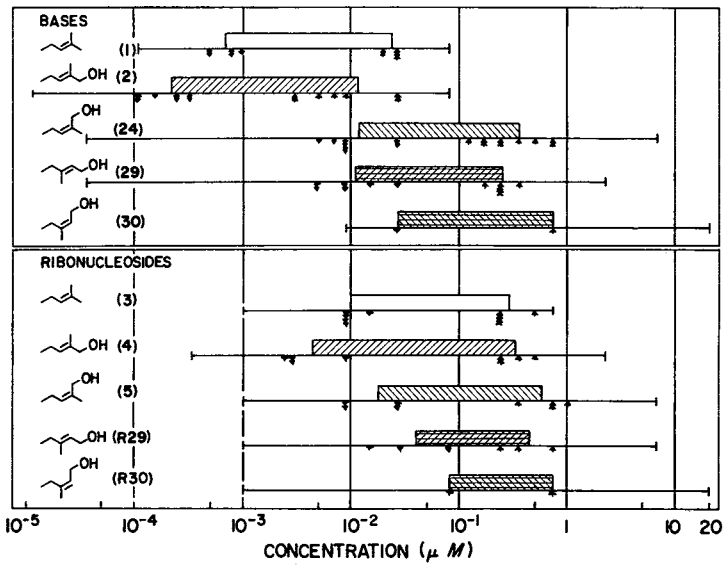


FIG. 9. Summary of cytokinin activities of *trans-*, *cis-*, and isozeatins and ribosylzeatins. The compounds are numbered as in the text. For easy reference the configuration of the substituent in the N^6-position has also been indicated in the margin. The base lines represent the test concentration ranges, the bars (▨ *trans*; ▧ *cis*; ▤ iso) represent mean values of the concentration range over which growth increased as a nearly linear function of the log of concentration of added cytokinin, and the arrows under the base lines represent the start and end points of this range in individual experiments. Schmitz *et al.* (1972b). Reprinted with the permission of the publisher of *Plant Physiology*.

tobacco bioassay corresponding roughly to that of the bases. However, higher concentrations were required for activity, and they are closely grouped on the logarithmic scale on which they are presented. The decrease in activity was least marked in the case of the isomers that already exhibited low activity. It should be noted that there is no means of distinguishing between the biological activity that may derive from the intact ribonucleosides and from bases set free by hydrolysis in the course of the bioassay. Possible differences in rates of hydrolysis are, *inter alia*, an additional complicating factor in attempts to compare biological activities of the ribonucleosides. These results confirm and amplify previous findings on the influence of size, configuration, etc., of the side chain on the cytokinin potency of N^6-substituted adenine derivatives and they permit a more detailed examination of their relative importance in compounds closely related to zeatin.

Comparison of 2-, 8-, and 2,8-Substituted Cytokinins

The finding that zeatin (**2**) had the highest activity of any known cytokinin offered the challenge of synthesizing a more active compound if possible. This was only partially realized when it was found that of the 2-substituted zeatins, 2-chlorozeatin was at least as active and possibly more active than zeatin itself (Hecht *et al.*, 1970a). The other 2-substituted derivatives were less active than their unsubstituted counterparts, and for both the zeatin and the 2iP derivatives, the activities were in decreasing order for 2-substituents: Cl > NH_2 ≥ CH_3S ≫ OH. When the terminal hydroxyl of zeatin was esterified with formic, acetic, propionic, and indole-3-acetic acids, these esters were consistently, although only slightly, more active than zeatin (Leonard *et al.*, 1969; Schmitz *et al.*, 1971). The formate, propionate, and indole-3-acetate esters of 2-chlorozeatin all showed activity about *twice* that of zeatin on a molar basis.

Another way of enhancing cytokinin activity is by means of 8-methyl substitution, and it was found that the 8-methyl derivatives of 6-benzylaminopurine and kinetin (6-furfurylaminopurine) showed enhanced cytokinin activity over the unsubstituted compounds (Kulaeva *et al.*, 1968). We have found that 6-(3-methyl-2-butenylamino)-8-methylpurine is likewise more active than the unsubstituted parent compound, 2iP (**1**) (Dammann *et al.*, 1973). For several series of 2-, 8-, and 2,8-substituted 6-(3-methyl-2-butenylamino)purines which have been synthesized, with the clear exception of the 8-methyl compound, substitution generally leads to lower cytokinin activity than that of the parent compound. Disubstitution at positions 2 and 8 gives lower cytokinin activity than monosubstitution by the same group at either the 2- or the 8-position, as illustrated in Table 1 by the minimum concentration at which linear growth response begins for selected, substituted 2iP (**1**) compounds. Exceptions seem to arise, however, if the substituent leads to high activity. For example, the 2-chloro-, 8-chloro-, and 2,8-dichloro-6-(3-methyl-2-butenylamino)purines all have about the same activity and are very active cytokinins.

The 2,8-dimethyl derivative shows an activity between that of the 2-methyl and 8-methyl compounds and all three are very active cytokinins. Bulky groups, such as methylsulfonyl and benzylthio, at the 2- and/or 8-positions decrease cytokinin activity (cf. CH_3S and C_7H_7S in Table 1). Substituents such as mercapto or hydroxyl, which exist mainly as "-one" tautomers, reduce cytokinin activity, and this trend may be reversed by *S*- or *O*-methylation. As in the series considered earlier, the effect of ribosidation throughout these series is to reduce the cytokinin activity, although the ribonucleosides show much the same order of decrease in activity as the free bases.

TABLE 1

TOBACCO CALLUS GROWTH RESPONSE TO 2-, 8-, AND 2,8-SUBSTITUTED N^6-(Δ^2-ISOPENTENYL)ADENINES

	Minimum concentration (μM), linear range		
Substituent	2-	8-	2,8-
CH_3S	0.0063	0.0022	0.0077
HS	0.030	0.0044	1.0
C_7H_7S	0.1	0.007	6.6
CH_3	0.0025	0.00044	0.0018
Cl	0.0011	0.0009	0.0024
CH_3SO_2	0.026	0.77	6.6

Changes in Structure

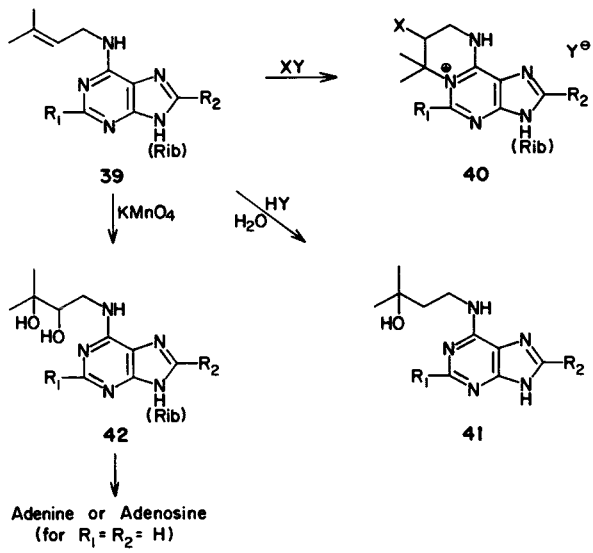
CONVERSIONS OF 6-(3-METHYL-2-BUTENYLAMINO)PURINES

Chemical Conversions of the Side Chain

Modifications of the side chain of 2iP (**1**) and 2iPA (**3**) were of interest because of changes in activity that might be produced and conversions that might be accomplished *in situ* within tRNA's containing the modified adenosine. Hall (1971) has summarized much of the work directed along these lines. Briefly, it was found that 2iPA (**3**) reacts in aqueous solution at room temperature with iodine to give a 1,N^6-ring-closed product (**40**, $R_1 = R_2 = H$; $X = Y = I$). Treatment of a dilute solution of 2iPA with hydrochloric acid (1*N*, 15 minutes reflux) did not give 2iP (**1**) but instead a mixture of two bases: compounds **41** ($R_1 = R_2 = H$) and **40** ($R_1 = R_2 = X = H$; $Y = Cl$). The reaction was concentration-, time-, and temperature-dependent (Martin and Reese, 1968). Mild oxidation of 2iPA with dilute aqueous permanganate gave several products, among which the dihydroxylated ribonucleoside (**42**; $R_1 = R_2 = H$) accounted for 52 percent and adenosine accounted for 30 percent. Chemical conversion of the 2iPA unit in yeast, rat liver, and *Lactobacillus acidophilus* tRNA's by means of permanganate has been reported (Kline *et al.*, 1969). The modification of the 2iPA unit in yeast serine tRNA with permanganate or iodine (Kline *et al.*, 1969; Hirsch and Zachau, 1970), reduces ribosomal binding of the iodine-treated tRNA by half. Bisulfite treatment, which included modification of the anticodon-adjacent 2iPA in yeast tyrosine

tRNA, was also found to decrease ribosomal binding (Furuichi *et al.*, 1970). At the base level, treatment of 2iP (**1**) with certain acids, e.g., HBF_4 and CF_3OOH, and under milder conditions with bromine or iodine gave cyclized salts of type **40**, and treatment of either the HBF_4 or CF_3COOH product with Dowex-1 (HCO_3^- or OH^-) gave the corresponding free base (Leonard *et al.*, 1966; Carraway *et al.*, 1968). The 1,N^6-cyclization of the isopentenyl side chain of **1** destroyed the cytokinin activity.

In terms of the requirements for the 1,N^6-cyclization reactions to occur, the substituted derivatives of compound **39** provided some information (Dammann, 1973). The mechanism of acid closure of 2iP (**1**) logically involves an equilibrium between ring-nitrogen protonation and side-chain protonation, and combination of the nucleophilic 1-nitrogen with the tertiary carbonium ion. The 2-chloro derivative (**39**, R_1 = Cl, R_2 = H) underwent only partial conversion to compound **40** (R_1 = Cl, R_2 = X = H, Y = BF_4) on heating with fluoboric acid, and the 2-methylsulfonyl derivative (**39**, R_1 = CH_3SO_2, R_2 = H) underwent no cyclization. The presence of a 2-methylthio group or a 2-methoxyl group in **39** did not prevent cyclization. Although precisely the same conditions could not be used in every case, it was apparent that the 2-substituent had a profound effect on the cyclization. As the electron-withdrawing ability of the 2-substituent increased, $SCH_3 < OCH_3 < Cl < SO_2CH_3$, the 1-nitrogen became less nucleophilic to the point where **39**, with R_1 = CH_3SO_2 and



R_2 = H, did not undergo cyclization. The 8-substituent, by contrast, was far enough removed so that it had little effect on the electronic character of the 1-position. Even the 8-methylsulfonyl derivative (**39**, R_1 = H, R_2 = CH_3SO_2) underwent cyclization with HBF_4 in 95 percent yield to give compound **40**, with R_1 = X = H, R_2 = CH_3SO_2, Y = BF_4. When either the 2-methylthio base (**10**) or riboside (**6**) was heated at reflux with *N* hydrochloric acid, only the hydrated base (**41**, with R_1 = CH_3S, R_2 = H) was isolated. Extensive conversion of the free base corresponding to **40**, with R_1 = CH_3S, R_2 = H, to the same product with a hydrated side chain was also realized by refluxing with *N* hydrochloric acid for 1 hour. Iodine treatment, at reflux in ethanol, did not bring about the cyclization of either ms2iP (**10**) or ms2iPA (**6**), unlike 2iP (**1**) and 2iPA (**3**). The addition of aqueous thiosulfate returned the starting material in each of the 2-methylthio cases. Thus, aqueous potassium iodide-iodine treatment cannot be depended on to cyclize the side chain of any ms2iPA (**6**) units in a tRNA (Faulkner and Uziel, 1971) although reversible iodine complexation is a possibility (Ramakrishnan, 1963). It has been found possible to desulfurize ms2iPA units in *Escherichia coli* tRNA with Raney nickel, leaving 2iPA residues in their stead (Hecht *et al.*, 1971a).

Biochemical Conversions

Permanganate cleavage of the isopentenyl side chain of modified adenosines in tRNA has been used by Bartz *et al.* (1970) to follow the isopentenylation of tRNA with isopentenyl pyrophosphate by means of an isopentenyl transferase enzyme [Δ^2-isopentenylpyrophosphate:tRNA-Δ^2-isopentenyltransferase (EC 2.5)] that acts at the tRNA level. Tritium-labeled isopentenyl pyrophosphate was used and the counts per minute incorporated based on A_{260} units of tRNA was compared for different *in vitro* substrate tRNA's (Table 2). *Mycoplasma* (Kid) tRNA, in which 2iPA is absent, was isopentenylated with isopentenyl pyrophosphate in the presence of the enzyme from *Escherichia coli*. Transfer ribonucleic acids from *E. coli*, rat liver, and yeast that had been treated with permanganate took on the labeled isopentenyl group to varying degrees, whereas *E. coli* ribosomal RNA and polyadenylic acid did not accept the group under enzymatic conditions (Bartz and Söll, 1972).

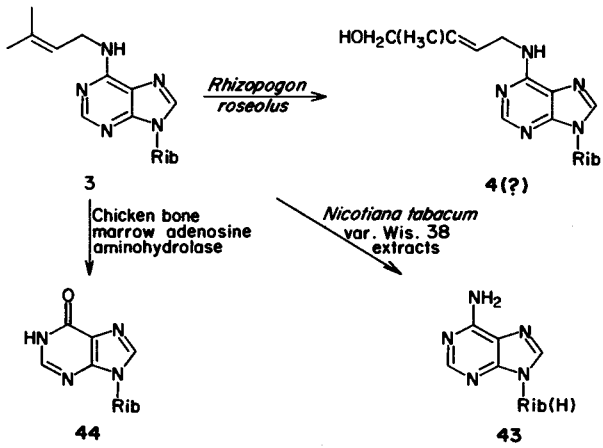
6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 2iPA (**3**), undergoes enzymatic conversions of the side chain. For example, Miura and Miller (1969) found that compound **3** was converted by the fungus *Rhizopogon roseolus* to zeatin riboside (**4**) (?), stereochemistry not established. Extracts of *Nicotiana tabacum*, var. Wisconsin No. 38, converted 2iPA to

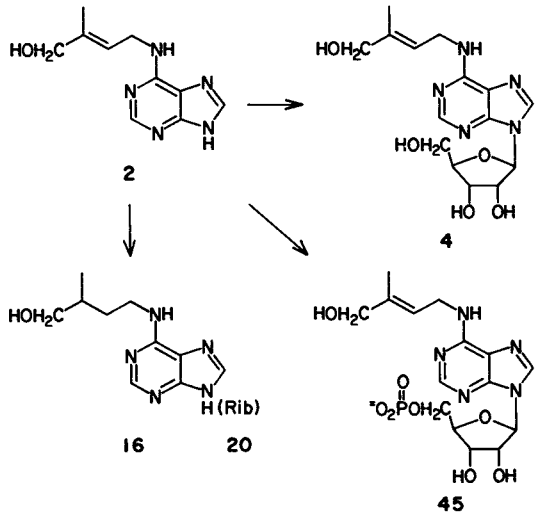
TABLE 2

INCORPORATION OF ISOPENTENYL GROUPS INTO VARIOUS TRANSFER RIBONUCLEIC ACIDS (tRNA's) INCUBATED FOR 40 MINUTES WITH ^{3}H-LABELED ISOPENTENYL PYRO PHOSPHATE AND TRANSFERASE ENZYME

Substrate	pmoles/A_{260}
Mycoplasma tRNA	9.2
Escherichia coli tRNA	1.6
Yeast tRNA	1.9
Yeast tRNA (permanganate treated)	32.9
Rat liver tRNA	6.5
Mycoplasma $tRNA^{Phe}$	0.5
Rat liver $tRNA_4^{Ser}$	634
Escherichia coli ribosomal RNA	0.0
f2 RNA	2.0
Polyadenylic acid	0.7

adenosine (**43**) and adenine (Pačes *et al.*, 1971). An adenosine aminohydrolase from chicken bone marrow, *inter alia*, converted 2iPA to inosine (**44**) (Hall *et al.*, 1971). The Δ^3-isomer, *n*-pentyl, isopentyl, and furfuryl derivatives are also cleaved. Thus, there exist in certain selected systems, enzymes that are capable of adding or removing the isopentenyl group, of removing the entire isopentenylamino side chain, and of hydroxylating the isopentenyl side chain.

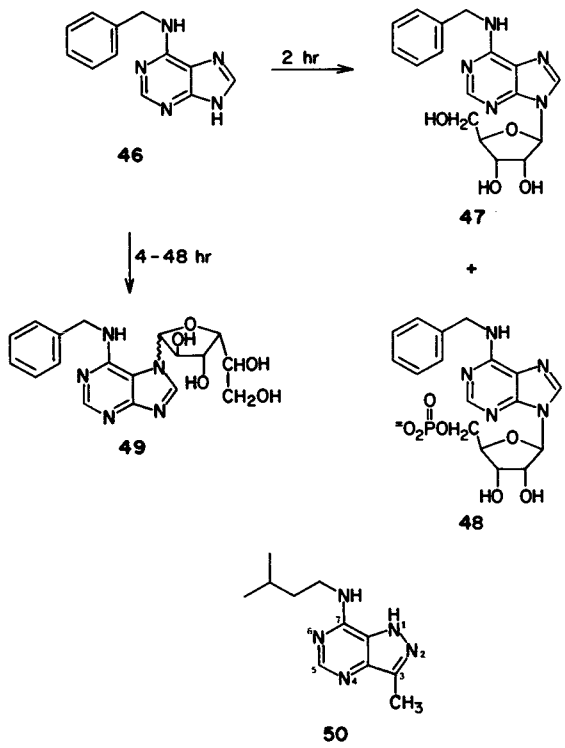




In the case of the hydroxyisopentenyl side chain, Sondheimer and Tzou (1971) followed the metabolism of zeatin (**2**) in excised bean axes and identified zeatin riboside (**4**) (?), zeatin ribotide (**45**), and dihydrozeatin (**16**) and its riboside (**20**) as products.

Conversions of Unnatural Cytokinins

The short-term metabolism of the unnatural cytokinin 6-benzylaminopurine (**46**) has been investigated (Dyson *et al.*, 1972; Deleuze *et al.*, 1972); 6-benzylaminopurine riboside (**47**) and ribotide (**48**) were identified as products of the conversion of compound **46** in contact with soybean tissue culture or potato tuber slices within 2 to 4 hours. Over a 4–48 hour period, a new metabolite accumulated, reported to persist for at least 60 days, which was assigned the structure **49**, a 7-glucofuranosyl derivative of benzylaminopurine. The position of substitution of the glucosyl group was indicated by the ultraviolet data and the furanose configuration, by the mass-spectrometric data. It was reported to possess cytokinin activity. Whether these metabolites are storage forms of the original benzylaminopurine or are "on line" to more specific sites of biological action has not been established. Work in the Wisconsin-Illinois laboratories has shown that compound **47** is less active in the tobacco bioassay than **46**, that compound **48** is slightly more active than **47**, and also that *N*-substituted (e.g., at least with benzyl at 1, 3, 7, or 9) benzylaminopurines are less active in this bioassay than compound **46** itself.



The tobacco bioassay represents a long-term experiment in terms of the efficacy of an exogenously added cytokinin in that the normal growing period runs from 3 to 5 weeks.

Since no one had previously approached the question of what "natural" cytokinin-active ribonucleosides are actually present in the tRNA of dependent tobacco tissue grown on a medium supplemented with a *synthetic* cytokinin, such as benzylaminopurine (**46**), the isolation and identification of the other active tRNA components produced under these conditions was regarded of special interest. We were able to characterize the "natural" cytokinin-active ribonucleosides isolated from a tRNA preparation of tobacco callus grown 3 weeks on medium supplemented with 6-benzylaminopurine as 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**5**), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**), and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**7**) (Burrows *et al.*, 1971; Playtis and Leonard, 1971). The last of this group (**7**) was present in the smallest amount. The first of the group, or ribosyl-*cis*-zeatin (**5**), was present in the greatest amount.

The origin of the three "natural" cytokinin-active ribonucleosides (**3**, **5**, **7**) in the cytokinin-dependent callus cultures supplied only with compound **46** indicates the stimulation of processes for their synthesis, since the benzyl group cannot of course provide directly for the isopentenyl chains in ribonucleosides **3**, **5**, and **7**. It is known that when tobacco pith or callus tissue is cultured *in vitro*, occasional pieces acquire the capacity to synthesize cytokinin. Although occasional habituated cultures may have been included in the material that was extracted, routine tests for habituation, done by transferring samples of the callus to medium without cytokinin, were negative. It is highly improbable, therefore, that detectable amounts of cytokinin-active ribonucleosides would have been derived from this source.

It is more likely that the biosynthesis of the "natural" cytokinin-containing nucleosides represents a part of the increased anabolic activity brought about and maintained by the exogenous supply of cytokinin. Such increased anabolic activity, in response to cytokinin, as reflected in measurements of nucleic acids (RNA and DNA), total protein content, specific enzymes, pigments, and other cell constituents, is well known (Skoog and Armstrong, 1970).

Also characterized by low- and high-resolution mass spectra, UV spectra, and chromatographic properties as present in the tRNA preparation, when cytokinin-dependent tobacco callus was cultured on a medium supplied with benzylaminopurine (**46**) as the only exogenous source of cytokinin, was the ribonucleoside of 6-benzylaminopurine (**47**) (Burrows *et al.*, 1971). These results demonstrate that cytokinins of both exogenous and endogenous origin are present as ribonucleoside constituents of tRNA preparations from cytokinin-dependent tobacco callus tissue cultured on media supplied with 6-benzylaminopurine.

The question as to whether or not the ribonucleoside **47**, isolated by hydrolysis of the tRNA, contained both benzyl and adenine units of the original cytokinin should be answerable by a double labeling experiment. Accordingly benzylaminopurine labeled with ^{3}H in the benzene ring and with ^{14}C at the 8-position in the adenine ring was used in growing experiments exactly like those employing "cold" **46** as the added cytokinin. The callus was harvested, the tRNA preparation was isolated and hydrolyzed, and the ribonucleosides were separated chromatographically. There was considerable randomization of the label into many compounds during the growth of the tobacco callus tissue. The radioactivity in the 6-benzylamino-9-β-D-ribofuranosylpurine (**47**) fraction showed the same proportion of $^{3}H/^{14}C$ as in the original benzylaminopurine (Fig. 10) and was well separated from the elution point of the latter. As a further check

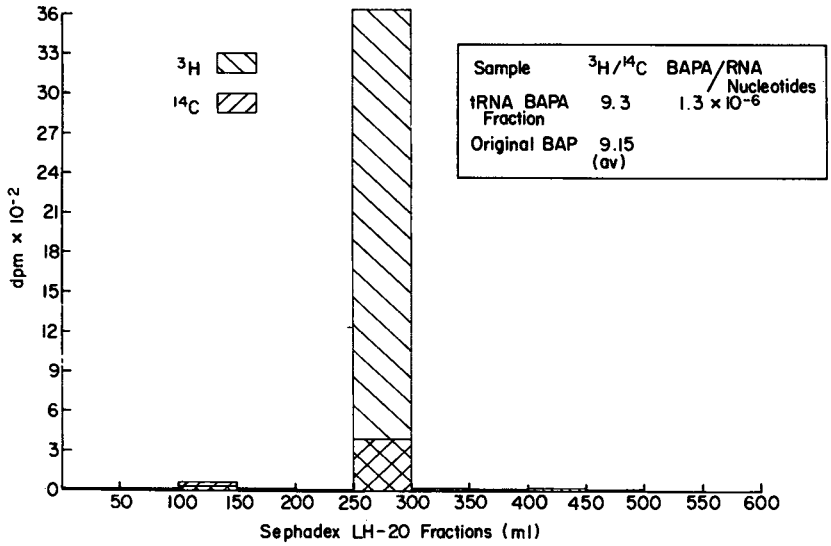


FIG. 10. Sephadex LH-20 chromatography of ethyl acetate extract of transfer ribonucleic acid (tRNA) ribonucleosides following the growing of *Nicotiana tabacum* var. Wisconsin No. 38 in the presence of $^{3}H/^{14}C$-labeled 6-benzylaminopurine (BAP) (**46**). G. Walker, N. J. Leonard, D. J. Armstrong, N. Murai, and F. Skoog, work in progress.

on the identity and radiochemical purity of the ribonucleoside isolated from the tRNA preparation, "cold" **47** was added to the fraction (250–300 ml, Fig. 10) and the fraction was rechromatographed on Sephadex LH-20 in distilled water. Both the ^{3}H and ^{14}C peaks recovered from the column coincided with the UV absorption peak from the unlabeled marker, and there was no change in the $^{3}H/^{14}C$ ratio. Since we recognized the difficulty of separating small molecules, including benzylaminopurine, from tRNA, we tried to circumvent this difficulty in our isolation, purification, and various control procedures, and we conclude that at least some of the ribonucleoside **47** that is in the tRNA preparations is nonexchangeable and therefore covalently incorporated, originating with the intact base (**46**). The constant $^{3}H/^{14}C$ ratio obtained in our experiment (current Illinois–Wisconsin investigation) rules out the possibility of transbenzylation taking place. However, the observed level of incorporation was extremely low, at most ca. 1 benzylaminopurine riboside molecule per 10,000 RNA molecules. This level is too low (by ca. 10^{3} times!) for the benzylaminopurine (**46**) to appear adjacent to the anticodon in the specific

tRNA species that would normally be modified, and the inference is that the incorporation is without particular physiological significance.

Antagonists of the Cytokinins

The synthesis of a potent cytokinin antagonist is of considerable interest, since it could extend the study of cytokinins to biological systms that do not require exogenously added cytokinins, presumably because they produce their own. The antagonist that blocks the action of the endogenous cytokinins in these systems would thus make the tissue cytokinin-dependent. The antagonist would be of greatest utility, of course, if it acted in a reversible manner on the same pathway through which the cytokinin itself exerts its effect. The first in a series of cytokinin antagonists was designed with the same approximation inherent in the design of any competitive inhibitor, namely, that the modified compound be sufficiently similar to the normal metabolite (cytokinin) in structure to allow participation in the same type of "receptor complexes" but that the modification render it ineffective as a cytokinin. Empirically, then, such a compound would be structurally related to the most active cytokinins, such as **1**, but would be inactive as a cytokinin itself. It was reported (Skoog *et al.*, 1967; Skoog and Armstrong, 1970) that modifications in the heterocyclic (adenine) moiety of cytokinins drastically lower cytokinin activity, suggesting that alkylated heterocycles structurally related to compound **1** might be useful as inhibitors of cytokinin activity.

The results of stepwise modification of structure, in terms of activity as determined by the tobacco assay, are shown in Fig. 11, in which the concentrations of exogenous compound that produce equivalent callus growth are compared (Hecht *et al.*, 1971b,c; Skoog *et al.*, 1973). Proceeding from the bottom line of the figure to the top line, one can see first the activity-lowering effect of side-chain saturation, **1** → **17**, as described earlier. The next stage represents the interchange of the 8- and 9-positions of the purine ring system of compound **1** to give a similarly substituted pyrazolo[4,3-*d*]pyrimidine. Blockage of the equivalent of the 9-position of a purine nucleus (actually the 3-position of the pyrazolo[4,3-*d*]pyrimidine nucleus) with a methyl group causes a further decrease in cytokinin activity, and the penultimate and ultimate stages of modification involve side-chain saturation and methyl incorporation. The compound shown on the top line of Fig. 11, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]-pyrimidine (**50**), is devoid of cytokinin activity. Thus, by saturating the isopentenyl side chain, reversing 8-CH and 9-N, and attaching the methyl

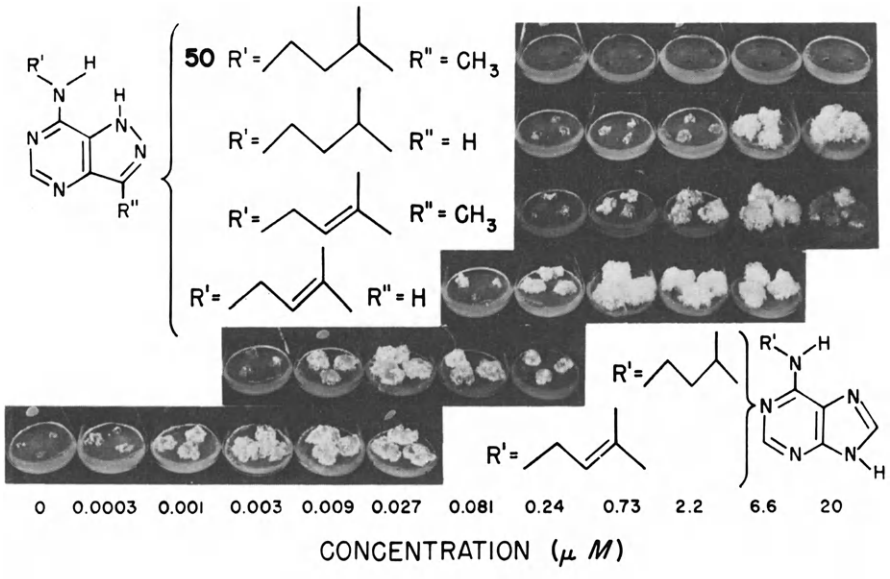


FIG. 11. Three steps converting cytokinin 2iP (**1**) to antagonist **50**. Diagram showing growth of tobacco callus on medium with serial concentrations of the following compounds (reading from the bottom up); 6-(3-methyl-2-butenylamino)purine (2iP) (**1**); 6-(3-methylbutylamino)purine (**17**), 7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine; 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine; 7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine; and 3-methyl-7-(3-methylbutylamino)pyrazolo-[4,3-*d*]pyrimidine (**50**). The bottom line represents growth obtained with 2iP, an active cytokinin; proceeding upward are shown the effect of saturating the side chain, the exchange of the C-8 and N-9 atoms, and the addition of the methyl substituent (R″). The top line represents compound **50**, an active cytokinin antagonist, incorporating all three modifications. Skoog *et al.* (1973). Reprinted with the permission of the publisher of *Phytochemistry*.

group, it has been possible to destroy cytokinin activity. But compound **50**, which was without growth-promoting activity, exhibited strong activity as an anticytokinin in appropriate tobacco bioassays: it caused complete inhibition of growth of tobacco callus supplied with optimal concentrations of either 6-(3-methyl-2-butenylamino)purine (Fig. 12) or 6-benzylaminopurine (Fig. 13), when it was added to the tissue in 100- to 200-fold excess over the cytokinins. At any given concentration at which the antagonist has an effect intermediate between no inhibition and total inhibition, a higher concentration of cytokinin has a restorative effect on growth, whereas a higher concentration of antagonist causes a further reduction in

growth. This implies a competition between the cytokinin and antagonist for the "receptor sites" associated with the promotion of cell division and growth.

An additional effect is apparent from the graph of fresh-weight yields corresponding to the cultures (Fig. 13). At the highest concentration of 6-benzylaminopurine (0.24 μM), where the cytokinin itself is becoming inhibitory, the addition of antagonist offsets the excess cytokinin and enhances growth, as though a smaller "effective" amount of cytokinin was being used. Thus, the antagonist is not merely toxic to the plant tissue but competes with the cytokinin in a reversible manner.

A strain of tobacco callus that grows without exogenously added cytokinin and has cytokinin activity (J. Einset, University of Wisconsin) was also inhibited by the antagonist at close to 1 μM. Strong evidence is thus provided that this tissue uses endogenous cytokinin. As in the case of the cytokinin-dependent tissue, when the concentration of the antagonist (**50**) required for effective competition was exceeded, the toxicity effect became apparent.

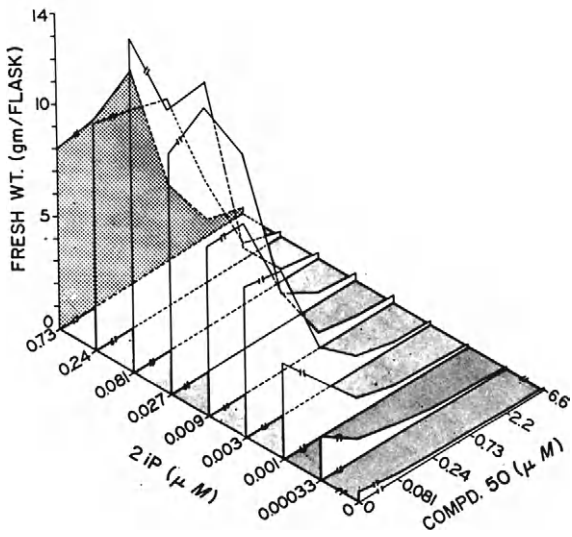


FIG. 12. Effect of various concentrations of cytokinin [6-(3-methyl-2-butenylamino)-purine (2iP) (**1**)] and cytokinin antagonist [3-methyl-7-(3-methylbutylamino)pyrazolo-[4,3-*d*] pyrimidine (**50**)] on the fresh-weight yield of tobacco callus. Growing period, 1/8/71–2/10/71. Skoog *et al.* (1973). Reprinted with the permission of the publisher of *Phytochemistry*.

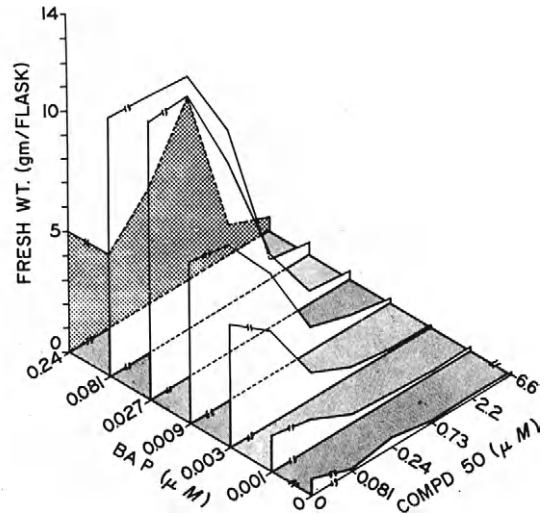


FIG. 13. Effect of various concentrations of cytokinin and cytokinin antagonist on the fresh-weight yields of tobacco tissue cultured on serial combinations of benzylaminopurine (BAP) (**46**) and the inhibitor (**50**). Growing period, 1/28/71–3/3/71.

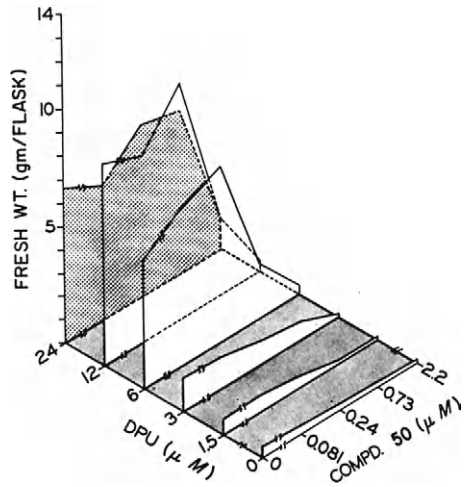


FIG. 14. Effect of various concentrations of cytokinin and cytokinin antagonist on the fresh-weight yields of tobacco tissue cultured on serial combinations of *N,N'*-diphenylurea (DPU) and the inhibitor (**50**). Growing period, 4/4/71–5/5/71.

Compound **50** also inhibited the action of diphenylurea as a cytokinin (Fig. 14) and the effect of 2iP (**1**) on budding (Skoog *et al.*, 1973). 3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**50**) was the first recognized example of a close structural analog of known cytokinins that is itself a potent anticytokinin. Additional candidates of this type have now been synthesized (Skoog *et al.*, 1973), and a fivefold increase in antagonist activity was realized with a change from the isopentyl (**50**) to the *n*-pentyl derivative.

The active 3-methyl-pyrazolo[4,3-*d*]pyrimidines appear to be highly specific cytokinin antagonists (as compared to abscisic acid), but in high concentrations they may interfere more generally in purine metabolism. The pyrazolo[4,3-*d*]pyrimidine derivatives promise to be useful tools in studies of the regulation of growth and the mechanism of cytokinin action in this process.

Summary

The present status of cytokinin chemistry has been described with special emphasis on the following subjects: isolation of natural cytokinins; methods of separation and structure elucidation; methods of synthesis of cytokinins; relation of structure to activity; comparison of 2-, 8-, and 2,8-substituted adenine derivatives in biological activity; stereoselective synthesis of *cis*-zeatin and ribosyl-*cis*-zeatin; identification of ribosyl-*cis*- and *trans*-zeatin from natural sources; geometric and position isomers of zeatin and ribosylzeatin; chemical modification of side-chain structure; biochemical modification of cytokinins; conversion of exogenous unnatural cytokinins in growing systems; cytokinin antagonists—design, synthesis, and determination of antagonist activity.

Acknowledgments

The author gratefully acknowledges the many contributions of his research colleagues at Illinois and of Professor Folke Skoog and his co-workers at Wisconsin who have made possible the writing of this paper on the present status of cytokinin chemistry.

The work at the University of Illinois has been supported by National Institutes of Health Research Grant GM-05829 and by an unrestricted research grant from the Hoffmann-La Roche Foundation. The work at the University of Wisconsin has been supported by National Institutes of Health Grant GM-12395, National Science Foundation Grant GB-25812, and the Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation.

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CHEMISTRY AND BIOCHEMISTRY OF ABSCISIC ACID

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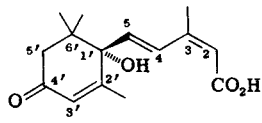
Introduction and Definition

To define the subject of this review we must necessarily start with the chemistry of abscisic acid (ABA), but it will not be covered exhaustively—the discussion will be confined to those aspects that have some bearing on research into the action and origin of the compound in plants.

The early literature has already been reviewed (Addicott and Lyon, 1969; Milborrow, 1969a). Suffice it to say that ABA was isolated from

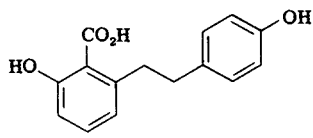
cotton plants (*Gossypium hirsutum*) as an abscission accelerant, and its structure (**1**) was proposed by Ohkuma *et al.* in 1965. It was confirmed by



(+)-Abscisic acid

1

synthesis (Cornforth *et al.*, 1965b), and an absolute configuration was proposed on the basis of an application of Mills' rule in 1967 (Cornforth *et al.*, 1967). We had isolated the same compound from sycamore (*Acer pseudoplatanus*) (Cornforth *et al.*, 1965a) where it had been implicated in the induction of dormancy. It was also isolated from yellow lupin (*Lupinus luteus*), where it caused abscission of immature fruitlets (Rothwell and Wain, 1964; Cornforth *et al.*, 1966b). Later it was isolated in crystalline form from pea (*Pisum sativum*) (Isogai *et al.*, 1970), yellow lupin (Koshimizu *et al.*, 1966), and avocado (*Persea gratissima*) (B. V. Milborrow, unpublished). It was also the major active ingredient in numbers of isolates of inhibitory material from other plant sources from which it was obtained by combinations of chromatographic techniques and identified by analysis of its optical properties and mass-spectrographic properties. It has now been detected in several score of flowering plants, two gymnosperms (Milborrow, 1969a; Le Page-Degivry *et al.*, 1969), a fern (Milborrow, 1967), a horsetail (D. R. Robinson, unpublished data), and a moss (Hiron, cited by Pryce, 1972). At this taxonomic level there appears to be a break because, so far, it has not been detected in liverworts or algae, and its physiological role in liverworts appears to be fulfilled by lunularic acid (**2**) (Pryce, 1972).



Lunularic acid

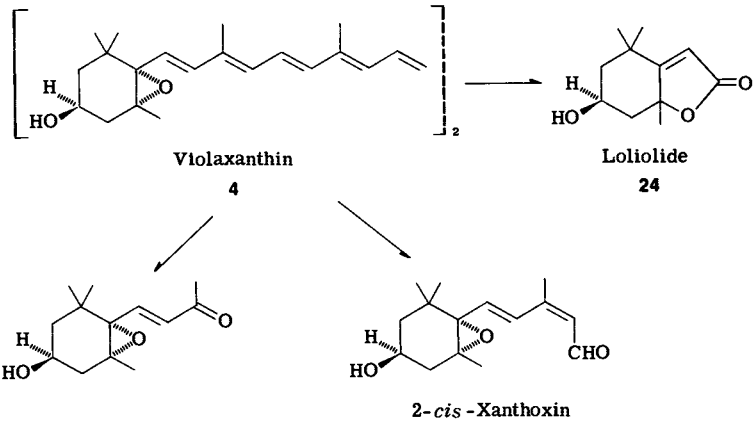
2

All determinations of the optical rotatory dispersion (ORD) spectra of isolates of natural ABA have shown it to be (+), and the specific rotation

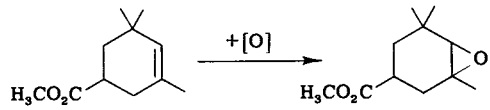
of the crystalline isolates are so similar that it appears that one enantiomer only is formed in nature.

Revision of the Absolute Configuration of Abscisic Acid

The absolute configuration of ABA was originally deduced by the application of Mills' rule to the 1′,4′-*cis*- and 1′,4′-*trans*-diols formed from the methyl esters of (+)- and (−)-ABA. G. Snatzke (personal communication to G. Ryback) first queried this because, although Mills' rule appeared to be obeyed in the ORD spectra of the diols in the region between ca. 230 and 340 nm, he observed ORD properties in the far ultraviolet (UV) which were incompatible with the designation.



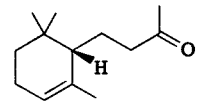
Later Burden and Taylor (1970) converted the 2-cis isomer of xanthoxin (**3**), a derivative of natural violaxanthin, into (+)-ABA by a method that maintained the stereochemistry of C-1′. The absolute configuration of violaxanthin had been proposed to be as shown in structure **4**, so that either (+)-ABA or violaxanthin required revision. The absolute configuration of the latter had been proposed by Bartlett *et al.* (1969) who found that permonophthalic acid preferentially epoxidizes compound **5** cis to the *O*-acetyl group (50 percent yield) rather than trans (20 percent). The



5

same preferential cis epoxidation was assumed to occur when zeaxanthin diacetate was treated with permonophthalic acid to form violaxanthin and, as the ORD spectrum of the major product was opposite to that of natural violaxanthin, the hydroxyl and epoxide groups of the latter were deduced to be of the trans form. The stereochemistry of the hydroxyl groups of zeaxanthin had been related by synthesis to the hydroxyl groups of fucoxanthin, the absolute configuration of a derivative of which had been determined by X-ray analysis. Consequently, the stereochemistry of the 3-hydroxyl group in violaxanthin was defined unambiguously but the stereochemistry attributed to the 5,6-epoxide group depended on the analogy between the ratio of cis and trans products formed from compound **5** and the ratio of the products formed from zeaxanthin by permonophthalic acid.

Recently, Oritani and Yamashita (1972) have also reported work which suggested that the absolute configuration of ABA as deduced by the application of Mills' empirical rule is incorrect. They synthesized ABA ethyl ester from the unnatural (−)-enantiomer of α-ionone (**6**) and obtained a preponderance of the (−)-enantiomer in the ABA ester product. They also concluded that natural (+)-ABA has the configuration shown in structure **1**. However, the crucial point of the synthesis is the retention of configuration during the oxidation of the tertiary hydrogen of (−)-α-ionone to a tertiary hydroxyl by selenium dioxide, and this reagent is not rigorously stereospecific.



6

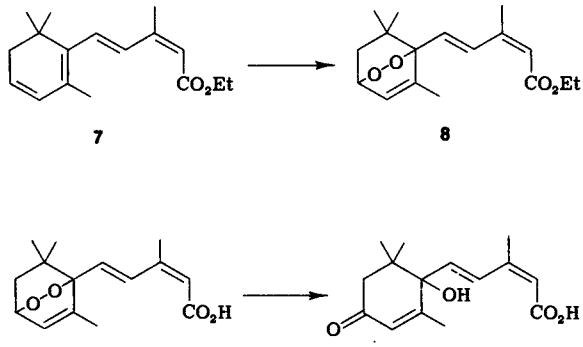
Ryback (1972) has now carried out a definitive chemical correlation of (+)-ABA with malic acid. He found that the original configuration deduced from the application of Mills's rule is incorrect and that (+)-ABA has the absolute configuration shown in **1**. This result, of course, also confirms the absolute configuration proposed for violaxanthin (**4**) by Bartlett *et al.* (1969). Koreeda *et al.* (1973) have also reported independent evidence requiring a revision of the absolute configuration of natural ABA.

The terminology of the stereochemistry of (+)-ABA is now somewhat confused because the Cahn, Ingold, Prelog rules, as they were originally formulated (Cahn *et al.*, 1956), gave the notation for the original configuration as (*S*). In 1966, they published a fuller account and changed the

rule of ligancy so that when their modified rules are applied to the old, incorrect configuration of (+)-ABA it is (*R*).

The absolute configuration of (+)-ABA has now been revised* and is as shown in structure **1**; thus, according to the Cahn, Ingold, Prelog notation of 1966, (+)-ABA is (*S*) (see also Weedon, 1971). The absolute configuration of natural (+)-α-carotene (Eugster *et al.*, 1969) is (*R*) according to the rules of the 1966 notation. The situation has been further complicated by the misapplication of the sequence rules to (+)-ABA (Moss, 1971; Oritani and Yamashita, 1972).

Synthesis



7 **8**

Epidioxide
8

(±)-Abscisic acid

The original synthesis (Cornforth *et al.*, 1965b) was accomplished by the light-sensitized epidioxidation of the known compound (**7**) to compound **8**. This was rearranged by the action of dilute base and hydrolyzed to give ABA. The main disadvantage of the method is that the light-sensitized epidioxidation forms a number of other products and the 2-cis bond is isomerized to give the inactive 2-trans isomer. These isomers can be separated readily by thin-layer chromatography (TLC) on silica gel using toluene:ethyl acetate:acetic acid (30:15:4 v/v), and the 2-trans isomer

* One possible explanation for the changes, reinterpretation, and general confusion concerning the absolute configuration of ABA is

Our stereochemist took fright
At the counterrotation of light.
He really was cruel,
To have broken Mills' rule.
What's left that was wrong is now right.

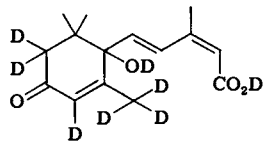
can be illuminated again to an equilibrium mixture comprising 50 percent 2-cis:50 percent 2-trans isomers.

Roberts *et al.* (1968) reported an improved synthesis starting with α-ionone. They oxidized the ring to 4-hydroxy-7-keto-α-ionone and then elaborated the side chain by a Wittig reaction. This route has been the basis for the synthesis of a large number of analogs (Oritani and Yamashita, 1970a,b, 1972; Tamura and Nagao, 1969a,b, 1970); it is also the preferred route for the synthesis of ^{14}C-labeled ABA (Cornforth *et al.*, 1968; Sondheimer and Tinelli, 1971) and for the 2-^{14}C analog (Ryback and Mallaby, cited by Milborrow and Noddle, 1970; B. V. Milborrow *et al.*, unpublished) because the reagent containing the label is introduced at a late stage in the synthesis and thereby minimizes losses of the most expensive ingredient.

Chemistry

The chemistry of ABA has been strangely neglected, and, apart from the incidental mention of some simple reactions to form derivatives used in the isolation and identification of labeled ABA, no publications have dealt with the reactions undergone by ABA itself until the report of Mallaby and Ryback (1972).

The commonest reactions routinely carried out to aid isolation and identification are methylation in ethereal diazomethane and reduction of the methyl ester in aqueous, methanolic sodium borohydride to an approximately equal mixture of the 1′,4′-*cis*- and 1′,4′-*trans*-diols (Milborrow and Noddle, 1970). The diols are readily reoxidized to ABA by manganese dioxide in dry chloroform (Milborrow, 1972b).

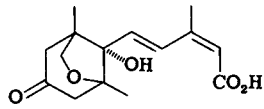


Octadeuterioabscisic acid

9

Abscisic acid exchanges six of its carbon-born hydrogen atoms with water at pH 11 and above, and this reaction has been used to remove tritium from ABA which had been formed by biosynthesis from stereospecifically tritiated mevalonolactones (Robinson and Ryback, 1969; Milborrow, 1972b). Hexadeuterioabscisic acid (**9**) has been fed to tomato plants as a substrate for phaseic acid formation to define which methyl

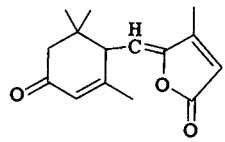
group of ABA is oxidized during the formation of phaseic acid (**10**) (Milborrow, 1969b) and thereby to revise the structure originally proposed for phaseic acid (MacMillan and Pryce, 1968).



Phaseic acid

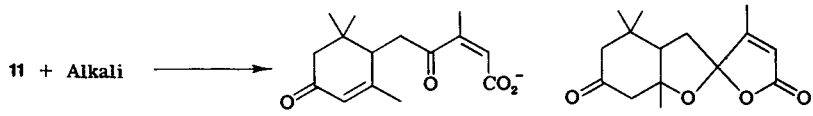
10

If ABA is heated with a mixture of formic and hydrochloric acids the major product is a lactone (**11**), although a range of other isomeric compounds is also formed (Mallaby and Ryback, 1972). Formation of the



11

lactone gives a convenient method for removing the hydrogen of C-4 (derived from C-2 of mevalonate), but its chief interest is the intense violet–red color produced with alkali. Unfortunately the color fades, the intensity is affected by the other materials present, and the lactone is not formed in constant yield and so cannot be readily used for quantitative assay. However, it provides a sensitive qualitative test for ABA and can be used on quantities as small as 0.1 μg. The lactone is believed to hydrolyze to a keto acid salt (**12**) which, on acidification, cyclizes to a spirolactone (**13**).

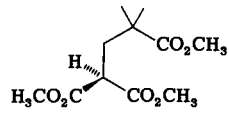


12 **13**

Indirect evidence supporting the existence of a ketone at C-4 comes from an experiment in which tritium, believed to be at C-5 in compound **12**, was exchanged with the medium on treatment with alkali (Milborrow, 1972a).

Partial chemical degradation of (+)-ABA via the 1′,4′-*trans*-diol to give compound **14** has now been accomplished by ozonolysis followed by

performate oxidation and methylation (Ryback, 1972). This technique will be elaborated and can be expected to give definitive information concerning the position of labeled atoms derived from precursors.



14

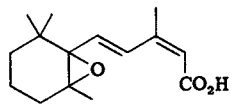
Biological Activity of Analogs

Chemical synthesis has produced many novel compounds which bear some structural relationship to ABA, they have been tested in a variety of bioassay systems, and almost all are less active than ABA and many are quite inactive. As yet there has not been found a synthetic compound that shows dramatically increased activity over that of the parent compound (as 2,4-dichlorophenoxyacetic acid does over indoleacetic acid). Most examples of slightly higher activity are esters or closely related compounds (Tamura and Nagao, 1969a) that can be expected to give rise to ABA over a period of time or, being less polar, may penetrate into the cells more efficiently.

The structure–activity field is beset with so many difficulties of interpretation that it is worth while to examine the bases of the arguments rather than to catalog the structures and their biological activities in the various bioassays employed. The first possible complication is penetration of the analog into the cells—inactivity can be caused by failure to penetrate to the active site—and, although ABA methyl ester is inactive in short-term tests (Milborrow, 1966), its greater activity in tests of long duration may, in part, be attributed to easier penetration followed by hydrolysis (Walton and Sondheimer, 1972b).

The biological effect observed, inhibition of growth, is a highly unspecific one, and, unless some attempt is made to show that a given, weakly inhibitory analog shows some unique feature of the ABA type of inhibition, it should not be considered as having the same mode of action. Reversibility of the inhibition by gibberellic acids, as used by Tamura and Nagao (1969b), or by cytokinin, or leaching in water to restore growth would go far toward doing this, but ideally the demonstration of the ABA type of activity should be carried out in a highly specific bioassay such as the ability of a compound to form dormant "turions" in *Lemna* (Stewart, 1969) or to close stomata (Mittelheuser and van Steveninck, 1969).

Even if a compound satisfies these requirements and its activity cannot be attributed to trace amounts of an active impurity, there is the possibility that the plant is able to convert it into another, active analog or into ABA itself. The activity of compound **15** (Anderson, 1969; Tamura and Nagao,



15

1970) was originally considered to indicate that the 4′-oxo group of ABA is not required for activity, but compound **15** has been shown by ^{14}C- and ^{18}O-labeling to be rapidly converted into ABA (Milborrow and Noddle, 1970). Thus the higher activity of **15** in some tests can be attributed to more efficient penetration or a "slow-release" effect by metabolism to ABA. It is interesting to note in this context that Mousseron-Canet *et al.* (1970) found that compound **15** was inactive in their biological test that measured the inhibition of fungal α-amylase, presumably because it was not metabolized to ABA in this system. Unfortunately they did not report its activity in the wheat coleoptile test that they also used.

Sondheimer *et al.* (1969) report that the growth-inhibitory activity of the epidioxide (**8**), an intermediate in the original synthesis of ABA, is caused by a metal-catalyzed rearrangement to ABA; they found no evidence that this compound requires the action of an enzyme for activity. We have also found that the 1′,4′-*trans*- and, in particular, the 1′,4′-*cis*-diols of ABA are oxidized to ABA by air in aqueous solutions (B. V. Milborrow and M. M. Garmston, unpublished). The aldehyde of ABA, which has been reported (Oritani and Yamashita, 1970b) as having virtually the same inhibitory activity as ABA, was also found to be oxidized to ABA in air (B. V. Milborrow, unpublished).

Many of the analogs that have been tested differ from ABA in several ways (e.g., ethyl esters of acids lacking a 4′-oxo group and a ring double bond) so it is difficult to deduce the minimum requirements for activity and the contribution of individual features. Furthermore, if an analog can be converted into ABA by a number of enzyme-catalyzed steps, some of which occur during normal biosynthesis, then it is possible that it will not inhibit growth because the flow of intermediates along the normal biosynthetic pathway of ABA is probably rigorously controlled and, therefore, the concentration of ABA formed from the added precursor would be kept within the normal range for the tissue.

The requirements for activity appear to be a carboxyl group—the nitrile is inactive (Tamura and Nagao, 1969a) and so is the methyl ester before hydrolysis (Milborrow, 1966). The 2-double bond must be cis (Cornforth *et al.*, 1965b). The effects of side-chain lengths are still in dispute. Oritani and Yamashita (1970a) found that shortening of the side chain yields inactive products, but the data of Popoff *et al.* (1972) indicate that citrylideneacetic acid, rather than ionylideneacetic acid skeletons, are sufficient for growth inhibition. The cyclohexane ring must contain a double bond in an α- or β-position, but the absence of the 4′-oxo group, and possibly the 1′-hydroxyl group also, is readily overcome by these compounds' being formed metabolically.

Finally there is the closest analog of all to (+)-ABA, namely the (−)-enantiomer. The first test of the pure resolved (−)-material (Milborrow, 1968) showed that, contrary to earlier reports that compared natural (+)-ABA with racemic material slightly contaminated with inactive 2-*trans*-ABA, it was as potent as the (+)-enantiomer.

The bioassay was carried out using excised, dry wheat embryos and the two enantiomers would be expected to exert their effect in maintaining the embryos in a dormant condition without being subjected to the same degree of differential metabolism that they would undergo in metabolically active and growing tissues. Sondheimer *et al.* (1971) found that both (+)- and (−)-enantiomers were active but differed in uptake in different tissues and were metabolized at different rates so that their relative effectiveness

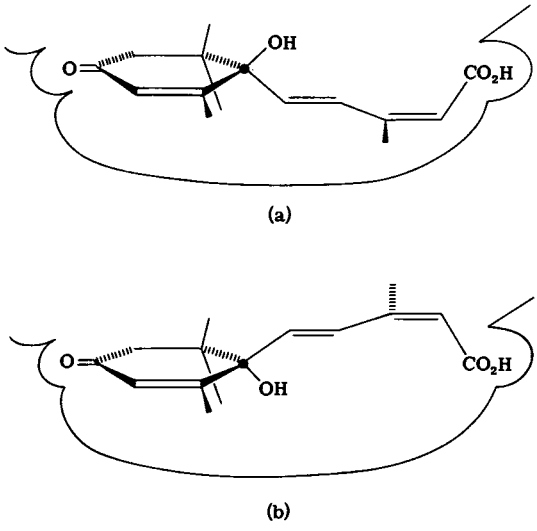


FIG. 1. (a) (−)-Abscisic acid in active site. (b) (+)-Abscisic acid in active site.

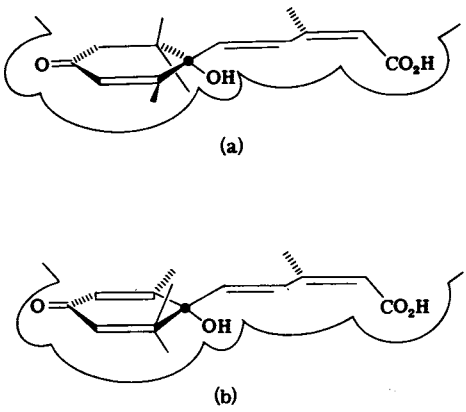


FIG. 2. (a) (+)-Abscisic acid in active site. (b) (−)-Abscisic acid in active site.

depended to some extent on the bioassay system used. The data of Sondheimer *et al.* do not permit the possibility of a racemase to be ruled out although they consider its presence unlikely.

Two hypotheses have been proposed to account for the activity of the (−)-enantiomer. According to one hypothesis (Fig. 1) the hydroxyl group is not involved with attachment to the active site, and this view is supported by the strong growth-inhibitory activity of the 1′-deoxyabscisic acid methyl ester (Milborrow, 1968). The 1′-hydroxyl is assumed to be present to stabilize the molecule and adjust its solubility characteristics. However, the 1′-deoxy derivative could owe its activity to metabolic hydroxylation to ABA.

The other hypothesis (Fig. 2) (proposed to me by J. W. Cornforth) considers the ring of ABA to be virtually symmetrical about the line of the 4′-oxo and 1′-hydroxyl groups and able to lie on either face at the active site. The 1′-hydroxyl group is envisaged as being necessary for binding at the active center but, whereas the *geminal* methyl groups of (+)-ABA are away from the observer in Fig. 2a, those of (−)-ABA lie toward the observer in Fig. 2b. By this change in position of the ring, the 1′-hydroxyl group of both (+)- and (−)-ABA can be brought into the same position in relation to a theoretical active site.

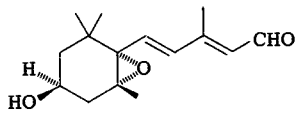
Pathway of Biosynthesis

One of the major controversies of ABA research has been the route by which the hormone is synthesized. Like many earlier controversies in science it has stimulated an intensive program of research and led to a rapid advancement of knowledge. The position of the two schools of

thought are, briefly, (1) that ABA is derived from a carotenoid, in particular violaxanthin, by photolytic cleavage or oxidative processes; and (2) that ABA is synthesized by a unique route from a C-15 precursor.

When the structure of ABA was proposed by Ohkuma *et al.* (1965), its structural similarity to the end portions of many carotenoids was noted. Circumstantial evidence—the occurrence of carotenoids in phototropic organs of fungi and the similarity of their action spectrum to the absorption spectrum of carotenoids, the occurrence of more inhibitory material in light-grown plants than in etiolated ones (Wright, 1954; Masuda, 1962), the identification of the major active constituent of this inhibitor β as ABA (Milborrow, 1967), and the work of Simpson and Wain (1961) suggesting that an inhibitor is formed in plants in the light—led to the hypothesis that carotenoids might be natural precursors of ABA (Taylor and Smith, 1967).

Taylor and Smith exposed a number of carotenoid pigments on damp filter paper to bright light and found that violaxanthin gave rise to a strongly inhibitory neutral material although other xanthophylls with a 3-hydroxy-5,6-epoxy ring also produced inhibitory products. The active constituent was isolated and characterized by Taylor and Burden (1970a,b; 1972). The active compound (**3**) is the 2-cis isomer of a mixture of this and approximately equal amounts of the inactive 2-trans isomer (**16**),



(+)-2-*trans*-Xanthoxin

16

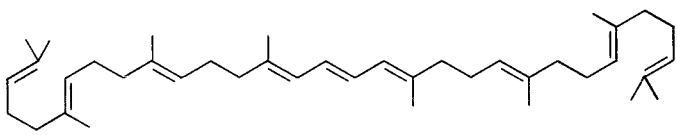
and it has now been found in extracts of dwarf bean (*Phaseolus vulgaris*) and wheat (*Triticum vulgare*). However, in the leaf extracts the ratio of 2-*cis*- to 2-*trans*-xanthoxin is similar to that present in the products of violaxanthin photolysis, and the inactivity of the 2-trans isomer suggests that, like the 2-*trans*-ABA, it cannot be isomerized enzymatically to the active form. The 2-*trans*-xanthoxin is unlikely, therefore, to be a precursor of the 2-cis isomer of ABA, and its presence in the leaf may indicate that it is an adventitious product formed by light.

The yield of ca. 2 percent xanthoxin from violaxanthin is so low and such high light intensities are required for photolysis *in vitro* that it seems unlikely that the extremely sensitive phototropic responses of some higher plant organs, which require the order of a few dozen quanta for activation, could be mediated by this means.

Recently, Firn and Friend (1972) have reported that soybean (*Glycine soja*) lipoxygenase is capable of cleaving violaxanthin oxidatively to form a similar range of products, in similar yield, to that formed during photolysis; whether the enzyme functions *in vivo* is unknown. The possibility that xanthoxin is produced by the action of lipoxygenase removes the requirement of light for its production, but the system then requires a photosensitive mechanism if the production of inhibitor is responsible for light-induced growth inhibition and phototropism.

The enzymatic production of xanthoxin from carotenoids could, if it is a normal precursor of ABA, account for the synthesis of ABA by avocado fruit and wheat leaves in darkness. A further possibility is that xanthoxin is a normal intermediate in ABA biosynthesis but that it is formed from a C-15 precursor and does not come from a carotenoid *in vivo*.

An experiment suggesting that ABA is not synthesized via a carotenoid has been carried out by D. R. Robinson (unpublished results). He prepared ^{14}C-labeled phytoene (**17**) and supplied it to an avocado fruit in the same



Phytoene
(The central double bond is shown here in the trans configuration.)

17

solution as [2-$^{3}H_2$]mevalonate. The tritium was incorporated, as expected, into carotenoids and ABA. The ^{14}C was incorporated into carotenoids so it had penetrated the cells and became metabolically available, but there was no ^{14}C label in ABA.

The simplest explanation of this result is that ABA is formed from mevalonate via a route not involving a carotenoid (Fig. 3). Unfortunately, the experiment had to be carried out using intact cells, and this always

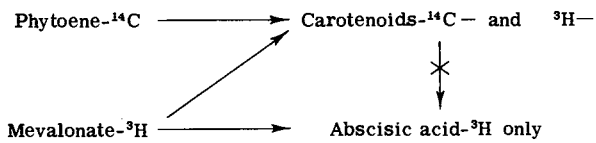
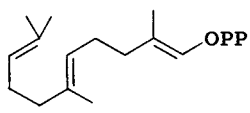


FIG. 3. Diagrammatic representation of the labeling of abscisic acid expected from operation of the "direct synthesis" pathway. Phytoene-^{14}C was converted into carotenoids. Mevalonate-^{3}H was converted into carotenoids and ABA but no ^{14}C was found in ABA suggesting that it is not formed via a carotenoid.

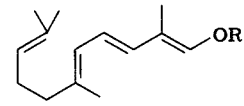
introduces the possibility of compartmentalization of different reactions in separate parts of the cells. Consequently, it is possible that in the tissue used the ^{14}C-phytoene failed to penetrate into a carotenoid pool which became heavily labeled by ^{3}H from mevalonate and which gave rise to ^{3}H-ABA.

Earlier this year my colleague, R. Mallaby, and I considered that the reactions of desaturation and cyclization could provide a useful analogy for these reactions in a hypothetical C-15 precursor of ABA. It appears probable that the desaturation of carotenoids occurs before cyclization (Porter and Lincoln, 1950; Kushwaha *et al.*, 1969), and the potential C-15 precursor of ABA, farnesyl pyrophosphate (**18**), could give rise to the first unique intermediate in the ABA pathway by undergoing desaturation to 4-dehydrofarnesol (**19**) or a derivative. This compound could then be expected, by analogy with carotenoid formation, to undergo cyclization.



Farnesyl pyrophosphate

18



Derivative of 2,4,6-*trans*-dehydrofarnesol

19

We have synthesized 2,4,6-*trans*-dehydrofarnesol and also 2-*cis*-4,6-*trans*-dehydrofarnesol and added them as "cold traps" to avocado fruit which were synthesizing labeled ABA from [2-^{14}C]mevalonate. In three experiments, approximately 0.1 percent of the label added was recovered in the 2-*trans*-dehydrofarnesol when this was reextracted. On the other hand, the 2-*cis*-dehydrofarnesol contained less than one-twentieth the amount of label present in the 2-trans isomer, and this could have arisen by isomerization from the 2-trans isomer during the extraction process. Dehydrofarnesol is unstable and polymerizes readily; at present the test of whether the cold trap material is labeled has been confined to three chromatographic separations and chromatography of the *O*-acetyl derivative. The putative trapped label cochromatographed with added cold material, the density of radioactivity of an radioautogram corresponded with the optical density of the carrier material, and no other radioactive bands were separated.

The two isomers have been synthesized with ^{14}C label at C-2 and supplied to avocado fruit. The ABA from the fruit supplied with 2-*trans*-dehydrofarnesol was found to contain ^{14}C and, by the criteria used by Milborrow

and Noddle (1970), to show that a labeled precursor had been incorporated. The criteria include chromatography of the ether-soluble acid fraction in two chromatographic systems; methylation, reduction with aqueous, methanolic borohydride to an equal mixture of 1′,4′-*cis*- and 1′,4′-*trans*-diols of ABA methyl ester, and reoxidation of both to methyl abscisate with manganese dioxide. After each of these procedures the derivative and the radioactivity cochromatographed with the appropriate marker of authentic material, and no other radioactive spots were found after chromatography of the methylated extract.

Although there is no doubt that ^{14}C-labeled ABA had been formed from [2-^{14}C]2-*trans*-dehydrofarnesol, we have not yet chemically degraded the ABA and demonstrated that the label is at C-2. The possibility remains, therefore, that the dehydrofarnesol is broken down by avocado fruit and the fragments are reassembled into ABA. However, the high incorporation of the 2-trans isomer (40 times more than of mevalonic acid added to another piece of the same fruit) and the virtual absence of incorporation from 2-*cis*-dehydrofarnesol suggest that this explanation is unlikely.

The true intermediate may not be 2-*trans*-dehydrofarnesol but a conjugate with glucose, pyrophosphate, or some other convenient grouping and to which the added [2-^{14}C]2-*trans*-dehydrofarnesol is converted. This reaction could maintain an equilibrium between a pool of free alcohol and the conjugate (Fig. 4). Alternatively, the free 2-*trans*-dehydrofarnesol could be conjugated within the cells and the cold trap could have become labeled by a "scavenging" of ^{14}C-mevalonate-derived material released by hydrolysis during extraction or by damage to the tissues.

It is particularly interesting that the 2-trans isomer is active and the 2 cis is inactive because other work (see next section) suggests that the 2-double bond of ABA is formed trans and isomerized at a subsequent stage of biosynthesis. The cis isomers only of compounds closely related to ABA are active (Tamura and Nagao, 1969a,b).

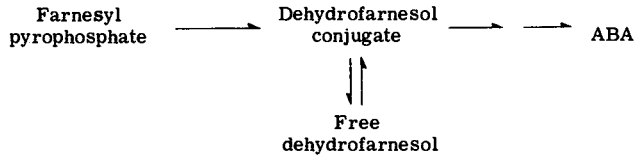


FIG. 4. Diagram of a possible interpretation of the labeling of dehydrofarnesol as a "cold trap" for mevalonate-^{14}C and for the incorporation of dehydrofarnesol-2-^{14}C into abscisic acid (ABA).

Stereochemistry of Biosynthesis

The structure of ABA immediately suggests that it is a terpenoid, and Noddle and Robinson (1969) indeed showed that labeled mevalonate could be incorporated into ABA in a number of fruits. Mevalonate could occur either by the synthesis of a carotenoid and then cleavage to give a 15-carbon fragment for elaboration into ABA (carotenoid pathway) or else synthesis of ABA could occur by a unique route from farnesyl pyrophosphate (direct synthesis pathway).

In an attempt to choose between these two alternative pathways, Robinson and Ryback (1969) made use of the stereochemistry of hydrogen elimination from C-4 of mevalonate (Fig. 5) which had been elucidated by Popják and Cornforth (1966) and associates. These workers had found that the 4(*S*) hydrogen was eliminated from C-4 when a trans double bond was formed during the junction of an isopentenyl pyrophosphate to a dimethylallyl pyrophosphate or geranyl pyrophosphate, and the 4(*R*) hydrogen was eliminated and the 4(*S*) retained when a cis double bond was formed during the incorporation of isopentenyl pyrophosphate into rubber hydrocarbon.

The C-2 of ABA is derived from C-4 of mevalonate so that, provided the enzymes that synthesize ABA follow the same stereochemical path as those of geranyl pyrophosphate and rubber biosynthesis, the hydrogen of the 2-cis double bond should be derived from the 4(*S*) hydrogen of mevalonate, but, if the bond was formed trans, then the 4(*R*) hydrogen should be retained (Fig. 6).

Goodwin (1971) and his collaborators have shown that the analogous bond of carotenoids is trans and retains the 4(*R*)-hydrogen; therefore, if the C-2 of ABA had carried a 4(*S*)-hydrogen of mevalonate, then this

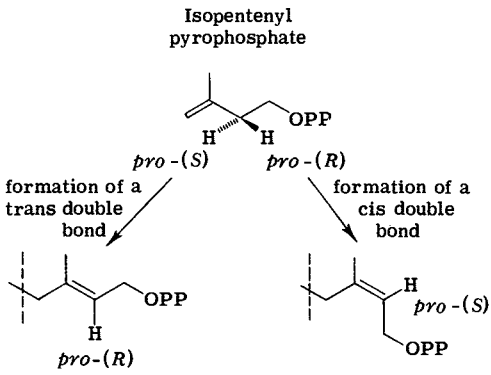
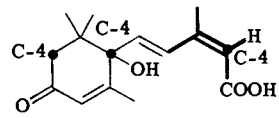
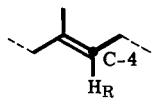


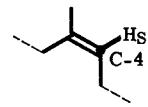
Fig. 5. Diagram of the fate of the hydrogen atoms of C-4 of mevalonate. The *pro*-(*R*) hydrogen of C-4 is retained in a trans double bond; the *pro*-(*S*) hydrogen of C-4 is retained in a cis double bond.



The position of C-4 of mevalonate in abscisic acid



trans Bond with retention of the 4(*R*)-hydrogen of mevalonate



cis Bond with retention of the 4(*S*)-hydrogen of mevalonate

FIG. 6. Position of C-4 atoms of mevalonate in abscisic acid. Retention of a *pro*-4(*S*) atom in the terminal residue of the chain would indicate that the 2-cis bond was formed in the cis configuration. The occurrence of the 4(*R*) tritium at this position indicated that it was formed trans and isomerized at a later stage of biosynthesis.

result would have excluded the carotenoid pathway. The 4(*R*)-hydrogen was retained at C-2 of ABA, and this result could have occurred by synthesis through the carotenoid pathway followed by isomerization or by the direct synthesis pathway also followed by isomerization. The result, therefore, could not be used to differentiate between the two alternative pathways.

More recently, the stereochemistry of the hydrogen eliminated from C-3′, C-4, and C-5 of ABA has been investigated (Milborrow, 1972a) using 3(*R*)-[2-^{14}C, 2(*R*)-2-$^{3}H_1$]- and 3(*R*)-[2-^{14}C, 2(*S*)-2-$^{3}H_1$]mevalonolactones (as racemates), synthesized by R. Cornforth, and 3(*RS*)-[2-^{14}C, 5(*S*)-5-$^{3}H_1$]mevalonolactone (Cornforth *et al.*, 1966a), synthesized by F. P. Ross (Cornforth and Ross, 1970). The nominally [2(*R*)-2-$^{3}H_1$]mevalonolactone was racemic but, whereas the natural 3(*R*)-enantiomer carried a tritium in the 2(*R*) position, the unnatural 3(*S*)-enantiomer carried a tritium in the 2(*S*) position. Experiments with animal systems have shown that the 3(*S*)-mevalonate is not utilized by mevalonate kinase and so is not incorporated into terpenoids. The racemate is incorporated as though it were tritiated in the nominal position only (Fig. 7). The [4(*R*)-4-$^{3}H_1$]-, [4(*S*)-4-$^{3}H_1$]-, and [5(*S*)-5-$^{3}H_1$]mevalonolactones were labeled analogously.

The data of Robinson and Ryback (1969) show that no tritium from the nominal 4(*S*)-tritiated mevalonate is incorporated into ABA and, therefore, the 3(*S*)-[4-(*R*)-4-$^{3}H_1$] enantiomer present in the mixture was not incorporated in the avocado fruit. The discrimination against 3(*S*)-mevalonate must operate in this tissue also.

3(*R*) Mevalonate incorporated into terpenoids

3(*S*) Mevalonate not incorporated

FIG. 7. The two enantiomers of mevalonate showing the stereochemistry of the hydrogens on C-2.

The data also show that from an initial $^{14}C/^{3}H$ ratio of 3:3 in the mevalonolactone the ratio dropped, as expected, to 3:1.93 (3:2) in ABA; after removal of the C-5′ hydrogens by exchange the ratio fell to 3:0.98 (3:1). Thus the three isoprene residues of ABA are derived equally from the added mevalonate.

This established that it was possible to investigate the stereochemical origin of the hydrogen from C-3′ and C-4 of ABA by feeding ^{14}C-2(*R*)- or ^{14}C-2(*S*)-tritiated mevalonate and then removing the hydrogens from C-3′ and C-4 of the ABA formed. This was done by monitoring the $^{14}C/^{3}H$ ratio before and after base-catalyzed exchange of the hydrogen on C-3′ (Table 1). The hydrogen at C-4 was removed by heating the ABA methyl ester recovered from the alkali-exchange experiment in formic acid to form the lactone (**11**). The remaining tritium in the molecule, which comprised the expected one-third of the amount originally present from the sum of [2(*R*)-2-$^{3}H_1$]- and [2(*S*)-2-$^{3}H_1$]mevalonate, was attributed to a methyl group on C-6′. This position is at present inaccessible to chemical degradation. The $^{14}C/^{3}H$ ratios show that there is a preponderance of 2(*R*) tritium at C-3′ and C-4. The results with [2-^{14}C, 2(*R*)-2-$^{3}H_1$]- and [2-^{14}C, 2(*S*)-2-$^{3}H_1$]mevalonate (Table 1) show variations from the expected $^{14}C/^{3}H$ ratios, but these can be accounted for by the action of isopentenyl pyrophosphate isomerase, and the differences observed are in good agreement with this explanation.

The conversion of isopentenyl pyrophosphate into dimethylallyl pyrophosphate, mediated by isopentenyl pyrophosphate isomerase, is reversible (Fig. 8). The activity of this enzyme relative to that catalyzing the next step in polyisoprenoid biosynthesis (isopentenyl pyrophosphate transferase) is much greater in plants than in animals; in plants, therefore, the dimethylallyl pyrophosphate is more likely to be reconverted into isopentenyl pyrophosphate and back again before being used for synthesis (Goad, 1970). Although the removal and replacement of the pro-4(*S*)-hydrogen of mevalonate by isopentenyl pyrophosphate isomerase is stereospecific, the three hydrogen atoms (the two originally present and one supplied from the medium) have become equivalent in the methyl group of di-

TABLE 1

THE $^{14}C/H^3$ RATIOS OF ABSCISIC ACID (ABA) AND DERIVATIVES FORMED FROM STEREOSPECIFICALLY TRITIATED MEVALONATES-^{14}C-BY AVOCADO FRUIT[a]

Substance	Observed ratio [2-^{14}C, (2*S*)-2-$^3H_1$] mevalonate	Expected ratio for the retention of the *pro*-2(*R*) hydrogen of mevalonate at C-4 and C-3′ of ABA	Observed ratio [2-^{14}C, (2*R*)-2-$^3H_1$] mevalonate	Expected ratio
Methyl abscisate	3:1.63	3:1.0	3:2.28	3:3.0
3′-Exchanged methyl abscisate	3:1.44	3:1.0	3:1.55	3:2.0
Lactone (**11**)	3:1.05	3:1.0	3:1.04	3:1.0
Aqueous exchange medium	3:0.23	3:0.0	3:0.58	3:1.0
Iodoacetamide sample				
Methyl abscisate	3:1.12	3:1.0	3:2.55	3:3.0
3′-Exchanged methyl abscisate	3:0.92	3:1.0	3:1.99	3:2.0
Lactone (**11**)	3:0.90	3:1.0	3:1.19	3:1.0
	Observed ratio [2-^{14}C, (5*S*)-5-$^3H_1$] mevalonate	Expected ratio for *pro*-5(*S*) hydrogen of mevalonate at C-5 of ABA		
Methyl abscisate	3:0.88	3:1.0		
Lactone (**11**)	3:0.87	3:1.0		
Repeat of experiment. Lactone $^{14}C/^3H$ ratio normalized to 3:1.				
Compound (**12**) after basecatalyzed exchange and rearrangement to (**13**)	3:0.16	3:0.0		

[a] The ratios in the mevalonates were normalized to 3:3. The 3′-exchanged methyl abscisate has lost any mevalonate-derived hydrogen from C-3′ and the lactone (**11**) has lost it from C-4 as well. The residual tritium is attributed to one of the *gem*-dimethyl groups.

methylallyl pyrophosphate. A second isomerization back to isopentenyl pyrophosphate is accompanied by a loss of one of these three hydrogen atoms of the methyl group, as shown in Fig. 8.

In pathway (a) the product will have the two hydrogen atoms H_A and H_B restored to their original positions. In pathway (b), H_A is lost

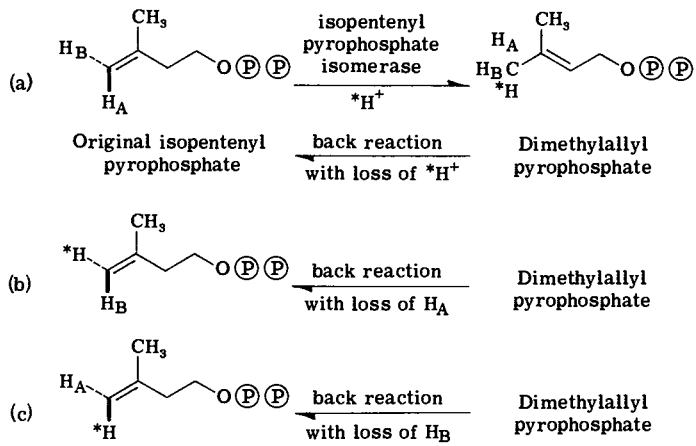


FIG. 8. Stereochemistry of the C-2-hydrogen atoms of mevalonate in isopentenyl pyrophosphate after the action of isomerase. In pathway (a), the original configuration of H_B is retained; in pathway (b), H_B occupies the position originally held in (a) by H_A; in pathway (c), H_B is lost.

and the position of H_B is reversed. In pathway (c), H_B is lost and H_A is reversed. Thus one cycle of the isomerase will randomize all the ^3H of isopentenyl pyrophosphate derived from stereospecifically 2-tritiated mevalonate, and further cycles will continue the loss of the ^3H to the medium. This racemization of the label between two positions will persist even if an isotope effect resists the elimination of ^3H (Cornforth *et al.*, 1970).

The dimethylallyl pyrophosphate derived from mevalonate-2-$^3H_1$ may have suffered some loss of ^3H from the methyl group before being utilized for biosynthesis, and the label in isopentenyl pyrophosphate similarly formed may be more-or-less randomized between the two hydrogen atoms of the terminal methylene group before being utilized for further synthesis. Randomization of the stereospecifically tritiated hydrogen from C-2 of mevalonate was observed at both C-4 and C-3′ of ABA. The presumed 6′-methyl label of ABA, originating from a methyl group of dimethylallyl pyrophosphate, showed little attenuation of its ^3H label, an indication either that a large isotope effect operated against removal of ^3H in the isomerization of dimethylallyl pyrophosphate to isopentenyl pyrophosphate or that no dimethylallyl pyrophosphate that had been through the double sequence (isopentenyl pyrophosphate → dimethylallyl pyrophosphate → isopentenyl pyrophosphate → dimethyl pyrophosphate) was used for further biosynthesis.

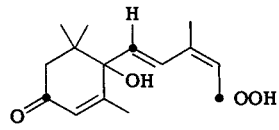
Isopentenyl pyrophosphate is particularly sensitive to inhibition by iodoacetamide; in a cell-free system isolated from liver, for instance, Cornforth *et al.* (1966a) reported that the isomerase was completely blocked by 5 m*M* iodoacetamide. This suggested that the enzyme could be inhibited *in vivo* by the application of a suitable concentration of the inhibitor. Accordingly, in another experiment, the 5 ml of aqueous mevalonic acid solution supplied to the avocado fruit contained 2 m*M* iodoacetamide in an attempt to lower the isomerase activity so that it became the rate-limiting step in the biosynthesis but was not completely blocked; it should not, therefore, completely prevent incorporation of labeled mevalonate into ABA because of a lack of dimethylallyl pyrophosphate.

The percentage incorporation of the labeled mevalonic acid into ABA per unit weight of avocado mesocarp in the presence of 2 m*M* iodoacetamide was similar to that in the other experiments, so that the overall rate of ABA synthesis did not appear to have been seriously affected. However, the amount of 2(*S*)-^{3}H incorporated at positions 3′ and 4 of ABA was much less than in the two previous experiments. Also, considerably more 2(*R*)-^{3}H was retained at these positions than in the absence of iodoacetamide. Thus it appears that less randomization of the 2(*R*)- and 2(*S*)-^{3}H of mevalonate takes place by recycling of the labeled precursors through the isopentenyl pyrophosphate → dimethylallyl pyrophosphate → isopentenyl pyrophosphate cycle under the action of isopentenyl pyrophosphate isomerase.

The ^{14}C/^{3}H ratios of ABA formed in the presence of iodoacetamide may not be exactly comparable with those of the other experiments because one of the basic assumptions may no longer hold, namely that all three isoprene residues of ABA are derived in the same proportion from the labeled mevalonate supplied. The decrease in the supply of dimethylallyl pyrophosphate could have altered the proportion of the *gem*-dimethyl isoprene residue of ABA derived from the labeled mevalonate. The proportions of the central and carboxyl-terminal isoprene residues would not be expected to differ from each other.

The results confirm that the hydrogen atoms on C-3′ and C-4′ of ABA are both derived from the *pro*-(*R*)-hydrogen of C-2 of mevalonate. The exaggeration of the differences in ^{14}C/^{3}H ratios by iodoacetamide, a potent inhibitor of isopentenyl pyrophosphate isomerase, supports the suggestion that this enzyme is responsible for the partial randomization of ^{3}H from C-2 of mevalonate. The amount of labeled mevalonate incorporated into ABA in the presence of 2 m*M* iodoacetamide shows that the other stages of ABA formation are not affected by iodoacetamide so that biosynthesis is prevented.

The tritium of only one of the three C-5 atoms of mevalonate would be expected to be retained in ABA because all the hydrogen of the other two are removed, during the formation of the C-4′-keto and the C-1 carboxyl groups (**20**).



Abscisic acid (showing the positions occupied by C-5 of mevalonate)

20

groups (**20**). Abscisic acid provides its own selection mechanism for determining which of the *pro*-(*R*) or *pro*-(*S*) hydrogen atoms is retained on C-5. [2-^{14}C, 5(*S*)-5-$^{3}H_1$]Mevalonate with a $^{14}C/^{3}H$ ratio of 3:3 gave ABA with a ratio of 3:1 showing that the C-5 hydrogen of ABA is derived from the *pro*-5(*S*) of mevalonate.

The stereochemistry of the hydrogen atoms on adjacent C-2 and C-5 atoms of mevalonate is reversed, in relation to their configuration in mevalonate, during the junction of the isoprene residues. Their stereochemistry in a hypothetical precursor of ABA would be as shown in Fig. 9 before the formation of the double bonds. The derivation of the hydrogen atoms of ABA from mevalonate is as shown in Fig. 10.

The retention of the 5-*pro*-(*S*) hydrogen of mevalonate as the hydrogen on C-5 of ABA parallels the retention of this same hydrogen at the analogous position in carotenoids (Walton *et al.*, 1969). The C-2 *pro*-(*R*) hydrogen of mevalonate is retained at C-3′ of ABA, and preliminary data of

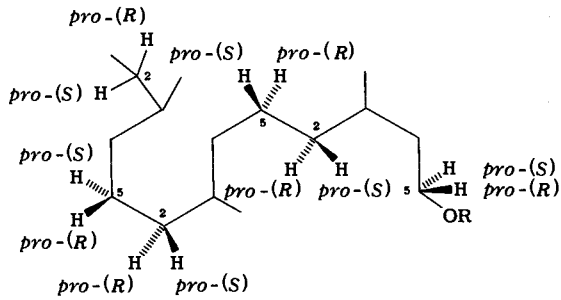


FIG. 9. Arrangement of hydrogen atoms from C-2 and C-5 of mevalonate on the carbon skeleton of a precursor of abscisic acid after junction of the isoprene residues.

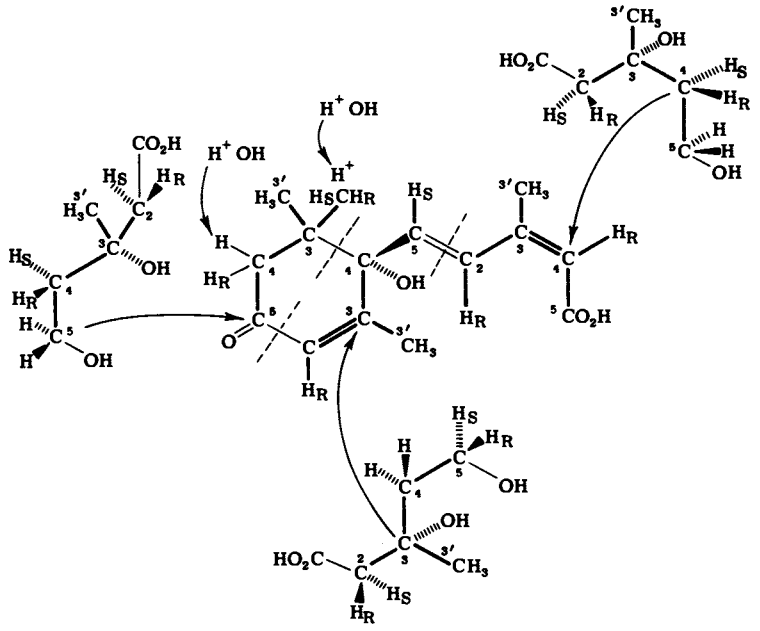


FIG. 10. The proposed derivation of the carbon and hydrogen atoms of abscisic acid from 3(*R*) mevalonate. The C and H atoms which remain in abscisic acid are drawn in heavy type.

J. R. Vose, G. Britton, and T. W. Goodwin (cited by Goodwin, 1971) indicate that the same hydrogen is retained at the analogous position in α-carotene. The stereochemistry of hydrogen elimination during the formation of all three double bonds of ABA is the same as that of carotenoids and cannot, therefore, be used to discriminate between their routes of synthesis.

An attempt was made (B. V. Milborrow, unpublished) to investigate the orientation of the isoprene chain during the cyclization of ABA by measuring the change in $^{14}C/^{3}H$ ratio of alkali-treated ABA biosynthesized from mevalonate-2-$^{3}H_2$ after metabolism to phaseic acid. No change was observed. If hydroxylation of the geminal methyl group of ABA occurred on the methyl derived from C-2 of mevalonate, then one-third of the tritium could be lost from this position (approximately two-ninths of the total in alkali-exchanged ABA), but the result is inconclusive because hydroxylation of the tritiated methyl group could occur without loss of tritium if there were a strong isotope effect discriminating against the removal of tritium. If the oxymethylene bridge of phaseic acid (**10**) is cis to the

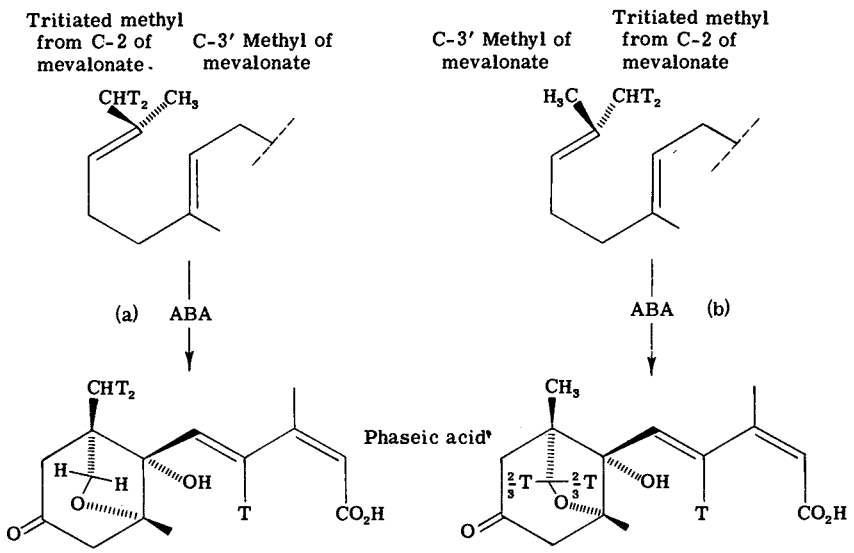
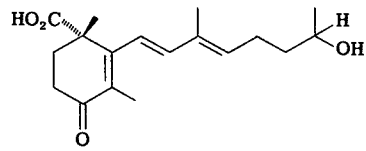


FIG. 11. Orientation of the terminal methyl groups prior to cyclization in a precursor of abscisic acid (ABA).

tertiary hydroxyl group, then the orientation of the chain before cyclization is as shown in Fig. 11a and is consistent with derivation of the *gem*-dimethyl groups of trisporic acid (**21**) formed from β-carotene by *Blakeslea trispora* (Bu'Lock *et al.*, 1970) and the usual direction of cyclization of terpenoids.



Trisporic acid

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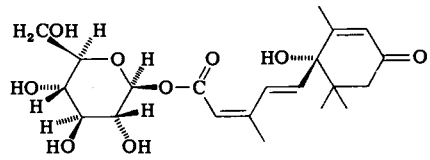
Regulation of Biosynthesis

Perhaps the most interesting physiological observation that has been made with ABA is the effect of wilting on the endogenous concentrations in leaves (Wright, 1969; Wright and Hiron, 1969). Normal turgid leaves contain between 10 and 20 μg/kg fresh weight, whereas the concentration

of ABA on wilting rises to between 400 and 500 μg/kg. It is obviously advantageous for a wilting plant to stop growing but the effect is probably more subtle, because ABA applied either via the sap stream or to the leaf surface causes the stomata to shut (Mittelheuser and van Steveninck 1969; Jones and Mansfield, 1970). Consequently, a rise in endogenous ABA concentration would be expected to have the same effect and would thereby reduce water loss and allow the plant to regain its turgor.

The mechanism by which this switch operates is at present unknown. All we can say at the moment is that it is operated fully by a 10 percent loss of fresh weight which would be unlikely to affect enzymes by an increase in concentration of a cellular constituent. However, the effect of such a water loss on the cell membrane in contact with a drying cell wall could be quite intense, and it is perhaps in the cell membrane that the mechanism (which senses the water loss) resides.

The rise in ABA concentrations that occur on wilting appear to result mainly, if not entirely, from synthesis rather than from release from a precursor, because the incorporation of mevalonate-^{3}H into ABA by wilting wheat leaves was 10 times greater than by turgid ones (Milborrow and Noddle, 1970). This result does not rule out mobilization from a stored precursor, but the only possible candidate for this known at present is the glucose ester (**22**) which usually occurs at a concentration of 10 to 30 percent of that of the free acid (Rudnicki and Pieniazek, 1971; B. V. Milborrow and D. R. Robinson, unpublished). This is, therefore, inadequate to produce the 4000 percent increase observed.



(+)-Abscisyl-β-D-glucopyranoside

22

The high concentration of ABA found in leaves of waterlogged plants (incipient wilting) is maintained at a constant level for some days (Wright and Hiron, 1972). There must, therefore, be a second control mechanism to stop the rapid synthesis once the requisite concentration has been formed. Experiments in which ^{14}C-labeled racemic 1′,4′-diols were supplied to samples of wheat shoots, some of which were then wilted, have shown that large amounts of ABA were formed from the diols, but measurements of the specific activity have shown that no further biosynthesis of natural

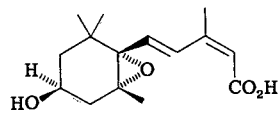
(+)-ABA took place on wilting. This result is interpreted as showing that the free ABA formed from the diols kept biosynthesis "switched off" by a product feedback-type mechanism (Milborrow, 1972b).

Compartmentalization

When intact cells are used for biochemical investigations there is always the possibility that added materials do not enter into the same parts of the cell as the compound that has been formed naturally. Other, and more complicated, situations can easily be envisaged.

The first example of compartmentalization of ABA and its derivatives can be deduced from the work of Koshimizu *et al.* (1968) and Milborrow (1970). The former authors isolated (+)-abscisyl-β-D-glucopyranoside (**22**) from yellow lupin fruit and reported that it had approximately half of the inhibitory activity of free ABA in a rice seedling bioassay (presumably almost equal on a molar basis). The latter paper reported that exogenously applied (±)-ABA-2-^{14}C was rapidly converted into the glucose ester by tomato shoots, but it was rapidly hydrolyzed by cell sap obtained from these shoots. Thus the glucose ester was apparently stable within the cells but was hydrolyzed when fed to rice seedlings or when mixed with a homogenate of the tomato shoots.

Recently we have investigated the conversion of (+)-xanthoxin acid-2-^{14}C (**23**) to ABA by avocado fruit in an attempt to define the absolute configuration of ABA.



(+)-Xanthoxin acid
23

B. V. Milborrow, R. S. Burden, and H. F. Taylor (unpublished) found that the 1′,4′-*trans*-diol of ABA added at the same time as a cold trap became heavily labeled, whereas the 1′,4′-*cis*-diol in the same solution retained about 3 percent of the amount of the label trapped by the trans isomer. In a control experiment the *trans*-diol, but not the *cis*-, trapped a similar amount of label when (±)-ABA-2-^{14}C itself was added to the avocado fruit. Thus, it appears that exogenous ABA or ABA formed from exogenous **23** can be reduced enzymatically to a 1′,4′-*trans*-diol when there is excess diol present to trap it.

The 1′,4′-*trans*-diols appear not to be natural intermediates of ABA biosynthesis because in five experiments both cis and trans isomers added to avocado fruit as cold traps failed to retain radioactivity (less than 4 cpm above background were present in the diols in all experiments although over 2×10^7 dpm mevalonate-2-^{14}C was added and about 6×10^4 dpm was recovered in ABA in each experiment). It appears from these results that ABA biosynthesized by the tissues from added mevalonate-^{14}C is in a separate cellular compartment from that into which the 1′,4′-diols penetrate. Labeled ABA added to the fruit and labeled ABA formed by oxidation and rearrangement of (+)-xanthoxin acid-2-^{14}C are converted into 1′,4′-*trans*-diol, and all these compounds must, therefore, be in a different cellular compartment from that in which ABA is biosynthesized.

Metabolites of Abscisic Acid

When ABA solution is absorbed by tomato shoots it is destroyed by two quite distinct mechanisms. Both (+)- and (−)-enantiomers are conjugated with glucose to form abscisyl-β-D-glucopyranoside (**22**), but the (+)-enantiomer is also hydroxylated on one of the geminal methyl groups to form the unstable "Metabolite C" (Milborrow, 1970). This compound rearranges extremely easily to give phaseic acid (**10**) by a nucleophilic attack of the hydroxymethyl on C-2′ to form a saturated furan ring (Milborrow, 1969b). Phaseic acid was originally isolated from bean seeds (*Phaseolus multiflorus*) by MacMillan and Pryce (1968), and a new structure was proposed for it on the basis of the nuclear magnetic resonance (NMR) spectrum of phaseic acid that had been formed from hexadeuterioabscisic acid in tomato plants (Milborrow, 1969b).

The occurrence of phaseic acid in plant extracts is no guarantee that it occurs naturally because it could be an artifact formed from Metabolite C during the extraction process. One experiment to discover whether or not phaseic acid is a normal degradation product of ABA has been carried out (Milborrow, 1972b). 2-^{14}C-labeled abscisic acid was fed to young tomato shoots and the phaseic acid-2-^{14}C isolated and purified. It was then refed to a further sample of young tomato shoots as was another sample of (±)-ABA-2-^{14}C.

The distribution of labeled products formed by the tomato shoots from the two compounds was analyzed by radioautography of extracts chromatographed on silica gel TLC plates. The products formed were quite different. Abscisic acid was metabolized to glucose ester and a number of other acids, whereas most of the phaseic acid was converted into a hydrolyzable water-soluble material, which may be a glucose ester, and a trace only of

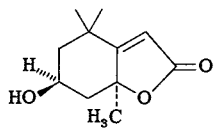
another acidic compound detected on the radioautogram. This metabolite of phaseic acid was absent from the extract of plants supplied with ABA.

It appears from this result that the fate of phaseic acid is different from that of ABA, but the experiment is not altogether satisfactory because equal amounts of ABA and one of its degradation products are unlikely to occur *in vivo*. Also great care must be taken when interpreting differences between the metabolic fate of compounds applied to the plant in comparison with that of the same compounds formed endogenously (see the preceding section on compartmentalization of ABA and its glucose ester). The differences between the fates of ABA-2-^{14}C and phaseic acid-2-^{14}C may represent differences between their uptake into, and position within, the cells. It is also possible that both are converted into the same unlableled products by removal of the carbon label at C-2 and these would not have been detected by the techniques employed. However, other derivatives of ABA are metabolized within a short time of application, and the stability of phaseic acid is unlikely to arise from a failure to penetrate into the cells because a large proportion was converted into a conjugate. At the moment the weight of the evidence suggests that phaseic acid is an artifact. Phaseic acid has now been found in bean, tomato, and sugar cane (Most, 1971).

Walton and Sondheimer (1972a,b) report that isolated bean axes convert (±)-ABA-2-^{14}C into two polar metabolites, M-1 and M-2, and suggest that M-1 is converted into the latter. The M-1 has now been identified as phaseic acid, and M-2 as 4′-dihydrophaseic acid (Tinelli *et al.*, 1973). Naturally occurring dihydrophaseic acid has been isolated from mature bean seed (Walton *et al.*, 1973).

Naturally Occurring Compounds Related to Abscisic Acid

The first difficulty one experiences in a discussion of naturally occurring compounds related to ABA is deciding how far from the structure one can go. We have already ventured into the carotenoid field in trying to find a precursor of ABA, and several of the closely related products that have been described could be formed by the breakdown of carotenoids as well as by the breakdown of ABA. Loliolide (**24**), the first of these compounds



Loliolide

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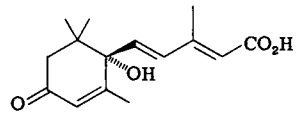
to be characterized [also isolated as digiprolactone from *Digitalis* leaves by Wada (1965)] was extracted from rye grass (*Lolium perenne*) by Hodges and Porte (1964). Its metabolic origin is in doubt since Taylor and Burden (1970a,b) found that it was one of the main products formed by the *in vitro* photolysis of violaxanthin (**4**). It is possible, therefore, that it is formed within the leaf by the effect of intense sunlight on violaxanthin rather than by a specific enzymatic process. Against this, Firn and Friend (1972) have found that a preparation from leaves of soybean contains a lipoxygenase capable of oxidatively cleaving violaxanthin to form xanthoxin and the other photolytic products. Whether this enzyme has the opportunity to cleave carotenoids within the normal leaf or whether it is in a different physiological compartment of the cells remains for investigation.

That light can effect a nonmetabolic change in leaves is suggested by the presence of the 2-trans isomer of ABA (**25**) in leaves that had been

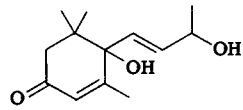


(+)-2-*trans*-Abscisic acid
25

exposed to bright sunlight for some days (Milborrow, 1970). Field rose (*Rosa arvensis*) leaves were harvested and carefully extracted in the dark with methanol to which radiochemically pure, racemic ABA had been added. When the acid fraction was chromatographed in a TLC system that separated the 2-cis from the 2-trans isomer, it was found that 4.4 percent of the total optically active ABA isomers was trans, whereas only 0.34 percent of the radioactivity occurred in this zone. Any isomerization of the optically active 2-cis isomer during extraction or isolation would have converted labeled ABA into 2-*trans*-ABA. The presence of 4 percent more (+)-2-trans isomer by its ORD than by its radioactivity shows that (+)-2-*trans*-ABA was originally present in the leaves before extraction. 2-*trans*-Abscisic acid cannot be reconverted to ABA by leaves, it is rapidly esterified by plants to its glucose ester. At equilibrium, ABA and its 2-trans isomer are present in equal proportions, so the 4 percent (+)-2-*trans*-ABA may represent a dynamic equilibrium between the rate of light-catalyzed isomerization and esterification. Thick opaque tissues into which light cannot penetrate, such as the fruit pulp (B. V. Milborrow, unpublished) and seeds (Milborrow, 1968) of avocado do not contain detectable amounts of 2-*trans*-ABA. We assume from these observations that 2-*trans*-ABA is produced by the action of sunlight—it may be the product of an

environmental accident. The presence of loliolide (**24**) and 2-*cis*- and 2-*trans*-xanthoxin in leaves (Taylor and Burden, 1970a,b) also favors the idea of their formation by photolysis.

Vomifoliol (**26**) was the first of several 13-carbon compounds that have been described recently; it was first isolated from leaves of *Rauwolfia*

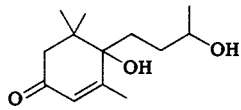


Vomifoliol (Blumenol A)

26

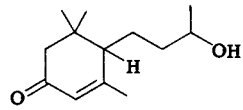
vomitoria by Pousset and Poisson (1969), and it has also been isolated (as blumenol A) from the leaves of *Podocarpus blumeii* by Galbraith and Horn (1972).

The positive rotation of vomifoliol (α_{578} + 223°; $CHCl_3$; C = 1) (Pousset and Poisson, 1969) and the data for the blumenols (Galbraith and Horn, 1972, and unpublished data) show that vomifoliol and the *Podocarpus* blumenols (**27**, **28**) have the same stereochemistry of the tertiary hydroxyl



Blumenol B

27

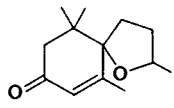


Blumenol C

28

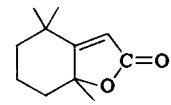
group. The similarity of the ORD spectrum of blumenol A to that of ABA indicates that all have the same absolute configuration of the tertiary hydroxyl group. Galbraith and Horn (1972) deduced the absolute configuration to be as shown by analogy with the NMR spectrum of α-carotene. This absolute configuration is in agreement with the revised configuration of (+)-ABA. The abnormality of the ORD spectrum of ABA and its diols, so that Mills' rule is apparently inapplicable, is probably associated with the presence of the 4-trans double bond in the side chain, because M. N. Galbraith and D. H. S. Horn (unpublished results) have found that saturation of this bond reverses the optical rotation of blumenol A. Reduction of the ketone of (+)-ABA reduces the specific rotation by approximately four-fifths, and saturation of the ring double bond reduces it to one-thirtieth of that of ABA but does not affect the sign (B. V. Milborrow, unpublished).

Another C-13 compound with a similar structure has been identified in black tea and is an important constituent of its aroma; it was named theaspirone (**29**) by Ina and Sakato (1968). It was isolated in a yield of 15 μg/kg which is about the concentration of ABA in normal leaves and well below the concentration in wilted ones. It is possible that it could have been formed from ABA during the fermentation of the tea leaves. A lactone that is also present in black tea extract (Bricout *et al.*, 1967), dihydroactinidiolide (**30**), gave a yield of 20 μg/kg. Its structure strongly suggests that it is derived from carotene, and it can be formed *in vitro* by photooxygenation of β-carotene (Isoe *et al.*, 1969). It is possible that it and theaspirone also are derived by oxidative processes from a carotenoid *in vivo*. So far there are no data on the method of formation of the compounds in the plants.



Theaspirone

29



Dihydroactinidiolide

30

The only other naturally occurring compounds related to ABA that have been isolated from plants are metabolites or potential precursors and these are dealt with in other sections.

Biochemical Role of Abscisic Acid

As stated earlier, ABA was first isolated by following its abscission-accelerating activity in explants of cotton seedlings (Ohkuma *et al.*, 1965), but it has subsequently been found to be much less effective when applied to intact mature plants (Smith *et al.*, 1968; El Antably *et al.*, 1967). It appears to cause the abscission of immature fruitlets of yellow lupin and accelerates the maturation and abscission of mature olive fruits (Barnsley *et al.*, 1968).

An independent line of research concerned with its dormancy-inducing effect led to its isolation from sycamore, and it has subsequently been found to induce the formation of resting buds in other woody perennials (El Antably *et al.*, 1967; Tinklin and Schwabe, 1970), the formation of turions (dormant, overwintering buds) in duckweeds (Stewart, 1969; Perry and Byrne, 1969), and the induction and maintenance of seed dormancy (Villiers, 1968; Le Page-Degivry, 1970; Ihle and Dure, 1972).

The massive increase in concentration of ABA in wilting leaves can be associated with its effect on stomata, but the high levels may also be responsible for the check on growth that is observed after plants have wilted. Abscisic acid is a potent inhibitor of growth of many plant organs when applied exogenously yet whether or not it has a slight growth-inhibitory effect in young, developing plant organs (which contain low concentrations of ABA) is uncertain.

The richest sources of ABA are fruit tissues, and they also incorporate added mevalonate into ABA more effectively than any other tissues (Noddle and Robinson, 1969). It is surprising, therefore, that the role of ABA in fruit has been largely ignored. In the avocado the most rapid synthesis occurs during incipient softening and after contact between the seed and the fruit tissues has been broken. The increase in ABA content during ripening and the high concentrations present at this time suggest that it is involved in some aspect of the ripening process.

How the one compound can regulate such a variety of physiological processes is at present unknown. The work of Varner and collaborators (Varner and Johri, 1968) has shown that, in barley aleurone, ABA inhibits the *de novo* synthesis of α-amylase. In wheat embryos, which contain preformed messenger ribonucleic acid, it appears to act by inhibiting translation (Chen and Osborne, 1970) as it does in the developing cotton seeds (Ihle and Dure, 1972). However, the primary reaction that is affected remains as obscure for ABA as it is for the other plant growth regulators.

Conclusions

Since 1965 when the structure of ABA was first reported, research interest, as expressed by the number of papers dealing with the subject, has almost doubled annually. During this time our knowledge of this hormone has advanced so rapidly that it now rivals that of the auxins, cytokinins, and gibberellins in most respects and even surpasses them in a few aspects. Some features of ABA make it suitable for detailed investigations that are impossible to carry out with other hormones (e.g., activity and differential metabolism of optical isomers); on the other hand, its biosynthetic intermediates have proved particularly refractory.

In 1972 appeared the unambiguous proof of the absolute configuration and the identification of what seems to be the first unique intermediate in ABA biosynthesis. Perhaps the next two most important advances will be a cell-free system capable of synthesizing it and, in common with the other hormones, the discovery of the mechanism by which ABA exerts its effects. Let us hope that they will not take too long.

Acknowledgments

Finally it remains for me to acknowledge the help I have received during the preparation of this review, to thank my co-workers for permission to cite unpublished work and for their continuing enthusiasm and skill. In particular, Professor J. W. Cornforth, Dr. G. Ryback, Dr. D. R. Robinson, Dr. R. C. Noddle, and Dr. R. Mallaby have done much to advance our knowledge of this intriguing compound.

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THE BIOCHEMISTRY OF THE ACTION OF INDOLEACETIC ACID ON PLANT GROWTH

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Introduction

Among plant growth regulators, the class whose mechanism of action has been longest under active investigation is the auxins, typified by in-

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doleacetic acid (IAA). The classic effect of auxins is to promote cell enlargement or cell elongation, a process that requires extension of the cell wall. Since the early days of auxin research there has been evidence that treatment of auxin-sensitive tissue with auxin increases the mechanical extensibility of its cell walls. This was, and often still is, assumed to be the cause of the promoted rate of cell enlargement induced by auxin. No convincing direct effects of IAA, at physiological concentrations, upon mechanical properties of isolated cell walls have been found. This plus the demonstration that cell wall growth requires energy metabolism, protein synthesis, and, at least in the longer term, ribonucleic acid (RNA) synthesis, have led to the generally accepted view that the effect of IAA on cell wall growth is a secondary consequence of a primary action exerted on the metabolic machinery of the cell. In this paper I shall discuss some current developments and points of view about primary auxin action and about the biochemistry of its effect on cell wall growth, without attempting a complete current review of this rapidly expanding subject. Much additional information as well as divergent opinion can be found in recently published conference proceedings (Carr, 1972) and in recent reviews (Galston and Davies, 1969; Cleland, 1971a; Davies, 1973).

Primary Action

Gene Activation Hypothesis

The central theme of research on action of auxin, as with most other hormones, during the past decade has been the gene activation theory, that hormones act by inducing transcription of genes coding for proteins specific to particular kinds of physiological performance. In the case of auxin stimulation of cell enlargement the theory predicts that auxin derepresses the gene(s) that code(s) for protein(s) that somehow act(s) on or lead(s) to an action on, the primary cell wall, causing the latter to become extended more rapidly by prevailing turgor forces.

The evidence that IAA stimulates growth by gene activation (Key, 1969) includes (*a*) effects of inhibitors of RNA and protein synthesis, (*b*) effects of auxin on incorporation of labeled ^{32}P or N bases into RNA and of amino acids into protein, and (*c*) evidence for auxin effects on RNA synthesis by chromatin-dependent RNA polymerase systems. The inhibitor work usually involved the fallacy of confusing a condition necessary for the phenomenon with a site of control of the phenomenon, and is inconclusive. The major effect of auxin on RNA synthesis was found to be on ribosomal

RNA (rRNA) synthesis (Ingle and Key, 1965; Trewavas, 1968a), but rRNA synthesis itself did not appear to be required for the growth response to auxin since growth was not prevented when rRNA synthesis was blocked by 5-fluorouracil (Key and Ingle, 1964). Moreover, except for a couple of unconfirmed reports, the IAA effect on RNA synthesis *in vivo* has been found to develop much more slowly than the elongation response to IAA, so the former could not be the cause of the latter (Key and Shannon, 1964; Trewavas, 1968a; Neumann and Palmer, 1971; P. Penny *et al.*, 1972). It was assumed by exponents of the gene activation hypothesis that auxin must have comparably large but more rapidly occurring stimulatory effects on the transcription of messenger RNA (mRNA) for particular "growth-specific proteins" (Trewavas, 1968b), but convincing evidence for such an effect has not been forthcoming (cf. Thompson and Cleland, 1971).

In soybean tissue, auxin treatment causes a large increase in RNA polymerase activity associated with chromatin preparations (O'Brien *et al.*, 1968). The large effect develops only after a period of hours, much more slowly than the elongation response (see p. 97).

Matthysse and Phillips (1969) claimed to have detected an effect of auxin *in vitro* directly upon an RNA polymerase system. According to this report, a promotion of transcription activity by auxins was obtained only in the presence of a soluble (presumably protein) factor. Although the reported promotion was relatively small and variable, this report might have been expected to lead to an intensive investigation of auxin regulation of gene function. However, only a few reports of a preliminary nature have appeared that give any indication of confirmation of this effect (Salomon and Mascarenhas, 1972a; Venis, 1972; Hardin *et al.*, 1972), and the effect was not found by Tautvydas and Galston (1972) using intact pea nuclei that were reported to bind IAA$-^{14}$C, nor by Hardin *et al.* (1970) using a soybean chromatin system sensitive *in vivo* to 2,4-dichlorophenoxyacetic acid (2,4-D) and capable of stimulation *in vitro* by an extractable factor.

Hardin *et al.* (1972) recently reported stimulation of soybean RNA polymerase *in vitro* by 2,4-D or IAA when particulate cell fractions were provided. They suggested the effect was due to interaction of auxins with plasma membrane fragments, and postulated that auxins cause a stimulatory factor for nuclear transcription to be released from the plasma membrane. However, the effects were marginal and it remains to be seen whether they have any significance in the regulation of gene function. The same may be said for recent reports that auxins *in vitro* promote dissociation of the complementary strands of double-stranded DNA, i.e., shift the "melting" curve to lower temperatures (Fellenberg, 1969; Bamberger, 1971; Spang & Platt, 1972).

Control of Translation

There are reports of interaction of IAA with RNA, which might suggest control at the translational level (cf. Kobayashi and Yamaki, 1972), but detailed studies have shown these indications to be misleading (Davies, 1971; Davies and Galston, 1971; Neumann and Palmer, 1971).

Trewavas (1968a) found that stimulation by IAA of amino acid incorporation into protein in pea stem segments lags far behind the elongation response to IAA. By sensitive double-label fractionation experiments Patterson and Trewavas (1967) tried to detect a specific IAA effect on rate of synthesis of particular species of protein, but the results suggest, instead, rather miscellaneous minor changes in incorporation into many proteins.

Cycloheximide, a powerful inhibitor of ribosomal protein synthesis in eukaryotic cells, inhibits elongation of coleoptiles and other auxin-sensitive tissues within minutes of its application. The timing of this inhibition has been interpreted as providing an assay for the pool size of a protein that is required for elongation (growth-limiting protein, GLP), and as indicating that IAA acts to increase substantially the size of the GLP pool (Cleland, 1971b), presumably by inducing the synthesis of GLP. Based on kinetic data indicating different kinds of interaction between IAA and the inhibitors actinomycin, cycloheximide and puromycin, in elongation tests, Yamamoto *et al.* (1973) also suggested that IAA stimulates coleoptile elongation by action at the translational level to stimulate production of GLP. This interpretation may be questioned. For example, plant cells might (indeed should) have a feedback regulation mechanism that shuts down cell wall growth when normal protein synthesis is restricted, and such a regulation need not depend on disappearance of specific protein pools.

D. Penny *et al.* (1972) have arrived, by techniques and interpretations similar to Cleland's (1971b), at the conclusion that in lupine hypocotyl segments IAA does *not* act to increase the pool size of GLP. Pope and Black (1972) and Penny (1971), who interpret cycloheximide inhibition data in a similar way, found a definite promotion of wheat coleoptile and lupine hypocotyl elongation by IAA under maximal (nearly complete) inhibition of protein synthesis by cycloheximide, and a similar finding with gherkin hypocotyls was reported by Addink and Meijer (1972). These authors conclude that IAA cannot be acting on elongation by inducing protein synthesis.

An example of IAA action at the biochemical level that does not depend on protein synthesis is the activation of glucan synthetase (Ray, 1973b), to be discussed later.

Timing of Auxin Response

This has attracted a swell of attention because of its implications regarding the mode of primary action of the hormone. Many workers, beginning with Ray and Ruesink (1962), have found that IAA induces elongation of coleoptiles and other auxin-sensitive tissues with a characteristic lag of 10 to 15 minutes after which the elongation rate rises quickly to its stimulated value (Fig. 1), or, in some cases, rises above the steady rate and returns to the latter by what appear to be damped oscillations (Barkley and Evans, 1970; P. Penny *et al.*, 1972; Addink and Meijer, 1972). The elongation effect of auxin is among the fastest, although not the fastest, of known hormone responses—its timing strains the credibility of the gene activation hypothesis of auxin action (Evans and Ray, 1969; P. Penny *et al.*, 1972).

Under special conditions Rayle *et al.* (1970a) reported obtaining an auxin effect on elongation substantially more quickly than the 10 to 15 minutes latent period typical for auxin action. Durand and Zenk (1972) denied these effects, but Nissl and Zenk (1969) reported that high concentrations of IAA given to oat coleoptiles at unphysiologically high temperature caused an immediate (within 1 minute) induction of rapid elongation. These authors concluded, and this work has been widely quoted as showing, that auxin does not stimulate elongation by inducing RNA or protein synthesis.

Nissl and Zenk (1969) considered that the latent period of about 10 minutes in IAA action seen by others is due to slow penetration of IAA into the tissue. However, in their work it was not satisfactorily shown that the immediate effect of high concentrations of IAA was not an instance of the rapid elongation response to acidic pH (discussed later). Other workers (Ray and Ruesink, 1962; Evans and Ray, 1969; etc.) have found that the latent period is not reduced appreciably by a substantial increase in the concentration of IAA, except that, at very high IAA concentrations in inadequately buffered media, an immediate response occurs which is attributable to acid pH (Evans, 1967). R. Cummins and P. B. Green (unpublished)(see Fig. 1) have found the typical latent period of 10 minutes by using coleoptiles that are treated to eliminate cuticular barriers to solute uptake and are vigorously perfused internally and externally in such a way that exchange between tissue and medium is several times more rapid than with normally treated coleoptile segments. Finally, the fact that coleoptile tissue that has been treated to maximize the efficiency of exchange and uptake still shows an *absolute* latent period of 10 minutes before *any* increase in elongation rate occurs after exposure to IAA and the fact that sufficient *uptake* of IAA to subsequently cause maximum elongation response occurs

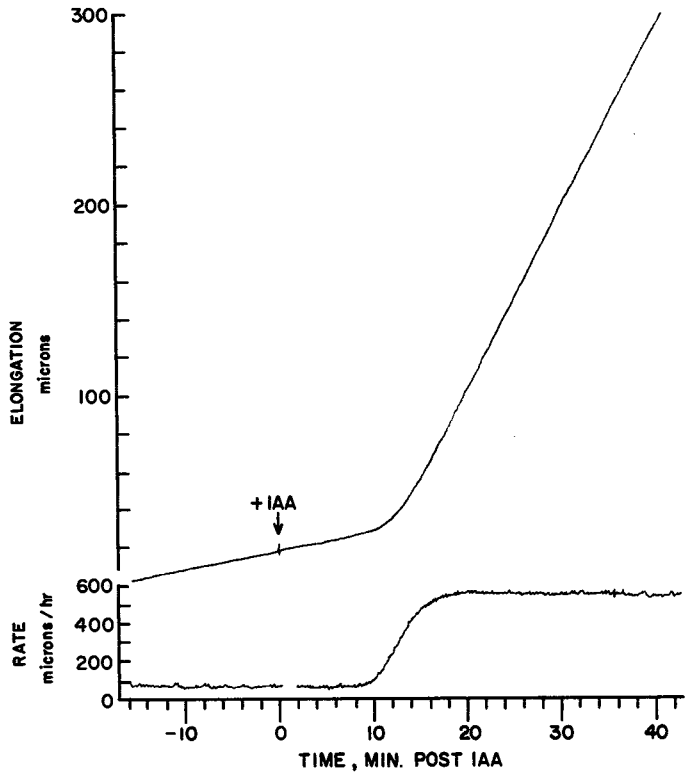

FIG. 1. Timing of elongation response to indoleacetic acid (IAA) in *Avena* coleoptile. A coleoptile segment 8 mm long, continuously perfused with growth medium from both inside and outside surfaces while mounted in contact with a position transducer to measure elongation, was transferred from minus-IAA medium to medium containing 0.5 mg/liter IAA at time shown by arrow. Upper curve shows elongation as plotted by a recorder that receives the output of the position transducer; lower curve shows rate of elongation, plotted simultaneously by the same recorder, from analog computer differentiation of the position transducer data. (Unpublished data of R. Cummins and P. B. Green.) Similar latency was repeatedly observed using coleoptile segments "scrubbed" with emery suspension to maximize solute exchange with the medium.

with only 2 minutes of exposure to IAA (Evans, 1967) indicate that the latent period does not represent mainly time required for IAA uptake.

A similar absolute latency phenomenon has been seen in the auxin elongation response of all tissues that have been carefully studied, thus suggesting that it is of fundamental importance relative to the nature of auxin action. None of the current theories of auxin action reviewed in the follow-

ing attempts to account for the latency phenomenon. The timing of change in elongation rate after exposure to IAA (lower curve in Fig. 1) resembles remarkably the timing expected theoretically for a sequence of six or more reaction steps following a site of primary stimulation by IAA (Evans and Ray, 1969). None of the current theories of auxin action envisages a causality sequence involving this many steps. Penny (1972) has shown that fewer steps need be involved if the sequence involves either "unmixed" compartments or substrate-activated steps. He analyzes the latency phenomenon in terms of an entirely hypothetical model by which IAA would cause conversion of the previously mentioned GLP into an active form on which the rate of cell wall expansion directly depends.

Rapid Effects versus Longer-Term Responses

The outcome of the auxin research of the 1960s is that, although regulation (directly or indirectly) of RNA and protein synthesis and, thus, possibly of gene expression by IAA is indicated by experimental evidence and is probably involved in the mechanism of longer-term auxin responses, such as cell division, differentiation, and morphogenesis, some other and more direct mode of action of IAA appears, in the view of most current workers, to be operating in cell enlargement. Hence, the current preoccupation with investigation of rapid auxin effects of any kind that might provide an explanation for the classic cell enlargement response. Both metabolic and transport effects of IAA have been visualized and pursued.

Protoplasmic Streaming Hypothesis

One of the most rapid auxin effects recorded in the literature is a stimulation by IAA of the rate of cytoplasmic streaming in oat coleoptile cells (Sweeney and Thimann, 1942). One might imagine such an effect to stimulate cell enlargement by promoting transport to the cell wall of whatever factors are needed for its extension. This hypothesis has, however, been ruled out by the finding that IAA induces cell enlargement in tissues whose streaming has been suppressed completely by cytochalasin B (Fig. 2) (Cande *et al.*, 1973).

Metabolic and Enzymatic Effects

An activation of citrate synthase by IAA has been claimed (Sarkissian, 1970, 1972) and denied (Zenk and Nissl, 1968). The effect apparently has not been confirmed by other workers, and was not mentioned in a recent paper by Greenblatt and Sarkissian (1973) on the properties of plant citrate

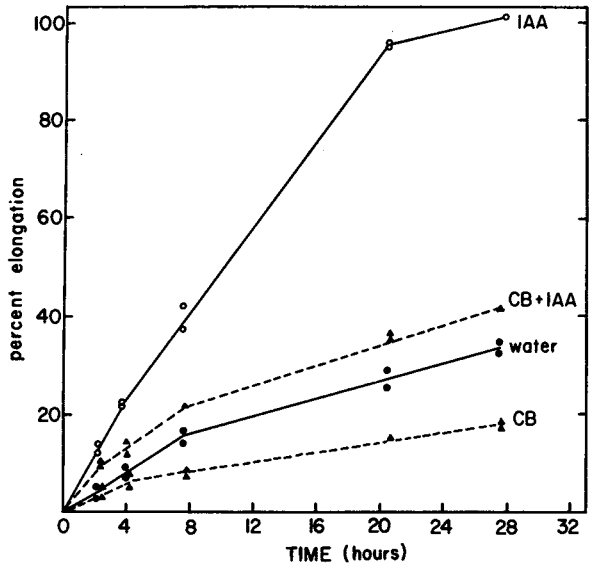

FIG. 2. Induction by indoleacetic acid (IAA) of elongation in normal *Avena* coleoptile segments (circles) and coleoptile segments whose streaming was completely inhibited by cytochalasin B (CB) (triangles). Cytochalasin B was given 2 hours prior to the start of the experiment. Note the nearly normal elongation response to IAA in CB-treated segments during the first 2 hours, with gradually developing stronger inhibition of elongation after 4 hours. (From Cande *et al.*, 1973.)

synthase. It is difficult to see how such an effect could induce plant cell enlargement. Sarkissian and McDaniel (1966) previously reported a dramatic *in vitro* promotion by IAA of oxygen consumption by mitochondria from maize scutellum, a tissue not known to show a physiological response to IAA. However, no such effect has been found with mitochrondria from auxin-responsive tissues despite efforts of a number of investigators in the 1950s, stemming from the then-popular belief that IAA acts on the connection between energy metabolism and growth (cf. Thimann, 1960).

Glucan synthetase (UDPG: β-1,4-glucan glucosyl transferase) activity rises dramatically over a 1-hour period after treatment of pea tissue with IAA (Ray, 1973a). Inhibitor experiments show that this effect is not dependent on RNA or protein synthesis; it appears to be an instance of enzyme activation (Ray, 1973b). Although the effect is much more rapid than previously known plant hormone effects on enzyme activities and commences within 15 minutes of IAA treatment (Fig. 3), it is not rapid enough to be a cause of the elongation response. It is probably involved in

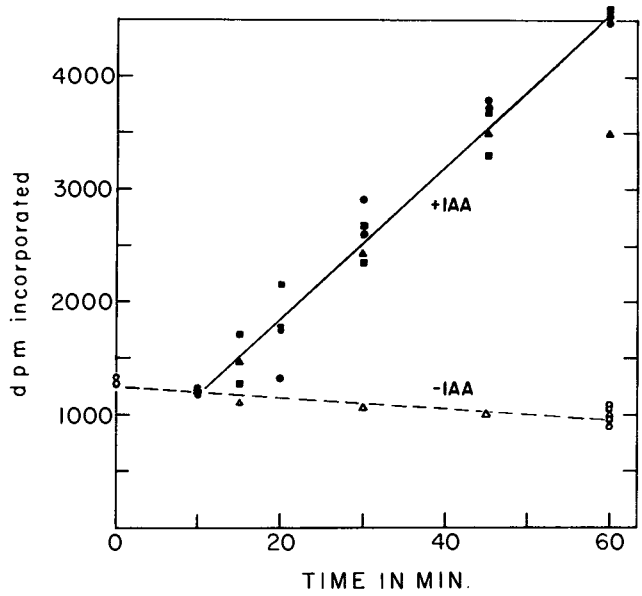

FIG. 3. Timing of response of β-glucan synthetase activity to indoleacetic acid (IAA) in pea stem tissue. Stem segments were incubated in 30 m*M* sucrose minus or plus 3 mg/liter IAA at 35°C, then chilled at the times shown, homogenized, and centrifuged for isolation of particulate β-glucan synthetase, which was assayed by incubating 10 minutes with uridine diphosphate–glucose-^{14}C (20,000 dpm) in presence of 50 m*M* Mg^{2+}. Data show ^{14}C incorporated into alcohol-insoluble glucan. (From Ray, 1973a.)

the stimulation of wall polysaccharide synthesis by IAA (Abdul-Baki and Ray, 1971). The effect depends on energy metabolism *and* on normal turgor pressure. Despite many trials, it has not so far been obtained *in vitro*; it may require intact cell function. Van der Woude *et al.* (1972), however, have claimed to detect an *in vitro* promotion of glucan synthetase activity of a particulate preparation from onion bulbs by 2,4-D. The reported effect was very small and variable, and one wonders further about its significance since it was found in a tissue not known to show a substantial cell enlargement response to auxin.

Neumann (1971) reported detecting a stimulating effect of IAA within 5 minutes on the incorporation of $^{32}PO_4$ by pea tissue into an unidentified "glycerol-phosphate compound." This recalls an earlier report of appearance of an unidentified major glucose metabolite in pea tissue treated with IAA (Winter, 1967).

Bidwell *et al.* (1968) described a transient promotion by IAA of photosynthesis in bean leaves, and recently (Tamas *et al.*, 1972) claimed to de-

tect a substantial promotion of photophosphorylation and of CO_2 fixation by isolated spinach and *Acetabularia* chloroplasts within 10 minutes of exposure to IAA. Whether this effect is a real promotion seems dubious in view of remarks (Tamas *et al.*, 1972; Tamas and Ware, 1972) suggesting that the action of IAA is to protect against photoinactivation of the chloroplasts, an effect that might be due to the antioxidant properties of IAA (Siegel and Porto, 1961). This possibility is reinforced by the statement that 2,4-D was inactive on photosynthesis (Tamas *et al.*, 1972) because, unlike IAA, 2,4-D is not capable of the facile 1-electron oxidation that confers antioxidant properties. Since 2,4-D is about as active an auxin as IAA, the chloroplast effect is not really an auxin effect. Moreover, there seems to be no evident possibility for a connection between the chloroplast effect and the stimulation of cell elongation of nongreen tissues in the dark which is the classic criterion of auxin action.

For similar reasons, a theory that IAA promotes growth by regulating cell redox balance by virtue of being an antioxidant (Siegel and Porto, 1961) has never gained appreciable acceptance.

Some additional metabolic effects are discussed in the section on action on the cell wall.

Membrane Effects

The currently favored view of auxin action appears to be that the rapid effects probably reflect an action at or on cellular membranes; for example, the regulation of export of growth-active materials across the plasma membrane into the cell wall space. This constitutes, in effect, a return to earlier hypotheses of auxin action (Veldstra, 1956; Van Overbeek, 1961).

Auxin action at the plasma membrane was a hypothesis advanced by Hertel and Flory (1968) and by Rayle *et al.*, (1970a), partly in the belief that auxin transport and auxin action on growth involve closely related if not identical interactions, presumably with some carrier site located in the plasma membrane. A hint of auxin action at the plasma membrane is the indication of IAA effects on membrane potentials (Etherton, 1970; Tanada, 1972), but this effect has not yet been adequately studied nor shown to relate to the elongation response. Another hint of auxin action at the plasma membrane is the induction of cell elongation by certain fungal toxins that are known to increase membrane permeability (Evans, 1973; Lado *et al.*, 1973).

Kang and Burg (1971) reported that IAA treatment of pea stem tissue reduced the half-time for exchange of tritium-labeled water between tissue and medium, which they ascribed to an influence on plasma membrane permeability. This effect was reported to be detectable within 6 minutes

after exposure to IAA and represents one of the most rapid physiological effects of IAA known and one provocative of much excitement in the current state of thinking on IAA action, even though a water permeability effect could not directly explain the cell enlargement response to IAA. Unfortunately, workers in three different laboratories have been completely unable to confirm this effect (Dowler *et al.*, 1973). It was found that the exchange of ^{3}HOH is controlled by the cuticle in this material and can be greatly speeded up by treatments that abrade the cuticle and eliminate it as an osmotic barrier. Even then, however, IAA had no effect whatever on the kinetics of ^{3}HOH exchange, although there was a normal elongation response to IAA. Possibly, with the peas used by Kang and Burg, IAA induction of elongation may have led to some kind of cracking of the cuticle and resultant enhancement of ^{3}HOH exchange.

Stimulations by auxins of K^+ and Rb^+ uptake (Higginbotham *et al.*, 1962; Ilan and Reinhold, 1963; Lüttge *et al.*, 1972) and of Cl^- uptake (Rubinstein and Light, 1972) have been inferred to represent an effect on carrier systems located at the plasma membrane, although there is no evidence that the effects are not a consequence of primary action elsewhere in the cell. In the case of pea and sunflower the effects were detected only 2 hours or more after giving IAA and are not shown to be rapid effects falling within the time scale needed to relate them to the auxin effect on cell enlargement. Rubinstein and Light (1972), however, found that stimulation by IAA of Cl^- uptake by *Avena* began about 10 minutes after exposure to IAA, i.e., at about the same time as promotion of elongation. Bentrup *et al.* (1973) on the other hand, found that IAA rapidly caused a strong *inhibition* of Cl^- uptake by auxin-sensitive parsley cells in culture.

These ion transport effects may be connected with auxin promotion of H^+ ion transport out of plant cells that has recently been demonstrated as one of the key pieces of evidence for the acid secretion theory of auxin action, which will be considered in a later section.

Membrane Binding and Possible Auxin Receptor

Lembi *et al.*, (1971) reported the binding of labeled naphthylphthalamic acid (NPA), a powerful inhibitor of polar auxin transport, to particle preparations from maize coleoptiles that were tentatively identified as plasma membrane-rich fractions. It was thought that interaction of NPA with plasma membrane sites might be the means by which NPA blocks auxin transport. Indoleacetic acid did not compete with NPA for these sites, which thus seem not to be the actual carrier sites for polar transport of IAA. Naphthylphthalamic acid binding can readily be detected also in peas, and density gradient centrifugation shows that the binding involves

a specific class of particles, with a density similar to that expected for plasma membrane fragments, rather than cellular membranes in general (Fig. 4; Cande, 1972).

Hertel *et al.* (1972) detected a "specific" binding of labeled IAA and naphthaleneacetic acid (NAA) to certain particles in maize homogenates. Specific binding was assayed as radioactivity retained by particles exposed to 2–10 $\times$ 10^{-7} M labeled auxin *minus* radioactivity retained when, in addition, 10^{-4} M unlabeled IAA or NAA was added to compete with and displace labeled auxin from saturatable binding sites. The radioactivity that remained in the latter case they termed unspecific binding. This difference-assay tests for sites with affinities in the range 10^{-7}–10^{-5} M. Specificity of the binding as an auxin phenomenon was indicated by evidence that

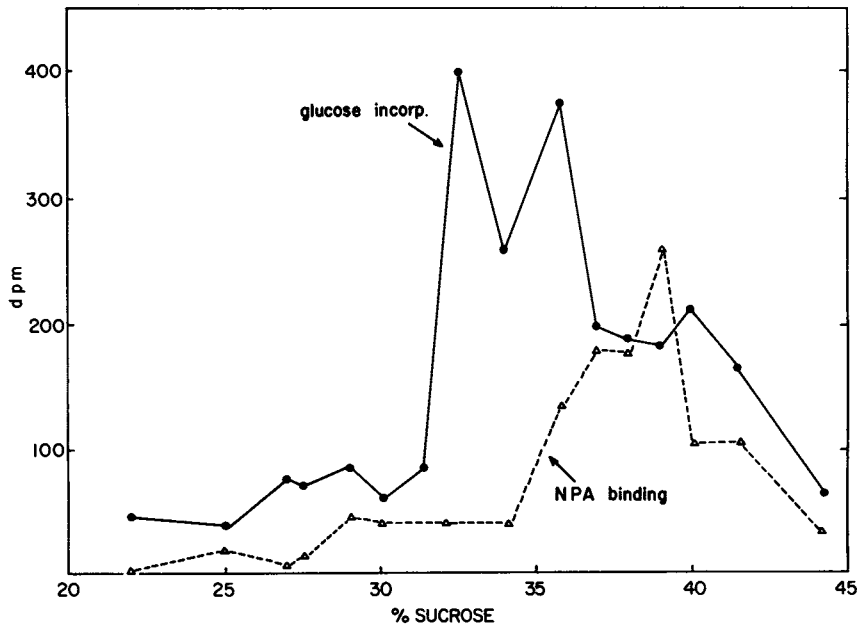

FIG. 4. Binding of naphthylphthalamic acid (NPA)-^{14}C by membrane particles from pea stem tissue. Cell particles from the microsomal fraction obtained by rate zonal centrifugation were fractionated by isopyknic density gradient centrifugation. The glucose-^{14}C incorporation profile shows radioactivity in polysaccharides formed during an 8-minute feeding of 2 gm of tissue with glucose-^{14}C just prior to homogenization; radioactivity is contained mainly in smooth-membrane vesicles of the Golgi type. The NPA curve shows radioactivity bound by each fraction, after correction for nonspecific binding and carryover of unbound activity in the particle pellet, when 3000 dpm of NPA-^{14}C was offered to particles derived from 10 gm of tissue. (From Cande, 1972.)

analogs of IAA or NAA that are active on growth and/or in polar transport can displace labeled IAA or NAA from the binding sites (and IAA and NAA can displace each other), whereas chemically similar but biologically inactive molecules do not compete in the binding assay. One defect was that 2,4-D, an active auxin, competed relatively weakly with IAA or NAA in the assay and, consistent with this, specific binding of labeled 2,4-D itself was not detected.

The concentration dependence of unlabeled/labeled auxin competition indicated an affinity (K_M) of 1–4 $\times$ 10^{-6} M for IAA and NAA, which is in the same range as for *in vivo* action. Scatchard plots of the data indicated an apparent concentration of binding sites in the homogenates of about 10^{-8} M. Unfortunately, this estimate differed by a factor of 2 between IAA and NAA, which should not be the case if they fully compete with one another. This and other problems with precision of the data are probably due to the very high background of "unspecific" binding, much exceeding the "specific" binding in most of the assays, the specific binding comprising at best only a few tenths of a percent of the labeled auxin used.

These defects might be grounds for skepticism about the significance of the observed binding, and it is to be hoped that the assay can be improved with further work, for the possibility that an auxin receptor site has actually been detected and is located on a cellular membrane system is an exciting one. R. Hertel (personal communication) has pointed out that, in view of the above mentioned results with 2,4-D, which is an active auxin on elongation but is only weakly transported, the receptor site seen in these experiments could well be that for polar transport rather than for auxin action on elongation. These two processes he now feels must involve different receptor sites. Whether a receptor involved in hormonal action of auxin can be detected by these methods must be determined by future work.

The identity of the responsible particles will also have to be clarified. Hertel *et al.* (1972) gave data suggesting that the sites that bind auxin and NPA may be localized in different membrane fractions. If NPA binding is to plasma membrane then implicitly auxin binding is not. Hertel *et al.* (1972) indicated that auxin-binding activity was entirely separable from nuclear material, contrary to reports of labeled auxin binding by nuclei and chromatin (Matthysse, 1970; Tautvydas and Galston, 1972).

Mediation of Auxin Action

Ethylene

Many classic auxin responses (but *not* the rapid cell-elongation responses that are the standard criteria for auxin action) are now believed to

involve ethylene as the active agent; that is IAA acts by stimulating the production of ethylene by the plant tissue. These phenomena are discussed by Yang in this volume, (Chapter 6) and have been reviewed by Pratt and Goeschl (1969) and Burg (1973).

In view of the evidence for ethylene mediation, the means by which IAA induces ethylene production by tissue is of enormous importance to any comprehensive explanation of auxin action. The effect develops over a period of hours, with a lag of about 1 hour (Kang *et al.*, 1971), and is therefore not a rapid response. According to Steen and Chadwick (1973) a minor part of the effect occurs more rapidly and may be a relatively immediate effect of auxin.

Mapson and Wardale (1972) reported a requirement for IAA in ethylene production by a peroxidase system functioning as an oxygenase. This effect is not entirely surprising since the peroxidase systems that generate ethylene are rather closely analogous to the peroxidase-catalyzed oxygenase oxidation of IAA itself. In the peroxidase-ethylene system, NAA and 2,4-D were ineffective, causing Mapson and Wardale to consider that the system may not closely model the *in vivo* auxin effect on ethylene production, in which these analogs of IAA are active.

Kang *et al.* (1971), Sakai and Imaseki (1971), and Steen and Chadwick (1973) give inhibitor data indicating that most of the IAA stimulation of ethylene production by tissue depends on protein synthesis, which would not be the case for the kind of catalytic effect described by Mapson and Wardale. As in similar earlier work discussed in the foregoing, these authors infer that IAA stimulates ethylene production by inducing the synthesis of some enzyme or other protein. Kang *et al.* believe that peroxidase is not the enzyme that is induced, because their assay for peroxidase-catalyzed ethylene formation in tissue extracts showed no increase in activity when tissue was treated with IAA. (This was a questionable assay for changes in peroxidase level because it depends on cofactors and substrates that could be limiting in the extracts.) They hold open, however, the possibility that changes in levels of cofactors or oxidants required for peroxidase-catalyzed ethylene formation could explain the IAA effect even though their assay did not give evidence for such changes. The mechanism by which IAA actually induces ethylene formation by tissues remains a major unsolved problem in the auxin field.

3-Methyleneoxindole Hypothesis

Tuli and Moyed (1969) proposed that auxin action on cell elongation is due not to IAA itself but to its oxidation product, 3-methyleneoxindole (3-MO) produced from IAA in tissue by peroxidase-catalyzed oxygenase

oxidation. Their principal evidence for this mediation was data to the effect that (*1*) 3-MO induces elongation of pea stem segments as strongly as IAA does, but at one-tenth the concentration, and (*2*) chlorogenic acid, a polyphenol that blocks peroxidase-catalyzed oxidation of IAA to 3-MO, also blocks the elongation response of pea stem segments to IAA but not the elongation response to 3-MO. Basu and Tuli (1972) extended the hypothesis to include IAA action on wheat coleoptile elongation.

This hypothesis has serious consequences for much of the current efforts to detect *in vitro* auxin effects, such as binding to membranes, that might be involved in primary action. If the physiological action were, in fact, due to 3-MO rather than to IAA, *in vitro* experiments with IAA would be quite beside the point. The 3-MO hypothesis may have been dismissed by many workers because it seems not to account for the high auxin activity of IAA analogs such as NAA and 2,4-D, which are *not* oxidized by peroxidase. However, Moyed and Williamson (1967) attempted to answer this by reporting that NAA and 2,4-D inhibit (at physiologically excessive concentrations) an enzyme that converts 3-MO to a growth-inactive compound; therefore, they concluded that these analogs induce elongation by causing a buildup of 3-MO derived from oxidation of endogenous IAA within the tissue.

Andersen *et al.* (1972) and Evans and Ray (1973) synthesized 3-MO possessing physical properties identical to those reported in the literature and found 3-MO to be totally inactive at any concentration in stimulating elongation of either peas or *Avena* coleoptiles. Furthermore, contrary to Tuli and Moyed but in agreement with much analogous data in the literature, chlorogenic acid does not block the elongation response to IAA but, if anything, enhances it (Evans and Ray, 1973). Therefore, we consider the 3-MO hypothesis of auxin action to be defunct by failure of the primary evidence advanced in its favor.

Cyclic Adenosine Monophosphate, Acetylcholine

Preliminary reports have appeared claiming that cyclic adenosine monophosphate (c-AMP) induces cell enlargement (weakly) in IAA-sensitive plant tissues (Kamisaka and Masuda, 1970; Salomon and Mascarenhas, 1971; Weintraub and Lawson, 1972; Hartung, 1972). These authors have proposed that c-AMP mediates plant hormone action as it does the action of many animal hormones (Sutherland, 1972). Some have claimed (Salomon and Mascarenhas, 1971, 1972b; Azhar and Krishna Murti, 1971; Brewin and Northcote, 1973; Janistyn, 1972) to have obtained a rapid increase in c-AMP content of plant tissue after administering IAA. The existence of this effect has, however, been denied by others (Ownby *et al.*, 1973), as has

the reality of a c-AMP effect on cell enlargement (Ockerse and Mumford, 1972; Brewin and Northcote, 1973). Kamisaka *et al.* (1973) obtained promotion of Jerusalem artichoke tissue growth by c-AMP only in the *presence* of auxin, so concluded that c-AMP does not mediate auxin action. The dispute continues as to whether c-AMP really exists in plants (cf. Raymond *et al.*, 1973; Bachofen, 1973) and whether the methods by which reports of hormone-induced changes in plants have been obtained are reliable. At present, mediation of auxin action by c-AMP has not been substantially demonstrated in any system.

It has been proposed that acetylcholine mediates certain phytochrome responses (Yunghans and Jaffe, 1972; Jaffe, 1972). Evans (1972) reported a promotion of oat coleoptile segment elongation by acetylcholine, but his data showed that action of IAA could not be mediated by acetylcholine, and the entire effect was later found to be spurious (Evans, 1973).

Hydrogen-Ion Secretion

Based on observations of acid pH effects on elongation to be discussed in the following, Hager *et al.* (1971) postulated that auxin acts by stimulating an outwardly-directed H^+ pump at the plasma membrane, which acidifies the cell wall, thereby causing a purely passive extension of the cell wall in response to low pH. The presumed energy requirement for a H^+ pump would serve to explain why auxin-induced growth is sensitive to inhibitors of energy metabolism. This hypothesis apparently occurred independently to Cleland and was briefly mentioned by him in the same year (Cleland, 1971a).

Since the 1930s it had been known that acidic media can stimulate elongation in auxin-sensitive tissue. Recent work showed that acidic buffers or CO_2 solutions in the pH range from 3 to 4 induce a rate of coleoptile cell elongation as great as or greater than that obtainable with auxin (Rayle and Cleland, 1970; Hager *et al.*, 1971; Evans *et al.*, 1971). Acid-induced elongation starts almost immediately upon treatment rather than after the 10–15 minute latent period characteristic of auxin action (Fig. 5). Acid-induced elongation is not suppressible by metabolic inhibitors, such as cyanide, mercurials, cycloheximide, and lack of oxygen, that block auxin-induced growth, and appears to be a passive process independent of metabolism. In later work it was found that when the epidermis is removed to improve H^+ entry into the tissue, the full acid-pH stimulation of elongation develops over the pH range from about 6.0 to about 5.0 (Rayle, 1973; Cleland, 1973).

Rayle *et al.* (1970b) found that cell wall skeletons of frozen-thawed, dead coleoptile tissue would elongate dramatically if treated with acidic media

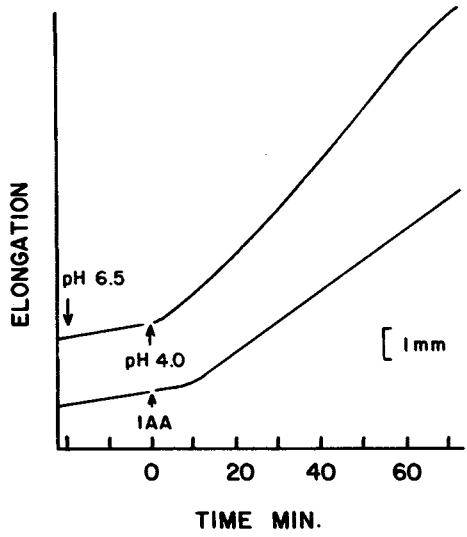

FIG. 5. Elongation response of *Avena* coleoptile to acid pH compared with response to IAA. Coleoptile segments were in 1-m*M* citrate buffer, pH 6.5, prior to transfer either to the same buffer containing 3 mg/liter IAA (lower curve) or to 1-m*M* citrate buffer, pH 4.0, without IAA (upper curve). (From Evans, 1967.)

while being held under tension by an applied load. Rayle and Cleland (1972) concluded that acid pH- and IAA-induced elongation must occur by the same mechanism, because the two kinds of elongation had similar rates, similar temperature dependences, and a similar yield threshold. While suggestive, these similarities do not establish identity of biochemical mechanism. The question of this mechanism will be considered in a later section; for the moment it is sufficient that acid pH induces a process of elongation which phenomenologically resembles that caused by IAA.

Hager *et al.* (1971) also felt, on grounds that IAA-induced elongation could be suppressed by alkaline media, that IAA-induced growth involves the same biochemical mechanism as acid-induced elongation. In support of their proposed auxin-stimulated H^+ pump they offered experiments showing rapid stimulation of coleoptile elongation by ATP, ITP, and GTP under anaerobic conditions, although these results may have represented merely the acid pH effect itself, since these compounds are strong acids and were applied in unbuffered, pH 5 media.

In recent work an auxin-induced release of H^+ ions from coleoptiles stripped of their epidermis (Cleland, 1973; Rayle, 1973) and from other

auxin-sensitive tissues (Marrè *et al.*, 1973; Ilan, 1973) has been detected. This is measured simply as a gradual fall in pH of the medium bathing the tissue, upon treatment with IAA, from near-neutral pH to pH 5 or below, i.e., into the range that by itself induces elongation. Detectable H^+ secretion by coleoptiles begins with 20 to 30 minutes after exposure to IAA and may be regarded as a "rapid response." To the extent so far studied, H^+ secretion by coleoptiles has a specificity for auxin analogs and antagonists similar to the specificity seen in growth, and a similar dependence on metabolism. Secretion is sensitive to inhibitors and uncouplers of energy metabolism and, perhaps unexpectedly for a transport process but just like cell enlargement, H^+ secretion is quickly inhibited by low concentrations of cycloheximide (Rayle, 1973; Cleland, 1973). Comparable results with pea stem segments have been reported by Marrè *et al.* (1973).

These findings, coupled with the observations that induction of elongation by auxin is prevented by sufficiently well-buffered media of pH 6 to 8 provided the cuticle is removed or rendered permeable by gentle abrasion (Rayle, 1973; P. M. Ray, unpublished), constitute presumptive evidence that the auxin effect on cell enlargement is mediated by externally secreted H^+ ions.

The simplicity and directness of the acid secretion theory of auxin action is appealing and the theory has quickly become popular. Various auxin effects on cation and anion transport noted previously were considered by their authors to be consistent with the H^+ secretion theory. H^+ secretion could involve either a parallel flow of anions, or a counterflow of, or exchange with, cations as has been inferred for H^+ pumping by beet cells (Poole, 1973). However, as yet no immediate dependence on external ions of either auxin-induced H^+ secretion or growth has been found. Rayle (1973) noted inhibition of H^+ release and elongation by valinomycin, implying some involvement of K^+, though it was not excluded that the effect could be due to energy uncoupling. From data of Ilan (1973, Exp. 1) one can calculate that IAA-induced release of about 15 μeq of H^+ by sunflower hypocotyl segments was accompanied by uptake of about 20 μeq of K^+ over and above that absorbed in the absence of IAA, with no change in uptake of phosphate, from a potassium phosphate solution. This suggests occurrence of auxin-induced K^+_{in}/H^+_{out} exchange, like the H^+ pump of beet cells (Poole, 1973). Such a mechanism could operate even in the absence of exogenous salts, by absorbing the exchangeable ions in the cell wall space that must be present as counterions for uronic acid groups of wall polysaccharides. Absorption of these counterions would set up a uronic acid/uronate buffer system in the cell wall that would bring the pH into the region around 5 that is effective for inducing rapid wall extension.

Cleland (1973) and Rayle (1973) observed that auxin-treated coleoptiles cease to release H^+ when the medium reaches pH 5.0 or slightly below, suggesting that auxin-stimulated H^+ secretion is inhibited by acid pH, like other H^+ pumps (Poole, 1973), or alternatively that, at pH 5, back-diffusion of H^+ into the cell offsets the action of the pump. This feature should allow extension-promoting pH values in the cell wall to be reached rapidly under auxin treatment, while shutting down further acidification that would probably be injurious to the cell (Cleland, 1973).

Some objections to the H^+ secretion theory of auxin action and weaknesses in the evidence for it should be mentioned. For example, it has not yet been shown that suppression of auxin elongation by neutral buffers is not due to inhibited uptake of auxin, or to failure of primary action [the auxin binding found by Hertel *et al.* (1972) had a pH optimum below pH 6.5]. Barkley and Leopold (1973) reported that auxin-responsive stem segments from light-grown peas show no elongation response to low pH. They concluded that the H^+ secretion hypothesis of auxin action is untenable. Logically, this conclusion would, of course, hold strictly only for green pea segments, not for systems used by other workers. Moreover, failure to get an acid pH elongation response in green pea segments could well have been due to failure of the applied citrate buffer to penetrate adequately, since no effort was made to render the cuticle permeable. This explanation of the results is supported by the fact that they obtained an elongation response of green segments to CO_2, an acid that should penetrate the cuticle better than citrate.

Ilan (1973) held that the H^+ secretion hypothesis is untenable because sunflower hypocotyl tissue placed in a pH 6.2 buffer without auxin grew much more slowly than did auxin-treated tissue which, by virtue of H^+ secretion, had lowered the pH of its medium from 6.8 to 6.2. However, this argument is questionable, because the minus-auxin tissue was at equilibrium with a medium of pH too high to cause wall extension, whereas the plus-auxin tissue was continuously releasing H^+ into the medium and must therefore have had in its cell wall space a pH lower than that of the medium.

This calls attention to the most important piece of evidence still needed for the acid secretion theory: that the pH in the tissue's free space falls to an extension-stimulating value at the time that rapid auxin-induced elongation commences, i.e., within 10–15 minutes after exposure to auxin, as against the hour or more that is required for an external bathing medium to reach pH 5.0 to 5.5 (Cleland, 1973; Rayle, 1973). At the very least it must be demonstrated that IAA induces H^+ secretion as quickly as it induces rapid elongation. Existing data fail this test and show an increase

in H^+ efflux from coleoptiles beginning, at the earliest, 20 or 30 minutes after exposure to IAA (Cleland, 1973; Rayle, 1973). These authors feel that the discrepancy may be attributed to time lags imposed by the free space volume and the diffusion path length for H^+ to reach the external medium. A worse timing discrepancy was seen with sunflower hypocotyl (Ilan, 1973; cf. Uhrström, 1969). A more sensitive method for measuring H^+ efflux needs to be employed to resolve these discrepancies. Better still, the pH within the free space of the tissue should be measured directly during response to auxin treatment.

Satisfaction of these tests would virtually prove the H^+ secretion theory. Such proof would not mean that the effect on H^+ transport is necessarily a primary action of the hormone in the sense of a process affected directly by the hormone molecule, even though it is popular today to expect this in view of the indications of membrane interactions mentioned earlier. Efforts will certainly be made to detect induction by IAA of H^+ transport across isolated plasma membrane vesicles, or stimulation by auxin of membrane-bound ATPases that might be involved in H^+ transport or coupled H^+/cation exchange. Other mediators or intermediate steps in auxin action may of course lie in the way of easily reaching these goals.

Regarding auxin action over the time scale involved in achieving substantial growth there is the objection that, in contrast to auxin-stimulated growth, acid-induced elongation fails to continue steadily for many hours. This might be explained (Rayle, 1973) as a longer-term need for other auxin-stimulated processes such as growth-essential enzyme synthesis, or cell wall synthesis to provide substrate for the action of H^+. This view in effect espouses multiple actions of auxin on cell enlargement over a time scale of hours, only the initial rapid stimulation of elongation rate being ascribable directly to H^+. Such a view may be needed also to account for *intra*cellular effects of auxin, such as on cell division, or the rapid activation by auxin of glucan synthetase mentioned earlier, which occurs in the Golgi membranes. A purist view, on the other hand, would be that intracellular effects of auxin result from an increase in cytoplasmic pH as H^+ ions are pumped out, or from changes in cytoplasmic ion concentrations due to ion transport that accompanies H^+-pumping. For example, with glucan synthetase activation might result from increase in cytoplasmic pH toward the pH optimum of enzyme. In this event, activation should be obtainable without auxin by exposing tissue to permeating weak bases like NH_3, which demonstrably raise internal pH, but we have not been able to obtain such an effect. So far as we can tell at present the activation of glucan synthetase is a separate effect of auxin.

Action on the Cell Wall

How the primary action of IAA, whatever that may be, leads to an effect on the cell wall that permits its extension and, thus, the occurrence of cell enlargement is an issue that can be divided into both biophysical and biochemical questions. Only the latter will be considered here (for discussions of the biophysics of plant cell growth see Cleland, 1971a; Green, 1972; Ray, 1969; Ray *et al.*, 1972.)

Regarding the biochemical basis of cell wall extension, much of the research activity during the past decade has been inspired by the gene activation hypothesis of auxin action, leading to the expectation that IAA must induce synthesis of enzymes that act to weaken the cell wall.

Hydrolase Hypothesis

The most dramatic auxin effect on enzyme formation known is the induction of cellulase, polygalacturonase, and other hydrolases in pea seedlings treated with a high concentration of IAA in lanolin paste (Datko and Machachlan, 1968). Detection of *in vitro* formation of cellulase by a ribosomal preparation from peas was claimed, and this was reported to be enhanced using a ribosomal system prepared from IAA-treated peas (Davies and Machlachlan, 1969). The large IAA effect on cellulase formation *in vivo* is a slow response, occurring over several days, and cannot be involved in rapid IAA action on elongation although it could be a cause of the lateral swelling response of pea cells to IAA that occurs under the treatment conditions used by these workers. However, a breakdown of cellulose *in vivo* under lateral swelling conditions has not been demonstrated. Cellulose is found to be quite stable during normal IAA-induced elongation (Katz and Ordin, 1967a; Labavitch and Ray, 1974).

With *Avena* coleoptiles an auxin effect on the level of cell wall-bound glucanases has been reported (Katz and Ordin, 1967b; Heyn, 1970; Masuda and Yamamoto, 1970) and held to be the basis of auxin action. Masuda and Yamamoto (1970) claimed that an increase in β-1,3-glucanase activity, which they believed to be due to enzyme synthesis, occurs in response to IAA within 10 minutes. At this time, however, the indicated effect was extremely small. The data indicate that glucanase activity rose steadily for at least 1 hour in *both* control and IAA-treated tissue, but more rapidly in the latter. After 1 hour, glucanase activity of the control (which was still slowly elongating) was much higher than the glucanase activity at 15 minutes in the presence of IAA (when maximum elongation rate had been attained). This makes it difficult to see how the assayed glucanase activity

could be the causal agent for wall extension. Masuda *et al.* (1970) nevertheless believed that they had discovered the enzymatic basis for cell wall growth in *Avena* coleoptiles, and they continue to maintain that a particular fungal β-1,3-glucanase induces rapid elongation of coleoptile and pea segments (Masuda and Yamamoto, 1972), this enzyme substituting for the effect of IAA. Cleland (1968) and Ruesink (1969) could not confirm this finding, however, and the entire question of glucanase action in coleoptiles seems to deserve further careful investigation in view of evidence to be discussed in the next section.

Wall Turnover

Much of the previous work on this subject, apropos of the hydrolase hypothesis, is fraught with serious questions (Ray, 1969; cf. Lamport, 1970) and will not be reviewed. However, Loescher and Nevins (1973) have reported a dramatic drop in noncellulosic wall glucan in *Avena* coleoptile segments given IAA in the absence of exogenous sugar, thus confirming earlier analytical data (Ray, 1963). The wall glucan breakdown has not yet been shown to parallel elongation in either inception or long-term time course. Alternatively the phenomenon might represent breakdown of storage reserve glucan under starvation conditions since the effect is not seen in the presence of exogenous sugar (despite good elongation) and was not clearly seen during IAA-induced elongation in pulse-chase labeling experiments (Katz and Ordin, 1967a). However, noncellulosic glucan breakdown still seems to be a possibility for the mode of action of IAA on elongation in *Avena* coleoptiles, and it is worth noting that this could be due to glucanase action even if IAA does not act by inducing glucanase synthesis; for example, IAA could activate the enzyme or induce its export into the cell wall space.

A detailed study of cell wall turnover and any auxin effect thereon in pea stem tissue has recently been performed using pulse/chase wall-labeling experiments (Labavitch and Ray, 1974). Considerable turnover of galactan occurs but this is not influenced by IAA. A wholesale breakdown of noncellulosic glucan does not occur, but a minor removal *in vivo* of glucose and xylose from a pectinase-degradable wall xyloglucan is observed, and this removal is promoted by IAA. Indoleacetic acid also causes, inversely, a notable buildup of cold water-soluble neutral xyloglucan, which is interpreted as derived from the wall-bound xyloglucan by some process of detachment or degradation. The action on soluble xyloglucan can be assayed with relative ease and precision, and has been positively demonstrated to be in progress within 30 minutes after exposure to IAA, and probably within 15 minutes, placing it among the most rapid metabolic

effects of IAA to be demonstrated and certainly the most rapid known IAA effect on a wall polymer (Table I). The effect is blocked by metabolic inhibitors that block elongation but persists under complete osmotic inhibition of elongation by mannitol; it seems likely that it is involved in the action on the cell wall that leads to elongation in pea cells (Labavitch, 1972). Involvement of xyloglucan is understandable in terms of a recent model for cell wall structure (Bauer *et al.*, 1973) according to which xyloglucan serves to bind matrix polysaccharides to cellulose microfibrils, thereby functionally cross-linking the latter.

Although discovered using methods for detecting polysaccharide turnover, the auxin effect on xyloglucan in pea has not been shown as yet to be due to breakage of covalent bonds, and could instead reflect a decrease in noncovalent interaction between xyloglucan and other wall polymers resulting from either chemical or physical changes in the latter. This possibility is suggested by the finding that auxin-induced increase in soluble xyloglucan can be completely reversed by subsequent treatment with Ca^{2+} in the cold (Labavitch, 1972), a treatment that seems likely to affect the physical properties of wall polyuronides but not to cause chemical changes.

TABLE 1

RELEASE OF SOLUBLE XYLOGLUCAN FROM CELL WALLS OF PEA TISSUE IN RESPONSE TO INDOLEACETIC ACID[a]

Time (hr)	Water-soluble xyloglucan-^{14}C (dpm)		Ratio
	−IAA	+IAA	
0.25	9050 ± 513	9870 ± 414	1.09
0.5	8770 ± 328	10790 ± 859	1.23
1	8920 ± 429	12960 ± 168	1.45
3	8650 ± 330	15610 ± 260	1.80
7	6560 ± 187	12700 ± 60	1.93

[a] Cell walls were prelabeled by 1 hour incubation of tissue in glucose-^{14}C, then tissue was incubated in unlabeled glucose for 3 hours to wash out soluble pools. At zero time, 3 mg/liter indoleacetic acid (IAA) was added to +IAA samples. Replicate tissue samples were chilled, ground in cold water (0°C), and soluble xyloglucan isolated and counted. Data of Labavitch, 1972.

Wall Synthesis

The timing of the glucan synthetase response to IAA in peas (Ray, 1973a) adds further weight to already existing evidence that IAA does not induce elongation by its promotion of total wall polymer synthesis (Ray, 1969). However, the possibility is still open that internal incorporation (intussusception) of polysaccharides into the cell wall is somehow regulated by IAA and contributes to wall extension. A new indication of requirement of elongation for wall polysaccharide synthesis is the inhibition of elongation by 2-deoxy-glucose (D. Penny *et al.*, 1972; P. M. Ray, unpublished) which in peas inhibits cell wall polysaccharide synthesis but not energy metabolism nor protein synthesis (Ray, 1973b).

Rayle *et al.* (1970b) concluded, to the contrary, that wall synthesis is totally unnecessary for cell wall growth or auxin action, visualized according to the H^+ secretion theory discussed in the foregoing, since low pH can induce wall extension in the absence of synthetic metabolism. More broadly construed (Rayle, 1973), the H^+ secretion theory implies that any dependence of wall extension on synthesis and auxin effects thereon would be secondary and come into play only over a longer time scale than that of the rapid elongation rate response to auxin.

Cell Wall Protein ("Extensin")

Lamport (1970) coined the name "extensin" for the hydroxyproline-rich polypeptide or protein associated with cell walls, in the belief that the protein must cross-link polysaccharide chains and thereby control cell wall growth. Despite effort in several laboratories, no findings have appeared that directly implicate a role of extensin in the control of wall growth by IAA (cf. Winter *et al.*, 1971; Cleland, 1971a).

Acid pH Effect

In the section on H^+ secretion we noted that media of pH 3 to 5 induce extension of cell walls held under tensile stress. The physiological importance of this depends of course upon whether auxin in fact induces cell enlargement by causing acidification of the cell wall, but even were this not so the mechanism of the acid pH effect would be of interest for whatever light it might shed on the molecular basis of the ability of the cell wall to support turgor stress and to undergo irreversible extension. The acid-secretion theory of auxin action predicts that whatever chemical changes in wall polymers accompany acid-induced extension, these changes should also occur during auxin-induced cell enlargement; this constitutes another potential test of the theory.

Quite divergent views are at large regarding the nature of the acid pH effect. Because Hager *et al.* (1971) found an optimum at pH 4 for a plasticizing effect of H^+ on sunflower hypocotyl specimens, and an inhibition by Cu^{2+}, they considered that acid pH acts by stimulating the action of a wall-softening enzyme, located within the cell wall, that has an acidic pH optimum. Thinking along these lines generally makes appeal to the known wall-bound hydrolases, mentioned above, as the model (cf. Johnson *et al.*, 1973). Rayle and Cleland (1972), however, found no pH optimum for acid-induced extension, which rose with increasing acidity and then simply reached a plateau, which later work showed begins just below pH 5.0 (Rayle, 1973).

Rayle and Cleland (1972) found that acid pH induces extension of wall material pretreated with protease or detergents that would be expected to destroy wall-bound enzymes. They considered that H^+ acts chemically to catalyze the hydrolysis of certain critical acid-labile bonds between wall polymers (Cleland, 1971a).

The concept of acid-labile covalent bonds seems improbable on energetic grounds. A very high negative free energy of hydrolysis is implied for acid-lability at physiological temperatures at a pH as high as 5. Moreover, such a highly exergonic reaction would have to be irreversible. However Rayle and Cleland (1972) found that acid pH treatment of walls in the absence of tensile stress did not make the walls capable of extending when they were later stressed at neutral pH. This implies that the critical bonds reform as fast as they are broken, i.e., a reversible reaction. This finding also tends to remove support from the concept of enzymatic hydrolysis induced by acid pH, because such hydrolase reactions are typically irreversible under normal conditions. A group-transfer reaction might, however, satisfy these observations. The concept of acid-labile bonds further clashes with the abovementioned observation of a plateau in extension rate at pH values below about 5.0, because the rate of an acid-catalyzed reaction increases with H^+ concentration without limit.

Although there is a widespread tendency to assume, as in the foregoing discussion, that wall extension must involve the breaking of covalent bonds within or between wall polymers, no direct evidence for such necessity has yet been given, nor for the occurrence of bond breaking during acid pH-induced extension. We should seriously consider the alternative that acid pH works by altering reversibly the conformation, ionic interactions, or hydrogen bonding between polymers that are not covalently linked, allowing them to slip past one another if the structure is under load. Such a reversible physical effect accounts readily for the lack of an extensibility after-effect when acid pH is given in the absence of load. The fact that

the acid pH effect develops over the pH range through which dissociation of uronic acid carboxyl groups becomes repressed is suggestive of such a basis for the phenomenon. Bauer *et al.* (1973) suggest that low pH might weaken hydrogen bonding between cellulose microfibrils and the xyloglucan component of the wall matrix, thereby allowing the microfibrils to slip through the matrix.

Summary

Because of evidence for very rapid auxin responses and for responses that are partly or entirely independent of nucleic acid and protein synthesis, there has been a marked change in research on auxin action, from being dominated in the 1960s by the concept of action at the gene level, to a currently prevailing expectation of action primarily at the level of membranes and transport processes. An apparently specific binding of auxin to as yet unidentified cellular membranes has been observed and may represent detection of a hormone receptor. Several mediator hypotheses have appeared. Ethylene definitely mediates a number of auxin effects, but not the effect on cell enlargement; the mechanism by which auxin stimulates ethylene formation remains unknown. A proposal that IAA works via oxidation to 3-methyleneoxindole appears to be ruled out. The idea that c-AMP mediates the auxin effect on cell enlargement has enjoyed a brief period of excitement but seems unlikely to be proven. A promising new hypothesis holds that auxin acts by stimulating an outwardly directed H^+ pump, thereby lowering the pH in the cell wall, which extends passively in response to low pH resulting in enlargement of the cell. Considerable evidence in favor of this acid secretion theory has been advanced, although certain critical tests remain to be satisfied. It is not yet understood how low pH causes cell walls to extend, but an apparent turnover or at least modification of cell wall xyloglucan in peas and of noncellulosic glucan in coleoptiles in response to auxin has been detected in recent work and may reflect the biochemical action on the cell wall that makes it capable of extension.

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GIBBERELLIN CONTROL OF A SECRETORY TISSUE

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The aleurone layer of the seeds of the Gramineae is a secretory tissue controlled by gibberellins from the embryo of the germinating seedling (for a recent review, see Yomo and Varner, 1971). The aleurone cells, in response to gibberellins, synthesize and secrete α-amylase, protease, and ribonuclease (Bennett and Chrispeels, 1972). β-Glucanase secretion (Taiz and Jones, 1970), but not its synthesis (Taiz and Jones, 1970; Bennett and Chrispeels, 1972), is also under gibberellin control.

The gibberellin-enhanced synthesis of hydrolases is preceded by a proliferation of rough endoplasmic reticulum (Yomo and Varner, 1971). Because control of proliferation of rough endoplasmic reticulum seems more likely than hydrolase synthesis and secretion to be close to the primary site of gibberellin action and more attractive as a possible general response of plant tissues to gibberellins, we shall examine rough endoplasmic reticulum proliferation in some detail.

There is a gibberellin-enhanced incorporation of labeled choline into the lipid-soluble material of endoplasmic reticulum and of other cell fractions (Fig. 1) (Evins and Varner, 1971), and an enhanced incorporation of $^{32}P_i$ into the phospholipids of several cell fractions (Table 1) (Koehler and Varner, 1972). This enhancement is first measurable about 4 hours after the addition of gibberellin (Fig. 2), is proportional to the log of the concentration between 10^{-9} and 10^{-7} M (Fig. 3), and is prevented by abscisic acid (Fig. 4), by 6-methylpurine, and by cycloheximide (Koehler and Varner, 1972).

There is also a gibberellin-induced enhancement of phosphorylcholine cytidylate-transferase and phosphorylcholine-glyceride transferase (Figs. 5 and 6) (Johnson and Kende, 1971) measurable as early as 2 hours after

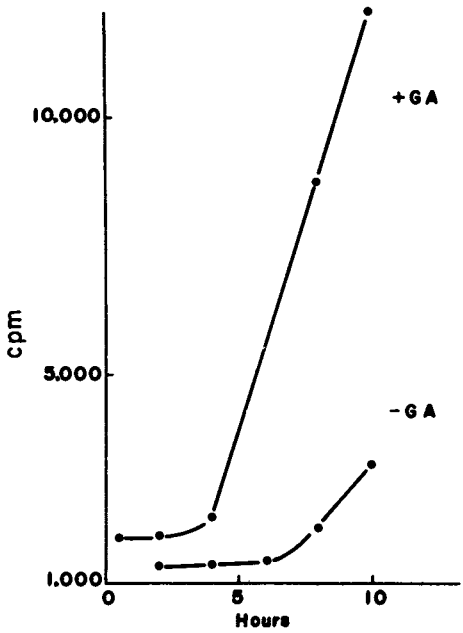

FIG. 1. Effect of a gibberellin (GA_3) on the rate of endoplasmic reticulum synthesis in barley aleurone layers. Forty aleurone layers were incubated at 25°C for various times in 1 m*M* acetate buffer (pH 4.8), 20 m*M* $CaCl_2$ with or without GA_3. The aleurone layers were transferred to a medium containing 5 μCi of (methyl-^{14}C) choline and casein hydrolyzate for the last 30 minutes of incubation. A semipurified endoplasmic reticulum fraction was prepared. Trichloroacetic acid-precipitable membrane material in this fraction was collected on a Millipore filter and counted. Each point is the average of duplicate samples. Similar results were obtained in three experiments. (Evins and Varner, 1971.)

treatment. The gibberellin-evoked increases in these membrane-bound (Table 2) enzyme activities are prevented by abscisic acid, actinomycin D, and cycloheximide. There is also a perturbation in the rate of $^{32}P_i$ incorporation into cytidine triphosphate within 1 hour of addition of gibberellins to isolated wheat aleurone layers (Collins *et al.*, 1972). These gibberellin-evoked increases in the lecithin synthesizing enzymes are not inhibited by concentrations of mannitol that prevent the increase in the incorporation of $^{32}P_i$ into phospholipids (Table 3; Fig. 7).

These early effects on phospholipid metabolism suggest (Johnson and Kende, 1971) that hydrolase synthesis occurs only on polysomes bound to newly synthesized endoplasmic reticulum and that the synthesis of hydrolases before the addition of gibberellins is limited not by messenger ribo-

TABLE 1

DISTRIBUTION OF LABELED PHOSPHOLIPIDS IN DIFFERENT CELL FRACTIONS[a,b]

Cell fraction	−GA		+GA		
	cpm	% of total	cpm	% of total	Enhancement by GA
4,000*g* Pellet	26,000	68	310,000	69	11.9×
10,000*g* Pellet	4,800	13	55,000	12	11.5×
Supernatant	7,400	19	86,000	19	11.6×

[a] From Koehler and Varner, 1972.

[b] Twenty aleurone layers per flask were incubated for 8 hours with or without 1 μM gibberellin (GA_3). At that time they were labeled with 225 μCi $^{32}P_i$ per flask for 30 minutes and chased with 50 mM KH_2PO_4 for an additional 15 minutes.

nucleic acid (mRNA) but by availability of appropriate membranes for attachment of polysomes that carry hydrolase-specific mRNA's (Evins, 1971). There may be a further clue as to the early effects of gibberellins in the finding that, within about 1 hour, GA_3 causes the aleurone cells to be

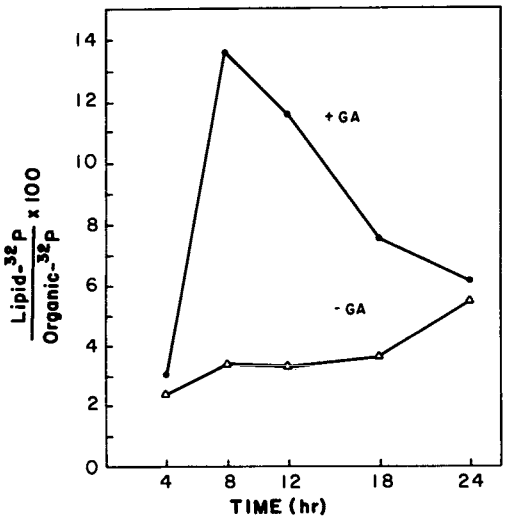

FIG. 2. Time course of phospholipid synthesis. Incorporation of $^{32}P_i$ into phospholipids is expressed as a percentage of the $^{32}P_i$ incorporation into organic phosphates. GA, gibberellic acid. (Koehler and Varner, 1972.)

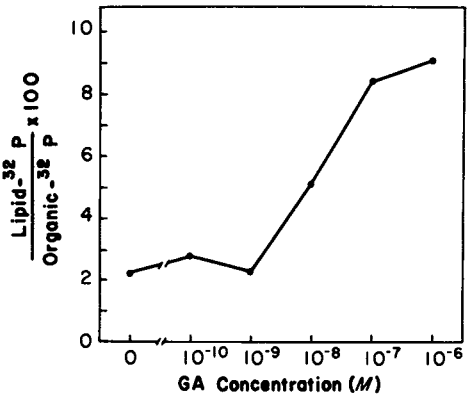

FIG. 3. Increase in phospholipid synthesis in response to increasing concentrations of gibberellic acid. Aleurone layers were incubated in solutions of the indicated concentration for 10 hours. They were then labeled with $^{32}P_i$ and the phospholipids extracted. (Koehler and Varner, 1972.)

sensitive to *O*-phenanthroline (Goodwin and Carr, 1972a) and changing temperatures.

If gibberellins control a rate-limiting step in phospholipid metabolism, why then are treatments such as the removal of gibberellins from the incubation medium, the addition of 6-methylpurine, and the addition of abscisic acid effective 9 hours after the addition of gibberellins? Is continued

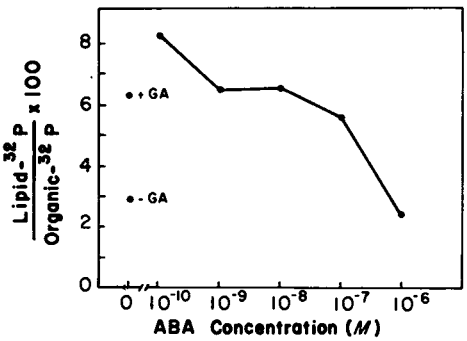

FIG. 4. Progressive inhibition of phospholipid synthesis by increasing concentrations of abscisic acid (ABA). Aleurone layers were incubated for 8 hours with both gibberellic acid (1 μM) and ABA at the indicated concentrations present from the start. The layers were then labeled with $^{32}P_i$ and the phospholipids extracted. (Koehler and Varner, 1972.)

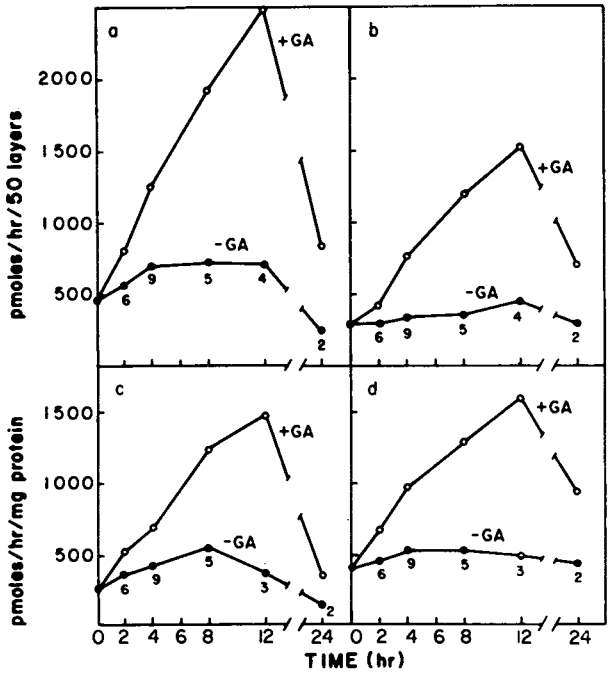

FIG. 5. Effect of gibberellic acid on phosphorylcholine-cytidyl transferase activity. Fifty half-seeds were incubated per treatment, with or without 1 μM gibberellic acid, for the times indicated. Aleurone layers were isolated and the enzyme extracted and assayed. Each set of points represents an average of a number, [indicated immediately below each control (data) point] of separate experiments. (a and c) 11,000*g* Pellet; (b and d) 44,000*g* pellet. (Johnson and Kende, 1971.)

synthesis or turnover of phospholipid necessary or do gibberellins have a function in addition to its control of endoplasmic reticulum synthesis? The reports that the aleurone cells are most sensitive to actinomycin D just before the end of the lag period (Goodwin and Carr, 1972b) and that the incorporation of labeled uridine into a putative mRNA fraction does not occur until after 4 hours incubation with gibberellins (Zwar and Jacobsen, 1972) favor the idea that it participates in hydrolase synthesis in a way over and above the gibberellin-enhanced phospholipid metabolism.

An examination of the secretion of hydrolases is of interest because it appears that gibberellin is required for the secretion of β-glucanase that was synthesized before its addition (Taiz and Jones, 1970). Gibberellin-enhanced release of β-glucanase into the medium is prevented by inhibitors and uncouplers of oxidative phosphorylation, by actinomycin D, by 6-

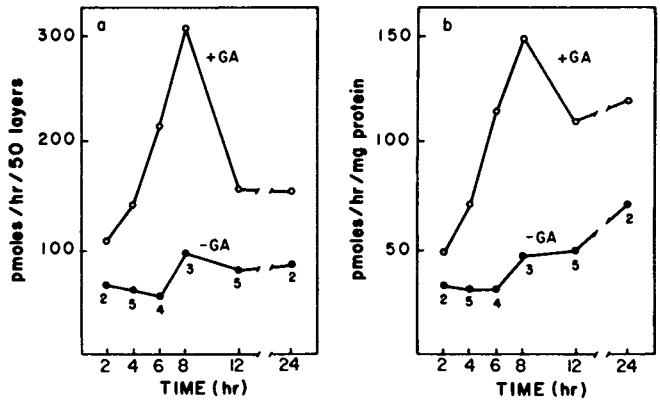

FIG. 6. Effect of gibberellin (GA) on phosphorylcholine-transferase activity. Fifty half-seeds were incubated per treatment, with or without 1 μM gibberellin, for the times indicated. The enzyme was prepared as a composite pellet (500g–44,000g pellet). (Johnson and Kende, 1971.)

methylpurine, and by cycloheximide (Jones, 1972). This is in contrast to the secretion (movement across the plasmalemma) of α-amylase which requires phosphorylative energy but is not affected by 6-methylpurine and cycloheximide (Varner and Mense, 1972). These differences in the secretion of β-glucanase and α-amylase may represent different modes of secretion for

TABLE 2

SUBCELLULAR DISTRIBUTION OF PHOSPHORYLCHOLINE-CYTIDYL AND PHOSPHORYLCHOLINE-GLYCERIDE TRANSFERASES[a,b]

Fraction tested	PC-cytidyl transferase (pmoles/hour/mg protein)		PC-glyceride transferase (pmoles/hour/mg protein)	
	50 Layers	Protein (mg)	50 Layers	Protein (mg)
11,000g Pellet	770	328	143	60
44,000g Pellet	744	886	189	224
100,000g Pellet	301	700	92	211
Postmicrosomal supernatant	107	11	36	4

[a] From Johnson and Kende, 1971.

[b] Enzymes prepared from aleurone layers after a 4-hour incubation of half-seeds. PC, phosphorylcholine.

TABLE 3

INFLUENCE OF 0.8 *M* MANNITOL ON GIBBERELLIN-ENHANCED ACTIVITIES OF PHOSPHORYLCHOLINE-CYTIDYL AND PHOSPHORYLCHOLINE-GLYCERIDE TRANSFERASES[a,b]

Substance	PC-cytidyl transferase (pmoles CDP-choline formed/hour/mg protein)	PC-glyceride transferase (pmoles lecithin formed/hour/mg protein)
Control	251	79
GA_3 (1 μm)	450	222
0.8 *M* Mannitol	261	152
Mannitol + GA_3	389	240

[a] From Koehler *et al.*, 1972.

[b] Expressing the data as enzyme activities per 50 layers gave a similar pattern. PC, phosphorylcholine; GA_3, gibberellin; CDP, cytidine diphosphate.

the two enzymes or it may be that the cellular apparatus required for the secretion of both enzymes is made during the first few hours of gibberellin treatment. There is evidence that intracellular α-amylase and protease are localized in vesicles (Gibson and Paleg, 1972). The role of these vesicles in secretion is unknown. Vesicular localization of β-glucanase has not yet been reported.

It has been reported that cyclic adenosine monophosphate (c-AMP) could replace or partly replace gibberellin in evoking the aleurone layer response. From the work in this laboratory it appears that c-AMP, dibutyryl c-AMP, cyclic guanosine monophosphate (c-GMP), N^2-monobutyryl c-GMP, theophylline, caffeine, and papaverine, when applied to isolated aleurone layers, cause no significant increase in α-amylase synthesis and secretion and, in many instances, cause inhibition of the usual response to gibberellin (R. A. B. Keates, unpublished results). Theophylline, caffeine, and papaverine are inhibitors of mammalian cyclic nucleotide phosphodiesterase and are used to increase endogenous levels of c-AMP. There is, however, no evidence that these compounds inhibit plant phosphodiesterase (Lin and Varner, 1972). These compounds do not prevent the breakdown of labeled c-AMP incubated with aleurone layers (R. A. B. Keates, unpublished). There is no effect of c-AMP on the incorporation of $^{32}P_i$ into phospholipids. If, as seems likely, increased phospholipid syn-

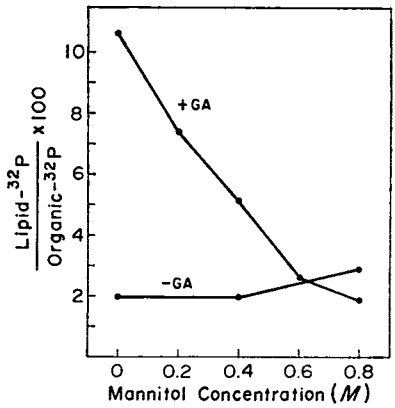

FIG. 7. Effect of increasing mannitol concentrations on gibberellin-enhanced incorporation of $^{32}P_i$ into phospholipids. (Koehler *et al.*, 1972.)

thesis is an essential part of the response to gibberellins, c-AMP could not increase α-amylase synthesis without first increasing the rate of $^{32}P_i$ incorporation into phospholipids. It also appears that gibberellic acid does not enhance the conversion of labeled adenosine to c-AMP, although it causes a twofold increase in the appearance of label in a compound that is chromatographically similar but not identical to c-AMP (R. A. B. Keates, unpublished).

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THE BIOCHEMISTRY OF ETHYLENE: BIOGENESIS AND METABOLISM

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Introduction

The interest in ethylene as a plant-growth regulator has extended over many years and, although it was clear even in the mid-nineteenth century that the presence of gaseous materials in the air could modify the growth of plants (Girardin, 1864), the identity of ethylene as an active agent was not established until the turn of the century by Neljubow (1901), who reported that ethylene was the component of illuminating gas causing reduction of elongation, increased radical expansion, and horizontal growth in pea seedlings. Flower growers also knew that traces of illuminating gas escaping in their glasshouses would result in premature closure of the blossoms (Crocker and Knight, 1908). An early report that ethylene could

exert physiological effects on fruits can be traced to Cousins (1910), who found that gases from oranges caused ripening of bananas in mixed commercial shipments. (It now is almost certain that the ethylene produced did not come from the oranges but from fungi growing on the oranges, since oranges produce very little ethylene.) Soon afterward, Sievers and True (1912) found that the emanations of incomplete combustion of kerosene hastened the degreening in citrus fruits. Twelve years later, Denny (1924) demonstrated that the ethylene in the emanations was the agent responsible for the coloring effect.

The first well-established indication that ethylene was a natural plant product came in 1932, when Elmer (1932) found that apples gave off a volatile substance that inhibited the growth of potato sprouts, and chemical proof that ethylene was indeed produced by ripe apples was provided by Gane (1934). Since then it has been shown that ethylene is produced essentially from all parts of higher plants such as leaves, stems, roots, flowers, fruits, tubers, and seedlings (Burg, 1962). The high production of ethylene from climacteric fruits during their ripening is well known (Biale, 1960a). Quantities of ethylene evolved by fruits range from 50 nl/kg/hour for Valencia oranges to 350 μl/kg/hour for passion fruit. However, higher plants are not the only biological source of ethylene. Biale (1940) and, independently, Miller *et al.* (1940) made the classic discovery that the common green mold on citrus furits, *Penicillium digitatum*, produced ethylene at a high rate. Many other fungi have also been shown to produce ethylene (Nickerson, 1948; Biale, 1960b; Ilag and Curtis, 1968; Chalutz and DeVay, 1969), as have some bacteria (Freebairn and Buddenhagen, 1964); it may even be evolved by human subjects (Chandra and Spencer, 1963a).

Ethylene as a plant hormone (endogenous plant growth regulator) initiating fruit ripening and regulating the growth of vegetative organs was first suggested by Crocker *et al.* (1935). Later, Hansen (1943) and Kidd and West (1945) concluded that endogenous ethylene is the factor responsible for the induction of the climacteric of respiration and associated ripening changes. On the other hand, Biale and his co-workers (1954), using the less sensitive manometric method available at that time(Young *et al.*, 1952), observed that some fruits underwent marked phases of ripening without evolving measurable amounts of ethylene and, therefore, put forward the view that native ethylene is a by-product of ripening. With the advent of sensitive gas chromatographic instruments, Lyons *et al.* (1962) and Burg and Burg (1962) have shown that fruits accumulate intracellular concentrations of ethylene high enough to stimulate ripening before the onset of the climacteric in respiration; these data and others (Burg and Burg, 1965;

Pratt and Goeschl, 1969; McGlasson, 1970) have confirmed the idea of ethylene acting as a ripening hormone. Once the ripening process is initiated, more ethylene is produced as part of the syndrome of ripening and this ethylene is certainly a by-product, as suggested by Biale. Since gas chromatographic techniques became available, work on ethylene physiology has been extended to other aspects of plant growth and development. The list of ethylene-regulated phenomena includes breaking of dormancy, regulation of swelling and elongation, hypertrophy, induction of adventitious roots, epinasty, hook closure, inhibition of leaf expansion, control of flower induction, exudation, ripening, senescence, and abscission. Many excellent reviews on general ethylene physiology (Burg, 1962; Pratt and Goeschl, 1969; Spencer, 1969; Mapson and Hulme, 1970) and fruit ripening (Biale, 1950; Burg and Burg, 1965; Hansen, 1966; Pratt and Goeschl, 1969; McGlasson, 1970) have appeared.

All earlier chemical methods for measuring ethylene production must now be considered obsolete since, with the flame-ionization gas chromatograph, one can assay 10^{-12} mole of the gas in 1 ml of gas sample. The absorption of ethylene by mercuric perchlorate and its release by the chloride ion (Young *et al.*, 1952), when used in conjunction with gas chromatography, increases the specificity of the method and also allows ethylene to be concentrated for subsequent analysis. The methods of ethylene assay have been discussed in the reviews of Burg (1962), Mapson (1969), and Pratt and Goeschl (1969).

The chemical structure required for ethylene-like action in higher plants has been investigated (Knight *et al.*, 1910; Knight and Crocker, 1913; Denny, 1935; Crocker *et al.*, 1935; Burg and Burg, 1967; Abeles and Gahagan, 1968), and the results obtained are in fair agreement, indicating that the order of activity is ethylene > propylene > carbon monoxide and/or acetylene. The relative biological activity of ethylene and its homologs on several different tissues are recorded in Table 1. Alkanes and halogenated alkanes are inactive. Burg and Burg (1967) have investigated this problem by assaying the inhibition of elongation of pea stem sections using chromatographically pure gases. They concluded that biological activity requires an unsaturated bond adjacent to a terminal carbon atom, is inversely related to molecular size, and is decreased by substitution which lowers the electron density in the unsaturated position. In kinetic studies on the effect of ethylene on the inhibition of elongation of pea stem sections, Burg and Burg (1967) estimated that the Michaelis-Menten constant for ethylene attaching to its receptor site was 6×10^{-10} M (or 0.14 ppm in the gas phase); this attachment is competitively inhibited by CO_2 with a K_I of 5×10^{-4} M (or 1.6 percent in the gas phase). In addition, oxygen is re-

TABLE 1

BIOLOGICAL ACTIVITY OF ETHYLENE AND OTHER UNSATURATED GASES[a]

Compound	Inhibition of growth			
	Pea stem[b]	Tobacco[c]	Abscission[d]	Epinasty[e]
$CH_2=CH_2$	1	1	1	1
$CH_2=CH-CH_3$	100	100	60	500
$C=O$	2,700	1,600	1,250	5,000
$CH\equiv CH$	2,800	100	1,250	500
$CH_2=CH-CH_2-CH_3$	270,000	2,000	100,000	500,000

[a] Data expressed as relative concentrations required for half-maximum activity. CH_4, CH_3-CH_3, and $CH_3-CH=CH-CH_3$ are inactive.

[b] Data from Burg and Burg, 1967.

[c] Data from Zimmerman, 1935.

[d] Data from Abeles and Gahagan, 1968.

[e] Data from Crocker *et al.*, 1935.

quired for ethylene action with a Michaelis-Menten constant of 40 μM (or 2.8 percent in the gas phase). On the basis that the biological activity of olefins is closely parallel to the binding affinity of those olefins to silver ion, Burg and Burg (1967) suggested that the receptor site contains a metal. There is so far no experimental evidence to prove or disprove this hypothesis (Abeles *et al.*, 1972; Beyer, 1972).

Research into the mechanism of action of ethylene has been active, resourceful, and stimulating. Nevertheless, the primary action of ethylene still remains unknown. The most important recent reviews covering this subject are those of Spencer (1969) and Abeles (1972).

In this review, I shall deal exclusively with the biogenesis and metabolism of ethylene. These topics have also been covered by a number of reviews (Jansen, 1965; Mapson, 1969; Pratt and Goeschl, 1969; Spencer, 1969; Yang and Baur, 1969; McGlasson, 1970; Abeles, 1972).

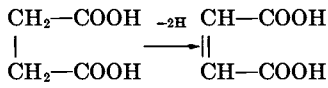
Biochemistry of Ethylene Production in Higher Plants

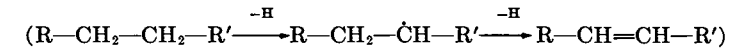
Although progress with this problem has been aided tremendously by the development of sensitive gas chromatographic and radiotracer techniques, studies on ethylene biogenesis have encountered difficulties. The amounts of ethylene produced by plant tissues are extremely low, indicating that its biosynthetic pathway represents a tiny fraction of the overall

TABLE 2

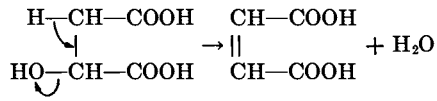
OLEFIN-FORMING BIOCHEMICAL REACTIONS

(1) Dehydrogenation

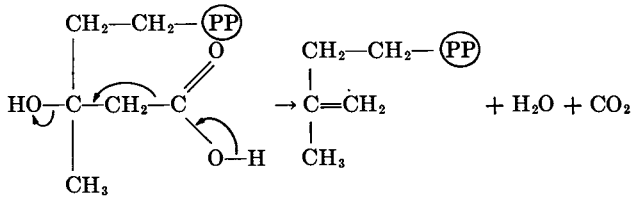




(2) Dehydration

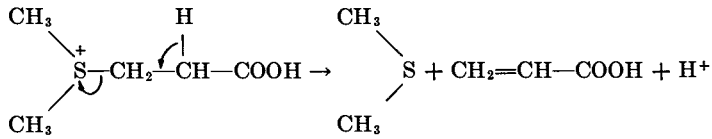


(3) Dehydration-decarboxylation



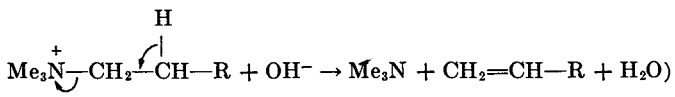
Mevalonic acid pyrophosphate Isopentenyl pyrophosphate

(4) Dethiomethylation



Dimethylpropiothetin Acrylic acid

(Hoffman degradation:



metabolic activities. For example, the rate of respiration is several hundred to several thousand times higher than that of ethylene production in fruits which are known to produce ethylene (Biale, 1960a) and more than 1000 times higher in *Penicillium digitatum* at its peak of ethylene production (Ketring *et al.*, 1968). Furthermore, due to the simple chemical structure

of ethylene, there are many compounds that could be converted to ethylene through various chemical reactions. For example, Faraday and Freeborn (1957) listed 668 methods of making ethylene, and this list is by no means complete. To discuss the biological precursors of ethylene, it is helpful to review current knowledge of the biochemical mechanism of olefin-forming reactions. Table 2 gives some examples of known olefin-forming biochemical reactions. From these it may be deduced that ethylene can be produced via two types of reaction mechanisms. One involves the formation of a $CH_3 \cdot CH_2 \cdot$ radical as an intermediate which is, in turn, oxidized to form ethylene:

$$R{-}CH_2{-}CH_3 \rightarrow \dot{C}H_2{-}CH_3 \rightarrow CH_2{=}CH_2$$

This reaction is analogous to the formation of fumarate from succinate by dehydrogenation as shown in reaction (1) of Table 2. The other type of reaction involves a concerted elimination process in which an ethylene precursor degrades into ethylene by a "push and pull" mechanism:

$$X{-}CH_2{-}CH_2{-}Y \rightarrow X^- + CH_2{=}CH_2 + Y^+$$

Reactions (2), (3), and (4) of Table 2 are examples of concerted elimination reactions.

A number of substances have been proposed as precursors of ethylene in higher plants. These include linolenic acid, propanal, β-alanine, acrylic acid, β-hydroxypropionic acid, ethionine, ethanol, ethane, acetic acid, fumaric acid, and methionine. Although it has now been established that methionine but none of the other proposed candidates just listed serves as the precursor of ethylene, a brief review of the work on these proposed precursors will show the way research in this area has progressed.

Linolenate-Propanal Pathway

The possibility that linolenic acid might serve as a precursor of ethylene was first suggested by Lieberman and Mapson (1964). Oxidation of linolenic acid to peroxidized linolenic acid is an important prerequisite for the breakdown of linolenic acid into ethylene. Evidence in favor of this proposal stems from the observation that (*a*) peroxidized linolenic acid was converted into ethylene in a model system consisting of Cu^{2+} and ascorbic acid (Lieberman and Mapson, 1964), (*b*) the rise in lipoxidase activity preceded the rise in ethylene evolution in ripening apple (Meigh *et al.*, 1967), (*c*) ethylene production by discs of apple peel was stimulated by the addition of linolenic acid and lipoxidase (Galliard *et al.*, 1968a), and (*d*) apple peel extracts catalyzed the enzymatic conversion of linolenic acid to

ethylene (Galliard *et al.*, 1968b). Subsequently, Lieberman and Kunishi (1967) found that propanal, a decomposition product of peroxidized linolenic acid, is a very effective precursor of ethylene in the model system and have, therefore, suggested that linolenic acid may be converted to ethylene via propanal *in vivo*. In the model system, Abeles (1966a) has shown that the terminal CH_3—CH_2—CH= group in linolenic acid is an essential structural feature for an active ethylene precursor. A reaction scheme for propanal formation from linolenic acid during its peroxidation reactions has been suggested (Bell *et al.*, 1951; Yang and Baur, 1969). Baur and Yang (1969a) prepared propanal-^{14}C labeled in C-1 or C-2 and studied the fate of the carbons in the Cu^{2+}-ascorbate-mediated model system. They found that propanal-2-^{14}C, but not propanal-1-^{14}C, yielded radioactive ethylene and ethane. The ratio of the yield of ethylene to ethane was about 7 : 1. Since the specific activity of the derived ethylene or ethane equalled that of the propanal, it was concluded that carbons-2 and -3 were converted to ethylene or ethane as a unit. When propanal-l-^{14}C was used as a substrate, formic acid was the major product. Based on the findings that carbon-1 of propanal gives rise to formic acid and carbons-2 and -3 to ethylene or ethane and that ascorbate is oxidized through a free radical mechanism, a reaction mechanism accounting for the reactions has been proposed (Baur and Yang, 1969a). This mechanism involves the formation of a $C_2H_5\cdot$ radical as an intermediate, which is, in turn, either oxidized by Cu^{2+} to yield ethylene (Kochi, 1967) or is reduced by ascorbate (AH_2) to yield ethane:

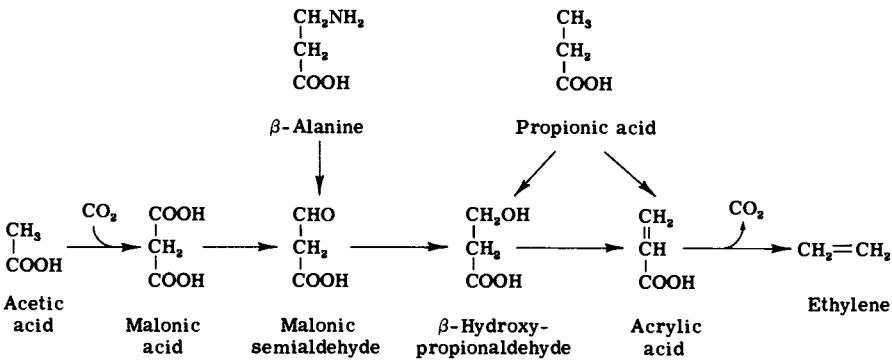
$$C_2H_5\cdot + Cu^{2+} \rightarrow CH_2{=}CH_2 + H^+ + Cu^+$$

$$C_2H_5\cdot + AH_2 \rightarrow CH_3{-}CH_3 + AH\cdot$$

In order to determine the efficiencies of propanal-2-^{14}C and linolenic acid-U-^{14}C as ethylene precursors *in vivo*, Baur and Yang (1969a,b) administered them to apple plugs along with methionine-U-^{14}C for comparison. Their results showed that propanal and linolenic acid were actively converted to CO_2, but there was no significant conversion to ethylene. In the same system, methionine was an excellent precursor of ethylene. In this regard, it is pertinent to note that breakdown of peroxidized linolenic acid (Lieberman and Mapson, 1964) or of propanal (Baur and Yang, 1969a) by the Cu^{2+}-ascorbate model system formed a large amount of ethane, which is not produced naturally in any significant amount by ripening fruits (Meigh, 1959). It was, therefore, concluded that a linolenate or linolenate-propanal pathway is not operative in apple tissue. This conclusion was confirmed by other investigators for apple and tomato tissues (Lieberman and Kunishi, 1969; Mapson *et al.*, 1970).

β-Hydroxypropionic Acid and Acrylic Acid Pathway

The possibility that β-alanine might serve as an ethylene precursor was proposed by Thompson and Spencer (1967) and Stinson and Spencer (1969), based on their observation that an enzyme preparation from bean cotyledon catalzyed a slight conversion of β-alanine to ethylene. From the cofactor requirements, they proposed that β-alanine is converted into ethylene via malonic semialdehyde, β-hydroxypropionic acid, and acrylic acid as intermediates. A similar reaction (Scheme 1) starting with acetate was proposed by Shimokawa and Kasai (1970a,b,c), largely based on their observations that (*a*) there was a slight conversion of acetic acid-2-^{14}C and propionic acid-2-^{14}C into the ethylene by banana slices, (*b*) the conversion of these acids to ethylene is inhibited by the addition of unlabeled β-hydroxypropionate or malonate, (*c*) β-hydroxypropionate stimulates ethylene production by the banana tissue, and (*d*) banana pulp extracts catalyze a very low conversion (less than 0.005 percent) of acrylic acid into ethylene.



Scheme 1

Although these authors presented indirect evidence in favor of Scheme 1, direct evidence supporting the operation of this scheme *in vivo* is lacking. For example, they failed to show that acrylic acid and β-hydroxypropionic acid, which would be direct precursors of ethylene, are converted into ethylene at a higher efficiency than are acetic acid or propionic acid which are distant precursors in their biosynthetic scheme. Furthermore, since methionine has been established as the most probable precursor of ethylene (as will be discussed later), this proposal has little significance because they failed to conduct a study comparing the ability of methionine and the precursors proposed in their scheme to serve as precursors of ethylene in their *in vivo* system. However, some investigators have compared the efficiencies

of methionine and those of intermediates in the scheme as precursors of ethylene in other fruit tissues. Lieberman and Kunishi (1969) found that although propionic acid, which is an intermediate in this scheme, stimulates ethylene production in tomato tissues, it was not converted to ethylene, whereas methionine was efficiently converted to ethylene by this tissue. Yang and Baur (1972) fed β-alanine-3-^{14}C and β-hydroxypropionic acid-3-^{14}C (which are the intermediates of Scheme 1) to apple tissue and found that neither was appreciably converted to ethylene, whereas methionine-U-^{14}C was an effective precursor. Earlier investigators (Jansen, 1965) also reported that malonic acid and propionic acid, which are closely related to malonic semialdehyde and acrylic acid in metabolism, are not efficient precursors of ethylene. Acetate-^{14}C as a precursor of ethylene has been studied by Burg and Burg (1964) in apple tissues and by Sakai *et al.* (1970) in sweet potato root tissue; they found a very low conversion efficiency. This conclusion was fully confirmed in this laboratory; whereas methionine was efficiently converted into ethylene in apple and avocado tissues, no measurable incorporation from acetate was observed. In apple tissues (Burg and Burg, 1964), as well as in healthy sweet potato root tissue (Sakai *et al.*, 1970), both carboxyl and methyl carbons of acetate were converted into ethylene with equal efficiency. These results are not compatible with the acrylic acid pathway as depicted in the foregoing, since the carboxyl carbon of acetate would be incorporated into the carboxyl group of acrylic acid and would then be lost as CO_2 during the decarboxylation step in which acrylic acid is converted to ethylene. It should be pointed out that if one assumes that acetate is converted to ethylene via methionine, then both carbons of acetate should be incorporated with equal facility (Yang, 1968). These results thus contradict the acrylic pathway but are fully compatible with the methionine pathway. In contrast to this finding, Sakai *et al.* (1970) reported that, in black rot-infected sweet potato root tissue, the methyl carbon of acetate was preferentially incorporated into ethylene over the carboxyl carbon. They, therefore, concluded that the pathways of ethylene production in healthy and in fungus-infected tissues are different. In view of the recent finding that the black rot fungus, *Ceratocystis fimbriata*, produces ethylene (Chalutz and DeVay, 1969), it is very likely that this difference was caused by ethylene production from the fungus in the infected tissues. As will be discussed in a later section, the pathway of ethylene production in higher plants is different from that in the fungus, *Penicillium digitatum*. In *P. digitatum*, the methyl carbon, but not the carboxyl carbon, of acetate is efficiently converted into ethylene (Gibson and Young, 1966). If the mechanism of ethylene biosynthesis in *C. fimbriata* should be identical to that in *P. digitatum*, it is understandable that the pattern of ethylene

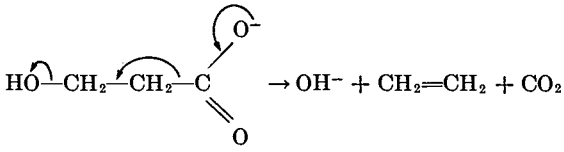
biosynthesis changes in sweet potato root tissue after it is infected with *C. fimbriata.*

If acrylic acid is the immediate precursor of ethylene as proposed, then the ethylene-anion, which is an intermediate of the decarboxylation, should pick up a proton from water to yield ethylene:

$$CH_2{=}CH{-}COO^- + H^+ \rightarrow CH_2{=}CH_2 + CO_2$$

Thus, one proton will be incorporated into the ethylene molecule. To examine this possibility, Yang and Baur (1969) administered tritiated water to apple plugs and determined the specific activity of the ethylene produced by the tissue. Although ethylene was found to be labeled, the specific activity of ethylene was only one-eighth that of the water within the tissue. These results tend to rule out the possibility that ethylene is formed directly from acrylic acid through a simple decarboxylation. In summary, there is no conclusive evidence supporting the idea that a β-alanine–acrylic acid pathway or an acetate–acrylic acid pathway operate to produce ethylene in higher plant systems.

β-Hydroxypropionic acid may undergo a concerted dehydration and decarboxylation, yielding ethylene in a manner analogous to the formation of isopentenyl pyrophosphate from mevalonic-5-pyrophosphate as suggested by Varner (1961):



However, since β-hydroxypropionate was not converted to ethylene by apple tissue, as already mentioned, this pathway should also be ruled out.

Ethionine Pathway

Shimokawa and Kasai (1967) suggested ethionine as a possible precursor of ethylene based on their finding that the ethyl moiety of ethionine was converted to ethylene in the presence of flavin mononucleotide (FMN) and light. Although we confirmed that, in the presence of FMN and light, the ethyl moiety was converted to ethylene *in vitro*, the ethylene formed from the ethyl moiety represented less than 1 percent of the total ethylene formed. The bulk (99 percent) of the ethylene was derived from carbons-3 and -4 of ethionine (Yang, 1970). In this system, the ethyl moiety of ethionine yields both ethylene and ethane in about equal amounts suggesting the formation of an ethyl radical as the common intermediate (Yang, 1970). When L-ethionine, labeled in either ethyl group or carbons-3 and -4, was

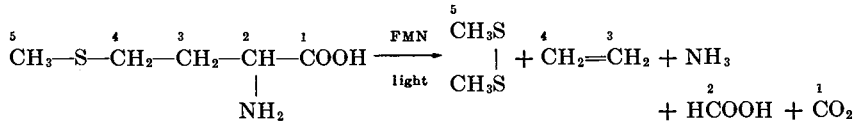
administered to apple plugs, no conversion to ethylene was observed (Yang and Baur, 1972). It is therefore concluded that ethionine is not a precursor of ethylene *in vivo*.

Other Precursors of Ethylene

Based on chemical reasoning, a number of other compounds have been speculated about as potential precursors of ethylene. For example, ethylene may be derived from ethanol, ethane, or fumaric acid via simple dehydration, dehydrogenation, or decarboxylation, respectively. When tested, none of these was found to be an immediate precursor of ethylene (Jansen, 1965; Biale, 1960b; Mapson, 1969). Bitancourt (1968) proposed that ethylene may be derived from the indole ring of indoleacetic acid (IAA). This suggestion is interesting since IAA, a natural plant hormone, promotes ethylene production in vegetative tissues (Zimmerman and Wilcoxon, 1935; Abeles and Rubinstein, 1964a; Burg and Burg, 1966). To test this hypothesis, we incubated subapical sections of pea seedlings with IAA labeled with ^{14}C on the acetic acid side chain or labeled with ^{3}H at the 5 position of the indole ring. Although ethylene production was greatly stimulated, there was no radioactive ethylene (S. F. Yang, unpublished results).

Methionine Pathway

It is now fairly well established that methionine is the precursor of ethylene in higher plants. Methionine as a possible precursor of ethylene was first suggested by Lieberman and Mapson (1964), based on the observation that methionine was rapidly converted into ethylene in the model system consisting of Cu^{2+} and ascorbic acid. In this conversion, methional (3-methylthiopropionaldehyde) appears to be an intermediate, and ethylene is derived from carbons-3 and -4 of methionine (Lieberman *et al.*, 1965). In 1964, Abeles and Rubinstein (1964b) observed that ethylene was formed nonenzymatically from the crude extract of pea seedlings in the presence of FMN. Yang *et al.* (1966) have since shown that this is a light-dependent reaction and identified the active substrate in the pea seedling extract as methionine. By using methionine-^{14}C labeled in different carbons, Yang *et al.* (1967) showed that methionine was efficiently degraded into ethylene and other products as represented by the following equation:



Kinetic data, as well as the results obtained from the analysis of the reaction products, are consistent with the assumption that the photochemical production of ethylene from methionine proceeds through the formation of methional as an intermediate. One possible mechanism accounting for the sequence of the reaction is described in Fig. 1. The first step consists of the transfer of an electron from the sulfur atom of methionine to the photoactivated FMN, followed by removal of hydrogen from the amino group. The mechanism that explains the formation of ethylene from methional consists of electron transfer from the sulfur atom of methional to the photoactivated FMN, followed by a nucleophilic attack by OH^- on the aldehyde group and a concerted elimination of the methylsulfonium group. It is interesting to note that 2-chloroethanephosphonic acid (Ethrel, ethephon), a synthetic growth regulator (Cooke and Randall, 1968), decomposes rapidly in neutral solution to give ethylene, chloride ion, and phosphate ion. The mechanism proposed for this reaction (Maynard and Swan, 1963) is essentially identical to that depicted in Fig. 1 for the methional

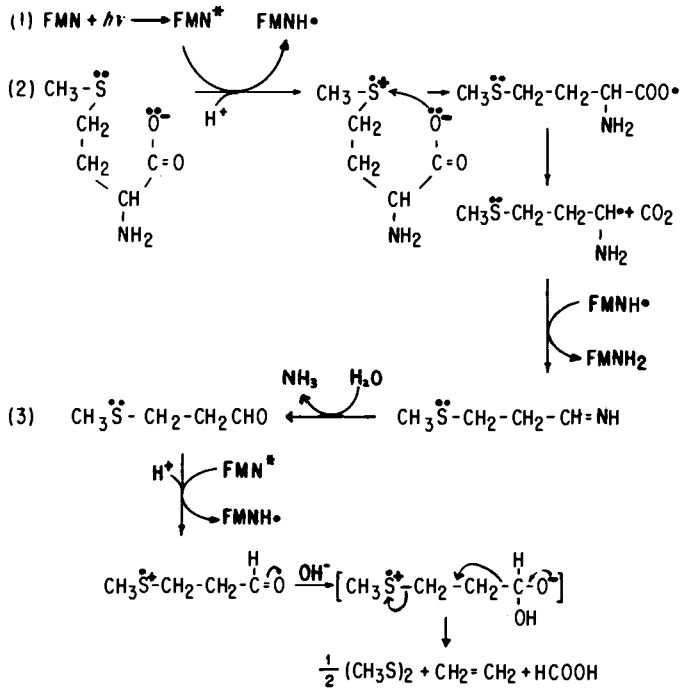
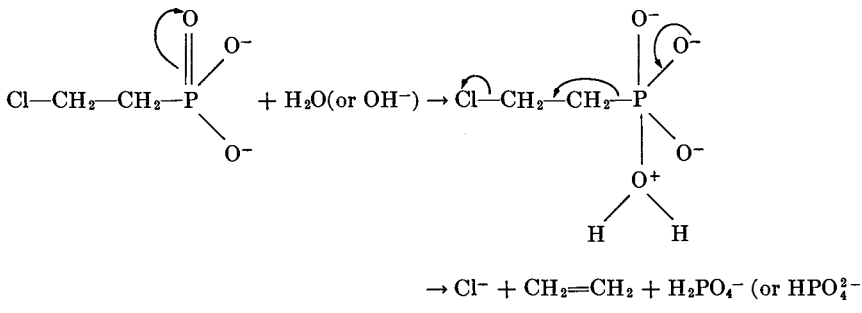


FIG. 1. Proposed scheme for the conversion of methionine to ethylene by flavin mononucleotide (FMN) and light. (From Yang *et al.*, 1967.)

radical, but with the electron-pulling $CH_3—\overset{+}{S}$ function replaced by a chlorine atom and the electrophilic function of carboxaldehyde replaced by a phosphonic acid group:



$$\rightarrow Cl^- + CH_2{=}CH_2 + H_2PO_4^- \text{ (or } HPO_4^{2-}\text{)}$$

It appears that a molecule possessing a $—CH_2—CH_2—$ group in the center of the molecule, with one end attached to an electron-withdrawing function and the other end attached to an electrophile (which is subject to an nucleophilic attack), is an excellent ethylene producer via a concerted elimination mechanism.

Direct evidence in support of the role of methionine as an ethylene precursor *in vivo* was first reported by Lieberman *et al.* (1966), who found that methionine-^{14}C was efficiently converted to ethylene by apple tissue; as in the model system, ethylene was derived from carbons-3 and -4 of methionine. These findings were subsequently confirmed by other investigators with apple and other plant tissues. In apple tissue, Burg and Clagett (1967) found that the conversion of methionine to ethylene represents the major pathway of methionine metabolism, when the amount of methionine supplied exogenously is low. In addition to apple tissue, the role of methionine as an ethylene precursor was also demonstrated in banana tissue and IAA-treated pea stem sections (Burg and Clagett, 1967), in IAA-treated mung bean stem sections (Sakai and Imaseki, 1972), in tomato and cauliflower floret tissues (Mapson *et al.*, 1970), in avocado tissue (Baur *et al.*, 1971), and in normal leaves of bean plants or leaves treated with 1-naphthaleneacetic acid, $CuSO_4$, 3,6-endoxohexahydrophthalic acid, or ozone (Abeles, 1972).

Methionine As a Physiological Precursor of Ethylene

There is little doubt that methionine can serve as a precursor of ethylene *in vivo*. It is pertinent to ask whether such conversion of methionine to ethylene occurs under natural physiological conditions and parallels the natural pattern of ethylene evolution, and to what extent the ethylene

produced *in vivo* is derived from methionine. The following observations are consistent with the proposal that methionine is indeed a physiological precursor of ethylene: (*a*) fruit tissues convert the L-form rather than the D-form of methionine to ethylene, indicating that the steps are controlled by a stereospecific enzymatic reaction (Baur and Yang, 1969a); (*b*) conversion of methionine to ethylene was observed only in the climacteric avocado, which actively produces endogenous ethylene, but not in the preclimacteric fruit which produces little ethylene (Baur *et al.*, 1971), and thus, the ability of fruit tissues at different stages of ripening to convert methionine into ethylene parallels the ability to produce ethylene endogenously; (*c*) conversion of methionine into ethylene occurs in pea or mung bean stems that have been pretreated with IAA and produce significant amounts of ethylene, whereas control tissues that produce little ethylene do not convert methionine to ethylene (Burg and Clagett, 1967; Sakai and Imaseki, 1972); (*d*) ethylene production from bean and tobacco leaves increased rapidly following the application of toxic compounds, such as $CuSO_4$, Endothal, and ozone, and these treatments that increased ethylene evolution also increased the conversion of methionine into ethylene (Abeles and Abeles, 1972); and (*e*) when the amount of methionine administered to apple tissues was increased, the specific radioactivity of ethylene recovered approached that of the administered methionine (Owens *et al.*, 1971; Yang and Baur, 1972). These results indicate that methionine is the major if not the sole precursor of ethylene in apple tissue. Although methionine has not been established as the major precursor of ethylene in plant tissues other than apples, no evidence has been put forward that ethylene may be synthesized from sources other than methionine.

It is well known that ethylene can be produced nonenzymatically or nonbiologically from a wide variety of compounds (Lieberman and Kunishi, 1967; Kumamoto *et al.*, 1969) including the degradation products of oxidized unsaturated fatty acids which exist abundantly in living organisms. The physiologically regulated ethylene production system produces ethylene in large amounts but produces negligible amounts of the other hydrocarbon gases. In contrast, the nonbiological systems produce extremely small amounts of ethylene along with other hydrocarbon gases such as ethane. It is very likely that such nonregulated, nonbiological ethylene production is derived from sources other than methionine and plays an insignificant physiological role. These systems may include the slight ethylene production from subcellular components of plants and animals (Chandra and Spencer, 1962, 1963b; Lieberman and Hochstein, 1966), from human subjects (Chandra and Spencer, 1963a), and possibly also that from preclimacteric fruits.

Substrate Specificity for Methionine

To study substrate specificity, Yang and Baur (1972) prepared several radioactive derivatives of methionine and administered them to apple tissues to determine their efficiency in producing ethylene relative to methionine. The results indicate that the structural requirements for methionine are very specific. When the carboxyl group is methylated (methionine methyl ester) or deleted (β-methylthiopropylamine), the substrate loses its activity as an ethylene precursor. When the amino group is acetylated (*N*-acetylmethionine), essentially all of its activity as an ethylene precursor is lost. Unlike methionine methyl ester, which is very poorly converted to CO_2, *N*-acetylmethionine is very actively converted to CO_2. When the sulfide function is oxidized (methionine sulfoxide), the activity as an ethylene precursor is reduced to 14 percent as compared to methionine. Since in plant tissues methionine sulfoxide is known to be reduced back to methionine, the active precursor could well be the methionine formed from the administered methionine sulfoxide. When the methyl group is substituted by an ethyl group (ethionine), activity as an ethylene precursor is completely lost. When the CH_3S— group is deleted from methionine (α-aminobutyric acid), all activity as a precursor is lost. Thus, all the functional groups of methionine (carboxyl, amino, ethylene, and methylmercapto) are essential. Other analogs of methionine that are active as ethylene precursors are the methionine keto analog, α-keto-γ-methylthiobutyric acid (KMBA), *S*-methylmethionine, and homoserine. Since they are converted into ethylene less efficiently than methionine and are metabolically closely related to methionine, it is likely that they are converted to methionine prior to their conversion into ethylene. Methional, however, was found to be an inactive substrate (Baur and Yang, 1969b).

Ku and Leopold (1970) and Demorest and Stahmann (1971) have suggested that ethylene may be derived from peptides or protein-containing C-terminal methionine. This proposal was based on their observations that *N*-acetylmethionine or other *N*-acylated derivatives of methionine were converted to ethylene in the peroxidase system of Yang (1967, 1969). However, since *N*-acetylmethionine was not converted into ethylene as already mentioned, and peroxidase is not involved in the *in vivo* system, there is no experimental support for this hypothesis.

Degradation Products of Methionine during Its Conversion to Ethylene

In apple tissue, it was shown that C-1 is converted to CO_2, C-2 to formic acid, and carbons-3 and -4 to ethylene; sulfur appears to be retained in the tissue, since no volatile radioactivity was detected with ^{35}S-labeled

methionine (Lieberman *et al.*, 1966; Burg and Clagett, 1967; Siebert and Clagett, 1969). Therefore, except for the CH_3S– group, the products of the *in vivo* conversion are identical to those of the FMN-light model system. Yang and Baur (1972) found that the level of free methionine plus protein methionine in apple is about 60 nmoles/gm, whereas its ethylene production rate is 5nmoles/gm-hour. Apples are known to sustain a high rate of ethylene production for months. Physiologically, it is probably important that no volatile sulfur compounds be evolved, since all the carbons of methionine can be derived from glucose, which is very abundant in apple, but sulfur would quickly become limiting if the methionine sulfur were lost as a volatile. These data indicate that the sulfur atom of methionine must be conserved and recycled during the continous synthesis of ethylene. Baur and Yang (1972) have presented evidence showing the recycling of methionine sulfur in apple tissue; these results are summarized in Fig. 2.

When methionine-^{35}S or methionine-[methyl-^{14}C] was administered to apple tissue, *S*-methylcysteine was identified as the major metabolic product. Although neither CH_3SH nor its derivatives were identified as intermediate products, when CH_3SH-^{14}C- or -^{35}S was administered to apple tissue, it was efficiently converted to S-methylcysteine. It is, therefore, thought that when methionine is degraded to yield ethylene, the CH_3S– group is transferred as a unit to serine derivatives to form S-methylcysteine. In the conversion of S-methylcysteine to methionine, sulfur is incorporated preferentially over the methyl group. S-Methylcysteine is first demethylated to yield cysteine, which then donates its sulfur to form methionine presumably through cystathionine and homocysteine. Such a view is supported by the observations that cysteine, homoserine, and homocysteine were all converted to methionine in a logical order of efficiency; although homoserine, homocysteine, and methionine are all converted into ethylene, methionine is the most efficient presursor, followed by homocysteine and homoserine. Methionine is thus shown to be closest to the immediate precursor of ethylene (Baur and Yang, 1972).

Enzymes and Intermediates Involved in the Conversion of Methionine to Ethylene

There is considerable confusion in the literature concerning the enzymes and intermediates involved in the conversion of methionine to ethylene. Although the peroxidase system was later shown not to be involved in ethylene biosynthesis *in vivo*, these peroxidase-catalyzed ethylene production systems are of much biochemical interest, and a brief summary of this work will serve to show the way research in this area has progressed.

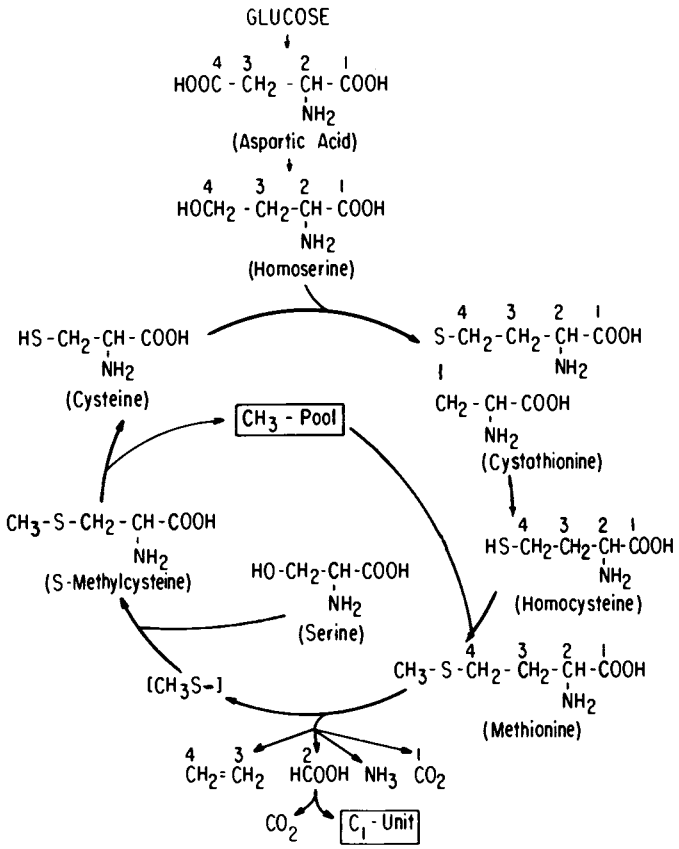
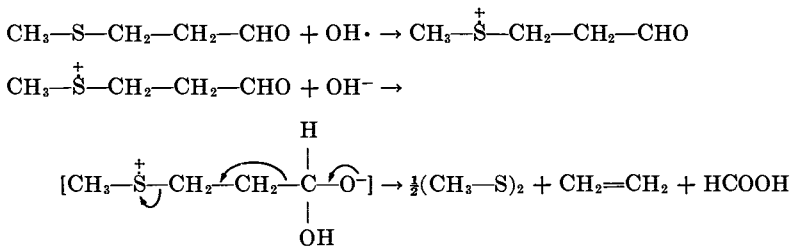


FIG. 2. Methionine–sulfur cycle in relation to the biosynthesis of ethylene in apple tissue. (From Baur and Yang, 1972.)

The enzymatic systems for conversion of methionine analogs to ethylene were first reported in 1967 by Mapson and Wardale (1967) from cauliflower florets and by Ku *et al.* (1967) from pea seedlings. Both systems produced ethylene from methional but not from methionine and required enzyme and cofactor fractions (Yang, 1968). Noting that these enzyme systems resemble peroxidase in many aspects and that peroxidase catalyzes the oxidation of a number of compounds through radical intermediates, Yang (1967) subsequently succeeded in reconstructing a very efficient system in which horseradish peroxidase catalyzed the conversion of either methional or KMBA to ethylene (Yang, 1967, 1968). The cofactors required are sulfite, monophenol, Mn^{2+}, and oxygen; H_2O_2 can replace Mn^{2+} and oxygen.

The biochemical mechanism accounting for this enzymatic reaction has been elucidated by Yang (1967, 1969) and by Beauchamp and Fridovich (1970). The OH· radical produced during the peroxidase-catalyzed sulfite oxidation serves as a 1-electron oxidant to oxidize methional to methional sulfonium radical, which is subsequently degraded to ethylene through a concerted elimination mechanism, as depicted in the following equations:



The resemblance of this horseradish peroxidase system to the cauliflower and pea seedling systems led Yang (1967) to speculate that both systems involved peroxidases. Since then Mapson and Wardale (1968) have re-examined their cauliflower enzyme and have found that their enzyme was in fact a peroxidase. It is interesting to note that the cofactors isolated from the cauliflower system were identified by Mapson and Mead (1968) and by Mapson *et al.* (1969a) as a monophenol (an ester of *p*-coumaric acid) and methanesulfinic acid; sulfite is effective in replacing methanesulfinic acid. They showed that their peroxide-generating system was probably a glucose oxidase. In identifying all these accessory factors and enzymes, cauliflower extracts were used. Ku *et al.* (1969) and Takeo and Lieberman (1969) also isolated peroxidase enzymes from tomato and apple tissue, respectively, which catalyzed the production of ethylene from methional or KMBA. In the presence of transaminase, methionine is converted to KMBA and can, therefore, serve as an ethylene precursor in the peroxidase system.

Since then, Baur and Yang (1969b) have found that, in apple tissues, methional was not converted to ethylene. Although KMBA was readily converted to ethylene, the efficiency of its conversion was less than that from methionine (Baur and Yang, 1969b; Baur *et al.*, 1971). They therefore concluded that KMBA is not an intermediate in the conversion of methionine to ethylene. On the contrary, Mapson *et al.* (1969b) reported that KMBA stimulated ethylene production in cauliflower floret tissue. The cauliflower floret tissue system showed greater incorporation of ^{14}C into ethylene from ^{14}C-labeled KMBA than from ^{14}C-labeled methionine. They concluded that KMBA is the intermediate in the biochemical pathway between methionine and ethylene. The first step in the conversion of

methionine to ethylene was considered to be a transaminase reaction which formed the KMBA intermediate. The apparent discrepancy in the findings of these two groups has been recently resolved in a study by Lieberman and Kunishi (1971a) who found that peroxidase and other components of an ethylene-forming model system leak from cauliflower tissue into the buffer solution surrounding the tissue under the experimental conditions employed by Mapson and his co-workers (1969b). As a result, in the external buffer solution, an artificial ethylene-forming system that can degrade KMBA to ethylene is created. When the cauliflower tissues were not soaked in buffer solution, the tissues failed to respond to KMBA. Therefore it appears that KMBA is not an intermediate in the conversion of methionine to ethylene.

The peroxidase-catalyzed ethylene production is also different from the natural *in vivo* ethylene production pathway in a number of respects. As just discussed the peroxidase system utilizes methional and KMBA, but not methionine, whereas in intact tissue, methional is a poor precursor, and methionine is the most efficient precursor. It is also known that monophenols promote ethylene production and that *o*-diphenols are inhibitors of the peroxidase system (Yang, 1967; Mapson *et al.*, 1969a). In contrast, ethylene production from intact tissue was not greatly influenced by the addition of either class of phenolic compounds (Gahagan *et al.*, 1968). Catalase is also known to be a potent inhibitor of the peroxidase system, but it failed to inhibit ethylene production by cauliflower tissue (Lieberman and Kunishi, 1971a). In the peroxidase system, Yang and Baur (1969) showed that C-2 of KMBA was converted to CO_2, whereas *in vivo* C-2 of methionine was not converted to CO_2 (Burg and Clagett, 1967; Siebert and Clagett, 1969). Finally, Kang *et al.* (1971) reported that there was no correlation between the amount of peroxidase in pea stem tissue and the rate of ethylene evolution. They found that IAA treatment caused a dramatic stimulation of ethylene production, but there was a slight decrease in peroxidase content in the tissue.

In conclusion, the available results fail to support the proposal that KMBA, methional, and peroxidase are involved in ethylene biosynthesis *in vivo*. Enzymatic systems for ethylene production from methionine have been sought actively in several laboratories. However, there is no sign of a breakthrough yet.

It was rather surprising to find that tissues, such as apple and tomato fruits, which evolve relatively large quantities of ethylene *in vivo*, gave virtually no ethylene upon homogenation, despite the use of various protective measures such as nitrogen atmosphere, addition of reducing agents, and polyvinylpyrrolidone (Lieberman and Kunishi, 1971b). It has been

generally assumed that the ethylene-producing system must be extremely labile. Indeed, the ethylene-producing system in IAA-treated pea sections has been shown to be labile and has a rapid turnover rate. Recently, Sakai and Imaseki (1973) reported that an extract of mung bean seedlings contains a heat-stable protein that greatly inhibits the ethylene production by the IAA-treated mung bean segments. Thus, it is quite possible that, during the processes of isolation of enzymes from tissues, the disruption of the cell causes mixing of enzymes and their inhibitors that would be rigidly compartmentalized in the living cells. As a result, no enzyme activity can be demonstrated until the inhibitors are removed.

Regulation of Ethylene Production

Ethylene production *in vivo* is known to be regulated by various physiological and environmental factors. For example, ethylene production is induced during certain stages of growth, such as germination, ripening of fruits, and abscission, by wounding, disease, radiation and other physical and chemical stresses, and by treatment with IAA or other growth regulators (Burg, 1962; Pratt and Goeschl, 1969; Spencer, 1969). It has been shown that new formation of enzymatic protein is required for the induction of ethylene evolution, since protein and RNA synthesis inhibitors effectively inhibit the induction of ethylene evolution in fruit tissues (Frenkel *et al*., 1968), in auxin-treated tissues (Abeles, 1966b), and in chemically stressed tissues (Abeles and Abeles, 1972). Until the enzyme system that catalyzes the conversion of methionine to ethylene is elucidated our current knowledge will be too limited to permit discussion of its regulation. Nevertheless, it is apparent that the regulation is exerted not on the synthesis of the ethylene precursor, methionine, but on the conversion of methionine to ethylene. Because methionine is not converted to ethylene in preclimacteric fruit tissues or in pea or bean seedling tissues, in which the normal endogenous ethylene production rate is low and because active conversion of methionine to ethylene is observed in fruit tissues during the climacteric rise or climacteric peak or in pea or bean seedling tissues that have been treated with IAA (Burg and Clagett, 1967; Baur *et al.*, 1971; Sakai and Imaseki, 1972), it is concluded that the control of ethylene production in those tissues occurs at a step subsequent to the synthesis or methionine. This conclusion was further supported by the results of Baur *et al.* (1971) who have shown that, during the climacteric peak, the ethylene production rate by avocado tissue increased 3000-fold, but the levels of methionine dropped by about 50 percent.

It is apparent that the level of methionine concentration is not a regulatory factor. The role of oxidative metabolism in ethylene formation was

first demonstrated by Hansen (1942) who found that ethylene production did not occur when oxygen was removed. He also noticed that there was a surge of ethylene production following the return of pear tissue from a nitrogen atmosphere to air. A similar phenomenon in apple tissue was observed by Burg and Thimann (1959). Baur *et al.* (1971) have studied the oxygen requirement for the conversion of methionine to ethylene by apple tissues and have found that oxygen is absolutely required for this conversion. Their data suggest that the methionine is converted into ethylene by apple tissues in two steps: the first step is an oxygen-independent reaction in which methionine is converted to an unidentified intermediate, followed by an oxygen-dependent reaction in which the intermediate is degraded into ethylene. Removal of oxygen from the fruit tissues causes immediate cessation of ethylene production without any observable lag phase, suggesting that molecular oxygen may participate directly in the conversion of methionine to ethylene.

The evolution of ethylene by sorghum seedlings, IAA- and kinetin-treated sorghum seedlings, and apple tissues is markedly inhibited by rhizobitoxine, a phytotoxin produced by certain strains of the bacterium, *Rhizobium japonicum* (Owens *et al.*, 1971). The conversion of methionine into ethylene by apple tissue was also inhibited by rhizobitoxine to about

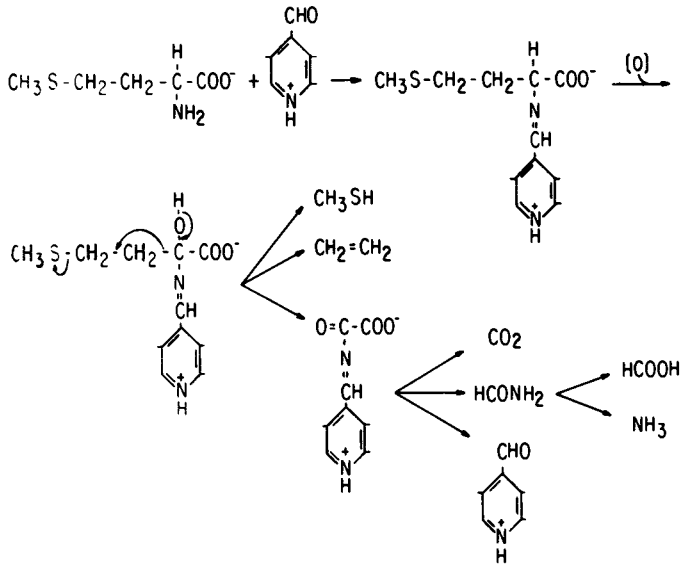


FIG. 3. Proposed mechanism of ethylene formation from methionine. (From Yang and Baur, 1972.)

the same extent as was total ethylene evolution, suggesting that rhizobitoxine interferes with ethylene biosynthesis by blocking the conversion of methionine to ethylene (Owens *et al.*, 1971). Since rhizobitoxine is known to inhibit other pyridoxal phosphate-dependent biochemical reactions (Giovanelli *et al.* (1971), Owens *et al.* (1971) suggest that the enzyme involved in converting methionine to ethylene also requires pyridoxal phosphate as a cofactor. It should be noted that pyridoxal phosphate-mediated γ-elimination reactions have been recognized in biological systems (Flavin, 1963). Based on the foregoing observations that pyridoxal phospate coenzyme and molecular oxygen may be involved in the reaction and on the available information with regard to the degradation products of methionine, Yang and Baur (1972) have proposed a concerted elimination reaction mechanism involving pyridoxal phosphate to account for the biological production of ethylene from methionine (as shown in Fig. 3). There is yet no direct evidence in support of such a scheme.

Biochemistry of Ethylene Production in Fungi

Ethylene is thought to be a common metabolic product of fungi (Ilag and Curtis, 1968), and it may play an important role in the growth of host plants. Among the microbes that produced ethylene, none is known to equal *Penicillium digitatum* in the rate of production from a comparable medium. This copious production of ethylene led to its being the first such fungus discovered (Biale, 1940; Miller *et al* ., 1940). Essentially all of the the work on the biosynthesis of ethylene by fungi has been carried out with *P. digitatum.*

The high rate of ethylene production and the ease of introducing proposed precursors offered distinct advantages to investigators working with this fungus. At first it was thought that this fungus could be used to determine easily the biosynthetic mechanism for ethylene and that this information could be applied to the biosynthetic pathway of higher plants, but this did not prove to be the case. It is now clear that the biosynthetic pathway in *P. digitatum* is entirely different from that of higher plants, (Jacobsen and Wang, 1968; Ketring *et al.*, 1968). Acetic acid has been shown to be an efficient precursor of ethylene in this fungus, and in its conversion C-2, but not C-1, is incorporated into ethylene (Gibson and Young, 1966), whereas in higher plants acetic acid is a very poor precursor of ethylene and in its conversion into ethylene both carbons are equally incorporated (Burg and Burg, 1964). Rhizobitoxine effectively inhibits ethylene evolution by tissues of higher plants but is ineffective in inhibition of ethylene

production by the fungus (Owens *et al.*, 1971). Using tritiated water, it has been shown that during the biosynthesis of ethylene by *P. digitatum* in glucose medium, three out of four ethylene hydrogens are derived from water (Chou and Yang, 1973), but in apple tissue there was little incorporation of hydrogen from water (Yang and Baur, 1969). More direct evidence is that *P. digitatum* utilizes glutamic acid but not methionine as its immediate precursor, whereas higher plants utilize methionine but not glutamic acid. Although considerable effort by many investigators has been devoted to the biosynthesis of ethylene in this fungus, the biosynthetic pathway has not been fully elucidated, nor has the enzymatic formation of ethylene been demonstrated. Nevertheless, glutamic acid is now established as an immediate precursor of ethylene, and some interesting biochemistry has been revealed.

Ethanol yields ethylene through dehydration and has been suggested as the immediate precursor of ethylene (Phan, 1962; Abeles, 1972). Chou and Yang (1973) have compared the conversion efficiencies of ethanol-2-^{14}C and acetate-2-^{14}C and have found that acetate was incorporated 10 times more efficiently. The observation that C-2 of ethanol, rather than C-1, was preferentially incorporated into ethylene excludes the possibility that ethylene is derived from ethanol through simple dehydration.

The operation of glycolysis and the Krebs cycle pathway in this mold have been previously demonstrated (Reed and Wang, 1959). With the use of specifically ^{14}C- labeled glucose and amino acids (glycine, alanine, glutamic acid, and aspartic acid) which are closely related to members of the Krebs cycle, Wang *et al.* (1962) demonstrated that all were actively converted to ethylene, and they proposed that conversion of glucose to ethylene occurred via glycolysis and the Krebs cycle. Unfortunately, the duration of the experiment conducted by Wang *et al.* (1962) was too long (ethylene production was followed daily for a period of 6 days) to permit critical evaluation as to which substrate is the most immediate precursor of ethylene. Although glutamic acid-3,4-^{14}C was found to be an efficient precursor of ethylene, they suggested that glutamate is first converted via the Krebs cycle to fumaric acid which then donates the middle carbons to give rise to ethylene (Wang *et al.*, 1962). Later, Jacobsen and Wang (1965) suggested that acrylic acid may be derived from fumaric acid and give rise to ethylene via decarboxylation. However, more recent work of Jacobsen and Wang (1968) showed that C-2 of acrylic acid was preferentially incorporated into ethylene over C-3, and that its conversion efficiency into ethylene was lower than that of acetate-2-^{14}C.

Gibson (1964) found that C-3 of pyruvate showed the highest incorporation into ethylene, but C-1 was not incorporated. Gibson and Young (1966)

found that C-2 of acetate, but not C-1, is extensively incorporated into ethylene and suggested that the path of ethylene biosynthesis is not via the steps of the Krebs cycle. Ketring *et al.* (1968) carried this line of work further and found that acetate-2-^{14}C and citrate-2,4-^{14}C were quite comparable as ethylene precursors in short-time incubation; citrate 1,5-^{14}C was not incorporated. Their data again suggest that ethylene production by this fungus is closely associated with the Krebs cycle. Since the condensation of acetate-2-^{14}C with oxalacetate to form citrate results in the labeling of the C-4 of citrate, they suggest that C-4 of citrate is converted to ethylene. Ketring *et al.* (1968) further showed that monofluoroacetate, which effectively blocks the conversion of citrate to isocitrate, markedly inhibits ethylene formation and the incorporation of label from acetate or citrate into ethylene. Addition of isocitrate restored ethylene production. Since other members of the Krebs cycle, including α-ketoglutarate, succinate, malate, *cis*-aconitate, or citrate, are ineffective in reversing the inhibition, isocitrate is considered to be the branching point from which ethylene is derived. These results led Ketring *et al.* (1968) to suggest the following sequence for the biosynthesis of ethylene by *P. digitatum:*

$$\text{acetate} \rightarrow \text{citrate} \rightarrow \text{isocitrate} \rightarrow \rightarrow \text{ethylene}$$

Chou and Yang (1973) confirmed the observation of Ketring *et al.* (1968) that fluoroacetate effectively inhibited ethylene production, but they were unable to confirm the observation that isocitrate could overcome the inhibition. We found that the inhibition of ethylene production by fluoroacetate can be overcome simply by adjusting the culture medium from pH 3.0 to around pH 6. Since the culture medium employed by Ketring *et al.* (1968) had little buffer capacity near pH 3 and required a relatively higher concentration of sodium isocitrate to overcome the fluoroacetate inhibition, we suspect that the effect of sodium isocitrate reported by Ketring *et al.* (1968) may well be no more than a pH effect. If this is the case, then their conclusion that isocitrate is the branching point had to be reexamined. Chou and Yang (1973) therefore reinvestigated the efficiency of other members of the Krebs cycle as ethylene precursors by *P. digitatum* and compared their conversion efficiency with that of acetate-2-^{14}C during short-time incubation (30 minutes). The conversion efficiency of glutamate-3,4-^{14}C was more than twice as much as that of acetate-2-^{14}C. Furthermore, the ratio of the specific radioactivity in ethylene to that in CO_2 was 10 for acetate-2-^{14}C but was 170 for glutamate-3,4-^{14}C. These data strongly indicate that glutamate is a more efficient precursor of ethylene than is acetate. This conclusion was further supported by the observation that the incorporation of acetate-2-^{14}C into ethylene was greatly reduced by the addition

of nonlabeled glutamate, but the incorporation of glutamate-3,4-^{14}C was not significantly reduced by the presence of nonlabeled acetate. Because glutamate and α-ketoglutarate (KGA) are interconvertible through transamination, and the latter is a member of the Krebs cycle, we also compared the conversion efficiency of these two substrates, both labeled on carbons-3 and -4. Since both substrates are incorporated into ethylene with equal efficiency, we are unable to reach a conclusion as to which substrate is closer to the immediate precursor of ethylene. The member of the Krebs cycle next to α-ketoglutaric acid is succinic acid, so the conversion efficiency of succinate-2,3-^{14}C was compared with that of glutamate-3,4-^{14}C. In the first 40-minute incubation, the conversion efficiency of succinate was only 3 percent of that of glumate. These results indicate that KGA is the branching point at which the path of ethylene production leads away from the Krebs cycle. To show whether ethylene is derived from carbon-3, carbon-4, or carbons-3 and -4 of glutamate, glutamate-3-^{3}H and glutamate-3,4-^{14}C were supplied to the *P. digitatum* culture in a dual-label experiment. Analysis of ethylene was followed every 30 minutes for 90 minutes. If ethylene is derived from C-3 only, C-4 only, or carbons-3 and -4 of glutamate, theory predicts that the ratio of ^{3}H/^{14}C in the ethylene should be 2, 0, or 1, respectively. The ratio found during the first, second and third 30-minute incubation was 1.02, 0.98 and 0.91, respectively (Table 3). It is therefore concluded that carbons 3 and 4 of glutamate are converted to ethylene as a unit. Wang *et al.* (1962) had previously shown that carbons-1, -2, and -5 of glutamic acid were not converted into ethylene. The proposed pathway for the biosynthesis of ethylene from glucose by *P. digitatum* is shown in Fig. 4. The earlier results to the effect that C-2 of acetate is a more efficient precursor of ethylene than C-2,3 of succinate or fumarate, whereas neither

TABLE 3

ETHYLENE FORMATION FROM DUAL-LABELED GLUTAMIC ACID HOOC—^{14}CH$_2$—^{14}C^{3}H$_2$—CH(NH$_2$)—COOH (^{3}H/^{14}C = 1)[a]

Incubation (minutes)	^{3}H/^{14}C Ratio in ethylene			
		Theory		
	Found	C-3	C-4	C-3,4
0–30	1.02			
30–60	0.98	2.0	0.0	1.0
60–90	0.91			

[a] From Chou and Yang (1973).

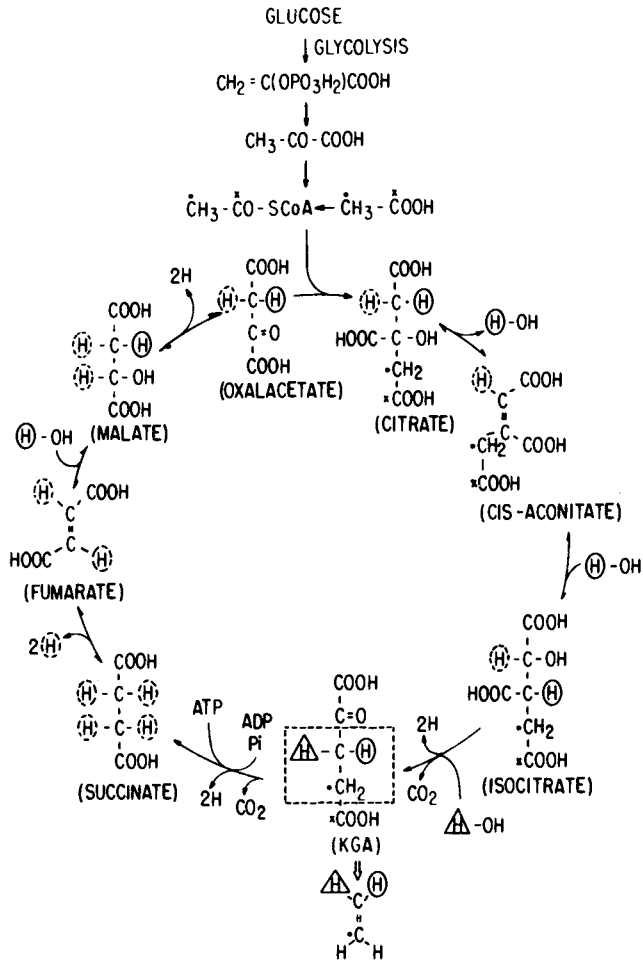


FIG. 4. Biosynthetic pathway of ethylene in *Penicillium digitatum* in relation to Krebs cycle. Acetyl CoA (with methyl carbon Ċ and carboxy-carbon $\overset{x}{C}$, respectively), which is formed from glucose via glycolysis, condenses with oxalacetate to form citrate. The C-4 of citrate is derived from C-2 of acetyl CoA. Action of aconitase leads to the formation of isocitrate, picking up a hydrogen marked Ⓗ at C-3 from the medium. In the course of the isocitrate dehydrogenese reaction, the hydrogen marked Ⓗ on C-3 is retained, and a new one, marked Ⓐ, is introduced from the medium due to decarboxylation. α-Ketoglutarate (KGA) is the branching point at which the path of ethylene biosynthesis leads away from the cycle. Thus, the two hydrogens attached to one of the ethylene carbons are derived from the medium protons, whereas the other methylene group is derived from the methyl group of acetyl CoA. For the purpose of discussion of the fate of carbons and hydrogens of succinate in relation to ethylene formation, the hydrogens attached to carbons-2 and -3 of succinate are designated Ⓗ. Following two

C-1 of acetate nor C-1,4 of those carboxylic acids are converted to ethylene (Gibson and Young, 1966; Jacobsen and Wang, 1968), can be explained well with this scheme. Chou and Yang (1973) observed that when acetate was supplied to the fungus culture grown on glucose at a concentration of 10 to 30 m*M*, the specific radioactivity of ethylene collected was occasionally equal or slightly higher (1.1 times) than that of the substrate, acetate-2-^{14}C. The data suggest that both carbon skeletons of ethylene are derived directly or indirectly from the methyl carbons of acetate. This anticipation is fully consistent with the pathway depicted in Fig. 4 in which C-4 of glutamate is directly derived from C-2 of acetate, whereas C-3 of glutamate is derived from C-2 of oxalacetate part of which is, in turn, derived from C-2 of acetate in the previous passage through the Krebs cycle. According to the scheme presented in Fig. 4, the two hydrogens attached to C-3 of KGA are derived from water. The C-4 of KGA is derived from the C-2 of acetyl CoA. In the conversion of PEP to pyruvate, there is an incorporation of one proton into the C-3 of the pyruvate molecule, and additional incorporation of protons can be anticipated if the pathway of the decarboxylation of oxaloacetate or malate into pyruvate becomes significant. If the scheme for the biosynthesis of ethylene in *P. digitatum* is as proposed, it is anticipated that there is incorporation of at least three protons into the ethylene molecule. Recent results of Chou and Yang (1973) confirm this prediction. They administered tritiated water in the culture medium of *P. digitatum* and measured the radioactive ethylene produced in successive 30-min periods. Chou and Yang (1973) found that the specific radioactivity of ethylene increased sharply and became constant after 60 minutes. By comparing the specific radioactivity of ethylene with that of the water in the medium, it was concluded that three protons were incorporated per molecule of ethylene produced. Finally, it should be noted that, whereas succinate-2,3-^{14}C was moderately converted to ethylene, succinate-2,3-^{3}H was not incorporated. This was in contrast to the incorporation of glutamate in which both glutamate-3,4-^{14}C and glutamate-3-^{3}H were equally incorporated in a short incubation period. These data can be fully explained by the scheme of Fig. 4: in the conversion of succinate to KGA via the Krebs cycle, all of the hydrogen in carbons 2 and 3 of succinate are lost (assuming that the pathway of the conversion of malate or ox-

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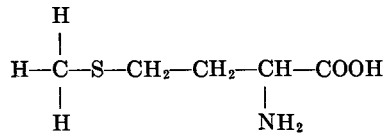
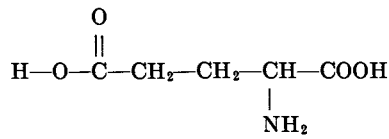
dehydrogenation steps, three of the four hydrogens in succinate molecule are removed, yielding oxalacetate. After condensation with acetyl CoA, the last remaining (H) from succinate is finally lost in the conversion of isocitrate to KGA. On the other hand, carbons-2 and -3 of succinate are converted to carbons-2 and -3 of KGA via the Krebs cycle, with C-3 of KGA being converted to ethylene. Thus, the middle carbons of succinate, but not the hydrogens attaching to them are incorporated into ethylene. (From Chou and Yang, 1973.)

alacetate into pyruvate is negligible), whereas one of the middle carbons of succinate will be incorporated into C-3 of KGA and therefore into ethylene.

In an attempt to examine whether there is any intermediate of KGA or glutamate that is a closer precursor of ethylene, Chou and Yang (1973) supplied a number of labeled substrates closely related to glutamate in metabolism. These substrates include L-proline-3,4-^{3}H, DL-ornithine-3-^{3}H, succinyl semialdehyde-2,3-^{14}C, DL-γ-aminobutyric acid-2,3-^{14}C, DL-α-aminobutyric acid-3-^{14}C, and L-glutamine-U-^{14}C. However, none of them was converted to ethylene with an efficiency comparable to that of glutamate-3-^{3}H or glutamate-3,4-^{14}C. It is, therefore, concluded that glutamate is the closest precursor of ethylene so far tested. Although the pathway of ethylene biosynthesis in higher plants is different from that in *P. digitatum*, it is interesting to note the similarity in chemical structure of methionine, precursor in higher plants, and that of glutamic acid, the precursor in *P. digitatum:*



Methionine



Glutamic acid

Whether such similarity bears any significance in the process of evolution between higher plants and fungi is an interesting question.

Although ethylene is produced in large amounts by *P. digitatum*, its physiological significance is unknown.

Metabolism of Ethylene

It was thought earlier that the study of ethylene metabolism in higher plants, especially in fruits, might lead not only to an understanding of the mechanism of ethylene action but might also provide a knowledge of the biosynthesis of ethylene, on the supposition that the enzyme reactions for metabolizing ethylene and synthesizing ethylene are reversible. Unfortunately, this was found not to be the case, because incorporation of ethylene into tissue was extremely small, and it was incorporated into a number of

metabolically unrelated compounds. Furthermore, it is thought that this slight metabolism of ethylene observed might be due to the metabolism of impurities in the applied ethylene rather than that of ethylene itself. Behmer (1958) exposed apple tissue to 1000 ppm of ethylene-^{14}C for 19 days and found no incorporation of radioactivity into the tissue. However, several workers have reported slight incorporation of ^{14}C- and ^{3}H-labeled ethylene into plant tissues. Buhler *et al.* (1957) found that ripe avocados and green pear fruits exposed to 1000 ppm of ethylene-^{14}C for several days incorporated 0.05 percent of the radioactivity, largely into the organic acid fraction, including fumaric and succinic acids. On the other hand, experiments with ripe oranges, limes, papayas, green apples, tomatoes, and grapes failed to show any incorporation into fruit tissues. Hall *et al* (1961) found that cotton and *Coleus* plants incorporated ethylene-^{14}C into a variety of compounds. The rate of incorporation was enhanced greatly by using ethylene-^{14}C that had been first absorbed on mercuric perchlorate and then regenerated with chloride ion. The extent of enhancement was 25-fold following a single trapping-regeneration procedure and 1000-fold following a second such procedure. Jansen (1965) also studied the difference in incorporation between fresh and mercuric perchlorate-regenerated ethylene. With ethylene-^{14}C, Jansen (1965) showed 0.02 and 0.04 percent incorporation into avocado fruit in a 4-hour exposure to atmospheres containing 250 and 2000 ppm of the labeled gas. Approximately one-quarter of the metabolites were volatile. Benzene and toluene were identified as metabolites. Compared with fresh ethylene, regenerated ethylene was incorporated to a much greater extent and produced much more labeled CO_2. These results led Jansen (1965) to conclude that ethylene metabolism is apparently obscured by the rapid metabolism of impurities in the regenerated ethylene, and the metabolism of regeneraged ethylene bears little resemblance to that of fresh ethylene. It seems reasonable to assume that the incorporation measured represents an artifact due to impurities in the applied ethylene. This would be especially true in the case where regenerated ethylene was used. It should be noted that the mercuric perchlorate solution used to trap ethylene contains 2 *M* perchloric acid (Young *et al.*, 1952), which is an oxidizing agent. Oxidation of ethylene by perchloric acid during the trapping process might account for the generation of impurities. Alternatively, impurities might arise from radiation decomposition (Tolbert and Lemmon, 1955).

In spite of the suggestions that the use of regenerated ethylene for ethylene metabolism experiments may not bear any physiological significance, Shimokawa and Kasai (1968) and Shimokawa *et al.* (1969) also used regenerated ethylene-^{14}C to study the fixation, translocation, and metab-

olism of ethylene in the Japanese morning glory. Intact seedlings were found to fix ethylene, and it was not transported to other parts of plant once it was incorporated. Isolation of labeled material indicated that ethylene had a high affinity for ribonucleic acid (RNA). They suggested that the binding of ethylene or its metabolite to RNA may cause conformational changes in 4*S* RNA which, in turn, plays a role in hormonal regulation by ethylene.

Beyer (1972) and Abeles *et al.* (1972) examined the physiological effect of deuterated ethylene and the possibility of exchange between the deuterium of the ethylene and cellular constituents. They found no exchange of hydrogen between the ethylene and the tissues. These results coupled with the facts that there was very little, if any, incorporation of ethylene into cellular components, suggest that ethylene functions catalytically, without being incorporated into cellular components in higher plants.

In animals, ethylene has generally been considered not to be metabolized. Although some conversion of ethylene-^{14}C to CO_2 and urinary products has been noted (Van Dyke and Chenoweth, 1965), ethylene is rapidly eliminated from the lungs by exhalation.

In *Penicillium digitatum*, ethylene appears to be a metabolic by-product, since no significant metabolism of ethylene by this mold has been observed when ethylene-^{14}C is supplied during various stages of growth (Chou and Yang, 1973).

Ethylene is one of the most serious air pollutants effecting vegetation. It is estimated that the total emission of ethylene by cars and other man related activities in the United States is 15 million tons annually (Table 4). The contribution from vegetation is estimated to be about 20 thousand tons or 0.1 percent of the total emission (Abeles *et al.*, 1971). In spite of

TABLE 4

ETHYLENE EMISSION FROM MAJOR SOURCES IN THE UNITED STATES, 1966[a]

Source	Million tons	(%)
Coal combustion	0.5	3
Fuel oil combustion	0.2	1
Motor fuels	14.2	93
Refuse burning	0.3	2
Vegetation	0.02	0.1

[a] For sources of values, see Abeles *et al.*, 1971.

this constant production of large amounts of ethylene, the levels of ethylene in rural areas are still very low. It is apparent that there are natural ways by which ethylene is removed or destroyed. The major mechanisms for removal include oxidation by ozone, photochemical reaction with nitrogen dioxide (Leighton, 1961), and aerobic microbial reactions in the soil (Abeles *et al.*, 1971). Abeles *et al.* (1971) estimated that 7 million tons of ethylene is removed annually by soil microorganisms. However, the species of microorganisms and the biochemical nature of the removal of ethylene have not been elucidated. In this respect, it is pertinent to note that many microorganisms including bacteria, yeasts, and molds have been shown to be able to utilize one or more kinds of hydrocarbons including olefins as carbon sources (ZoBell, 1950; Allen *et al.*, 1971).

ACKNOWLEDGMENTS

Our work, cited herein, has been supported by research grants from the National Science Foundation. I wish to express my sincere thanks to Professor H. K. Pratt who has inspired me to work in this field and has critically read this manuscript.

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