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THE CONTROL OF GIBBERELLIN BIOSYNTHESIS IN HIGHER PLANTS

by

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SUMMARY

Some of the mechanisms by which gibberellin biosynthesis in plants is controlled were investigated by studying the efficiency with which young barley and dwarf rice seedlings and young apricot fruits converted exogenous radioactive metabolites to gibberellins.

A large part of the work was directed at improving the methods for extracting and purifying gibberellins from plant tissue; particular emphasis was placed on reducing the tedium and variability with which these procedures had been previously associated. The methods developed for gibberellin extraction produced consistent results with recoveries of added gibberellin A_3 of 50-60%. Purification of extracts, which was achieved with paper chromatography, paper electrophoresis and thin-layer chromatography, produced dry weight reductions of the crude extracts of 200-fold without any detectable loss of gibberellin. Techniques for subsequent gas-liquid chromatographic analysis of the extracts were also developed which, aided by collection and bioassay of the appropriate portions of the effluent, permitted partial identification of the biologically active compounds in the extracts.

Compounds akin to gibberellins A_1 and A_3 were found in extracts of young barley seedlings in amounts of 3-10 ng/plant or

30-330 ng/g dry weight. Evidence for the presence of conjugated gibberellins in barley seedlings was obtained, although their chemical nature was not established. A number of biologically active gibberellins were extracted from young apricot fruits, the most prominent one of which accounted for 70-80% of the total biological activity in the extracts and behaved during gas-liquid chromatography in a manner identical to gibberellin A_{32}. The concentration of gibberellins in the apricot fruits was in the range 5-10 µg/g fresh weight.

Incorporation experiments with $^{14}CO_2$ showed young barley seedlings were biosynthesising gibberellins A_1 and A_3 de novo from small molecular weight precursors. These experiments showed the biosynthesised gibberellin was turning over rapidly such that within 12 hours a complete turnover had resulted. The results indicated that gibberellin A_1, which was present in the seedlings in much smaller amounts than gibberellin A_3, was turning over faster than gibberellin A_3; this suggests these two compounds may not be interconverted.

Exogenous ^{14}C-mevalonic acid was not incorporated into the gibberellins of young barley seedlings or young apricot fruits. Experiments with cell-free preparations of young barley seedlings showed ^{14}C-mevalonic acid was converted into a number of non-polar isoprenoid compounds, but no radioactivity was found

associated with gibberellins A_1 or A_3.

Barley seedlings, cv. Prior, failed to elongate in response to exogenous ent-kaurenoic acid, and failed to convert exogenous ^{14}C-ent-kaurenoic acid to gibberellins A_1 or A_3.

Dwarf rice seedlings, cv.Tan-ginbozu, elongated significantly in response to exogenous ent-kaurenoic acid, and metabolised exogenous ^{14}C-ent-kaurenoic acid to a number of radioactive compounds, one of which behaved during gas-liquid chromatography in a manner identical to gibberellin A_1 in that it showed radioactivity and biological activity at the same retention time. This result, which for the first time verifies the proposed pathway of gibberellin biosynthesis in plants, indicates that the growth of the dwarf rice cultivar in response to exogenous ent-kaurenoic acid is a consequence of conversion of part of the applied compound to a true gibberellin, which is the actual stimulant of the growth response.

The significance of these findings in relation to the control of gibberellin biosynthesis in plants is discussed.

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(KYM FRANCIS FAULL)

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LIST OF ABBREVIATIONS

The following abbreviations have been used in this thesis:

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	N,O-Bis-(Trimethylsilyl)Acetamide
b.p.	Boiling point
cv.(s)	Cultivar (s)
D.W.	Dry Weight
EDTA	Ethylenediamine tetra-acetic acid (disodium salt)
F.W.	Fresh weight
g	Gram (s)
kg	Kilogram (s)
mg	Milligram (s)
µg	Microgram (s)
ng	Nanogram (s)
HMDS	Hexamethyldisilazane
hr	Hour
ml	Millilitre (s)
µl	Microlitre (s)
GA_3	Gibberellin A_3
GLC	Gas-liquid chromatography
I.D.	Internal diameter
M	Molar
m.p.	Melting point
mmole	Millimole (s)
µmole	Micromole (s)
nmole	Nanomole (s)

cm	Centimetre (s)
mm	Millimetre (s)
N	Normal
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NAD(H)	Nicotinamide Adenine Dinucleotide (reduced)
p.p.m.	Parts per million or mg/litre
PPO	2,5-diphenyloxazole
dimethyl-POPOP	1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene
RF	Distance migrated relative to the solvent front
RT	Retention time in minutes
S.E.	Standard error
s.g.	Specific gravity
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TONY	p-Tolysulphonylmethylnitrosoamide
TMS	Trimethylsilyl derivative
TMS ether/methyl ester	Trimethylsilyl ether of the methyl ester
TMS ether/ester	Trimethylsilyl ether of the trimethylsilyl ester
'Tween-20'	Polyoxyethylene(2)sorbitan monolaurate
U.V.	Ultra violet

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I.

LITERATURE REVIEW

Review Approach

The scope of this review is intended to be limited and is directed primarily toward providing a background for the research project which has been undertaken.

The first part of the review (A) is meant to serve merely as an introduction to the chemistry and physiology of the gibberellins and their natural occurrence in plants. As such no attempt will be made to evaluate in any depth any of the topics discussed in this section.

Emphasis has been placed on Part B, which is devoted to a description and evaluation of the published research on gibberellin biosynthesis in *Gibberella fujikuroi* and higher plants.

A. Introduction to the Gibberellins.

1. Historical Outline.

The history of investigations which has led to the recognition of gibberellins as an important class of plant hormones stems originally from the work of a number of Japanese botanists and plant pathologists who, in the period 1910-1940, investigated a disease of rice known now as bakanae disease. Characterised by the appearance of tall thin seedlings which markedly overgrew their uninfected neighbours this disease was shown by Sawada, in 1912, to be associated with a fungus on the affected plants (Phinney and West, 1960). In 1926 Kurosawa showed that a cell-free culture filtrate of the fungus Gibberella fujikuroi, which had been isolated from the affected rice plants, was able to reproduce the symptoms of abnormal elongation noted in the field (Stowe and Yamaki, 1957); in the following years Japanese chemists attempted to purify and identify the components of the filtrates which were responsible for the overgrowth symptoms. Their work culminated in the isolation of two crystalline, biologically active principles which were named gibberellins A and B (Yabuta and Sumiki, 1938). It has since been shown that these preparations were in fact a mixture of several similar but different gibberellins, and for this reason the concerted efforts of the Japanese toward structure elucidation of these compounds was unsuccessful

(Takahashi et al., 1955).

Little further research on gibberellins was done for the next 10-12 years until interest was revived outside of Japan. In 1951 a group in the United States Department of Agriculture commenced work on the isolation of gibberellin from the fungus Gibberella fujikuroi, and at about the same time a group at the Akers and Butterwick Research Laboratories of Imperial Chemical Industries Ltd., England, independently undertook the isolation of gibberellin. In due course a pure gibberellin was isolated from culture filtrates and its structure determined (Cross, 1954; Stodola et al., 1955; Grove, 1961; Aldridge et al., 1963).

The first indication that gibberellins or gibberellin-like substances are present in higher plants came when extracts from plants were found to mimic the effects of gibberellic acid, which had been isolated from the fungus, when applied to certain dwarf species of plants (West and Phinney, 1956; Radley, 1956).

Since then a large number of structurally different gibberellins have been isolated and identified from plants. They have been found to be widely distributed among higher plants and a considerable volume of literature has now been published on many aspects of their biosynthesis and metabolism. Their effects on plant growth and metabolism have also been extensively documented.

2. Plant Responses to Exogenous Gibberellin.

Gibberellins are of considerable interest to plant physiologists and biochemists because of the profound and often dramatic morphological, anatomical and biochemical changes which follow the application of minute doses of these compounds to plants. These changes are characterised by the absence of marked growth abnormalities and include effects on shoot growth, particularly internode elongation, seed and fruit development, dormancy, dwarfism, flowering and endosperm mobilisation (Stowe and Yamaki, 1957; Brian et al., 1960; Phinney and West, 1961; Brian, 1966; Paleg and West, 1972).

In general, higher plants have been found to have very few areas of development which are insensitive to exogenous gibberellin. Roots, however, are one notable exception. Roots of intact plants usually show no response or slight inhibition as a result of gibberellin treatment (Brian et al., 1960; Phinney and West, 1961).

3. Gibberellins as Plant Hormones.

There is now little doubt that gibberellins in higher plants conform to the classical definition of a hormone; that is, "a substance which, being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process" (Went and Thimann, 1937).

The role of gibberellin as an endosperm mobilising hormone during germination of a number of monocotyledenous species has now been firmly established (Paleg, 1960a, 1960b; Yomo, 1960a, 1960b). Gibberellin, released by the embryo, has a dramatic effect on the aleurone layer (the layer of cells surrounding the starchy endosperm) in consequence of which the aleurone layer liberates into the endosperm quantities of hydrolytic enzymes which hydrolyse the polymeric reserve materials contained therein (starch, protein etc.) to monomeric units (monosaccharides and amino acids, etc.) which are required by the embryo for growth. This is the clearest and most completely understood example of gibberellin acting precisely in a hormonal manner in plants.

Since 1950 a considerable volume of circumstantial evidence has been accumulating which suggests gibberellins act as hormones which control plant processes in addition to that of germination. These lines of evidence can be briefly summarised into three broad categories. Firstly, gibberellins are of ubiquitous occurrence

in all stages of the life cycle of higher plants, where with few exceptions they occur in very low concentrations. Secondly, exogenous application of gibberellin to plants induces a variety of responses involving growth and differentiation. The amounts necessary to produce these effects are very small and are usually of the order of micrograms. Finally, positive correlations between the levels of endogenous gibberellins and certain developmental trends, particularly for seed and fruit development, dormancy, flowering and stem growth have been obtained (Corcoran and Phinney, 1962; Brian, 1966; Ross and Bradbeer, 1971a). These lines of evidence suggest, but do not prove, that gibberellins are functional hormones in growing plants. To conform more precisely to the classical definition of a hormone it is necessary to demonstrate biosynthesis in the plant at a location which is spatially removed from the site of action of the hormone, and transport of the hormone to this site of action. Positive evidence on this point has been difficult to obtain, although recently Crozier and Reid (1971; 1972) have been able to demonstrate that the gibberellins in the shoots of normal Phaseolus coccineus seedlings originate in the roots, and biosynthesis in the root tips is followed by translocation to other parts of the plant. As has already been mentioned, roots are uniquely non-responsive to gibberellins. This is clearly a case which conforms well with

the classical definition of a hormone, since after production in the root tip the biologically active compound is translocated to other parts of the plant and is then capable of bringing about its biochemical and physiological response.

4. Chemistry of the Gibberellins.

The gibberellins are tetracyclic diterpenoid acids which have structures based on the ent-gibberellane skeleton shown in Figure 1. As is customary, substituents which are above or below the plane of the paper will be referred to as β- or α- respectively, although systematically these substituents will be ent-α and ent-β respectively. The gibberellins can be conveniently subdivided into two groups, the C20-gibberellins or ent-gibberellanes and the C19-gibberellins or ent-20nor-gibberellanes. The structures of some of the more commonly occurring gibberellins are also presented in Figure 1.

There is a trivial nomenclature for this group of compounds in which they are afforded A numbers. Such numbers are only given to naturally occurring fully identified compounds with the ent-gibberellane skeleton and appropriate biological properties (MacMillan and Takahashi, 1968). At the time of writing, 38 structurally different gibberellins have been identified in plants and the fungus Gibberella fujikuroi (Lang, 1970; Bearder and MacMillan, 1972; Hiraga et al., 1972). According to the trivial nomenclature these are described as gibberellins A_1 to A_{38}. In this system gibberellic acid, the gibberellin produced in greatest abundance by the fungus Gibberella fujikuroi and one of the most widely occurring plant gibberellins (Lang, 1970), is designated

FIGURE 1.

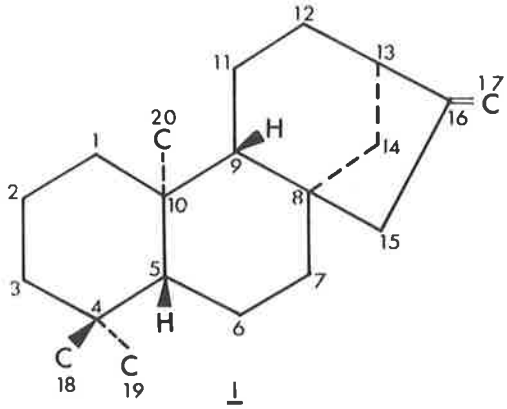
STRUCTURE OF THE GIBBERELLINS

The basic skeletons of *ent*-kaurene and *ent*-gibberellane and the structures of some gibberellins.

In this and the following 5 figures, wedges indicate bonds lying above the plane of the ring; broken lines indicate bonds lying below the plane of the ring.

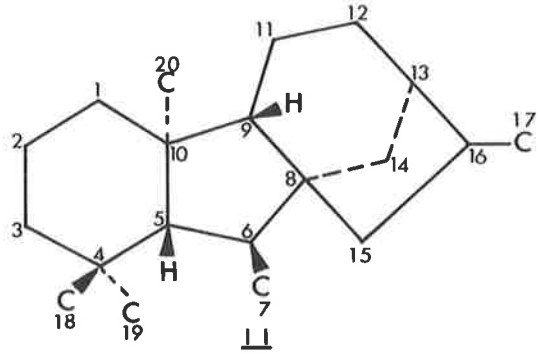
FIGURE 1.

BASIC SKELETONS



I

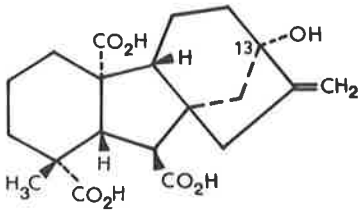
ent-KAURENE



II

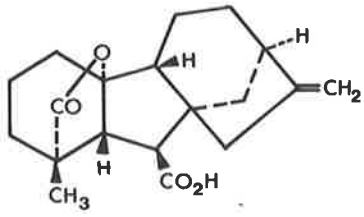
ent-GIBBERELLANE

GIBBERELLIN STRUCTURES



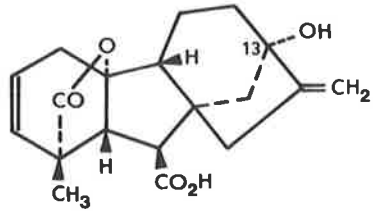
III

A_{17}



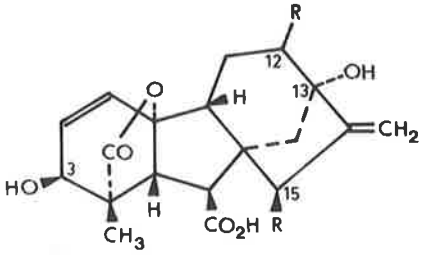
IV

A_9

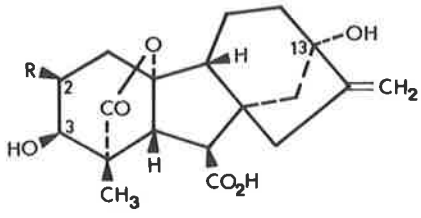


V

A_5



	R	
VI	H	A_3
VII	OH	A_{32}



	R	
VIII	H	A_1
IX	OH	A_8

gibberellin A_3. It should be noted that the A numbers of gibberellins do not necessarily follow their order of discovery.

In spite of the basic similarity the compounds which have been identified show a considerable degree of heterogeneity with respect to chemical behaviour and biological activity.

In general, the most important single factor contributing to the range of chemical behaviour covered by the known gibberellins is determined by the degree of hydroxylation of the basic skeleton. Thus the range extends from gibberellin A_9 (IV, Fig.1) the least polar gibberellin, which has a γ- lactone function, a C16-methylene and a C7-carboxyl function with no hydroxyls, to gibberellin A_{32} (VII, Fig.1) the most polar known gibberellin, which has in addition to the same γ- lactone, methylene and carboxyl functions, four hydroxyls on positions 3, 12, 13 and 15 and a C1 unsaturation (Yamaguchi et al., 1970; Coombe and Tate, 1972). Gibberellin A_3 (VI, Fig 1) has the same γ-lactone, methylene and carboxyl groupings, with two hydroxyls (positions 3 and 13) and a C1 unsaturation. As such, gibberellin A_3 has a degree of polarity intermediate between gibberellins A_9 and A_{32}.

5. Natural Occurrence of the Gibberellins.

Having been originally isolated from the fungus Gibberella fujikuroi (conidial state Fusarium moniliforme) gibberellins are now known to be virtually ubiquitous among higher plants. They have been detected in over 100 species of dicotyledons and 30 species of monocotyledons and in several conifers (MacMillan, 1971). Their occurrence in a wide variety of plant tissues and organs, including roots and all organs of the shoots, has been noted (Phinney and West, 1961).

Evidence for their presence in plants other than angiosperms and micro-organisms other than the fungus Gibberella fujikuroi has been put forward. They have been reported to occur in gymnosperms, ferns, algae and bacteria (Vancura, 1961; Kato et al., 1962; Katznelson et al., 1962; Mowat, 1963; Jackson et al., 1964; Katznelson and Cole, 1965; Schraudolf, 1966; Gairola et al., 1972), although in none of these cases has rigorous identification been achieved, and furthermore, in all cases only a limited number of examples have been reported.

The concentrations in which gibberellins occur in plants have been found to vary considerably, depending on the organ or tissue and the stage of development of the organ or tissue. Amounts of gibberellins in vegetative plant tissue invariably fall within

the range 1-20 µg/kg fresh weight (Crozier and Audus, 1968; Murakami, 1968; Grigorieva et al., 1971), while amounts of gibberellins up to 100 mg/kg fresh weight in immature seeds have been found (Corcoran and Phinney, 1962; Jackson and Coombe, 1966; Yokota et al., 1969; Takahashi et al., 1972; MacMillan, 1972). This high level of gibberellin in immature seeds is only a transitory phase as mature seeds are found to have low levels of gibberellins (Corcoran and Phinney, 1962; Jackson and Coombe, 1966; Sembdner et al., 1968). Mature seeds are, however, a good source of conjugated gibberellins, and their structure and function will be discussed in a following section.

A given species of plant has the ability to synthesise, and in some cases accumulate, several different gibberellins. Immature seed of Phaseolus coccineus contains 8, and possibly more, of the known gibberellins (Durley et al., 1971). Dark-grown seedlings of the same species contain a number of gibberellins of which 3 have been identified (Crozier et al., 1971). Immature seed of Pharbitis nil and Calonyction aculeatum have been found to contain 6 and 14 different gibberellins respectively (Murofushi et al., 1968; Takahashi et al., 1972). As pointed out by Paleg and West (1972), it is not clear which of these gibberellins function as plant hormones, which are present as biosynthetic intermediates leading to the production of other gibberellins,

which are de-activated gibberellins having no biological activity, and which, if any, are metabolic by-products of no utility to the plant.

It seems that while there may be a considerable number of gibberellins present in a given tissue at any one time, they are not all equally abundant and one or two compounds usually predominate. Thus gibberellins A_8 and A_1 comprise 67% and 22% respectively of the total gibberellin content of immature Phaseolus coccineus seed; gibberellins A_{27} and A_{26} comprise 63% and 23% respectively of the total gibberellin content of immature Calonyction aculeatum seed; gibberellin A_4 is the dominant gibberellin in dark-grown Phaseolus coccineus seedlings and gibberellin A_1 is the dominant gibberellin in light-grown Phaseolus coccineus seedlings (Murofushi et al., 1968; Yokota et al., 1969; Takahashi et al., 1972; Crozier and Reid, 1971, 1972; Crozier et al., 1971; MacMillan, 1972).

The fungus Gibberella fujikuroi has the ability to accumulate relatively large amounts of gibberellins in its culture medium. Stowe and Yamaki (1957) report levels in the range 8-544 mg/litre of culture medium. Gibberellic acid is often the dominant gibberellin in the filtrates, although, depending on the culture conditions, other gibberellins can also be produced in abundance. Sixteen chemically different gibberellins have now been identified

in culture filtrates (Lang, 1970; Bearder and MacMillan, 1972). However, the fungus is not an obligatory producer of gibberellins and it can completely lose the ability for production, particularly when grown for several generations on media devoid of rice grain nutrients (Stoll, 1954; Phinney and Spector, 1967).

6. Relative Biological Potency of the Gibberellins.

With respect to biological activity the known gibberellins show a considerable range of activity. Of the compounds represented in Figure 1, gibberellins A_1 (VIII), A_3 (VI) and A_{32} (VII) are amongst the most active, while A_8 (IX) is one of the least active known gibberellins (Crozier et al., 1970; Coombe, 1971). Attempts have been made to correlate biological activity with structure (Brian et al., 1962; 1967; Crozier et al., 1970; Yamane et al., 1973), but the correlation is confused by two compounding factors. Firstly, there are now at least nine different gibberellin bioassays in common usage, and structural requirements for activity in any one bioassay are not necessarily the same as structural requirements for activity in another bioassay. Secondly, some gibberellins can be metabolically inter-converted in plant tissues (see I.B.2(6)). Thus, if a structurally inactive gibberellin is converted to a structurally active one by the test tissue, the measured response will in fact not be due to the former compound, although it may appear to elicit a response.

An attractive hypothesis, originally proposed by Brian et al., (1964), to explain the range of biological activities demonstrated by the gibberellins, and the structural requirements for activity shown by the various bioassays, is that

gibberellins which show high biological activity have a molecular configuration which fits a receptor molecule in the plant cell. The greater the variation in structure from this, the lower the biological activity. Furthermore, variation in shape of the receptor molecule from one plant species to another could account for the species specific activity that some of the gibberellins demonstrate.

In spite of the compounding factors mentioned above, it is clear that certain structural features are necessary for biological activity. Oxygenation at the C-3 position or a C-2,3 double bond is often associated with high biological activity, as is a C-4,10 γ-lactonic function and a C-7 carboxyl group. These structural features are present in the most biologically active gibberellins A_1, A_3, A_7 and A_{32}. There seems to be a requirement for a free C-2 position, and hydroxylation here can account for reduced biological activity as in the case of gibberellin A_8 (Crozier et al., 1970; Coombe, 1971; Yamane et al., 1973).

The correlation between structure and biological activity is particularly good in the case of the cucumber hypocotyl bioassay where a 13-deoxylactonic structure is a prerequisite for high biological activity (Brian et al., 1967).

As mentioned in the previous section, gibberellins can be divided into the categories of precursors, active and deactivated gibberellins. From this point of view then it is not surprising that high biological activity is a property not common to all gibberellins.

7. Conjugated Gibberellins.

(1) Occurrence and Structure.

Beginning originally with the work of McComb (1961), considerable attention has been focused on the so-called "bound" forms of gibberellins, in which gibberellins are said to be conjugated with other low molecular weight compounds or bound to macromolecules and cell structures.

Nine different gibberellin conjugates have now been structurally identified from higher plants, and 3-O-β acetyl A_3 has been identified in culture filtrates of Gibberella fujikuroi (TABLE 1). The structures of some of these compounds are given in Figure 2.

The conjugates from higher plants include six glucosides which have been found to be particularly abundant in seeds. So far, gibberellin A_8-glucoside is the only conjugate to have been identified in tissue other than seeds.

Three glucosyl esters of gibberellins have been identified in mature Phaseolus vulgaris seed. As pointed out by Hiraga et al. (1972) it is quite possible that these conjugates correspond to the neutral gibberellins reported by other workers (Hayashi and Rappaport, 1962; Hashimoto and Rappaport, 1966).

Typically the gibberellin glucosides are completely hydrolysed by hot dilute mineral acid to yield glucose and the aglycone; the latter may be the intact gibberellin or the acid-rearranged product of it. Enzymatic hydrolysis of 2-O-β glucosides takes place readily

TABLE 1.

CONJUGATED GIBBERELLINS

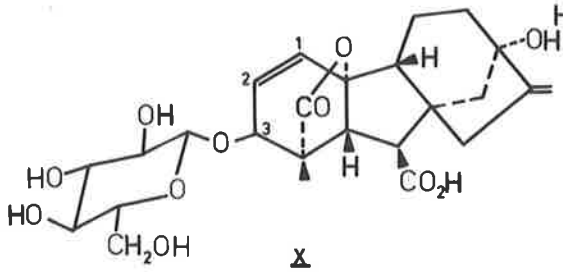
NAME	SOURCE	REFERENCE
3-O-β-Acetyl A_3	culture filtrates *Fusarium moniliforme*	Schreiber *et al.*, 1966.
3-O-β-D-glucopyranosyl A_3	immature seeds *Pharbitis nil*	Tamura *et al.*, 1968.
	immature fruits *Phaseolus coccineus*	Sembdner *et al.*, 1972.
2-O-β-D-glucopyranosyl A_8	immature fruits *Phaseolus coccineus*	Sembdner *et al.*, 1968.
	immature seed *Pharbitis nil*	Yokota *et al.*, 1969.
	shoot apices *Althaea rosea*.	Harada and Yokota, 1970.
2-O-β-D-glucopyranosyl A_{26}	immature seed *Pharbitis nil*	Yokota *et al.*, 1971.
2-O-β-D-glucopyranosyl A_{27}	immature seed *Pharbitis nil*	Yokota *et al.*, 1971.
2-O-β-D-glucopyranosyl A_{29}	immature seed *Pharbitis nil*	Yokota *et al.*, 1971.
11-O-β-D-glucopyranosyl A_{35}	immature seed *Cystisus scoparius*	Yamane *et al.*, 1971.
7-O-β-D-glucopyranosyl gibberellate A_4	mature seed *Phaseolus vulgaris*	Hiraga *et al.*, 1972.
7-O-β-D-glucopyranosyl gibberellate A_{37}	mature seed *Phaseolus vulgaris*	Hiraga *et al.*, 1972.
7-O-β-D-glucopyranosyl gibberellate A_{38}	mature seed *Phaseolus vulgaris*	Hiraga *et al.*, 1972.

FIGURE 2.

STRUCTURES OF CONJUGATED GIBBERELLINS

FIGURE 2.

GIBBERELLIN A_3 - GLUCOSIDE

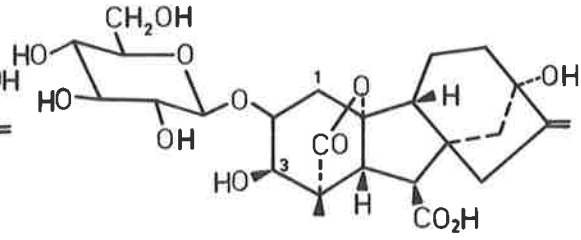


X

O-3-β-D-GLUCOPYRANOSYL

GIBBERELLIN A_3

GIBBERELLIN A_8 - GLUCOSIDE

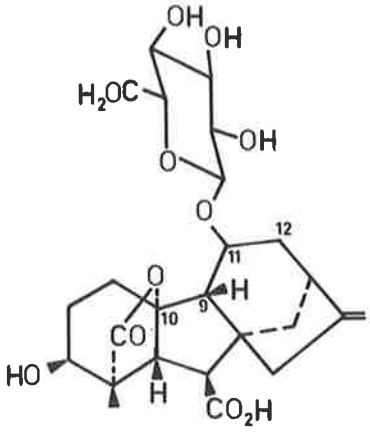


XI

O-2-β-D-GLUCOPYRANOSYL

GIBBERELLIN A_8

GIBBERELLIN A_{35} - GLUCOSIDE

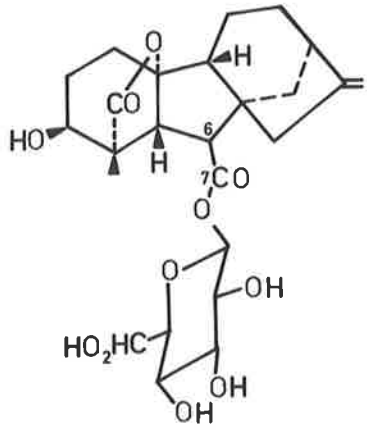


XII

11-O-β-D-GLUCOPYRANOSYL

GIBBERELLIN A_{35}

GIBBERELLIN A_4 GLUCOSE ESTER



XIII

7-O-β-D-GLUCOPYRANOSYL

GIBBERELLATE A_4

with β-glucosidases, but it is noteworthy that the same enzymes are very inefficient in hydrolysing the 3-O-β glucosides (Sembdner et al., 1972; Yokota et al., 1971).

The glucose esters are readily hydrolysed under acid and alkaline conditions (Hiraga et al., 1972), but enzymatic hydrolysis has yet to be reported.

There are a signifigant number of references which purport to demonstrate the existence of protein-bound gibberellins in plants. These claims are based on work in which plant extracts are incubated with proteolytic enzymes, which results in the appearance of more "free" gibberellin than had been present previously (McComb, 1961; Jones, 1964; Reinhard and Sacher, 1967; Pegg, 1966; Dale, 1969; Hayashi et al., 1971; Loveys and Wareing, 1971). For two reasons caution must be exercised in interpreting this work. Firstly, the enzymes which have been used (ficin and pronase) are invariably non-homogeneous (Smith and Kimmel, 1960), and, secondly, the specificity of the enzymes concerned is such that a wide range of amino acid derivatives, including dipeptides, tripeptides, amides and esters, are as readily cleaved as the bonds in peptide chains (Smith and Kimmel, 1960; Hagihara, 1960). Thus the inference of protein-bound gibberellins does not necessarily follow. In fact, the suggestion that what is being observed is the liberation of gibberellin from relatively simple amino acid or small

peptide residues finds some substantiation in the report of Sembdner and Schreiber (1965) who found shoot apices and flower buds of Nicotiana tobaccum contain, in addition to gibberellins A_1 and A_3, three polar substances, one of which upon acid hydrolysis yielded the gibberellin degradation product gibberic acid as well as amino acids and the carbohydrates glucose and rhamnose.

Evidence relating to the occurrence and structure of conjugated gibberellins is still being accumulated. Their presence in diverse materials of plant origin, for example bleeding sap of trees (Sembdner et al., 1968) and apices and seeds of plants (Harada and Yokota, 1970; Yokota et al., 1971) suggests they are a form of gibberellin of widespread occurrence among higher plants.

(2) Biological Activity and Function.

(a)Gibberellin Glucosides.

It seems quite clear that the gibberellin glucosides *per se* are biologically inactive, and any activity they elicit in a bioassay is due to the free aglycone which arises from the glucoside by prior hydrolysis in the test tissue. The very low biological potency of the glucosides forms the basis of the function or functions to which they have been ascribed in plants.

Sembdner *et al*. (1972) showed activity of gibberellin A_8-glucoside, in the dwarf pea bioassay, was equal to that of gibberellin A_8 alone. When condurit-B-epoxide, a specific inhibitor of β-glucosidases was included, the response from the glucoside was abolished although there was no effect on the response from gibberellin A_8. It was also shown that the potency of gibberellin A_3-glucoside, in four different bioassays, relative to its free aglycone, was between 0.05%-0.5%. Only when the glucoside was applied as a root drench was significant activity observed; this was thought to be due to hydrolysis of the glucoside prior to penetration into the plant.

Yokota *et al*. (1971) investigated the biological activity of the glucosides of gibberellins A_{26}, A_{27} and A_{29}, in addition to those of A_3 and A_8 in six different bioassays. They found in all cases the glucosides showed less activity than the free aglycones.

Yamane et al. (1973) investigated the biological activity of gibberellin A_{35}-glucoside in seven different bioassays and found it was virtually inactive. This was in contrast to the free aglycone which in general showed high activity.

It has been suggested that the glucosides represent a de-activated pool or storage depot of gibberellin, and a form in which gibberellins can be conveniently transported in plants.

The role as a de-activated pool or storage depot has been quite well established for gibberellin A_8-glucoside in Phaseolus coccineus seeds (Sembdner et al., 1968, 1972). Immature seed of Phaseolus coccineus is a rich source of free gibberellins (MacMillan, 1972), but during seed maturation the content of free gibberellins declines, and the content of gibberellin A_8-glucoside increases, such that in ripe seeds the glucoside is the only detectable component (Sembdner et al., 1968). During subsequent germination, both the imbibed seed and the cotyledons contain a mixture of free gibberellins and A_8-glucoside (Sembdner et al., 1968). Work with ^{14}C-gibberellin A_3 and ^{3}H-gibberellin A_6 has shown that during seed maturation these free gibberellins are converted in part to labelled A_8-glucoside, and during subsequent germination the decline in labelled A_8-glucoside is paralleled by an increase in labelled free gibberellin (Sembdner et al., 1972). These results suggest that gibberellin A_8-glucoside in bean seeds is reconvertible and

therefore possesses a depot function. Similar results have been obtained with pea seeds (Pisum sativum) and Japanese Morning Glory seeds (Pharbitis nil) (Barendse et al., 1968).

The possible function of the glucosides as a translocated form of the gibberellins rests with the report of Sembdner et al. (1968) in which gibberellin glucosides were found to occur in the bleeding sap of trees (Norway maple, Acer plantanoides and Wych elm, Ulmus glabra). Bleeding sap from these trees was collected in early spring and preliminary results showed the maple sap contained a form of gibberellin A_8 which was liberated by treatment with β-glucosidase. More detailed work was carried out on the gibberellin components of the sap of Wych elm, and evidence for the presence of gibberellin A_3-hexopyranoside was obtained.

The formation in plants of glycosides of hydroxylated compounds is a well known phenomenon and is sometimes considered to be a de-toxication mechanism (McIlroy, 1951). It seems a similar role is played by glycosylation of exogenous gibberellins in plants. Murakami (1961) found exogenous gibberellin A_3 was converted to gibberellin A_3-glucoside by leaf discs of seven different plant species. Sembdner et al. (1972) reported that the application of ^{14}C-gibberellin A_3 to mature seed of Phaseolus coccineus resulted in the production of ^{14}C-gibberellin A_3-glucoside; neither gibberellin A_3 nor its glucoside, are normally found in these

seeds (Sembdner et al., 1968; MacMillan, 1972). Glycosylation of ^{14}C-gibberellin A_3 also resulted after exogenous application to Cucumis sativis cotyledons, and glycosylation of ^{3}H-gibberellin A_4 resulted after application to dwarf d1 of Zea mays (Sembdner et al., 1972).

In this respect glycosylation of exogenous gibberellins would represent a normal reaction by the plant to a foreign chemical, and is perhaps a phenomenon distinct from the glycosylation of endogenous naturally-occurring gibberellins.

(b) Gibberellin Glucose Esters.

The occurrence of gibberellin glucose esters has only recently been established, and information relating to their biological activity and function has yet to be reported (Hiraga et al., 1972).

B. Biosynthesis of the Gibberellins.

An understanding of the characteristics of the biosynthetic pathway responsible for the formation of gibberellins in plants would obviously be of value in understanding the overall role of these regulators in the control of plant growth and development. The characteristics of the pathway include, in addition to the sequence of intermediate structures leading from simple precursors to the biologically active compound(s), the nature of the enzymes involved in the various steps and their localization in the tissues of the plant and the organelles within the plant cells, and the factors involved in the regulation of activity of the biosynthetic sequence.

In this section of the literature review these characteristics of the gibberellin biosynthetic pathway in Gibberella fujikuroi and higher plants will be discussed.

1. Biosynthesis of Gibberellins in Gibberella fujikuroi.

(1) Introduction.

All of the definitive work on gibberellin biosynthesis has been done using cultures in which the fungus Gibberella fujikuroi has been growing. This follows from the ability of the fungus to produce and the culture to accumulate large quantities of gibberellin, and from the technical ease associated with biosynthetic studies using fungal cultures as opposed to growing plants.

The sequence of reactions which participate in gibberellin biosynthesis in this fungus have largely been elucidated by examining the efficiency with which various radio-active precursors are converted to gibberellin by fungal cultures. The radio-active precursors which have been used include mevalonic acid, ent-kaurene derivatives and ent-gibberellane derivatives (for structures of mevalonic acid, ent-kaurene and ent-gibberellane see Figures 1 and 3). ^{14}C-mevalonic acid labelled on carbon-2, and ^{3}H-mevalonic acid labelled at positions 2, 4 and 5 have been used. The ent-kaurene and ent-gibberellane derivatives have invariably been labelled with ^{14}C at the exocyclic methylene group (carbon-17). This has been done by reacting a 17-norketone derivative with the Wittig reagent prepared from ^{14}C-methyliodide (Geissmann et al, 1966; Cross, 1968).

The possibility of randomisation of the radio-active

carbon atoms during the metabolism of the precursors by the fungal cultures has been eliminated by degradation studies carried out on the radioactive products. In the case of 17-^{14}C-methylene derivatives this has been easily accomplished by ozonolysis of the purified product to yield ^{14}C-formaldehyde which has been isolated and identified as its dimedone derivative (Cross et al., 1964). Such studies have shown the fungus does not randomise label from any of the above mentioned precursors, and in all cases the specificity of incorporation has exceeded 95%.

Several different groups of workers have contributed to the work on gibberellin biosynthesis in Gibberella fujikuroi. It is hazardous however, to compare the relative incorporations of precursors reported by the different groups because of the widely different fermentation conditions and times used. Furthermore, failure to achieve incorporation in these types of biosynthetic studies must always be interpreted with caution since failure may be due to the labelled substrate failing to reach the site of the appropriate enzyme systems, or the labelled substrate competing poorly at the active site of the enzymes with non-labelled enzyme-bound intermediates.

Substantiation of the results of feeding experiments will be achieved when the various enzymes in the pathway are isolated, purified, and studied in in vitro systems. As yet, however,

little work on the enzymology of this pathway in Gibberella fujikuroi has been reported.

Gibberellin A_3 is the most abundant gibberellin in the fungal cultures, and for this reason a large proportion of the work done on gibberellin biosynthesis has been directed toward gibberellin A_3 production. At least fifteen other ent-gibberellane compounds co-occur with A_3 in the culture filtrates, but there is only fragmentary information available relating to their biosynthesis.

(2) Outline of the Biosynthetic Pathway.

The work of Birch et al. (1958, 1959) in which it was shown that label from 1-^{14}C-acetate and 2-^{14}C-mevalonate was incorporated (with specificity) into gibberellin A_3 by growing cultures of Gibberella fujikuroi provided the first experimental proof of the diterpenoid origin of the gibberellins, and substantiated the earlier proposals of Ruzicka (1953), Wenkert (1955) and Cross et al. (1956) which were based on structural considerations.

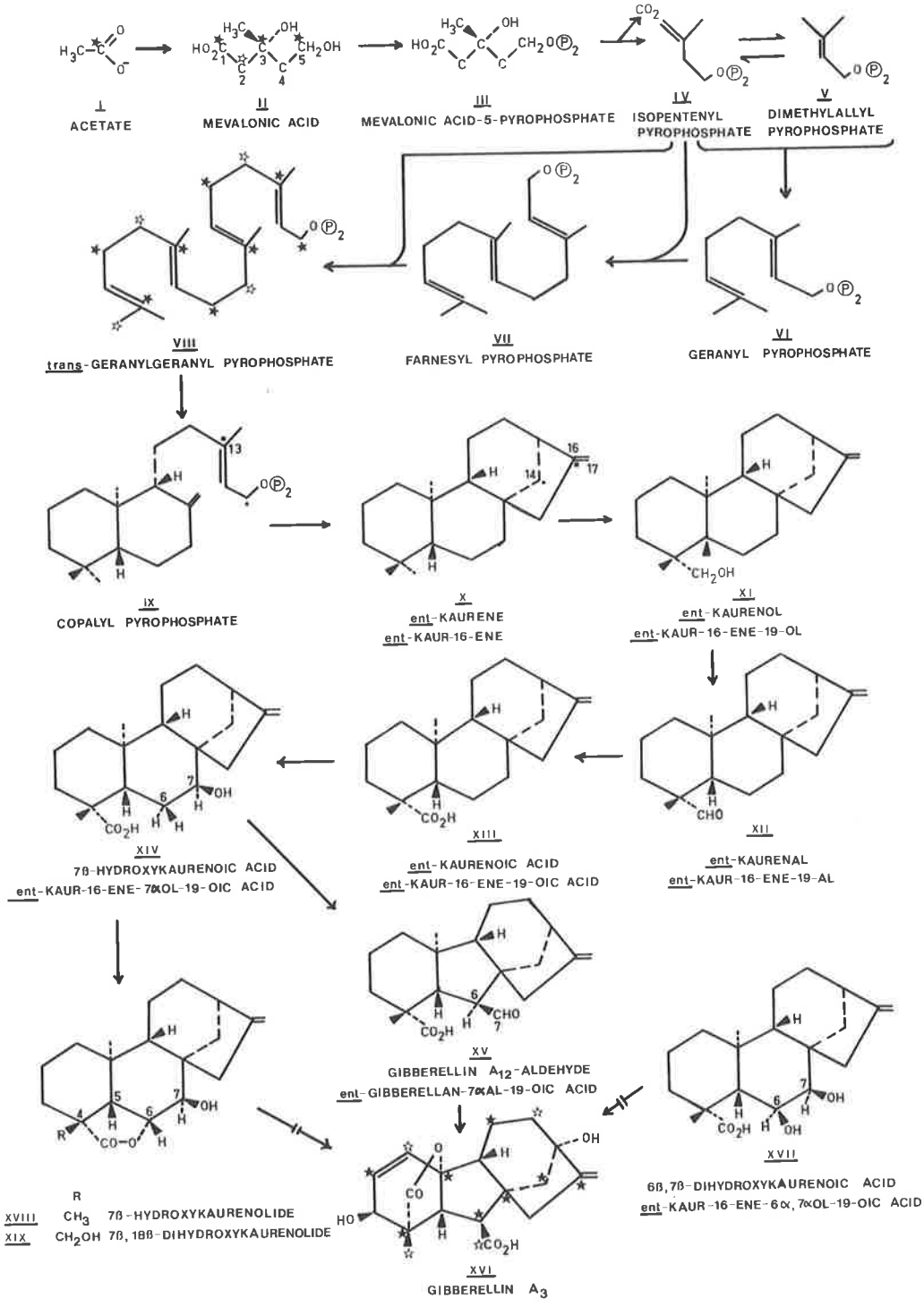
The overall pathway proposed for gibberellin A_3 biosynthesis in Gibberella fujikuroi is outlined in Figure 3. In this pathway R-mevalonic acid (II) is phosphorylated via mevalonic acid-5-monophosphate to the pyrophosphate (III), which then undergoes decarboxylation, with the loss of carbon-1, to yield isopentenyl pyrophosphate (IV). Dimethylallyl pyrophosphate (V), arises from rearrangement of isopentenyl pyrophosphate (IV), and these two 5-carbon compounds undergo a series of head to tail condensation reactions which results, via the intermediates geranyl pyrophosphate (VI) and farnesyl pyrophosphate (VII) in the production of C20 trans-geranylgeranyl pyrophosphate (VIII) which is the last non-cyclized intermediate in the pathway. trans-Geranylgeranyl pyrophosphate (VIII) then undergoes cyclisation to yield eventually ent-kaurene (X), the first tetracyclic intermediate. The further metabolism of ent-kaurene (X) involves a unique series of

FIGURE 3.

SEQUENCE OF REACTIONS LEADING TO GIBBERELLIN A_3 BIOSYNTHESIS IN GIBBERELLA FUJIKUROI

The labelling patterns of trans-geranylgeranyl pyrophosphate (VIII) and gibberellin A_3 (XVI) from 1-^{14}C-acetate and 2-^{14}C-mevalonate are shown with solid and open stars respectively.

Broken arrows indicate reactions which have been shown not to occur.


I
ACETATE
II
MEVALONIC ACID
III
MEVALONIC ACID-5-PYROPHOSPHATE
IV
ISOPENTENYL PYROPHOSPHATE
V
DIMETHYLALLYL PYROPHOSPHATE
VI
GERANYL PYROPHOSPHATE
VII
FARNESYL PYROPHOSPHATE
VIII
trans-GERANYLGERANYL PYROPHOSPHATE
IX
COPALYL PYROPHOSPHATE
X
ent-KAURENE
ent-KAUR-16-ENE
XI
ent-KAURENOL
ent-KAUR-16-ENE-19-OL
XII
ent-KAURENAL
ent-KAUR-16-ENE-19-AL
XIII
ent-KAURENOIC ACID
ent-KAUR-16-ENE-19-OIC ACID
XIV
7β-HYDROXYKAURENOIC ACID
ent-KAUR-16-ENE-7αOL-19-OIC ACID
XV
GIBBERELLIN A12-ALDEHYDE
ent-GIBBERELLAN-7αAL-19-OIC ACID
XVI
GIBBERELLIN A3
XVII
6β,7β-DIHYDROXYKAURENOIC ACID
ent-KAUR-16-ENE-6α,7αOL-19-OIC ACID
R
XVIII CH3 7β-HYDROXYKAURENOLIDE
XIX CH2OH 7β,18β-DIHYDROXYKAURENOLIDE

reactions in which oxidation of carbon-19 results finally in the formation of ent-kaur-16-ene-19-oic acid (XIII), which is then hydroxylated to yield ent-kaur-16-ene-7α ol-19-oic acid (XIV). The hydroxy-acid (XIV) then undergoes B-ring contraction with expulsion of carbon-7 to give ent-gibberellan-7al-19-oic acid (XV). The final stages in production of gibberellin A_3 (XVI), which are perhaps the least understood, involve hydroxylation and oxidation of the aldehyde (XV), loss of the angular methyl substituent (carbon-20), formation of the γ -lactone grouping, and introduction of the double bond into the A-ring.

The intermediates in this pathway, up to and including trans-geranylgeranyl pyrophosphate (VIII) are not unique to the gibberellin biosynthetic pathway. Geranyl pyrophosphate (VI) is an important intermediate in monoterpene biosynthesis, farnesyl pyrophosphate (VII) is an intermediate in both sesquiterpene biosynthesis and sterol biosynthesis, and trans-geranylgeranyl pyrophosphate (VIII) is an intermediate in the biosynthesis of carotenoids and other higher terpenoids.

(a) Biosynthesis of ent-Kaurene.

The original demonstration by Birch et al. (1958,1959) that 1-^{14}C-acetate and 2-^{14}C-mevalonate are specifically incorporated into gibberellin A_3 by Gibberella fujikuroi cultures has since been substantiated by Cross et al. (1964) using 2-^{14}C-mevalonate, and Hanson and White (1969a, 1969b) using 4(R)-(4-^{3}H,2-^{14}C) and 2(R)-^{3}H-mevalonate and 5(R)-^{3}H-mevalonate.

The sequence of reactions in which acetate (I) is converted via mevalonate (II) to trans-geranylgeranyl pyrophosphate (VIII) has not been investigated in depth in Gibberella fujikuroi. However, these reactions are common to a great number of organisms which biosynthesise a variety of terpenes, and there is no reason to doubt that the precursors mevalonic acid-5-pyrophosphate (III), isopentenyl pyrophosphate (IV), dimethylallyl pyrophosphate (V), geranyl pyrophosphate (VI) and farnesyl pyrophosphate (VII), which have been shown to occur in other organisms (Cornforth, 1968), also participate in the early stages of gibberellin biosynthesis in Gibberella fujikuroi.

The cyclisation of trans-geranylgeranyl pyrophosphate (VIII) to form eventually ent-kaurene (X), the first tetracyclic compound in this sequence of reactions, proceeds via the formation of the bicyclic intermediate copalyl pyrophosphate (IX) (Shechter and

West, 1969). The stereo-specificity of this cyclisation has been known since the early work of Birch when it was shown that carbon-19 of gibberellin A_3 (XVI), and therefore of the tetracyclic intermediate (ent-kaurene (X)), was not labelled from 2-^{14}C-mevalonate; thus, cyclisation of trans-geranylgeranyl pyrophosphate (VIII) is stereospecific.

Both copalyl pyrophosphate (IX) and ent-kaurene (X) are specifically incorporated into gibberellin A_3 to the extent of 5.13% and 5.7% respectively by cultures of Gibberella fujikuroi (Hanson and White, 1969a; Cross et al., 1964). The ability of the fungus to incorporate trans-geranylgeranyl pyrophosphate (VIII) directly into gibberellin A_3 has not been investigated, but Hanson and White (1969a) found 1-^{3}H-geranylgeraniol was specifically incorporated to the extent of 0.02% into gibberellin A_3. The low incorporation obtained was presumably due to the low rate at which the alcohol was taken up and phosphorylated by the fungus prior to incorporation.

Considerable rearrangement of the atoms involved in C and D-ring formation of the ent-gibberellane skeleton takes place during the cyclisation of the bicyclic intermediate copalyl pyrophosphate (IX). Birch et al. (1959) observed that label from 1-^{14}C-acetate, and therefore from carbon-13 in copalyl pyrophosphate (IX), appeared at carbon-16 in gibberellin A_3(XVI), and hence at position 16 in

ent-kaurene (X). Furthermore, Hanson and White (1969a) found 15-^{3}H-copalyl pyrophosphate (IX) appeared exclusively at carbon-14 in gibberellin A_3 (XVI), and hence at the same position in ent-kaurene (X).

No tricyclic intermediates have been isolated from the fungus, so presumably the direct rearrangement of the bicyclic intermediate copalyl pyrophosphate (IX) takes place while it is still attached to the enzyme (Evans and Hanson, 1972).

(b) Conversion of ent-Kaurene into the first ent-gibberellane precursor.

An unusual ~~A unique~~ sequence of reactions follows the formation of ent-kaurene (X): contraction of ring-B with, in the case of the C19-gibberellins, loss of the angular carbon-20 group, formation of the γ-lactone (carbons 4 → 10), and hydroxylation of the skeleton at two sites (carbons 3 and 13).

This sequence of events commences with oxidation of carbon-19 of ent-kaurene (X) to give finally ent-kaur-16-ene-19-oic acid (XIII). The acid (XIII) is then hydroxylated in a stereo-specific fashion to give ent-7α hydroxykaurenoic acid (XIV). Both the acid (XIII) and the 7-hydroxy-derivative (XIV) have been isolated from cultures of the fungus (Cavell and MacMillan, 1967; Hanson and White, 1969c) and both are specifically incorporated into gibberellin A_3 (XVI) in amounts which exceed 30% (Geissmann et al., 1966; Hanson and White, 1969c).

The oxidation of carbon-19 of ent-kaurene (X) is thought to proceed in a stepwise manner via the formation of the alcohol (XI) and the aldehyde (XII). Although neither of these compounds have been isolated and identified in fungal cultures, they are both incorporated into gibberellin A_3 (West et al., 1968).

The hydroxy-acid (XIV) is converted to the aldehyde (XV) which

is the first intermediate with the ent-gibberellane skeleton, and as such is characterised by the 5-membered B-ring. The aldehyde (XV) has been shown to be incorporated into gibberellin A_3 to the extent of 15.4% (Cross et al., 1968b) and is known to be a normal component of culture filtrates (Hanson and White, 1969c).

Details of the ring contraction of ent-7α-hydroxy-kaurenoic acid (XIV) have been studied with 2(R) and 5(R)-^{3}H-mevalonic acid (Hanson and White (1969b). The process of ring contraction involves the loss of the 5(R) mevalonoid hydrogen from carbon-6 (the 6β-hydrogen in XIV), which suggests the group initiating the ring contraction process possesses the 6β configuration. β-Oxygenation at carbon-6 would seem a likely preliminaty to ring contraction, but it is noteworthy that 6β,7β-dihydroxy-kaurenoic acid (XVII) is not incorporated into gibberellin A_3 by fungal cultures (Cross et al., 1970; Hanson and Hawker, 1971). Perhaps a more likely process is the loss of the 6β-oxygen group as a phosphate ester (MacMillan, 1971).

From a stereo-chemical point of view it is interesting to note that while gibberellin A_3 arises by loss of the 5(R)-mevalonoid hydrogen at carbon-6 (the 6β-hydrogen in XIV), the kaurenolides (XVIII and XIX), which are diterpenes present with the gibberellins in the culture filtrates, arise by retention of the 5(R)-mevalonoid hydrogen at carbon-6. The kaurenolides are

not precursors of gibberellin A_3 (Cross et al., 1968a), so it seems that the stereo-chemistry of attack on carbon-6 of ent-7 α-hydroxy-kaurenoic acid (XIV) determines whether the molecule is destined for kaurenolide formation or ent-gibberellane formation (MacMillan, 1971).

(c) Final Stages in Gibberellin A_3 Biosynthesis.

Information relating to the further conversion of ent-gibberellan-7al-19-oic acid is somewhat scattered, but several pieces of information suggest a possible series of reactions which involves β-hydroxylation at carbon-3, oxidation of the C7-aldehyde to the carboxyl, followed by γ-lactone formation which occurs in conjunction with the loss of the angular C20-substituent, and finally further hydroxylation at the carbon-13 position and introduction of the double bond into the A-ring. The evidence relating to this series of reactions will be discussed in conjunction with the structures and proposed sequence of reactions outlined in Figure 4A.

Hedden and MacMillan (1971) found the aldehyde (XV) was efficiently converted to gibberellin A_{14} (XXII) by cultures of Gibberella fujikuroi. Under the same conditions gibberellin A_{12} (XX) was not converted very efficiently to gibberellin A_{14} (XXII). This result indicated direct β-hydroxylation at carbon-3 of the aldehyde (XV) as the next step in the sequence to gibberellin A_3 (XVI), and suggests the intermediacy of gibberellin A_{14}-aldehyde (XXI) in this reaction.

Cross and Norton (1965, 1966) and Cross et al. (1968b) investigated the efficiency with which the C-20 gibberellins A_{12} (XX) and A_{14} (XXII) are converted to A_3 (XVI) by the fungus. They

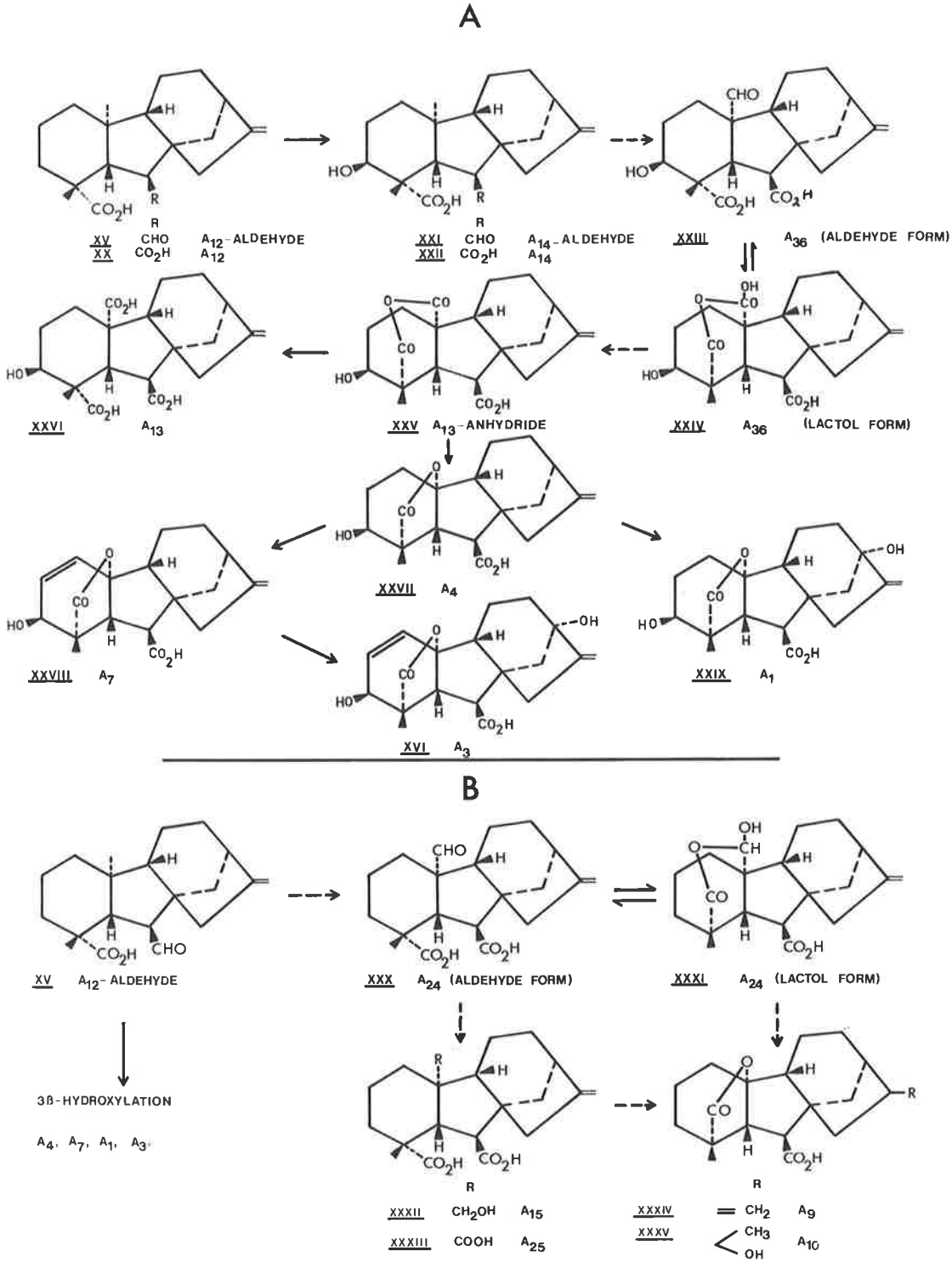
FIGURE 4A.

FINAL STAGES OF BIOSYNTHESIS OF GIBBERELLIN A_3
IN GIBBERELLA FUJIKUROI

FIGURE 4B.

POSSIBLE ORIGIN OF THE NON-HYDROXYLATED GIBBERELLINS
IN GIBBERELLA FUJIKUROI

In Figure 4A and Figure 4B reactions which have been shown to occur are indicated with complete arrows; postulated conversions are shown with dashed arrows.


A
XV CHO A12-ALDEHYDE
XX CO2H A12
XXI CHO A14-ALDEHYDE
XXII CO2H A14
XXIII A36 (ALDEHYDE FORM)
XXIV A36 (LACTOL FORM)
XXV A13-ANHYDRIDE
XXVI A13
XXVII A4
XXVIII A7
XXIX A1
XVI A3
B
XV A12-ALDEHYDE
3β-HYDROXYLATION
A4, A7, A1, A3
XXX A24 (ALDEHYDE FORM)
XXXI A24 (LACTOL FORM)
XXXII CH2OH A15
XXXIII COOH A25
XXXIV =CH2 A9
XXXV CH3 OH A10

found neither of these two C20-gibberellins were incorporated as efficiently into gibberellin A_3 as the aldehyde (XV), although A_{12} (XX) and A_{14} (XXII) were converted to the extent of 0.7% and 4.7% respectively. This low efficiency of conversion could be indicative of the fact that these compounds are not the true intermediates, but are oxidation products of the true intermediates. It is possible that many of the reactions involved in the conversion of the aldehyde (XV) to the C19-gibberellins take place at the aldehyde oxidation level (MacMillan, 1971; Paleg and West, 1972).

Gibberellin A_{13} (XXVI) is not converted at all to gibberellin A_3 (Cross et al., 1968b, Hanson and Hawker, 1972), and from the way it slowly accumulates in fermentation broths it must arise from an irreversible oxidation of a true intermediate (MacMillan, 1971).

Hanson and Hawker (1972) found gibberellin A_{13}-anhydride (XXV) was incorporated into gibberellins A_4 plus A_7 (XXVII, XXVIII) and gibberellin A_3 (XVI) to the extent of 0.07% and 0.4% respectively, during an 18-hour incubation. This result was obtained in spite of the tendency for the anhydride (XXV) to revert to A_{13} (XXVI) over a period of hours in the conditions of the fermentation, and suggests that loss of the angular substituent (carbon-20) is involved in the γ-lactone formation and takes place via the formation of a carbon 4 $\longrightarrow$ 10 substituent oxygen bridge.

The structure of gibberellin A_{36} (XXIII) and its ability to

exist in solution in equilibrium between the free aldehyde form (XXIII) and the lactol form (XXIV) (Bearder and MacMillan, 1972) could be pertinent here. This gibberellin is a fungal metabolite, and it is conceivable that oxidation of carbon-20 of gibberellin A_{14} (XXII) or A_{14}-aldehyde (XXI) to produce gibberellin A_{36} (XXIII) is part of the sequence of reactions which culminates in loss of the carbon-20 group and formation of the γ-lactone. This sequence of reactions could be imagined to operate through oxidation of carbon-20 of the lactol (XXIV) to the anhydride (XXV), which leads to the production of gibberellin A_4 (XXVII). This postulated series of reactions is quite speculative but is currently being pursued as a line of research (Bearder and MacMillan, 1972).

More concrete information exists relating to the interconversions of gibberellin A_4 (XXVII), A_7 (XXVIII), A_1 (XXIX) and A_3 (XVI). The time course studies of Geissmann et al. (1966), Verbiscar et al. (1967) and Pitel et al. (1971) have shown that A_4 (XXVII) can be converted to A_7 (XXVIII) by introduction of a C1-double bond, and A_4 (XXVII) can be converted to A_1 (XXIX) by α-hydroxylation at carbon-13. It is clear that A_3 (XVI) arises by α-hydroxylation at carbon-13 of A_7 (XXVIII) (Pitel et al., 1971). Production of A_3 (XVI) from A_1 (XXIX) does not occur at all rapidly in Gibberella fujikuroi.

The introduction of the double bond into the A-ring of

gibberellin A_3 (XVI) has been shown by Hanson and White (1969b) to involve elimination of hydrogen from the α-face of the skeleton.

(d) Origin of Other Gibberellins in the Culture Filtrates.

It was mentioned earlier that the research on gibberellin biosynthesis in Gibberella fujikuroi has been largely directed toward production of gibberellin A_3. Other gibberellins including A_{12}, A_{13}, A_{14}, A_{36}, A_4, A_7 and A_1 have already been implicated as either precursors of gibberellin A_3 or as products arising from the oxidation of the true precursors. There remains a number of gibberellins of uncertain origin which have been found to occur in the culture filtrates. Structural and chemical resemblances of some of these to the more commonly occurring gibberellins suggest possible routes by which they may arise. The pertinent structures and possible reaction sequences are presented in Figure 4B.

Cross et al. (1968a) found 17-^{14}C-gibberellin A_9 (XXXIV) was not converted to gibberellin A_3 in the fungal cultures, but it was converted in part to 17-^{14}C-gibberellin A_{10} (XXXV). Clearly these non-hydroxylated gibberellins are not on the main route of gibberellin A_3 biosynthesis, but they could conceivably arise by a series of reactions, similar to that outlined in Figure 4B, in which it is suggested the non-hydroxylated gibberellins (A_{15}, A_{24}, A_{25}, A_9 and A_{10}) arise from oxidation of the C20-group of the aldehyde (XV). In this case the aldehyde (XV) is situated at a branch point in gibberellin biosynthesis, one branch leading to the 3 β -hydroxylated series, and the other branch leading to the

non-hydroxylated series.

The structure of gibberellin A_{24} (XXX) and its ability to exist in solution in equilibrium between the free aldehyde form (XXX) and the lactol form (XXXI) (Harrison and MacMillan, 1971) suggests it may be a precursor of A_9 (XXXIV), in which case A_{24} will serve a function in the biosynthesis of the non-hydroxylated γ-lactone gibberellins (A_9 and A_{10}) directly analogous to that which has been proposed for A_{36} in the biosynthesis of the hydroxylated γ-lactone series (see previous section).

(3) Enzymology of the Pathway.

The enzymes involved in the reactions which lead to gibberellin A_3 biosynthesis in Gibberella fujikuroi have thus far received scant attention. The reactions responsible for the transformation of mevalonate into the prenyl phosphates up to and including trans-geranylgeranyl pyrophosphate (Figure 3) are common to a great variety of organisms, and the enzymes which mediate these reactions in animals and yeast have been studied in some detail (Popják and Cornforth, 1960). The general properties of these enzymes do not vary greatly from different sources. They are derived from the soluble fraction of cell extracts, and require only ATP and divalent magnesium or manganese ions as low molecular weight co-factors.

The enzymes which are involved in the cyclisation of trans-geranylgeranyl pyrophosphate, and the subsequent metabolism of the cyclised products, are of greater interest here. Of these enzymes only ent-kaurene synthetase has been studied in any detail (Fall and West, 1971; West and Fall, 1972). This enzyme, which catalyses the cyclisation of trans-geranylgeranyl pyrophosphate to copalyl pyrophosphate, and also the cyclisation of copalyl pyrophosphate to ent-kaurene, has been purified 170-fold from cell-free extracts of the fungus. These two separate enzyme activities are associated with a protein complex of molecular weight

$4.3\text{-}4.9 \times 10^5$, as measured by sucrose gradient centrifugation and Sepharose-4B gel filtration. Polyacrylamide gel electrophoresis of the purified protein at pH 8.0 showed one major protein band from which both activities could be recovered. Electrophoresis at pH 10.2, or in sodium dodecyl sulphate, revealed the presence of multiple protein bands, none of which retained any catalytic activity. The purified protein complex showed no evidence for activity of prenyl transferase, squalene synthetase, 2,3-oxido-squalene cyclase or ent-kaurene oxidase. Both enzymic activities were stimulated by 1-2 mMolar dithiothreitol. Gibberellin A_3 at 10^{-3} Molar was found to have no inhibitory effect on either activity, which suggests the absence of feed-back inhibition by gibberellin A_3 at this site.

2. Biosynthesis of Gibberellins in Higher Plants.

(1) Introduction.

Knowledge and understanding of the complete biosynthetic pathway which gives rise to gibberellin production in plants has been hampered by the lack of a system which is capable of incorporating radioactively labelled precursors directly into gibberellins. This can largely be attritubed to the low concentrations in which gibberellins occur in plants and the inherent technical difficulties associated with biosynthetic studies in plants. In spite of this major difficulty several somewhat unrelated lines of evidence suggest that at least the broad outline of the pathway in plants is the same as in the fungus. These lines of evidence can be conveniently divided into four points.

The first and probably most important point is that a number of the key fungal intermediates, including ent-kaurene and its oxidised derivatives, have now been identified as either normal constituents of plants, or have been identified as products arising originally from 2-^{14}C-mevalonic acid in cell-free plant extracts.

Secondly, several of these intermediates have been shown to be converted by plants to more polar compounds, akin at least on TLC to some of the known gibberellins. Because of the small amounts of material which have been obtained, positive identification of the conversion products has not been possible.

Thirdly, experiments with certain dwarfed single-gene mutants of maize (Zea mays) and rice (Oryza sativa) suggest some of the intermediates in gibberellin biosynthesis in these plants are the same as some of the biosynthetic intermediates in the fungus.

Finally, "growth retardants" which have been shown to inhibit gibberellin biosynthesis in the fungus have marked effects on the growth and development of plants.

The evidence relating to these four points will be discussed in greater depth in the following sections. The ent-kaurene and ent-gibberellane structures mentioned in these sections can all be referred to the structures given in Figure 3.

(2) Biosynthesis and Metabolism of ent Kaurene in Plants.

(a) Biosynthesis of ent-Kaurene.

ent-Kaurene, the first tetracyclic intermediate of the fungal gibberellins, has been shown to be produced from 2-^{14}C-mevalonic acid by cell-free enzyme preparations from several plants during incubation with ATP and magnesium ions. This activity has been found in preparations from the cotyledons of immature seeds of Pisum sativum, nucellar-endosperm preparations of immature seed of Echinocystis macrocarpa, endosperm preparations of immature seed of Cucurbita pepo, and in extracts from young castor bean seedlings (Ricinus communis).

The system from the endosperm of immature seed of Cucurbita pepo is capable of converting 2-^{14}C-(RS)-mevalonic acid to ^{14}C-ent-kaurene with a yield of 40% of the active isomer (Graebe, 1969). It is now clear, however, that under certain conditions several other more polar diterpenoids are produced by this system (Graebe, 1972; Graebe et al., 1972). The nature and importance of these will be discussed in more detail in section I.B.2.(2)c.

The characteristics of the system from Echinocystis macrocarpa have been investigated by C.A.West and his colleagues. They found the enzymes responsible for the sequence of reactions leading to ent-kaurene production were found only in the nucellar-endosperm of the immature seed (Graebe et al., 1965): homogenates of the

embryos completely failed to catalyse the formation of ent-kaurene. The enzymes were soluble and were not sedimented by centrifugation at 105,000xg for 30 minutes (Upper and West, 1967). Evidence for the participation of mevalonic acid-5-phosphate, mevalonic acid-5-pyrophosphate, isopentenyl pyrophosphate, geranyl pyrophosphate, farnesyl pyrophosphate and trans-geranylgeranyl pyrophosphate in this sequence of reactions has been obtained (Oster and West, 1968; West et al., 1968).

The enzyme system from immature pea seeds has been investigated by T.C.Moore and his colleagues. This system has properties very similar to the system from Echinocystis macrocarpa. Coolbaugh and Moore (1971a) found the enzymes responsible for the synthesis of ent-kaurene from 2-^{14}C-mevalonic acid were localised exclusively in the cotyledons of the immature seeds, no activity being present in the isolated seed coats and embryonic shoot axes. Furthermore, the enzymes were soluble, remaining in the supernatant after centrifuging at 100,000xg for one hour. Coolbaugh and Moore (1969) found the capacity of the system to produce ent-kaurene varied markedly with the stage of seed development, with a maximum being reached about 13 days after anthesis, at which time the fresh weight of the seed was about one-half of the fresh weight of a mature seed. This pattern of ent-kaurene synthesising activity closely resembles the pattern of gibberellin content of seeds at

various stages of development (Corcoran and Phinney, 1962) suggesting a relationship between the two.

The system from young seedlings of Ricinus communis has been investigated by Robinson and West (1970a, 1970b). They found soluble enzyme preparations from 60-hour old seedlings converted 2-^{14}C-mevalonic acid, in the presence of ATP, into a mixture of hydrocarbons and more polar compounds, via the formation of trans-geranylgeranyl pyrophosphate. The hydrocarbon fraction consisted of at least 5 diterpenes, the structures of which are given in Figure 5A. Three of these diterpenes were identified as ent-beyerene (XXXVI), ent-kaurene (XXXVII) and ent-isopimaradiene (XXXVIII). ent-13,16-Cycloatisane (trachylobane) (XXXIX) was incompletely characterised as the fourth hydrocarbon, while the structure of the fifth, named casbene (XXXX), was only tentatively described. The results of purification of the enzymes responsible for cyclisation of trans-geranylgeranyl pyrophosphate to these cyclic diterpenes suggested that separate enzymic components participated in the cyclisation reactions, except for ent-kaurene (XXXVII) and ent-13,16-cycloatisane (XXXIX) where no evidence for the separation of activities was obtained.

Shechter and West (1969) found copalyl pyrophosphate, the bicyclic intermediate which immediately precedes ent-kaurene formation in Gibberella fujikuroi, was converted to ent-kaurene by

the enzyme system from Echinocystis macrocarpa, and was converted to a mixture of diterpenoids including ent-kaurene by the enzyme system from Ricinus communis. Although copalyl pyrophosphate has not been identified as a normal constituent of plants, it seems likely that it is involved in ent-kaurene formation in plants in the same way that it is in Gibberella fujikuroi.

FIGURE 5A.

DITERPENE HYDROCARBONS FROM RICINUS COMMUNIS SEEDLINGS

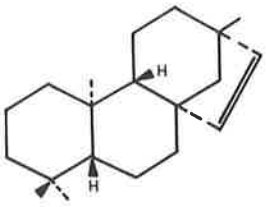
FIGURE 5B.

BIOSYNTHESIS OF STEVIOL IN LEAVES OF STEVIA REBAUDIANA

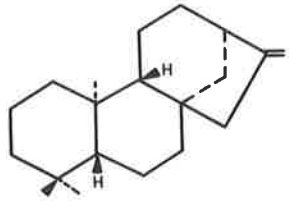
FIGURE 5C.

STRUCTURES OF PLANT GROWTH RETARDANTS

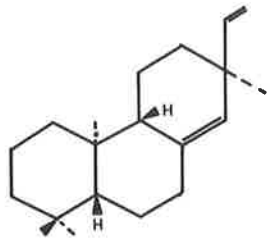
A



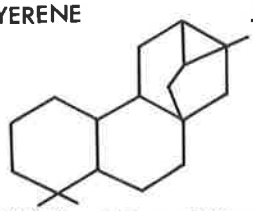
XXXVI ent-BEYERENE



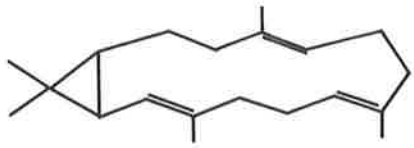
XXXVII ent-KAURENE



XXXVIII ent-ISOPIMARADIENE



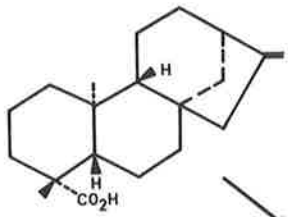
XXXIX ent-13,16-CYCLOATISANE



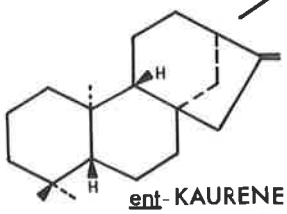
XXXX CASBENE

B

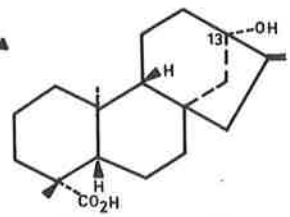
MEVALONIC ACID



ent-KAURENOIC ACID

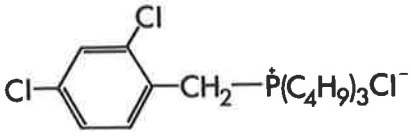


ent-KAURENE

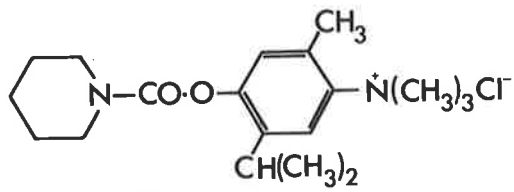


XXXXI STEVIOL

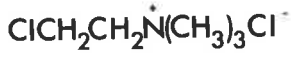
C



PHOSPHON D



AMO-1618



CCC

(b) Oxidation and Hydroxylation of ent-Kaurene in Plants.

The reactions which proceed from the formation of ent-kaurene in plants involve the sequential oxidation of the carbon-19 methyl group via the alcohol and aldehyde to give finally ent-kaur-16-ene-19-oic acid. Further metabolism of ent-kaurenoic acid to ent-7α hydroxy-kaurenoic acid has been shown to occur in some plants. This sequence of reactions is identical to that which participates in gibberellin biosynthesis in Gibberella fujikuroi.

The enzyme system from Echinocystis macrocarpa catalyses this sequence of reactions and culminates in the formation of ent-7α hydroxy-kaurenoic acid (Murphy and West, 1969; West et al., 1968; Lew and West, 1971). Each step in this system is catalysed by a microsomal fraction obtained by centrifuging the homogenate at 105,000xg for one hour, and requires reduced pyridine nucleotide and molecular oxygen as reactants. These reactions, which are subject to inhibition by carbon monoxide, are thought to involve the direct participation of a pigment which has the spectral properties of cytochrome P-450. The characteristics of these reactions indicate their close similarity to the mixed-function oxygenases which have been described in mammalian microsomes. The further metabolism of ent-7α hydroxy-kaurenoic acid by this enzymic system has yet to be reported.

A similar sequence of reactions is involved during the

oxidation of ent-kaurene by the enzyme system from peas, where evidence for the formation of ent-kaurenol, ent-kaurenal and ent-kaurenoic acid has been obtained (Coolbaugh and Moore, 1971b). The participation of a non-catalytic "carrier protein" which renders the lipophilic ent-kaurene accessible for oxidation by the microsomal mixed-function oxidases is involved in this system (Moore et al., 1972). To date this system has not been reported to metabolise ent-kaurene beyond the ent-kaurenoic acid stage.

By feeding ^{3}H-ent-kaurene and ^{3}H-ent-kaurenol to four-day old Pharbitis nil seedlings, and analysing the metabolism of these compounds at various times following application, by TLC of the extractable materials, Takeba and Takimoto (1971) obtained evidence for the sequential oxidation of ent-kaurene via the alcohol and aldehyde to ent-kaurenoic acid. They suggested that ent-kaurenoic acid was further metabolised to unidentified substances which remained at the origin during TLC, but in the absence of any further information the nature of these polar substances remains unknown.

(c) ent-Gibberellane Biosynthesis in Plants.

In Gibberella fujikuroi the sequence of reactions beyond the formation of ent-7 ∝ hydroxy-kaurenoic acid involves contraction of the B-ring with expulsion of carbon-7, the next recognised intermediate being gibberellin A_{12}-aldehyde. At the time of writing, only one system from higher plants has been shown to catalyse the further metabolism of ent-7 ∝ hydroxy-kaurenoic acid. (Graebe et al. (1972) have shown a cell-free system prepared from the endosperm of immature seeds of Cucurbita pepo has the ability to incorporate label from 2-^{14}C-mevalonate into ent-kaurenoic acid, ent-7 ∝ hydroxy-kaurenoic acid and gibberellin A_{12}-aldehyde. The products of conversion were identified by GLC and combined GLC-mass spectrometry of the methyl esters and TMS ethers/methyl esters. Gibberellin A_{12} was also detected in the system, but it is thought this may have been an artifact derived from gibberellin A_{12}-aldehyde by non-enzymic conversion. This is the first report of gibberellin A_{12}-aldehyde in higher plants, and is the only report of a plant system catalysing the oxidation and metabolism of ent-kaurene beyond the stage of contraction of the B-ring. This finding lends support to the view that gibberellin biosynthesis in higher plants at least partly follows the same steps as in the fungus.

The characteristics of the enzyme or enzymes which mediate this interesting set of reactions have yet to be reported, but the

absolute requirement for ATP in the production of ent-kaurenoic acid and gibberellin A_{12}-aldehyde is clear (Graebe, 1972). In this respect this system differs from the Echinocystis macrocarpa system where the oxidation of ent-kaurene to ent-7 ∝ hydroxy-kaurenoic acid occurs in the absence of added ATP. Graebe (1972) also found that only seeds at a certain stage of development were capable of efficiently converting 2-^{14}C-mevalonic acid to gibberellin A_{12}-aldehyde.

The identification of compounds arising from the further metabolism of the aldehyde by this system has yet to be reported, but Graebe et al. (1972) did observe that several radioactive peaks resulted when ^{14}C-ent-kaurenoic acid was used as a substrate for the enzyme system. One of these peaks was identified as gibberellin A_{12}-aldehyde and the RF values of the others on TLC indicated a more polar nature, suggesting they may be more advanced intermediates which have possibly undergone hydroxylation. Thus it seems likely that the preparations from Cucurbita pepo endosperm have the necessary enzyme complement for the conversion of mevalonic acid to gibberellins.

(d) The Normal Occurrence of ent-Kaurenoid Compounds in Plants.

ent-Kaurenoid diterpenes are now known to be normal constituents of a number of plant species. ent-Kaurene has been isolated and identified from Agathis australis, Dacrydium franklini, Phyllocladus trichananoides, Araucaria auracana, Cupressus macrocarpa, Sciadopitys vercicillata, and a number of species of Podocarpus (Hanson, 1968). ent-Kaur-16-ene-19-oic acid has been identified as a normal constituent of other plants, including members of the Euphorbiaceae (Henrick and Jeffries, 1964) and Espeletia species (Usubillaga and Morales, 1972).

In this connection the occurrence of steviol in the Paraguayan compositae Stevia rebaudiana is of interest. Steviol, which normally occurs in the form of a glycoside, has been shown to possess gibberellin activity in several bioassays (Ruddat and Lang, 1963; Murakami, 1972). Chemically characterised as ent-kaur-16-ene 13β-ol-19-oic acid (structure XXXXI, Figure 5B), it has been shown to arise from 2-^{14}C-mevalonic acid via ent-kaurene and ent-kaurenoic acid (Bennett et al., 1967; Hanson and White, 1968). However, the possession of a 13 α-hydroxyl group with a 6-membered B-ring indicates that it is likely to be an offshoot of the normal gibberellin biosynthetic sequence. This is supported by the fact that it was not converted to gibberellin A_3 by cultures of Gibberella fujikuroi (Ruddat et al., 1965).

(3) Direct Incorporation of Precursors into Plant Gibberellins.

Aside from the work which has been discussed above (Sections (2)a, (2)b and (2)c) several other attempts have been made to incorporate radioactively labelled precursors directly into plant gibberellins, either by feeding the precursors to intact plants, or excised organs, or by using the precursor as a substrate for cell-free enzyme incorporation studies. Attempts have been made to do this using 2-^{14}C-mevalonic acid, ^{14}C-*ent*-kaurene and 17-^{14}C-*ent*-kaurenoic acid.

Sandhu *et al*. (1972) fed 2-^{14}C-mevalonic acid to dwarf and tall cucumber (*Cucumis sativis*) by growing germinated seeds for one week on filter paper soaked with an aqueous solution of the labelled substrate. The materials extractable from the shoots with aqueous-acetone were partly purified by basic lead acetate treatment, solvent partitioning and TLC on silica gel H. The spectrum of radioactivity along the length of the TLC plates showed two peaks, one of which coincided with gibberellin A_3 standard, suggesting to the authors that this represented production of gibberellin A_3 from mevalonic acid.

Barendse and Kok (1971) prepared ^{14}C-*ent*-kaurene by feeding 2-^{14}C-mevalonic acid to the enzyme preparation from *Cucurbita pepo*. The ^{14}C-*ent*-kaurene thus formed was extracted and purified to some extent on TLC. Three-day old seedlings of *Pharbitis nil* were then treated by injection of 10-20 μl of a solution of this

material containing approximately 600 d.p.m. After a further 24 hours the plants were extracted with methanol, and the acidic materials were concentrated in an ethyl acetate extract. Significant amounts of radioactivity were recovered in this extract, and TLC of this fraction on silica gel G revealed several peaks of radioactivity. One of these peaks coincided with gibberellin A_3 standard after development in two different solvent systems. These results suggested to the authors that Pharbitis nil seedlings had converted some of the injected ^{14}C-ent-kaurene into gibberellin A_3.

Stoddart (1969) found that sonicated chloroplast preparations from Brassica oleraceae showed increased gibberellin-like activity, as measured by the α-amylase bioassay, after incubation with ent-kaurenoic acid and the cofactors NADPH and magnesium chloride. Incubation without ent-kaurenoic acid did not result in any increased gibberellin activity. Furthermore, he found when 17-^{14}C-ent-kaurenoic acid was substituted in the incubations for non-labelled substrate, and the products of incorporation analysed by TLC on silica gel H, several peaks of radioactivity were found. Some of these peaks were found to coincide with peaks of biological activity, thus suggesting that during the incubation the ^{14}C-ent-kaurenoic acid had been converted to a biologically active gibberellin.

Stoddart and Lang (1968) fed 17-^{14}C-*ent*-kaurenoic acid to the uppermost faces of the leaflets of *Trifolium pratense* plants, and after 24 hours extracted the gibberellin-like material from the leaves and purified it to some extent by chromatography on Sephadex-G10 TLC plates, followed by thin-layer electrophoresis on silica gel H. Extracts from non-radioactive plants, during electrophoresis, were found to separate into 3 zones possessing gibberellin biological activity. When the plants were treated with ^{14}C-*ent*-kaurenoic acid, radioactivity was found associated with these 3 zones. Again this result suggests conversion of *ent*-kaurenoic acid to biologically active gibberellin by the plants.

In all these cases the claims of conversion of the precursors to the biologically active gibberellins must be considered with reservation, because of the inadequate methods of identification which have been used. The minute amounts of material which have been obtained makes positive identification extremely difficult. Nevertheless these results are consistent with the proposed scheme of gibberellin biosynthesis in plants.

(4) Experiments with Dwarf Rice and Dwarf Maize.

The dwarf variety of maize (Zea mays) known as d5, and the dwarf variety of rice (Oryza sativa) known as Tan-ginbozu, which are both used in gibberellin bioassays (Phinney and West, 1960; Phinney, 1961; Murakami, 1972) have proven to be particularly useful in helping to elucidate the gibberellin biosynthetic pathway in plants.

The phenotypic characteristics of these two dwarfs, which are both single-gene recessive mutants, include reduced stem height and very short internodes. They both respond in such a way to exogenous gibberellin that they become virtually indistinguishable from their normal counterparts (Phinney and West, 1960). Furthermore, neither of these dwarfs have been found to have any detectable level of gibberellin in their shoots (Phinney, 1961; Suge and Murakami, 1968). These findings are consistent with the hypothesis that the dwarfism is a consequence of the plant's inability to synthesise the amounts of gibberellin required for normal growth because of a metabolic block in the gibberellin biosynthetic pathway (Phinney, 1961; Murakami, 1972).

The interesting feature of these dwarfs is their ability to respond to exogenous ent-kaurene and some of its derivatives in a fashion which is qualitatively identical to the response elicited by exogenous gibberellin A_3. Katsumi et al. (1964) found

seedling elongation of d5 maize was significantly stimulated by ent-kaurenoic acid and ent-kaurenol, while West et al. (1968) reported the length of leaf sheaths of d5 maize were stimulated by ent-kaurenoic acid and ent-7α hydroxy-kaurenoic acid. Murakami (1972) found elongation of the second leaf sheath of Tan-ginbozu rice was stimulated by the application of ent-kaurene, ent-kaurenol and ent-kaurenoic acid.

The effectiveness with which the ent-kaurenoid derivatives mimic the gibberellin A_3 response in these two species is somewhat variable, but in general these derivatives are between 1/100th and 1/1000th as effective as gibberellin A_3 in stimulating elongation (Katsumi et al., 1964; Murakami, 1972).

The ability of ent-kaurenoid derivatives to stimulate elongation in dwarf rice and dwarf maize has been taken as evidence that these derivatives are converted by the plants to physiologically active gibberellin, which is the actual stimulant of stem elongation (West et. al., 1968; Murakami, 1972). The inference is that ent-kaurene, ent-kaurenol, ent-kaurenoic acid and ent-7α hydroxy-kaurenoic acid are precursors of gibberellins in these species, and the metabolic block in their gibberellin biosynthetic pathway must occur before ent-kaurene biosynthesis.

There is an alternative explanation for the growth effects of the ent-kaurenoid derivatives which should be considered. This is

that the ent-kaurene derivatives actually simulate gibberellin A_3 at the active site of gibberellin A_3 action, which results in a promotion of stem elongation. In this case prior conversion of the applied compounds to a true gibberellin would not be necessary for growth stimulation, and the inference that ent-kaurene and its oxidised derivatives are therefore gibberellin precursors in these plants does not necessarily follow. At the moment it is not known if these plants are capable of converting radioactively labelled ent-kaurenoid derivatives to true gibberellins, but it is pertinent that West et al. (1968) found ent-kaur-15-ene-7α ol-19-oic acid and ent-7β hydroxy-kaurenoic acid were ineffective in stimulating leaf sheath elongation in d5 maize. ent-7β -Hydroxy-kaurenoic acid is not a gibberellin precursor in Gibberella fujikuroi (Cross et al., 1968a), and the position of the D-ring unsaturation in ent-kaur-15-ene-7α ol-19-oic acid precludes it as a potential gibberellin precursor.

(5) Experiments with Growth Retardants.

Growth retardants, or dwarfing agents as they are otherwise known, constitute a somewhat diverse array of synthetic organic chemicals which have been found to reduce the growth of many plants. Structures for three of the more widely used growth retardants, the common names for which are AMO-1618, Phosphon D and CCC, are presented in Figure 5C.

These compounds have excited considerable interest among plant physiologists because, in general, they affect plant growth through a reduction in internode elongation, a behaviour which is opposite to that of the gibberellins. In a number of cases this effect can be completely reversed by the application of gibberellin (Tolbert, 1961; Cathey, 1964), which suggests that these compounds may produce their effects on plants by inhibiting endogenous gibberellin biosynthesis.

In low concentrations the growth retardants are capable of strongly inhibiting gibberellin production by growing cultures of Gibberella fujikuroi (Harada and Lang, 1965; Barnes et al., 1969; Cross and Meyers, 1969; Sembdner et al., 1972). The site of action of these compounds in the fungus is at the stage of cylisation of trans-geranylgeranyl pyrophosphate to ent-kaurene (Barnes et al., 1969; West and Fall, 1972).

Work with cell-free enzyme systems from higher plants has verified the effect of these compounds on the cyclisation reaction. Dennis et al. (1965) found AMO-1618 and Phosphon D were capable of inhibiting the cyclisation of trans-geranylgeranyl pyrophosphate to ent-kaurene in the enzyme system from Echinocystis macrocarpa seed, but CCC at the concentration tested failed to have a significant effect on this reaction.

Anderson and Moore (1967) have shown the enzyme system from Pisum sativum seeds which synthesises ent-kaurene from 2-^{14}C-mevalonate was inhibited by AMO-1618 and CCC, although a 1000-fold greater concentration of the latter was necessary to evoke the same percentage inhibition as AMO-1618. Graebe (1968) also found AMO-1618 inhibited ent-kaurene biosynthesis from mevalonate in a cell-free system from Pisum sativum fruits.

Robinson and West (1970a) found AMO-1618, Phosphon D and CCC all inhibited the formation of the cyclic diterpenes ent-beyerene, ent-isopimaradiene, ent-kaurene and ent-13,16-cycloatisane from trans-geranylgeranyl pyrophosphate in the enzyme system from Ricinus communis seedlings.

It seems clear then, in both Gibberella fujikuroi and cell-free enzyme preparations from higher plants, the growth retardants are capable of inhibiting the cyclisation reaction which precedes the formation of ent-kaurene. In the fungus this inhibition

results in a marked reduction in gibberellin production, and by direct extrapolation to growing plants the growth inhibition observed would be due to a similar reduction in gibberellin production.

Attempts have been made to verify this hypothesis in growing plants by correlating the levels of extractable gibberellins obtained from plants which have been treated with growth retardants with the growth of the plants. Some cases have been reported in which growth retardant treatment of plants has resulted in a decrease of extractable gibberellin level, paralleling to some extent a decline in the growth rate of the plant (Baldeva and Lang, 1965; Zeevart, 1966; Jones and Phillips, 1967; Barendse, 1971; Van den Ende and Zeevart, 1971). Other cases have been reported in which the correlation between growth inhibition and decreased extractable gibberellin levels following growth retardant application is not upheld (Reid and Crozier, 1970, 1972).

However, it would seem doubtful if verification of the hypothesis can be achieved by this approach. The validity of the basic assumption that plant growth and endogenous gibberellin concentrations are always positively correlated can be questioned on the grounds that endogenous gibberellin concentration is a summation of several competing processes, including biosynthesis, translocation, hydrolysis of conjugated forms, metabolism,

de-activation and breakdown. Hence endogenous gibberellin concentration will not always reflect the rate of biosynthesis alone.

If, in some plants, growth retardants do inhibit stem elongation by inhibiting ent-kaurene production and hence gibberellin biosynthesis, as is the case in Gibberella fujikuroi, then this demonstrates that gibberellin biosynthesis in these plants proceeds through ent-kaurene in the same way as it does in Gibberella fujikuroi.

(6) Final Stages of Gibberellin Biosynthesis in Plants.

The sequence of intermediates involved in the final stages of gibberellin biosynthesis in plants is entirely unknown, but some information is available relating to the interconversions of some of the plant gibberellins. One difference between the fungal and plant gibberellins, which may prove to be quite important, is that no fungal C20-gibberellins have a 13-hydroxyl group, whereas many, though not all C20-gibberellins from plants possess a 13-hydroxyl group. Clearly 13-hydroxylation can occur at an earlier stage in some plants, and this could be indicative of the operation of two different major pathways in plants, one characterised by initial hydroxylation at the 13-position and the other characterised by initial hydroxylation at the 3-position as in Gibberella fujikuroi (Katsumi and Phinney, 1969; Lang, 1970).

The situation in plants is further complicated by the fact that different plants tend to biosynthesise and accumulate different gibberellins. Twenty-two structurally different gibberellins have now been identified in higher plants (Lang, 1970; Hiraga et al., 1972), and many of these have only been found in a few species. Consequently the same sequence or sequences of reactions will not necessarily operate during biosynthesis in all plants.

The metabolic interconversions of gibberellins in plants

which have been shown to take place are described structurally in Figure 6. These include hydroxylation and hydration reactions, and in one instance the formation of the γ-lactone group in ring-A.

Jones (1968a) fed gibberellin A_5 to light-grown pea (Pisum sativum) seedlings which had been previously treated with AMO-1618. The plants were extracted 3 days after the feeding. Bioassays, with the d5 dwarf corn bioassay, following TLC of the extracts indicated two distinct zones of growth promotion, one corresponding to the position of gibberellin A_5 and the other corresponding to the position of gibberellin A_1. Seedlings which had been treated with AMO-1618 in the absence of added gibberellin A_5, possessed no significant levels of extractable gibberellin. Jones was also able to show, by using similar TLC-bioassay procedures, that crude enzyme preparations from light-grown normal peas, in the presence of NADH, ferrous ions, magnesium ions, EDTA and ATP, were also capable of catalysing the hydration of gibberellin A_5 to gibberellin A_1.

Crozier and Reid (1971, 1972) examined the native gibberellins present in light-grown Phaseolus coccineus seedlings by bioassay of purified plant extracts. They found root apex removal of the seedlings resulted in the disappearance of gibberellin A_1 in the leaves, apical buds and root remnants of the seedlings.

Concurrent with the disappearance of gibberellin A_1 in these tissues was the accumulation of gibberellin A_{19}. The authors considered the most likely explanation for these results was the conversion of gibberellin A_{19} to A_1 in the root tips. Gibberellin A_{19} was thought to arise in the leaves. Furthermore the authors suggested that likely intermediates in this conversion could be gibberellins A_{20} or A_{23}. Gibberellin A_{20} has already been identified in immature Phaseolus coccineus seed (MacMillan, 1972).

Nadeau and Rappaport (1972) imbibed seed of Phaseolus vulgaris for 30 hours in an aqueous solution containing ^{3}H-gibberellin A_1, after which they extracted the seed with methanol and made a thorough search for radioactive metabolic products. The primary conversion product of ^{3}H-gibberellin A_1, detected by GLC of the TMS ethers/methyl esters, was ^{3}H-gibberellin A_8-glucoside along with traces of ^{3}H-gibberellin A_8. In this case 2β -hydroxylation of gibberellin A_1 was followed by glycosylation. The absence of ^{3}H-gibberellin A_3 and ^{3}H-gibberellin A_3-glucoside was indicated, which shows that the conversion of gibberellin A_1 to gibberellin A_3 did not take place.

Durley and Pharis (1973) applied ^{3}H-gibberellin A_4 to dwarf rice (Oryza sativa cv. Tan-ginbozu) seedlings, and found, within 24 hours of the application, conversion of gibberellin A_4 to gibberellins A_1 and A_{34} had taken place. Identification was made

by direct comparison of the TMS ethers/methyl esters on GLC with gibberellin A_1 and A_{34} standards using three different column packings. The conversion of gibberellin A_4 to A_1 and A_{34}, which involved single hydroxylations, accounted for 0.3%-0.8% of the applied gibberellin A_4.

FIGURE 6.

METABOLIC INTERCONVERSIONS OF GIBBERELLINS IN PLANTS

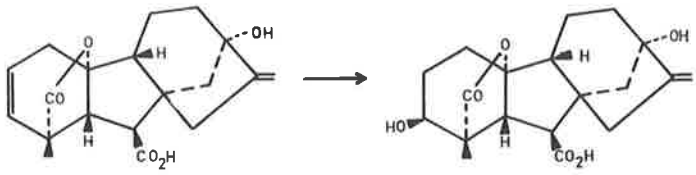
A. Conversion of gibberellin A_1 to A_5 by light-grown pea seedlings (Pisum sativum) (Jones, 1968a).

B. Conversion of gibberellin A_{19} to A_1, with the postulated intermediates A_{20} and A_{23}, by light-grown Phaseolus coccinius seedlings (Crozier and Reid, 1971, 1972).

C. Conversion of gibberellin A_1 to A_8 and A_8-glucoside by imbibed Phaseolus vulgaris seeds (Nadeau and Rappaport, 1972).

D. Conversion of gibberellin A_4 to A_1 and A_{34} by Tan-ginbozu dwarf rice seedlings (Oryza sativa) (Durley and Pharis, 1973).

A



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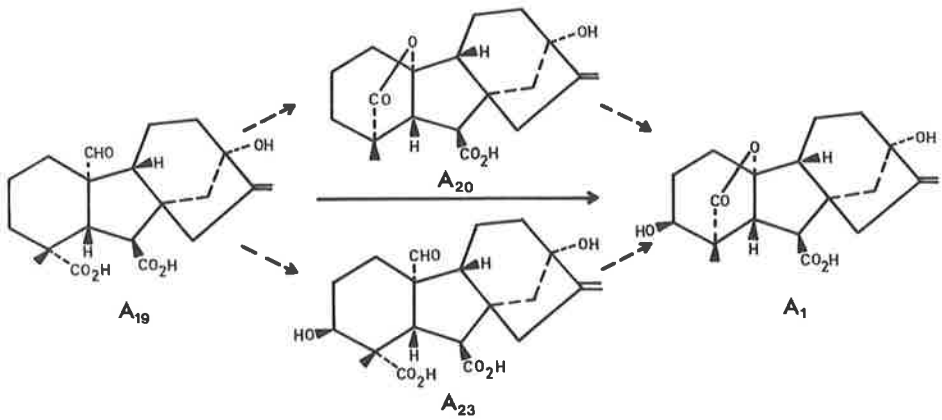

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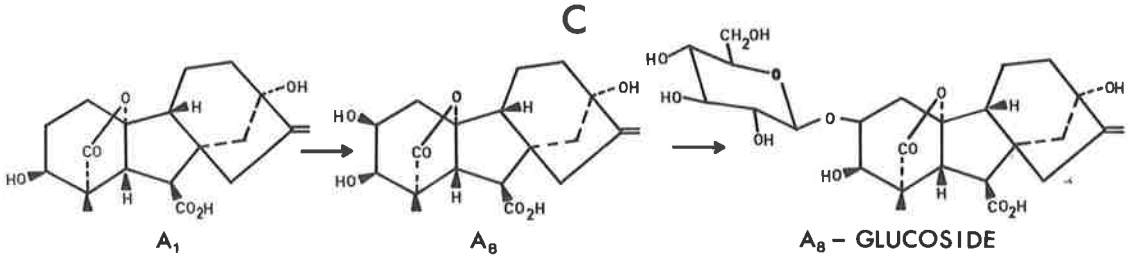
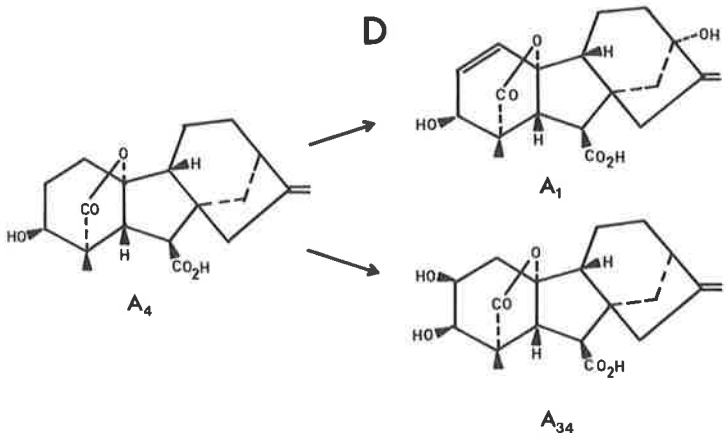


D



(7) Sites of Gibberellin Biosynthesis in Plants.

As has been mentioned before in this review, understanding of the gibberellin biosynthetic pathway in plants has been hampered by the lack of a system which is capable of incorporating radioactively labelled precursors directly into gibberellins. In spite of this major difficulty a large amount of indirect evidence has been obtained for several distinct sites of gibberellin biosynthesis in plants.

The work of Jones and Phillips (1966), and Phillips (1971), has demonstrated that apical buds, including young leaves, are important areas of gibberellin biosynthesis. The results of apical bud excision and exogenous gibberellin application experiments by Jones and Phillips (1966) with sunflower plants (Helianthus annus) suggested the petiolate leaves of the apical bud were the major site of biosynthesis of gibberellin-like substances necessary for normal internode elongation. This was substantiated by a combination of diffusion and extraction techniques, using the dwarf pea and lettuce hypocotyl bioassays, in which it was shown that diffusion of excised apical buds on agar for 20 hours revealed a level of gibberellin in the agar greater than was obtained by solvent extraction of the same number of apices. Also the gibberellin levels of apices extracted following a 20-hour diffusion period was the same as that obtained from buds extracted

immediately following excision from the plant. Similar experiments with young internode sections indicated they were not sites of gibberellin biosynthesis. Similar results of this type have been obtained with peas (Pisum sativum) (Lockhardt, 1957; Jones and Phillips, 1966).

Phillips (1971) extended the work on Helianthus annus by following the diffusible gibberellin yields from excised green apical buds supported on agar which was supplemented with either sucrose or mevalonic acid. Under normal circumstances the yield of diffusible gibberellin in the agar blocks supporting the apical buds fell within 48 hours of excision of the bud. If the agar contained either 2% sucrose or 0.1% (RS)-mevalonic acid, gibberellin continued to diffuse from the buds into the agar for at least 72 hours. The inclusion of sucrose in the agar resulted in a maintenance of the same rate of gibberellin diffusion over a longer period of time; when mevalonic acid was supplied the rate of gibberellin diffusion into the agar blocks was enhanced over the 72 hour period. On the basis of paper chromatography in one solvent, there appeared no qualitative change in the nature of the gibberellin-like component in the diffusate. These results strongly suggest that the apical buds of sunflower contain all the enzymes necessary for the incorporation of mevalonic acid into biologically active gibberellin, and the gibberellin produced in

the apical buds is largely responsible for the internode elongation in the stems of the plant.

The function of roots in the biosynthesis and metabolism of gibberellins has been receiving increasing attention since it became known that gibberellins occur in the roots of plants (Butcher, 1963; Phinney and West, 1961; Murakami, 1968), and that free gibberellins and gibberellin glycosides occur in significant quantities in the bleeding sap of plants and trees (Skene, 1967; Kende and Sitton, 1967; Sitton et al., 1967; Reid and Carr, 1967; Sembdner et al., 1968; Carr and Reid, 1968; Reid and Burrows, 1968).

The origin of the root and bleeding sap gibberellins is not clear. They could be biosynthesised in the roots, or they could be biosynthesised in the shoots and transported to the roots. Sitton et al. (1967) found root tips of Helianthus annus were capable of biosynthesising ent-kaurenol, a gibberellin precursor in Gibberella fujikuroi, when incubated with 2-^{14}C-mevalonic acid. However, this is not conclusive evidence of gibberellin biosynthesis as there is no indication that roots can convert ent-kaurenol to gibberellin.

Jones and Phillips (1966) observed the appearance of diffusible gibberellins in agar blocks when the apical 3-4 mm of roots of Helianthus annus were supported on agar blocks. Again, however, this is no proof of biosynthesis in the roots as the diffusible

gibberellins could arise from hydrolysis of conjugated gibberellins.

Butcher (1963) detected gibberellin-like compounds in excised roots which had been growing in culture for 4-5 weeks. These results show that excised roots are capable of gibberellin biosynthesis, but it does not necessarily follow that roots *in situ* carry out the same function.

Crozier and Reid (1971, 1972) provided data which supported a shoot $\longrightarrow$ root $\longrightarrow$ shoot recycling system being involved in gibberellin biosynthesis in *Phaseolus coccineus* seedlings. Normal light-grown seedlings were found to contain predominantly gibberellin A_1, while seedlings from which the root tips had been removed by shaving the main root were found to contain predominantly gibberellin A_{19}. The authors suggest these results indicate that the root tips are responsible for the conversion of gibberellin A_{19} to A_1.

The suggestion of the involvement of a recycling system in gibberellin biosynthesis in plants of the type proposed by Crozier and Reid is attractive inasmuch as it has the potential, on a hormonal basis, of ensuring sympathy between root growth and shoot growth, a characteristic of plant growth which has long been known.

Developing seeds are another site in which active gibberellin biosynthesis takes place. The ability of immature seeds to

accumulate large amounts of free gibberellins has already been mentioned (see I.A.5), and immature seeds possess the necessary enzymes for conversion of mevalonic acid to ent-kaurene and the oxidised derivatives of ent-kaurene, including gibberellin A_{12}-aldehyde (see I.B.2).

The lack of growth of apricot pericarp when seeds are prevented from growing, and the resumption of this growth with exogenous gibberellin A_{32}, the predominant gibberellin in these seeds, is good presumptive evidence for gibberellin A_{32} being biosynthesised in developing apricot seed (Coombe, 1971).

(8) Control of Gibberellin Biosynthesis in Plants.

The mechanism or mechanisms by which plants control the rate and type of gibberellin production, and in this way influence their growth and development, is potentially one of the more fascinating aspects of the physiology and biochemistry of the gibberellins. There is, at the moment, virtually a complete absence of factual information in this area, but some speculations which have been made are noteworthy.

One popular hypothesis regarding control of gibberellin biosynthesis is that certain environmental factors to which plants respond, bring about their effects by directly controlling gibberellin production. Brian (1958) was the first to recognise the similarity in growth response between gibberellin treatment and certain environmental treatments, for example, long-day photoperiods and exposure to low temperatures. These correlations have been extended and broadened (Phinney and West, 1961; Paleg, 1965; Chailakhyan, 1968), but there is no deeper insight into the respective mechanisms controlling the growth responses.

Significant changes in the levels of extractable gibberellins from plants following various photoperiodic and vernalization treatments have been recorded (Nicholls and May, 1964; Reid and Clements, 1968; Zeevart, 1969; Suge, 1970; Cleland and Zeevart, 1970; Beevers et al., 1970; Loveys and Wareing, 1971a, 1971b;

Zeevart, 1971; Van der Ende and Zeevart, 1971), but, as has already been mentioned, the concentrations in which gibberellins occur in plant tissues is a summation of several competing processes including translocation, biosynthesis and liberation from conjugated forms, and utilisation, breakdown and deactivation. Consequently, changes in concentrations of endogenous gibberellins will not necessarily reflect changes in rates of gibberellin biosynthesis. Attempts have been made to overcome this problem by following changes in gibberellin content with and without the growth retardants CCC and AMO-1618 (Reid et al., 1968), but the concentrations of growth retardants which have been used (100 mg/litre of AMO-1618 and 1-10 g/litre of CCC) are so high that the specificity of effects of these compounds at this level on plant tissue must be seriously questioned.

It does seem clear, however, that future thinking on the control of gibberellin biosynthesis in plants will have to take account of factors which pertain to root growth and root activity, as roots are now known to be important sites of gibberellin biosynthesis and/or metabolism. (See I.B.2(7)).

3. Summary of Gibberellin Biosynthesis in Higher Plants and Gibberella Fujikuroi.

It is evident that the present state of awareness of gibberellin biosynthesis in plants is heavily dependent upon analogy with the results of research on gibberellin biosynthesis in the fungus Gibberella fujikuroi. In practical experimental terms the principal difference between these two sources of gibberellin resides in the enormous disparity in concentration in which gibberellins are found in plants and fungal cultures. However, there is really a marked fundamental distinction between the occurrence of gibberellins in plants, where they function as hormones capable of producing in small doses dramatic effects on plant growth, and in Gibberella fujikuroi, where they have no hormonal function and are produced in relatively large amounts. This distinction may not have any significant bearing on the biosynthetic pathway leading to gibberellin production in plants and the fungus, indeed the pathways in these two organisms seem to be very similar, but it would be expected to have some bearing on the control mechanisms which operate during gibberellin biosynthesis in these two types of organism.

Consequently it would be expected that while studies with Gibberella fujikuroi are likely to be highly informative with regard to the sequence of intermediates which lead up to

gibberellin production, these studies would not be expected to make any contribution toward our understanding of the control of the rate of gibberellin production in plants. To further our understanding of the mechanisms involved in this control process it is therefore imperative that a system be developed from higher plants in which gibberellin biosynthesis can be easily and conveniently studied.

II.

OBJECTIVES OF THE PROJECT

The fundamental aim of the project was to develop a system from plants which is capable of actively incorporating radioactive precursors into gibberellins. Once having developed such a system the intention was to study the sites of gibberellin biosynthesis within the growing plant (tissues involved), and within the cells of the tissues (intracellular localisation of enzymes). Finally some of the possible mechanisms operating in the control of gibberellin production were to be studied by following the rates of conversion of precursors to gibberellin by plants grown or subjected to different environmental conditions.

The results contained in the literature suggested that the development of a convenient system from plants for studying the incorporation of radioactive precursors into gibberellins would be the major problem of the project; this proved to be the case.

Because of the desire ultimately to study the control of gibberellin production in relation to plant growth, and in particular stem elongation, growing barley seedlings (Hordeum vulgare) were normally used as the source of tissue. Experiments were also conducted on developing apricot fruits (Prunus armeniaca) and dwarf rice seedlings (Oryza sativa). How these experiments pertain to the general theme of the project is explained in the relevant results and discussion sections.

III.

MATERIALS AND METHODS

A. Materials.

1. Chemicals and Reagents.

The chemicals and reagents used in this project were analytical grade. The source of the chemicals and reagents are listed below:-

*Allogibberic acid; L. Mander, Organic Chemistry Dept., University of Adelaide.

Anakrom ABS; Analabs Inc., Connecticut, U.S.A.

^{14}C-Benzoic acid (standard); Packard Instrument Co., Illinois, U.S.A.

Bovine serum albumen (crystalline); Sigma Chemical Co., Missouri, U.S.A.

BSA (in 1 ml ampoules); Pierce Chemical Co., Illinois, U.S.A.

*[1] n-Butyl lithium (20% in n-hexane); Merck & Co., Darmstadt, Germany.

Cholesterol (A grade); Calbiochem, California, U.S.A.

Creatine phosphate (disodium salt, A grade); Calbiochem, California,U.S.A.

Chloramphenicol; Parke, Davis and Co., Sydney, Australia.

Creatine phosphokinase (A grade); Calbiochem, Calif., U.S.A.

DEAE-Sephadex (A 50); Pharmacia, Uppsala, Sweden.

Folin and Ciocalteu reagent; British Drug Houses, England.

β -Emulsin; Merck and Co., Darmstadt, Germany.

Gaschrom Q; Applied Science Laboratories, Pennsylvania, U.S.A.

Gibberellin A_3 (97.4% pure); Merck and Co., New Jersey, U.S.A.

*Gibberellin A_1; Rural Group, Imperial Chemical Industries of Australia and New Zealand.

Gibberellin A_4 plus A_7 (71% A_7); G.N.Turner, Imperial Chemical Industries, England.

*[2] Gibberellins A_1, A_5, A_6, A_8, A_9; L.G.Paleg, University of Adelaide.

D.L.-Glutamic acid; British Drug Houses Ltd., England.

Glutamic dehydrogenase (Type II); Sigma Chemical Co., Missouri, U.S.A.

HMDS: Applied Science Laboratories, Pennsylvania, U.S.A.

ent-Kaurenoic acid; Organic Chemistry Dept., University of Western Australia.

ent-17nor-Kauran-16-on-19-oic acid; Organic Chemistry Dept., University of Western Australia.

Lanosterol; Mann Research Laboratories, New York, U.S.A.

2-Mercaptoethanol; Fluka and Co., Buchs, Switzerland.

Methyl iodide; British Drug Houses, England.

^{14}C-Methyl iodide; Radiochemical Centre, Amersham, England.

2-^{14}C-(RS)-Mevalonic acid; Radiochemical Centre, Amersham, Eng.

Molecular sieve 5A ($\frac{1}{16}$th inch pellets); British Drug Houses of Australia.

OV-17; Applied Science Laboratories, Pennsylvania, U.S.A.

OV-101; Applied Science Laboratories, Pennsylvania, U.S.A.

'Parafilm'; Gallenkamp, England.

dimethyl-POPOP; Packard Instrument Co., Illinois, U.S.A.

PPO; Packard Instrument Co., Illinois, U.S.A.

*[3] Pronase; Calbiochem, California, U.S.A.

QF-1; Applied Science Laboratories, Pennsylvania, U.S.A.

Silica gel H (Chromatography grade); Merck and Co., Darmstadt, Germany

^{14}C-Sodium carbonate; Radiochemical Centre, Amersham, England.

Streptomycin sulphate; Glaxo-Allenburys Pty. Ltd., Sydney, Australia.

n-Tetracosane (pure); Kock-Light Laboratories, England.
TMCS; Applied Science Laboratories, Pennsylvania, U.S.A.
TONY; Kock-Light Laboratories, England.
Triphenylphosphine (pure); Kock-Light Laboratories, England.
'Tween-20'; Merek and Co., New Jersey, U.S.A.
'Silyl-8'; Pierce Chemical Co., Illinois, U.S.A.
Squalene; Sigma Chemical Co., Missouri, U.S.A.

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* Gifts of these compounds are hereby gratefully acknowledged.

*[1] n-Butyl lithium was donated by the Organic Chemistry Dept., University of Adelaide.

*[2] Small amounts of these gibberellins were made available by L.G.Paleg, University of Adelaide.

*[3] A sample of pronase was donated by the Department of Agricultural Biochemistry and Soil Science, University of Adel.

Gibberic acid was prepared from gibberellin A_3 by a method similar to that described by Cross (1954) in which 97 mg of gibberellin A_3 was suspended in 10 ml of I N HCl at 100°C for 2 hours, after which the material was extracted into ethyl acetate and crystallised from a mixture of ethyl acetate and light petroleum (b.p. 60-80°C) to give approximately 50 mg of needles (m.p. 137-145°C). Upon recrystallisation from the same solvent the melting point of the needles was raised to 153-154°C.

Gibberellins A_1, A_3, A_4, A_5, A_6, A_7, A_8, A_9, allogibberic acid and cholesterol, all showed the presence of single peaks when run as the methyl esters or TMS ethers/esters during GLC analysis, except allogibberic acid, which was contaminated to the extent of 5% with gibberic acid.

2. Solvents.

All solvents used for extraction of gibberellins and for chromatography were distilled in a glass fractionating column before use.

Petroleum spirit, tetrahydrofuran and pyridine were dehydrated by the following procedures: petroleum spirit (b.p. 60-80°C) by storing the freshly distilled solvent over freshly extruded sodium wire in a tightly capped container according to the method described by Vogel (1956); tetrahydrofuran was dried in an identical fashion after it had been distilled from ferrous sulphate; pyridine was dried by refluxing for several hours over KOH and storing the distillate in a tightly capped container over Molecular Sieve 5A which had been previously activated by heating to 450°C for 2-3 hours. Routine checks on the water content of the pyridine were carried out according to the method described by Sherman et al. (1971a) in which a few µl of HMDS and TMCS were added to a small quantity of pyridine: normally a slight haze was produced, but on occasions an excessive precipitate formed, in which case the solvent was re-distilled and dried again over freshly activated Molecular Sieve 5A.

3. Plant Material.

Seedlings of barley (Hordeum vulgare cv. Prior), which were used for gibberellin extractions, were obtained from a supply of barley grain provided by D. Aspinall of the University of Adelaide.

Tan-ginbozu dwarf rice (Oryza sativa) seeds were supplied by Y. Murakami of The National Institute of Agricultural Sciences, Nishigahara, Kita-Ku, Tokyo, Japan.

Young apricot fruits (Prunus armeniaca cv. Moorpark) were obtained from the orchard of the Waite Institute during September 1971 and September 1972.

Barley seed (Hordeum vulgare cvs. Prior and Clipper), used in the gibberellin endosperm bioassay, were obtained from the South Australian Department of Agriculture, and were cleaned and graded for uniform size before use.

4. Treatments for Chromatography.

Whatman paper, used for chromatography and electrophoresis, was washed before use by sequential elution with 0.1 M EDTA (brought to pH 8.5 with ammonia, s.g. 0.89), distilled deionised water, 2 N acetic acid, distilled deionised water and distilled methanol. The papers were then air dried before use.

For TLC silica gel H was coated 1 mm thick on clean glass plates as an aqueous film using a Shandon Chromatogram Spreader according to the procedure described by Stahl (1962). The plates were then dried in a stream of air at room temperature before use. Only in specifically mentioned cases were the silica gel H plates activated in a hot oven before use.

DEAE-Sephadex (A50) was allowed to swell in distilled deionised water and was then washed before use with excess amounts of 0.5 N NaOH, distilled deionised water, 0.5 N acetic acid and distilled deionised water.

5. Equipment.

Barley and rice seedlings were raised in controlled environment growth cabinets (Zankel Scientific Equipment Co.). The cabinets were not equipped with humidity control, but maintained a relatively constant temperature within 2°C of the set reading. Light was supplied in the cabinets by double banks of fluorescent tubes; light intensity at plant level was maintained at a constant level by adjusting the distance between the plants and the light bank.

High voltage paper electrophoresis was carried out on a water-cooled flat-bed electrophoresis unit (Paton Industries Ltd.). Electrophoretic analysis of nucleotides was carried out with a smaller unit which used a tank of carbon tetra-chloride as coolant.

Results of barley endosperm bioassays were read directly from a Waters R4 Differential Refractometer supplied by Waters Associates Ltd., Cheshire, England.

A Nuclear Chicago Actigraph II gas-flow radioactive scanner was used to monitor radio-activity along the length of TLC plates and strips of paper chromatograms.

Liquid scintillation radioactive counting was carried out on a Packard Tricarb Liquid Scintillation Spectrometer.

Fraction collecting from column chromatograms was carried out with a LKB Ultrorak Fraction Collector, set for collection

on a time basis, and used in conjunction with a LKB Recychrom Peristaltic pump, adjusted to pump at a rate of 1 ml/minute.

Routine GLC analysis was carried out on a Perkin-Elmer Model 801 fitted with glass columns and a flame ionisation detector. A stream splitter and collection apparatus was contrived; when open, in excess of 98% of the carrier gas was diverted to the collector. The collector consisted of a short length of heated stainless steel tubing to the open end of which was attached a steel Luer-Lock fitting. Glass Luer-Lock syringes were used as collecting tubes by packing with glass wool moistened with methanol/water (1/1). Fractions were collected by manually exchanging the glass syringes at convenient intervals.

GLC analysis of ent-kaurenoic acid and synthesised 17-^{14}C-ent-kaurenoic acid was carried out on a Shimadzu GC-1C machine fitted with glass columns and a flame ionisation detector. Fraction collecting of the column effluent was carried out as above.

B. Methods.

1. Introduction.

General methods applicable to all experiments will be described here. Specific methods will be detailed with each experiment in the results section.

2. Culture of Barley and Rice Plants.

Barley (Hordeum vulgare cv. Prior) seeds were germinated on moist filter paper in petri dishes in the dark at 20°C for 24-36 hours. They were then planted out into 5" plastic pots, 5 seeds/pot, containing moist pearlite. The seedlings were raised in a growth cabinet, without humidity control, at 20°C ($\pm$ 1°C) with light intensity of 4.1-5.4 milliwatts/cm^2/hour at the level of the seedlings. Continuous lighting was used except when specified otherwise.

The seedlings were irrigated every other day with a modified Hoagland nutrient solution containing the following salts, with the concentration in p.p.m. given in brackets: $Ca(NO_3)_2.4H_2O$ (1653), KNO_3 (505), KH_2PO_4 (348), $MgSO_4.7H_2O$ (493), Fe.EDTA (1.0 Fe), $MnSO_4$ (0.25 Mn), H_3BO_3 (0.25 B), $ZnSO_4$ (0.25 Zn), $CuSO_4$ (0.02 Cu) and Na_2MoO_4 (0.02 Mo).

Seedlings were usually used 9-10 days after sowing, by which time the third leaf was just beginning to emerge from the

second leaf and the plant apex was at the double ridge stage of development.

Tan-ginbozu dwarf rice (Oryza sativa) seeds were germinated as described by Murakami (1970) in a beaker of water at 30°C for 2 days, after which the seeds were planted out in small plastic pots containing moist pearlite, 5 seeds/pot, and the seedlings were raised in a controlled environment cabinet at 30°C ($\pm$ 1°C) with a light intensity of 4.1 milliwatts/cm^2/hour at the level of the seedlings. A regular alternation of 16 hours light and 8 hours dark was used, and the pots were irrigated every other day with nutrient solution as described above. The plants were used for experiments 3 days after sowing, by which time the first leaf was just emerging from the coleoptile sheath.

3. Extraction of Gibberellins.

(1) Barley and Rice Seedlings.

The methods used to extract the free gibberellins from barley and rice seedlings were developed after particular reference had been made to the work of Jones et al. (1963), Radley (1966), Radley (1968) and Jones (1968b). The same methods were used for barley and rice seedlings.

The seedlings were harvested, washed free of pearlite, divided into tops and roots (if necessary), weighed, and either wrapped in aluminium foil and immersed in liquid nitrogen after which they were stored in a cold room ($-20^{o}C$) before extraction, or extracted immediately following harvesting.

The tissue was diced, suspended in cold potassium phosphate buffer (pH 7.5, 0.05 M) and homogenised with a mortar and pestle using acid-washed sand as an abrasive. For each 4 g fresh weight of tissue, 10 ml of buffer was used. An equal volume of distilled acetone was added to the homogenate and after further homogenisation the mixture was centrifuged (3000xg for 15 minutes).

The supernatant was decanted, and the pellet consisting of cell debris and protein was re-extracted in an identical fashion.

The pooled supernatant solutions were shaken with $\frac{1}{3}$rd volume of distilled chloroform after the pH of the aqueous-acetone mixture had been adjusted to 8.5 with 1 N NaOH. The mixture was again

centrifuged (3000xg for 15 minutes) and the aqueous phase was removed with a pasteur pipette. The lower chloroform—acetone phase was washed by shaking with a small volume of potassium phosphate buffer (pH 7.5, 0.05 M), and centrifuged again.

The pooled aqueous fractions were acidified to pH 2.5 with 1 N HCl and were then extracted three times with $\frac{1}{3}$rd volume of ethyl acetate using centrifugation to aid separation. The ethyl acetate layers were pooled.

This procedure yielded three extracts: chloroform-acetone extract (C); acid ethyl acetate extract (E), and water residue fraction (A), in addition to the cell debris and protein pellet.

Before these extracts were further purified they were rotary evaporated at 25^{o}C under reduced pressure until dry. Fractions E and A were neutralised with dilute NaOH before rotary evaporation, and fraction A was warmed to 35^{o}C during rotary evaporation.

(2) Apricot Fruits.

The procedure for the extraction of free gibberellins from young apricot fruits was based on the work of Coombe and Tate (1972).

Young apricot fruits were harvested, weighed, wrapped in aluminium foil, frozen by immersion in liquid nitrogen and stored in a cold room ($-20^{o}C$) before extraction.

The fruits were homogenised in potassium phosphate buffer (pH 7.5, 0.05 M) and distilled acetone in a mortar and pestle with acid-washed sand as described above for barley and rice seedlings.

The homogenate was centrifuged (3000xg for 15 minutes) and the pellet was re-extracted three more times in the same fashion.

The pooled soluble extracts were shaken with $\frac{1}{3}$rd volume of chloroform after the pH of the aqueous-acetone mixture had been adjusted to 8.5 with 1 N NaOH, and the mixture was centrifuged (3000xg for 15 minutes) to separate the phases. The upper aqueous layer was removed with a pasteur pipette and the lower chloroform-acetone phase was washed with a small volume of potassium phosphate buffer (pH 7.5, 0.05 M).

The combined aqueous phases were subjected to brief rotary evaporation at $25^{o}C$ under reduced pressure to remove any residual chloroform and acetone, and the extract was then subjected to column chromatography (see III.B.4(3)).

4. Preliminary Purification of Extracts.

(1) General.

Purification of the gibberellin extracts obtained from barley and rice seedlings and young apricot fruits was carried out using paper chromatography, paper electrophoresis, TLC, and column chromatography, as a preliminary to quantification of the gibberellin content of the extracts by the barley endosperm bioassay procedure, or as a preliminary to GLC analysis.

Freshly distilled methanol was used as a solvent for the extracts. Paper chromatograms and electrophoretograms were eluted with a mixture of water and methanol (20% methanol in water). The eluates were dried by rotary evaporation at 25°C under reduced pressure. Recovery from silica gel H TLC plates was accomplished in small glass columns with methanol as an eluant. The eluates were again dried by rotary evaporation at 25°C under reduced pressure.

Glucose and gibberellin mixtures containing about 5 µg each of gibberellins A_4 plus A_7 and A_3, and occasionally A_1 and A_9 were used as side markers on chromatograms and electrophoretograms. The markers were located by spraying with 0.5% aqueous potassium permanganate; the gibberellins appeared within a few seconds as yellow spots, while glucose became evident as a clear area in 30-60 minutes. Location of the standards on silica gel H TLC

plates was achieved either with the potassium permanganate spray, in which case the gibberellins were immediately visible as yellow spots which faded within 2-3 minutes, or with a 5% sulphuric acid in ethanol spray followed by heating to 120°C for 10 minutes. In the latter case glucose charred and showed as a darkened area, while the gibberellins were seen as fluorescent spots under U.V. light.

Usually methyl red was used as a side marker and superimposed on the extract during chromatography and electrophoresis; this coloured marker provided a useful internal standard for assessing the migration of the compounds in the extract relative to the standards run beside the extract.

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(2) Extracts from Barley and Rice Seedlings.

Crude acid ethyl acetate extracts from barley and rice seedlings were purified by a combination of the following three techniques:-

(a) Descending paper chromatography on washed 3MM Whatman chromatography paper with isopropanol/ammonia (s.g. 0.89)/water (10/1/1, v/v/v) as solvent.

(b) High voltage paper electrophoresis on washed 3MM Whatman chromatography paper in ammonium bicarbonate buffer (0.1 M, pH 8.9) at 2,300 volts for 1 hour.

(c) TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1, v/v/v), and occasionally in n-butanol/acetic acid/water (85/5/10, v/v/v).

(3) Extracts from Apricot Fruits.

The aqueous extract from apricot fruits was loaded on to a DEAE-Sephadex (A50) column (30 cm x 2.4 cm) (see III. A.4). The column was eluted with a linear gradient of 500 ml of water and 500 ml of 2 N acetic acid. One hundred 10 ml fractions were collected from the column, and the location of peaks of biological activity was made by bioassaying small aliquots of each fraction (usually 1/100th).

Those fractions possessing biological activity were pooled, rotary evaporated, and the dried residue was subjected to paper electrophoresis on washed Whatman 3MM chromatography paper in ammonium bicarbonate buffer (0.1 M, pH 8.9) at 2,300 volts for 1 hour, followed by descending paper chromatography in isopropanol/ammonia (s.g. 0.89)/water (10/1/1, v/v/v).

5. Gas-Liquid Chromatographic Techniques.

(1) General.

The techniques used in GLC of standard gibberellins, acidic plant extracts and ent-kaurenoic acid were developed with reference to the work of Cavell et al. (1967), Davis et al. (1968), Pacakova et al. (1971), Sherman et al. (1971a, 1971b), Aung et al. (1971), Perez and Lachmann (1971), Butts (1972) and Coombe and Tate (1972).

(2) Column Preparation.

(a) Preparation of 3% OV-17 on Silanised Gaschrom Q.

The technique of coating solid supports with thin films of stationary phase described by Horning et al. (1963) was used to coat Gaschrom Q with OV-17. Fifty grams of Gaschrom Q (mesh size 100-120) was silanised by treatment with 200 ml of distilled benzene containing 0.5% HMDS (v/v) in a 1 litre round-bottomed flask. The benzene and excess HMDS were removed under reduced pressure at 55-60^{o}C on a rotary evaporator using a slow and even rotation. This procedure was repeated twice more.

The silanised Gaschrom Q was then suspended in 200 ml of distilled acetone to which was added 200 ml of acetone containing 1.5 g of OV-17. The slurry was thoroughly mixed by swirling, and the acetone was removed under reduced pressure at 55-60^{o}C on a rotary evaporator as before. The OV-17 coated support was then dried in an oven at 85^{o}C.

(b) Preparation of 2% QF-1 on Anakrom ABS.

2% QF-1 on Anakrom ABS (mesh size 80-90) was prepared (by R.D.Firn and J.A.Considine) using a filtration method from a 2% (w/v.) solution of QF-1 in 80 ml of distilled methyl ethyl ketone which was slurried with 20 g of Anakrom ABS. The slurry was de-gassed, allowed to stand for 5 minutes and filtered under reduced pressure in a Buchner funnel until foaming had ceased. The support was then spread on a filter paper and dried for a few hours at 120°C.

(c) Preparation of 2.5% OV-101 on Gaschrom Q.

Packing of 2.5% OV-101 on Gaschrom Q (mesh size 80-100) was prepared (by T.J.Douglas) using the filtration method as in III.B. 5(3)b above, except distilled chloroform was used as the solvent.

(3) Preparation of Derivatives.

(a) Methyl esters.

Methyl esters of gibberellin standards, plant acids and ent-kaurenoic acid were prepared with diazo-methane by the method of Powell (1964) as described by Firn (1968). Diazo-methane, prepared by reacting 2 ml of carbitol, 2 ml of 60% (w/v) potassium hydroxide, and about 100 mg of TONY, was carried in a stream of dry nitrogen saturated with methylene chloride, through small bore 'Teflon' tubing, and bubbled into a methanolic solution of the compounds to be methylated. Bubbling was stopped after the solution had turned yellow, indicating the presence of excess diazo-methane, and the solution was concentrated or dried completely by blowing with dry nitrogen.

(b) Trimethylsilyl ethers of methyl esters.
(TMS ethers/methyl esters)

TMS ethers/methyl esters of gibberellins and plant acids were prepared by the method of Cavell et al. (1967) in which the dry methyl ester sample was dissolved in 100 µl of dry pyridine to which was added 10 µl each of HMDS and TMCS. The reaction, carried out in a small stoppered tube, was stopped after 20-30 minutes by removing the solvent and excess silylating reagents in a gentle stream of dry nitrogen.

The sample was re-dissolved in a small quantity of dry petroleum spirit (b.p. 60-80°C) when required for GLC analysis.

(c) Trimethylsilyl ethers of trimethylsilyl esters.
(TMS ethers/esters)

TMS ethers/esters of gibberellins and plant acids were prepared in glass ampoules (6.0 cm x 0.5 cm I.D.). Methanolic solutions of the materials to be derivatised were injected into open glass tubes and the methanol was removed by blowing with a fine stream of dry nitrogen. The sample was then thoroughly dried in a desiccator over activated Molecular Sieve 5A for 12-24 hours.

A small quantity (10-12 µl) of freshly prepared BSA/dry pyridine (1/1, v/v), containing 2% TMCS (v/v) as catalyst, was introduced into the tube with a dry syringe and the contents of the tube were quickly frozen by partial immersion in a liquid nitrogen bath. The open end of the tube was then sealed in a hot flame. Upon thawing the reagents were mixed by agitating the ampoule. Derivatisation was completed by heating to 85°C for 2-3 hours or leaving the tube at room temperature for 12 or more hours.

For injection onto the GLC column the ampoule was broken open and the contents sucked into a dry microsyringe.

(d) Choice of Derivatives.

The use of the three derivatives was governed by their individual characteristics.

The methyl esters were easy to prepare, but poly-hydroxylated gibberellins were found to run poorly and break down on OV-17 columns. Gibberellin A_8 methyl ester (3 free hydroxyls) failed to elute from OV-17 columns (Figure 7).

The TMS ethers/methyl esters were easy to prepare and gave sharp symmetrical peaks on OV-17. However, methylation of gibberellin A_3 is known to result in at least a 300-fold decline in the potency of this molecule in the barley endosperm bioassay (Coombe, private communication). For this reason these derivatives were not used for routine work, but were used for calibrating and conditioning new columns.

TMS ether/ester derivatives of gibberellins have the advantage in that the parent gibberellin can be recovered by hydrolysis with water. These derivatives also gave sharp symmetrical peaks on OV-17, and were the derivative of choice for most of the routine analyses.

The stability of the derivatives was another factor which affected their usefulness. The methyl esters were found to be stable for several weeks. The TMS ether/methyl ester derivatives were similarly stable provided they were kept dry in a tightly

capped container. The TMS ether/ester derivatives, in sealed ampoules, were stable for several weeks, but once the ampoule was opened the derivatives disappeared within a few minutes (presumably by hydrolysis with atmospheric moisture). For this reason it was found necessary, following the opening of each ampoule, to inject the mixture onto the column immediately, using a dry microsyringe.

(4) Retention Times of Standard Gibberellins.

Almost all of the GLC work reported in this thesis was done on 3% OV-17 columns. The retention times of a number of standard gibberellins on these columns are presented in Table 2 using the Kovats Retention Index System (Ettre, 1964). The separation of these standard gibberellins with the operating conditions routinely used are shown in Figure 7.

The Kovats Retention Indices were determined using a solution of 'parafilm' in petroleum spirit as the source of n-alkane standards, and standardising this source with n-tetracosane as described by Gaskin et al. (1971).

(5) Use of Internal Standards.

The range of retention times of the gibberellins on OV-17 columns extends from gibberellin A_9 to gibberellin A_{32} (Figure 7, Table 2). Allogibberic acid elutes before gibberellin A_9 and cholesterol elutes after gibberellin A_{32}.

It has been found that the relative retention times of the gibberellin standards between allogibberic acid and cholesterol can be calculated precisely. Hence known amounts of these two compounds (2 µg each) were routinely included with plant extracts prior to derivatisation. Besides permitting the calculation of

FIGURE 7.

GLC SEPARATION OF STANDARD GIBBERELLINS ON OV-17 COLUMNS

Column type: 3% OV-17 on silanised Gaschrom Q.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 210°C and rising to 270°C at a rate of 1.7°C/minute.

Detector: flame ionisation at 220°C.

Derivatives: prepared as described in III.B.5(3).

Attenuation: the same for each figure (X20).

Abbreviations: IAA indole acetic acid.
ABA abscisic acid (cis trans isomer).
AG allogibberic acid.
GBA gibberic acid.

FIGURE 7A. Methyl esters (2 µg of each compound).

FIGURE 7B. TMS ethers/methyl esters (2 µg of each compound with the exception of allogibberic acid (4 µg)).

FIGURE 7C. TMS ethers/esters (2 µg of each compound with the exception of allogibberic acid (4 µg)).

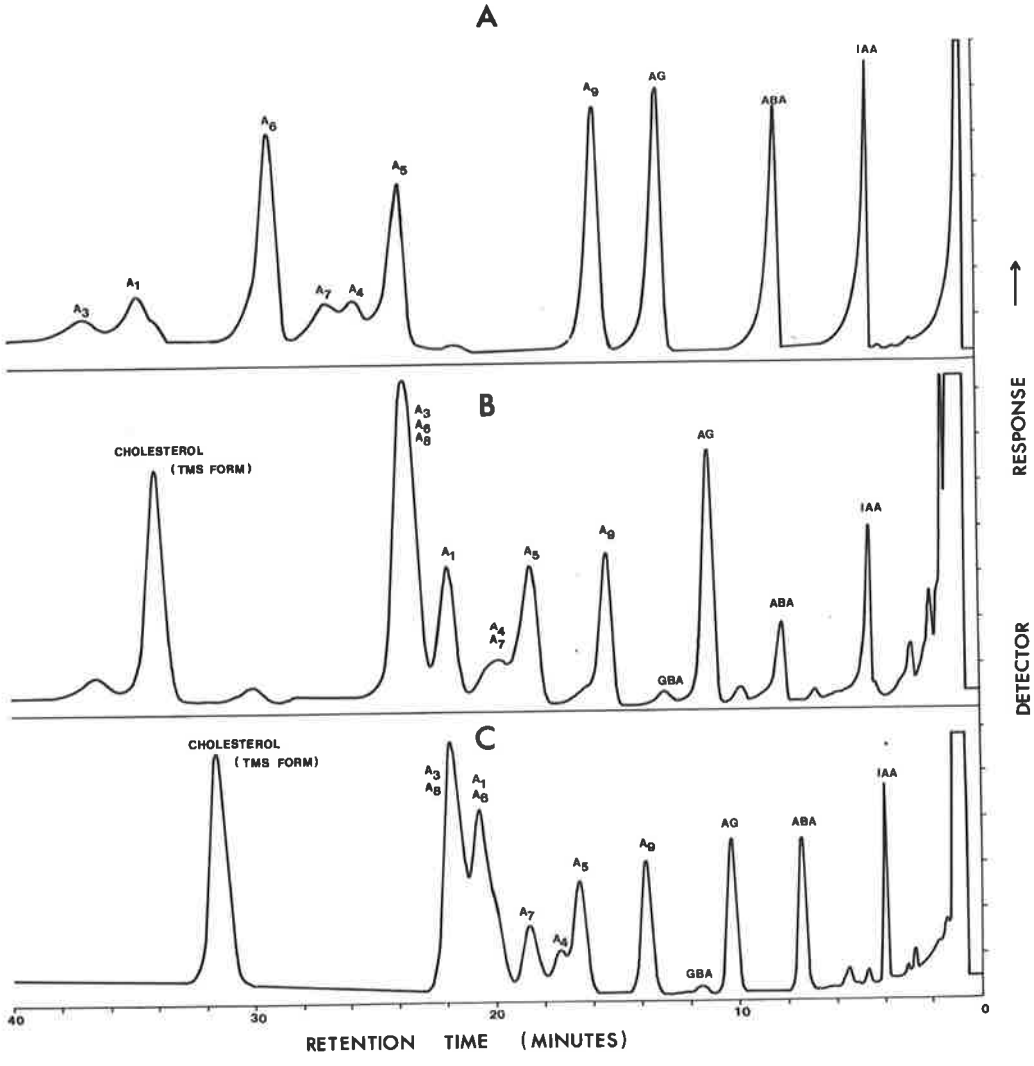

A
B
C
CHOLESTEROL
(TMS FORM)
CHOLESTEROL
(TMS FORM)
A3
A6
A8
A1
A4
A7
A5
A9
AG
GBA
ABA
IAA
RESPONSE
DETECTOR
40
30
20
10
0
RETENTION TIME (MINUTES)

TABLE 2.

The retention indices were calculated according to the procedure described by Ettre (1964) and Gaskin et al.(1971).

* Not Detected.

** Calculated from the retention index of a compound possessing biological activity in the barley endosperm bioassay present in an extract from young apricot fruits (see Figure 19), and checked against the data of Coombe and Tate (1972).

TABLE 2.

KOVÁTS RETENTION INDICES FOR STANDARD COMPOUNDS ON OV-17

Compound	Derivative		
	Methyl Ester	TMS Ether/ Methyl Ester	TMS Ether/ Ester
Indole acetic acid	2213	2220	2192
Abscisic acid (cis trans isomer)	2434	2443	2430
Allogibberic acid	2618	2543	2528
Gibberellin A_9	2705	2691	2665
A_5	2950	2787	2762
A_4	2997	2806	2790
A_7	3026	2849	2830
A_1	3245	2893	2877
A_6	3094	2933	2892
A_3	3298	2940	2923
A_8	*	2945	2935
A_{32} **	not done	not done	3063
Cholesterol	3323 (free hydroxyl)	3223 (TMS form)	3223 (TMS form)

the retention times of other gibberellins in the extract, comparison of the peak areas of the markers when run with an extract, and when run alone, permitted calculation of the efficiency of the derivatisation process, and provided a useful check on the proportion of each sample which was being successfully injected onto the column.

(6) Collection of Fractions.

Fraction collecting was carried out by the method already described (see III.A.5). Fractions were collected every 15 or 30 seconds commencing when allogibberic acid had eluted and ending just before cholesterol (TMS form) appeared; using a linear temperature program beginning at 210°C and rising to 260°C at a rate of 1.7°C/minute, this usually took 10 minutes.

The glass syringes were eluted with a small volume of distilled methanol. The eluate was concentrated under a stream of air, and portions distributed for measurements of radioactivity and biological activity.

(7) Problems with the Gas-Liquid Chromatographic Procedure.

Two major difficulties were encountered during the development of this methodology.

The first concerned formation of the TMS ether/ester derivatives. If either incompletely dry pyridine, incompletely dry extract or old BSA solution were used, the derivatisation process often failed. This failure was evidenced by the appearance of two liquid phases in the reaction mixture. The lower, oily, heavily coloured phase, and the upper less viscuous, clear phase, were immiscible; they were difficult to suck into a glass syringe, and in the few instances when they were able to be injected onto the GLC column, no peaks appeared from the extract, the internal standard allogibberic acid was not present, and cholesterol was present but in the free form, not in the TMS form. By experience it was found that once the two phases had formed no amount of manipulation or addition of fresh BSA/pyridine could overcome the problem. This problem, which has been described by other workers preparing similar derivatives with similar reagents (Sherman *et al.*, 1971b), only occurred when plant extracts were being derivatised, and could be avoided by using dry pyridine, fresh BSA reagent and thoroughly drying the extract prior to derivatisation.

The other major difficulty which developed after columns had

been used for some time (40-50 analyses) concerned the high background of biological activity which appeared in the collected fractions. TLC analysis of the bleed material obtained from old columns showed that it contained the gibberellins normally used as standards in the conditioning and running-in process. This problem was to some extent circumvented by regularly injecting small aliquots (5-10 µl) of the column conditioner 'Silyl-8', and by using TMS ether/methyl ester derivatives during the conditioning process. However, when the background of biological activity became too high the column was repacked.

6. Barley Endosperm Bioassay.

(1) Introduction.

Throughout this work the basis for detection and measurement of extractable plant gibberellins was the barley endosperm bioassay of Coombe et al. (1967a, 1967b) as modified by Coombe (1971).

(2) Preparation of Barley Half-Seeds.

Barley seed (cvs. Prior or Clipper) was dehusked by soaking in 50% sulphuric acid (v/v) for 4 hours at room temperature, then thoroughly washed in autoclaved deionised distilled water with vigorous shaking to dislodge husks. The dehusked seed was then soaked in autoclaved water for a further 20-24 hours at 2-4^{o}C before use; in some cases this period was reduced to 2-4 hours. Endosperm pieces were prepared by cutting the seed transversely 4 mm from the end away from the embryo as described by Coombe et al. (1967a).

(3) Procedure.

The bioassays were carried out in heat sterilised 2" x 1" specimen vials. Into each vial 0.6 ml of autoclaved citrate-phosphate buffer (0.005 M, pH 4.0), 0.2 ml of chloramphenicol/streptomycin mixture (25 µg each) and 0.2 ml of autoclaved water containing known amounts of gibberellin A_3 standard or unknown extract, were pipetted. All the reagents were dispensed with autoclaved automatic syringes. Two embryo-less barley half seeds were then placed in each vial and the vials were capped, briefly agitated to mix the reagents, and incubated for 40-42 hours at 30°C.

After incubation the caps were removed, 9 ml of water was added and the refraction of the diluted solution was measured with a Waters R4 Differential Refractometer, set to read digitally at maximum sensitivity and with water in the reference cell. The change in refractometer units was obtained by subtracting the readings obtain with a reagent blank without any half-seeds.

Silica gel H TLC plates, paper chromatograms and paper electrophoretograms of plant extracts were bioassayed for gibberellin activity directly by dividing into segments. Provided the chromatograms had been adequately air-dried to remove all traces of residual solvents, the inclusion of small amounts of silica gel H or chromatography paper had no effect on the bioassay response. When silica gel H was present in the vials, the diluted solution

was filtered through filter paper cups (Whatman Number 1) before refraction was read.

When it was inconvenient to take the bioassay readings at the completion of the 40-42 hour incubation, the vials were stored at $-20^{o}C$. They were subsequently read by thawing the contents, adding 9 ml of water and measuring refraction. The freezing and thawing process had no effect on the bioassay response.

In the early stages of the project, microbial growths developed in some vials during incubation, producing turbid, smelly solutions. This was avoided once the dispensing pipettes had been autoclaved, and the standard gibberellin solutions millipore filtered (0.22μ millipore). As an added precaution the cutting block and blades with which the half-seeds were prepared were regularly swabbed with aqueous methanol (methanol/water, 60/40) during the cutting process.

(4) Standard Bioassay Response.

Of the known compounds possessing ent-gibberellane skeletons, only gibberellins $A_1 - A_7$, A_{22}, A_{23} and A_{32} have been found to produce significant responses in amounts of 1 µg or less in the barley endosperm bioassay (Crozier et al., 1970; Coombe, 1971). Structural similarities between these compounds suggest the requirements for activity include a γ-lactone bridge between C-4 and C-10, a C-3 hydroxyl group and a free C-2 position, or alternatively a double bond or epoxide between C-2 and C-3.

A typical dosage/response curve for the barley endosperm bioassay with gibberellin A_3 is shown in Figure 8. A significant response was produced by 10^{-9}g of gibberellin A_3, and the magnitude of the response continued in an approximately linear manner to 10^{-7}g of gibberellin A_3.

A diverse array of other compounds not possessing an ent-gibberellane skeleton have been reported to elicit responses in this bioassay, thus simulating gibberellin activity. ent-Kaurene, mevalonic acid and kinetin have all been reported to produce a gibberellin-like response in this bioassay (Boothby and Wright, 1962; Jones, 1968c; Van der Groenpetridis et al, 1968). In addition, the nucleotides 3',5'-cyclic AMP and ADP, and the amino acids glutamate and aspartate have been found to induce α-amylase liberation by barley aleurone layers (Galsky and Lippincott, 1969a,

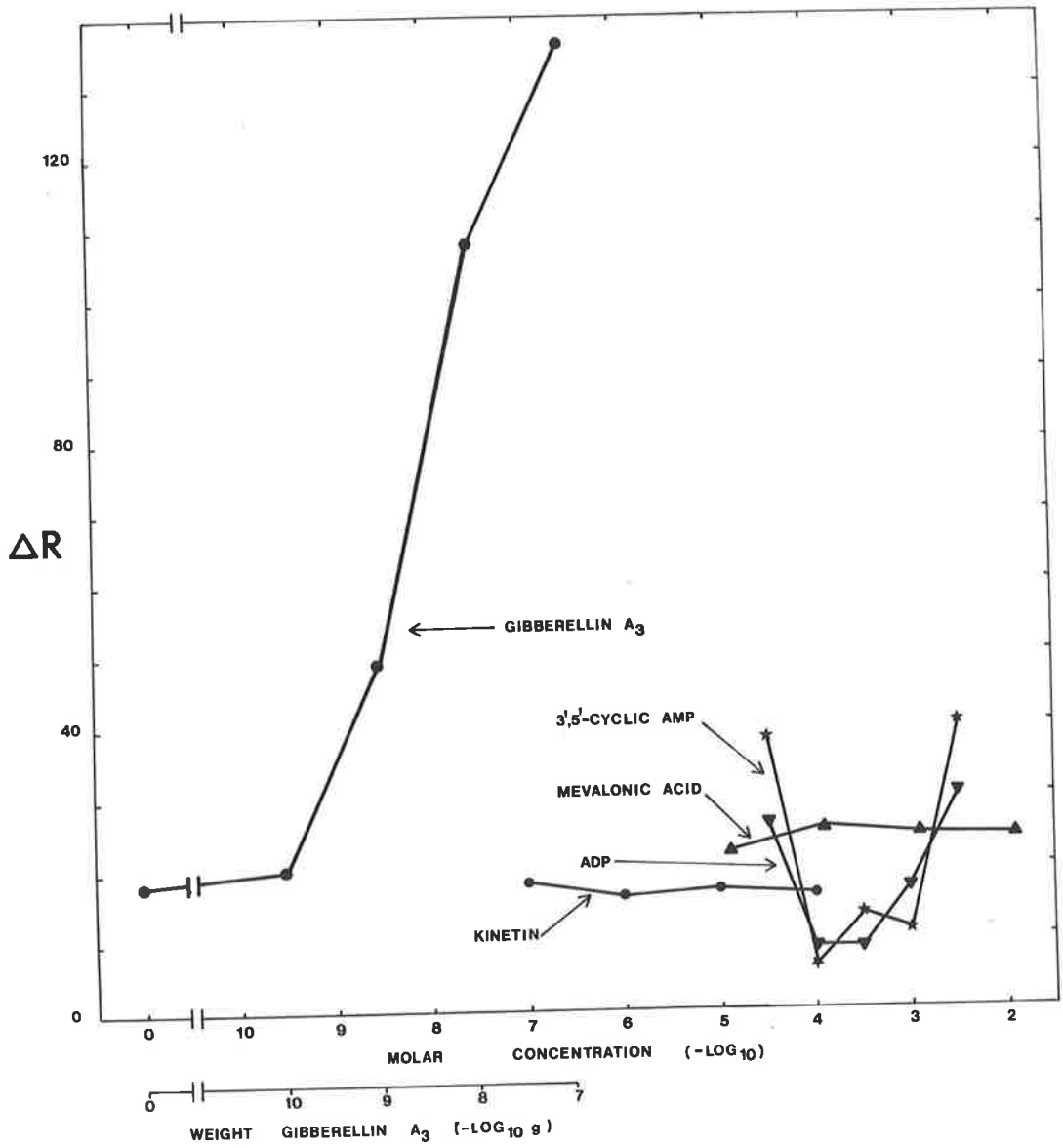
FIGURE 8.

STANDARD BIOASSAY RESPONSE

Bioassays were carried out as described in III.B.6.

Abscissa values refer to the concentrations of the various compounds.

Ordinate values (ΔR) refer to the increase in refraction of the ambient bioassay solution following a 40-hour incubation.


ΔR
120
80
40
0
GIBBERELLIN A3
3',5'-CYCLIC AMP
MEVALONIC ACID
ADP
KINETIN
0
10
9
8
7
6
5
4
3
2
MOLAR CONCENTRATION (-LOG10)
0
10
9
8
7
WEIGHT GIBBERELLIN A3 (-LOG10 g)

1969b, 1971; Alvarez, 1969; Duffus and Duffus, 1969; Pollard, 1971). All these responses, however, require high levels of the inducer, or they require long incubation times for the response to become manifest.

The relative potencies of mevalonic acid, kinetin, ADP and 3',5'-cyclic AMP, under the conditions of the bioassay as described here, have been investigated and the results are shown in Figure 8. None of these compounds showed any activity in the bioassay over the concentration ranges tested.

Certain solvent impurities and condensation products have been reported to show activity in some gibberellin bioassays. Hartley et al. (1969) reported activity in the d5-dwarf maize bioassay, and lettuce hypocotyl bioassay, in various solvents, but they found no activity in redistilled solvents. Mitchell et al. (1969) reported gibberellin-like activity in a bean elongation test of some acetone condensation products, while Briggs (1966) found certain solvents, particularly ethyl acetate, left highly variable biologically active residues when rotaty evaporated at 20°C.

Crozier et al. (1972), and Coombe (private communication) have found the acetone condensation products phorone, diacetone alcohol and mesityl oxide possess no biological activity in the barley endosperm bioassay.

The nature of the activity reported by Briggs (1966) remains obscure, but in the experience obtained during this project residues from distilled ethyl acetate, and other solvents used, did not possess any significant potency in the barley endosperm bioassay.

(5) Quantification of the Bioassay.

Quantitative determinations of the gibberellin content of extracts and fractions, in gibberellin A_3 equivalents, have been made from the linear portions of the dosage/response curves of gibberellin A_3 standard and the unknown extract, according to the procedure described by Bliss (1952). All doses on both curves have been replicated at least twice, and as many points as possible have been included in the linear portions of each curve (this usually amounts to two and sometimes 3 points).

The computer program prepared by the Biometry Section of the Waite Institute, which incorporates the relevant equations of Bliss (1952), computes from the bioassay readings the pertinent statistics including mean dose, mean response, slope, standard error of slope, residual mean square of the standard and unknown, the log ratio of potency, and its standard error and confidence limits, and the combined slope and its variance ratio; from these are computed the amounts of gibberellin A_3 equivalents and the

errors.

To be valid, Bliss analyses require parallelism between the dose/response curves of the unknowns and the standard. In all but 5 out of 104 analyses this condition was met; the 5 exceptions are referred to specifically in the results.

7. Preparation of Cell-free Extracts.

Cell-free extracts of young barley seedlings were prepared from both tops and roots by removing the plants from the pot and washing them free of pearlite. The remnants of the seed coats were removed, and the plants were homogenised in cold Tris-HCl buffer (0.05 M, pH 7.8) containing 2-mercaptoethanol (2 mM), either with a mortar and pestle using washed sand as an abrasive, or with an Ultra-Turrax Probe, operating at two thirds maximum voltage for 1½-2 minutes.

The homogenate was then filtered through four layers of clean cheesecloth and centrifuged (3000xg for 15 minutes) in a 2-4°C cold room to remove the larger particulate matter. The final yellow/green supernatant, which was used as a source of enzymes, was kept on ice until used.

For each 5 g fresh weight of plant material 25 ml of buffer was used during the homogenisation. The final yellow/green supernatant had a total protein content of 1.5-2.5 mg/ml as measured, after precipitation with an equal volume of ice-cold 5% (v/v) trichloroacetic acid, by the method of Lowry et al. (1951) using Bovine serum albumen as a standard.

8. Synthesis of 17-^{14}C-ent-Kaurenoic Acid.

17-^{14}C-ent-Kaurenoic acid was prepared by the method of Geissman et al. (1966). Glassware was dried in a hot oven (120^{o}C) and nitrogen was dried by passing over freshly activated Molecular Sieve 5A. Dilute HCl was standardised by titration to pH 5.1 against a known weight of re-crystallised sodium tetraborate according to the method described by Vogel (1961). n-Butyl lithium was standardised by titration to pH 7.0 against the standard HCl.

^{14}C-Methyl iodide (500 µcuries, 60 µcuries/µmole) in benzene, was introduced to 170 mg of triphenylphosphine (0.645 mmoles) also in a benzene solution in a stoppered test tube. 38 µl of non-radioactive methyl iodide (0.636 mmoles) was added, and the solution mixed and allowed to stand. After 5 days the ^{14}C-triphenylphosphonium methyl iodide crystals (which weighed 203 mg when dried) were collected on a glass fibre disc in a Buchner funnel and thoroughly dried for 13 days in a desiccator over freshly activated Molecular Sieve.

Dried ^{14}C-triphenylphosphonium methyl iodide crystals (100 mg, 0.248 mmoles) were placed in a 3-necked, 100 ml round-bottomed flask, with 10 ml of dry tetrahydrofuran, to which was connected a Leibig condensor. A slow steady stream of dry nitrogen was continuously directed through the condensor and flask. 250 µl of n-butyl lithium (0.548 mmoles, 2.2 N) in n-hexane was added slowly

to the reaction mixture with continuous slow stirring. When all the solid material had dissolved, the dark orange solution of the ^{14}C-Wittig reagent was treated with 25 mg (0.082 mmoles) of ent-17nor-kauran-16-on-19-oic acid in 1.5 ml of dry tetrahydrofuran. The reaction mixture was stoppered, the flow of dry nitrogen was stopped, and the mixture stirred at room temperature for 7 hours, after which it was gently refluxed for 9 hours. The tetrahydrofuran was then removed under a stream of nitrogen, water was added and the mixture acidified to pH 2.5 by slow addition of dilute HCl. The acidified aqueous suspension was then extracted by four successive partitions against ethyl acetate.

The pooled ethyl acetate extracts were taken to dryness on a rotary evaporator (total weight 143 mg), dissolved in a small volume of ethyl acetate and streaked on to 20 cm heat-activated silica gel H TLC plates. The plates were developed in chloroform with 4% methanol (v/v), dried, then sprayed with water which revealed the hydrophobic ent-kaurenoic acid as a white band. The ent-kaurenoic acid was eluted from the silica gel H with ethyl acetate and re-run twice more in the same solvent.

TLC of this purified 17-^{14}C-ent-kaurenoic acid showed the presence of only one radioactive peak (Figure 9A), and GLC of the methyl ester on OV-101 columns showed the presence of one major and two minor compounds in the mixture (Figure 9B(A)). On the

basis of the relative areas of the 3 peaks in Figure 9B(A) the prepared sample was judged to contain 62% of 17-^{14}C-ent-kaurenoic acid.

Collection of fractions from the GLC showed only one radioactive peak which coincided with authentic ent-kaurenoic acid standard; all of the radioactivity was contained in this compound (Figure 9B(C)).

The total weight of the sample was 1.65 mg, and from the above data the specific activity of the 17-^{14}C-ent-kaurenoic acid was calculated to be 0.62 μcuries/umole (or 2.06 μcuries/mg).

FIGURE 9.

IDENTIFICATION OF SYNTHESISED 17-^{14}C-*ent*-KAURENOIC ACID

FIGURE 9A.

TLC on heat-activated silica gel H plates developed in chloroform with 4% methanol. The radioactivity scan was obtained at a scanning speed of 0.75 cm/minute at a setting of 1 K for full scale deflection.

Standards

1. Synthesised material which showed as a brown charr with yellow fluourescence under U.V.light after spraying with 5% H_2SO_4 in ethanol and heating to 120°C for 15 minutes.
2. Authentic *ent*-kaurenoic acid which showed up in an identical fashion as 1.
3. Authentic starting material (*ent*-17nor-kaur-16on-19-oic acid) which showed as a blue/white fluourescence under U.V.light after the ethanol/sulphuric acid spray and heat treatment.

FIGURE 9B.

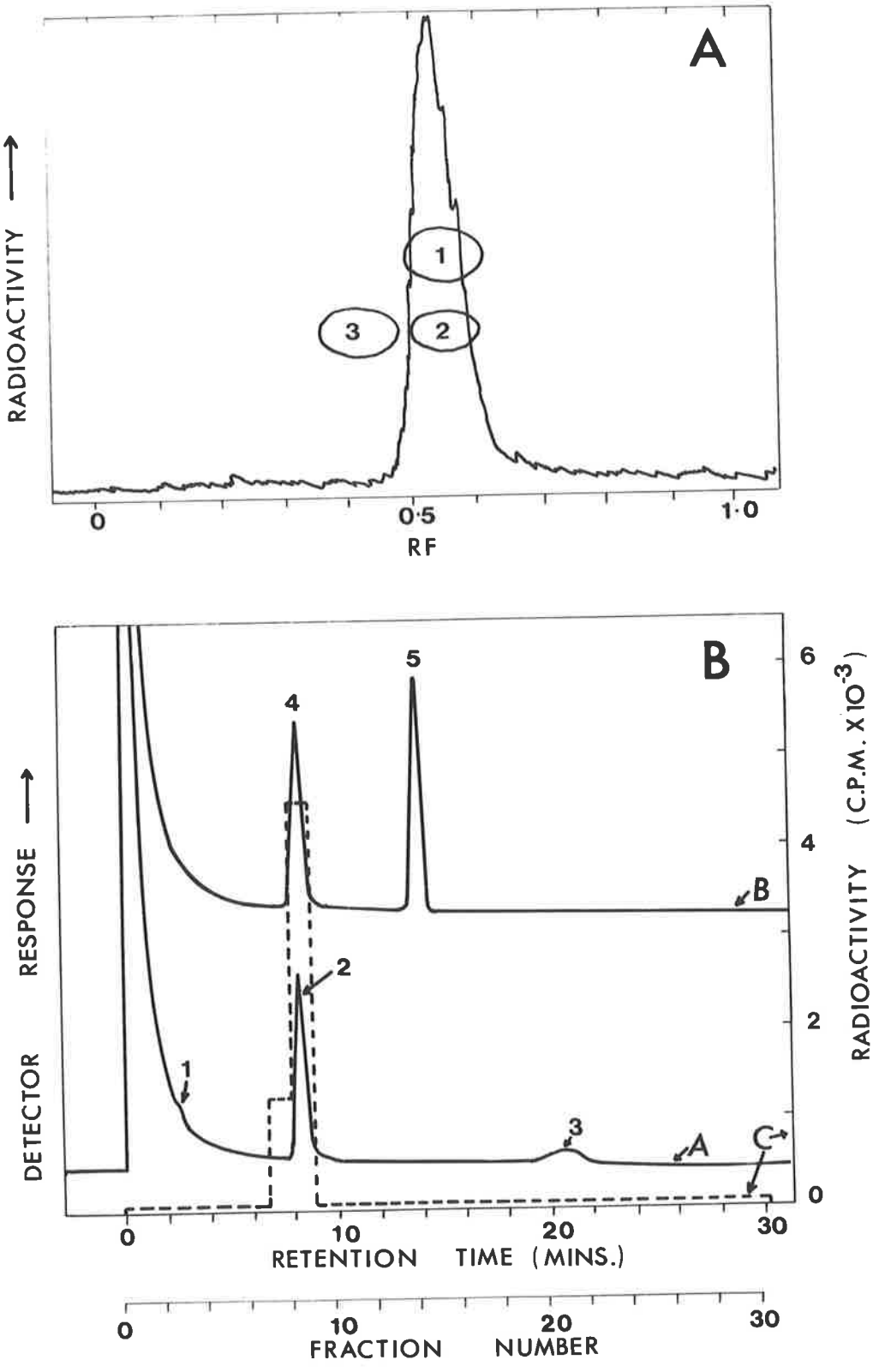
GLC of the methyl esters on OV-101 columns. Running Conditions: nitrogen flow rate 100 ml/minute; column temperature 210°C; flame ionisation detector at 300°C.

Scan A: Synthesised and purified 17-^{14}C-*ent*-kaurenoic acid which gave three peaks, labelled 1, 2 and 3.

Scan B: Sample of authentic *ent*-kaurenoic acid (peak 4) and starting material (*ent*-17nor-kaur-16on-19-oic acid) (peak 5).

Scan C: Radioactivity in fractions (broken black line) of the column effluent collected during the running of another sample of the synthesised 17-^{14}C-*ent*-kaurenoic acid.

The RT of peaks 2 and 4 coincided exactly; the only radioactive peak in Scan C had the same RT as peak 2, and in excess of 90% of the injected radioactivity was recovered in this peak.


A
RADIOACTIVITY
1
2
3
0
0·5
1·0
RF
B
DETECTOR RESPONSE
RADIOACTIVITY (C.P.M. X 10⁻³)
1
2
3
4
5
A
B
C
0
2
4
6
0
10
20
30
RETENTION TIME (MINS.)
0
10
20
30
FRACTION NUMBER

9. Administration of Radioactive Precursors to Plants.

(1) ^{14}C-Carbon dioxide.

^{14}C-Carbon dioxide was administered to barley seedlings in an air tight glass container by mixing in a small petri dish, 2 mmoles of orthophosphoric acid for every mmole of aqueous ^{14}C-sodium carbonate. Air circulation was maintained by a continuously rotating magnetic flea and the experiments were carried out in a growth cabinet with the lights on.

(2) ^{14}C-Mevalonic acid.

Aqueous solutions of 2-^{14}C-(RS)-mevalonic acid were administered to young barley seedlings in three ways: immersing the roots of the seedlings in the solution of mevalonic acid; immersing the ends of severed roots in the solution; and by a cotton wick method. In the last-named method two threads of wet cotton were drawn through the stem of the seedling with a fine needle. The needle was then cut from the cotton, one end of which was drawn flush with the stem of the plant, and the other end was dipped into the solution of mevalonic acid. Presumably by capillary action, the mevalonic acid migrated along the cotton and into the plant. In this way up to 50 µl of an aqueous solution

could be fed to a young barley seedling in 4-5 hours. The uptake of the solution could be hastened if the plant was placed in an air draught.

Aqueous solutions of 2-^{14}C-(RS)-mevalonic acid were supplied to young apricot fruits by three methods. These were: injection of 2 µl of radioactive solution into the centre of each fruit via the side; wick feeding solutions (as above) through cotton wicks which pierced the fruit's pedicel; allowing excised young fruits to absorb drops of solution placed on the cut surface of the pedicel. In the last-named case, after all the radioactive solution had been absorbed, the cut ends were pressed into agar plates containing 2% sucrose.

(3) 17-^{14}C-*ent*-Kaurenoic acid.

Radioactive and non-radioactive *ent*-kaurenoic acid was applied to young barley seedlings and dwarf rice seedlings in a 50% acetone/water (v/v) solution containing 0.025% 'Tween-20', by placing small droplets of this solution in the axils of leaves at daily intervals.

10. Counting Radioactive Solutions.

Radioactive solutions were routinely counted by absorbing the solutions on glass fibre discs which were then dried and counted in a Liquid Scintillation Spectrometer with 1 ml of toluene fluor containing 3.5 g/litre of PPO and 0.4 g/litre of dimethyl-POPOP.

Volatile compounds in solutions of organic solvents were counted directly in the toluene based scintillation liquid, and on occasions aqueous solutions were counted directly in Bray's scintillation liquid (Bray, 1960).

Counting paper chromatograms was done by cutting thin strips of the chromatogram into 1 cm pieces, placing each piece in a scintillation vial and counting with 1 ml of toluene based scintillation liquid. Counting materials on silica gel H was carried out in a similar fashion with 1 ml of scintillation liquid.

Counting efficiency was always determined by the channels ratio method (Bush, 1963), and quenching curves were constructed using standard ^{14}C-benzoic acid of known specific activity.

IV.

RESULTS AND DISCUSSION

A. Extraction and Identity of Barley Gibberellins.

1. Introduction

The purpose of this section of the project was to identify and characterise the gibberellins which were normally present in barley seedlings using the techniques which had been developed for the extraction, purification and measurement of these compounds.

2. Biologically Active Compounds in Extracts of Barley Seedlings.

The methods used for the extraction of seedlings (see III.B. 3(1)) produced 3 extracts: a chloroform-acetone extract, an acid ethyl acetate extract, and the remaining water phase. The content of biologically active compounds in these three fractions was investigated with the barley endosperm bioassay. The results are presented in Figure 10.

(1) Chloroform-Acetone Extract.

The results for this extract are shown in Figure 10B. In no instance was any significant response obtained when aliquots of this fraction were bioassayed.

(2) Acid Ethyl Acetate Extract.

The results for this fraction are shown in Figures 10C and 10D. Extracts from both tops and roots of seedlings always produced a highly significant response in the barley endosperm bioassay. The dosage/response curves of the crude extracts (Figure 10C) showed poor parallelism to the standard gibberellin A_3 curve (Figure 10A), and as such were unsatisfactory for estimating amounts of gibberellins present in the extract. However, after the extract had been partially purified by TLC on silica gel H,

FIGURE 10.

OCCURRENCE OF BIOLOGICALLY ACTIVE COMPOUNDS IN EXTRACTS OF BARLEY SEEDLINGS.

Extractions and bioassays were carried out as described in III.B.3(1) and III.B.6. Ordinate values (Δ R) refer to the increase in refraction of the ambient bioassay solution after a 40-hour incubation. Abscissa values refer to the relative concentrations of the solutions being tested.

Background refraction of the chloroform-acetone extract (C) and ethyl acetate extract (E and E1) was negligible, but subtractions were made for the background refraction of the aqueous fraction (A).

Symbols: Solid circles (●) refer to root extracts.
Solid triangles (▲) refer to leaf and stem extracts.

The results presented here were obtained from extracts of twenty 11-day old seedlings.

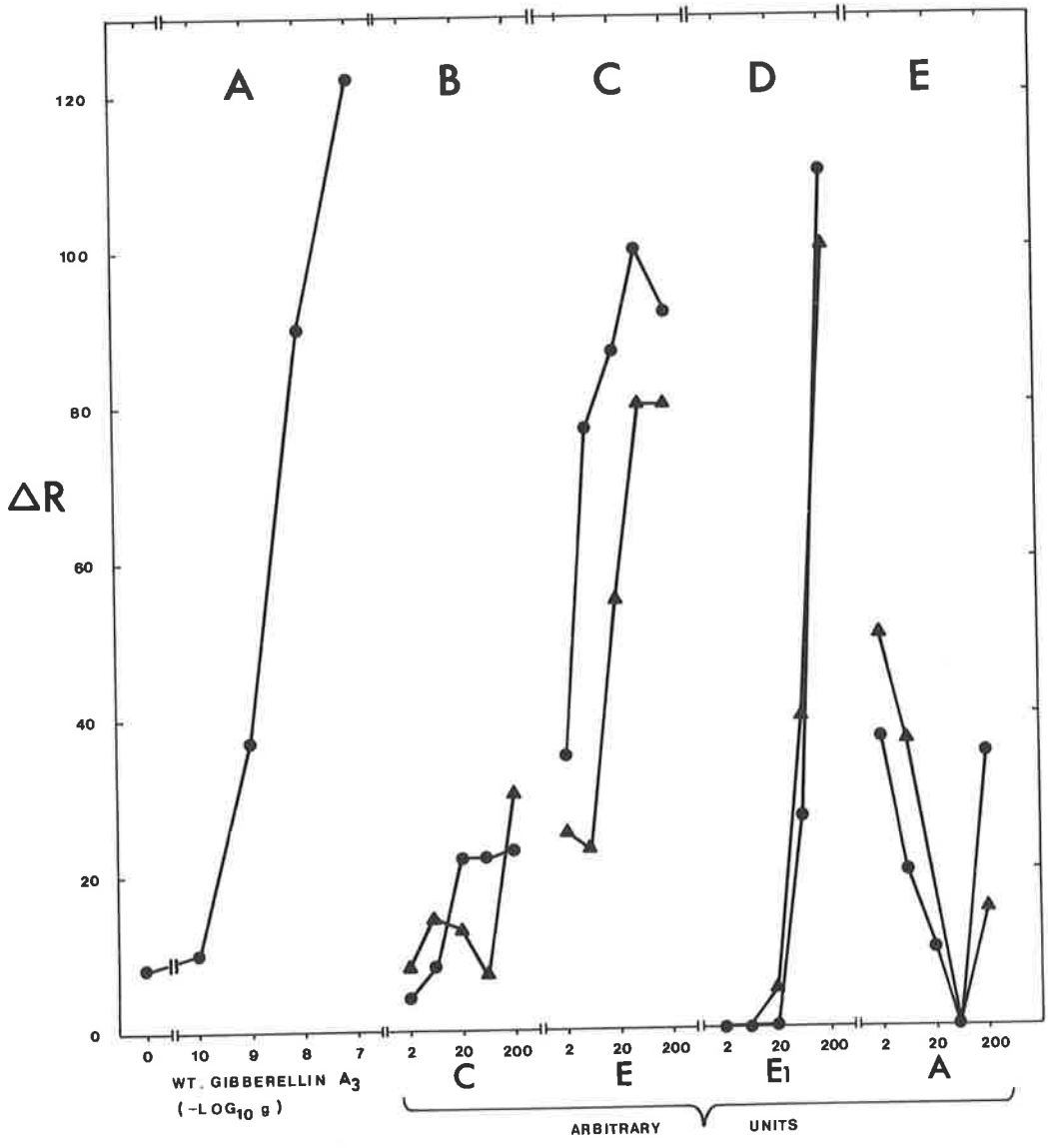
FIGURE 10A. Response from gibberellin A_3.

FIGURE 10B. Response from the chloroform-acetone extract (C).

FIGURE 10C. Response from crude acid ethyl acetate extract (E).

FIGURE 10D. Response from acid ethyl acetate extract partially purified by TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1) (E1).

FIGURE 10E. Response from residual aqueous fraction (A).


A
B
C
D
E
120
100
80
60
40
20
0
ΔR
0
10
9
8
7
WT. GIBBERELLIN A3
(-LOG10 g)
2
20
200
C
E
E1
A
ARBITRARY
UNITS

using chloroform/ethanol/formic acid (85/15/1), and the biologically active zone eluted, the dosage/response curve of the eluant (Figure 10D) accurately matched that of the standard gibberellin A_3 curve. The improvement in the response is presumably due to the removal of various substances from the crude extract which interfered with the bioassay. For quantification of amounts of gibberellins present in extracts the crude acid ethyl acetate extracts were henceforth purified in this way before dosage/response curves were constructed.

(3) Remaining Aqueous Extract.

Highly variable results were obtained when aliquots of the aqueous fraction, which remained after ethyl acetate extraction, were tested for biological activity with the barley endosperm bioassay. Dosage/response curves for this fraction from the tops and roots of barley seedlings are shown in Figure 10E. At low concentrations a significant response was often obtained, but at higher concentrations this response disappeared.

This inconsistency could be due to compounds in the extract which interfered with the bioassay. An attempt was made to separate some of these compounds by descending paper chromatography using isopropanol/ammonia/water (10/1/1) as solvent. Bioassays of the developed and dried chromatograms gave inconsistent results. In some cases a significant peak of biological activity appeared in the region corresponding to gibberellin A_3 standard, and in a few cases other peaks of biological activity were present. In 50% of the cases no peaks of biological activity appeared on the chromatograms.

3. Preliminary Identification of the Gibberellins Present in the Acid Ethyl Acetate Fraction.

The compound or compounds responsible for the biological activity in the acid ethyl acetate fraction were characterised to some extent by paper electrophoresis, paper chromatography and TLC on silica gel H according to the methods already described (III.B.4(1), III.B.4(2), III.B.6).. The results are presented in Figures 11 and 12.

During paper electrophoresis in ammonium bicarbonate buffer (0.1 M, pH 8.9) the biologically active principles from extracts of whole seedlings migrated as a mono-carboxylic acid with the same mobility as standard gibberellins A_3, A_4 and A_7 (Figure 11A).

TLC on silica gel H of extracts from the tops and roots of seedlings showed a single peak of biological activity which had the same RF as gibberellins A_1 and A_3 (Figures 11B and 11C).

Descending paper chromatography in isopropanol/ammonia/water (10/1/1) of extracts from the tops and roots of seedlings showed the presence of a single peak of biological activity which had the same RF as gibberellins A_1 and A_3 (Figure 12A).

When extracts from tops and roots of seedlings were subjected to descending paper chromatography with the solvent system of Bird and Pugh (1958), two peaks of biological activity appeared,

the smaller one of which migrated like gibberellin A_1 and the larger one migrated like gibberellin A_3 (Figure 12B).

Extracts from the tops and roots of seedlings aged 8, 11 and 15 days all produced the same results when subjected to the preliminary purification procedures shown in Figures 11 and 12.

FIGURE 11.

PRELIMINARY IDENTIFICATION OF THE GIBBERELLINS PRESENT IN ACID ETHYL ACETATE EXTRACTS OF BARLEY SEEDLINGS

Paper electrophoresis and TLC on silica gel H.

Left hand ordinate values refer to the increase in refraction of the ambient bioassay solution after a 40-hour incubation.

Right hand ordinate values refer to the amounts of gibberellin A_3, in grams, required to produce the given responses.

Abscissa values refer to the distance migrated in cm. (Figure 11A), or to the RF (Figures 11B and 11 C).

The procedures used for preparation of extracts, chromatography, electrophoresis and bioassay are described in sections III.B.3(1), III.B.4(1), III.B.4(2) and III.B.6.

FIGURE 11A. Paper electrophoresis (2,300 volts, 1 hour) of a whole plant extract in ammonium bicarbonate buffer (0.1 M, pH 8.9).

FIGURE 11B. TLC on silica gel H of extracts from the tops and roots of seedlings in n-butanol/acetic acid/water (85/5/10).

FIGURE 11C. TLC on silica gel H of extracts from the tops and roots of seedlings in chloroform/ethanol/formic acid (85/15/1).

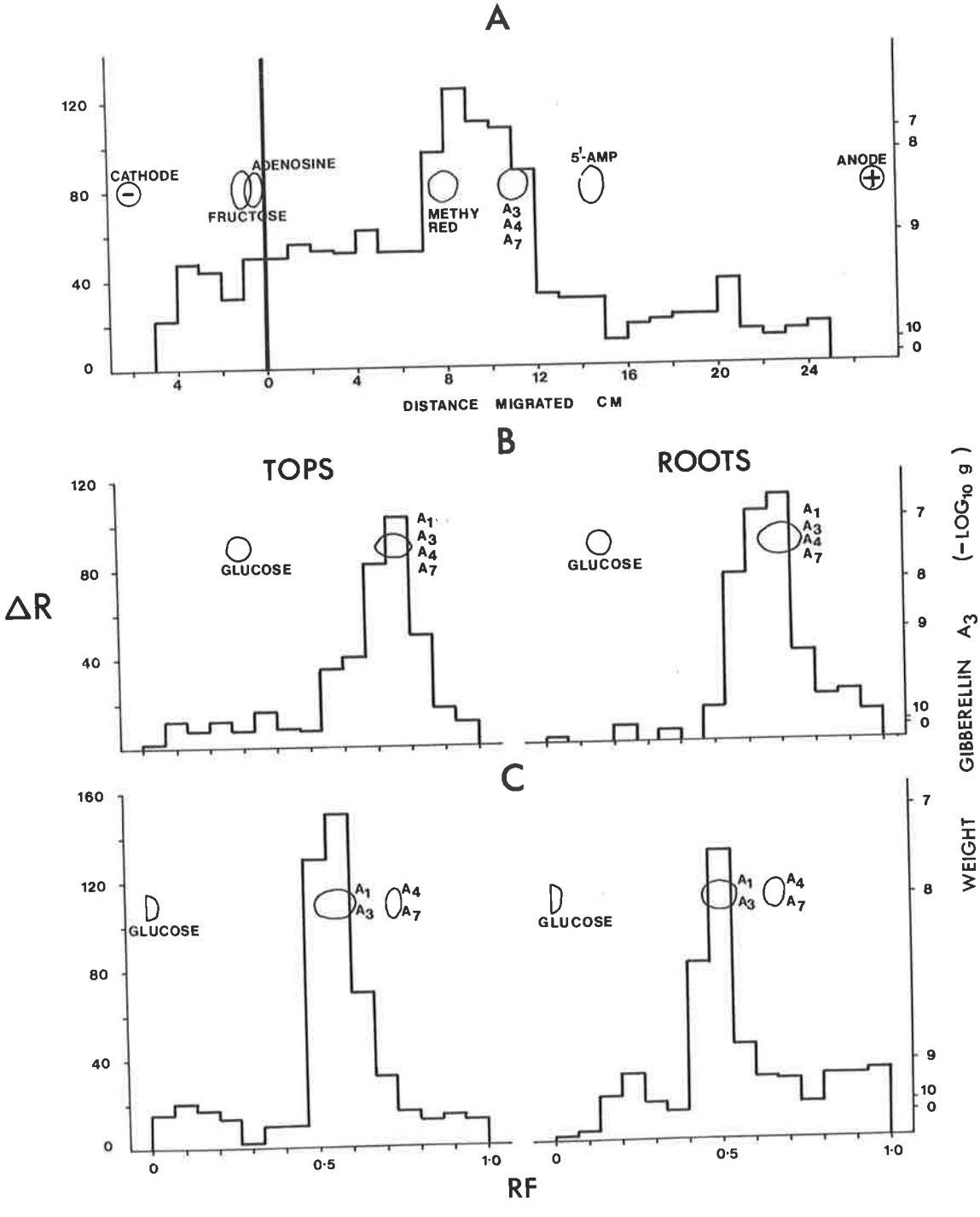

A
CATHODE
ADENOSINE
FRUCTOSE
METHY RED
A3 A4 A7
5'-AMP
ANODE
DISTANCE MIGRATED CM
B
TOPS
ROOTS
ΔR
GLUCOSE
A1 A3 A4 A7
C
RF
WEIGHT GIBBERELLIN A3 (-LOG10 g)

FIGURE 12.

PRELIMINARY IDENTIFICATION OF GIBBERELLINS PRESENT IN ACID ETHYL ACETATE EXTRACTS OF BARLEY SEEDLINGS

Descending paper chromatography.

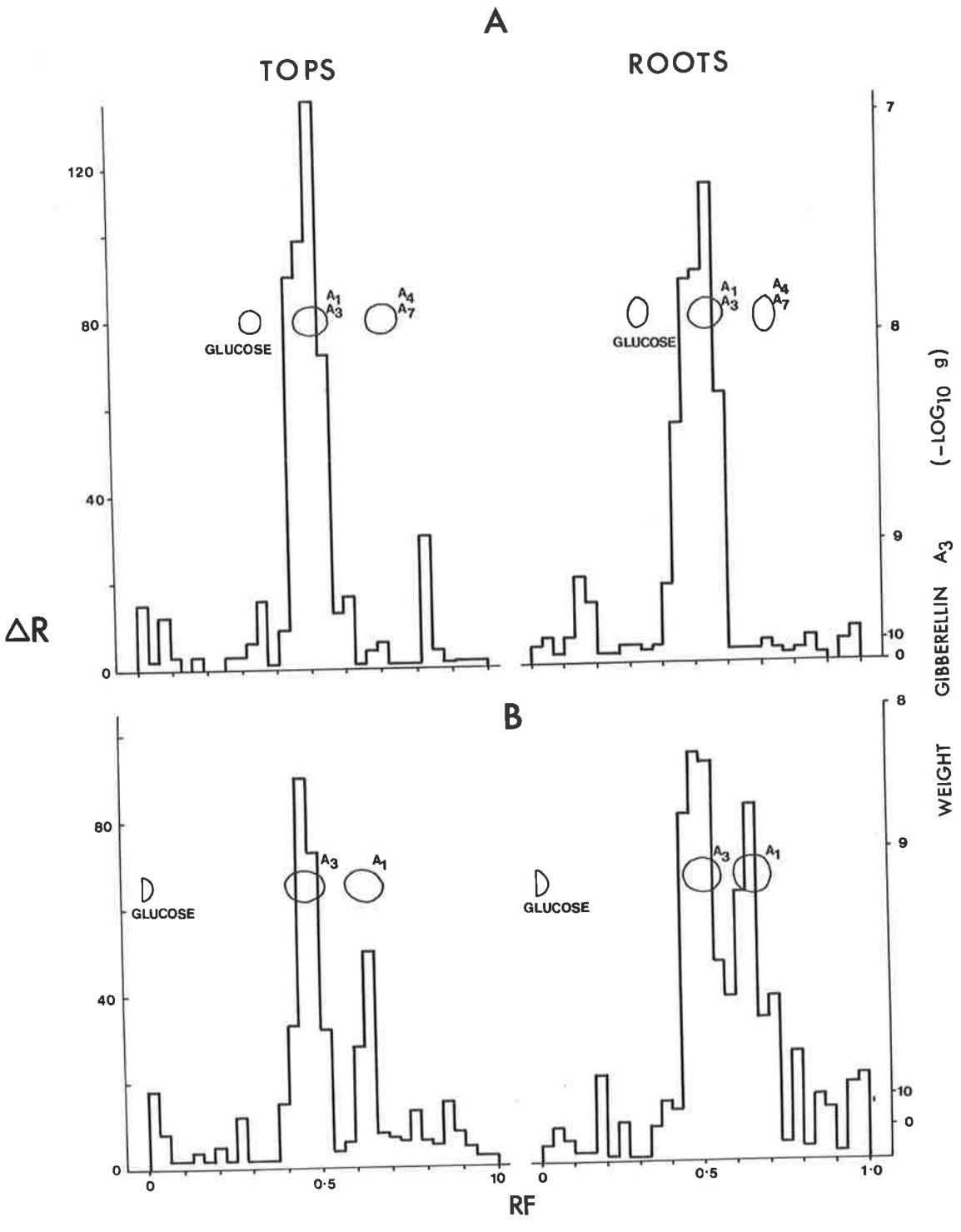
Left hand ordinate values refer to the increase in refraction of the ambient bioassay solution after a 40-hour incubation. Right hand ordinate values refer to the amounts of gibberellin A_3, in grams, required to produce the given responses.

Abscissa values refer to the RF values of the chromatograms.

The procedures used for the preparation of extracts, chromatography and bioassay are described in sections III.B.3(1), III.B.4(1), III.B.4(2) and III.B.6.

FIGURE 12A. Descending paper chromatography of extracts from the tops and roots of seedlings in isopropanol/ammonia/water (10/1/1).

FIGURE 12B. Descending paper chromatography of extracts from the tops and roots of seedlings in the benzene/proprionic acid/water (8/3/5) system of Bird and Pugh (1958).


A
TOPS
ROOTS
ΔR
120
80
40
0
GLUCOSE
A1
A3
A4
A7
7
8
9
10
0
(-LOG10 g)
GIBBERELLIN A3
WEIGHT
B
80
40
0
GLUCOSE
A3
A1
0·5
10
1·0
RF

4. Recovery of Gibberellin During Extraction and Preliminary Purification.

(1) Recovery of Added Gibberellin A_3.

To test the efficiency with which gibberellin A_3 was recovered during extraction, a known amount of gibberellin A_3 was added to the mortar and pestle during the homogenisation of batches of barley seedlings. The extraction was carried out as described (III.B.3(1)) and control extracts from identical batches of seedlings to which no gibberellin A_3 had been added were also prepared.

The crude acid ethyl acetate extracts were partly purified by TLC on silica gel H (see IV.A.2(2)), and the zone of biological activity, corresponding to the position of gibberellin A_3 standard, was eluted and tested for potency in the barley endosperm bioassay. Quantification of the results, which are presented in Table 3, was carried out by the procedure of Bliss (1952) as described in III.B.6(5).

Percentage recoveries of 58% and 59% of added gibberellin A_3 into the acid ethyl acetate extract were obtained.

TABLE 3.

Extracts were prepared and purified as described in III.B.3(1) and IV.A.2(2); bioassays were carried out as described in III.B.6.

Log relative potency estimates were made on 67% of the total extract. The remaining 33% was used to locate the zone of biological activity on the TLC plates.

5 barley seedlings (tops and roots) were used for each replicate.

TABLE 3.

RECOVERY OF ADDED GIBBERELLIN A_3 IN THE ACID ETHYL ACETATE EXTRACT

Age of Plants (days)	Fresh wt. (g)	Extract	Log Relative Potency ± S.E.	Gibb. A_3 Equiv. (ng)	% Recovery
8	2.0	Control Extract (mean of 4 replicates)	-7.89 ± 0.18	20	58%
8	1.8	Control Extract + 500 ng A_3 (mean of 3 replicates)	-6.69 ± 0.11	310	
11	3.7	Control Extract (mean of 4 replicates)	-7.95 ± 0.13	17	59%
11	4.1	Control Extract + 500 ng A_3 (mean of 4 replicates)	-6.68 ± 0.14	310	

(2) Recovery of Barley Gibberellin.

The recovery of endogenous barley gibberellin through the preliminary purification procedures which were routinely used was tested. A large scale extract from 200 g fresh weight of 8-day old barley seedlings was prepared (see III.B.3(1)) and purified in four steps involving chromatography and electrophoresis, using the methods described in sections III.B.4(1) and III.B.4(2). At each purification stage 0.0167% of the total extract was expended on a bioassay to locate the zone of activity on the chromatogram. In each case the zone of biological activity coincided with the position of gibberellin A_3 standard. Also at each purification stage a further 0.0167% of the total eluant was expended on a dosage/response curve with the barley endosperm bioassay, from which the quantity of gibberellin in the eluant was determined (see III.B.6(5)). The results are presented in Table 4.

The initial crude ethyl acetate extract (fraction 1) contained 1 µg of gibberellin A_3 equivalents, and a similar amount was also present in the more purified fractions 2, 3 and 4. An increase in the gibberellin content of the final fraction (fraction 5) was obtained, although this increase was not statistically significant.

The slight increase in weight after TLC on silica gel H (fraction 4), was probably only an apparent one due to the presence of silica gel H particles in the eluant. The final purification

step, which was a repeat of the second step in the sequence, was used to remove the silica gel H particles from the eluant.

The five purification steps produced a total dry weight reduction of the crude extract of approximately 200-fold without any loss of biological activity. The final fraction which weighed about 1 mg, had a gibberellin component as active as 3 µg of gibberellin A_3. Assuming the barley gibberellin is equally as potent as gibberellin A_3 in the endosperm bioassay, then the biologically active material in the final fraction comprised about 0.3% of the weight of the extract.

TABLE 4.

The procedures used for ethyl acetate extract preparation, chromatography, and bioassays, are described in sections III.B.3(1), III.B.4(1), III.B.4(2) and III.B.6.

Dry weights were determined in carefully wiped dry glass containers. The material obtained in Fraction 5 gained weight during the weighing process, presumably by absorbing moisture from the atmosphere, and for this reason the dry weight could not be determined accurately.

*The dosage/response curve of this extract was significantly different in slope from the slope of the standard gibberellin A_3 curve. For this reason Bliss analysis of the response is not legitimate.

At the 95% confidence level none of the comparisons of log relative potency are different.

TABLE 4.

PURIFICATION OF LARGE SCALE EXTRACT

Purification Step	Total dry wt. (mg)	Purification by dry weight reductn.	Log relative potency ± S.E.	Gibb. A_3 Equiv. (µg)
Plant tissue: 8-day old seedlings (tops & roots)	20,000			
1. Acid Ethyl Acetate Extract	192	1X	*-5.99 ± 0.10	1.0
2. Paper chromatography (isopropanol/ammonia/ water)	25	8X	-5.82 ± 0.17	1.5
3. Paper electrophoresis (ammonium bicarbonate)	6	32X	-5.94 ± 0.16	1.1
4. TLC on silica gel H (chloroform/ethanol/ formic acid)	8	24X	-5.89 ± 0.11	1.3
5. Paper chromatography (isopropanol/ammonia/ water)	approx. 1	192X	-5.49 ± 0.10	3.2

5. Gas-Liquid Chromatography.

(1) Recovery of Standard Gibberellins.

The efficiency with which gibberellins could be recovered in the fractions collected during GLC analysis was tested by two methods.

In the first, known amounts of the TMS ether/methyl ester derivative of gibberellins A_1 and A_3 were injected onto OV-17 columns, and fractions of the columns effluent were collected in the described way (III.B.5(6)). The collected fractions were eluted, taken to dryness in a stream of nitrogen, re-derivatised with HMDS and TMCS (as described in III.B.5(3)b) and re-injected onto the GLC. The efficiency of recovery during collection was measured by comparing the peak areas of the gibberellins in the collected samples with the peak area of the gibberellins in the original mixture. Recovery efficiencies, measured in this way, of 50-62% were obtained.

In the second method, known amounts of the TMS ether/ester derivative of gibberellins A_1 and A_3 were injected onto OV-17 columns, and the fractions of the column effluent which were collected were eluted and tested for potency in the barley endosperm bioassay (see III.B.6). Comparisons were made with identical gibberellin samples which had not been derivatised, and

with samples which had been derivatised, but were removed from the glass ampoules and directly tested for potency.

Results of this work showed that no loss of biological activity occurred during the derivatisation process, and recoveries of biologically active gibberellin in the collected fractions of 50% were obtained.

(2) Gas-Liquid Chromatography of Barley Gibberellins.

Aliquots of the final fraction from the large scale extract of barley seedlings (fraction 5, Table 4) were analysed by GLC on OV-17 columns of the TMS ether/ester derivatives. The results are presented in Figure 13. Numerous peaks are evident in the gibberellin zone between the internal standards allogibberic acid and cholesterol. Two of the smaller peaks in this region had retention times which were identical to gibberellins A_1 and A_3, and the added area of these two peaks agreed with the peak area which was expected from the estimated quantity of gibberellin which had been injected onto the column.

Bioassays of fractions of the column effluent, collected between the internal standards during the running of another aliquot of fraction 5 (Table 4), revealed two peaks of activity which had the same RT as the peaks noted in the first run (inset Figure 13).

The total amount of gibberellin A_3 equivalents recovered in the two peaks of activity accounted for 40% of the estimated amount of gibberellin which had been injected onto the column.

GLC analysis, on QF-1 columns, of the TMS ether/ester derivative of a partly purified extract from 11-day old barley seedlings showed the presence of one large peak of biological activity with the same RT as gibberellins A_1 and A_3 (Figure 14).

FIGURE 13.

GLC OF BARLEY GIBBERELLINS ON OV-17 COLUMNS

Column type: 3% OV-17 on silanised Gaschrom Q.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 210°C and rising at a rate of 1.7°C/minute to 260°C.

Detector: flame ionisation at 220°C.

Derivatives: TMS ether/ester derivatives prepared as described in III.B.5(3)c, with 2 µg each of cholesterol and allogibberic acid.

Fraction collecting: carried out as described in III.B.5(6).

The retention times of the standard gibberellins were calculated from the positions of the internal standards.

The complete scan represents 20% of fraction 5 (Table 4).

The inset refers to fractions collected during the running of another aliquot of fraction 5 (Table 4). The dried eluates of the collected fractions were tested for potency with the barley endosperm bioassay (III.B.6); quantification of the results was carried out by the Bliss (1952) method (III.B.6(5)). Only the significantly active fractions are shown as histograms in the inset; their dilution curves had a slope significantly greater than horizontal and not significantly different from the slope of the gibberellin A_3 curve.

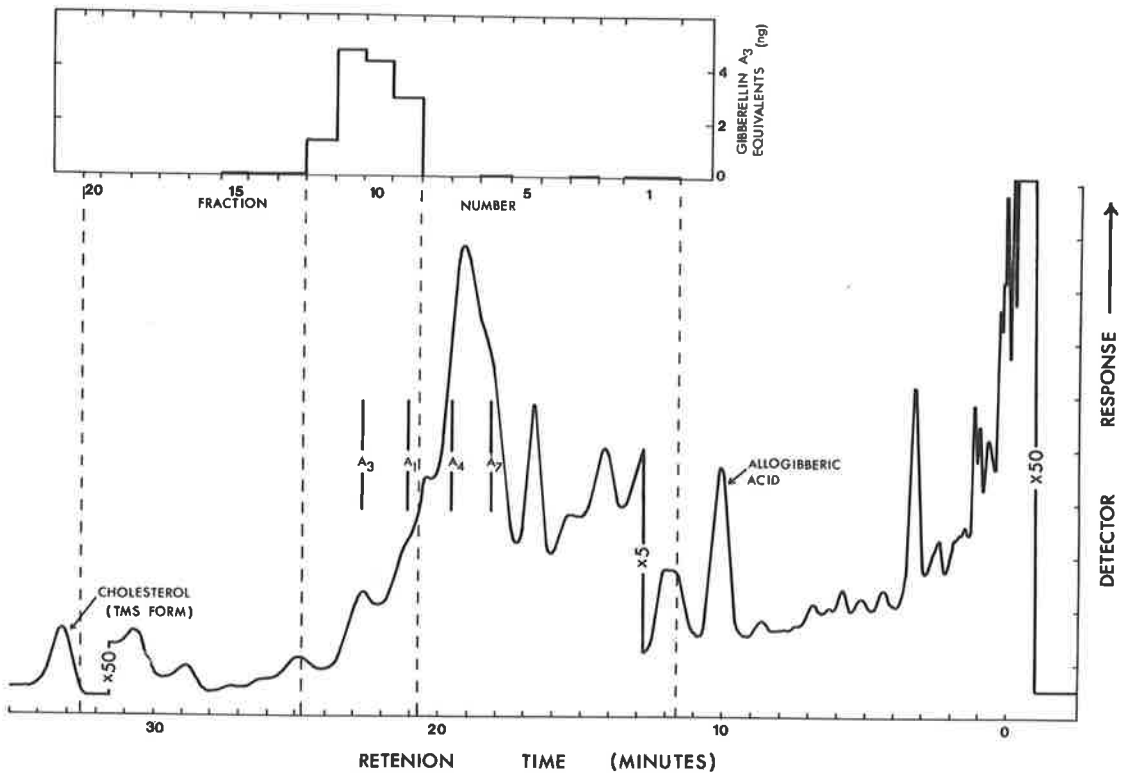

GIBBERELLIN A_3 EQUIVALENTS (ng)
4
2
0
20
15
FRACTION
10
NUMBER
5
1
A_3
A_{11}
A_4
A_7
ALLOGIBBERIC ACID
CHOLESTEROL (TMS FORM)
x50
x5
x50
DETECTOR RESPONSE
30
20
10
0
RETENION TIME (MINUTES)

FIGURE 14.

GLC OF BARLEY GIBBERELLINS ON QF-1 COLUMNS

Column type: 2% QF-1 on Anakrom ABS.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 150°C and rising at a rate of 3.3°C/minute to 230°C.

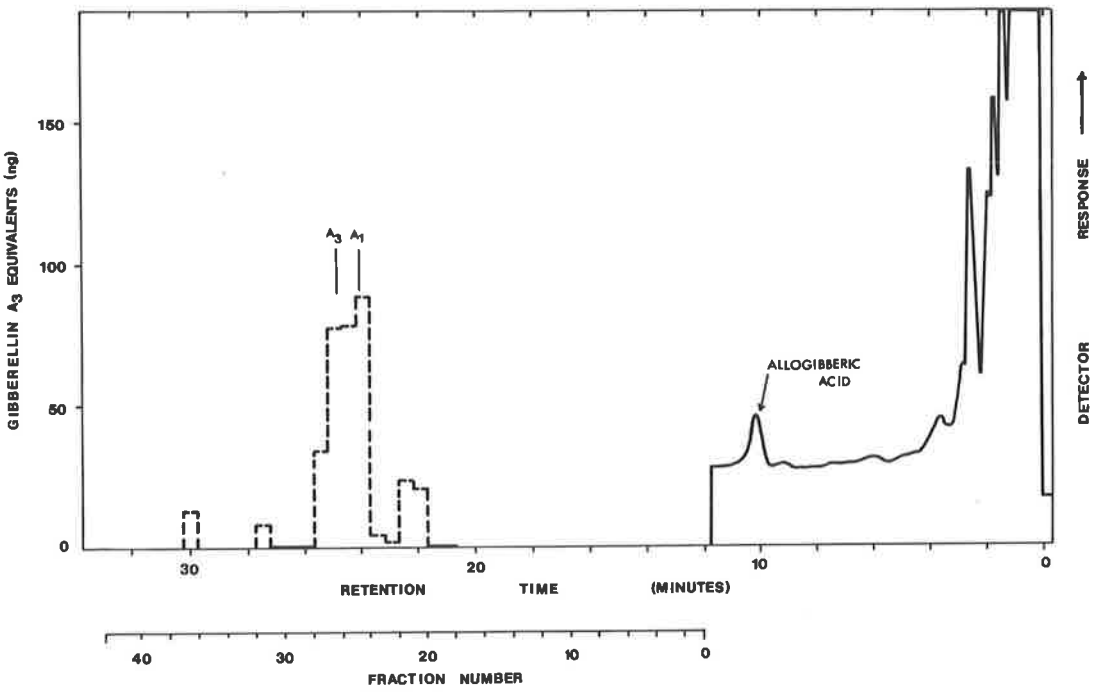
Detector: flame ionisation at 220°C.

Derivatives: TMS ether/ester derivatives prepared as described in III.B.5(3)c with 2 µg of allogibberic acid.

Fraction collecting: carried out as described in III.B.5(6).

Retention times of standard gibberellins were calculated from the position of allogibberic acid.

The sample was a purified ethyl acetate extract from 11-day old barley seedlings. The dried eluates of the collected fractions were tested for potency with the barley endosperm bioassay (III.B.6). Quantification of the bioassay results was carried out by the Bliss (1952) method (III.B.6(5)). Only the significantly active fractions are shown as histograms; their dilution curves had a slope significantly greater than horizontal and not significantly different from the slope of the gibberellin A_3 curve.


GIBBERELLIN A_3 EQUIVALENTS (ng)
150
100
50
0
A_3 A_1
ALLOGIBBERIC ACID
DETECTOR RESPONSE
30
20
10
0
RETENTION TIME (MINUTES)
40
30
20
10
0
FRACTION NUMBER

6. Amounts of Biologically Active Gibberellin Extractable from Barley Seedlings.

In Table 5 the results of a time course experiment in which the amounts of gibberellin A_3 equivalents in the acid ethyl acetate extract from the tops and roots of seedlings at three different stages of development are tabulated.

At each stage examined significant amounts of gibberellin were detected in the extracts. Roots were found to contain a major proportion of the total plant gibberellin, and in all cases the concentration in the roots (amount per g dry weight or fresh weight) exceeded the concentration in the tops. Between days 11 and 15 there was a net increase of free gibberellin on a per plant basis, but this increase, which was particularly evident in the root fraction, was not statistically significant.

Attempts were made by various treatments to increase the level of extractable gibberellin obtained from barley seedlings. The results of this work are compiled in Tables 6 and 7.

Application of low concentrations (1 or 20 mg/litre) of the plant growth retardant AMO-1618 as a root drench to the seedlings two days prior to extraction, reduced the level of biologically active gibberellin extractable from the seedlings to about one-half of the control level (Table 6A). This reduction was not statistically significant; there was no change in the growth or

TABLE 5.

Extracts were prepared, purified, and bioassayed for gibberellin content as described in sections III.B.3(1), III.B.6 and IV.A.2(2).

Two replicates per harvest were used; for each replicate duplicate dosage/response curves were constructed. One hundred seedlings were used for each replicate.

The 8-day old seedlings had vegetative apices; the apices of the 11-day old seedlings were at the double ridge stage of development; the apices of the 15-day old seedlings were about to commence internode elongation.

*The slope of the dosage/response curves for these extracts were significantly different from the slope of the standard gibberellin A_3 curve, and for this reason Bliss analysis (III.B.6(5)) of these curves is invalid.

None of the values of log relative potency are significantly different from one another at the 95% confidence level.

TABLE 5.

AMOUNTS OF GIBBERELLINS EXTRACTED INTO ETHYL ACETATE FROM BARLEY SEEDLINGS AT DIFFERENT STAGES OF DEVELOPMENT

Plant Age (days)	Part of Plant	Log Relative Potency ± S.E.	Total ng GA_3 Equiv. per Plant Part	ng GA_3 Equiv. per g F.W.	ng GA_3 Equiv. per g D.W.
8	Tops	*-6.17 ± 0.14	6.81	25.6	201.5
	Roots	*-6.38 ± 0.15	4.18	28.1	321.5
	Total		10.99	26.7	234.8
11	Tops	-6.43 ± 0.18	3.73	8.5	70.6
	Roots	-6.53 ± 0.22	2.99	10.2	137.8
	Total		6.72	9.2	90.2
15	Tops	-6.36 ± 0.12	4.30	4.6	31.6
	Roots	-6.27 ± 0.17	5.38	6.0	65.1
	Total		9.68	5.3	44.2

TABLE 6.

Extracts were prepared, purified, and bioassayed for gibberellin content as already described in sections III.B.3(1), III.B.6 and IV.A.2(2).

8-day old seedlings were used with 2 replicates per treatment; for each replicate duplicate dosage/response curves were constructed. Five seedlings (tops and roots) were used for each replicate.

TABLE 6A. Plants were treated with a root drench of AMO-1618 two days before they were harvested and extracted. At the 95% confidence level, log relative potency for Treatments 1, 2 and 3 were not different from one another.

TABLE 6B. Plant homogenates were prepared in 0.1 N HCl, and hydrolysis was carried out at 60°C for 1 hour, after which the usual extraction procedure was followed.
At the 95% confidence level, log relative potency for Treatments 1 and 2 were not different from Treatments 3 and 4 respectively.

TABLE 6.

AMOUNTS OF BIOLOGICALLY ACTIVE GIBBERELLIN EXTRACTABLE FROM BARLEY SEEDLINGS FOLLOWING GROWTH RETARDANT TREATMENT AND ACID HYDROLYSIS

TABLE 6A.

Treatment	Fresh wt. of Seedlings (g)	Log relative potency ± S.E.	Gibb. A_3 Equiv. (ng)	% Recovery.
1. Control Extract	2.2	-7.49 ± 0.10	39.2	
2. AMO-1618 (1 mg/litre)	2.2	-7.79 ± 0.11	16.2	
3. AMO-1618 (20 mg/litre)	2.1	-7.82 ± 0.12	15.2	

TABLE 6B.

1. Control Extract	2.0	-7.89 ± 0.17	12.9	
2. Control Extract + 334 ng A_3	1.8	-6.69 ± 0.11	207	55%
3. Hydrolysed Extract	1.7	-8.35 ± 0.17	4.5	
4. Hydrolysed Extract + 334 ng A_3	1.7	-7.29 ± 0.19	51	14%

fresh weight of the seedlings.

Acid hydrolysis of seedling homogenates in dilute hydrochloric acid (0.1 N) at 60°C for one hour reduced the level of biologically active gibberellin obtainable from the homogenates to about one-third of the control level (Table 6B). This reduction, which was not statistically significant, was reflected in a similar reduction in the recovery of added gibberellin A_3 following this treatment.

The results of enzymatic hydrolyses of seedling homogenates for 18 hours at 30°C with the proteolytic enzyme pronase and the glycosidic enzyme β-emulsin are present in Table 7. Hydrolysis with these enzymes produced small increases over controls in the amounts of biologically active material obtainable from the homogenates (Table 7; compare treatments 5 and 8 with treatments 3 and 6 respectively), although these increases were not statistically significant.

Relatively low amounts of biologically active gibberellin were obtained from the homogenates following 18 hour incubation in phosphate buffer and acetate buffer at 30°C (Table 7, treatments 3 and 6), and this result is reflected in the low recovery of added gibberellin A_3 following similar treatment (Table 7, treatments 4 and 7).

TABLE 7.

Extracts were prepared, purified, and bioassayed for gibberellin content as already described in sections III.B.3(1), III.B.6 and IV.A.2(2). 11-day old seedlings were used with 2 replicates per treatment; for each replicate duplicate dosage/response curves were constructed. Five seedlings (tops and roots) were used for each replicate.

Buffers: potassium phosphate buffer, 0.05 M, pH 7.4.
sodium acetate buffer, 0.05 M, pH 5.4.

Enzymes: β-emulsin and pronase were added to the homogenates to give a final concentration of 1 mg/ml.

At the 95% confidence level, log relative potency for Treatments 4 and 7 were both different from Treatment 2, while log relative potency for Treatments 1, 3, 5, 6 and 8 were not different from one another.

TABLE 7.

AMOUNTS OF BIOLOGICALLY ACTIVE GIBBERELLIN OBTAINED FROM BARLEY SEEDLING HOMOGENATES FOLLOWING HYDROLYSIS WITH PRONASE AND β -EMULSIN

Preparation and Treatment of Homogenate	Fresh wt. of Plants (g)	Log relative potency ± S.E.	Gibb. A_3 Equiv. (ng)	% Recovery.
1. Phosphate buffer, extracted immediately	3.7	-7.95 ± 0.13	11.3	
2. Phosphate buffer + 500 ng A_3, extracted immediately	4.1	-6.68 ± 0.14	310	59%
3. Phosphate buffer, incubated 18 hrs./30°C before extraction	3.9	-8.27 ± 0.16	5.4	
4. Phosphate buffer + 500 ng A_3, incubated 18 hrs/30°C before extraction	3.5	-7.30 ± 0.08	50.4	9%
5. Phosphate buffer with pronase, incubated 18 hrs/30°C before extraction	3.7	-8.03 ± 0.14	9.3	
6. Acetate buffer, extracted immediately	4.0	-8.46 ± 0.09	3.5	
7. Acetate buffer + 500 ng A_3, incubated 18 hrs/30°C before extraction	4.0	-7.51 ± 0.17	31.4	6%
8. Acetate buffer with β -emulsin, incubated 18 hrs/30°C before extraction	4.1	-8.39 ± 0.13	4.0	

7. Discussion.

(1) The Techniques Used in the Extraction, Purification and Measurement of Gibberellins.

The acid ethyl acetate extract from barley seedlings was consistently found to contain biologically active principles, and good agreement was always obtained between replicate extractions for the gibberellin content of this fraction. Recoveries of added gibberellin A_3 into the ethyl acetate fraction of 55-60% were obtained (Tables 3, 6B and 7).

With no detectable losses of biological activity, dry weight reductions of the crude ethyl acetate extracts of 200-fold were obtained by TLC on silica gel H, paper chromatography and paper electrophoresis (Table 4).

GLC of the TMS ether/ester derivatives of the partly purified, biologically active extract showed the presence of numerous compounds (Figure 13), and emphasised the need for the superimposition of another technique to single out the gibberellin peaks from the other organic acids. Fraction collecting of the GLC column effluent, and biological activity testing of the collected fractions permitted a more precise description of the gibberellins present in the extract, and could be carried out with recoveries of biologically active gibberellin in the range 40-50% (Section IV.A.5).

Precise quantitative measurement of the gibberellin content of the extracts by the methods used here, presented some difficulties. The combined effects of the log-based bioassay response and the inherent variability in bioassay readings, resulted in the estimations of log relative potency having a relatively large standard error. In general, differences in amounts of gibberellin in two extracts which were less than 4-5 fold could not be proven statistically significant. However, with greater replication and by using more points on the linear portion of the dosage/response curves, the minimal differences which can be proven to be statistically significant can be reduced to 2-fold (Coombe, 1971).

In each case, when non-parallelism between the dosage/response curves for an extract and gibberellin A_3 occurred (Tables 4 and 5), the extract had been obtained from 8-day old seedlings. However, no particular significance is attributed to this because, in several other cases, the dosage/response curves obtained from extracts of 8-day old seedlings were parallel to the dosage/response curve for gibberellin A_3 (Tables 3 and 6). The reason for the non-parallelism in the 3 cases mentioned (Tables 4 and 5) is not known.

(2) Characterisation and Quantification of Gibberellins in the Ethyl Acetate Extract.

With one exception all the 38 known free gibberellins can be readily extracted from acidified aqueous solutions with ethyl acetate. The known exception is gibberellin A_{32}, the four substituent hydroxyl groups of which impart sufficient polarity to the molecule such that it remains in the acidified aqueous phase following ethyl acetate extraction (Jackson and Coombe, 1966; Coombe and Tate, 1972).

The only biologically active substances obtainable in ethyl acetate extracts of tops and roots of barley seedlings aged 8-15 days behaved in a manner identical with gibberellins A_1 and A_3 in a variety of chromatographic, electrophoretic and GLC systems (Figures 11, 12, 13 and 14). There can be little doubt then that the biologically active principles extractable with ethyl acetate from barley seedlings are gibberellins A_1 and A_3.

On the basis of chromatography and bioassay techniques, and in one instance a fluorimetry method, Jones et al, (1963), Cohen and Paleg (1967) and Radley (1968) suggested gibberellins A_1 and/or A_3 were present in immature barley grain, germinating barley embryos, and young barley seedlings. These reports lend credence to the findings obtained in this project. No evidence was found that gibberellin A_4/A_7-like material, reported by Radley (1966) to be present in immature barley seed, was present in barley seedlings.

Accurate measurements of the amounts of gibberellins A_1 and A_3 in barley seedlings have not been made, but as judged by the peak areas on chromatograms, the relative amounts of these two compounds in the tops and roots of seedlings aged 8, 11 and 15 days were the same, and were of the order 10:1 (A_3:A_1). In arriving at this ratio, the 2-fold difference in potency of these two compounds in the barley endosperm bioassay has been taken into account (Coombe, 1971).

The finding that significant amounts of gibberellin occurred in the roots of seedlings (Figures 10, 11 and 12) was not unexpected in view of the reports of Murakami (1968) and others (see I.B.2(7)), but concentrations in roots which regularly exceed concentrations in shoots (Table 5) have not been previously reported.

Under normal circumstances the total gibberellin concentration per plant was not found to vary a great deal with the age of the seedling or the stage of seedling development (Table 5), and was generally in the range 3-10 ng/plant or 0.03-0.30 µg/g dry weight. Similar concentrations of gibberellins from comparable tissues have been reported by Crozier and Audus (1968) for dark-grown Phaseolus coccineus seedlings, Dale (1969) for light-grown Phaseolus vulgaris seedlings, and Reid and Crozier (1972) for Pisum sativum, Hordeum vulgare and Phaseolus vulgaris seedlings.

Nicholls and May (1964) found the levels of extractable gibberellins in the apices of barley seedlings increased 80-fold between days 11 and 15 after germination. The seedlings used in work reported here were grown under identical conditions, but between days 11 to 15 there was no significant increase in the gibberellin content of the tops or the roots (Table 5). The ability of barley apices to accumulate up to 2 ng of gibberellin per apex just prior to the onset of internode elongation, as described by Nicholls and May (1964), could be a consequence of a rapid but localised increase in gibberellin biosynthesis in the apex or surrounding tissue, or a sudden change in gibberellin distribution in the tops of the plants.

(3) Attempts to Increase the Amount of Extractable Gibberellin from Barley Seedlings.

Reid and Crozier (1972) reported that treatment of young barley seedlings with a root drench of the growth retardant AMO-1618 (1 mg/litre) resulted in a 6-fold increase in the extractable gibberellin content of the seedlings. As shown in Table 6A similar treatment of young barley seedlings with root drenches of AMO-1618 had a depressing, although not statistically significant, effect on the gibberellin content of the seedlings. This result is in accord with the concept of AMO-1618 as an inhibitor of gibberellin biosynthesis in plants (see I.B.2(5)), but the reason for the disparity between these results and those reported by Reid and Crozier (1972) is not known.

Working on the plausible assumption that some form or forms of conjugated gibberellins are present in barley seedlings, attempts to hydrolyse the conjugates by acidic and enzymic procedures were made (Tables 6B and 7). Hydrolysis of barley seedling homogenates with dilute HCl (0.1 N) at 60°C for 1 hour resulted in a 3-fold reduction in the level of extractable gibberellin obtained from the homogenates, while the recovery of added gibberellin A_3 declined by a factor of 4-fold during this procedure (Table 6B).

Acid treatment is known to degrade some gibberellins to forms

which are not biologically active. Gibberellin A_3 is almost completely degraded to allogibberic acid in 1 N HCl at 55-60°C for 2.25 hours (Cross, 1954), while gibberellenic acid, an intermediate in the formation of allogibberic acid, forms readily from gibberellin A_3 under milder conditions (0.1—1.0 N HCl) (Gerzon et al., 1957). Allogibberic acid has a very low biological potency in most bioassays including the barley endosperm bioassay (Paleg et al., 1964; Brian et al., 1967), and from consideration of the proposed structure of gibberellenic acid (Griffiths et al., 1964) it seems highly unlikely that this compound would be biologically active. Jones et al. (1963) hydrolysed 5 µg of purified gibberellin obtained from barley seedlings in 0.1 N HCl for 0.5 hours, and found unchanged gibberellin A_3, allogibberic acid and gibberic acid in the hydrolysate.

Not all gibberellins are broken down by acidic conditions. Gibberellin A_8 seems to be relatively stable to 1 N sulphuric acid at 100°C for up to 1 hour (Sembdner et al., 1968). Consequently the success with which acid hydrolysis can be used to hydrolyse gibberellin conjugates to release biologically active gibberellin depends on the nature of the gibberellin and its susceptibility to degradation under acidic conditions. Dale (1969) and Loveys and Wareing (1971a, 1971b) have reported the successful

use of acid hydrolysis to release free, biologically active gibberellin from conjugated forms from Phaseolus vulgaris seedlings and etiolated wheat leaves (Triticum aestivum) respectively.

Hydrolysis of barley seedling homogenates with the proteolytic enzyme pronase, obtained from the bacterium Streptomyces griseus, in phosphate buffer (0.05 M, pH 7.4) for 18 hours at 33°C resulted in a small, but not statistically significant, increase in the level of biologically active gibberellin extracted from the homogenate (Table 7). This increase was obtained in spite of a very low recovery of added gibberellin A_3 (9%).

Essentially identical results were obtained with the β-glycosidic enzyme β-emulsin, for which acetate buffer was used (0.05 M, pH 5.4).

A number of workers have reported the successful use of proteolytic and β-glycosidic enzymes to hydrolyse gibberellin conjugates from plants, and thereby obtain an estimate of the conjugated gibberellin pool in plants (see I.A.7). However this work could not be successfully repeated with barley seedlings using pronase and β-emulsin.

The fundamental difficulty found with the hydrolysis experiments (Tables 6B and 7) is the very low recovery of added gibberellin A_3 obtained after hydrolysis. This result complete obscures further interpretation about the occurrence of potentially

hydrolysable gibberellin conjugates in barley seedlings. It seems clear that acidic degradation of barley gibberellin to non-biologically active forms can account for the failure of acidic hydrolysis to result in the accumulation of more gibberellin. However, the reasons for the low recovery of added gibberellin A_3 from 18-hour incubations at 33°C in either phosphate or acetate buffer are not so clear. Enzymatic breakdown or alteration of the gibberellin molecules under these conditions could account for these results, an effect which must only occur in plant homogenates in which mixing of enzymes and potential substrates takes place readily. This would not normally occur in growing plants because of inter-cellular and intra-cellular compartmentalization.

(4) Gibberellin Principles in the Remaining Aqueous Fraction.

Of the chemically defined gibberellins and gibberellin conjugates, only gibberellin A_{32}, gibberellin glycosides and gibberellin glucose esters would remain in the acidified aqueous phase after ethyl acetate extraction (Yokota et al., 1971; Coombe and Tate, 1972; Hiraga et al., 1972).

The variable occurrence of gibberellin activity in this fraction derived from barley seedlings has already been described (IV.A.2(3)). On paper chromatography the peak of biological activity migrated in a manner similar to gibberellin A_3. The lack of a peak of biological activity at an RF less than that of gibberellin A_3 eliminates gibberellin A_{32} as a component of this fraction (Coombe and Tate, 1972).

It is suggested that the biologically active principles in this fraction arose from hydrolysis of a conjugated gibberellin, and an explanation for the inconsistency of the results obtained with this fraction could lie in the methods which have been used. Because good agreement between replicates was obtained for the gibberellin content of the ethyl acetate fraction, it appears that at this stage all the free gibberellin was removed with the ethyl acetate. The remaining acidified aqueous fraction was often left standing at room temperature for several hours before it was

neutralised and taken to dryness. During this time liberation of more biologically active gibberellin from a conjugated form could have occurred. The mild hydrolytic conditions set up could have been sufficient in some cases to hydrolyse at least part of the gibberellin conjugate without degrading the liberated gibberellin to a non-biologically active form.

Confirmation of this hypothesis with acid hydrolysis experiments (Table 6B) was not achieved because the stronger hydrolytic conditions used resulted in breakdown of the gibberellin to a non-biologically active form.

The nature of the proposed gibberellin conjugate in the aqueous phase is unknown. To date gibberellin glucosides and gibberellin glucose esters are the only conjugates which have been chemically characterised (See I.A.7). Experiments with pronase and β -emulsin failed to clarify this problem (Table 7 and Section IV.A.7(3)).

No further work was done on the biologically active principles obtainable from this fraction, and for the remainder of the project emphasis was placed on gibberellins A_1 and A_3 which could be consistently found in the ethyl acetate fraction.

B. ^{14}C-Carbon Dioxide Incorporation into Barley Seedlings.

1. Introduction

To test the hypothesis that young barley seedlings were biosynthesising gibberellins de novo from precursors of small molecular weight, experiments were conducted with ^{14}C-carbon dioxide to see if radioactivity from this source could be incorporated into the extractable gibberellins A_1 and A_3.

2. Methods.

The experiments were conducted as described in III.B.9(1) using an aqueous solution of ^{14}C-sodium carbonate (total activity 1 millicurie, specific activity 28.4 mcuries/mmole) and five 11-day old barley seedlings.

To follow the time-course of $^{14}CO_2$ uptake, gas samples (500 µl) from inside the jar were taken, and after bubbling through 1 ml of ethanolamine, to which was added 2 ml of 2-methoxy-ethanol and 7 ml of toluene fluor, radioactive measurements were made according to the methods described in III.B.10.

Twelve hours after the commencement of each experiment the remaining $^{14}CO_2$ inside the jar was trapped in a KOH solution. The jar was then opened and the plants were prepared for extraction immediately (12-hour incorporation experiment), or they were kept in the growth cabinet for a further 12 hours before extraction (24-hour incorporation experiment).

Extraction of the whole plants (roots and tops) was accomplished as already described (III.B.3(1)). The gibberellin A_1/A_3 component of the crude ethyl acetate extract was purified in the following four systems:-

(a) Descending paper chromatography in isopropanol/ammonia/water.

(b) Paper electrophoresis in 0.1 M ammonium bicarbonate (pH 8.9).

(c) TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1).

(d) Repeat of system (a) above.

The procedures followed were those described in III.B.4(2). The final fraction was analysed by GLC of the TMS ether/ester derivatives on OV-17 columns, according to the methods already described (III.B.5). The column effluent between the internal standards allogibberic acid and cholesterol was collected, and the eluted fractions were dispensed for bioassay analysis (III.B.6) and radioactivity measurement (III.B.10).

3. Results.

(1) Uptake of ^{14}C-Carbon Dioxide.

The uptake of $^{14}CO_2$ by the barley seedlings is shown by the results presented in Table 8. Four hours after the commencement of the experiment the amount of $^{14}CO_2$ in the atmosphere in the jar had fallen to less than 10% of the original level. The experiments were terminated after 12 hours, and although gas samples were not taken at this time, there was still some radioactivity in the atmosphere as evidenced by the reading obtained with a hand-monitor.

(2) Distribution of ^{14}C in the Acid Ethyl Acetate Fraction.

Large amounts of radioactivity were recovered in the acid ethyl acetate extracts, much of which was lost during the subsequent purification of gibberellins A_1 and A_3. The distribution of radioactivity on the TLC plates and final paper chromatograms for both the 12-hour and 24-hour experiments is shown in Figure 15.

In the case of the 12-hour experiment relatively large amounts of radioactivity were still associated with the gibberellin A_1/A_3 zone on the final chromatogram (Figure 15B), but in the 24-hour experiment most of the radioactivity had been removed from the gibberellin A_1/A_3 zone on the final chromatogram (Figure 15D).

TABLE 8.

The description of the experiment and method of counting the gas samples is given in IV.B.2.

The values of c.p.m. per 500 µl of gas are the means of 3 replicates, uncorrected for counting efficiency.

TABLE 8.

UPTAKE OF $^{14}CO_2$ BY BARLEY SEEDLINGS

Time following Commencement of Experiment	C.P.M. per 500 µl of gas	Percentage of Initial Value
5 minutes	6058	100%
25 minutes	6544	108%
1 hour	1696	28%
2 hours	1513	25%
3 hours	672	11%
4 hours	557	9%

FIGURE 15.

DISTRIBUTION OF RADIOACTIVITY ON TLC SILICA GEL H PLATES AND FINAL PAPER CHROMATOGRAMS OF ETHYL ACETATE EXTRACTS FROM $^{14}CO_2$ EXPERIMENTS.

The experiment was carried out as described in IV.B.2. Ethyl acetate extracts were prepared and purified as described in III.B.3(1) and III.B.4(1).

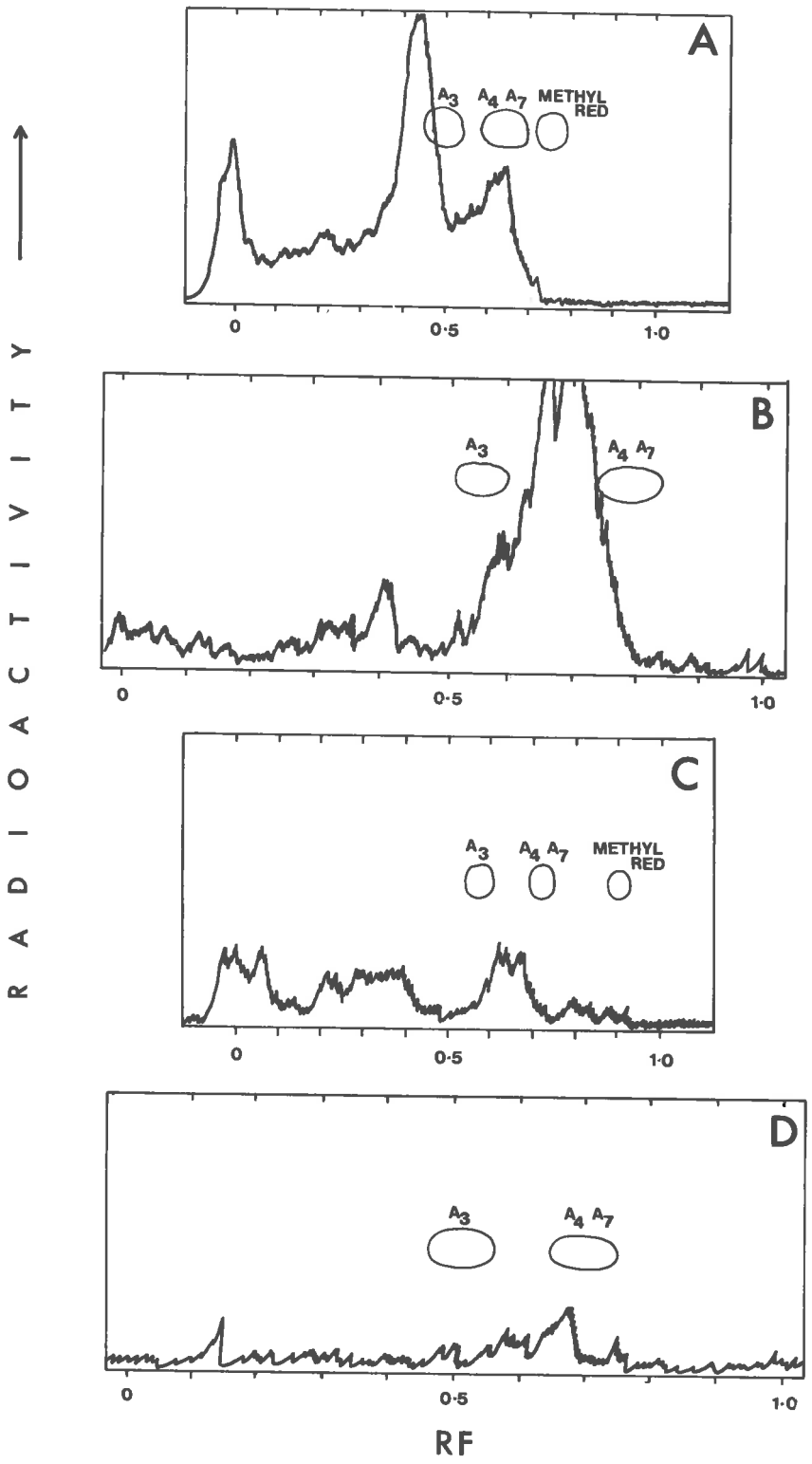
Radioactive scans of the chromatograms were obtained at a scanning speed of 0.75 cm/minute.

FIGURE 15A. TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1) of the ethyl acetate extract from a 12-hour incorporation experiment. Scanner setting 3K for a full scale deflection.

FIGURE 15B. Descending paper chromatography in isopropanol/ ammonia/water (10/1/1) of the extract from a 12-hour incorporation experiment. Scanner setting 1K for a full scale deflection.

FIGURE 15C. TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1) of the ethyl acetate extract from a 24-hour incorporation experiment. Scanner setting 1K for a full scale deflection.

FIGURE 15D. Descending paper chromatography in isopropanol/ ammonia/water (10/1/1) of the extract from a 24-hour incorporation experiment. Scanner setting 1K for a full scale deflection.


A
A3
A4 A7
METHYL RED
0
0·5
1·0
B
A3
A4 A7
0
0·5
1·0
C
A3
A4 A7
METHYL RED
0
0·5
1·0
D
A3
A4 A7
0
0·5
1·0
RADIOACTIVITY
RF

The results of GLC analysis of the TMS ether/ester derivatives of the partly purified extracts are shown in Figure 16. The results of the 12-hour incorporation experiment (Figure 16A) show there is a peak of radioactivity exactly coinciding with the gibberellin A_1 peak of biological activity, and there is a smaller amount of radioactivity associated with the larger gibberellin A_3 peak of biological activity.

In a repeat of this experiment, done under identical conditions with seedlings of the same age using the same amount of ^{14}C-sodium carbonate, very similar results were obtained, with a peak of radioactivity occurring in the fraction with gibberellin A_1 biological activity, and a smaller amount of radioactivity occurring in the fractions with gibberellin A_3 biological activity. The collected results for the two 12-hour $^{14}CO_2$ incorporation experiments are presented in Table 9.

The results of the 24-hour incorporation experiment (Figure 16B) show the two peaks of biological activity are not associated with any radioactivity. In a repeat of the 24-hour incorporation experiment identical results were obtained.

FIGURE 16.

GLC ANALYSIS OF PURIFIED EXTRACTS FROM $^{14}CO_2$-FEEDING EXPERIMENTS WITH BARLEY SEEDLINGS

Column type: 3% OV-17 on silanised Gaschrom Q.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 210°C. and rising at a rate of 1.7°C/minute to 260°C.

Detector: flame ionisation at 220°C.

Derivatives: TMS ether/ester derivatives prepared as described in III.B.5(3)c, with 2 µg each of cholesterol and allogibberic acid.

Fraction collecting: carried out as described in III.B.5(6).

The RT of the standard gibberellins was calculated from the position of the internal standards.

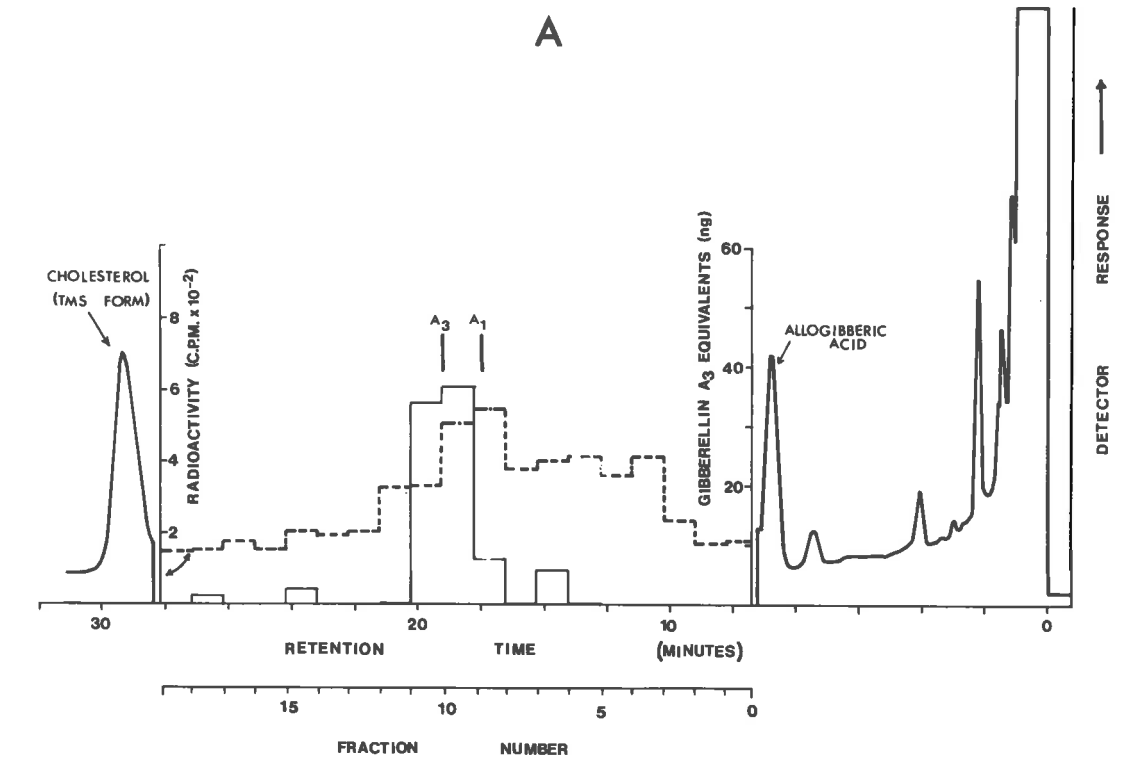
The dried eluates of the collected fractions were dissolved in a known volume of methanol, 60% of which was measured for radioactivity as described in III.B.10. The remainder of each fraction was bioassayed as described in III.B.6. Quantification of the bioassay response was achieved by Bliss analysis as described in III.B.6(5). Only the significantly active fractions are shown as histograms; their dilution curves had a slope significantly greater than horizontal and, with the exception of the fraction denoted by a closed star (Figure B), not significantly different from the slope of the gibberellin A_3 curve. The dosage/response curve of the eluate of the fraction denoted by a closed star (fraction 21, Figure B) had a slope significantly less than the gibberellin A_3 curve, and for this reason the Bliss analysis of this response is not legitimate.

Figure A is the analysis of the extract from a 12-hour feeding experiment.

Figure B is the analysis of the extract from a 24-hour feeding experiment.

Radioactivity measurements are presented with broken black lines; biological activity measurements are presented with continuous black lines.

A


CHOLESTEROL
(TMS FORM)
RADIOACTIVITY (C.P.M. x 10⁻²)
A3
A1
GIBBERELLIN A3 EQUIVALENTS (ng)
ALLOGIBBERIC ACID
DETECTOR RESPONSE
30
20
10
0
RETENTION TIME (MINUTES)
15
10
5
0
FRACTION NUMBER

B

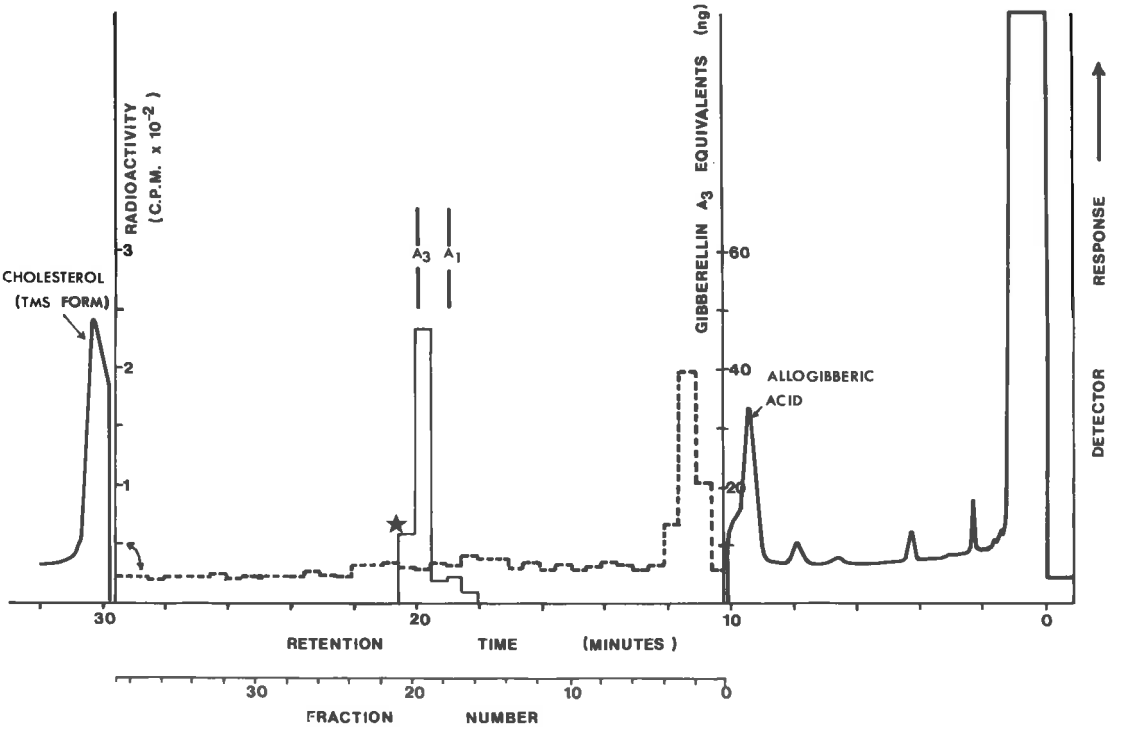

RADIOACTIVITY (C.P.M. x 10⁻²)
CHOLESTEROL
(TMS FORM)
A3
A1
GIBBERELLIN A3 EQUIVALENTS (ng)
ALLOGIBBERIC ACID
DETECTOR RESPONSE
30
20
10
0
RETENTION TIME (MINUTES)
30
20
10
0
FRACTION NUMBER

TABLE 9.

Description of the experiments are given in IV.B.2. The figures for total biological activity were obtained from the Bliss analysis (III.B.5(6)) of the dosage/response curves of the biologically active GLC fractions.

The figures for total radioactivity (in d.p.m.) were calculated from the c.p.m. in the biologically active fractions. The range of values is presented because the variable radioactivity of the background eluting from the GLC column made precise measurement impossible. Corrections for counting efficiency were made as described in III.B.10.

After dilution the specific activity of the $^{14}CO_2$ in the initial experiment was 8 μcuries/μmole: the specific activity of the $^{14}CO_2$ in the repeat experiment was 16 μcuries/μmole.

TABLE 9.

SPECIFIC ACTIVITY OF THE GIBBERELLIN IN THE BIOLOGICALLY ACTIVE GLC FRACTIONS FROM THE 12-HOUR $^{14}CO_2$ EXPERIMENTS

GLC FRACTION	Corresponding Gibberellin Standard	Gibb. A_3 Equiv. (ng)	D.P.M. above background	Specific Activity (μcuries/ μmole).
Initial Experiment				
19	A_1	7.4	150-300	3.1-6.3
20 + 21	A_3	61.0	170-345	0.4-0.9
Repeat Experiment				
17	A_1	27	400-800	2.3-4.6
18 + 19	A_3	124	500-1000	0.5-1.0

4. Discussion.

Incorporation of ^{14}C-Carbon Dioxide into the Gibberellins of Barley Seedlings.

The difficulty associated with drawing conclusions from these results is the uncertainty that the peak of radioactivity in fractions 19-21 of Figure 16A is associated with gibberellins A_1 and A_3, and is not associated with a compound or compounds which happen to have the same or similar RT as gibberellins A_1 and A_3 under the GLC conditions used.

Reference to Figure 13 shows that of the compounds present in an extract of barley which had been purified in an identical manner, the two peaks due to gibberellins A_1 and A_3 (see IV.A.5(2)) are well separated from the other compounds in the extract, thus permitting the conclusion that the peak of radioactivity in fractions 19-21 of Figure 16A is associated with gibberellins A_1 and A_3. There is the possibility that the peak of radioactivity is due to a compound, or compounds, with a relatively high specific radioactivity which escaped detection by flame ionisation in Figure 13 because they are present in very small amounts. However, this possibility can be discounted for the following reason. The maximum possible specific radioactivity of compounds produced in the experiment with radioactive carbon dioxide cannot exceed 8 μcuries/μmole, the specific radioactivity of the $^{14}CO_2$ at the

start of the experiment (Table 9). This means that the peak of radioactivity in fractions 19-21 of Figure 16A, which totals 320-635 d.p.m. (Table 9), cannot represent less than a total of 0.028 to 0.037 nmoles of a compound or compounds. Considering that the scan in Figure 13 represents the compounds in an extract from an amount of plant tissue 10-fold greater than that used in the experiments with $^{14}CO_2$, then peaks representing 0.28 to 0.37 nmoles of material would be the smallest expected in the gibberellin A_1/A_3 region of Figure 13. This amount of material would be readily detected by flame ionisation, and the absence of such peaks additional to those of gibberellins A_1 and A_3 supports the conclusion that the peak of radioactivity in fractions 19-21 of Figure 16A can only be associated with gibberellins A_1 and A_3 and therefore represents incorporation of $^{14}CO_2$ into these compounds.

If this is accepted, then the results of these experiments show that within 12 hours the barley seedlings had converted some $^{14}CO_2$ into these gibberellins. Furthermore, during the 12-hour period following the $^{14}CO_2$ feeding, the entire population of gibberellin molecules which were biosynthesised during the initial 12 hours were either broken down, de-activated or otherwise metabolised such that a complete turnover resulted.

So far as I am aware there are no comparable experiments

reported in the literature with which these results and conclusions can be compared. A number of reports have been made on the rapidity with which plant tissues metabolise exogenous radioactive gibberellin (Rappaport et al., 1967; Kende, 1967; Barendse et al., 1968; Musgrave and Kende, 1970; Barendse and Lang, 1972; Nadeau and Rappaport, 1972; Nadeau et al., 1972; Durley and Pharis, 1973). In general, this work has shown that radioactive gibberellin applied externally to plant tissues is metabolised to different compounds, but at a comparatively slow rate, such that 24 or more hours after the hormone application significant amounts of radioactivity are still associated with the applied compound.

The rapidity with which exogenous gibberellin is metabolised by plants depends on the rate of penetration into the plant and the proportion which reaches the site of the appropriate enzymes. These factors are not involved in the metabolism of endogenous gibberellin, and this must be the reason for the discrepancy between the results reported here for turnover rate, and those obtained by the authors cited above.

The estimations of specific activity of the gibberellins in the GLC fractions from the 12-hour incorporation experiment, presented in Table 9, show in both experiments gibberellin A_1 has a higher specific activity than gibberellin A_3. This suggests gibberellin A_1 is turning over at a faster rate than gibberellin

A_3, and implies these two compounds may not be interconvertible. In Gibberellin fujikuroi interconversion of gibberellins A_1 and A_3 does not take place readily (see I.B.1(2)c).

C. ^{14}C-Mevalonic Acid Incorporation.

1. ^{14}C-Mevalonic Acid Incorporation into whole Barley Seedlings.

(1) Introduction.

Mevalonic acid is a general terpenoid precursor in plants, animals and micro-organisms. In yeast and animals it is known to be biosynthetically derived from amino acids, fatty acids and acetate, via the formation of β-hydroxy-β-methyl-glutaryl-Coenzyme A. The irreversibility, under physiological conditions, of the last reaction leading to the formation of mevalonic acid means it cannot be used as a carbon source for growth or energy production (Popjack and Cornforth, 1960).

Birch et al.(1958, 1959), Cross et al. (1964) and Hanson and White (1969a, 1969b) have shown ^{14}C-mevalonic acid, supplied in the culture medium, is incorporated (with specificity!) into the gibberellins produced by growing cultures of Gibberella fujikuroi.

Experiments were conducted to test the hypothesis that 11-day old barley seedlings could incorporate exogenous ^{14}C-mevalonic acid into the extractable gibberellins A_1 and A_3.

(2) Methods.

Unless otherwise stated experiments were conducted in a controlled environment cabinet at 20°C (± 1°C) under continuous fluorescent lighting with an intensity of 4.1 milliwatts/cm^2/hr. at the level of the seedlings.

Aqueous solutions of 2-^{14}C-(RS)-mevalonic acid (specific activity 10.3 mcuries/mmole) were administered to 11-day old barley seedlings, as described in III.B.9(2), by feeding the radioactive solution to the intact roots, by feeding the radioactive solution to the severed ends of the roots, and by feeding the radioactive solution to the seedlings through a wet cotton wick.

Up to 10 µcuries of the substrate were fed to each plant, and five 11-day old seedlings were used for each experiment.

The period of time which elapsed during which the substrate was fed to the seedlings was varied from a few hours to two days.

At the completion of the experiments the plants were washed free of pearlite, weighed and extracted as described in III.B.3(1). The gibberellin A_1/A_3 component of the crude ethyl acetate fraction was purified as described in III.B.4(1) and III.B.4(2) by descending paper chromatography in isopropanol/ammonia/water, followed by paper electrophoresis in ammonium bicarbonate buffer, and

redevelopment in the paper chromatography system used initially. The final fraction was analysed by GLC of the TMS ether/ester derivatives on OV-17 columns according to the methods described in III.B.5. The column effluent between the internal standards allogibberic acid and cholesterol was collected, and the eluted material was dispensed for barley endosperm bioassay analysis (III.B.6) and radioactivity measurement (III.B.10).

(3) Results.

Brief examination of the chloroform-acetone extract on heat-activated silica gel H TLC plates in n-hexane/ethyl acetate (75/25) showed the presence of several large peaks of radioactivity. This indicated that the mevalonic acid was being incorporated by the barley seedlings into a variety of non-polar isoprenoid compounds.

The crude ethyl acetate extracts were found to contain relatively large amounts of radioactivity, the majority of which behaved during the preliminary purification steps in a manner identical to mevalonic acid. The purified extracts which were analysed by GLC contained no more than a few hundred c.p.m. of radioactivity.

The results of GLC analysis of the purified extracts always showed the presence of two peaks of biological activity, corresponding in retention times to gibberellins A_1 and A_3, but in no instance was any peak of radioactivity ever found to coincide with the peaks of biological activity. These results were obtained in

spite of a concerted effort to achieve incorporation by using a variety of feeding procedures and a variety of incorporation times.

Experiments were also carried out by feeding mevalonic acid to 11-day old seedlings in the dark; by feeding mevalonic acid to etiolated seedlings, and by raising seedlings in a short day photoperiod (8 hours light), transferring them to continuous light, and feeding mevalonic acid. In none of these experiments were any peaks of radioactivity found associated with the peaks of biological activity in the fractions collected from the GLC.

2. ^{14}C-Mevalonic Acid Incorporation with Cell-free Preparations from Barley Seedlings.

(1) Introduction.

Cell-free preparations from developing seeds are capable of converting ^{14}C-mevalonic acid into ent-kaurene and a number of its oxidised derivatives, including gibberellin A_{12}-aldehyde (see I.B.2(2)c). Cell-free preparations from young castor bean seedlings have been shown to incorporate ^{14}C-mevalonate into ent-kaurene (Robinson and West, 1970a, 1970b).

Attempts were made to incorporate ^{14}C-mevalonic acid into gibberellins A_1 and A_3 using cell-free preparations from barley seedlings.

(2) Methods.

Cell-free homogenates of barley seedlings were prepared in Tris-HCl buffer (0.05 M, pH 7.8) or phosphate buffer (0.05 M, pH 7.5) as described in III.B.7. Incubations were carried out in glass centrifuge tubes (11 cm x 2.5 cm); the contents of each tube were:- Tris-HCl buffer (pH 7.8, 50 µmoles), NAD (0.98 µmoles), NADPH (0.74 µmoles), ATP (1.13 µmoles), $MnCl_2$ (5µmoles), creatine phosphate (50 µmoles), creatine kinase (0.5 mg), D.L.glutamic acid (73 µmoles), glutamic dehydrogenase (0.1 mg) and 2-^{14}C-(RS)-mevalonic acid (4.85 nmoles, 50 ncuries). The reactions were commenced

by the addition of 0.4 ml of enzyme (0.8 mg protein) which gave the incubation a total volume of 1 ml. The reactions were allowed to continue for up to 4.5 hours at 30°C in a shaking water bath.

The ability of the nucleotide regenerating systems of creatine phosphate/creatine kinase and glutamic acid/glutamic dehydrogenase to maintain a supply of NADH, NADPH and ATP was followed by electrophoresis (at 1500 volts for 1 hour) of 5 µl aliquots of each reaction mixture on Whatmann chromatography paper in Tris-citrate buffer (0.05 M, pH 4.8). The presence of the nucleotides was determined by inspection of the electrophoretogram under U.V. light and comparison with authentic nucleotide standards.

Reactions were terminated by addition of an equal volume of distilled acetone to the reaction mixture. The components were then separated according to the methods described in III.B.3(1).

The radioactive materials present in each extract were examined by TLC on silica gel H and paper chromatography.

(3) Results.

Very low incorporation of ^{14}C-mevalonic acid was obtained with cell-free preparations from 11-day old barley seedlings. Extracts from such seedlings were found to have a high level of phosphatase, NADPH oxidase and NADH oxidase activity, as evideneed

by the rapid disappearance of ATP, NADH and NADPH following commencement of the incubations.

Significant incorporations of ^{14}C-mevalonic acid into radioactive metabolites was obtained with cell-free preparations from 6-day old seedlings. In these incubations the nucleotides ATP, NADPH and NADH were still present after 3 hours, indicating the regenerating systems were able to maintain a supply of these compounds.

Cell-free homogenates of 6-day old seedlings prepared either in phosphate buffer (0.05 M, pH 7.5), or Tris-HCl buffer (0.05 M, pH 7.8), in a mortar and pestle, or with an Ultra-Turrax Probe (see III.B.7) were equally active. So long as the nucleotide regenerating systems were included, the pattern of incorporation did not seem to be affected by varying the nucleotide concentration.

The results of a typical experiment in which the homogenate was prepared in phosphate buffer with a mortar and pestle are shown in Figure 17. Aliquots of the total reaction mixture, separated by the descending paper chromatography system of Bloch et al. (1959) showed several peaks of radioactivity (Figure 17A), the most prominent one of which had an RF similar to that of mevalonic acid. Substantial amounts of radioactive compounds more polar than mevalonic acid and less polar than mevalonic acid

FIGURE 17.

CHROMATOGRAPHY OF METABOLITES FROM CELL-FREE INCORPORATIONS WITH ^{14}C-MEVALONIC ACID

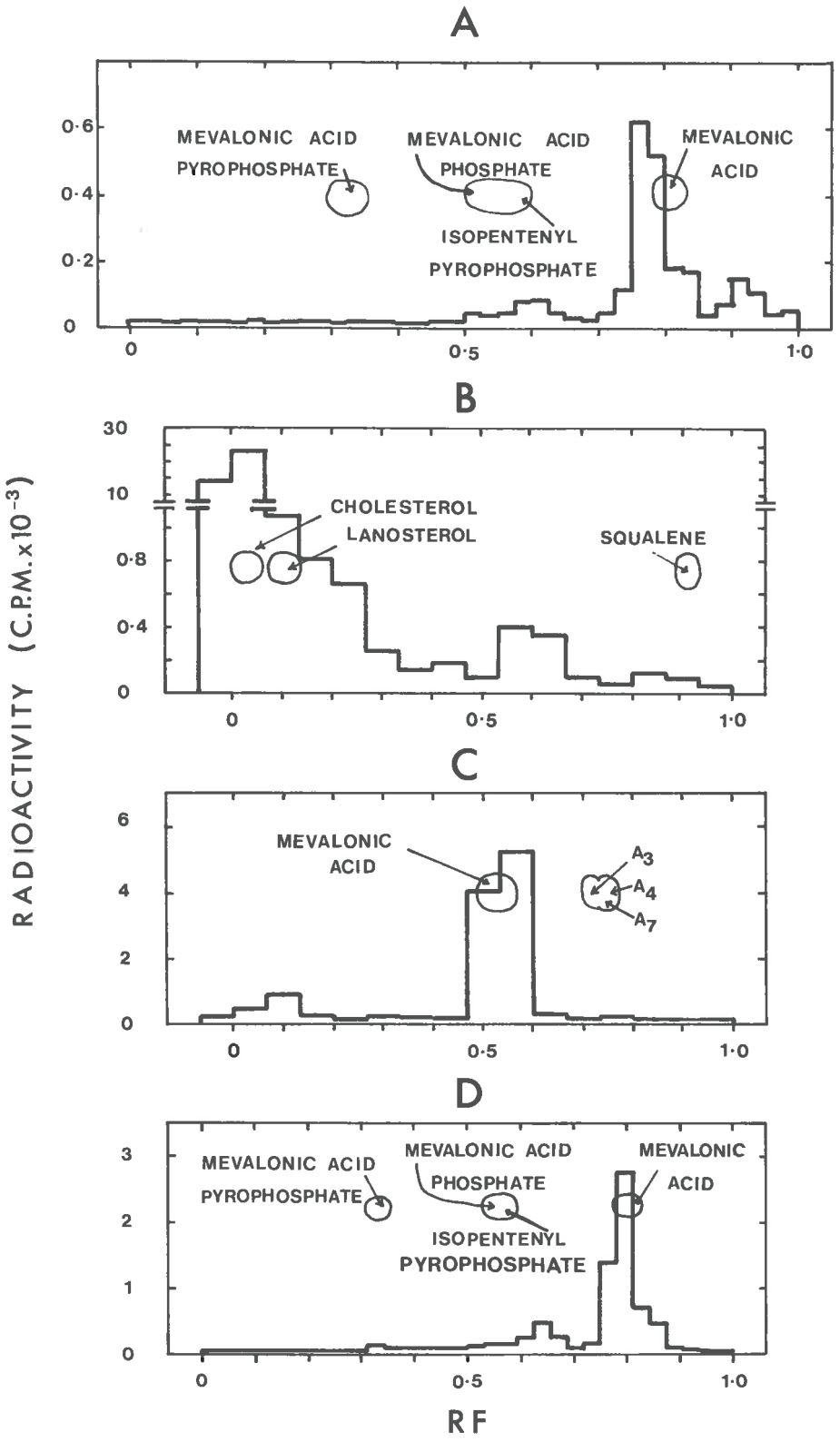
The cell-free homogenate was prepared from 6-day old barley seedlings in phosphate buffer (0.05 M, pH 7.5) with a mortar and pestle as described in III.B.7. Incubations were carried out as described in IV.C.2(2). After 4-5 hours the components of the incubations were fractionated as described in III.B.3(1)

FIGURE 17A. Descending paper chromatography in tert. butanol/formic acid/water (40/10/16) of 5 µl of the total reaction mixture. RF's of standards are those given by Bloch et al. (1959).

FIGURE 17B. TLC on silica gel H (heat activated) in n-hexane/ethyl acetate (75/25) of part of the acetone-chloroform extract. Standards were made visible with iodine vapour.

FIGURE 17C. TLC on silica gel H in n-butanol/acetic acid/water (85/5/10) of part of the ethyl acetate fraction.

FIGURE 17D. Descending paper chromatography in tert. butanol/formic acid/water (40/10/16) of part of the remaining aqueous fraction. RF's of standards are those given by Bloch et al. (1959).


A
MEVALONIC ACID
PYROPHOSPHATE
MEVALONIC ACID
PHOSPHATE
ISOPENTENYL
PYROPHOSPHATE
MEVALONIC
ACID
0·6
0·4
0·2
0
0
0·5
1·0
B
30
10
0·8
0·4
0
CHOLESTEROL
LANOSTEROL
SQUALENE
0
0·5
1·0
C
6
4
2
0
MEVALONIC
ACID
A3
A4
A7
0
0·5
1·0
D
3
2
1
0
MEVALONIC ACID
PYROPHOSPHATE
MEVALONIC ACID
PHOSPHATE
ISOPENTENYL
PYROPHOSPHATE
MEVALONIC
ACID
0
0·5
1·0
RADIOACTIVITY (C.P.M.x10⁻³)
RF

are evident on this chromatogram.

The chloroform-acetone extract, when separated on heat activated silica gel H TLC plates in n-hexane/ethyl acetate (75/25), showed the presence of several large radioactive peaks (Figure 17B) one of which had an RF similar to that of cholesterol. Small amounts of radioactivity migrated on the squalene region of the silica gel H plates, and other peaks of radioactivity were evident.

The acid ethyl acetate extract was examined by TLC on silica gel H in n-butanol/acetic acid/water (85/5/10), and always showed the presence of a large peak of radioactivity which had the same RF as mevalonic acid (Figure 17C). Some more polar radioactive compounds were present in this extract, but no significant peak of radioactivity was ever found to be associated with the gibberellin A_1/A_3 region on the chromatogram.

The aqueous phase remaining after ethyl acetate extraction, when separated by descending paper chromatography with the system of Bloch et al. (1959) showed the presence of a large peak of radioactivity which had the same RF as mevalonic acid, and a smaller, more polar peak of radioactivity (Figure 17D). This latter peak could possibly be phosphorylated isoprenoid intermediates.

3. ^{14}C-Mevalonic Acid Incorporation into Young Apricot Fruits.

(1) Introduction.

Young apricot fruits accumulate gibberellin A_{32} during the first 20 days following anthesis (Jackson and Coombe, 1966; Coombe and Tate, 1972), and as stated earlier (I.B.2(7)), there is good presumptive evidence that the gibberellin A_{32} originates in the developing seed (Coombe, 1971).

Growing fruits and seeds from several plant species have been shown to have the necessary enzyme complement for the conversion of mevalonic acid to ent-kaurene, ent-kaurenoic acid and gibberellin A_{12}-aldehyde (see I.B.2(2)), all of which are gibberellin precursors in the fungus Gibberella fujikuroi (Figure 3). If apricot fruits have a similar complement of enzymes, then they would be expected to have the capacity to incorporate radioactive mevalonic acid into gibberellin A_{32}.

Experiments were conducted to see if young apricot fruits could incorporate exogenous 2-^{14}C-(RS)-mevalonic acid into gibberellin A_{32}.

(2) Methods.

Experiments were conducted during September 1971 and September 1972 but only the latter are reported here. Three separate batches of 10 apricot fruits (cv. Moorpark), 0.5-1 cm in diameter, were selected on the trees in the Waite Institute orchard. Aqueous solutions of ^{14}C-mevalonic acid were fed to each batch of fruit in a different way as described in III.B.9(2). Batch 1 was injected with a small volume (2 µl) of radioactive solution, batch 2 was wick-fed with the radioactive solution, and batch 3 was excised from the trees and the radioactive solution fed to the fruits through the cut pedicel. 2.5 µcuries of 2-^{14}C-(RS)-mevalonic acid (specific activity 10.3 mcuries/mmole) were fed to each fruit.

The fruits in batch 2 took 24-36 hours to absorb all the radioactive solution. Those in batch 3 took 6-8 hours to absorb all the solution, after which they were implanted on a plate of agar containing 2% sucrose until they were extracted.

Three days after the comencement of feeding the fruits were harvested, frozen by immersion in liquid nitrogen, and extracted in the manner described in III.B.3(2). The extracts were partly purified by column chromatography on DEAE-Sephadex, followed by paper chromatography and electrophoresis as described in III.B. 4(1) and III.B.4(3).

The final biologically active eluate was analysed by GLC of the TMS ether/ester derivatives on OV-17 columns as described in III.B.5. The column effluent between the internal standards (allogibberic acid and cholesterol) was collected, and the eluted material was dispensed for barley endosperm bioassay analysis (III.B.6) and radioactivity measurement (III.B.10).

(3) Results.

An example of the results of DEAE-Sephadex column chromatography of the extracts is given in Figure 18A. Aliquots (1%) of each fraction were bioassayed for activity, and the histogram shows fractions 25-36 were associated with a peak of biological activity. The extracts from the 3 batches of fruit all produced similar results.

An example of the radioactivity scan of the final paper chromatogram of the extracts is given in Figure 18B. The only significant peak of radioactivity occurred near the origin of the chromatogram. The chromatograms of the extracts from all 3 batches of fruit produced similar results. Gibberellin A_{32} is known to migrate between glucose and gibberellin A_3 in this system (Coombe and Tate, 1972), and this zone was eluted from each chromatogram.

The results of GLC analysis of the final fraction from each batch of fruit are presented in Figure 19. A large peak of biological activity is evident in the expected location for gibberellin A_{32} in each of the extracts, and this was accompanied by other smaller peaks of biological activity. In no instance were any peaks of radioactivity found to coincide with the peaks of biological activity.

FIGURE 18.

PURIFICATION OF EXTRACTS FROM APRICOT FRUITS FED ^{14}C-MEVALONIC ACID

The description of the experiment is given in IV.C.3(2).

FIGURE 18A.

DEAE-SEPHADEX COLUMN CHROMATOGRAPHY

Extracts of the fruits were prepared for column chromatography as described in III.B.3(2). The preparation of the columns is given in III.B.4(3).

After loading, the column was eluted with a linear gradient of acetic acid (0-2.0 N). The eluant, collected in 100 ten ml fractions, was tested for activity in the barley endosperm bioassay (III.B.6) by bioassaying a total of 1% of each fraction.

The left hand ordinate values refer to the increase in refraction of the ambient bioassay solution following a 40-hour incubation. The right hand ordinate values refer to the amounts of gibberellin A_3, in grams, required to produce the given responses.

FIGURE 18B.

RADIOACTIVE SCAN OF THE FINAL PAPER CHROMATOGRAM

Descending paper chromatography in isopropanol/ammonia/water (10/1/1). Scanning speed 0.75 cm/minute with a setting of 1K for a full scale deflection.

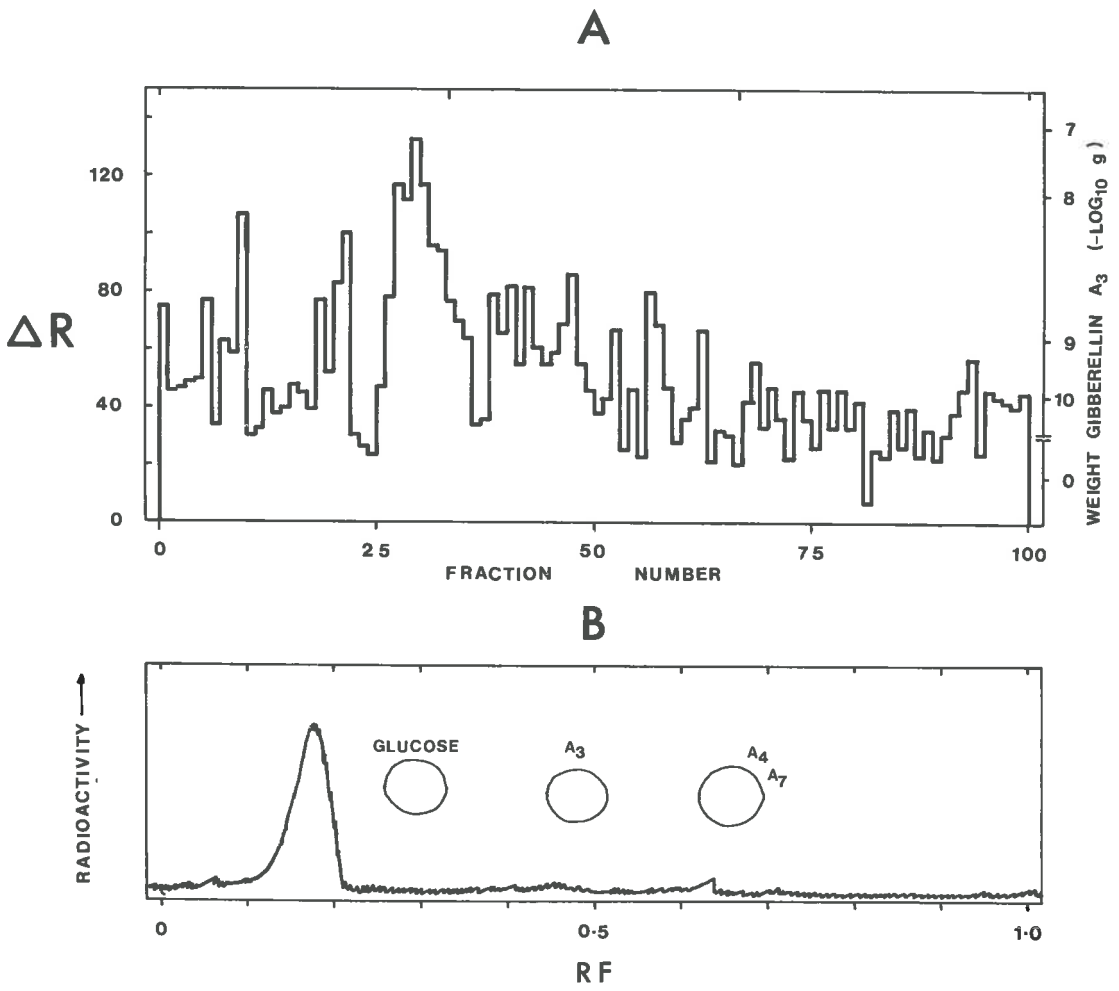

A
ΔR
120
80
40
0
0
25
50
75
100
FRACTION NUMBER
7
8
9
10
0
WEIGHT GIBBERELLIN A3 (-LOG10 g)
B
RADIOACTIVITY
GLUCOSE
A3
A4
A7
0
0·5
1·0
RF

FIGURE 19.

GLC OF THE PARTLY PURIFIED EXTRACTS FROM APRICOT FRUITS FED ^{14}C-MEVALONIC ACID

Column type: 3% OV-17 on silanised Gaschrom Q.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 210°C and rising at a rate of 1.7°C/minute to 260°C.

Detector: flame ionisation at 220°C.

Derivatives: TMS ether/ester derivatives, prepared as described in III.B.5(3)c, with 2 µg each of cholesterol and allogibberic acid.

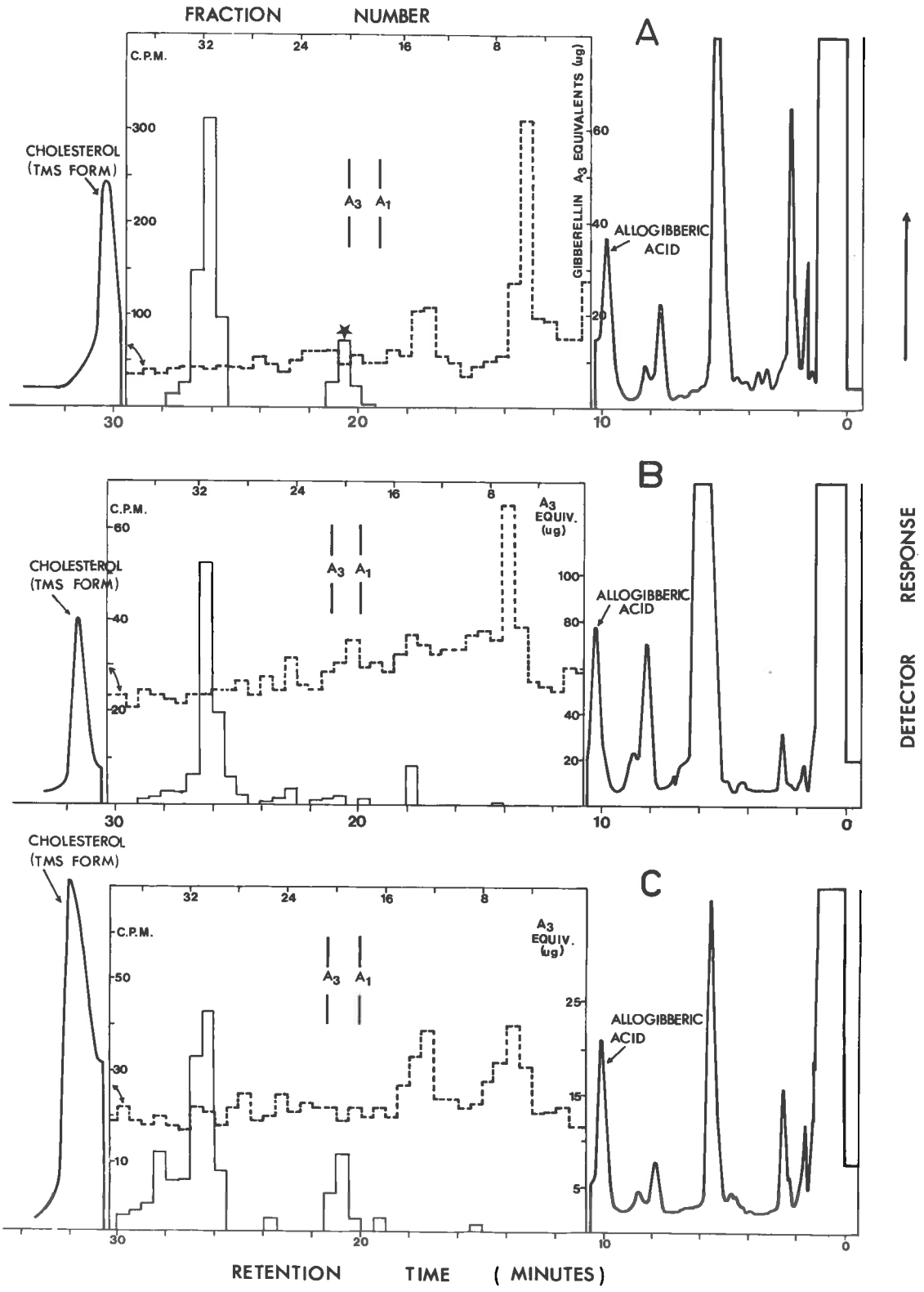
Fraction collecting: carried out as described in III.B.5(6).

The RT of the standard gibberellins was calculated from the position of the internal standards.

The dried eluates of the collected fractions were dissolved in a known volume of methanol, 87.5% of which was measured for radioactivity as described in III.B.10. The remainder of each fraction was tested for biological activity as described in III.B.6. Quantification of the results was carried out by the Bliss (1952) method as described in III.B.6(5). Only the significantly biologically active fractions are shown as histograms; their dilution curves had a slope significantly greater than horizontal and, with the exception of fraction 21 Figure A (denoted by a closed star), were not significantly different from the slope of the gibberellin A_3 curve. The dosage/response curve for fraction 21 had a slope significantly greater than the gibberellin A_3 curve, and for this reason the Bliss analysis of this response is not legitimate.

Figures A, B and C refer to the extracts from the batches of fruit fed ^{14}C-mevalonic acid by injection, detached feeding and wick feeding respectively (III.B.9(2)).

Radioactivity measurements are given in broken black lines; biological activity measurements are given with continuous black lines.


FRACTION NUMBER
A
B
C
C.P.M.
GIBBERELLIN A3 EQUIVALENTS (ug)
A3 EQUIV. (ug)
CHOLESTEROL (TMS FORM)
A3 A1
ALLOGIBBERIC ACID
DETECTOR RESPONSE
RETENTION TIME (MINUTES)

4. Discussion on the Incorporation of ^{14}C-Mevalonic Acid into the Gibberellins of Barley Seedlings and Young Apricot Fruits.

It is suggested that two factors are primarily responsible for the failure of mevalonic acid to act as a precursor of gibberellins in barley seedlings and young apricot fruits. Firstly, mevalonic acid is used as a precursor in the biosynthesis of such a large number of compounds that tremendous dilution of gibberellin precursors derived from exogenous radioactive mevalonic acid would occur. This results in only a small amount of the applied radioactivity being available for incorporation into gibberellin.

The second, and probably the most important point, is that exogenous radioactive mevalonic acid, or a precursor produced from it, must fail to reach the site or sites of the enzyme systems which are biosynthesising gibberellin.

The failure of exogenous ^{14}C-mevalonic acid to act as a precursor for gibberellin biosynthesis could be taken as evidence that mevalonic acid is not on the gibberellin biosynthetic pathway. In consideration of the evidence presented in the literature review (I.B.2) this possibility is discounted.

The results obtained from ^{14}C-carbon dioxide incorporation into the gibberellins of barley seedlings (IV.B.3) suggest these plants are actively biosynthesising gibberellins A_1 and A_3 from

simple precursors, and there is good circumstantial evidence that young apricot fruits are also actively biosynthesising gibberellin A_{32} from simple precursors (see IV.C.3(1)). Hence the failure of exogenous mevalonic acid to act as a gibberellin precursor in these plants is unlikely to be due to the fact that gibberellin biosynthesis is not occurring.

In the experiments with whole barley seedlings, the ethyl acetate fraction was found to contain small amounts of radioactive metabolites which behaved, at least during preliminary purification on TLC and paper chromatography, in a manner identical to gibberellins A_1 and A_3. During GLC of the TMS ether/esters, these radioactive compounds did not co-chromatograph with the gibberellins. Sandhu et al. (1972) extracted similar compounds from Cucumis sativis when seedlings were grown on a source of ^{14}C-mevalonic acid, but in this case identification of these compounds past the stage of TLC on silica gel H was not reported.

Barley seedlings contain very small amounts of gibberellin (less than 300 ng/g dry weight), and do not accumulate gibberellin between days 8 and 15 after germination (Table 5). Hence the first factor mentioned above, concerning dilution of radioactive gibberellin precursors by use in the biosynthesis of other terpenoid compounds, is likely to be important in this case. On the other hand, there is a greater concentration of gibberellin in

young apricot fruits (5-10 µg/g fresh weight), and these fruits are actively accumulating gibberellin A_{32} (Jackson and Coombe, 1966). In this case the most plausible reason for the failure of exogenous ^{14}C-mevalonic acid to act as a precursor of gibberellin A_{32} is that exogenous mevalonic acid, or a derivative of it, must be excluded from the site or sites of gibberellin biosynthesis in these fruits.

Compartmentalization of monoterpene, sesquiterpene, sterol and carotenoid biosynthesis in plants is now established (Rogers et al., 1965; Treharne et al., 1966; Rogers et al., 1966a, 1966b; Shah and Rogers, 1969; Croteau and Loomis, 1972; Croteau et al., 1972), and is regarded as one factor in the regulation of the biosynthesis of these compounds (Rogers et al., 1968; Croteau et al., 1972). The basis of this regulatory mechanism is the segregation of enzymes within the plant cells, and the impermeability of certain cellular membranes, including the chloroplast membrane, to mevalonic acid. By analogy, compartmentalization of the enzymes involved in gibberellin biosynthesis in organelles or structures from which exogenous mevalonic acid, or a product derived from it, is excluded, would explain the negative results obtained with mevalonic acid incorporation into gibberellins.

The only other report which indicates exogenous mevalonic acid can act as a substrate for gibberellin biosynthesis in plant

tissues is that of Phillips (1971) who cultured excised apical buds of Helianthus annus on agar blocks, and measured by bioassay the amount of gibberellin diffusing into the agar blocks. If the agar were supplemented with 0.1% (RS)-mevalonic acid more gibberellin diffused into the blocks than if the agar contained no additional ingredient, or 2% sucrose. It has yet to be shown however, that radioactivity from the mevalonic acid is incorporated (with specificity) into the released gibberellin.

One way of circumventing the problems associated with compartmentalization of enzymes and substrates in tissues is to prepare cell-free homogenates. This has been done with success in the study of the biosynthesis of ent-kaurene, ent-kaurenoic acid, and gibberellin A_{12}-aldehyde by enzyme systems extracted from developing seeds of a number of plant species (see I.B.2(2)). Provided an adequate supply of NADPH, NADH and ATP was maintained, cell-free homogenates of barley seedlings incorporated significant amounts of ^{14}C-mevalonic acid into a number of non-polar and polar terpenoid compounds (Figure 17), but no gibberellin-like radioactive products were produced. There are probably 20 to 30 separate enzymic reactions involved in the conversion of mevalonic acid to the gibberellins (Figure 3), and it would be very difficult, if not impossible, to produce a cell-free preparation from plants which is capable of catalysing this complete sequence of reactions.

D. Experiments with ent-Kaurenoic Acid.

1. Introduction.

ent-Kaurenoic acid, a precursor of the gibberellins produced by growing cultures of the fungus Gibberella fujikuroi (see I.B.1.) has been reported to stimulate leaf sheath elongation in d5 dwarf maize (Zea mays) and Tan-ginbozu dwarf rice (Oryza sativa) (see I.B.2(4)), an effect which has implicated this compound as a precursor of gibberellins in these two species. However, it is not clear if this effect is due to conversion by the plant tissue of ent-kaurenoic acid to a gibberellin, which is the actual stimulant of leaf sheath elongation, or if ent-kaurenoic acid actually mimics gibberellin at the site of action of gibberellin, and in this way simulates the effect of gibberellin on leaf sheath elongation,

Experiments were conducted to see if exogenous ent-kaurenoic acid stimulated leaf sheath elongation of Prior barley seedlings and Tan-ginbozu dwarf rice seedlings. Another series of experiments were conducted to see if these species converted exogenous ^{14}C-ent-kaurenoic acid to gibberellin.

2. Methods.

Tan-ginbozu dwarf rice seedlings and barley seedlings, grown as already described (III.B.2), were treated with solutions of radioactive and non-radioactive ent-kaurenoic acid and gibberellin A_3 as described in III.B.9(3). Treatment of barley commenced when the seedlings were 7 days old; treatment of rice commenced when the seedlings were 5 days old; treatments were made daily and were continued for 7 days, after which the seedlings were harvested and the length of the leaf sheaths measured. In experiments in which 17-^{14}C-ent-kaurenoic acid was used (specific activity 0.62 μcuries/μmole), the seedlings were extracted as described in III.B.3(1), and preliminary purification and identification of the radioactive compounds in the extracts was carried out by TLC on silica gel H, paper chromatography and paper electrophoresis, as described in III.B.4(1) and III.B.4(2).

GLC on OV-17 columns of the TMS ether/ester derivatives was carried out as described in III.B.5. The column effluent between the internal standards (allogibberic acid and cholesterol) was collected, and the collected fractions were dispensed for bioassay analysis and radioactivity measurement as described in III.B.6 and III.B.10.

3. Results.

(1) Effect of ent-Kaurenoic Acid on Leaf Sheath Elongation of Tan-ginbozu Dwarf Rice and Prior Barley Seedlings.

The effects of exogenous ent-kaurenoic acid and gibberellin A_3 on the elongation of the third leaf sheath of Prior barley and Tan-ginbozu dwarf rice seedlings are shown in Figure 20.

At low concentrations gibberellin A_3 significantly stimulated leaf sheath elongation in both species; 0.034 nmoles of gibberellin A_3 was the smallest amount which produced a significant response in both barley and dwarf rice.

ent-Kaurenoic acid significantly stimulated leaf sheath elongation in dwarf rice, 0.34 nmoles being the smallest amount which produced a significant response. Over the concentration range tested, ent-kaurenoic acid had no effect on leaf sheath elongation in barley.

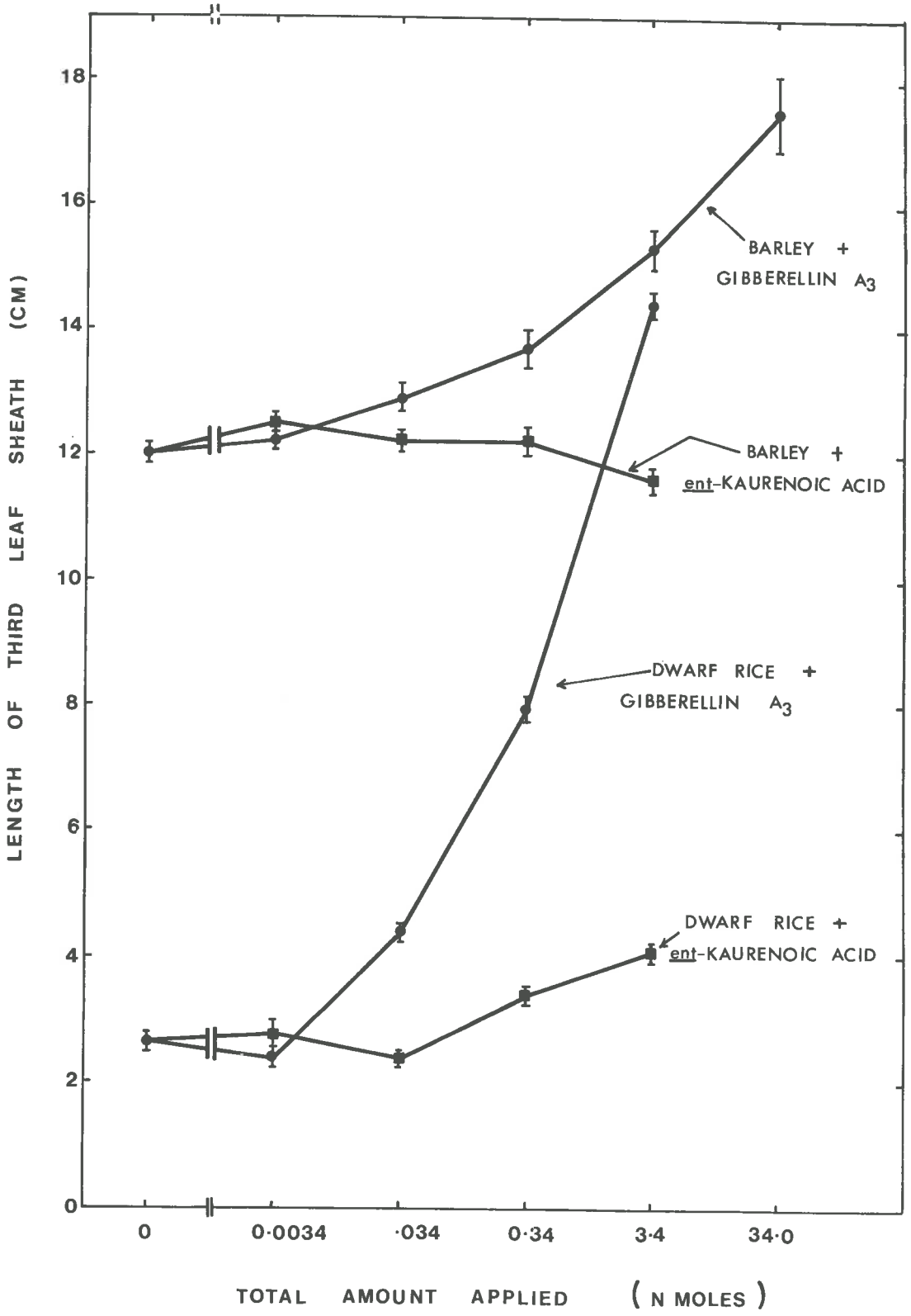
FIGURE 20.

EFFECT OF *ent*-KAURENOIC ACID AND GIBBERELLIN A_3 ON THE ELONGATION OF THE THIRD LEAF SHEATH OF BARLEY AND DWARF RICE SEEDLINGS

Seedlings were raised as described in III.B.2, and treated at daily intervals for 1 week with solutions of gibberellin A_3 and *ent*-kaurenoic acid as described in III.B.9(3).

Treatments began on rice seedlings when they were 5 days old. Treatments began on barley seedlings when they were 7 days old.

The vertical lines refer to the standard error for each mean. The means for dwarf rice treated with 0.34 and 3.4 nmoles of *ent*-kaurenoic acid were significantly different from the control at the 95% confidence level.


LENGTH OF THIRD LEAF SHEATH (CM)
18
16
14
12
10
8
6
4
2
0
BARLEY + GIBBERELLIN A3
BARLEY + ent-KAURENOIC ACID
DWARF RICE + GIBBERELLIN A3
DWARF RICE + ent-KAURENOIC ACID
0
0·0034
·034
0·34
3·4
34·0
TOTAL AMOUNT APPLIED (N MOLES)

(2) Metabolism of 17-^{14}C-ent-Kaurenoic Acid by Prior Barley and Tan-ginbozu Dwarf Rice Seedlings.

The effect of 17-^{14}C-ent-kaurenoic acid on the elongation of the third leaf sheath of barley and Tan-ginbozu dwarf rice seedlings is shown in Table 10A. The radioactive ent-kaurenoic acid had no significant effect on the elongation of the third leaf sheath of barley, but significantly stimulated elongation of the third leaf sheath of dwarf rice.

The distribution of radioactivity in the three extracts derived from the seedlings is shown in Table 10B. Considerable amounts of radioactivity were found in each extract from both barley and dwarf rice seedlings.

TLC, on heat activated silica gel H plates, of the chloroform-acetone extracts (Figure 21A) showed the extract from dwarf rice seedlings contained several radioactive metabolites, one of which had the same RF as authentic ent-kaurenoic acid. The extract from barley seedlings contained only one radioactive metabolite, in addition to a peak of radioactivity which remineed at the origin, and a large peak coinciding with the position of ent-kaurenoic acid.

TLC on silica gel H of the remaining aqueous phase from both barley and dwarf rice seedlings (Figure 21B) showed only one peak of radioactivity which remained at the origin of the plates during

TABLE 10A.

17-^{14}C-ent-Kaurenoic acid (specific activity 0.62 μcuries/μmole) was prepared and administered to the seedlings as described in III.B.8 and III.B.9(3). Control seedlings were treated with the same volume of solvent without any ent-kaurenoic acid.

*At the 95% confidence level treated not significantly different from control.

**At the 95% confidence level treated significantly longer than control.

TABLE 10B.

Experiment described in IV.D.2 and the seedlings were extracted as described in III.B.3(1); aliquots of each extract were measured for radioactivity as described in III.B.10.

TABLE 10A.

EFFECT OF 17-^{14}C-*ent*-KAURENOIC ACID ON THE ELONGATION OF THE THIRD LEAF SHEATH OF PRIOR BARLEY AND TAN-GINBOZU DWARF RICE SEEDLINGS

	Barley cv. Prior	Rice cv. Tan-Ginbozu
Number of plants treated	10	13
Total amount of 17-^{14}C-*ent*-kaurenoic acid applied per seedling (µg)	13.95	4.34
Length of 3rd leaf sheath: ratio of treated/control	*0.95	**1.69

TABLE 10B.

DISTRIBUTION OF RADIOACTIVITY IN THE 3 EXTRACTS FROM BARLEY AND DWARF RICE SEEDLINGS FOLLOWING ADMINISTRATION OF 17-^{14}C-*ent*-KAURENOIC ACID

Extract	Barley cv. Prior		Rice cv. Tan-Ginbozu	
	Total D.P.M.	% of Applied	Total D.P.M.	% of Applied
Chloroform-acetone	83,000	13.4	66,300	26.5
Acid ethyl acetate	180,450	29.1	28,192	11.3
Remaining water phase	192,600	31.1	34,640	13.8
Total Recovered (soluble)	456,050	75.3	129,132	51.6

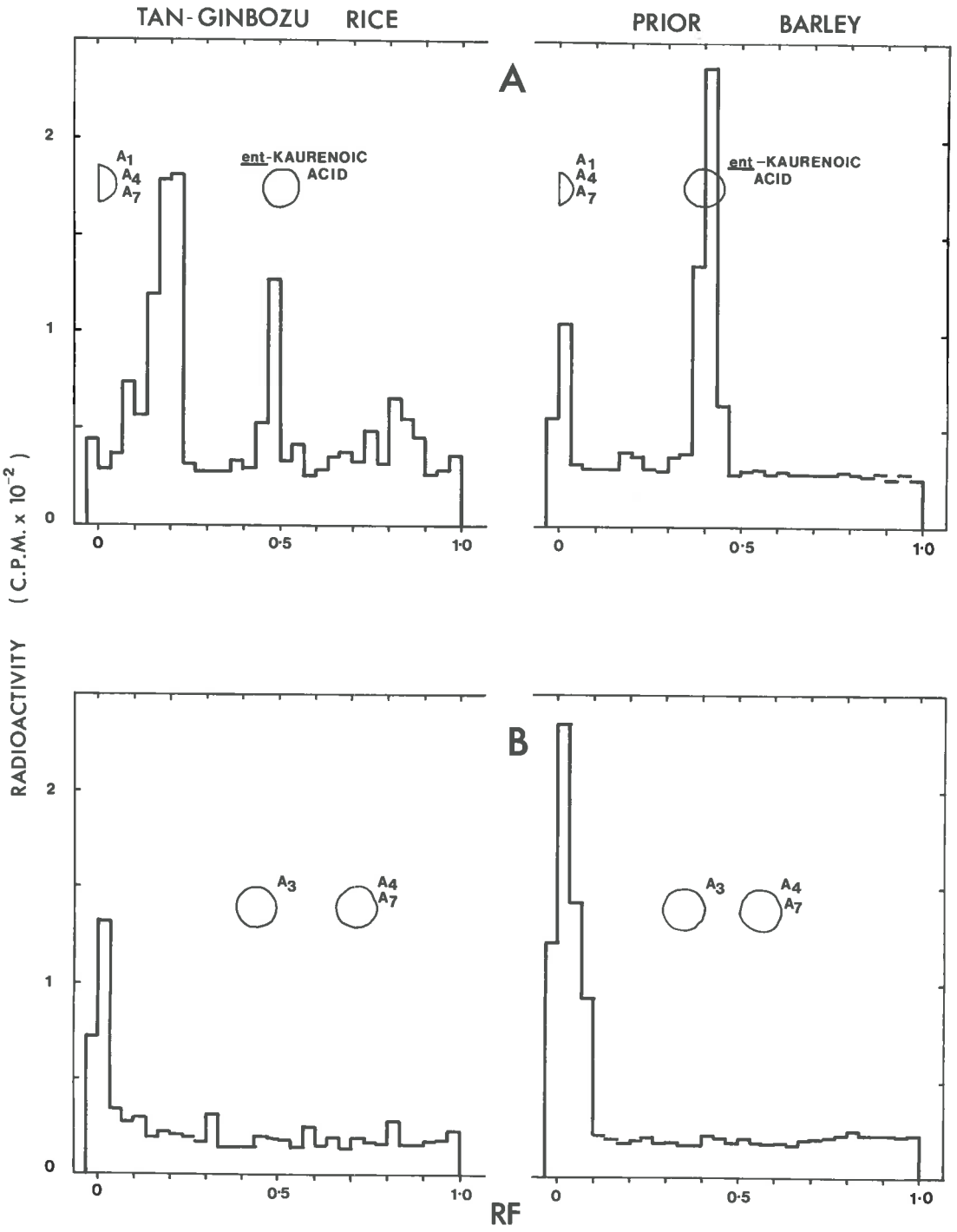
FIGURE 21.

TLC OF THE CHLOROFORM-ACETONE EXTRACT AND REMAINING AQUEOUS PHASE FROM PRIOR BARLEY AND TAN-GINBOZU DWARF RICE SEEDLINGS FED 17-^{14}C-*ent*-KAURENOIC ACID

Counting of silica gel H plates was carried out as described in III.B.10.

A. TLC on heat-activated silica gel H, in chloroform with 4% methanol, of aliquots of the chloroform-acetone extracts.

B. TLC on silica gel H in chloroform/ethanol/formic acid (85/15/1) of aliquots of the remaining aqueous phase.


TAN-GINBOZU RICE
PRIOR BARLEY
A
B
A1
A4
A7
ent-KAURENOIC ACID
A3
RADIOACTIVITY (C.P.M. x 10⁻²)
RF
0
1
2
0·5
1·0

development in chloroform/ethanol/formic acid (85/15/1). Further characterisation of the radioactive compounds in this extract was not carried out.

The results of TLC on silica gel H of the ethyl acetate extract from barley seedlings is shown in Figure 22. The radioactivity measurements revealed the presence of one large peak (RF 0.08) and one very minor peak (RF 0.59). The biological activity measurements on the same plate showed a peak of activity which coincided with gibberellin A_3 standard. No peak of radioactivity was associated with this peak of biological activity, and the main radioactive peak (RF 0.08) was not associated with a peak of biological activity.

The result of TLC on silica gel H of the ethyl acetate extract from Tan-ginbozu dwarf rice seedlings is shown in Figure 23. The scan of radioactivity along the length of the TLC plate showed several peaks of radioactivity, some of which had an RF similar to that of the gibberellin standards. The plate was divided into 3 zones, each of which was eluted separately.

The radioactivity eluted from zone 1 did not migrate from the origin during paper electrphoresis in ammonium bicarbonate buffer (0.1 M, pH 8.9), and during descending paper chromatography in isopropanol/ammonia/water (10/1/1) 3 peaks of radioactivity were produced (RF values 0.24, 0.44, 0.52 and 0.77).

FIGURE 22.

TLC ON SILICA GEL H OF THE ETHYL ACETATE EXTRACT FROM PRIOR BARLEY SEEDLINGS FED 17-^{14}C-ent-KAURENOIC ACID

The solvent used was chloroform/ethanol/formic acid (85/15/1).

One fifth of the plate was scraped in 0.5 cm zones, and each zone was measured for radioactivity as described in III.B.10. The scale for the radioactive measurements (c.p.m.) is given on the left hand ordinate, and the results are presented with broken black lines.

Another fifth of the plate was scraped in 0.5 cm zones and the biological activity in each zone was measured as described in III.B.6. The scales for the biological activity measurements are given on the right hand ordinate. One scale (Δ R) refers to the increase in refraction of the ambient bioassay solution after a 40-hour incubation: the other scale refers to the amount of gibberellin A_3, in grams, required to produce the given responses. The results of bioassay measurements of the chromatogram are given in continuous black lines.

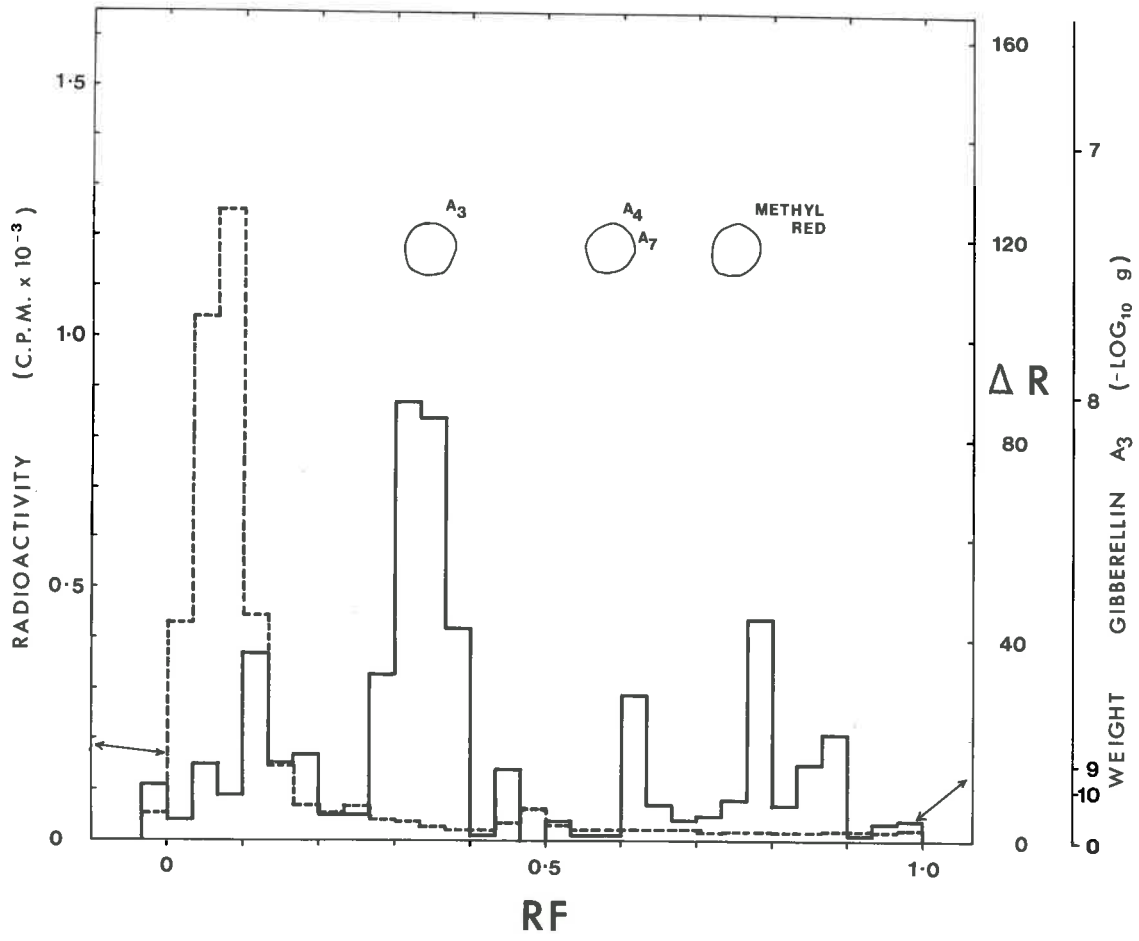
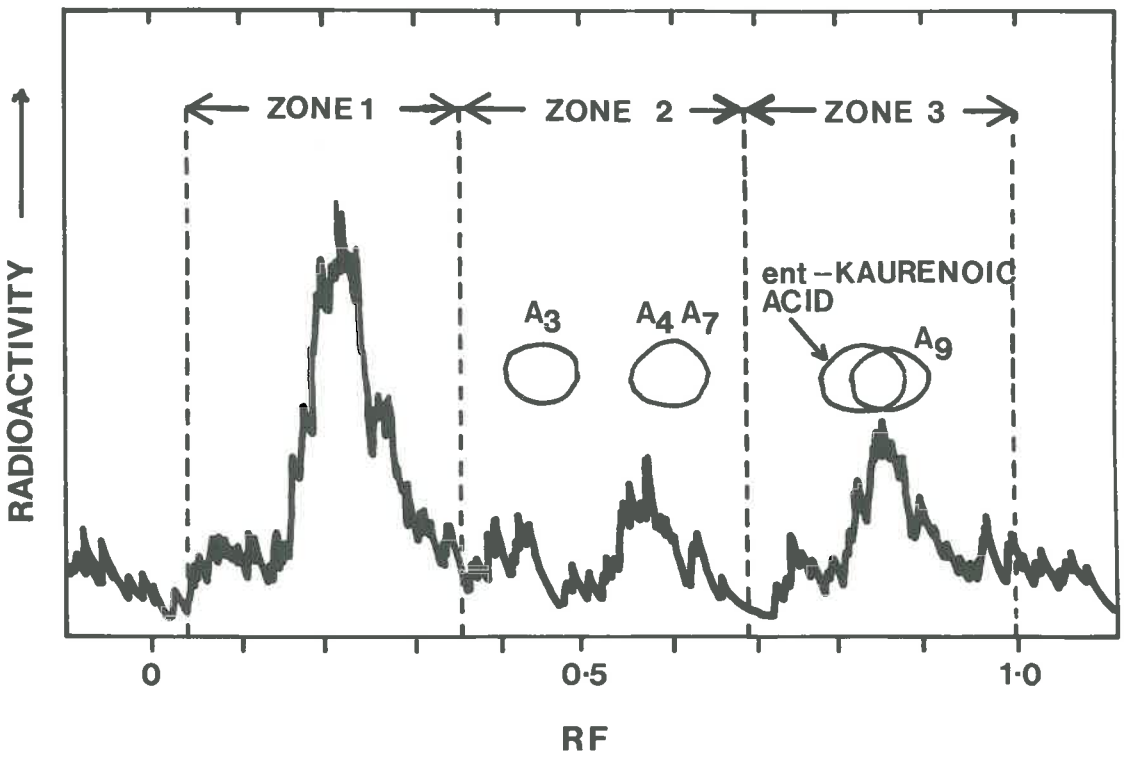

RADIOACTIVITY (C.P.M. x 10⁻³)
1·5
1·0
0·5
0
0
0·5
1·0
RF
A3
A4
A7
METHYL RED
160
120
80
40
0
ΔR
7
8
9
10
0
WEIGHT GIBBERELLIN A3 (-LOG10 g)

FIGURE 23.

TLC ON SILICA GEL H OF THE ETHYL ACETATE EXTRACT FROM TAN-GINBOZU DWARF RICE SEEDLINGS FED 17-^{14}C-ent-KAURENOIC ACID

The solvent used was chloroform/ethanol/formic acid (85/15/1).

The plate was scanned for radioactivity with a gas-flow scanner, set to give a full scale deflection with 1K, at a scanning speed of 0.75 cm/minute. Elution in the 3 marked zones was carried out as described in III.B.4(1).


ZONE 1
ZONE 2
ZONE 3
RADIOACTIVITY
A3
A4 A7
ent-KAURENOIC ACID
A9
0
0·5
1·0
RF

The radioactivity eluted from zones 2 and 3 migrated with the same mobility as gibberellins A_3, A_4 and A_7 during electrophoresis in ammonium bicarbonate buffer (0.1 M, pH 8.9).

GLC analysis of the TMS ether/ester derivatives of the radioactivity eluted from zone 2 is shown in Figure 24. A small but significant peak of radioactivity, with an RT the same as gibberellin A_1, was found in the collected fractions, and a small peak of biological activity was found to occur in the same fractions.

No radioactivity was found in the fractions collected between the internal standards (allogibberic acid and cholesterol) in the GLC analysis of the zone 3 eluate.

FIGURE 24.

GLC OF THE ZONE 2 ELUATE FROM TAN-GINBOZU DWARF RICE SEEDLINGS TREATED WITH 17-^{14}C-*ent*-KAURENOIC ACID

(Description of Zone 2 eluate is given in Figure 23)

Column type: 3% OV-17 on silanised Gaschrom Q.

Carrier gas: nitrogen at a flow rate of 50 ml/minute.

Column temperature: initially 210°C and rising at a rate of 1.7°C/minute to 260°C.

Detector: flame ionisation at 220°C.

Derivatives: TMS ether/ester derivatives, prepared as described in III.B.5(3)c, with 2 µg each of cholesterol and allogibberic acid.

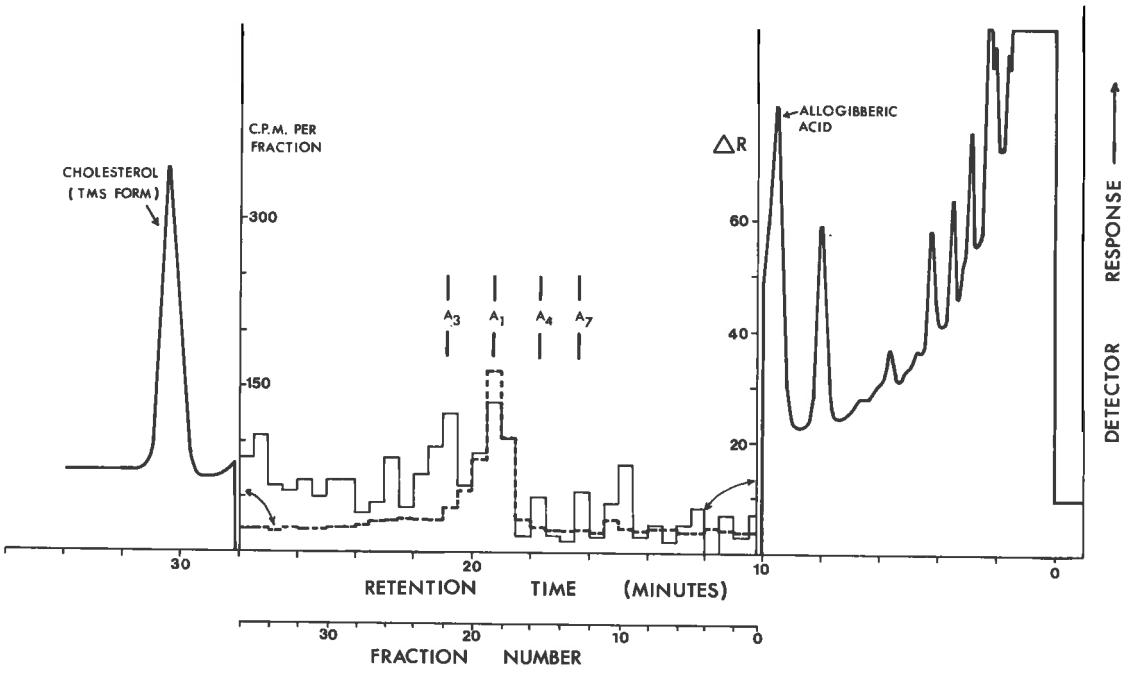
Fraction collecting: carried out as described in III.B.5(6).

Retention times of the standard gibberellins were calculated from the positions of the internal standards.

The dried eluates of the collected fractions were dissolved in a known volume of methanol, 50% of which was measured for radioactivity as described in III.B.10. The results of the radioactive measurements are given in broken black lines.

10% of the remainder of each fraction was tested for biological activity in the barley endosperm bioassay as described in III.B.6. The bioassay results presented (Δ R), in continuous black lines, are the mean refractometer readings obtained above control from 3 different dilutions of each fraction; the relative concentrations used for the dilutions were 1, 3 and 10. The responses obtained, above control, from 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} g of gibberellin A_3 were 3, 20, 91 and 138 Δ R units respectively.

The mean Δ R values for fractions 18, 19 and 20 were each significantly greater, at the 0.1% confidence level, than the mean Δ R value for fractions 1 to 17.


CHOLESTEROL
(TMS FORM)
C.P.M. PER
FRACTION
300
150
A3
A1
A4
A7
ΔR
ALLOGIBBERIC
ACID
60
40
20
DETECTOR RESPONSE
30
20
10
0
RETENTION TIME (MINUTES)
30
20
10
0
FRACTION NUMBER

4. Discussion on Experiments with ent-Kaurenoic Acid

Exogenous ent-kaurenoic acid significantly stimulated leaf sheath elongation of Tan-ginbozu dwarf rice seedlings (Figure 20), a result which agrees with that of Murakami (1972). These seedlings metabolised 17-^{14}C-ent-kaurenoic acid to a number of radioactive compounds (Table 10B, Figures 21 and 23) such that only 3.8% of the total radioactivity applied to the seedlings was re-extractable in the chloroform-acetone extract as ent-kaurenoic acid (Figure 21A, Table 10B).

One of the radioactive compounds in the acid ethyl acetate extract chromatographed on GLC in a manner identical to gibberellin A_1 (Figure 24). This compound also produced a small but significant response in the barley endosperm bioassay (Figure 24). The structural requirements for activity in the barley endosperm bioassay, which have already been described (III.B.6(4)), include a γ-lactonic function on the A-ring, a C-3 hydroxyl group and a free C-2 position, or alternatively a double bond or epoxide between C-2 and C-3. These requirements, taken together with the RT on GLC, leads to the conclusion that the radioactive compound in Figure 24 is a hydroxylated gibberellin possessing a γ-lactonic function on the A-ring.

This conclusion is consistent with the hypothesis that the stimulation of leaf sheath elongation in response to ent-kaurenoic

acid is a consequence of conversion by the seedlings of part of the applied compound to a gibberellin, which is the actual stimulant of the growth response. Of the total radioactivity originally applied to the seedlings, 0.2% was recovered as a gibberellin (Figure 24). This conversion rate is in general agreement with the potency of ent-kaurenoic acid, relative to that of gibberellin A_3, in stimulating leaf sheath elongation in the seedlings.(Figure 20).

The type of gibberellin which is present in normal rice seedlings is not known, although on the basis of a differential growth response in two bioassays Murakami (1972) concluded gibberellin A_{19} was present. This compound does not have a γ-lactonic function on the A-ring (Figure 6), and is inactive in the barley endosperm bioassay (Crozier et al., 1970). In this context it may be pertinent that Durley and Pharis (1973) found Tan-ginbozu dwarf rice seedlings could convert exogenous ^{3}H-gibberellin A_4 to ^{3}H-gibberellin A_1 and ^{3}H-gibberellin A_{34}.

TLC analysis of the chloroform-acetone extract and the acid ethyl acetate extract (Figures 21 and 23) showed the dwarf rice seedlings converted ent-kaurenoic acid to a number of other radioactive compounds. Some of these compounds are presumably intermediates between ent-kaurenoic acid and gibberellin.

Prior barley seedlings failed to elongate in response to exogenous ent-kaurenoic acid (Figure 20), but metabolised ^{14}C-ent-

kaurenoic acid such that the extracted radioactivity was evenly distributed between the chloroform-acetone, ethyl acetate and remaining water extracts (Table 10B). Of the total applied radioactivity, 10% was recovered in the chloroform-acetone extract as ent-kaurenoic acid (Figure 21,,Table 10B). The acid ethyl acetate extract contained only one prominent radioactive metabolite (Figure 22, RF 0.08); this metabolite was inactive in the barley endosperm bioassay. TLC analysis showed none of the radioactivity in the acid ethyl acetate extract was associated with gibberellins A_1 or A_3 (Figure 22).

Both Prior barley and Tan-ginbozu dwarf rice seedlings converted a significant proportion of the applied radioactivity to metabolites which were extracted with ethyl acetate and migrated with a low RF on silica gel H in chloroform/ethanol/formic acid solvent (Figures 22 and 23). The nature or these compounds is unknown, but they failed to move from the origin during electrophoresis in ammonium bicarbonate buffer (0.1 M, pH 8.9), which indicates the lack of a free carboxyl group. All the known gibberellin precursors derived from ent-kaurenoic acid possess at least one carboxyl function, either at the C-19 or C-7 position (Figures 3 and 4). The lack of a free carboxyl function can therefore be taken as evidence that these compounds are unlikely to be gibberellin precursors.

It is difficult to explain why exogenous 17-^{14}C-ent-kaurenoic acid served as a gibberellin precursor in Tan-ginbozu dwarf rice seedlings, yet failed to do so in Prior barley seedlings. There are two possible explanations for this result, one that different gibberellin biosynthetic pathways operate in these two species and another that exogenous ent-kaurenoic acid reached the sites of the appropriate enzyme systems for conversion to gibberellin when applied to dwarf rice, but failed to do so when applied to barley because of a permeability barrier. The evidence presented in the literature review (I.B.2) suggests that it is unlikely that the gibberellin biosynthetic pathways operating in barley and dwarf rice are different. It would seem more likely that exogenous ent-kaurenoic acid is excluded from the sites of gibberellin biosynthesis in barley seedlings, but is not excluded from the sites in dwarf rice seedlings, and that this fact must account for the difference in the ability of these two species to incorporate this compound into gibberellin.

One interesting possibility, albeit speculative, is that endogenous gibberellin exerts a controlling effect on the rate at which the gibberellin biosynthetic pathway operates, such that when the endogenous gibberellin concentration is ~~high~~ low the biosynthetic pathway operates more rapidly than when the endogenous gibberellin concentration is ~~low~~ high. Control systems which have a

similar effect through the regulation of the production of enzymes (enzyme repression), and through the regulation of the activity of already existing enzymes ("feed back" inhibition), have been shown to control the rate of biosynthesis of a number of metabolites in bacteria (Mahler and Cordes, 1966; Conn and Stumpf, 1972), and examples of these control mechanisms have been found in higher plants (Glasziou, 1969; Marcus, 1971; Ong and Jackson, 1972a, 1972b). Tan-ginbozu rice does not contain gibberellin (Suge and Murakami, 1968), and therefore the enzymes in the biosynthetic pathway in this cultivar would not be subjected to the postulated suppressing influence of gibberellin. Prior barley contains gibberellins A_1 and A_3 and the biosynthetic pathway in these seedlings would be under the control of these compounds. For this explanation to account for the difference with which Tan-ginbozu rice and Prior barley seedlings converted exogenous ent-kaurenoic acid to gibberellin, the site or sites of gibberellin control on the biosynthetic pathway must occur after ent-kaurenoic acid formation.

As interesting as this postulated control mechanism is, it would seem to be inadequate to explain all the results which have been obtained. The experiments with radioactive carbon dioxide have shown gibberellin biosynthesis is taking place in barley seedlings (see IV.A.), yet ent-kaurenoic acid failed to serve as a

precursor. This may indicate that the biosynthetic pathway in barley seedlings is operating at a much slower rate than the pathway in dwarf rice seedlings which have been supplied ent-kaurenoic acid. An alternative explanation, which has already been mentioned above, is that exogenous ent-kaurenoic acid is transported less efficiently in barley than in dwarf rice to the sites of gibberellin biosynthesis.

V.

GENERAL DISCUSSION ON THE CONTROL OF GIBBERELLIN BIOSYNTHESIS IN PLANTS

There are several mechanisms by which cells, tissues and organisms control the rate at which biochemical pathways operate. These range from mechanisms which operate at the level of enzymes in the cell, to mechanisms which operate during the transport of intermediates between cells and tissues, and mechanisms which are influenced by the environmental conditions to which the organism responds. The problem of metabolic regulation of a biosynthetic pathway should be considered as an integration embracing all of the contributing factors, but for the sake of simplicity the subject is more easily discussed if each of the contributing factors is considered individually. With reference to the control of gibberellin biosynthesis in plants, the problem assumes particular interest because the end product of the biosynthetic pathway, in very small amounts, is capable of dramatically affecting plant growth and development.

At the level of the enzymes involved in a biosynthetic pathway, a number of control mechanisms have now been clearly described; these include compartmentation of enzymes and substrates, kinetic factors involving interactions of substrates, cofactors and enzymes,

and the control of enzyme concentration (Conn and Stumpf, 1972). The data presented in this thesis provides evidence for the involvement of some of these mechanisms during gibberellin biosynthesis in plants.

Compartmentation of enzymes and substrates is an important feature of the cells of eukaryotic organisms, and as already stated is an integral part of metabolic regulation in these organisms (Oaks and Bidwell, 1970; Conn and Stumpf, 1972). The important features of compartmentation are, firstly, that considerable simplification of the control mechanisms which integrate all the reactions occurring in a cell can be achieved by segregating certain reactions and closely related groups of reactions; secondly, if the entry of potential substrates into the compartment is regulated by the membrane enclosing the compartment then some control of the rate of the reactions taking place in the compartment can be achieved.

Incorporation experiments with $^{14}CO_2$ showed 11-day old barley seedlings were biosynthesising gibberellin de novo from small molecular weight precursors. The failure of exogenous 2-^{14}C-mevalonic acid to act as a gibberellin precursor in both 11-day old barley seedlings and immature apricot fruits indicates that some of the reactions involved in gibberellin biosynthesis are compartmentalised such that exogenous mevalonic acid, or gibberellin

precursors derived from it, is prefententially excluded. Mevalonic acid is known to be incapable of penetrating across the chloroplast membrane (Rogers et al., 1968), and it is interesting to note that Stoddart (1969) found sonicated chloroplast preparations from Brassica oleraceae had the necessary enzyme complement to metabolise 17-^{14}C-ent-kaurenoic acid to compounds akin on TLC to known gibberellins. It would therefore seem plausible to postulate that some of the reactions involved in gibberellin biosynthesis are confined to chloroplasts. This hypothesis however, is untenable in the case of developing fruits where the accumulated gibberellin originates in the seed (Coombe, 1971), a tissue which is probably non-photosynthetic. In this case other membrane bound organelles, which are impermeable to exogenous mevalonic acid or gibberellin precursors derived from exogenous mevalonic acid, must be invoked as the site of some of the gibberellin biosynthetic reactions.

As already mentioned, the simplification of the mechanisms required to regulate a pathway would seem to be the major significance of segregating into a compartment the enzymes involved in gibberellin biosynthesis. The singularly salient feature of such an arrangement would be the possibility of regulating the reactions by controlling the entry of substrate into the compartment. The preference with which exogenous mevalonic acid, and the isoprenoid

precursors derived from mevalonic acid, are excluded from the proposed compartment suggests that the substrates for gibberellin biosynthesis must come from CO_2 via acetyl-Coenzyme A, or some of the other compounds which are precursors of mevalonic acid (Popják and Cornforth, 1960).

A comparison of the structure of the ent-gibberellane skeleton with the structures of the other known tetracyclic diterpenes (Hanson, 1968) reveals that the stage of contraction of the B-ring, to form the ent-gibberellane skeleton, is probably the first reaction in the whole gibberellin biosynthetic pathway peculiar to the gibberellins. No other tetracyclic diterpenes possess a 5-membered B-ring and kaurenoic acid and 7β-hydroxy-kaurenoic acid, the two intermediates which immediately precede the B-ring contraction step (see Figure 3 and Graebe et al., 1972) are also precursors of other compounds (the kaurenolides, steviol, and probably the other kaurene acids which have been isolated from plants (Hanson, 1968)). Gibberellin A_{12}-aldehyde is the first known gibberellin precursor with the 5-membered B-ring; as such the formation of this compound is an important step in the biosynthesis of gibberellins. Not only is gibberellin A_{12}-aldehyde the first ent-gibberellane compound in the pathway, but it has already been postulated as being situated at a branch point in the biosynthesis of the hydroxylated and non-hydroxylated gibberellins (Figure 4B).

The "feed-back" inhibition and enzyme repression mechanisms which have been described for certain bacterial and plant biosynthetic systems (Mahler and Cordes, 1966; Conn and Stumpf, 1972; Glaziou, 1969; Marcus, 1971; Ong and Jackson, 1972a, 1972b) are both directed at controlling the first reaction in the biosynthetic sequence. By analogy with the gibberellin biosynthetic pathway, it is possible that the inhibition which gibberellin has been postulated to exert on the pathway (see IV.D.4) is directed at the reaction which produces gibberellin A_{12}-aldehyde, the first true gibberellin intermediate. Such a scheme is only hypothetical and while the experiments reported here offer no clue as to the nature of the inhibition which gibberellin exerts on the pathway, or the exact location of the site of inhibition, the observation that Tan-ginbozu rice seedlings can convert exogenous ent-kaurenoic acid to gibberellin provides research workers, for the first time, with a system from plants in which these reactions can be studied.

While the results from work with mevalonic acid suggest intracellular compartmentation is a feature of some of the reactions involved in gibberellin biosynthesis in plants, the results of Crozier and Reid (1971, 1972) suggest a different sort of control, based on separation of enzymes between organs, is also involved. These workers produced results which indicated that not all of the

reactions involved in gibberellin biosynthesis occur in one tissue; they suggested that precursors were transported from shoots to roots where some of the final oxidative modifications of the ent-gibberellane skeleton takes place.

Such a scheme, in which the biosynthesis of the final biologically active compound requires the participation of enzymes in the shoots and roots of the plant, and the efficient transport of precursors between these tissues, is another feature of the control which may be imposed on gibberellin biosynthesis in plants.

The results which have been presented in this thesis bear testimony of the point made initially (Chapter II) that the major problem of the project was the development of a convenient system from plants for studying the reactions involved in gibberellin biosynthesis. Nevertheless, the data which has been obtained has permitted some discussion and conclusions regarding some of the mechanisms by which plants control the rate of gibberellin biosynthesis. However, no information has been obtained on the problem of the influence of environmental factors, such as day-length and temperature, on the rate of gibberellin biosynthesis.

VI

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